UNIVERSIDADE FEDERAL DO CEARÁ

CENTRO DE CIÊNCIAS

DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

STELAMARIS DE OLIVEIRA PAULA MARINHO

RESPOSTAS FISIOLÓGICAS E METABÓLICAS MEDIADAS PELA FONTE DE NITROGÊNIO: PAPEL DO NH_4^+ NA TOLERÂNCIA DE PLANTAS DE SORGO SOB SALINIDADE

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Tese apresentada ao Programa de Pós-graduação em Bioquímica da Universidade Federal do Ceará como requisito parcial para obtenção do grau de Doutor em Bioquímica. Área de concentração: Bioquímica Vegetal.

Orientador: Prof. Dr. Enéas Gomes Filho.
Coorietador: Dr. Rafael de Souza Miranda

FORTALEZA
2020

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)

M291r Marinho, Stelamaris de Oliveira Paula.
Respostas fisiológicas e metabólicas mediadas pela fonte de nitrogênio : Papel do NH4+ na tolerância de plantas de sorgo sob salinidade / Stelamaris de Oliveira Paula Marinho. – 2020.
101 f. : il. color.

Tese (doutorado) – Universidade Federal do Ceará, Centro de Ciências, Programa de Pós-Graduação em Bioquímica , Fortaleza, 2020.
Orientação: Prof. Dr. Enéas Gomes Filho.
Coorientação: Prof. Dr. Rafael de Souza Miranda.

1. Bioquímica vegetal. 2. Fisiologia vegetal. 3. Estresse salino. 4. Metabolômica. 5. Amônio. I. Título.
CDD 572

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A Deus.

Aos meus pais, Ernilson Paula e Anailza Maia

e meu esposo, Italo Marinho.

AGRADECIMENTOS

A Deus, meu melhor amigo e Senhor da minha vida, que sempre esteve ao meu lado e me sustentou em todos os momentos, sem Ele nada disso seria possível.

Aos meus pais, Francisco Ernilson de Oliveira Paula e Maria Anailza Maia de Oliveira Paula, pelo amor incondicional, pelo apoio e incentivo durante toda essa jornada, a eles minha eterna gratidão e amor.

Ao meu esposo, Italo Freire Marinho, por sempre me acompanhar nessa caminhada, por seu amor e cuidado, e por sempre me incentivar, acreditar na minha capacidade e por compreender minhas ausências. Meu amor pra sempre.

Ao professor Dr. Enéas Gomes Filho, pela orientação, confiança e pela paciência de ensinar-me durante esses 7 anos.

Ao professor, Dr. Rafael de Souza Miranda, por toda ajuda e instrução para execução deste trabalho, por sua amizade e por sempre me incentivar e acreditar no meu potencial.

Às professoras Dra. Maria Raquel Alcântara de Miranda e Dra. Maria Izabel Gallão, por sempre permitirem o uso de seus laboratórios para a realização das análises.

À professora, Dra. Valdineia Soares Freitas, pelos ensinamentos e conselhos durante a convivência no laboratório, e por sua amizade.

Aos membros da banca examinadora já mencionados, por aceitarem o convite, e pelas críticas e sugestões ao trabalho.

Aos professores, Dr. Humberto Henrique de Carvalho e Dr. Danilo de Menezes Daloso, por todos os ensinamentos e pelo tempo dispendido para orientar-me na execução das análises de metabolômica.

À professora, Dra. Rosilene Oliveira Mesquita, por toda ajuda com as análises do IRGA, bem como por sua valiosa amizade.

Ao professor, Dr. Emílio de Castro Miguel, pela orientação durante as análises de microscopia confocal e eletrônica de transmissão.

Aos meus avós, Raimundo Maurício de Oliveira e Rita Maia de Oliveira, e minha tia/madrinha Silvia Helena Maia de Oliveira, por sempre torcerem pelo meu sucesso.

Aos meus irmãos, Anderson de Oliveira Paula e Ernilson Paulo Barbosa, meus cunhados Regina Celi Lopes, Ivna Marinho e William Marinho e minha sogra Jucilene Freire.

Às minhas amigas Gyedre dos Santos Araújo e Karollyny Roger Pereira Lima, por toda ajuda e carinho, por sempre escutarem meus desabafos e me apoiarem em todos os momentos. Vocês são um presente de Deus em minha vida.

Aos amigos do Laboratório de Fisiologia Vegetal, Lineker Lopes, Cibelle Gadelha, Daniel Farias, Isabelle Mary Pereira, Lucas Pacheco, Igor Rafael Costa, Francisco Dalton Barreto, como também aos que já passaram pelo laboratório, Daniel Coelho, Bruno Silva, Lindefânia Melo, Valéria Batista, Nara Lídia Alencar, Alexcyane Feijão, Natália Amaral, Elton Marques e Luckas Huriel, por toda a ajuda e momentos de descontração.

Aos queridos amigos Cíntia Barros, Rafaela Alves, João Fellypy Carvalho, Regys Albuquerque, Alessandra Queiroz, Otania Freire, Duillys Chaves, Rachel Sanabio, Luiz Walter Holanda, José Cleomon da Silva, Doris Maia, Tiago Nogueira, Juliana Barbosa, Sarah Nobre, Delano Borges, Amanda Coelho, João Victor Nunes, Mayara Patrícia Matos, Frederico Garcia, Lívia Lopes e Evanildo Júnior.

Aos professores do Programa de Pós-graduação em Bioquímica, e funcionários do Departamento de Bioquímica e Biologia Molecular, bem como aos meus colegas do curso de mestrado e doutorado da bioquímica.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pela concessão de bolsa de doutorado durante os anos de 2015 a 2018.

Ao Instituto Nacional de Ciência e Tecnologia em Salinidade (INCTSal), a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e a Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP), que concederam auxílio financeiro para execução deste trabalho.

À Central Analítica da UFC, especialmente aos técnicos João Victor Nunes, Marlos de Medeiros e Sergimar Kennedy de Paiva, por todo auxílio e orientação prestados no uso dos equipamentos.

Ao Instituto Agronômico de Pernambuco (IPA), representado pelo Dr. José Nildo Tabosa, por sempre fornecer as sementes para este e outros trabalhos do laboratório.

E por fim, a todas as pessoas que contribuíram de alguma forma para o desenvolvimento deste trabalho e que não foram citados.

“The cell never acts; it reacts” Ernest Haeckel

RESUMO

A salinidade é um dos fatores abióticos que mais limitam o crescimento e desenvolvimento das plantas, causando distúrbios metabólicos, morfológicos e moleculares, sendo a fotossíntese um dos processos fisiológicos mais afetados pelo estresse. Recentes estudos reportam a ação benéfica das fontes de nitrogênio, em especial o NH_4^+, na redução destes efeitos deletérios por induzir mecanismos de aclimatação que conferem maior tolerância ao estresse. Entretanto, seus efeitos na eficiência fotossintética e regulação de metabólitos ainda são pouco conhecidos. Portanto, este trabalho teve por objetivo testar a hipótese de que a aclimatação à nutrição com NH_4^+ aciona mecanismos de defesa e causa alterações metabólicas que conferem maior tolerância à salinidade. Para isso, plântulas de sorgo, cv. CSF 20, foram crescidas em solução de Hoagland modificada, contendo 5 mM de N, nas formas de NO_3^- ou NH_4^+ isoladamente, ou na mistura equimolar NO_3^-:NH_4^+ e após 12 dias de aclimatação, as plantas foram submetidas ao estresse salino (75 mM NaCl) durante 12 dias. A salinidade reduziu severamente o crescimento das plantas para todas as formas ou regimes de nutrição nitrogenada, porém esse efeito inibitório foi menor nas plantas nutridas com NH_4^+, que apresentaram maior área foliar e massa seca da parte aérea. Além disso, plantas nutridas com NH_4^+ apresentaram menor potencial osmótico, menor vazamento de eletrólitos e maior relação K^+/Na^+ em comparação com os outros regimes de nutrição sob salinidade. Embora a nutrição com NH_4^+ tenha induzido elevado conteúdo de H_2O_2 basal, não foi observado danos à integridade dos cloroplastos, nem mesmo após o estresse salino. Adicionalmente, o NH_4^+ promoveu melhor desempenho fotossintético, em relação às outras formas de nutrição nitrogenada, sob salinidade. Isto foi relacionado com a maior taxa de assimilação de CO_2 e eficiência da carboxilação da rubisco (*A/Ci*), bem como manutenção da eficiência do fotossistema II. A salinidade ocasionou diversas mudanças no perfil metabólico de folhas de sorgo, no entanto o grau de variação, bem como o conjunto de metabolitos alterados foi dependente da fonte de nitrogênio. Aminoácidos, ácidos orgânicos, açúcares, polióis e outros grupos de metabolitos foram significativamente aumentados ou reduzidos pela salinidade. Plantas nutridas com regime misto apresentaram incrementos em vários aminoácidos, que podem estar associados com a degradação de proteínas e clorofila, evidenciado pela perda da integridade dos cloroplastos e significativas reduções na assimilação do CO_2 e eficiência fotoquímica. O aumento no conteúdo de prolina foi observado sob condições salinas em plantas nutridas com regime misto e apenas NO_3^-, enquanto outros metabolitos (sacarose, trealose, maltitol) foram responsáveis pelo ajustamento osmótico em planta crescidas com NH_4^+. O aminoácido asparagina teve importante contribuição na

separação dos perfis metabólicos entre as diferentes fontes de N, bem como mostrou-se responsivo ao estresse salino em todos os regimes de N. Sob nutrição com NH_4^+, a salinidade aumentou o conteúdo de ácido ascórbico e ácido desidroascórbico, os quais podem ter significativo efeito na homeostase redox celular e na proteção dos cloroplastos contra danos oxidativos. De modo geral, plantas nutridas com NH_4^+ exibiram poucas alterações nos metabolitos em comparação com as outras fontes após o estresse salino. Isto pode estar relacionado às prévias mudanças ocorridas durante a aclimatação ao NH_4^+, permitindo rápidas repostas à estresses posteriores, como a salinidade. Portanto, a nutrição com NH_4^+ foi capaz de ativar mecanismos envolvidos na manutenção da eficiência fotossintética e regulação de importantes metabólitos, que atenuaram os efeitos deletérios da salinidade nas plantas de sorgo.

Palavras-chave: Salinidade. Amônio. Nitrato. Metabolômica. *Sorghum bicolor*.

ABSTRACT

Salinity is an abiotic factor that most limits plant growth and development, causing metabolic, morphological and molecular disorders, it is photosynthesis one of the physiological processes most affected by stress. Recent studies report the beneficial action of nitrogen sources, especially NH_4^+, reducing these deleterious effects through induced acclimatization mechanisms that confer greater stress tolerance. However, their effects on photosynthetic efficiency and metabolite regulation are still poorly understood. Therefore, this study aimed to test the hypothesis that acclimatization to NH_4^+ nutrition triggers defense mechanisms and causes metabolic changes that confer greater salt tolerance. Herein, sorghum seedlings, cv. CSF 20, were grown in a modified Hoagland solution to contain 5 mM N in the form of sole NO_3^- or NH_4^+, or in the equimolar mixed-N (NO_3^-:NH_4^+) and, after 12 days of acclimatization, the plants were subjected to saline stress (75 mM NaCl) for more 12 days. Salinity severely reduced plant growth for all forms or N regimes nutrition, but this inhibitory effect was lower in NH_4^+-fed plants, which had higher leaf area and shoot dry mass. Besides, NH_4^+-fed plants had lower osmotic potential and electrolyte leakage and higher K^+/Na^+ ratio compared to other N nutrition under saline conditions. Although NH_4^+ nutrition induced high basal H_2O_2 content, no damage to chloroplast integrity was observed, even after saline stress. Additionally, NH_4^+ nutrition promoted better photosynthetic performance compared to other N forms under salinity. This was related to higher CO_2 uptake rate and rubisco carboxylation efficiency (*A*/*Ci*), as well as maintenance of photosystem II efficiency. Salinity caused several changes in the metabolic profile of sorghum leaves. However, the degree of variation, as well as the profile of altered metabolites was dependent on the N source. Amino acids, organic acids, sugars, polyols, and other groups of metabolites were significantly increased or decreased by salinity. Plants fed with mixed N showed increases in several amino acids, which may be associated with protein and chlorophyll degradation, which can be evidenced by the loss of chloroplast integrity and significant reductions in CO_2 assimilation and photochemical efficiency. The increase of proline content was observed under saline conditions in plants fed with mixed N and sole NO_3^- nutrition, while other metabolites (sucrose, trehalose, maltitol) were responsible for osmotic adjustment in plants grown with NH_4^+ nutrition. Asparagine had an important contribution to the separation of metabolic profiles among different N sources, as well as was responsive to salt stress in all N nutrition. Under NH_4^+ nutrition, salinity increased the content of ascorbic acid and dehydroascorbic acid, which may have a significant effect on cellular redox homeostasis and the protection of chloroplasts against oxidative damage. Overall, NH_4^+-fed

plants exhibited few changes in metabolites compared to other N sources after salt stress. This may be related to previous changes during acclimatization to NH_4^+, allowing quick responses to secondary stresses, such as salinity. Therefore, NH_4^+ nutrition was able to activate mechanisms involved in the maintenance of photosynthetic efficiency and regulation of important metabolites, which attenuated the deleterious effects of salinity on sorghum plants.

Keywords: Salinity. Ammonium. Nitrate. Metabolomics. *Sorghum bicolor*.

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LISTA DE ABREVIATURAS E SIGLAS

A	Taxa de assimilação do CO_2
APX	Peroxidase do ascorbato
CAT	Catalase
Ci	Concentração interna de CO_2
DCFH-DA	2',7'- dichlorofluorescein diacetate
E	Transpiração
ETR	Taxa de transporte de elétrons
EXC	Excesso de energia relativa no PS II
Fv/Fm	Eficiência fotoquímica máxima do PS II
GC-MS	Gas chromatography–mass spectrometry
GPOD	Peroxidase do guaiacol
GR	Redutase da glutationa
g_s	Condutância estomática
MDA	Malondialdeído
NPQ	Quenching não fotoquímico
PCA	Análise de componente principal
PS II	Fotossistema II
qP	Quenching fotoquímico
ROS	Reactive oxygen species
RWC	Relative water content
SOD	Dismutase do superóxido
TCA cycle	Ciclo do ácido tricarboxílico
Ψs	Potencial osmótico
ΦPSII	Eficiência fotoquímica efetiva do PS II

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1 JUSTIFICATIVA

O abastecimento de alimentos tem se tornado uma grande preocupação mundial, tendo em vista o crescimento populacional e a escassez de água para a agricultura. Adicionalmente, o uso de técnicas de manejo inapropriadas e o uso de água de baixa qualidade intensificam a salinização dos solos, acarretando consequências drásticas para o crescimento e a produtividade das plantas. Segundo estimativas da FAO (do inglês, *Food and Agriculture Organization of the United Nations*), aproximadamente 20% das áreas cultivadas no mundo, o que corresponde a 45 milhões de hectares, estão com altas concentrações de sais. Esse problema é alarmante, uma vez que cerca de 1 a 1,5 milhões de hectares de terras agricultáveis são perdidos a cada ano devido a salinidade (FAO, 2019). Nesse contexto, estudos voltados para o desenvolvimento de técnicas de manejo e/ou para a seleção de cultivares que apresentem maior capacidade de crescer em solos salinos são extremamente importantes para contornar o problema do estresse salino.

O estresse salino afeta o crescimento e o desenvolvimento das plantas através de fatores de natureza osmótica e iônica, provocados pela alta concentração de sais no solo, e posteriormente pelo acúmulo dos íons nos tecidos vegetais (NEGRÃO; SCHMÖCKEL; TESTER, 2017). Esses fatores causam diversos danos ao metabolismo, incluindo mudanças no *status* hídrico das plantas, redução da absorção de nutrientes, acúmulo em excesso de íons tóxicos nos tecidos, desbalanço hormonal, perda da eficiência fotossintética e alterações de várias vias metabólicas (CHEN *et al.*, 2019; DIAS *et al.*, 2016; KUMAR *et al.*, 2017; MAHLOOJI *et al.*, 2017). Além disso, esse rompimento no metabolismo normal promove a geração excessiva de espécies reativas de oxigênio (ROS, *Reactive Oxygen Species*), caracterizando o estresse oxidativo, que pode causar danos a biomoléculas e componentes celulares (AHMAD *et al.*, 2019; GAO *et al.*, 2015), interferindo em importantes processos metabólicos, como a fotossíntese (ASHRAF; HARRIS, 2013).

Para lidar com os efeitos deletérios da salinidade, as plantas desenvolveram mecanismos fisiológicos e bioquímicos para mitigar esses danos e manter o crescimento e desenvolvimento. Dentre eles, destacam-se (i) os mecanismos voltados para a redução do potencial osmótico celular (ou ajustamento osmótico), envolvendo o acúmulo de íons inorgânicos (K^+, Na^+ e Cl^-) no vacúolo e de solutos orgânicos no citosol (açúcares, ácidos orgânicos, aminoácidos, dentre outros), que resultam na manutenção da atividade metabólica das células (DI MARTINO *et al.*, 2003); (ii) a ativação de mecanismos de controle da

homeostase iônica (HORIE; KARAHARA; KATSUHARA, 2012; MIRANDA *et al.*, 2017); bem como (iii) aqueles voltados para a eliminação de ROS, incluindo enzimas e moléculas de defesa.

Além disso, as plantas também reprogramam o conteúdo de metabólitos, como aminoácidos, açúcares, ácidos orgânicos, moléculas sinalizadoras, compostos secundários que podem ser parte de uma resposta adaptativa ao estresse (PARIDA; PANDA; RANGANI, 2018). Neste contexto, os estudos de metabolômica são bastante promissores para decifrar mecanismos metabólicos que são indicadores de danos induzidos pelo estresse, bem como pode revelar respostas que atenuem os efeitos deletérios, promovendo tolerância ao estresse (BORELLI *et al.*, 2018; CHANG *et al.*, 2019; DIAS *et al.*, 2015; WIDODO *et al.*, 2009). Desta forma, tais informações podem ser fonte de pesquisa em programas de melhoramento genético voltados ao aumento da tolerância à salinidade, como também na seleção de cultivares com maior capacidade fotossintética em solos com concentrações elevadas de sais.

Estudos recentes reportam a relação entre a tolerância ao estresse salino e a fonte de N prevalecente no meio de crescimento. Neste contexto, a nutrição com NO_3^- ou a substituição parcial pelo NH_4^+ tem sido mais eficaz em atenuar os efeitos da salinidade na maioria das plantas, promovendo maior acúmulo de massa seca, maior eficiência fotossintética, menor acúmulo de íons tóxicos e maior acúmulo de solutos compatíveis sob condições salinas (FRECHILLA *et al.*, 2001; KANT *et al.*, 2007; MENG *et al.*, 2016; RIOS-GONZALEZ; ERDEI; LIPS, 2002). Embora o NH_4^+ seja tóxico para um grande número de espécies, estudos demonstram a atuação da nutrição com NH_4^+ como um estresse leve, acionando mecanismos de defesa que contribuem para reduzir os efeitos deletérios de um estresse secundário, um fenômeno conhecido como tolerância cruzada (FERNÁNDEZ-CRESPO; CAMAÑES; GARCÍA-AGUSTÍN, 2012; HESSINI *et al.*, 2013).

Em *Sorghum bicolor*, nosso grupo de pesquisa vem demostrando o papel da nutrição com NH_4^+ em promover maior tolerância à salinidade, através da regulação da homeostase iônica, melhor eficiência fotossintética e acúmulo de solutos compatíveis (COELHO *et al.*, 2020; MIRANDA *et al.*, 2013). Miranda *et al.* (2017) destacam o papel da nutrição com NH_4^+ conferindo maior tolerância à salinidade, através do acionamento de mecanismos que regulem a homeostase de Na^+, como maior atividade das bombas de prótons e transportadores Na^+/H^+ nas raízes de plantas de sorgo. Além disso, o maior acúmulo de aminoácidos e melhor relação K^+/Na^+ também foram relacionados com o melhor desempenho na assimilação de CO_2 em plantas de sorgo nutridas com NH_4^+ quando comparadas com aquelas

nutridas com NO_3^-, conferindo maior tolerância as condições salinas (MIRANDA *et al.*, 2016). No entanto, o envolvimento da fonte de nitrogênio na manutenção da maquinaria fotossintética, bem como seu efeito no sistema antioxidante e nas vias metabólicas ainda permanecem desconhecidos.

Portanto, o presente trabalho teve como alvo investigar o papel das fontes de nitrogênio, em particular do NH_4^+, na manutenção da maquinaria fotossintética, bem como na modulação do perfil metabólico, visando esclarecer as respostas envolvidas na atenuação dos efeitos do estresse salino em plantas de sorgo.

2 HIPÓTESE

O NH_4^+ como fonte de nitrogênio promove o efeito *priming* em plantas de sorgo, diferente de outras fontes nitrogenadas, induzindo mecanismos que condicionam as plantas a responderem de modo mais eficiente ao estresse salino.

3 OBJETIVOS

3.1 Objetivo geral

Verificar o papel da fonte de nitrogênio, em particular a nutrição com NH_4^+, na ativação de mecanismos que atenuem os danos induzidos pelo estresse salino, envolvendo o sistema antioxidativo, a modulação de metabólitos, bem como a manutenção da integridade e eficiência da maquinaria fotossintética de plantas de sorgo sob salinidade.

3.2 Objetivos específicos

Em plantas de sorgo, cv. CSF 20, foram avaliados os efeitos das diferentes fontes de nitrogênio (NO_3^-, NH_4^+ e mistura equimolar de NO_3^-:NH_4^+) e da salinidade (75 mM NaCl):

- nos parâmetros de crescimento (área foliar e massa seca);
- no conteúdo relativo de água, no vazamento de eletrólitos, bem como no potencial osmótico de folhas;
- nos parâmetros de trocas gasosas e de fluorescência da clorofila *a*, bem como nos teores dos pigmentos fotossintéticos (clorofilas e carotenoides);
- na ultraestrutura dos cloroplastos das células do mesofilo;
- na geração e localização de ROS nos tecidos foliares, através de microscopia confocal;

- nos teores de superóxido, peróxido de hidrogênio e peroxidação de lipídeos de membrana em extratos foliares;
- na ação dos mecanismos antioxidantes, em particular na atividade das enzimas dismutase do superóxido, peroxidase do ascorbato, catalase, peroxidase do guaiacol e redutase da glutationa;
- no perfil metabólico de folhas, especificamente nos metabolitos primários.

4 ESTRATÉGIA EXPERIMENTAL

Para testar a hipótese levantada, este estudo foi desenvolvido em três etapas consecutivas, nas quais plantas de sorgo foram cultivadas em soluções nutritivas, contendo três diferentes regimes, NO_3^-, NH_4^+ e a mistura equimolar NO_3^-:NH_4^+ (1:1), e mantidas na ausência (0 mM NaCl) ou na presença de estresse salino (75 mM NaCl). A **primeira** etapa teve o objetivo de investigar as implicações da fonte de nitrogênio na eficiência da maquinaria fotossintética das plantas de sorgo frente à salinidade, sendo mensurados os parâmetros de trocas gasosas, a fluorescência da clorofila *a*, os teores dos pigmentos fotossintetizantes, bem como as respostas relacionadas com o componente osmótico do estresse, conteúdo relativo de água e potencial osmótico foliar. A partir do melhor desempenho fotossintético e crescimento em plantas nutridas com NH_4^+ como única fonte de N, foi avaliado a ultraestrutura dos cloroplastos e a homeostase redox nos tecidos fotossintetizantes, o que constituiu a **segunda** etapa do estudo. Nessa ocasião, foram mensurados os danos oxidativos e os componentes enzimáticos do sistema antioxidante, bem como o acúmulo de ROS e sua localização *in situ* nos tecidos foliares. Esses resultados foram descritos com detalhes no **item 6**. Sugeriu-se então, verificar as mudanças no perfil metabólico relacionadas à fonte de nitrogênio e à salinidade, bem como compreender que metabólitos podem estar relacionados à tolerância ao estresse salino. Diante disso, na **terceira** etapa, foram obtidos os perfis metabólicos de extratos foliares através de cromatografia gasosa acoplada à espectrômetro de massas (GC-MS), com posterior comparação e análise dos diferentes perfis aplicando método de análise multivariada e análise do principal componente (PCA). Tais resultados foram apresentados no **item 7** desse trabalho.

5 FUNDAMENTAÇÃO TEÓRICA

5.1 Aspectos gerais dos efeitos da salinidade em plantas

A salinidade é um dos principais fatores abióticos que limitam a produção agrícola em razão dos seus efeitos no crescimento e no desenvolvimento das plantas. Em todo o mundo, cerca de 397 milhões de hectares são de solos salinos, compreendendo 20% das áreas cultivadas (FAO, 2019). São regiões áridas e semiáridas, comumente atingidas pela salinidade devido ao baixo índice pluviométrico e à elevada taxa de evapotranspiração na região, que favorecem o acúmulo de sais no solo. Além disso, a ação antrópica agrava o problema das áreas salinizadas devido ao uso de água de baixa qualidade e as más práticas de irrigação (BELTRÁN, 2016).

Na planta, o estresse salino pode causar distúrbios ao balanço hídrico, iônico e bioquímico, que podem ser de natureza osmótica e/ou iônica. O fator osmótico é observado logo nos primeiros minutos após exposição ao estresse, devido a redução do potencial osmótico da solução do solo, dificultando a absorção de água e nutrientes pelas raízes (DIAS *et al.*, 2016; MUNNS; TESTER, 2008). Enquanto que o fator iônico ocorrerá com a exposição prolongada ao estresse, resultando na absorção excessiva dos íons, principalmente Na^+ e Cl^-, que, ao serem acumulados em excesso no citosol, afetam vários processos bioquímicos, fisiológicos e moleculares (BERNSTEIN, 2019; NEGRÃO; SCHMÖCKEL; TESTER, 2017). Todos esses efeitos provocam alterações nos processos fisiológicos essenciais, podendo trazer consequências graves ao crescimento e desenvolvimento, incluindo a morte das plantas.

A redução do *status* hídrico da planta é decorrente do efeito do estresse osmótico induzido pela salinidade (HESSINI *et al.*, 2009; KHAYYAT *et al.*, 2014; PARIHAR *et al.*, 2015). A resultante perda de turgor celular leva à inibição do crescimento, causando reduções na área foliar e no acúmulo de massa seca da parte aérea, como observado em *Gossypium hirsutum* e *Eugenia myrtifolia* (ACOSTA-MOTOS *et al.*, 2015; ZHANG *et al.*, 2014). Por outro lado, algumas espécies são mais tolerantes ao componente osmótico da salinidade, sofrendo maiores danos decorrentes dos efeitos tóxicos do acúmulo dos íons em seus tecidos (KUMAR *et al.*, 2017). Em cevada (*Hordeum vulgare* L.), o aumento na concentração de Na^+ afetou a balanço de outros íons, como Ca^{2+}, K^+ e Mg^{2+}, devido os efeitos na absorção e toxicidade nos tecidos (MAHLOOJI *et al.*, 2017). Segundo os autores, a tolerância entre genótipos de cevada foi dependente da capacidade de manter baixo o conteúdo de Na^+ nos tecidos das plantas. Além dos efeitos no crescimento, a salinidade também afeta outros processos, provocando a inibição de enzimas durante a germinação (MARQUES *et al.*, 2013), redução da absorção de outros íons

essenciais (FREITAS *et al.*, 2019), decréscimos no conteúdo dos pigmentos fotossintéticos (TAÏBI *et al.*, 2016), além de distúrbios no processo fotossintético (ARAÚJO *et al.*, 2018) e alterações metabólicas (CHEN *et al.*, 2019).

Para lidar com os efeitos deletérios da salinidade, as plantas desenvolveram mecanismos fisiológicos e bioquímicos a fim de atingir a homeostase e evitar a toxicidade nas células, dentre os quais pode-se citar: exclusão de íons tóxicos dos tecidos, compartimentação destes íons no vacúolo e acúmulo de solutos compatíveis (MIRANDA *et al.*, 2013; MUNNS *et al.*, 2019; RANJIT; MANISH; PENNA, 2015). Em *Beta macrocarpa*, o acúmulo dos íons Na^+ e Cl^- nos vacúolos foi crucial para o aumento da tolerância à alta salinidade (HAMOUDA *et al.*, 2016). Para os autores, a compartimentalização iônica no interior dos vacúolos mitigou os efeitos tóxicos dos íons, bem como contribuiu para o ajustamento osmótico e balanço hídrico. Já para *Acacia auriculiformis*, o acúmulo de açúcares, aminoácidos livres e prolina foram determinantes na osmoproteção dos tecidos foliares e tolerância ao estresse salino (RAHMAN *et al.*, 2017). Tais mecanismos de tolerância também foram reportados em diferentes variedades de *Triticum aestivum* (WU *et al.*, 2015a), *Spinacia oleracea* (DI MARTINO *et al.*, 2003) e *Arachis hypogaea* L. (CHAKRABORTY *et al.*, 2016).

Como resultado dos distúrbios no metabolismo normal, o estresse salino também induz o estresse oxidativo, por meio da produção excessiva de ROS, tais como o peróxido de hidrogênio (H_2O_2), e os radicais livres superóxido ($^{\bullet}O_2^-$) e hidroxil (OH•). As ROS são altamente prejudiciais aos componentes celulares, pois promovem oxidação de biomoléculas e interferem nos processos metabólicos, como a fotossíntese (DE LIMA *et al.*, 2014; GAO *et al.*, 2015; HOSSAIN; DIETZ, 2016). Esses danos provocados pelo estresse oxidativo são comumente associados com elevado conteúdo de peroxidação de lipídeos de membrana e vazamento de eletrólitos, como reportado nas espécies *Zea mays* (GONG *et al.*, 2011), *Lycopersicon esculentum* (GONG *et al.*, 2013), *Panicum turgidum* (KOYRO *et al.*, 2013) e *Dianthus superbus* (MA *et al.*, 2017) em condições salinas.

As ROS são produzidas naturalmente pelo metabolismo celular, em diversos compartimentos celulares, como apoplasto, parede celular, peroxissomos, mitocôndrias e cloroplastos. A fuga de elétrons das cadeias transportadoras de elétrons para o oxigênio é a principal origem da formação das ROS, porém reações bioquímicas também podem contribuir na geração dessas moléculas (DEMIDCHIK, 2015; SHARMA *et al.*, 2012). Na literatura, ainda se discute o duplo papel das ROS no metabolismo da planta, seja como um efeito danoso

resultante do estresse, bem como sua função sinalizadora na tolerância a condições adversas (BAXTER; MITTLER; SUZUKI, 2014; LIM *et al.*, 2019; NOCTOR, REICHHELD, FOYER, 2018). Sob condições normais, em baixas concentrações, as ROS participam da sinalização de processos fisiológicos importantes para o crescimento e desenvolvimento do vegetal (DINAKAR *et al.*, 2010; PITZSCHKE; FORZANI; HIRT, 2006; RODRÍGUEZ; GRUNBERG; TALEISNIK, 2002). Dentre as ROS, apenas o H_2O_2 é capaz de atravessar membranas e ser transportado, atuando em outro sítio celular distante de onde foi produzido. Isto é possível devido suas características frente às demais espécies reativas, como superior meia-vida (1 ms) e moderada reatividade, favorecendo-o como molécula sinalizadora na comunicação célula-célula e entre organelas (SHARMA *et al.*, 2012). Estudos recentes apontam o papel sinalizador do H_2O_2 nas respostas ao estresse salino (ABDELGAWAD *et al.*, 2016; FREITAS *et al.*, 2018; GONDIM *et al.*, 2013). Portanto, o balanço entre a produção e desintoxicação de ROS é crucial na resposta da planta ao estresse, que para isso dispõem de mecanismos que neutralizam e regulam os níveis dessas moléculas no interior das células.

Os mecanismos antioxidantes podem ser classificados em dois tipos: mecanismos enzimáticos e não enzimáticos. O primeiro é composto pelas enzimas dismutase do superóxido, catalase, peroxidase do ascorbato e peroxidase do guaiacol (AHMAD *et al.*, 2019). Enquanto, o sistema antioxidante não enzimático é formado por compostos de baixa massa molecular, incluindo o ascorbato reduzido, a glutationa reduzida, os tocoferois e carotenoides (SHARMA *et al.*, 2012). Em *Brassica juncea*, o efetivo acionamento das enzimas antioxidantes foi um fator determinante para amenizar os danos provocados pela salinidade (MITTAL; KUMARI; SHARMA, 2012). Resposta semelhante foi obtida também em plantas de *Dianthus superbus* pré-tratadas com ácido salicílico a 0,5 mM, onde foi observado o aumento da atividade das enzimas antioxidantes, com consequente redução do acúmulo de ROS e dos danos de membrana (MA *et al.*, 2017). Srivastava *et al.* (2015) comparando duas espécies com diferente tolerância à salinidade (halófita e glicófita), observaram que além de ativarem as enzimas antioxidantes, as halófitas também apresentam enzimas mais robustas e mais estáveis em condições de salinidade em comparação com as de glicófitas. Além disso, os autores reportam que a maior eficiência do sistema antioxidante foi fator crucial para determinar o grau de tolerância entre as espécies estudadas.

As respostas das plantas ao estresse salino são bastante complexas e podem variar em função de uma série de fatores, desde aqueles relacionados ao estresse (concentração e composição dos sais, tempo de estresse), como os intrínsecos ao vegetal (espécie, genótipo,

estádio de desenvolvimento), bem como da interação entre eles (KHARE; KUMAR; KISHOR, 2015; SHOUKAT *et al.*, 2019).

5.2 Eficiência fotossintética de plantas C4 sob estresse salino

O crescimento da planta envolve processos bioquímicos, fisiológicos e moleculares, e dentre esses, a fotossíntese é o processo chave, capaz de converter energia luminosa em energia química utilizável no crescimento e desenvolvimento da planta. O processo fotossintético baseia-se em duas etapas de reações que ocorrem nos cloroplastos: a chamada "etapa fotoquímica", onde os elétrons retirados da oxidação da molécula de água são transferidos ao $NADP^+$ ao longo dos centros de reações dos fotossistemas (PSI e PSII, do inglês *photosystem*), resultando na produção de NADPH e ATP, sendo este último formado pela atividade dos complexos ATP sintase. Por fim, os produtos dessa etapa inicial são encaminhados à etapa bioquímica, localizada no estroma dos cloroplastos, onde ocorre a fixação do CO_2 em trioses fosfato no ciclo de Calvin-Benson (TAIZ *et al.*, 2015).

As plantas com metabolismo C4 são conhecidas por apresentarem melhor desempenho no balanço de carbono fixado por massa de água utilizada, promovendo uma melhor economia de água em relação às plantas que apresentam metabolismo C3 (HAMIM, 2005). Isto é favorecido pela capacidade de elevar a concentração de CO_2 disponível para a enzima rubisco (ribulose-1,5-bifosfato carboxilase/oxigenase), principal fixadora de CO_2. Em plantas C4, a reação de oxigenação da rubisco é evitada através da ação da enzima fosfoenolpiruvato carboxilase (PEPC), responsável pela carboxilação primária nas células do mesofilo (BRÄUTIGAM; GOWIK, 2016). Além disso, as folhas das plantas C4 apresentam uma anatomia diferenciada, denominada anatomia do tipo Kranz, em que se observa dois tipos de células fotossintéticas, as do mesofilo e aquelas da bainha vascular. Nas células do mesofilo ocorre a reação de fixação do CO_2 (HCO_3^-) pela PEPC, cujo produto de 4 C (malato ou aspartato) é transportado para as células da bainha com conseguinte descarboxilação e liberação de CO_2, onde predomina a reação de carboxilação da rubisco (GHANNOUM, 2009). Plantas de sorgo, cana-de-açúcar e milho são classificadas no subtipo NADP-ME (enzima málica dependente de NADP), cujo ácido de 4 C transportado é o malato.

Em condições ambientais adversas, como seca e salinidade, a fotossíntese é um dos processos mais rapidamente afetados, podendo sofrer alterações na ultraestrutura de organelas, na concentração de pigmentos, na atividade de enzimas e na regulação estomática (ASHRAF;

HARRIS, 2013). Durante o estresse salino, as mudanças nas relações hídricas juntamente com o efeito tóxico do acúmulo de íons no interior dos tecidos fotossintéticos têm implicações consideráveis para a atividade fotossintética das plantas (CHAVES; FLEXAS; PINHEIRO, 2009).

A limitação da abertura estomática constitui um dos primeiros efeitos da salinidade, que é decorrente do elemento osmótico (MUNNS; TESTER, 2008). Nessa condição, um sinal químico a partir das raízes pode induzir o fechamento estomático, reduzindo a difusão dos gases (ASHRAF; HARRIS, 2013). A limitação da entrada de CO_2 nos tecidos pode afetar negativamente a taxa de carboxilação, como observado em plantas de *Ocimum basilicum* tratadas com NaCl, em que a menor eficiência de carboxilação comparada com as plantas não estressadas foi atribuída à redução na condutância estomática (TARCHOUNE *et al.*, 2012). Para os autores, esse decréscimo na taxa da fotossíntese, não esteve relacionado com a perda da integridade dos fotossistemas de *O. basilicum*, uma vez que a eficiência máxima do PS II foi mantida inalterada.

De acordo com Ghannoum (2009), a redução na concentração interna de CO_2 resultante da restrição estomática tem sido considerada para explicar a limitação de CO_2 para a fotossíntese em plantas C4. Contudo, esta situação ocorre principalmente nos primeiros estágios do estresse, sendo a redução na capacidade fotossintética também relacionadas com limitações não estomáticas, como reduções na atividade de enzimas, reduções na produção de energia, danos estruturais, dentre outros (ASHRAF, HARRIS, 2013; GHANNOUM, 2009; WUNGRAMPHA *et al.*, 2018). Em *Phragmites karka*, Shoukat *et al.* (2019) reportaram que a taxa fotossintética foi reduzida inicialmente pela limitação estomática durante curta exposição ao estresse, sendo esses efeitos somados aos dos fatores não estomáticos com a exposição prolongada aos sais. Portanto, além da resposta estomática, a alta concentração de íons Na^+ e Cl^- nos tecidos afeta a capacidade fotossintética da planta através da inativação de enzimas (ASHRAF; HARRIS, 2013).

Estudos recentes mostraram significativas reduções no conteúdo e atividade da rubisco em plantas submetidas ao estresse salino (GALMÉS *et al.*, 2013; HE *et al.*, 2014). Da mesma forma, outras enzimas do metabolismo do carbono, como a PEPC, também sofreram efeitos negativos das altas concentrações de sais nos tecidos (BOUTHOUR *et al.*, 2012). Em plantas de milho estressadas com solução de NaCl à 3% durante 5 dias, além da limitação estomática, reduções na atividade de enzimas relacionadas ao processo fotossintético também

foram responsáveis pela restrição observada na fotossíntese (OMOTO; TANIGUCHI; MIYAKE, 2012). Neste estudo, Omoto, Taniguchi e Miyake (2012) observaram redução significativa na atividade e conteúdo da rubisco com a imposição do estresse salino, bem como decréscimo na atividade da enzima málica (dependente de NADP), esta última associada à regulação da razão $NADPH/NADP^+$, uma vez que o consumo de redutores foi prejudicado. Além do desbalanço consequente de interferências no funcionamento normal da etapa bioquímica da fotossíntese, a salinidade também pode provocar danos à maquinaria fotossintética através das alterações nos componentes da etapa fotoquímica.

O cloroplasto, sítio da fotossíntese, é uma organela muito propensa a danos oxidativos, não só a devido suas características químicas, mas também pela alta demanda por poder redutor e o excesso de energia na cadeia transportadora de elétrons (CTE) (ASADA, 2006). Assim, o comprometimento da etapa de carboxilação induzido pela salinidade, resulta no acúmulo de moléculas com poder redutor nas membranas dos tilacóides, excedendo as necessidades para as reações de carboxilação (SUZUKI *et al.*, 2012). Esse excesso de energia favorece a redução do oxigênio, com consequente geração de ROS em ambos os fotossistemas (I e II) (GARCÍA-CAPARROS; HASANUZZAMAN; LAO, 2019).

No interior dos cloroplastos, a síntese de ROS ocorre devido à fotoredução do oxigênio molecular em diferentes componentes da CTE. O oxigênio singleto ($^1O_2$) é produzido no fotossistema II a partir do estado tripleto da clorofila ($^3P680^*$), que é decorrente da carência de aceptores oxidados [$^3P680^* + {}^3O_2 \rightarrow {}^1P680 + {}^1O_2$] (FISCHER; HIDEG; KRIEGER-LISZKAY, 2013). Já o radical superóxido ($^{\bullet}O_2^-$), é formado no fotossistema I pela reação de Mehler [$2O_2 + 2Fd_{red} \longrightarrow 2{}^{\bullet}O_2^- + 2Fd_{ox}$] (MAKINO; MIYAKE; YOKOTA, 2002); enquanto que o H_2O_2 é produzido a partir da dismutação deste radical, através da atividade da enzima superóxido dismutase (SHARMA *et al.*, 2012). Como um dos mais reativos e sem local definido nos cloroplastos, o radical hidroxil (OH•) é gerado pelo processo descrito por Haber-Weiss [$^{\bullet}O_2^- + H_2O_2 \longrightarrow OH\bullet + OH^- + O_2$], envolvendo uma reação intermediária, chamada reação de Fenton [$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH\bullet$], sendo dependente da disponibilidade de ambos os substratos, $^{\bullet}O_2^-$ e H_2O_2 (SHARMA *et al.*, 2012). Dentre os componentes da CTE, o fotossistema II é um dos mais sensíveis ao estresse, cuja integridade é perdida principalmente devido à degradação da proteína D1, comprometendo o fluxo linear de energia ao longo da cadeia transportadora na membrana dos tilacóides. A taxa de regeneração da proteína D1, no centro de reação do fotossistema II, é influenciada tanto pelo controle de sua síntese quanto pelo acúmulo de ROS (MITTAL; KUMARI; SHARMA, 2012).

Os danos consequentes do estresse oxidativo comprometem o funcionamento dos fotossistemas principalmente por reduções na eficiência fotoquímica (KHATRI; RATHORE, 2019), mas também podem causar alterações na morfologia foliar e ultraestrutura dos cloroplastos (NAVARRO *et al.*, 2007; OMOTO *et al.*, 2013). Em quinoa (*Chenopodium quinoa*), a alta salinidade provocou a dilatação dos tilacóides, reduzindo seu empilhamento, bem como reduziu o número de grana e aumentou a quantidade de plastoglobulos (MANAA *et al.*, 2019). Neste estudo, os autores associaram a perda da integridade dos cloroplastos e o decréscimo do conteúdo de clorofilas com a redução na eficiência fotoquímica máxima (Fv/Fm), o que levou a menor eficiência do fotossistema II em comparação com as plantas não tratadas (MANAA *et al.*, 2019).

Mudanças estruturais nos cloroplastos induzidas pela salinidade também já foram reportadas em milho (OMOTO *et al.*, 2016), cevada (ZAHRA *et al.*, 2014) e *Sulla carnosa* (BEJAOUI *et al.*, 2016). Diante disso, os cloroplastos também apresentam mecanismos protetores contra as ROS, a fim de evitar ou minimizar os danos à sua estrutura e funcionamento. Apresentam um eficiente sistema enzimático antioxidativo, incluindo as enzimas SOD, APX, GR já mencionadas, além das enzimas redutase do monodesidroascorbato (MDHAR) e a redutase do desidroascorbato (DHAR) que completam o ciclo ascorbato-glutationa (FOYER; NOCTOR, 2011). Além disso, apresentam antioxidantes não enzimáticos, incluindo o ascorbato (ASC), a glutationa (GSH), os tocoferois e os carotenoides (SHARMA *et al.*, 2012).

5.3 Perfil metabólico e estresse salino

Nas últimas décadas, pesquisas envolvendo as "ômicas" vêm ganhando destaque por fornecerem uma visão abrangente em informações da célula, tecido ou organismo (ARBONA *et al.*, 2013; FAN *et al.*, 2018; FUKUSHIMA *et al.*, 2009; SATOU *et al.*, 2014). As abordagens mais utilizadas incluem a genômica, a transcriptômica, a proteômica e a metabolômica, sendo a interação entre elas uma estratégia de estudo para compreender a biologia de sistemas (RAZZAQ *et al.*, 2019). Dentre essas abordagens, a metabolômica envolve a identificação e a quantificação da composição química da amostra biológica, sendo as variações nos metabólitos um reflexo dos processos regulatórios nas células.

O perfil metabólico tem sido aplicado para diversas finalidades no estudo de plantas, incluindo diferenciação entre genótipos (SUN *et al.*, 2020), estudo do desenvolvimento (TEH *et al.*, 2013; ZHANG *et al.*, 2018), caracterização das variações induzidas por fatores externos (GRIESSER *et al.*, 2015; OBATA; FERNIE, 2012) e para avaliação da qualidade

genética de culturas (GAMBOA-BECERRA *et al.*, 2019). Dentre os métodos analíticos de estudo do perfil metabólico, a cromatografia gasosa acoplada à espectrometria de massa (GC-MS) é amplamente difundida por ser de fácil aplicação, ter boa reprodutibilidade e por promover uma eficiente separação dos compostos (BOWNE *et al.*, 2018). Portanto, os estudos metabolômicos ajudam na compreensão da regulação do metabolismo induzida pelo estresse abiótico, caracterizando os alvos moleculares envolvidos nas respostas ao estresse, particularmente ao estresse salino.

Diversos estudos reportaram alterações nos metabolitos primários, como aminoácidos, carboidratos, ácidos orgânicos, em resposta ao estresse salino (BATISTA *et al.*, 2019). No entanto, o metabolismo secundário também tem grande papel nas respostas à salinidade, principalmente as relacionadas aos mecanismos de tolerância ao estresse (BENJAMIN *et al.*, 2019). A regulação do perfil metabólico em plantas sob estresse salino já foi descrita para as espécies *Hordeum vulgare* (WIDODO *et al.*, 2009), *Oryza sativa* (ZHAO *et al.*, 2014), *Glycine soja* (JIAO *et al.*, 2018) e *Apocyni veneti* (CHEN *et al.*, 2019). Tais alterações metabólicas podem estar associadas com a tolerância à salinidade. Por exemplo em *Atriplex halimus*, a tolerância à salinidade foi associada com o acúmulo de prolina e sacarose, que podem funcionar como solutos compatíveis (BENDALY *et al.*, 2016). Ao comparar as respostas metabólicas de três cultivares de arroz (*Oryza sativa*), os metabólitos shikimato, quinato e malato, e os carboidratos trealose, rafinose e sacarose foram considerados marcadores de tolerância ao estresse salino (CHANG *et al.*, 2019). Os autores também destacaram o manitol como metabolito chave na tolerância ao estresse salino, por atuar como soluto compatível mitigando os efeitos da salinidade. Já Dias *et al.* (2015), ao confrontar dois cultivares de *Cicer arietinum* com diferente grau de tolerância ao estresse salino, reportaram que as principais diferenças metabólicas incluíram alterações em metabolitos ligados ao metabolismo do carbono e de aminoácidos, bem como com o ciclo do ácido tricarboxílico (TCA).

Recentes estudos têm demonstrado estratégias voltadas para induzir vias de defesa envolvendo mudanças metabólicas relacionadas a tolerância à salinidade. Em plantas de *Egletes viscosa*, o pré-tratamento com ácido salicílico atenuou os efeitos da salinidade através de alterações nos metabólitos primários, incluindo aumento no conteúdo de prolina, valina e alanina, bem como de inositol e sacarose (BATISTA *et al.*, 2019). Para os autores, esses metabólitos além de atuarem no ajustamento osmótico, também participam da manutenção da homeostase celular e na proteção contra o estresse oxidativo. O *halopriming* de sementes de *Cajanus cajan* promoveu um melhor desempenho das plântulas sob estresse salino, sendo

alterações metabólicas induzidas pelo estresse menores em comparação com aquelas de plântulas não pré-tratadas (BISWAS; BISWAS; DE, 2018). Muitos metabólitos têm sido considerados como críticos nas respostas à salinidade, os quais são também relacionados a mecanismos de tolerância ao estresse. No entanto, embora uns sejam bem conhecidos, a influência de outros e de suas respectivas vias metabólicas ainda carecem de estudos. Portanto, uma maior compreensão dessas interações e da relação desses metabólitos com a tolerância ao estresse pode auxiliar na seleção de espécies mais tolerantes à salinidade.

5.4 Papel da fonte de nitrogênio no aumento da tolerância à salinidade

O nitrogênio (N) é um macronutriente determinante para o crescimento e produtividade das plantas, por constituir biomoléculas essenciais, incluindo proteínas, clorofila, ATP, NAD(P)H e outros metabólitos (MASCLAUX-DAUBRESSE *et al*., 2010). Na solução do solo, este elemento encontra-se disponível para a planta nas formas NO_3^- e NH_4^+. Ao serem absorvidos pelas raízes, podem ser estocados nesses tecidos, assimilados em aminoácidos e/ou translocados para a parte aérea onde serão assimilados ou acumulados (BLOOM, 2015; TEGEDER; MASCLAUX-DAUBRESSE, 2018). Durante a assimilação, o NO_3^- é reduzido a NH_4^+ através das enzimas do metabolismo do nitrogênio. Primeiramente, NO_3^- é reduzido pela redutase do nitrato à nitrito, que por ser tóxico é rapidamente convertido à NH_4^+ no interior dos plastos. Por fim, o NH_4^+ é incorporado em aminoácidos, que podem ser translocados para as outras partes da planta (WANG *et al.*, 2012). Em contrapartida, a absorção direta de NH_4^+ pode ocorrer nas raízes através de canais de baixa afinidade (aquoporinas, canais de potássio) e transportadores de alta afinidade (AMT, *ammonium transportes*) (TEGEDER; MASCLAUX-DAUBRESSE, 2018), e a assimilação decorre da atividade da atividade das enzimas sintetase da glutamina e sintase do glutamato, que compõem a via GS-GOGAT, com produções de glutamato e glutamina, num processo com menor custo energético de absorção e assimilação comparado ao do NO_3^- (VEGA-MAS *et al.*, 2019). Além disso, a enzima desidrogenase do glutamato (GDH) também desempenha importante papel nas raízes com a assimilação direta do NH_4^+, principalmente em condições com alta concentração deste íon no meio de crescimento (SETIÉN *et al.*, 2014; VEGA-MAS *et al.*, 2019).

Em condições de estresse, como a salinidade, o adequado suprimento de N é determinante na sobrevivência e performance das plantas em resposta à condição adversa. Essa interação entre o metabolismo do N e o estresse salino tem sido estudado nas espécies *Brassica juncea* (IQBAL; UMAR; KHAN, 2015), *Glycine max* (GUO *et al.*, 2017), *Sorghum bicolor*

(MIRANDA *et al*., 2013, 2016, 2017) e *Solanum lycopersicum* (SINGH; SINGH; PRASAD, 2019). Em híbridos de *Populus,* o aumento no fornecimento de N esteve ligado à maior eficiência fotoquímica sob condições de salinidade, bem como a estabilidade das membranas dos tilacoides (WANG *et al.*, 2019). Diante das claras diferenças entre a assimilação das duas fontes, as plantas apresentaram preferência entre as formas nitrogenadas, principalmente ao NO_3^- devido os efeitos tóxicos do NH_4^+ no seu metabolismo (BITTSÁNSZKY *et al.*, 2015; BRITTO; KRONZUCKER, 2002). Entretanto, os resultados encontrados na literatura em plantas submetidas ao estresse salino são bastante controversos, pois, enquanto alguns relatos sugerem que o NO_3^- é determinante para a maior tolerância ao estresse, outros demonstram que a nutrição com amônio (NH_4^+) ou uma combinação entre as duas fontes é mais favorável para o crescimento das plantas sob salinidade.

Certas espécies como *Pisum sativum* (FRECHILLA *et al.*, 2001), *Helianthus annuus* e *Zea mays* (RIOS-GONZALEZ; ERDEI; LIPS, 2002), e *Populus simonii* (MENG *et al.*, 2016) apresentaram melhor performance sob condições salinas quando supridas com NO_3^-. Em plantas de *Gossypium hirsutum*, o melhor desempenho das plantas nutridas com NO_3^- comparadas àquelas nutridas com NH_4^+ foi associado com a maior assimilação de N e menor acúmulo de Na^+ nos tecidos, o que contribuiu para aliviar os efeitos do estresse salino (DAI; DUAN; DONG, 2015). No entanto, alguns estudos apontam que o ajustamento entre as fontes de N, como a substituição parcial de NO_3^- por NH_4^+, pode mitigar os efeitos da salinidade no crescimento de algumas espécies (BYBORDI, 2012; KANT *et al.*, 2007). Em *Catharanthus roseus* tratadas com 200 mM NaCl, o fornecimento da nutrição combinada forneceu maior resistência aos efeitos do estresse salino, que foi relacionada com maior atividade fotossintética, maior acúmulo de carboidratos e aminoácidos, bem como o menor acúmulo de Na^+ nos tecidos induzido pela presença do NH_4^+ no meio de crescimento (ZHONGHUA *et al.*, 2011).

Entretanto, algumas espécies apresentam elevada tolerância à nutrição com amônio, por acionarem mecanismos que evitam seus efeitos tóxicos nos tecidos (ESTEBAN *et al.*, 2016). Diante disso, a nutrição com NH_4^+ já mostrou ser determinante para à aclimatação ao estresse salino em plantas de *Carrizo citrange*, *Spartina alterniflora* e *Sorghum bicolor* (FERNÁNDEZ-CRESPO; CAMAÑES; GARCÍA-AGUSTÍN, 2012; HESSINI *et al.*, 2013; MIRANDA *et al.*, 2013, 2017). Os estudos forneceram indícios que o NH_4^+ pode influenciar em pelo menos dois mecanismos de aclimatação à salinidade: (1) na redução do acúmulo de íons tóxicos nos tecidos; e/ou (2) atuando como *priming* para o acionamento dos sistemas de defesa antioxidante e outras vias metabólicas. Em citrus, a nutrição com NH_4^+ não somente reduziu o acúmulo de íons Cl^-

nos tecidos fotossintéticos (FERNÁNDEZ-CRESPO; CAMAÑES; GARCÍA-AGUSTÍN, 2012), mas também ativou eficientemente os mecanismos antioxidantes enzimáticos e não enzimáticos, reduzindo os danos oxidativos ocasionados pelas ROS (FERNÁNDEZ-CRESPO *et al.*, 2014). De modo similar, a nutrição com NH_4^+ da halófita *S. alterniflora* intensificou o sistema de defesa antioxidante, reduzindo a peroxidação de lipídeos e o acúmulo de ROS, culminando na tolerância à salinidade extrema de NaCl a 500 mM (HESSINI *et al.*, 2013).

Em sorgo, pesquisas do nosso grupo de estudo evidenciaram que a nutrição com NH_4^+ reprogramou as repostas das plantas ao estresse salino (MIRANDA *et al*., 2013, 2014, 2016, 2017). Na presença de NaCl, plantas nutridas com NH_4^+ apresentaram maior acúmulo de compostos nitrogenados, o que coincidiu com o aumento nas taxas de assimilação de CO_2 (MIRANDA *et al*., 2016). Os autores sugeriram que o aumento da fotossíntese foi acionado para atender a demanda por esqueletos de carbono requeridos para a assimilação de nitrogênio. Em contrapartida, além de evitar o acúmulo do íon NH_4^+ em níveis tóxicos na célula, os aminoácidos exerceram papel importante no ajustamento osmótico das plantas expostas aos sais (MIRANDA *et al*., 2016). Mais recentemente, esses autores observaram que o controle do acúmulo de Na^+ mediado pelo NH_4^+ envolve uma regulação coordenada, nas raízes, da atividade e da expressão gênica das bombas P-ATPase e V-ATPase e transportadores SOS1 e NHX de membrana plasmática e de tonoplasto, culminando no controle do acúmulo e transporte de Na^+ na planta (MIRANDA *et al.*, 2017).

Baseado no exposto, a nutrição com NH_4^+ pode ser uma estratégia importante para o cultivo de plantas em ambientes com altas concentrações de sais, haja vista que o fornecimento desse íon como fonte de N economiza grande quantidade de energia. Tal economia de energia permitiria que mais recursos sejam direcionados para mecanismos de aclimatação ao estresse salino, culminando sobre a manutenção da maquinaria fotossintética que favoreceria a crescimento das plantas em condições salinas. No entanto, o papel do NH_4^+ nos mecanismos de regulação redox, na reprogramação dos metabólitos primários, bem como na integridade dos cloroplastos em plantas C4, ainda permanecem pouco claros

5.5 O sorgo

O sorgo (*Sorghum bicolor* L. Moench) é uma espécie anual, gramínea, com metabolismo fotossintético do tipo C4. É uma cultura relevante em todo o mundo, sendo as principais utilidades desta cultura voltadas para a nutrição de ruminantes (BORBA *et al*., 2012), a alimentação humana (MORAES *et al*., 2012) e a produção de etanol e açúcar (WHITFIELD;

CHINN; VEAL, 2012). O sorgo é o quinto cereal mais produzido no mundo, ficando atrás do milho (*Zea mays* L.), do trigo (*Triticum aestivum* L.), do arroz (*O. sativa* L.) e da cevada (*H. vulgare* L.) (FAO, 2019). No Brasil, a produtividade média de sorgo vem aumentando nos últimos anos e as projeções para a safra de 2019 foram de 2,5 milhões de toneladas, com crescimento de 10,2% em relação a 2018, sendo o estado de Goiás o maior produtor deste grão (IBGE, 2019).

Estudos prévios mostraram que plantas de *S. bicolor* quando cultivadas com NH_4^+, apresentaram manutenção na capacidade fotossintética, como também a nutrição com NH_4^+, quando comparada com outros regimes de nutrição nitrogenada, reduziu efetivamente o acúmulo de Na^+ nos tecidos e manteve inalterado o teor de K^+ sob condições de estresse salino (MIRANDA *et al.*, 2013; 2014). Contudo, o envolvimento da fonte de nitrogênio na manutenção da integridade estrutural do aparato fotossintético, seu efeito no acionamento do sistema antioxidante, bem como a regulação de metabolitos primários, principalmente ao interagir com o estresse salino, ainda permanecem desconhecidos.

6 AMMONIUM-MEDIATED ENHANCED PHOTOSYNTHETIC PERFORMANCE UNDER SALINITY IS RELATED TO IMPROVED K^+/Na^+ HOMEOSTASIS RATHER THAN ROS SCAVENGING IN SORGHUM PLANTS

(Artigo submetido no periódico *Photosynthesis Research*)

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Abstract

Salt stress is widely known to promote drastic reduction in growth and development of plants, mainly through the harmful effects on photosynthetic machinery. In this study, it was assessed the role of external nitrogen source against salinity-promoted damages on photosynthetic

apparatus of *Sorghum bicolor* L. The plants were grown in modified Hoagland's nutrient solutions with different N nutrition (NO_3^-, NH_4^+, and NO_3^-:NH_4^+) at 5 mM, and the assays were performed 12 days after 75 mM NaCl-stress imposition. Sorghum growth was severely decreased by salinity; however, the reduction of leaf area and shoot dry mass was less pronounced in NH_4^+-fed plants. Closely, NH_4^+ nutrition promoted an important decrease in leaf osmotic potential for osmotic adjustment purposes, associated with higher and lower accumulation of K^+ and Na^+ in plant tissues, respectively. In parallel, however, there were high H_2O_2 and malondialdehyde content in the plant, the NH_4^+ did not aggravate the effect of salt stress, exhibiting lower electrolyte leakage and mild injury in thylakoid structure in comparison to other N nutrition. Metabolic adjustment reflected in elevated photosynthetic performance, which was attributed to biochemical (improved CO_2 assimilation and rubisco carboxylation efficiency) and photochemical efficiency (with an increase in photochemical quenching and reduced energy excess at the PSII). Despite unchanged antioxidant enzymes activities, the results suggested that NH_4^+-induced H_2O_2 production acted in signaling events promoting low ion toxic accumulation, maintain photochemical efficiency and chloroplast integrity, which resulted in better performance under salt stress.

Keywords: Photosynthesis · Nitrogen nutrition · Salt stress · Oxidative stress · Ionic homeostasis

6.1 Introduction

Salinity stress imposes severe damages in plants occurring in two time-dependent phases. The first one is the ion-independent, happening within minutes or hours after salt stress imposition, resulting in osmotic shock and disturbing important plant processes like cellular expansion, stomatal conductance and then CO_2 assimilation (CHAVES; FLEXAS; PINHEIRO, 2009; NEGRÃO; SCHMÖCKEL; TESTER, 2017). The second phase occurs after long period (days to weeks) of salt exposure and may vary depending on the ion toxic concentration into the tissues (NEGRÃO; SCHMÖCKEL; TESTER, 2017; PARIHAR *et al.*, 2015). Excessive accumulation of toxic ions promotes damage in ionic homeostasis, cell constituents and photosynthetic process (ASHRAF; HARRIS, 2013; ISAYENKOV; MAATHUIS, 2019). These changes can lead to secondary effects, the oxidative stress, characterized by excessive reactive oxygen species (ROS) production and accumulation. ROS may injury nucleic acids, proteins,

membrane lipids, enzymes functioning and activate programmed cell death (AHMAD *et al.*, 2019; GILL; TUTEJA, 2010).

ROS production, such as the superoxide radical ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2), commonly occur under optimal conditions in different sites of plant cells, especially in organelles with high oxidant activity, such as chloroplast, mitochondria, and peroxisomes (DEMIDCHIK, 2015). It is known that the increase of ROS production is a clearly plant response to adverse conditions, such as drought (HESSINI *et al.*, 2017), salinity (GONDIM *et al.*, 2013) and others. Therefore, recent studies have shown that concept of ROS related to damage has changed, as its production might play a function in response to stressful condition (FREITAS *et al.*, 2018; HUANG *et al.*, 2019; SHARMA *et al.*, 2019); thus, the balance between ROS generation and scavenge is fundamental to plant response under abiotic stress. In cucumber, brassinosteroid-induced H_2O_2 generation has a key role in the increases of genes expression and salt stress tolerance, evidencing its central role in mediating stress responses (XIA *et al.*, 2009).

Salinity-induced oxidative stress can promote severe consequences to photosynthetic machinery, including degradation or decreased synthesis of photosynthetic pigments, complications in activity and content of enzymes, and photochemical efficiency (HE *et al.*, 2014; KHATRI; RATHORE, 2019; MITTAL; KUMARI; SHARMA, 2012). These oxidative damages are associated to increase in electrolyte leakage and lipid peroxidation in plants (KOYRO *et al.*, 2013; MA *et al.*, 2017). In addition, some studies have reported disruption of chloroplast integrity due to salt stress (GAO *et al.*, 2015; GOUSSI *et al.*, 2018; OMOTO *et al.*, 2013, 2016), whose ion toxic effect lead to unstacking of grana and swelling of thylakoids, contributing to impairment of photochemical efficiency and electron transport (KOTULA *et al.*, 2019; ZAHRA *et al.*, 2014). At the same time, to cope with oxidative stress, plants have an efficient set of antioxidants, enzymatic and non-enzymatic, including mainly the enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), guaiacol peroxidase (GPOD) and glutathione reductase (GR), and the antioxidant molecules, such as glutathione and ascorbate (GARCÍA-CAPARRÓS; HASANUZZAMAN; LAO, 2019). Many studies reveal that approaches in enhancing and triggering of antioxidant defense system are effective in the stress response and can confer salt tolerance for crops (GONDIM *et al.*, 2013; WU *et al.*, 2015b; YAN *et al.*, 2016).

Nitrogen (N) nutrition has showed to be an important strategy to mitigate deleterious effects of salinity (IQBAL; UMAR; KHAN, 2015; SINGH; SINGH; PRASAD, 2019), so that tolerance degree of plants depending on N form, nitrate (NO_3^-) and ammonium

(NH_4^+) (ASHRAF *et al.*, 2018). Studies report the effect of NO_3^- nutrition favoring growth and ion balance in *Pisum sativum* (FRECHILLA *et al.*, 2001), *Gossypium hirsutum* (DAI; DUAN; DONG, 2014) and *Brassica napus* (GAO *et al.*, 2016) under salinity; whereas sole NH_4^+ or the partial replacement of NO_3^- by NH_4^+ were more advantageous to other species, such as *Hordeum vulgare* (KANT *et al.*, 2007), *Catharanthus roseus* (ZHONGHUA *et al.*, 2011) and *Zea mays* (HESSINI *et al.*, 2019), suggesting that the preference of N form under environmental stress is species-dependent. In these terms, although NH_4^+ nutrition is a stressful condition for many plants, the positive role of NH_4^+ in the cross-tolerance is investigated (MARINO; MORAN, 2019). In *Spartina alterniflora* and *Carrizo citrange*, NH_4^+ nutrition led to the "acclimation stage", contributing to better responses to salt stress, by stimulating high antioxidant enzyme activity (FERNÁNDEZ-CRESPO *et al.*, 2014; HESSINI *et al.*, 2013). The effect of nitrogen available and different N sources in antioxidant defenses under salt stress have been reported in some species (MISRA; GUPTA, 2006; RIOS-GONZALEZ; ERDEI; LIPS, 2002; SINGH; SINGH; PRASAD, 2019).

Sorghum has emerged as a moderate-salt tolerant crop, displaying expressive response under NH_4^+ nutrition. Miranda *et al.* (2013, 2016, 2017) have shown that the salt-responses of *Sorghum bicolor* are multiple, highlighting that ionic homeostasis and osmotic adjustment; nevertheless, the beneficial effects of nitrogen nutrition in antioxidative defense and protection of photosynthetic apparatus of salt-stressed sorghum remain unclear. Thus, our working hypothesis was that external nitrogen source, mainly NH_4^+ nutrition, efficiently activates mechanisms to protect the photosynthetic apparatus and promotes salt tolerance in sorghum plants. Therefore, we assessed growth, photosynthesis, chloroplast integrity, photochemical efficiency, and production and scavenging ROS in leaves of sorghum under salt stress and different N sources.

6.2 Materials and Methods

6.2.1 Plant material and growth condition

Forage sorghum seeds [*Sorghum bicolor* (L.) Moench] cv. CSF 20, obtained from the Instituto Agronômico de Pernambuco (IPA, Pernambuco, Brazil), were superficially sterilized with 2% sodium hypochlorite and sown in vermiculite moistened with distilled water. After four days of sown, uniform seedlings were transferred to nutrient solution at 1/3 ionic strength. Hoagland's nutrient solution (HOAGLAND; ARNON, 1950) was modified to contain 5.0 mM nitrogen, supplied with the isolated forms of NO_3^-, NH_4^+ or NO_3^-:NH_4^+ ratio (1:1)

(TABLE 1). Salt treatments were imposed 12 days after sowing, by adding two daily doses of 37.5 mM NaCl until reach 75 mM NaCl. Nutrient solutions were renewed every three days, and the harvest occurred 12 days after the salt addition. During the experiments, environmental conditions were as following: midday photosynthetic photon flux density at approximately 1,200 μmol m^{-2} s^{-1}, mean temperature of 32.2 ± 2 °C during the day and 25.9 ± 1 °C at night and mean relative humidity of 63.4 ± 16%.

Table 1 - Components concentration of the modified nutrient solutions (based in Hoagland and Arnon, 1950), containing different proportions in the 5.0 mM inorganic nitrogen source

Nitrogen source	**NO_3^-**	**NO_3^-:NH_4^+**	**NH_4^+**
Stock solution	**Volume (mL) to 1 liter of nutrient solution**		
KCl (1.0 M)	0.75	0.75	0.75
$Ca(NO_3)_2.4H_2O$ (1.0 M)	2.50	-	-
NH_4NO_3 (1.0 M)	-	2.5	-
NH_4Cl (1.0 M)	-	-	4.50
$CaCl_2$ (1.0 M)	-	2.5	2.50
$MgSO_4.7H_2O$ (1.0 M)	0.35	0.35	0.35
NaH_2PO_4 (1.0 M)	0.25	0.25	-
$(NH_4)_2HPO_4$ (1.0 M)	-	-	0.25
Fe – EDTA (10 μM)	1.00	1.0	1.0
Micronutrients*	1.00	1.0	1.0
NaCl (1.0 M)**	75.0	75.0	75.0

*$CaCl_2$ a 50 μM, H_3BO_3 a 12.5 μM, $MnSO_4$ a 1 μM, $ZnSO_4.7H_2O$ a 1 μM, $CuSO_4$ a 0.5 μM e H_2MoO_4 a 0.1 μM.
** Solution applied only in saline condition
Source: made by the author

6.2.2 Growth parameters and relative water content

During the harvest, the leaf area was measured using a leaf area meter (LI-3100C, Li-Cor®). The plant material was divided into shoot and roots and dried in air circulation oven at 60 °C for 72 h to provide the shoot and root dry mass. The relative water content (RWC) was determined using ten leaf discs of fully expanded leaves. Briefly, after measure fresh mass (FM), the discs were immediately hydrated by floating for 6 h on deionized water to obtain turgid mass (TM) under room temperature. Then, the dry mass (DM) was obtained after drying in an oven at 60 °C for 48 h. The RWC was measured using the formula: $RWC\ (\%)=100\times(\frac{FM-DM}{TM-DM})$ (GONDIM *et al.*, 2013).

6.2.3 Leaf osmotic potential and electrolyte leakage

Leaf osmotic potential (Ψ_s) was measured in the last fully expanded leaf (0.1 g) using a vapor pressure osmometer (model 5600, Vapro®). The data were converted to MPa using the Van't Hoff equation: $\pi = (-R \times T \times Ci)$, where R is universal gas constant (0.00831 MPa kg mol^{-1} K^{-1}), T is absolute temperature (298 K) and Ci is the molar concentration of solute (mmol kg^{-1}) (BAO *et al.*, 2014).

Electrolyte leakage was determined in fully expanded leaves according to Dionisio-Sese and Tobita (1998). The samples were incubated in deionized water and the electrical conductivity (EC1) was measured after 24 h using an electrical conductivity meter (Mp513, Sanxin®). Then samples were incubated in water bath at 95 ºC, for 30 min, to provoke total destructive plasma membrane and measure electrical conductivity again (EC2). The EL was determined using the formula: $EL\ (\%) = 100 \times (\frac{EC1}{EC2})$.

6.2.4 Inorganic ions contents

Samples form dried leaf or root were incubated in deionized water at 45 °C for 1 h, and then centrifuged at 3,000 x g for 15 min. The supernatants were collected and used as crude extracts to determine the contents of K^+ and Na^+. Na^+ and K^+ contents were measured by flame photometry (model B462, Micronal®), according to Malavolta, Vitti and Oliveira (1989).

6.2.5 Gas exchange, chlorophyll fluorescence, and photosynthetic pigments

Gas exchange and chlorophyll fluorescence were measured in last fully expanded leaves using a portable photosynthesis system (IRGA, model Li-6400XT, Li-Cor®) with an artificial light source [photosynthetic photon flux density (PPFD = 1,200 µmol photon m^{-2} s^{-1})] and coupled to a fluorometer (model 6400-40, Li-Cor®). The CO_2 concentration in the chambers was approximately 380 µmol mol^{-1}. Values of CO_2 assimilation rate (*A*), stomatal conductance (g_s), intercellular CO_2 concentration (*Ci*), transpiration rate (*E*) and instantaneous carboxylation efficiency (*A/Ci*) were estimated from 9:00 to 11:00 h a.m. Chlorophyll fluorescence parameters were measured after 30 min in the dark, being assessed Fm (dark maximum fluorescence), Fo (dark minimum fluorescence), Fm' (light maximum fluorescence), Fs (light steady-state fluorescence) and Fo' (light minimum fluorescence after the far-red illumination). Then, the values were used to estimated effective quantum yield of PSII (ΦPSII), maximum quantum yield of PSII (Fv/Fm), photochemical [qP = (Fm' - Fs)/(Fm' - Fo')] and non-photochemical

[NPQ = (Fm - Fm')/Fm'] quenching, electron transport rate [ETR = ΦPSII × PPFD × 0.5 × 0.84] and relative energy excess at the PSII (EXC).

Concentrations of photosynthetic pigments were estimated as described by Wellburn (1994). Extracts were prepared incubating three leaf discs of fully expanded leaves in dimethyl sulfoxide (DMSO) saturated with calcium carbonate ($CaCO_3$). After 24 h in the dark, samples were incubated at 65 °C for 45 min and then subjected to absorbance readings at 665, 649 and 480 nm. Pigment contents were determined using the equations: Chlorophyll *a* = 12.47 (A_{665}) – 3.62 (A_{649}); Chlorophyll *b* = 25.06 (A_{649}) – 6.50 (A_{665}); Chlorophyll (*a* + *b*) = 7.15 (A_{665}) – 18.71 (A_{649}); Carotenoids = [1000 (A_{480}) – 1.29 (chlorophyll *a*) – 53.78 (chlorophyll *b*)]/220.

6.2.6 Ultrastructure of chloroplasts

Fully-expanded leaf sections (1-2 mm^2) were collected and fixed with 5% glutaraldehyde in 50 mM phosphate buffer (pH 7.2) and post-fixed in 2% (w/v) OsO_4 in the same buffer (OMOTO *et al.*, 2009). After dehydrated in a graded acetone series, the sections were infiltrated and embedded in epoxy resin (Embed 812) according to the methodology described by Yamane *et al.* (2012), with some modifications. Ultra-sections (70 nm) were obtained using an ultramicrotome (model UC7, Leica®) and contrasted using uranyl acetate and lead citrate. Then the sections were examined with a transmission electron microscopy (JEM 1011, JEOL®) at an accelerating voltage of 100 kV.

6.2.7 ROS production in leaves by confocal laser scanning microscope

Detection of ROS in freehand sections of sorghum leaves was performed as described by Freitas *et al.* (2018) with minor modifications. The sections were incubated on a solution of 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma Aldrich®) at 50 µM on 10 mM phosphate buffer (pH 7.0) for 30 min at dark. After forth washed with the same buffer, the sections were immediately observed under Ar laser (λ = 488 nm) by confocal laser scanning microscopy (CLSM) (LM 710, Zeiss®). Excitation signals were collected between 500 and 580 nm. The control images were performed only under HeNe laser (λ = 633 nm) related to chlorophyll *b* autofluorescence, producing excitation signals between 638 and 721 nm. Digital images were acquired using 20x magnification objective lens and serial images were reconstructed with Carl Zeiss LSM software.

6.2.8 Contents of $\bullet O_2^-$, H_2O_2 and lipid peroxidation

Crude extracts were prepared by homogenizing 0.2 g of fresh mass from leaves in 50 mM phosphate buffer (pH 7.8) (XU *et al.*, 2010) and in 0.1% (w/v) trichloroacetic acid (TCA), containing 5 mM KCN (CHEESEMAN, 2006), respectively, for superoxide radical ($\bullet O_2^-$) and hydrogen peroxide (H_2O_2) extraction. Spectrophotometric readings at 530 nm were carried out in supernatant to measure $\bullet O_2^-$ content using $NaNO_2$ solution as standard curve (ELSTNER; HEUPEL, 1976); whereas, H_2O_2 concentration was determined according to Sergiev, Alexieva and Karanov (1997), using a standard curve prepared with H_2O_2 solutions.

Lipid peroxidation was assayed by monitoring the absorbance of malondialdehyde (MDA) (HEATH; PACKER, 1968). Fresh leaves (0.2 g) were briefly ground in 5% TCA, at 4 °C. After centrifuged at 10,000 x g for 15 min, an aliquot of the supernatant was mixed with solution of 0.5% thiobarbituric acid (TBA) in 20% TCA and heated at 95°C in water bath for 30 min. MDA content was calculated through the difference between the absorbance at 532 and 600 nm, using the molar extinction coefficient of 155 mM^{-1} cm^{-1}.

6.2.9 Antioxidant enzymes

For antioxidant enzyme activities, 0.15 g of leaves was homogenized in 100 mM phosphate buffer (pH 7.0) with 0.1 mM ethylenediaminetetraacetic acid (EDTA) at 4°C. After centrifugation at 12,000 x *g*, at 4°C, the supernatant was collected and used to measure the enzyme activity. Protein concentration in the crude extracts was determined according to Bradford (1976), using bovine serum albumin as standard. Enzyme activities were assayed in a 96-well microplate reader (Synergy HTX, BioTek®), performing a kinetic activity during 10 min at 30 °C, except for superoxide dismutase (SOD; EC 1.15.1.1). Ascorbate peroxidase (APX; EC 1.11.1.11) activity was measured as described by Nakano and Asada (1981). The APX activity was calculated using the molar extinction coefficient for ascorbate (2.8 mM^{-1} cm^{-1}). Catalase (CAT; EC 1.11.1.6) activity was determined by consumption of H_2O_2 monitored at 240 nm as described for Beers and Sizer (1952), using the molar extinction coefficient (36 M^{-1} cm^{-1}). Guaiacol peroxidase (GPOD; EC 1.11.1.7) activity was measured according to Urbanek, Kuzniak-Gebarowska and Herka (1991), using the molar extinction coefficient for tetraguaiacol (26.6 mM^{-1} cm^{-1}). APX, CAT and GPOD activities were expressed as μmol H_2O_2 min^{-1} mg^{-1} protein. Glutathione reductase (GR; EC 1.6.4.2) activity was assayed according to Foyer and Halliwell (1976), monitoring oxidation of NADPH at 340 nm. GR activity was determined

using the molar extinction coefficient for NADPH (6.2 mM^{-1} cm^{-1}) and expressed as nmol NADPH min^{-1} mg^{-1} protein.

The SOD activity was determined according to Beyer and Fridovich (1987) by absorbance reading of blue formazan at 560 nm, produced by photoreduction of the nitroblue tetrazolium (NBT), after 15 min under light (two 20-W fluorescent tubes) at 25 °C. SOD activity was determined as the amount of enzyme required to cause 50% inhibition of the NBT photoreduction, it was expressed as enzyme unit (EU mg^{-1} of protein).

6.2.10 Experimental design and statistical analyses

The experimental design was a completely randomized, in a 3 × 2 factorial scheme, composed of three nitrogen sources (NO_3^-, NO_3^-:NH_4^+ and NH_4^+) and two salt levels [(0 mM and 75 mM NaCl], with five biological replicates. Data were subjected to ANOVA and the mean values were compared by Tukey's test ($p < 0.05$) using Sisvar 5.6 program.

6.3 Results

6.3.1 Plant growth parameters, hydric status, and membrane damage in leaves

Salinity promoted significant decreases in *S. bicolor* growth as compared to plants grown in non-saline condition (0 mM NaCl) (FIGURE 1, TABLE 2). Moreover, salt negative effects in dry mass and leaf area were influenced by N source. After 12 days of salinity, NH_4^+-fed stressed plants showed higher shoot dry mass than NO_3^-- and NO_3^-:NH_4^+-fed stressed plants, while the lowest leaf area was exhibited by plants grown with mixed N source (TABLE 2). Moreover, the highest root dry mass was observed on plants fed only NO_3^- under saline conditions.

Figure 1 - Sorghum plants cv. CSF 20 grown in the absence (0 mM NaCl) or presence of 75 mM NaCl under different nitrogen nutrition (NO_3^-, NO_3^-:NH_4^+ or NH_4^+) during 12 days of treatments. Source: made by the author.

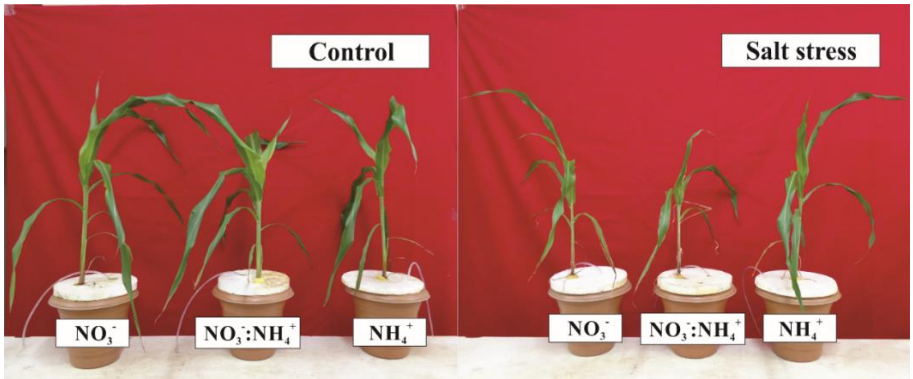


Table 2 - Leaf area, shoot and root dry masses, leaf osmotic potential and leaf electrolyte leakage of sorghum plants cv. CSF 20 grown in the absence (0 mM NaCl) or presence of 75 mM NaCl under different nitrogen nutrition (NO_3^-, NO_3^-:NH_4^+ or NH_4^+) during 12 days of treatments.

NaCl treatment	Nitrogen nutrition	Leaf area	Dry mass (g plant^{-1})		Osmotic potential	Electrolyte leakage
		(cm^2 plant^{-1})	Shoot	Root	(MPa)	(%)
0 mM	NO_3^-	524.8 ± 26.5^{Ba}	3.37 ± 0.07^{Aa}	0.85 ± 0.02^{Aa}	-0.80 ± 0.03^{Aa}	19.5 ± 1.0^{Ab}
	NO_3^-:NH_4^+	670.9 ± 39.4^{Aa}	3.63 ± 0.07^{Aa}	0.75 ± 0.03^{Ba}	-0.85 ± 0.03^{Aa}	16.5 ± 1.7^{Ab}
	NH_4^+	611.6± 48.4^{ABa}	3.36 ± 0.10^{Aa}	0.69 ± 0.01^{Ba}	-1.27 ± 0.05^{Ba}	21.7 ± 0.4^{Aa}
75 mM	NO_3^-	270.2 ± 17.9^{ABb}	1.79 ± 0.15^{Bb}	0.58 ± 0.02^{Ab}	-1.06 ± 0.04^{Ab}	27.6 ± 1.8^{Ba}
	NO_3^-:NH_4^+	198.7 ± 19.3^{Bb}	1.32 ± 0.05^{Cb}	0.50 ± 0.02^{Bb}	-1.13 ± 0.01^{Ab}	45.7 ± 4.6^{Aa}
	NH_4^+	379.0 ± 39.2^{Ab}	2.16 ± 0.10^{Ab}	0.50 ± 0.02^{Bb}	-1.40 ± 0.04^{Bb}	28.3 ± 2.2^{Ba}

Data are means ± SE of five repetitions. In the same NaCl treatment, different capital letters indicate significant differences due to nitrogen nutrition, whereas different lowercase letters denote significant differences due to NaCl concentration in the same nutrition, according to Tukey's test ($p < 0.05$). Source: made by the author.

Salt-stressed plants showed a decrease in Ψ_s as compared to those grown in NaCl absence, irrespective of external nitrogen source (TABLE 2). NH_4^+-fed plants displayed lowest Ψ_s values in conditions with and without salinity in comparison to others N nutrition. Unexpectedly, modulation of Ψ_s was not reflected in RWC, once no significant difference (mean of 89.27%) was observed in sorghum plants neither by salinity nor by nitrogen nutrition (data not shown). Sorghum leaves presented increased membrane damage (evidenced by electrolyte leakage) under 75 mM NaCl-stress in comparison to respective controls, except for NH_4^+-fed plants (TABLE 2). The highest membrane damage caused by salinity was exhibited in plants grown with mixed nutrition (NO_3^-:NH_4^+), displaying an electrolyte leakage increase of 177% in comparison to non-stressed plants.

6.3.2 Ion homeostasis in leaves and roots

Salt stress condition dramatically increased Na^+ content in leaves and roots of sorghum plants regardless of the nitrogen nutrition (TABLE 3). Furthermore, the lowest Na^+ contents in leaf and root under salinity were exhibit by NH_4^+-fed plants. An opposite response was observed for K^+ accumulation, in which salinity reduced the K^+ content, but the highest K^+ contents under salinity were found in leaves and roots of NH_4^+-fed plants. As result of ion accumulation, salt stress provoked a reduction in K^+/Na^+ ratio of sorghum tissues, with the more prominent effects in NO_3^--fed plants, showing a decrease of 92 and 80% in leaves and root, respectively (TABLE 3). Despite the reduction in K^+/Na^+ ratio, NH_4^+-fed plants showed higher K^+/Na^+ ratio than those from other nitrogen treatments.

Table 3 - Na^+ and K^+ contents, K^+/Na^+ ratio of leaves and roots of sorghum plants cv. CSF 20 grown in the absence (0 mM NaCl) or presence of 75 mM NaCl under different nitrogen nutrition (NO_3^-, NO_3^-:NH_4^+ or NH_4^+) during 12 days of treatments.

NaCl treatment	Nitrogen nutrition	Na^+content (µmol g^{-1} DM)		K^+content (µmol g^{-1} DM)		K^+/Na^+ratio	
		Leaf	Root	Leaf	Root	Leaf	Root
0 mM	NO_3^-	116.5 ± 3.3^{Ab}	378.3 ± 13.5^{Ab}	992.3 ± 17.7^{Aa}	726.3 ± 5.9^{Ba}	8.21 ± 0.19^{Aa}	1.97 ± 0.13^{Ca}
	NO_3^-:NH_4^+	120.0 ± 1.7^{Ab}	402.2 ± 12.9^{Ab}	920.7 ± 30.1^{Ba}	930.9 ± 19.6^{Aa}	7.97 ± 0.28^{Aa}	2.33 ± 0.13^{Ba}
	NH_4^+	114.8 ± 3.5^{Ab}	282.6 ± 14.8^{Bb}	838.9 ± 22.1^{Ca}	966.7 ± 22.7^{Aa}	7.32 ± 0.18^{Ba}	3.30 ± 0.10^{Aa}
75 mM	NO_3^-	578.3 ± 10.9^{Aa}	1182.6 ± 27.5^{Aa}	414.3 ± 14.7^{Bb}	455.2 ± 6.6^{Cb}	0.70 ± 0.02^{Bb}	0.40 ± 0.01^{Bb}
	NO_3^-:NH_4^+	513.0 ± 35.8^{Ba}	1034.8 ± 28.8^{Ba}	445.0 ± 8.9^{Bb}	698.2 ± 7.7^{Bb}	0.81 ± 0.07^{ABb}	0.66 ± 0.00^{ABb}
	NH_4^+	417.4 ± 17.4^{Ca}	900.0 ± 20.5^{Ca}	526.8 ± 8.9^{Ab}	754.5 ± 14.1^{Ab}	1.27 ± 0.04^{Ab}	0.82 ± 0.02^{Ab}

Data are means ± SE of five repetitions. In the same NaCl treatment, different capital letters indicate significant differences due to nitrogen nutrition, whereas different lowercase letters denote significant differences due to NaCl concentration in the same nutrition, according to Tukey's test ($p < 0.05$). Source: made by the author.

6.3.3 Modulation of the photosynthetic machinery

The net photosynthetic rate was decreased by 24.4% under salinity only in plants grown with NO_3^-:NH_4^+ nutrition (TABLE 4). In addition, the salt stress significantly reduced the g_s (↓47.4%), *Ci* (↓23.7%) and *E* (↓18.6%) only in NO_3^-:NH_4^+-fed plants. Also, under ammonium nutrition, sorghum plants showed higher values of Rubisco carboxylation efficiency (*A/Ci*) than plant from other nitrogen treatments, in both levels of NaCl (TABLE 4).

In salt absence, the NO_3^-:NH_4^+-fed plants showed higher effective quantum yield of PSII (ΦPSII), photochemical quenching (qP) and electron transport rate (ETR), and lower relative energy excess at the PSII (EXC) than those grown with the other N source (TABLE 5). Whereas, the salt stress decreased the ΦPSII, qP and ETR parameters, and increased EXC in plants grown with mixed regime as compared to non-saline condition. The salinity significantly reduced Fv/Fm and increased the NPQ of sorghum plants with exception for NH_4^+-fed plants, which exhibited no change in these parameters (TABLE 5). In addition, the EXC was decreased by salinity in sorghum plants supplied with NO_3^- (↓22.7%) or NH_4^+ (↓25.0%) as a sole nitrogen source.

Photosynthetic pigment content was similar in plants from all nitrogen treatments in absence salinity, except for chlorophyll *b* content in NO_3^--fed plants, whereas salt stress decreased all photosynthetic pigments of sorghum leaves when mixed regime (NO_3^-:NH_4^+) was supplied (FIGURE 2). In NH_4^+-fed plants, only chlorophyll *b* content was affected by salinity, with values found to be 17% lower than those of non-stressed plants (FIGURE 2b). In addition, NO_3^-:NH_4^+-fed plants exhibited lower chlorophyll *total* (*a* + *b*) than those grown with other N nutrition (FIGURE 2c), while there was no significant difference in carotenoids content among N sources under salinity (FIGURE 2d). Salt stress had no significant effect on photosynthetic pigments content of NO_3^--fed plants.

Table 4 - CO_2 assimilation rate (*A*), stomatal conductance (g_s), intercellular CO_2 concentration (*Ci*), transpiration rate (*E*) and instantaneous carboxylation efficiency (*A*/*Ci*) of sorghum plants cv. CSF 20 grown in the absence (0 mM NaCl) or presence of 75 mM NaCl under different nitrogen nutrition (NO_3^-, NO_3^-:NH_4^+ or NH_4^+) during 12 days of treatments.

NaCl treatment	Nitrogen nutrition	*A* ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	g_s ($mol\ H_2O\ m^{-2}\ s^{-1}$)	*Ci* ($\mu mol\ CO_2\ mol^{-1}$)	*E* ($mmol\ H_2O\ m^{-2}\ s^{-1}$)	*A/Ci* [($\mu mol\ m^{-2}\ s^{-1}$) ($\mu mol\ mol^{-1}$)$^{-1}$]
0 mM	NO_3^-	25.6 ± 1.9^{Aa}	0.21 ± 0.02^{Ba}	188.4 ± 10.7^{Bb}	5.96 ± 0.29^{Ca}	0.12 ± 0.01^{Ba}
	NO_3^-:NH_4^+	31.6 ± 2.4^{Aa}	0.38 ± 0.04^{Aa}	229.2 ± 4.5^{Aa}	10.21 ± 0.56^{Aa}	0.15 ± 0.01^{Ba}
	NH_4^+	30.0 ± 2.2^{Aa}	0.20 ± 0.02^{Ba}	112.6 ± 2.4^{Cb}	7.66 ± 0.38^{Bb}	0.27 ± 0.03^{Aa}
75 mM	NO_3^-	21.0 ± 0.6^{Ba}	0.22 ± 0.02^{Aa}	215.0 ± 7.0^{Aa}	6.40 ± 0.15^{Ba}	0.10 ± 0.00^{Ca}
	NO_3^-:NH_4^+	23.9 ± 2.3^{Bb}	0.20 ± 0.02^{Ab}	174.9 ± 9.5^{Bb}	8.31 ± 0.36^{Ab}	0.16 ± 0.01^{Ba}
	NH_4^+	32.4 ± 1.2^{Aa}	0.22 ± 0.02^{Aa}	136.7 ± 9.8^{Ca}	9.03 ± 0.43^{Aa}	0.25 ± 0.02^{Aa}

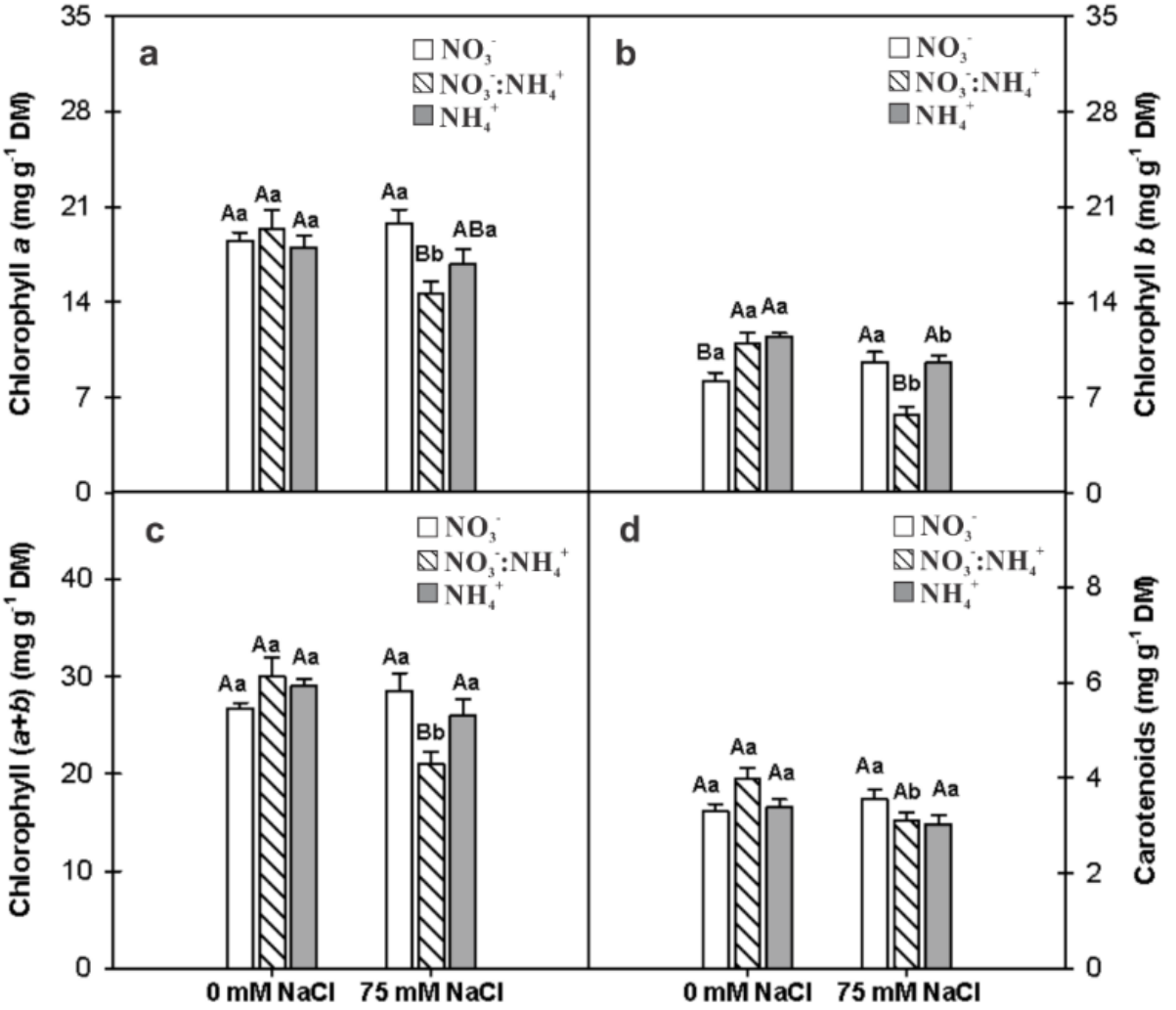
Data are means ± SE of five repetitions. In the same NaCl treatment, different capital letters indicate significant differences due to nutrition, whereas different lowercase letters denote significant differences due to NaCl concentration in the same nutrition, according to Tukey's test ($p < 0.05$). Source: made by the author.

Table 5 - Effective quantum yield of PSII (ΦPSII), maximum quantum yield of PSII (Fv/Fm), photochemical quenching (qP), electron transport rate (ETR), non-photochemical quenching (NPQ) and relative energy excess at the PSII level (EXC) of sorghum plants cv. CSF 20 grown in the absence (0 mM NaCl) or presence of 75 mM NaCl under different nitrogen nutrition (NO_3^-, NO_3^-:NH_4^+ or NH_4^+) during 12 days of treatments.

NaCl treatment	Nitrogen nutrition	Photochemical parameters					
		ΦPSII	Fv/Fm	qP	ETR	NPQ	EXC
0 mM	NO_3^-	0.44 ± 0.01^{Ba}	0.79 ± 0.00^{Aa}	0.71 ± 0.01^{Ba}	228.6 ± 4.0^{Ba}	0.57 ± 0.04^{Bb}	0.22 ± 0.01^{Aa}
	NO_3^-:NH_4^+	0.47 ± 0.01^{Aa}	0.77 ± 0.00^{ABa}	0.76 ± 0.01^{Aa}	249.2 ± 4.9^{Aa}	0.49 ± 0.02^{Bb}	0.16 ± 0.01^{Bb}
	NH_4^+	0.43 ± 0.01^{Ba}	0.76 ± 0.00^{Ba}	0.73 ± 0.00^{Bb}	225.5 ± 3.3^{Ba}	0.71 ± 0.04^{Aa}	0.20 ± 0.01^{Aa}
75 mM	NO_3^-	0.44 ± 0.00^{Aa}	0.76 ± 0.01^{Ab}	0.72 ± 0.00^{Ba}	229.5 ± 1.5^{ABa}	0.74 ± 0.02^{Aa}	0.17 ± 0.00^{ABb}
	NO_3^-:NH_4^+	0.41 ± 0.02^{Bb}	0.75 ± 0.00^{Ab}	0.73 ± 0.01^{Bb}	216.5 ± 8.2^{Bb}	0.74 ± 0.05^{Aa}	0.22 ± 0.02^{Aa}
	NH_4^+	0.45 ± 0.00^{Aa}	0.75 ± 0.00^{Aa}	0.76 ± 0.01^{Aa}	237.8 ± 2.1^{Aa}	0.71 ± 0.02^{Aa}	0.15 ± 0.00^{Bb}

Data are means ± SE of five repetitions. In the same NaCl treatment, different capital letters indicate significant differences due to nitrogen nutrition, whereas different lowercase letters denote significant differences due to NaCl concentration in the same nutrition, according to Tukey's test ($p < 0.05$). Source: made by the author.

Figure 2 - Photosynthetic pigments contents in leaves of sorghum plants cv. CSF 20 grown in the absence (0 mM NaCl) or presence of 75 mM NaCl under different nitrogen nutrition (NO_3^-, NO_3^-:NH_4^+ or NH_4^+) during 12 days of treatments. (a) Chlorophyll *a*, (b) chlorophyll *b*, (c) chlorophyll (*a* + *b*) and (d) carotenoids



Data are means ± SE of five repetitions. In the same NaCl treatment, different capital letters indicate significant differences due to nitrogen nutrition, whereas different lowercase letters denote significant differences due to NaCl concentration in the same nutrition, according to Tukey's test ($p < 0.05$). Source: made by the author.

6.3.4 Ultrastructure of chloroplasts

The role of nitrogen source on ultrastructure of chloroplasts from mesophyll cells of sorghum grown under salinity was investigated by transmission electron microscopy (FIGURE 3). Images of sorghum mesophyll cells revealed well-developed grana in all nitrogen treatments and similar presence of plastoglobuli in absence of salinity (FIGURE 3a, c, e). Moreover, there was a significant change in the ultrastructure of thylakoids and increase the number of plastoglobuli under 75 mM NaCl stress. Salt stress provoked the swelling in grana (arrow in FIGURE 3), resulting in wavy thylakoids (FIGURE 3b, d, f), with less conspicuous effect in NH_4^+-fed plants (FIGURE 3f).

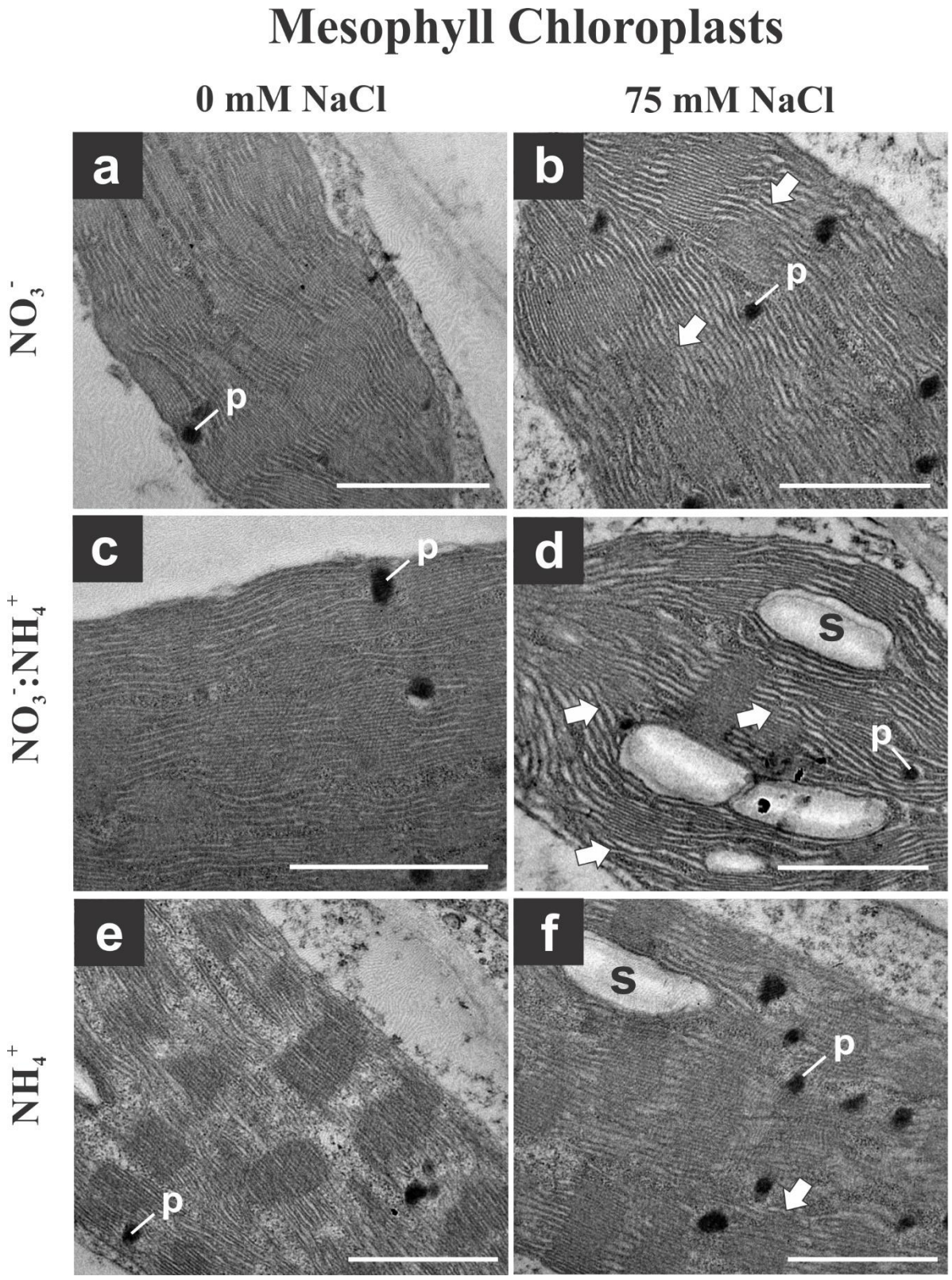
6.3.5 ROS production by confocal laser scanning microscopy

Herein, the probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) was employed to monitor the ROS production, mainly H_2O_2 (green colour), in leaf cells of sorghum plants subjected to different treatments (FIGURE 4). In absence of salinity, H_2O_2 production was detected mainly in mesophyll cells of sorghum plants from all nitrogen nutrition (FIGURE 4b, d, f), but a high fluorescence emission was found in NH_4^+-fed plants, implying that NH_4^+ nutrition promoted higher H_2O_2 synthesis than other nitrogen sources (FIGURE 4f). An over emission of green fluorescence was detected in leaves of plants under salt stress, in both mesophyll and bundle sheath cells (FIGURE 4c, e, g), especially in NO_3^-- and NH_4^+-fed plants (FIGURE 4).

6.3.6 Contents of $\bullet O_2^-$, H_2O_2 and lipid peroxidation

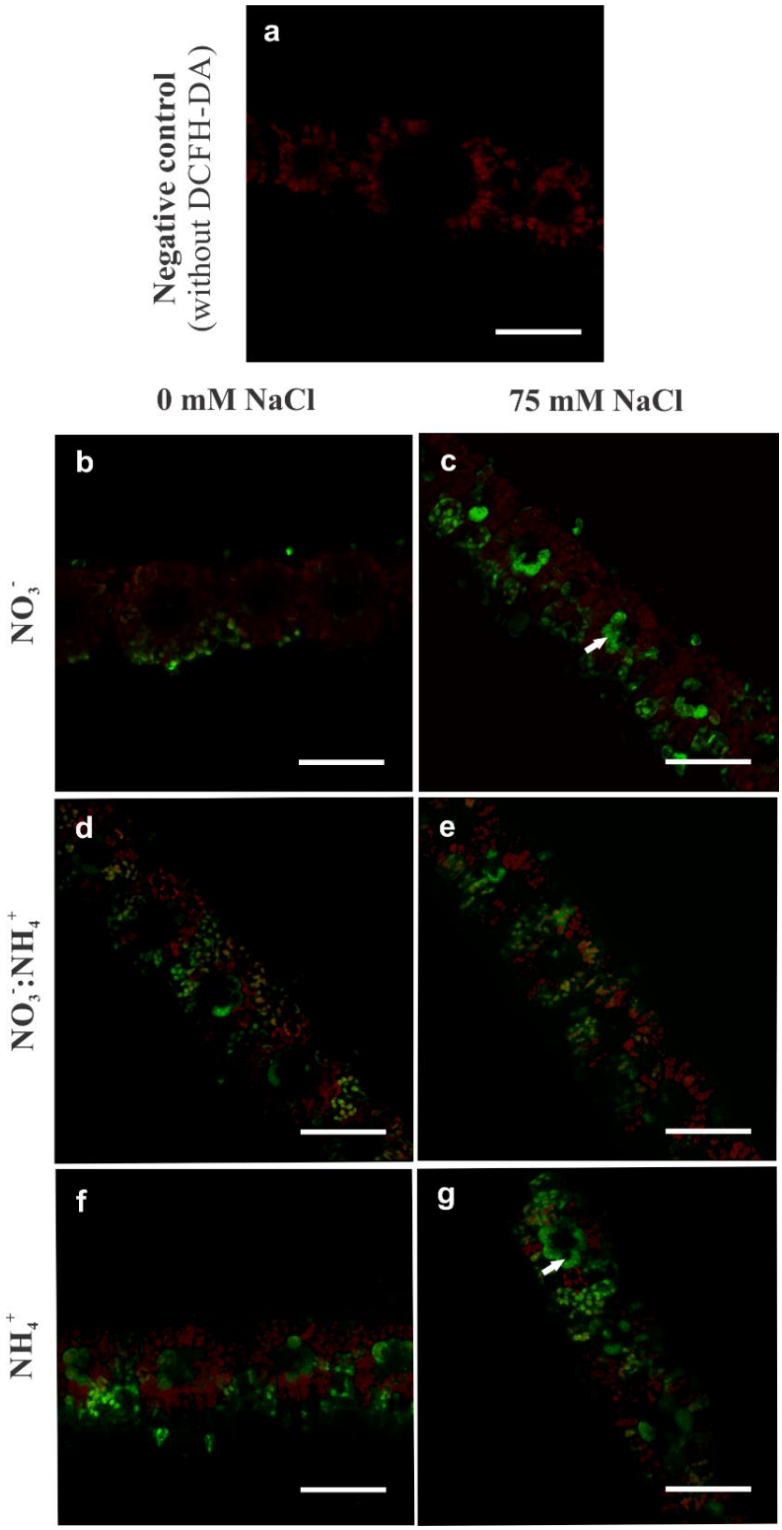
In general, under non-saline conditions, superoxide radical ($\bullet O_2^-$) production in leaves was not significantly influenced by nitrogen source (FIGURE 5a), while H_2O_2 production and membrane lipid peroxidation (estimated by MDA content) were greater in NH_4^+-fed plants than in NO_3^- and NO_3^-:NH_4^+-fed ones (FIGURE 5a, b). Salinity induced a slight increase in $\bullet O_2^-$ content of plants growing with NO_3^-:NH_4^+ (↑19%) and NH_4^+ (↑13%). Yet, H_2O_2 content was drastically increased by salt stress only in leaves from NO_3^- (↑70.5%) and NO_3^-:NH_4^+-fed (↑28.5%) plants; whereas leaf MDA content was increased by salinity in NO_3^-:NH_4^+-fed (↑51.0%) and NO_3^--fed (↑34.5%)stressed plants. Interestingly, NH_4^+-fed plants showed no significant alteration by NaCl stress in leaf H_2O_2 and MDA contents (FIGURE 5b, c).

Figure 3 - Transmission electron micrographs of chloroplasts from mesophyll of sorghum plants cv. CSF 20 grown with nitrogen nutrition (NO_3^-, NO_3^-:NH_4^+ or NH_4^+) and under different NaCl levels during 12 days. (a) NO_3^- + 0 mM NaCl; (b) NO_3^- + 75 mM NaCl; (c) NO_3^-:NH_4^+ + 0 mM NaCl; (d) NO_3^-:NH_4^+ + 75 mM NaCl, (e) NH_4^+ + 0 mM NaCl and (f) NH_4^+ + 75 mM NaCl.



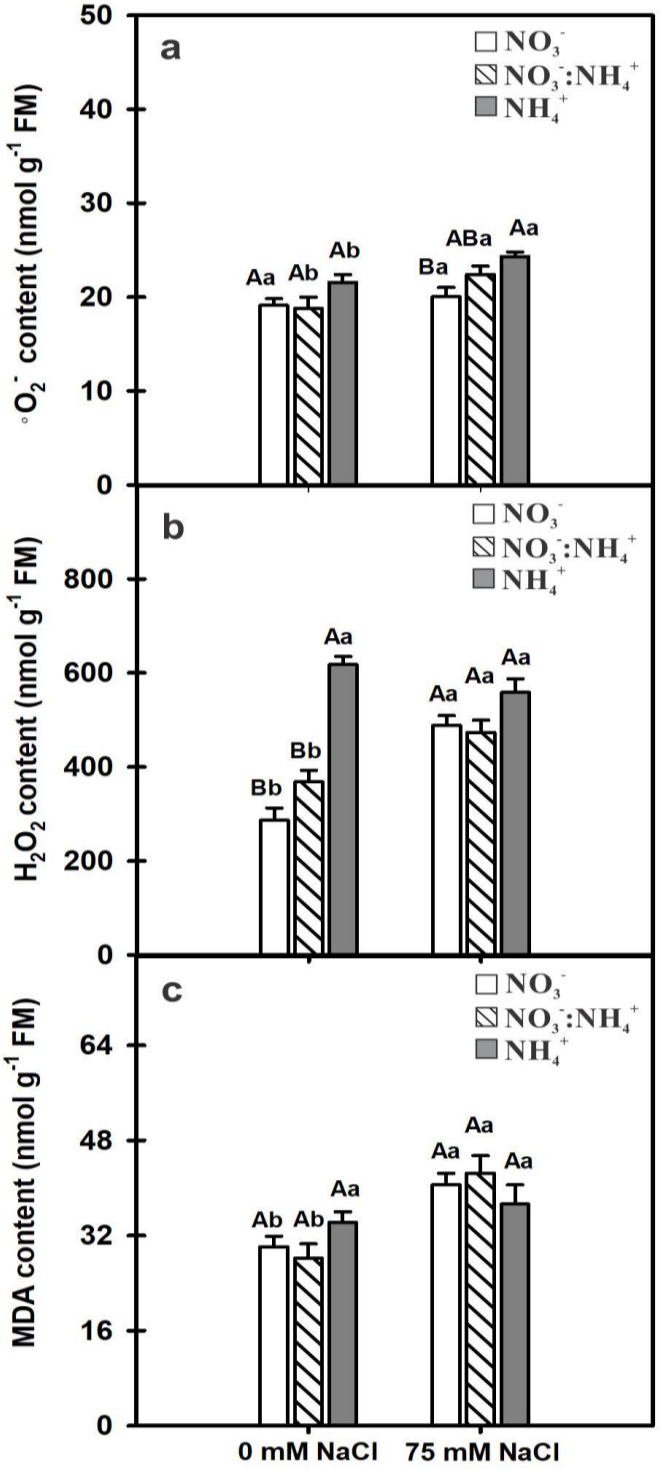
Arrows – Wavy thylakoids, p – Plastoglobule, S – Starch grain. Bar = 1 µm. Source: made by the author.

Figure 4 – Reactive oxygen species production (*in vivo* – green fluorescence) in leaves of sorghum plants cv. CSF 20 grown under different NaCl levels and nitrogen nutrition (NO_3^-, NO_3^-:NH_4^+ or NH_4^+) during 12 days. Leaf sections were obtained after incubation with 2',7'-dichlorofluorescein diacetate (DCFH-DA) using confocal laser scanning microscopy. (a) negative control, without DCFH-DA; (b) NO_3^- + 0 mM NaCl; (c) NO_3^- + 75 mM NaCl; (d) NO_3^-:NH_4^+ + 0 mM NaCl; (e) NO_3^-:NH_4^+ + 75 mM NaCl, (f) NH_4^+ + 0 mM NaCl and (g) NH_4^+ + 75 mM NaCl.



White arrow = H_2O_2 production in bundle sheath cell, and red color = chlorophyll *b* autofluorescence. Bar = 100µm. Source: made by the author.

Figure 5 - Superoxide radical ($\bullet O_2^-$, a), hydrogen peroxide (H_2O_2, b) and malondialdehyde (MDA, c) contents in leaves of sorghum plants cv. CSF 20 grown in the absence (0 mM NaCl) or presence of 75 mM NaCl under different nitrogen nutrition (NO_3^-, NO_3^-:NH_4^+ or NH_4^+) during 12 days of treatments.



Data are means ± SE of five repetitions. In the same NaCl treatment, different capital letters indicate significant differences due to nitrogen nutrition, whereas different lowercase letters denote significant differences due to NaCl concentration in the same nutrition, according to Tukey's test ($p < 0.05$). Source: made by the author.

6.3.7 ROS scavenging

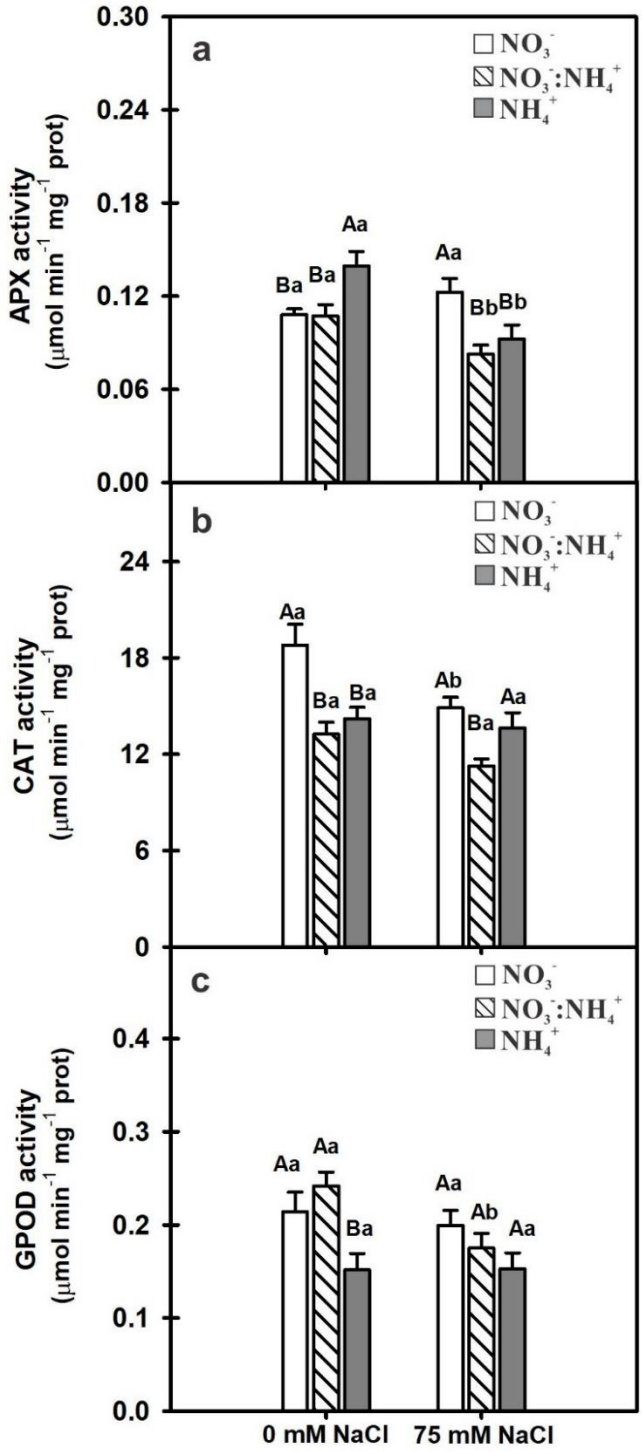
In NaCl absence, NH_4^+-fed plants displayed elevated APX activity and decreased CAT and GPOD activity in relation to plants grown with nitrate as well as for mixed N source with the exception of CAT activity which was similar to NH_4^+-fed plants (FIGURE 6). Unexpectedly, the activity of antioxidant enzymes remained unaltered or decreased in response to salt stress, irrespective of nitrogen nutrition. APX activity was reduced by 33 and 29% in leaves of stressed plants grown with NH_4^+ and mixed N source, respectively (FIGURE 6a). In a similar way, CAT activity was decreased by 21% in NO_3^--fed plants under NaCl stress (FIGURE 6b). Furthermore, only GPOD activity of NO_3^-:NH_4^+-fed plants was affected negatively (↓28%) by salt stress (FIGURE 6c). On the other hand, SOD and GR activities were not affected by salinity, exhibiting mean values of 28.65 (EU mg^{-1} protein) and 13.05 (nmol min^{-1} mg^{-1} protein), respectively (TABLE 6).

Table 6 - Antioxidant enzymes activity, superoxide dismutase (SOD) and glutathione reductase (GR), in sorghum plants grown in the absence (0 mM NaCl) or presence of 75 mM NaCl under different nitrogen nutrition (NO_3^-, NO_3^-:NH_4^+ or NH_4^+) for 12 days of treatments.

NaCl treatment	Nitrogen nutrition	SOD (EU mg^{-1} of protein)	GR (nmol min^{-1} mg^{-1} protein)
0 mM	NO_3^-	28.97 ± 2.32^{Aa}	13.51 ± 0.46^{Aa}
	NO_3^-:NH_4^+	25.01 ± 0.46^{Aa}	12.94 ± 0.28^{Aa}
	NH_4^+	30.19 ± 1.59^{Aa}	14.01 ± 0.86^{Aa}
75 mM	NO_3^-	31.27 ± 1.87^{Aa}	12.50 ± 0.61^{Aa}
	NO_3^-:NH_4^+	24.43 ± 0.55^{Ba}	11.72 ± 0.48^{Aa}
	NH_4^+	32.08 ± 2.87^{Aa}	13.66 ± 0.90^{Aa}

Data are means ± SE of five repetitions. In the same NaCl treatment, different capital letters indicate significant differences due to nitrogen nutrition, whereas different lowercase letters denote significant differences due to NaCl concentration in the same nutrition, according to Tukey's test ($p < 0.05$). Source: made by the author.

Figure 6 - Antioxidant enzymes activity in leaves of sorghum plants cv. CSF 20 grown in the absence (0 mM NaCl) or presence of 75 mM NaCl-stress under different nitrogen nutrition (NO_3^-, NO_3^-:NH_4^+ or NH_4^+) during 12 days of treatments. Ascorbate peroxidase (APX, a), catalase (CAT, b), guaiacol peroxidase (GPOD, c).



Data are means ± SE of five repetitions. In the same NaCl treatment, different capital letters indicate significant differences due to nitrogen nutrition, whereas different lowercase letters denote significant differences due to NaCl concentration in the same nutrition, according to Tukey's test ($p < 0.05$). Source: made by the author.

6.4 Discussion

Salinity is a serious environmental factor that negatively influences the growth, yield and physiological mechanisms of several plant species (NEGRÃO; SCHMÖCKEL; TESTER, 2017). Adjustments to the ratio of nitrogen sources have emerged as a promisor strategy to improve salt tolerance to some plant species, suggesting that plant species might activate defense mechanisms in order to withstand with deleterious salt effects (ASHRAF *et al.*, 2018). Herein, we provide evidence into how ammonium nutrition alleviates salt damage in photosynthetic machinery and increases salt tolerance of *S. bicolor* plants.

6.4.1 Ammonium-induced salt tolerance is associated with favorable ionic/osmotic homeostasis and CO_2 assimilation

Numerous studies have shown that the ability to water retention and turgor maintenance is determinant for leaf area expansion, a response closely related to capacity to mitigate the salt deleterious effects on plant growth (HNILIČKOVÁ; HNILIČKA; MARTINKOVÁ, 2017; MA *et al.*, 2012; NAVARRO *et al.*, 2007; PARIDA *et al.*, 2016;). In this study, imposition of 75 mM NaCl-stress reduced growth plant by decreasing leaf area, and shoot and root dry mass (TABLE 2). However, NH_4^+ nutrition was able to decrease Ψ_s of cells from sorghum leaves, which contributed to maintenance of water uptake and turgor, resulting in increased leaf expansion and leaf area under salinity. The low Ψ_s in NH_4^+-fed stressed plants was most likely due to a burst of free amino acids (mainly Gln, Asn, and Ser) in tissues, as previously reported by Di Martino *et al.* (2003) and Miranda *et al.* (2016).

In addition, modulation of Ψ_s can also be related to ion accumulation (like K^+) in tissues (CHAKRABORTY *et al.*, 2016; HAMOUDA *et al.*, 2016; WANG *et al.*, 2013). Herein, although K^+ content was decreased by salt stress in plants from all nitrogen treatments, NH_4^+-fed stressed plants showed elevated K^+ accumulation (in both shoot and roots) in comparison to NO_3^- and NO_3^-:NH_4^+-fed stressed ones, which might have contributed to lowest Ψ_s (TABLES 2, 3). In parallel to osmotic adjustment, NH_4^+ nutrition also promoted lower Na^+ accumulation in tissues of salt-stressed plants and, consequently, greater K^+/Na^+ ratio (TABLE 3). Our findings are in accordance with the studies reported for sorghum plants grown under different nitrogen sources (MIRANDA *et al.*, 2013; 2016; 2017). Therein, the authors provide strong evidence that NH_4^+ activates of Na^+ extrusion of plant cells by coordinate activity of SOS transporters and proton pumps in roots of sorghum plants, regulating the ionic homeostasis and improving salt tolerance. Moreover, this suggests that distinct NH_4^+ capacity in the reduction

of ions uptake and controlled translocation to leaves can promote salt-tolerance and favor the growth in plants, as observed in maize plants (HESSINI *et al.*, 2019).

Under salinity, the poor performance of sorghum plants supplied under mixed-N (NO_3^-:NH_4^+) nutrition may arise from water restriction associated with stomatal limitation and Ψ_s reduction, as well as the decline in CO_2 assimilation rate (TABLE 4). On the contrary, salt-stressed sorghum plants fed with NH_4^+ maintained stomatal conductance and CO_2 assimilation unaltered (TABLE 4). In previous studies with different plant species, numerous researchers highlighted that photosynthetic capacity and maintenance of CO_2 availability were used as physiological tools to determine the degree of salt-tolerance in plants (CHAVES; FLEXAS; PINHEIRO, 2009; MIRANDA *et al.*, 2016; NAJAR *et al.*, 2018). However, the CO_2 limitation commonly occurs in the early phases of osmotic stress, highlighting that photosynthesis activity can be reduced due to stomatal and/or biochemical limitations, such as inhibition of enzymes activity associated to CO_2 fixation (GHANNOUM, 2009; HE *et al.*, 2014; OMOTO; TANIGUCHI; MIYAKE, 2012).

6.4.2 Ammonium promotes priming phenomenon and maintains photosynthetic machinery efficiency for salt tolerance

Salt damage on biochemical phase of photosynthesis is frequently associated with injury to ultrastructure of chloroplasts, reduced photosynthetic pigments and metabolites, and impaired enzyme activities (ASHRAF; HARRIS 2013; MENG *et al.*, 2016; WUNGRAMPHA *et al.*, 2018). Here, in order to investigate the role of external N nutrition in photosynthetic machinery efficiency of sorghum plants subjected to salinity, the photosynthetic pigments, chlorophyll *a* fluorescence parameters, ultrastructure of chloroplasts and ROS production were examined (TABLE 5; FIGURES 2, 3, 4, 5). In NO_3^-:NH_4^+-fed stressed plants, the low CO_2 available (stimulated by reduced salt-induced stomatal conductance) (TABLE 4) occasioned an over reduction of electron transport chain, inducing an energy excess (↑EXC) along photosystem II (TABLE 4). Our results indicated that NO_3^-:NH_4^+-fed plants were not able to avoid photoinhibition, which was reflected in decreased Fv/Fm and qP (TABLE 5). In addition, reduced photochemical efficiency may be associated to damages on the structure of chloroplasts (SHEN *et al.*, 2019; ZAHRA *et al.*, 2014), as also observed in plants fed with mixed-N nutrition. This might be related to salt-induced ROS accumulation, resulting in enhanced lipid peroxidation as compared to non-saline condition, that suggest damages on chloroplast integrity (FIGURES 5b, c and 3d). Thus, the unsatisfactory functioning of PSII culminated in low ΦPSII, as evidenced by minor ETR (TABLE 5) (HUANG *et al.* 2014; QU *et al.*, 2012). Also, salt-

stressed plants grown with NO_3^-:NH_4^+ exhibited decrease in photosynthetic pigments (FIGURE 2), suggesting downregulation of biosynthetic pathways or improved catabolism (ASHRAF; HARRIS, 2013; HU *et al.*, 2016) most likely due to toxic effects of Na^+ overaccumulation in the leaves (TABLE 3) (NAJAR *et al.*, 2018).

Yet, under NO_3^- nutrition, restrictions for photosynthetic efficiency were not due to photochemical limitations originated from photoinhibition, once ΦPSII, qP, and ETR were unaltered by salinity (TABLES 4, 5). This suggests that Fv/Fm decline may be more related to increasing NPQ than possible damages to PSII reaction centers (GUIDI; LO PICCOLO; LANDI, 2019). Nevertheless, salinity-induced oxidative stress was marked by high H_2O_2 generation and accumulation, and enhanced lipid peroxidation (FIGURES 5b, c), that might result in the observed alterations on chloroplast integrity as compared to non-saline condition (FIGURES 3a, b). For all cases, salt-stressed sorghum plants fed with NO_3^- or NO_3^-:NH_4^+ displayed an increase in NPQ to improve thermal dissipation, trying to avoid photodamage in PSII and protecting, at least in part, the photosynthetic apparatus from excess energy under salinity. This phenomenon was also demonstrated in *Boehmeria nivea*, *Panicum turgidum* and barley plants under stressful conditions (ALLEL; BEN-AMAR; ABDELLY, 2018; HUANG *et al.*, 2014; KOYRO *et al.*, 2013).

In a different way, NH_4^+ nutrition was beneficial for sorghum plants under salinity, allowing elevated photosynthetic performance and growth under limiting conditions (TABLES 2, 4, 5). Surprisingly, under control conditions, NH_4^+ as solely nitrogen source promoted a greater accumulation of H_2O_2 (one ROS) (FIGURES 4f, 5b), resulting in a slightly damaged membrane (FIGURE 5c), without a drastic alteration in thylakoids membranes after salt stress (FIGURE 3e). These findings indicate that NH_4^+ promoted H_2O_2 generation fine-tuning for signaling events, which in turn acts as a second messenger activating downstream targets to cope with salt harmful effects (HUANG *et al.*, 2019; MITTLER, 2017). Concordantly, NH_4^+-fed sorghum plants neither present ROS overproduction nor suffer oxidative damage under salinity (FIGURES 4, 5), preserving the integrity of thylakoids (FIGURE 3f) and photosynthetic performance (TABLES 4, 5). The priming effect connecting H_2O_2 basal production was also reported in citrus and *Spartina alterniflora* plants, where NH_4^+ nutrition may act as a mild stressor, inducing cross-tolerance to salt stress (FERNÁNDEZ-CRESPO *et al.*, 2014; HESSINI *et al.*, 2013).

6.4.3 Antioxidant enzymatic system is not determinant for nitrogen-mediated salt tolerance in sorghum plants

The salinity-induced oxidative stress in plants may be assessed through markers of oxidative damage of cell membranes, reflecting in increase of lipid peroxidation (MDA content) and electrolyte leakage (ABDELGAWAD *et al.*, 2016). In this study, salt stress induced an increase of $\bullet O_2^-$ and H_2O_2 in leaves of NO_3^-:NH_4^+-fed plants as compared to control condition, which collaborated to extensive damages in chloroplast structure (FIGURES 3d and 5a, b) (OMOTO *et al.*, 2013), as evidenced by drastic increase in lipid peroxidation (FIGURE 5c) and electrolyte leakage (TABLE 2) (SINGH; SINGH; PRASAD, 2019). Furthermore, our results suggest that NO_3^-:NH_4^+-fed plants failed to activate antioxidant system for ROS scavenging (showed decreased or unchanged APX, GPOD and CAT activity) (FIGURE 6), as well exhibited lower SOD activity under salinity in comparison to other N sources (TABLE 6), resulting in elevated sensibility to NaCl-stress (SINGH; SINGH; PRASAD, 2019).

In *Arabidopsis*, the long-term NH_4^+ nutrition promoted oxidative damage by increasing ROS production linked to increase of respiratory metabolism, which results in excess of reducing power (PODGÓRSKA *et al.*, 2013). Therefore, more studies are necessary for understanding this mechanism and its function on retrograde signaling to salinity acclimate (FAROOQ *et al.*, 2019). In the current study, the superior performance of NH_4^+-fed sorghum plants subjected to salt stress was not closely related to functioning and the need for an enzymatic antioxidant system. Our findings indicate that the protection of photosynthetic machinery and performance triggered by ammonium is related to ionic homeostasis in leaves rather than N-induced antioxidant mechanisms.

6.5 Conclusion

Sorghum bicolor subjected to salinity exhibited better performance under NH_4^+ nutrition than other N nutrition. Increased photosynthetic performance is a result of (i) low ion toxic accumulation and (ii) prevention of increase in salt-induced ROS and lipid peroxidation content, that (iii) maintain photochemical efficiency and chloroplast integrity. Antioxidant enzymatic defense was not determinant for tolerance to salinity of sorghum plants. Further research is necessary to make clear the downstream targets (signaling pathways and metabolites) involved in regulation salt-tolerance of photosynthetic apparatus mediated by NH_4^+.

7 METABOLIC ANALYSIS OF LEAVES SORGHUM GROWN WITH DIFFERENT NITROGEN SOURCE REVEALS DISTINCTS ADAPTIVE RESPONSES TO SALT STRESS

(Artigo a ser submetido)

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Abstract

This study aimed to assess how the adjustment of N source (NO_3^-, NH_4^+ or mixed-N equimolar) influences the metabolic response to salt stress of sorghum plants, comparing metabolic profiles using gas chromatography-mass spectrometry. According to physiological results, NH_4^+-fed plants promoted higher CO_2 assimilation and dry mass accumulation in comparison to other N treatments under salinity. Metabolites regulation after salt stress was dependent of N source, producing distinct metabolic profile. The salinity up-regulated many amino acids in mixed-N nutrition, which was regarded an index of salt damages. Many sugars were increased by salt stress in all N nutrition. Moreover, only in NO_3^--fed plants, fumaric acid and citric acid, were decreased by salt stress, reflecting the salt effects in the energy metabolism. NH_4^+ nutrition promoted higher amino acids pool and antioxidants (ascorbic acid) in both saline conditions, which contributed to high tolerance to salinity in comparison with other N nutrition. Our findings provide new insights into metabolic changes to better understanding of complex interplay between N metabolism and salinity response.

Keywords: Ammonium · Salt tolerance · Asparagine · Organic acids · Metabolism

7.1 Introduction

Nitrogen (N) is a crucial element to plant metabolism, acting as structural component of diverse molecules, such as amino acids, proteins, nucleic acids. In soil solution, N is available as nitrate (NO_3^-) and ammonium (NH_4^+), which are absorbed by channels and

transporters in roots and assimilated into amino acids in plant cells (MASCLAUX-DAUBRESSE *et al.*, 2010).

The majority of plant species prefer NO_3^- or mixed-N (NO_3^-:NH_4^+) nutrition, once NH_4^+ nutrition can disturb plant growth and metabolism (BRITTO; KRONZUCKER, 2002; BITTSÁNSZKY *et al.*, 2015). However, some species showed tolerance mechanism that allowing growth under NH_4^+ nutrition (ESTEBAN *et al.*, 2016), highlighting the role of N forms as benefit strategy to improve tolerance to abiotic stress in plants, mainly salinity (MIRANDA *et al.*, 2014; IQBAL, UMAR, KHAN, 2015; FERNÁNDEZ-CRESPO *et al.*, 2014; HESSINI *et al.*, 2017). For example, *Populus simonii* had better performance against salt stress under NO_3^- nutrition (MENG *et al.*, 2016). However, some studies reported that partial NH_4^+ addition in the growth medium is more efficient to alleviated salinity damages compared to sole N form application (KANT *et al.*, 2007; ZHONGHUA *et al.*, 2011). As emergent application, the contribution of sole NH_4^+ nutrition was reported to *Spartina alterniflora* and *Citrus sinensis* under salt stress, suggesting that physiologic and metabolic changes promoted by NH_4^+-induced mild stress may effectively trigger salt tolerance responses in plants (HESSINI *et al.*, 2013; FERNÁNDEZ-CRESPO, CAMAÑES, GARCÍA-AGUSTÍN, 2012).

Salt stress is widely cited as a severe stress that affects the growth and development of plants, imposing both osmotic complications associated with reduction of plant water status, and ionic effect, involving the ion toxic concentration into the tissues (PARIHAR *et al.*, 2015, NEGRÃO; SCHÖCKEL; TESTER, 2017). This growth reduction is a phenotype trait, commonly associated to impairment in the CO_2 assimilation net (ASHRAF; HARRIS 2013; MAHLOOJI *et al*., 2017). In addition, disturbed ionic homeostasis, impaired metabolism, oxidative stress and nutritional imbalance were also reported in many species under salt stress (KHARE, KUMAR, KISHOR, 2015; SHAO *et al.*, 2015; ALLEL, BEN-AMAR, ABDELLY, 2018; ALAM *et al.*, 2019). To cope with deleterious effects of stress, diverse strategies are triggered by plants. For example, the Na^+ exclusion from roots in *Sorghum bicolor* (MIRANDA *et al*., 2017), accumulation of osmolytes for osmotic adjustment by *Nerium oleander* plants (KUMAR *et al.*, 2017), enhancement of antioxidant system as observed in *Oryza sativa* (KHARE, KUMAR, KISHOR, 2015). The plant response to salinity is very complex, being the phenotypic differences a result of the interaction of different elements with stress, such as transcriptomic changes, proteomic variation and metabolic alteration (FORMENTIN *et al.*, 2018; JIA *et al.*, 2019).

The metabolomic studies recently gained attention as they allow an overview of complex metabolism, improving the understanding of the metabolic pathway regulation and alterations in key metabolites under saline conditions (PARIDA *et al.*, 2018). Recently metabolomics studies report the salt impact on metabolism of plants, such as alteration in pathways involving energy metabolism, sugar metabolism, biosynthesis of phytohormones and amino acids profile (DIAS *et al.*, 2015; GUPTA; DE, 2017; BENJAMIN *et al.*, 2019; CHEN *et al.*, 2019). Based on the changes in the organic acids, amino acids and sugar, different *Oryza sativa* cultivars could be compared and the degree of salt tolerance among them was observed (CHANG *et al.*, 2019). For the authors, the mannitol and trehalose were considered the key metabolites, being accumulations of them associated to salt tolerance in *O. sativa*. There is many information using metabolomic approach to provide the plant responses to salinity, as well as the impact of N nutrition on the plant metabolism (BEATTY *et al.*, 2016; ZHAO *et al.*, 2019). However, the effect of the N nutrition, specifically N source, on the plant metabolic profile under saline condition remain unclear. The assessment of the changes in metabolites induced by N source may provide insight of plant molecular and physiologic responses to nutrition that may improve the tolerance response to salt stress.

Researches demonstrated that *Sorghum bicolor* L. showed different degree of tolerance depending on nitrogen regime when applied under salt stress (MIRANDA *et al.*, 2013; 2016). Herein, sorghum was employed as a model species to test the hypothesis that N source promotes punctual metabolite changes to alleviate the harmful effects of secondary stress imposed by salt stress, resulting in elevated salt tolerance in sorghum plants. We compared the metabolite profile from leaves of sorghum plants grown with different N source (NO_3^-, NH_4^+ and mixed NO_3^-:NH_4^+) under absence and presence of salt stress, following GC-MS based metabolomics approach, searching biomarkers of salt responses.

7.2 Materials and Methods

7.2.1 Plant material and growth conditions

Sorghum seeds [*Sorghum bicolor* (L.) Moench], cv. CSF 20, obtained from Instituto Agronômico de Pernambuco (IPA, Pernambuco, Brazil), were sown in vermiculite moistened with distilled water during four days. Then, uniform seedlings were transferred to hydroponic system with modified Hoagland's nutrient solution (HOAGLAND; ARNON, 1950) at 1/3 ionic strength, containing N at 5.0 mM with different N source [isolated forms of NO_3^-, NH_4^+ or mixed NO_3^-:NH_4^+ (1:1)]. Salt stress was imposed after 12 days of germination, consisting of 0

(control) and 75 mM NaCl (salt stress) for each N nutrition. Nutrient solutions were renewed every three days, and pH was adjusted daily to 6.0 using HCl or NaOH at 1 mM. Plants were grown in greenhouse under the following environmental conditions: midday PPFD at approximately 1,200 μmol m^{-2} s^{-1}, with a mean temperature of 32.2 ± 2 °C during the day and 25.9 ± 1 °C at night, and mean relative humidity of 63.4 ± 16%. The plant material was harvested 12 days after the salt addition, using four replicates per treatment.

7.2.2 Physiological traits and NO_3^- and NH_4^+ content in leaves

Before the harvest, gas exchange parameters [net photosynthetic rate (*A*) and stomatal conductance (g_s)] were measured in first fully-expanded leaf from 9:00 to 11:00 h using a portable photosynthesis system (IRGA, model Li-6400XT, Li-Cor®) with an artificial light source (PPFD = 1,200 μmol m^{-2} s^{-1}) and coupled to a fluorometer (model 6400-40, Li-Cor®). The CO_2 concentration in the chambers was approximately 380 μmol mol^{-1}. The total dry mass (shoot and root) was obtained after the whole-plant was oven-dried at 60 °C for three days.

Dried leaf powder (0.1 g) was incubated in deionized water at 45 °C for 1 h and then centrifuged at 3,000 x g for 15 min to obtain crude extracts. The supernatant was saved and used to measure NO_3^- and NH_4^+ contents. NO_3^- content was measured as described by Cataldo *et al.* (1975) using absorbance reading at 410 nm and standard curve of KNO_3 solution. Also, NH_4^+ content was estimated at 625 nm by spectrophotometry using $(NH_4)_2SO_4$ solution as standard (WEATHERBURN, 1967).

7.2.3 Metabolite profile by GC-MS and data analysis

Leaf samples were collected from the same leaf type used for gas exchange measurements at 10:00 to 11:00 h. Plant material was immediately frozen in liquid nitrogen and stored at -80 °C until processing. The extracts of polar metabolites were developed as described by Lisec *et al.* (2006) with minor modifications. Fifty milligrams of powdered sample were extracted with solution containing methanol, chloroform and ultrapure water (2:1:2, v/v). Aliquot of 150 μL of the upper water-methanol (polar) phase was transferred to a new tube and dried in a vacuum concentrator at room temperature. For derivatization step, the dried samples were treated with methoxylamine hydrochloride (20 mg mL^{-1}) in anhydrous pyridine with shake at 37 °C for 2 h, followed by the addition of N-methyl-N-(trimethylsilyl)- trifluoro acetamide (MSTFA) with shake at 37 °C for 30 min.

The profile of metabolites was obtained using gas chromatography coupled to mass spectrometry (GC-MS, QP-PLUS 2010, Shimadzu, Japan). One microliter sample was injected in split mode (1:5 ratio). Helium was used as the carrier gas with a flow rate of 1.2 mL min^{-1}. The RTX-5MS capillary column (30 m × 0.25 mm × 0.25 um) was used to separate metabolites, being programed with initial temperature at 80 °C for 2 min, then ramped of 10 °C per min to 315 °C, and held for 8 min. The injection and ion source temperature were kept at 250 °C and the MS interface temperature was set at 230 °C. The mass spectrometer was operated at 70 eV (EI) and used scan range of 40-700 (*m/z*), initiated after solvent cut time of 3 min. Both chromatogram and mass spectra analysis were evaluated using Xcalibur™ 2.1 software, being compared with mass spectrum of library. The files with relative concentration of metabolites for leaves tissues of sorghum under control and salt stress conditions and grown with different N nutrition (NO_3^-, NH_4^+ or NO_3^-:NH_4^+) after 12 days were uploaded to the MetaboAnalyst 4.0 server (http://www.metaboanalyst.ca) for posterior analysis.

7.2.4 Experimental design and statistical analyses

The experimental design was completely randomized with three nitrogen nutrition (NO_3^-, NO_3^-:NH_4^+ e NH_4^+) and two salt levels (0 and 75 mM NaCl), corresponding to factorial scheme 3 x 2, with four replicates. For physiological traits and ion content measurements, the mean values were compared by Tukey's test ($p < 0.05$), using Sisvar 5.6 program.

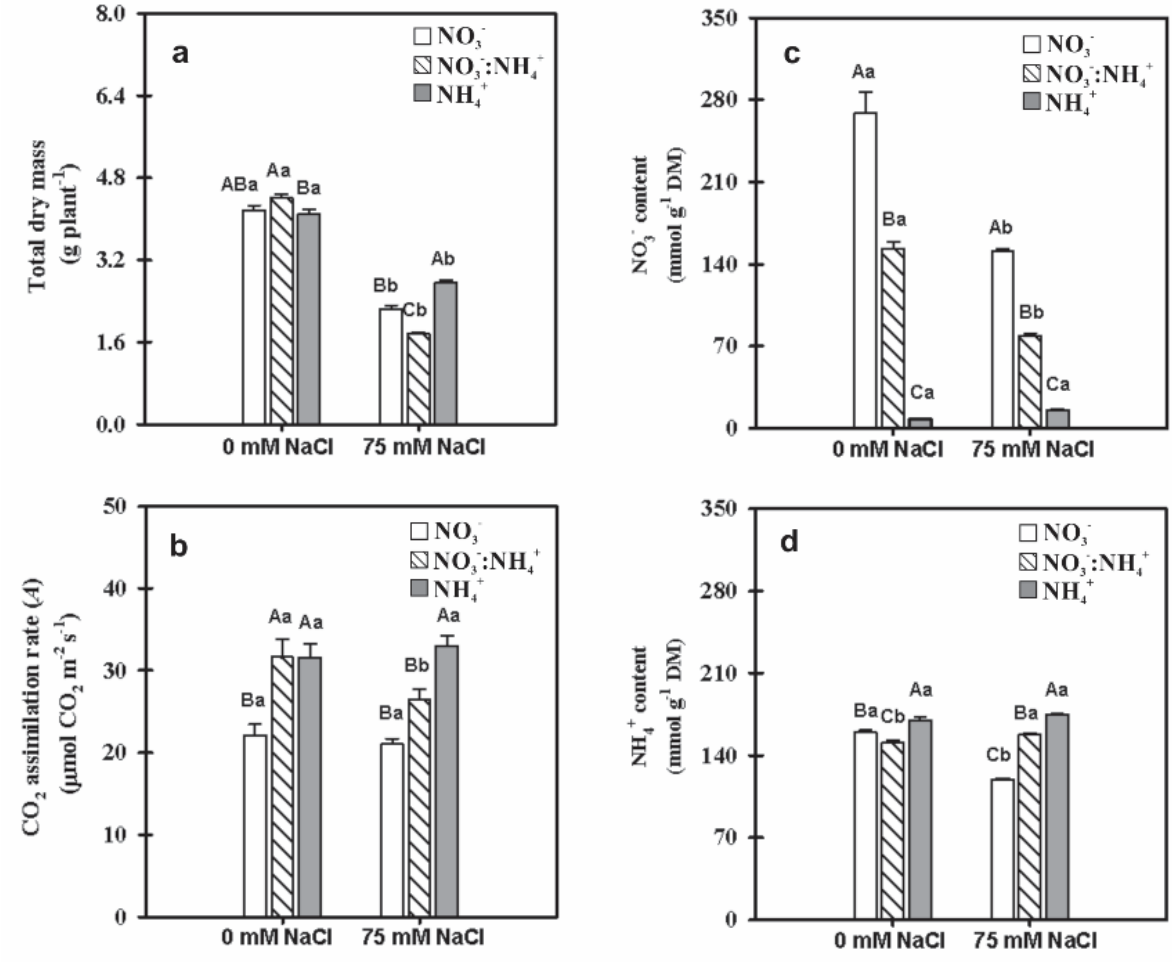
For metabolic analysis, the data were normalized to the internal standard (ribitol) in each chromatogram, and the sample fresh mass, being expressed as the relative area concentration. To improve the data quality for statistical analysis by MetaboAnalyst, the data were normalized by cube root transformation and pareto scaling. The mean values of the metabolites were compared using two tests performed separately by MetaboAnalyst. Tukey's test was used to compare the N source in each saline condition, whereas T-test was applied within each N nutrition to assess the salt stress effect. Principal Component Analysis (PCA) was performed to identify the differences in metabolic composition among the N source (NO_3^-, NO_3^-:NH_4^+ and NH_4^+) in absence (0 mM NaCl) and presence of salt stress (75 mM NaCl). The effect of salt stress in metabolic profiling in each N nutrition was performed by Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA). The pathway analysis was based in changes in relative concentration of detected metabolites in comparison with the corresponding control, using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway data base. The heatmap analysis was performed using MEV software 4.9.

7.3 Results

7.3.1 Regulation of physiological traits and ion homeostasis in response to salt stress

In this study, salt stress drastically reduced dry mass accumulation in plants from all N treatments (FIGURE 7a). Salinity reduced CO_2 assimilation only in NO_3^-:NH_4^+-fed plants (FIGURE 7b), which contributed to the highest reduction (by 60.2%) in total dry mass. Under NaCl-stress, nutrition with solely NH_4^+ promoted higher CO_2 assimilation and dry mass accumulation in comparison to other N treatments (FIGURE 7a, b).

Figure 7 - Total dry mass, CO_2 assimilation rate and NO_3^- and NH_4^+ content in leaves of sorghum, cv. CSF 20, under absence (0 mM NaCl) and presence of salt stress (75 mM NaCl) during 12 days and grown with different N source (NO_3^-, NO_3^-:NH_4^+ and NH_4^+). (a) total dry mass; (b) CO_2 assimilation rate; (c) NO_3^- content and (d) NH_4^+ content in leaves.



Data are means ± SE of four repetitions. In the same NaCl treatment, different capital letters indicate significant differences due to nitrogen nutrition, whereas different lowercase letters denote significant differences due to NaCl concentration in the same nutrition, according to Tukey's test ($p < 0.05$). Source: made by the author.

Salinity decreased N-NO_3^- content in leaves, except for NH_4^+-fed plants (FIGURE 7c); however, NO_3^--fed plants showed higher NO_3^- content, followed by NO_3^-:NH_4^+-fed and NH_4^+-fed plants under both control and salt treatments. NH_4^+ content was increased and decreased by salt stress in leaves of NO_3^-:NH_4^+-fed and NO_3^--fed plants, respectively (FIGURE 7d), whereas no significant effect of salinity was registered in NH_4^+-fed plants.

7.3.2 Different metabolic responses from leaves of sorghum to N nutrition and salinity

Metabolic profile analysis identified a total of 67 metabolites from leaves of sorghum grown with different nitrogen source (NO_3^-, NO_3^-:NH_4^+ and NH_4^+) under salinity and non-salinity condition, including amino acids, sugar and derivates (sugar phosphate and sugar alcohol), amines, organic acids, phenolic compounds and vitamins (TABLE 7). Sugar and derivates (26) were the major identified metabolites, followed by organic acids (19) and amino acids (16).

Table 7 - List of detected metabolites in leaves of sorghum with their classification in compound types, their retention time, respectively, with the mass fragment used for relative quantitative analysis of each compound and with their Kyoto Encyclopedia of Genes and Genomes identifier number (KEGG ID) [a]

	Name of metabolites	Compound type	Retention time (min)	Mass fragment (m/z)	Compound ID[a]
1	Glycolic acid	Organic acid	4.25	161	C00160
2	Pyruvic acid	Organic acid	5.15	174	C00022
3	Lactic acid	Organic acid	5.30	117	C00186
4	Oxalic acid	Organic acid	5.92	190	C00209
5	Valine	Amino acid	7.66	218	C00183
6	Leucine	Amino acid	8.52	158	C00123
7	Phosphoric acid	Organic acid	8.61	314	C00009
8	Proline	Amino acid	8.90	130	C00148
9	Glycine	Amino acid	9.07	248	C00037
10	Succinic acid	Organic acid	9.13	247	C00042
11	Glyceric acid	Organic acid	9.45	189	C00258
12	Fumaric acid	Organic acid	9.58	143	C00122
13	Serine	Amino acid	9.82	278	C00065
14	Alanine	Amino acid	9.86	188	C00041
15	4-Hydroxybutyric acid	Organic acid	10.08	117	C00989
16	Threonine	Amino acid	10.27	218	C00188
17	Beta-Alanine	Amino acid	10.71	160	C00099
18	Malonic acid	Organic acid	11.45	247	C00383
19	Malic acid	Organic acid	11.63	233	C00149

Table 7 continue

	Name of metabolites	Compound type	Retention time (min)	Mass fragment (m/z)	Compound ID[a]
20	Aspartic acid	Amino acid	12.05	202	C00049
21	Pyroglutamic acid	Amino acid	12.10	156	C01879
22	Erythronic acid	Organic acid	12.42	292	-
23	Threonic acid	Organic acid	12.63	220	C01620
24	Glutaric acid 2-oxo	Organic acid	12.72	198	C00026
25	Glutamic acid	Amino acid	13.28	246	C00025
26	Phenylalanine	Amino acid	13.41	218	C00079
27	Glutamine	Amino acid	13.77	227	C00064
28	Asparagine	Amino acid	13.94	188	C00152
29	Xylose	Sugar	14.12	103	C00181
30	Ribose	Sugar	14.62	189	C00121
31	Putrescine	Amine	14.73	174	C00134
32	Cis-aconitic acid	Organic acid	14.80	211	C00417
33	Glycerol-3-phosphate	Sugar phosphate	15.05	445	C00093
34	Galactonic acid	Sugar	15.16	292	C00880
35	Gluconic acid	Sugar	15.26	292	C00257
36	Shikimic acid	Organic acid	15.50	204	C00493
37	Glyceric acid 3-phosphate	Sugar phosphate	15.57	299	C00597
38	Tartaric acid	Organic acid	15.65	333	C00898
39	Citric acid	Organic acid	15.68	273	C00158
40	Dehydroascorbic acid	Organic acid	16.07	173	C00425
41	Quinic acid	Phenolic compound	16.21	345	C06746
42	Fructose	Sugar	16.36	217	C02336
43	Sorbose	Sugar	16.47	277	C00247
44	Galactose	Sugar	16.58	319	C00984
45	Glucose	Sugar	16.64	160	C00031
46	Lysine	Amino acid	16.73	156	C00047
47	Tyrosine	Amino acid	16.94	218	C00082
48	Mannitol	Sugar alcohol	17.06	319	C00392
49	Ascorbic acid	Vitamins	17.12	449	C00072
50	Pantothenic acid	Vitamins	17.52	157	C00864
51	Myo-inositol	Sugar alcohol	18.62	305	C00137
52	Ribulose-5-phosphate	Sugar phosphate	19.50	357	C00199
53	Methyl alpha-D-glucopyranoside	Sugar	20.41	290	-
54	Fructose-6-phosphate	Sugar phosphate	20.68	315	C00085
55	Glucose-6-phosphate	Sugar phosphate	20.79	387	C00668

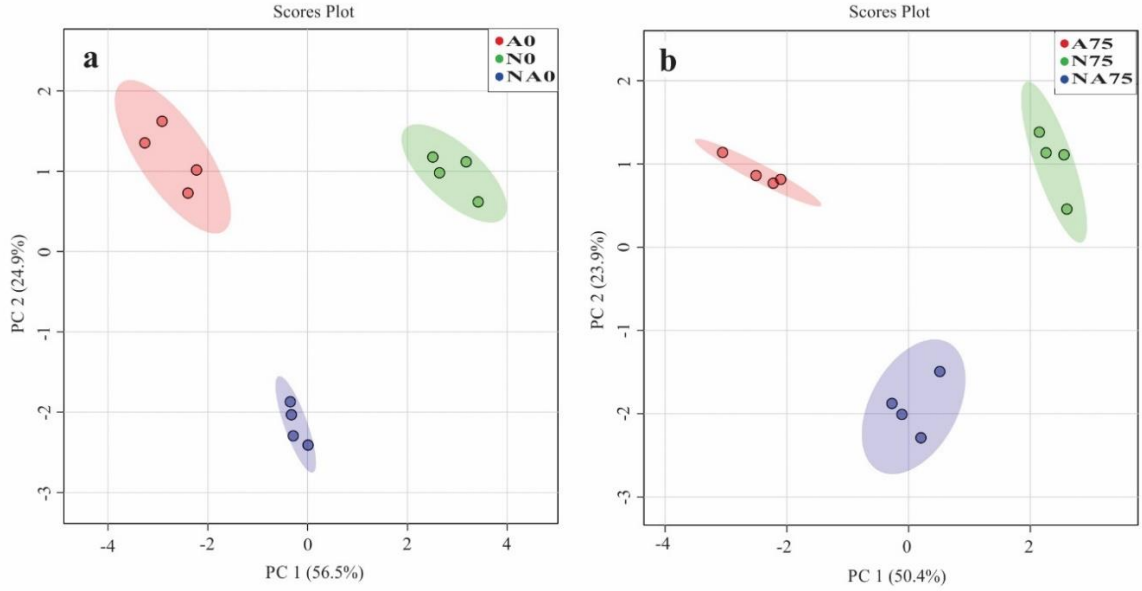
Table 7 continue

	Name of metabolites	Compound type	Retention time (min)	Mass fragment (m/z)	Compound ID[a]
56	Sucrose	Sugar	23.48	361	C00089
57	Caffeic acid	Phenolic compound	23.79	219	C01481
58	Lactitol	Sugar alcohol	24.21	204	C13542
59	Cellobiose	Sugar	24.38	169	C06422
60	Threalose	Sugar	24.53	361	C01083
61	Maltose	Sugar	24.98	361	C00208
62	Maltitol	Sugar alcohol	25.05	204	-
63	Galactinol	Sugar alcohol	26.11	204	C01697
64	Caffeyolquinic acid	Phenolic compound	26.90	345	C00852
65	Palatinose	Sugar	27.05	361	C01742
66	Raffinose	Sugar	30.03	437	C00492
67	Maltotriose	Sugar	31.67	204	C01835

Source: made by the author.

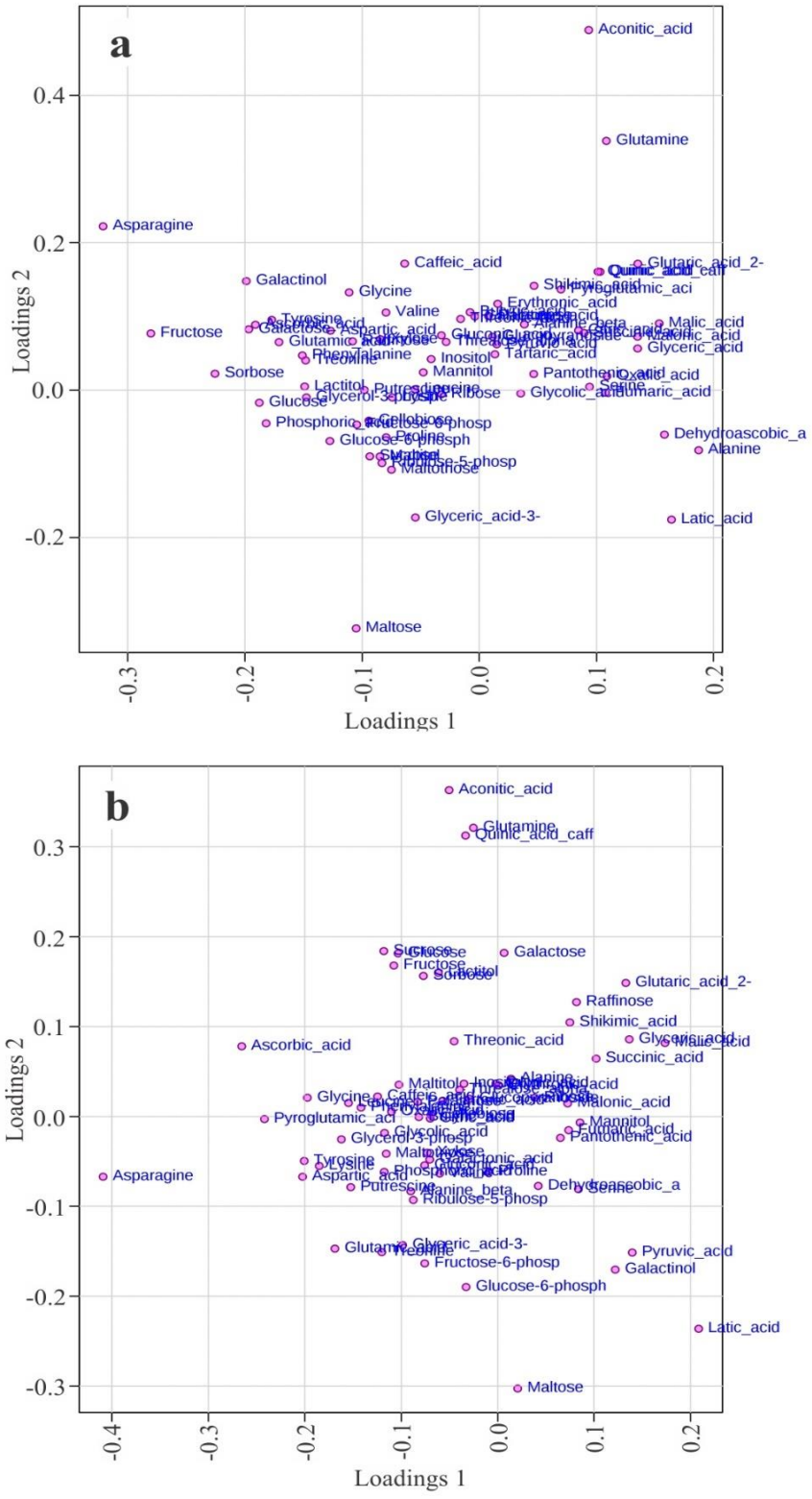
PCA analysis of metabolic profile from sorghum plants under control and salt stress influenced by nitrogen source in shown Figures 8 and 9. The Score plot results indicated a good separation of the three N nutrition in both NaCl conditions, as PC1 showed a 56.5% variance among N source in absence of salinity (FIGURE 8a), and a 50.4% difference among metabolic profiles under salt stress (FIGURE 8b).

Figure 8 - Principal Component Analysis (PCA) of metabolic profiling with score plot in leaves of sorghum, cv. CSF 20, grown with different N nutrition (NO_3^-, NO_3^-:NH_4^+ and NH_4^+) under absence (0 mM NaCl; a) and presence of salt stress (75 mM NaCl; b) during 12 days. Score plot for N sources under non-saline condition [(N0) NO_3^- + 0 mM NaCl; (NA0) NO_3^-:NH_4^+ + 0 mM NaCl and (A0) NH_4^+ + 0 mM NaCl] and under saline condition [(N75) NO_3^- + 75 mM NaCl; (NA75) NO_3^-:NH_4^+ + 75 mM NaCl and (A75) NH_4^+ + 75 mM NaCl].



Source: made by the author.

Figure 9 - Loading plot of metabolites in leaves of sorghum, cv. CSF 20, grown with different N nutrition (NO_3^-, NO_3^-:NH_4^+ and NH_4^+) under absence (0 mM NaCl; a) and presence of salt stress (75 mM NaCl; b) during 12 days.



Source: made by the author.

The loading plots of each PCA showed the contribution of detected metabolites to separate the groups (FIGURE 9). Herein, the most dominating metabolites in salt absence were asparagine, alanine, aconitic acid, glutamine and maltose (FIGURE 9a). On other hand, under saline condition, the metabolites which most contributed to separate N sources showed a similar trend as asparagine, glutamine, aconitic acid, maltose and caffeoylquinic acid (FIGURE 9b). To assess the metabolic dynamic of N nutrition and salinity, the relative concentrations of detected metabolites were statistically analyzed (TABLE 8) and their abundance are visualized in a heatmap, based on the mixed (NO_3^-:NH_4^+) nutrition in absence of NaCl (NA0) as reference group (FIGURE 10).

Ammonium nutrition alone promoted higher accumulation of amino acids compared to other N nutrition, as evidenced by elevated contents of threonine, glycine, aspartic acid, glutamic acid, asparagine, phenylalanine, and tyrosine, irrespective of salt treatment. Despite few amino acids accumulation in salt absence, NO_3^--fed plants exhibited higher abundance in organic acids in comparison to other N nutrition, including tricarboxylic acid (TCA) cycle intermediates, such as succinic acid, fumaric acid, malic acid, aconitic acid and citric acid (TABLE 8; FIGURE 10). On the contrary, NO_3^- nutrition promoted lower accumulation of sugar and derivates than other N sources, however sucrose, being the most abundant metabolite, showed no significant effect due to N nutrition under non-saline condition. Ascorbic acid content was greater in NH_4^+-fed plants in both saline levels, while putrescine content was pooler accumulated in NO_3^--fed plants.

Table 8 - Relative concentration values of metabolites in leaves of sorghum cv. CSF 20 with grown different nitrogen source (NO_3^-, NO_3^-:NH_4^+ and NH_4^+) under absence (0 mM NaCl) and presence of salt stress (75 mM NaCl) during 12 days. Values represent the means of four repetitions ± standard error. Different capital letters indicate significant difference due nitrogen source in the same saline condition, using Tukey's test ($P < 0.05$). Whereas different lowercase letters showed significant difference due to salt stress in the same nitrogen source, using T-test ($p < 0.05$).

	Metabolite	**Relative concentration[a]**					
		NO_3^-		**NO_3^-:NH_4^+**		**NH_4^+**	
	Amino acids	**0 mM NaCl**	**75 mM NaCl**	**0 mM NaCl**	**75 mM NaCl**	**0 mM NaCl**	**75 mM NaCl**
1	Valine	0.31 ± 0.04^{Ba}	0.42 ± 0.04^{Aa}	0.27 ± 0.03^{Bb}	0.57 ± 0.03^{Aa}	0.56 ± 0.05^{Aa}	0.56 ± 0.08^{Aa}
2	Leucine	0.48 ± 0.04^{Aa}	0.39 ± 0.04^{Ba}	0.56 ± 0.02^{Aa}	0.59 ± 0.06^{Ba}	0.61 ± 0.06^{Ab}	0.97 ± 0.07^{Aa}
3	Proline	0.96 ± 0.08^{Bb}	1.54 ± 0.10^{Aa}	1.39 ± 0.08^{Ab}	1.83 ± 0.10^{Aa}	1.36 ± 0.13^{Aa}	1.62 ± 0.21^{Aa}
4	Glycine	0.98 ± 0.09^{Ba}	0.91 ± 0.10^{Ca}	0.92 ± 0.05^{Bb}	1.56 ± 0.18^{Ba}	1.82 ± 0.17^{Aa}	2.72 ± 0.33^{Aa}
5	Serine	1.14 ± 0.12^{Aa}	0.93 ± 0.05^{Aa}	0.87 ± 0.07^{ABa}	0.97 ± 0.07^{Aa}	0.67 ± 0.05^{Ba}	0.67 ± 0.06^{Ba}
6	Alanine	7.16 ± 0.63^{Aa}	3.09 ± 0.39^{Ab}	5.55 ± 0.51^{Aa}	2.77 ± 0.14^{Ab}	2.60 ± 0.17^{Ba}	3.01 ± 0.30^{Aa}
7	Threonine	0.20 ± 0.02^{Cb}	0.70 ± 0.08^{Ba}	0.39 ± 0.05^{Bb}	1.59 ± 0.17^{Aa}	0.78 ± 0.05^{Ab}	1.44 ± 0.12^{Aa}
8	Beta-Alanine	1.93 ± 0.11^{Aa}	1.62 ± 0.20^{Aa}	1.50 ± 0.14^{Ab}	2.30 ± 0.16^{Aa}	1.71 ± 0.15^{Aa}	2.32 ± 0.21^{Aa}
9	Aspartic acid	0.16 ± 0.01^{Ba}	0.19 ± 0.01^{Ca}	0.21 ± 0.03^{Bb}	0.66 ± 0.07^{Ba}	0.53 ± 0.05^{Ab}	1.09 ± 0.13^{Aa}
10	Pyroglutamic acid	30.22 ± 3.83^{Aa}	25.58 ± 1.44^{Ca}	22.50 ± 1.03^{Ab}	34.63 ± 2.94^{Ba}	24.98 ± 1.83^{Ab}	46.17 ± 1.59^{Aa}
11	Glutamic acid	1.96 ± 0.07^{Bb}	3.54 ± 0.54^{Ba}	2.84 ± 0.24^{Bb}	7.40 ± 0.86^{Aa}	4.97 ± 0.57^{Aa}	6.98 ± 0.83^{Aa}
12	Phenylalanine	0.13 ± 0.01^{Cb}	0.29 ± 0.03^{Ba}	0.26 ± 0.03^{Bb}	0.43 ± 0.05^{Ba}	0.62 ± 0.06^{Aa}	0.69 ± 0.07^{Aa}
13	Glutamine	2.31 ± 0.10^{Aa}	1.54 ± 0.15^{Ab}	0.10 ± 0.01^{Cb}	0.36 ± 0.03^{Ba}	1.01 ± 0.09^{Bb}	1.68 ± 0.07^{Aa}
14	Asparagine	0.10 ± 0.03^{Cb}	1.00 ± 0.06^{Ca}	0.37 ± 0.03^{Bb}	7.78 ± 0.88^{Ba}	9.17 ± 0.65^{Ab}	20.69 ± 1.16^{Aa}
15	Lysine	0.53 ± 0.04^{Ba}	0.52 ± 0.05^{Ca}	0.67 ± 0.02^{ABb}	1.13 ± 0.08^{Ba}	0.78 ± 0.08^{Ab}	1.59 ± 0.07^{Aa}
16	Tyrosine	1.21 ± 0.10^{Ba}	1.28 ± 0.11^{Ba}	1.66 ± 0.07^{Bb}	2.48 ± 0.22^{Aa}	3.72 ± 0.32^{Aa}	3.45 ± 0.34^{Aa}
	Organic acids						
17	Glycolic acid	0.19 ± 0.01^{Aa}	0.26 ± 0.03^{Ba}	0.18 ± 0.01^{Ab}	0.38 ± 0.03^{ABa}	0.16 ± 0.01^{Ab}	0.50 ± 0.05^{Aa}
18	Pyruvic acid	9.50 ± 1.22^{Aa}	11.45 ± 0.84^{Aa}	8.32 ± 0.70^{Aa}	12.04 ± 1.21^{Aa}	8.75 ± 0.85^{Aa}	7.29 ± 0.25^{Ba}
19	Lactic acid	2.54 ± 0.31^{Aa}	2.31 ± 0.09^{Aa}	2.68 ± 0.37^{Aa}	2.78 ± 0.24^{Aa}	0.60 ± 0.06^{Ba}	0.34 ± 0.01^{Ab}
20	Oxalic acid	1.83 ± 0.09^{Aa}	1.75 ± 0.23^{Aa}	1.34 ± 0.17^{ABa}	2.08 ± 0.21^{Aa}	1.07 ± 0.08^{Bb}	2.50 ± 0.21^{Aa}
21	Phosphoric acid	10.68 ± 0.63^{Cb}	15.94 ± 1.43^{Ba}	16.47 ± 0.57^{Bb}	19.09 ± 0.31^{ABa}	19.87 ± 0.81^{Aa}	20.31 ± 0.63^{Aa}
22	Succinic acid	0.12 ± 0.01^{Aa}	0.12 ± 0.01^{Aa}	0.04 ± 0.00^{Bb}	0.06 ± 0.00^{Ba}	0.05 ± 0.00^{Ba}	0.05 ± 0.00^{Ba}

Table 8 continue

	Metabolite	Relative concentration[a]					
		NO_3^-		NO_3^-:NH_4^+		NH_4^+	
	Organic acids	**0 mM NaCl**	**75 mM NaCl**	**0 mM NaCl**	**75 mM NaCl**	**0 mM NaCl**	**75 mM NaCl**
23	Glyceric acid	2.87±0.20^{Aa}	3.33±0.37^{Aa}	1.74±0.14^{Ba}	2.14±0.15^{Ba}	1.49±0.10^{Ba}	1.87±0.20^{Ba}
24	Fumaric acid	0.18±0.02^{Aa}	0.12±0.01^{Ab}	0.11±0.01^{Ba}	0.10±0.01^{ABa}	0.06±0.01^{Ca}	0.08±0.01^{Ba}
25	4-Hydroxybutyric acid	0.90±0.06^{Aa}	0.81±0.03^{Ba}	0.72±0.01^{Bb}	0.91±0.02^{ABa}	0.92±0.04^{Aa}	1.03±0.03^{Aa}
26	Malonic acid	0.56±0.07^{Aa}	0.38±0.03^{Aa}	0.20±0.01^{Bb}	0.32±0.01^{Aa}	0.18±0.02^{Ba}	0.27±0.03^{Aa}
27	Malic acid	18.12±1.35^{Aa}	17.38±1.36^{Aa}	11.62±0.79^{Ba}	12.13±0.31^{Ba}	11.47±0.96^{Ba}	10.53±1.21^{Ba}
28	Erythronic acid	0.77±0.05^{Aa}	0.72±0.06^{Aa}	0.55±0.02^{Ba}	0.66±0.06^{Aa}	0.73±0.05^{Aa}	0.71±0.08^{Aa}
29	Threonic acid	0.23±0.02^{ABa}	0.27±0.03^{Aa}	0.18±0.01^{Bb}	0.23±0.01^{Aa}	0.26±0.02^{Aa}	0.31±0.02^{Aa}
30	Glutaric acid 2-oxo	3.59±0.21^{Aa}	3.60±0.33^{Aa}	1.32±0.10^{Bb}	1.99±0.10^{Ba}	1.75±0.16^{Ba}	2.08±0.09^{Ba}
31	Cis-aconitic acid	19.57±0.32^{Aa}	15.86±1.47^{Aa}	1.69±0.30^{Cb}	6.96±0.69^{Ba}	13.09±0.56^{Bb}	17.92±1.21^{Aa}
32	Shikimic acid	1.69±0.08^{Aa}	1.68±0.11^{Aa}	1.05±0.10^{Ba}	1.22±0.06^{Ba}	1.43±0.13^{ABa}	1.29±0.09^{Ba}
33	Tartaric acid	0.29±0.02^{Aa}	0.32±0.03^{Aa}	0.25±0.02^{Ab}	0.35±0.03^{Aa}	0.27±0.01^{Aa}	0.40±0.04^{Aa}
34	Citric acid	2.22±0.06^{Aa}	1.83±0.08^{Ab}	1.55±0.10^{Ba}	1.99±0.19^{Aa}	1.58±0.13^{Ba}	2.24±0.20^{Aa}
35	Dehydroascorbic acid	2.48±0.25^{Aa}	3.51±0.28^{Aa}	1.79±0.11^{Bb}	3.87±0.35^{Aa}	0.86±0.08^{Cb}	3.07±0.21^{Aa}
	Amine						
36	Putrescine	0.49±0.04^{Ba}	0.31±0.02^{Bb}	0.67±0.05^{ABa}	0.70±0.05^{Aa}	0.82±0.08^{Aa}	0.83±0.07^{Aa}
	Sugar and derivates						
37	Xylose	0.66±0.04^{Ba}	0.66±0.04^{Ba}	0.70±0.02^{Bb}	0.81±0.04^{Aa}	0.97±0.09^{Aa}	0.83±0.03^{Aa}
38	Ribose	4.10±0.07^{Aa}	4.32±0.06^{Aa}	4.21±0.05^{Aa}	4.17±0.02^{Ba}	4.24±0.05^{Aa}	4.15±0.02^{Ba}
39	Galactonic acid	0.39±0.03^{Aa}	0.43±0.03^{Aa}	0.30±0.00^{Bb}	0.55±0.04^{Aa}	0.39±0.02^{Ab}	0.55±0.04^{Aa}
40	Gluconic acid	0.05±0.00^{ABa}	0.05±0.01^{Ba}	0.04±0.00^{Bb}	0.08±0.01^{Aa}	0.06±0.01^{Aa}	0.09±0.00^{Aa}
41	Fructose	28.99±3.18^{Ca}	42.95±3.58^{Aa}	43.80±2.03^{Ba}	39.72±0.65^{Aa}	74.30±7.72^{Aa}	50.97±4.02^{Aa}
42	Sorbose	2.44±0.10^{Cb}	5.18±0.51^{ABa}	5.04±0.30^{Ba}	4.43±0.08^{Ba}	8.95±0.89^{Aa}	6.27±0.47^{Aa}
43	Galactose	1.46±0.18^{Cb}	3.33±0.23^{Aa}	2.34±0.27^{Ba}	2.16±0.16^{Ba}	4.99±0.34^{Aa}	3.16±0.41^{ABa}
44	Glucose	18.88±1.49^{Bb}	29.02±1.78^{ABa}	26.44±1.28^{Aa}	26.27±0.63^{Ba}	31.53±0.52^{Aa}	34.35±1.98^{Aa}
45	Methyl alpha-D-glucopyranoside	0.79±0.06^{Aa}	0.80±0.04^{Aa}	0.66±0.02^{Ab}	0.80±0.03^{Aa}	0.76±0.04^{Aa}	0.85±0.03^{Aa}
46	Sucrose	126.62±9.51^{Ab}	169.85±7.43^{Ba}	149.60±6.34^{Aa}	158.87±2.08^{Ba}	142.84±4.57^{Ab}	193.22±4.49^{Aa}

Table 8 continue

	Metabolite	Relative concentration[a]					
		NO_3^-		NO_3^-:NH_4^+		NH_4^+	
	Sugar and derivates	**0 mM NaCl**	**75 mM NaCl**	**0 mM NaCl**	**75 mM NaCl**	**0 mM NaCl**	**75 mM NaCl**
47	Cellobiose	0.93 ± 0.05^{Bb}	1.33 ± 0.03^{Aa}	1.29 ± 0.05^{Aa}	1.44 ± 0.10^{Aa}	1.36 ± 0.09^{Aa}	1.59 ± 0.07^{Aa}
48	Threalose	5.28 ± 0.26^{Ab}	7.02 ± 0.33^{Aa}	5.06 ± 0.33^{Ab}	6.94 ± 0.45^{Aa}	5.60 ± 0.36^{Ab}	7.39 ± 0.29^{Aa}
49	Maltose	4.70 ± 0.24^{Cb}	8.77 ± 0.54^{Ba}	15.27 ± 0.97^{Aa}	14.75 ± 1.50^{Aa}	7.76 ± 0.55^{Ba}	8.39 ± 0.55^{Ba}
50	Palatinose	0.68 ± 0.04^{Aa}	0.59 ± 0.05^{Ba}	0.51 ± 0.02^{Ab}	0.66 ± 0.04^{Aa}	0.65 ± 0.07^{Aa}	0.79 ± 0.04^{Aa}
51	Raffinose	2.06 ± 0.15^{Ab}	5.18 ± 0.26^{Aa}	2.36 ± 0.11^{Ab}	3.85 ± 0.26^{Ba}	3.44 ± 0.60^{Aa}	4.12 ± 0.30^{ABa}
52	Maltotriose	0.27 ± 0.01^{Ba}	0.29 ± 0.02^{Ba}	0.51 ± 0.04^{Aa}	0.45 ± 0.03^{Aa}	0.42 ± 0.02^{Aa}	0.53 ± 0.04^{Aa}
53	Glycerol-3-phosphate	0.20 ± 0.02^{Cb}	0.49 ± 0.04^{Ca}	0.45 ± 0.04^{Bb}	0.86 ± 0.02^{Ba}	0.72 ± 0.04^{Ab}	1.29 ± 0.09^{Aa}
54	Glyceric acid 3-phosphate	0.15 ± 0.01^{Cb}	0.28 ± 0.03^{Ba}	0.46 ± 0.04^{Aa}	0.63 ± 0.05^{Aa}	0.25 ± 0.04^{Bb}	0.52 ± 0.02^{Aa}
55	Ribulose-5-phosphate	0.56 ± 0.07^{Ba}	0.79 ± 0.04^{Ba}	0.98 ± 0.05^{Aa}	1.16 ± 0.04^{Aa}	0.86 ± 0.08^{Aa}	1.11 ± 0.06^{Aa}
56	Fructose-6-phosphate	0.43 ± 0.04^{Bb}	0.73 ± 0.06^{Ca}	0.75 ± 0.07^{Ab}	1.33 ± 0.04^{Aa}	0.84 ± 0.05^{Aa}	1.08 ± 0.06^{Ba}
57	Glucose-6-phosphate	0.72 ± 0.07^{Bb}	1.47 ± 0.08^{Ba}	1.43 ± 0.12^{Ab}	2.43 ± 0.12^{Aa}	1.59 ± 0.13^{Aa}	1.71 ± 0.08^{Ba}
58	Mannitol	0.45 ± 0.01^{Ab}	0.63 ± 0.03^{Aa}	0.48 ± 0.03^{Aa}	0.54 ± 0.05^{ABa}	0.55 ± 0.05^{Aa}	0.44 ± 0.03^{Ba}
59	Myo-inositol	22.81 ± 2.12^{Aa}	23.61 ± 1.35^{Aa}	22.84 ± 0.91^{Aa}	23.42 ± 0.38^{Aa}	24.31 ± 1.48^{Aa}	24.33 ± 0.41^{Aa}
60	Lactitol	78.02 ± 3.14^{Ba}	78.76 ± 8.77^{Aa}	89.45 ± 3.18^{ABa}	72.69 ± 5.67^{Aa}	98.23 ± 3.43^{Aa}	86.25 ± 2.59^{Aa}
61	Maltitol	0.56 ± 0.05^{Ba}	0.78 ± 0.07^{Ba}	0.91 ± 0.02^{Aa}	0.87 ± 0.01^{Ba}	0.84 ± 0.07^{Ab}	1.13 ± 0.03^{Aa}
62	Galactinol	13.01 ± 0.44^{Bb}	19.32 ± 1.16^{Aa}	14.98 ± 0.69^{Bb}	21.38 ± 1.18^{Aa}	27.98 ± 3.44^{Aa}	14.08 ± 0.48^{Bb}
	Phenolic compounds						
63	Quinic acid	5.71 ± 0.29^{Aa}	3.73 ± 0.24^{Ab}	3.17 ± 0.16^{Ca}	3.57 ± 0.15^{Aa}	3.91 ± 0.17^{Ba}	3.77 ± 0.32^{Aa}
64	Caffeic acid	2.65 ± 0.19^{ABa}	1.87 ± 0.09^{Bb}	2.02 ± 0.20^{Ba}	2.16 ± 0.19^{Ba}	3.49 ± 0.35^{Aa}	2.79 ± 0.12^{Aa}
65	Caffeyolquinic acid	11.20 ± 1.22^{Aa}	7.38 ± 0.62^{Aa}	6.69 ± 0.80^{Ba}	3.63 ± 0.15^{Bb}	7.72 ± 0.55^{ABa}	7.97 ± 0.20^{Aa}
	Others						
66	Ascorbic acid	0.03 ± 0.00^{Cb}	0.11 ± 0.01^{Ca}	0.10 ± 0.01^{Bb}	0.32 ± 0.03^{Ba}	0.63 ± 0.06^{Ab}	1.83 ± 0.13^{Aa}
67	Pantothenic acid	0.15 ± 0.01^{Aa}	0.13 ± 0.00^{Aa}	0.11 ± 0.01^{ABa}	0.13 ± 0.01^{Aa}	0.11 ± 0.00^{Ba}	0.10 ± 0.01^{Aa}

[a] *Ratio of metabolic peak area to ribitol peak area*

Source: made by the author.

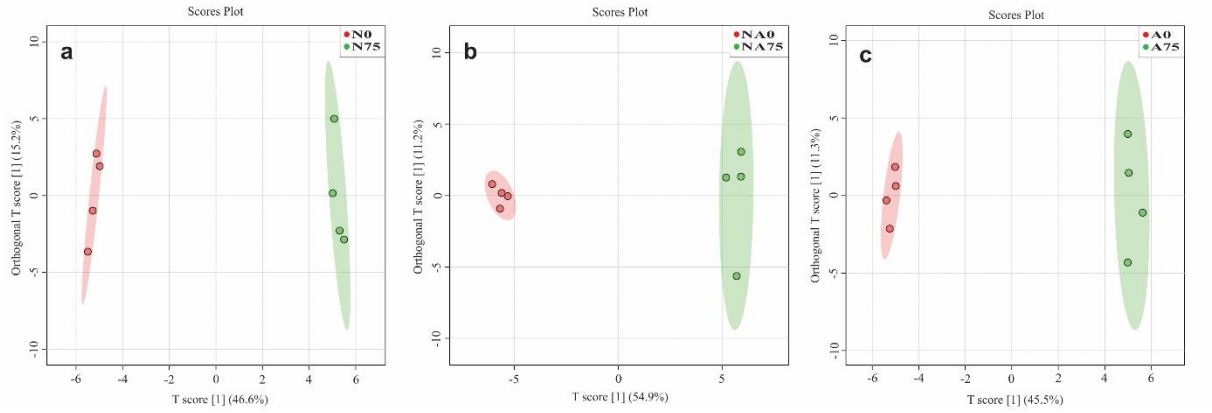
Figure 10 - Heat map representation of the relative abundance of 67 detected metabolites in leaves of sorghum, cv. CSF 20, under absence (0 mM NaCl) and presence of salt stress (75 mM NaCl) during 12 days; and grown with different N source (NO_3^-, NO_3^-:NH_4^+ and NH_4^+). (NA0) NO_3^-:NH_4^+ + 0 mM NaCl; (NA75) NO_3^-:NH_4^+ + 75 mM NaCl; (N0) NO_3^- + 0 mM NaCl; (N75) NO_3^- + 75 mM NaCl (A0) NH_4^+ + 0 mM NaCl and (A75) NH_4^+ + 75 mM NaCl. Transformed means (log_2) based in the value of NA0 for each metabolite.

Source: made by the author.

7.3.3 Metabolite changes of sorghum plants grown with different N source in response to salinity

The OPLS-DA method was employed for each N nutrition to determine significant differences due to salt stress (FIGURES 11, 12). These results indicated a clear separation between groups, although the higher variance was observed within NO_3^-:NH_4^+ nutrition (FIGURE 11). Out of these 67 identified metabolites, salt stress differentially modulated 37 in leaves of NO_3^-:NH_4^+-fed plants; whereas 28 and 20 metabolites were significantly altered in NO_3^-- and NH_4^+-fed plants, respectively. The fold change of metabolites and their significance degree were assessed by comparison (T-test, $p < 0.05$) between salt-stressed plants and non-stressed plants for each N nutrition (FIGURE 13 and TABLE 9).

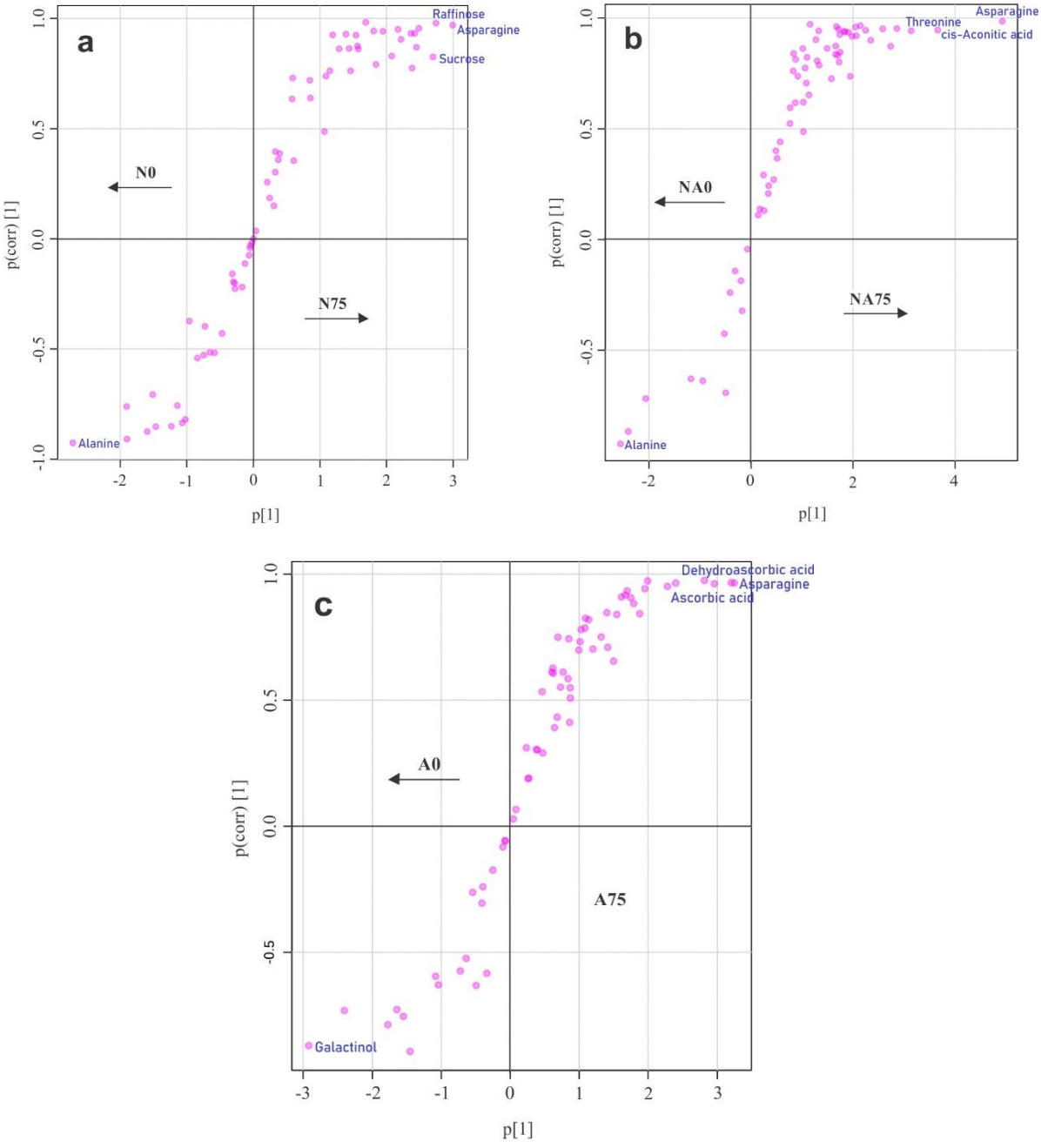
Figure 11 – Orthogonal Partial Least-Discriminate Analysis (OPLS-DA) of metabolic profiling of plants of sorghum cv. CSF 20 grown with NO_3^- (a), NO_3^-:NH_4^+ (b) and NH_4^+ (c) comparing absence (0 mM NaCl) and presence of salt stress (75 mM NaCl). (N0) NO_3^- + 0 mM NaCl; (N75) NO_3^- + 75 mM NaCl; (NA0) NO_3^-:NH_4^+ + 0 mM NaCl; (NA75) NO_3^-:NH_4^+ + 75 mM NaCl; (A0) NH_4^+ + 0 mM NaCl and (A75) NH_4^+ + 75 mM NaCl.



Source: made by the author.

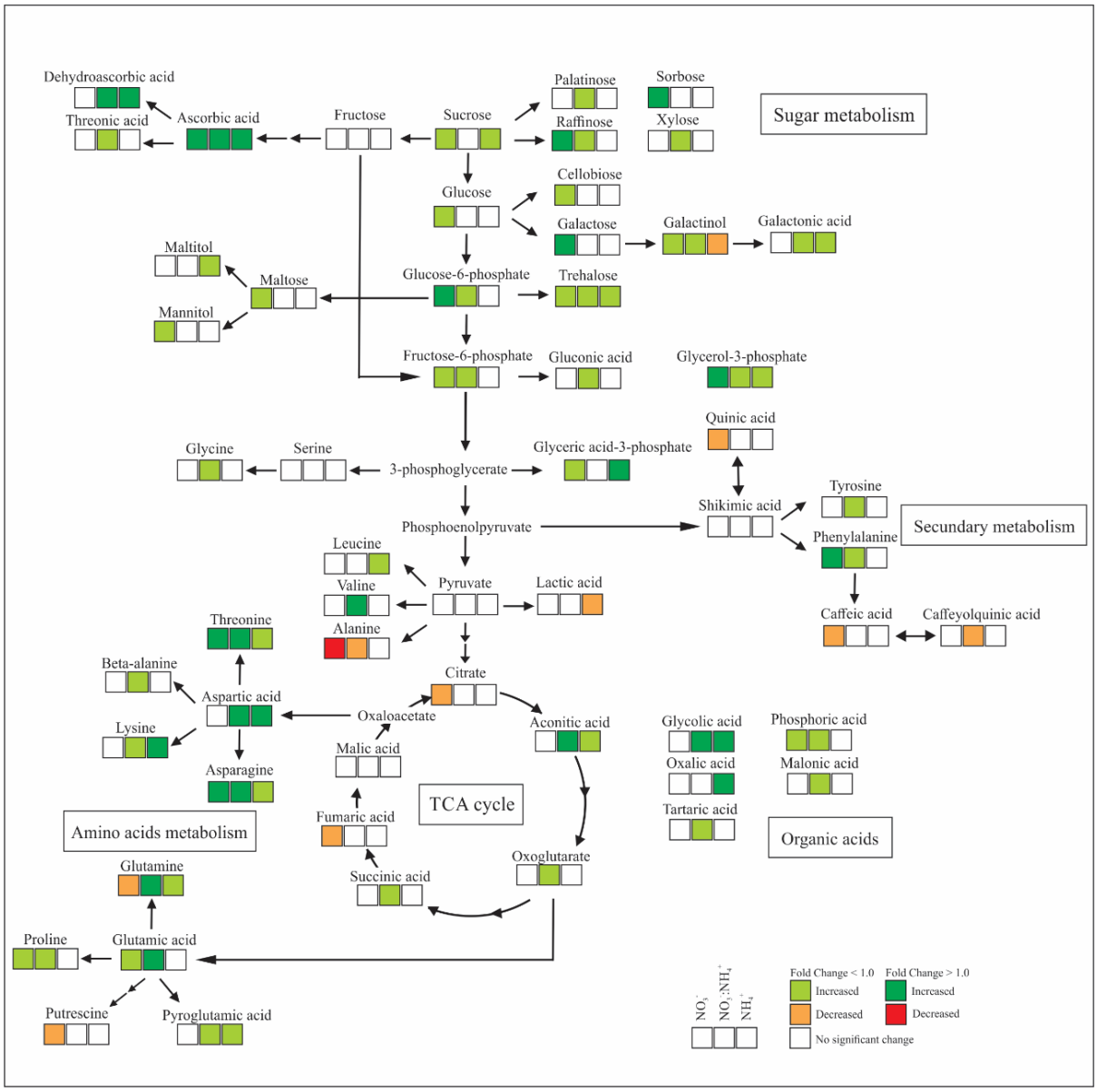
Interestingly, salt stress promoted few effects on metabolomic profile of leaves of NH_4^+-fed plants. Just twenty metabolites were significantly altered by salinity with higher changes of amino acids and sugars. Under saline condition, N metabolism-related amino acids (asparagine, glutamine, aspartic acid, pyroglutamic acid), leucine, threonine, phenylalanine and lysine were increased in NH_4^+-fed plants. In organic acid terms, four metabolites were increased by salinity, including glycolic acid, oxalic acid, cis-aconitic acid and dehydroascorbic acid, while lactic acid content was reduced. Furthermore, salinity increased sugar and derivates, such as sucrose, trehalose-alpha, glycerol-3-phosphate, glyceric acid-3-phosphate and maltitol, while galactinol content was reduced. Ascorbic acid was also significantly increased under salt stress in NH_4^+-fed pants (TABLE 9).

Figure 12 - S-plots of metabolites in plants of sorghum cv. CSF 20 grown with NO_3^- (a), NO_3^-:NH_4^+ (b) and NH_4^+ (c) comparing absence (0 mM NaCl) and presence of salt stress (75 mM NaCl). (N0) NO_3^- + 0 mM NaCl; (N75) NO_3^- + 75 mM NaCl; (NA0) NO_3^-:NH_4^+ + 0 mM NaCl; (NA75) NO_3^-:NH_4^+ + 75 mM NaCl; (A0) NH_4^+ + 0 mM NaCl and (A75) NH_4^+ + 75 mM NaCl.



Source: made by the author.

Figure 13 - Metabolic pathway changed by salt stress in leaves of sorghum cv. CSF 20 grown with different N nutrition (NO_3^-, NO_3^-:NH_4^+ and NH_4^+). The colors indicate the significant fold change of metabolites according to T-test ($p < 0.05$).



Source: made by the author.

Table 9 - Fold changes of metabolites of leaves of sorghum cv. CSF 20 grown with different N nutrition after salt stress. Values of log_2 (salt stressed/non-stressed), N0 (NO_3^- + 75 mM NaCl), N75 (NO_3^- + 75 mM NaCl), NA0 (NO_3^-:NH_4^+ + 0 mM NaCl), NA75 (NO_3^-:NH_4^+ + 75 mM NaCl), A0 (NH_4^+ + 0 mM NaCl) and A75 (NH_4^+ + 75 mM NaCl); * and ** indicate significant difference by T-test with $p < 0.05$ and $p < 0.01$, respectively.

Metabolites	**Fold change (log_2)**		
Amino acids	**N75/N0**	**NA75/NA0**	**A75/A0**
Valine	0.44	1.08**	0.00
Leucine	-0.30	0.08	0.67*
Proline	0.68*	0.40*	0.25
Glycine	-0.11	0.76*	0.58
Serine	-0.29	0.16	0.00
Alanine	-1.21**	-1.00**	0.21
Threonine	1.81**	2.03**	0.88**
Beta-Alanine	-0.25	0.62*	0.44
Aspartic acid	0.25	1.65**	1.04*
Pyroglutamic acid	-0.24	0.62*	0.89**
Glutamic acid	0.85*	1.38**	0.49
Phenylalanine	1.16**	0.73*	0.15
Glutamine	-0.58*	1.85**	0.73**
Asparagine	3.32**	4.39**	1.17**
Lysine	-0.03	0.75**	1.03**
Tyrosine	0.08	0.58*	-0.11
Organic acids			
Glycolic acid	0.45	1.08**	1.64**
Pyruvic acid	0.27	0.53	-0.26
Lactic acid	-0.14	0.05	-0.82*
Oxalic acid	-0.06	0.63	1.22**
Phosphoric acid	0.58*	0.21*	0.03
Succinic acid	0.00	0.58*	0.00
Glyceric acid	0.21	0.30	0.33
Fumaric acid	-0.58*	-0.14	0.42
4-Hydroxybutyric acid	-0.15	0.34**	0.16
Malonic acid	-0.56	0.68**	0.58
Malic acid	-0.06	0.06	-0.12
Erythronic acid	-0.10	0.26	-0.04
Threonic acid	0.23	0.35*	0.25
Glutaric acid 2-oxo	0.00	0.59*	0.25
Cis-aconitic acid	-0.30	2.04**	0.45*
Shikimic acid	-0.01	0.22	-0.15
Tartaric acid	0.14	0.49*	0.57
Citric acid	-0.28*	0.36	0.50
Dehydroascorbic acid	0.50	1.11**	1.84**
Amine			
Putrescine	-0.66*	0.06	0.02
Sugar and derivates			
Xylose	0.00	0.21*	-0.22
Ribose	0.08	-0.01	-0.03
Galactonic acid	0.14	0.87**	0.50*
Gluconic acid	0.00	1.00**	0.58
Fructose	0.57	-0.14	-0.54
Sorbose	1.09**	-0.19	-0.51
Galactose	1.19**	-0.12	-0.66
Glucose	0.62*	-0.01	0.12
Methyl alpha-D-glucopyranoside	0.02	0.28*	0.16
Sucrose	0.42*	0.09	0.44**

Table 9 continue

Metabolites	Fold change (log_2)		
Sugar and derivates	**N75/N0**	**NA75/NA0**	**A75/A0**
Cellobiose	0.52**	0.16	0.23
Threalose-alpha	0.41*	0.46*	0.40*
Maltose	0.90**	-0.05	0.11
Palatinose	-0.20	0.37*	0.28
Raffinose	1.33**	0.71**	0.26
Maltotriose	0.10	-0.18	0.34
Glycerol-3-phosphate	1.29**	0.93**	0.84**
Glyceric acid 3-phosphate	0.90*	0.45	1.06**
Ribulose-5-phosphate	0.50	0.24	0.37
Fructose-6-phosphate	0.76*	0.83**	0.36
Glucose-6-phosphate	1.03**	0.76**	0.10
Mannitol	0.49**	0.17	-0.32
Myo-inositol	0.05	0.04	0.00
Lactitol	0.01	-0.30	-0.19
Maltitol	0.48	-0.06	0.43*
Galactinol	0.57**	0.51**	-0.99*
Phenolic compound			
Quinic acid	-0.61**	0.17	-0.05
Caffeic acid	-0.50*	0.10	-0.32
Caffeyolquinic acid	-0.60	-0.88*	0.05
Others			
Ascorbic acid	1.87**	1.68**	1.54**
Pantothenic acid	-0.21	0.24	-0.14

Source: made by the author.

7.3.4 OPLS-DA for identification of salt stress markers

Possible salt stress biomarkers were analyzed between control and stressed plants using S-plots for each OPLS-DA, which showed distribution of metabolites and their contribution to non-saline and saline condition in the respective NO_3^-, mixed (NO_3^-:NH_4^+) and NH_4^+ nutrition (FIGURE 12). In NO_3^--fed plants, asparagine, raffinose and sucrose ($p < 0.01$) contributed to the clustering of salt stressed plants, while alanine was more related to non-saline condition (FIGURE 12a). Asparagine, threonine and aconitic acid ($p < 0.00$) were the metabolites that most been related to stress saline in plants NO_3^-:NH_4^+-fed plants (FIGURE 12b); whereas, ascorbic acid, dehydroascorbic acid and asparagine were significant in NH_4^+-fed plants under saline condition ($p < 0.05$), and galactinol was more related to non-saline condition (FIGURE 12c).

7.4 Discussion

Salt stress is one of the environmental factors that affects the growth, development and physiological mechanisms of many species. Salt-induced damages are initially perceptible in complications of growth parameters, which is commonly associated with decreases in

photosynthetic efficiency (ASHRAF; HARRIS, 2013; PARIHAR *et al.*, 2015). Our results demonstrated that NH_4^+-fed plants were less affected by salt stress (75 mM NaCl) as indicated by less reduction in total dry mass and higher CO_2 assimilation rate compared to others N regimes. Thus, our results confirmed which NH_4^+ nutrition can ameliorate the deleterious effects of salinity and improve tolerance in sorghum plants, as previously demonstrated by Miranda *et al.* (2013; 2016; 2017). Herein, we assessed metabolic profile of sorghum leaves in relation to supplied N source to understanding the metabolic changes in salt tolerance.

7.4.1 External N source promotes differential regulation in metabolomic profile in sorghum leaves

Plant metabolism alterations are a common response to stressful conditions, including drought, salinity, nutrient deficiency and biotic stress, being a complex variation of plants responses (ARBONA *et al.*, 2013; PARIDA *et al.*, 2018). In this context, the supplied N, as well its deprivation, can promote significant variations in the metabolites, mainly C and N metabolism (URBANCZYK-WOCHNIAK; FERNIE, 2005). In the current study, the N sources promoted different effects on amino acids, organic acids, sugars and others metabolite content, leading to distinguished metabolic profiling (FIGURES 8, 9, 10). Consisting with our data, the amino acids content was closely regulated by availability and type of supplied N form, thereby greatly enhanced in plants under NH_4^+ nutrition (PASQUALINI *et al.*, 2001; QUAN *et al.*, 2016). In addition, the higher asparagine content in NH_4^+ nutrition may play a role in the reduction of NH_4^+ toxic effects in leaves, as well as controlling the C/N status (GUO; ZU; TANG, 2012). These results are in accordance to those of González-Hernández *et al.* (2019) and Miranda *et al.* (2016), that reported significant increase in amino acids content in NH_4^+-fed plants, mainly asparagine. These findings suggest that asparagine display a key contribution to distinguish the metabolic profile among N nutrition (FIGURE 9).

During N assimilation, NO_3^- is reduced to nitrite, which is rapidly reduced to ammonium following incorporation in amino acids through GS/GOGAT cycle (MASCLAUX-DAUBRESSE *et al.*, 2010). The first phase of NO_3^- reduction demands high energy consume, depending on part of the photosynthesis reductants. However, production of OH^- as result of NO_3^- reduction alters the charge balance inside cells, thus demanding higher organic acids accumulation (KANDLBINDER; CRUZ; KAISER, 1997; TURAN; SEVIMLI, 2005), as evidenced by high organic acids accumulation (succinic acid, glyceric acid, fumaric acid, malonic acid, aconitic acid, oxoglutaric acid) in NO_3^--fed sorghum plants (FIGURE 10 and TABLE 8). This may be supported by higher PEPC activity in NO_3^--fed plants (data not shown),

which play a key reaction to organic acids synthesis, like malate acid (KANDLBINDER, CRUZ, KAISER, 1997; CRUCHAGA *et al.*, 2013).

Under non-saline condition, the low sugar content (fructose and glucose) in NO_3^--fed plants may be related to higher C skeleton transport and energy to roots, as sucrose, in order to maintain growth and development (QUAN *et al.*, 2016). Contrarily, the aconitic acid was a key metabolite in the separation of N source-related profiles, being clearly associated with NO_3^- nutrition that induce higher abundance of organic acids and TCA cycle activity, whereas fructose, maltose and other sugars exhibited contrary trend (FIGURE 9). This suggest the distinct regulation of organic acid and sugar metabolism in the plants grown with NO_3^- nutrition.

Up-regulating the antioxidants systems (enzymatic and/or non-enzymatic mechanisms) has been investigated an effective strategy to improve plant performance against abiotic stresses (GONDIM *et al.*, 2013; JIANG *et al.*, 2012). More specifically, ascorbate is an important antioxidant molecule to regulate the redox status and act as cofactor in biochemical pathway (FOYER; NOCTOR, 2011). In wheat, the enhancement of ascorbate and glutathione contents due to salicylic acid and cold pre-treatment alleviated oxidative damages under subsequent cold stress (WANG *et al.*, 2020). Similar effect was observed in sorghum plants, where the NH_4^+-induced basal pool of ascorbic acid was higher in comparison to other N nutrition in absence of salt stress (FIGURE 10), thus contributing to mitigate the deleterious effects of salt stress as reported to *Vigna angularis* (AHANGER *et al.*, 2020), mainly to maintenance of chloroplast integrity (data not published), promoting better photosynthetic activity and mitigate the decline in growth compared to another N regime (FIGURE 7).

The N source also showed significant influence over amine compounds, as increasing putrescine content in NH_4^+ presence in growth medium (sole NH_4^+ and mixed regime), (FIGURE 10 and TABLE 8), suggesting that enhancement of putrescine, mainly in NH_4^+-fed plants, may be related with arginine metabolism, an important precursor of polyamines biosynthesis, controlling the NH_4^+ accumulation in plants (ESTEBAN *et al.*, 2016; HOUDUSSE *et al.*, 2008). Yet, the phenylpropanoids metabolism was mainly induced by sole NO_3^- and NH_4^+, as observed by higher accumulation of intermediates (quinic acid, caffeic acid and caffeoylquinic acid) in leaves, although some alterations compared to mixed-N nutrition showed no significant difference (FIGURE 10 and TABLE 8). Then, based in the production of distinguished metabolite profiles, our findings suggest that N metabolism is linked with diverse metabolic pathway depending on nitrogen source.

7.4.2 Increased amino acids pool in mixed-N nutrition is a potential index for salt damages

In salt-stressed sorghum plants, amino acids were significantly altered by salinity in all N treatments, mainly in NO_3^-:NH_4^+-fed plants with increased 13 amino acids, including valine, aspartic acid, asparagine and glutamic acid. These changes may be correlated with slower growth rather than an adaptive response to salt stress due to elevated protein and chlorophyll degradation (FAN *et al.*, 2019; HUANG *et al.*, 2018), as evidenced by higher growth inhibition (FIGURE 7a), reduced CO_2 assimilation (FIGURE 7b) and chlorosis (data not shown) in salt-stressed plants grown with mixed-N nutrition. On the other hand, the decrease in some amino acids of NO_3^--fed plants (TABLE 8) may be related to damage of salt stress over NO_3^- uptake (COELHO *et al.*, 2020), that was consistent with the significant reduction in NO_3^- and NH_4^+ content in leaves (FIGURE 7c, d).

Specifically, proline is an important metabolite accumulated in root and leaves under salinity which act as osmoprotectant, reducing oxidative effects and contributing with osmotic adjustment (IQBAL; UMAR; KHAN, 2015; PER *et al.*, 2017). Herein, we reported a proline accumulation in NO_3^-- and NO_3^-:NH_4^+-fed plants (FIGURE 10 and TABLE 8), however it needs further research to elucidate its contribution in the osmotic adjustment. In *Stevia rebaudiana*, proline was considered a potential biochemical marker for tolerance under salt stress (DEBNATH *et al.*, 2018).

Our study revealed that salinity significantly reduced the alanine levels in sorghum plants grown with NO_3^- and NO_3^-:NH_4^+ nutrition (FIGURE 13). In *Triticum aestivum*, Che-Othman *et al.* (2019) reported similar response, which was associated with impairment in the TCA cycle and consequent pyruvate accumulation. However, in sorghum, only NO_3^--fed plants displayed significant decrease of some TCA cycle intermediates, such citric acid and fumaric acid (FIGURE 13).

Asparagine also displays important role in the N transport and storage due to its high N/C ratio, as well as avoid the excess ammonium in the tissues (AMELIA *et al.*, 2018; COELHO *et al.*, 2019; WOODROW *et al.*, 2016). In our study, asparagine content was increased in all N nutrition after salt stress, which should be important marker for salinity stress (FIGURES 12, 13), except for NO_3^-:NH_4^+-fed plants, which showed elevated sensibility to stress. In this plant group, elevated asparagine content was most likely result of N re-assimilating originated from protein degradation (TABLE 9), as reported in *Camellia sinensis* (HUANG *et al.*, 2018). Although, studies related the increased asparagine level with tolerant

stress, indicating that the role of amino acid in supplying core nitrogen metabolism might mitigate salt stress-induced damages (HU *et al.*, 2015), corroborating with our results to NH_4^+ nutrition under salinity which showed better physiologic performance (dry mass and CO_2 assimilation rate, FIGURE 7a, b) than other N regime.

7.4.3 Energy and antioxidant metabolism are modulated by N source in response to salinity

The organic acids are important C skeletons source for biochemical processes, mainly N metabolism. They can also enter in TCA cycle to produce energy for plant metabolism, as well as supplying other metabolic pathway, including amino acids biosynthesis (QUAN *et al.*, 2016). Our data showed that changes in the organic acids levels were higher in NO_3^-:NH_4^+-fed plants, whose increased punctual metabolites (phosphoric acid, succinic acid, threonic acid, glutaric acid and glycolic acid) involved in the charge balance (CHANG *et al.*, 2019). In *Poa pratensis*, the accumulation of organic acids acted in the regulation of charge imbalance induced by alkalinity and salinity stress (HU *et al.*, 2015). Among the organic acids, aconitic acid accumulation was superior in NO_3^-:NH_4^+-fed plants compared to other N nutrition under saline condition (FIGURE 13), acting as potential indicators to salinity in sorghum plants (FIGURE 12b).

Ascorbic acid and dehydroascorbic acid are related with redox potential in plants, having their levels increased in response to salinity (TAÏBI *et al.*, 2016). Despite its role as antioxidant, ascorbic acid also acts in the growth and developmental process, promoting cross-talk among redox-regulated pathway and hormones (ORTIZ-ESPÍN *et al.*, 2018). In our study, NH_4^+ nutrition promoted higher basal level of ascorbic acid compared to other nutrition as mentioned previously. Furthermore, salinity induced significant increase in ascorbic acid and dehydroascorbic acid contents in plant from all N treatments, however the ratio ascorbic/dehydroascorbic acid was higher in NH_4^+-fed plants after salt stress imposition (TABLE 8), suggesting that high levels of antioxidants contribute to alleviate the oxidative stress and promote salt tolerance, being potential biomarkers to salinity in plants grown with NH_4^+ nutrition (FIGURE 12c). Similar responses were observed in barley cultivar, where higher ascorbic acid levels may had promoted the better performance of tolerant cultivar under high salinity (WIDODO *et al.*, 2009). Ascorbic acid also has significant influence in the photosynthesis, protecting photosynthetic apparatus of ROS-damages and avoiding photoinhibition (IVANOV, 2014). In mixed-N plants, salinity induced a reduction in the photosynthesis that may be associated with structural damages in the chloroplasts aggravated

by low antioxidant capacity. Although there was increased ascorbic acid content in all N nutrition after salt stress, NO_3^-:NH_4^+-fed plants also had significant increase in tartaric acid and threonic acid levels under salt stress, indicating major degradation of ascorbic acid (TABLE 8) (SAITO *et al.*, 1997).

NO_3^- nutrition also showed significant alterations in the organic acids content, mainly reduction in the intermediates of TCA cycle (fumaric acid and citric acid) under salt stress (FIGURE 13). This was consistent with the reduction of energy molecules generation through pyruvate oxidation into TCA cycle, that reflected in the growth reduction of plants under saline condition (FIGURE 7a) (DAS *et al.*, 2019).

7.4.4 Sugar metabolism may be correlated with osmotic adjustment in NO_3^- and NO_3^-:NH_4^+nutrition under salinity

To cope with salinity-induced osmotic stress, the plants accumulate compatibles solutes as sugar to maintain turgor and alleviate the damages induced by salinity (PARIDA *et al.*, 2018). Many other functions are associated with sugar, including regulation of metabolism, growth, senescence, signaling pathway, as well as stress responses (SAMI *et al.*, 2016). Sugars, such as fructose, xylose, sucrose, maltose, melezitose are also accumulated in response to salt stress (CHANG *et al.*, 2019; LIU *et al.*, 2019).

In our study, NO_3^- and mixed-N nutrition promoted an increase in sugars, as xylose, galactose, sucrose, cellobiose, maltose and trehalose under salinity (FIGURE 13), while few changes were observed in NH_4^+-fed plants, suggesting that sugar metabolism was not determinant to salt response in these plants. Interestingly, raffinose and sucrose were significantly increased in NO_3^--fed plants, being potential markers to salinity for sorghum grown with NO_3^- nutrition (FIGURE 12a), that could contribute with reduction of osmotic potential in leaves, as well as be storing energy (GUPTA; HUANG, 2014; ZHAO *et al.*, 2019). In addition, the sucrose also has showed a key role in the regulation of stomatal opening (DALOSO *et al.*, 2015). In this context, the increase of sucrose level in response to salinity can had attenuated the salt effects on the stomatal conductance in NO_3^-- and NH_4^+-fed plants, that promoted maintenance of CO_2 assimilation net under salt stress (FIGURE 7b).

Polyols are metabolites with reactive hydroxyl groups that act in the osmotic adjustment and ROS scavenging, as well as stabilizing proteins and membranes (GUPTA; HUANG, 2014). In plants from all N nutrition, salinity induced an increase of polyols (mannitol,

galactinol and maltitol) in leaves of sorghum plants, that may had contributed to osmotic regulation and reduction of salinity-induced damages.

7.5 Conclusion

Salinity affected the performance of sorghum plants, but external N source was determinant to salt tolerance, with NH_4^+ nutrition more powerfully to alleviate salt harmful effects. Different N source promoted distinct metabolic profiles related to specific mechanism to achieve the salt tolerance. Several compounds were identified as markers to salt stress triggered by N nutrition, mainly asparagine that was common among the nutrition. NH_4^+ nutrition acted as mild stressor promoting upregulation of specific metabolites, charactering as potential biomarkers, such as ascorbic acid, dehydroascorbic acid, pyroglutamic acid and sucrose. In addition, amino acids pool and antioxidants in the NH_4^+-fed plants contributed to high tolerance to salinity. Our findings provide new insights into metabolic changes to better understanding of complex interplay between N metabolism and salinity response.

8 CONSIDERAÇÕES FINAIS

A partir dos dados fisiológicos, fotoquímicos, ultraestruturais e de metabolômica, podemos concluir que a nutrição com NH_4^+ como única fonte de N aciona previamente mecanismos para aclimatação a nutrição nitrogenada, que contribuem para melhor resposta à um estresse secundário, no caso a salinidade. O melhor desempenho das plantas de sorgo nutridas com NH_4^+ envolveu o menor acúmulo de Na^+ nos tecidos com consequente aumento da relação K^+/Na^+, manutenção de altas taxas de assimilação de CO_2, melhor desempenho fotoquímico com aumento do quenching fotoquímico e redução do excesso de energia nas membranas dos tilacóides, preservando a integridade e bom funcionamento dos cloroplastos. Além disso, a maior síntese de aminoácidos e de antioxidante contribuíram para melhor performance sob salinidade em comparação com os outros regimes de nutrição estudados.

Estes resultados fornecem informações importantes sobre a influência da fonte de nitrogênio, em particular do NH_4^+, na aclimatação de plantas de sorgo à salinidade, com destaque a identificação de potenciais biomarcadores de salinidade que podem ser auxiliar na compreensão do metabolismo da planta sob estresse salino e no desenvolvimento de estratégias para melhorar a tolerância das culturas à salinidade.

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