UNIVERSIDADE FEDERAL DO CEARÁ
CENTRO DE CIÊNCIAS
DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR
DOUTORADO EM BIOQUÍMICA

ASSIMILAÇÃO DE NITRATO E AMÔNIO NA PROTEÇÃO OXIDATIVA, ATIVIDADE FOTOQUÍMICA E ASSIMILAÇÃO DE CO_2 EM PLANTAS DE *Jatropha curcas* EXPOSTAS AO ESTRESSE SALINO.

RAFAEL MAGALHÃES DE ARAGÃO

FORTALEZA-CE
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Dedico

A minha família

Há três métodos para ganhar sabedoria:

primeiro, por reflexão, que é o mais nobre;

segundo, por imitação, que é o mais fácil;

e terceiro, por experiência, que é o mais amargo.

Confúcio

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A Deus, por iluminar os meus caminhos e me fortalecer diante de todas as decisões que tenho tomado ao longo de minha vida;

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Muito obrigado!

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RESUMO GERAL

A salinidade é um fator conhecido por limitar o crescimento e a produtividade das plantas. Dentre os processos afetados está a absorção e assimilação de Nitrato (NO_3^-). Além disso, a salinidade induz a produção de espécies reativas de oxigênio que levam a danos oxidativos e aumento da pressão de elétrons na cadeia transportadora de elétrons fotossintéticos. Assim, neste presente estudo foram utilizadas diferentes abordagens para testar a hipótese de que o processo de assimilação do nitrato pode aliviar a pressão de elétrons atuando como um dissipador e assim mitigar os efeitos negativos da salinidade e toxicidade do amônio em plantas de *Jatropha curcas*. Plantas submetidas à salinidade (NaCl 100 mM) e supridas com NO_3^- 10 mM mostraram maior absorção, atividade de NR e conteúdo de proteínas solúveis quando comparada com plantas supridas com NO_3^- 1mM. A alta assimilação de nitrato de alta foi associada com um maior crescimento foliar, assimilação de CO_2 e menor dano de membrana em plantas tratadas com NaCl. Além disso, uma melhor performance das plantas sob salinidade e supridas com NO_3^- 10 mM foi indicada por maior rendimento quântico efetivo do PSII e taxa de transporte de elétrons e menor excesso de energia ao nível de PSII e quenching não-fotoquímico. Em outra abordagem, os dados mostraram que o NO_3^- exógeno e sua assimilação podem mitigar a toxicidade do NH_4^+, especialmente quando combinado com a salinidade, conforme mostrado pelos distúrbios no metabolismo oxidativo, atividade fotoquímica e assimilação de CO_2. Estes efeitos foram demonstrados quando diferentes razões de NO_3^-/NH_4^+ foram aplicadas em plantas intactas e discos foliares incubados com tungstato (um inibidor da NR) e dose única de NH_4^+ na presença ou ausência de MSO (um inibidor de GS). A atividade da GS *em Jatropha curcas* foi fortemente aumentada pelo NH_4^+ permitindo manter níveis não-tóxicos de amônia em tecidos sob moderadas/altas concentrações de amônio externos. Paralelamente, quando a relação exógena de NO_3^-/NH_4^+ diminuiu, as plantas sofreram redução no acúmulo de matéria seca associada com acumulação de espécies de oxigênio, diminuição da eficiência fotoquímica, surgimento de uma aparente fotoinibição e redução na assimilação de CO_2. Tomados em conjunto, os dados obtidos indicam que a assimilação de NO_3^- é capaz de mitigar os efeitos negativos da salinidade e de NH_4^+ , visto pelo seu efeito de proteção contra danos oxidativos e por atuar como um dissipador de elétrons das membranas tilacóides minimizando fotodanos e estimulando a assimilação de CO_2 em plantas de *Jatropha Curcas*.

1. Introdução geral

1.1. Caracterização da região semiárida

As regiões semiáridas do mundo são definidas como zonas de transição entre as regiões áridas e subúmidas. Geralmente, as características mais conhecidas que propiciam definir essas regiões como semiáridas são as baixas precipitações e temperaturas elevadas. Segundo Pessarakli & Zabolcs (1999), as regiões áridas e semiáridas representam um terço da superfície da terra e um levantamento geográfico estimou que as regiões semiáridas, principalmente dentro dos trópicos, cobrem maior parte das nações em desenvolvimento no mundo, incluindo a América Latina, grande parte do leste e sul da África, Índia e o sudoeste da Ásia (Fig. 1) (ICRISAT, 1998).

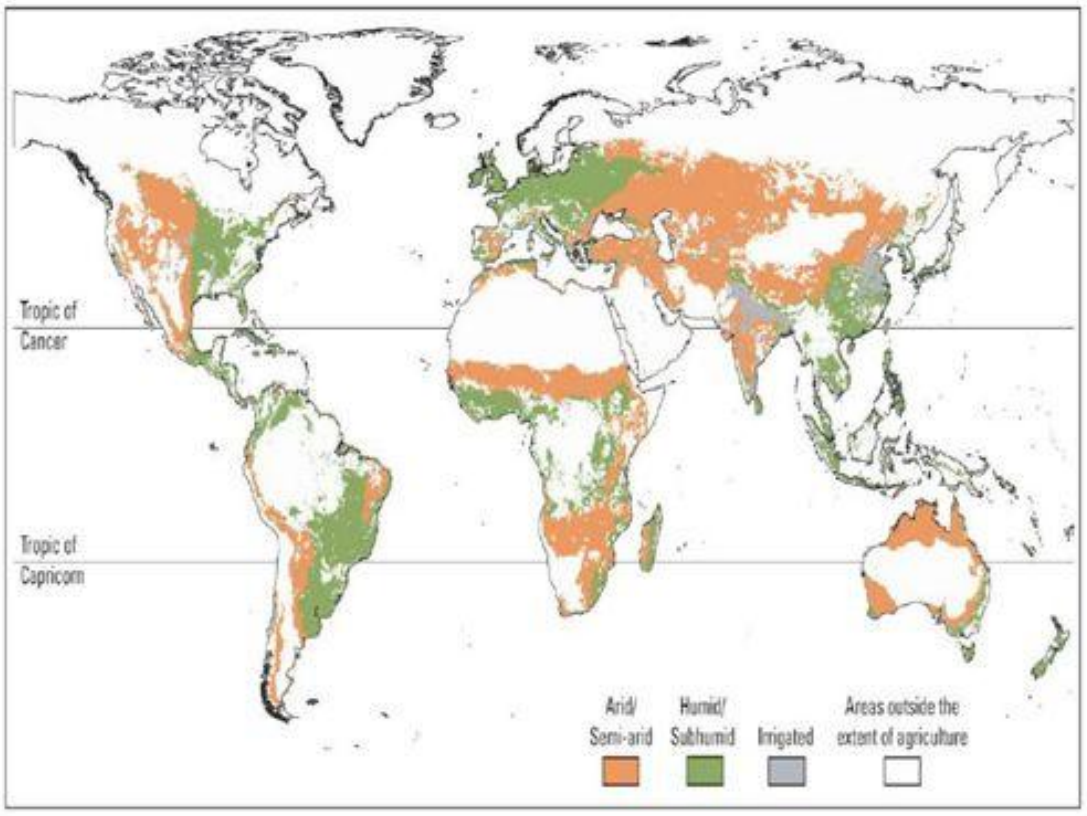

Figura 1 – Áreas semiáridas, subúmidas, irrigadas e fora da extensão da agricultura. (Fonte: www.syngetafoundation .org).

De acordo com estatísticas da população mundial, cerca de um bilhão de pessoas dependem economicamente dessas regiões e este número cresce a cada dia. No entanto, estas terras são desfavorecidas devido a condições climáticas adversas tais como: elevadas

temperaturas, baixas precipitações, salinidade e baixa fertilidade do solo (FLOWERS, 2004). As áreas semiáridas apresentam pelo menos um 3/4 do ano totalmente sem chuvas, sendo que na maioria delas a quantidade de chuvas varia de 500 – 1000 mm/ano. Dentro deste contexto, é importante salientar que por conta da baixa e má distribuição da precipitação nessas áreas, aumentou-se intensivamente o uso da irrigação para compensar a falta de precipitações, sendo que esta prática tem sido alvo de preocupação, pois leva ao acúmulo de sais pela alta evaporação, principalmente, quando associada com água de baixa qualidade, o que agrava os efeitos da salinidade e fertilidade do solo, afetando diretamente a produtividade das principais culturas adaptadas a essa região (MUNNS, 2002).

As características da região semiárida associadas às desigualdades sociais condicionam fortemente a população das áreas rurais a sobreviver principalmente de atividades econômicas ligadas à agricultura e a pecuária. Estas atividades se realizam sempre buscando o melhor aproveitamento possível das condições naturais desfavoráveis, ainda que apoiadas em base teórica frágil, utilizando na maior parte dos casos, tecnologias tradicionais (SUDENE, 2008). De modo geral, as regiões semiáridas continuam sendo um problema para a agricultura mundial constituindo uma notável necessidade e dificuldade que a população enfrenta em obter tecnologias mais avançadas para enfrentar as adversidades impostas pela natureza, principalmente no semiárido brasileiro.

1.2. Salinidade

Segundo Munns & Tester (2008) estima-se que 6% das terras do mundo e 30% das áreas irrigadas sejam afetadas pela salinidade. No entanto, uma porção significante das terras destinadas à agricultura tem se tornado salina por causa de desmatamentos e irrigação de má qualidade (MUNNS, 2005). A qualidade da água para irrigação é, geralmente, muito indesejável. Por isso, essa forte ligação entre irrigação e salinização tem provocado uma discussão imediata sobre o seu uso para o aumento da produção agrícola (FLOWERS, 2004).

No Brasil, cerca de 20-25% das áreas irrigadas têm problemas de salinidade ou de drenagem (SUDENE, 2008). Além destas áreas, cerca 2,5% da superfície total do nosso território possuem áreas naturalmente afetadas por sal. De acordo com uma prospecção de solo feita para os estados de Alagoas, Bahia, Ceará, Paraíba, Rio Grande do Norte e Sergipe, compreendendo 110 milhões de hectares, as áreas afetadas por sal foram estimadas em 9,1 milhões de hectares, cerca de 9% da área pesquisada, incluindo solos salinos e sódicos (Fig.2).

Irrigação excessiva, ineficiente gerenciamento do solo e dos recursos hídricos, invasão de águas marinhas e drenagem insuficiente, com consequente aumento nos níveis dos lençóis freáticos e de áreas de alagamento, solos rasos e com baixa fertilidade e irrigação com águas salinas sem análises adequadas, são as principais causas da salinidade nos solos do território brasileiro (SUDENE, 2008).

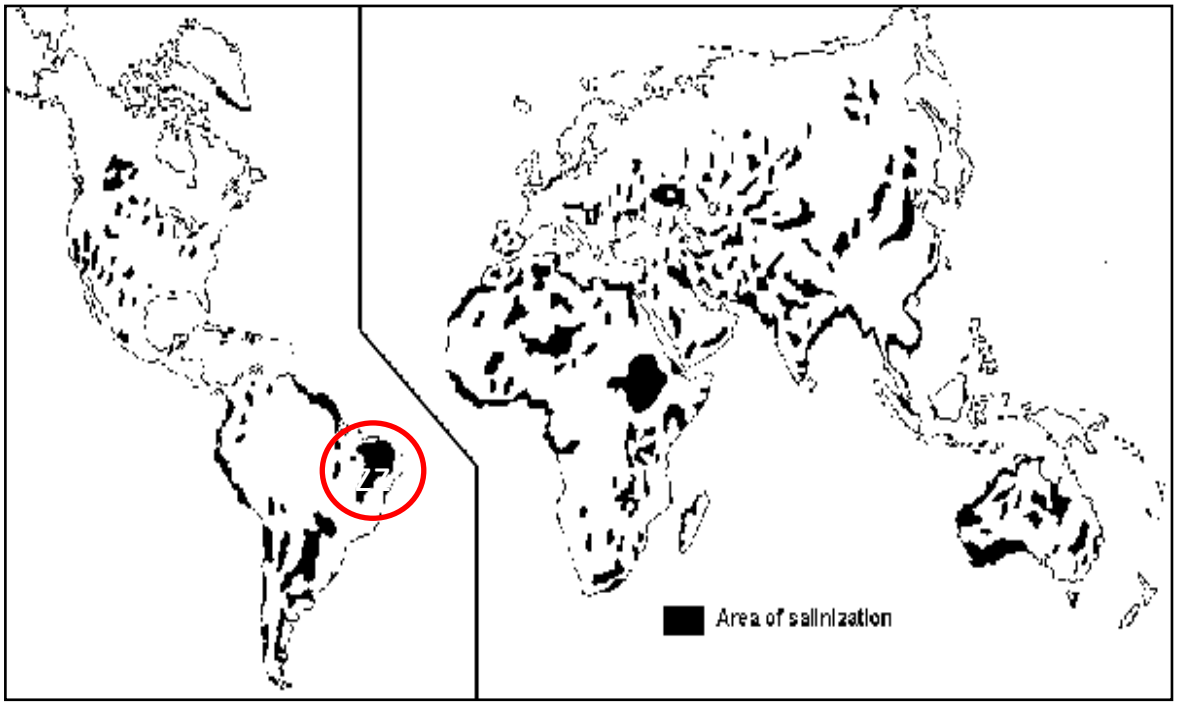

Figura 2 – Visão geral das áreas afetadas pela salinidade em todo mundo. (Fonte: Rhoades et al., 1996).

A salinidade é um fator conhecido por limitar o crescimento e a produtividade das culturas. Os mecanismos de tolerância à salinidade ainda não estão bem elucidados, pelo fato deste fenômeno ser extremamente complexo, podendo envolver alterações morfológicas e de desenvolvimento, bem como processos fisiológicos e bioquímicos (SHANKER; VENKATESWARLU, 2011). Os efeitos da salinidade no crescimento e desenvolvimento das plantas são assim enumerados: 1º- efeito osmótico, posto que uma elevada concentração salina diminua o potencial osmótico do solo, fazendo com que este retenha mais água, disponibilizando-a em menor quantidade para a planta (GHEYI, 2000); 2º- efeitos iônicos, caracterizados pelo acúmulo de íons específicos na planta.

Por exemplo, um excesso de Na^+ e de Cl^- no protoplasma leva a distúrbios na fotofosforilação, cadeia respiratória, assimilação de nitrogênio e o metabolismo proteico (MUNNS, 2002); 3º- efeito nutricional, no qual o excesso de um íon no solo inibe a absorção de outros íons; por exemplo, quando a concentração de Na^+ e Cl^- no solo é alta, a absorção de nutrientes minerais, especialmente NO_3^-, K^+ e Ca^{2+} são quase sempre reduzidas (MARSCHNER, 1995).

Em resposta à salinidade, muitas plantas acumulam osmólitos compatíveis no citoplasma de suas células, na tentativa de combater o efeito osmótico produzido por esse estresse (MUNSS; TESTER, 2008). Em outras palavras, o fenômeno de ajustamento osmótico é mantido pela acumulação e perda regulada de íons inorgânicos e de solutos orgânicos de baixa massa molecular, o que torna possível a manutenção da absorção de água e da pressão de turgescência da célula. Dessa forma, o ajustamento osmótico pode contribuir para a manutenção de processos fisiológicos, tais como: abertura estomática, fotossíntese, alongamento e divisão celular (SERRAJ & SINCLAIR, 2002; STRANGE, 2004). O ajustamento osmótico representa, assim, um importante mecanismo de aclimatação das plantas às condições de seca ou salinidade.

É válido destacar que, entre as espécies vegetais e dentro de cada espécie, pode-se encontrar variabilidade genética em relação à tolerância à salinidade (AYERS; WESTCOT, 1999), a qual pode estar relacionada com diferenças em respostas morfo-fisiológicas (KUIPER, 2000; LACERDA et al., 2003). Em muitas espécies o Na^+ e Cl^- são efetivamente excluídos da parte aérea através de retenção nas raízes independente da transpiração. Ao contrário, nas plantas halófitas, constituintes da flora natural de solos altamente salinos, esse mecanismo geralmente não existe, ocorrendo grande acumulação de íons salinos nas folhas (MUNNS, 2005).

Assim, de maneira geral, as plantas têm mostrado alternativas adaptativas por meio de mecanismos fisiológicos e bioquímicos para crescerem e se reproduzirem em ambientes salinos; segundo especialistas no assunto, duas estratégias são essenciais às plantas e uma delas é evitar o acúmulo excessivo de sais, principalmente no tecido fotossintetizante. Desta forma, as plantas não sofreriam consequências de efeitos iônicos e osmóticos (MUNNS; TESTER, 2008). A outra seria acumular sal no tecido fotossintetizante, porém de forma compartimentalizada, evitando que os mesmos afetem os processos e as funções essenciais do vegetal (JACOBY, 1999).

1.3. A importância do nitrato para as plantas

O nitrogênio é considerado um elemento fundamental para o crescimento e desenvolvimento de todas as plantas cultivadas, que o requerem em grandes quantidades. Normalmente, o nitrogênio é absorvido da solução do solo, na forma de íons de nitrato (NO_3^-) ou amônio (NH_4^+) (SMITH; GALLON, 1993). De todos os elementos químicos necessários às plantas, o nitrogênio (N) é o que é requerido em maiores quantidades, perfazendo até 2% de sua massa seca total (MILLER; CRAMER, 2004). Além da sua grande importância, o NO_3^- é a principal fonte de N devido sua maior disponibilidade no solo em comparação ao NH_4^+. Entretanto, a solução do solo frequentemente apresenta baixas concentrações de NO_3^-, o que limita o crescimento das plantas (SILVEIRA et al., 2001). Ao ser absorvido pelas raízes, o NO_3^- é transportado para as partes em crescimento da planta. Por fim, o nitrogênio é armazenado nas sementes e das culturas agronomicamente importantes, como os cereais e as leguminosas, que são de considerável valor comercial.

Em resposta aos diversos habitats, as plantas evoluíram múltiplas estratégias para adquirirem o nitrogênio, dentre elas, a absorção de nitrato e a fixação do nitrogênio. Em muitos solos, especialmente aqueles com cultivos anuais, o nitrato é a fonte nutricional mais abundante de nitrogênio, sendo que, sua concentração no solo varia entre 1 e 5 mM, podendo alcançar valores de até 5 μM, em consequência de sua ampla utilização pelas plantas e microrganismos e, adicionalmente, de sua lixiviação do solo (MILLER; CRAMER 2004). Na agricultura, baseada em altas produtividades, pressupõe elevadas aplicações de insumos, a fim de suprir a demanda nutricional das plantas. Desta forma, a adubação nitrogenada é, normalmente, a mais consumida devido à alta exigência em N das culturas. Entretanto, segundo Mortvedt et al. (1999), a eficiência no aproveitamento do fertilizante nitrogenado adicionado ao solo, em particular nas regiões tropicais, está em torno de 50-70%, fazendo com que parte do investimento em adubação não tenha o retorno esperado.

A variação do nível de NO_3^- no solo pode afetar a taxa de crescimento e a concentração de nitrato armazenada nas plantas. Quando fornecidos em grandes quantidades, as concentrações na raiz e parte aérea podem atingir até 100 mM, mais do que é armazenado no interior dos vacúolos (MILLER & SMITH, 1996). O íon NO_3^- também serve como um importante sinalizador para o crescimento de plantas, em função da alteração de seu metabolismo e pela indução de genes relacionados a sua absorção e assimilação. Estes genes codificam transportadores que reconhecem o nitrato da solução do solo e enzimas específicas convertem o nitrato a amônio dentro das células (MILLER e al., 2007). Outros dados sugerem

que o NO_3^- atua como um sinalizador para a regulação do metabolismo do carbono, por exemplo, pela modulação da expressão de genes envolvidos na biossíntese de ácidos orgânicos (FOYER et al., 2001).

O NO_3^- presente no solo é absorvido pelas raízes através da membrana plasmática de células da epiderme e do córtex da raiz, principalmente em regiões mais jovens (TAIZ; ZEIGER, 2008). Após a absorção, o NO_3^- poderá tomar quatro destinos: (a) ser imediatamente reduzido por ação de enzimas como redutase de nitrato (NR) e redutase de nitrito (RNi). A amônia produzida por RNi é então assimilada pela via GS/GOGAT (glutamina sintetase/glutamato sintase); (b) A segunda alternativa será a mobilização do NO_3^- das raízes para a parte aérea, a partir do descarregamento no xilema. (c) O terceiro caminho será o influxo do nitrato para os vacúolos das raízes ou de outra parte vegetativa, constituindo o "pool" de armazenamento e (d), sofrer efluxo para o apoplasto, através da membrana plasmática (Fig. 3) (FORDE, 2000; TISCHNER; KAISER, 2007). A fração de nitrato no citosol é bastante pequena, a qual constitui o "pool" metabólico, que é ativo para a indução de síntese de proteínas e atividade de RN.

Fatores abióticos como a salinidade podem interferir na absorção, assimilação e transporte de NO_3^- via xilema. Alguns autores têm relatado uma redução da absorção de NO_3^- pela salinidade resultando em níveis reduzidos deste íon nos tecidos (SILVEIRA et al., 2001; PARIDA; DAS, 2005). Estudos com plantas de feijão caupi tratadas com 100 mM de NaCl exibiram uma grande sensibilidade nos processos de absorção e assimilação de NO_3^- (SILVEIRA et al., 1999). Recentemente, o mesmo grupo também mostrou que salinidade afeta mais negativamente o fluxo de nitrato no xilema do que os processos de absorção e assimilação desse íon nas folhas de feijão caupi (ARAGÃO et al., 2010).

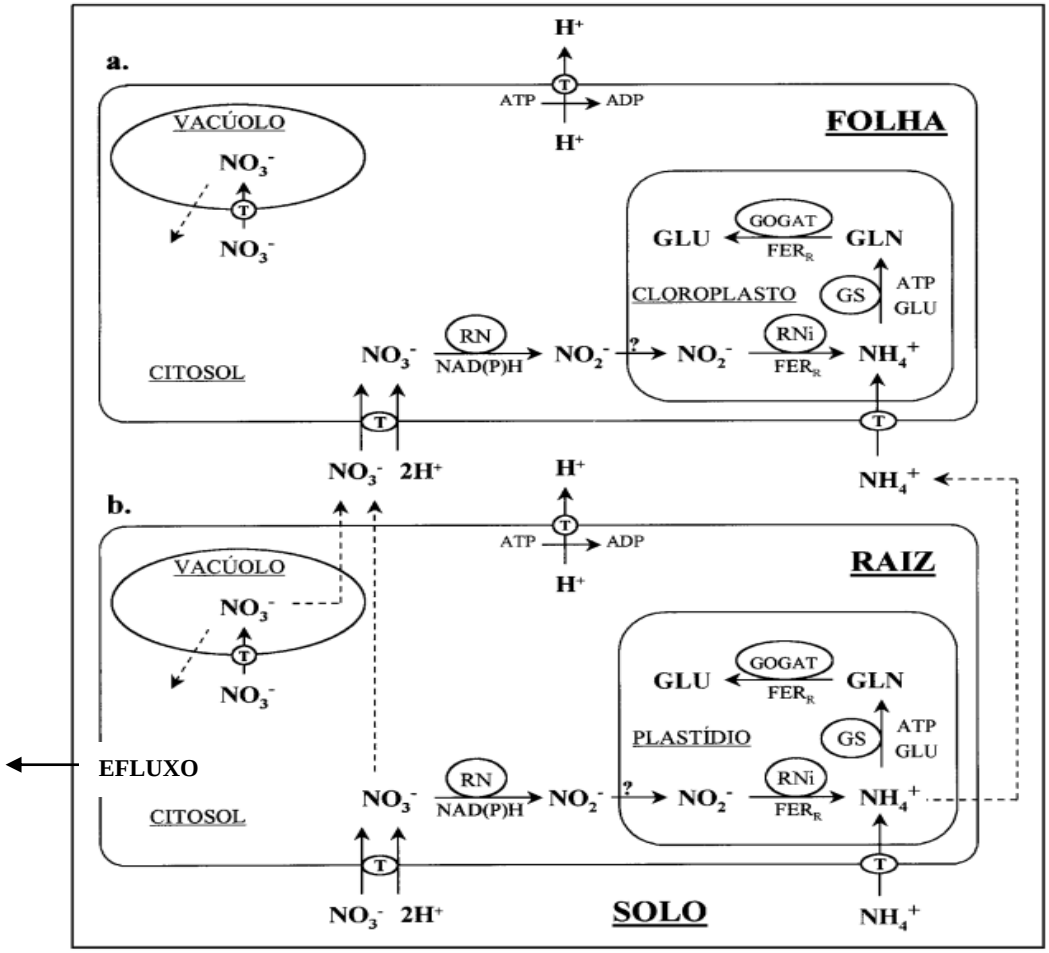

Figura 3 – Nitrato e os caminhos percorridos. (Fonte: adaptado de Bredemeier & Mundstock, 2000).

1.4. A cultura do Pinhão Manso (*Jatropha curcas.*L).

O pinhão manso (*Jatropha curcas* L.), pertencente à família das Euforbiáceas, a mesma da mamona e da mandioca, é considerada uma opção agrícola para a região nordeste por ser uma espécie nativa, exigente em insolação e com forte resistência à seca (Fig. 10) (OPENSHAW, 2000). O pinhão manso é amplamente cultivado no México, Nicaragua, Tailândia e em partes da Índia. Atualmente tem-se encontrado produção na África do Sul, Brasil, Mali e no Nepal (OPENSHAW, 2000). No Brasil essa espécie já é conhecida comercialmente como é uma planta oleaginosa conhecida pela sua utilização para a extração de óleo que serve para a fabricação de sabão, como purgativo para o gado bovino e principalmente, como fonte de energia (Biodiesel) (PRAMANIK, 2003). Ensaios feitos com o óleo extraído do pinhão-manso (óleo-de-purgueira), comparando-o com o diesel, deram bons resultados mostrando ser um óleo com características promissoras (CARNIELLI, 2003). Assim, acompanhando o movimento mundial, o governo brasileiro tem aumentado cada vez mais sua atenção para as culturas oleaginosas e aos projetos destinados à pesquisa do biodiesel. No entanto, foi a partir do lançamento do Programa Nacional de Produção e Uso do Biodiesel (PNPB), em dezembro de 2004, pelo Governo Federal, que o biodiesel avançou significativamente, tornando-se um instrumento de geração de riqueza e inclusão social.

A produtividade do pinhão manso pode variar em função da região de plantio, método de cultivo, tratos culturais, idade da cultura, bem como da quantidade de chuva e da fertilidade do solo. O pinhão manso produz, no mínimo, duas toneladas de óleo por hectare/ano. Adam (1953) relatou um rendimento de 4 a 5 kg de frutos por planta e Peixoto (1973) afirma que o rendimento dessa cultura varia de 500 a 1.200 kg de sementes limpas por hectare. O início da produção leva de três a quatro anos para atingir a fase economicamente produtiva, que pode se estender por longos períodos. Experiências recentes com o pinhão manso, a cargo de algumas instituições agrícolas do país, cujos resultados definitivos ainda demandam algum tempo, comprovam o interesse crescente no conhecimento agronômico da cultura. Em escala mundial, existe pouco conhecimento sobre esta planta, cujo gênero tem mais de 170 espécies, sendo a mais importante a *Jatropha curcas* L. e somente nos últimos 30 anos é que foi iniciado estudos agronômicos sobre a mesma, sendo ainda uma espécie considerada não domesticada (SATURNINO *et al.*, 2005).

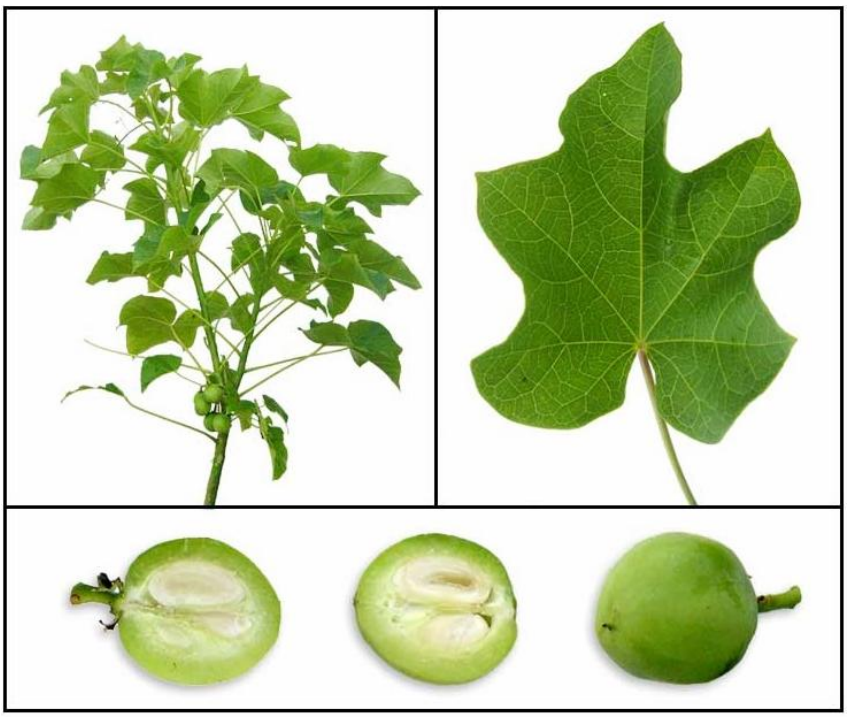

Figura 4 – Pinhão manso (*Jatropha curcas* L.).

As perspectivas que favorecem a implantação racional da cultura do pinhão manso decorrem não somente dos baixos custos de sua produção agrícola, conforme se deve esperar diante das vantagens anunciadas, mas, sobretudo porque ela poderá ocupar solos pouco férteis e arenosos e, de modo geral, reduzir os impactos sociais, econômicos e ambientais favoráveis à agricultura de subsistência. Não há duvida de que a cultura racional do pinhão manso, desenvolvida com o emprego de melhores técnicas, deverá constituir-se entre as mais promissoras fontes de grãos oleaginosos para fins carburantes. Além do alto índice de produtividade, as maiores facilidades de seu manejo agrícola e de colheita das sementes, com relação a outras espécies como palmáceas, tornam a cultura do pinhão manso bastante atrativa e especialmente recomendada para um programa de produção de óleos vegetais (Biodiesel). Outros aspectos positivos referem-se à possibilidade de armazenagem das sementes por longos períodos de tempo, sem os inconvenientes da deterioração do óleo por aumento da acidez livre, conforme acontecem com os frutos de dendê ou de macaúba, ambos os quais devem ser processados o mais depressa possível (PRAMANIK, 2003).

Apesar de tolerante à seca, o pinhão manso pode ter a produtividade comprometida, em regiões com precipitações pluviais abaixo de 600 mm ano^{-1} (SATURNINO *et al.*,, 2005), o que freqüentemente ocorre no semi-árido brasileiro. Nos mais recentes trabalhos publicados o pinhão manso tem mostrado uma série de características como aparente sensibilidade à salinidade devido a um grande acúmulo de Na^+ e Cl^- nas folhas (SILVA *et al.*, 2009). No

entanto, o mesmo grupo evidenciou um mecanismo de recuperação após a substituição da solução salina por outra sem NaCl (SILVA *et al.*, 2010; 2011) Mais recente ainda, foi mostrado que plantas jovens de *J.curcas* submetidas à salinidade apresentam danos fotossintéticos intensos causados tanto por limitações estomáticas como por limitações bioquímicas (SILVA *et al.*, 2011). Em condições de estresse combinado (Calor + Sal) foi demonstrada uma estimulação de enzimas antioxidantes como APX e SOD, ao passo que a expressão da CAT foi estimulada somente por sal (SILVA et al., 2012).

Mesmo tendo como ponto positivo a facilidade de cultivar o pinhão manso em solos poucos férteis, poucas informações sobre os aspectos nutricionais são conhecidas. Além disso, ainda não existem trabalhos que relatam os efeitos da adubação nitrogenada sobre o crescimento e desenvolvimento de *J.curcas*. De modo geral, a carência de informações relevantes deixa evidente a necessidade de mais pesquisas sobre a cultura do pinhão manso no sentido de se conhecer alguns aspectos fisiológicos e bioquímicos desta cultura promissora.

2. Justificativa

O pinhão manso (*Jatropha curcas* L.) pertence à família das euforbiáceas e é distribuído nas regiões áridas e semiáridas da América do Sul e em todas as regiões tropicais do Planeta (KUMAR et al., 2008). Recentemente, com o advento do Programa Brasileiro de Biodiesel e o surgimento de grande demanda por óleos vegetais, o pinhão manso tem sido divulgado como uma alternativa para fornecimento de matéria-prima. Esta escolha se baseia na expectativa de que a planta possua alta produtividade de óleo, tenha baixo custo de produção (por ser perene) e seja resistente ao estresse hídrico, o que seria uma vantagem significativa principalmente na região semiárida do país (FRANCIS et al., 2005; SEVERINO et al., 2006).

As perspectivas favoráveis à implantação racional da cultura do pinhão manso decorrem não somente dos baixos custos de sua produção agrícola, mas, sobretudo porque ela poderá ocupar solos pouco férteis e arenosos e, de um modo geral, diminuir os impactos sociais, econômicos e ambientais encontrados especialmente na agricultura de subsistência.

Apesar de ser uma cultura promissora, a caracterização fisiológica e bioquímica dessa espécie em condições de estresse como salinidade ainda permanece pouco explorada. Menos explorado ainda são os estudos direcionados ao entendimento dos componentes metabólicos diretamente relacionados com os mecanismos de danos e proteção celular, sob essas condições estressantes típicas das regiões semiáridas. Adicionalmente, ainda são poucos os estudos sobre aspectos nutricionais desta espécie, principalmente, sobre a nutrição nitrogenada. Esta ênfase a nutrição nitrogenada deve-se a estudos anteriores que demonstraram que sob condições de salinidade, os processos de absorção e assimilação do nitrogênio são afetados (SILVEIRA et al., 2001; ARAGÃO et al., 2010), bem como os processos fotossintéticos (SILVA et al., 2010; 2011).

Dessa forma, alguns questionamentos são extremamente pertinentes para o pinhão manso. Plantas de pinhão manso apresentam mecanismos de tolerância (modulação de solutos orgânicos) à salinidade? Qual a fonte de nitrogênio dentre as mais comuns promovem maior crescimento da cultura? A nutrição nitrogenada pode proteger o pinhão manso contra os efeitos deletérios da salinidade e danos oxidativos? A assimilação do nitrato pode proteger o sistema fotossintético contra o aumento da pressão de elétrons na cadeia transportadora de elétrons fotossintéticos, atuando como dreno de elétrons? Até que ponto a nutrição por amônio pode ser benéfica sem causar danos tóxicos a esta espécie? Essas perguntas pertinentes são respondidas utilizando abordagens experimentais como as descritas neste presente estudo.

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4. Objetivos

4.1. Objetivo geral

Diante do exposto, o presente estudo visou à obtenção de dados que possam auxiliar na compreensão do metabolismo do nitrogênio sobre os processos de assimilação de CO_2 em plantas de *Jatropha curcas* expostas ao estresse salino

4.2 Objetivos específicos

- Realizar uma revisão de literatura sobre Nitrogênio x assimilação de CO_2 x salinidade;

- Avaliar se o processo de assimilação do nitrato pode mitigar efeitos negativos da salinidade, bem como os danos oxidativos induzidos por este estresse;

- Avaliar se o processo da assimilação de nitrato pode atuar como um importante dreno alternativo de elétrons na cadeia transportadora de elétrons fotossintéticos, minimizando fotodanos e estimulando a assimilação de CO_2;

- Caracterizar a nutrição de amônio e nitrato em *J.curcas* quando expostas à salinidade e avaliar seus efeitos sobre a proteção oxidativa, assimilação de CO_2 e atividade fotoquímica;

- Avaliar o efeito da inibição de duas enzimas da assimilação de nitrato (NR) e amônio (GS) sobre a proteção oxidativa, assimilação CO_2 e atividade fotoquímica.

5. Estratégia experimental

O presente estudo foi dividido em experimentos sequenciais e independentes que resultaram na produção de três capítulos, sendo um capítulo inicial de revisão de literatura e outros dois que foram arranjados em forma de artigos científicos. O primeiro capítulo trata-se de uma revisão que descreve os principais conceitos que envolvem o presente estudo, os achados na literatura e os pontos ainda não explorados (Capítulo I). O segundo trata-se de um artigo que tem como título "**High supply of NO_3^- mitigates salinity effects through an enhancement in the efficiency of photosystem II and CO_2 assimilation in *Jatropha curcas* plants**" (Capítulo II). O terceiro tem o título de "**Nitrate alleviates NH_4^+-toxicity and Salinity by Restricting of Oxidative Damage, Improvement of Photochemistry Activity and CO_2 Assimilation in *Jatropha plants***" (Capítulo 3). A descrição detalhada de cada um dos capítulos mencionados acima é mostrada a seguir.

Capítulo I

Revisão de Literatura

1. Assimilação fotossintética do nitrogênio

O nitrogênio (N) é um dos principais elementos requeridos pelos vegetais exercendo importantes papéis na nutrição e crescimento das plantas. Este elemento é responsável pela formação de biomoléculas importantes como ATP, NADH, NADPH, clorofila, proteínas e inúmeras enzimas (MORALES et al., 2006). Grande parte desta alta demanda por N reflete a grande importância deste elemento sobre o aparato fotossintético (ABROL et al., 1999) e sua disponibilidade é um ponto determinante para a capacidade fotossintética e rendimento das culturas agrícolas (KUMAR et al., 2004). Assim, as plantas podem absorver e assimilar várias formas de N como amônio, nitrato e uréia.

Na maioria dos sistemas de produção a disponibilidade de N é quase sempre um fator limitante, razaão pelo qual influencia o crescimento da planta mais do que qualquer outro nutriente. Além de ser um fator limitante ao crescimento, o N apresenta alta mobilidade no solo e por isso tem sido intensamente estudado no sentido de maximizar a eficiência do seu uso (BRITTO; KRONZUNCKER, 2005). Para tanto, tem-se procurado diminuir as perdas de N no solo, bem como melhorar a sua absorção e metabolização no interior da planta (FAN *et al*., 2009). Além desses aspectos limitantes, fatores ambientais como a salinidade são grandes problemas que afetam não somente os processos de absorção e assimilação de N (Fig. 1) (ARAGÃO et al., 2011), mas principalmente, o uso deste elemento para outros processos vitais ligados ao fechamento estomático e fotossíntese (Fig. 2) (SILVA et al., 2011)

No tocante a fotossíntese, o método mais utilizado para compreender como a fotossíntese em plantas C_3 responde a perturbações é o de Farquhar (FARQUHAR et al., 1980). Neste modelo, as reações bioquímicas da fotossíntese são consideradas em dois estados estacionários distintos. Em um estado, a taxa de fotossíntese pode ser prevista pelas propriedades de ribulose 1,5-bisfosfato carboxilase/oxigenase (rubisco), assumindo um suprimento saturante de substrato, RuBP; e este estado é chamado de fotossíntese limitada pela rubisco e normalmente ocorre quando a $[CO_2]$ é baixa. A limitação pela Rubisco está associada mais com menor $[CO_2]$ do que com a V_{max} da enzima (SHARKEY et al., 2007). No outro estado, as taxas fotossintéticas são previstas assumindo que a taxa de regeneração de RuBP é limitada, e então a RuBP é utilizada a uma taxa constante. Este estado é chamado de fotossíntese limitada pela regenração da RuBP. Esta condição ocorre em maior $[CO_2]$. A fotossíntese limitada pela regeneração da RuBP inclui as condições onde a intensidade da luz limita a taxa da fotossíntese, mas pode também incluir condições em que enzimas do ciclo de Calvin (exceto Rubisco) limitam a taxa de fotossíntese (Figura 3) (SHARKEY *et al.*, 2007).

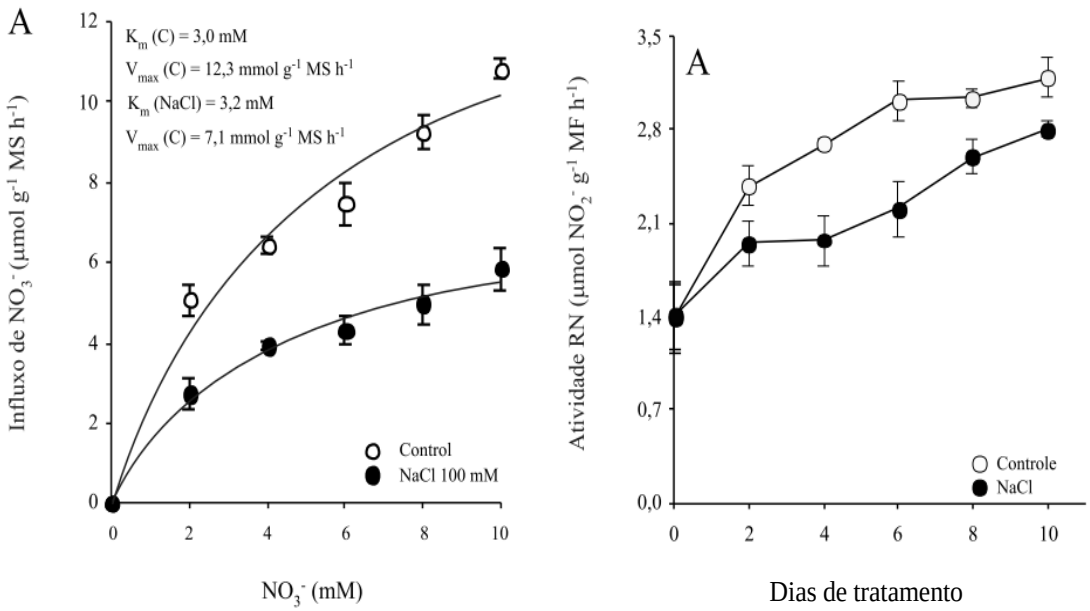

Figura 1 – Influxo de NO_3^- em função da concentração crescente de NO_3^- e atividade da enzima redutase do nitrato – RN ao longo do tempo em folhas de feijão caupi submetidas ao tratamento com NaCl 100 mM. (Fonte: Aragão et al., 2011).

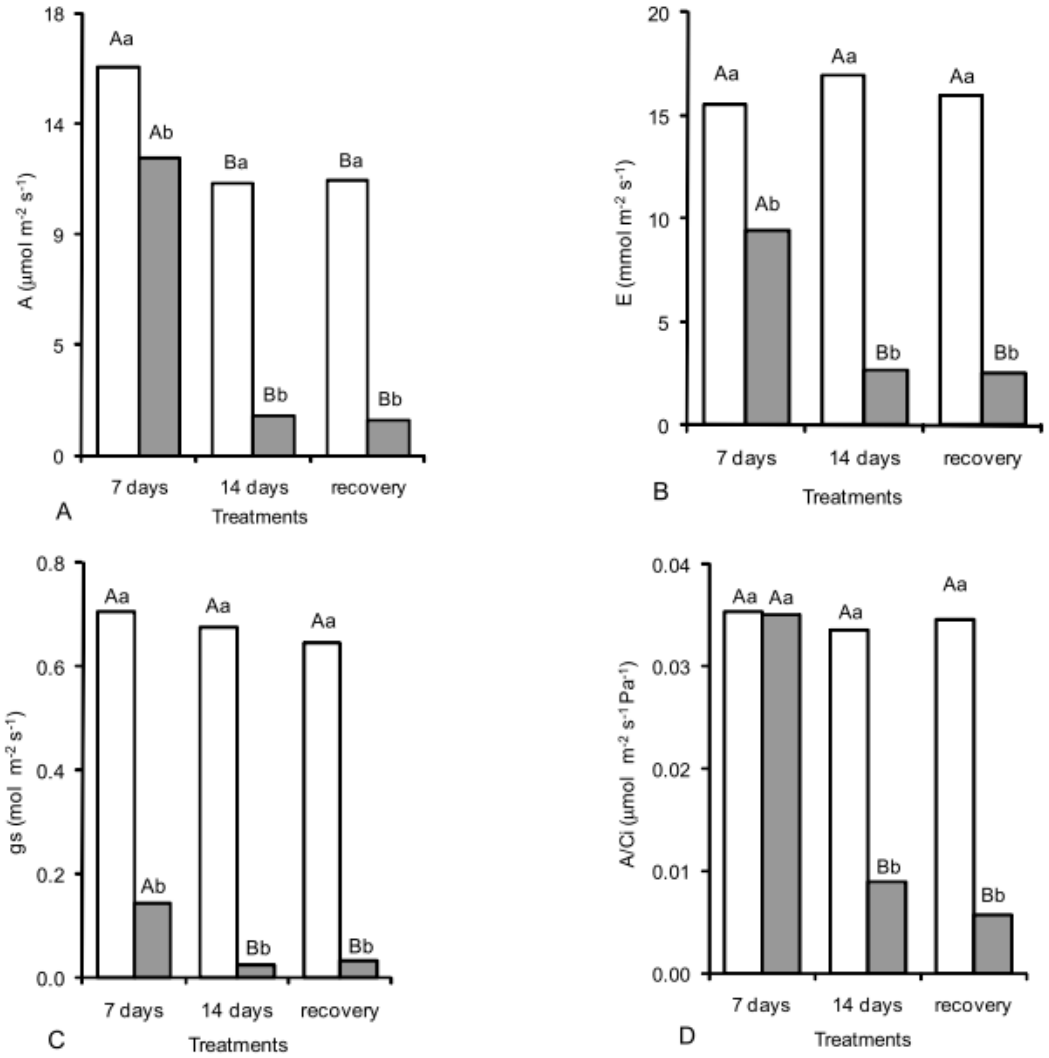

Figura 2 – Taxa de assimilação de CO_2 (A), transpiração (B), condutância estomática (C) e eficiência instantânea de carboxilação (D) em planta de *J.curcas* cultivadas em ausência e presença de NaCl (100 mmol L^{-1}) durante 7-d and 14-d e sua recuperação após 3-d. Barras brancas representam plantas controle a barras cinzas representam plantas tratadas com NaCl. (Fonte: Silva et al., 2011).

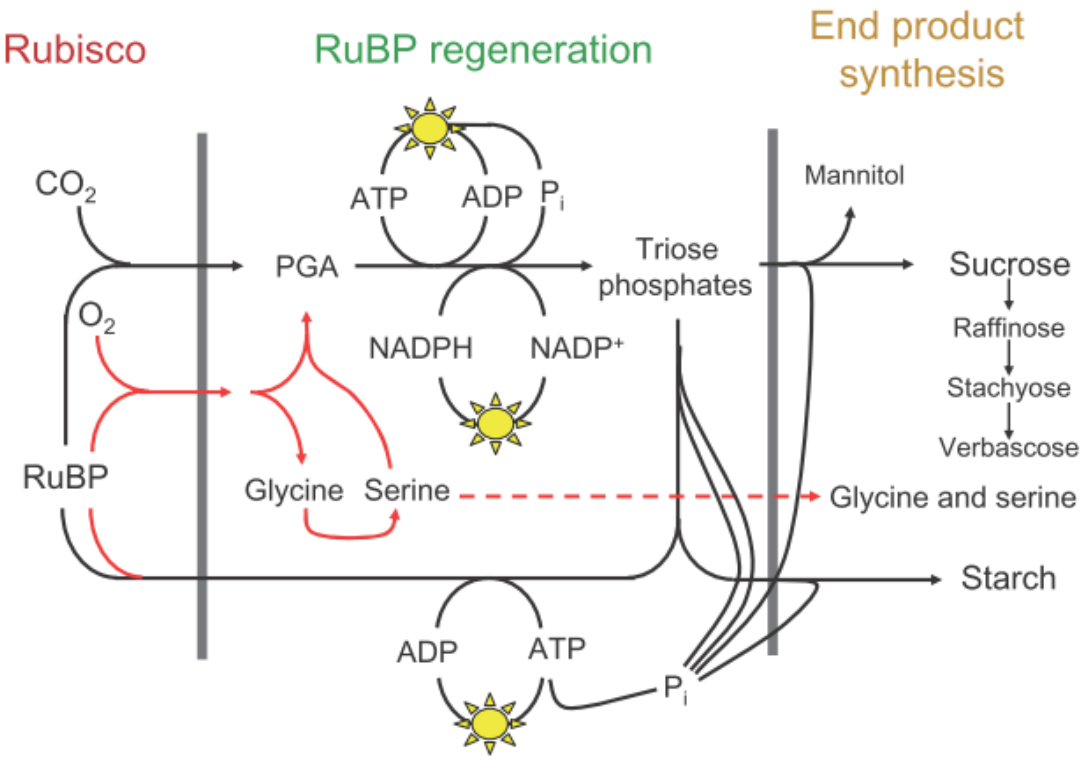

Figura 3- Esquema mostrando os extágios estacionários das reações bioquímicas da fotossíntese. (Fonte: Sharkey et al., 2007).

Uma maneira de se estudar o potencial da síntese de rubisco é por análise de sua variação espacial em lâminas de folhas expandidas porque nesta fase, a degradação da Rubisco é mínima (SENEWEERA E CONROY, 2005). Em geral, o crescimento das folhas apresenta um padrão de divisão celular, alongamento e maturação, com aumento da distância da lígula (MACADAM; NELSON, 2002). Na zona de divisão celular, a exigência de N é alta porque as rápidas taxas de síntese protéica são necessárias para a produção de novas células. Para a zona de expansão celular, o N é necessário para a síntese de proteínas fotossintéticas (GASTAL; NELSON, 1994; SKINNER; NELSON, 1994). Assim, a alocação de N para dentro de células das zonas de expansão e maturação pode ser a chave para síntese de proteínas fotossintéticas (SENEWEERA et al., 2011). Quick et al. (1992) mostraram uma forte relação entre nitrogênio e rubisco quando cultivou plantas de tabaco em condições limitadas de N. Assim, essas plantas crescidas com menores níveis de nitrogênio apresentavam menores conteúdo de rubisco, não em termos absolutos, mas também em relação a outras proteínas. Esses resultados evidenciaram que plantas de tabaco diminuem o investimento de rubisco em condições limitantes de N (Figura 4).

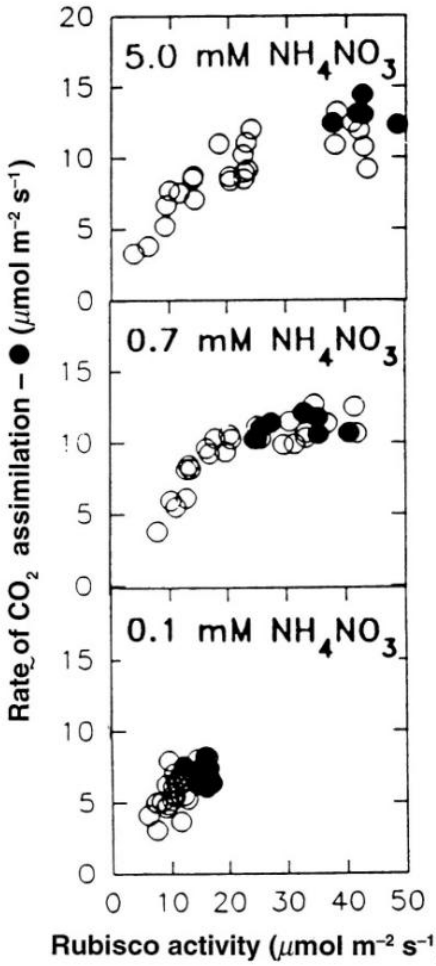

Figura 4 – Relação entre rubisco, fotossíntese e nitrogênio em plantas de Tabaco. (Fonte: Quick et al., 1992).

Nos cloroplastos, a bio-assimilação do N depende de poder redutor, produzido pela transferência de elétrons gerados pela fotossíntese através de uma série de complexos de proteínas na membrana do tilacóide (WILHELM; SELMAR, 2011). O vetor final dos destes elétrons a partir da membrana do tilacóide para o estroma dos cloroplastos é a proteína ferredoxina (Fd). A Fd atua sobre enzimas envolvidas na assimilação do nitrogênio, tais como a nitrito redutase (NiR) e a glutamina-oxoglutarato amidotransferase Fd-dependente (Fd-GOGAT). Em células fotossintéticas, 80% do poder redutor necessário para assimilação do N vêm diretamente da ferredoxina (FOYER; NOCTOR, 2002). Já o poder redutor para a atividade da redutase do nitrato (NR), enzima responsável pela assimilaçao do nitrato, é fornecido pela cadeia transportadora de elétrons fotossintéticos através da operação das lançadeiras ou pela via de oxidação respiratória (Fig. 5) (FOYER; NOCTOR, 2002).

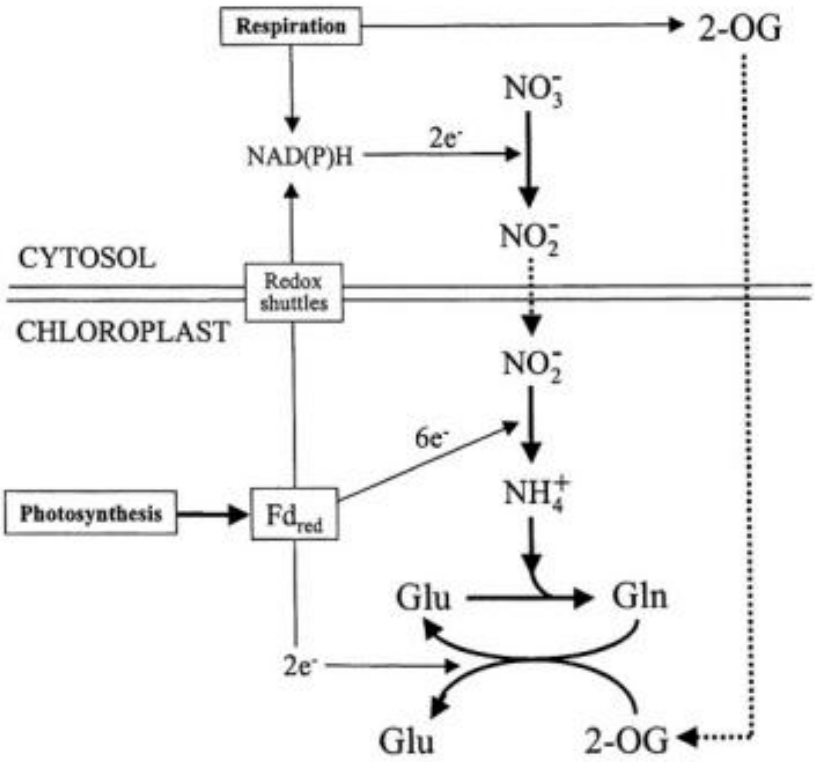

Figura 5 - Esquema mostrando a Ferredoxina (Fd) como vetor final de elétrons a partir da membrana do tilacoide para o estroma. A Fd atua sobre enzimas envolvidas na assimilação do nitrogênio, tais como nitrito redutase (NIR) e Fd-dependente glutamina-oxoglutarato amidotransferase (Fd-GOGAT). (Fonte: Foyer & Noctor, 2002.)

Evidências recentes têm destacado o papel da assimilação no nitrato como um importante dreno alternativo de elétrons para aliviar a pressão de elétrons na CTE decorrentes de fatores estresssantes (FOYER et al., 2009). Uma vez dentro das células, o nitrato pode ser armazenado dentro dos vacúolos para ser utilizado conforme a necessidade da planta, enquanto que outra fração é imediatamente metabolizada no citoplasma para reduzir-se a nitrito, via enzima NR, que consome 2e- por meio do NAD(P)H como doador de elétrons. O nitrito produzido é então transportado para o cloroplasto e reduzido a amônio pela enzima N_iR. Somente nessa reação são consumidos $6e^-$ por meio da Fd_{red} como doador de elétrons (Figura 6). Assim, a via de assimilação do nitrato até a formação de amônio é descrita abaixo:

Nitrato redutase (NR)

$$NO_3^- + NAD(P)H + 2e^- + H^+ \rightarrow NO_2^- + NAD(P)^+ + H_2O$$

Nitrito redutase (N_iR)

$$NO_2^- + 6Fd_{red} + 6e^- + 8H^+ \rightarrow NH_4^+ + 6Fd_{ox} + H_2O$$

Os íons nitrito e amônio não podem ser acumulados dentro das células, uma vez que apresentam toxicidade pela mudança de pH ao nível de membrana e por induzirem espécies reativas de nitrogênio (RNS) e danos oxidativos (CHOW, 2012). Consequentemente, sua incorporação a compostos orgânicos são relativamente rápidos, evitando acumulação e toxicidade. A assimilação de amônia-N em compostos de carbono (aminoácidos) ocorre primariamente pela ação da enzima glutamina sintetase (GS) e glutamina 2-oxoglutarato aminotransferase ou glutamato sintase (GOGAT), dentro dos cloroplastos. A assimilação do amônio pela GS requer glutamato e ATP como substrato. Já a GOGAT está presente em duas regiões. Uma primeira ocorre nos plastídios e tem o NADH como doador de elétrons para produzir glutamato. A NADH-GOGAT é presente em tecidos não fotossintéticos tais como raízes. A segunda está presente nas mitocôndrias e tem a Fd_{red} como doadora de elétrons. A Fd-GOGAT é encontrada no cloroplasto e atua no metabolismo fotorrespiratório (TAIZ; ZEIGER, 2008). As reações da assimilação de amônio são descritas abaixo:

Glutmina Sintetase (GS)

$$NH_4^+ + \text{glutamato (Glu)} + ATP \rightarrow \text{glutamina (Gln)} + ADP + Pi$$

Glutamato sintase (GOGAT)

$$\text{Glutamina} + \text{2-oxoglutarato} + NADH + H^+ + \rightarrow 2\ \text{Glutamato} + NAD^+$$

$$\text{Glutamina} + \text{2-oxoglutarato} + Fd_{red} \rightarrow 2\ \text{glutamato} + Fd_{ox}$$

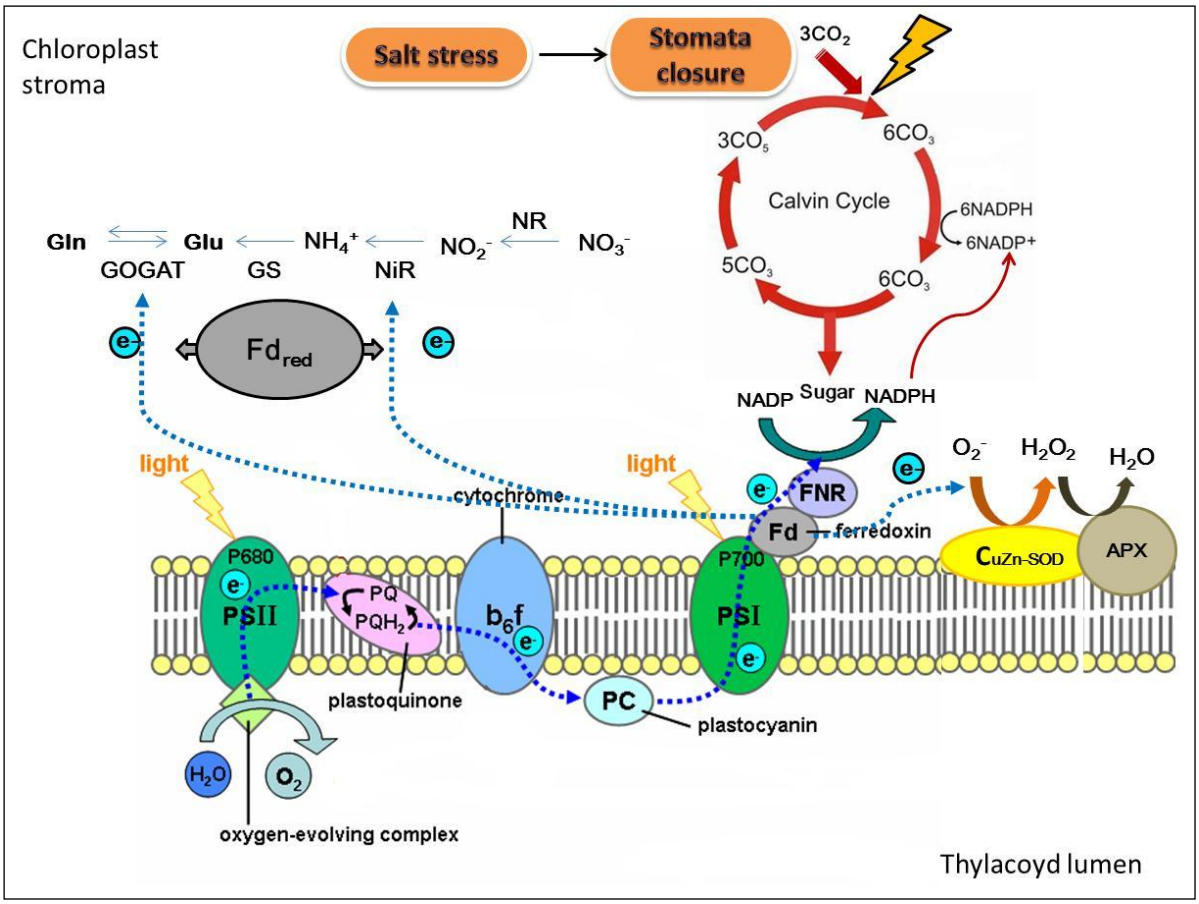

Figura 6 - Esquema mostrando a Ferredoxina (Fd) como dreno de elétrons a partir da membrana do tilacoide para o estroma em situação de fechamento estomático induzido por estresse salino. Os elétrons são utilizados por enzimas envolvidas na assimilação do nitrogênio, tais como nitrito redutase (NiR) e glutamina-oxoglutarato amidotransferase Fd-dependente (Fd-GOGAT).

A assimilação de nitrogênio também está integrada com a atividade fotorrespiratória. Um exemplo disso são os ácidos orgânicos que são modulados com a regulação do pH celular durante a redução do nitrato e também como aceptores de grupos amino para a síntese de aminoácidos (FOYER et al., 2001). Assim, o ciclo de fotorrespiratório é uma chave mais de interação entre C fotossintéticos e o metabolismo do N, envolvendo a regeneração e reassimilação de amônia através dos pools de Glicina (Gly), Serina (Ser), glutamina (Gln) e glutamato (Glu) em folhas (Fig. 6). Na maioria das espécies C3 pelo menos, a transferência de amônia pela fotorrespiração parece ser mais rapida do que a produção de amônio proveniente da redução de nitrato (FOYER et al., 2012).

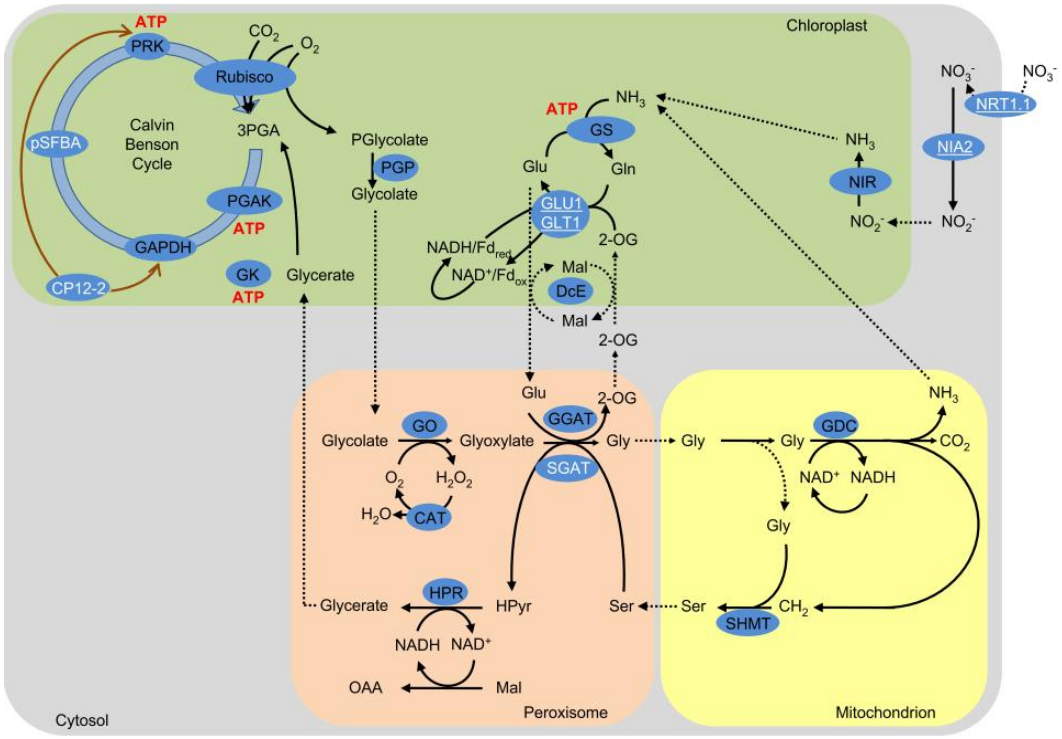

Figura 7 – Esquema simplifacado mostrando a redução fotossintética de carbono via ciclo de Calvin-Benson e o ciclo de oxidação de carbonos fotossintéticos associado com regenração e reassimilação de amônia. (Fonte: Foyer et al., 2012).

Existem algumas eventuais restrições de drenos de ATP mitocondriais e citosólicos no fluxo de elétrons respiratório e, assim, algumas interações entre fotossíntese e assimilação de N são descritas sobre esta situação. Um exemplo é o acoplamento redox que pode influenciar a integração da assimilação de N e metabolismo de C. A formação líquida de um 2-oxoglutarato (2-OG) a partir de açúcar fosfatado envolve uma produção líquida de quatro NAD(P)H (duas pelo glyceraldehyde3-fosfato-desidrogenase, um pela piruvato desidrogenase e outra pela isocitrato desidrogenase). Uma prporção de 50 a 75% deste poder redutor pode ser formado no citosol, dependendo da localização da oxidação do isocitrato. Mesmo se o nitrato atuar como receptor de elétrons para um quarto do poder redutor formado, ainda haverá um excesso que deve ser oxidado por outros meios, presumivelmente através da cadeia transportadora de elétrons. Logo, o transporte de elétrons mitocondrial e a enzima NR podem competir por redutores. Além disso, um número insufuciente de drenos poderia dificultar a produção de 2-OG e levar a uma acumulação de Glutamina (Gln) e, possivelmente, amônia no cloroplasto que pode ser tóxico dependendo da concentração (FOYER et al., 2001). Neste

contexto, aumento na atividade de algumas enzimas como a GS e GOGAT é de grande valor para mitigar os efeitos tóxicos da amônia.

Em geral, embora a redução de nitrato nas folhas seja geralmente apenas uma fração da taxa de fixação de C, a assimilação de nitrato pode ser um dreno potencialmente significativo para a energia fotossintética (LEWIS et al., 2000). Sob a maioria das condições, a asimilação de N é um verdadeiro processo fotossintético, na qual energia da luz é usada para alimentar a incorporação de redutores de uma molécula orgânica simples em compostos orgânicos. O requisito para a energia fotossintética se reflete na acentuada estimulação de redução de nitrato pela luz em muitas espécies vegetais. Adicionalmente, deve-se considerar que o produto final da atividade da NR, o nitrito, é um substrato da enzima nitito redutase (N_iR) que utiliza a Fd como doador de elétrons para produzir amônio. Nesse contexto, a assimilação de nitrato torna-se mais eficiente como dreno alternativo de elétrons do que a assimilação direta de amônio, pois o amônio limitaria a atividade da N_iR.

2. Efeitos da salinidade sobre a fotossíntese

A salinidade é conhecida por reduzir a produtiviadade das plantas (Das, 2012) A redução da produtividade observada para a maioria das espécies vegetais submetidas à salinidade é associada com mudanças nos processos fisiológicos, tais como a fotossíntese, devido tanto a fatores estomáticos quanto não estomáticos (LORETO et al., 2003). Em condiçoes de alta salinidade, os sais podem acumular-se nas folhas em nívies elevados causando toxicidade. Todavia, como ocorre este efeito tóxico a nível celular ainda permanece desconhecido. Os sais podem acumular-se no apoplasto desidratando células, bem como acumular-se no citoplasma e inibir as enzimas envolvidas no metabolismo de carboidratos ou acumulando-se nos cloroplastos exercendo um efeito tóxico direto sobre os processos fotossintéticos (MUNNS; TESTER, 2008).

As reduções na fotossíntese pela salinidade estão relacionadas a consequências fisiológicas indiretas impostas por este estresse, pois os sais absorvidos pelas plantas não podem diretamente controlar o crescimento somente por afetar o turgor, fotossíntese ou atividades enzimáticas. O acúmulo de sal está envolvido com a aceleração do processo de senescência de folhas mais velhas, que por sua vez afeta o crescimento diminuindo o fornecimento de assimilados e/ou hormônios para as regiões de crescimento (MUNNS, 2002). Outras possibilidades que envolvem reduções na fotossíntese sob salinidade são relacionadas à

inibição do crescimento e regulação por "feedback", porém esses mecanismos ainda não estão bem esclarecidos (OSMOND E FORSTER, 2006).

Sabe-se que a atividade fotoquímica apresenta resistência ao estresse salino de curta duração (NETONDO et al., 2004), sem redução da eficiência quântica potencial do fotossistema II (F_v/F_m) (PRAXEDES et al., 2010). Contudo, sob uma exposição prolongada a sais e quando altas concentrações de íons se acumulam nos tecidos, a atividade fotoquímica também pode ser afetada. Na grande maioria das pesquisas, o estresse salino parece afetar a relação F_v/F_m em plantas cultivadas sob alta PPFDs (Densidade de Fluxo de Fótons Fotossintéticos) . Isto tem sido demonstrado tanto em plantas halófitas tais como *Artimisia anethifolia* (LU et al., 2003) como em cevada (BELKHODJA et al., 1999). O decréscimo em Fv/Fm pode ser interpretado como fotodano, que pode ser devido a uma inativação do centro de reação do PSII, uma inibição do transporte de elétrons em ambos doadores e receptores do PSII e uma distribuição da energia de excitação em favor do PSI, aumentando o fluxo cíclico de elétrons ao redor do PSI. Estes decréscimos são identificados como um processo adpatativo de baixa regulação do PSII, ou seja, um mecanismo de proteção que ajuda a dissipar o excesso de energia a partir do aparato fotossintético (KRAMER et al., 2004; HILL; RALPH, 2005). De modo geral, as causas exatas que relatam danos fotoquímicos pela salinidade como fotoinibição ainda não são bem esclarecidas.

Um fator importante na determinação da capacidade fotoquímica do PSII é o estado redox das quinonas (Q_A). Frequentemente, o fator de eficiência do PSII (coeficiente de extinção fotoquímica ou quenching fotoquímico - qP) representa a proporção da energia dos fótons capturada pelos centros de reação do PSII abertos e dissipada via transporte de elétrons (JUNEAU et al., 2005), refletindo o grau de oxidação e redução de Q_A. O qP representa a utilização da energia luminosa para os processos fotoquímicos da fotossíntese (doação do elétron proveniente da molécula de água para o aceptor NADP (Nicotinamida adenina dinucleotídio fosfato)). Este processo é a base da fotossíntese. Esta energia dissipada é usada para a formação do poder redutor e da molécula de ATP, os quais serão utilizados na fase bioquímica do processo fotossintético (SCHREIBER et al., 1986). Paralelamente, o coeficiente de extinção não-fotoquímico (NPQ) representa a queda na fluorescência devido a processos não-fotoquímicos, sendo que corresponde a um aumento na perda de energia absorvida via dissipação térmica, ou seja, associado a todos os processos de excitação não-radiativos (JUNEAU et al., 2005). O NPQ é constituído pela soma de três componentes: coeficiente de extinção dependente de energia (qE), coeficiente de extinção fotoinibitórrio (qI) e coeficiente de extinção do estado de transição (qT) (BAKER, 2008). Na prática, estudos com

salinidade tem mostrado que o qP parece manter-se constante ou então reduzir, dependendo da severidade do estresse. De modo contrário, tém-se observado frequentemente que o coeficiente de extinção não fotoquímica (NPQ) pode aumentar sob condições salinas (CAMBROLLÉ et al., 2011; SILVA et al, 2011; SOUZA et al., 2011). No entanto, pouco se conhece como a salinidade exerce reduções no qP e consequentemente aumentos no NPQ.

Outro parâmetro que frequentemente aumenta em função da salinidade é a razão ETR/P_N que está relacionado com a capacidade de proteção de processos como do metabolismo antioxidante contra a formação de espécies reativas de oxigênio (MELONI et al.,2003). O aumento na razão ETR/P_N está relacionado com drenos alternativos de elétrons que possivelmente aumentam devido às condições estressantes impostas pela salinidade (RIBEIRO et al., 2009). Em outras palavras, o aumento desta razão indica que mais elétrons são dirigidos para outros drenos (fotorespiração, metabolismo do nitrigênio ou reações de Mehler), sugerindo uma condição de estresse. Alguns trabalhos tem reportado essa característica em diferentes espécies de plantas como cevada (BELKHODJA et al., 1999) e pinhão manso (SILVA et al., 2010), suportando a existência de dissipação de elétrons por outros processos de fixação de CO_2. Segundo Morales et al. (2006), nesta fase não é difícil diferenciar entre respiração, fotorrespiração, a reação de Mehler, transporte cíclico de elétrons-PSII ou quaisquer outros processos que consomem elétrons.

Estudos recentes têm revelado que a interação entre salinidade e seca levou a uma menor regulação de alguns genes fotossintéticos (CHAVES et al., 2009). Desingh e Kanagaraj (2007) mostraram que a taxa fotossintética e a atividade da Rubisco decresceram com o aumento da salinidade. Estas consequências foram relacionadas com a produção de espécies reativas de oxigênio (ERO_S) tais como ânion superoxido ($O_2^{\cdot-}$), peróxido de hidrogênio (H_2O_2) e radicais hidroxilas (·OH), particularmente, nos cloroplastos e mitocôndrias (ASADA 1994; PROCHAZKOVA; WILHELMOVA, 2007). É notável que diversas informações sejam encontradas quando as plantas são submetidas à salinidade. Os diversos efeitos sobre os mecanismos que envolvem a capacidade de assimilar de CO_2, capacidade fotoquímica e a presença de drenos alternativos de elétrons para mitigar os danos causados pela salinidade são ainda desconhecidos e merecem maior atenção.

3. Danos oxidativos

O oxigénio é essencial para a viabilidade da maioria dos organismos, mas também pode ser potencialmente tóxico. Em organismos aeróbicos, o oxigênio é utilizado como aceptor final de elétrons permitindo elevada produção de energia na respiração, em consequência de seu alto potencial eletroquímico. Entretanto, devido a sua configuração eletrônica, o oxigênio pode sofrer reduções parciais e levar à formação de radicais livres, de forma que as Espécies Reativas de Oxigênio (ERO_S) estão constantemente presente nas células eucarióticas (FOYER et al., 2009).

No seu estado fundamental, o oxigênio molecular ou tripleto é uma molécula que geralmente não é reativa. A ativação de oxigênio em seu estado fundamental por várias reações supera a restrição de spins e conduz à formação do radical superóxido ($O_2^{\cdot-}$), peróxido de hidrogênio (H_2O_2) e o radical hidroxil (·OH), enquanto a excitação eletrônica conduz à formação do singuleto-estado de oxigênio (oxigênio singleto; $^1O_2$). Estes vários tipos de ERO_S podem danificar muitos componentes celulares, tais como proteínas, lipídios e ácidos nucléicos (Fig. 7) (HALLIWELL; GUTTERIDGE, 1990; DAS, 2012).

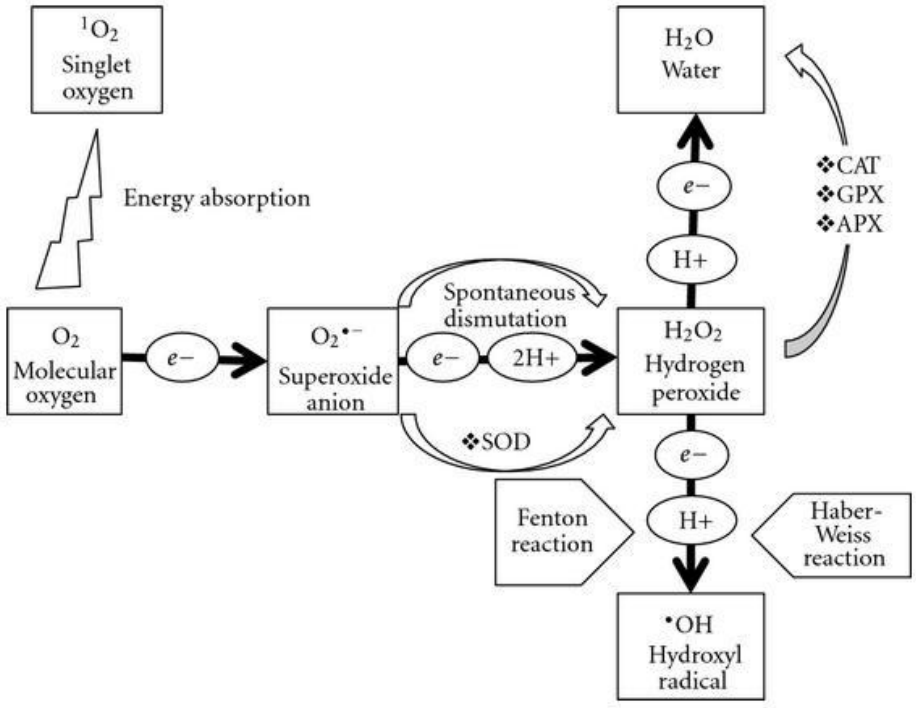

Figura 8 - Representação esquemática da geração de espécies reativas de oxigênio (EROs) em plantas. (Fonte: hindawi.com).

O maquinário fotossintético também é uma fonte de EROs, e isto ocorre predominantemente nas membranas do tilacóide (ASADA, 1994, 1999). Ambos H_2O_2 e ·OH são gerados durante a redução do O_2^- no lado aceptor do fotossistema I como um resultado do transporte fotossintético de elétrons. O H_2O_2 e O_2^- são também gerados no PSII e o $^1O_2$ é gerado pela transferência de energia a partir de pigmentos excitados, tais como clorofila (ZOLLA; RINALDUCCI, 2002), de centros Fe-S excitados no PSI e a partir de fotodanos no PSII (Fig. 8) (CHUNG; JUNG, 1995; ANDERSON, 2001). A geração de EROs é promovida quando a disponibilidade de CO_2 ou de NADP é limitada (ASADA, 1999).

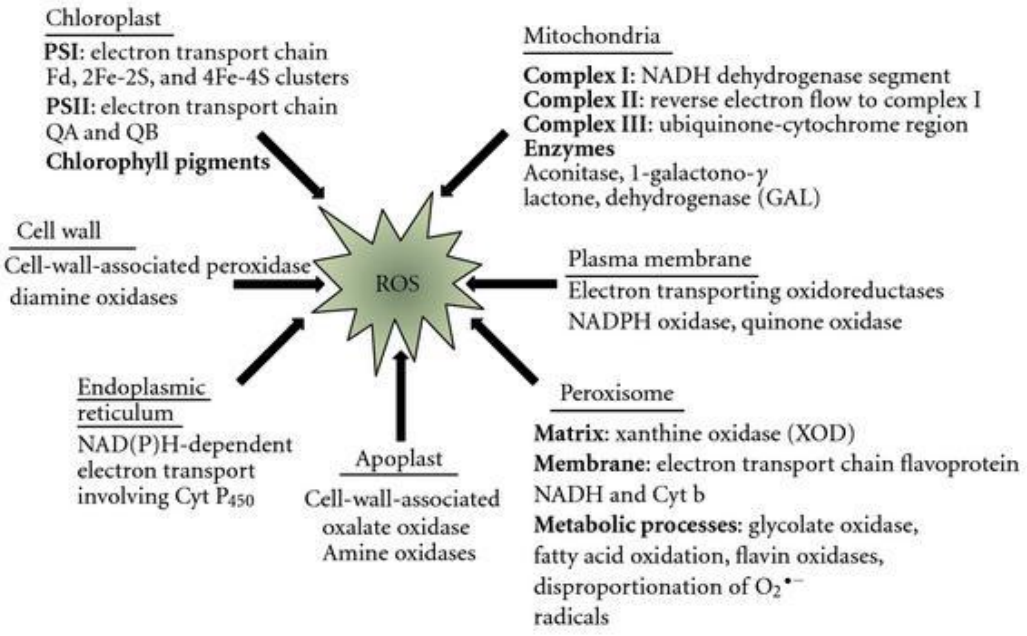

Figura 9 – Sítios de produção de espécies reativas de oxigênio (EROs) em plantas. As EROs são produzidas em várias locais como cloroplastos, mitocôndrias, membrana plasmática, peroxissomos, apoplasto, retículo endoplasmático e parede celular. (Fonte: hindawi.com).

Parte da energia é dissipada pelo uso do O_2 como um dreno de elétrons nos processos que produzem espécies reativas de oxigênio. Alguns sintomas de danos oxidativos são frequentes em plantas estressadas, incluindo peroxidação de lipídios e proteínas e a presença de quantidades detectáveis de H_2O_2 (MORALES et al., 2006). Sintomas de estresse oxidativo têm sido encontrados em plantas cultivadas em condições salinas. A peroxidação lipídica por meio do método TBARS tem mostrado-se como um bom indicador de danos oxidativos em várias espécies de plantas submetidas à salindade (GÓMEZ et al., 1999;. SAIRAM;

SRIVASTAVA, 2002; SILVA et al, 2012), assim como danos oxidativos às proteínas. Além disso, aumentos na concentração de H_2O_2 e TBARS têm sido relatados frequentemente em plantas submetidas à salinidade (GÓMEZ et al., 1999; SAIRAM E SRIVASTAVA, 2002; SILVA et al., 2012).

A alta salinidade é conhecida por causar efeitos iônicos e osmóticos às plantas, levando a uma desorganização de membranas e consequentemente induzindo a produção de ERO_S. Além disso, a salinidade reduz o fornecimento de CO_2, reduzindo mais ainda a relação CO_2/O_2 nos cloroplastos. O consequente acúmulo de poder redutor, por conta da redução CO_2/O_2 causa um excesso de energia eletroquímica ao nível de membrana. Essa energia extra é canalizada através da reação de Mehler que gera ERO_S tais como O_2^-, H_2O_2 e OH^-, provocando estresse oxidativo (PEREZ-LOPEZ et al., 2009). Na reação de Mehler (Fig. 9A), o superóxido (O_2^-) formado no PSI, através da ferredoxina, é convertido em H_2O_2 por meio da enzima superóxido dismutase (SOD). Em seguida, a enzima peroxidase do ascorbato (APX) reduz o H_2O_2 a água através da oxidação do ascorbato (Asc) até radicais de monodehidroascorbato (MDHA), que são reduzidas de volta para o ascorbato através da glutationa. Sob condições de vários stresses, a concentração celular de EROs aumenta numa condição que os mecanismos de defesa antioxidantes não conseguem dar conta da quantidade produzida. Nesta situação, a reação de Fenton (Fig. 9B) transforma parte do excesso de H_2O_2 produzido em radicais hidroxil ($^{\bullet}OH$) por meio de íons de ferro divalente (Fe^{2+}) como catalisadores.

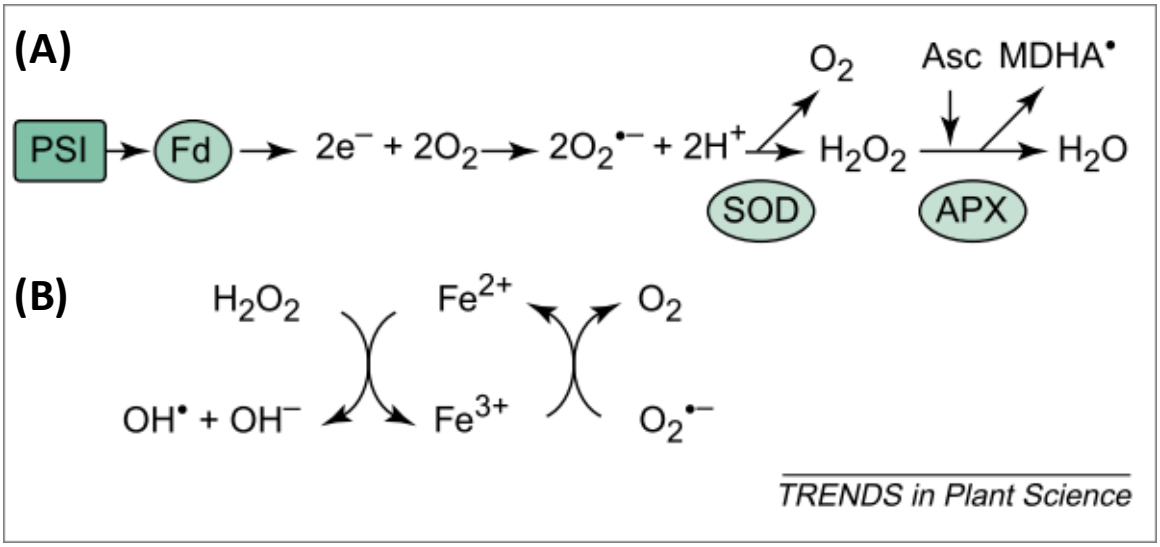

Figura 10 – Reação de Mehler (A) e reação de Fenton (B); ambas são fontes de ROS. (Fonte: Pfannschmidt, 2003).

Estes radicais, uma vez produzidos, causam danos oxidativos por oxidação de ácidos graxos e aminoácidos, o que pode prejudicar a estrutura das membranas ou a função de proteínas (PFANNSCHMIDT, 2003). Para prevenir os danos oxidativos, as plantas possuem sistemas compostos de antioxidantes de baixo peso molecular como Ascorbato e Glutationa, e enzimas protetoras como ascorbato peroxidase (APX), catalase (CAT) e superóxido dismutase (SOD) (PEREZ-LOPEX et al., 2009). A correlação entre a capacidade antioxidante e tolerância à salinidade é bem conhecida e estudos indicam que plantas com elevados níveis constitutivos ou induzidos de antioxidantes têm mostrado maior resistência aos danos oxidativos, como descrito por Parida e Das (2005).

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ORIGINAL PAPER

High supply of NO_3^- mitigates salinity effects through an enhancement in the efficiency of photosystem II and CO_2 assimilation in *Jatropha curcas* plants

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Abstract This study was performed to determine if a high supply of N-NO_3^- is capable of mitigating negative salinity effects on photosynthesis and growth through the stimulation of nitrate assimilation, which could act as an sink from photosynthetic electron transport chain and restrict the over reduction in thylakoid membrane in *Jatropha curcas* leaves. The experiment was arranged in a factorial design with two nitrate concentrations (1 and 10 mM) and two NaCl levels (0 and 100 mM). Salt-stressed plants supplied with high NO_3^- demonstrated a higher nitrate uptake rate, nitrate reductase activity and soluble-protein content when compared with plants that presented low nitrate uptake. High nitrate assimilation was associated with higher leaf growth, CO_2 assimilation and lower membrane damage in salt-stressed plants. The superior performance of salt-stressed plants grown with high NO_3^- was indicated by a higher effective quantum yield of PSII and electron transport rate and lower energy excess at the PSII level and non-photochemical quenching. Interestingly, a high NO_3^- level in the absence of NaCl did not alter the leaf growth, photochemical activity and gas exchange parameters when compared with plants supplied with low nitrate. The proline and glycinebetaine contents were similarly increased in both low- and high-NO_3^- salt-stressed plants. Our data suggest that the favorable effects induced by high nitrate supply were possibly associated with stimulation in the nitrate assimilatory pathway. This process might have acted as a sink of electrons from the thylakoid membranes minimizing photo-damage and stimulating CO_2 assimilation under salinity in *J. curcas*.

Keywords *Jatropha curcas* · Nitrate assimilation · Organic solutes · Photochemical activity · Photosynthesis · Salt stress

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Introduction

High salinity and low soil nitrogen (N) availability are important growth-limiting factors for most plants species (Villa-Castorena et al. 2003). Increasing the availability of N in soils through fertilizer utilization can improve crop productivity and mitigate some stressful factors (Albassam 2001). In saline soil, by mechanisms yet unknown, the supply of N might alleviate the adverse effects of salinity (Flores et al. 2003). Nitrate is often the main nitrogen source in agricultural soil, and the uptake and assimilation of nitrogen are strongly affected by salinity (Silveira et al. 2001). Nitrate reductase (NR, E.C.1.7.1.1) is the most important enzyme involved in the nitrate assimilation pathway (Campbell 1999). The second step, nitrite reduction to ammonia, occurs in chloroplasts and is a strong consumer of electrons from reduced ferredoxin (Campbell 1999).

The final step of N assimilation involves the glutamine synthetase/glutamate synthase activities (the GS/GOGAT cycle), which also occurs in chloroplasts. The reactions of this cycle consume ATP and reduced ferredoxin (Lea and Azevedo 2007). The GS/GOGAT cycle produces glutamine and glutamate, which can act as amino-acids initiators for several pathways involved with the synthesis of amino acids, proteins and other compounds essential for

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High supply of NO_3^- mitigates salinity effects through an enhancement in the efficiency of photosystem II and CO_2 assimilation in Jatropha curcas plants

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Abstract - This study was performed to determine if a high supply of N-NO_3^- is capable of mitigating negative salinity effects on photosynthesis and growth through the stimulation of nitrate assimilation, which could act as an sink from photosynthetic electron transport chain and restrict the over reduction in thylakoid membrane in *Jatropha curcas* leaves. The experiment was arranged in a factorial design with two nitrate concentrations (1 and 10 mM) and two NaCl levels (0 and 100 mM). Salt-stressed plants supplied with high NO_3^- demonstrated a higher nitrate uptake rate, nitrate reductase activity and soluble-protein content when compared with plants that presented low nitrate uptake. High nitrate assimilation was associated with higher leaf growth, CO_2 assimilation and lower membrane damage in salt-stressed plants. The superior performance of salt-stressed plants grown with high NO_3^- was indicated by a higher effective quantum yield of PSII and electron transport rate and lower energy excess at the PSII level and non-photochemical quenching. Interestingly, a high NO_3^- level in the absence of NaCl did not alter the leaf growth, photochemical activity and gas exchange parameters when compared with plants supplied with low nitrate. The proline and glycinebetaine contents were similarly increased in both low- and high-NO_3^- salt-stressed plants. Our data suggest that the favorable effects induced by high nitrate supply were possibly associated with stimulation in the nitrate assimilatory pathway. This process might have acted as a sink of electrons from the thylakoid membranes minimizing photo-damage and stimulating CO 2 assimilation under salinity in J. curcas.

Key words: Jatropha curcas · Nitrate assimilation · Organic solutes · Photochemical activity · Photosynthesis · Salt stress

1. Introduction

High salinity and low soil nitrogen (N) availability are important growth limiting factors for most plants species (VILLA-CASTORENA *et al.*,, 2003). Increasing the availability of N in soils through fertilizer utilization can improve crop productivity and mitigate some stressful factors (ALBASSAM, 2001). In saline soil, by mechanisms yet unknown, the supply of N might alleviate the adverse effects of salinity (FLORES *et al.*,, 2003). Nitrate is often the main nitrogen source in agricultural soil, and the uptake and assimilation of nitrogen are strongly affected by salinity (SILVEIRA *et al.*,, 2001). Nitrate reductase (NR, E.C.1.7.1.1) is the most important enzyme involved in the nitrate assimilation pathway (CAMPBELL, 1999). The second step, nitrite reduction to ammonia, occurs in chloroplasts and is a strong consumer of electrons from reduced ferredoxin (CAMPBELL, 1999).

The final step of N assimilation involves the glutamine synthetase/glutamate synthase activities (the GS/GOGAT cycle), which also occurs in chloroplasts. The reactions of this cycle consume ATP and reduced ferredoxin (LEA; AZEVEDO, 2007). The GS/GOGAT cycle produces glutamine and glutamate, which can act as amino-acids initiators for several pathways involved with the synthesis of amino acids, proteins and other compounds essential for plant growth (ROCHA *et al.*,, 2012). Most of these reactions are consumers of energy and electrons as NAD(P)H, reduced ferredoxin and ATP. Thus, the whole nitrate assimilation process is a strong consumer of electrons in the photosynthetic electron-transport chain, and can thus, alleviate the ‘‘electron pressure’’ on photosystem II (OSMOND; FORSTER, 2006).

The electron excess and over-reduction of the thylakoid membranes is frequent under the combined conditions of high light and abiotic stress. Stress conditions, such as salinity, induce stomatal closure and impairment in CO_2 assimilation (Adams *et al.*, 2006). Under these conditions, the plants can deploy several mechanisms to avoid or minimize photo-damage, photo-inhibition and oxidative stress (SILVA *et al.*,, 2010a). The most common processes utilized are photorespiration, heat dissipation and the xanthophyll cycle. Recently, Osmond and Forster (2006) have suggested other processes, such as N metabolism, growth and respiration. However, the mechanisms associated with nitrate assimilation and energy dissipation in photosystems are currently unknown.

Because a large fraction of leaf nitrogen is allocated to the photosynthetic apparatus, especially for Rubisco content, the leaf N content can also influence the photosynthetic capacity (HIKOSAKA; HIROSE, 2000). An N deficit necessarily promotes the remobilization

of nitrogen from Rubisco (PAUL; FOYER, 2001) the salinity might decrease the nitrate acquisition, and as a consequence, the Rubisco content and the photosynthesis rates could decrease (SOUSSANA *et al.*,, 2000). There are reports that nitrate improves the growth of salt-stressed citrus by improving photosynthetic activity and reducing chloride accumulation (Iglesias *et al.*, 2004). However, in mate´ plants supplied with different forms of N, a higher photosynthetic efficiency was observed in the plants supplied with $N\text{-}NH_4^+$ when compared to plants supplied with $N\text{-}NO_3^-$ (GAIAD *et al.*,, 2006).

Another important strategy used by plants in response to abiotic stress is the accumulation of compatible solutes (SILVA *et al.*,, 2010b). High levels of N might stimulate the accumulation of nitrogenous solutes, such as proline and glycinebetaine (CHEN *et al.*, 2007). Thus, the N-induced accumulation of these solutes could mitigate the negative effects of salinity by increments of osmotic adjustment and the protection of proteins and membranes against damage caused by reactive oxygen species (ROS) (SILVEIRA *et al.*, 2009). Our recent studies have demonstrated that amino acids, glycinebetaine and soluble sugars are effective and important mediators of the osmotic adjustment of J. curcas young plants under conditions of high salinity and drought (SILVA *et al.*, 2009, 2010b).

Jatropha curcas is a species that grows in marginal areas where other crop species are not able to survive (FRANCIS *et al.*, 2005). In addition, J. curcas has high economic potential due to its seed-oil quality, which can be converted to biodiesel for industrial use (KING *et al.*, 2009). Although this species has shown satisfactory yield under the constraining conditions of semiarid regions, such as drought and high temperature (SILVA *et al.*, 2010a), there are few studies regarding the salt tolerance associated with nitrate nutrition. In this study, we tested the hypothesis that a high supply of NO_3^- can mitigate the negative effects of the salinity by increasing the nitrate assimilation rate. This process might contribute to an improvement in the photosynthetic electron transport and CO_2 assimilation, because it acts as an electron sink, thus attenuating thylakoid over-reduction. Our study evaluated changes in nitrate assimilation, photosystem II efficiency and CO_2 assimilation, and it evaluated proline and glycinebetaine accumulation in the absence and presence of salinity in J. curcas plants cultivated under high and low exogenous nitrate levels.

2. Materials and methods

2.1. Plant material and experimental conditions

The experiment was conducted in a greenhouse under natural conditions (3°44´S; 38°33W, at sea level), and the environmental conditions were as follows: an average air temperature of 29 °C, a mean air relative humidity of 65%, an average maximum photosynthetic photon flux density (PPFD) of approximately 1,300 μmol m^{-2} s^{-1} and a photoperiod of 12 h. *Jatropha curcas* seeds were supplied by the Fazenda Tamandua´ (Santa Terezinha, PB, Brazil), and they were selected by taking into account the seed size and weight. Eight days after the germination in sand, the seedlings were transferred to plastic pots (2L) containing quarter-strength Hoagland and Arnon (1950) nutrient solution (pH 6.0) in the first week and were given half-strength nutrient solution for the remainder of the experiment. Twenty-three days after germination, the NO_3^- and NaCl treatments were applied. The plants were divided into two groups and supplied with 1 mM NO_3^- as 0.25 mM $Ca(NO_3^-)_2$ and 0.5 mM KNO_3, which represented the N1 treatment, or 10 mM NO_3^- in the form of 2.5 mM $Ca(NO_3^-)_2$ and 5 mM KNO_3, which represented the N10 treatment, dissolved in a complete nutrient solution. The Ca_2^+ and K^+ concentrations were kept at 3.0 mM and 6.0 mM, respectively, in all nutrient solutions by utilization of $CaCl_2$ and KCl. The other nutrients were utilized according to a half-strength Hoagland and Arnon (1950) nutrient solution. These two groups of plants were simultaneously subjected to salt stress over 10 days by the dissolution of 100 mM NaCl in the nutritive solution. To avoid osmotic shock, the NaCl was added to the solution in two subsequent steps (50 mmol NaCl L^{-1} day^{-1}). Two new treatments were then initiated (N1+ Salt and N10 + Salt). The N10 treatment was used as the control or reference. Nitrate concentration was monitored daily and adjusted to 1 or 10 mM as necessary. The in vivo photosynthesis measurements were performed at 10:00 a.m. in a fully expanded leaf. A similar leaf was subsequently used to determine the NR activity. After 5 hours of sunshine, the plant material was harvested at 11:00 a.m. to allow for the induction of nitrate reductase.

2.2. Relative water content, membrane damage, dry weight and chlorophyll content in leaves

The leaf relative water content (RWC) was calculated as follows: RWC = [(FW - DW)/(TW - DW)] x 100, where FW is the fresh weight, TW is the turgid weight measured after 6 h of saturation in deionized water at 4 °C in the dark, and DW is the dry weight determined after 48 h in an oven at 75 °C (SILVEIRA *et al.*, 2009). The electrolyte leakage (EL) in the leaves was determined as previously described (SILVA *et al.*, 2010a). At the end of the experiment, the plants were collected, and the total leaf dry matter was obtained by drying the leaves in an oven at 75 °C for 48 h. The total chlorophyll concentration was calculated as previously described (SILVA *et al.*, 2010c).

2.3. Leaf gas exchange and chlorophyll fluorescence measurements

The leaf gas exchange and chlorophyll fluorescence parameters were measured using a portable photosynthesis system (LI-6400-XT) and a leaf chamber fluorometer (6400-40), respectively (LI-COR, USA). The measurements were performed in fully expanded and mature leaves under constant CO_2 concentration and PPFD (~380 µmol mol^{-1} CO_2 and 1,000 µmol photons m^{-2} s^{-1}, respectively). The air-flow rate was 300 µmol s^{-1}. The actinic light intensity was 1,000 µmol photons m^{-2} s^{-1}. Measurements were recorded when the total coefficient of variation (CV) was <5 %. To reduce the time for measurement stabilization, the air pumped into the LI-6400-XT was passed through a buffering gallon (5 L). There was an approximate 1- to 2-min time lag to acquire the steady-state level of fluorescence. Measurements of the leaf CO_2 assimilation rate (P_N, in µmol m^{-2} s^{-1}), transpiration (*E*, in mmol m^{-2} s^{-1}), stomatal conductance (g_s, in mol m^{-2} s^{-1}) and intercellular CO_2 concentration (C_I) in Pa were taken, and the instantaneous carboxylation efficiency (P_N/C_I , in µmol m^{-2} s^{-1} Pa^{-1}) and the ratio between apparent electron-transport rate (ETR) and leaf CO_2 assimilation rate (ETR/P_N in µmol $µmol^{-1}$) were calculated. For leaves that were in the light or that had adapted to the dark for 30 min, the chlorophyll fluorescence measurements were taken using the saturation pulse method. Prior to this determination, a test subjecting the leaf to different dark periods (10, 20, 30, 40, 50 and 60 min) was performed to obtain the best time for the F_v/F_m measurement. After the optimization of the dark adaptation time, the measured values of F_m and $F_m´$ were used to calculate the non-photochemical quenching (NPQ). The intensity and duration of the saturation light pulse were 8,000 µmol m^{-2} s^{-1} and 0.7 s, respectively. To

maximize the stomatal opening, the amount of blue light was 10 % of the PPFD (FLEXAS *et al.*, 2007). The following parameters were assessed: the maximum quantum yield of PSII in dark-adapted leaves [$F_v/F_m = (F_m - F_o)/F_m$], the effective quantum yield of PSII [$\Delta F/F_{m'} = (F_m' - F_s)/F_m'$], the photochemical [$qP = (F_m' - F_s)/(F_m' - F_o')$] and non-photochemical [$NPQ = (F_m - F_m')/F_m'$] quenching, the apparent ETR [$ETR = (\Delta F/F_m' \times PPFD \times 0.5 \times 0.84)$], and the relative energy excess at the PSII level [$EXC = (F_v/F_m) - (\Delta F/F_m')/(F_v/F_m)$]. To evaluate the ETR, 0.5 was used as the fraction of the excitation energy distributed to PSII, and 0.84 was used as the fraction of incoming light absorbed by the leaves. PPFD is the PPFD. The minimum (F_o), maximum (F_m) and variable ($F_v = F_m - F_o$) fluorescence intensities were sampled in dark-adapted leaves. In addition, measurements were taken under light-adapted conditions, with a sampling of the minimum (F_o') and maximum (F_m') fluorescence intensities. The F_o' signal was measured after PSI excitation using far-red light. The measurement of F_m' was performed by supplying far-red illumination after the actinic light flash removal. The fluorescence signal measured immediately before the saturation pulse is referred to as F_s', and the variable fluorescence signal under light conditions is $\Delta F' = F_m' - F_s'$ (FLEXAS *et al.*, 2007; SILVA *et al.*, 2010a).

2.4. Net nitrate uptake by roots and nitrate reductase activity, nitrate content and soluble-protein content in leaves

The net nitrate uptake was evaluated by NO_3^- depletion in the nutrient solution after a 24-h interval of root uptake (SILVEIRA *et al.*, 2001). The nitrate concentration was measured by the Cawse (1967) method. Over the experimental period (10 days), the nitrate concentration was measured daily in the nutrient solution and the initial concentrations of each treatment (1 and 10 mM) were restored by addition of $CaCl_2$ 1 M and KNO_3. The nitrate reductase activity was measured by an in vivo method according to Hageman and Hucklesby (1971), with minor modifications described in details by Silveira *et al.*, (2001). The leaf nitrate was extracted with hot water (100 °C), and the concentration was determined using the method of Cataldo *et al.*, (1975). The total soluble protein was extracted with a 100 mM Tris–HCl buffer (pH 8.0) containing 30 mM DTT, 20 % (v/v) glycerol and 3 % (w/v) PEG-6000 (ZIMMERMAM *et al.*, 2006). The total soluble-protein concentration was measured using the Bradford (1976) method with a standard curve obtained using bovine serum albumin (BSA).

2.5. Determination of organic solutes

Lyophilized leaf samples were transferred to hermetically closed tubes containing deionized water, and the tubes were placed in a 100 °C water bath for 1 h. After the supernatant extraction, the total soluble sugar was determined using the phenol–sulfuric method (DUBOIS *et al.*, 1956). Sucrose determination was performed using the method described by van Handel (1968). The total free amino acids (TFAA) and the proline and glycinebetaine (GB) concentrations in the leaves were determined as previously described (SILVEIRA *et al.*, 2009).

2.6. Experimental design and data analysis

The experiment was arranged in a factorial (2 x 2) design. The experiment had two nitrate concentrations (1 and 10 mM) and two NaCl levels (0 and 100 mM) with four replicates (an individual pot containing one plant was one replicate). The data were analyzed by an ANOVA, and the means were compared with Tukey's test at the 0.05 level of significance. The standard deviation is plotted in each of the tables and figures.

3. Results

3.1. Leaf dry weight accumulation and membrane damage

Salt stress promoted strong changes in the physiological parameters of the *J. curcas* plants under high and low nitrate concentrations during medium growth. For example, the leaf dry weight was reduced by 57 and 43 % in the N1+Salt and N10+Salt treatments, respectively, compared to their respective controls (Table 1). In contrast, the electrolyte leakage (EL) and membrane damage were significantly increased by 57 and 33 %, respectively, in the same salt treatments compared to the controls (Table 1). The degree of leaf hydration, expressed as the relative water content and the concentrations of photosynthetic pigments in the salt-stressed plants, was not affected by salinity or nitrate (Table 1). It is important to note that, in terms of growth and membrane integrity, a high NO_3^- level (10 mM) in the nutrient solution was able to minimize the damage caused by NaCl.

Table 1 - Leaf dry weight, electrolyte leakage, relative water content and chlorophyll content in the J. curcas plant leaves exposed to salt stress with high or low nitrate contents.

Treatment	Leaf DW (g Plant^{-1})	EL (%)	RWC (%)	Chlorophyll (mg g^{-1}FW)
N1	11.13±2.21	24.90±1.96	67.37±8.95	0.48±0.01
N1 + Salt	4.73±0.83	39.30±1.78	74.92±4.50	0.46±0.01
N10	10.73±0.84	26.03±3.20	65.28±4.40	0.51±0.01
N10 + Salt	6.09±0.44	34.73±2.25	68.03±1.91	0.49±0.01

The values are the means of four replicates ± SD

3.2. Leaf gas exchange and chlorophyll fluorescence

All of the leaf gas exchange parameters evaluated in this study were also affected by salinity. The leaf CO 2 assimilation was decreased by 55 and 30 % in the N1+Salt and N10+Salt treatments, respectively, compared to their respective references (Table 2). Similarly, transpiration (E), stomatal conductance (g_s) and carboxilation instantaneous

efficiency (P_N/C_I) showed reductions of 20, 53 and 65 %, respectively to N1+Salt and 25, 37 and 35 %, respectively, to N10+Salt treatment compared to the respective references (Table 2). In contrast, the ETR/P_N ratio increased by 42 and 28 %, respectively, to N1+Salt and N10+Salt treatments, compared to the reference plants (Table 3). Based on the leaf DW and EL results, the plants supplied with 10 mM NO_3^- suffered less-severe salt stress than the plants treated with 1 mM NO_3^-. Regarding the chlorophyll fluorescence parameters, when compared to the reference plants, the effective quantum yield of PSII ($\Delta F/Fm'$) and the photochemical quenching (qP) were reduced by 28 and 18 %, respectively, in N1+Salt treatment and by 13 and 20 %, respectively, in the N10+Salt treatment (Table 3). Similarly, for N1+Salt and N10+Salt treatments, the apparent ETR was reduced by 36 and 17 %, respectively, when compared to the reference plants (Table 3). However, the non-photochemical quenching coefficient (NPQ) and the relative energy excess at the PSII level (EXC) reached values approximately 200 and 120 % higher than those of the reference plants, respectively, for N1+Salt treatment, and they reached values approximately 130 and 40 % higher, respectively, for N10+Salt treatment (Table 3). In addition, neither stressed nor non-stressed plants showed significantly changes ($p > 0.05$) in the maximum quantum yield of PSII in dark-adapted leaves (F_v/F_m) (Table 3) and minimum fluorescence (F_o) (data not shown).

Table 2 - Leaf CO_2 assimilation rate, transpiration, stomatal conductance and carboxylation instantaneous efficiency in the *J. curcas* plant leaves exposed to salt stress with high or low nitrate contents.

Treatment	P_N ($\mu mol\ m^{-2}\ s^{-1}$)	E ($mmol\ m^{-2}\ s^{-1}$)	g_s ($mol\ m^{-2}\ s^{-1}$)	P_N/C_I ($\mu mol\ m^{-2}\ s^{-1}\ Pa^{-1}$)
N1	12.73±0.66	1.86±0.15	0.15±0.01	0.60±0.06
N1 + Salt	5.65±0.88	1.50±0.04	0.07±0.01	0.21±0.04
N10	12.61±1.38	2.31±0.21	0.16±0.01	0.66±0.08
N10 + Salt	8.85±0.62	1.73±0.12	0.10±0.01	0.43±0.06

The values are the means of four replicates ± SD

Table 3 - The effective quantum yield of PSII, the maximum quantum yield of PSII in dark-adapted leaves, photochemical and non-photo-chemical quenching, the apparent electron-transport rate, the relative energy excess at the PSII level and the ratio between the apparent electron transport rate and the leaf CO_2 assimilation rate in the *J. curcas* plant leaves exposed to salt stress with high or low nitrate contents.

Treatment	ΔF/Fm´	qP	NPQ	F_v/F_m	ETR (µmol µmol^{-1})	EXC (µmol µmol^{-1})	ETR/P_N (µmol µmol^{-1})
N1	0.63±0.01	0.91±0.02	0.44±0.04	0.79±0.01	27.26±0.50	0.22±0.01	2.15±0.14
N1 + Salt	0.45±0.01	0.74±0.02	1.25±0.19	0.76±0.02	17.48±2.09	0.48±0.03	3.06±0.72
N10	0.61±0.01	0.94±0.01	044±0.08	0.80±0.01	26.36±0.35	0.24±0.01	2.04±0.26
N10 + Salt	0.53±0.02	0.75±0.03	1.00±0.15	0.77±0.03	21.80±1.69	0.33±0.02	2.62±0.80

The values are the means of four replicates ± SD

3.3. Nitrate assimilation and nitrogenous compounds accumulation

Regardless of the salt present, the nitrate uptake was strongly enhanced in plants supplied with high NO_3^- concentration when compared to those that received a low nitrate level (Fig. 1a). The leaf nitrate concentration was slightly higher in both salt-treated and non-treated (reference) plants grown under high nitrate levels compared to the respective plants cultivated in the presence of a low nitrate level (Fig. 1b). Nitrate reductase activity was strongly decreased by salinity in both low and high NO_3^- fed plants (Fig. 1c). Moreover, compared with the plants grown under a low nitrate level, a high supply of nitrate strongly increased the nitrate reductase only in salt-stressed plants. The soluble-protein concentrations indicated a similar pattern to those observed by nitrate uptake and nitrate reductase activity. That is, in both salt-treated and non-treated conditions, the plants treated with 10 mM NO_3^- had higher leaf soluble-protein concentrations than the plants grown under low nitrate levels (Fig. 1d). The contents of the TFAA in leaves were slightly increased by the effect of salinity under a low supply of nitrate, but the contents were significantly increased by salinity under a high supply of nitrate. However, when compared with the plants grown under a low nitrate level, the plants grown under a high level of nitrate alone demonstrated a discrete increase in the concentrations of amino acids.

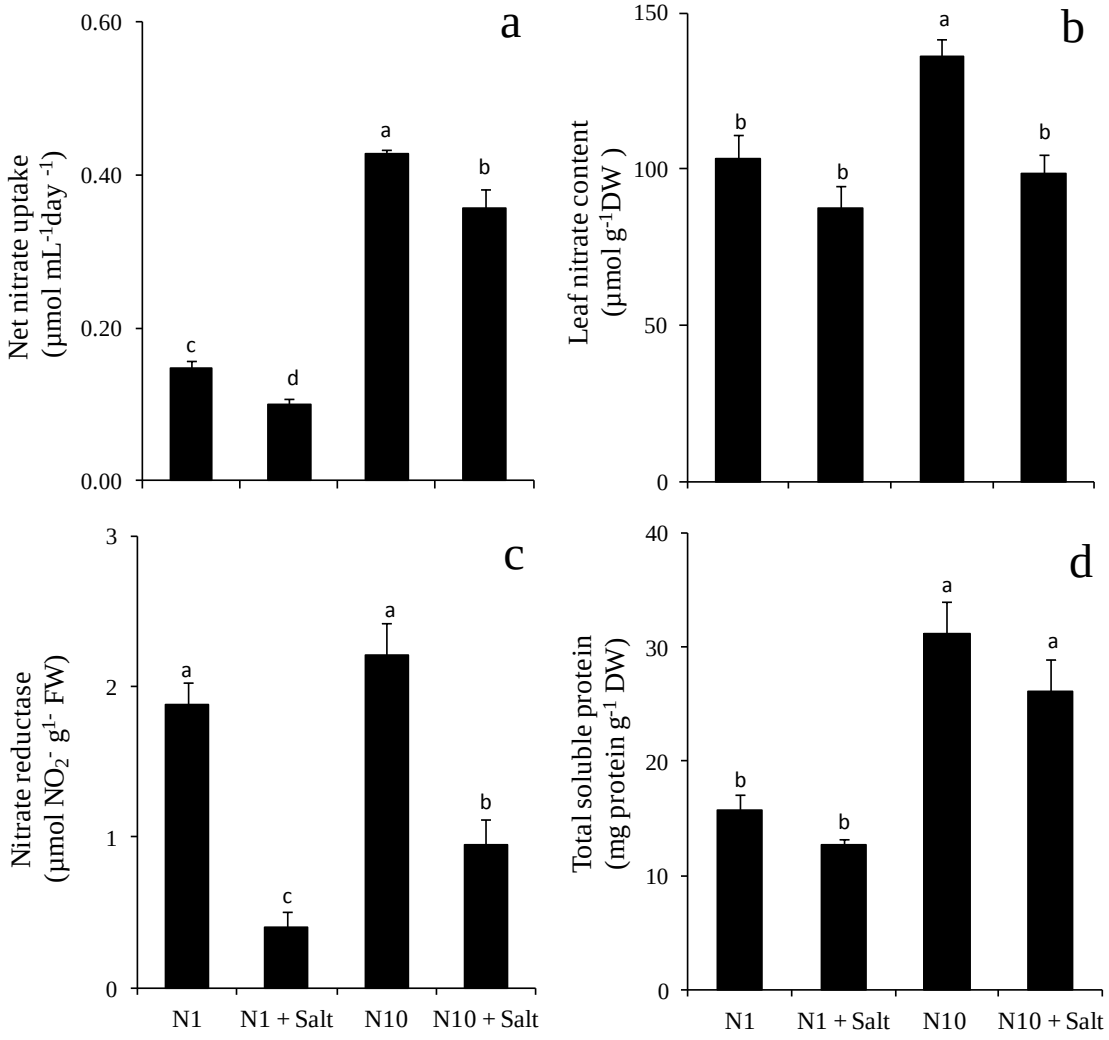

Figure 1 - Net nitrate uptake (a), nitrate content (b), nitrate reductase activity (c) and total soluble-protein levels (d) in the J. curcas leaves exposed to salt stress under high and low nitrate conditions. The values are the means of four replicates ± SD. The bars represent the mean values (n = 4) ± SD. The same letters represent differences that are not significant based on a significance cutoff of 0.05, as assessed by Tukey's test.

3.4. Proline, glycinebetaine and soluble-sugar accumulation

In regard to the studied osmo-solutes, proline and glycinebetaine (GB), the salinity significantly increased the proline levels in low and high nitrate-treated plants compared to their respective references (Fig. 2b) Nitrate alone did not exhibit any effect on the proline concentration. However, the salt stress promoted moderate increases in the GB content in both high and low nitrate-treated plants. In addition, in the

absence of NaCl, the exogenous nitrate levels had no effect on the GB concentrations (Fig. 2c). It is important to note the low levels of all obtained proline concentrations (from 0.25 to 0.38 μmol g^{-1}

DW when compared with GB, which changed from approximately 205–245 μmol g^{-1} DW). In spite of this high GB concentration, salt stress was slightly stimulated at both nitrate levels. In contrast to other solutes, neither the nitrate levels nor the salinity changed the soluble-carbohydrate contents (Fig. 2d).

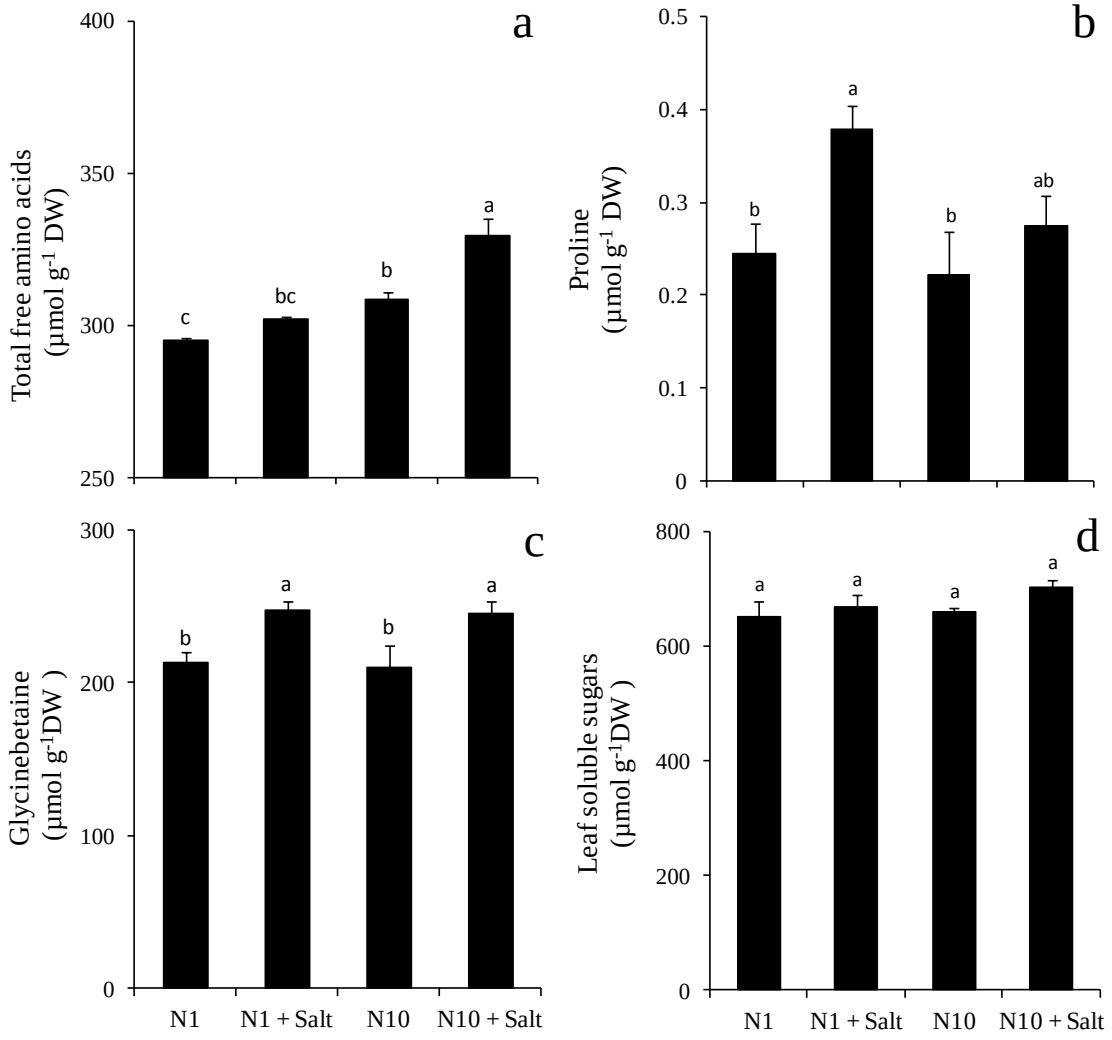

Figure 2 - The levels of total free amino acids (a), proline (b), glycinebetaine (c) and soluble sugar (d) in the *J. curcas* leaves exposed to salt stress under high and low nitrate conditions. The values are the means of four replicates ± SD. The bars represent the mean values (n = 4) ± SD. The same letters represent differences that are not significant based on a significance cutoff of 0.05, as assessed by Tukey's test.

4. Discussion

In this study, we demonstrated that a high supply of exogenous NO_3^- was more effective in mitigating, at least partially, the negative effects caused by salinity when compared with the low NO_3^- in the root medium of *J. curcas*. Interestingly, because the endogenous NO_3^- concentrations in the leaf tissues were almost similar among the two exogenous nitrate levels studied, these effects were essentially triggered by the nitrate flux. These results allow us to propose the following hypothesis: in alleviating the negative effects of salinity, the nitrate flux from the roots to the leaves is more important than the nitrate endogenous level or N status in leaf tissues. As the nitrate flux might control the nitrate reductase activity (ABD-ELBAKI *et al.,* 2000; BYBORDI 2010), it is plausible to assume that the nitrate assimilatory reduction process, by acting as a sink of electrons in the photosynthetic electron-transport chain, is essential in minimizing the deleterious effects caused by salinity on the photosynthesis of *J. curcas.*

This current hypothesis is reasonably supported by our obtained data. First, the highest supply of nitrate favored leaf growth and photosynthesis (CO_2 assimilation and photochemical activity) only in salt-stressed plants. Second, the concentrations of the two nitrogenous solutes involved in osmotic adjustment and cell protection, proline and glycinebetaine, were similarly increased following both the low and high NO_3^- supply. The free amino acids and soluble proteins had their concentrations increased by high nitrate concentrations in both salt- and non-stressed plants. It is important to note that both low and high NO_3^- -fed plants exhibited similar leaf growth and photosynthesis. Thus, low NO_3^- -fed plants previously stored sufficient amounts of N, which, when combined with 1 mM NO_3^- supplied by the nutrient solution, was likely sufficient to maintain an adequate rate of growth and photosynthesis.

The nitrate assimilatory reduction by nitrate reductase and nitrite reductase are the reactions that consume high amounts of electrons in the cytosol from NAD(P)H (two electrons) and in the chloroplasts from reduced ferredoxin (six electrons), respectively (CAMPBELL, 1999). This fact can explain the more severe effects on photosynthesis, growth and membrane damage caused by NaCl on the plants grown under low nitrate concentrations, which exhibited low nitrate uptake and assimilation. In other words, the higher rates of nitrate assimilation could contribute to the consummation of a part of the electron excess in the thylakoid membrane, thus inducing a lower $NADPH/NADP^+$ ratio. This process might allow a higher electron-transport rate

from photosystem II to CO 2 assimilation under conditions of restrictions in the stomatal opening caused by salt stress (KATO *et al.,* 2003). Nitrate assimilation could then act as an additional chloroplast electron sink under low CO_2 assimilation conditions.

Although Kato *et al.,* (2003) have demonstrated that a high N level favors photochemical activity, these authors neither discussed nor explained the underlying mechanisms involved with photo-inhibition and photosynthesis improvement by N. Thus, to the best of our knowledge, our study is the first to demonstrate a proposed mechanism for explaining the favorable effects triggered by high N in photo-acclimation under salt stress. Indeed, in an excellent review on the mechanisms involved with the protection of photosystems, Osmond and Forster (2006) suggested that N utilization might attenuate over-reduction of the photosystem II and to improve photo-acclimation. However, these authors did not propose any explanation of the bio-chemical mechanism involving N and the improvement in photosystem II efficiency and CO_2 assimilation.

All photochemical and gas exchange parameters obtained in the current study corroborate with the notion that high rates of NO_3^- flux and assimilation might attenuate the adverse effects caused by an imbalance between the high rates of electron supply from photosystems I and II and the low rates of utilization for the most important electron sink, CO 2 assimilation. Moreover, as our experiments were conducted under natural conditions with high light levels, high temperature and a high vapor pressure deficit, these factors might have interacted strongly with the salinity, thus allowing for a large accumulation of electrons in the photosystem II and over-reduction in the thylakoid membrane due to low rates of CO_2 assimilation (SILVA *et al.,* 2010a). Under these conditions, *J. curcas* frequently suffers membrane damage and oxidative stress (SILVA *et al.,* 2010a). Interestingly, as revealed by the high values of F_o and F_v/F_m, the significant photochemical alterations induced in the *J. curcas* salt-stressed plants were not sufficient to cause photo-inhibition and photo-damage in PSII.

In our current study, the higher NO_3^- uptake and nitrate reductase activity in salt-stressed plants grown under high NO_3^- occurred in parallel to the high nitrate assimilation and amino-acids synthesis. Nitrate assimilation and amino-acids synthesis are processes that consume considerable amounts of energy, reducing power (electrons) and ATP (CABELLO-PASINI *et al.,* 2011). Of course, protein synthesis requires amino acids that have originated from nitrate and ammonia assimilation, followed by an

intense interconversion among amino acids. Thus, nitrate assimilation, amino acids and protein synthesis are important processes involved in the utilization of electrons and ATP production by the photochemical machinery reducing the ‘‘energy pressure’’ on the photosystems and the thylakoid membrane. As the nitrate and ammonia assimilation depends on the supply of carbon skeletons, electrons and ATP produced by the Calvin cycle and photochemical reactions, it is important to emphasize that both CO_2 and NO_3^- assimilation might operate in balance (ROBREDO *et al.*, 2011).

Although decreases in photochemical activity and increases in the ETR/P_N ratio (an indicator of an alternative sink for photosynthetic electrons) have been observed in *J. curcas* plants under conditions of high salinity (evidenced by reductions in $\Delta F/F_m'$, qP and ETR), such conditions suggest that these changes are an acclimation mechanism rather than an indicator of dangerous effects on the chloroplast's machinery (RIBEIRO *et al.,* 2009a, b; SILVA *et al.,* 2011). In the current study, the NPQ, a parameter associated with heat dissipation, was strongly increased, especially in low-nitrate grown plants. These results again reinforce our hypothesis that high rates of nitrate assimilation might act as an important sink for the electron excess in the PSII, and thus, protect chloroplasts against photo-inhibition and oxidative damages.

The reduction in the actual quantum yield of PSII and apparent electron-transport rates might be associated with a down-regulation in the electron-transport rate at the PSII level (RIBEIRO *et al.*, 2009a, b), especially in high NO_3^- fed salt-stressed plants. Down-regulating the linear electron transport between the two photosystems to match the demand of CO_2 fixation could decrease the electron transport to O_2 on the acceptor side of PSII (i.e., in the Mehler reaction), thus minimizing the production of reactive oxygen species (DRODZOVA *et al.,* 2004; FOYER *et al.,* 2009). Alternatively, an up-regulation of other electron sinks, such as photorespiration and nitrate assimilation, also could minimize photo-damage, photo-inhibition and/ or oxidative stress (FOYER *et al.*, 2009).

5. Conclusion

Our results demonstrate that a high supply of NO_3^- and the nitrate assimilation process can mitigate the negative effects of salinity. Nitrite assimilatory reduction and ammonia assimilation in the chloroplast might act as a sink of electrons from the thylakoid membrane, thus minimizing photo-inhibition and photo-damage and stimulating CO_2 assimilation under conditions of stomatal limitations imposed by salt stress in *J. curcas.*

Author contributions

J. A. G. Silveira was the master-mind of this project, planning all of the experiments and writing the manuscript. R. M. Aragão conducted all of the experiments in the greenhouse and performed chemical and biochemical determinations. E. N. Silva measured all of the parameters of leaf gas exchange and chlorophyll fluorescence and helped in drafting the manuscript and in interpreting the results. C. F. Vieira performed the statistical analysis and helped with both the chemical measurements and the experiments in the greenhouse.

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Capítulo III
(Artigo submetido)

Nitrate alleviates NH_4^+-toxicity and salinity by restricting of oxidative damage, improvement of photochemistry activity and CO_2 assimilation in *Jatropha plants*

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Abstract – This study was assessed to test the hypothesis the nitrate assimilatory process contribute to mitigate the negative effects caused by salinity and ammonia on the photosynthesis and oxidative damage in young *Jatropha curcas* plants. In addition, we studied the role of NH_4^+, single or in combination with NO_3^- and salinity, in the oxidative and photosynthetic response. Our data clearly showed that exogenous nitrate and NO_3^- assimilation can mitigate the NH_4^+-toxicity and salinity as expressed by disturbances in the oxidative metabolism, photochemical activity and CO_2 assimilation. These effects were demonstrated when different NO_3^-/NH_4^+ ratios were employed in intact plants, leaf discs incubated with tungstate (a NR inhibitor) and single NH_4^+ in presence or absence of methionine sulphoximine (MSO, a GS inhibitor). The glutamine synthetase (GS) activity in *J. curcas* leaves is strongly increased by NH_4^+ concentrations allowing maintain non-toxic levels of ammonia in tissues under moderate/high external ammonium concentrations. In this manner, *J. curcas* leaves are resistant relatively to ammonia in terms of oxidative damage, photochemical activity and CO_2 assimilation. When the exogenous NO_3^-/NH_4^+ ratios were progressively decreased the plants suffered decrease in dry matter accumulation associated with reactive oxygen species accumulation, decrease in the photochemical efficiency, appearance of photoinhibition and decrease in the CO_2 assimilation. The antioxidant enzymes superoxide dismutase, ascorbate peroxidase and catalase are more active in presence of single NO_3^- compared with NH_4^+. Altogether, our data strongly suggest that NO_3^- nutrition is very important to *J. curcas* plants because the whole nitrate reduction assimilatory process is capable to act as an important sink for the electron excess in photosynthetic electron transport chain in the thylakoid. This nitrate effect is capable to mitigate the negative effects of NH_4^+ toxicity and salinity. Single ammonium exhibits toxicity in terms of oxidative damage and photoinhibition only when in high concentrations in *J. curcas* leaves.

Key words: Ammonium, oxidative damage, CO_2 assimilation, nitrate assimilation

1. Introduction

Nitrogen is a key element for plant growth, reproduction and is a vital nutrient and more required fertilizer in all agricultural crops. Plants can store nitrogen in large amounts within enzymes involved in process such amino acids production, carbon fixation, such as leaf Rubisco and gene regulation (TISCHNER; KAISER, 2007). However, nitrogen is also often a limiting nutrient in many natural environments, and different plant species have evolved specific strategies to acquire nitrogen (nitrate and ammonium) from their environments and assimilate it into organic compounds (BERNARD; HABASH, 2009). Some environmental factors such as high salinity can limit the nitrogen acquisition due osmotic changes which affecting nitrate uptake and assimilation, principally, through reduction in nitrate reductase (NR, E.C.1.7.1.1), the most important enzyme involved in the nitrate assimilation pathway (SILVEIRA et al., 2001).

The availability of N in soils through fertilizer utilization can improve crop productivity and mitigate some stressful factors (ALBASSAM, 2001) such salinity, but this mechanisms yet unknown (FLORES et al., 2003). In glycophytes species, there is a wide spectrum of salt sensitivity and others factors that can affect the response of plants to salt-stress (FRECHILLA, et al., 2001). Thus, the form in which N (nitrate or ammonium) is supplied to salt-stressed plants can influence the salinity response, although these responses still remain inconclusive.

The effects of NO_3^- and NH_4^+ nutrition on plant growth are intensively studied but results are not consistent and was mainly dependent on plant species, some plant species such as maize, wheat and bean, prefer nitrate nutrition, however, some species such as rice, pine, and hydrophyte, prefer ammonium nutrition (GUO et al., 2007). The ammonium in excess can lead to toxicity by potential difference across the plasma membrane and some studies have showed that this ion induces harmful changes on leaf CO_2 assimilation rate in Fraser fir (ROTHSTEIN; CREGG, 2005) and barley (KANT et al., 2007), in others species such as: French been (GUO et al., 2002); Maté (GAIAD et al., 2006), rice (GUO et al., 2007) and tomato (HORCHANI et al., 2010). Plants supplied with NH_4^+ had a CO_2 higher assimilation rate and stomata conductance than those supplied with NO_3^-, suggesting that N forms have different effects on gas exchange parameters. According Guo et al. (2007b) the effects of N form on

photosynthesis are related to ether photo-energy coast, nitrogen translocation and enzyme activity.

The NH_4^+ assimilation involves the glutamine synthetase/glutamate synthase activities (the GS/GOGAT cycle), which also occurs in chloroplasts. The reactions of this cycle consume ATP, reduced ferredoxin to synthesize amino acids, proteins and other compounds essential for plant growth (ROCHA et al., 2012). Unlike, the NH_4^+ accumulation in tissues causes deleterious effects on plant growth and can result in toxicity symptoms in many plants (Britto and Kronzucker, 2002). The reported symptoms of NH_4^+ toxicity range widely and generally appear with external NH_4^+ concentrations above 0.5 mmol/L (BRITTO; KRONZUCKER, 2002).

Several controversial effects of N form on plant growth are related to photosynthesis as well as to stomatal conductance (g_S) and intercellular CO_2 partial pressure (C_I) (GUO et al., 2007a). Recently we demonstrated that *J.curcas* plants supplied with endogenous NO_3^- high (10 mM) in nutrient solution when compared to low nitrate (1 mM) were able to mitigate the negative effects caused by salinity on photosynthetic apparatus (ARAGÃO et al, 2012). In this context, nitrate assimilation process is a strong consumer of electrons in the chloroplast electron transport chain and can thus alleviate the "electron pressure" on photosystem II (OSMOND; FORSTER 2006). The electron excess and over-reduction of the thylakoid membranes is frequent under combined conditions of high light and abiotic stress. Stress conditions, such as salinity, induce stomatal closure and impairment in CO_2 assimilation (ADAMS III et al., 2006). Under these conditions, the plants can deploy several mechanisms to avoid or minimize photo-damage, photo-inhibition and oxidative stress (SILVA et al. 2010a). The most common processes utilized are photorespiration, heat dissipation and the xanthophyll cycle. Some years ago, Osmond and Forster (2006) have suggested other processes, such as N metabolism, growth and respiration. However, the mechanisms associated with nitrate and ammonium assimilation and energy dissipation in photosystems are currently unknown.

It is true that negative effects associated with NH_4^+ nutrition in certain plant species can also be mitigates with the presence of NO_3^- in the nutrient solution (KANT et al., 2007; JUAN et al., 2007; HORCHANI et al., 2010). However, the mechanism responsible for this beneficial effect of NO_3^- on NH_4^+ remains unknown. Certain studies suggest that it might be related to changes in the physiological pH, the maintenance of appropriate carboxylate levels in plants or to specific changes in the plant content of

certain hormones and/or polyamines (GARNICA et al., 2009). This action of NO_3^- on the development of plants supplied with NH_4^+ might also be mediated by effects on the root uptake and further assimilation of NH_4^+ (BLOOM et al., 2012).

Jatropha curcas is a species that grows in marginal areas where other crop species are not able to survive (FRANCIS et al. 2005). In addition, it has high economic potential due to its seed oil quality, which can be converted to biodiesel for industrial use (KING et al. 2009). Moreover, this species has shown satisfactory yield under the constraining conditions of semiarid regions such as drought and high temperature (SILVA et al., 2010). On the other hand, the knowledge about the physiological responses of plant cultivated under salinity and with different N source remains still scarce.

The salt-induced responses on growth and potosynthesis in plants grown with different N source have been widely investigated (KANT et al., 2007; DLUZNIEWSKA et al., 2007), however to our knowledge this is the first work that evaluate in combination the degree of toxicity of NH_4^+ and the protective role of NO_3^- to alleviate these negative effects associating coordinately keys physiological mechanisms such as: growth, N metabolism, CO_2 assimilation rate, photochemical activity and oxidative protection. Here, we describe an investigation into the effects of toxicity of NH_4^+ on physiological and biochemical mechanisms of *J. curcas*. Moreover, we discuss the role of NO_3^- to mitigate the negative effects caused by ammonium and salinity.

2. Material and Methods

2.1. Plant material, growth conditions and treatments

The experiments were carried out under greenhouse conditions (3°44'S; 38°33'W, at sea level), where the environmental conditions were: minimum and maximum mean air temperature of 24 and 36°C, respectively; mean air relative humidity of 65%; means photosynthetic photon flux density (PPFD) of approximately 700 μmol m^{-2} s^{-1}. *Jatropha curcas* seeds supplied by Fazenda Instituto Tamanduá (Santa Terezinha, PB, Brazil) were previously selected taking into account the seed size and weight. Eight days after germination in sand, seedlings were transferred to plastic pots (2L) containing Hoagland and Arnon (1950) nutrient solution (pH 6.0) with one-four strength in the first week and one-half strength afterward.

Thirty days after transference to nutrient solution, the treatments with different NO_3^-/NH_4^+ ratios and salinity were imposed. Thus, the plants were divided in four ratios supplied with nitrate (NO_3^-) and/or ammonium (NH_4^+). The ratios were: KNO_3^- 5mM (100/0), KNO_3^- 3.75 mM/NH_4Cl^- 1.25 mM (75/25), KNO_3^- 1.25 mM/ NH_4Cl^- 3.75 mM (25/75) and NH_4Cl^- 5 mM (0/100). For all NO_3^-/NH_4^+ ratios, the total nitrogen concentration was 5 mM. To the salt treatment, half plants of each group were subjected to salt stress NaCl 100mM by addition in nutritive solution. NaCl was added gradually (50 mmol NaCl L^{-1} d^{-1}) into the solution in order to avoid osmotic shock. The nitrate and ammonium consumption were monitored daily and repaired when necessary. After 10 days of treatment, the plant material was collected for further analysis in the fourth hour of the photoperiod (10:00), justly to obtain a maximum activity RN.

A second experiment with leaf discs were tested with lower concentrations of NH_4^+ (0, 0.5, 1.0, 1.5 and 2.0 mM) and inhibitors specifics of NR and GS activities, tungstate 500 μM and methionine sulphoximine - 1 mM, respectively. In the study with inhibitor NR, leaf discs of *Jatropha curcas* were treated for six hours in incubation solution MES-Tris 1.5m pH 6.0 (Control), tungstate 500 μM (W), NaCl 100 mM (NaCl) or Tungstate 500 μM plus NaCl 100 mM (W + NaCl). The plants were pretreated with $Ca(NO_3^-)_2$ 10mM for 24h to induce NR activity. For study with GS inhibitor, leaf discs of *J.curcas* were treated for six hours in incubation solution MES-Tris 1.5m pH 6.0 (C), MSO 1.0 mM (MSO), or NH_4^+ 5.0 mM plus MSO 1.0 mM (NH_4^+ + MSO). The leaf discs of 10 mm diameter were obtained from the third leaf occurring after the cotyledon leaf expansion by plants with 30 days of age. The assays were

performed in Petri dishes containing incubation solution MES-Tris 1.5 mM, pH 6.0 and respective treatments for 6h, under controlled growth chambers (Phytotron) set to 27 ° C, 70% RH, CO_2 380 ppm and PAR 1000 µmol m^{-2} s^{-1}.

2.2. Nitrate and ammonium content

For nitrate and ammonium concentrations in leaves, lyophilized samples were extracted with deionised water (50mg DW/ 5 mL) at 100°C for 30 minutes and then centrifuged for 10 min at 5.000 x g. Extract aliquots were used to quantify the nitrate (CATALDO et al., 1975) and ammonium concentration (WEATHERBURN, 1967).

2.3. Enzyme assays

The nitrate reductase Activity (NR) "in vitro" was performed from frozen plant material was homogenized in chilled mortar and pestle with 100 mM potassium phosphate buffer (pH 7.4) containing 7.5 mM cysteine, 1 mM EDTA and 1.5% (w/v) casein. The homogenate was centrifuged at 30,000g for 15 min at 4 °C. After, the extract of 0.1 ml was incubated in a reaction mixture containing 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.4), 0.1 ml of 0.15 mM NADH, and 0.1 ml of 0.1 M KNO_3 at 30 °C for 30 min. Nitrate reductase (NR) was incubated with $MgCl_2$ 10 mM (for actual NRA determination) or with excess of 15 mM EDTA (for maximum NRA determination). The nitrite ion was estimated spectrophotometrically as described by Hageman and Hucklesby (1971). The glutamine synthetase activity (GS), frozen samples was were homogenized in a cold mortar and pestle with grinding medium containing 0.1 M potassium buffer (pH 8.0), EDTA 5 mM, β-mercaptoethanol 10 mM, DTT 10 mM and polyvinylpyrrolidone PVP 1%. The homogenate was centrifuged at 14, 000g for 30 min at 4º C. GS activity was determined using hydroxylamine as substrate, and the formation of λ-glutamylhydroxamate (λ -GHM) was quantified with acidified ferric chloride (ELLIOTT, 1955). SOD activity was assayed according to the methods of Giannopolitis and Ries (1977), with some modifications. CAT activity was determined according to Havir and Mchale (1987). APX was assayed following Nakano and Asada (1981).

2.4. Protein content and total free amino acids determination

For total soluble proteins determination, fresh samples of leaves were subjected to extraction (100 mg FW/ 20 mL) at 100°C for 30 minutes. The concentrations of total soluble proteins were calculated based on adjusted to a standard curve from increasing doses of bovine serum albumin (BSA) at 595 nm as described by Bradford (1976). The concentration of free amino acids was determined by the method of Peoples et al. (1989). Leaf samples were lyophilized previously subjected to extraction with MCW 12:5:3 (methanol: chloroform: water) and then centrifuged at 5,000 x g for 10 minutes. The methanolic fraction was collected and the determination of free amino acids was quantified spectrophotometrically.

2.5. Leaf H_2O_2 content and lipid peroxidation (TBARS)

Samples of fresh leaves (0.1 g) were powdered in liquid nitrogen and extracted with 100 mM potassium phosphate buffer (pH 6.4) containing 5 mM KCN, according to Cheeseman (2006). The reaction was carried out at 25ºC for 30 min and the absorbance was read at 560 nm. The H_2O_2 concentration was calculated according to a standard curve and expressed as µmol g^{-1} FW. Lipid peroxidation was determined by measuring the thiobarbituric acid-reactive substances (TBARS), according to Heath and Packer (1968). The TBARS concentrations were calculated using the molar extinction coefficient (155 mM^{-1} cm^{-1}) and are expressed as nmol g^{-1} FW.

2.6. Leaf gas exchange and chlorophyll fluorescence measurements

Leaf gas exchange was measured with an infrared gas analyzer (LCi, ADC, Hoddesdonm, UK), operating in open system and with air flow of 200 mL min^{-1}. Measurements of leaf CO_2 assimilation rate (P_N), transpiration (E), stomatal conductance (g_s) and intercellular CO_2 concentration (Ci) were taken. The instantaneous carboxylation efficiency (P_N/Ci) was calculated. The chlorophyll fluorescence was evaluated with a modulated fluorometer (FMS2, Hansatech, King's Lynn, UK). Minimum (Fo), maximum (Fm) and maximum variable ($F_v = F_m - F_o$) fluorescence

intensities were sampled under steady-state conditions in dark-adapted (30 min) leaves. In addition, measurements were also taken under light-adapted conditions, being referred as F_o' (minimum) and F_m' (maximum). The F_o' signal was measured after PSI excitation by far-red light. The fluorescence signal under light-adapted conditions before the saturation pulse is referred as Fs' and the variable fluorescence signal under light conditions is $\Delta F=F_m'-F_s'$. The following photochemical variables were calculated: maximum (F_v/F_m) and actual ($\Delta F/F_m'$) quantum yield of primary photochemistry. Apparent electron transport rate (ETR= $\Delta F/F_m' \times PPFD \times 0.5 \times 0.84$), photochemical [$qP = (F_m'-F_s)/(F_m'-F_o')$] and non-photochemical [$NPQ= (F_m-F_m')/F_m'$] quenching (FLEXAS et al., 2007). For ETR calculation, 0.5 was used as the fraction of excitation energy distributed to PSII and 0.84 as the fraction of the incoming light absorpted by leaves. Fo' is the basal fluorescence yield measured after PSI excitation by far-red light. The ETR/P_N ratio was calculated to estimate the use of electrons in other processes not related to the photosynthetic CO_2 assimilation rate (RIBEIRO et al., 2009, SILVA, et al., 2010).

2.7. *In situ* detection of hydrogen peroxide and superoxide

In situ detection of H_2O_2 was done following the protocols proposed by Thordal-Christensen et al. (1997). Leaf discs were vacuum infiltrated under dark conditions with 10 mM potassium phosphate buffer, 10 mM $NaNO_3$ and 0.1% (w/v) 3, 3'-diaminobenzidine (DAB), pH 7.8. Leaf discs were incubated overnight under dark conditions and then clarified with 0.15% (w/v) trichloroacetic acid in 4:1 (v/v) ethanol:choloroform for 48 h before being photographed. *In situ* detection of superoxide (O_2^-) was done essentially as described by Jabs et al. (1996). Detached leaves were vacuum infiltrated with 10 mM potassium phosphate buffer, 10 mM NaN_3, 0.1% (w/v) nitro blue tetrazolium (NBT) and 0.05% (v/v) Tween 20, pH 7.8. The detached, infiltrated and NBT-treated leaves were then maintained for 30 min under daylight conditions, prior to discoloration of the leaves using the same method described above for detection of H_2O_2.

2.8. Experimental design and data analysis

All experiments were arranged in a Factorial design with four replicates (an individual pot containing one plant represented one replicate). Data were analyzed by ANOVA, and means were compared by the Tukey's test at the 0.05 level of confidence. The standard deviation is plotted in all tables and figures.

3. Results

3.1 Toxic effects of ammonium and salinity

Different NO_3^-/NH_4^+ ratios affect distinctly the growth by matter dry production and were able to induce oxidative stress through increasing in NH_4^+ concentration. The matter dry productions were higher in presence of single NO_3^- (100/0) and lower in presence of single NH_4^+ (0/100) treatment. (Fig.1a). Indeed, comparing the extreme ratios (100/0 and 0/100) was observed a significantly reduction in control conditions and, in presence of salinity, this reduction was more expressive for all NO_3^-/NH_4^+ ratios tested (Fig.1a). The content of H_2O_2 and TBARS also was modulated by the different NO_3^-/NH_4^+ ratios. The H_2O_2 and TBARS showed a significantly increases as NO_3^-/NH_4^+ ratio decreased in control conditions. Comparing the extreme ratios (100/0 and 0/100) under salinity conditions, was noted a significantly increases in H_2O_2 and TBARS compared to control plants (Fig. 1b and c). Interestingly, the H_2O_2 and TBARS content were exacerbated in presence of salinity only in the 0/100 ratio. Thus, theses parameters showed that ammonium was toxic as well as salinity by reduction in growth and to induce oxidative stress in *J.curcas*.

3.2. Study of nitrate and ammonium content and enzymatic relationships

We investigated whether salinity and different NO_3^-/NH_4^+ ratios modulates the NO_3^- and NH_4^+ content, soluble proteins content and total free amino acids. As expected, in control conditions the nitrate content was higher at single NO_3^- (100/0)

well as the ammonium content was higher in single NH_4^+ (0/100). Comparing the extreme treatments 100/0 and 0/100 under salinity conditions was observed a significant reduction for both NO_3^- and NH_4^+ contents and, interestingly, the salinity effects as able to further reduce more the nitrate than ammonium content (Table 1).

The levels of soluble proteins were affected by different NO_3^-/NH_4^+ ratios. In the presence of single NO_3^- (100/0), the soluble proteins content was maximum and lower in single NH_4^+ (0/100) for control conditions. The presence of salinity reflects in lower soluble proteins levels for all treatments, compared to control (Table 1). In contrast, responses to total free amino acids showed lower levels in presence of single NO_3^- (100/0) and higher in presence of single NH_4^+ (0/100). Comparing the extreme treatments (100/0 and 0/100) can be observed that salinity conditions induced a significant increase in total free amino acids levels in comparison to control (Table 1).

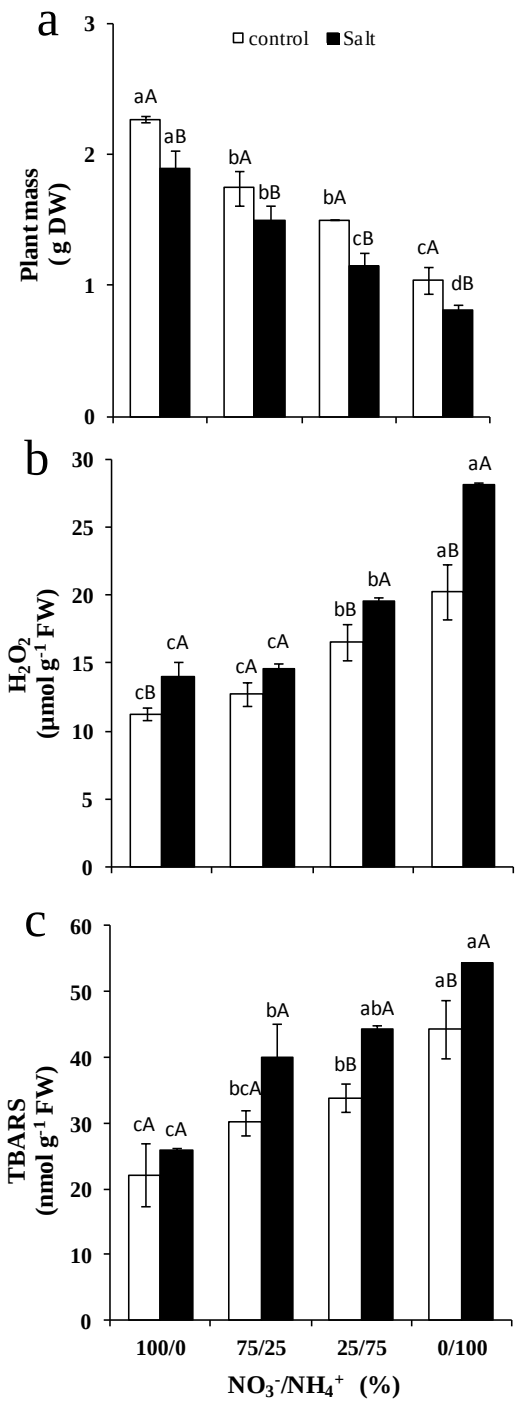

Fig 1 – Plant dry weight (a), H_2O_2 content (b) and lipid peroxidation-TBARS (c) in leaf of *Jatropha curcas* pre-treated for 10 days in solution nutritive with KNO_3^- 5mM (100/ 0), KNO_3^- 3.75 mM plus NH_4Cl^- 1.25 mM (75/ 25), KNO_3^- 1.25 mM plus NH_4Cl^- 3.75 mM (25/ 75) or only NH_4Cl^- 5 mM (0/ 100). To the salt treatment, half plants of each group were subjected to salt stress of 100 mM NaCl. Vertical bars represent standard errors (n = 4). Different lowercase indicate significant differences between the variables and uppercase indicate differences between the treatments tested at the 0.05 level by Tukey's.

Table 1- NO_3^- e NH_4^+ content, soluble proteins and total free amino acids in leaf of *Jatropha curcas* pre-treated for 10 days in solution nutritive with KNO_3^- 5mM (100/0), KNO_3^- 3.75 mM plus NH_4Cl^- 1.25 mM (75/25), KNO_3^- 1.25 mM plus NH_4Cl^- 3.75 mM (25/75) or only NH_4Cl^- 5 mM (0/100). To the salt treatment, half plants of each group were subjected to salt stress of 100 mM NaCl. Different lowercase indicate significant differences between the variables and uppercase indicate differences between the treatments tested at the 0.05 level by Tukey's.

	NO_3^- (µmol g^{-1} DW)		NH_4^+ (µmol g^{-1} DW)		Soluble proteins (mg protein g^{-1} DW)		Total free amino acids (µmol g^{-1} DW)	
NO_3^-/NH_4^+ (%)	Control	Salt	Control	Salt	Control	Salt	Control	Salt
100/0	137.91±9.38aA	102.13±5.02aB	4.92±0.61cA	3.99±0.54bA	25.97±0.48aA	18.23±0.72aA	256.22±6.14cA	389.66±1.67cB
75/25	122.74±1.34aA	98.57±0.67aB	6.29±0.20cA	5.83±0.33aA	22.36±0.48abA	15.69±2.00aB	294.12±28.47bcA	469.41±6.14bB
25/75	106.39±3.01bA	79.85±4.35bB	7.98±0.35bA	6.08±0.42aB	19.91±2.73bA	14.45±0.48aB	370.71±58.62bA	519.55±20.09bB
0/100	91.94±4.69bA	40.04±0.20cB	10.83±0.54aA	6.07±0.36aB	19.87±0.72bA	16.81±0.02aB	550.76±47.45aA	580.75±2.00aB

±represent standard errors (n = 4).

The enzymes activities related to assimilation of NO_3^- (NR) and NH_4^+ (GS) were analyzed to identify the source of nitrogen is more efficient and what the effects of salinity on them. As expected, the NR activity was maximum in single nitrate (100/0) and decreased progressively as the NO_3^-concentration decreased in the nutrient solution. Indeed, very little activity was detected in 0/100 treatment and under salinity conditions there was no activity (Fig. 2a). Interestingly, the salinity was able to reduce NR Activity in more than half in 100/0 and, the combined effect of NH_4^+ x salinity led to a drastic reduction in NR activity. Unlike, the GS activity was lower in 100/0 treatment and increased progressively as the NH_4^+concentration increment in the nutrient solution. The highest values for GS activity were found in 75/25 and 0/100 treatments and under salinity conditions, the GS activity were higher in all NO_3^-/NH_4^+ ratios, compared to control (Fig.2b). Curiously, the GS Activity was very efficient due to high levels of NH_4^+, principally in the presence of salinity. These data showed that high dry matter productions were related to high NR activity and low GS activity. Moreover, the low soluble proteins and high free amino acids levels indicating a toxic tendency by NH_4^+ and salinity.

3.3. Gas exchange analysis under different NO_3^-/NH_4^+ ratios and salinity

A study of gas exchange was then performed in order to observe how the processes of CO_2 assimilation and photochemistry operate under main sources nitrogen (nitrate and ammonium) and salinity. The CO_2 assimilation (P_N) was highly modulated by different NO_3^-/NH_4^+ ratios and also by salinity. The P_N was higher at in single NO_3^- (100/0) and decreased progressively to less than half in in single NH_4^+ (0/100). The salinity conditions negatively affected in all NO_3^-/NH_4^+ ratios. Indeed, the salinity reduced P_N to less than half in 100/0 and this tendency was aggravated as the NH_4^+ concentration increment in nutritive solution (Fig. 3a). The stomatal conductance (g_s) showed a trend similar to P_N where the 100/0 was higher than 0/100 and the salinity effect reduced drastically in all NO_3^-/NH_4^+ ratios. Interestingly, the g_s was not detected in 0/100 under salinity conditions (Fig. 3b). The P_N/Ci showed similar behavior to the P_N which was higher in 100/0 and lower in 0/100. The salinity drastically reduced to less than half of control conditions in all NO_3^-/NH_4^+ ratios tested (Fig. 3c). As expected, the ratio ETR/P_N was lower at 100/0 and increased progressively with the increment of NH_4^+concentration in nutrient solution (Fig. 3d). The salinity was able to considerably

increase the ETR/P_N in approximately two folds for all NO_3^-/NH_4^+ ratios. The analyzes of these data indicated that the presence of NO_3^- was essential to keep the P_N and g_s in 100/0 and 75/25 treatments, but the extent to which the NO_3^- was becoming limited in nutrient solution, the harmful effects caused by ammonia toxicity alone or combined with salinity were increased. This harmful effect by NH_4^+ was able to induce lower instantaneous carboxilation efficiency (P_N/C_I) and increases in ETR/P_N (an indicator of alternative sink for photosynthetic electrons). The presence of salinity combinated with single NO_3^- (100/0) reduced P_N and g_s, but in the presence of single NH_4^+ (0/100) these reductions were more pronounced.

The photochemical parameters $\Delta F/F_m'$, ETR, qP and NPQ were monitored on the different NO_3^-/NH_4^+ ratios. The responses were varied and indicated that both N sources affect differently the photochemical processes. The ΔF/Fm' was maximum in 100/0 and decreased as the NO_3^- concentration was diminished in the nutrient solution. In presence of salinity, the reduction of ΔF/Fm' was more pronounced in all NO_3^-/NH_4^+ ratios, principally in the 0/100 combination (Fig. 4a). The ETR showed a trend similar to ΔF/Fm' where the 100/0 had higher than 0/100 treatment and salinity conditions was able to reduce in all NO_3^-/NH_4^+ ratios, principally in the 0/100 ratio (Fig. 4b). The qP moderately decreased in the direction of 100/0 to 0/100 and under salinity conditions, the qP showed greater reductions with emphasis to 0/100 which reduced two folds compared to its respective control (Fig. 4c). Unlike, the NPQ increased in the direction of 100/0 to 0/100 and in presence of salinity, the NPQ was more pronounced for all NO_3^-/NH_4^+ ratios, principally in the 0/100 ratio (Fig. 4d). As expected, the salinity decreased qP and increased NPQ in all NO_3^-/NH_4^+ ratios. Interestingly, the photochemistry damage in 0/100 was more intense compared to other treatments.

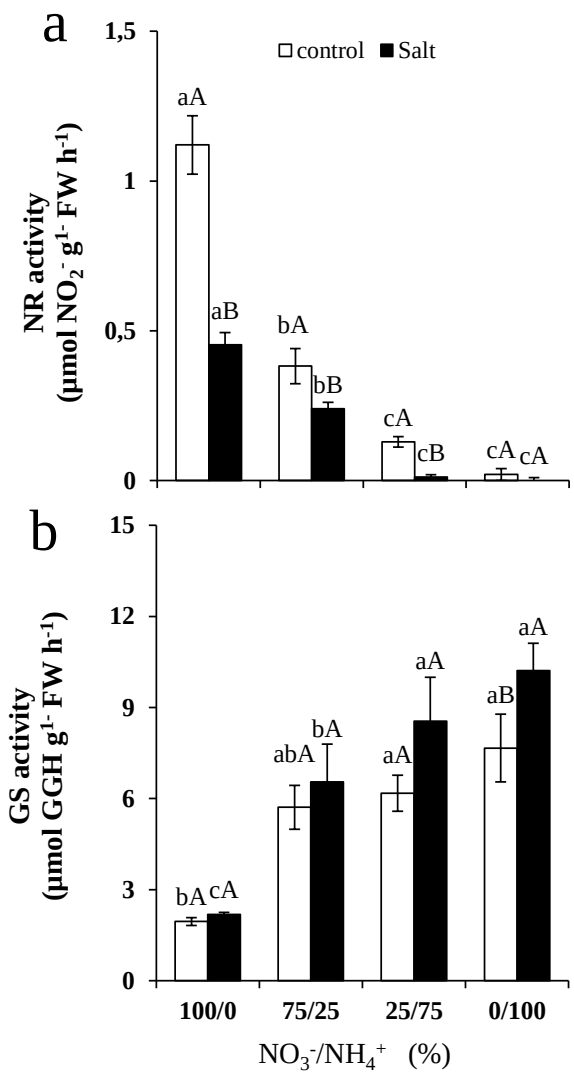

Fig 2 – NR activity (a) and GS activity in leaf of *Jatropha curcas* pre-treated for 10 days in solution nutritive with KNO_3^- 5mM (100/ 0), KNO_3^- 3.75 mM plus NH_4Cl^- 1.25 mM (75/ 25), KNO_3^- 1.25 mM plus NH_4Cl^- 3.75 mM (25/ 75) or only NH_4Cl^- 5 mM (0/ 100). To the salt treatment, half plants of each group were subjected to salt stress of 100 mM NaCl. Vertical bars represent standard errors (n = 4). Different lowercase indicate significant differences between the variables and uppercase indicate differences between the treatments tested at the 0.05 level by Tukey's.

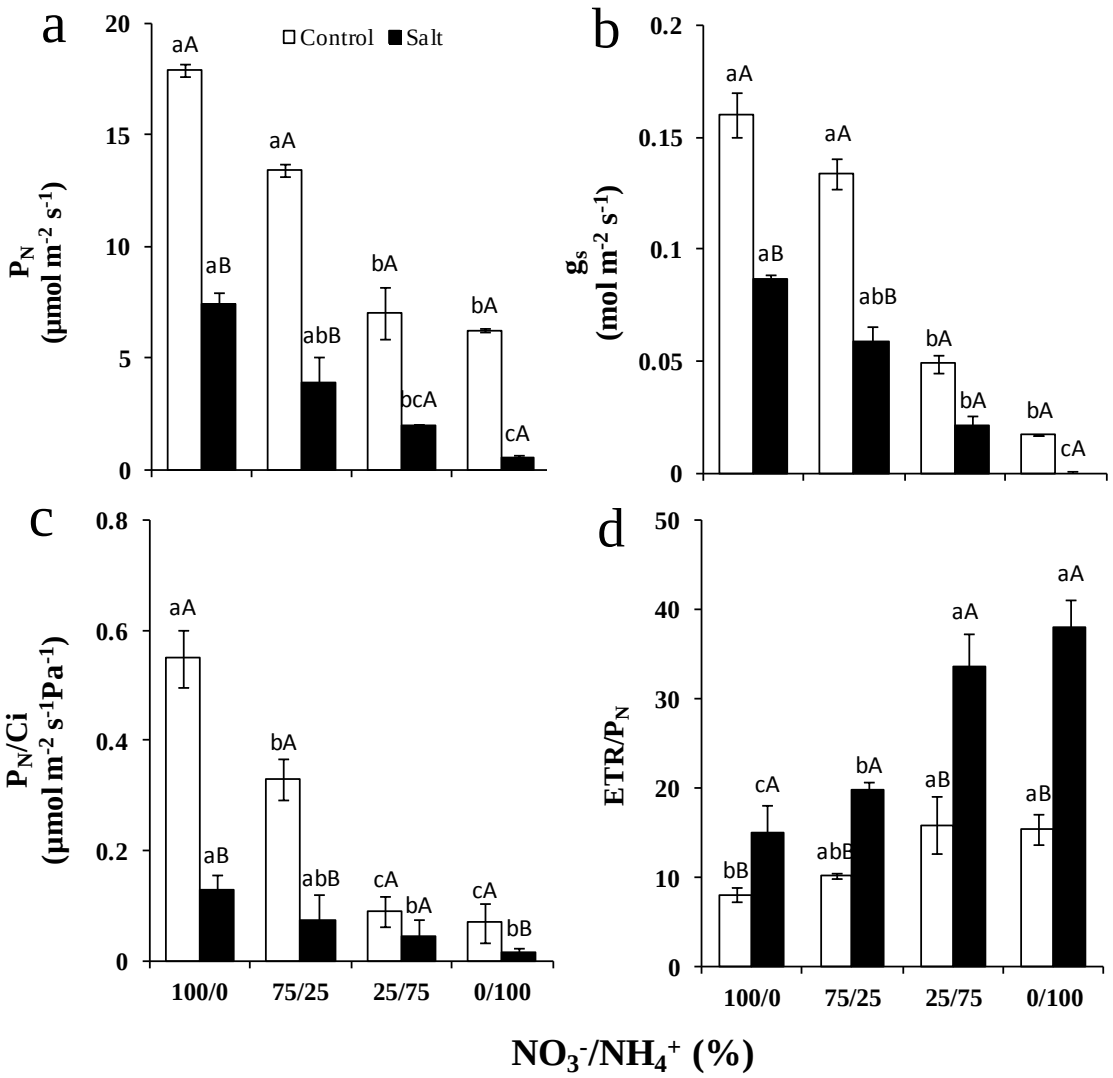

Fig. 3 - Photosynthesis – P_N (a), stomatal conductance – g_s (b), P_N/Ci ratio (c) and (d) ETR/P_N ratio in leaf of *Jatropha curcas* pre-treated for 10 days in solution nutritive with KNO_3^- 5mM (100/ 0), KNO_3^- 3.75 mM plus NH_4Cl^- 1.25 mM (75/ 25), KNO_3^- 1.25 mM plus NH_4Cl^- 3.75 mM (25/ 75) or only NH_4Cl^- 5 mM (0/ 100). To the salt treatment, half plants of each group were subjected to salt stress of 100 mM NaCl. Vertical bars represent standard errors (n = 4). Different lowercase indicate significant differences between the variables and uppercase indicate differences between the treatments tested at the 0.05 level by Tukey's.

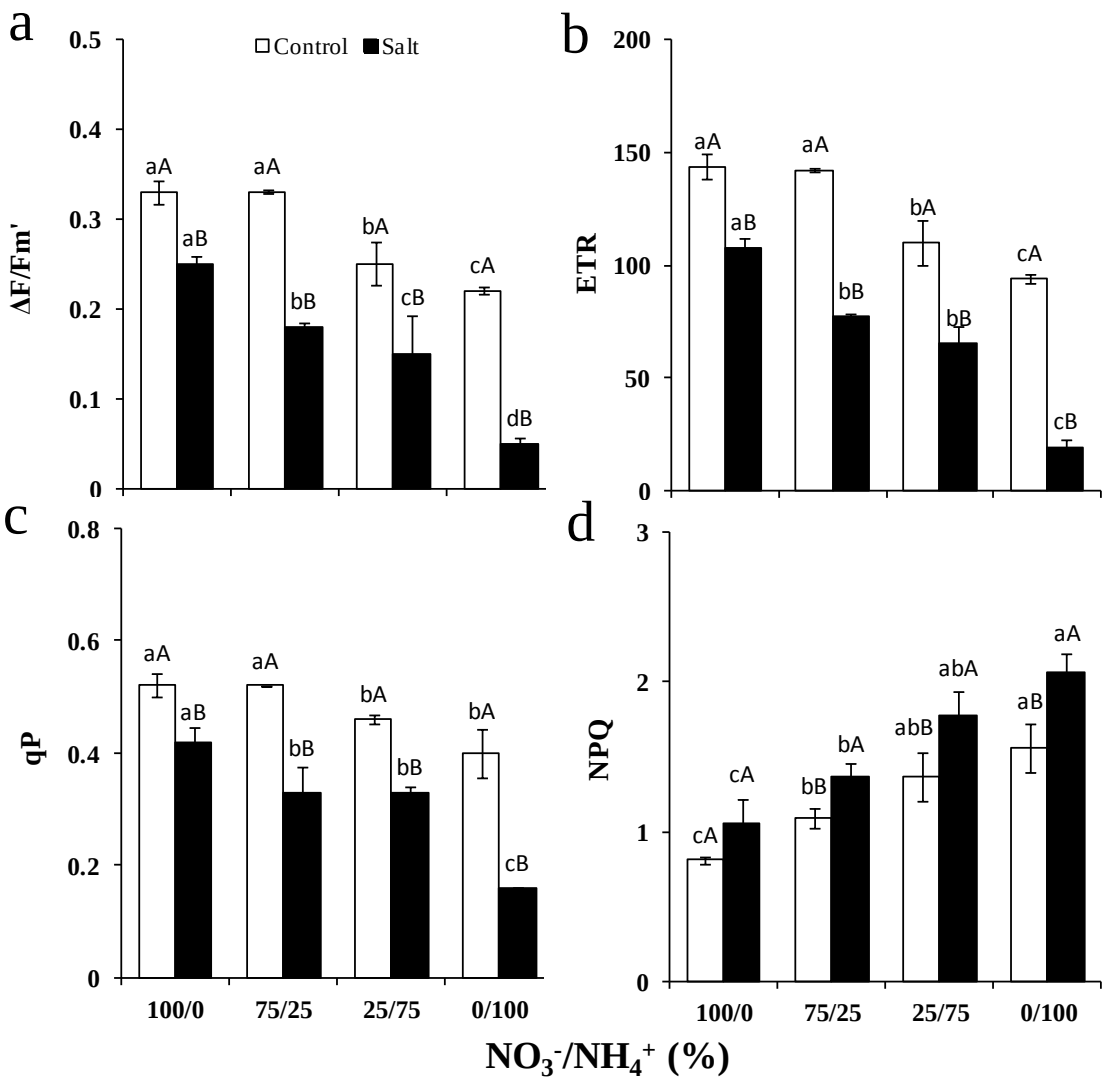

Fig.4 - The effective quantum yield of PSII - ΔF/Fm´ (a), the apparent electron transport rate – ETR (b), photochemical – qP (c) and non-photochemical quenching – NPQ (d) in leaf of *Jatropha curcas* pre-treated for 10 days in solution nutritive with KNO_3^- 5mM (100/ 0), KNO_3^- 3.75 mM plus NH_4Cl^- 1.25 mM (75/ 25), KNO_3^- 1.25 mM plus NH_4Cl^- 3.75 mM (25/ 75) or only NH_4Cl^- 5 mM (0/ 100). To the salt treatment, half plants of each group were subjected to salt stress of 100 mM NaCl. Vertical bars represent standard errors (n = 4). Different lowercase indicate significant differences between the variables and uppercase indicate differences between the treatments tested at the 0.05 level by Tukey's.

The photoinhibition indicators F_o and F_v/F_m did not change significantly even when comparing the extreme 100/0 and 0/100 ratios. However, the salinity effect increased significantly F_o only in single NH_4^+ (0/100) (Fig. 5a). Unlike, the salinity decreased significantly F_v/F_m only 0/100, which indicates a tendency of photoinhibition when there is interaction between NH_4^+ and salinity (Fig. 5b). The reductions induced by NH_4^+ shown in $\Delta F/F_m'$, ETR, qP and increase in NPQ indicate that NO_3^--N was the preferred source of nitrogen and has a protective action against photochemical damage. Indeed, comparing the toxic effects caused by salinity, the 100/0 was less affected than the end 0/100 as showed in $\Delta F/F_m'$, ETR and qP. In addition, the F_o and F_v/F_m showed that the interaction between NH_4^+ and salinity were harmful leading to a small but significant photoinhibition.

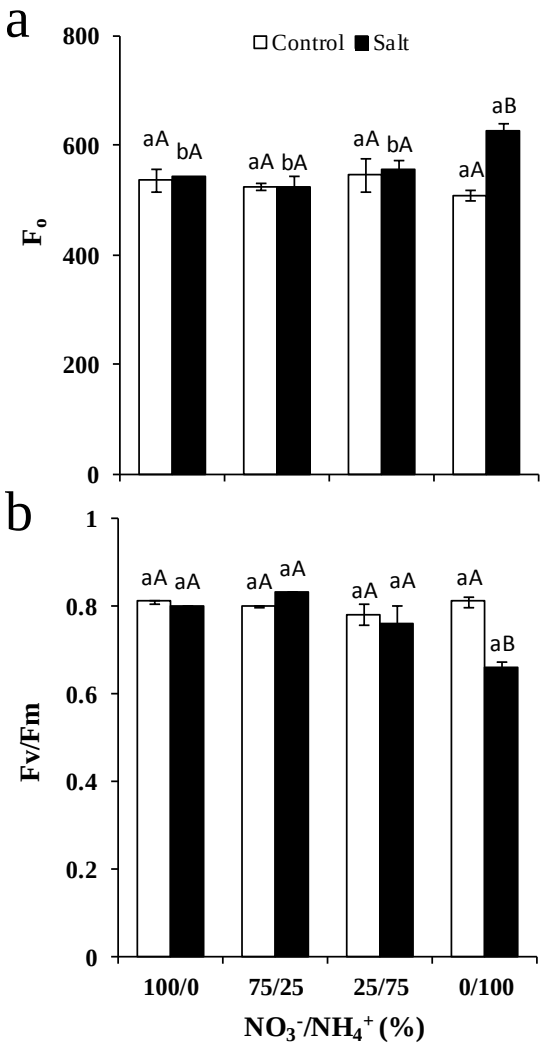

Fig. 5 – Fluorescence minimum-Fo (a) maximum quantum yield of PSII -F_V/F_M (b) in leaf of *Jatropha curcas* pre-treated for 10 days in solution nutritive with KNO_3^- 5mM (100/ 0), KNO_3^- 3.75 mM plus NH_4Cl^- 1.25 mM (75/ 25), KNO_3^- 1.25 mM plus NH_4Cl^- 3.75 mM (25/ 75) or only NH_4Cl^- 5 mM (0/ 100). To the salt treatment, half plants of each group were subjected to salt stress of 100 mM NaCl. Vertical bars represent standard errors (n = 4). Different lowercase indicate significant differences between the variables and uppercase indicate differences between the treatments tested at the 0.05 level by Tukey's.

3.4. Antioxidant enzymes analysis under different NO_3^-/NH_4^+ ratios and salinity

Due to an increased production of H_2O_2 and TBARS, we tested whether the antioxidant enzymes (APX, CAT and SOD) were modulated by different NO_3^-/NH_4^+ ratios and salinity. In the presence of single NO_3^- (100/0), the APX activity was maximum and decreases gradually until 0/100. The salinity condition was able to induce higher APX activity for all ratios, except for 0/100, compared to control (fig. 6a). Similar to APX, the CAT activity was maximum in 100/0 and decreases progressively until 0/100. Comparing the NO_3^-/NH_4^+ ratios can be observed that salinity conditions significantly reduced the CAT activity in 100/0 and 75/25, compared to control (fig. 6b). Interestingly, the CAT activity in 100/0 was two folds higher than 0/100 for both control and salinity conditions (Fig. 6b). The SOD activity was little affected by different NO_3^-/NH_4^+ ratios. However, in the presence of salinity the 0/100 increased its activity (Fig. 6c). Overall, the single NO_3^- showed high APX, CAT and SOD activities. The effect of salt stress on SOD was contrasting for to induce reduction under controlled conditions and to increase under conditions of salinity. This increase was most prominent in plants that had NH_4^+ in nutrient solution.

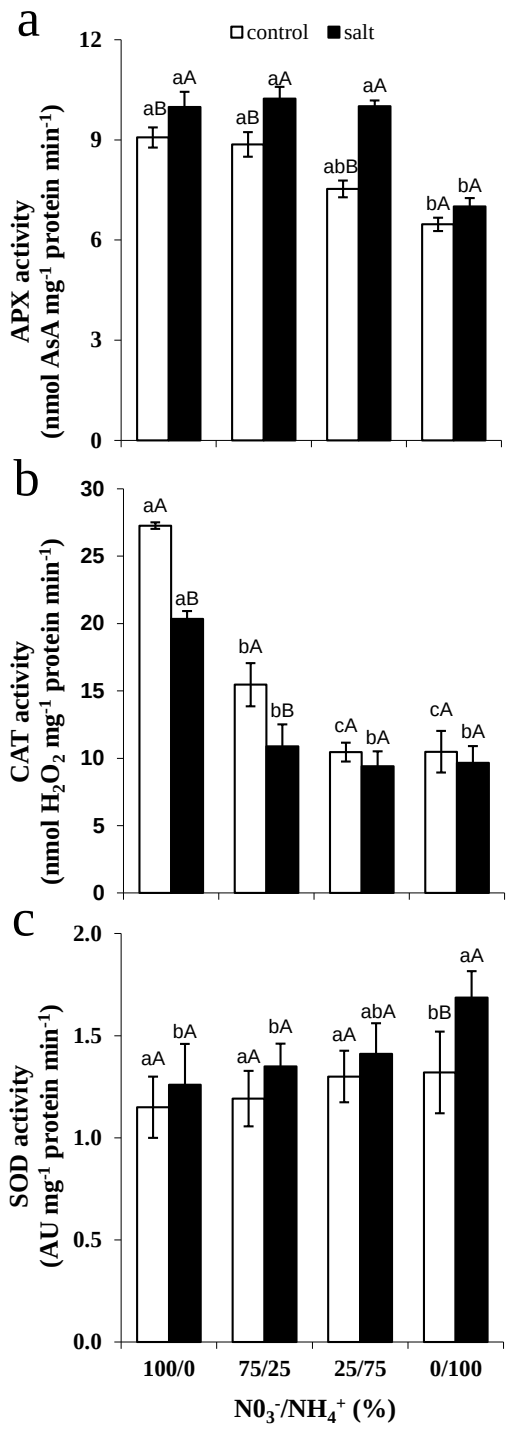

Fig.6 – APX activity (a), CAT activity (b) and SOD activity (c) in leaf of *Jatropha curcas* pre-treated for 10 days in solution nutritive with KNO_3^- 5mM (100/ 0), KNO_3^- 3.75 mM plus NH_4Cl^- 1.25 mM (75/ 25), KNO_3^- 1.25 mM plus NH_4Cl^- 3.75 mM (25/ 75) or only NH_4Cl^- 5 mM (0/ 100). To the salt treatment, half plants of each group were subjected to salt stress of 100 mM NaCl. Vertical bars represent standard errors (n = 4). Different lowercase indicate significant differences between the variables and uppercase indicate differences between the treatments tested at the 0.05 level by Tukey's.

Correlation analyzes were conducted using the parameters evaluated in this experiment. In table 2 shows the highest correlations calculated with bases in their respective values of regression (r). According to the calculated regressions can be noted that the parameters such as electrolyte leakage, H_2O_2 and TBARS were positively correlated. In addition, data such as mass, P_N, ETR and qP were better correlated with higher content of NO_3^- in tissue and NR activity. The ETR/P_N was better correlated with GS Activity (Table 2). Although the responses obtained in this experiment showed positive effects to NO_3^- nutrition and negative effects to NH_4^+ nutrition, is not yet clear whether these effects were due to NO_3^- protection or NH_4^+ toxicity. Thus, the next experiments were designed to answer this question.

Table 2 - Regression analysis of parameters measured with different NO_3^-/ NH_4^+ ratios combined with salinity.

Correlations	Equations	Regression (r)	Significance
H_2O_2 x TBARS	Y= 2.2018x – 0.9143	0.96	p<0.05
H_2O_2 x EL	Y= 1.1798x + 4.3893	0.76	p<0.05
TBARS x EL	Y= 0.4929x + 8.1913	0.95	p<0.05
Mass x NO_3^-	Y= 38.629x + 51.38	0.98	p<0.05
Mass x NH_4^+	Y= -4.455x + 15.11	0.90	p<0.05
P_N x NR	Y= 0.0158x + 0.1789	0.88	p<0.05
P_N x GS	Y= -0.0057x + 0.1728	0.76	p<0.05
P_N x NO_3^-	Y= 0.1771x + 9.9522	0.89	p<0.05
P_N x NH_4^+	Y= -1.7383x + 24.711	0.79	p<0.05
NO_3^- x ETR	Y = 1.3616x – 37.804	0.97	p<0.05
NH_4^+ x ETR	Y = -0.2549x + 96.543	0.01	p<0.05
NO_3^- x qP	Y = 0.0039x + 0.0126	0.96	p<0.05
NH_4^+ x qP	Y = 0.0026x + 0.3756	0.05	p<0.05
NR x ETR/P_N	Y = -18.921x + 25.026	0.66	p<0.05
GS x ETR/P_N	Y = 2.966x + 1.2933	0.79	p<0.05

3.5. NR activity inhibition induces low oxidative damage and photodamage

In this study we focused on the influence of NO_3^- assimilation in the protection against oxidative damage, photochemical and CO2 assimilation efficiency. Therefore, a specific inhibitor of NR enzyme (tungstate -WO_4^-) was used and the results compared to presence of salt stress (NaCl). Initially, the NR activity was harmfully affected by all treatements, but single WO_4^- (W) and combinated with NaCl (W+NaCl). The activation state between actual and maximum NR activity was average 80% for all treatments (data not show) (Fig. 7a). As a consequence, the NR inhibition was able to increase the electrolyte leakage (Fig. 7b). The H_2O_2 content and TBARS increased in response to NR inhibition being more pronounced when combined with NaCl (W+NaCl) (fig. 7c e d). In accordance with H_2O_2, analysis in situ of staining leaf discs using DAB or NBT as indicators of H_2O_2 or O_2^-, respectively, showed that H_2O_2 was present in all treatments, but the highest level were detected in W+NaCl treatment, similar to tendency of the data of figure 7c (Fig. 8). Additionally, high levels of the O_2^- radical were detected in all treatments being more distinguished at W+NaCl treatment (Fig. 8).

The gas exchange analyses showed that P_N was lightly modulated by WO_4^- and salinity. Statistical analysis for P_N showed that its reduction was only significant among treatments combined NaCl and W+NaCl compared to control (C) and the other parameter settings g_s, E and P_N/Ci did not show significant changes compared to control (Table 3). The photochemical parameters ΔF/Fm', qP, NPQ, ETR, EXC and the sink alternative indicator (ETR/P_N) were monitored in presence of WO_4^- and salinity. The responses indicate that no significant difference occurred when WO_4^- or NaCl were applied separately, compared to control, except to W+NaCl interaction that showed high changes in the parameters analyzed (Table 4) The Fo and Fv/Fm values remained constant indicating no photoinhibition (Table 4). In general, these data confirm that the NR inhibition induced oxidative damage and increased leakage of electrolyte, principally, in presence if salinity. However, treatment with single WO_4^- (W) showed less photodamage and oxidative damage than when combined with NaCl (W + NaCl). This behavior is due, at principle, to pretreatment with NO_3^- 10 mM for to active NR activity. It was essential to protect against photodamage and reduction CO_2 assimilation efficiency.

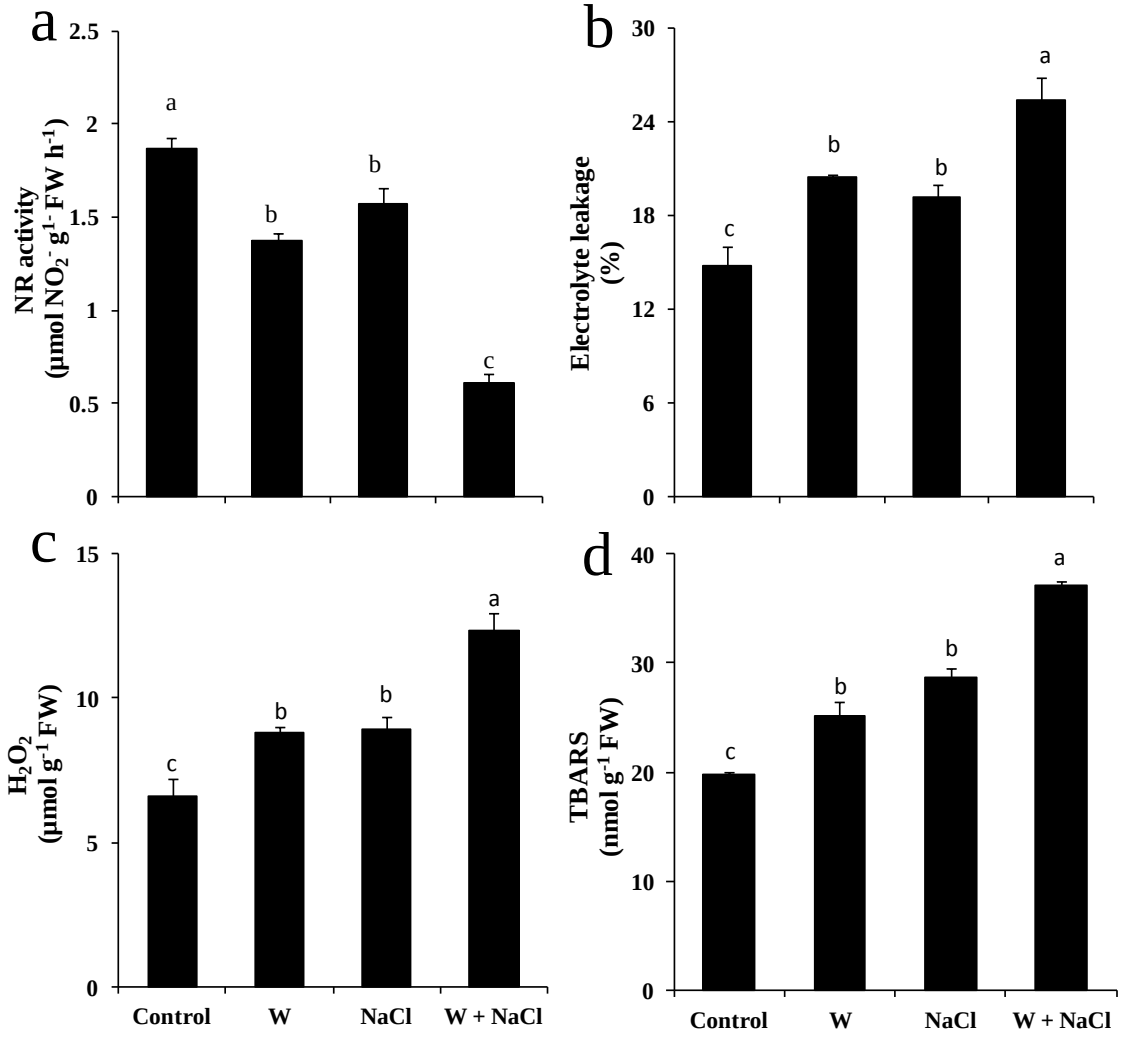

Figure 7 – NR activity (a), Electrolyte leakage (b), H_2O_2 content (c) and TBARS content (d) in leaf discs of *Jatropha curcas* treated for six hours in incubation solution MES-Tris 1.5m pH 6.0 (Control), or containing tungstate 500 µM (W), or containing NaCl 100 mM (NaCl) or Tungstate 500 µM plus NaCl 100 mM (W + NaCl). The plants were pretreated with $Ca(NO_3^-)_2$ 10mM for 24h to induce NR activity . Vertical bars represent standard errors (n = 4). Different lowercase indicate significant differences treatments and the uppercase indicate differences between the actual and maximum NR activity in each treatment tested at the 0.05 level by Tukey's.

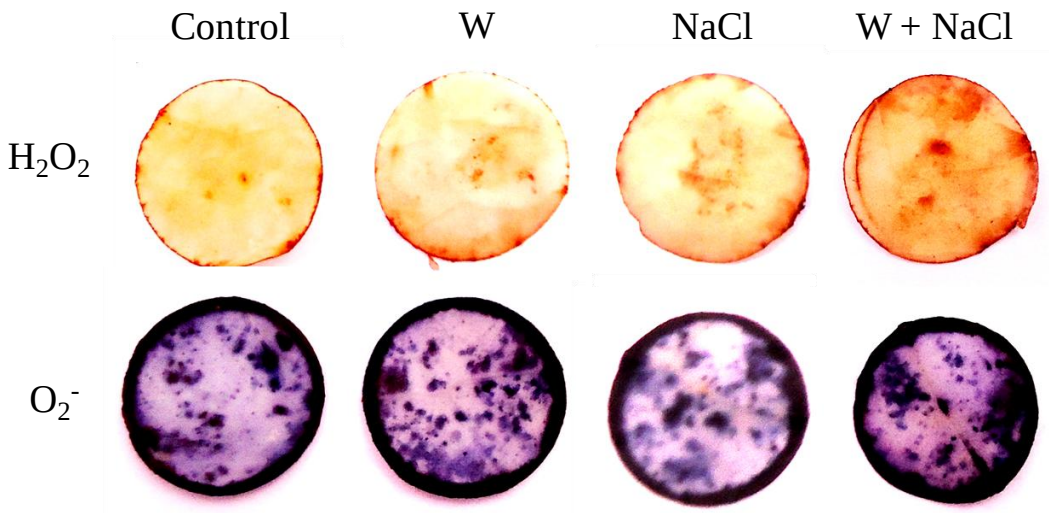

Fig. 8 – In situ determination of H_2O_2 and $O_2^{\bullet -}$ by staining leaf discs with DAB or NBT, respectively. The leaf discs of *Jatropha curcas* were treated for six hours in incubation solution MES-Tris 1.5m pH 6.0 (Control), or containing tungstate 500 µM (W), or containing NaCl 100 mM (NaCl) or Tungstate 500 µM plus NaCl 100 mM (W + NaCl). The plants were pretreated with $Ca(NO_3^-)_2$ 10mM for 24h to induce NR activity . The leaf discs are representatives of five biological replicates.

Table 3 - Photosynthesis (P_N), stomatal conductance (g_s), Transpiration (E) and P_N/Ci ratio in leaf discs of *Jatropha curcas* treated for six hours in incubation solution MES-Tris 1.5m pH 6.0 (Control), tungstate 500 µM (W), NaCl 100 mM (NaCl) or Tungstate 500 µM plus NaCl 100 mM (W + NaCl). The plants were pretreated with $Ca(NO_3^-)_2$ 10mM for 24h to induce NR activity.

	P_N µmol m^{-2} s^{-1}	g_s mol m^{-2} s^{-1}	E mmol m^{-2} s^{-1}	P_N/Ci µmol m^{-2} s^{-1} Pa^{-1}
C	17.76±0.32	0.36±0.03	5.59±0.48	0.06±0.01
W	17.22±0.45	0.37±0.02	5.53±0.30	0.06±0.00
NaCl	15.76±1.04*	0.36±0.04	5.54±0.77	0.05±0.01
W+NaCl	13.42±1.03*	0.35±0.01	4.82±0.16	0.05±0.01

± represent standard errors (n = 4)

Table 4 - The effective ($\Delta F/F_m´$) and maximum (F_v/F_m) quantum yield of PSII, photochemical (qP) and non-photo-chemical quenching (NPQ), the apparent electron-transport rate (ETR), the relative energy excess at the PSII level (EXC), the ratio between the apparent electron-transport rate and the leaf CO 2 assimilation rate (ETR/P_N) and the minimum fluorescence (Fo) in leaf discs of *Jatropha curcas* treated for six hours in incubation solution MES-Tris 1.5m pH 6.0 (Control), tungstate 500 µM (W), NaCl 100 mM (NaCl) or Tungstate 500 µM plus NaCl 100 mM (W + NaCl). The plants were pretreated with $Ca(NO_3^-)_2$ 10mM for 24h to induce NR activity.

	$\Delta F/Fm´$	qP	NPQ	ETR	EXC	ETR/P_N	Fo	Fv/Fm
C	0.21±0.02	0.44±0.03	1.36±0.02	95.19±7.76	0.72±0.02	5.29±0.36	538.44±4.88	0.74±0.01
W	0.23±0.02	0.45±0.07	1.39±0.23	96.82±7.17	0.69±0.02	5.53±0.26	527.71±6.69	0.76±0.01
NaCl	0.22±0.01	0.44±0.03	1.51±0.33	96.01±0.84	0.71±0.01	6.16±0.73	526.22±14.16	0.76±0.03
W+NaCl	0.18±0.02*	0.38±0.04*	1.54±0.02*	79.09±9.58*	0.75±0.04	5.89±0.13*	554.56±17.78	0.74±0.02

± represent standard errors (n = 4)

3.6. Low NH_4^+ concentrations are able to induce oxidative damage.

In the first experiment it was found that moderate NH_4^+ concentration (25/75 treatment) was capable of inducing oxidative damage and photochemical, even in the absence of salinity. Thus, to confirm if low NH_4^+ nutrition may be associated with the damage previously shown, we performed a dose-dependent study with leaf discs treated with crescent levels of NH_4^+. The analyzes for the NH_4^+ content showed that the leaf discs responded to increasing levels through its accumulation in tissue (Fig. 9a) and the electrolytes leakage showed that discrete increases in NH_4^+ induced more membrane damage (Fig. 9b). The H_2O_2 and TBARS accumulations were responsive to lower concentrations of NH_4^+. The H_2O_2 content was significantly increased from 0.5 mM and did not differ significantly of 1.0, 1.5 and 2.0 mM treatments (Fig. 9c). The Data for TBARS showed that was able to enhance from 1.0 mM and between the treatments 1.0, 1.5 and 2.0 mM no significant difference was observed (Fig. 9d). In accordance with H_2O_2, analysis in situ of staining leaf discs using DAB or NBT as indicators of H_2O_2 or O_2^-, respectively, showed that H_2O_2 was present in all treatments, but the highest levels were detected in 1.0, 1.5 e 2.0 mM treatments, likely to figure 9c (Fig. 10). Additionally, high levels of the O_2^- radical were detected in all treatments being more distinguished at higher concentrations of NH_4^+ (Fig. 10). These experiments indicate that ammonium is toxic to *J.curcas* at low concentrations such as 1.0 mM and this toxicity is observed from experiments in a short time, as shown by the leaf discs in this experiment.

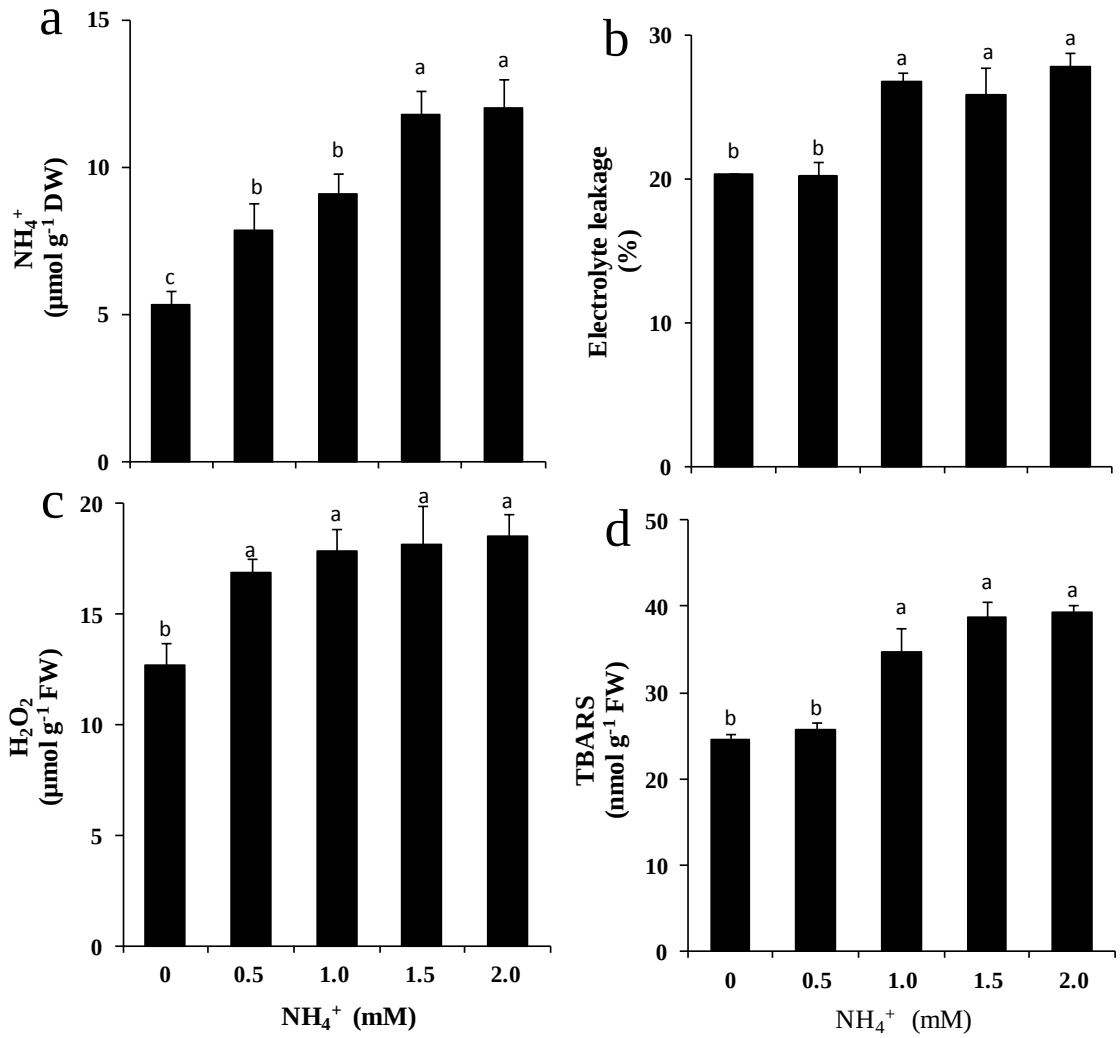

Figure 9 – NH_4^+ content (a), electrolyte leakage (b), H_2O_2 content (c) and TBARS content (d) in leaf discs of *Jatropha curcas* treated for six hours in incubation solution MES-Tris 1.5m pH 6.0 with NH_4Cl^- 0, 0.5, 1.0, 1.5 and 2.0 mM. Vertical bars represent standard errors (n = 4). Different lowercase indicate significant differences between the variables and the letters indicate differences between the treatments tested at the 0.05 level by Tukey's.

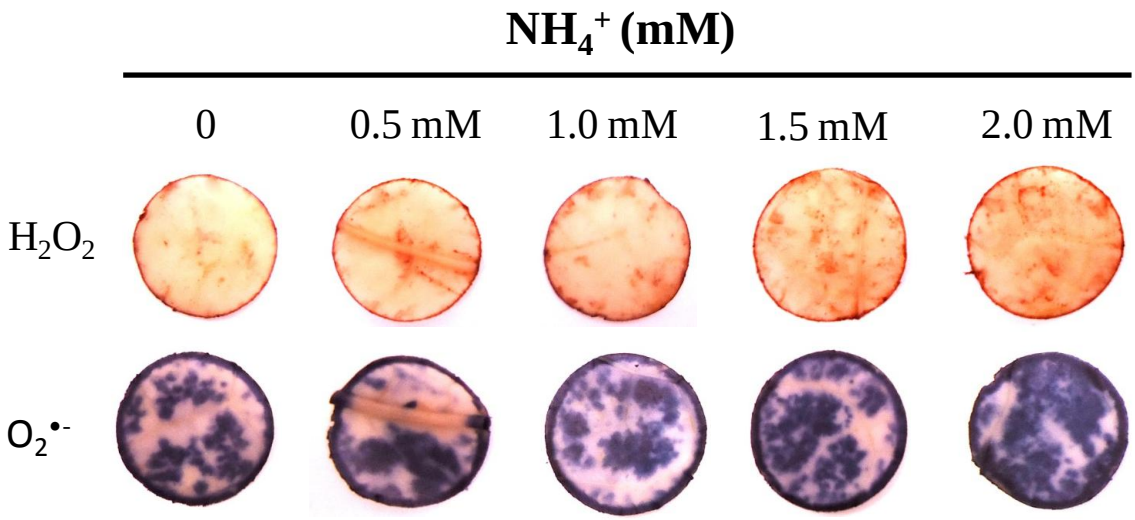

Figure 10 – In situ determination of H_2O_2 and $O_2^{\bullet-}$ by staining leaf discs with DAB or NBT, respectively. The leaf discs of *Jatropha curcas* were treated for six hours in incubation solution MES-Tris 1.5m pH 6.0 with NH_4Cl^- 0, 0.5, 1.0, 1.5 and 2.0 mM. The leaf discs are representatives of five biological replicates.

3.7. Increases in NH_4^+ concentration by GS activity inhibition induce severe damages

In the first study with different NO_3^-/NH_4^+ ratios, the GS activity was strongly induced by NH_4^+, but the main cause for the excessive increase of GS activity was not understood. Thus, we conducted a study with MSO (An inhibitor GS) in leaf discs to understand how GS activity was associated with photochemical damage and reduction in the CO_2 assimilation. The analysis for NH_4^+ content showed increments in all treatments principally, when NH_4^+ was combined with MSO (NH_4^+ + MSO) (Fig. 11a). The MSO was able to induce a harmfully reductions in GS activity when treated by single MSO (MSO) and combined with NH_4^+ (NH_4^+ + MSO) (Fig. 11b). As a consequence, the GS inhibition was able to increase the electrolyte leakage (Fig. 12a) and accumulation in H_2O_2 and TBARS, but this responses were more pronounced in NH_4^+ + MSO treatment (Fig. 12b e c). In accordance with H_2O_2, analysis in situ of staining leaf discs using DAB as indicators of H_2O_2, has show that H_2O_2 was present in all treatments, but the highest levels were detected in NH_4^+ + MSO treatment, likely to figure 12b (Fig. 13).

The gas exchange analyses showed that P_N significantly reduced by treatment with single MSO and, unexpectedly, the NH_4^+ + MSO treatment was able to reduce photosynthesis in approximately two folds. The g_s, E and P_N/Ci was lightly modulated

by single MSO, but the NH_4^+ + MSO combination induced detrimental changes, compared to control (Table 5). The photochemical parameters ΔF/Fm', qP, NPQ, ETR, EXC and the sink alternative indicator (ETR/P_N) showed changes significant only in NH_4^+ + MSO treatment (Table 6). Interestingly, these results specifically indicate photochemical breakdown such as lower NPQ, which would normally be the opposite. The Fv/Fm reduced significantly indicating a photoinhibition by NH_4^+ + MSO treatment. Curiously, the EXC and Fo did not change in NH_4^+ + MSO, which leads us to believe that this combination led to photochemical disturbances abnormal in photosynthetic apparatus (Table 6). Overall, the responses indicate that NH_4^+ accumulation in tissues and GS activity inhibition by MSO is capable of inducing oxidative damage and photoinhibition, particularly when combined with high levels of NH_4^+. Moreover, high activity of GS is an indication that *J.curcas* has an efficient mechanism to prevent accumulation and consequently toxicity by NH_4^+.

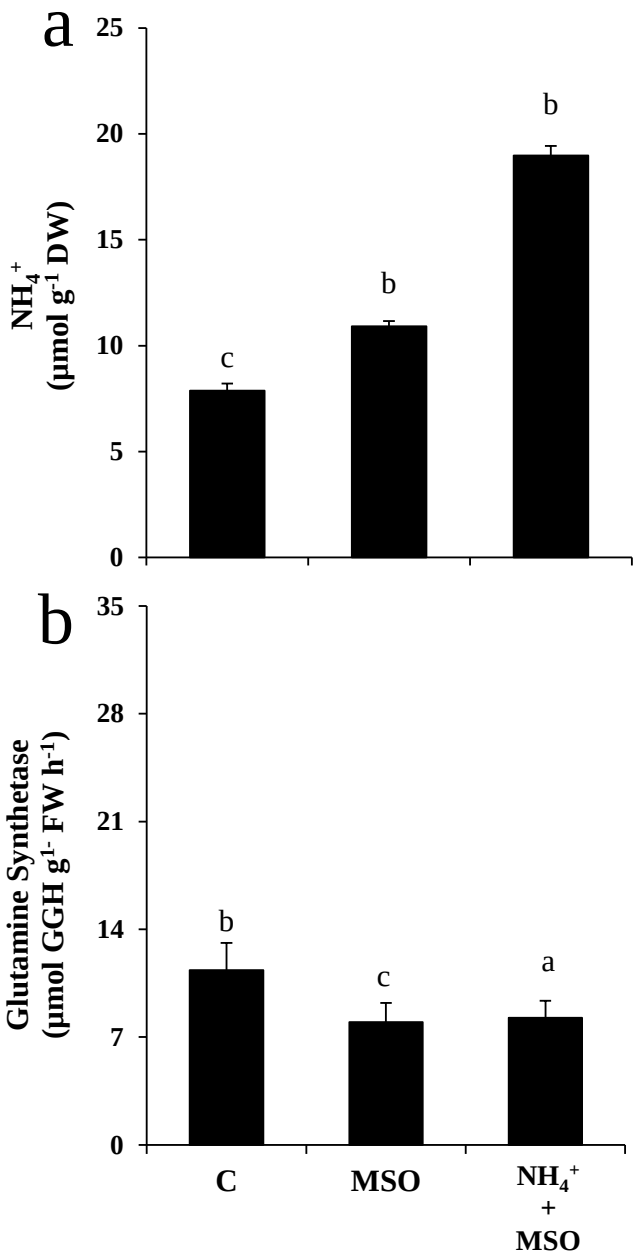

Figure 11 – NH_4^+ content (a) and GS activity (b) leaf discs of *Jatropha curcas* treated for six hours in incubation solution MES-Tris 1.5m pH 6.0 (C), or containing MSO 1.0 mM (MSO), or NH_4^+ 5.0 mM plus MSO 1.0 mM (NH_4^+ + MSO). Vertical bars represent standard errors (n = 4). Different lowercase indicate significant differences tested at the 0.05 level by Tukey's.

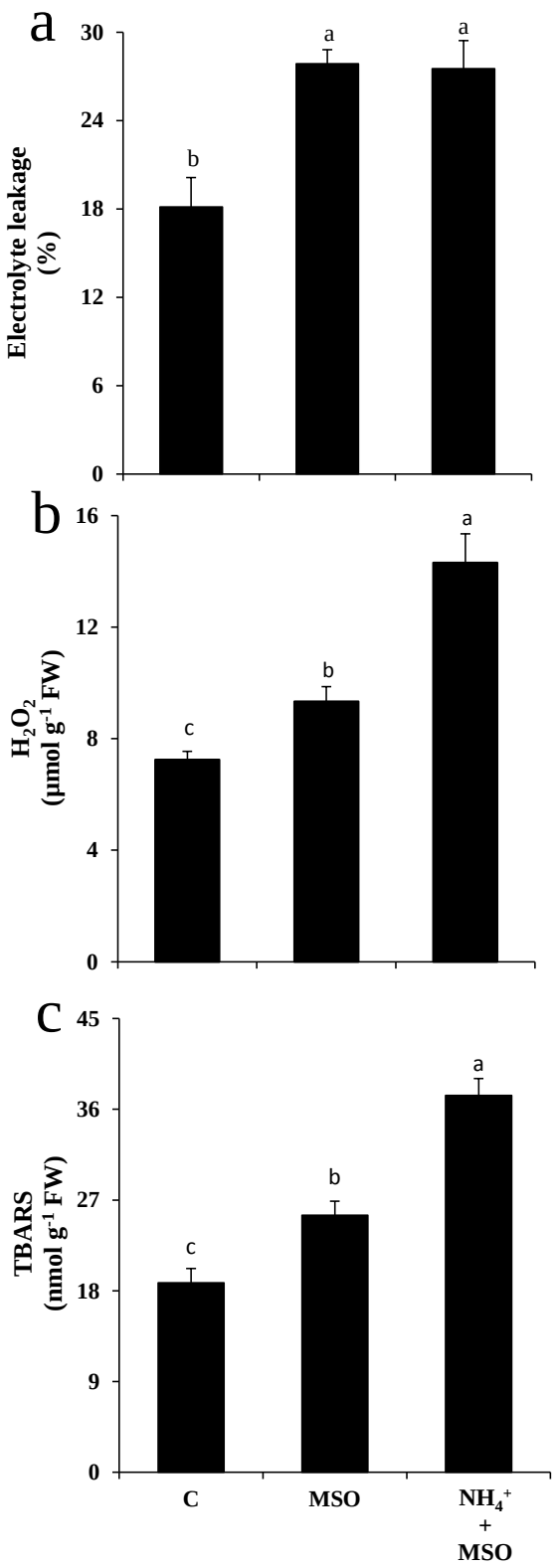

Figure 12 – Electrolyte leakage (a), H_2O_2 content (b) and TBARS content (c) in leaf discs of *Jatropha curcas* treated for six hours in incubation solution MES-Tris 1.5m pH 6.0 (C), or containing MSO 1.0 mM (MSO), NH_4^+ 5.0 mM plus MSO 1.0 mM (NH_4^+ + MSO). Vertical bars represent standard errors (n = 4). Different lowercase indicate significant differences tested at the 0.05 level by Tukey's

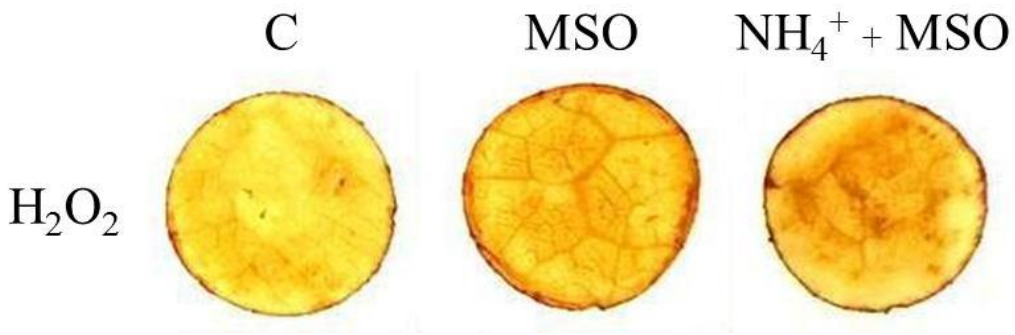

Fig. 13 – In situ determination of H_2O_2 by staining leaf discs with. The leaf discs of *Jatropha curcas* were treated for six hours in incubation solution MES-Tris 1.5m pH 6.0 (C), or containing MSO 1.0 mM (MSO), or NH_4^+ 5.0 mM plus MSO 1.0 mM (NH_4^+ + MSO). The leaf discs are representatives of five biological replicates.

Table 5 - Photosynthesis (P_N), stomatal conductance (g_s), Transpiration (E) and P_N/Ci ratio in leaf discs of *Jatropha curcas* treated for six hours in incubation solution MES-Tris 1.5 mM pH 6.0 (C), or containing MSO 1.0 mM (M) or NH_4^+ 5.0 mM plus MSO 1.0 mM (N+ M).

	P_N µmol m^{-2} s^{-1}	g_s mol m^{-2} s^{-1}	E mmol m^{-2} s^{-1}	P_N/Ci µmol m^{-2} s^{-1} Pa^{-1}
C	11.53±0.32*	0.13±0.03	2.79±0.45	0.05±0.01
M	10.05±0.64*	0.13±0.01	2.70±0.16	0.04±0.01
N+M	4.06±0.66*	0.03±0.01*	0.80±0.18*	0.02±0.01*

± represent standard errors (n = 4).

Table 6 - Effective ($\Delta F/F_m'$) and maximum (F_v/F_m) quantum yield of PSII, photochemical (qP) and non-photo-chemical quenching (NPQ), apparent electron-transport rate (ETR), relative energy excess at the PSII level (EXC), ratio between apparent electron-transport rate and leaf CO_2 assimilation (ETR/P_N) and minimum fluorescence (Fo) in leaf discs of *Jatropha curcas* treated for six hours in incubation solution MES-Tris 1.5m pH 6.0 (C), or containing MSO 1.0 mM (MSO), or NH_4^+ 5.0 mM plus MSO 1.0 mM (NH_4^++ MSO).

	ΔF/Fm´	qP	NPQ	ETR	EXC	ETR/P_N	Fo	Fv/Fm
C	0.18±0.01	0.45±0.01	1.98±0.08	76.13±3.08	0.76±0.01	6.61±0.45	559.87±5.34	0.74±0.01
MSO	0.19±0.01	0.37±0.08	1.96±0.29	80.48±3.68	0.75±0.01	8.01±0.20*	556.81±3.37	0.73±0.02
NH_4^+ + MSO	0.06±0.01*	0.22±0.05*	0.73±0.17*	26.10±6.05*	0.86±0.05*	6.67±0.09	548.10±8.47	0.45±0.05*

± represent standard errors (n = 4).

4. Discussion

Our study showed that a higher NO_3^-/NH_4^+ ratio was able to induce higher growth and lower oxidative damage even under salinity. Indeed, the single NO_3^- treatment (100/0) was two folds greater than single NH_4^+ treatment (0/100), regardless of the salinity. Is useful to highlight that 25/75 and 0/100 treatments already showed leaf necrosis symptoms and loss of turbidity and, under salinity, these effects were exacerbated. The most of the cultivated plants show high growth and development when supplied with nitrate and this preference is usually associated with ammonia toxicity under high and moderate concentrations (FORDE, 2002). Apparently, our results showed that a moderate concentration of exogenous NH_4^+ (25/75) has been able to interfere in the growth and to induce oxidative damage.

The answers to the content of NO_3^- and NH_4^+ in leaves of *J.curcas* were as expected. In 100/0 treatment, the soluble protein levels were higher compared to 0/100. In the context, some studies have shown that plants well supplied with nitrate produce higher amounts of soluble proteins and consequently lower production of amino acids (CARILLO et al., 2008; TEXEIRA et al., 2009). Curiously, the level of total free amino acids in 0/100 has not changed compared to treatment with NaCl. This trend indicates an apparent saturation in amino acid levels induced by high NH_4^+ content and GS activity in tissues. On the other hand, low NH_4^+ concentration (75/25) has been able to reduce NR activity by more than half , certainly due to its great capacity to modulate NR activity (GARNICA et al., 2009). The salinity also drastically reduced the NR activity and this can be due to indirect effects related to the reduction in NO_3^- uptake and xylem flux (SILVEIRA et al., 2001; ARAGÃO et al., 2010).

The P_N indicated that photosynthesis was two folds higher in 100/0 compared to 0/100 treatment. Some studies with healthy plants of *J.Curcas* showed P_N in the range of 10-12 µmol m^{-2} s^{-1}, i.e., lower than the values found in our plants treated with single NO_3^- (SILVA et al., 2010; 2012) Within this context, we can indicate that the single NO_3^- (100/0) was able to protect the photosynthetic apparatus and lead to improvement of CO_2 assimilation. Moreover, the g_s and P_N/C_i ratio values in 100/0 were higher compared to 0/100 indicating a CO_2 assimilation system more effective when supplied NO_3^-. The low P_N in salt-stressed plants is associated to the responses of stomatal closure, loss of carboxylation efficiency of Rubisco and CO_2 assimilation capacity reduction, characteristic of stressed plants (ZHAO et al., 2007; RIBEIRO et al., 2009).

These similar responses in salinity conditions were found in *J.curcas* grown under drought, heat and salinity (SILVA et al., 2010, 2012).

The increase of ETR/P_N in relation to high NH_4^+ concentration showed damage to the photosynthetic apparatus. In this situation, more electrons sinks were activated to protect the thylakoid membranes against an over-reduction in the electron transport chain induced by oxidative damage and NH_4^+ toxicity. The ETR/P_N ratio represents an imbalance between electron flow and CO_2 assimilation during photosynthesis, which is often associated with increased activity of Rubisco oxigenase and may indicate the existence of electron flow to other physiological processes rather than the reactions of CO_2 assimilation (RIBEIRO et al., 2009). Thus, reductions in P_N/C_i and increases in ETR/P_N indicate that there was loss of the photosynthetic efficiency in *J.curcas* when supplied with NH_4^+ and salinity.

The 100/0 treatment was able to protect the primary electron acceptors (plastoquinone pool) and photosynthetic electron flow at single NO_3^- (100/0) and low NH_4^+ concentration (75/25), as shown by parameters $\Delta F/Fm'$, ETR and qP. However, at higher levels of NH_4^+ (25/75 and 0/100) there was photochemical damage and activation of protective mechanisms that dissipate the excess energy not used in photochemical reactions, as shown by the increase in NPQ. Additionally, toxicity by NH_4^+ was also able to induce photoinhibition was shown as indicator parameters Fo and F_v/F_m, but this photoinhibition was only significant when combined with salinity, as occurred in 0/100 treatment. The increases in Fo values occurs when the PSII reaction centers are damaged, or the transfer of excitation energy of the complex antenna to the reaction centers of PSII are under stress (BOLHAR-NORDENAMPF et al., 1993). The value for F_v/F_m in non-photoinhibitory condition is approximately 0.832 and lower values can imply in photoinhibition and oxidative damage, particularly when associated with increases in Fo (BOLHAR-NORDENAMPF et al., 1993).

Responses to understand whether these effects were due the protective effects of nitrate assimilation through or due to toxicity of ammonia have been discussed differently. First, the antioxidant enzymes superoxide dismutase, ascorbate peroxidase and catalase are more active in presence of single NO_3^- compared with single NH_4^+. Second, the experiment with leaf discs in low concentrations of NH_4^+ (0.5 and 1.0 mM) resulted in increases in the oxidative damage and electrolyte leakage, which become evident that the low NH_4^+ external concentrations is able to cause toxicity regardless of the NO_3^- assimilation. Additionally, the inhibition of NO_3^- and NH_4^+ assimilation by

WO_4^- and MSO, respectively, can also induce oxidative damage and electrolyte leakage. However, the induction of this damage by NR activity inhibition was probably due to reduced consumption of electrons by NAD(P)H to transform NO_3^- to NO_2^-. In this case, the lower nitrite production leads to lower activity NiR which reduces the consumption of electrons through the reduced ferredoxin. Thus, the NO_3^- assimilation showed to be an important key against photodamage, indicating that the enzyme NR is essential to mitigate against the effects of ammonia toxicity and salinity, mainly by acting as a major power sink of photosynthetic electrons. The validity of this assertion can be evidenced when comparing the values of P_N, ETR and ETR/P_N with the experiment that used MSO as an inhibitor of the GS activity.

In relation to GS activity inhibition, unexpectedly, the NH_4^+ + MSO treatment dramatically reduced the values of P_N, g_s, E and P_N/C_i. In addition, indicative of dissipation of electrons by non-photochemical reactions (NPQ) was lower, i.e., the opposite of what was expected. More unexpected still was the reduction of nearly twice the F_v/F_m ratio, which indicated a strong photoinhibition by increased in NH_4^+ content and GS activity inhibition. These results showed that GS activity is very active to compensate the NH_4^+ toxicity. The inhibition in GS activity can be led to a metabolic disorder that possibly induced disruption of proton gradient at the membrane, limiting the electron transport in photosynthesis and photorespiration.

5. Conclusion

In summary, our data showed that NO_3^- reduction assimilatory process is very important to *J. curcas* plants because is capable to act as an important sink for the photosynthetic electron transport chain in the thylakoid. Additionally, the NO_3^- assimilation induces the improvement of photochemical activity, CO_2 assimilation and mitigates the deleterious effects of NH_4^+ toxicity and salinity. The use of NH_4^+ as the only N source is toxic at high concentrations and induces photoinhibition by GS activity inhibition and presence of salinity.

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ANEXO

Outras Publicações

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Acúmulo de íons e crescimento de pinhão-manso sob diferentes níveis de salinidade[1]

Ion uptake and growth of physic nut under different salinity levels

Evandro Nascimento da Silva[2]*, Joaquim Albenísio Gomes Silveira[3], Cícera Raquel Rodrigues Fernandes[4], Antônia Tathiana Batista Dutra[5] e Rafael Magalhães de Aragão[6]

Resumo - Objetivou-se caracterizar diferenças no padrão de absorção e partição dos íons sódio (Na^+), cloreto (Cl^-) e potássio (K^+) em folhas e raízes, além de variáveis de crescimento em plantas de pinhão-manso expostas ao estresse salino. Plântulas de pinhão-manso com 23 dias de idade foram cultivadas em solução nutritiva contendo 0; 25; 50; 75 e 100 mM de NaCl durante quinze dias em condições de casa de vegetação com as seguintes condições ambientais: temperatura de 28 a 36 °C durante o dia e de 24 a 27 °C durante a noite e a umidade relativa média de 40 a 80% (dia/noite). A intensidade de radiação fotossinteticamente ativa máxima nas proximidades das folhas foi aproximadamente 1.200 μmol m^{-2} s^{-1}. O acúmulo de Na^+ e Cl^- nas folhas e raízes aumentou proporcionalmente ao incremento de NaCl, contudo o conteúdo de K^+ foi reduzido tanto em folhas quanto em raízes em função do aumento da salinidade. Nas folhas, o acúmulo de Na^+ e Cl^- foi de 2.493 e 980 mmol kg^{-1} MS enquanto nas raízes 1.681 e 1.458 mmol kg^{-1} MS, respectivamente, para a dose de 100 mM. Os conteúdos de K^+ em folhas e raízes, no maior nível de salinidade, foram de 188 e 1.043 mmol kg^{-1} MS, respectivamente. A relação K^+/Na^+ diminuiu significativamente tanto em folhas quanto em raízes com o aumento da dose de NaCl. Uma mesma tendência foi observada na quantidade de massa seca total da planta. Os dados evidenciam que plantas jovens de pinhão-manso são sensíveis à salinidade.

Palavras-chave - *Jatropha curcas*. Estresse salino. Salinização do solo.

Abstract - The objective of this work was to characterize the uptake and partitioning of sodium (Na^+), chlorate (Cl^-) and potassium (K^+) ions in leaves and roots of physic nut plants exposed to different NaCl levels. 35-day-old seedlings were exposed to 0; 25; 50; 75 and 100 mM of NaCl supplied in the nutritive solution during 15 days under greenhouse conditions (day/night) temperatures from 28 to 36 and 24 to 27 °C, average relative humidity 40-80% (day/night) and maximum PAR 1,200 mmol m^{-2} s^{-1}. The accumulation of Na^+ and Cl^- in leaves and roots increased proportionally to external NaCl levels. In contrast, the K^+ content was prominently reduced in both organs as salinity increased. Na^+ and Cl^- concentrations in leaves were 2,493 and 980 mmol kg^{-1} DM while in roots they achieved 1,681 and 1,458 mmol kg^{-1} DM, respectively, under 100 mM NaCl treatment. The K^+ content in leaves and roots was 188 and 1,043 mmol kg^{-1} DM, respectively. The K^+/Na^+ ratios decreased significantly in leaves and roots as salinity increased, in parallel to the dry matter yield. These data strongly suggest that young plants of physic nut are sensitive to salinity.

Key words - *Jatropha curcas*. Salt stress. Soil salinity.

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Absorção, fluxo no xilema e assimilação do nitrato em feijão-caupi submetido à salinidade[1]

Nitrate uptake, xylem NO_3^- flux, and nitrate assimilation in cowpea exposed to salinity

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Resumo - O presente trabalho foi desenvolvido com o objetivo de avaliar qual etapa da aquisição de nitrato (absorção, fluxo no xilema ou redução assimilatória) é mais influenciada pela presença do NaCl em feijão-caupi. Plântulas com 12 dias de idade foram tratadas com NaCl 50 mM em solução nutritiva durante 4 dias e as avaliações realizadas sob duas condições ambientais contrastantes: dia típico (pleno sol) e dia completamente nublado (nublado). A salinidade afetou mais intensamente o fluxo da seiva e o fluxo de nitrato no xilema do que a transpiração sob as duas condições ambientais. Plantas tratadas com NaCl mostraram uma intensa redução na taxa de absorção de nitrato e na atividade da redutase de nitrato nas folhas, tanto em pleno sol como em dia nublado, quando comparadas com as plantas não tratadas. A transpiração foi reduzida pela nebulosidade, enquanto que os fluxos da seiva e de nitrato permaneceram inalterados. A absorção de nitrato e a atividade da redutase de nitrato foram menos afetadas pela nebulosidade do que a transpiração, tanto no controle como nas plantas estressadas. A nebulosidade afetou mais intensamente as variáveis de estudo das plantas controle do que de plantas tratadas. Em adição, o NaCl reduziu a concentração de nitrato em raiz, caule e folha nos dois ambientes, enquanto que a nebulosidade afetou somente nas folhas, tanto no controle como nas plantas estressadas. A salinidade afeta mais negativamente o fluxo de nitrato no xilema do que os processos de absorção e assimilação desse íon nas folhas de feijão-caupi.

Palavras-chave - Estresse salino. Nitrogênio-efeito sobre as plantas. *Vigna unguiculata*. Xilema.

Abstract - This work was carried out to evaluate what is the nitrate acquisition stage (nitrate uptake, xylem nitrate flux or assimilatory reduction) most influenced by the presence of NaCl in cowpea. Twelve day-old seedlings were treated with 50 mM of NaCl in nutrient solution during four days and measurements carried out under two contrasting environmental conditions: typical day (full sun) and completely cloudy day (cloudiness). The salinity affected more intensely the xylem sap flux and nitrate flux than transpiration. Plants treated with NaCl showed a strong decrease in both nitrate uptake rate and leaf nitrate reductase activity as in the full sun as in cloudy day. Transpiration was reduced by the cloudiness while xylem sap flux and nitrate flux remained unchanged, in both salt-treated and control. Moreover, nitrate uptake and nitrate reductase activity were less affected by cloudiness than the transpiration. In addition, NaCl negatively affected nitrate accumulation in roots, stems and leaves while the cloudiness affected only the leaf nitrate accumulation, both in control and stressed plants. Salinity affects more negatively the nitrate xylem flux, as compared with the nitrate uptake and nitrate assimilatory reduction in cowpea leaves.

Key words - Salt stress. Nitrogen- effects on the plants. *Vigna unguiculata.* Xylem.

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Temperaturas elevadas afetam a distribuição de íons em plantas de feijão caupi pré-tratadas com NaCl[1]

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RESUMO

Objetivou-se com este trabalho caracterizar o efeito de diferentes temperaturas sobre a distribuição de Na^+, Cl^- e K^+ em raízes, caules e folhas de feijão caupi pré-tratadas com NaCl. Após o período de aclimatação as plantas foram divididas em 2 grupos: 0 NaCl e 100 mM de NaCl por 2 dias. Posteriormente, as plantas foram divididas em 4 subgrupos e submetidas a temperaturas crescentes de 27, 32, 37 e 42 °C, separadamente, por um fotoperíodo de 12 h para cada temperatura. O tratamento sem NaCl a 27 °C foi adotado como referência. As concentrações de Na^+ e Cl^- nos diferentes órgãos foram aumentadas pelo pré-tratamento com NaCl e esta acumulação foi intensificada com a exposição a altas temperaturas. Nas raízes, por exemplo, observou-se que na temperatura de 42 °C as concentrações de Na^+ foram 4 vezes maiores do que nas folhas, enquanto o Cl^- se acumulou principalmente nas folhas, tendo um aumento de 20 vezes na concentração deste íon em relação às plantas referência. Inversamente, as concentrações de K^+ foram reduzidas em todos os órgãos analisados. Desta forma, conclui-se que temperaturas elevadas afetam a distribuição de íons nos diferentes órgãos afetando diretamente a homeostase iônica das plantas.

Palavras-chave: *Vigna unguiculata*, estresse salino, calor

High temperatures affect ion distribution in NaCl-pretreated cowpea plants

ABSTRACT

The purpose of this study was to characterize the effect of temperature on the distribution of Na^+, Cl^- and K^+ in roots, stems and leaves of cowpea pre-treated with NaCl. After the acclimation period, plants were divided into two groups: 0 NaCl and 100 mM NaCl for 2 days. Subsequently, the plants were divided into 4 sub-groups and subjected to increasing temperatures of 27, 32, 37 and 42 °C, separately, for a photoperiod of 12 hours at each temperature. The treatment without NaCl at 27 °C was used as reference. The concentrations of Na^+ and Cl^- in different organs were increased by pretreatment with NaCl and this accumulation was intensified by exposure to high temperatures. In roots, for example, at temperature of 42 °C concentrations of Na were four times higher than in leaves. While Cl^- accumulated mainly in leaves, with a 20-fold increase in the concentration of this ion in relation to reference plants. Inversely, K^+ concentrations were reduced in all organs analyzed. Thus, it is conclude that high temperatures affect the distribution of ions in different organs directly affecting ion homeostasis in plants.

Key words: *Vigna unguiculata*, salt stress, heat

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Salinidade modula negativamente a absorção e assimilação de NO_3^- em plantas de feijão de corda[1]

Salinity modulates negatively nitrate uptake and assimilation in cowpea plants

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Resumo - Realizou-se um estudo sobre a absorção e assimilação de NO_3^- em plantas de feijão de corda com o objetivo de analisar como os dois processos se relacionam frente a condições de salinidade, bem como, esclarecer possíveis mecanismos de modulação das plantas sob estresse salino. Plantas com 14 dias, crescidas em solução nutritiva (Sem NH_4^+), foram submetidas a KNO_3 e NaCl 100 mM por dez dias. Avaliou-se a absorção e o conteúdo de NO_3^-, atividade da Redutase do Nitrato (RN), transpiração, proteínas solúveis e aminoácidos livres totais (ALT). A aplicação do NaCl resultou na redução da absorção líquida e no conteúdo de NO_3^-, principalmente, em raízes. A redução da atividade da RN foi positivamente correlacionada com a redução da transpiração, mas nenhuma relação foi estabelecida com a redução nos níveis e proteínas solúveis, que foi mais eminente em raízes. Os níveis de ALT foram superiores em raízes de plantas controle e tratadas com NaCl. Os resultados indicam um possível mecanismo de modulação devido a menores níveis de proteínas e maiores níveis de ALT em raízes sob condições de salinidade. Em resumo, a salinidade modula negativamente a absorção e assimilação de NO_3^- em plantas de feijão de corda, a princípio pela redução nos níveis de proteínas solúveis e pelo acúmulo de aminoácidos em raízes, e a inibição do tipo não-competitiva pelo NaCl indica que a competição entre NO_3^- e Cl^- pelos sítios de absorção não é o fator mais limitante para modular a absorção no NO_3.

Palavras-chave - Aminoácidos livres totais. Estresse salino. Nitrogênio. *Vigna unguiculata.*

Abstract - This present work aims to perform a study on the NO_3^- uptake and assimilation in cowpea plants, analyzing how the two processes are related front to salinity conditions and clarify possible mechanisms of plant modulations under salt stress. Plants with 14 days old grown in nutrient solution (Absence NH_4^+) were subjected to KNO_3 10 mM and NaCl 100 mM for ten days. Was evaluated NO_3^- net uptake and content, nitrate reductase activity (RN), transpiration, soluble proteins and total free amino acids (ALT). Application of NaCl resulted in reduced net uptake and the content of NO_3^-, mainly in roots. The reduction of NR activity was positively correlated with the reduction of transpiration, but no relation was established with the reduction in the soluble proteins levels, which was more conspicuous on roots. ALT levels were higher in roots of control plants and treated with NaCl. The results indicate a possible mechanism of modulation due to lower protein levels and higher ALT levels in roots under saline conditions. In summary, salinity modulates the uptake and assimilation of NO_3^- in cowpea plants, in principle, by the reduction in the soluble proteins and amino acids accumulation in roots and the non-competitively inhibition by NaCl indicates that competition between NO_3^- and Cl^- by the sites is not the most limiting factor to modulate the NO_3^- uptake.

Key words - Total free amino acids. Salt stress. Nitrogen. *Vigna unguiculata.*

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BRIEF COMMUNICATION

Salinity affects indirectly nitrate acquisition associated with glutamine accumulation in cowpea roots

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Abstract

The aim of this study was to test the hypothesis that salinity can affect indirectly the nitrate acquisition by a negative modulation triggered by glutamine accumulation. Cowpea plants were exposed to a mild NaCl concentration (50 mM) in order to restrict growth and N-demand. After 21 d, pretreated plants and control plants were supplied with 0, 5 and 10 mM of $Ca(NO_3)_2$ for 3 d in absence of NaCl. Salt pretreated plants showed a great limitation in acquisition of NO_3^-, indicated by decline in the nitrate uptake rate, NO_3^- accumulation, nitrate reductase activity and protein content. The restriction of NO_3^- utilization was positively associated with increased glutamine synthetase activity and glutamine accumulation, especially in roots.

Additional key words: glutamine synthetase, NaCl stress, nitrate reductase, nitrate uptake, *Vigna unguiculata.*

Experimental results have shown that salt stress affects several metabolic steps of nitrate utilization in plants, such as NO^-_3 influx (Peuke and Jeschke 1999), loading of nitrate into the root xylem (Peuke *et al*. 1996), nitrate reductase activity (Abd-El Baki *et al*. 2000), amino acid metabolism (Silveira *et al*. 2003) and protein synthesis (Aslam *et al*. 1996). On the other hand, salt stress induces an increase in the glutamine synthetase activity (Silveira *et al*. 2003, Veeranagamallaiah *et al*. 2007). The salt-induced imbalance between ammonia assimilation and protein synthesis frequently induces a significant increase in the free amino acid pool in the roots and shoots (Silveira *et al*. 2001, 2003). This increase in the content of amino acids, which is associated with a lower N demand for plant growth, could cause a negative control of the nitrate influx (Foyer and Noctor 2004). The negative feedback model has become generally accepted, but the precise nature or mechanism of the signal(s) is not known yet (Miller *et al*. 2007a). The free amino acids, especially glutamine, are the strongest candidates as the signaling molecule for regulation of nitrate uptake (Krapp *et al*. 2004, Thornton 2004, Miller *et al*. 2007b).

Although free glutamine is involved in the negative modulation of nitrate reductase gene expression (Fan *et al*. 2006), its involvement in the regulation of nitrate uptake is yet not confirmed, although several works have suggested its participation (for a review, see Miller *et al*. 2007a). Experimental results have shown that when glutamine is exogenously supplied to the root medium or is artificially increased in the root tissues and phloem sap, it negatively modulates both the nitrate influx and NO_3^- transporter expression (Pal'ove-Balang and Mistrík 2002, Thornton 2004). Likewise, salinity controls both the nitrate uptake and growth in parallel to accumulation of amino acids (Silveira *et al*. 2001). However, to the best of our knowledge, there are no published reports concerning the mechanisms underlying the salt-induced growth restriction associated with the reduction in nitrate uptake

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Abbreviations: AA - amino acids; Gln - glutamine; GS - glutamine synthetase.
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ORIGINAL PAPER

Salt resistance in two cashew species is associated with accumulation of organic and inorganic solutes

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Abstract This study establishes relationships between salt resistance and solute accumulation in roots and leaves of two contrasting cashew species. The sensitive (*Anacardium microcarpum*) and resistant (*A. occidentale*) species showed maximum root LD_{50} values (the external NaCl concentration required for a 50% reduction in dry weight) of 63 and 128 mM NaCl, whereas the shoot LD_{50} values were 90 and 132 mM, respectively. The salt sensitivity was directly associated with Na^+ accumulation and especially with the Cl^- content in leaves and to a minor extent in roots. The accumulation of saline ions was associated with higher net uptake rates by roots and transport rates from root to shoot in the sensitive cashew species. The K^+/Na^+ ratios were not associated with salt resistance either in roots or leaves. Proline and free amino acid concentrations were strongly increased by salinity, especially in the leaves of the resistant species. The soluble sugar concentrations were not influenced by NaCl treatments in leaves of both species. In contrast, the root soluble sugar content was significantly decreased by salinity in the sensitive species only. In conclusion, the higher salt sensitivity of *A. microcarpum* is associated to an inefficient salt exclusion system of the leaves, especially for Cl^-. On the other hand, the resistant species displays higher concentrations of organic solutes especially a salt-induced accumulation of proline and free amino acids in leaves.

Keywords *Anacardium occidentale* · *Anacardium microcarpum* · Ionic toxicity · Osmo-solutes · Salinity

Abbreviations

DW	Dry weight
FW	Fresh weight
LD_{50}	External concentration of NaCl required for a 50% reduction in dry weight
RGR	Relative growth rate
FAA	Free amino acids
TSS	Total soluble sugars

Introduction

High concentrations of salts in soils largely account for the decrease in yield of a wide variety of crops worldwide (Munns and Tester 2008). This problem is more acute in arid and semi-arid regions, including areas in Brazilian northeast where the salinity is one of the major limiting factors for productivity. Salt excess causes a reduction in water potential, ion imbalance, disturbances in ion homeostasis and ionic toxicity. Salinity stress involves both osmotic and ionic effects, and plant growth suppression is directly related to the total concentration of salt ions and/or the decrease in the soil osmotic potential (Silva-Ortega et al. 2008).

The major saline ions, Na^+ and Cl^-, can affect nutrient uptake through competitive interaction or by affecting membrane selectivity. For example, a high level of Na^+

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