UNIVERSIDADE FEDERAL DO CEARÁ

CENTRO DE CIÊNCIAS

DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR

ALTERAÇÕES FISIOLÓGICAS INDUZIDAS POR ESTRESSES ABIÓTICOS EM PLANTAS JOVENS DE PINHÃO-MANSO

EVANDRO NASCIMENTO DA SILVA

FORTALEZA

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Tese de Doutorado submetida à Coordenação do Curso de Pós-Graduação em Bioquímica, da Universidade Federal do Ceará, como requisito parcial para a obtenção do grau de Doutor em Bioquímica.

FORTALEZA-CEARÁ
2009

S578a Silva, Evandro Nascimento da
Alterações fisiológicas induzidas por estresses abióticos em plantas jovens de pinhão manso / Evandro Nascimento da Silva, 2009.
144f. ;il. color. enc.

Orientador: Prof. Dr. Joaquim Albenísio Gomes da Silveira
Área de concentração: Bioquímica vegetal
Tese (Doutorado) - Universidade Federal do Ceará, Centro de Ciências, Depto. de Bioquímica e Biologia Molecular, Fortaleza, 2009.

1. *Jatropha curcas.* 2. Estresse hídrico. 3. Estresse salino. 4. Calor. 5. Estresse combinado. I. Silveira, Joaquim Albenísio Gomes da (Orient.) II.Universidade Federal do Ceará – Programa de Pós-graduação em Bioquímica. IV.Título.

CDD 574.192

Tese de Doutorado apresentada a Coordenação do Programa de Pós-Graduação em Bioquímica como parte dos requisitos para a obtenção do grau de Doutor em Bioquímica, área de concentração Bioquímica Vegetal, outorgado pela Universidade Federal do Ceará e se encontra a disposição dos interessados na Biblioteca desta Universidade.

A transcrição de qualquer trecho desta Tese é permitida, desde que feita de acordo com as normas da ética científica.

Tese aprovada em 16 / 10 / 09

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AGRADECIMENTOS

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

A minha querida esposa Mara, pelo seu amor, dedicação e incentivo nas horas mais difíceis durante o meu curso de doutorado;

Aos meus pais, José Vieira e Enedina por terem me dado a oportunidade de realizar muitos dos meus sonhos.

A Capes pelo apoio financeiro para realização deste trabalho.

Ao Prof. Joaquim Albenísio Gomes da Silveira, pela amizade, incentivo, paciência e inestimável contribuição para a minha formação profissional;

Ao Prof. Sérgio Luiz Ferreira Silva, pela amizade, parceria e valiosas sugestões para a melhoria da qualidade desse trabalho;

Ao Prof. Ricardo Almeida Viégas, pela concessão de materiais, participação na banca de avaliação e valiosas sugestões para a melhoria da qualidade desse trabalho;

Ao Prof. Enéas Gomes Filho, pelos ensinamentos ao longo do curso, pela sua participação na banca de avaliação e valiosas sugestões para a melhoria da qualidade desse trabalho;

Ao Dr. Marlos Alves Bezerra, pela valiosa contribuição na realização deste trabalho e pela sua participação na banca de avaliação.

Aos colegas do LABPLANT, Raquel, Rafael, Tathiana, Cristina, Márcio, Milton, Nívea, Adilton, Naélia, Cíntia e Suyanne pela convivência saudável e espírito de companheirismo;

Aos professores do departamento de Bioquímica da UFC, pelos exemplos de profissionalismo e contribuição para a minha formação profissional;

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ALTERAÇÕES FISIOLÓGICAS INDUZIDAS POR ESTRESSES ABIÓTICOS EM PLANTAS JOVENS DE PINHÃO-MANSO

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RESUMO

Neste trabalho foram estudados vários mecanismos fisiológicos, como o ajustamento osmótico, a fotossíntese, incluindo trocas gasosas e parâmetros de fluorescência da clorofila, além das respostas oxidativas em plantas de pinhão-manso (*Jatropha curcas* L.) submetidas a diferentes estresses abióticos, como: salinidade, seca e temperatura elevada. O primeiro experimento visou o estudo dos efeitos das concentrações de NaCl (0, 25, 50, 75 e 100 mM) no acúmulo dos íons (Na^+, Cl^- e K^+), e em algumas variáveis de crescimento, bem como avaliou o estado hídrico e os principais solutos (orgânicos e inorgânicos) envolvidos no ajustamento osmótico de plantas de pinhão-manso sob esta condição estressante. Plantas de pinhão-manso se mostraram sensíveis ao estresse salino, mostrando uma redução de 50% na massa seca com uma concentração estimada de 47 mM de NaCl durante 15 dias. Esta sensibilidade deve-se, principalmente, a uma grande acumulação de Na^+ e Cl^- nas folhas, associada com uma grande redução na concentração de K^+. Por outro lado, plantas de pinhão-manso foram capazes de se ajustar osmoticamente à salinidade devido a uma intensa redução no potencial osmótico e aumento do estado hídrico das folhas, principalmente nos níveis mais elevados de NaCl. Dentre os solutos estudados, observou-se que os íons salinos Na^+ e Cl^- contribuíram com a maioria do ajustamento osmótico, enquanto que a contribuição do K^+ foi diminuída intensamente pelo NaCl. A glicina-betaína comparada à prolina, foi muito mais importante para o ajustamento osmótico de folhas de pinhão-manso. O segundo experimento avaliou a resistência do aparato fotossintético de plantas de pinhão-manso submetidas a diferentes tempos de exposição (7 e 14 dias e após recuperação de 3 dias) ao estresse salino (100 mM de NaCl). As mudanças causadas sobre a atividade fotoquímica e as trocas gasosas foram avaliadas em função da acumulação de Na^+ e Cl^- e da diminuição da relação K^+/Na^+ nas folhas. Após sete dias de tratamento ficou evidente uma maior ação de efeitos osmóticos. Contudo, após 14 dias de tratamento, os efeitos iônicos causados pela acumulação excessiva dos íons Na^+ e Cl^- e pela intensa redução na relação K^+/Na^+ nas folhas, provocaram danos permanentes na fotossíntese tanto devido a limitações estomáticas quanto não-estomáticas. O terceiro experimento visou estudar os efeitos comparativos dos estresses salino (50 mM de

NaCl) e hídrico (induzido por PEG 6000 com potencial osmótico correspondente ao da solução salina) na fotossíntese, relações hídricas e crescimento de plantas de pinhão-manso. Os efeitos do estresse hídrico induzido por PEG no crescimento foliar, vazamento de eletrólitos e trocas gasosas foliares foram mais deletérios do que os causados pelo NaCl. Nos dois estresses foi observada perda na assimilação de CO_2 foliar devido tanto a fatores estomáticos quanto não estomáticos. Todavia, eles não foram capazes de afetar os parâmetros de fluorescência da clorofila. O quarto experimento avaliou a contribuição relativa dos solutos orgânicos e inorgânicos no ajustamento osmótico de folhas e raízes de pinhão-manso sob diferentes níveis de restrição hídrica. Dentre os solutos analisados, o K^+ e os açúcares solúveis foram os mais envolvidos no ajustamento osmótico tanto em folhas quanto em raízes. Outros solutos, como Na^+, Cl^-, aminoácidos livres totais e glicina-betaína, também apresentaram um efeito na redução do potencial osmótico em ambos os órgãos. Por outro lado, o conteúdo de prolina nas folhas, embora tenha aumentado significativamente, não foi suficiente para promover uma participação efetiva desse aminoácido no ajustamento osmótico de plantas de pinhão-manso. O mesmo experimento também visou observar os efeitos isolados e combinados do estresse hídrico e da temperatura elevada na fotossíntese e avaliar o sistema de defesa contra danos oxidativos em plantas de pinhão-manso. O aparato fotossintético se mostrou mais sensível aos efeitos isolados da seca do que o do calor, sendo que a combinação deles proporcionou efeitos deletérios maiores neste complexo. Adicionalmente, os danos oxidativos também foram mais marcantes na combinação dos estresses. No geral, os resultados mostram que plantas de pinhão-manso, embora apresentem capacidade de se ajustar osmoticamente à salinidade e seca, tem seu aparato fotossintético bastante afetado nessas condições estressantes. Assim como, o sistema de defesa contra danos oxidativos parece não ter sido eficiente em plantas expostas aos estresses isolados e combinados de seca e calor.

Palavras-chave: Jatropha curcas. Estresse hídrico. Estresse salino. Calor. Estresse combinado.

PHYSIOLOGICAL CHANGES INDUCED BY ABIOTIC STRESSES IN PHYSIC NUT YOUNG PLANTS

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ABSTRACT

In this work were studied diverse physiological mechanisms, as the osmotic adjustment, photosynthesis, including gas exchange and chlorophyll fluorescence parameters, as well the oxidative responses in physic nuts submitted to different abiotic stresses as: salinity, drought and high temperature. The first experiment aimed to study the effects of increase of the NaCl concentrations (0, 25, 50, 75 and 100 mM) in the ions accumulate (Na^+, Cl^- and K^+) and some growth variables, as well evaluate the water state and the principals solutes (organic and inorganic) involved on the osmotic adjustment of physic nuts plants under this stressful condition. Physic nuts plants showed sensibility to salt stress, presenting a reduction by 50% in dry matter from by 47 mM of NaCl concentration during 15-d. This sensibility should be due the leaf Na^+ and Cl^- high accumulation, associated a strong reduction in the K^+ concentration, induced by high Na^+ content. On the other hand, physic nuts plants were able osmotic adjust to salinity due a severe decrease on the osmotic potential and increase of leaf water state, principally in the higher NaCl levels. Of the solutes studied, was observed that salt ions (Na^+ and Cl^-) contributed with the most of the osmotic adjustment, while that the K^+ contribution was decreased strongly by NaCl. The glycinebetaine compared to proline was more important to the osmotic adjustment of physic nuts leaves, as in the absence as in presence of different NaCl levels in the nutrient solution. The second experiment evaluated the resistance of photosynthetic apparatus of physic nuts plants submitted to different time of exposure (7-d and 14-d of treatment and 3-d of recovery) to salt stress (100 mM of NaCl).The changes caused on photochemistry activity and leaf gas exchange were evaluate by Na^+ and Cl^- accumulation and decrease of K^+/Na^+ ratio in the leaves. After 7-d of treatment was observed a major action of osmotic effects. However, after 14-d of treatment, the ionic effects caused by Na^+ and Cl^- excessive accumulation and by K^+/Na^+ ratio strong reduction in the leaves, caused permanent damages on the photosynthesis of physic nuts plants due as the stomatal limitations as non-stomatal ones. The third experiment aimed to study the comparative effects between the salt stress (50 mM of NaCl) and water stress (induced for

PEG 6000), both with osmotic potential of –0.22 MPa on the photosynthesis, water relations and growth of physic nuts plants. The water stress effects induced for PEG in the leaf growth, electrolyte leakage and leaf gas exchange were more deleterious than by NaCl ones. In the both stresses was observed decrease in the leaf CO_2 assimilation due the stomatal and non-stomatal limitations. However, the chlorophyll fluorescence parameters did not affect. The fourth experiment aimed to evaluate the relative contribution of organic and inorganic solutes on the osmotic adjustment of leaves and roots physic nuts plants in different water restriction levels. Of the solutes studied, the K^+ and soluble sugar were the most involved in the osmotic adjustment as in the leaves as in roots. Others solutes as, Na^+, Cl^-, total amino acids and glycibetaine, also presented a effective role in the reduction of osmotic potential in both organs. On the other hand, the leaf proline content, although has increased significantly, was not sufficient to promote an effective participation of this amino acid in the osmotic adjustment of physic nuts plants. The same experiment aimed to observe the isolated and combined effects of water stress and high temperature on the photosynthesis and evaluate the oxidative defenses system in physic nuts plants. The photosynthetic apparatus was more sensitive to water stress than heat ones, been that the combination of them caused deleterious effects yet large in this complex. Additionally, the oxidative damages also were more marked in the combined stress. In general, the data shown that physic nuts plants, although present ability to adjust osmotically to salinity and drought, have their photosynthetic apparatus very affected in this stressful conditions. Even as, the defense system against oxidative damages appears has not been efficient in plants exposure at the drought and heat isolated and combined stresses.

Keywords: *Jatropha curcas.* Water stress. Salt stress. Heat. Combined stress.

1. Introdução e revisão de literatura

1.1. O que é o semiárido?

As regiões semiáridas do mundo são definidas como zonas de transição entre as zonas áridas e subúmidas. Podem também ser definidas como áreas onde a precipitação é inferior a evaporação potencial, caracterizada por temperaturas elevadas (30-45 ºC) nos meses mais quentes. As regiões semiáridas ao lado das áridas representam um terço da superfície da terra (FAO, 2005). Segundo dados do Icrisat (1998), estima-se que as regiões semiáridas, especialmente dentro dos trópicos, cobrem a maior parte das nações em desenvolvimento no mundo, incluindo a América Latina, grande parte do leste e do sul da África e partes da Índia e sudoeste da Ásia.

De acordo com as estatísticas da população mundial, cerca de um bilhão de pessoas vivem nessas regiões em todo o mundo e o número de pessoas que dependem da produtividade destas terras está a crescer a cada dia. Essas terras são desfavorecidas devido a condições climáticas limitadas, tais como: elevadas temperaturas, baixas precipitações e fertilidade do solo. As áreas semiáridas apresentam pelo menos um mês ao ano totalmente sem chuvas, sendo que na maioria delas a quantidade de chuvas varia de 500-1000 mm/ano. Isso significa que condições de déficit hídrico são extremamente comuns nestas regiões (FAO, 2005).

No semiárido nordestino, um dos traços principais são as constantes secas que podem ser caracterizadas pela ausência, escassez, alta variabilidade espacial e temporal das chuvas. As características desse ambiente condicionam fortemente a população a sobreviver principalmente de atividades econômicas ligadas à agricultura e a pecuária. Estas se realizam sempre buscando o melhor aproveitamento possível das condições naturais desfavoráveis, ainda que apoiadas em base técnica frágil, utilizando na maior parte dos casos, tecnologias tradicionais (SUDENE, 2008).

Além das vulnerabilidades climáticas do semiárido, grande parte dos solos encontra-se degradada. Os recursos hídricos caminham para a insuficiência ou apresentam níveis elevados de poluição. A flora e a fauna vêm sofrendo a ação predatória do homem. E os frágeis ecossistemas regionais não estão sendo protegidos, ameaçando a sobrevivência de muitas espécies vegetais e animais e criando riscos à ocupação humana, inclusive associados a processos, em curso, de desertificação (SUDENE, 2008).

1.2. Estudo dos estresses abióticos

1.2.1. Estresse salino

Segundo Munns and Tester (2008) estima-se que 6% das terras do mundo e 30% das áreas irrigadas sejam afetadas pela salinidade. Grande parte dessa salinidade e toda a sodicidade são de procedência natural. No entanto, uma porção significante das terras destinadas à agricultura tem se tornado salina por causa do desmatamento e da irrigação (MUNNS, 2005). A qualidade da água para irrigação nas regiões áridas e semiáridas é geralmente muito pobre. Essa forte ligação da irrigação com a 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 (FAO, 2005). Além destas áreas, cerca de 2,4% da superfície total de 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. Irrigação excessiva, ineficiente gerenciamento do solo e dos recursos hídricos, invasão de águas marinhas e drenagem insuficiente, com conseqüente aumento nos níveis dos lençóis freáticos e de áreas de alagamento, solos rasos e com baixa fertilidade e irrigação com águas salinas sem gerenciamento adequado são as principais causas da salinidade nos solos do território brasileiro (FAO, 2005).

A presença de sal no solo tem dois principais efeitos no crescimento da planta. Primeiro, a alta concentração na solução do solo reduz a capacidade da planta absorver água e isto leva a uma redução no crescimento. Este é conhecido como o efeito osmótico ou déficit hídrico causado pela salinidade. Segundo, o sal pode entrar na corrente transpiratória e eventualmente danificar as células foliares, reduzindo ainda mais o crescimento e provocando um grande distúrbio metabólico. Este é o efeito iônico ou específico da presença de sal (MUNNS; TESTER, 2008).

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 (MUNNS; TESTER, 2008). Esses solutos compatíveis são importantes para o balanço osmótico e para o metabolismo celular, uma vez que além da função principal no

ajustamento osmótico, os solutos compatíveis podem ajudar na estabilização de macromoléculas (osmoprotetores) e proteção contra danos oxidativos sob condições adversas (PÉREZ et al., 2009).

Sob estresse salino, um fator chave na acumulação de solutos em células vacuoladas é a sua compartimentalização subcelular, visto que as quantidades excessivas de íons salinos no citoplasma inibem as atividades de muitas enzimas. Assim a compartimentalização desses íons salinos no vacúolo contribui para o ajustamento osmótico sem afetar os sistemas enzimáticos do citoplasma. Nestas células, o balanço hídrico entre o vacúolo e o citoplasma é mantido pela síntese e acúmulo de compostos orgânicos. Em adição, o acúmulo destes compostos no citoplasma pode proteger as membranas celulares, as proteínas e a maquinaria metabólica, o que pode preservar a estrutura subcelular dos danos resultantes da desidratação (SERRAJ; SINCLAIR, 2002).

A fotossíntese, dentre os processos primários, é um dos mais afetados pela salinidade (CHAVES et al., 2009; MUNNS; TESTER, 2008). Os efeitos podem ser diretos como a redução do CO_2 disponível causados pela limitação na difusão dos estômatos (FLEXAS et al., 2004; 2007) ou pelas alterações no metabolismo fotossintético (LAWLOR; CORNIC, 2002) ou podem surgir a partir de efeitos secundários como o estresse oxidativo. Esse tipo de estresse é mais freqüente sob condições de estresses múltiplos (CHAVES; OLIVEIRA, 2004) e pode afetar seriamente a maquinaria fotossintética da folha (ORT, 2001). O conteúdo total de clorofila e carotenóides (PARIDA; DAS, 2005), bem como o acúmulo nos cloroplastos dos íons Na^+ e/ou Cl^-, que afetam desfavoravelmente os processos bioquímicos e fotoquímicos envolvidos na fotossíntese assim como decréscimos na atividade da ribulose-bisfosfato carboxilase/oxigenase são alguns dos processos afetados pela salinidade (TAIZ; ZEIGHER, 2009).

1.2.2. Estresse hídrico

O estresse hídrico nas plantas é uma das principais limitações ambientais que afeta a produção das culturas no mundo todo (REDDY et al., 2004; SOUZA et al., 2004), sendo o fator que rege a distribuição das espécies nas diferentes zonas climáticas do globo (PIMENTEL, 2004). Nas regiões áridas e semiáridas, a disponibilidade de água é o principal fator limitante que define o crescimento das plantas e a produção das culturas nestas áreas, normalmente carentes de grande produção de alimentos, que não é suficiente para a sua crescente população (NGUYEN; SUTTON, 2009). Além disso, a alta demanda

evapotranspiratória e a baixa capacidade de retenção de água no solo fazem com que as zonas semiáridas tropicais sejam mais susceptíveis à seca do que as zonas semiáridas de clima temperado.

Em condições de estresse hídrico ou sob uma excessiva demanda evapotranspiratória, a sobrevivência da planta dependerá do conjunto de suas respostas metabólicas e fisiológicas (PIMENTEL, 2004). Tais respostas são complexas, e envolvem tanto mudanças aclimatativas e adaptativas, como efeitos danosos (CHAVES et al., 2009), daí a existência de inúmeras dúvidas sobre as suas vantagens e desvantagens para a planta. Os efeitos da seca são bastante variáveis em função da sua intensidade, velocidade de imposição do estresse e do estádio de desenvolvimento da planta (PIMENTEL, 2004), além da espécie da planta e do genótipo que também são determinantes no estudo da resposta ao estresse hídrico (BRAY et al., 2000).

A redução da absorção de água e de nutrientes do solo, associada à intensa restrição fotossintética, são alguns dos impactos negativos do estresse hídrico no metabolismo vegetal, que resulta na redução da produtividade agrícola de várias espécies de importância econômica no mundo todo (CATTIVELLI et al., 2008). Sob condições moderadas de estresse hídrico, a diminuição da fotossíntese ocorre principalmente devido ao fechamento estomático, e com a progressão da intensidade do estresse, alterações bioquímicas podem comprometer diretamente a eficiência da fixação do CO_2 (CHAVES et al., 2009).

Segundo Bray et al. (2000), as plantas possuem mecanismos de resistência à deficiência hídrica, dentre eles: escape à seca, que se refere à habilidade da planta de completar o ciclo antes que ocorra uma falta de água severa, tolerância à seca, que inclui o ajustamento osmótico, fechamento estomático, indução de antioxidantes e por fim resistência à seca, no qual as plantas podem aprofundar suas raízes, impermeabilizar suas folhas e fechar seus estômatos. Estes mecanismos podem levar à limitação estomática e redução da fotossíntese pela limitação da fixação de CO_2. Essa limitação resulta no aumento da relação $NADPH/NADP^+$ no estroma dos cloroplastos, diminuindo a concentração de $NADP^+$, o principal aceptor de elétrons fotossintéticos. Nessas condições, ocorre rapidamente uma sobrecarga de energia na cadeia transportadora de elétrons cloroplástica e o excesso de elétrons pode ser desviado para redução do O_2 (FOYER; NOCTOR, 2000).

Embora a resposta estomática seja provavelmente o fator mais importante no controle da fixação do carbono, o decréscimo na fotossíntese em condições de déficit hídrico tem sido atribuído tanto a limitações estomáticas, como não-estomáticas (PARIDAS, DAS, 2005). Alguns estudos mostram que a limitação da atividade fotossintética é mais um efeito metabólico, pois a concentração intercelular de CO_2 na folha (Ci) se mantém alta ou mesmo

aumenta com a seca (TAIZ; ZEIGHER, 2009). Contudo, Souza et al. (2004) afirmam que a limitação da fotossíntese é devida principalmente à menor disponibilidade de CO_2 para a enzima Rubisco. Ainda segundo tais autores, essa limitação só ocorreria em condições de estresse severo.

1.2.3. Estresse com calor

O estresse térmico, induzido por temperaturas elevadas, provoca uma série de alterações morfofisiológicas e bioquímicas. Essas alterações afetam o crescimento e o desenvolvimento, podendo levar a uma drástica redução no rendimento econômico (WAHID; CLOSE, 2007). Esse estresse ocorre pelo aumento da temperatura acima de um limiar e por período de tempo suficiente para causar dano irreversível no desenvolvimento vegetal. No geral, elevações transientes da temperatura acima da temperatura ambiental são consideradas causadores de choque térmico. Por outro lado, o estresse térmico é uma função complexa, envolvendo interações de intensidade, duração e taxa de incremento da temperatura (WAHID et al., 2007).

O efeito do calor pode diferir em função dos diferentes estádios fenológicos da planta. Na germinação, reduz a velocidade podendo levar a completa inibição deste processo, dependendo da espécie e do estresse imposto (GALLIE et al., 1998). Em etapas posteriores do desenvolvimento vegetal, o excesso de calor pode afetar diferentes processos metabólicos, incluindo fotossíntese, respiração, relações hídricas, fluidez e estabilidade dos sistemas de membranas, além de modular os níveis de hormônios e de metabólitos primário e secundário (WAHID et al., 2007).

A fotossíntese é um dos processos mais sensíveis ao excesso de calor e a manutenção de uma alta capacidade fotossintética é importante para as plantas tolerarem altas temperaturas (XU; HUANG, 2000). A inibição de sua atividade devido ao calor excessivo está associada com a interrupção do transporte de elétrons, redução na eficiência fotoquímica do fotossistema II, fixação e assimilação de CO_2 e em algumas espécies a inibição da atividade carboxilase da rubisco (XU; HUANG, 2000). Na verdade, em plantas C_3, a redução na fotossíntese que ocorre durante a exposição das plantas a temperaturas relativamente altas é atribuída ao aumento de atividade de oxigenase da rubisco (SHARKEY, 2005). O excesso de calor aumenta a solubilidade do O_2 mais do que a do CO_2 e favorece o incremento da relação O_2/CO_2, reduzindo a capacidade carboxilase da rubisco e favorecendo a da oxigenase (LIU, HUANG, 2008).

Além de alterar a relação O_2/CO_2 e induzir fotorrespiração, as alterações nas reações fotoquímicas, na membrana dos tilacóides, no metabolismo do carbono, e no estroma dos cloroplastos, são distúrbios primários do metabolismo vegetal induzidos por temperaturas elevadas (WISE et al., 2004). A atividade do fotossistema II (PSII) é intensamente reduzida, ou até mesmo inibida, pelo calor (CAMEJO et al., 2005), resposta atribuída às propriedades da membrana do tilacóide, onde está localizado o PSII (LIU, HUANG, 2008). O estresse térmico pode induzir a dissociação do complexo protéico de liberação de O_2, resultando em desbalanço do fluxo de elétrons fotoquímico entre esse complexo e os centros de reação do PSI e PSII, levando à geração de espécies reativas de oxigênio (WAHID et al., 2007).

1.2.4. Estresses abióticos combinados

Estresses abióticos como seca, salinidade e temperaturas elevadas são estresses ambientais responsáveis por perdas significativas da produtividade agrícola em diversas partes do mundo (MUNNS; TESTER, 2008). Isoladamente, esses estresses afetam importantes aspectos morfológicos, bioquímicos e fisiológicos da maioria das espécies vegetais, particularmente das culturas agrícolas, e por esta razão são intensamente estudados (WAHID et al., 2007; CATTIVELLI et al., 2008; MUNNS; TESTER, 2008). A maioria desses estudos utiliza abordagens experimentais que avaliam os efeitos isolados desses fatores no metabolismo vegetal, sem considerar os diferentes aspectos interativos entre os próprios estresses, nem destes com o ambiente (MITTLER, 2006).

Apesar da grande contribuição desses trabalhos para a compreensão atual dos distúrbios metabólicos induzidos pelos estresses abióticos, esses estudos são, ainda, relativamente limitados e não explicam, completamente, os diferentes tipos de interações desses estresses sobre o metabolismo vegetal. No campo, as plantas estão expostas a uma combinação variada de diversos fatores abióticos, os quais interagem fortemente, resultando numa combinação múltipla de fatores adversos que afetam a produtividade (MUNNS; TESTER, 2008; MITTLER, 2002). Recentemente, estudos têm demonstrado que as respostas metabólicas atribuídas aos efeitos combinados dos estresses hídrico e de temperatura elevada, por exemplo, são únicas, e não podem ser obtidas com base nas respostas associadas aos seus efeitos isolados (RIZHSKY et al., 2004; MITTLER, 2006).

A combinação de seca com calor é um exemplo de condição ambiental que comumente ocorre nas áreas agrícolas, particularmente nas regiões áridas e semiáridas. Essa combinação é um caso típico de interação ambiental onde um fator abiótico possui interação

antagônica sobre o outro. Por exemplo, em plantas submetidas à temperatura elevada ocorre aumento da condutância estomática, resposta que está associada à dissipação de calor para redução da temperatura foliar (WAHID et al., 2007; RIZHSKY et al., 2002). No entanto, sob condições de estresse hídrico, o aumento da condutância será limitado pela restrição de água do solo, e nessas condições o efeito do estresse térmico no metabolismo celular será potencializado (RIZHSKY et al., 2004).

A seca e/ou temperaturas elevadas são estresses abióticos comumente relacionados com a indução de estresse oxidativo em plantas (WANG et al., 2003; GUO et al., 2006). O estresse hídrico reduz a condutância estomática e limita a fixação de CO_2, promovendo um desbalanço entre as fases bioquímicas e fotoquímicas da fotossíntese (WAHID et al., 2007). Por outro lado, temperaturas elevadas afetam a fotossíntese por alterações bioquímicas diretas, pelo aumento da atividade oxigenase da rubisco e pela dissociação do complexo protéico de liberação de O_2 nos cloroplastos, alterando o fluxo de elétrons entre os PSII e PSI (WAHID et al., 2007). Nos dois casos pode ocorrer produção excessiva de espécies reativa de oxigênio (EROs), por fotorrespiração ou por redução direta do O_2 (MITTLER, 2002).

Atualmente, apesar do número relativamente reduzido de estudos focados na compreensão das mudanças metabólicas induzidas pela presença simultânea de dois ou mais estresses em plantas, os resultados preliminares são contundentes e demonstram que o metabolismo é afetado diferentemente pelos efeitos isolados e combinados dos estresses. Os resultados demonstram, ainda, que os mecanismos de proteção ou defesa para uma combinação de estresses podem ser distintos daqueles atribuídos aos fatores isolados, podendo responder, exclusivamente, à combinação (RIZHSKY et al., 2002). Por fim, os estudos envolvendo os diferentes aspectos metabólicos em plantas, indicam, cada vez mais, que a combinação de estresses deve ser considerada como um novo estado do estresse abiótico, e não apenas uma combinação de estresses isolados (MITTLER, 2006).

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, essa espécie recebeu tremenda atenção por ser uma oleaginosa viável para a obtenção do biodiesel. Na verdade, acredita-se que o pinhão-manso apresente uma produção anual de óleo que varie entre 1100 a 1700 litros de óleo/ha, levando de três a quatro anos para atingir a idade economicamente produtiva, que

pode se estender por 40 anos (GLOBO RURAL, 2006). Além das características já mencionadas, acrescenta-se o fato do pinhão manso ser perene e nativo das Américas, resistente às condições adversas de solo e clima (baixa pluviosidade e temperaturas elevadas) e boa qualidade do óleo produzido (FRANCIS et al., 2005). Essas particularidades tornam esta espécie uma excelente oleaginosa para ser adotada como alternativa na produção de biodiesel.

As 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. No mundo todo existe pouco conhecimento sobre esta planta, cujo gênero tem mais de 170 espécies, sendo a mais importante a *Jatrofa 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).

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. Além disso, 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 (MEIRELES, 2003)

Apesar de ser uma cultura promissora, pouco se conhece sobre a caracterização fisiológica e bioquímica da espécie em condições de estresses como salinidade, seca e temperatura elevada. Mais raros ainda são os estudos direcionados ao entendimento dos componentes metabólicos diretamente relacionados com os mecanismos de danos e proteção celular, sob essas condições estressantes. Dessa forma, alguns questionamentos são extremamente pertinentes para o pinhão-manso sob essas condições ambientais adversas. Plantas de pinhão-manso apresentam mecanismos de tolerância (ajustamento osmótico) a seca e a salinidade? Salinidade, seca e temperatura elevada provocam danos significativos no aparato fotossintético (trocas gasosas e parâmetros de fluorescência da clorofila) de plantas de pinhão-manso? Existe similaridade de danos oxidativos causados pela seca, calor e a

combinação de ambos nessa espécie? Qual (is) sistema (s) de proteção é (são) mais importante(s)? Essas perguntas só poderão ser respondidas utilizando abordagens experimentais como o do presente estudo, capazes de contemplar os efeitos dos diferentes fatores estressantes.

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4. OBJETIVOS

4.1. OBJETIVO GERAL

Diante do exposto, o presente trabalho visou à obtenção de dados que possam auxiliar na compreensão dos principais mecanismos fisiológicos e bioquímicos induzidos por diferentes estresses abióticos em plantas jovens de pinhão-manso.

4.2. OBJETIVOS ESPECÍFICOS

- Caracterizar a acumulação de Na^+, Cl^- e K^+ em folhas e raízes de plantas jovens de pinhão-manso, em associação com variáveis de crescimento, visando conhecer o grau de sensibilidade dessa espécie ao estresse salino (Capítulo I).

- Avaliar a acumulação de solutos orgânicos e inorgânicos bem como suas respectivas contribuições para o ajustamento osmótico em folhas de pinhão-manso em diferentes níveis de NaCl (Capítulo II).

- Avaliar a resistência do aparato fotossintético de plantas de pinhão-manso submetidas ao estresse salino. A atividade fotoquímica e as trocas gasosas foliares serão estudadas durante o estresse salino e recuperação, sendo as respostas fisiológicas analisadas junto com as mudanças nos conteúdos foliares de Na^+ e Cl^- e na relação K^+/Na^+ (Capítulo III).

- Observar os efeitos comparativos entre os estresses hídrico e salino nas tocas gasosas foliares e na fluorescência da clorofila em plantas jovens de pinhão-manso (Capítulo IV)

- Determinar a contribuição relativa de solutos orgânicos e inorgânicos no ajustamento osmótico de plantas de pinhão-manso submetidas ao estresse hídrico (Capítulo V).

- Avaliar as alterações fotossintéticas e os mecanismos de proteção oxidativa em plantas de pinhão-manso submetidas aos estresses isolados e combinado de seca e calor (Capítulo VI).

4. ESTRATÉGIA EXPERIMENTAL

O presente trabalho foi dividido em experimentos seqüenciais e independentes que resultaram na produção de seis capítulos, cada um, correspondendo a um artigo científico. O primeiro artigo é intitulado "**Acúmulo de íons e crescimento de pinhão-manso sob diferentes níveis de salinidade"** (Capítulo I). O segundo artigo tem como título "**Contribuição de solutos orgânicos e inorgânicos no ajustamento osmótico de pinhão-manso submetido à salinidade"** (Capítulo II). O terceiro artigo trata do estudo sobre "**Salt stress induced damages on the photosynthesis of physic nut young plants"** (Capítulo III). O quarto artigo tem como título "**Photosynthetic acclimation and physiological responses to water stress and salinity in *Jatropha curcas* young plants"** (Capítulo IV). O quinto artigo é intitulado "**The role of organic and inorganic solutes in the leaves and roots osmotic adjustment in drought-stressed** *Jatropha curcas* **plants**" (Capítulo V) e o sexto é **Photosynthetic changes and protective mechanisms against oxidative damages during drought and heat isolated and combined stresses in** *J. curcas* **plants** (Capítulo VI). A descrição detalhada de cada um dos capítulos mencionados acima se encontra no segmento "material e métodos" dos respectivos capítulos a seguir.

Capítulo I

(Artigo publicado na Revista Ciência Agronômica, v.40, n.2, p.240-246, abr-jun, 2009)

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

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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 (*Jatropha curcas* L.) 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 conteúdo 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.

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[1] Recebido para publicação em 22/09/2008; aprovado em 03/03/2009
Projeto de pesquisa financiado pela Capes

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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 (*Jatropha curcas* L.) exposure to different NaCl levels. 35-day-old seedlings were exposure to 0 ; 25; 50; 75 and 100 mM of NaCl suppplied in the nutrient 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 μmol 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 μmol g^{-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.

Introdução

A salinização dos solos é um sério problema no mundo inteiro e tem crescido substancialmente, causando perdas na produtividade das culturas. Estima-se que 6% das terras cultivadas no mundo e aproximadamente 30% das terras irrigadas estejam afetadas por sais (MUNNS; TESTER, 2008). Esse problema é mais agudo nas regiões semiáridas onde a baixa pluviosidade e a elevada demanda evaporativa contribuem decisivamente para o agravamento da salinização dos solos (VIÉGAS et al., 2001). As altas concentrações de sais no solo, além de reduzir o potencial hídrico do solo, podem provocar efeitos tóxicos nas plantas, causando distúrbios funcionais e injúrias no metabolismo.

A redução no potencial hídrico dos tecidos causada pelo excesso de sais provoca restrição no crescimento, uma vez que as taxas de elongação e de divisão celular dependem diretamente do processo de extensibilidade da parede celular (ASHRAF; HARRIS, 2004). Portanto, a resposta imediata das plantas ao estresse salino é uma forte diminuição na expansão foliar (PARIDA; DAS, 2005). Dessa forma, o balanço osmótico é essencial para o crescimento dos vegetais em meio salino e qualquer falha neste balanço resultará em injúrias semelhantes aos da seca, como a perda de turgescência e a redução no crescimento,

resultando em plantas atrofiadas, desidratação e finalmente a morte das células (ASHRAF; HARRIS, 2004).

A inibição do crescimento das plantas sob salinidade ocorre por duas razões. A primeira é devido ao efeito osmótico ou déficit hídrico provocado pela salinidade, que reduz a absorção de água. A segunda é devido ao efeito específico dos íons ou excesso de íons, que entram no fluxo de transpiração e eventualmente causam injúrias nas folhas, reduzindo assim o crescimento (MUNNS, 2005). A redução no crescimento das plantas em resposta ao estresse salino tem sido observada por diversos autores (TÁVORA et al., 2001; VIÉGAS et al., 2001, VIÉGAS et al., 2003). As espécies cultivadas podem ser classificadas em tolerantes ou sensíveis e o nível de tolerância, assim como os níveis de sais que são letais, varia grandemente entre as diferentes espécies vegetais e dentro de uma mesma espécie (PARIDA; DAS, 2005).

A acumulação de íons salinos pode causar problemas de toxicidade iônica, deficiências nutricionais ou ambos. Diversos trabalhos na literatura demonstram que a salinidade promove um aumento nos teores de sódio e cloreto, tanto em glicófitas como em halófitas (GREENWAY; MUNNS, 1980). A injúria provocada pelo acúmulo excessivo de íons tóxicos, Na^+ e Cl^-, se manifesta como clorose marginal e causa o surgimento de zonas necróticas, o que contribui para aceleração dos processos de senescência e abscisão foliar (MUNNS, 2002). Em plantas que crescem em solos salinos, as células podem apresentar distúrbios na homeostase iônica não só devido ao aumento da concentração de Na^+ como também pela diminuição da concentração de K^+ no citosol, causando a conseqüente redução da relação K^+/Na^+ (ZHU, 2003).

O pinhão-manso (*Jatropha curcas* L.) é uma espécie oleaginosa, de fácil propagação e que pode apresentar relevante importância social e econômica para o Brasil, especialmente como fonte de bicombustível. Infelizmente, essa espécie ainda é muito pouco estudada nos diversos aspectos agronômicos, especialmente na fisiologia vegetal ligada ao estresse salino.

O objetivo do presente trabalho foi caracterizar a acumulação de Na^+, Cl^- e K^+ em folhas e raízes de plantas jovens de pinhão-manso, em associação com variáveis de crescimento, visando conhecer o grau de sensibilidade dessa espécie ao estresse salino.

Materiais e métodos

Condições de crescimento

A fase de germinação e o desenvolvimento das plantas foram conduzidos em casa de vegetação, pertencente ao Departamento de Bioquímica e Biologia Molecular, na

Universidade Federal do Ceará (UFC), em Fortaleza, Ceará, Brasil (latitude 3°44' S, longitude 38°33' W). Durante o experimento as condições ambientais no interior da casa de vegetação foram: fotoperíodo de 12 horas, temperaturas média mínima 24 °C, máxima 36 °C, temperatura média diária 28 °C, umidade relativa do ar média de 65% e radiação fotossinteticamente ativa máxima média de aproximadamente 700 µmol m^{-2} s^{-1}.

Material vegetal e condução das plantas

Sementes de pinhão-manso foram postas para germinar em bandejas com areia e irrigadas diariamente com água destilada até a queda dos cotilédones (± 8 dias após o plantio). Em seguida, foram transferidas para vasos de 2 L onde passaram a receber solução nutritiva de Hoagland e Arnon (1950) modificada, apresentando a seguinte composição de macronutrientes (mM): 2,5 $Ca(NO_3)_2$; 1,0 NH_4Cl; 0,5 K_2HPO_4; 0,5 $MgSO_4$ e 2 KNO_3 e micronutrientes (µM): 40 H_3BO_3; 9 $MnCl_2$; 3 $CuSO_4$; 7 $ZnMoO_4$; 0,1 Na_2MoO_4 e 100 Fe-EDTA com ajuste do pH para 6,0. As plantas permaneceram em solução nutritiva por duas semanas com diluição de ¼ na primeira semana e solução sem diluição na segunda semana.

Aos 20 dias após a germinação foram aplicados os tratamentos salinos de maneira parcelada (25 mM de NaCl por dia) e ao final de quatro dias tínhamos 5 tratamentos (0; 25; 50; 75 e 100 mM de NaCl) com quatro repetições. Os tratamentos se estenderam por quinze dias quando se coletou o material vegetal, sendo separados em raízes e folhas para a determinação das concentrações de Na^+, Cl^- e K^+ .

As determinações das concentrações de sódio e potássio foram realizadas segundo Brilhante (2006). A extração foi feita utilizando 50 mg de massa seca do tecido para 20 mL de H_2O deionizada em banho-maria a 100 ºC por 1 hora. O extrato obtido foi centrifugado e seu sobrenadante foi analisado em fotômetro de chama (Micronal B462) para determinação dos conteúdos de Na^+ e K^+.

A determinação da concentração de cloreto foi determinada segundo Malavolta et al. (1997); 100 mg de massa seca do tecido foram submetidos à extração com 25 mL de água deionizada com agitação ocasional durante 30 minutos. Em seguida, alíquotas de 20 mL do extrato foram filtradas em papel de filtro e adicionadas 1 mL da solução indicadora de cromato de potássio K_2CrO_4 5%(p/V). Cada amostra foi titulada lentamente com nitrato de prata $AgNO_3$ 28 mM em bureta até a viragem do indicador através da formação do precipitado de Ag_2CrO_4 (coloração marrom pálido persistente). Cada 1 mL de nitrato de prata gasto na titulação correspondeu a 2,5 mg de cloreto em 100 mg de matéria seca. Foi utilizado

um branco com 20 mL de água deionizada + indicador + algumas gotas de Ag_2NO_3 até obtenção da coloração marrom pálido. O volume do branco foi subtraído de cada amostra.

As variáveis de crescimento avaliadas foram as seguintes: massa seca total, área da folha meristemática apical, altura da planta e diâmetro do caule. Essas variáveis foram medidas a cada dois dias até o final do experimento, com exceção da massa seca total que só foi determinada após os quinze dias de tratamento salino.

Delineamento experimental

Os tratamentos foram dispostos em delineamento inteiramente casualizado com cinco tratamentos (0; 25; 50; 75 e 100 mM de NaCl) e quatro repetições, onde cada parcela experimental foi composta de uma planta por vaso. Os resultados foram submetidos ao teste F a 5% de significância, através da análise de variância, e as médias comparadas pelo teste de Tukey ($p<0,05$).

Resultados e discussão

Acúmulo de íons inorgânicos em folhas e raízes expostas à salinidade

Foi observado acúmulo excessivo de Na^+ nas folhas, sendo este proporcional ao aumento das doses de NaCl (Figura 1A) chegando a 661% na dose mais elevada de sal (100 mM). A acumulação do íon Cl^- nas folhas obedeceu a mesma similaridade do sódio, todavia com 100 mM de NaCl ocorreu um acúmulo de 1400% (Figura 1B).

Os resultados obtidos sugerem não ter havido mecanismos de exclusão dos íons tóxicos (Na^+ e Cl^-) após o processo de absorção, resultando em acúmulo na parte aérea, com surgimento de cloroses e necroses nas folhas. Essas respostas resultaram, provavelmente, de alteração no balanço hormonal, na perda de turgescência das células-guarda e na redução generalizada da atividade metabólica da planta (GORHAM et al., 1988).

O elevado acúmulo de Na^+ e Cl^- no tecido vegetal durante a exposição das plantas ao estresse salino representou um dos principais efeitos desse estresse sobre o metabolismo vegetal. O componente iônico da salinidade pode causar danos irreparáveis em estruturas celulares as quais podem comprometer a eficiência metabólica e até mesmo provocar a morte celular (SHI et al., 2002). O efeito tóxico causado pelo excesso de Na^+ oriundo do meio externo pode ser reduzido pelos seguintes mecanismos: restrição da entrada de Na^+ na célula através da absorção seletiva; exclusão ou compartimentalização no vacúolo do excesso de Na^+ citosólico, bem como um sistema eficiente de partição deste íon na planta (ASHRAF;

AHMAD, 2000). A concentração de K^+ nas folhas foi reduzida pelos tratamentos salinos (Figura 1C). Tal redução foi proporcional ao incremento das doses de NaCl, obtendo na maior dose (100 mM) reduções de 87%. Diminuição na concentração de K^+ nas folhas com o aumento da salinidade também foi encontrado em plantas do gênero *Atriplex* (BRILHANTE, 2006), em milho (AZEVEDO NETO et al., 2004) e sorgo (LACERDA, 2000; NETONDO et al., 2004).

As concentrações de K^+ nas folhas podem ter sido reduzidas pelas altas concentrações de sódio através do antagonismo que existe entre esses dois íons. Na realidade, alguns autores têm observado a existência de múltiplos sistemas de absorção com diferentes seletividades para Na^+ e K^+ o que pode refletir a necessidade da planta para coordenar o influxo desses cátions (SCHACHTMAN; LIU, 1999). Para Lacerda (2005), a duração do estresse e a idade da folha amostrada podem produzir diferentes resultados e interpretações.

Nas raízes o aumento nas concentrações de Na^+ e Cl^- foi proporcional ao incremento de NaCl sendo estes de 580% e 1250% respectivamente, na dose mais elevada de sal (Figuras 2A e 2B). Por outro lado, a acumulação de K^+ foi reduzida em 35% na dose de 100 mM (Figura 2C). Essa redução pode estar relacionada à exposição direta das raízes ao sal, o que provoca alterações na integridade e permeabilidade seletiva da membrana plasmática (GRATTAN; GRIEVE, 1998; VIÉGAS et al., 2001).

A relação K^+/Na^+ foi reduzida com o aumento da salinidade (Figura 3), proporcionando reduções de 99,9% nas folhas (Figura 3A) e 99,8% nas raízes (Figura 3B). Esses resultados estão de acordo com os dados obtidos por Viégas et al. (2001) e Alves et al. (2008) que observaram reduções da relação K^+/Na^+ em plantas de cajueiro submetidas à salinidade.

Diversos autores (MAATHUIS; AMTMANN, 1999) têm correlacionado a resistência à salinidade com a manutenção de uma adequada nutrição potássica dentro de uma planta, podendo a relação K^+/Na^+ ser utilizada como critério de seleção de materiais sensíveis e resistentes ao estresse salino. Múltiplos sistemas de absorção com seletividades para K^+ e Na^+ podem refletir a necessidade da planta em coordenar o influxo desses cátions (SCHACHTMAN; LIU, 1999).

Ao final do experimento foram analisadas algumas variáveis de crescimento onde se observou um comportamento linear decrescente em todas com o incremento das doses de NaCl. A concentração de NaCl capaz de reduzir em 50% a massa seca das plantas foi de aproximadamente 47 mM, valor esse estimado por regressão utilizando a equação do gráfico (Figura 4). Na dose mais elevada de sal (100 mM de NaCl) o decréscimo chegou a

aproximadamente 64%. Resultados semelhantes foram encontrados por Dantas et al. (2002) e Costa et al. (2003) trabalhando com feijão-de-corda. As reduções e/ ou inibições constatadas no crescimento dos vegetais, à medida que eram submetidas a concentrações crescentes de salinidade (NaCl), é atribuído ao efeito osmótico, à toxicidade pela absorção excessiva dos íons Na^+ e Cl^- e ao desequilíbrio nutricional causado pelos distúrbios na absorção dos nutrientes essenciais (RODRIGUES, 2007). As demais variáveis de crescimento estão representadas na Tabela 1.

A área foliar também apresentou um decréscimo linear com o aumento dos níveis de sal. Esse chegou a aproximadamente 53% na dose de 100 mM de NaCl . Uma das primeiras respostas das plantas submetidas ao estresse salino é a redução na taxa de crescimento foliar, reduzindo a área foliar disponível para a fotossíntese (TERRY; WALDRON, 1984). Essa redução foi observada por Aragão et al. (2005) em plantas de *Phaseolus vulgaris* e Carmo et al. (2003) em plantas de bananeira. O tamanho da planta e o diâmetro do caule apresentaram reduções de 46 e 50% respectivamente, na dose mais elevada de NaCl (100 mM) em relação às plantas controle. De acordo com a classificação de Mass (1986), utilizada para indicar à tolerância de várias culturas a salinidade, pode-se classificar o pinhão-manso como uma espécie sensível à salinidade devido a redução significativa de todas essas variáveis de crescimento analisadas neste estudo já a partir da dose de 50 mM durante o período de estresse.

Conclusões

Plantas jovens de pinhão-manso são sensíveis ao excesso de NaCl na solução externa, mostrando uma redução de 50% na massa seca com uma concentração estimada de 47 mM de NaCl.

A sensibilidade das plantas de pinhão-manso ao estresse salino está relacionada, a uma grande acumulação de Na^+ e Cl^- nas folhas, associada com grande redução na concentração de K^+ induzida pelo excesso de Na^+.

Agradecimentos

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq) e Fundação Cearense de Apoio ao Desenvolvimento Cientifico e Tecnológico (FUNCAP) pelo auxilio

financeiro e bolsas concedidas e ao Instituto Fazenda Tamanduá, Santa Terezinha (PB), pela concessão de sementes selecionadas de pinhão-manso.

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Lista de tabelas e figuras

Tabela 1 - Variáveis de crescimento em plantas de pinhão-manso submetidas a diferentes doses de NaCl durante 15 dias. Letras diferentes dentro da coluna significam diferenças significantes entre médias no nível de 5% de probabilidade pelo teste de Tuckey.

Tratamentos (mM)	Área foliar (cm^2)	Altura da planta (cm)	Diâmetro do caule (cm)
0	134,52a	19,64a	1,50a
25	102,54b	16,53b	1,25b
50	89,83c	14,25c	1,06c
75	68,54d	12,01d	0,98c
100	62,85d	10,51e	0,75d

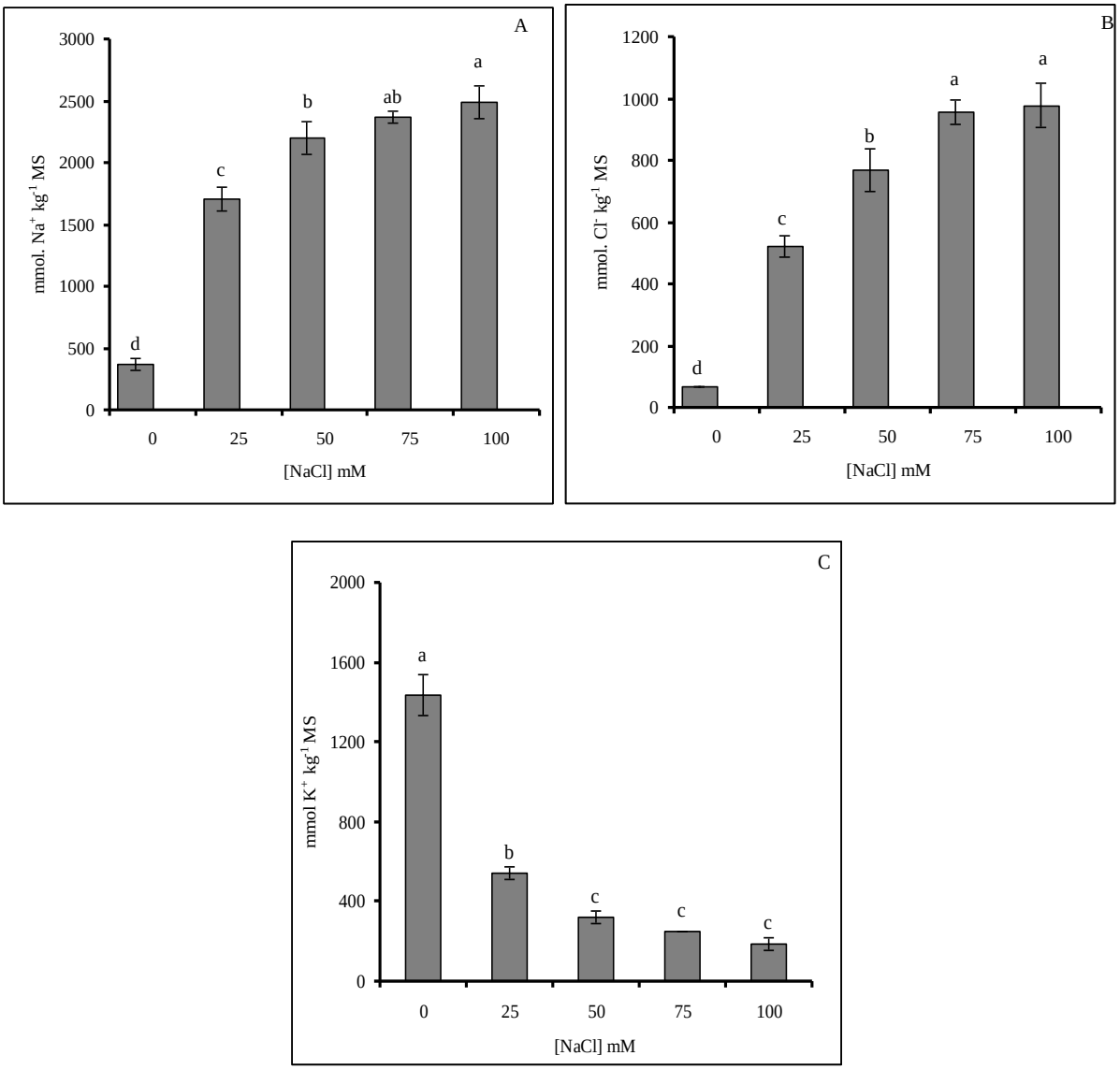

Figura 1 - Concentrações de Na^+ (A) Cl^- (B) e K^+ (C) em folhas de pinhão-manso submetidas a diferentes doses de NaCl. Os valores representam médias de 4 repetições (n=4) ± desvio padrão. Letras minúsculas iguais não diferem estatisticamente ao nível de significância pelo teste de Tukey.

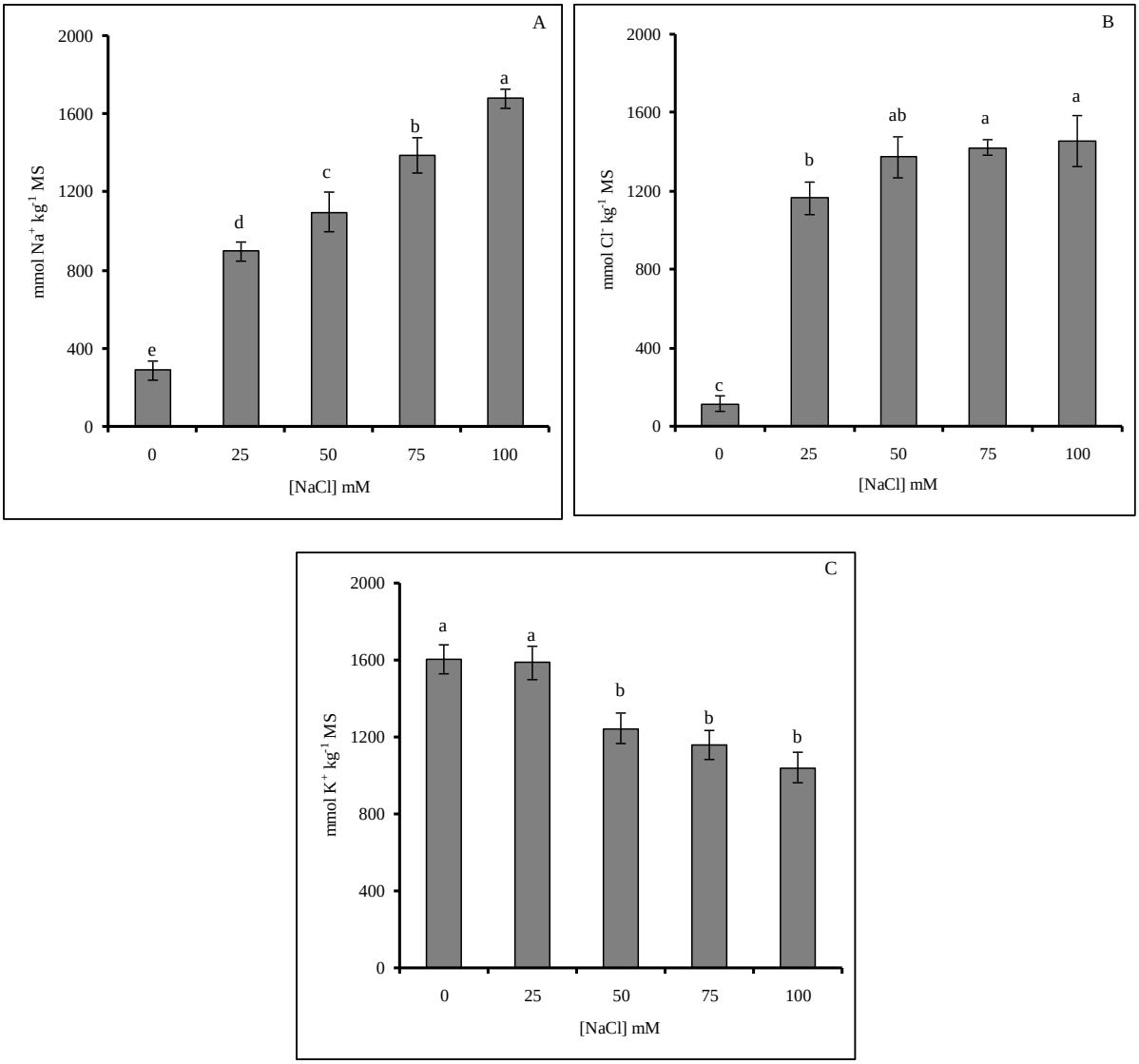

Figura 2 - Concentrações de Na^+ (A) Cl^- (B) e K^+ (C) em raízes de pinhão-manso submetidas a diferentes doses de NaCl. Os valores representam médias de 4 repetições (n=4) ± desvio padrão. Letras minúsculas iguais não diferem estatisticamente ao nível de significância pelo teste de Tukey.

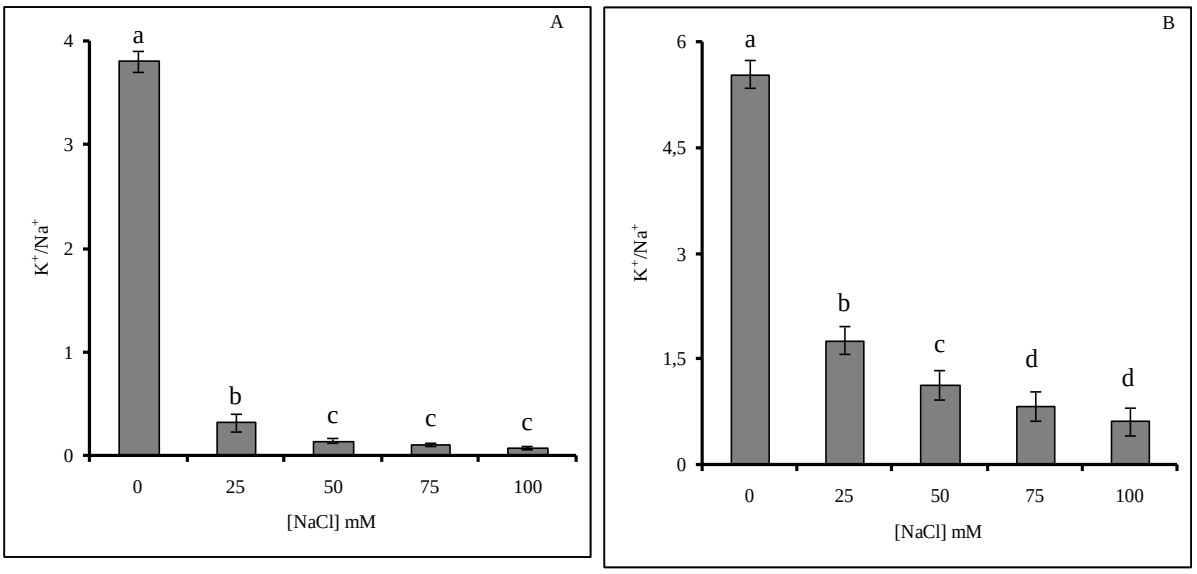

Figura 3 - Relação K^+/Na^+ em folhas (A) e raízes (B) em plantas de pinhão-manso submetidas a diferentes doses de NaCl. Os valores representam médias de 4 repetições (n=4) ± desvio padrão. Letras minúsculas iguais não diferem estatisticamente ao nível de significância pelo teste de Tukey.

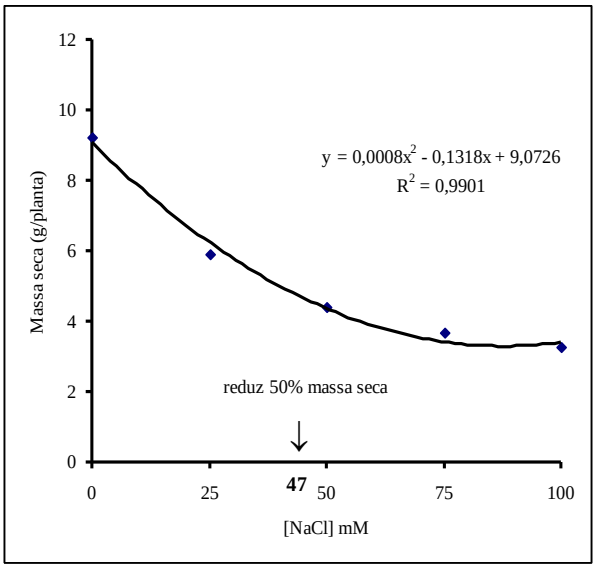

Figura 4 - Análise de regressão para estimativa da massa seca de plantas de pinhão-manso submetidas a diferentes doses de NaCl. Os valores representam médias de 4 repetições (n=4).

Capítulo II

(Artigo publicado na Revista Pesquisa Agropecuária Brasileira, v.44, n.5, p.437-445, 2009)

Contribuição de solutos orgânicos e inorgânicos no ajustamento osmótico de pinhão-manso submetido à salinidade

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Resumo – O objetivo deste estudo foi avaliar a acumulação de solutos orgânicos e inorgânicos e suas contribuições para o ajustamento osmótico de folhas de pinhão-manso (*Jatropha curcas* L.) submetido à salinidade. O experimento foi conduzido em casa de vegetação, em delineamento experimental inteiramente casualizado, com cinco tratamentos (0; 25; 50; 75 e 100 mM de NaCl) e quatro repetições. O potencial osmótico das folhas decresceu progressivamente e variou de -0,84 a -2,05 MPa, enquanto o conteúdo relativo de água aumentou nos tratamentos com 75 e 100 mM. Os íons Na^+ e Cl^- foram os mais importantes, em termos quantitativos e contribuíram com cerca de 52% e 20%, respectivamente, para o ajustamento osmótico das folhas de plantas tratadas com NaCl. A contribuição do K^+ decresceu de modo acentuado e foi de 17% e 5% com 25 e 100 mM de NaCl. A contribuição média dos solutos orgânicos, açúcares, aminoácidos, glicina-betaina e prolina, foi de 5,5, 6%, 4% e 0,03%, respectivamente. Os resultados mostram que folhas de pinhão-manso ajustam-se osmoticamente em presença de salinidade e mantêm bom nível de hidratação, principalmente por meio da acumulação de Na^+ e Cl^-. A glicina-betaina tem um papel quantitativo mais importante do que prolina no ajustamento osmótico, tanto em presença quanto em ausência de salinidade.

Palavras chaves: *Jatropha curcas*, Estresse salino, Potencial osmótico, Solutos osmoticamente compatíveis.

Contribution of organic and inorganic solutes on the osmotic adjustment of physic nut under salinity

Abstract – The objective of this study was to evaluate the organic and inorganic solutes accumulation and their contribution to the osmotic adjustment of physic nut (*Jatropha curcas* L.) leaves under salinity. The experiment was carried out in a greenhouse using a completely randomized design with five treatments (0, 25, 50, 75 and 100 mM of NaCl) and four replications. The osmotic potential decreased progressively, changing from – 0.84 to -2.05 MPa, while the relative water content was increased in the 75 and 100 mM treatments. The relative contribution of Na^+ and Cl^- ions were the most important quantitatively for osmotic adjustment of salt-treated plants, showing rough average of 52% and 20%, respectively. The K^+ relative contribution decreased significantly by salt treatments, changing from 17% to 5% when the NaCl level was increased from 25 to 100 mM. The average contribution of the organic solutes sugars, amino acids, glycinebetaine and proline was approximately 5.5%, 6%, 4% and 0.03%, respectively. The data evidence that physic nut leaves exhibit an effective osmotic adjustment under salinity, maintaining a good hydration status, mainly via Na^+ and Cl^- accumulation. The glycinebetaine is more important to osmotic adjustment than is proline, in both salt-treated and untreated plants.

Key words: *Jatropha curcas*, salt stress, osmotic potential, organics solutes.

Introdução

O pinhão-manso (*Jatropha curcas* L.) é uma espécie oleaginosa viável para a obtenção do biocombustível, pois produz, no mínimo, 2 Mg de óleo por hectare (SATURNINO et al., 2005). Os mesmos autores relatam que os graves problemas a ser enfrentados pelos produtores no cultivo da espécie em região semiárida, são a salinidade e a salinização dos solos. No caso de cultivo de sequeiro, será provável a expansão para solos naturalmente salinizados que são amplamente distribuídos na região Nordeste do Brasil. Na agricultura irrigada, as condições de elevada evapotranspiração, baixa qualidade das águas e baixa pluviosidade em muito deverão contribuir para a salinização secundária. Tal cenário, especialmente no médio e longo prazos, deverá não somente limitar a produtividade de pinhão-manso assim como inviabilizar outras culturas.

Para suportar o estresse salino, as plantas têm desenvolvido mecanismos complexos que contribuem para a adaptação ao estresse osmótico e iônico provocado pela alta salinidade (MELONI et al., 2004). Estes mecanismos incluem o ajustamento osmótico que é usualmente acompanhado pela absorção de íons inorgânicos bem como pela acumulação de solutos orgânicos compatíveis (osmoprotetores) (STRANGE, 2004). Íons inorgânicos são seqüestrados no vacúolo, enquanto os solutos orgânicos são compartimentalizados no citoplasma para balancear o baixo potencial osmótico nos vacúolos (TAIZ; ZEIGHER, 2004).

O acúmulo de compostos inorgânicos (Na^+, K^+ e Cl^-) é bem evidenciado em condições de estresse salino, embora esses elementos tenham um papel importante no crescimento de plantas superiores nessas condições, suas contribuições relativas variam entre as espécies, entre as cultivares de uma mesma espécie, entre órgãos e tecidos de uma mesma planta e até entre diferentes compartimentos de uma célula (ASHARAF; HARRIS, 2004). Recentemente, Silveira et al. (2009) observaram que os íons Na^+ e Cl^- são os solutos mais importantes no ajustamento osmótico de folhas e raízes de *Atriplex nummularia* e que K^+ tem sua contribuição diminuída intensamente pela salinidade.

Entre os compostos orgânicos, os nitrogenados (aminoácidos, inclusive a prolina e compostos quaternários de amônio, entre eles a glicina-betaina) e os poliidroxílicos (carboidratos e poliálcoois) são os solutos mais comumente acumulados em plantas sob condições de estresse (ASHARAF; HARRIS, 2004). Nesse contexto, o acúmulo de aminoácidos e de carboidratos solúveis tem sido estudado intensamente em plantas sob condições de estresse salino (AZEVEDO NETO et al., 2004), visto que são os solutos orgânicos de maior contribuição para o potencial osmótico.

O objetivo deste trabalho foi avaliar a acumulação de solutos orgânicos e inorgânicos e suas respectivas contribuições para o ajustamento osmótico em folhas de pinhão-manso em diferentes níveis de NaCl.

Materiais e métodos

O experimento foi conduzido em casa de vegetação no Departamento de Bioquímica e Biologia Molecular da Universidade Federal do Ceará, em Fortaleza-CE (3° 44’ S e 38° 33’ W). O clima local é do tipo AW, de acordo com a classificação de Koeppen. O período experimental foi compreendido entre março e abril de 2008. Durante o experimento as condições ambientais no interior da casa de vegetação foram: fotoperíodo de 12 horas,

temperaturas média mínima 24 °C, máxima 36 °C, temperatura média diária 28 °C, umidade relativa do ar média de 65% e radiação fotossinteticamente ativa máxima média de aproximadamente 700 μmol m^{-2} s^{-1}.

Foram utilizadas sementes de pinhão-manso (*Jatropha curcas* L.) fornecidas pelo Instituto Fazenda Tamanduá, São João do Bonfim, Paraíba (PB). Sementes previamente selecionadas por tamanho e peso, foram germinadas em areia e as plântulas mantidas por oito dias até posterior transferência para vasos de 2 L com solução nutritiva de Hoagland e Arnon (1950) diluída ¼ na primeira semana e solução sem diluição na segunda semana, com pH 6,0, ajustado a cada dois dias com NaOH 0,1M ou HCl 0,1M. Na terceira semana, iniciaram-se os tratamentos com NaCl por meio da adição diária de 25 mM até atingir os níveis de 25, 50, 75 e 100 mM, no quarto dia. Em seguida, as plantas permaneceram por mais 11 dias em exposição aos níveis de NaCl. As plantas cultivadas na ausência de NaCl foram adotadas como controle.

O delineamento experimental foi inteiramente casualizado com cinco tratamentos (0, 25, 50, 75 e 100 mM de NaCl) e quatro repetições. A parcela experimental foi representada por um vaso contendo uma planta, perfazendo no total 20 parcelas experimentais. Os resultados foram submetidos ao teste F a 5% de significância por meio da análise de variância e as médias comparadas pelo teste de Tukey ($p<0,05$).

Após a coleta, foi feita pesagem da massa fresca das folhas que foram posteriormente transferidas para secagem em estufa com circulação de ar quente a 75 °C, por 48 horas. Após esse período, foi determinada a massa de matéria seca. Para a determinação do conteúdo relativo de água (CRA) foram coletados 30 discos foliares de 1 cm, dos quais foi determinada a massa de matéria fresca (MF1). Os discos foliares foram transferidos para placas de Petri, com água destilada e deixados sobre uma bancada por 6 h. A seguir, os discos foram removidos e colocados entre folhas de papel de filtro, pressionados para a eliminação do excesso de água e, em seguida foram pesados novamente (MF2). Logo após, foram colocados em sacos de papel, para secar em estufa a 75ºC por 48 horas. Foi então determinada a massa seca do material (MS). O conteúdo relativo de água (CRA) foi calculado pela equação: CRA = [(MF1-MS)/(MF2-MS)] x 100 e a suculência foliar (SF) pela equação (MF1)/A onde A representa a área de 30 discos cujo diâmetro é 1 cm.

Para a determinação da osmolalidade total do tecido foliar foram coletadas folhas do terço médio da parte aérea da planta que foram maceradas em almofariz com pistilo. A seiva obtida do tecido foi filtrada e centrifugada a 10.000 x g por 10 min a 4 ºC. Uma alíquota de 10 μL do sobrenadante foi utilizada para a determinação da osmolalidade do tecido usando

um osmômetro de pressão de vapor modelo Vapro 5520 (Wescor, Inc., Logan, UT, USA). Os valores obtidos em mmol kg^{-1} foram convertidos em potencial osmótico por meio da equação de Van't Hoff usando a fórmula: 1 mmol kg^{-1} = -c (mosmoles kg^{-1}) x 2,58 x 10^{-3}. O ajustamento osmótico foi estimado pela diferença no potencial osmótico entre plantas estressadas e não estressadas.

Os solutos inorgânicos e os orgânicos foram extraídos a partir de tecidos foliares liofilizados previamente congelados em presença de N_2 líquido. Amostras pulverizadas foram transferidas para tubos hermeticamente fechados na presença de água deionizada e colocados em banho-maria a 100 ºC por 1 h. Os extratos foram filtrados e armazenados em freezer a -20 ºC para determinações posteriores. Os conteúdos de sódio e potássio foram determinados por fotometria de chama e o conteúdo de cloreto pela titulação com $AgNO_3$. Os teores de nitrato foram determinados segundo método de Cataldo et al. (1975). A determinação dos teores de açúcares solúveis totais foi realizada pelo método de Dubois et al. (1956). Os teores de aminoácidos livres totais e glicina-betaína foram determinados segundo Silveira et al. (2009), enquanto as concentrações de prolina segundo método de Silveira et al. (2003).

Todas as concentrações de solutos foram expressas em mmol soluto kg^{-1} de água no tecido após correção da umidade (SILVEIRA et al., 2009). A contribuição de cada soluto para o potencial osmótico foi estimada como percentagem da osmolalidade, por meio da seguinte relação: conteúdo do soluto (mmol soluto kg^{-1} de água no tecido)/osmolalidade (mmol kg^{-1}do solvente) x 100.

Resultados e Discussão

O estresse salino acarretou decréscimo significativo no crescimento foliar, em plantas jovens de pinhão-manso, após 15 dias de tratamento. Mesmo na concentração mais baixa de NaCl (25 mM), a massa de matéria seca sofreu uma redução de aproximadamente 25% em comparação com o controle (Figura 1A). Entre os tratamentos com 50, 75 e 100 mM de NaCl não houve diferenças significativas. Essa redução foi acompanhada por intensos sintomas visuais de toxicidade causados pelo estresse salino, caracterizados por clorose seguida de necrose, inicialmente em áreas localizadas das folhas com progressiva expansão em consequência da dose de NaCl e do tempo de exposição.

Apesar dos efeitos tóxicos da salinidade sobre as folhas de pinhão-manso, os indicadores do estado hídrico determinados, conteúdo relativo de água (CRA) e suculência foliar, não diminuíram na presença do sal. Ao contrário, nos tratamentos com 75 e 100 mM

de NaCl houve um aumento significativo destas variáveis em relação ao controle (Figuras 1B e 1C). Portanto, o estresse salino não afetou negativamente o grau de hidratação das folhas, e o excesso de íons acumulados contribuiu para a retenção de água no tecido.

O aumento nos valores da suculência foliar e no conteúdo relativo de água induzido pelo NaCl são indicativos de ter ocorrido um efetivo ajustamento osmótico em plantas estressadas, conforme observado por Martínez et al. (2004). Os efeitos da salinidade sobre o potencial osmótico em folhas de plantas de pinhão-manso são apresentados na Figura1D, em que se observa que esse parâmetro foi sistematicamente reduzido em plantas tratadas com NaCl. A variação foi de – 0,84 (0 mM) até -2,05 MPa em 100 mM de NaCl. Vários autores relatam que a redução do potencial osmótico foliar em plantas lenhosas submetidas à salinidade tem como principal causa à maior absorção dos íons Na^+ e Cl^- (OTTOW et al., 2005; SILVEIRA et al., 2009).

Os conteúdos de Na^+ e Cl^- foram significativamente maiores em folhas expostas ao sal em comparação ao controle. Os aumentos ocorreram a partir da concentração 25 mM e apresentaram uma tendência de estabilização a partir de 50 mM (Figuras 2A e 2B). O conteúdo de Na^+ na concentração mais elevada de sal atingiu aproximadamente 373 mM (expressa na base de água no tecido), cerca de quatro vezes a concentração de Na^+ na solução nutritiva, enquanto a soma das concentrações de Na^+ + Cl^- nas folhas nesse mesmo tratamento alcançou 520 mM, valor aproximadamente cinco vezes maior que a concentração externa na solução. Isso indica que essa espécie não possui mecanismos eficientes de redistribuição e exclusão de Na^+ e Cl^- para atuar na redução do excesso de íons salinos acumulados na parte aérea de plantas sob salinidade (GARCÍA-SÁNCHES et al., 2002).

Os íons Na^+ e Cl^- contribuíram com aproximadamente 17% e 4%, respectivamente, para o ajustamento osmótico das plantas de pinhão-manso não tratadas com NaCl enquanto que as tratadas apresentaram contribuições médias de 52% e 20%, respectivamente (Tabela 1). Esses dados mostram que os próprios íons salinos são, quantitativamente, os solutos mais importantes para o ajustamento osmótico de folhas de pinhão-manso submetido à salinidade. Esse tipo de resposta ocorre freqüentemente em glicófitas (OTTOW et al., 2005) e em halófitas submetidas ao estresse salino (SILVEIRA et al., 2009).

Os conteúdos de K^+ e NO_3^- nas folhas foram significativamente menores em plantas expostas ao sal quando comparadas às do controle (Figura 2C e 2D). Essas reduções foram significativas já a partir da dose de 25 mM e apresentaram uma tendência à estabilização a partir da dose de 50 mM em diante. Na dose mais elevada de sal, as reduções de K^+ e NO_3^- foram de 89% e 37%, respectivamente. Diminuição na concentração de K^+ nas folhas com o

aumento da salinidade também foi encontrada em milho por Azevedo Neto et al. (2004) enquanto Meloni et al. (2004) observaram diminuições nos teores de nitrato sob estresse salino em plantas de algaroba. Isso demonstra que reduções nos conteúdos de NO_3^- e K^+ devem estar relacionadas ao antagonismo por Cl^- e Na^+, respectivamente, durante a absorção (WHITE; BROADLEY, 2001).

A redução intensa nos conteúdos de K^+ causadas pela salinidade do NaCl deverá ter sérias implicações no ajustamento osmótico do citosol e organelas de folhas de pinhão-manso. De fato, nas plantas controle esse soluto mostrou uma contribuição de aproximadamente 50% no ajustamento osmótico enquanto que nas plantas tratadas com 100 mM de NaCl essa contribuição foi de apenas 5% (Tabela 1). Esse tipo de efeito é observado em outras glicófitas (LACERDA et al., 2003), mas não com essa intensidade observada em pinhão-manso. Na maioria das espécies a redução no conteúdo de K^+ causado pelo NaCl é muito mais intenso nas raízes do que nas folhas (FERREIRA-SILVA et al., 2008) enquanto que nas halófitas do gênero *Atriplex* esse efeito ocorre nos dois órgãos (SILVEIRA et. al., 2009). Adicionalmente, a contribuição do nitrato para o potencial osmótico foliar de plantas de pinhão-manso também foi reduzido pela salinidade. Esta variou de 4,0 (0 mM) para 1,5 (100 mM de NaCl), conforme Tabela 1.

Os conteúdos de aminoácidos livres totais em plantas estressadas, quando comparado as plantas controle, permaneceram praticamente inalterados até a dose de 50 mM de NaCl, todavia nos níveis mais elevados de sal (75 e 100 mM) houve um aumento significativo de 28 e 52%, respectivamente (Figura 3A). Esse aumento deve ter sido causado pela degradação e/ou redução na síntese protéica, já que ocorreu uma queda nos níveis de proteínas totais (dado não mostrado). Dados semelhantes foram obtidos por Lacerda et al. (2003) em plantas de sorgo forrageiro e por Silveira et al. (2003) trabalhando com plantas de cajueiro expostas a 100 mM de NaCl.

Nas plantas controle de pinhão-manso os aminoácidos livres totais mostraram uma contribuição relativa de aproximadamente 8% para o ajustamento osmótico enquanto que nas estressadas a contribuição média caiu para em torno de 6% (Tabela 2). Assumindo que os aminoácidos livres estão localizados principalmente no citosol e organelas e que nesta fração celular a concentração pode ser até 10 vezes maior em comparação com aquela determinada na fração celular total (SILVEIRA et. al., 2009), pode-se inferir que os aminoácidos mostram grande importância no ajustamento osmótico de folhas de pinhão manso submetido ou não ao estresse salino.

O conteúdo de prolina aumentou gradativamente até a dose de 75 mM de NaCl e no nível mais elevado de sal, houve um aumento bastante significativo (94%) em relação ao controle. A despeito desse aumento nas plantas estressadas, a contribuição desse soluto compatível para o potencial osmótico em folhas de pinhão-manso foi desprezível, alcançando valor máximo de 0,04% (Tabela 2). Existe muita controvérsia sobre o papel osmótico efetivo da prolina nas plantas submetidas ao estresse salino, isto é, se sua acumulação seria simplesmente uma decorrência de distúrbios metabólicos no metabolismo de aminoácidos e proteínas (SILVEIRA et al., 2003) ou de caráter genético adaptativo. No presente estudo, devido as concentrações muito baixas atingidas por esse aminoácido, é mais provável que o aumento na acumulação tenha sido induzidas mais pelas injúrias causadas pelo NaCl (SILVEIRA et al., 2009).

Para os solutos orgânicos acumulados em tecidos de plantas expostas a salinidade tem sido atribuída participação no ajustamento osmótico e proteção de estruturas celulares (STRANGE, 2004). Diversos estudos têm demonstrado que a acumulação de solutos compatíveis, como prolina e glicina-betaína, está relacionada à resistência a estresses abióticos, o que indica que esses solutos têm papel na osmoproteção (ASHARAF; HARRIS, 2004). No entanto, outros estudos têm indicado que a acumulação de solutos orgânicos em condições ambientais adversas deve estar mais relacionada a distúrbios metabólicos do que ao ajustamento osmótico (LUTTS et al., 1999).

O conteúdo de glicina-betaína não apresentou mudanças significativas quando comparadas plantas controle e estressadas (Figura 3D). Esse soluto contribuiu com aproximadamente 6% no ajustamento osmótico das plantas não tratadas e em média com 4% nas plantas tratadas com NaCl (Tabela 2). Como a glicina-betaína está situada principalmente no citosol e cloroplastos, que ocupam um volume de 5 a 10% do volume celular, sua concentração pode estar subestimada em relação às frações encontradas para esse soluto, podendo ser até dez vezes maiores do que os valores indicados na Figura 3D. (SILVEIRA et al., 2009). Sendo assim, na dose mais elevada de NaCl, a glicina-betaína pode atingir uma concentração de 28 mM na base de água de tecido que corresponde a aproximadamente 280 mM. Essa concentração é suficiente para que esse soluto exerça efeitos osmóticos e protetores nas células de folhas de pinhão-manso como já observado em outras espécies acumuladoras de glicina-betaina. Dessa maneira, é provável que folhas de pinhão-manso utilizem esse osmo-soluto protetor de uma maneira constitutiva para auxiliar o ajustamento osmótico de citosol e organelas (especialmente cloroplastos), além da proteção de estruturas celulares, como membranas (SAKAMOTO; MURATA, 2002).

O conteúdo de açúcares solúveis apresentou um ligeiro decréscimo com a imposição do tratamento salino (Figura 3C). Esses solutos contribuíram com aproximadamente 10% do ajustamento osmótico das plantas não tratadas e em média com 5,5% nas plantas tratadas com NaCl (Tabela 2). Resultados semelhantes foram encontrados por Ferreira-Silva et al. (2008) ao trabalhar com clones de cajueiro. Esses autores concluíram que o papel desse soluto orgânico na osmorregulação foi pouco expressivo.

Diferentemente, Lacerda et al. (2003) observaram acréscimos nos teores de açúcares em plantas de sorgo forrageiro submetidas ao estresse salino o que determinou um papel mais efetivo para o ajustamento osmótico e crescimento dessas plantas. Segundo Sanches et al. (1998) não é possível saber a real contribuição de açúcares no ajustamento osmótico durante o estresse salino sem o conhecimento da proporção entre monossacarídeos, dissacarídeos e oligossacarídeos. A redução no conteúdo de açúcares solúveis assim como na sua contribuição relativa no potencial osmótico em folhas de pinhão-manso em condições de salinidade deve estar relacionada a uma queda na fotossíntese ou mesmo numa maior taxa de migração via floema para outras partes da planta (TAIZ; ZEIGHER, 2004).

No tocante à contribuição relativa dos solutos inorgânicos e orgânicos para o ajustamento osmótico, a maior contribuição relativa de Na^+ e Cl^- em folhas de plantas estressadas são indicadores que o pinhão-manso possui algumas características semelhantes a de espécies halófitas que exibem grande avidez por íons salinos (SILVEIRA et. al., 2009). A contribuição excessiva dos íons salinos sugere que pinhão-manso se ajusta osmoticamente a concentrações elevadas de NaCl basicamente pelo uso do sódio e do cloreto, principalmente o Na^+. Estes resultados corroboram os de Alarcón et al. (1993) que observaram em algumas espécies de tomate não domesticadas, expostas à salinidade por um período longo, um ajustamento osmótico quase exclusivamente à custa dos íons sódio e cloreto.

Ao contrário dos íons salinos (Na^+ e Cl^-), o potássio se caracterizou por apresentar uma baixa contribuição relativa para o ajustamento osmótico em folhas de pinhão-manso a elevados níveis de salinidade. Sob concentrações altas de Na^+, a absorção de K^+ é inibida por meio de um transportador com afinidade alta a K^+-Na^+ e esse transportador opera como um sistema de absorção para Na^+ (TAIZ; ZEIGHER, 2004). Esses resultados corroboram com Patade et al. (2008) que observaram em cana-de-açúcar uma contribuição pouco efetiva do K^+ no ajustamento osmótico em condições de alta salinidade. Embora o potássio desempenhe um importante papel no ajustamento osmótico em espécies glicófitas, plantas de pinhão-manso utilizaram intensamente esse íon para se ajustar osmoticamente na ausência ou a baixos níveis de NaCl.

Dos solutos orgânicos estudados no presente trabalho somente a fração aminoácidos livres e prolina tiveram seus conteúdos aumentados nos níveis mais elevados de salinidade em proporção acima daquela observada na massa seca das folhas. Isso deve indicar que os aminoácidos livres totais, incluindo prolina, tiveram suas sínteses aumentadas e/ou suas utilizações metabólicas diminuídas. Entretanto, em termos quantitativos, para o ajustamento osmótico, a participação de prolina mostra menor importância quando comparada com a glicina-betaina, apesar desse último soluto não ter sua acumulação aumentada por efeito do estresse salino.

Conclusões

1. O pinhão-manso é capaz de se ajustar osmoticamente em presença de salinidade, por redução intensa no potencial osmótico e aumento do estado hídrico das folhas em concentrações elevadas de NaCl.
2. Os íons salinos Na^+ e Cl^- contribuem para a maioria do ajustamento osmótico, enquanto que a contribuição do K^+ é diminuída intensamente pelo NaCl.
3. Aminoácidos livres e açúcares solúveis mostram contribuição quantitativamente semelhante entre si para o ajustamento osmótico das plantas estressadas.
4. A glicina-betaína é mais importante quantitativamente do que a prolina para o ajustamento osmótico de folhas de pinhão-manso, tanto na ausência como na presença de diferentes níveis de NaCl na solução nutritiva.

Agradecimentos

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP) pelo auxilio financeiro e bolsas concedidas e ao Instituto Fazenda Tamanduá, São João do Bonfim (PB), pela concessão de sementes selecionadas de pinhão-manso.

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Lista de tabelas e figuras

Tabela 1. Contribuição relativa (%) de solutos inorgânicos na osmolalidade total das folhas de plantas de pinhão-manso expostas a diferentes concentrações de NaCl durante 15 dias.

[NaCl] mM	Na^+	K^+	Cl^-	NO_3^-
0	17,40	50,54	3,79	4,24
25	48,57	17,01	14,15	2,70
50	54,73	7,92	18,78	1,90
75	53,64	5,46	23,38	1,60
100	50,80	5,30	23,22	1,49

Tabela 2. Contribuição relativa (%) de solutos orgânicos na osmolalidade total das folhas de plantas de pinhão-manso expostas a diferentes concentrações de NaCl durante 15 dias.

[NaCl] mM	AA totais(1)	Prolina	Açúcares solúveis	Glicina-betaína
Controle	7,92	0,05	10,07	5,88
25	6,59	0,04	6,45	4,07
50	6,05	0,03	5,73	3,70
75	5,69	0,03	4,93	3,93
100	5,58	0,04	4,60	3,67

(1) Aminoácidos livres totais

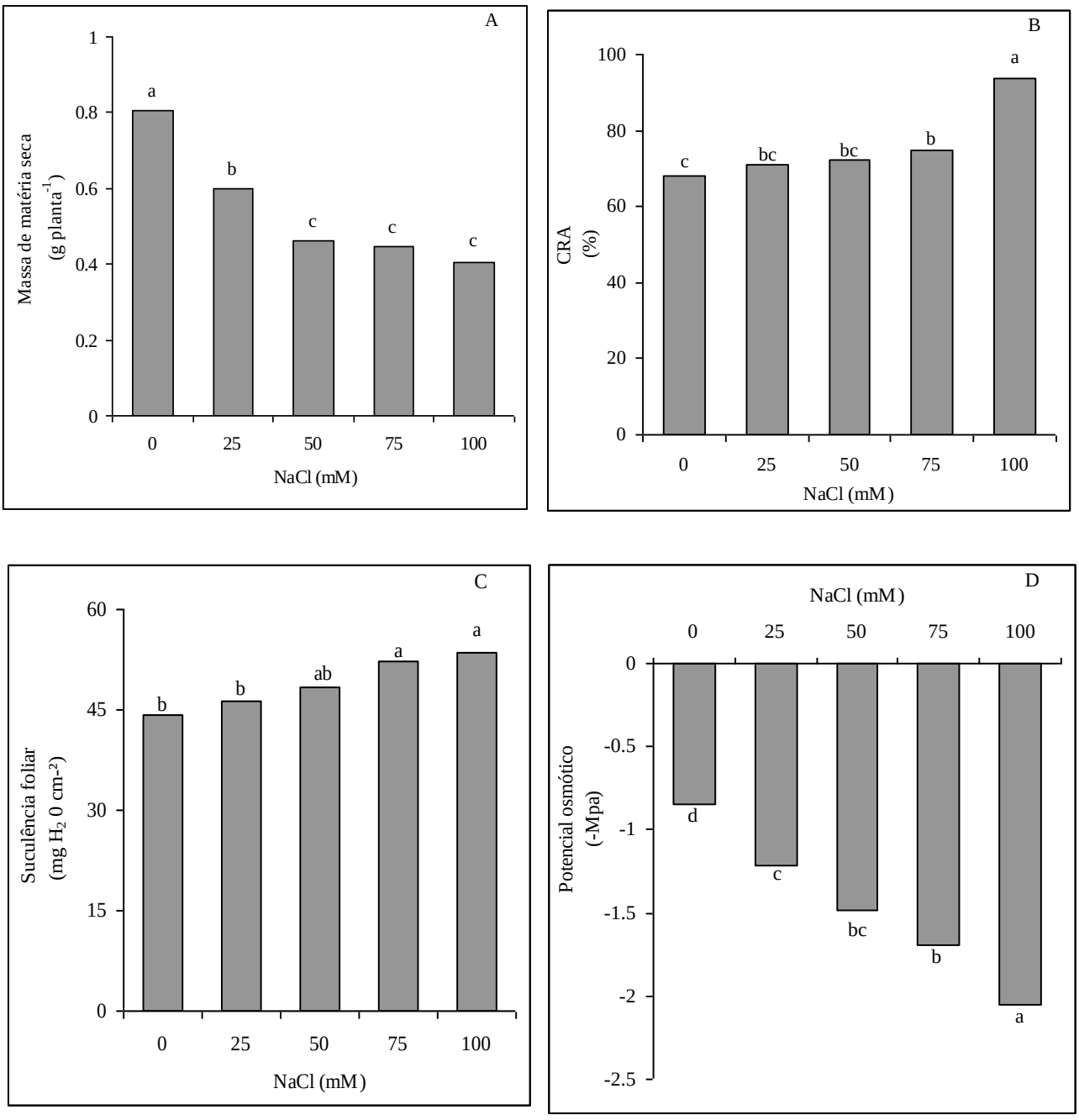

Figura 1. (A) Massa seca; (B) Conteúdo relativo de água; (C) Suculência foliar; (D) Potencial osmótico em folhas de pinhão-manso expostas a diferentes concentrações de NaCl durante 15 dias. Letras minúsculas iguais não diferem estatisticamente ao nível de 5% de significância pelo teste de Tukey.

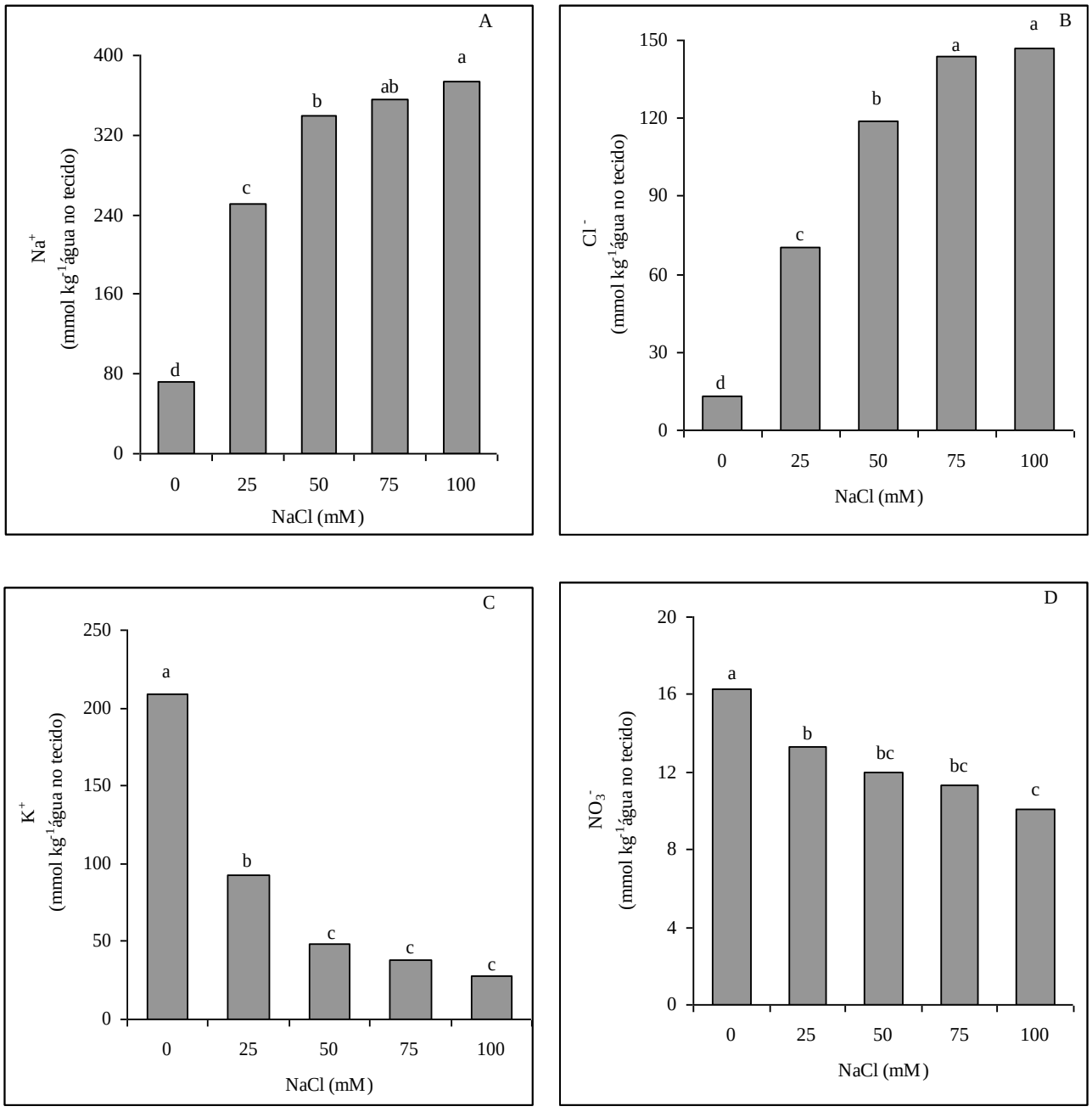

Figura 2. Conteúdos de (A) Sódio; (B) Cloreto; (C) Potássio; (D) Nitrato em folhas de pinhão-manso expostas a diferentes concentrações de NaCl durante 15 dias. Letras minúsculas iguais não diferem estatisticamente ao nível de 5% de significância pelo teste de Tukey.

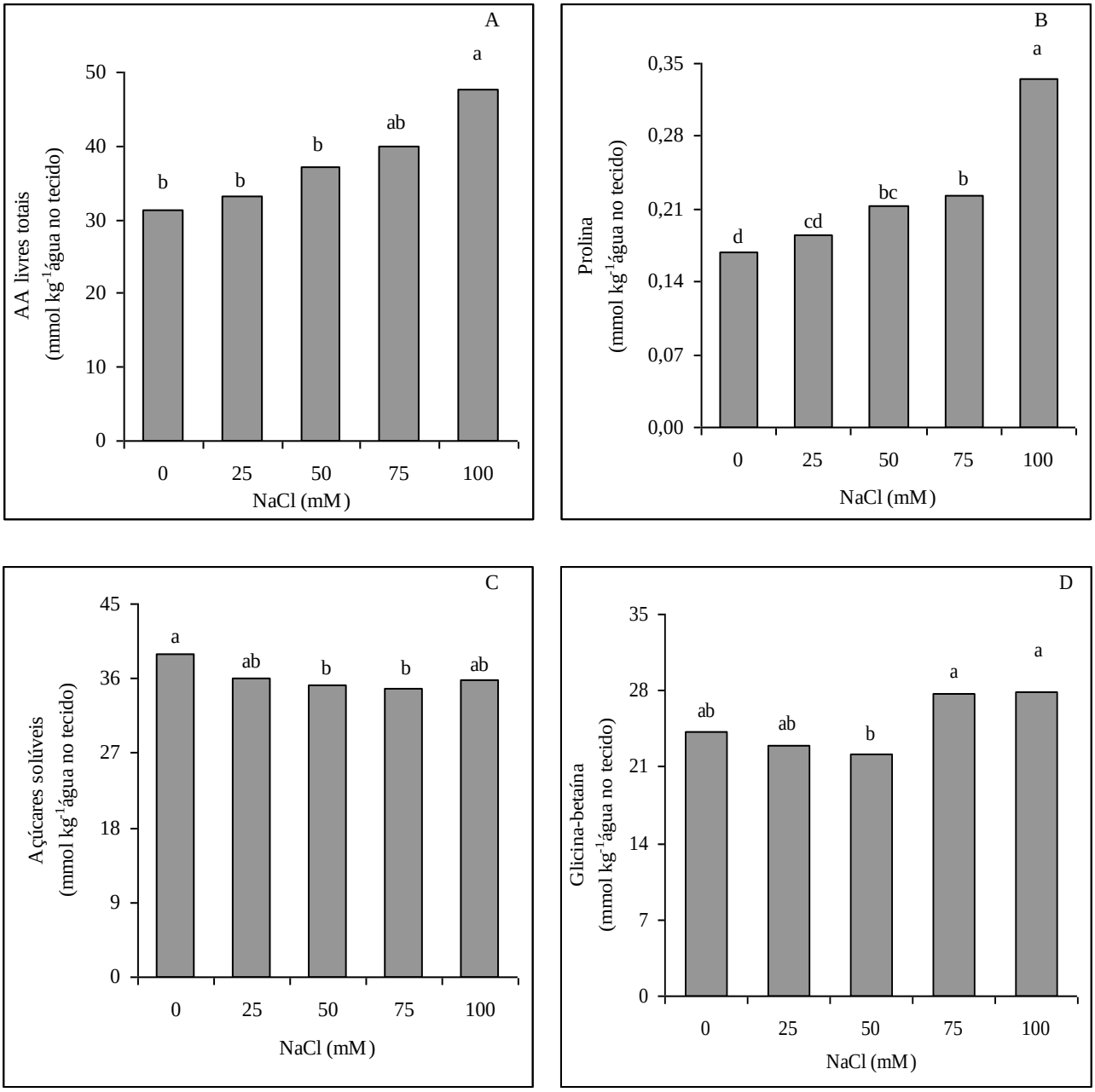

Figura 3. Conteúdos de (A) AA livres totais; (B) prolina; (C) açúcares solúveis; (D) glicina-betaína em folhas de pinhão-manso expostas a diferentes concentrações de NaCl durante 15 dias. Letras minúsculas iguais não diferem estatisticamente ao nível de 5% de significância pelo teste de Tukey.

Capítulo III

(Artigo submetido na Revista Scientia Agricola em 23/09/09)

Salt stress induced damages on the photosynthesis of physic nut young plants

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Salt stress induced damages on the photosynthesis of physic nut young plants

Abstract – The aim of this study was to evaluate the resistance of the photosynthetic apparatus of *Jatropha curcas* plants under salt stress. The experiment was carried out in a completely randomized design with treatments in a 2x3 factorial: two NaCl levels (0 and 100 mM) and three harvest times: 7 and 14 days of exposure and 3 days of recovery. The Na^+ and Cl^- accumulation and the reduction in the K^+/Na^+ ratio after 7 days of salt exposure did not indicate ionic toxicity in leaves but the salt stress caused significant reduction in leaf gas exchange parameters, such as CO_2 fixation, stomatal conductance and transpiration and, in contrast, it did not change the photochemical efficiency of the photossystem II. After 14 days of treatment, the saline ions achieved very high concentrations, reaching toxic levels in leaves. In such conditions, both leaf gas exchange and photochemistry suffered strong impairment caused by the ionic stress. The recovery treatment did not relieve the ionic toxicity and none improvement was observed in photosynthetic performance. In general, the photochemical activity is tolerant to the osmotic stress but both leaf gas exchange and photochemistry are strongly inhibited by NaCl-induced ionic stress in physic nut leaves.

Key words: chlorophyll fluorescence, gas exchange, ionic toxicity, *Jatropha curcas*, salinity.

Danos causados por estresse salino sobre a fotossíntese de plantas jovens de pinhão-manso

Resumo - O objetivo do estudo foi avaliar a resistência do aparato fotossintético de pinhão-manso (*Jatropha curcas* L.) submetido ao estresse salino. O experimento foi realizado em delineamento inteiramente casualizado com tratamentos em fatorial 2x3: duas concentrações de NaCl (0 e 100 mM) e três tempos de avaliação (7 e 14 dias de exposição e 3 dias de recuperação). A acumulação de Na^+ e Cl^- e a redução na relação K^+/Na^+ após 7 dias de exposição ao sal não indicou toxidade iônica nas folhas mas o estresse salino causou redução significativa nos parâmetros de trocas gasosas, como fixação de CO_2, condutância estomática e transpiração, e em contraste, não alterou a eficiência fotoquímica do fotossistema II. Após 14 dias de tratamento, os íons salinos atingiram concentrações muito elevadas nas folhas. Em tais condições, trocas gasosas foliares e fotoquímica sofreram forte redução causada pelo estresse iônico. O tratamento de recuperação não aliviou a toxidade iônica e nenhuma melhoria foi observada no desempenho fotossintético. Em geral, a atividade fotoquímica é tolerante ao estresse osmótico, mas ambos, trocas gasosas foliares e fotoquímica são fortemente inibidas pelo estresse iônico induzido pelo NaCl em folhas de pinhão-manso.

Palavras-chaves: fluorescência da clorofila, trocas gasosas, toxicidade iônica, *Jatropha curcas*, salinidade.

Introduction

Salinity adversely affects plant growth and development, with nearly 6% of the world's cultivated area and nearly 30 of the world's irrigated lands being affected by salt stress (MUNNS; TESTER, 2008). This problem is more relevant in semiarid regions where rainfall and high evaporative demand contribute intensely to the aggravation of soil salinization (VIÉGAS et al., 2001). In these regions, the problem of soil secondary salinization is exacerbated by the use of low quality water associated with inadequate techniques of soil management (FERREIRA-SILVA et al., 2009).

The accumulation of salt ions in plants can cause osmotic stress, ionic toxicity and induce nutritional deficiencies (MUNNS, 2002). When in high concentrations, Na^+ and Cl^- ions cause impairments in both biochemical and photochemical processes of photosynthesis (MUNNS; TESTER, 2008). Sorghum plants exhibited reduction in stomatal opening, which was the main limiting factor for photosynthesis under salt stress conditions (NETONDO et al., 2004). However, damage on the photosynthetic machinery may also occur due to non-stomatal limitation, i.e., decrease in Rubisco activity (DELFINE et al., 1999).

Physic nut (*Jatropha curcas*) is a species adapted to semiarid environmental conditions with high economic importance due to the seed oil quality, which can be converted in biodiesel by industry (SILVA et al., 2009a). Although this species had shown satisfactory yield under constraining conditions of semiarid regions, the physiological mechanisms controlling its salt stress tolerance are poorly understood.

Thus, this study was designed to evaluate the tolerance of photosynthetic apparatus of physic nut plants to salt stress. Photochemistry activity and leaf gas exchange were studied during salt stress and recovery, being the physiological response analyzed together with changes in the Na^+ and Cl^- leaf contents and K^+/Na^+ ratios.

Material and Methods

The experiment was 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%; maximum photosynthetic photon flux density (PPFD) of approximately 700 μmol m^{-2} s^{-1}. *Jatropha curcas* L. seeds supply 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 (2 L), containing Hoagland and Arnon (1950) nutrient solution (pH 6.0) with one-four strength in the first week and full strength afterward.

After this period, nutrient solution was supplied with 100 mM NaCl and then plants were subjected to stressful treatment during 14 days. The NaCl was added gradually (50 mmol NaCl L^{-1} d^{-1}) into solution in order to avoid osmotic shock. The treatment with nutrient solution in absence of NaCl was taken as control. At the end of two weeks of treatment, the salt-stressed plants were returned to control conditions for three days. In previous experiment, a three-day period was sufficient for full recovery of physic nut young plants subjected to 50 mM NaCl.

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 (A), transpiration (E), stomatal conductance (gs) and intercellular CO_2 concentration (Ci) were taken. The instantaneous carboxilation efficiency (A/Ci) was calculated (ZHANG et al., 2001).

The chlorophyll fluorescence was evaluated with a modulated fluorometer (FMS2, Hansatech, King's Lynn, UK). Basal (Fo) and maximal (Fm) fluorescence yields were measured in dark-adapted (30 min) leaves, whereas steady-state (Fs) and maximal (Fm') fluorescence yields were sampled in light-adapted tissues. Variable fluorescence yields were determined in dark-

adapted (Fv=Fm-Fo) and in light-adapted (ΔF=Fm'-F) leaf tissues. The following photochemical variables were calculated: potential (Fv/Fm) and effective (ΔF/Fm') quantum efficiency of PSII. Apparent electron transport rate (ETR= ΔF/Fm' x PPFD x 0.5 x 0.84), photochemical [qP= (Fm'-Fs)/(Fs-Fo')] and non-photochemical [qN=(Fm-Fm')/(Fm-Fo')] quenching (Rohácek, 2002). For ETR calculation, 0.5 was used as the fraction of excitation energy distributed to PSII and 0.84 as the fraction of incoming light absorpted by leaves (SCHREIBER et al., 1998). Fo' is the basal fluorescence yield measured after PSI excitation by far-red light. The ratio ETR/A was calculated to estimate the use of electrons in other processes not related to the photosynthetic CO_2 assimilation (RIBEIRO et al., 2009).

Leaf gas exchange and chlorophyll fluorescence were measured simultaneously, in fully expanded and mature leaves of plants exposure to 25 °C and PPFD of 590 μmol m^{-2} s^{-1} into growth chamber at 9:00h a.m. Those measurements were taken after 7-d and 14-d of treatment (0 and 100 mM NaCl), and repeated again three days after returning plants to the nutrient solution without the presence of NaCl.

At the end of experiment, leaves were sampled, frozen and stored at -80 ºC. After lyophilization, samples were placed into hermetically closed tubes containing deionized water and heated under water-bath at 100 ºC for 1 h. The extracts were then filtered and used for determination of Na^+ and K^+ contents by flame photometry. The Cl^- content was determinate through titration with $AgNO_3$ as described for Silveira et al. (2009).

The experiment was arranged in a completely randomized design, with two factors: NaCl (0 and 100 mM); time (seven and 14 days of salt stress and three days of recovery). Data were analyzed by ANOVA and mean values of four replications were compared by the Tukey test at the 0.05 level of confidence.

Results and Discussion

Leaves of physic nut young plants treated with NaCl (100 mM) showed significant changes in the Na^+, Cl^- and K^+ concentrations compared to control plants (Table 1). After seven days of treatment, the Na^+ and K^+ concentrations reached values around 603 and 581 mmol (kg DM)$^{-1}$ respectively, while the K^+/Na^+ ratio was reduced from 5.4 to 0.96 in stressed plants. In spite of the Na^+ accumulation associated with a reduction in K^+ concentration and consequent decrease in K^+/Na^+ ratio in treated plants, the saline condition was not sufficient to induce an ionic stress and a great change in the leaf ion homeostasis. In fact, the toxicity symptoms in physic nut plants appeared only when the leaf K^+/Na^+ ratio was much lower than 1.0 (SILVA et al., 2009b).

After seven days of treatment with 100 mM NaCl, the leaf Cl^- content was just 326 mmol (kg DM)$^{-1}$ (Table 1). This result, together with the Na^+ content reinforces that the stress imposed by saline condition was predominantly osmotic. Nevertheless, after 14-d of exposure to salt stress, the Na^+ and Cl^- accumulations were intense and reached toxic levels (SILVA et al., 2009b). The Na^+ and Cl^- concentrations were about 1,721 and 1,498 mmol (kg DM)$^{-1}$ respectively, in plant under salt stress. On the other hand, leaf K^+ content was decreased suddenly from 1,071 to 423 mmol (kg DM)$^{-1}$, when considering control and stressed plants. As a consequence, the K^+/Na^+ ratio was reduced from 5.0 (untreated plants) to 0.24 (treated plants).

The salinity toxic effects in physic nut leaves were also evidenced by the appearance of leaf necrotic areas and even after the NaCl removal from the nutrient medium (recovery), the toxicity symptoms persisted. After the recovery time, the Na^+ and Cl^- contents decreased only by 15% and 17%, respectively while that the K^+/Na^+ ratio was not changed when compared to stressed plants after 14-d of exposure to NaCl (Table 1). Thus, these results indicate that the salt stress was caused mainly by osmotic component after seven days of treatment. However, a strong ionic stress was established in physic nut leaves in the following seven days, i.e., after 14-d of exposure to NaCl.

Our results suggest that physic nut young plants have not efficient mechanisms for salt ions redistribution and/or exclusion, which could contribute to impede excessive accumulation of Na^+ and Cl^- in plant shoot under salinity (SILVA et al., 2009b). Differently, cowpea plants (*Vigna unguiculata*) showed an efficient system for remove Na^+ from leaf tissues. The leaf Na^+ content in cowpea plants submitted for six days to NaCl (200 mM) was 6-fold higher than control plants but after 3-d of recovery, the ion concentration was reduced in similar magnitude (CAVALCANTI et al., 2007).

Leaf CO_2 assimilation was strongly reduced in plants exposure to salt stress compared to control ones, with plants submitted to NaCl showing reductions of 23% and 85% after 7 and 14-d of treatment, respectively (Figure 1A). Salt stress also affected transpiration, stomatal conductance and intercellular CO_2 concentration decreases. The transpiration was reduced by 39% and 84% (Figure 1B), the stomatal conductance by 80% and 97% (Figure 1C) and the intercellular CO_2 concentration by 23% and 47% (data not shown) at 7 and 14-d of treatment, respectively. In contrast, the instantaneous carboxilation efficiency, estimated by the A/Ci ratio (Figure 1D), remained unchanged after seven days of treatment but was strongly reduced (73%) after 14-d of exposure to NaCl. After 3-d of recovery, the A/Ci ratio in stressed plants did not return to control level (Figure 1D).

Our results indicate that the reduction of photosynthesis until the 7th day was caused only by stomatal limitation, i.e. reduced CO_2 availability to carboxylation, while both stomatal and non-

stomatal limitations were observed in the 14th day of exposure to salt stress. Leaf gas exchange variables showed a significant perturbation of plant physiology even after a mild stress (seven days) and evidences of irreversible photosynthetic damage as stressful condition was prolonged (14 days under salt stress).

The decrease of photosynthesis associated with low stomatal conductance in plants subjected to saline conditions (Figure 1) is in accordance to Meloni et al. (2003), who observed impairment in the photosynthetic efficiency of cotton cultivars exposed to 50 and 100 mM NaCl due to stomatal limitation. On the other hand, significant decreases in intercellular CO_2 concentration and A/Ci ratio also indicate that salt stress affected the photosynthesis by metabolic limitation. Reductions of A/Ci ratio are probably associated with a decrease of the carboxylase Rubisco activity, which occurred in parallel with a Na^+ and Cl^- intense accumulation in leaf tissues (Table 1). Thus, the reduction of photosynthesis may, at least in part, be a direct effect of Na^+ and Cl^- ions on the photosynthetic apparatus, as observed in sorghum (NETONDO et al., 2004) and orange (LÓPEZ-CLIMENT et al., 2008) plants. In fact, stressed leaves of physic nut plants showed visual symptoms of injury, evidencing intense and irreversible cell damages.

Regarding to photochemistry effects, the potential quantum efficiency of PSII (Fv/Fm) was not affected by salinity, whereas the effective quantum efficiency (ΔF/Fm') decreased significantly (39%) after 14-d of exposure to NaCl (Figure 2A,B). The photochemical quenching (qP) was not affected by salt stress, while the non-photochemical quenching (qN) increased significantly in plants subjected to 100 mM NaCl (Figure 3C,D). The increase of qN was already significant on the 7th day of treatment, showing increasing trend until the 14th day of salt stress (Figure 3D).

The reduction of ΔF/Fm' accompanied by decrease in apparent electron transport rate (data not shown) in stressed plants differs from results reported by Lu et al. (2002), who did not observe effects of salt stress in photochemical reactions of *S. salsa*. Impairments in photochemistry of plants exposed to salinity may be related to possible damages in primary electron acceptors, such as plastoquinone pool (FOYER; NOCTOR, 2000). In addition, the continuous increase of qN during stress and recovery treatments suggest the activation of a protective mechanism for dissipation of excessive energy not used in photochemical reactions (RIBEIRO et al., 2009; ROHÁCEK, 2002). In fact, full inhibition of photochemistry occurs on the PSII apparatus just under severe oxidative damage (CHAGAS et al., 2008).

After 14-d under salt stress, plants showed ETR/A ratio 4-fold higher than in control ones. This difference was maintained even after recovery period (Figure 3). The increase in ETR/A ratio represents an imbalance between the electron flow and the CO_2 fixation during photosynthesis, which is associated with increases in oxygenase activity of Rubisco and represent electron flow to

other physiological processes rather than CO_2 fixation (BAKER et al., 2007; RIBEIRO et al., 2009). Occurrence of increases in ETR/A and decreases in A/Ci indicate loss of photosynthetic efficiency in physic nut plants under salt stress.

The accumulation of toxic ions (Na^+ and Cl^-) accompanied by decreases in K^+ content and severe photosynthetic damage show that physic nut young plants are sensitive to salinity caused by NaCl. The absence of any recovery even after three days of salt removing also indicates that high Na^+ and Cl^- leaf contents cause permanent damages on photochemical and carboxylation reactions of photosynthesis.

Conclusion

Physic nut plants are sensitive to saline conditions, showing high leaf Na^+ and Cl^- contents and low K^+/Na^+ ratio and permanent photosynthetic damage due to stomatal and non-stomatal limitations.

Acknowledgements

We thank to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP) for financial support. J.A.G.S. and R.V.R. are CNPq research scientist and E.N.S. and S.L.F.S. hold CNPq fellowships, respectively. The authors gratefully acknowledge the Tamanduá Farm Institute, Santa Terezinha-PB (Brazil), especially to Dr. R.A.V. for supplying physic nut seeds.

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Table and Figures List

Table 1. Concentrations of Na^+, Cl^- e K^+ and K^+/ Na^+ ratio in leaves of physic nut young plants cultivated in absence and presence of NaCl (100 mM) during 7-d and 14-d and after 3-d of recovery. Values are the mean for four replicates. The values represented by the same upper case letters, between time of treatment and same lower case letters, into of each time of treatment are not significantly different to 0.05 by Tukey test.

Parameter	NaCl	Days after treatment		
	(mM)	7 days	14 days	Recovery
Na^+	0	133 Bb	215 Ab	215 Ab
(mmol kg^{-1}DM)	100	603 Ca	1721 Aa	1458 Ba
Cl^-	0	102 Bb	147 Ab	139 Ab
(mmol kg^{-1}DM)	100	326 Ca	1498 Aa	1240 Ba
K^+	0	716 Ba	1071 Aa	1123 Aa
(mmol kg^{-1}DM)	100	581 Ab	423 Cb	496 Bb
K^+/Na^+	0	5.4 Aa	5.0 Aa	5.2 Aa
	100	0.96 Ab	0.24 Bb	0.34 Bb

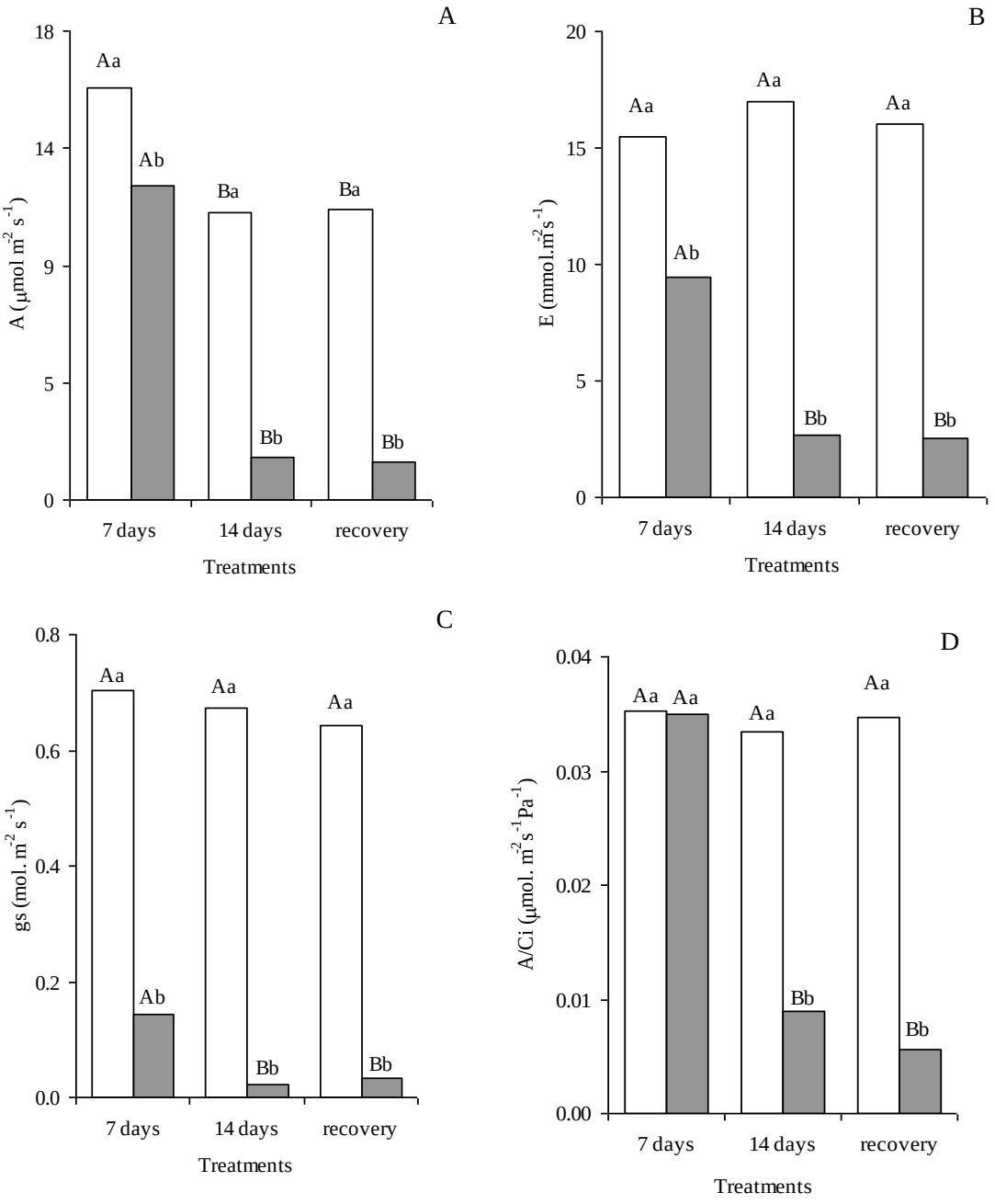

Figure 1. Leaf CO_2 assimilation (A), transpiration (B), stomatal conductance (C) and instantaneous carboxilation efficiency (D) in *Jatropha curcas* young plants cultivated in absence and presence of NaCl (100 mM) during 7-d and 14-d and after 3-d of recovery. White bars represent control plants and gray bars represent stressed plants. Values are the mean for four replicates. The values

represented by the same upper case letters, between time of treatment and same lower case letters, into of each time of treatment are not significantly different to 0.05 by Tukey test.

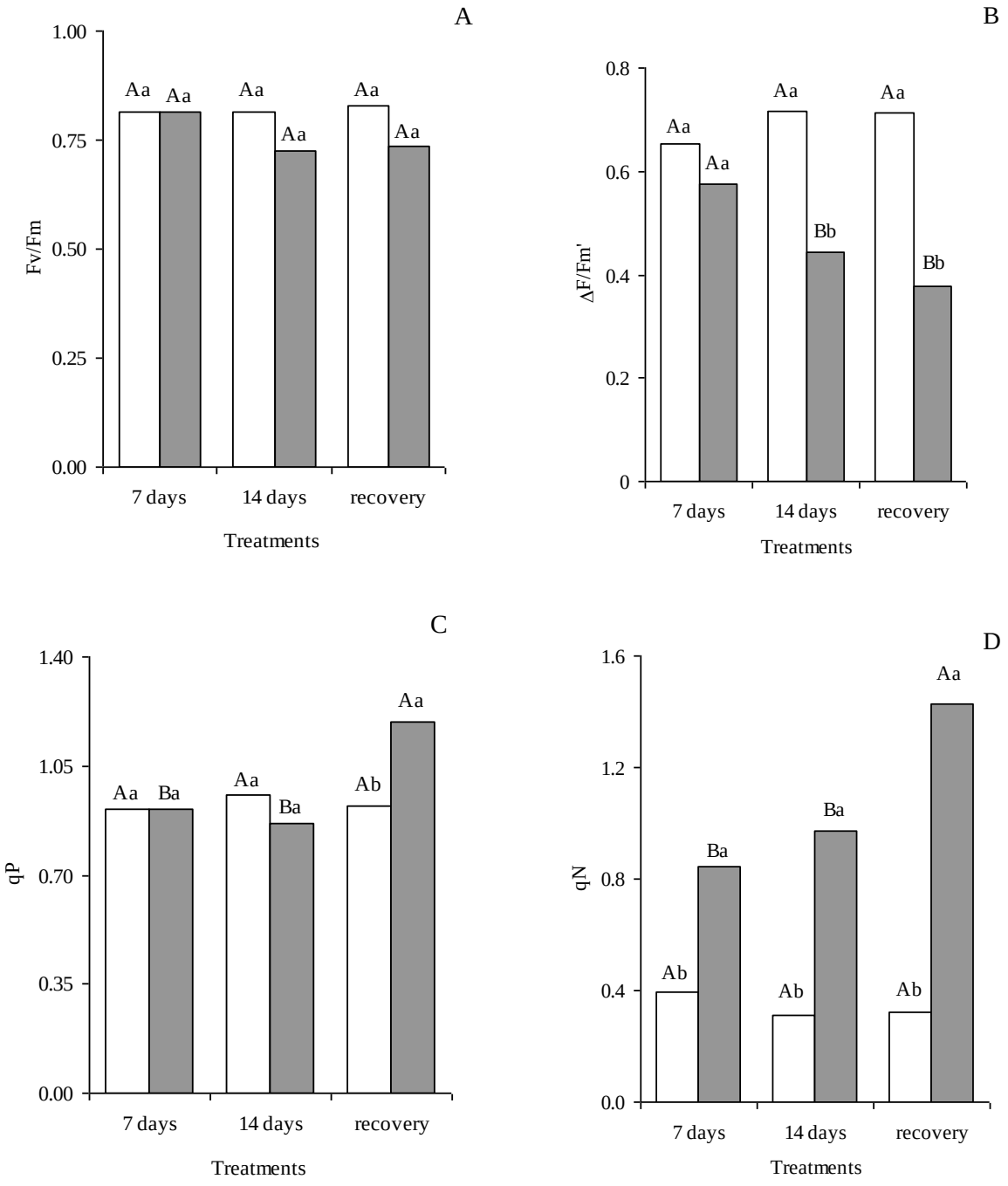

Figure 2. Effective (A) and potential (B) quantum efficiency, photochemical (C) and non-photochemical (D) quenching in *Jatropha curcas* young plants cultivated in absence and presence of NaCl (100 mM) during 7-d and 14-d and after 3-d of recovery. White bars represent control

plants and gray bars represent stressed plants. Values are the mean for four replicates. The values represented by the same upper case letters, between time of treatment and same lower case letters, into of each time of treatment are not significantly different to 0.05 by Tukey test.

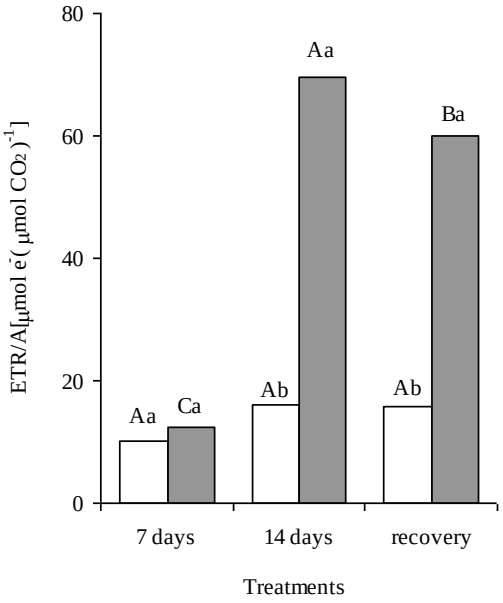

Figure 3. Ratio between apparent electron transport rate and CO_2 assimilation (ETR/A, in C) in *Jatropha curcas* young plants cultivated in absence or presence of NaCl (100 mM) during 7-d and 14-d and after 3-d of recovery. White bars represent control plants and gray bars represent stressed plants. Values are the mean for four replicates. The values represented by the same upper case letters, between time of treatment and same lower case letters, into of each time of treatment are not significantly different to 0.05 by Tukey test.

Capítulo IV

(Artigo submetido no Journal of Arid Environments em 26/10/09)

Comparative effects of salinity and water stress on photosynthesis, water relations and growth of physic nut plants

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Abstract

The aim of this study was to evaluate the physiological responses of physic nut (*Jatropha curcas* L.) plants exposed to water stress and salinity in order to elucidate some acclimatory mechanisms. Mild water and salt stresses were imposed by plant exposure to -0.22 MPa iso-osmotic solutions with PEG 6,000 or NaCl 50 mM for 8 days. Stress recovery was evaluated under control conditions after three and eight days. PEG treatment caused higher reductions in Ψw and Ψs, but both RWC and succulence were not affected by the two stressing treatments, compared to the control. The PEG-stressed plants suffered higher restrictions in leaf growth compared to the salt-stressed ones. Moreover, only the PEG treatment caused a pronounced effect on leaf electrolyte leakage. Both treatments caused similar impairment of the CO_2 assimilation, but the PEG stressed plants showed higher restriction in g_s and E. Although both stresses caused significant decreases on the leaf chlorophyll content, the photochemical activity was not affected. Since the plants subjected to mild water and salt stresses showed rapid and complete recovery in all the studied parameters, these physiological alterations could represent a set of adaptive mechanisms employed by *Jatropha curcas* to cope with these stressful conditions.

Key words: *Jatropha curcas*, gas exchange, chlorophyll fluorescence, salt stress, drought.

Introduction

Plants are often subjected to periods of soil and atmospheric water deficits during their life cycle. In many agricultural areas, plants also face soil salinity, another environmental constraint. It is estimated that 6% of the world's land and 30% of the world's irrigated areas already suffer from salinity problems (MUNNS; TESTER, 2008). Expansion of agriculture to semi-arid and arid regions using intensive irrigation and fertilization will increase secondary salinization as a result of changes in the soil water balance considering the water applied and the water used by crops (CHAVES et al., 2008).

In this context, photosynthesis and plant growth are among the primary processes affected by drought (CHAVES, 1991) and salinity (MUNNS et al., 2006). The water stress and salinity can affect photosynthesis directly or indirectly by decreases in CO_2 availability caused by diffusion limitations (FLEXAS et al., 2007), alterations in photosynthetic metabolism (LAWLOR; CORNIC, 2002) or restrictions in the photochemical system apparatus under severe stress conditions (SOUZA et al., 2004). In parallel to impairment of photosynthesis, salinity and water stress induce strong alterations of leaf water relations and osmotic homeostasis. It is widely accept that after short term (days) exposure the salinity induces osmotic effects, while under long terms it can cause ionic damages to the plant cells (MUNNS, 2002).

Under salt stress, an irreversible impairment of the photosynthetic apparatus, associated with a reduction of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity, occurs when the stress is prolonged and salt continues to accumulate in the leaves (DELFINE et al., 1999). Under drought conditions, there are controversies about the effects of this stress on RuBP content and Rubisco activity (LAL et al., 1996). Some authors did not observe water stress effects on the parameters mentioned above (DELFINE et al., 2001), whereas others observed a significant reduction of RuBP content and Rubisco activity in plants subjected to drought (MAROCCO et al., 2002). This apparent discrepancy is due to the fact that such studies were done under different environmental conditions, using different species subjected to different drought intensities (BOTA et al., 2004).

Both salt and drought stresses cause impairments of photosynthesis related to the low electron transport through PSII (ETR) and to changes in the structure and function of the photochemical apparatus (HURA et al., 2007). As an indirect consequence of stomatal

closure, restriction in intercellular CO_2 concentration should increase the susceptibility to photochemical damages as excessive light energy at PSII increases due to low photosynthetic rates. Among the photochemical responses, reduction in the potential (Fv/Fm) and effective (ΔF/Fm') quantum efficiency of PSII, loss of PSII activity and increases in minimum fluorescence (Fo) are expected under environmental stresses (TEZARA et al., 2005). In sorghum plants under saline conditions, Netondo et al. (2004) reported that the ΔF/Fm', ETR and photochemical quenching (qP) decreased significantly, whereas the non-photochemical quenching (qN) increased substantially. Under water stress conditions, Souza et al. (2004) observed decreases in the ΔF/Fm', qP and ETR with an increase in the qN of cowpea plants after 6-d of treatment.

Although the seeds of physic nut plants represent a promising bioenergy source, knowledge about the plant's physiological responses to drought and salt stresses is poorly developed. This understanding is essential in order to adopt competitive strategies for improving crop production, and the objective of this work is to evaluate the comparative effects of water stress and salinity on leaf gas exchange, water relations, growth and chlorophyll fluorescence in *J. curcas* young plants.

Material and Methods

Plant material and experimental conditions

The experiment was carried out under greenhouse conditions, where the environmental conditions were: minimum and maximum mean air temperature of 24 and 36°C, respectively; mean air relative humidity of 65%; maximum photosynthetic photon flux density (PPFD) of approximately 700 μmol m^{-2} s^{-1}; and 12 h photoperiod. Physic nut seeds were previously selected, taking into account the seed size and weight standard. Eight days after germination in sand, the seedlings were transferred to plastic pots (2 L) containing Hoagland and Arnon (1950) nutritive solution (pH 6.0) with one-fourth strength in the first week and full strength afterward. The seedlings were subjected to stressful treatments over eight days, in which nutritive solution was supplied with 50 mM NaCl or PEG-6000 11.96% (m/v), both with Ψ_{os} = -0.22 MPa. NaCl and PEG were added gradually (25 mmol NaCl L^{-1} d^{-1} and 59.8 g PEG L^{-1} d^{-1}) into the solution in order to avoid osmotic shock. The treatment with the nutrient solution in the absence of NaCl and PEG was taken as the control.

Leaf gas exchange and chlorophyll fluorescence

Leaf gas exchange was monitored with an infrared gas analyzer (LCi, ADC, Hoddesdonm, UK), operating in the open system and with an air flow of 200 mL min^{-1}. Measurements of leaf CO_2 assimilation (A), transpiration (E), stomatal conductance (gs) and intercellular CO_2 concentration (Ci) were taken. The instantaneous carboxilation efficiency (A/Ci) was calculated. The chlorophyll fluorescence was evaluated with a modulated fluorometer (FMS 2, Hansatech, King's Lynn, UK). Basal (Fo) and maximal (Fm) fluorescence yields were measured in dark-adapted (30 min) leaves, whereas steady-state (Fs) and maximal (Fm') fluorescence yields were sampled in light-adapted tissues. Variable fluorescence yields were determined in dark-adapted (Fv=Fm-Fo) and light-adapted (ΔF=Fm'-F) leaf tissues. The following photochemical variables were measured: potential (Fv/Fm) and effective (ΔF/Fm') quantum efficiency of PSII, apparent electron transport rate (ETR= ΔF/Fm' x PPFD x 0.5 x 0.84), and photochemical [qP= (Fm'-Fs)/(Fs-Fo')] and non-photochemical [qN=(Fm-Fm')/(Fm-Fo')] quenching (Rohácek, 2002). For ETR calculation, 0.5 was used as the fraction of excitation energy distributed to PSII and 0.84 as the fraction of incoming light absorbed by the leaves (Schreiber et al., 1998). Fo' is the basal fluorescence signal measured after PSI excitation by far-red light.

The ratio ETR/A was calculated to estimate the use of electrons in other processes not related to the photosynthetic CO_2 assimilation (Ribeiro et al., 2009). Leaf gas exchange and chlorophyll fluorescence were measured simultaneously at 9:00 h a.m., in the fully expanded and mature leaves of plants exposed to 25°C and PPFD of 260 μmol m^{-2} s^{-1}. Those measurements were taken after eight days of treatment (NaCl and PEG), and they were repeated again three and eight days after returning plants to the nutritive solution without the presence of NaCl and PEG.

Determination of inorganic solutes

Lyophilized leaf samples were transferred into hermetically closed tubes containing deionized water and placed in a 100ºC water bath for 1 h. The extracts were then filtered and stored at -20ºC for later determinations. Na^+ and K^+ contents were determined by flame photometry and Cl^- content through titration with $AgNO_3$, as previously described (Silveira et al., 2009).

Water relations parameters

The leaf water potential (Ψw) was evaluated immediately after sampling using the pressure chamber method (Scholander et al., 1965) at pre-dawn (Ψw, at 6:00 h) in leaves similar to those used for leaf gas exchange and chlorophyll fluorescence measurements. The leaf relative water content (RWC) was determined after the determination of leaf discs fresh, turgid and dry weight as previously described (Silveira et al., 2009). The leaf succulence (LS) was calculated by the formula (FW)/A, where FW and A are the fresh weight and area of thirty leaf discs (diameter 1.0 cm), respectively.

For determination of the osmolality, small segments from fully expanded leaves and terminal 5 cm-segments of roots were macerated in a mortar. After extract filtration in a miracloth membrane, the sap was centrifuged at 10.000 x *g* for 10 min at 4ºC. After, the supernatant was collected and utilized to determine the osmolality (c) using a vapor pressure osmometer (Vapro 5520, Wescor, USA). The osmotic potential was determined using the formula: Ψs (MPa) = -c (mosmol kg-1) x 2.58 x 10^{-3}, according to the Van't Hoff equation.

Electrolyte leakage, leaf area and chlorophyll content

Electrolyte leakage was assessed as described by Lutts et al. (1996). Leaf discs were placed in closed tubes containing 10 mL of deionized water and incubated at 25°C in a water bath for 6 h; subsequently, the electrical conductivity of the solution (L1) was determined. Samples were then boiled at 100°C for 1 h, and the last electrical conductivity (L2) was obtained after equilibration at 25°C. The electrolyte leakage (EL) was defined as follows: EL (%) = (L1/L2) x 100. The total chlorophyll content was calculated according to Lichtenthaler (1987): Chl (mg g MF^{-1}) = (7.15 x A_{663}) + (18.61 x A_{647}) x V, where A is the absorbance and V the final volume of the extract (mL). The relative leaf area (LA) was measured in the newly developing leaf previously labeled at the beginning of experimental period. The measurements were made with a paquimeter as LA = length x width, and the leaf area was expressed in cm^2 $leaf^{-1}$.

Experimental design and data statistical analyses

The experiment was arranged in a completely randomized design with three treatments and four replicates (an individual pot containing one plant). Data were analyzed by ANOVA, and means were compared by the Tukey test at the 0.05 level of confidence. The standard deviation is plotted in all figures.

Results

In this study, physic nut young plants were subjected to mild salt and water stress induced after 8-d exposure to 50 mM NaCl and 11.96% PEG (-0.22 MPa iso-osmotic solutions). Leaf RWC and succulence did not change under both stressful conditions compared to controls, and after three (3-d) and eight (8-d) days of recovery, the plants pretreated with NaCl and PEG showed similar values of RWC and LS compared to controls (Table 1). Moreover, the leaf water content of both stressed plants was not altered after 8-d treatments (data not shown). These results, taken together, indicate that the leaves of the physic nut were able to maintain a good hydration status after 8-d of exposure to both NaCl and PEG treatments.

In contrast to leaf hydration status, both treatments were able to induce decreases in the leaf water free energy, as indicated by the reductions in both Ψw and Ψs (Table 1). However, the values achieved for water potential (-0.96 and -1.10 MPa for salt and PEG treatments, respectively) evidenced that physic nut plants suffered only a mild water stress after 8-d treatments. However, PEG treatment caused a more pronounced decrease on both Ψw and Ψs when compared with NaCl. The higher reduction in the osmotic potential as compared to Ψw evidences that the stressed leaves displayed positive turgor potential in both treatments (Table 1). The water free energy of both the salt- and PEG-pretreated leaves was completely restored after only 8-d of recovery (Table 1).

Physic nut plants treated with 50 mM NaCl showed an increase in both Na^+ and Cl^- contents in the leaves as compared to the controls and the PEG-treated plants, after 8-d of treatments (Table 2). However, the Na^+ and Cl^- concentrations did not achieve high levels (approximately 441 and 560 mmol kg^{-1} DM, respectively) in the leaf tissues. If these concentrations had been expressed in a tissue water basis (considering the water content as approximately 90%), the new values will be 49 and 62 mM, respectively, which are considered to be moderate and non-toxic for physic nut leaves (Silva et al., 2009a). Interestingly, these salt ion concentrations in the leaves remained practically unchanged even after 8-d of NaCl withdrawing from the root medium (Table 2).

The leaf K^+ concentration was significantly reduced (33%) by NaCl treatment and increased (59%) by PEG, as compared to controls, after 8-d of treatments (Table 2). Moreover, after 3-d of recovery only the NaCl-pretreated plants restored the K^+ content to control levels, while the PEG-pretreated plants showed slight recovery in this period and only presented similar values to untreated plants after 8-d (Table 2). On the other hand, the leaf dry

matter (DM) was decreased by 26% and 39% due to NaCl and PEG treatments, respectively, in comparison to controls (Table 2). Nevertheless, although the NaCl- and PEG-pretreated plants showed a progressive recuperation, they did not allow the complete restoration of dry matter accumulation even after 8-d of recovery (Table 2).

After 8-d of exposure to stressful conditions, the leaf electrolyte leakage, a membrane damage indicator, increased significantly (±75 %) in PEG-stressed plants but remained unchanged in salt-treated plants compared to controls (Figure 1A). After 3-d under non-stressful conditions, the PEG-pretreated plants showed limited recovery and only presented similar values to both controls and salt-stressed plants after 8-d (Figure 1B-C). In contrast to electrolyte leakage, the total chlorophyll content decreased significantly ($p \leq 0.05$) under both treatments, with 40% due to NaCl and 38% due to PEG, when compared to controls (Figure 1D). Moreover, stressed plants showed only a partial recovery in their chlorophyll content after 3-d and 8-d under recovery conditions (Figure 1E-F). At end of the experimental period, the chlorophyll content was significantly lower ($p \leq 0.05$) in previously stressed plants when compared to controls.

In parallel to leaf dry matter reduction, the leaf area (LA) was significantly ($p \leq 0.05$) decreased by 28% (NaCl) and 39% (PEG) in stressed plants as compared to controls (Figure 1G). Again similar to the results for leaf dry matter, PEG- and NaCl-stressed plants showed only a partial recovery in leaf area after 8-d, achieving values of approximately 85% and 79% in relation to the LA of control plants (Figures 1H-I). The comparative analysis among the effects of salt and PEG treatments in terms of variables associated with plant stress intensity (membrane damage and leaf growth) strongly suggests that the latter treatment caused more negative effects on the physic nut young plants than the NaCl salinity.

After 8-d of exposure to stressful conditions, CO_2 assimilation (A) decreased significantly in both treatments as compared to controls. This reduction was around 80% and 85% for the salt and water stress treatments, respectively (Figure 2A). After 3-d and 8-d of recovery, the stressed plants showed similar A values as the control plants (Figure 2B-C), evidencing a full restoration of photosynthesis after 3-d. Additionally, stomatal conductance (g_s) also decreased significantly, by around 43% and 50% due to the salt and water stress treatments, respectively (Figure 2D). As observed for A, the treated and control plants had similar gs values after 3-d and 8-d of recovery (Figure 2E-F). Compared to control conditions, transpiration (E) decreased significantly in NaCl (55%) and PEG (84%) treatments (Figure 2G). As noted for g_s, all plants showed similar values of E after those recovery periods, regardless of the previous conditions (Figure 2H-I).

As compared to control plants, the A/Ci ratio of the stressed plants was decreased by around 80%, regardless of the stressing condition, after 8-d of treatment (Figure 3A). However, this parameter was rapidly recovered after 3-d under non-stressful conditions, achieving a full restoration after 8-d when compared to untreated plants (Figures 3B-C). Inversely, the ETR/A ratio was increased by 5- and 6.5-fold due to NaCl and PEG, respectively, compared to controls (Figure 3D). Similarly to the A/Ci ratio, the ETR/A ratio was also partially restored after 3-d under non-stressful conditions, achieving a full restoration after 8-d in comparison to untreated plants (Figures 3E-F). As taken together, the gas exchange results reinforce that PEG treatment, when compared with salinity, induced higher restrictions on transpiration and higher disturbances in the ETR/A ratio, reflecting an unbalance between the photochemical and CO_2 assimilation biochemical phases.

In this present study, the photochemical activity, as evaluated by the potential (Fv/Fm) and effective (ΔF/Fm') quantum efficiencies of PSII, the photochemical (qP) and non-photochemical (qN) quenching and the apparent electron transport rate (ETR) parameters, was not affected by the NaCl and PEG treatments (Table 3).

Discussion

The results of this study indicate that both NaCl and PEG treatments were able to induce significant and reversible alterations of the physiological stress indicators associated with water relations, growth and leaf gas exchange, but they were unable to cause any changes on the photochemical activity of *J. curcas* young plants. On the other hand, the results demonstrate that physic nut leaves were able to avoid, at least after 8-d of salt exposure, toxic accumulation of Na^+ and Cl^- and were also able to trigger a salt- and PEG-induced K^+ accumulation in the leaves under stressful conditions. This strategy is very important for the osmotic adjustment and ionic homeostasis as previously reported by Silva et al. (2009b), who observed an effective participation of Na^+ and Cl^- ions in the osmotic adjustment of physic nut leaves under salinity, while Patakas et al. (2002) observed a great importance of K^+ in the osmotic adjustment of grapevine plants under water stress.

The occurrence of the osmotic adjustment mechanism in physic nut leaves in this present study is also reinforced by the maintenance of the leaf water status, evidenced by both leaf RWC and LS values that were not altered under the stressful conditions. In parallel to this, physic nut plants also exhibited other avoidance strategies to both stressful conditions, such as the strong restriction of photosynthesis via stomatal closure associated with growth

restriction and the leaf area and leaf dry matter reductions. Similar results were found in cotton cultivars exposed to 50 and 100 mM of NaCl (Meloni et al., 2003), as well as in maize plants under water stress induced by PEG (Wang et al., 2008). Cowpea, a species adapted to cope with drought and salinity, displays similar avoidance strategies (Souza et al., 2004; Cavalcanti et al., 2004) as those displayed by physic nut.

The decreases in both leaf CO_2 assimilation and transpiration can be partially attributed to the stomatal closure induced by NaCl and PEG (Figure 2). However, the maintenance of similar intercellular CO_2 concentration (data not shown) associated with low CO_2 assimilation under both treatments strongly suggests the occurrence of a non-stomatal limitation of photosynthesis in young physic nut plants subjected to salinity and water deficit. Furthermore, reductions in instantaneous carboxylation efficiency (A/Ci) also indicate some mesophyll limitation on the photosynthesis of stressed plants. On the other hand, an increase in the ETR/A ratio indicates that electron alternative sinks, such as photorespiration (Ribeiro et al., 2009), were increased due to the stressful conditions caused by NaCl and PEG.

Increases in photorespiration have been considered as a protective mechanism consuming photochemical products and impeding damages at the PSII level (Chaves et al., 2008). This physiological strategy was efficient since no damage was noticed in the photochemical apparatus of *Jatropha curcas* leaves, as also verified by Ribeiro et al. (2004) working with *Citrus* plants subjected to biotic and abiotic stresses. According to Foyer et al. (1994), the increase in the ETR/A ratio is related to the capacity of protective processes, such as the action of antioxidant metabolism for scavenging reactive oxygen species.

Although the stressful effects of PEG and NaCl treatments on the leaf chlorophyll content of physic nut have been noted, they did not affect the photochemical activity, suggesting a high resistance of the photochemistry apparatus to such stresses. The reduction in the photosynthetic pigments in plants subjected to salinity and drought may be a strategy of protection and/or acclimation, in which the reduction of energy waste, carbon skeletons and nutrients in chlorophyll synthesis may favor other physiological processes (Taiz and Zeiger, 2006).

The photochemical (qP) and non-photochemical (qN) quenching were similar between the plants subjected to NaCl and PEG treatments and the control plants. Those results are in accordance with Lu et al. (2002), who reported non-significant changes in the photochemical activity of *S. salsa* plants treated with NaCl. In addition, Guóth et al. (2008) did not notice considerable changes in the chlorophyll fluorescence of wheat plants under water stress induced by PEG.

The great inhibition of CO_2 assimilation associated with the effective restriction in water lost by transpiration control and maintenance of leaf water status, in parallel with intense leaf growth reduction (given by both leaf area and leaf dry matter), strongly suggests that this response is an acclimating physiological mechanism employed by the *J. curcas* species to cope with moderate levels of salinity and water deficit. Under these conditions, the photochemical apparatus is fully preserved, and the plants are able to rapidly recovery their photosynthesis, water relations and growth after stress relief.

Further studies are needed to explain why the stressful effects caused by PEG on the growth and electrolyte leakage of physic nut leaves were more deleterious than those caused by iso-osmotic NaCl concentration; that is, is this species in fact more sensitive to drought stress or could the PEG solution have caused some toxic effects? Although physic nut plants are considered to be a species adapted to cope with drought and hot environments like those widespread in semi-arid regions (Kumar et al., 2008), it is possible to infer that the young plants of *J. curcas*, like those utilized in the present study, are as sensitive to PEG-induced water stress as they are also sensitive to drought (water withdrawal), as recently observed in our lab (data not published).

In conclusion, young *J. curcas* plants are able to cope with the mildly stressful conditions of salinity and water stress by employing an acclimating mechanism of stress avoidance strongly restricting the CO_2 assimilation and leaf growth, which is associated with the maintenance of leaf water status and photochemical activity. This response might represent a set of adaptive mechanisms employed by *J. curcas* to survive under those abiotic stressful conditions.

Acknowledgements

We thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP) for financial support. J.A.G.S. and R.V.R. are CNPq research scientists, and E.N.S. and S.L.F.S. hold CNPq fellowships, respectively. The authors gratefully acknowledge the Tamanduá Farm Institute, Santa Terezinha-PB (Brazil) for supplying physic nut seeds.

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Table e figures List

Table 1. Relative water content, leaf succulence, water potential and osmotic potential in physic nut young plants subjected to the salt and osmotic stresses induced by NaCl and PEG, respectively. Measurements were taken after 8-d of exposure to stressful conditions and 3-d and 8-d after transferring plants to control conditions. Data refer to mean values (n=4). The same letters are not significantly different at $p \leq 0.05$ by Tukey's test.

Condition	Evaluation time*	RWC (%)	Leaf succulence (mg H_2O cm^{-2})	Ψw (-MPa)	Ψs (-MPa)
Control	Stress-8d	65.91 ab	47.52 ab	-0.60 c	-0.75 d
NaCl		70.66 a	48.69 a	-0.96 b	-1.09 b
PEG		74.71 a	49.58 a	-1.10 a	-1.24 a
Control	Recovery-3d	67.83 a	50.99 a	-0.70 c	-0.91c
NaCl		69.39 a	50.42 a	-0.90 b	-1.12 b
PEG		70.18 a	50.69 a	-1.03 b	-1.15 b
Control	Recovery-8d	69.46 a	54.14 a	-0.81 b	-1.02 c
NaCl		70.71 a	53.54 a	-0.85 b	-1.06 c
PEG		69.65 a	53.64 a	-0.95 b	-1.09 c

Table 2. Concentrations of Na^+, Cl^-, K^+ and leaf dry matter in physic nut young plants subjected to the salt and osmotic stresses induced by NaCl and PEG, respectively. Measurements were taken after 8-d of exposure to stressful conditions and 3-d and 8-d after transferring plants to control conditions. Data refer to mean values (n=4). The same letters are not significantly different at $p \leq 0.05$ by Tukey's test.

Condition	Evaluation time *	Na^+	Cl^-	K^+	Leaf DM (g $plant^{-1}$)
		mmol kg^{-1} MS			
Control	Stress-8d	139.1 bc	70.0 b	411.8 bc	1.19 a
NaCl		440.6 a	560.0 a	276.4 d	0.88 b
PEG		173.9 b	70.0 b	654.4 a	0.72 c
Control	Recovery-3d	115.9 bc	70.0 b	383.6 c	1.27 a
NaCl		428.9 a	490.0 a	428.7 b	0.95 b
PEG		104.4 bc	70.0 b	496.4 b	0.89 b
Control	Recovery-8d	99.6 c	70.0 b	456.9 b	1.35a
NaCl		417.4 a	420.0 a	394.9 c	1.01 b
PEG		92.7 c	70.0 b	445.6 b	0.98 b

Table 3. Chlorophyll fluorescence parameters in physic nut young plants subjected to the salt and osmotic stresses induced by NaCl and PEG, respectively. Measurements were taken after 8-d of exposure to stressful conditions and 3-d and 8-d after transferring plants to control conditions. Data refer to mean values (n=4). The same letters are not significantly different at $p \leq 0.05$ by Tukey's test.

Condition	Evaluation time*	ΔF/Fm'	Fv/Fm	ETR (μmol $e^{-}m^{-2}s^{-1}$))	qP	qN
Control	Stress-8d	0.79 a	0.85 a	86.80 a	1.00 a	0.34 a
NaCl		0.79 a	0.86 a	86.90 a	0.97 a	0.30 a
PEG		0,78 a	0.83 a	85.30 a	0.97 a	0.31 a
Control	Recovery-3d	0.79 a	0.84 a	86.10 a	0.96 a	0.35 a
NaCl		0.79 a	0.84 a	86.30 a	0.99 a	0.33 a
PEG		0.78 a	0.85 a	85.50 a	0.96 a	0.32 a
Control	Recovery-8d	0.78 a	0.85 a	86.10 a	0.97 a	0.36 a
NaCl		0.78 a	0.85 a	85.60 a	0.96 a	0.36 a
PEG		0.77 a	0.85 a	85.10 a	0.97 a	0.34 a

*From the beginning of experimental period.

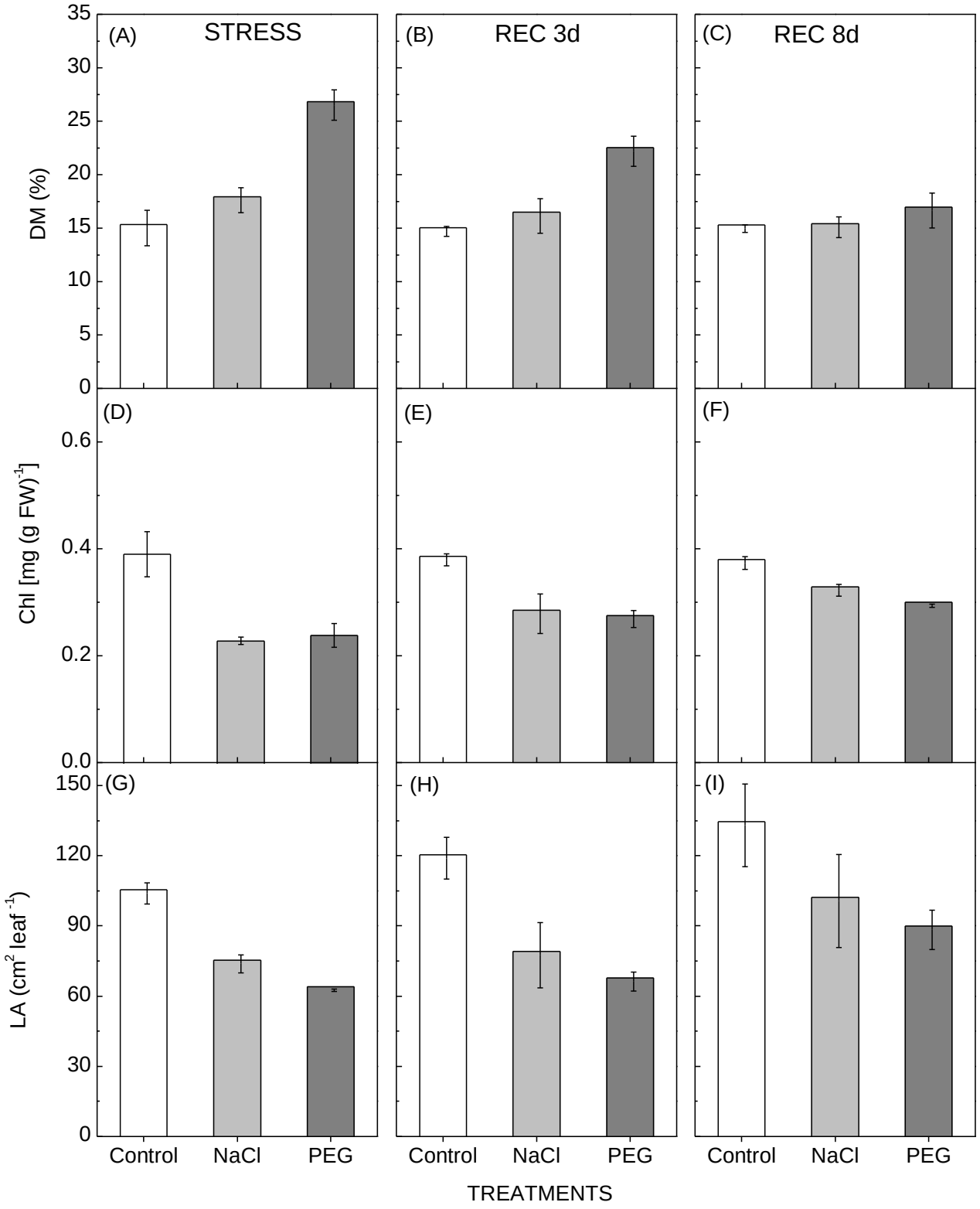

Figure 1. Membrane damage (MD, in A-C), total chlorophyll content in leaves (Chl, in D-F) and leaf area (MLA, in G-I) in *Jatropha curcas* young plants after 8-d of exposure to NaCl and PEG treatments (A, D, G), after 3-d (B, E, H) and 8-d (C, F, I) of recovery under control conditions. Bars represent mean values (n = 4) ± SD.

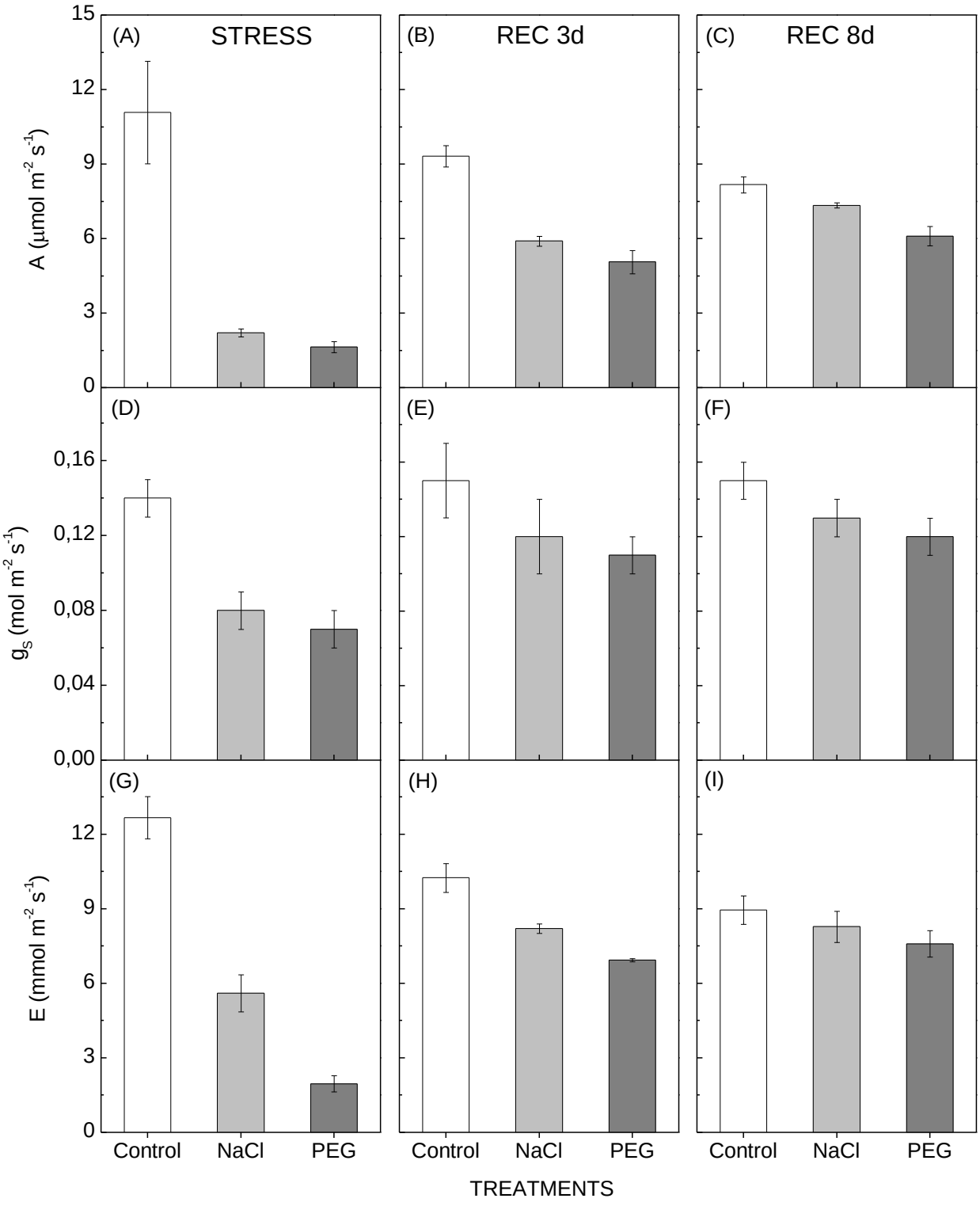

Figure 2. Leaf CO_2 assimilation (A, in A-C), stomatal conductance (gs, in D-F) and transpiration (E, in G-I) in *Jatropha curcas* young plants after 8-d of exposure to NaCl and PEG treatments (A, D, G), after 3-d (B, E, H) and 8-d (C, F, I) of recovery under control conditions. Bars represent mean values (n = 4) ± SD.

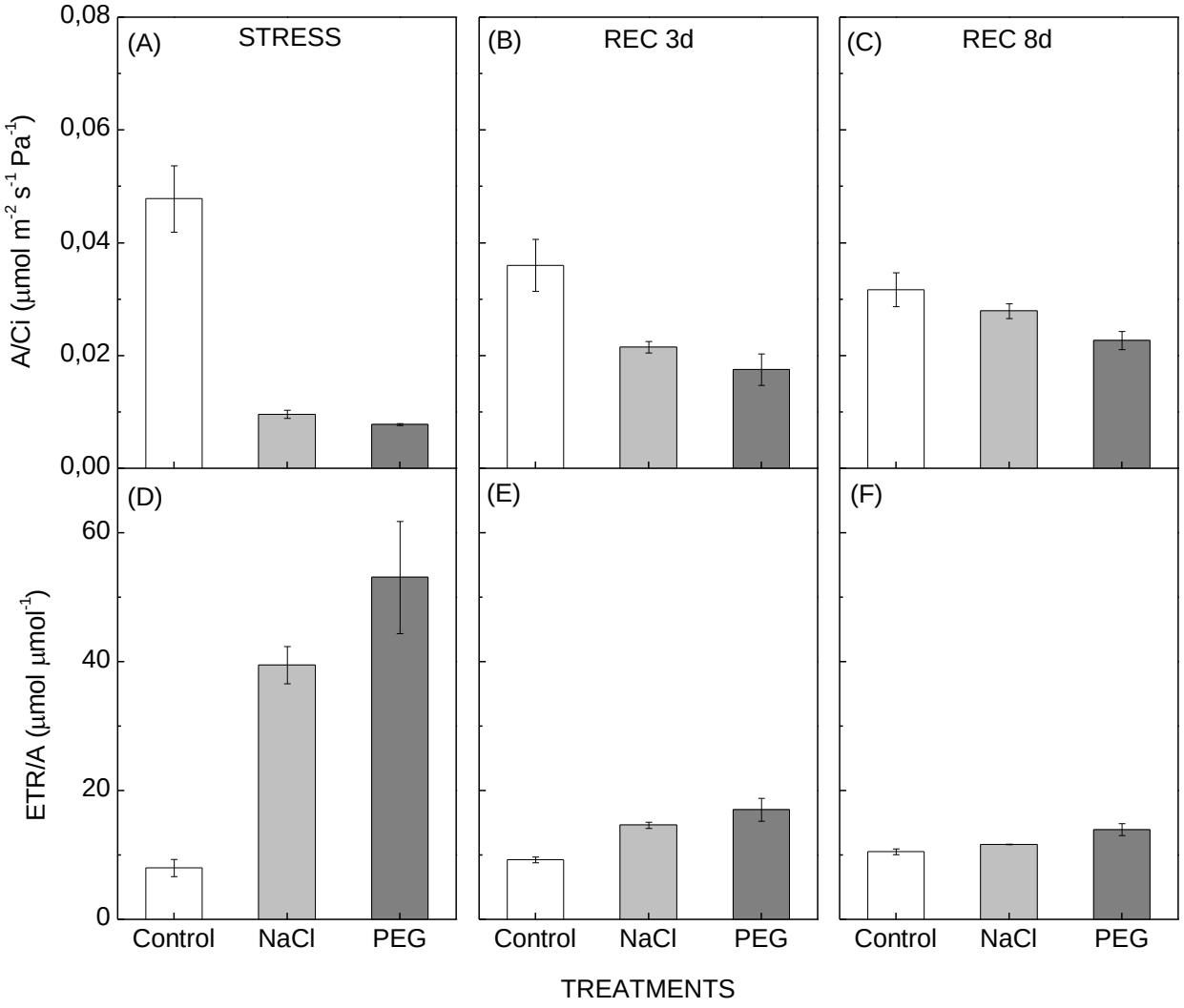

Figure 3. Instantaneous carboxilation efficiency (A/Ci, in A-C) and ratio between apparent electron transport rate and CO_2 assimilation (ETR/A, in D-F) in *Jatropha curcas* young plants after 8-d of exposure to NaCl and PEG treatments (A, D, G), after 3-d (B, E, H) and 8-d (C, F, I) of recovery under control conditions. Bars represent mean values (n = 4) ± SD.

Capítulo V

(Artigo submetido no Environmental and Experimental Botany em 04 /11/09)

The role of organic and inorganic solutes in the osmotic adjustment of drought-stressed *Jatropha curcas* plants

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Abstract - This study aimed to assess the accumulation of organic and inorganic solutes and their relative contribution to osmotic adjustment in roots and leaves of *Jatropha curcas* plants subjected to different substrate moisture levels under greenhouse conditions. Water stress was progressively imposed by withholding irrigation until the water content of the substrate reached 70% (control), 56%, 43%, 26% and 14% of its holding capacity, corresponding to approximately 50, 40, 30, 20 and 10 cm^3 H_2O per cm^3 of substrate, respectively. Both the leaf water potential (Ψw) and osmotic potential (Ψs) decreased progressively as the water deficit increased while the leaf pressure potential showed positive values in all treatments. Moreover, the relative water content and leaf succulence did not show differences between the control and stressed plants indicating osmotic adjustment in the water-stressed leaves. The K^+ ions had greater participation in the osmotic adjustment in both leaves and roots followed by Na^+ and Cl^-, while the NO^-_3 ion only showed minor (approx. 5% of osmolality) involvement. Of the organic solutes studied, the total soluble sugars (TSS) showed the highest relative contribution to the osmotic adjustment in both organs and its concentration positively increased with more severe water stress. The free amino acids (AA) and glycinebetaine (GB) presents in the organic solute fraction also effectively contributed to the osmotic potential reduction of both the root and leaves. The role of proline was insignificant in terms of osmotic adjustment (less than 0.1% of osmolality) in both the control and stressed roots and leaves. The sum of inorganic and organic solutes represented an approximately fifty-fifty contribution in both control and stressed roots and leaves. Our data reveal that roots and leaves of *J. curcas* young plants display osmotic adjustment mechanisms in response to drought by means of the participation of some inorganic (K^+, Na^+, Cl^-, and NO^-_3) and organic

solutes (TSS, TFAA, and GB). Of all the solutes studied, soluble sugars uniquely display a prominent drought-induced synthesis and/or accumulation in both roots and leaves.

Key words: *Jatropha curcas*, osmotic adjustment, osmolytes, solutes, water stress.

Abbreviations: GB – glycinebetaine; OA – osmotic adjustment; RWC – relative water content; TSS – total soluble sugars; TFAA – total free amino acids; Ψw water potential; Ψs osmotic potential

1. Introduction

Water deficit is one of the most important environmental stresses affecting agricultural productivity around the world (HESSINI et al., 2009). Therefore, the knowledge of physiological and biochemical mechanisms involved with the whole plant level responses to water stress generate considerable interest (SLAMA et al., 2007). Consequently, many breeding programs and intense studies have been carried out in order to identify physiological markers which can be used for the selection of plants resistant to drought (LIZANA et al., 2006).

The physiological and developmental mechanisms which allow a species to tolerate prolonged periods of water deficit can involve numerous attributes. One means of increasing drought tolerance is by accumulation of osmotically active solutes, so that turgor and turgor-dependent processes may be maintained during episodes of dry-down. The osmotic adjustment allows water uptake, cell enlargement and plant growth during water stress associated with partial stomata opening allowing the CO_2 assimilation at low water potentials that are otherwise inhibitory (ALVES; SETTER, 2004).

In this context, the osmotic adjustment (OA) has been considered as an important physiological adaptation character associated with drought tolerance and it has drawn much attention during the last years (HESSINE et al., 2009). OA involves the net accumulation of solutes in plant cells in response to falls in water potential in the root medium. As a consequence, the cell's osmotic potential is diminished which in turn attracts water into the cell by tending to maintain turgor pressure (PÉREZ-PÉREZ et al., 2009). According to Martínez et al (2005), compatible soluble like sugars, glycerol, proline or glycinebetaine can also contribute to this process.

Species and varieties of crop plants differ greatly in respect to the types of solutes accumulated and their relative contribution in lowering the osmotic potential and the primary osmolyte involved in OA is species-dependent (RHODES et al., 2002). In order to achieve osmotic balance at the cellular level, these substances have to be allocated between the cytoplasm and the vacuole, and also between the cytoplasm and the apoplast. Cellular osmotic homeostasis can result from sequestration of a major fraction of the toxic substances in the vacuolar compartment, while the non-toxic solutes should be preferentially located in the cytoplasm where they can act as compatible osmo-solutes (GAGNEUL et al., 2007).

In several studies, it has been reported that OA occurs in some species in response to water stress in both field and controlled environmental conditions (CHIMENTI et al., 2006). However, substantial differences also have been reported between species, cultivars or landraces in terms of OA capacity and with respect to the nature of the major solutes contributing to osmotic potential (BAJJI et al., 2001). The degree of OA also could be affected by the rate of stress intensity and most particularly by organ type and plant age (ALVES; SETTER, 2004). The osmotic adjustment in the root axis of plants cultivated in dry soils is crucial to growth and drought resistance (SERRAJ; SINCLAIR, 2002).

It has been evidenced that the accumulation of proline (GARCÍA-SÁNCHES et al., 2007) and glycinebetaine (BAJJI et al., 2001) are commonly observed metabolic response of higher plants to water deficit. Similarly, changes in the potassium content may contribute substantially to osmo-regulation (SHABALA; CUIN, 2007) and may occur in concert with changes in sugars and amino acids (PÉREZ-PÉREZ et al., 2009). In some cases, however, changes in sugars, amino acids, or organic acids were not accompanied by changes in potassium concentrations (SLAMA et al., 2007).

Jatropha curcas is distributed over the arid and semiarid areas of South America and in all tropical regions and in the last years it has recently received tremendous attention because its high seed oil content which can be converted to biodiesel. This manner, it is being considered as a universally accepted energy source crop (KUMAR et al., 2008). This species grows in areas with extreme climates and soil conditions that could not be habited by most of the agriculturally important plant species (FRANCIS et al., 2005).

Recently, we reported that *J. curcas* displayed an effective osmotic adjustment in response to salinity (SILVA et al., 2009a) and, interestingly, it exhibited some mechanisms similar to those employed by the halophyte *Atriplex nummularia* (SILVEIRA et al., 2009). However, the involvement of the osmotic adjustment in the water stress resistance of *J.*

curcas is still unknown. In addition, the comprehension of the role that process in the drought tolerance in cultivated plants is also poorly understood.

This study was carried out to test the hypothesis that *Jatropha curcas* roots and leaves display an effective osmotic adjustment by the use of a drought-induced net accumulation of K^+ ions and organic solutes especially, glycinebetaine. Although the *J. curcas* young plants have exhibited indicators of effective osmotic adjustment in response to a wide range of water deficit, of all the solutes studied, soluble sugars uniquely display a prominent drought-induced synthesis and/or accumulation in both roots and leaves.

Material and Methods

Plant material and Growth conditions

Jatropha curcas L. seeds, kindly provided by the Instituto Fazenda Tamandua, Brazil and previously selected for size and weight, were surface sterilized for 1 minute with a 5% sodium hypochlorite solution and germinated in sand. Eight days after germination, a homogeneous group of seedlings in height and having the same morphological aspects was transplanted into plastic pots (2 L) filled with vermiculite and allowed to grow for 15 d. One seedling was maintained in each pot. Every two days, the pots were watered with half strength Hoagland and Arnon solution (1950) in quantities enough to bring the substrate holding capacity to 70%. The study was carried out in a greenhouse with the following environmental conditions: mean air temperature of 29°C; mean air relative humidity of 65%; maximum photosynthetic photon flux density (PPFD) of approximately 700 μmol m^{-2} s^{-1} and 12 h photoperiod.

Water deficit treatments and harvesting

In order to have a wide range of water availability to 23-d-old seedlings (± eight leaves stage), a regime from well-watered to severely water-stressed conditions was imposed. The pots were transferred from the greenhouse into a growth chamber with environmental conditions of: PFFD of 400 μmol m^{-2} s^{-1}, air temperature of 27ºC, air humidity of 70% and photoperiod of 12 h. The water stress was progressively induced in the plants by withholding the water supply until the water content of the substrate reached 70% (control), 56%, 43%, 26% and 14% of its holding capacity, corresponding to approximately 50, 40, 30, 20 and 10 cm^3 of H_2O per cm^3 of substrate, respectively. This point was the start of the experiment, which finished ten days later. During the experiment all of the pots were daily weighed and

corrected for water loss with full-strength Hoagland and Arnon (1950) solution when needed. At the end of the experiment, the plants were harvested, separated into leaves and roots, then frozen and stored at -80ºC for lyophilization and further chemical and biochemical analyses.

Water status and osmotic adjustment measurements

The leaf water potential (Ψw) was evaluated immediately after sampling using the pressure chamber method (SCHOLANDER et al., 1965) at pre-dawn (Ψw, at 6:00 h) in leaves similar to those used for leaf gas exchange and chlorophyll fluorescence measurements. The leaf relative water content (RWC) was determined as previously described (SILVEIRA et al 2009). Thirty leaf discs (diameter 1.0 cm) were sampled and immediately weighed (FW). Then, they were immersed in distilled water in Petri dishes for 6 h at 25°C under a photon flux density of 40 μmol m^{-2} s^{-1}, blotted on filter paper, and the turgid weight (TW) was determined. The discs were dried in an oven at 75°C for 48 h and the dry weight (DW) was obtained. The RWC was calculated using the following equation: RWC = (FW – DW) / (TW – DW) x 100. The leaf succulence (LS) was calculated by the equation (FW)/A, where A is the area of thirty leaf discs (diameter 1.0 cm), as described by Silveira et al. (2009).

For determining the osmolality, small segments from fully expanded leaves and 5 cm-segments of terminal roots were macerated in a mortar. After extract filtration in a miracloth membrane, the sap was centrifuged at 10,000 *g* for 10 minutes at 4ºC. The resultant supernatant was used to determine the osmolality (c) with a vapor pressure osmometer (Vapro 5520, Wescor, USA). The osmotic potential was determined using the formula: Ψs (MPa) = -c (mosmol kg-1) x 2.58 x 10^{-3}, according to the Van't Hoff equation. For the measurement of osmotic potential at full turgor (Ψs^{100}), intact leaves and root segments of stressed and control plants were fully hydrated on moistened filter paper in Petri dishes for 24 h at 4ºC in the dark. The total OA was calculated as the difference of osmotic potential at full turgor between the control (Ψsc^{100}) and salt stress (Ψss^{100}) conditions (MARTINEZ-BALLESTA et al., 2004).

Determination of organic and inorganic solutes

Lyophilized leaf and root samples were transferred into hermetically closed tubes containing deionized water and placed in a 100ºC water bath for 1 h. The extracts were then filtered and stored at -20ºC for later analyses. The Na^+ and K^+ contents were determined by flame photometry and the Cl^- content through titration with $AgNO_3$. The NO_3^- concentration was determined by the method described by Cataldo et al. (1975). The total soluble sugar

content was determined using the phenol-sulfuric method (Dubois et al., 1956) and the total free amino acids were measured by reaction with ninhydrin (YEMM; COCKING, 1955). The proline concentration was determined according to the method of Bates et al. (1973). Quaternary ammonium compounds (QACs) were extracted and measured as glycinebetaine equivalents according to Grieve and Grattan (1983). All of the results of solute concentration are expressed as mmol solute kg^{-1} tissue water after correction for the humidity in the leaf and root samples. The relative contribution (RC) of each solute to the osmotic potential was estimated as % of the osmolality calculated by following ratio: RC=solute concentration (mmol kg^{-1} water tissue)/osmolality (mmol kg^{-1} solvent) according to Silveira et al. (2009).

Statistical analysis

The experiment had a completely randomized design, with five treatments and four independent replicates, each one consisting of an individual plant per pot. Data were analyzed by ANOVA and means were compared by Tukey's test at the 0.05 confidence level. The standard deviation is plotted in all of the graphics.

Results

In this study, well-watered and water-stressed *Jatropha curcas* young plants were studied to assess the effects of water deficit over 10 days on the osmotic adjustment mechanisms in both the roots and leaves. As expected, the leaf water potential (Ψw) declined gradually with decreasing water supply and it varied between -0.55 MPa and -1.05 MPa (Table 1) over the range of water availability used during the experiment. The same standard was observed for the osmotic potential (Ψs), which varied between -0.85 and -1.10 MPa in the leaves and between -0.78 and -0.91 MPa in the roots (Table 1).

The leaf turgor potential also decreased progressively as the water deficit increased, varying from +0.30 to +0.05 MPa (calculated from the differences on between the Ψs and the Ψw, data not shown). In addition, the osmotic adjustment of stressed plants, calculated from the differences on between the full turgor Ψs of the control and water-stressed tissues, showed positive values (Table 1). Moreover, reinforcing that *Jatropha curcas* young plants were able to display osmotic adjustment in response to water stress, no significant effect of drought levels on either the leaf RWC or leaf succulence was observed (Table 1).

The Na^+ concentration in the leaves of the water-stressed treated plants was slightly higher than in plants grown with an adequate water supply (Figure 1A) whereas the Cl^- ions

accumulated more intensively than Na^+ in response to drought level. Conversely, the concentrations those ions in the roots remained practically unaffected in all treatments (Figure 1B). The importance of these inorganic solutes in carrying out osmotic adjustment in the *Jatropha curcas* plants exposed to water deficit is highlighted by their relative contribution to the total osmolality. The relative contribution of the Na^+ + Cl^- sum to the total osmotic potential reached approximately 30% in the leaves and 26% in the roots (Table 2).

The K^+ content was not significantly altered in the leaves or in roots by any water-deficit treatment (Figure 1C). In spite of this, the K^+ ion contributed most to the osmotic adjustment in both organs in all treatments (Table 2). In general, the relative contribution of K^+ to the osmotic potential in water-stressed plants was approximately 25% in the leaves and 29% in the roots. By contrast, increased NO_3^-concentrations were present in leaves with more severe water-deficit treatments (Figure 1D). However, our data for the analyzed inorganic solutes showed that NO_3^- did not contribute as much to the osmotic potential in the stressed plants (~5% in leaves and 4% in roots) (Table 2).

The total free amino acid (AA) content in the leaves increased significantly ($p<0.05$) at and below 20 cm^3 H_2O per cm^3 substrate, but was not significantly different in the roots with reduced water availability (Figure 2A). The AA fraction accounted for 9% and 8.5% of the overall osmotic adjustment in the leaves and roots of the water-stressed plants, respectively (Table 3). Conversely, the total soluble sugar content (TSS) of these two organs increased with reduced water availability to be three-fold higher in the leaves of the most stressed plants, compared to control (Figure 2B). The contribution of AA and TSS to the osmotic potential of leaves and roots of *J. curcas* plants was higher than the other analyzed organic solutes and made up 21% of the total osmotic adjustment in both organs (Table 3).

The proline content in the leaves was greater in the water-stressed plants than in the well-watered control; however, a significant change was not observed in the roots with different treatments (Figure 2C). Although water-stressed plants accumulated significantly more proline in the leaves, the amount recorded (approximately 0.1% of osmolality) was at a level very low to osmotic adjustment of *J. curcas* plants would be expected (Table 3). Conversely, leaf and root glycinebetaine concentrations (GB) were higher at and below a substrate moisture of 20 cm^3 H_2O per cm^3 substrate (Figure 2D). Unlike proline, GB played a role in reducing the osmotic potential of *J. curcas* plants in both the water-stressed and well-watered plants with a relative contribution of approximately 8% in the leaves and 9% in the roots (Table 3).

The above-mentioned results indicate that K^+ contributed most to the osmotic adjustment in the leaves and much more in the roots in the water-stressed *Jatropha curcas* plants (Table 2). Interestingly, Na^+ and Cl^- also had a significant contribution to the osmotic potential in both organs for all of the treatments (Table 2). Except for proline, all of the other organic solutes analyzed in the current study contributed significantly to the osmotic adjustment as in well-watered as in drought-stressed plants (Table 3). Altogether TSS, TFAA and GB had a relative contribution to the osmotic potential of approximately 30% in both leaves and roots of water-stressed plants (Table 3).

Discussion

Osmotic stress is a physiological event often associated with excessive water deficit that can reduce plant growth through mechanisms not yet fully known. Osmotic adjustment is a cellular adaptive mechanism vital for water-stress-tolerant plants, allowing for plants to continue growing in the case of drought. Osmotic adjustment is usually defined as a decrease in the cell sap osmotic potential resulting from a net increase (discounted the concentration effect due to drought-induced reduction in cell volume) of intracellular solutes rather than from a loss of cell water (KUSAKA et al., 2005). That phenomenon in plants is very difficult to measure as well as evaluate its importance for beneficial effects in drought tolerance (SERRAJ; SINCLAIR, 2002).

The results of this study evidence that *Jatropha curcas* plants have an adaptive mechanism to avoid the drought stress by maintaining good leaf water status. This strategy is also associated with a rapid growth restriction and impairment of photosynthesis (data not shown). Under these stressing conditions the leaf water potential is slightly reduced whereas both the leaf relative water content (RWC) leaf succulence are maintained at level of well-watered plants. That strategy is common in semi-arid adapted species like cowpea when subjected to drought stress (SOUZA et al., 2004) and salinity (CAVALCANTI et al., 2004). Similar results were found for lemon plants exposed to drought for 15-d (PÉREZ-PÉREZ et al., 2009) and for annual clovers under water stress imposed by withholding water (IANUCCI et al., 2002). According to Bajji et al. (2001), in response to water stress, the reduction in Ψw is influenced by leaf age, and the effect of leaf age on Ψw seems to be due to the leaf capacity to adjust osmotically and to maintain a good RWC.

Our data reveal that inorganic solutes are effectively involved in the OA of *Jatropha curcas* plants, especially K^+, Na^+ and Cl^-, in the leaves and roots, although increased concentrations triggered by water deficits have not been commonly found in the tissue of

higher plants (IANUCCI et al., 2002). The K^+ concentration in these two organs was high and its relative contribution to the osmotic potential of *Jatropha curcas* plants under water stress was higher than other inorganic ions. The K^+ ion is known to be quite soluble and to play a key osmo-regulatory role in guard cells and similarly in turgor maintenance (SHABALA; CUIN, 2007). Our results are in line with those obtained by Patakas et al. (2002), who reported on the importance of K^+ in the osmotic adjustment of grapevine plants under water stress.

In this study, we observed increased leaf and root Na^+ and leaf Cl^- concentrations in the stressed plants compared to the control. These results are unusual for plants under water stress, but not for plants under salt stress. Our data show that the Na^+ and Cl^- accumulation in plants exposed to drought may be due to decreased dry matter yield (data not shown) – a "concentration effect" – and/or by the fact that *J. curcas* plants are characterized by as a salt includer species, especially for Na^+ and Cl^-, even when cultivated with low concentrations of those ions (SILVA et al., 2009b). This involvement of Na^+ and Cl^- ions in OA is demonstrated by the high relative contribution of Na^+ and Cl^- ions in the osmotic potential of *J. curcas* in both organs in all of the tested conditions.

As already mentioned, our current results are in agreement with those obtained by Patakas et al. (2002), who observed an increase in the Na^+ and Cl^- concentrations in grapevine leaves under water stress. Conversely, Pérez-Pérez et al. (2009) demonstrated a negligible participation of Na^+ and Cl^- in the osmotic adjustment in lemon plants subjected to drought. An increase in the NO_3^- content probably comes from the inhibition of reductase activity, which has been observed in many species, even under mild stress (KAMELI et al., 1995). Although the NO_3^- contribution to the osmotic adjustment is not quantitatively comparable to other solutes, its relative participation to the osmotic potential can be important in both organs in the untreated and treated plants.

The organic solutes similarly participated in the osmotic adjustment in the leaves and roots of *J. curcas* plants, especially TSS, TFAA and glycinebetaine. The increase in the leaf TFAA contents in the more severe drought treatments (20 and 10 cm^3 H_2O per cm^3 substrate) indicates major protein degradation as viewed by the reduction in protein content (data not shown). On the other hand, the leaf and root TSS contents increased in all of the water-stress treatments provably due to restriction in sucrose translocation from leaves and diminished use of assimilates by leaf and root growth induced by water stress rather than an inhibitory effect from sucrose synthase or invertase activities (STURM; TANG, 1999). Our data reinforce the hypothesis that carbohydrates are the organic solutes that most contribute to the osmotic

adjustment in the leaves and roots of higher plants in water-stressed conditions (IANUCCI et al., 2002).

Although the proline content is not changed in roots, proline accumulation is higher in leaves under the more severe drought treatment. Nevertheless, the leaf proline content has not significant effect on the osmotic potential in *Jatropha curcas* plants. Proline may have different roles in drought mechanisms, such as in the scavenging of free radicals and thereby protecting cellular structures against oxidative damage and denaturation (GIRIJA et al., 2002), and can also serve as a carbon and nitrogen reserve for growth after stress relief (SILVEIRA at al., 2003). However, our data showed that proline not accumulates in *J.curcas* tissues in sufficient quantities to function either as an osmolyte or a protein protectant. The accumulation of proline in this study is likely due to the response of the tissue to stress-induced damage than to acclimation or adaptation for drought tolerance.

In spite of the *J. curcas* plants not undergoing prominent changes in the leaf and root GB concentrations in stressed plants under water stress (it was significantly increased only at the lowest moisture level), the GB contribution to osmotic adjustment of well-watered and drought stresses is significant. There is circumstantial evidence that water stress-induced GB synthesis is an adaptive response since it may function as a non-toxic osmolyte or an osmoprotectant primarily in the cytoplasm (BAJJI et al., 2001). The subcellular location of GB in the cell cytoplasm is important for drought tolerance, and improved GB synthesis is triggered by both water and salt stress in most Chenopodiaceae species (MARTÍNEZ et al., 2005; HESSINE et al., 2009).

If the GB accumulation is exclusively confined into the cytosol, which usually makes up 10% of the total cell volume, then its contribution to OA can be 10-fold higher (SILVEIRA et al., 2009) in all of the studied treatments. In this case, GB jointly with soluble sugars and K^+ would contribute mostly to OA in the cytoplasm of leaf and root cells. Besides being considered as an efficient osmolyte, GB is thought to improve tolerance to dehydration (SAKAMOTO; MURATA, 2002), to stabilize the protein structure of the PSII complex and to prevent damages in the cell membranes of drought-stressed plants (YANG et al., 2007; HASSINE et al., 2008). In this context, our data reinforces the suggestion that GB may have a central role in both cellular protection and cytosol OA in leaf and root cells of *Jatropha curcas* exposed to water stress. Previously, we demonstrated that GB is an important solute for osmotic adjustment of *J. curcas* leaves subjected to salt stress (SILVA et al., 2009a).

In conclusion, roots and leaves of *J. curcas* young plants display an effective osmotic adjustment mechanism in response to a wide range of drought stresses involving the

participation of some inorganic (K^+, Na^+, Cl^-, and NO^-_3) and organic solutes (TSS, TFAA, GB). However, of all of the studied solutes, soluble sugars uniquely display a prominent drought-induced synthesis and/or accumulation in both roots and leaves. The osmotic adjustment in *J. curcas* is associated with mechanisms linked to restriction of water loss and maintaining a good water status in leaves.

Acknowledgements

We thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP) for financial support. J.A.G.S. is a CNPq research scientist and E.N.S holds a CNPq fellowship. The authors gratefully acknowledge the Tamanduá Farm Institute, Santa Terezinha-PB (Brazil) for supplying the physic nut seeds.

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Tables and figures list

Table 1. Leaf water potential, relative water content, leaf and root osmotic potential, leaf succulence and leaf and root osmotic adjustment in *J. curcas* plants exposed to different substrate moisture levels for ten days. Data refer to mean values (n=4). The same letters are not significantly different to 0.05 by Tukey's test.

Substrate moisture (cm^3 H_20 cm^{-3} substrate)	Ψw (-MPa)	RWC (%)	Ψs (-MPa) leaf	Ψs (-MPa) root	Leaf Succulence (mg H_2O cm^{-2})	OA leaf	OA root
50	-0.55a	64.04a	-0.85a	-0.78a	40.07a		
40	-0.71b	64.77a	-0.96b	-0.85a	40.18a	0.21d	0.14
30	-0.89c	65.81a	-0.97b	-0.85a	40.39a	0.43c	0.24
20	-0.98c	66.13a	-1.05b	-0.84a	40.54a	0.55b	0.33
10	-1.05d	72.49b	-1.10c	-0.91b	40.68a	0.65a	0.38

Table 2. Relative contribution of inorganic solutes in the osmotic adjustment of leaves and roots of *J. curcas* plants exposed to different substrate moisture levels for ten days. Data refer to mean values (n=4).

Substrate moisture	Na^+		Cl^-		K^+		NO_3^-	
	% osmolality							
(cm^3 cm^{-3})	Leaf	root	leaf	root	leaf	root	Leaf	root
50	15.7	17.2	13.7	9.5	32.7	30.1	5.1	5.0
40	17.9	16.0	13.2	12.0	30.4	32.9	5.0	4.3
30	16.4	18.7	15.4	9.9	23.9	29.0	4.6	3.8
20	16.4	16.3	13.9	8.3	23.3	28.0	4.8	3.9
10	13.3	16.7	16.4	6.6	21.3	25.6	5.3	4.1

Table 3. Relative contribution of organic solutes in the osmotic adjustment of leaves and roots of *J. curcas* plants exposed to different substrate moisture levels for ten days. Data refer to mean values (n=4).

Substrate moisture	TFAA		TSS		GB		Proline	
	% osmolality							
(cm^3 cm^{-3})	Leaf	root	leaf	root	leaf	root	leaf	root
50	8.7	10.1	7.7	10.6	7.4	8.6	0.1	0.1
40	7.7	7.7	9.8	9.7	7.1	9.2	0.1	0.1
30	7.9	9.7	16.2	12.4	6.6	8.7	0.1	0.1
20	10.2	8.1	18.2	19.6	8.4	8.3	0.1	0.1
10	9.5	9.1	20.7	21.4	8.8	10.0	0.2	0.2

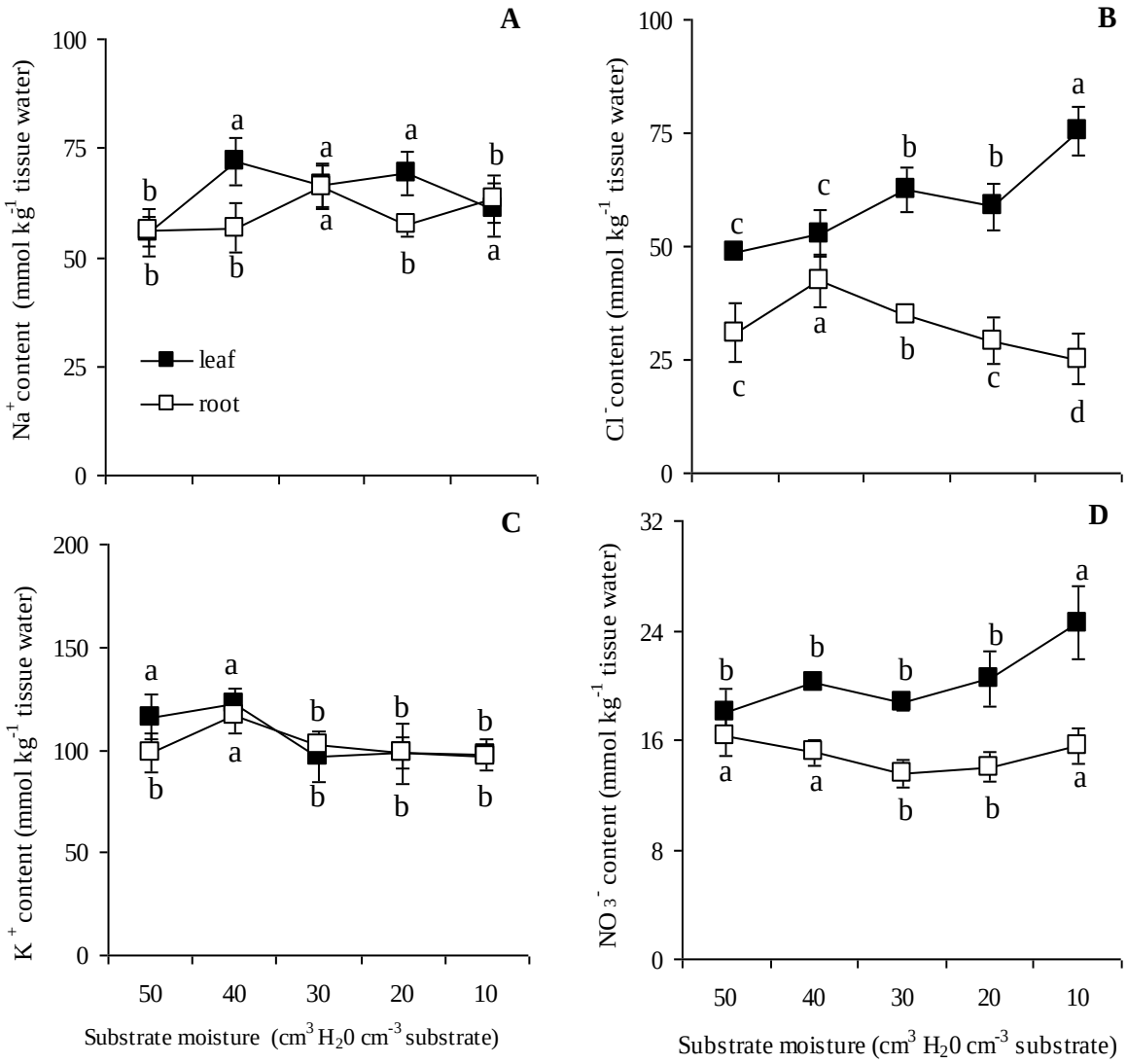

Figure 1. (A) Na^+ content (B) Cl^- content (C) K^+ content and (D) NO_3^- content in the leaves (■—■) and roots (□—□) of *Jatropha curcas* plants exposed to different drought levels. Data are means of four replicates ± SD. Same letters are not significantly different to 0.05 by Tukey's test.

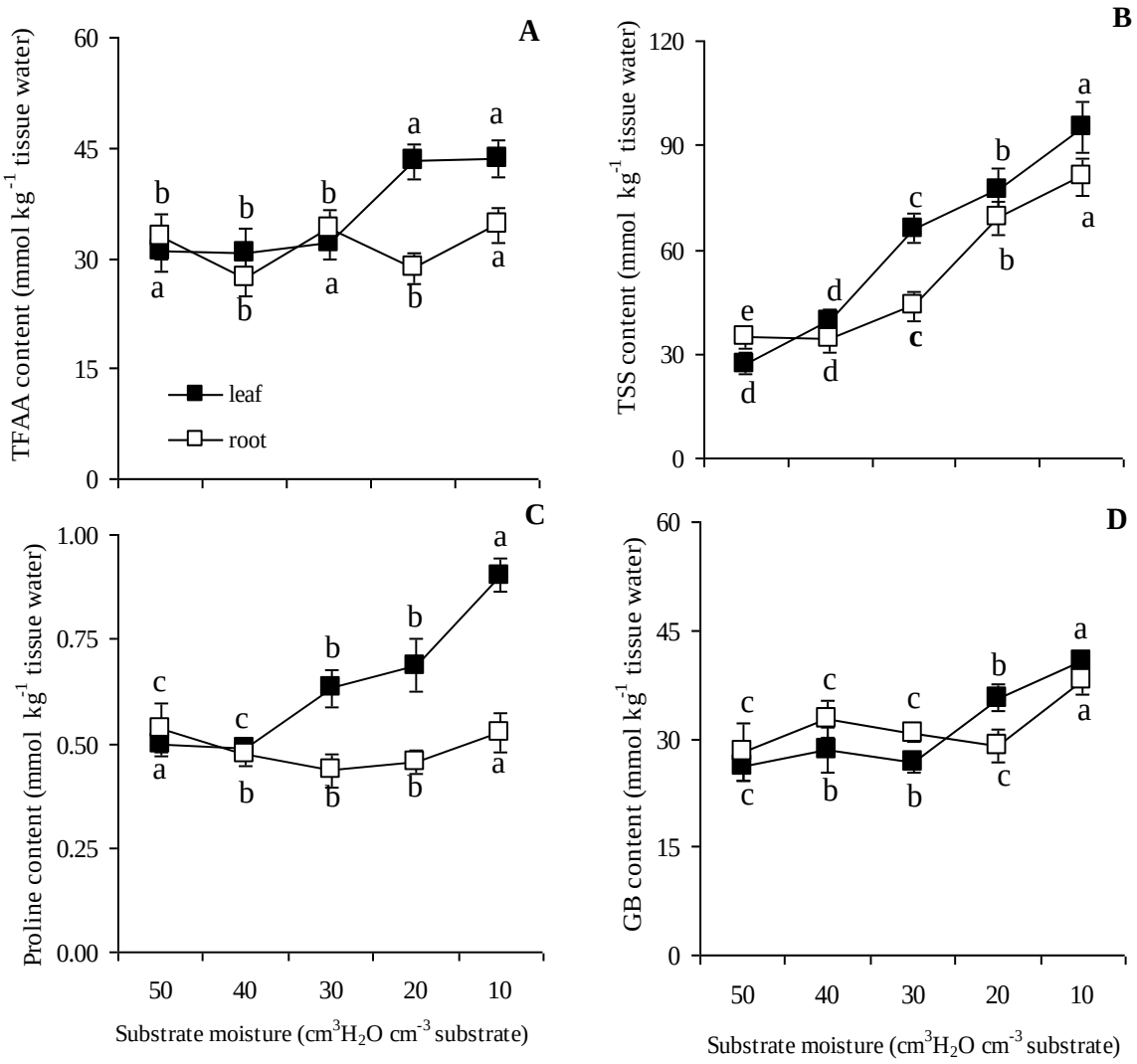

Figure 2. Concentrations of amino acids (A), total soluble sugar (B), proline (C) and glycinebetaine (D) in the leaves (■—■) and roots (□—□) of *Jatropha curcas* plants exposed to different drought levels. Data are means of four replicates ± SD. Same letters are not significantly different to 0.05 by Tukey's test.

Capítulo VI

(Artigo submetido no Journal of Plant Physiology em 19/11/09)

Photosynthetic changes and protective mechanisms against oxidative damage under isolated and combined drought and heat stresses in *Jatropha curcas* plants

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Abstract - Photosynthetic changes and protective mechanisms against oxidative damage were evaluated in leaves of *Jatropha curcas* subjected to drought and heat stresses, both in isolation and combined, in order to elucidate synergistic and antagonistic mechanisms involved with these abiotic factors. Both the drought and heat stresses caused significant damage to the leaf membrane integrity and lipid peroxidation, but the combination of these stresses greatly enhanced these physiological disturbances. CO_2 assimilation by leaves as well as stomatal conductance and instantaneous carboxilation efficiency (A/Ci) were decreased significantly in all plants subjected to stressful conditions when compared to unstressed plants. In contrast, reduction in photochemical activity was observed only under drought and drought + heat conditions. CAT and SOD activities were stimulated only under heat stress, whereas APX activity increased in all treated plants when compared to the controls. Moreover, the leaf H_2O_2 content was increased similarly under all studied stresses. Interestingly, the balance of reduced and oxidized ascorbate did not show significant differences between control and stressed plants. Although *J. curcas* plants exhibit mechanisms for acclimating to the stresses studied, they do not present an efficient

mechanism for protection against drought- and heat-induced oxidative stress, especially under combined stress conditions. Also, the combination of drought and heat causes the greatest disturbances in the photosynthetic fixation of CO_2 and on the photochemical apparatus. The results show that drought greatly disturbs the photochemical apparatus and oxidative metabolism and that these negative effects are strongly stimulated by heat stress. The data also evidence that the combination of heat and drought triggers an intricate response involving antagonistic and synergistic interactions.

Keywords: Abiotic stress, antioxidant enzymes, oxidative stress, photochemical activity, photosynthesis.

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; ASA, ascorbic acid; DHA,dehydroascorbate; ROS, reactive oxygen species; Ψ_w, water potential; EL, electrolyte leakage.

Introduction

Drought and heat are two major abiotic stresses that affect crop growth and yield in agricultural areas, especially in tropical regions. Although the isolated effects of those stresses on plant metabolism have been extensively studied, relatively little is known about the simultaneous impact of drought and heat on biochemical processes (MITTLER, 2006). In fact, both stresses occur simultaneously under field conditions in semi-arid regions and in drought-stricken areas. Recent studies have revealed that the molecular and metabolic responses to the combination of drought and heat are unique and cannot be directly extrapolated when considering plant responses to drought or heat stresses applied individually (MITTLER, 2006).

Photosynthesis is one of the primary processes most affected by abiotic stresses (LIU; HUANG, 2008). Under high temperatures, photosynthesis can be inhibited by impairment of electron transport, reduction in photochemical efficiency of PSII and inhibition of Rubisco activity. In fact, the state of Rubisco activation decreases under moderate heat stress due to impairment of Rubisco activase activity, and is exacerbated with increasing temperature (LIU; HUANG, 2008). On the other hand, the reduction of photosynthesis in drought-stressed plants may be caused by decreased CO_2 availability through increased resistance to CO_2 diffusion

from the atmosphere to the leaves or from the sub-stomatal cavity to carboxilation sites (FLEXAS et al., 2007). In addition, low photosynthesis under a water deficit may be imposed by alterations in photosynthetic metabolism, such as a reduction in biochemical and/or photochemical activity (LAWLOR; CORNIC, 2002).

High temperatures are often accompanied by water deficits and stomatal closure under field conditions, which reduce CO_2 availability and may decrease the CO_2/O_2 ratio in the chloroplasts (FOYER; NOCTOR, 2000). Once plants present low NADPH and ATP consumption due to reduced photosynthetic rates, the $NADP^+$/NADPH ratio is also decreased and the photosynthetic electron transport chain becomes over-reduced. This condition facilitates electron flow to molecular oxygen (O_2) and superoxide radical ($O_2^{\cdot-}$) production by the Mehler reaction (FOYER; NOCTOR, 2003). Additionally, the photorespiration pathway may be enhanced when C3 plants are subjected to stressful conditions since it is a potential source of reactive oxygen species, especially hydrogen peroxide (FOYER; NOCTOR, 2000).

Reactive oxygen species (ROS) are generated as natural products of plant cellular photosynthetic and aerobic metabolism. At low concentrations, ROS can serve as signaling molecules in the redox signal transduction pathway of plants (ZHU et al., 1997). However, the overproduction of ROS in plant cells under stress can damage cellular components, including DNA, proteins and membrane lipids (MITTLER, 2002). Plants have evolved efficient anti-oxidant systems that can protect them from the damaging effects of oxidative stress (ASADA, 1999). These mechanisms employ ROS-scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) as well as low molecular weight anti-oxidants such as ascorbic acid and glutathione (NOCTOR; FOYER, 1998).

Although there has been an immense amount of recent research involving oxidative stress associated with abiotic factors, few studies have addressed combined stresses like drought and heat. Moreover, those results are frequently fragmentary and do not link oxidative responses with key processes like photochemistry and overall photosynthesis. Indeed, to date, the triggering of oxidative metabolism induced by isolated and combined abiotic stresses is poorly understood, especially considering the interaction with photosynthesis and related processes. In other words, the current knowledge about this issue is still incipient and fragmentary (MITTLER, 2006). However, this information is essential to crop breeding programs aiming to develop tolerant genotypes capable of displaying high yields under stressful conditions.

Although *Jatropha curcas* has been reported as a species that grows in marginal areas where important crop species are not able to develop (FRANCIS et al., 2005), the plant's physiological responses to abiotic stresses are not yet known. To address this question, we evaluated the leaf gas exchange, chlorophyll fluorescence and antioxidative response of *J. curcas* plant leaves subjected to drought and heat stress alone or in combination in order to test the hypothesis that this species has mechanisms for tolerance and/or efficient acclimation to protect against oxidative stress and photochemical damages caused by those stressful conditions.

The data this study evidence that the combination of heat and drought triggers an intricate response involving antagonistic and synergistic interactions. The importance these plant responses to abiotic stress tolerance is discussed.

Materials and Methods

Plant material and experimental conditions

The initial phase of the experiment was carried out under greenhouse conditions, where mean air temperature varied between 24°C (minimum) and 36°C (maximum) with a mean temperature of 29°C, a mean air relative humidity of 65%, a maximum photosynthetic photon flux density (PPFD) of 700 μmol m^{-2} s^{-1} and a photoperiod of around 12 h. *Jatropha curcas* L. seeds were previously selected, taking into account the seed size and weight. Eight days after germination in sand substrate, seedlings were each transferred to plastic pot (2 L) containing vermiculite. Plants were watered every two days with 250 mL of half-strength Hoagland and Arnon's solution (1950), which was sufficient to reach 70% of the water-holding capacity of the vermiculite substrate. When plants were 23 d old, they were transferred to a growth chamber at 27ºC with PPFD of 400 μmol m^{-2} s^{-1}. At this time, two treatments were imposed: well-watered + 27°C (control) and drought + 27°C (drought). Drought was imposed by withholding water and after 5 days of treatment, the temperature was gradually elevated to 43°C (4ºC h^{-1}) and plants were subjected to this temperature for 6 h. Therefore, two more conditions were defined at this time: well-watered + 43°C (heat) and drought + 43 °C (drought + heat). Before increasing the temperature (on the 5^{th} day) and after the heat treatment, leaf discs were harvested to determine of membrane damage (K^+ leakage) and relative water content. Then, leaves were frozen in liquid nitrogen and stored at -80□C until the biochemical determinations were performed.

Water status, electrolyte leakage and dry matter yield of leaves

The leaf water potential (Ψw) was evaluated immediately after sampling using the pressure chamber method (SCHOLANDER et al., 1965) at pre-dawn (Ψw, at 6:00 h) in leaves similar to those used for leaf gas exchange and chlorophyll fluorescence measurements. The leaf relative water content (RWC) was determined as previously described (SILVEIRA et al., 2009). The leaf succulence (LS) was calculated by the equation (FW)/A, where A is the area of thirty leaf discs (diameter 1.0 cm), as described by Silveira et al. (2009). Electrolyte leakage in leaves was measured as previously described (CAVALCANTI et al., 2004) and the leaf dry matter was obtained by drying the leaves in an oven at 75°C for 48 h.

Leaf gas exchange and chlorophyll fluorescence

Leaf gas exchange was measured with an infrared gas analyzer (LCi, ADC, Hoddesdonm, UK), operating as an open system and with an air flow rate of 200 mL min^{-1}. Leaf CO_2 assimilation (A), stomatal conductance (gs) and intercellular CO_2 concentration (Ci) were measured. The instantaneous carboxilation efficiency (A/Ci) was calculated. The chlorophyll fluorescence was evaluated with a modulated fluorometer (FMS 2, Hansatech, King's Lynn, UK). Basal (Fo) and maximal (Fm) fluorescence yields were measured in dark-adapted (30 min) leaves. Steady-state (Fs) and maximal (Fm') fluorescence yields were sampled in light-adapted tissues. Variable fluorescence yields were determined in both dark- (Fv=Fm-Fo) and light-adapted (ΔF=Fm'-F) leaf tissues. The following photochemical variables were calculated: potential (Fv/Fm) and effective (ΔF/Fm') quantum efficiency of PSII; apparent electron transport rate (ETR= ΔF/Fm' x PPFD x 0.5 x 0.84) and non-photochemical quenching [qN=(Fm-Fm')/(Fm-Fo')]. For the ETR calculation, 0.5 was used as the fraction of excitation energy distributed to PSII and 0.84 as the fraction of incoming light absorbed by leaves. Fo' is the basal fluorescence signal measured after PSI excitation by far-red light. The ratio ETR/A was calculated to estimate the use of electrons in other processes not related to the photosynthetic CO_2 assimilation (RIBEIRO et al., 2009). Leaf gas exchange and chlorophyll fluorescence were measured simultaneously, in fully expanded and mature leaves under PPFD of 400 μmol m^{-2} s^{-1} into growth chamber at 9:00 h a.m. Those measurements were taken after five days of treatment at 27ºC (well-watered + 27ºC and drought + 27ºC), and repeated again after 6-h of heat treatment at 43ºC (well-watered + 43ºC and drought + 43ºC).

Leaf hydrogen peroxide content and lipid peroxidation

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 et al. (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 the Heath and Packer (1968) method. The TBARS concentration was calculated using the molar extinction coefficient (155 mM^{-1} cm^{-1}) and was expressed as nmol g^{-1} FW.

Ascorbate content

The content of total ascorbate (ASA+DHA) was determined by adding the leaf extract to a mixture of potassium phosphate buffer (pH 7.4) containing 2 mM DTT, 0.5% (w/v) N-ethylmaleimide, 10% (w/v) TCA, 45% (w/v) $H_2PO_4^-$, 4% (w/v) bipyridyl, and 5% (w/v) $FeCl_3$. The reaction was performed at 40°C for 30 min, and the absorbance was read at 525 nm. The contents of ASA+DHA (total ascorbate) and ASA (reduced ascorbate) were estimated using L-ascorbate as a standard and expressed as μmol g^{-1} FW. The oxidized ascorbate (DHA) content was obtained by subtracting the reduced fraction from the total content (KAMPFENKEL et al., 1995)

Enzyme extraction and activity assays

Samples of frozen leaves (0.1 g) were macerated in liquid nitrogen and extracted with 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). For superoxide dismutase (SOD) and ascorbate peroxidase (APX) extraction, the pH of buffer was adjusted to 7.0 and 30 mM DTT was replaced by 1 mM ascorbate. The extract was centrifuged at 14,000 *g* for 30 min at 4°C. Soluble protein was quantified using bovine serum albumin as the standard.

The activity of superoxide dismutase (SOD; EC: 1.15.1.1) was determined by adding leaf extract to a mixture containing 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM L-methionine, 2 μM riboflavin and 75 μM p-nitro blue tetrazolium chloride (NBT) in the dark. The reaction was carried out under illumination (using a 30 W fluorescent lamp) at 25 °C for 6 min. The absorbance was measured at 540 nm. One SOD activity unit (AU) was defined as the amount of enzyme required to inhibit 50% of the NBT

photoreduction (BEAUCHAMP; FRIDOVICH, 1971), and the activity was expressed as AU g^{-1} FW min^{-1}. The APX activity (APX; EC: 1.11.1.1) was assayed after reaction of the extract in the presence of 50 mM potassium phosphate buffer (pH 6.0) and 0.5 mM ascorbic acid. The reaction was started by the addition of 0.1 mL of 30 mM H_2O_2, and the decreasing absorbance at 290 nm was monitored for 300 s (NAKANO; ASADA, 1981). The APX activity was estimated considering the molar extinction coefficient of ascorbate (2.8 mM^{-1} cm^{-1}) and expressed as μmol ASA g^{-1} FW min^{-1}. Catalase activity (CAT; EC: 1.11.1.6) was determined after the reaction of the extract in the presence of 50 mM potassium phosphate buffer (pH 7.0) containing 20 mM H_2O_2. The reaction took place at 30 °C and the absorbance at 240 nm was monitored for 300 s (HAVIR; MCHALE, 1987). The CAT activity was calculated according to the molar extinction coefficient of H_2O_2 (36 mM^{-1} cm^{-1}) and expressed as nmol H_2O_2 g^{-1} FW min^{-1}.

Experimental design and statistical analysis

The treatments studied were water conditions (well-watered and drought) and temperature (27 and 43 ºC). Data were subjected to ANOVA procedure and mean values (four replicates) were compared by the Tukey test at the 0.05 level of confidence. The standard deviation is plotted in all figures.

Results

In this study *Jatropha curcas* plants were subjected to the isolated and combined stresses of drought and heat. Although the drought, heat and drought+heat stresses caused 35%, 10% and 41% reduction in the leaf dry matter, respectively, in comparison with the well-watered control plants (data not shown), none of the stressed plants exhibited visual symptoms of injury. However, the heat + drought stressed plants showed slight signs of drying in the leaf margins and incipient chlorotic areas in the leaf blades (data not shown). The leaf water potential (Ψ_w) was significantly reduced ($p<0.05$) only in the drought treatment, remaining similar to the control in the other treatments (Figure 1A). The relatively high leaf Ψ_w value of drought-stressed plants (-1.05 MPa) was associated with a good leaf hydration status, as also indicated by the relative water content and succulence values. Those values, which were similar to the values in well-watered plants, were around 72% for relative water content and 42 mg H_2O per cm^2 of leaf for succulence (data not shown).

The electrolyte leakage, a membrane damage indicator, was increased by 15%, 28% and 122% in drought, heat and drought + heat treatments, respectively (Figure 1B). In parallel, the stressful conditions also markedly enhanced lipid peroxidation, with leaves showing increases of 100%, 50% and 200% in plants subjected to drought, heat and drought + heat respectively (Figure 1C). These results demonstrate that the combination of drought and heat enhanced the oxidative damages and physiological injuries suggesting an antagonistic or negative interaction.

After exposure to stressful conditions, leaf CO_2 assimilation (A) decreased appreciably in all treatments when compared to the control condition. These reductions were around 86%, 41% and 89% in plants subjected to drought, heat and combined stresses, respectively (Figure 2A). Similarly, the stomatal conductance (gs) and the instantaneous carboxilation efficiency (A/Ci) were also reduced due to drought and heat. Compared to control plants, gs was decreased by 80%, 47% and 94%, while the A/Ci ratio was reduced by 84%, 35% and 82% in drought, heat and drought + heat treatments, respectively (Figures 2B,C). The combination of drought and heat did not show interactive effects on leaf gas exchange, and apparently only the drought effects were predominant, regardless of temperature. Also, the effects of heat were less pronounced than those of drought stress.

The photochemical activity was evaluated by the effective (ΔF/Fm') quantum efficiencies of PSII, non-photochemical quenching (qN) and apparent electron transport rate (ETR). The ΔF/Fm' and ETR values decreased by 56% and 83% in plants subjected to drought and combined stresses, respectively, when compared to controls (Figures 3A, B). However, the plants did not show significant changes ($p>0.05$) in Fv/Fm when stressed and non-stressed plants were compared, varying between 0.774 and 0.703 (data not shown). The qN increased significantly ($p<0.05$) only in plants subjected to drought and combined stresses when compared to control ones (Figure 3C).

The ETR/A ratio increased by approximately 50% with heat and drought + heat treatments and 300% with drought treatment when compared to control conditions (Figure 3D). Such data evidence a strong positive interaction (synergism) between drought and heat in terms of allocation of the electron excess of the photosystem II to CO_2 fixation. Interestingly, the heat treatment alone did not alter the majority of photochemical parameters (Figure 3). However, the damages to the photochemical apparatus caused by drought stress were strongly intensified by heat condition (antagonism effect).

The activities of the antioxidant enzymes, catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) presented differential response patterns to stressful

conditions. Total CAT activity was notably stimulated by heating, with plants showing increases of 38%. On the other hand, the activity of that enzyme was inhibited strongly by 60% and 36% in the drought and drought + heat treatments, respectively, when compared to the control condition. This suggests a synergetic effect or positive interaction between heat and drought (Figure 4A). The total APX activity was increased in all stressful conditions when compared to non-stressed plants. This increase was approximately 100%, 100% and 300% in plants under drought, heat and combined stresses, respectively (Figure 4B). Again, a positive interaction between the two stress factors was verified. Total SOD activity was stimulated in the heat treatment, showing an increase of around 36%. However, it was inhibited by 42% due to combined stresses in relation to the controls, and it was not affected by drought treatment alone, indicating a strong negative interaction (antagonism) of the combination of heat + drought (Figure 4C).

The leaf H_2O_2 concentration increased substantially in all stressed plants when compared to non-stressed ones. These increases were similar among the stress treatments (between 26% and 28%) (Figure 5A), suggesting non-additive and non-interactive effects between drought and heat. Inversely, the content of total ascorbate (ASA+DHA) was reduced by approximately 13% in plants exposed to drought and heat. Non-significant changes were noted in plants subjected to combined stresses when compared to controls (Figure 5B). In fact, these changes occurred due to variations in the concentrations of both reduced and oxidized ascorbate. The leaf ASA/DHA ratio was 0.35 in the control plants and it was not changed in plants exposed to drought and drought + heat (data not shown). Conversely, plants under heat stress had a 71% increase in the ASA/DHA ratio in relation to the controls.

Discussion

This study reveals that drought, heat and drought + heat stresses were able to induce significant alterations in the physiological indicators of stress associated with membrane integrity, leaf gas exchange and chlorophyll fluorescence as well as in the oxidative damage indicators and the ROS scavenging system of *Jatropha curcas* plants.

The reductions in plant growth and leaf CO_2 assimilation, mainly under drought and drought + heat, may be attributed to an avoidance strategy to deal with stressful conditions or deleterious effects of symptoms of stress. The decreases in leaf CO_2 assimilation were partially attributed to diffusive (low gs) and metabolic limitations (low A/Ci) under constraining conditions, mainly drought (Figure 2). The maintenance of intercellular CO_2

concentration (data not shown) associated with low leaf CO_2 assimilation was additional evidence of non-stomatal limitation of photosynthesis in *Jatropha curcas* under stressful conditions. Curiously, reductions in gs were not associated with low leaf water potential during heat treatment, suggesting a physiological response to the increasing temperature itself or to an increasing air vapor pressure deficit (RIBEIRO et al., 2004).

In addition to changes in leaf gas exchange, photochemical activity was also affected by drought and combined stress but not by heat stress alone. The effective quantum efficiency of PSII and the apparent electron transport rate were strongly decreased (Figure 3). As ETR may be considered an overall index of photochemical activity, our data suggest that drought and drought + heat treatments imposed significant de-activation of the electron transport chain in the thylakoid membranes (CHAGAS et al., 2008). This is probably associated with damage to the primary electron acceptors of PSII (plastoquinone) due to reduced Qa accumulation (FOYER; NOCTOR, 2000), which is indicated by the significant reduction in ΔF/Fm' (Figure 3).

Increases in qN were noticed when plants were subjected to drought conditions, alone or combined with heat. High qN indicates that the non-radiative energy dissipation mechanism is active and that a higher proportion of energy is lost as heat instead of being used to drive photosynthesis (RIBEIRO et al., 2009). Such thermal dissipation of the excessive excitation energy is considered a photo-protective mechanism, which maintains the primary electron acceptors of PSII in an oxidative state and reduces the probability of photo-damage (SOUZA et al., 2005). In fact, this mechanism is more active in drought-stressed plants.

The highest ETR/A ratios were found in plants subjected to drought, which suggests that more electrons are driven to alternative sinks rather than to the photosynthesis (RIBEIRO et al., 2009). Those alternative sinks were able to give additional protection to PSII activity, as ΔF/Fm' and ETR were less affected in drought-stressed plants when compared to ones subjected to drought + heat. Drought stress had the greatest effect on leaf gas exchange and photochemistry in *J. curcas*. However, the simultaneous occurrence of heat + drought stress increased the negative effect of drought on photochemistry, as indicated by the lowest values of ΔF/Fm' and ETR in drought combined with heat treatment (Figure 3).

Considering the leaf gas exchange and the photochemical activity, the most deleterious condition was drought + heat, which is in accordance with the membrane integrity (electrolyte leakage) and lipid peroxidation (TBARS levels) results (Figures 1,3). The

increased electrolyte leakage showed a positive correlation with TBARS accumulation, suggesting that the injuries induced in the plasmalemma by drought + heat stress result from oxidative damage. Numerous investigations have demonstrated that injuries caused by abiotic stresses on the plant cells are triggered in part by oxidative stress (ASADA, 1999).

The SOD-APX-CAT system protects the photosynthetic machinery from oxidative damage in plants exposed to environmental stresses (CAVALCANTI et al., 2004). SOD scavenges the $O_2^{\bullet-}$ generated by the electron transport chain into the chloroplasts and mitochondria, and the H_2O_2 produced by SOD activity is then eliminated by APX in different cell compartments (SHIGEOKA et al., 2002). However, CAT removes the H_2O_2 generated in the photorespiration pathway inside the peroxisomes (MITTLER, 2002). In the current work, oxidative stress was clearly established, as indicated by lipid peroxidation (Figure 1C). The intensity of lipid peroxidation was higher in all stressed plants when compared to control ones, suggesting the development of oxidative damages in cell membranes. In fact, Cavalcanti et al. (2007) reported that TBARS production is a good indicator for evaluation of oxidative damages in plant tissues.

CAT and SOD activities were stimulated by the heat treatment but are strongly inhibited in the combined stress (Figures 4B,C). The inhibitions of CAT and SOD activities by drought and by the combination of drought and heat may be consequences of down-regulation of gene expression or of degradation, denaturation and/or inhibition/inactivation of these proteins. For instance, a light-dependent decrease in total catalase protein and activity has been reported in response to salinity (HERTWIG et al., 1992). Besides, ongoing protein synthesis is required to maintain CAT activity under conditions in which degradation exceeds resynthesis, otherwise the enzyme activity will decrease (CAVALCANTI et al., 2004). Conversely, the increases in SOD and CAT activities in *J. curcas* leaves subjected to heat stress could be attributed to heat-induced up-regulation of gene expression and/or activation of protein isoforms.

In contrast to CAT and SOD, APX activity was increased in all stressed plants compared to the controls, especially in the drought + heat combination. Several APX isoforms are widely distributed in almost all cell organelles, and abiotic stresses frequently induce increases in both the gene expression and APX total activity in order to compensate for deficiencies in CAT activity (PALATNIK et al., 2002). However, apparently the increase in total APX activity did not compensate for the restriction presented by CAT activity since H_2O_2 accumulated in leaf tissue. The excess of H_2O_2 produced in peroxisomes and chloroplasts might diffuse to the cytosol and be converted to hydroxyl radicals by the Fenton

reaction (MØLLER et al., 2007). These are the most toxic ROS and are directly involved in lipid peroxidation (FOYER; NOCTOR, 2000).

Our results indicate that the ROS-scavenging system was not sufficient to protect J. *curcas* leaves against oxidative damages induced by isolated drought, isolated heat or the combined stresses. This fact is confirmed by the high levels of lipid peroxidation and H_2O_2 accumulation (Figures 1C, 5A). The hydrogen peroxide accumulated in leaves of *J. curcas* under stressful conditions may be generated mostly by photorespiration, a process which is located mainly inside peroxisomes. This assumption is supported by the decreases in CAT activity (Figure 4) and the increases in the ETR/A ratios (Figure 3D) under constraining conditions. In fact, photorespiration is the major alternative electron sink in C3 plants (RIBEIRO et al., 2004; 2009).

The data from this study evidence the complexity of the relationships between photochemical damage and oxidative stress under conditions of isolated and combined drought and heat stresses. In fact, the data regarding photosynthesis and antioxidative enzyme activities are not enough to explain some responses showed by *J. curcas* plants, especially the responses to oxidative damages. Clearly, drought, as an isolated factor, is more stressful than heat, in terms of photosynthesis disturbances (CO_2 fixation and photochemical activity) and oxidative damages. These more severe negative effects are associated with the highest loss of electrons from photosystem II to carboxylation reactions, inhibition of CAT and SOD activities and higher H_2O_2 accumulation.

In contrast to drought stress alone, the heat treatment greatly stimulated CAT and SOD activities and preserved APX activity at the level of the controls. Collectively, this response is extremely favorable for scavenging H_2O_2 and superoxide radicals, especially in chloroplasts and peroxisomes. The maintenance of a favorable balance between SOD, APX and CAT is essential to avoid ROS accumulation, preserving the photochemical apparatus, and to avoid prominent oxidative damages (GUO et al., 2007) as noted in heat-treated plants (Figures. 3, 1C). In fact, the impairment of photochemical activity and the abrupt increase in photorespiration are the main causes of ROS accumulation in leaves (CHAGAS et al., 2008).

Concerning the combined effects of drought + heat on *J. curcas* leaves, our data clearly evidence that this stress condition induced the most negative alterations in membrane integrity, photosynthetic fixation of CO_2, photochemical activity and oxidative metabolism. In fact, photosystem II activity was impaired, CAT and SOD activities were markedly inhibited and hydrogen peroxide accumulated. Unexpectedly, the intense increase in APX activity in plants subjected to the combined stress was insufficient to avoid prominent oxidative damages

and peroxide accumulation. It is plausible that the combination of stresses could trigger an up-regulation of gene isoforms located mainly in the cytosol while not significantly altering the expression of chloroplastic isoforms. The cytosolic APX isoforms are the most important for maintenance of redox homeostasis in the plant cell (SHIGEOKA et al., 2002; MITTLER, 2002).

Surprisingly, total APX activity was prominently up-regulated, but the total ascorbate concentration and its redox balance were not altered under the combined stresses. This result suggests that the fraction of reduced ascorbate consumed as a substrate for APX activity was probably derived from a new synthesis of ascorbate. Under these conditions, the oxidized ascorbate produced after the reaction of reduced ascorbate with hydrogen peroxide could be catabolized to produce two- and four-carbon products, such as oxalate and tartrate, which can accumulate at relatively high levels in plant cells (NOCTOR; FOYER, 1998). Thus, the measurement of the concentrations of reduced and oxidized forms, at a specific time, could only reflect a stationary condition of the ascorbate redox balance.

Our data agree with Mittler (2006) who observed that the simultaneous exposure of plants to different abiotic stress conditions will result in the co-activation of different stress-response pathways. Also, the response to combined stresses is quite different of the sum of the responses to individual abiotic factors. The stress factors might display antagonistic or synergistic interactions with each other. In this present study, for some specific processes (photosynthesis, enzymatic antioxidative response, and oxidative damages) heat and drought in combination trigger different response types, i.e. antagonism or synergism. But, in general, the combined effects of drought and heat are much more damaging than the individual stresses.

In summary, *Jatropha curcas* plants subjected to the combination of drought and heat exhibited very different oxidative and photosynthetic (leaf gas exchange and photochemistry) responses from those triggered by the isolated stresses or the sum of both stress responses, that is, they showed a negative interactive response. Moreover, drought is more damaging in terms of oxidative stress and photosynthetic damage than heat stress. Heat was less deleterious because plants exhibited an up-regulation in the activities of catalase, ascorbate peroxidase, superoxide dismutase and had a favorable redox balance between the reduced and oxidized forms of ascorbate. In conclusion, *J. curcas* plants exhibit acclimatizing mechanisms to the stresses studied; however, they do not present an efficient mechanism for protection against drought- and heat-induced oxidative stress, and are unable to avoid H_2O_2 accumulation and significant oxidative stress, mainly under the combined stress conditions.

Acknowledgements

We thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP) for financial support. J.A.G.S and R.V.R. are CNPq researcher scientists and E.N.S. holds a CNPq fellowship. The authors gratefully acknowledge the Tamanduá Farm Institute, Santa Terezinha-PB (Brazil) for supplying the seeds.

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Figures list

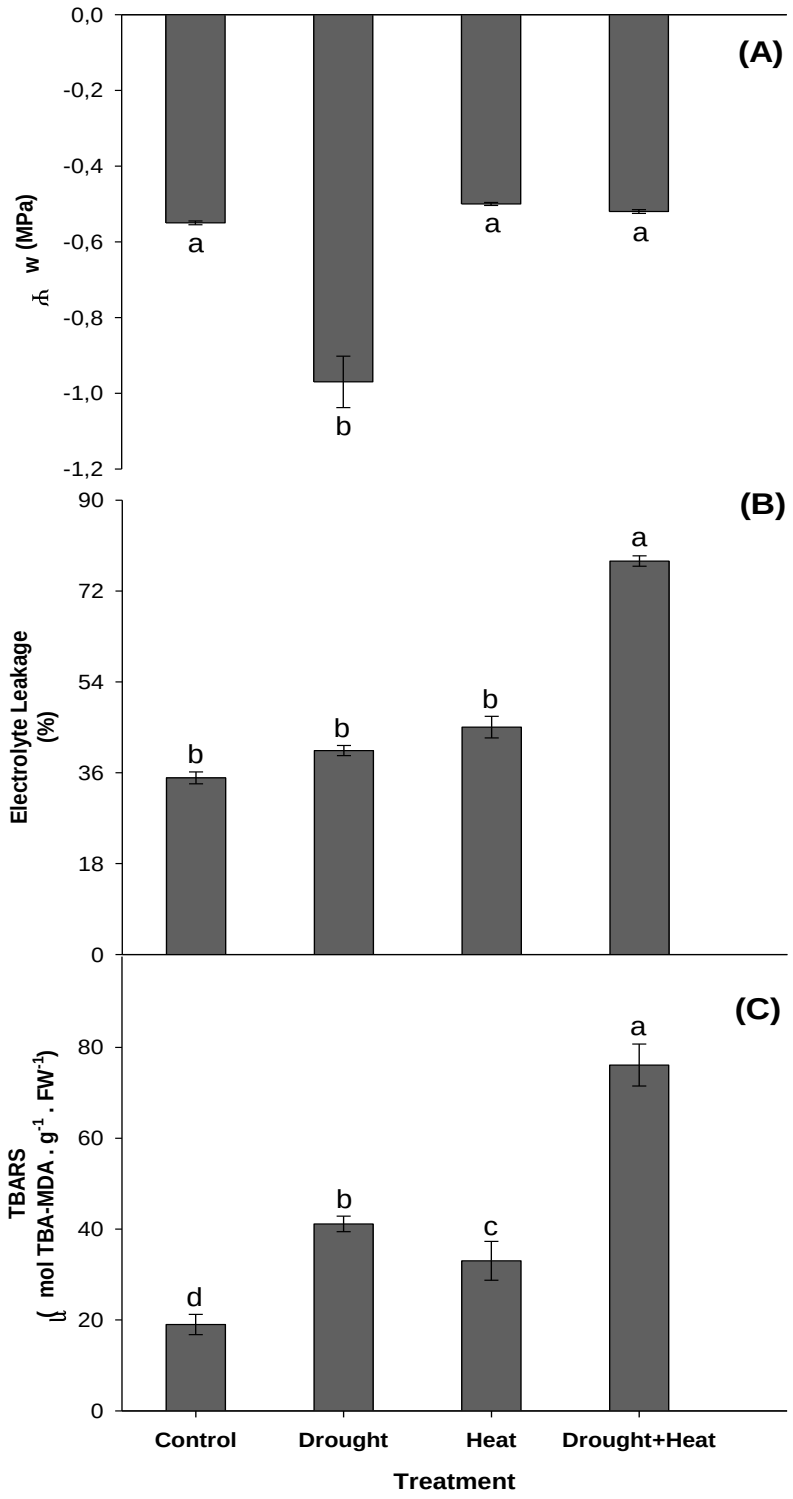

Figure 1. Leaf water potential (Ψw, in A), electrolyte leakage (B) and TBARS content (C) in *Jatropha curcas* plants subjected to isolated and combined stresses of drought and heat. Data are mean values of four replicates ± SD. Same letters are not significantly different to 0.05 by Tukey's test.

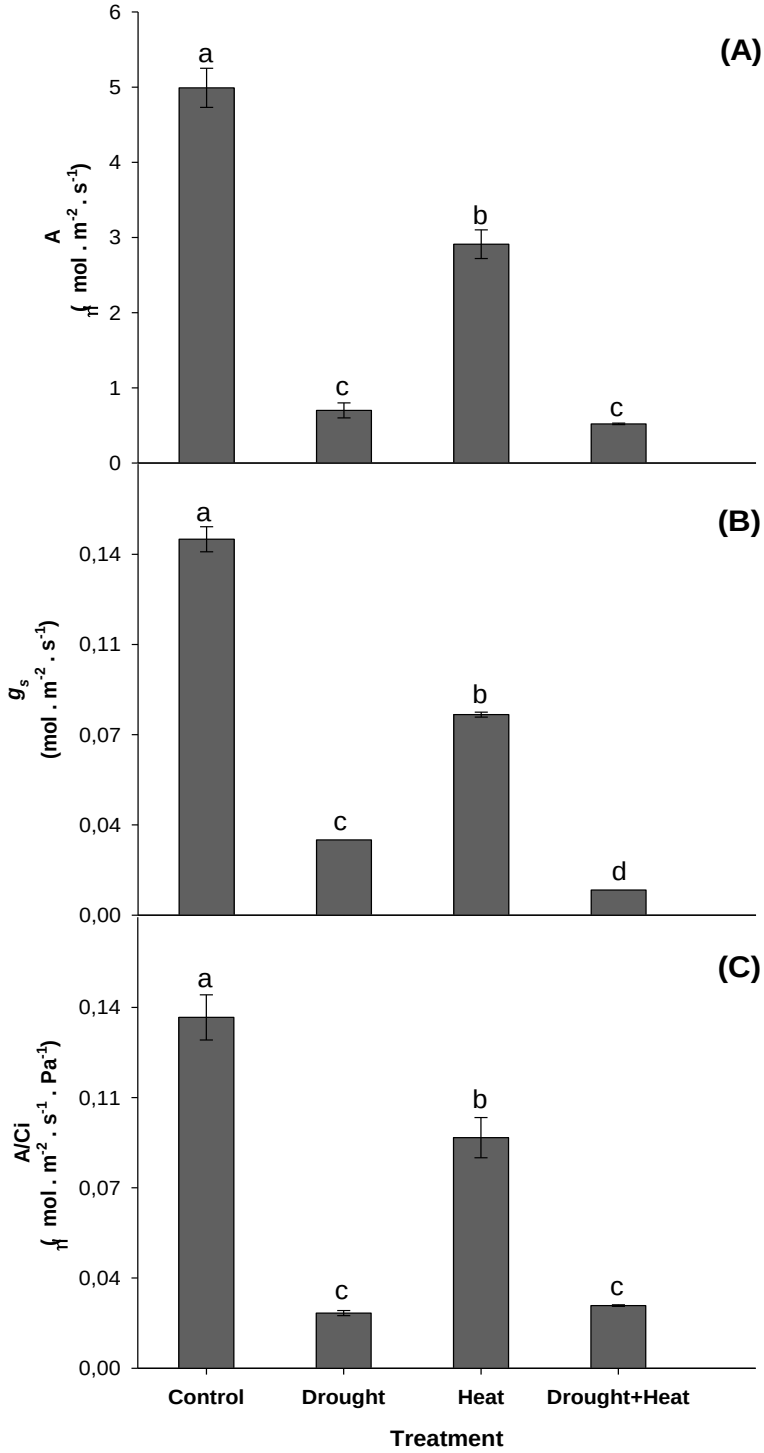

Figure 2. Leaf CO_2 assimilation (A, in A), stomatal conductance (gs, in B) and instantaneous carboxilation efficiency (A/Ci, in C) in *Jatropha curcas* plants subjected to isolated and combined stresses of drought and heat. Data are mean values of four replicates ± SD. Same letters are not significantly different to 0.05 by Tukey's test.

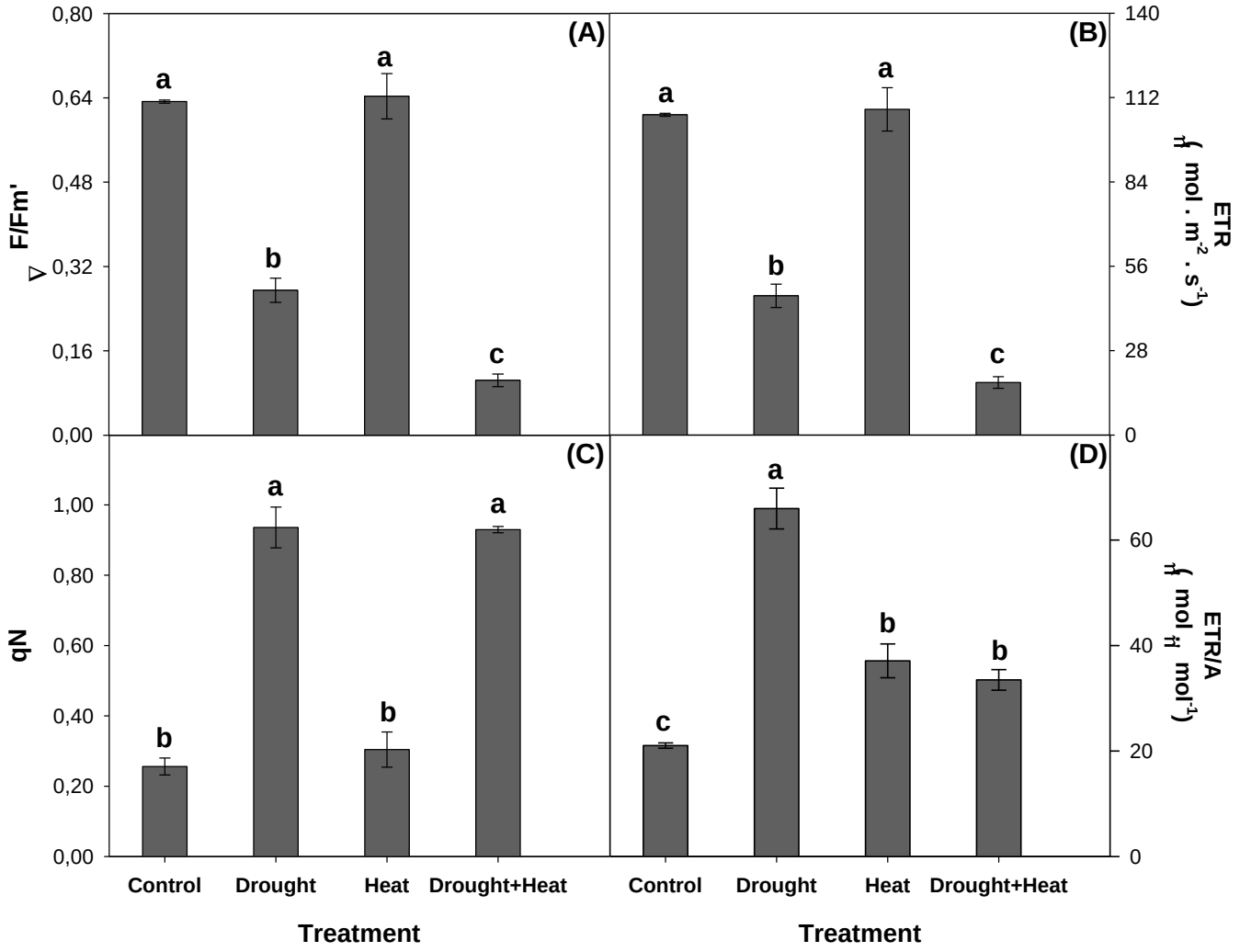

Figure 3. Effective quantum efficiency of PSII (ΔF/Fm', in A), apparent electron transport rate (ETR, in B), non-photochemical quenching (qN, in C) and ratio between apparent electron transport rate and CO_2 assimilation (ETR/A, in D) in *Jatropha curcas* plants subjected to isolated and combined stresses of drought and heat. Data are mean values of four replicates ± SD. Same letters are not significantly different to 0.05 by Tukey's test.

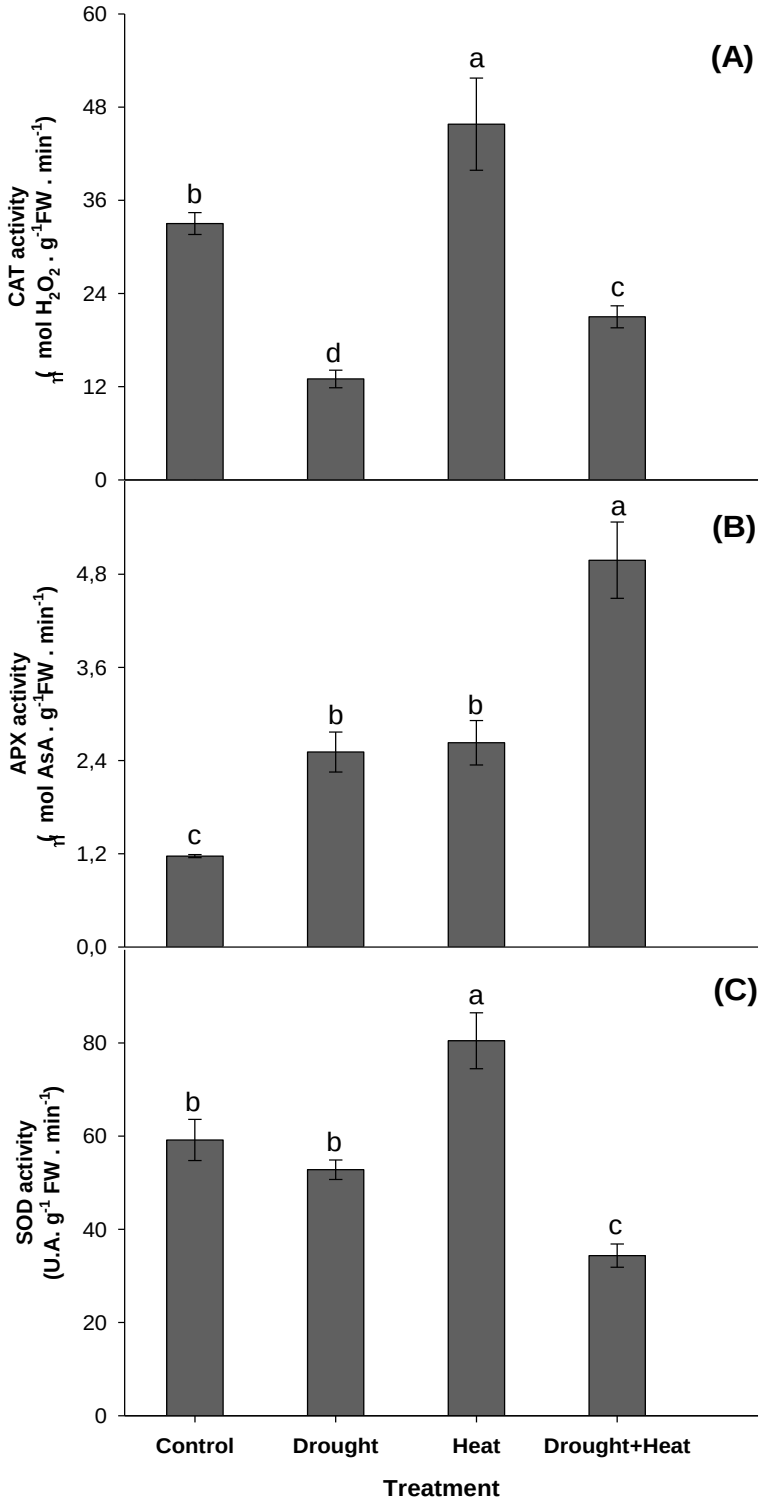

Figure 4. Catalase activity (CAT, in A), ascorbate peroxidase activity (APX, in B) and superoxide dismutase activity (SOD, in C) in *Jatropha curcas* plants subjected to isolated and combined stresses of drought and heat. Data are mean values of four replicates ± SD. Same letters are not significantly different to 0.05 by Tukey's test.

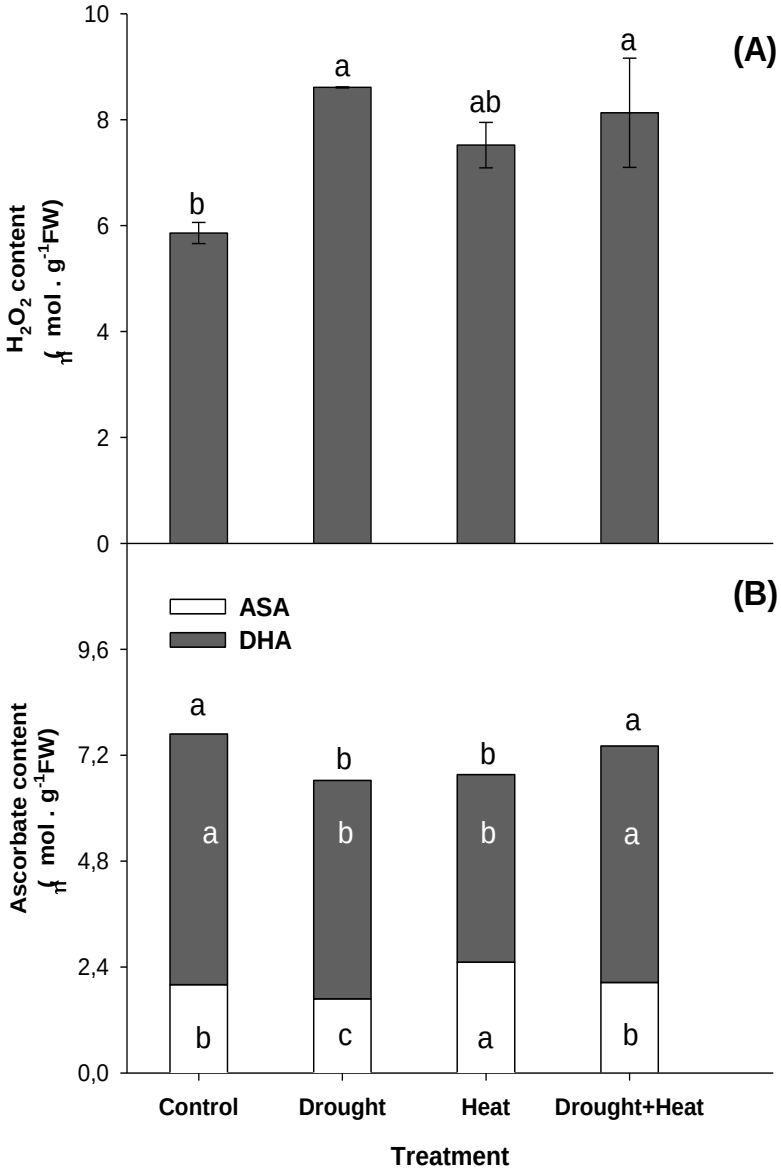

Figure 5. Hydrogen peroxide content (H_2O_2, in A) and reduced (ASAred) and oxidized (ASAox) ascorbate (in B) in *Jatropha curcas* plants subjected to isolated and combined stresses of drought and heat. Data are mean values of four replicates ± SD. Same letters are not significantly different to 0.05 by Tukey's test.

CONSIDERAÇÕES FINAIS

Os resultados indicam que o pinhão-manso quando submetido à salinidade, apresenta um caráter de planta incluidora de sais, especialmente de Na^+, o que leva a um desequilíbrio na homeostase iônica e conseqüente aumento da relação K^+/Na^+. Por outro lado, o íon sódio assim como o cloreto, em números quantitativos são os que mais contribuem com o ajustamento osmótico nessa espécie, tanto na ausência quanto na presença de NaCl. É claro que alguns solutos orgânicos também estão envolvidos nesse processo, dentre eles, a glicina-betaína, que em comparação com a prolina, está bem mais envolvida na redução do potencial osmótico de folhas de pinhão-manso expostas à salinidade.

Nestas condições estressantes, o acúmulo de sódio e cloreto nas folhas levou a uma intensa redução em alguns parâmetros de trocas gasosas e fluorescência da clorofila da espécie. Esta redução apresentou magnitudes diferentes, variando com a intensidade e duração do estresse. Sob condições de estresse salino severo, como mostrado no capítulo III, os danos causados no aparato fotossintético foram irreversíveis, provenientes de um desbalanço entre as duas fases da fotossíntese devido tanto a efeitos osmóticos quanto iônicos.

Semelhantemente ao ocorrido com plantas expostas à salinidade, o estresse hídrico também induziu uma forte redução em alguns parâmetros fisiológicos analisados, especialmente nas trocas gasosas e fluorescência da clorofila. Por outro lado, quando se avaliou a participação dos principais solutos envolvidos no ajustamento osmótico da espécie nessa condição estressante, observou-se que, ao contrário do estresse salino, a seca induziu um aumento significativo no conteúdo de açúcares solúveis e de potássio tanto em folhas quanto em raízes. Isto fez com que estes dois solutos fossem os mais envolvidos na redução do potencial osmótico de pinhão-manso sob estresse hídrico.

Quando se avaliou os efeitos isolados e combinados dos estresses hídrico e de temperatura elevada, observou-se que a combinação dos estresses apresentou um efeito deletério aditivo sobre o aparato fotossintético, integridade de membranas e conteúdo de TBARS. Paralelamente a isto, o sistema de remoção de EAO, através da ação das enzimas CAT, APX e SOD parece não ter sido eficiente no controle contra danos oxidativos. Os resultados demonstram que plantas de pinhão-manso foram mais afetadas pela seca do que pelo calor, todavia a combinação de ambos os estresses causou danos mais significativos no aparato fotossintético assim como no metabolismo oxidativo das plantas.

No geral, os resultados demonstram que plantas jovens de pinhão-manso apresentam maior sensibilidade aos estresses de seca e salinidade do que a temperatura elevada.