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PROTEÔMICA DIFERENCIAL E RESPOSTA DO METABOLISMO ANTIOXIDATIVO NO POTENCIAL FITORREMEDIADOR DE LEMNACEAE

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“Não é sobre chegar no topo do mundo e saber que venceu.
É sobre escalar e sentir que o caminho te fortaleceu [...]”

Ana Vilela

RESUMO

As lentilhas-d'água são plantas capazes de crescer densamente na superfície das águas, gerando rápida produção de biomassa com alto teor de proteínas e amido. Além disso, apresenta características naturais relevantes para estudos de biologia e biotecnologia de plantas e aplicações práticas, como exemplo, o estresse salino que é capaz de induzir a produção e armazenamento de amido em níveis elevados. Por outro lado, a tolerância à salinidade pode contribuir para expandir as aplicações biotecnológicas potenciais destas plantas, as quais apresentam ampla variação intraespecífica. Neste trabalho, objetivou-se identificar mecanismos antioxidativos que favorecem a aclimatação das lentilhas-d'água, visando investigar estratégias de tolerância da espécie ao estresse oxidativo ocasionado pelo estresse salino, bem como a identificação de proteínas significativamente associadas com aplicações biotecnológicas. Análises bioquímicas, fisiológicas e moleculares foram realizadas em quatro clones (M1, U1, RC e DI) da espécie *Lemna aequinoctialis* submetidos a diferentes concentrações de NaCl (0, 25 e 50 mM). No geral, o estresse estimulou nos clones o acúmulo de amido, açúcares redutores e reduziu o teor dos pigmentos fotossintéticos, além de provocar inibição do crescimento e respostas antioxidantes nos clones submetidos a 50 mM de NaCl. A inibição no crescimento das plantas foi reflexo do estresse oxidativo, evidenciado pelo significativo incremento no conteúdo de malondialdeído (MDA) e peróxido de hidrogênio (H_2O_2). No entanto, os clones M1 e DI cultivados sob estresse moderado (25 mM), apresentaram um incremento na atividade e no acúmulo das enzimas antioxidativas, paralelo a manutenção dos níveis de MDA. Ademais, o clone M1 destacou-se por apresentar maior extração de Na^+, o que reflete a eficiência da aclimatação. A partir dos dados bioquímicos foram selecionados os clones M1 (mais tolerante) e U1 (mais sensível) para análise proteômica. Os géis bidimensionais apresentaram um total de 188 *spots* diferencialmente acumulados nos clones M1 e U1 (82 e 106, respectivamente), dos quais 131 (67 em M1 e 64 em U1) foram identificados a partir da análise por espectrometria de massa. Os perfis proteômicos tiveram coeficiente de correlação maior que 0,90. As proteínas responsivas ao estresse salino foram principalmente associadas à produção de energia, aos componentes de fotossistemas e indução de resposta a estímulos ambientais, sugerindo a existência de mecanismos de ajuste fisiológico aos fatores do estresse estudado. A redução de crescimento associado à elevada produção de amido indica que estas macrófitas podem ser cultivadas sob condições salinas moderadas, podendo ser útil no processo de dessalinização, produzindo matéria-prima potencial para a produção de biocombustíveis, além de boa fonte de proteínas vegetais que pode ser direcionada para outros produtos de interesse, como consumo animal.

Palavras-chave: Cultivo *in vitro.* Enzimas antioxidativas. Estresse salino. Macrófitas.

ABSTRACT

Duckweed are plants that grow densely on the water surface, creating fast production of biomass with high content of protein and starch. In addition to present relevant natural characteristics for biology studies and plant biotechnology and practical applications. For example, the salt stress is capable of inducing high levels of starch accumulation, while the salinity tolerance can contribute to expanding the potential biotechnological applications of these plants. However this feature, tolerance to salinity has wide intraspecific variation. The purpose of this work is to identify antioxidant mechanisms that benefit the duckweed, aiming to investigate mechanisms of tolerance of the specie to the oxidative stress caused for the salt stress, and identification of proteins Lemnoiceae significantly associated to biotechnogical applications. Biochemical analyses, physiological and molecular were performed in four clones (M1, U1, RC e DI) *Lemna aequinoctialis* (water lentils), subjected to different concentrations of NaCl (0, 25 e 50 mM of NaCl). To the most of the clones, the effects of salt stress stimulated accumulation of starch and reducing sugars and reduced the concentration of photosynthetic pigments. Furthermore, caused growth inhibition and antioxidant responses in 50 mM of NaCl. The inhibition in growth of the plants is reflex of oxidative stress evidenced by the significant increment in the malondialdehyde content (MDA) and hydrogen peroxide (H_2O_2). However, the clones M1 and DI cultivated under moderate stress (25mM) presented a increment in the activity and in accumulation of antioxidative enzymes parallel to the maintenance of MDA levels, besides that M1 presented higher extraction of Na^+ which reflects the efficiency of acclimatization. The results of two-dimensional electrophoreses presented that a total of 188 spots differentially accumulated in clones M1 and U1 (82 and 106, respectively) of which 131 (67 in M1 and 64 in U1) were identified from mass spectrometry analysis. Proteomic profiles had a correlation coefficient higher than 0.90. The proteins responsive to the salt stress were mainly associated to energy production, components of photosystems and induction of response to environmental stimuli. The pattern of behavior of the identified protein suggests the existence of mechanisms of physiological adjustment to the studied stress factors. The reduction of growth associated to high starch production indicates that macrophytes can be grown under moderate salt conditions, and may be useful in the desalination process, producing material with potential biofuel, as well as a good source of plant proteins that can Products of interest, such as animal consumption.

Keywords: In vitro Cultivation. Antioxidative enzymes. Salt stress. Macrophytes.

LISTA DE FIGURAS

CAPÍTULO I

CAPÍTULO II

LISTA DE TABELAS

CAPÍTULO I

CAPÍTULO II

LISTA DE ABREVIATURAS E SIGLAS

APX – Peroxidase de Ascorbato

APS – Persulfato de amónio

ATP – Trifosfato de adenosina

ATPase ATP sintetase

BSA – Albumina de soro bolvino

CAT – Catalase

CuSOD – Enzima superóxido dismutase contendo cobre

DTT – Detiotreitol

EDTA – Ácido etilenodiaminotetracético

FeSOD – Enzima superóxido dismutase contendo ferro

$HO_2^{\bullet}$ – Radical hidroperóxido

H_2O_2 – Peróxido de hidrogênio

IEF – Focalização isoelétrica

IPG – Gradiente de pH imobilizados em géis de poliacrilamida

MDA – Malodialdeído

MnSOD – Enzima superóxido dismutase contendo manganês

NADPH – Nicotinamida adenina dinucleotídeo fosfato

NBT – Nitro blue tetrazolium

$^1O_2$ – Oxigênio singleto

O_2 – Oxigênio molecular

$O_2^{\bullet -}$ – Radical superóxido

$OH^{\bullet}$ – Radical hidroxila

pI – Ponto isoelétrico

PMF – ‘Peptide mass fingerprinting’

ROS – ‘Reactive Oxygen Species’

SOD – Superóxido dismutase

SDS – Dodecil Sulfato de Sódio

TRA – Teor relativo de água

TCA – Ácido tricloroacético

TCR – Taxa de crescimento relativo

TBA – Ácido 2-tiobarbitúrico

2D – PAGE Eletroforese bidimensional em gel de poliacrilamida

SUMÁRIO

INTRODUÇÃO GERAL E REVISÃO DE LITERATURA

1 INTRODUÇÃO GERAL

A região semiárida brasileira caracteriza-se pela precipitação pluviométrica limitada, associada à elevada demanda evaporativa. A tendência é de agravamento dessa situação com as expectativas crescentes dos efeitos do clima associados à reduzida disponibilidade de água superficial e subterrânea, as quais apresentam, frequentemente, alta concentração de sais dissolvidos (GHEYI et al., 2012). A escassez deste recurso leva à utilização, na agricultura, desta água rica em sais, mediante o uso de poços rasos característicos da região supracitada (ARAUJO et al., 2014).

Diante desta problemática, as inovações tecnológicas e as pesquisas são indispensáveis, tanto para o enfrentamento de desafios presentes e futuros da sociedade, no que compete à disponibilidade de água de boa qualidade para manutenção da vida, quanto são igualmente importantes para a compreensão dos mecanismos de sobrevivência das plantas à salinidade. São os dados de base científica, que formam o alicerce para tomada de decisões nos programas de melhoramento vegetal.

Existe uma crescente demanda por plantas resistentes/tolerantes a ambientes salinizados, uma vez que as projeções futuras sobre alterações climáticas e aumento da população mundial também sugerem aumento da extensão da área afetada pela salinidade (PITMAN; LÄUCHLI, 2002; ZHAO et al., 2014). Neste sentido, as macrófitas aquáticas, popularmente conhecidas como lentilhas-d'água, pertencentes ao grupo de plantas aquáticas da família Lemnaceae, têm despertado interesse mundial devido a sua utilização em ampla gama de aplicações, como por exemplo, na restauração de corpos de água (DHOTE; DIXIT, 2009). Ademais, a rápida velocidade de crescimento associada à capacidade de acumular amido dessas plantas, apresenta promissor potencial biotecnológico, podendo ser prontamente convertido em etanol, usando as tecnologias já existentes (XU; SHEN, 2011, XU et al., 2012).

Como forma de atenuar as consequências da poluição ambiental, as lentilhas-d'água já vêm sendo utilizadas com sucesso em outros países no tratamento de águas residuais (IQBAL, 1999), devido à capacidade de retirar compostos nitrogenados e fosfatados com grande eficiência, reduzindo concentrações de matéria orgânica e de

sólidos em suspensão (MOHEDANO et al., 2012, 2014). Neste aspecto, possuem aptidão de crescimento em água com salinização moderada sem a perda da capacidade de remoção de compostos nitrogenados e fosfatados (LIU; DAI; SUN, 2016), além da capacidade comprovada de dessalinização de água pela captação do sal dissolvido (BALLA et al., 2014) sendo, portanto, uma candidata promissora para a fitorremediação de ambientes aquáticos.

A deficiência hídrica e o excesso de sais conduzem a uma condição estressante em plantas glicófitas, provocando alterações metabólicas nas plantas e acarreta aumento da geração de espécies reativas de oxigênio (ROS - *Reactive Oxygen Species*). Para tanto, as plantas desenvolveram mecanismos de defesa com um sistema antioxidativo composto por enzimas e metabólitos capazes de regular o nível de ROS (DEWIR et al., 2006), que podem atuar como sinalizadoras ou, quando em excesso, provocar danos celulares. O estresse oxidativo se estabelece em decorrência do desequilíbrio entre o acúmulo de ROS e a ação de compostos antioxidantes.

Espera-se que os resultados obtidos neste trabalho possam aprimorar o conhecimento sobre crescimento e o potencial fitorremediador da espécie *Lemna aequinoctialis* em de águas salinas, viabilizando o tratamento e valorização de águas salobras ou moderadamente salinizadas, bem como a produção de plantas com elevado teor de amido.

A investigação fisiológica, bioquímica e molecular de lentilhas-d’água tornou-se essencial para a exploração do potencial da biomassa, bem como uma melhor compreensão dos mecanismos de ativação das respostas de aclimatização às novas condições impostas, principalmente aquelas que favorecem sua aplicação biotecnológica. Estas características podem variar significativamente entre as espécies de lentilha d’água. Portanto, objetivou-se avaliar a atividade do sistema antioxidante, visando investigar estratégias de tolerância da espécie *Lemna aequinoctialis* ao estresse oxidativo ocasionado pelo estresse salino, bem como identificar proteínas de Lemnaceae significativamente associados com aplicações biotecnológicas.

2 REVISÃO DE LITERATURA

2.1 Problematização

2.1.1 Salinidade

A salinidade, um grave problema ambiental, pode ser caracterizada pelo acúmulo, no solo e/ou na água, de sais solúveis, em especial do cloreto de sódio, em quantidades que podem prejudicar o desenvolvimento e rendimento econômico da maioria das culturas, afetando a sustentabilidade ambiental (RENGASAMY, 2006). Em função dessa redução da capacidade produtiva das culturas, a salinidade, associada à seca, tem crescido substancialmente e é descrita como um dos mais disseminados riscos ambientais, em especial nas regiões áridas e semiáridas (PITMAN; LÄUCHLI, 2002; ZHAO et al., 2007), constituindo uma ameaça visível para a sobrevivência das populações terrestres e ecossistemas aquáticos.

Os problemas associados à salinização do solo datam do início da civilização, porém, nos últimos dois séculos, o impacto da salinidade pela irrigação sobre a produtividade agrícola tem sido reconhecido em muitas partes do mundo (RENGASAMY, 2008). Estima-se que aproximadamente 20% da área com solos irrigados no planeta, estão afetados pela salinização (MUNNS; TESTER, 2008; ZHAO et al., 2013). Segundo dados do mapa mundial de solos da FAO/UNESCO, o total da área com solos afetados pela salinidade é de 397 milhões de hectares, que representam cerca 3,1% da área terrestre no mundo (FAO, 2005). Vale salientar que projeções da década passada já apontavam alterações climáticas e aumento da população mundial induzindo aumento da extensão da área com solos salinos (RENGASAMY, 2008), problema este que também afeta os corpos de água doce, nos quais esse processo vem sendo, de fato, acelerado pelas atividades antrópicas (KAUSHAL et al., 2016).

A salinidade é frequentemente mencionada como estresse salino, tendo por base que é considerado estresse o fato de fatores bióticos e abióticos serem capazes de alterar o estado fisiológico de um organismo vegetal a ponto de prejudicar funções essenciais para o desenvolvimento (GASPAR et al., 2002). O estresse salino pode afetar, mais especificamente, processos do metabolismo vegetal, como a fotossíntese, que refletem na inibição ou mesmo na redução do crescimento

(KIRSCHBAUM, 2011). Esse evento é o efeito mais visível, o que torna a taxa de crescimento um dos parâmetros mais utilizados nos estudos comparativos de tolerância salina entre plantas, ainda que outras alterações morfológicas também sejam indicadoras de estresse salino (FLOWERS et al., 2010).

No Brasil, a salinização induzida pelo homem é mais perceptível em regiões caracterizadas pela irregularidade e baixa taxa de precipitação pluvial, associada à baixa atividade bioclimática. O manejo inadequado da água de irrigação na região semiárida somado às condições de drenagem deficientes contribui para a aceleração do processo de salinização de terras (e ambientes aquáticos) e vem se tornando motivo de grande preocupação (PEDROTTI et al., 2015). Neste contexto, a desertificação associada à salinização dos solos ocorre em maior ou menor proporção em quase todas áreas irrigadas do Nordeste, em que a salinização é a principal causa da degradação dos solos e, consequente, desertificação nas áreas de agricultura irrigada.

Considerando-se o despejo no solo de rejeitos de dessalinizadores sem tratamento prévio, tem-se outro agravante que propicia alto acúmulo de sais nas camadas superficiais dos terrenos onde são instalados os equipamentos (PEDROTTI et al., 2015). A dessalinização por osmose reversa é uma das técnicas de purificação de água de poços altamente salina e imprópria para consumo humano e animal, porém, apresenta baixa qualidade (atingindo cerca de 50% de água potável) e altos riscos ambientais (WANDERLEY, 2009).

Frente à situação descrita, faz-se de extrema importância o desenvolvimento e a aplicação de tecnologias com modelo produtivo ambientalmente sustentável. Neste aspecto, uma técnica que vem ganhando bastante prestígio nos últimos tempos é a fitorremediação que, diferente das demais, possui baixo custo sem deixar prejuízos no ambiente. Nesse caso, o cultivo de macrófitas aquáticas é atraente para a restauração de corpos de água (DHOTE; DIXIT, 2009).

2.2 Lentilhas-d’água

2.2.1 Aspectos gerais

As pequenas macrófitas aquáticas flutuantes conhecidas como lemnáceas, lentilhas-d’água, “duckweeds” ou apenas lemnas, são consideradas as menores

angiospermas do mundo (JOURNEY et al., 1993). Apesar de possuírem flores e frutos, são de rápida propagação vegetativa, podendo apresentar taxa de crescimento quase exponencial quando comparadas a outras espécies (SREE et al., 2015).

As lentilhas d'água são monocotiledôneas aquáticas classificadas em cinco gêneros: *Lemna, Spirodela*, *Wolffia, Landoltia* e *Wolffiella.* Essas plantas de pequeno porte, apresentam diminuto sistema radicular, suas folhas se distribuem de forma a facilitar a dispersão da planta pelo vento e os rebentos são liberados da planta-mãe precocemente. Com estas características, se deslocam no reservatório pelas ações dos fluxos de água e pelo vento. Neste aspecto, possuem distribuição geográfica e climática mundial, portanto, sendo consideradas cosmopolitas, com centro de dispersão tropical e subtropical na América do Sul e com a maioria das espécies encontrada em climas moderados de zonas tropicais e subtropicais (POTT; CERVI, 1999). Assim, elas se ajustam as mais variadas condições climáticas, com exceção às regiões desérticas e polares (SKILLICORN et al., 1993), além de uma ampla faixa de pH, temperatura e elevadas concentração de nutrientes (LANDOLT; KANDELER, 1987; FEDLER; DUAN, 2011).

As lentilhas-d'água são pouco conhecidas no Brasil, principalmente pela falta de coleta botânica (POTT; CERVI, 1999), porém, apesar dos estudos escassos, o clima e a presença de espécies nativas desta família no país apontam um grande aliado na sustentabilidade do ambiente rural. Essas plantas, são geralmente encontradas na superfície de águas paradas e ricas em nutrientes, sendo confundidas muitas vezes com algas. Possuem pouca fibra, quando comparadas com outras plantas, pois não precisam de tecido estrutural para suportar folhas, o que torna a totalidade dos seus tecidos metabolicamente ativos e atrativos como alimento (JOURNEY et al., 1993).

Apesar do pequeno tamanho das lentilhas-d'água, quando são cultivadas em condições ideais podem dobrar sua massa em aproximadamente 48 horas (LANDESMAN et al., 2005), caracterizando-as como as plantas de crescimento mais rápido entre as angiospermas (ZIEGLER et al., 2015). As lentilhas-d'água têm sido estudadas de forma crescente apenas nas últimas décadas, embora possuam utilização bastante variada e apresentem importância devido ao alto teor inerente de

proteína bruta e amido, chegando a mais de 40% de proteína (HASAN; EDWARDS, 1992) e, dependendo da espécie, até 75% de amido (LANDOLT; KANDELER, 1987).

A espécie utilizada neste trabalho foi a *Lemna aequinoctialis* (Figura 1), cuja classificação é apresentada a seguir:

Reino: Plantae
Divisão: Angiospermae
Classe: Monocotyledoneae
Ordem: Arales
Família: Lemnaceae
Sub-família: Lemnoideae
Gênero: *Lemna*
Espécie: *Lemna aequinoctialis* Welw

Figura 1 - População de lentilhas-d'água dominando a superfície de uma lagoa de tratamento de efluentes (ETE), Recife-PE (a); Imagens de *Lemna aequinoctialis* (b e c). Escala = 1 mm.

Fonte: O autor (2016)

Devido essa rápida velocidade de crescimento associada à capacidade de acumular amido, as plantas da família Lemnaceae podem ser fonte de matéria-prima promissora. Neste aspecto, alterações das condições de crescimento que induzam o estresse, como limitação de nutrientes ou aumento da concentração de sal, geram reduções na taxa de crescimento e, concomitantemente, um significativo acúmulo de

amido, que pode ser convertido em etanol (XU; SHEN, 2011; XU et al., 2012), agregando mais valor ao sistema pelas aplicações biotecnológicas.

Por se tratar de uma alternativa promissora, têm despertado interesse não apenas no âmbito da pesquisa como também dos governos. O Departamento de Energia dos Estados Unidos apoiou, em 2009, um projeto para sequenciar o genoma de *Spirodela polyrhiza*, a qual tem sido investigada como alternativa para complementar a produção de etanol à base de milho (CHENG; STOMP, 2009). A China, por sua vez, lançou um projeto para produzir biocombustíveis líquidos a partir da biomassa de lemnáceas, e apoiado pelo ministro da Ciência e Tecnologia, a primeira conferência internacional sobre aplicação e pesquisa de lemnas foi realizada em 2011 (TAO et al., 2013).

Em ambientes naturais, essas plantas constituem um importante recurso alimentar para aves e peixes, mas também são utilizadas há séculos na alimentação de humanos, principalmente no leste e sudeste asiático. Neste aspecto, um estudo recente realizado, com pelo menos uma espécie de cada gênero, destaca aspectos das lentilhas para consumo humano (APPENROTH et al., 2017). Embora, a forma mais comum e difundida de aplicação da biomassa produzida seja a alimentação de peixes, fornecida em sua forma fresca ou desidratada, em combinação com outras fontes de alimento (IQBAL, 1999). Outra aplicação é a utilização destas plantas como fitoindicadores de toxidez ou genotoxicidade em ambientes contaminados (MARCHAND et al., 2011).

As lentilhas-d'água também são ampla e eficientemente utilizadas com sucesso na biorremediação de águas contaminadas (DHOTE; DIXIT, 2009), por se tratar de uma tecnologia potencial que combina o tratamento de efluentes e a produção de alimento com elevado valor nutricional (APPENROTH et al., 2017), gerando como subproduto, biomassa com potencial energético (ZHAO et al., 2012).

2.2.2 Utilização de Lemnaceae no tratamento de efluentes salinizados

As pesquisas acerca da salinidade dos solos, bem como o aproveitamento dos recursos hídricos, auxiliam nas intervenções sobre como promover a remediação e a implementação de culturas em territórios propensos a estresses abióticos (PEQUENO

et al., 2014). As técnicas de recuperação de solos e águas salinos são de fundamental importância para possibilitarem o retorno ao processo de produção. A fitoextração, técnica de fitorremediação de baixo custo que utiliza espécies de plantas que absorvem e acumulam o sódio na parte aérea, é uma estratégia eficiente de recuperação de solos salinos, apresentando desempenho comparável ao uso de corretivos químicos (QADIR et al., 2007).

O cultivo de lentilhas apresenta dentre outras vantagens, a aptidão de crescimento em água com salinização moderada sem a perda da capacidade de remoção de nitrogênio (N) e fósforo (P) (LIU; DAI; SUN, 2016). Acredita-se, portanto, na potencialidade do uso dessas plantas para tratamento de águas salobras a partir de sua capacidade comprovada de dessalinização pela captação de sal dissolvido (BALLA et al., 2014).

Em condições favoráveis, as macrófitas aquáticas apresentam elevadas taxas de crescimento, devido à eficiência de reprodução vegetativa e alta capacidade de absorção de nutrientes (LIU; DAI; SUN, 2016), o que exige um manejo periódico de retirada de plantas, para que não haja queda na eficiência do tratamento. Assim, a biomassa vegetal pode se transformar em resíduo sólido, que dependendo do destino dado, pode ser considerada um problema ambiental (MOHEDANO, 2010). Todavia, quando trabalhada adequadamente, a biomassa de lentilhas-d'água pode passar da condição de resíduo à matéria-prima com elevado potencial quanto à demanda por biomassa alternativa para produção de etanol (CHENG; STOMP, 2009).

A utilização da biomassa gerada com potencial energético pelas lentilhas-d'água, agrega valor ao processo de fitorremeação como indutora na síntese de amido, com potencial para produção de biocombustíveis, tornando-se uma solução ecológica e econômica para o tratamento de águas (MURADOV et al., 2014). Esse sistema encoraja a produção de biomassa aliado a sistemas de cultivo e processamento ambientalmente sustentáveis (XU et al., 2012).

2.2.3 Utilização de Lemnaceae na produção de biocombustível

A necessidade de combustíveis alternativos surge para minimizar os problemas de energia dos dias atuais como por exemplo, o declínio das reservas de combustíveis

fósseis. Existe a demanda pelo desenvolvimento de processos tecnológicos que apresentem eficiência para a conversão de biomassa em combustíveis de transporte, que sejam tecnologias ambientalmente não nocivas (MURADOV et al., 2010). Nesta discussão, os biocombustíveis ganham destaque e vêm sendo amplamente investigados por serem considerados uma alternativa promissora aos combustíveis tradicionais.

O etanol, por exemplo, é tido como uma alternativa promissora aos combustíveis fósseis. A produção de bioetanol é realizada principalmente em escala industrial a partir de matérias-primas que contêm amido e açúcar, como o milho nos EUA e a cana-de-açúcar no Brasil (SÁNCHEZ; CARDONA, 2008). Este processo de produção pode ser visto como uma forma de reduzir o consumo do petróleo bruto e a poluição ambiental, além de ser um recurso renovável, pois a biomassa vegetal representa um fornecimento potencialmente inesgotável de matéria-prima (DEMIRBAS, 2008). Contudo, o elevado custo de biocombustíveis é considerado um obstáculo à sua comercialização. A matéria-prima constitui cerca de 80% do custo operacional total (DEMIRBAS, 2008), sendo o principal fator a considerar para os custos de produção que limita a comercialização em larga escala. Além disso, possui outros problemas inerentes, incluindo a insuficiência de terras agrícolas (CHEN et al., 2012), o que evidencia, neste caso, que a exploração de matérias-primas alternativas consiste em um tema urgente.

A biomassa das lentilhas-d'água vem sendo considerada ideal por excluir a necessidade de insumos agrícolas e o uso de terras produtivas. Além disso, sua taxa de crescimento é superior a de outras plantas, incluindo culturas agrícolas (LANDOLT; KANDELER, 1987) e, como característica mais importante, são capazes de acumular amido, o que evidencia que elas são candidatas promissoras para a produção de etanol através da hidrólise enzimática do amido e da celulose e posterior fermentação dos açúcares produzidos, usando as tecnologias já existentes para a conversão do amido de milho (XU; SHEN, 2011; XU et al., 2012).

Comprovadamente, o acúmulo de amido, em lentilhas-d'água, pode ser induzido mediante situações adversas, como limitação de nutrientes ou aumento da concentração de sal (XU; SHEN, 2011, CUI et al., 2011; XU et al., 2012; SREE et al. 2015). As plantas, como organismos sésseis, precisam de ajustes frente aos

estresses a que são expostas frequentemente em ambientes naturais, o que pode gerar influência negativa em seu desenvolvimento (SHAO et al., 2007). Assim, o estudo e a compreensão dos efeitos do estresse salino são importantes para aprofundar e ampliar a compreensão sobre o metabolismo de tolerância ambiental.

2.3 Efeitos adversos do estresse salino nas plantas

O estresse, do ponto de vista fisiológico, é a condição causada por fatores que podem alterar o equilíbrio. Neste sentido, pode ser considerado desfavorável quando a demanda de energia pela planta, para sua sobrevivência, é maior que a produção. Em contrapartida, o estresse pode ser importante no desenvolvimento dos vegetais, sem causar danos quando significa uma restrição, no sentido de sobrevivência. Desse modo, quando uma condição de estresse suave e estimulante que ativa o metabolismo celular e incrementa a atividade fisiológica da planta, comporta-se como um fator positivo que impulsiona o crescimento vegetal (LICHTENTHALER, 2004).

Fatores abióticos são capazes de ocasionar estresse a ponto de alterar o estado fisiológico de um organismo vegetal. O estresse salino, por exemplo, pode interferir negativamente no desenvolvimento das plantas através de mecanismos formados por dois componentes: osmótico e iônico. O componente osmótico é resultado da elevada concentração de solutos na solução do solo, ocasionando um déficit hídrico pela redução do potencial osmótico (BRITTO et al., 2010; WILLADINO; CAMARA, 2010). O componente iônico refere-se ao acúmulo de íons no citosol das células, provocando problemas de toxidade, principalmente dos íons Na^+ e Cl^-, e desequilíbrio nutricional, decorre da interferência na absorção de nutrientes, destacando-se a redução da concentração de K^+ (WILLADINO et al., 2011; NASCIMENTO et al., 2015),

O sódio (Na^+) é considerado um íon potencialmente tóxico às plantas (SILVEIRA et al., 2012). Sua influência negativa à absorção de potássio (K^+), diminuído pela redução do influxo do nutriente para a célula, altera a relação K^+/Na^+ nos tecidos (BRITTO et al., 2010). Tanto Na^+ quanto K^+ apresentam o mesmo raio iônico e utilizam os mesmos carreadores nas membranas celulares (APSE; BLUMWALD, 2007; WILLADINO; CAMARA, 2010; NASCIMENTO et al., 2015), assim, a capacidade das células vegetais para preservarem o K^+ citosólico frente à salinidade

tem sido apontada como uma das características de tolerância ao estresse salino (SHABALA et al., 2005, 2010).

O acúmulo de sais pode gerar várias alterações nas células vegetais, tais como: restrição das trocas gasosas (parcial ou totalmente), limitando os processos fotossintéticos mediante um efeito tóxico direto sobre os processos bioquímicos e fotoquímicos nos cloroplastos (MUNNS; TESTER 2008; SARAVANAVEL et al., 2011); menor disponibilidade de carboidratos, substratos necessários para o crescimento do vegetal; reduz o teor de proteínas, devido ao aumento da proteólise (PARIDA; DAS, 2005) e altera o metabolismo de lipídios em função da maior intensidade de oxidação. Ademais, provoca desequilíbrio no sistema redox, gerando estresse fisiológico, que representa grande empecilho ao crescimento e desenvolvimento de várias espécies de plantas (SREE et al., 2015).

Para enfrentar as injúrias causadas pelo excesso de sal, a planta precisa direcionar energia metabólica para absorção de água pelas raízes e para realizar os ajustes bioquímicos necessários à sobrevivência sob estresse, desviando, portanto, a energia do crescimento e dos processos produtivos (RHOADES et al., 2000). As espécies vegetais diferem bastante na resposta à salinidade, deste modo, após a percepção da salinidade, aquelas mais sensíveis sofrem de imediato os efeitos, refletindo negativamente no metabolismo, crescimento e desenvolvimento (LICHTENTHALER, 2004). Sob esta ótica, a tolerância envolve um processo de ajustamento resultante de uma alteração no potencial osmótico causado pelo acúmulo de solutos compatíveis (MAHAJAN; TUTEJA, 2005). Essa estabilização osmótica condiciona o vegetal para suportar situações adversas e inesperadas (ZHU, 2002; BECK et al., 2007; WANG et al., 2012).

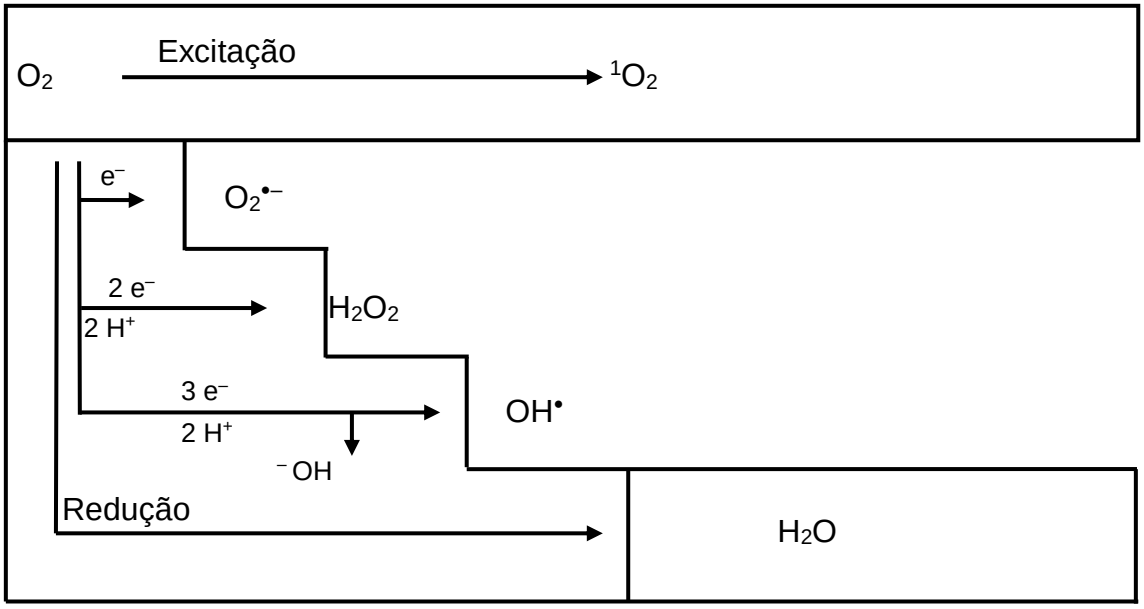
O ajuste osmótico favorece a manutenção do turgor e do volume celular, garantindo o crescimento vegetal ainda que a uma taxa reduzida (WILLADINO; CAMARA, 2010). No processo de tolerância à salinidade, no que se refere à toxidez iônica pelo sódio, a planta evita ou reduz os danos através de três mecanismos: exclusão iônica, que diminui a concentração de sal no citoplasma (ZHU, 2001; MUNNS, 2005); restrição da entrada de Na^+ pela seletividade na absorção de íons e compartimentalização vacuolar de Na^+ e Cl^- (LEBAUDY et al., 2007; MAHAJAN et al., 2008), evitando assim a citotoxicidade destes íons.

2.3.1 A salinidade como fator de estresse oxidativo, um estresse secundário.

A presença do oxigênio molecular (O_2) na atmosfera terrestre é consequência do metabolismo fotossintetizante. No processo de respiração celular, o oxigênio é completamente reduzido ao longo da cadeia respiratória por quatro elétrons, gerando duas moléculas de água (SOARES; MACHADO, 2007) (Figura 2). Entretanto, na cadeia respiratória, por exemplo, podem escapar alguns, resultando em uma redução parcial do O_2, formando moléculas extremamente reativas (MITTLER, 2002).

As espécies reativas de oxigênio (ROS) são geradas como subprodutos de vias metabólicas em distintos compartimentos celulares e podem causar danos oxidativos a diversos componentes celulares, são elas: o radical aniônico superóxido ($O2^{\bullet-}$), o peróxido de hidrogênio (H_2O_2), o radical hidroxílico ($\bullet OH$) e oxigênio “singleto” ($^1O_2$), formado pela excitação do oxigênio molecular (MITTLER, 2002; FOYER; NOCTOR, 2005). As ROS participam de uma sofi sticada rede de vias de sinalização em plantas, em resposta a situações de estresse. Essas espécies químicas têm influência na expressão de vários genes envolvidos no metabolismo e em vias de transdução de sinais (BARBOSA et al., 2014).

Figura 2. Formação de espécies reativas de oxigênio: oxigênio singleto ($^1O_2$), radical superóxido ($O_2^{\bullet-}$), peróxido de hidrogênio (H_2O_2) e radical hidroxila ($OH^{\bullet}$).



Fonte: adaptado de Bartosz, 1997.

Estresse salino pode provocar a geração excessiva dessas espécies reativas. Em condições de equilíbrio, as ROS são eliminadas por vários mecanismos antioxidativos (FOYER; NOCTOR, 2005; BARBOSA et al., 2014). Essas espécies químicas podem, inclusive, atuar na sinalização celular nos processos de crescimento, desenvolvimento e aclimatação ao estresse, agindo, portanto, como “moléculas sinalizadoras” ou “mensageiros secundários” (DEL RÍO et al., 2006; BARBOSA et al., 2014; PASSAIA; MARGIS-PINHEIRO, 2015).

A atividade biológica das ROS em plantas já foi evidenciada com ênfase na função do H_2O_2 como molécula sinalizadora (HUNG et al., 2005). Porém, quando a planta é exposta a condições adversas como seca, salinidade, alta temperatura, patógenos etc., ocorrem alterações na homeostase celular e a produção de ROS torna-se maior do que a capacidade antioxidante da célula, caracterizando assim o estresse oxidativo (PANDHAIR; SEKHON, 2006; GIL; TUTEJA, 2010). O estresse oxidativo, portanto, pode ser definido como um desequilíbrio, em qualquer compartimento celular, entre os níveis endógenos de compostos antioxidantes e compostos oxidantes, ocasionando o acúmulo de ROS (CASSELLS; CURY, 2001, MØLLER, 2001).

As ROS podem ser encontradas no apoplasto, citosol, cloroplasto, mitocôndria e peroxissomo (NOCTOR et al., 2004). Vários fatores bióticos ou abióticos podem quebrar a homeostase celular e causar o acúmulo de ROS, que acarretam distúrbios metabólicos graves, com alterações no sistema redox e danos a macromoléculas (GIL; TUJEDA, 2010). No processo fotossintético, a fotorredução do oxigênio em ânion superóxido, conhecida como Reação de Mehler, bem como a formação de oxigênio singleto quando a energia armazenada na clorofila não é dissipada, e sim transferida para o O_2, promovem um incremento na produção de ROS (BHATTACHARJEE, 2010).

A toxicidade dos compostos oxidantes está na sua capacidade de iniciar cascatas de reações que resultam em danos, como por exemplo, a peroxidação lipídica (PORPÍSIL, 2016), que envolve um conjunto de reações em cadeia resultante da ação das ROS sobre os ácidos graxos insaturados dos lipídeos das membranas celulares, resultando na formação de hidroperóxidos lipídeos e aldeídos, tais como o malondialdeído, que pode ser detectado em amostras biológicas e utilizado para

avaliar o grau de dano membranar (HALLIWELL; CHIRICO, 1993). A peroxidação lipídica pode ser mediada por •OH e $^1O_2$ (TRIANTAPHYLIDÈS et al., 2008; PORPÍSIL, 2016). Além disso, o acúmulo de ROS pode afetar severamente a funcionalidade e integridade celular com alterações na homeostase redox, danos a macromoléculas e desnaturação de proteínas (PARIDA et al., 2004; BEN-AMOR et al., 2006; GILL; TUTEJA, 2010).

Para desintoxicar a célula das ROS, várias estratégias são utilizadas pelas plantas e outros organismos, interrompendo as cascatas de oxidação (FOYER; NOCTOR, 2009). Uma dessas estratégias consiste na síntese de compostos de baixo peso molecular e enzimas antioxidativas, que constituem o sistema antioxidante e faz parte do metabolismo natural, mas pode ser intensificado em condições de estresse, sendo considerado um dos principais mecanismos de defesa (FOYER; NOCTOR, 2005).

2.3.2 Sistema antioxidativo

Para as plantas, a estratégia de sobrevivência às condições adversas é atingir um estado de equilíbrio. Para tanto, uma série de processos são ativados a partir de alterações da expressão gênica, resultando em alterações fisiológicas e bioquímicas. A evolução de diferentes respostas metabólicas e a habilidade na percepção dos sinais ambientais externos são considerados mecanismos desenvolvidos e aprimorados (FUJITA et al., 2006).

O processo pelo qual a planta percebe os diferentes sinais de estresse e os transmite para toda a maquinaria celular é chamado de transdução de sinais (XIONG; ZHU, 2002). Foi necessário às plantas desenvolverem, ao longo da escala evolutiva, essa maquinaria de proteção para manter a homeostase redox celular, resultando em processos como a ativação de enzimas do sistema antioxidativo e síntese de metabólitos de baixo peso molecular, como o ascorbato e a glutationa (STASOLLA, 2010), a prolina (RADYUKINA et al., 2008) entre outros. Esse complexo sistema constituído por enzimas e metabólitos antioxidantes atua no combate aos efeitos prejudiciais do acúmulo das ROS (DEWIR et al., 2006), seja na remoção ou na neutralização dessas moléculas.

As enzimas do sistema antioxidante são bastante sensíveis às condições de estresse abiótico e servem, inclusive, como indicadora do estresse. Destacam-se dentre elas: superóxido dismutase (SOD), catalase (CAT) e ascorbato peroxidase (APX). Trabalhos realizados com lentilhas-d'água submetidas a estresse mostraram alterações no padrão das enzimas supracitadas (PEVALEK-KOZLINA, 2010; CHENG, 2011; CHANG et al., 2012; CHENG; CHENG, 2012; PARLAK; YILMAZ, 2012).

A acumulação de Na^+ e Cl^- em plantas pode modular as atividades de enzimas antioxidantes. As superóxidos dismutases dismutam o $O_2^{\bullet-}$, ocasionando a formação de outra ROS, o H_2O_2. As SOD são divididas em três grupos: a CuSOD e ZnSOD (contendo cobre e zinco, respectivamente) estão localizadas no citosol e cloroplastos; a FeSOD (contendo ferro) está presente nos cloroplastos; a MnSOD (contendo manganês) é encontrada nas mitocôndrias e peroxissomos (ALSCHER et al., 2002). As enzimas APX e CAT, que comandam a eliminação de H_2O_2, possuem diferentes afinidades por essa ROS. A APX tem alta afinidade (μM) e está presente em praticamente todos os compartimentos da célula, enquanto a CAT, está localizada nos peroxissomos/glioxissomos, possui baixa afinidade (mM) (MITTLER, 2002). Além disso, as APX podem eliminar H_2O_2 que estão inacessíveis à CAT, como no caso dos cloroplastos (FOYER; NOCTOR, 2011).

A distribuição subcelular dessas enzimas sugere que a APX cloroplástica remove o H_2O_2 produzido durante a reação de Mehler e outros processos cloroplásticos (NAKANO; ASADA, 1981), enquanto a CAT elimina o H_2O_2 fotorrespiratório. Porém, vale ressaltar que o H_2O_2 não é estritamente compartimentalizado, sendo capaz de se difundir livremente através das membranas.

A regulação redox tem sido demonstrada em diversos processos, como o movimento estomático (CHEN; GALLIE, 2004). Os produtos gênicos ativados nessas respostas podem estar envolvidos na transdução de sinais e na regulação da expressão de genes associados aos mecanismos de tolerância a (FOYER; NOCTOR, 2009; FOYER et al., 2012). Dentre eles estão os genes que codificam e modulam as proteínas da síntese metabólica, antioxidantes, proteínas estabilizadoras de membrana e síntese de osmoprotetores, chaperonas e detoxificação (MAHAJAN; TUTEJA, 2005; GRENNAN, 2006; SHAO et al., 2007).

As vias de transdução específicas de sinais são ativadas em nível celular e influenciam diretamente a expressão de proteínas. No entanto, a revisão realizada por Boareto e Mazzafera (2013) aponta que poucos estudos têm sido realizados na aplicação da proteômica, que envolve o estudo em larga escala das proteínas expressas, frente à demanda do tema biocombustível. Esse quadro não teve muitos avanços nos últimos anos, o que destaca, a necessidade de estudos associados à abrangência da complexidade genética de culturas bioenergéticas.

2.4 Proteômica

As plantas respondem ao estresse ambiental ativando mecanismos de expressão gênica diferencial, o que resulta em mudanças em nível transcricional, traducional e metabólico. Neste contexto, o aumento dos estudos genômicos e pós-genômicos vem possibilitando a disponíbilidade de maior quantidade de informação na literatura. Contudo, é possível constatar que apenas os dados de expressão genética não revelam a complexidade de respostas moleculares às adversidades, tal como o estresse salino, e dessa forma, o nível da expressão dos transcritos nem sempre é correlacionada com o padrão celular das proteínas expressas (PIQUES et al., 2009; BAERENFALLER et al., 2012).

A justificativa para tal perspectiva é o fato de que alterações no comportamento celular podem ser reguladas por proteínas pré-existentes, que são modificadas pós-traducionalmente ou são degradadas, além disso, a possibilidade de variações no processamento do RNAm, por *splicing* alternativo, permite a expressão de proteínas variantes a partir de uma mesma sequência gênica (QUIRINO et al., 2010). Uma vez que as proteínas são os efetores diretos da resposta ao estresse e estão mais próximas dos fenômenos fisiológicos, há vantagens em estudos de proteômica visando conhecer o mapa proteômico expresso em determinada condição estressante, buscando assim uma compreensão mais profunda das alterações de regulação gênica e, assim, dos mecanismos de regulação mediante as condições adversas (HUANG et al., 2014).

A análise proteômica, portanto, é uma técnica disponível para o estudo de proteínas em larga escala (PANDEY; MANN, 2000) e pode ser utilizada para estudar

a expressão diferencial de genes associados a respostas em condições adversas. A nível conceitual, proteoma refere-se às proteínas expressas pelo genoma de uma célula, sob condições específicas ou estádio de desenvolvimento (HUANG et al., 2014). Assim, a proteômica é um conjunto de metodologias utilizadas para caracterizar um proteoma, permitindo a criação de mapas de proteínas para organismos inteiros com determinações qualitativas ou quantitativas de um grande número de proteínas envolvidas no metabolismo celular (CAO et al., 2008).

O estudo proteômico pode fornecer importantes informações para identificar, quantificar e estudar as modificações pós-traducionais das proteínas (PANDEY; MANN, 2000). Os avanços tecnológicos permitiram o aumento da cobertura do proteoma e a inferência de aspectos quantitativos de expressão de proteínas, dando suporte a proteômica quantitativa, a qual vem se destacando nas pesquisas com plantas de interesse biotecnológico e comercial (VADERSCHUREN et al., 2013).

A eletroforese bidimensional (2D-PAGE) permite a separação de misturas complexas de proteínas. Essa ferramenta é comumente utilizada para revelar o proteoma de células, tecidos e organismos (QUIRINO et al., 2010). Ela consiste em analisar as proteínas por eletroforese bidimensional e posteriormente sua identificação via espectrometria de massas (MS), sendo, portanto, técnicas utilizadas para análise de proteomas diferenciais (ROCHA, 2005).

A técnica 2D-PAGE é atualmente um dos métodos mais comumente utilizados em estudos proteômicos (QUIRINO et al., 2010; GAUCI et al., 2013). Nessa técnica é possível separar as proteínas de uma determinada amostra de interesse, que inicialmente são separadas pelo seu ponto isoelétrico (p*I*) (na primeira dimensão), por meio da focalização isoelétrica, e posteriormente, pela sua massa molecular (na segunda dimensão). A combinação dessas duas etapas possibilita a separação de inúmeras proteínas, culminando com a construção de ricos mapas proteicos (ANDRADE, 2006). Na 2D-PAGE, as proteínas (*spots*) podem ser visualizadas, o que permite o controle da qualidade e reprodutibilidade do processo de preparação da amostra (SMITH, 2009).

As amostras biológicas, bem como, as repetições técnicas são comparadas usando vários programas computacionais, por exemplo, o ImageMaster, gerando resultados com maior confiabilidade. Com base na análise estatística dos géis, as

proteínas consideradas diferencialmente expressas são excisadas e processadas, posteriormente, são identificadas por análise de espectrometria de massas (MS) (QUIRINO et al., 2010). A MS é considerada uma tecnologia indispensável para interpretação da informação codificada do genoma obtidas através da eletroforese bidimensional.

A espectrometria de massas é capaz de determinar a massa de moléculas a partir da relação entre a massa e a carga (*m/z*) de espécies ionizadas em fase gasosa, além de ser uma técnica sensível e rápida (CUNHA et al., 2006). O espectrômetro de massas é um instrumento formado por um analisador de massas, uma fonte de íons, um detector e um sistema de aquisição de dados. Ainda de acordo com os autores, os analisadores de massas podem ser acoplados entre si, permitindo a ocorrência de experimentos em sequência (MS/MS), sendo possível detectar determinado peptídeo e, na sequência, submetê-lo a uma etapa de fragmentação para determinação da sequência de aminoácidos (CUNHA et al., 2006).

A informação da sequência de aminoácidos dos peptídeos fragmentados (MS/MS), bem como os dados da massa molecular dos peptídeos originados da digestão enzimática (*Peptide Mass Fingerprint* - PMF), são comparados por meio de programas da bioinformática com um banco de dados contendo sequências de proteínas conhecidas ou o genoma do organismo (ELIAS et al., 2005). Isto é possível por meio de programas como o MASCOT, que tem a capacidade de simular as sequências primárias potenciais das proteínas, baseados na suposta predição da informação contida em bancos de genes, clivando teoricamente estas supostas proteínas em peptídeos, e posteriormente, calculando a massa absoluta dos peptídeos a partir de cada proteína. Em resumo, o MASCOT compara massas de peptídeos da proteína alvo de estudo, desconhecida, à massa teórica de peptídeos da proteína depositada em cada base de dados (QUIRINO et al., 2010).

De maneira geral, a análise proteômica identifica não apenas o conjunto de proteínas de uma amostra, mas também caracteriza as inúmeras isoformas das proteínas, produtos de modificações pós-traducionais e a interação dessas proteínas entre si (TYERS; MANN, 2003). O objetivo de se traçar um perfil proteômico é a obtenção de proteínas que sirvam como marcadores moleculares funcionais relacionados ao estresse em questão. Esses marcadores podem ser obtidos

diretamente do DNA ou podem ser marcadores bioquímicos, como são chamados os marcadores de origem protéica (KOSOVÁ et al., 2011). Assim, a identificação de proteínas (genes) responsáveis por qualidades agronomicamente desejáveis e sua posterior manipulação por meio de técnicas de biologia molecular pode-se acelerar o ritmo do melhoramento e reduzir as perdas na agricultura, além de permitir o aproveitamento de áreas.

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CAPÍTULO I

Efeito do estresse salino e acúmulo de amido em lentilhas-d'água (*Lemna aequinoctialis*, Lemnaceae)

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Salt Stress Induces Increase in Starch Accumulation and Different Biochemical and Physiological Responses in Lesser Duckweed (*Lemna aequinoctialis*, Lemnaceae)

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Abstract

Duckweed has emerged as promising crop plants for biomass production, in addition to present relevant natural characteristics for biology studies and plant biotechnology and compatible processes and practical applications. For example, the salt stress is capable of inducing high levels of starch accumulation, while the salinity tolerance can contribute to expanding the potential biotechnological applications of these plants. However this feature, tolerance to salinity has wide intraspecific variation. In this study, antioxidant processes were searched in macrophyte duckweed in order to investigate tolerance mechanisms in this species against oxidative damage caused by salinity stress. Biochemical and histological analyses were performed to four *Lemna aequinoctialis* (lesser or common duckweed) grown in different NaCl concentrations (0, 25 and 50 mM). For most clones, the salt stress effects caused growth inhibition and antioxidant responses at 50 mM NaCl. Also, starch and reducing sugars accumulation were increased with salt, while the photosynthetic pigments content was reduced in clone U1. The plants growth inhibition reflects the oxidative stress, shown by the significant increase in malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content. In RCclone, with the highest MDA levels, no antioxidant enzymatic activity was observed. However, a significant increase in superoxide dismutase (SOD) was detected in the other clones. The M1 clone presented, in parallel, higher ascorbate peroxidase (APX) and catalase (CAT) activities, indicating that efficiency of defense mechanism relies on synchrony between such enzymes activity towards successive elimination of reactive oxygen species (ROS) and resulting in the assurance of some level of protection to metabolism from oxidative damage. Considering moderate salt stress (25mM), the maintenance of MDA content and the small growth diminishing associated to the high starch production suggested the acclimation efficiency of M1 and DI clones, pointing them as suitable for cultivation under moderate saline conditions, serving as biofuels feedstock.

Keywords: antioxidant system, aquatic macrophyte, biomass, duckweed, *in vitro* culture.

Introduction

Duckweeds belong to monocotyledon class and are aquatic plants known as the smallest angiosperms. Those macrophytes are classified in five genera: *Lemna, Spirodela, Wolffia, Landoltia* and *Wolffiella*; they present cosmopolitan distribution and have been described in tropical and subtropical environments with or without seasonal occurrence. In brazil were found in the northeast semiarid '*Caatinga'*, the swamp *'pantanal mato-grossense'* and the Atlantic rainy forest [1]. Despite the wide distribution, the geographic differentiation of duckweeds has significant role on their genetic diversity, even in regional scale [2], what influences in characterization and application studies.

The rapid propagation, with almost exponential growth rate with short doubling time (2-3 days), is the physiological basis for practical usages of duckweed [3]. Its cultivation also stands out due to the ability to grow on brackish and wastewaters [4] and to the usage for animal feeding, considered a good protein source [5]. The excellent growth associated to the ability to accumulate starch have pointed growing attention to duckweeds as feedstock for bioethanol production [6–8], a proven fuel to attend renewable energy demand. In such aspect, Lemnaceae starch content can be increased in plant exposure to stressing factors, like nutrient deprivation, high or low temperature, pH, light intensity, photoperiod and salinity [4,6–8], and make possible the use of its biomass [6].

Plants, in their natural habitat, are subjected to several abiotic conditions that change their metabolism, among which the salinity is noteworthy. The increase in the amount of waters disturbed by salinization may trigger imbalance in plant redox system and cause physiological stress, which may represent a big impairing to growth and development [4], in addition to induce changes and reprogramming of biochemical pathways, physiological and morphological processes [9,10]. The study of oxidative stress related to plant abiotic stressing factors has advanced considerably [11], and with the elucidation of mechanisms responsible for the adaptation, the identification of plants with higher tolerance/resistance to saline environments becomes increasingly important [4,12].

The production of reactive oxygen species (ROS) is a common feature in aerobic organisms during normal growth process, however, in plants under stress there is an excessive ROS production that leads to oxidative stress [13], able to cause damages in cell structures and even result in plant death. Salinity induces stress by its osmotic component as well as through ionic toxicity [14,15], and can specifically activate the antioxidant defense system [16], the mechanism which acts by the synthesis of metabolites and the activation of an specific enzymatic system [17]. In this system, the enzymes catalyze the decomposition of oxidant molecules, that might impair the cell integrity and functionality, frequently causing irreversible damages [18].

Here are presented effects of salt stress on growth, physiological responses and antioxidant defense of four clonal axenic cultures of lesser duckweed (*Lemna aequinoctialis*). The obtained information are relevant tools that may improve the understanding of duckweed biological mechanisms adopted to circumvent the stress induced by exposure to higher NaCl concentrations, and the resulting starch accumulation.

Material and Methods

Plant material and pre-cultivation

The *L. aequinoctialis* clones used in this work, named M1, U1, RC and DI, integrate a duckweed collection maintained in sterile conditions in the Plant Tissue Culture Laboratory at Universidade Federal Rural de Pernambuco (Recife, PE, Brazil). Plant material, listed in Table 1, was collected in Recife metropolitan region and kept in local effluent until asepsis. Fronds were submitted to disinfection during 30 sin 1% sodium hypochlorite (NaClO) solution. In sterile conditions, about 200 fronds were washed three times in sterile distilled water and were transferred onto half-strength SH culture medium (0.5 x SH [19]), added with 10 $g.L^{-1}$ sucrose and pH adjusted to 5.8 prior autoclaving. After plants recovery and novel fronds sprouting, a unique frond was transferred to fresh

0.5 x SH culture medium, in order to assure clonal propagation and genetic uniformity. Cultivation was kept in growth room at 25 ± 2ºC, under light intensity of 57,5 µmolm^{-2} s^{-1} and photoperiod of 16 h.

Table 1. Duckweed harvests in Recife metropolitan region, PE, Brazil. Clones: identification code in clonal collection. Geographic coordinates of harvest locations (latitude, longitude).

Localização	Mês/Ano	Latitude e Longitude	Acess
ETE, Recife-PE	12/2013	S 8º4'37", O 34º55'30"	M1
CCEN-UFPE, Recife-PE	12/2013	S 8º4'27", O 34º57'8"	U1
Rio Capibaribe, São Lourenço da Mata	01/2015	S 7º59'59", O 35º2'45"	RC
Dois Irmãos, Recife-PE	01/2015	S 8º1'22", O 34º50'20"	DI

Cultivation under salt stress

Initially, 100 fronds of each pre-cultivated clonal population were randomly selected as inoculum for experimental phase as well as for growth measurements. To determine the growth rate, fronds were transfered to 350 mL glass flasks containing 50 mL 0.5 x SH sterile culture medium, and submitted to treatments: no NaCl (T0 = control) and NaCl (at T1= 25 mM; or T2 = 50 mM; moderate and severe stress, respectively). The NaCl concentrations was 0, 25 and 50 mM, and correspond to electrical conductivity (EC) of 1.81, 5.09 and 7.57 dS cm^{-1}, respectively, as measured with a probed conductivimeter (Soil Control, Inc.).

The relative growth rate was determined from three samples in each curve point, clone and stress condition (every two days). Each sample weight was determined after rapid quick superficial drying the fronds on sterile paper towel. Experimental period comprised 10 days, and growth curve was observed until 15th day to ensure the monitoring of the exponential phase. Design was completely randomized in 4x3 factorial treatment arrangement (four clones and three NaCl concentrations), with three replicates (n=3). Data were submitted to variance analysis and the averages were compared by

Tukey test at 5% probability. In addition, were also used for principal component analysis (PCA) and grouping analysis with the software FITOPAC 2.1 [20]. PCA was performed for different variables groups and based on their correlation matrix.

Growth rate, biomass yields and growth inhibition

Growth was evaluated from relative growth rates (RGR) that express the mass increase, by initial weight unit, in a time gap. Relative growth rates per point (equation 1) were calculated according to Ziegler et al. [21], where 'ln' = neperian logarithm; Wl and W2 represent the fresh weight in T1 and T2 (time 1 and time 2), respectively. The biomass yield was evaluated from the equation 2, 'S' is the area occupied by the culture at the available substrate for each replicate separately:

$$RGR = (\ln W2 - \ln W1) / (T2 - T1) \quad (1)$$

$$(W2 - W1) / S / (T2 - T1) \quad (2)$$

Growth inhibition rate (GIR) was estimated based on the comparison between control plants and the remaining treatments and results were expressed in percent of inhibition, according to the following formula:

$$GIR\ (\%) = (1 - \text{fresh weight of treated plants} / \text{weight of control plants}) \times 100 \quad (3)$$

Quantitation of H_2O_2

Hydrogen peroxide content was quantitated according to Alexieva et al. [22] protocol. Fresh samples were grinded with trichloroacetic acid (TCA) 0.1% in 1 g/10 mL (w:v) relation. Samples were centrifuged at 10,000x*g* for 15 min, at 4ºC. Supernatant (200 µL) were transferred to new tube and added by 200 µL of potassium phosphate buffer 100 mM (pH 7.5) and 800 µL of 1 M potassium iodide. Samples were kept on ice and dark during 1 h. After that period, they were removed from ice

and kept in room temperature for reaction stabilization, followed by reading in spectrophotometer at 390 nm.

Quantitation of malondialdehyde in plant tissue (MDA)

Lipidic peroxidation was determined according to Health and Packer [23], with modifications. Reaction was determined by the production of MDA, ametabolite reactive to 2-thiobarbituric acid (TBA). Biological samples were grinded in TCA 0.1% in the proportion of 0.2 g/2 mL buffer, with 20% PVPP (polyvinylpolypirrolydone). After homogenization, sample was centrifuged at 10,000 x*g* during 5 min. A 200 µL aliquote from supernatant was transferred to other tube with 1.0 mL solution containing 20% and 0.5% TBA. The mix was kept in water bath at 95ºC during 30 min and, in following, was rapidly cooled for 10 min. Before reading in spectrophotometer at 535 and 600 nm, samples were centrifuged for additional 10 min at 10,000 x*g*.

Protein extraction

Frozen samples were grinded and homogenized in potassium phosphate buffer 100 mM (pH 7.5), 1 mM EDTA, 3 mM DTTand 20% PVPP on ice, in a 1 g/3mL (w:v) relation [24]. The homogenized was centrifuged at 10,000x*g* during 30 min and the supernatant was separated in aliquotes and stored at -20 ºC until the determination of enzymatic activities.

Protein quantitation

Total proteins concentration was determined according to Bradford [25] method, using BSA (bovine serum albumin) as standard. Reaction was performed by adding 20 µL sample (previously diluted) to 1 mL Bradford reagent and incubated at room temperature for 5 min.

Catalase (CAT)

The activity of CAT was determined by the method described by Havir and Mchale [26] with modifications according to Azevedo et al. [24], in a solution containing 1.5 mL potassium phosphate buffer 100 mM (pH 7.5) and 60 µL hydrogen peroxide at 0.1 M. The reaction was started by adding 50 µL protein extract and the activity was determined following the H_2O_2 decomposition for 60 s, through alterations at 240 nm, 25ºC, in spectrophotometer absorbance read. Results were expressed in µmol.mg de protein^{-1}.min^{-1}, considering one unity of CAT activityable to decompose 1 µmol H_2O_2 per mg protein in 1 min at pH 7.5.

Ascorbate peroxidase (APX)

The activity of APX was determined in accordance to Nakano and Asada [27]. The reaction medium was composed by 650 µL potassium phosphate buffer 80 mM, pH 7.5, 100 µL ascorbate 5 mM, 100 µL EDTA 1 M, 100 µL H_2O_2 1 mM and 50 µL protein extract. APX activity was determined by monitoring the ascorbate oxidation rate at 290 nm, 30ºC, during 60 s, in spectrophotometer. Results were expresses in µmol.mg de protein^{-1}.min^{-1}, considering one unity of APX activity able to convert 1 µmol ascorbic acid in monodehydroascorbate during 1 min.

Superoxide dismutase (SOD)

The protocol was followed according to Giannopolitis and Ries [28], with minor modifications. By this method, it is determined the inhibition of NBT (nitro blue tetrazolium) reduction by enzymatic extract, thus avoiding the formation of the formazan chromophore. The reaction solution (3 mL) was composed by 85 mM phosphate buffer (pH 7.8), 75 µM NBT, 5 µM riboflavin, 13 mM methionine, 0.1 mM EDTA and 50 µl enzymatic extract. The solution was transferred to glass tubes and irradiated with white light (fluorescent 15 W lamp) for 5 min. After exposure time, the solution was analyzed

inspectrophotometer at 560 nm. One activity unit was defined as the amount of enzyme that inhibits 50% of formazan formation per gram of protein, with results expressed in U.mg protein^{-1}.

Photosynthetic pigments

The determination of chlorophyll and carotenoids contents was performed by the method with extraction in acetone 80%, according to Lichtenthaler and Wellburn [29]. Based on absorbance obtained from samples in spectrophotometer in wavelengths 470, 645 and 663 nm, the content of chlorophyll *a*, *b*, total [30] and carotenoids [29] was determined.

Starch content

The amount of starch was determined by colorimetric method as defined by Appenroth et al. [31]. Fresh plant material was grinded in HCl 18% (w/v), incubated at 5ºC for 1 h. Following, the suspension was centrifuged at 5,000x*g* for 20 min. An aliquote from supernatant was added in equal volume of lugol solution [KI 0.5% (w/v) and I_2 0.25% (p/v) in distilled water] to determine absorbance at 530 and 605 nm. Absorbance values were used in equation described for starch quantitation [31].

Reducing sugars content

The calorimetric method was used with 3,5-dinitrosalicylic acid (DNS), described in [32], for 250 mg samples, by dilution in water and addition DNS reagent. The mix was kept under warming in water bath at 100°C (boiling) during 5 min. Samples were cooled and had their absorbance determined at 540nm. For spectrophotometric standard curve preparation for reducing sugars, it was used a solution of glucose 1g.L^{-1}.

Inorganic solutes

The Na^+ and K^+ concentrations was obtained from dry biomass of grinded fronds submitted to nitroperchloric digestion, followed by flame emission photometry analysis according to [33] and [34].

Anatomical analysis

A part of the fresh fronds were cut freehand and submitted to lugol for starch detection [35]. Frond samples were fixed in FAA70 [36] being dehydrated in an ethylic series [36] an then included in paraffin. The samples were cut with a rotary microtome (LUPTEC/MRP09) being obtained with 5 μm cross sections were subjected to double staining with Alcian blue and safranin, mounted on permanent blades with Canada's balm [37]. Part of fixed fronds was separated for stomatal density analysis, immersed in solution of hydrogen peroxide and acetic acid, washed in water [38]. Images were registered from light microscope (Coleman), with corresponding scales obtained in the same optical conditions. The stomatal densitywas determined from paradermal sections and from in an area of 200 μm^2 with 400-fold increase (n = 36 per treatment), calculating the average amount of stomata per mm^2.

Results

NaCl effects on growth rate, Na^+ accumulation, pigments content, starch content and stomatal density

The electrical conductivity dynamics in the culture medium was monitored along the experiment, showing a continuous reduction with the time passing (Table 2). Electrical conductivity presented higher values in treatments with 25and 50 mM due to the increase of Na^+ and Cl^-, which may interfere in nutrient and water absorption in plants.

Table 2. Average values of electrical conductivity (dS.cm $^{-1}$.g $^{-1}$) in *Lemna aequinoctialis* submitted to different NaCl levels (Average ± SE, n=3).

Clones	Days	NaCl (mM)		
		0	**25**	**50**
M1	0	1.81 ± 0.00	5.09 ± 0.00	7.57 ± 0.00
	10	0.70 ± 0.04	3.65 ± 0.05	6.42 ± 0.02
	15	0.24 ± 0.02	3.32 ± 0.01	6.32 ± 0.05
U1	0	1.81 ± 0.00	5.09 ± 0.00	7.57 ± 0.00
	10	1.06 ± 0.04	3.61 ± 0.16	6.46 ± 0.12
	15	0.77 ± 0.09	3.54 ± 0.05	6.27 ± 0.08
RC	0	1.81 ± 0.00	5.09 ± 0.00	7.57 ± 0.00
	10	1.62 ± 0.04	3.61 ± 0.1 6	6.56 ± 0.05
	15	0.80 ± 0.07	3.58 ± 0.08	6.51 ± 0.06
DI	0	1.81 ± 0.00	5.09 ± 0.00	7.57 ± 0.00
	10	1.34 ± 0.04	3.82 ± 0.03	6.56 ± 0.13
	15	0.34 ± 0.04	3.09± 0.03	5.58 ± 0.17

Exposure of *L. aequinoctialis* to moderate and severe stress lead to significant Na^+ increase in plants. In contrast, independently of the clone, there was a progressive K^+ reduction according to NaCl level increase in culture medium. The clone U1 stood out by presenting half the K^+ amount when compared to the other plants treated with 50 mM. The Na^+/K^+ ratio increased notably with growing salinity (Table3).

Table 3. Effect of different NaCl concentrations on Na and K (g/kg) accumulation in *Lemna aequinoctialis* submitted to different NaCl levels (Average ± SE, n=3).

Clones	NaCl (mM)	Na^+	K^+	Na^+/K^+
M1	0	0.56 ± 0.03 aB	64.89 ± 1.42 bA	0.01
	25	14.79 ± 0.20 aA	34.22 ± 1.86 bB	0.44
	50	16.28 ± 0.43 aA	15.20 ± 1.91 abC	1.13
U1	0	0.86 ± 0.05 aB	66.64 ± 2.35 bA	0.01
	25	13.38 ± 0.91 aA	32.04 ± 1.74 bB	0.42
	50	14.99 ± 0.07 aA	8.87 ± 0.07 bC	1.69
RC	0	0.70 ± 0.04 aC	55.08 ± 0.55 cA	0.01
	25	8.62 ± 0.76 bB	38.86 ± 2.35 bB	0.23
	50	14.78 ± 1.04 aA	15.58 ± 0.24 abC	0.95
DI	0	0.73 ± 0.70 aC	76.62 ± 2.13 aA	0.01
	25	13.77 ± 1.14 aB	48.91 ± 0.20 aB	0.28
	50	17.21 ± 0.70 aA	16.63 ± 1.84 aC	1.06

Equal lowercase letters among clones in the same treatment do not differ ($p<0.05$).

Salt stress effect caused a growth diminishing in studied clones. Although all the clones presented significant decrease in relative growth rate (RGR), at any NaCl concentration tested, and differences were observed in growth potential among clones. On the tenth day, limit of exponential phase for most clones, it was observed in control plants an average RGR of 0.218 $gFW.day^{-1}$ with individual values ranging from 0.180 (RC) and 0.263 (M1) $gFW.day^{-1}$. In this sense, the clone M1 had higher accumulated biomass, reaching 69.5 $gFW.d^{-1}.m^{-2}$. Despite the sharp reduction in 25 mM treatment (36%), M1 stood out by about biomass even when compared to the remaining clones cultivated in the control treatment (Fig 1A), a behavior that suggests higher acclimation efficiency and yield. On the other hand, all the other clones had growth reduction/inhibition when treated with 50 mM salt, indicating sensitiveness to such high saline concentration (Fig 1B, 1C and 1D).

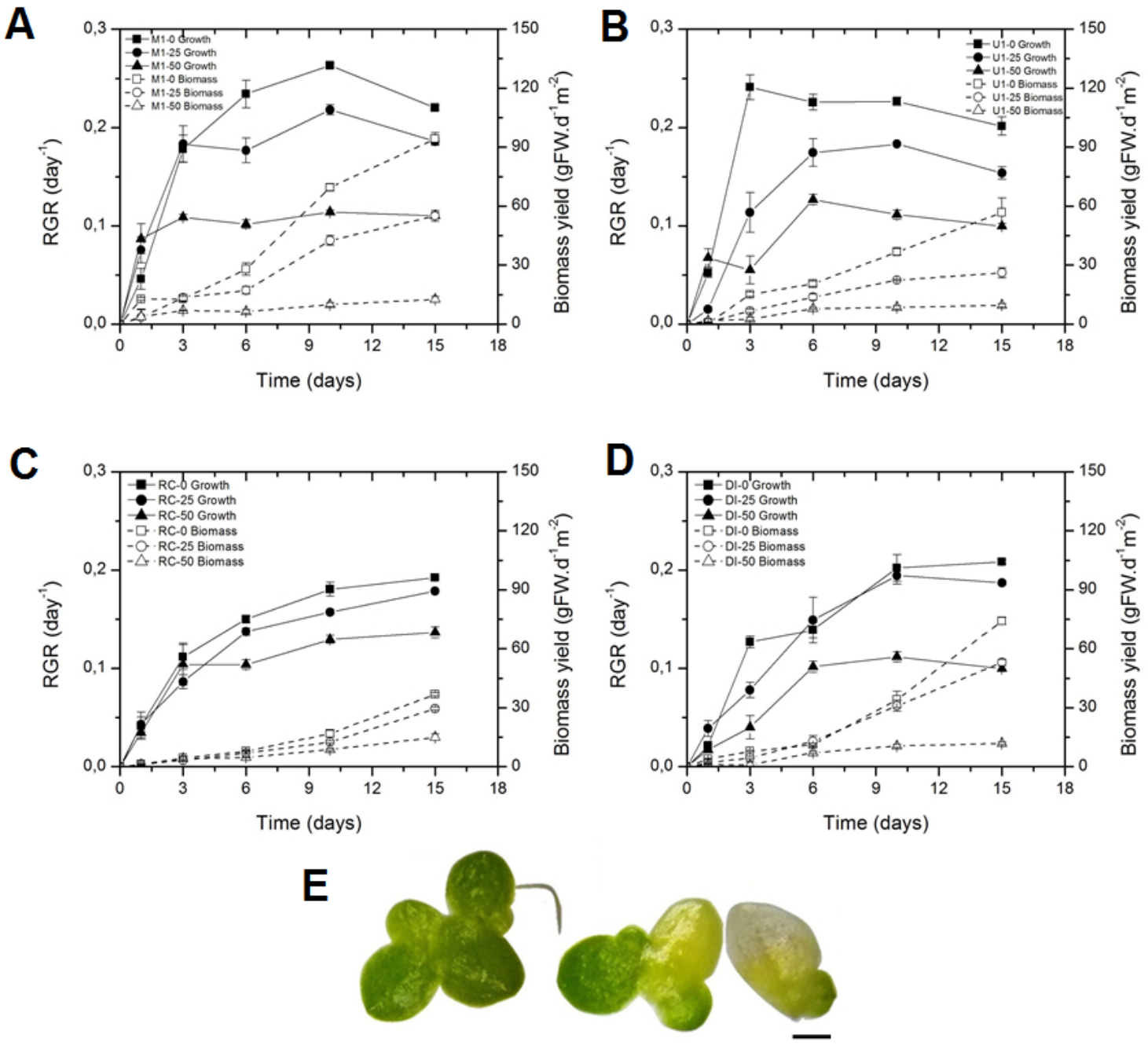


Fig. 1. Relative growth rate and biomass production of *Lemna aequinoctialis* clones in response to different NaCl concentrations. Each data point represents the mean of triplicate values; error bars indicate the standard deviation. (a-M1; b-U1; c-RC; d-DI). (e) M1 fronds showing NaCl effect, with appearance of stains in 25 mM and 50 mM treatments. Scale bar = 1 mm.

Control plants for all the clones showed a vigorous aspect, without damage or senescence signals. In contrast, it was observed in clone U1 chlorosis on the edges of young fronds treated with 25 mM from the third day on, and from the fifth day on in the other clones (M1, DI and RC). In the

more severe treatment (50 mM) this effect was observed in some fronds of all the clones, being more intense in U1 along the experiment, evolving in some individuals to a generalized chlorosis and initial necrosis (Fig 1E).

In clone U1, the severe treatment promoted a drastic reduction (35%) of chloroplast pigments, indicating that the photochemical apparatus may have been damaged by salinity. On the other hand, the clone M1 under moderate stress treatment presented significant increase in chlorophyll content (Table 4).

Table 4. Chlorophylls *a*, *b*, *total* (mg.g $^{-1}$ fresh material) and carotenoids content in *Lemna aequinoctialis* submmited to different NaCl concentrations (average ± SE, n=3)

Clones	NaCl (mM)	Chloroplastid pigments			
		Chlorophyll *a*	**Chlorophyll *b***	**Chlorophyll total**	**Carotenoids**
M1	0	0.633 ± 0.01 bB	0.289 ± 0.02 aA	0.919 ± 0.03 aAB	201.53 ± 9.77 aAB
	25	0.755 ± 0.02 aA	0.290 ± 0.01 aA	1.042 ± 0.03 aA	215.29 ± 6.60aA
	50	0.551 ± 0.01 bcB	0.250 ± 0.01aB	0.799 ± 0.02 bB	167.30 ± 3.57 bB
U1	0	0.770 ± 0.03 aA	0.324 ± 0.01 aA	1.091 ± 0.04 aA	232.57 ± 11.07 aA
	25	0.751 ± 0.04 aA	0.297 ± 0.02 aA	1.045 ± 0.05 aA	225.09 ± 12.02 aA
	50	0.500 ± 0.04 cB	0.199 ± 0.02 aB	0.697 ± 0.06 bB	149.93 ± 17.54 bB
RC	0	0.728 ± 0.01 abA	0.334 ± 0.05 aA	1.059 ± 0.05 aA	240.46 ± 14.75 aA
	25	0.823 ± 0.01 aA	0.324 ± 0.01 aA	1.144 ± 0.01 aA	251.07 ± 3.44 aA
	50	0.814 ± 0.05 aA	0.307 ± 0.02 aB	1.118 ± 0.07 aA	255.30 ± 11.94 aA
DI	0	0.648 ± 0.03 abA	0.364 ± 0.04 aA	1.009 ± 0.06 aA	204.72 ± 8.80 aA
	25	0.606 ± 0.03 aA	0.348 ± 0.05 aA	0.951 ± 0.05 aA	212.96 ± 15.56 aA
	50	0.632 ± 0.00 aA	0.262 ± 0.00 aB	0.891 ± 0.01 bA	183.60 ± 0.97 bA

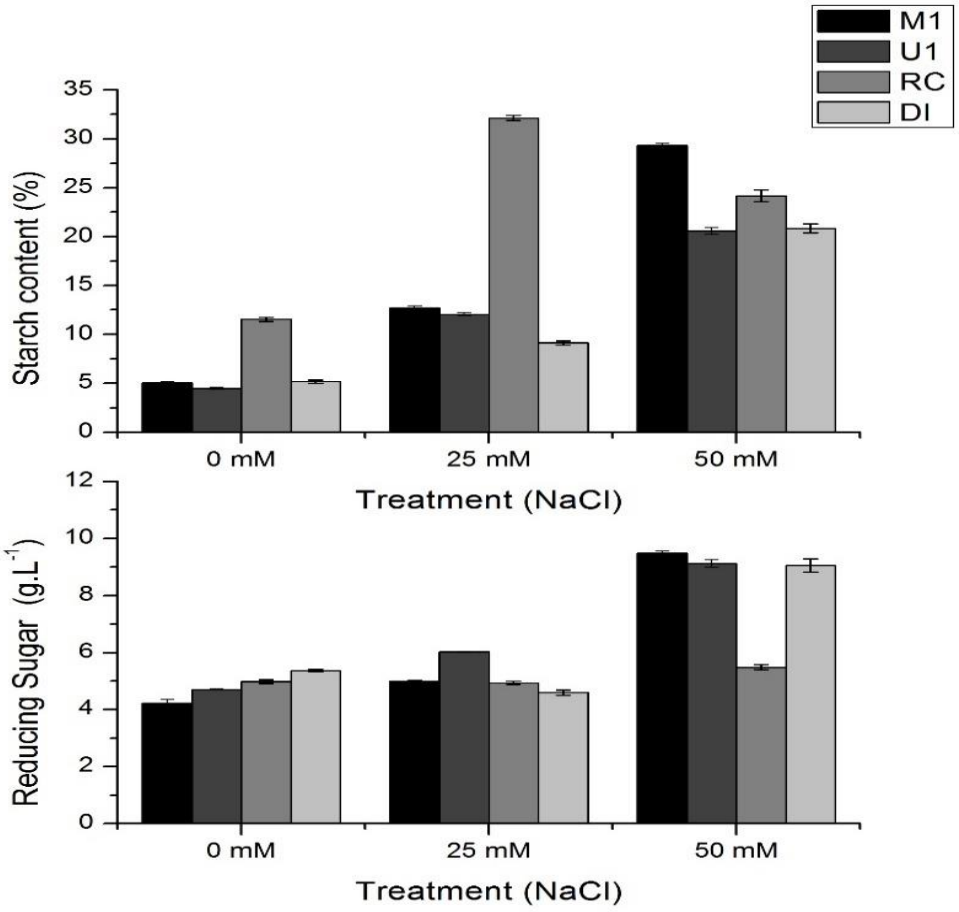
Equal lowercase letters amongclones in the same treatment do not differ (p<0,05).

The starch content in control plants, NaCl free, was similar for most clone, except RC which presented a significantly higher content than the other clones. In opposite, it was observed an increase in starch amount accordingly to applied salinity level, shown by a linear increase in M1,U1 and DI. The clone M1 varied from 5% to 29.3% in control and 50 mM NaCl treatments, respectively. The

298 only clone that presented a distinct behavior on starch content in response to salt stress was RC. For
299 this one, the 25 mM NaCl concentration seemed to have stimulated a higher starch biosynthesis if
300 compared to control, but the effect of 50 mM NaCl subsequently decreased (Fig 2A).

301 Regarding reducing sugars content, a significant increase occurred in fronds of plants
302 submitted to 50 mM NaCl from clone M1, U1 and DI, but not in RC (Fig 2B).

303



304

305 **Fig. 2. Starch and reducing sugars content in fresh biomass of *Lemna aequinoctialis.*** Starch (a)
306 and reducing sugars (b) content in fresh biomass of *Lemna aequinoctialis* under influence of salt
307 stress. (average ± SE, n=3).

308

309 The clone M1 stood out in 25 mM as the best extraction of Na^+ (6.30 $gNa.FW.d^{-1}.m^{-2}$) since
310 it had the highest biomass and a higher content of Na^+ in this treatment, and similar to DI, showed
311 the smallest decrease in potassium levels. In addition, M1 is the biggest accumulator starch and

312 reducing sugars with 5.32 and 2.13 g.FW.$d^{-1}m^{-2}$, respectively. The worst puller was the RC clone due
313 to its lower content of Na^+, the same treatment (Table 5).

314

315 **Table 5.** Mean values of starch extraction, reducing sugars, sodium and potassium (gFW.d^{-1}.m^{-2}),
316 based on nutritional analysis and the yield of fresh weight *Lemna aequinoctialis*.

Clones	**NaCl (mM)**	**Starch**	**Red. Sug.**	**Na^+**	**K^+**
M1	25	5.40 aA	2.13 aA	6.30 aA	14.65 aA
	50	2.90 aB	0.9413 aB	1.60 aB	1.54 aB
U1	25	2.70 cA	1.35 bA	3.01 bA	7.22 bA
	50	1.76 bB	0.78 abB	1.28 aB	0.76 aB
RC	25	4.01 bA	0.62 cA	1.07 cA	4.89 bA
	50	2.06 abB	0.48 bA	1.29 aA	1.36 aB
DI	25	2.83 cA	1.42 bA	4.26 bA	15.15 aA
	50	2.20 abA	0.98 aB	1.81 aB	1.79 aB

317 Equal lowercase letters among clones in the same treatment do not differ (p<0,05).

318

319 Quantitative data of starch content were corroborated by histochemical analysis, which
320 revealed amyloplasts with expressive amount of starch granules in the treatment where plants were
321 submitted to the most severe salt stress level. In control fronds were visualized few starch granules,
322 in inverse pattern. Amyloplasts with several sizes and shapes, from oval to elliptical (Fig 3).

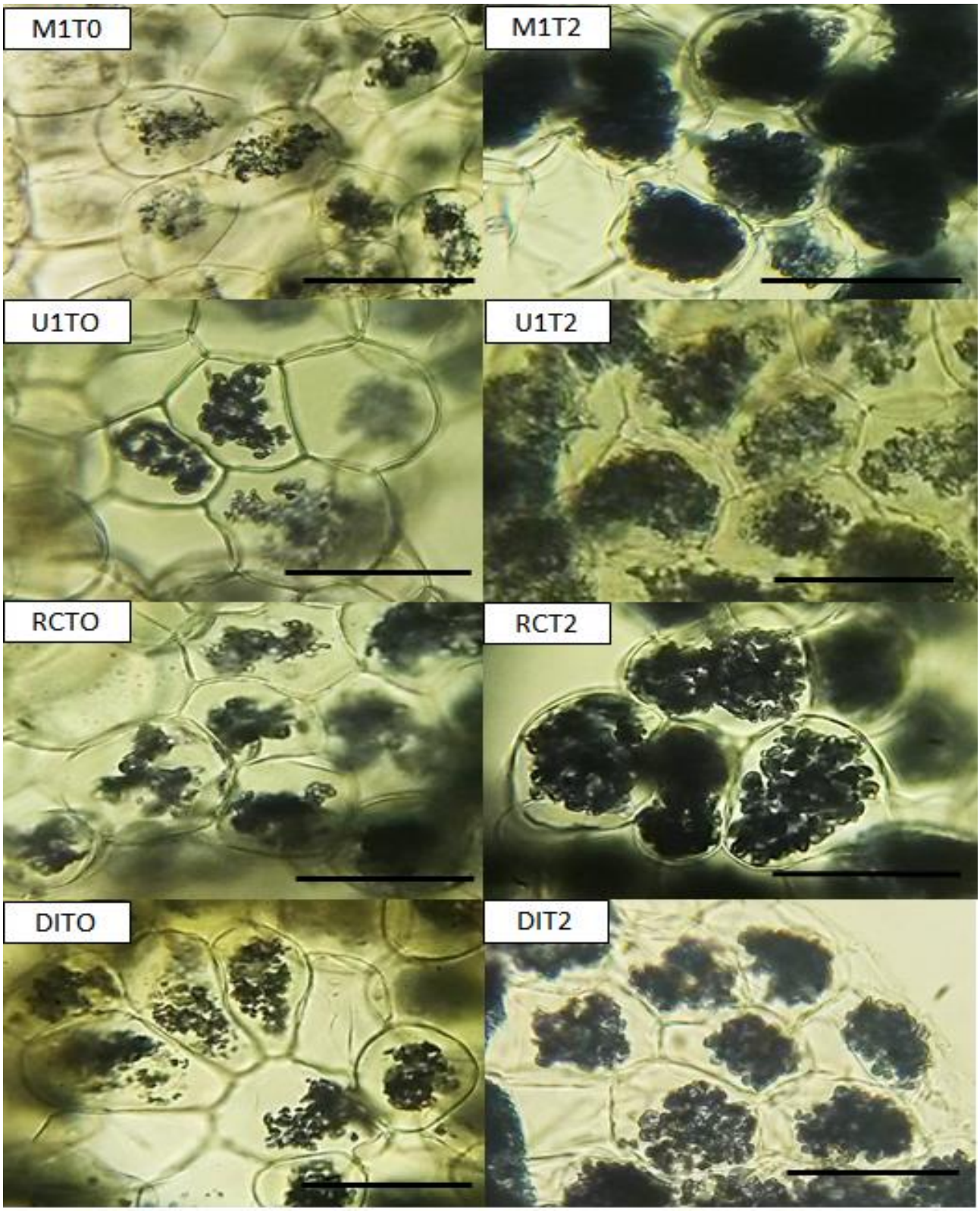


Fig. 3. Histochemical analysis. Transversal fronds section of *Lemna aequinoctialis* submitted to different NaCl levels stained with lugol. Some amyloplasts in the parenchyma tissue containing starch granules can be visualized in fronds of control from all the clones (left column: T0 - 0 mM), while there was starch presence in these plastids in treated plants (right column: T2 - 50 mM NaCl); Scale bar = 5 µm.

329 The analysis cross view of adaxial face epidermis revealed presenting stomata arranged in
330 organized longitudinal rows (Fig 4A), and small tabular cells (Fig 4B). Stomatal complexes show
331 edreniform guard cellsand absence of subsidiary cells comprising the anomocitic pattern (prevailing
332 type) (Fig 4C). In addition, it was possible to detect idioblasts containing raphids, the tissue
333 parenchyma (Fig 4D). Large aerenchyme gaps that facilitate floating (Fig 4E). Furthermore, stomata
334 present in adaxial face (emersed fronds), showed variation associated to NaCl concentration increase
335 (Fig 5).

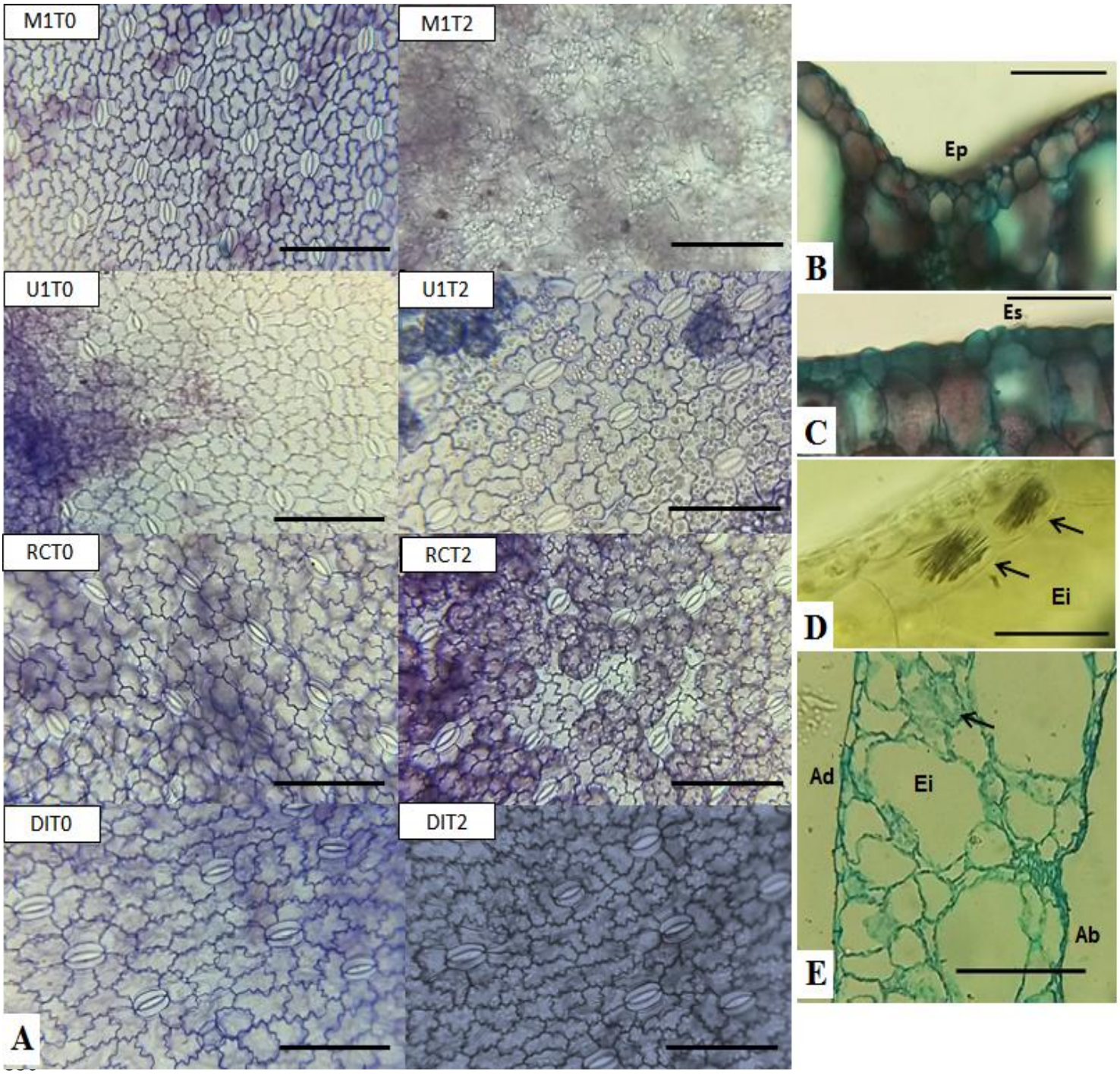


Fig. 4. Paradermic sections of *Lemna aequinoctialis* fronds submitted to salt stress. (A) Front view of adaxial face containing stomatal apparatus of anomocitic types, shape and sinuosity of the cell walls of parenchyma (left column T0 - 0 mM; right column T2 – 50 mM de NaCl); (B) Transversal section of adaxial face showing the compact arrangement of epidermal cells (without stomata presence); (C) Transversal section demonstrating adaxial face stomata; (D) Idioblasts with raphid crystals (arrows), in series; (E) Tranversal section of control plant frond, with parenchyma tissue containing chloroplasts distributed in peripherical cytoplasm, as well as the aerenchyma gapes. Scale bar = 10 µm (A, E); 5 µm (B, C, D). Abbreviations: stomata (Es); adaxial (Ad); abaxial (Ab); epidermis (Ep); intercellular space (Ei).

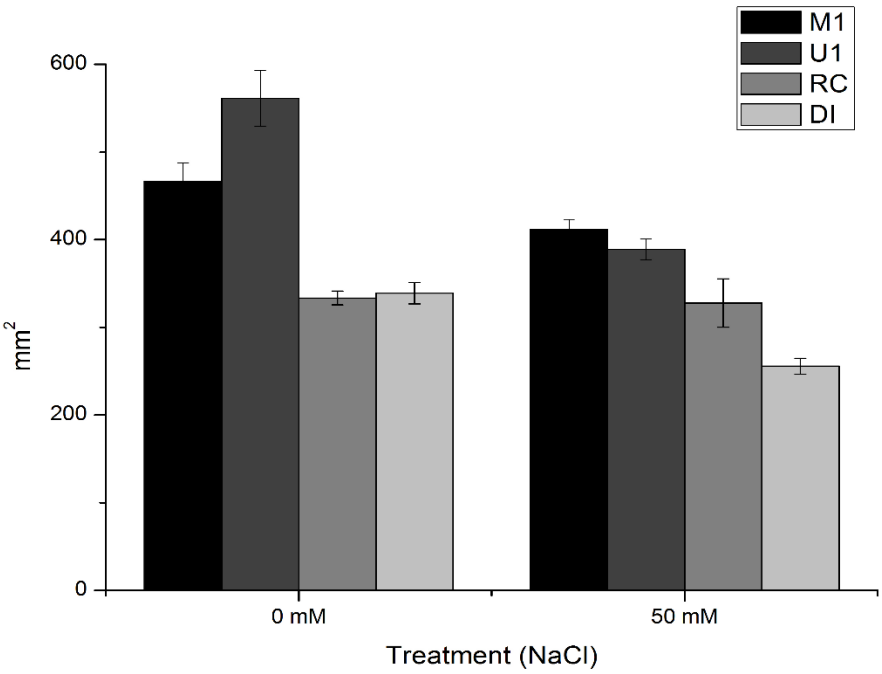
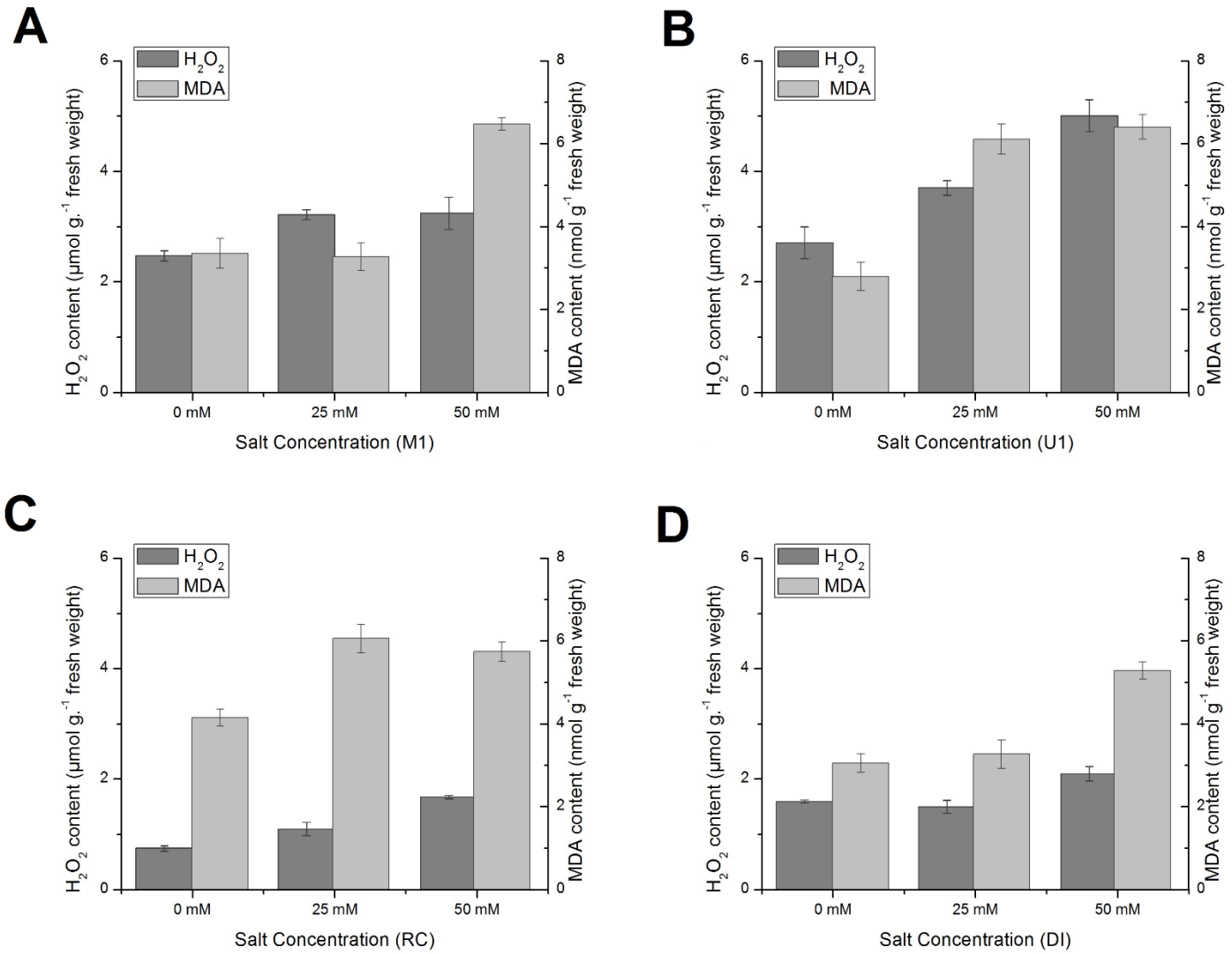


Fig. 5. Stomatal density of *Lemna aequinoctialis* clones under influence of salt stress. Error bars indicate the standard deviation.

Oxidative stress

A significant increase in MDA content, product of polyunsaturated fatty acids peroxidation from biomembranes, was detected in plant samples of clones U1 and RC already in moderate stress (25 mM), in the order of 2.29 and 1.38 times, respectively (Fig 6B and 6C). However, the lipidic peroxidation increased in clones M1 and DI, and was observed just under the most severe stress (Fig 6A and 6D). The consistent MDA accumulation was always accompanied by significant increase of H_2O_2 ($p<0.05$) in all the clones.



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359 **Fig. 6. Effect of salt stress in H_2O_2 content and lipidic peroxidation in *Lemna aequinoctialis.***
360 Lipidic peroxidation expressed by malondialdehyde (MDA) content (A-M1; B-U1; C-RC; D-DI).
361 (average ± SE, n=3).

362 The antioxidant enzymatic system, represented by SOD, APX and CAT activities, showed
363 significant differences in responses depending on clones and treatments, especially in the clones M1,
364 U1 and DI. In both salt concentrations, 25 and 50 mM, were observed significant increases in SOD
365 activity (mainly in U1 plants), a H_2O_2 generator (100 and 145%, respectively), which suggests the
366 presence of superoxide radical (Fig 7).

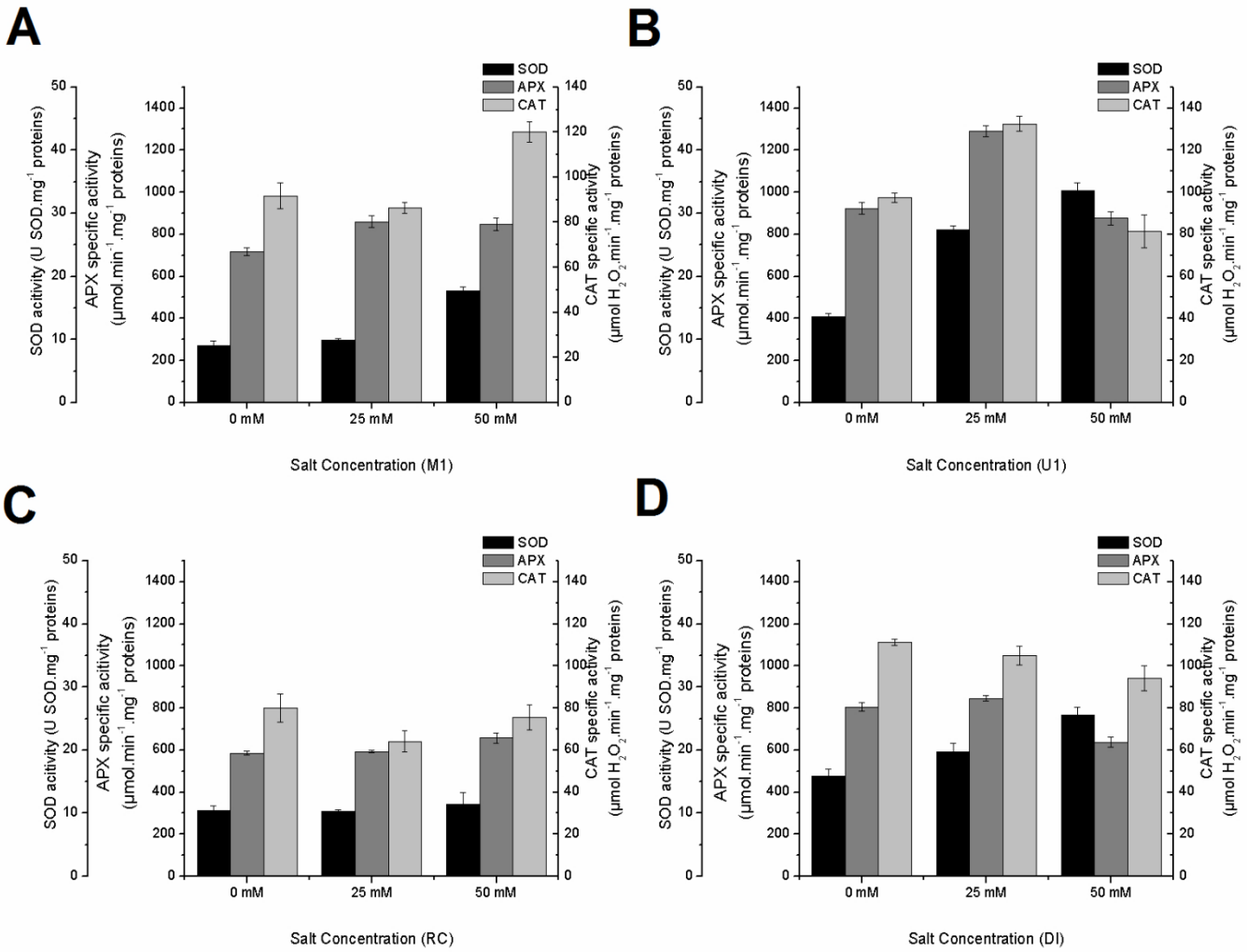


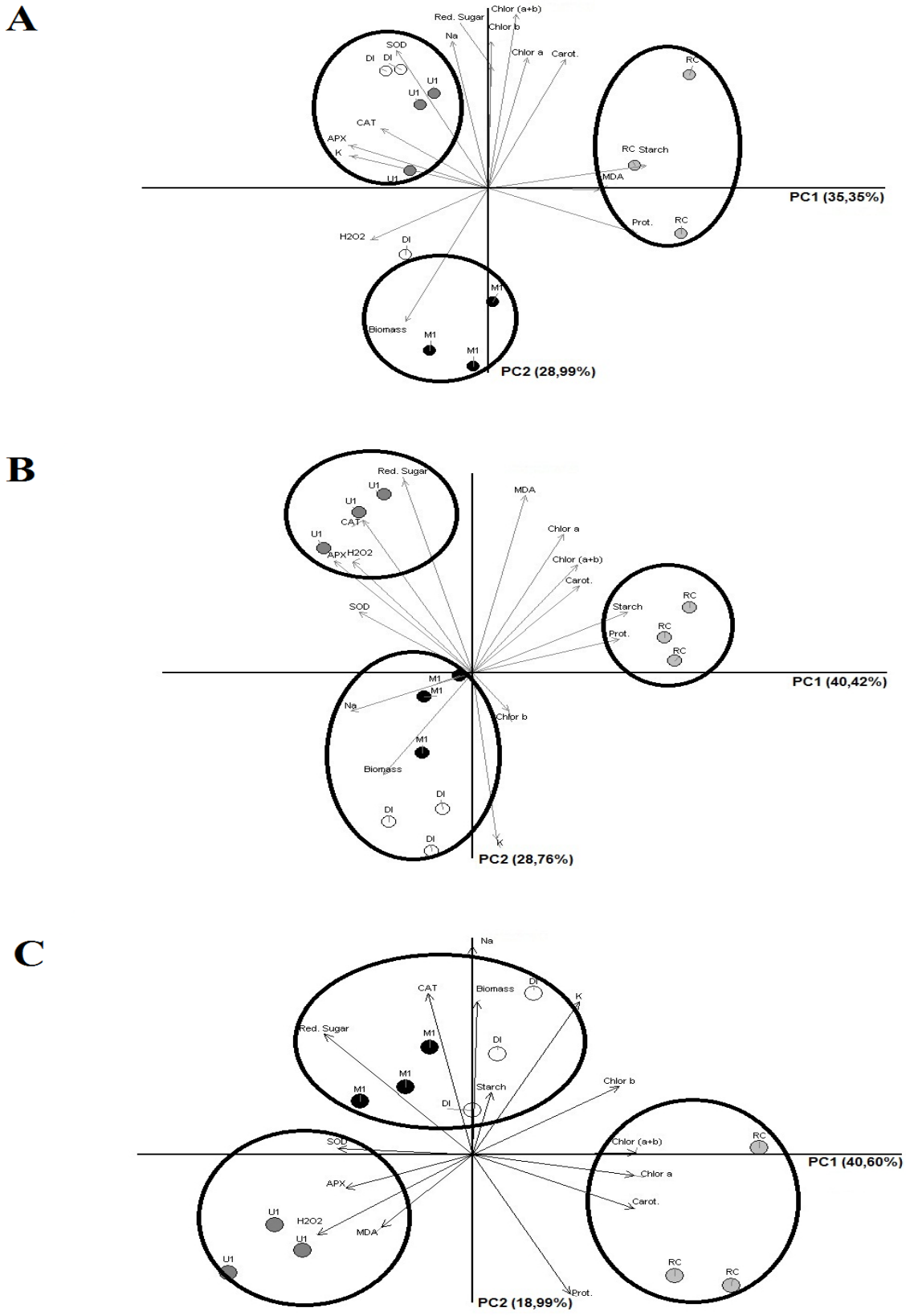
Fig. 7. Effect of NaCl on the activity of oxidative metabolism enzymes in *Lemna aequinoctialis.* superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (A-M1; B-U1; C-RC; D-DI). (average ± SE, n=3).

In the clone M1 occurred the maintainance of SOD and CAT activities, in parallel to APX activity increase; in opposite, in DI occurred SOD increases positively correlated to APX (r =0.70*, p<0.05). Such antioxidant responses were enough to keep constant the MDA levels in the two clones under moderate stress (Fig 7A). On the other hand, in clone U1 plants, in this 25 mM level, it was observed a synchronic increase in SOD, APX and CAT enzymes activities, being possible to detect a highly significant positive correlation between CAT and APX (r=0.92**, p<0.01), as well as between SOD and H_2O_2 content (r=0,90**, p <0.01) (Fig 7B). Still, even with the synchrony of these

379 enzymes activities, was observed the increase in H_2O_2 and MDA content. In the clone RC, the
380 antioxidant system enzymes seemed not to be activated in function of the stress, moderate or severe
381 (Fig 7C).

382 In the severe stress, APX activity was kept constant while SOD and CAT ones seemed to have
383 gained relevance in the fight against ROS in M1, showing positive correlation (r=0.76*, p<0.05).
384 However, it was simultaneous to almost doubling the MDA content and a considerable decrease in
385 growth rate (Fig 1). In this salt concentration, it was observed an increase in SOD activity in clone
386 U1, also verified in clone DI (Fig 7D), in parallel to a significant reduction of APX and CAT, what
387 may have contributed to elevate H_2O_2 content. CAT, in turn, seems to be more sensitive to salt stress.

388 The principal component analysis and its respective relative demonstrated that the two first
389 components were able to explain 64.27, 69.18 and 64.59% of total variation in the control, 25 and 50
390 mM NaCl treatments, respectively (Figs 8A, 8B and 8C). This model revealed the formation of three
391 distinct groups in response to stress, according to differential behavior among clones (Fig 8B and
392 8C). In the 25 mM treatment, the variables that had the highest contribution for discriminating the
393 groups, strongly influencing the first axis, were: starch content (0.94), protein (0.88), SOD (-0.68),
394 APX (-0.83), H_2O_2 (-0.72) and sodium (-0.73). In opposite, in the concentration of 50 mM, the
395 variables that stood out were: SOD (-0.77), H_2O_2 (-0.88), reducing sugars (-0.84) and chlorophylls *a*,
396 *b* and carotenoids (>0.90).



397

398 **Fig. 8. Projection in the first two main components of the biochemical variables in *Lemna***
399 ***aequinoctialis.*** a) 0 mM; b) 25 mM and c) 50 mM of NaCl.

Discussion

Confirming reports widely described in literature, the exposure of duckweed to salt stress promoted an increase in Na^+ concentration in the plant tissues. The Na^+ accumulation frequently generates severe disturbances in plant growth [39], as observed here in studied distinct clones of *Lemna aequinoctialis*. Concomitant with the increase in Na^+ content, it occurred as expected an expressive decrease in K^+ content due to competition of Na^+ for the K^+ carrier [39].

Although the plants submitted to 50 mM NaCl had presented intense lowering of potassium levels, 71% in average, these values were superior than 10 $g.kg^{-1}$, considered the minimum content for a suitable plant growth. The question focus is the increase in Na^+/K^+ ratio, whose value in this work exceeded 0.6, a value reported as maximum to keep efficient the metabolism in glycophytes [40]. When considering the Na^+/K^+ higher values were observed for M1 and U1 clones. The U1 clone features chlorosis, this level of salt, followed by necrosis in 50 mM while the clone M1, like the others, has chlorosis only to 5 days.

Initial decrease in the plant growth provoked by sodium excess effects is commonly associated with osmotic stress, and can cause a loss in relative water content (RWC) that may result in decrease of cell turgor [14]. In a second moment, the growth reduction is attributed to Na^+ accumulation in tissues, that can reach toxic levels and lead to chlorosis and, as observed in plants under severe salt stress, necrosis. At the molecular level, growth reduction may be related to inhibition of cell division due to oxidative stress [41]. Lowering growth rates have also been reported in *Lemna* under salt stress [4,14,16]. The magnitude of decrease in growth varies in function of the species and even inside the species [4], as verified in this work for the clones of *L. aequinoctialis*. The clone M1 excelled by presenting simultaneously the highest biomass production and RGR in control and moderate stress treatments; the clone RC, in turn, had the lowest values for RGR and accumulated biomass.

In this study, the clones showed the ability to keep photosynthetically active in moderate salinity condition. The clone M1 was able to increase its chloroplast pigments content under moderate stress, usually an important fact for plant development under stress, it has been treated as an important variable in determining genotypes tolerance [42]. However, more severe saline conditions seemed to have promoted adverse effects over chlorophyll contents in clone U1, in which the salt stress caused effective reduction in pigments content. Chlorophyll *b* content showed to be more sensitive to the stress than chlorophyll *a*, a fact also observed in other Lemnaceae: *Spirodela polyrhiza* in 100 mM NaCl [14] and *Lemna minor* in 150 mM NaCl [43]. This clone, U1, presented chlorosis that evolved to necrosis in the 50 mM NaCl treatment. The chlorophylls and carotenoids content reduction is one of the primary effects of salinity over photosynthesis in stress-sensitive plants and has been considered as a physiological marker for abiotic stress in duckweeds [31,44–46]. This pigment loss may derive from thylakoids membrane damages and direct oxidative disintegration of pigments, inducing the instability on the protein-pigment complexes in duckweed photosynthesis reaction centers [14].

In plants, sugar is produced by the metabolism related to growth, providing energy through biological oxidation process, as well as carbon precursors. The increase in carbohydrate content observed under severe stress (in clones M1, U1 and DI) may reflect an accumulation of osmoregulators, to minimize the salt stress effects [47]. This metabolic strategy is performed by *Lemna minor*, which accumulates soluble sugars (mainly monosaccharides glucose and galactose), in response to NaCl concentration increase in solution [15]. In contrary, the inhibition of duckweed growth in function of salt stress or heavy metals occurs in larger scale than the inhibition of photosynthesis, what generates a carbon reserve that can be converted to starch and stored in plastids [4,48,49], as clearly observed in the plants submitted to 25 mM and, noteworthy, 50 mM NaCl. The high starch content detected in the RC clone in 25 mM treatment (32.1%) is more than double the others, but does not reflect the greater accumulation of this carbohydrate (4.01 $g.FW.d^{-1}m^{-2}$). On the other hand, clone M1 was higher starch accumulator, reducing sugars and being able to extract higher

449 Na^+ amounts without giving further damage (chlorosis) compared to the other clones. M1 also showed
450 less reduction in the potassium content, indicating more efficient compartmentalisation of Na^+ and
451 minimize damage to the plant tissue.

452 It has been recently reported for several duckweed species that the growth reduction
453 concomitantly with a rapid starch accumulation, in response to salt stress, is a strategy that can be
454 used to increase starch biomass in plants towards bioethanol production [4]. Starch storage in plastids
455 of the clones cultivated in 50 mM NaCl were easily visualized by histochemical analysis. The absence
456 of soluble reducing sugars increase observed only in clone RC may point that, under severe stress,
457 those sugars have been immediately consumed by plants in order to keep survival [50]. Probably in
458 clone RC a fraction of starch granules had to be converted to trioses and hexoses during salt stress,
459 what could explain the intense lowering in starch content in more severe treatment compared to
460 moderate stress condition.

461 Regarding stomata, the clones presented sinuosity of the epidermal walls, besides lower
462 density of such anatomical structures when submitted to severe salt stress (except RC), indicating the
463 existence of different regulatory pathways involved in stomatal formation and responsive to salt
464 excess. In natural environments, these features may lead to low transpiration rates, which is essential
465 for water saving in plants at osmotic stress, however, it may affect photosynthetic relevant gas
466 exchanges (CO_2 and O_2) and interfere on yield [51], as observed in plant growth rate inhibition in
467 severe stress treatment. There was no damage to the cell morphology, indicating tolerance to saline
468 stress and phytoremediation power.

469 The observed anatomic structure is typical of aquatic plants: the epidermal is unisseriate layer
470 withair cavity formed by cells that facilitate floating (aerenchymas tubes). However, to our
471 knowledge, the presence of raphids in parenchyma tissue of *L .aequinoctialis* verified in this work is
472 not found in the description proposed by Potter and Cervi [52], and so is here unprecedently described
473 for the species. Raphids also common in monocotyledons, suggesting the presence of calcium-rich
474 compounds, probably oxalate. The calcium oxalate crystals in raphids shape are defined as a final

product of cell metabolism and are related to different roles like calcium storage and regulation, ionic balance and osmoregulation [53–55]. However, in some aquatic plants the raphids can perform more specialized roles, as like to promote the formation of spaces in parenchyma tissues, thus increasing cell wall plasticity around aerenchymae [54]. This could facilitate cells/organ growth until reaching maturity. Such hypothesis explains better the results from this work, since the observed raphids were detected only in parenchyma tissue neighboring intercellular spaces (aerenchymae).

In the clones M1 and DI, which had the highest growth rates and biomass production when submitted to moderate stress, no increase in MDA concentration was detected, likely reflecting the efficiency of acclimation of these clones. Hence, one may consider that the increase or the maintenance of APX and CAT activities indicate the effectiveness of these enzymes on H_2O_2 detoxifying and protection against oxidative damage [56]. The increase in H_2O_2 content observed in M1 suggests the production of this ROS from other metabolic processes cellular signaling in the processes of growth and development. The stress in 25 mM seemed to have already induced significant increase in MDA content in clones U1 and RC, evidencing metabolism damaging after oxidative stress. MDA has been considered a common biomarker for NaCl toxicity in plants [57–60].

The severe stress was noted in clones U1 and DI by the SOD activity increase, followed by significant reduction of APX and/or CAT activity. Depending on the salt level and on the clone, CAT showed changes as activity reduction compared to control, similarly to the previously observed by Panda and Upadhyay [61] in *Lemna minor* roots submitted to 50 mM NaCl. This picture in severe stress contributes with peroxide content increase, which suggests a redox system imbalance. Thus, it seems to be relevant that SOD activity is in synchrony with CAT and/or APX activities, since the product of $O_2^{-\cdot}$ dismutation by SOD is H_2O_2, and its accumulation may be as harmful as O_2^{-} one.

Although non-marine aquatic plants usually have higher sensitiveness to salinity by harboring no metabolic strategy to protect against osmotic stress [14], clones of *L. aequinoctialis* submitted to moderate salt stress (25 mM) showed metabolic response associated with adjustments in antioxidant

metabolism, towards new homeostasis. On the other hand, the results suggest that 50 mM NaCl can be considered a toxic concentration for *L. aequinoctialis*. To circumvent such stress level, the lesser duckweed practically ceased growth (related or not with photochemical apparatus damage), and also takes place the hydrogen peroxide accumulation and increased lipid peroxidation. These alterations can be attributed to oxidative damages caused by salt stress, reinforcing results obtained by Cheng [14] and Chang et al. [16] with *Spirodela polyrhiza* (giant duckweed) under salt stress. It is stated that oxidative stress is an important mechanism that indicates the level of toxicity in duckweeds in salt stress response. However, the sensitiveness to salt cannot be screened only in species level, since natural variations can be easily detected in clones from a same duckweed species, reflecting possibly associated genetic variability [4].

The principal component analysis helped the understanding of differences and similarities among the clones of *L. aequinoctialis* in response to salt stress, according to the proximity among distinct sample of clones, as well as the proximity among clones. The distance between U1 and RC points out the responses divergence between them, and specially the negative correlation with the group formed by DI and M1, which presented high similarity. Although the evidences for oxidative stress are in the same axis, clone U1 samples were more related to enzymatic metabolism, while the clone RC was more related to photosynthetic pigments, suggesting metabolic responses differentially modulated. In opposite, the set of biochemical responses was enough to ensure the better performance of clones M1 and DI under moderate stress. It is graphically noted that there is a significant similarity between the clones and the higher correlation, in every treatments, with biomass accumulation. The different clones analyzed present alternative pathways and molecular mechanisms which, in turn, may produce different phenotypical responses.

Conclusions

NaCl induced different biochemical and physiological responses with relevant variations among clones of *Lemna aequinoctialis*.

Salt stress causes growth inhibition simultaneously with increase in starch content, making the studied clones (M1 and DI specially) potential candidates for bioethanol production if cultivated in moderate salinity conditions.

The increase in H_2O_2content and the lipid peroxidation are important biomarkers of toxicity, and higher activity and synchrony of antioxidant enzymes may assure protection against oxidative damages.

L. aequinoctialis presents growth ability in moderately saline aquatic environments. Additionally, the usage of residual NaCl may be economically feasible in order to produce high starch content duckweeds in saline conditions.

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CAPÍTULO II

Proteômica diferencial de lentilha-d'água (*Lemna aequinoctialis*, Lemnaceae) em resposta ao estresse salino

Submissão: Jornal of Proteome Research

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Differential proteome of duckweeds (*Lemna aequinoctialis*, Lemnaceae) in response to saline stress

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ABSTRACT: The objective of this work was to identify differentially expressed proteins in *Lemna aequinoctialis* (duckweed) through two-dimensional electrophoresis (2-DE) associated with mass spectrometry (MS). Clones of the duckweed species were cultivated *in vitro* under laboratory conditions and submitted to different concentrations of NaCl (0, 25 and 50 mM NaCl) for 10 days, and submitted to protein extraction and quantification. Integrity was evaluated on SDS-PAGE gel and for the 2D-PAGE 250 μg of proteins were used, focused in 13 cm immobilized pH 3-10 drystrips in the IPGPhor system. After staining with Coomassie G-250 blue, the 2D gels images were analyzed in ImageMaster 2D Platinum v.7.05 program. The differential spots were excised, digested by trypsin and identified by mass spectrometry. Different responses to saline stress were observed in the clones proteome and potentially associated with tolerance mechanisms. Clone M1, partially tolerant, presented accumulation of proteins involved in the metabolism of carbohydrates and energy, antioxidant metabolism and protein protection. These stress response pathways seem to play a role in salinity tolerance in duckweed and their efficiency depends on the detection of stress and the activation of the expression of the coding genes.

KEYWORDS: Mass spectrometry, duckweeds, salinity, proteins, tolerance

INTRODUCTION

Plants, in their natural habitat, are subject to a number of abiotic conditions that alter their metabolism. Associated with drought, salinity is considered one of the most widespread and severe environmental risks, restricting agriculture especially in arid and semi-arid regions.[1,2] Therefore, there is an increasing demand for plants tolerant to salinized environments, since projections from the past decade had already pointed to climate change and an increase in the world population, suggesting an increase in the extent of the area affected by salinity.[3] The increase in salinization, which also affects freshwater resources, has been accelerated by anthropogenic activities, [4] In this case, as an ecological method, the cultivation of aquatic macrophytes is attractive for the restoration of water resources.[5]

Duckweeds are small floating aquatic macrophytes that have a rapid vegetative propagation and, depending on the environmental conditions, may show an almost exponential growth rate when compared to other species.[6] These plants acquired interest by their use for animal consumption, being considered as a good source of vegetable protein.[7] In addition, the rapid growth and consequent high biomass production associated with high starch production capacity in duckweeds have attracted increasing attention as feedstock with potential for bioenergy production.[8-11] The cultivation of duckweeds also present advantages due to the capacity of growth in water with moderate salinization, while keeping the capacity of N and P removal,[12] besides the proven capacity of water desalination by the capture of dissolved salt,[13] being therefore also a promising candidate for the phytoremediation of salinized aquatic environments.

Plants generally detect and respond to stressing abiotic factors by modulating gene expression, resulting in biochemical and physiological changes that allow their survival in the environment.[14] This perception results in specific changes in signal transduction pathways that are activated at the cellular level and directly influence the production and acumulation of proteins. Because they are closer to the physiological phenomena, there are, therefore, advantages in studies of functional genomics aimed to a deeper understanding of the changes in gene expression and consequently on

the regulatory mechanisms that may lead to tolerance in adverse conditions.[15] However, in spite of the relevance and importance of biofuels production,[16] few studies have been especially associated with the elucidation of the genetic mechanisms underlying the adaptation of energy crops under stress conditions.

Salt stress is an environmental factor that limits the growth and productivity of plants. Since it is known that the starch content in duckweed can be increased by stressing situations, such as salinity[17] as well as that these plants have the ability to desalinate water by the uptake of dissolved salt,[13] it was aimed, in this work, to identify proteins differentially accumulated in duckweed submitted to saline stress. The obtained informations are important tools that can improve the understanding on the duckweed physiological strategies to response to the stress induced by the NaCl exposure and the consequent accumulation of starch, that can make this species more useful as bioenergy feedstock.

MATERIAL AND METHODS

Plant cultivation

The clones used in this research (M1, U1, RC and DI) were harvested in the metropolitan region of Recife, and are part of the duckweed collection kept under axenic conditions in the collaborator Plant Tissue Culture Laboratory of Federal Rural University of Pernambuco (Recife, PE, Brazil). The clones were cultivated in SH culture medium,[18] at half-strength (0.5x) nutrient concentration, plus 10 gL^{-1} sucrose at pH 5.8, kept in 25±2 ºC under light intensity of 57.5 μmol $m^{-2}s^{-1}$ and photoperiod of 16 h.

Saline Stress Cultivation

Initially, 100 fronds from each clone pre-cultured collection were randomly selected as inoculum and transferred to glass containers with 350 mL capacity containing 50 mL of 0.5x SH

culture medium, autoclaved prior to use, and submitted to treatments: absence of NaCl (T0 = control) and presence of NaCl (T1 = 25 mM, T2 = 50 mM, moderate and severe stress, respectively). The levels of NaCl, 0, 25 and 50 mM corresponded to the electrical conductivities (ECs) of 1.81 ; 5.09 and 7.57 dS cm^{-1}, respectively, measured with a digital conductivimeter.

The experimental period was 10 days (exponential phase) growth curve was observed until 15^{th} day to ensure the monitoring of the exponential phase long. After harvesting the plant material was immediately frozen in liquid nitrogen and kept at -80°C until the analyzes were performed. For the biochemical and physiological parameters, the design was completely randomized in a 4 x 3 factorial scheme (four clones and three concentrations of NaCl), with three replicates. Based on the results obtained (data not shown article 1), the most tolerant (M1) clone presented efficiency of defense mechanism relies on synchrony between such enzymes activity towards successive elimination of reactive oxygen species. The more sensitive (U1) clones presented the photosynthetic pigments content was reduced, significant increase in malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content. These clones were selected for proteomic analysis, in which three distinct replicates were considered for the comparison between contrasting concentrations (0 and 50 mM) in each selected clone, so that the gel images were grouped into two sets of three comparisons (matches), making a total of six comparisons for each clone, in which only the treatment parameter was considered as variable.

Protein Extraction and Quantification

Total proteins were extracted according to Hurkman and Tanaka,[19] with some modifications. A sample of 1 g of plant material, after brief drying excess moist on paper towel, was grounded in liquid nitrogen and then homogenized in 10 ml of extraction buffer (500 mM Tris-HCl, pH 7.5; 0.7 M sucrose, 0.1 M KCl, 50 mM EDTA, 2 mM PMSF, 2% β-mercaptoethanol) and kept under constant stirring (70 rpm) for 30 min in an ice bath at 6ºC. After incubation, 10 mL of Tris-HCL (pH 8.5)

equilibrated phenol was added and subsequently the tubes were shaken and again kept under stirring for 30 min at 4 ° C.

The tubes were centrifuged at 7,200x*g* for 50 min at 4°C for recovery of the supernatant (organic phase). The recovered phenol containing the proteins was transferred to a new tube and kept overnight at 4°C in 100 mM ammonium acetate solution in 100% methanol for complete precipitation of proteins overnight. Tubes were centrifuged at 7,200x*g*, 4ºC for 1 h. The protein precipitate was washed once with 100 mM ammonium acetate solution in ice-cold methanol, once in ice-cold methanol and twice in 80% ice-cold acetone solution. After drying, the pellet was solubilized in 600 µL of urea:thiourea buffer (7M:2M). Protein quantitation was performed according to Bradford method,[20] in triplicates and using bovine serum-albumin for standard curve. The integrity of the proteins was visually checked on 12% gel SDS-PAGE.

Two-dimensional electrophoresis

Isoelectric focusing (IEF)

IEF was performed on 13 cm immobilized pH gradient 3-10 IPG drystrips using the Ettan IPGphor 3 (GE Healthcare Life Sciences) platform for each replicate from each treatment. The strips were hydrated with 250 µg of proteins dissolved in rehydration solution [7M urea, 2M thiourea, 2mM DTT, 1.0% IPG pH 3-10 buffer (GE Healthcare Life Sciences) and 0.005% bromophenol blue]. IEF was performed at 15°C in five steps: i) 200 V - 150 V h^{-1}; ii) 500 V - 500 V h^{-1}; iii) 1,000 V - 1,000 V h^{-1} in gradient; iv) 8,000 V - 14,500 V h^{-1} in gradient; and v) 8,000 V - 4,550 V h^{-1} with current of 50 µA per strip. After focusing, the strips were stored at -80°C until the second dimension was performed.

Electrophoresis (2D-PAGE)

For the second dimension (electrophoresis), the reduction and alkylation of the focused IEF strips were carried out. In the reduction step, the strips were immersed in equilibrium solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS) containing 1% dithiothreitol (DTT) and bromophenol traces for 20 min. Subsequently, in the alkylation step, the strips were transferred for immersion in equilibrium solution containing 2.5% iodoacetamide (IAA) for 20 min. After equilibration, the electrophoresis was performed in 12.5% (20x20 cm) gel at 15°C in two steps: 300 V gradient, 15 mA and 30 W for 20 min; 300 V, 30 mA and 30 W for up to 2 h. 2D-PAGE gels were stained with 0.01 $g.L^{-1}$ Coomassie Brilliant Blue G-250 solution as described by Candiano et al.[21]

Scanning, image analysis and selection of differential spots

Images of the 2D gels were obtained by scanning the gels in ImageScanner III (GE Healthcare Life Sciences) with the LabScan 6.0 program (GE Healthcare Life Sciences) according to the standard parameters. The image files were analyzed in the Image Master 2D Platinum v7.05 software (GE Healthcare Life Sciences). All replicates were submitted to the same parameters in comparative analysis for detection and selection of spots, with visual inspection to eliminate interfering bubbles and staining artifacts, according to Pacheco et al.[22] In the comparisons between the treatments, the spots with a ratio ≥ 1.5 for percentage of volume (% Vol) and significant ANOVA ($p \leq 0.05$) were selected as differentially accumulated. These selected spots were called DEPs (differentially expressed proteins), representing differentially accumulated proteins.

Digestion and identification of DEPs by mass spectrometry

The spots with significant variation between treatments were manually excised from the gel were their %Vol was more intense, followed by in gel digestion using trypsin according to the protocol described by Shevchenko et al.[23] The peptides were extracted in solution containing 5% trifluoroacetic acid (TFA) and 50% acetonitrile, which were concentrated in a vacuum rotoevaporator and stored at 20°C until MS analysis.

For MALDI-TOF MS analysis, the peptide pellet was solubilized in 10 µl of 0.1% TFA solution, then mixed in 1:1 ratio with matrix solution (1% α-cyano-4-hydroxycinnamic acid; 50% acetonitrile; 0.3% TFA) and poured onto a MTP 384 target plate (Bruker Daltonics). The plate was incubated at room temperature until samples were dried and complete crystallization of the matrix. The MS and MS/MS spectra were acquired in the Autoflex III MALDI-TOF (Bruker Daltonics) in the manual mode through the FlexControl ™ software (Bruker Daltonics). The parameters were adjusted to the positive ion reflection mode with acceleration voltage of 20 kV, admitting: trip rate of 100 Hz; range of mass of 700.0-5,000,0 Da; laser intensity 25-50%; and 2,100-4,000 accumulated shots per spectrum. The equipment was calibrated using a peptide mixture [M+H] + ions for standard MALDI-ToF/MS calibration. The peaklist.xml files were generated from the FlexAnalysis 3.4 program (Bruker Daltonics). Protein identification was performed using the local Mascot platform against the Lemnaceae protein database obtained from UniProt (http://www.uniprot.org). When unidentified, identification was performed on the Mascot opensource (http://www.matrixscience.com/) platforms, peptide mass fingerprint for MS data tool considering SwissProt database (www.uniprot.org/), from the database Viridiplantae and post-translational modifications: oxidation of methionine and carbamidomethyl. Only proteins with p-value ≤ 0.05 (significant score) were considered with positive identification.

Gene Ontology Analysis (GO)

The amino acid sequences of the differentially accumulated proteins were obtained in the MULTIFASTA format in the UniProtBETA database (http://beta.uniprot.org/uploadlists/). This file was used for comparative mapping to the species *Arabidopsis thaliana* (model for plant organisms) through the Mercator[24] program (http://www.plabipd.de/portal/mercator-sequence).

In addition, we performed an analysis of GO-terms enrichment with Panther software (http://www.pantherdb.org, accessed from http://geneontology.org) with standard parameters for mapping data against the protein GO database of *A. thaliana*. For this analysis, each duckweed DEP

UniProt access identifier was necessarily mapped to its corresponding most similar UniProt access in the *A. thaliana* proteome database. The set of more accumulated DEPs in the salt treatment was considered as the analyzed list, which was compared to: i) the reference proteome deduced *in silico* from the *A. thaliana* genome; and ii) the set of more accumulated DEPs in the control treatment. The type of analysis performed in the Panther program was the over-representation test, with Bonferroni correction and ontology database (complete for biological processes) updated in June 2015.

Network of interactions of differentially expressed proteins

In addition to the functional classification, we also carried out a search to predict the interactions among identified proteins, through the STRING program version 9.05 (Search Tool for the Retrieval of Interacting Genes/Proteins), available online (http: // string -db.org/), which provides a system for searching interactions predicted or already described in the literature among proteins or coding genes. The list of differentially accumulated proteins was explored against the *Arabidopsis thaliana* database available in STRING. Interactions may include direct (physical) and indirect (functional) associations, which are derived from four sources: genomic context, high-throughput, co-expression (conserved) and prior knowledge. Therefore, these interactions data are quantitatively integrated by STRING forming a functional protein network.

Starch content

The starch content was determined by the calorimetric method standardized by Appenroth et al.[25] The fresh plant material was macerated in 18% HCl (w/v), incubated at 5°C for 1 h. The suspension was then centrifuged at 5,000x*g* for 20 min. An aliquot of the supernatant was added in equal volume by lugol solution [0.5% (w/v) KI and 0.25% (w/v) I_2 in distilled water] and was used for absorbance read at 530 and 605 nm. The absorbance values were applied to the described equation for the determination of starch content.

RESULTS AND DISCUSSION

Proteomic Analysis

Phenolic extraction method allowed to recover and solubilize proteins in a large amplitude of molecular mass. The efficiency of the method and the integrity of the proteins can be observed through the electrophoretic migration profile in SDS-PAGE gel (Figure 1), revealing low concentration of interferers, compatible with the subsequent analyzes. Protein extraction was performed from a bulk of replicates of each treatment. Besides the advantage of the possibility of carrying out a larger number of analyzes on a smaller scale of time and material consumption, the grouping of the complex samples reduces the biological variation, thus increasing the capacity to detect differences between the treatments.[26]

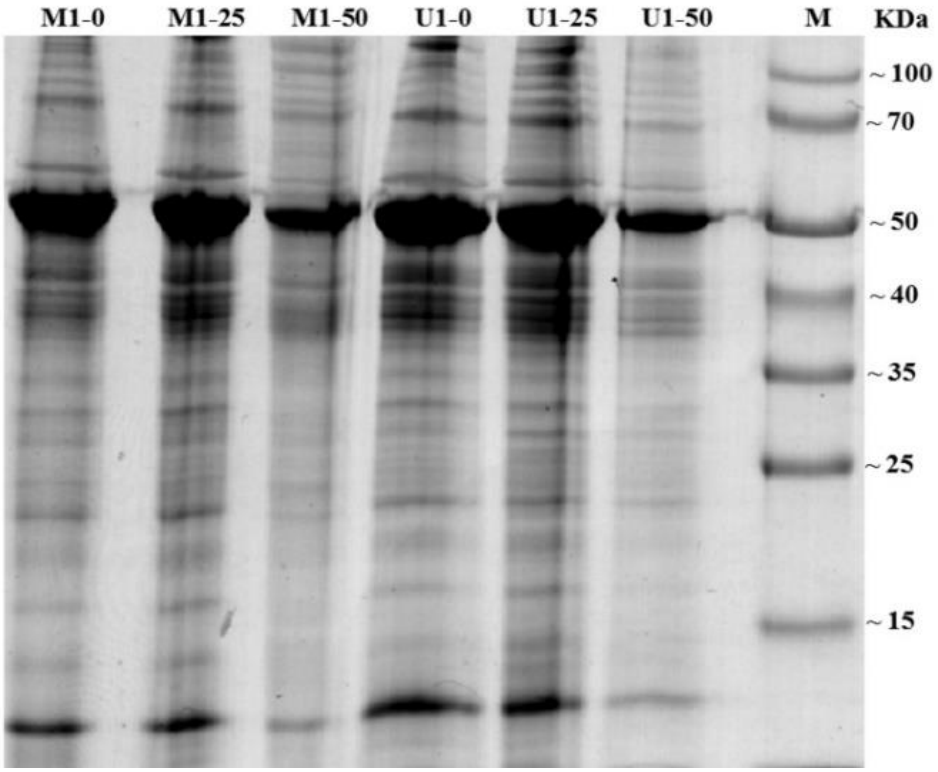

Figure 1. SDS-PAGE of total soluble proteins from samples of the clones M1 and U1 of *Lemna aequinoctialis* in response to NaCl: 0 mM; 25 mM and 50 mM NaCl. High molecular weight marker (MM) in KDa.

The correlation coefficient between the three replicates of the 2D gels of each treatment was significant, varying from 0.917 to 0.967, showing low experimental variation among the replicates of the same treatment, which favors the reliability of the results. In the analyzes of the gels (Figure 2) performed in the Image Master program, it was possible to verify the distribution of spots with molecular mass between 14.4 and 97.0 kDa with the most frequent concentration between 30 and 50 kDa (55.22% in M1 and 56.25% in U1), and pI between 4 and 7 (76.12% in M1 and 81.25% in U1) (Figure 2; Figures 3A and B).

The 2D electrophoresis allowed the detection of 1,302 spots of the M1 clone, with an average of 211 spots per gel (ranging from 187 to 258) in the T0 (0 mM NaCl) treatment, and 222 spots per gel (ranging from 179 to 260) in the T2 (50 mM NaCl) treatment, of which 82 were detected as DEPs, totaling 46 exclusive spots: 22 in T0 treatment and 24 in T2. The remaining 36 spots were detected in both treatments (common spots), out of which 8 were more accumulated in the control condition and 28 showed greater accumulation in the samples from the T2 treatment (Figure 3C).

In the U1 clone 1,242 spots were detected, with an average of 204 spots per gel (ranging from 183 to 218) in the T0 and 210 spots per gel (ranging from 186 to 248) in T2, of which 106 were exclusive: 35 in the T0 and 41 in T2. The remaining 30 DEPs were common to both treatments (5 in control treatment plants and 25 in stress plants) (Figure 3D).

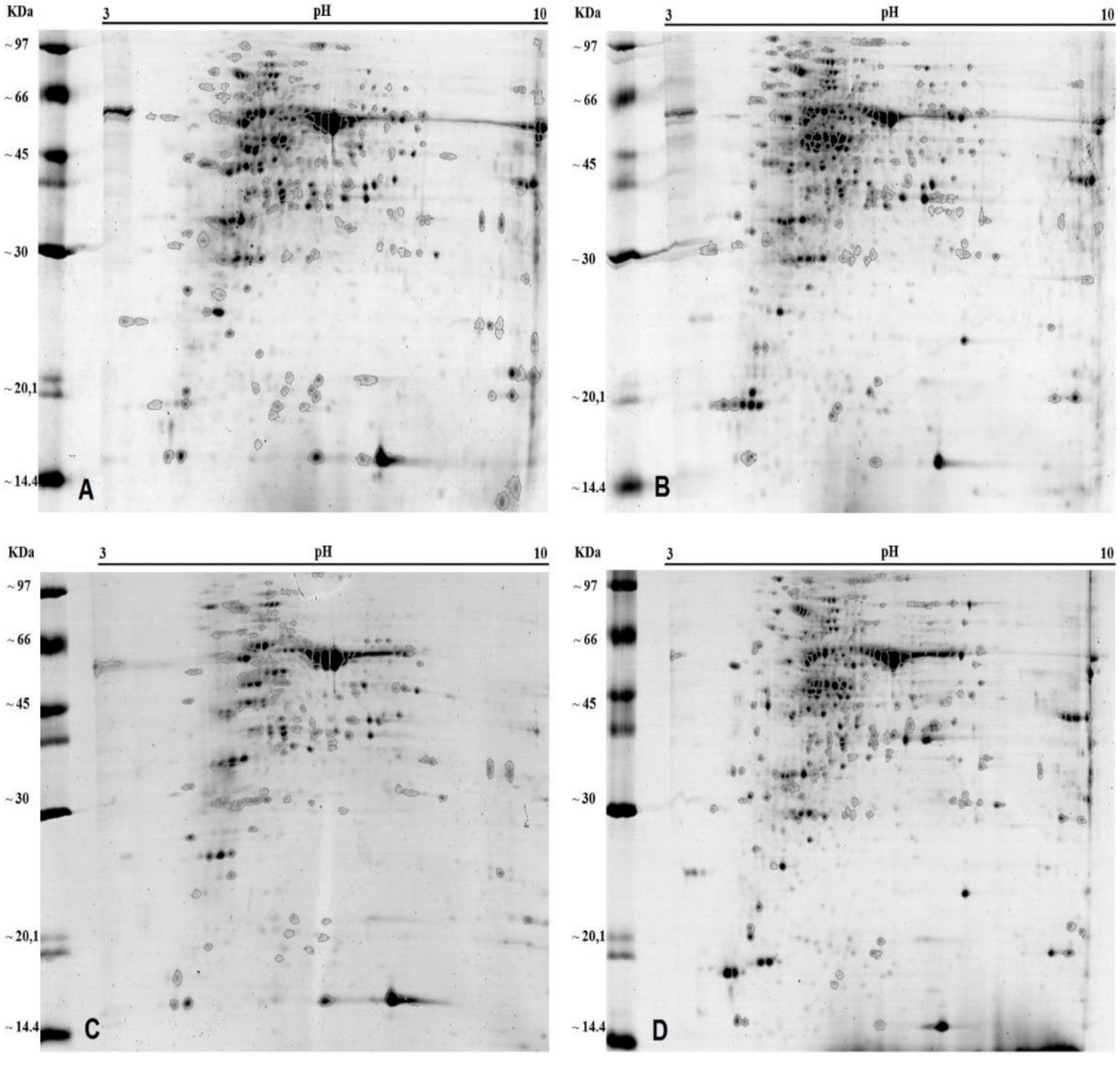

Figure 2. Images of 2D-PAGE locating spots using total protein extract from samples of the clones M1 (tolerant; A, B) and U1 (sensitive; C, D) of *Lemna aequinoctialis* in response to NaCl. The proteins were separated in 13 cm IPG drystrips, pH 3 to 10. (A, C) 0 mM NaCl; (B, D) 50 mM NaCl.

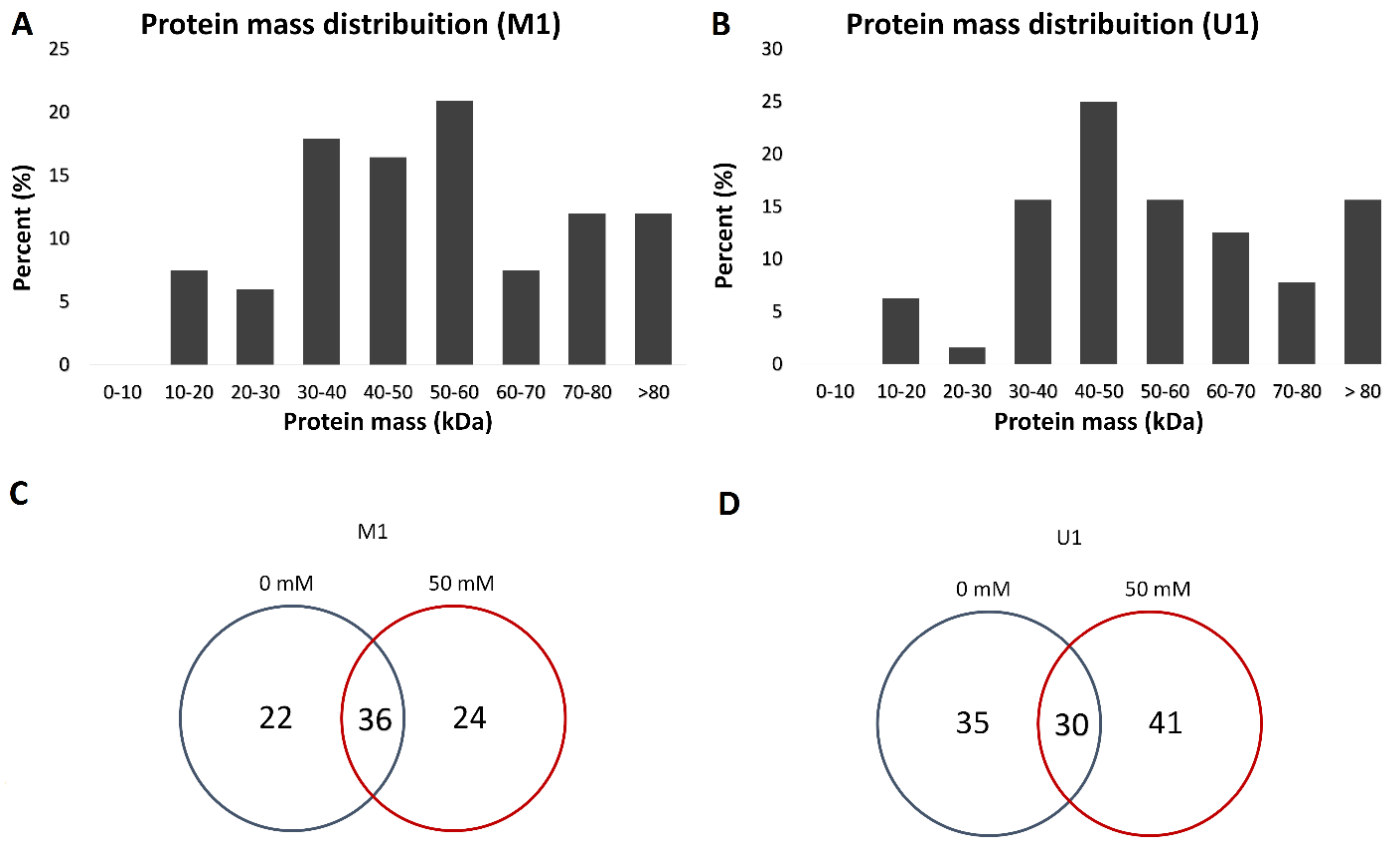

Figure 3. Distribution of identified proteins among different molecular weights (M1, A; U1, B); Venn diagrams presenting the number of differentially accumulated annotated proteins (DEPs) between contrasting NaCl concentration treatments (M1, C; U1, D).

The analysis of the 82 spots selected from the M1 clone by PMF (peptide mass fingerprinting) allowed the identification of 67 spots, in a mass spectra identification efficiency of 81.7%. Out from the 67 identified spots, six were matched to unknown proteins whose cellular roles are not clear, while 52 non-redundant proteins were putatively annotated, allowing to infer possible functional aspects of the differential proteome, as well as its association with physiological and metabolic processes. In the clone U1, 64 spots out from 106 were annotated to 64 non-redundant proteins (efficiency of 60.4%), from which 59 have known predicted functions and only one is unknown.

Some proteins with similar annotations were identified from different spots, and some of them presented changes in the values of molecular mass and isoelectric point when compared to the predicted values in databases, likely indicating post-translational modification events (Tables 1 and 2).

Table 1. Presumed annotation of the DEPs from clone M1 of *Lemna aequinoctialis*, according to the significant similarity scores found after Mascot program ($p \leq 0.05$). ANOVA refers to the calculated *p*-value for statistical significance of the difference in the spot %Vol between the treatments, being significant when $p \leq 0.05$; ratio refers to the variation between the spot %Vol in control related to NaCl treatment. For each spot the isoelectric point (pI) and the molecular mass (MM, in Da) calculated from the similar access and observed in the 2D-PAGE are shown.

Spot ID	Ratio M1-O: M1-2	ANOVA	Accession	Protein identified	Seq. Coverage (%)	Score	PI	MM (Da)	e-Value
More abundant in control (down-accumulated)							**Cal/Obs**	**Cal/Obs**	
1	2.27	1,58E-03	P19310	Ribulose bisphosphate carboxylase small chain SSU40B, chloroplastic	49%	63	7.57/7.45	20146/15939	5,40E-04
46	2.06	4,67E-03	H6THB6	ATP synthase subunit beta (Fragment)	26%	46	4.95/5.14	52490/58349	2,70E-02
61	1.85	1,50E-02	A9L9A4	Ribulose bisphosphate carboxylase large chain	44%	120	6.09/6.62	53594/57829	1,10E-09
106	4.26	2,91E-02	F8SVU2	Ribulose bisphosphate carboxylase small chain	58%	65	8.84/6.38	19976/15915	0,00036
133	1.81	3,23E-02	G1FBM3	50S ribosomal protein L2, chloroplastic	27%	49	10.93/9.34	30081/35984	1,30E-02
167	4.22	9,65E-03	A9L9A4	Ribulose bisphosphate carboxylase large chain	45%	129	6.09/6.52	53594/55770	4,80E-09
247	Unique	4,32E-06	O49931	Protein TIC 55, chloroplastic	18%	58	8.72/4.30	62771/27370	3,20E-02
248	Unique	4,11E-04	Q8WHG7	Ribulose-1,5-biphosphate carboxylase (Fragment)	25%	73	6.32/6.97	49976/34045	5,60E-05
239	Unique	1,46E-02	F4K1D2	Protein agamous-like 26	35%	63	9.14/4.14	42109/16226	0,026
251	Unique	7,54E-03	Q9FKW6	Ferredoxin-NADP reductase, leaf isozyme 1, chloroplastic	23%	58	8.32/5.99	40643/37207	2,40E-02
252	Unique	9,86E-03	E4MWF4	CASP-like protein 2B1	33%	59	9.62/5.57	21534/37443	2,20E-02
253	Unique	2,74E-02	H6THB6	ATP synthase subunit beta (Fragment)	12%	56	4.95/6.03	52490/39702	2,90E-03
255	Unique	2,00E-02	A2XD35	Adenylosuccinate synthetase	16%	58	6.39/7.66	52546/42279	8,10E-03
258	Unique	1,50E-02	Q8LPR9	Protein TIC110, chloroplastic	12%	57	5.73/7.76	112565/48317	3,20E-02
260	Unique	9,51E-04	O82264	NPL4-like protein 2	18%	55	5.00/6.63	46368/51742	4,70E-02
261	Unique	5,11E-05	Q9S7E9	Glutamate-glyoxylate aminotransferase 2	20%	70	6.21/8.02	53980/55659	0,0038
267	Unique	5,81E-04	A9L9D3	50S ribosomal protein L16, chloroplastic	25%	45	11.59/7.27	15321/62797	3,30E+01
289	Unique	6,91E-03	B6UG02	TIFY20 (Uncharacterized protein)	35%	65	9.92/8.46	17034/47540	3,00E-02
290	Unique	4,53E-03	Q8WHG7	Ribulose-1,5-biphosphate carboxylase (Fragment)	33%	91	6.32/7.09	49976/58515	8,60E-07
292	Unique	4,40E-03	Q66GI4	Proteinaceous RNase P 1, chloroplastic/mitochondrial	13%	61	9.20/5.24	65747/77683	2,90E-02
293	Unique	4,66E-03	P53684	Calcium-dependent protein kinase 7	11%	50	5.50/9.97	61627/49831	4,00E-02

297	Unique	3,67E-03	G1FBM3	50S ribosomal protein L2, chloroplastic	29%	45	10.93/9.08	30081/35609	3,30E-02
Most abundant in the stress (Up-accumulated)									
2	2.73	1,58E-02	Q9SB79	Putative B3 domain-containing protein REM4	22%	66	6.02/4,37	57946/20279	9,40E-03
12	3.57	1,09E-02	Q40186	NPR1 protein (Fragment)	40%	45	10.39/7,16	16515/39158	3,30E-02
18	1.67	4,54E-02	P0C378	Translation initiation factor IF-1, chloroplastic	65%	51	9.84/6,26	12410/41603	3,70E-02
20	2.48	2,90E-02	A0A096U9Z0	Uncharacterized protein	27%	66	8.40/7,28	66000/41677	2,10E-02
22	1.87	1,87E-02	A0A0C6WCT2	ScMYB17 protein	42%	56	9.65/6,04	27781/42065	6,80E-03
23	1.62	2,78E-02	O48905	Malate dehydrogenase, cytoplasmic	34%	61	6.39/5,77	35866/42248	3,10E-02
33	2.27	1,49E-03	B8AR30	GATA transcription factor 19	34%	57	6.14/5,74	29479/50675	9,10E-03
35	1.94	4,99E-02	Q3E9T2	B3 domain-containing protein REM2	19%	59	8.82/5,17	55515/51099	2,10E-02
39	2.18	1,35E-02	Q8MA05	ATP synthase subunit alpha, chloroplastic	21%	59	5.42/5,55	55844/53589	4,60E-02
40	2.82	2,18E-03	Q9C638	Protein weak chloroplast movement under blue light-like 2	12%	55	5.11/5,41	84516/53786	5,10E-02
41	2.15	2,79E-02	Q8M9U0	Photosystem I iron-sulfur center	79%	62	5.65/5,83	9382/53617	2,30E-02
52	1.94	2,99E-02	Q01859	ATP synthase subunit beta, mitochondrial	33%	62	5.95/5,36	59012/61108	3,20E-03
54	2.00	1,06E-02	Q9FFI0	Homeobox-leucine zipper protein HDG9	18%	57	7.31/4,89	81644/59806	0,03
70	2.98	3,03E-02	Q8WHL6	Maturase K	13%	46	9.45/4,86	61031/68458	3,00E-02
71	2.85	3,85E-02	P52416	Glucose-1-phosphate adenylyltransferase small subunit 1, chloroplastic	16%	58	6.43/5,40	55935/72595	3,20E-02
72	2.47	3,38E-03	F4K1D2	Protein agamous-like 26	29%	60	9.14/5,71	42109/73424	5,20E-02
73	3.06	9,72E-03	Q9S726	Probable ribose-5-phosphate isomerase 3, chloroplastic	34%	54	5.72/5,61	29401/73517	5,30E-02
77	1.70	3,09E-02	O23654	V-type proton ATPase catalytic subunit A	19%	61	5.11/5,68	69111/77362	4,30E-02
79	2.57	4,61E-02	P55238	Glucose-1-phosphate adenylyltransferase small subunit, chloroplastic/amyloplastic	14%	56	6.11/6,05	56413/79288	4,50E-02
83	3.21	2,35E-02	P22953	Probable mediator of RNA polymerase II transcription subunit 37e (HSP 70)	15%	73	5.03/5,16	71712/81213	2,00E-03
86	1.87	1,83E-02	C4J8G5	Uncharacterized protein	77%	67	11.27/5,59	8807/89245	1,70E-02
87	2.39	6,48E-03	Q9ZVT1	DUF724 domain-containing protein 1	22%	55	5.33/5,66	77211/89032	0,05
103	3.25	1,39E-02	O48905	Malate dehydrogenase, cytoplasmic	20%	58	6.39/6,24	35866/20102	3,30E-02
118	1.86	3,87E-02	I1KX44	Uncharacterized protein	27%	67	8.90/5,52	30837/30619	1,50E-02

126	4.76	6,30E-03	Q6AUR2	Nitrogen regulatory protein P-II homolog	31%	53	9.91/5,96	22744/41990	2,20E-02
144	2.97	4,71E-02	Q6AUR2	Nitrogen regulatory protein P-II homolog	27%	54	9.91/4,17	22687/34909	0,017
150	7.45	4,71E-03	Q9M3A8	Pentatricopeptide repeat-containing protein At3g49240	17%	58	5.41/4,41	71703/19387	2,50E-02
169	2.94	2,18E-02	P25819	Catalase-2	24%	65	6.63/5,85	57237/50930	0,0051
171	Unique	7,23E-03	Q9SD12	Probable protein phosphatase 2C 46	18%	64	8.51/7,76	42381/24253	1,50E-02
172	Unique	1,07E-03	-	Lignin-associated protein	11%	60	5.29/5,77	27359/36176	1,70E-02
175	Unique	1,13E-02	Q7FAH2	Glyceraldehyde-3-phosphate dehydrogenase	21%	67	6.34/5,74	36921/50675	6,90E-03
176	Unique	3,87E-04	A0A059Q220	Formin-like protein	9%	63	9.31/5,84	90605/46049	1,30E-03
179	Unique	6,80E-03	Q8LD63	Elicitor peptide 5	60%	60	10.02/5,94	10109/75952	1,60E-02
180	Unique	5,64E-03	P27323	Heat shock protein 90-1	12%	58	4.97/4,97	80870/95210	2,50E-02
182	Unique	7,39E-04	P37229	Malate dehydrogenase [NADP] 2, chloroplastic	18%	60	5.97/5,99	47124/45392	2,00E-02
185	Unique	2,36E-02	F4K1D2	Protein agamous-like 26	34%	62	9.14/4,47	42119/67516	2,90E-02
184	Unique	3,67E-03	Q01859	ATP synthase subunit beta, mitochondrial	20%	66	5.95/5,27	59012/64687	8,50E-03
188	Unique	1,09E-02	P13548	V-type proton ATPase catalytic subunit A	17%	65	5.30/5,56	68923/84619	0,012
190	Unique	9,59E-03	Q0DKF1	Thioredoxin H4-2	33%	52	5.70/3,86	14976/31183	3,20E-02
194	Unique	4,83E-03	G4WMV6	Putative peroxisome-type ascorbate peroxidase (Fragment)	25%	45	6.46/5,60	27953/38331	3,10E-02
215	Unique	8,38E-03	Q7XJ02	Probable L-ascorbate peroxidase 7, chloroplastic	10%	63	8.76/5,31	38473/72467	2,10E-03
224	Unique	9,99E-04	P36472	30S ribosomal protein S4, chloroplastic (Fragment)	46%	57	11.07/5,51	22643/99895	4,00E-02
226	Unique	1,70E-02	P36465	30S ribosomal protein S4, chloroplastic (Fragment)	48%	58	10.97/5,42	22706/93463	2,70E-02
233	Unique	8,28E-04	G1FB90	50S ribosomal protein L32, chloroplastic	59%	47	11.67/7,78	6417/87152	2,20E-02
236	Unique	2,92E-02	Q0DKF1	Thioredoxin H4-2	31%	51	5.70/4,14	14976/19584	3,70E-02

Table 2. Presumed annotation of the DEPs of the U1 clone Lemna aequinoctialis, according to the significant similarity scores found after Mascot program ($p \leq 0.05$). ANOVA refers to the calculated *p*-value for statistical significance of the difference in the spot %Vol between the treatments, being significant when $p \leq 0.05$; ratio refers to the variation between the spot %Vol in control related to NaCl treatment. For each spot the isoelectric point (pI) and the molecular mass (MM, in Da) calculated from the similar access and observed in the 2D-PAGE are shown.

Spot ID	Ratio U1-O: U1-2	ANOVA	Accession	Protein identified	Seq. Coverage (%)	*Score*	PI	MM (Da)	e-Value
More abundant in control (down-accumulated)							**cal/Obs**	**Cal/Obs**	
2	2,51623	2,20E-02	F8SVU2	Ribulose bisphosphate carboxylase small chain	51%	69	8.84/6.41	19976/16010	1,30E-04
3	3,97317	2,55E-03	F8SVU2	Ribulose bisphosphate carboxylase small chain	51%	75	8.84/7.42	19976/15963	3,10E-05
42	1,75882	4,43E-03	Q6ZH85	Plant intracellular Ras-group-related LRR protein 2	26%	64	5.51/7.88	55345/48187	0,0019
69	1,96243	4,93E-03	A9L9A3	ATP synthase subunit beta, chloroplastic	45%	82	5.01/5.29	53637/57471	7,30E-06
121	3,57387	3,93E-02	O48791	Stomatal closure-related actin-binding protein 1	28%	56	8.25/7.60	55840/43298	3,40E-02
227	Unique	4,05E-04	Q6Z1G7	Pyruvate dehydrogenase E1 component subunit beta-1, mitochondrial	18%	54	5.25/6.06	40204/42349	1,80E-02
228	Unique	9,29E-05	Q9FGM9	23.5 kDa heat shock protein, mitochondrial	33%	56	8.96/4.98	23454/44903	3,80E-02
229	Unique	1,24E-02	A2Z928	Glutamyl-tRNA reductase, chloroplastic	26%	60	8.80/5.77	58912/53852	5,10E-03
230	Unique	7,32E-05	Q9FIT7	Pentatricopeptide repeat-containing protein At5g61990, mitochondrial	13%	58	5.70/5.27	110278/55051	2,30E-02
235	Unique	1,98E-02	Q8WHG8	Ribulose-1,5-biphosphate carboxylase (Fragment)	28%	56	6.32/4.95	50041/61579	0,0027
237	Unique	2,09E-02	Q8WHI1	Ribulose-1,5-biphosphate carboxylase (Fragment)	17%	48	6.32/6.26	50122/67246	1,80E-02
244	Unique	1,38E-02	A0A059Q317	Transcription factor	21%	52	5.45/5.01	27100/31458	1,60E-02
274	Unique	2,03E-05	Q8WHG7	Ribulose-1,5-biphosphate carboxylase	40%	90	6.32/6.96	49976/61438	1,00E-06
275	Unique	7,71E-05	Q8WHG8	Ribulose-1,5-biphosphate carboxylase (Fragment)	39%	101	6.32/7.08	50041/62555	8,4E-08
296	Unique	1,50E-02	J7QZ92	Ribulose bisphosphate carboxylase large chain (Fragment)	26%	45	5.79/7.87	21807/61698	3,50E-02
Most abundant in the stressed (Up-accumulated)									
13	2,1776	3,10E-02	P29618	Cyclin-dependent kinase A-1	34%	50	6.52/5.19	34221/30641	4,20E-02
14	2,46559	5,81E-04	Q9XGX8	Mitochondrial import inner membrane translocase subunit Tim9	69%	62	5.85/5.35	11099/30829	2,60E-02
18	3,28602	3,14E-03	A2XMV1	Glutamate dehydrogenase 1, mitochondrial	15%	51	6.15/4.39	44599/32699	3,50E-02

26	2,50659	5,03E-03	B6UGG4	Ninja-family protein 4	40%	58	9.42/7.16	28096/39480	3,30E-02
27	2,97796	8,12E-04	Q7FAH2	Glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic	19%	62	6.34/6.84	36921/39583	2,60E-03
22	1,86701	2,95E-02	A0A096RA71	Uncharacterized protein (GT1)	28%	67	5.01/5.86	39100/36947	1,70E-02
34	2,30415	4,97E-02	P55229	Glucose-1-phosphate adenylyltransferase large subunit 1, chloroplastic	21%	58	8.03/6.08	58037/41889	2,70E-02
35	1,62508	1,26E-02	A0A067ZYH9	Catalase	25%	48	6.32/7.29	56820/41990	4,20E-02
56	2,51623	4,74E-03	Q8M9U0	Photosystem I iron-sulfur center	79%	61	5.65/5.61	9382/52676	3,30E-02
57	3,89852	4,74E-03	Q9M462	Glucose-1-phosphate adenylyltransferase small subunit, chloroplastic	30%	71	5.87/5.78	57294/52986	3,30E-03
58	2,33058	3,57E-02	B3GGB4	Fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase	12%	49	6.06/5.50	77642/53180	3,60E-02
68	1,83172	3,06E-02	O23255	Adenosylhomocysteinase 1	21%	57	5.66/6.02	53971/59189	3,10E-02
81	2,18757	3,27E-02	Q9M0N8	Mitotic-spindle organizing protein 1B	40%	56	4.77/6.03	7887/70743	4,00E-02
82	2,64472	4,52E-03	A0A059Q2G9	Potassium channel	18%	51	5.42/5.76	42338/72322	2,20E-02
83	2,50618	3,52E-03	Q9LU73	Protein SMAX1-LIKE 5	14%	58	8.01/5.66	116116/72074	2,40E-02
85	3,49609	1,90E-02	P09469	V-type proton ATPase catalytic subunit A	21%	92	5.29/5.58	69077/74909	2.6e-05
94	2,44187	1,60E-04	P36465	30S ribosomal protein S4, chloroplastic (Fragment)	42%	63	10.97/5.70	22706/87838	8,70E-03
99	2,74475	4,29E-02	O48905	Malate dehydrogenase, cytoplasmic	20%	58	6.39/5.87	35866/41732	3,30E-02
102	3,39784	3,05E-02	P11143	Heat shock 70 kDa protein	22%	66	5.22/5.08	70871/82474	7,50E-04
107	4,50269	1,27E-02	F2Q7G7	Allene oxide synthase	23%	46	8.11/5.84	53012/50137	2,70E-02
124	2,14398	1,49E-02	H6U804	40S ribosomal protein S10	49%	51	9.92/5.90	20334/87690	9,00E-03
125	1,85194	4,49E-02	Q01859	ATP synthase subunit beta, mitochondrial	43%	72	5.95/5.42	59012/60223	0,0024
126	3,62706	3,58E-02	Q9SD76	Alpha-glucan phosphorylase 2, cytosolic	28%	64	5.79/5.34	95499/60734	1,70E-02
134	1,76099	1,28E-02	P51119	Glutamine synthetase cytosolic isozyme 2	14%	60	5.69/5.56	39298/44560	2,10E-02
146	Unique	9,45E-04	Q3C1L6	30S ribosomal protein S3, chloroplastic	42%	60	9.73/4.59	25241/19288	3,60E-02
147	Unique	1,76E-04	Q9SSR7	F-box/LRR-repeat protein At1g52650	33%	57	7.53/7.79	53980/23338	3,10E-02
150	Unique	6,23E-03	Q9ZUE9	Pentatricopeptide repeat-containing protein At2g06000	24%	65	8.59/7.78	60988/31284	0,011
151	Unique	8,55E-03	Q6ZKC0	14-3-3-like protein GF14-C	39%	57	4.78/4.89	28979/33551	8,50E-03
152	Unique	1,61E-02	Q0DBS1	Putative DEAD-box ATP-dependent RNA helicase 51	18%	54	9.50/4.39	67153/19387	1,70E-02
154	Unique	5,99E-03	Q9LE73	Nudix hydrolase 4	36%	55	5.27/5.83	23857/43487	4,50E-02

155	Unique	1,15E-03	P38948	Major pollen allergen Aln g 1	41%	59	5.46/6.06	17385/43788	4,70E-02
157	Unique	3,84E-05	Q9M462	Glucose-1-phosphate adenylyltransferase small subunit, chloroplastic	16%	61	5.87/5.95	57294/48177	3,30E-02
159	Unique	1,53E-03	Q8WHH9	Ribulose-1,5-biphosphate carboxylase (Fragment)	38%	134	6.29/7.45	50052/57209	4,20E-11
161	Unique	5,03E-03	P36688	50S ribosomal protein L12, chloroplastic	28%	60	5.99/7.77	19675/70777	3,70E-02
162	Unique	4,18E-03	Q9LMP6	Probable disease resistance protein At1g15890	18%	59	6.24/7.85	97209/81711	1,90E-02
163	Unique	1,13E-03	Q6H9K5	30S ribosomal protein S4, chloroplastic	45%	58	10.46/7.05	24013/81658	0,033
172	Unique	7,17E-03	O22714	Pentatricopeptide repeat-containing protein At1g60770	28%	56	8.92/4.80	56098/65059	4,00E-02
177	Unique	9,43E-04	P52839	Cytosolic sulfotransferase 12	27%	57	5.36/7.58	37515/82440	2,80E-02
182	Unique	2,27E-03	Q0IY07	Origin of replication complex subunit 3	10%	59	6.98/6.21	81269/98872	5,50E-03
186	Unique	1,11E-02	V5J389	Drought responsive protein 2	49%	54	9.60/6.41	8935/99071	1,10E-02
187	Unique	1,42E-03	Q2QLI6	Microtubule-associated protein 70-1	27%	53	8.11/7.19	63689/81164	2,50E-02
190	Unique	2,42E-03	H6U7Z6	Glycolate oxidase	21%	46	9.06/9.57	40229/40924	2,50E-02
192	Unique	7,91E-03	Q9LZI2	UDP-glucuronic acid decarboxylase 2	31%	63	9.19/9.71	50111/40916	7,00E-03
194	Unique	1,97E-03	Q39067	Cyclin-B1-2	24%	56	9.32/4.11	50100/34457	0,039
195	Unique	3,51E-02	B5WX55	Ribulose-biphosphate carboxylase (Fragment)	40%	123	6.14/9.90	51976/54469	5,30E-09
201	Unique	3,01E-05	P34923	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	19%	65	6.19/5.37	36910/49596	1,30E-02
214	Unique	1,66E-03	Q8M9U0	Photosystem I iron-sulfur center	79%	57	5.65/5.81	9382/47913	4,00E-02
220	Unique	4,05E-04	Q9SRQ5	BTB/POZ domain-containing protein At3g03510	30%	55	8.02/5.53	59074/84872	1,80E-02
221	Unique	2,31E-02	Q8H2D1	B3 domain-containing protein REM8	28%	63	6.25/8.02	52448/48737	4,80E-02

272

Based on GO categories distribution obtained from differentially expressed proteins analysis regarding biological processes, a functional enrichment analysis was performed based on each sub-ontology, so that M1 and U1 derived DEPs were distributed into functional groups whose frequencies are presented in Figure 4A, B. In this section, the categorization of the total proteins the M1 identified showed that the most expressive functional groups during stress were: photosynthesis / energy production (15%), protein metabolism (11%), RNA (17%), stress response (8%) and plant defense (11%). In U1 it was observed a greater variety of functional groups, among them photosynthesis / respiration (15%), carbohydrate metabolism (13%), protein metabolism (17%) and cell cycle (7%). The GO categories of some proteins were classified into two or more functional groups.

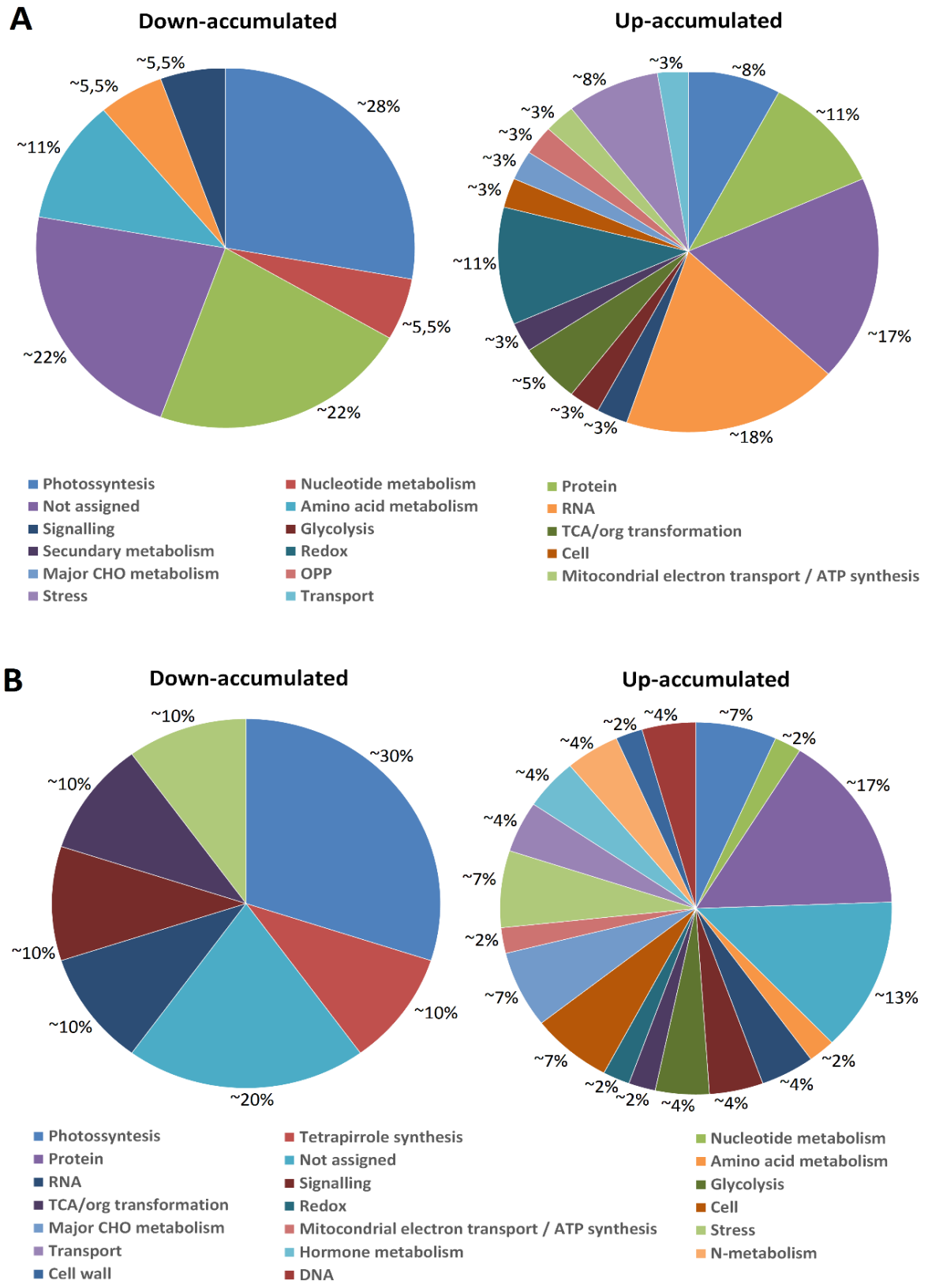

Figure 4. Categorization of the gene ontology of the DEPs of the clones M1 (A) and U1 (B) of *Lemna aequinoctialis* expressed in 0 mM NaCl (down-accumulated) and under the salt stress 50 mM NaCl (up-accumulated). The frequency (%) indicated in the sectors of the graph is identifiable of the categories of biological process according to the number of GO terms found for each set of DEPs, of each treatment, also represented in the respective legends. Category OPP (oxidative pentose phosphate pathway).

A number of proteins involved in photosynthesis were negatively regulated in the salt stress condition for both clones. Glutamyl-tRNA reductase (A2Z928), for example, is a protein involved in the chlorophyll biosynthesis and was exclusive of the control condition of the U1 clone, which might explain the chlorosis observed in the first 72 h of exposure to the stressor agent, that evolved to necrosis in some plants throughout the experiment. Chlorosis has also been attributed to the loss of photosynthetic pigments in other studies with duckweed.[28,29,12]

In clone M1, another component of the photochemical phase was observed only in the control condition: ferredoxin-NADP reductase (Q9FKW6), the last member of the photosynthetic electron transport before the reduction of $NADP^+$ to NADPH, which is used for CO_2 fixation.[30] However, even with the appearance of chlorosis on fronds submitted to the most severe treatment (50 mM NaCl) since the fifth day of experiment, this clone was biochemically noteworthy due to the ability to increase the content of chloroplast pigments in the treatment of 25 mM NaCl (data not shown), pointing the capacity to remain photosynthetically active under moderate stress, an important feature towards genotype tolerance.[31]

Salinity stress induced the accumulation of the Fe-S protein from the reaction center of the PSI (Q8M9U0), an intermediate in the electron flow, suggesting a possible attempt to minimize the effects of stress by the maintenance such flux, probably related to the avoidance of the formation and, consequently, the accumulation of oxygen radicals,[32] that can generate greater damage to a cell already damaged by salinity effects. However, the expression of glycolate oxidase (H6U7Z6) in the stress-stressed U1 clone indicates the need for the reduction and adjustment of photosynthesis, simultaneously with the activation of photorespiration and oxidative pathways.[33] In this process, due to RuBisCO oxygenase activity, 2-phospho-glycolate is formed within the chloroplasts, and subsequently translocated to the peroxisomes where it is oxidized by the glycolate oxidase generating the glyoxylate and most of the H_2O_2 produced during photosynthesis,[33] which is then deoxygenated by the peroxisomal catalase . Regarding RuBisCO in the light-independent phase for CO^2 fixation, there was also a difference in the

316 diversity and accumulation of proteins. In the control treatment of the two clones, there was a
317 higher accumulation of this protein considered very important for catalyzing the first step in the
318 fixation of CO2 in the photosynthesis.[15] Several DEPs were annotated as the major (M1: 2x
319 A9L9A4; U1: J7QZ92) and minor (M1: P19310, F8SVU2; U1: 2x F8SVU2) RuBisCO
320 subunits, in addition to the fragments (M1: 2x Q8WHG7; U1: 2x Q8WHG8 and Q8WHI1).

321 In general, these results confirmed the biochemical and physiological data that
322 evidenced the osmotic agent promoting an effective reduction in the content of photosynthetic
323 pigments and negatively affecting the photosystem I and II, especially in the more severe stress
324 (50 mM NaCl), leading to growth reduction (data not shown), since photosynthesis plays a
325 determinant role in the growth rate of plants.[34] Thus, the decrease in the expression level of
326 most of the identified proteins involved in photosynthesis suggests that this is the main
327 biological process suppressed during the exposure to saline stress, corroborating several reports
328 described in literature, including works with duckweeds.[12,28,29,35,36] In this aspect yet, saline
329 damage causes oxidative harms and directly affects the photosystems components, specifically
330 promoting adverse effects on chlorophyll synthesis, reduction of photosynthetic activity,
331 alteration in electron transport and impairing the organization of photosynthetic
332 complexes.[37,38,39] Reprogramming protein expression may indicate a possible redirecting of cell
333 metabolism to the synthesis of compounds involved in other biological processes,[40] e.g., the
334 accumulation of reserve and/or defense molecules.

335 In the M1 clone was observed higher frequency and variety of proteins associated to
336 the synthesis of ATP molecules, photosynthetic energy for growth, represented by spots
337 annotated as plastid ATP synthase α (Q8MA05), and mitochondrial ATP synthase β,
338 respectively annotated from different spots (2x Q01859, 2x H6THB6), as well as the subunit A
339 of vacuolar ATPase V/H^+ (O23654; P13548) associated with H^+ transport. In the results
340 obtained from gene ontology terms enrichment analysis, a significant variation was identified
341 between the saline treatment DEPs set and the reference *A. thaliana* proteome: exactly the

metabolic process of ATP (generation of precursors/energy) and the trans-membrane transport of hydrogen ions were significantly different (*p*-values of 0.0145 and 0,0282, respectively).

The higher efficiency and performance of M1 clone through exposure to saline stress, observed in biochemical and physiological parameters, may be associated with the differential activation of ATP synthases. The increase in the expression of the ATP complex in other proteomic studies in wheat[41] and rice[42] was related to adaptation to saline stress, as well as the positive regulation and higher frequency of vacuolar ATPase was related to cellular homeostasis during adaptation to salinity,[43] Due to the vital and effective strategy of osmotic adjustment from vacuolar sequestration and Na^+ compartmentalization, consequently reducing the concentration of these ions in the cytosol.[43] In this sense, plants vary in their ability to exclude Na^+ ions or to enclose them in the vacuole. This feature is determinant for tolerance, since it defines the capacity to avoid changes in the Na^+/K^+ ratio that could be harmful to the plant.[44]

In parallel, in the fronds of the two clones submitted to salinity, differential expression related to control was observed for enzymes associated with the carbohydrate metabolism, as well as with the glycolytic and tricarboxylic acid (TCA) pathways: glyceraldehyde-3-P dehydrogenase (Q7FAH2 in M1 and U1; P34923 in U1), a glycolysis/gluconeogenesis enzyme with reversible catalysis was also differentially accumulated; the glucose-1-P adenylyl transferase (P52416 and P55238 in M1, P55229 and 2x Q9M462 in U1), located in the chloroplast and/or amyloplast, a key enzyme that targets starch biosynthesis; and malate dehydrogenase (P37229 in M1, O48905 in U1), cytosolic (TCA cycle key enzyme) and chloroplast (2x O48905 in M1) forms. The functional performance of the enzymes involved with the glycolytic pathway represents the first stage of cellular respiration, followed by the citric acid cycle (TCA) and the electron transport chain. These interconnected pathways function to generate energy equivalents and carbon skeletons that can be used in the biosynthesis of various metabolites.[45]

This picture demonstrates an apparent energy metabolism reprogramming and seems to highlight the physiological costs of the stress condition. Possibly in clone M1 the production of metabolic energy occurs more dependent on the photochemical phase of photosynthesis than on glycolysis. In addition, the change in the levels of malate dehydrogenase under stress was observed mainly in M1. The mechanism of regulation of the malate dehydrogenase coding gene (*MdcyMDH*) has recently been investigated and its overexpression has been correlated to the increase in salicylic acid and redox content, conferring greater tolerance of plants to salt and cold.[46]

Proteomic data confirm that starch biosynthesis in duckweed is favored by situations that may trigger stress, as already observed[8,9,10,17] also confirming the biochemical analyzes (Figure 5). In this sense, when the comparison of the frequency of GO terms associated with the DEPs from saline treatment of M1 and U1 were compared to the frequency of the same GO terms associated with the DEPs from respective control treatments, the metabolic process of organic substances, such as carbohydrates, for example, showed to be significantly variable between the tested saline conditions (*p*-value of 0,0043 and 0,0109 , respectively).

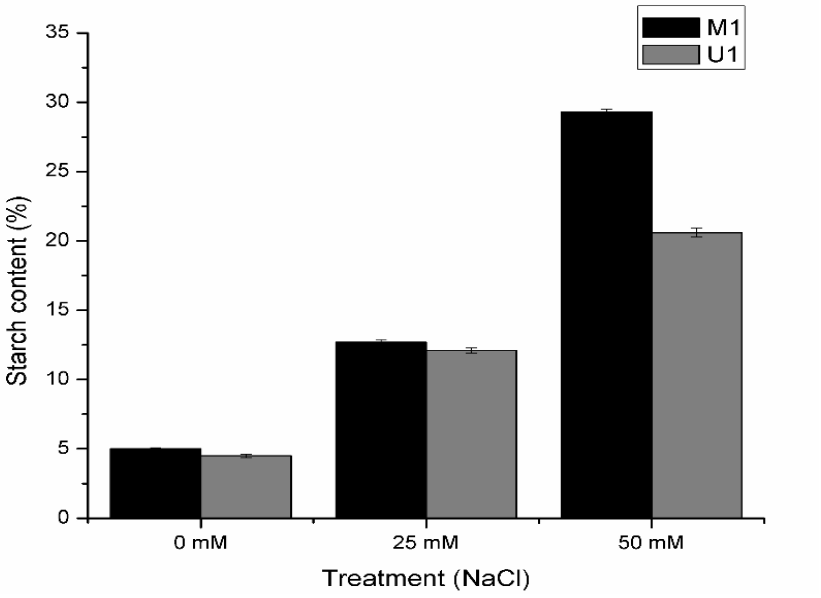

Figure 5. Starch content of the fresh biomass of *Lemna aequinoctialis* under the influence of saline stress. (Mean ± SD, n = 3).

On the other hand, only in clone U1 the activation of the glycolytic pathway was evidenced during stress by the accumulation of the bifunctional enzyme phosphofructokinase-2 / fructose 2,6-bisphosphatase (B3GGB4), which stimulates glycolysis and inhibits gluconeogenesis, indicating that the most sensitive clone probably takes a longer period to activate tolerance mechanisms, a behavior also observed in sugarcane subjected to saline stress.[22] Additionally, only in this clone submitted to NaCl the overexpression of cytosolic alpha-glucan phosphorylase 2 (Q9SD76), important in the degradation of starch, was observed, suggesting that under severe stress conditions the need for immediate consumption of carbohydrate reserve may be necessary to keep survival.[47]

Proteins categorized as stress responsive or involved in redox metabolism corresponded to another group of proteins with altered expression and triggered up-regulation after the detection of stress, especially in clone M1. The maintenance of cellular homeostasis is a determining characteristic, since salinization can trigger imbalance in the redox system of plants and cause physiological stress, which represents a major impairing for growth and development.[17] Stress resulted in greater accumulation (M1: P22953; U1: P11143) or activation (M1: P27323) of a group of responsive polypeptides known as heat shock proteins (Hsp), corresponding to the highly conserved families of Hsp70 and Hsp90, respectively. The accumulation of these proteins has already been detected by proteomics in response to salinity and water deficit.[48] In the cellular environment, the positive regulation of Hsp occurs when molecular mechanisms are activated to prevent or repair damage to proteins or membranes.[49] Under stress conditions, Nitric oxide (NO) and hydrogen peroxide (H_2O_2) participate in the transduction of signals related to Hsps expression, but the type of response depends on the ability of the plant to activate the defense mechanisms.[50]

In agreement with the distribution of oxidative molecules in the possible regulation of Hsps production, there was a strong trend to reprogram the expression of proteins involved in the ROS (reactive oxygen species) scavenging process: catalase (M1: P25819; U1:

A0A067ZYH9), ascorbate peroxidase (M1: G4WMV6, Q7XJ02) and thioredoxin (2x Q0DKF1 in M1). Early signaling events, including ion flow through the membrane, increases in Ca^{2+} levels in the cytoplasm, and the activation of mitogen-activated protein kinases (MAPKs), may lead to the production of ROS in the cell's natural metabolism, which may be increased during exposure to stress, causing imbalance in cellular homeostasis.[51] Therefore, the altered expression of these proteins may indicate an overload of oxidative molecules in the cell, providing oxidative stress which required a counterbalance from the antioxidant system. In this case, the coordinated activity of CAT and APX, distributed in different cell compartments, can ensure the effective elimination of H_2O_2, preventing them from diffusing through the membranes to other sites and inducing oxidative changes in several biological molecules.[52] Parallel to the expression of CAT and APX, another protein involved with redox homeostasis was expressed only in M1: thioredoxin, considered essential for controlling the flow of cytosolic and mitochondrial H_2O_2.[53]

In addition to the impact of salinity on the activity of antioxidant enzymes, the comparison between clones in the treatments with or without addition of NaCl showed accumulated or repressed DEPs in the saline condition related to signaling and transport, secondary metabolism, amino acid metabolism, nucleotides, RNA, protein metabolism and other functional categories. The accumulation of nitrogen-regulating homologous P-II protein (2x Q6AUR2), responsible for signal transduction and coordination of several mechanisms in nitrogen metabolism by interacting with ATPase,[54] shows that, in the presence of salinity, clone M1 increases the capacity of nitrogen assimilation, which is present in the composition of the most important biomolecules, such as ATP, NADH, NADPH, chlorophyll, proteins and nucleic acids.

A protein that acts on the formation of lignin precursors has been identified exclusively in M1 under stress (spot 172) which acts on the formation of precursors for lignin, a secondary polymer that when accumulated in cell walls can increase thickness and

permeability under stress, providing more structural rigidity and thickening in order to reduce water loss.[22,55] The stimulation of the phenylpropanoid pathway is a mechanism of adaptation to abiotic stress, such as drought, salinity, ozone poisoning and heavy metals.[56,57] In the case of saline stress, the greater the lignin deposition, the more difficult it becomes for the radial oxygen loss, a fundamental element in the process of sodium exclusion in plants submitted to saline stress.[58] In *Lemna minor* it was suggested that the toxicity of NH_4^+ stimulates the phenylpropanoid pathway inducing a change in metabolism towards lignin.[59] In addition to the role in monolignol biosynthesis for lignin formation, this enzyme is directly related to plant defense by promoting the increase of phenolic polymers that associate with and reinforces the cell wall.[55]

Activation of the alternative pathway of glucose degradation, pentose phosphate pathway, which accumulates about 3x plus ribose-5-P (Q9S726), a precursor of ribose and deoxyribose required in the synthesis of RNA And DNA, respectively.[60] In this respect, it was possible to observe that during the regulation of the proteins in face of the metabolic challenges, the enrichment of the ontologies associated with the metabolism occurred to both RNA metabolism and protein metabolism (Figure 4).

The signaling and transport proteins were more expressed in the saline condition in the two clones, where curiously the detected 14-3-3 (Q6ZKC0) protein, as well as a potassium ion channel (A0A059Q2G9) were found only in U1. Pacheco et al.[22] and Cheng et al.[61] reported the inhibition of 14-3-3 protein in tolerant genotypes, despite their interaction with ion transporters such as potassium channels and stress-related hormones such as abscisic acid (ABA), thus presenting an important role in the regulation of plant development and response to salt stress. In addition, the activity of the potassium channel allows the opening of a channel present in the cell membrane causing an increase in the K^+ ion output from the intracellular medium by passive diffusion,[62] which may justify the increase of Na^+/K^+ to 1.69 in the tissues

of the U1 clone, exceeding the maximum value (0.6) for the efficient maintenance of metabolism in glycophytes.[63]

It is known that proteins do not act in cellular processes as single entities, but form a functional network based on protein-protein interactions. The differentially expressed proteins in this study were analyzed using the database available in the STRING program, aiming to understand how salinity affects may alter protein interactions and, consequently, their cellular functions. Functional partners of these proteins were predicted and, based on this, predicted binding or relation of such cell actors was elaborated (Figure 6). The abbreviations referring to the specific names of the proteins shown in the network are indicated in Table 4, highlighting proposed functional partners based on described information available for such proteins and their links.

The proposed interaction network model includes, in brief, the direct relationship between photosensitive proteins: Fe-S from the PSI reaction center (PSAC) and the ATP complex proteins, including beta (AT5G08680) and alpha (ATPA). In turn, these proteins interact with the alpha subunit of ATPase (HAV-A), involved in the transport of protons through the membrane, which plays an important role for the synthesis of ATP. In addition, despite the lower frequency and abundance of proteins from the cellular respiration process in M1, it was possible to detect the interaction of the alpha subunit of ATP (identified only in M1) with the TCA cycle, through the two proteins identified as malate dehydrogenase AT5G58330, c-NAD-MDH1), which have a direct link with glyceraldehyde 3-P (GAPC2), important in carbohydrate metabolism and representative of the first phase of glycolysis, as well as interacting with catalase, corroborating with the literature.[46]

These observed putative interactions are confirmed by several evidences, derived from, for example, experimental data, neighborhood and gene co-expression. A link between the aforementioned proteins with the chaperone Hsp70 (HSC70-1) and Hsp90 (HSP90.1) was proposed, which in turn interact on one side of the network with the stress defense system

(AT4G35090, APX3, SAPX , TH9) and, on the other side of the network, there is a prediction that Hsps interact with the 30S and 50S subunits (ATCG00380.1 and RPL32, respectively), which constitute structural subunits of the ribosomes, related to protein translation.

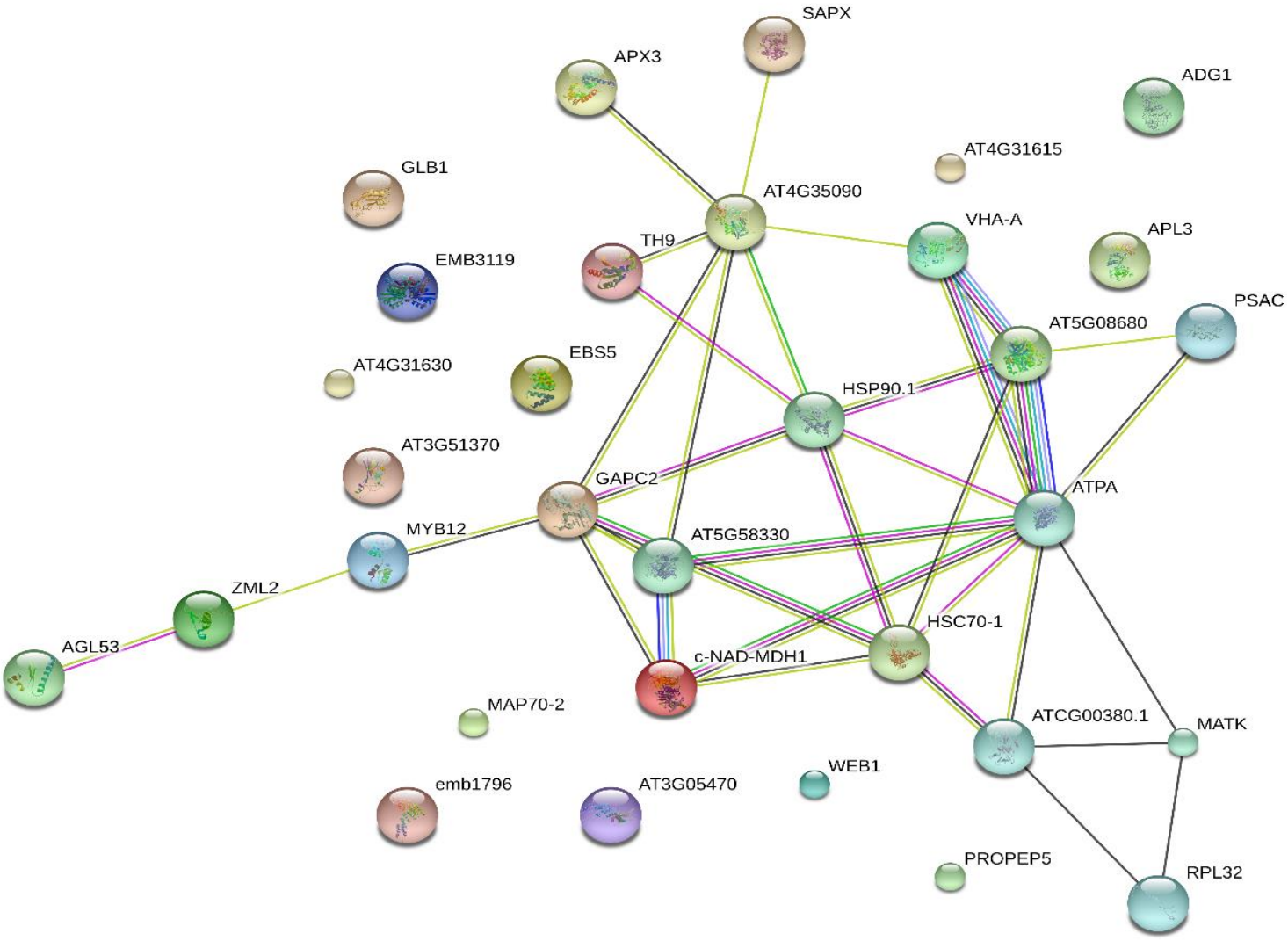

Figure 6. Protein-protein interaction network analyzed by the STRING software for the M1 clone of *Lemna aequinoctialis* up-accumulated DEPs. Lines of different colors represent different evidence of association: green, evidence of neighborhood; red, evidence of fusion; blue, evidence of co-occurrence; black, evidence of coexpression; purple, experimental evidence; light blue, evidence in database; yellow, evidence in text mining; light purple, evidence of homology.

Table 3. Abbreviations of the specific names of the functional interaction network proteins (Fig. 6), where a prediction of the interaction between the proteins identified with existing or predicted proteins, proposed by the STRING v. 9.05.

Nº do *spot*	String ID	Functional category
		Photosynthesis
41	PSAC	Photosystem I iron-sulfur center
39	ATPA	ATP synthase subunit alpha
52	AT5G08680	ATP synthase subunit beta
		Glycolysis
175	GAPC2	Glyceraldehyde-3-phosphate dehydrogenase 2
		TCA
182	AT5G58330	Malate dehydrogenase
23	c-NAD-MDH1	Malate dehydrogenase
		Redox
169	AT4G35090	Catalase-2
215	APX3	L-ascorbate peroxidase 7
194	SAPX	L-ascorbate peroxidase 7
190	TH9	Thioredoxin H4-2
		Protein
224	ATCG00380.1	30S ribosomal protein S4
233	RPL32	50S ribosomal protein L32
171	AT3G51370	Probable protein phosphatase 2C 46
73	EMB3119	Probable ribose-5-phosphate isomerase 3
		RNA
2	AT4G31630	Putative B3 domain-containing protein REM4
35	AT4G31615	B3 domain-containing protein REM2
22	MYB12	ScMYB17 protein
33	ZML2	GATA transcription factor 19
72	AGL53	Protein agamous-like 26
70	MATK	Maturase K
		Transport
77	VHA-A	V-type proton ATPase catalytic subunit A
		Stress
83	HSC70-1	Probable mediator of RNA polymerase (heat shock 70kDa protein)
180	HSP90.1	Heat shock protein 90-1
179	PROPEP5	Elicitor peptide 5
		Carbohydrate metabolismo
79	APL3	Glucose-1-phosphate
		Cell
20	MAP70-2	Microtubule-associated protein 70
		Signaling
126	GLB1	Nitrogen regulatory protein P-II homolog
		Not assigned
176	AT3G05470	Formin-like protein

During the occurrence of biotic stresses, there may or may not be the induction of an effective response in plants through a complex network of perception, amplification and signal transduction. Overall, the clones shared some common actions in response to salt stress as a consequence of comprehensive regulation. However, in earlier study, the changes induced by saline stress showed important intraspecific variations in duckweed.[17] Clone U1, for example, presented a variety of ontologies linked to glycolysis and protein metabolism, suggesting an increased need for metabolic precursors, as well as energy from cell respiration. In addition, it presented a greater reduction of chlorophyll content, and also the activation of photorespiration is possibly linked to a delayed response of the redox system, which may have contributed to the oxidative damages caused by the stress.

On the other hand, some of the responses seem to be differentially regulated in M1, which selects alternative metabolic pathways that require less mineral nutrition, probably due to the deleterious effects of salinity that make it difficult to redistribute elements between the organs to reallocate the biomass proportion. Thus, the rate of photosynthesis, cellular respiration and growth rate were adjusted, concomitant with the intense accumulation of starch, protein and activation of the redox system, which is, under stress conditions, the absorption of light energy, fixation of CO_2 and lowering respiratory consumption. The plants may have redirected the metabolic flow to the biosynthesis and storage of carbohydrates, as observed by Huang et al.[15]

The results suggest that the partial tolerance to salinity in the M1 clone may be associated with the more effective regulation of the sensing mechanisms that trigger modulation of the molecular responses, since this clone presents pathways that can confer potential tolerance with greater efficiency, which shows that the natural variations between clones of the same species regarding salt sensitivity can be characterized at the species level. In this sense, the accumulation of proteins of the antioxidant system may have guaranteed protection to the membranes, proteins and nucleic acids. The M1 clone was able to maintain organization and

synchronization of the metabolic machinery, accumulating signaling proteins, energy, synthesis and proteic protection, as well as evidences of the vacuolar sequestration and Na^+ compartmentalization. Concentration of these ions in the cytosol may help to assure the better performance against adversity by stress (Figure 7).

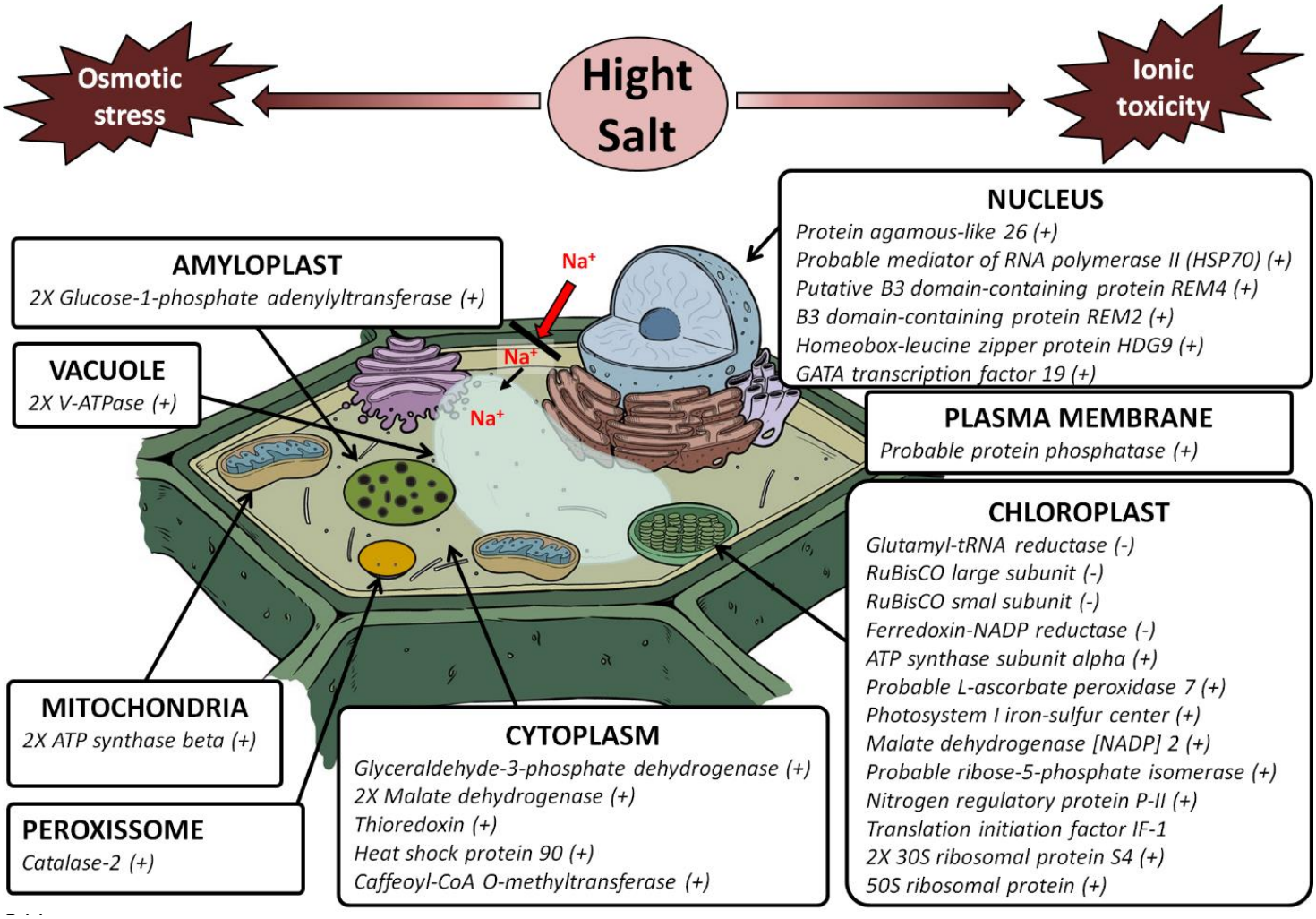

Figure 7. Schematic model of the foliar protein regulation processes in *Lemna aequinoctialis* M1 clone submitted to saline stress using the control treatment as reference. (+) Proteins more abundant in the control treatment; (-) Proteins more abundant in saline treatment.

CONSIDERATIONS

The set of results obtained in this work leads to the observation that the species *L. aequinoctialis* has reproduction capacity in aquatic environments with moderate salinization, and may be useful in desalination processes in water bodies. Saline stress affects photosynthesis reducing growth rate, but induces high starch and protein contents, making NaCl use a

commercially viable procedure for stress induction to produce bioethanol. In addition, in parallel to water phytoremediation, the biomass with suitable high protein amount can be directed to other applications, such as animal feeding. Among the identified DEPs, those that presented more significant association with salt stress tolerance were CAT, APX, ATPase, thioredoxin and a protein directly involved in lignin biosynthesis, which could be the focus of further investigation. It is therefore suggested that they may be candidates as auxiliar functional molecular markers towards salinity and stress tolerance.

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4 CONSIDERAÇÕES FINAIS

Sabe-se que já ocorreram muitos avanços do conhecimento a respeito dos mecanismos de tolerância à salinidade em plantas, em termos fisiológicos, bioquímico e molecular. Neste sentido, muito genes envolvidos com os mecanismos de tolerância à salinidade já foram identificados e utilizados em transformação genética. Entretanto, a tolerância à salinidade é um sistema complexo que resulta da expressão de diversos genes e muitas alterações podem ocorrer até a observação do fenótipo.

Os principais mecanismos de tolerância envolvem: exclusão iônica, tolerância do tecido aos íons tóxicos e tolerância osmótica modulados por complexas vias de transdução de sinal. Neste trabalho, a identificação de várias proteínas de categorias funcionais e metabólicas distintas, no capítulo II, sugere relevância diferente entre as atividades moleculares, fisiológicas e bioquímicas em lentilhas-d'água, corroborando os resultados obtidos previamente nas análises descritas no capítulo I. A extração comprovada de sais associada a ativação do sistema redox e acúmulo de amido evidenciam que *Lemna aequinoctialis* podem ser cultivadas em condições salinas moderas atuando na fitorremediação e assim sendo útil no processo de dessalinização de corpos hídricos. Por outro lado, o estresse salino afeta a fotossíntese atingindo as taxas de crescimento, mas induz elevado teor de amido e proteínas, tornando a utilização de NaCl um procedimento comercialmente viável na indução de estresse em lentilhas-d'água, gerando biomassa que podem viabilizar o aproveitamento de produtos de interesse.

Poucos estudos foram capazes de examinar os efeitos das modificações nos caracteres de tolerância à salinidade e seu reflexo no rendimento das culturas. Neste sentido, as variações detectadas no proteoma sugerem alvos com elevado potencial de utilidade em estratégias de seleção, melhoramento genético e estudos moleculares em lentilha-d'água. Assim, avanços futuros nas pesquisas, modificações genéticas e seleção assistida por marcadores são aguardados para que sejam superadas as dificuldades impostas pela complexidade da tolerância ao estresse salino e sejam obtidas plantas otimizadas e capazes de manter sua produção mesmo quando cultivadas em ambientes salinizados.

APÊNDICE

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1. FORMAÇÃO ACADÊMICA

1.1. Graduação

Licenciatura em Ciências Biológicas

Universidade do Estado do Rio Grande do Norte – UERN

Campus Central de Mossoró-RN

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1.2. Pós-Graduação

Mestrado em Melhoramento Genético de Plantas

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Título da dissertação: Ação combinada de fatores abióticos de estresse em variedades de cana-de-açúcar: variáveis fisiológicas e bioquímicas.

Orientadora: Lilia Gomes Willadino

2. PRODUÇÃO CIENTÍFICA

2.1. Artigos publicados ao longo do doutorado

ULISSES, C.; PEREIRA, J. A. F.; SILVA, S. S.; ARRUDA, E. C. P.; **MORAIS, M. B.** Indução e histologia de embriões somáticos primários e secundários do híbrido *Phalaenopsis* Classic Spotted Pink (Orchidaceae). Acta Biologica Colombiana, v. 21, p. 571-580, 2016.

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2.2. Artigos submetidos ao longo do doutorado

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Effect of multiple stresses on the oxidative metabolism of sugarcane varieties. Revista Ciência Rural, 2014.

2.3. Capítulos de livros submetidos ao longo do doutorado

WILLADINO, L.; **MORAIS, M. B.**; MELO, G. M.; CAMARA, T.
A cultura de tecidos e o pré-condicionamento ao estresse como estratégia de redução dos efeitos dos sais na planta. In: GHEYI, H. R.; DIAS, N. S.; LACERDA, C.F.; GOMES FILHO, E. (Eds.). Manejo da Salinidade na agricultura: Estudos básicos e aplicados. Fortaleza-CE, 2016.

MORAIS, M. B.; SILVA, F. A. C.; SOUZA, A. E. R.; CALSA, M. C. P.; CALSA JUNIOR, T.
Ômicas e genética. In: POMPELLI, M. F. Práticas laboratoriais em biologia vegetal. Capítulo 9, 2016 **(Versão online: https://biologiavegetal.com/capitulo-9/).**

2.4. Resumos publicados em anais de eventos

BARBOSA NETO, A. G.; **MORAIS, M. B**.; CALSA JUNIOR, T. Avaliação da plasticidade fenotípica de *Lemna aequinoctialis* (L.) para fitorremediação e produção de bioenergia. XXI Encontro de Genética do Nordeste, Recife-PE, 2016.

BARBOSA NETO, A. G.; **MORAIS, M. B.;** CALSA JUNIOR, T. Perfil proteômico de *Lemna aequinoctialis* (L.) cultivada em meio SH e efluente de estação de tratamento de esgoto. XXI Encontro de Genética do Nordeste, Recife-PE, 2016.

VASCONCELOS, S. R.; **MORAIS, M. B.**; BARBOSA NETO, A. G.; WILLADINO, L.; CALSA JUNIOR, T. Micropropagação de lentilhas-d'água (*Lemna aequinoctiales*): efeito do meio nutritivo e sistema de iluminação no metabolismo antioxidativo. In: XVI Jornada de Ensino, Pesquisa e Extensão da UFRPE (JEPEX 2016), 2016, Recife-PE.

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Desempenho de duas variedades micropropagadas de cana-de-açúcar submetidas a diferentes ambientes. In: XIX Congresso Brasileiro de Floricultura e Plantas Ornamentais e VI Congresso Brasileiro de Cultura de Tecido de Plantas, 2013, Recife-PE.

MORAIS, M. B.; FERREIRA, L. T.; ULISSES, C.; CAMARA, T.; WILLADINO, L.
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Metabolismo oxidativo de uma variedade de cana-de-açúcar (RB855453) submetida ao estresse múltiplo. In: XIX Congresso Brasileiro de Floricultura e Plantas Ornamentais e VI Congresso Brasileiro de Cultura de Tecido de Plantas, 2013, Recife-PE.

MACÊDO, C. R.; SILVA, M. M. A.; **MORAIS, M. B.**; CAMARA, T.; WILLADINO, L.
Micropropagação de cana-de-açúcar: Efeito das trocas gasosas e sistema de iluminação. In: XIX Congresso Brasileiro de Floricultura e Plantas Ornamentais e VI Congresso Brasileiro de Cultura de Tecido de Plantas, 2013, Recife-PE.

2.5. Participação em eventos técnico-científicos durante o doutorado

- Fórum de Oportunidade de Negócios em Biotecnologia, 2016. (Outro)

- XV Congresso Brasileiro de Fisiologia Vegetal, 2015. (Congresso)

- GE Day na FIOCRUZ Aggeu Magalhães, 2014. (Outro)

- I Simpósio de Fisiologia Vegetal na Unidade Acadêmico de Garanhuns - PE, 2014. (Simpósio)

- VI Congresso Brasileiro de Cultura de Tecido de Plantas, 2013. (Congresso)

- I Simpósio Regional de Melhoramento Genético de Plantas, 2013. (Simpósio)

- VI Seminário Estadual de Biotecnologia e Home Show, 2013. (Seminário)