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RESPOSTAS MORFOFISIOLÓGICAS E ANATÔMICAS DE *Dizygostemon riparius* AO ESTRESSE SALINO EM CONDIÇÕES DE CULTIVO *IN VITRO*

São Luís - MA
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Tese apresentada ao Programa de Pós-graduação em Ciências Agrárias da Universidade Estadual do Maranhão.
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Orientadora: Prof. Dra. Thais Roseli Corrêa
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RESUMO

O crescimento populacional e as mudanças climáticas intensificam os estresses abióticos, com a salinidade emergindo como um dos principais fatores limitantes à produtividade agrícola e à sobrevivência de espécies nativas. Neste contexto, os objetivos desta pesquisa foram investigar as respostas fisiológicas, morfológicas e anatômicas da Melosa (*Dizygostemon riparius*), ao estresse salino induzido por NaCl em condições de cultivo *in vitro*, e avaliar o potencial da biotina (vitamina B7) na mitigação de estresse salino. No Experimento I, a exposição a 0, 50 e 100 mM de NaCl demonstrou que a salinidade impôs um estresse em *D. riparius*, pois as plantas apresentaram redução no comprimento e a massa seca da parte aérea e o conteúdo relativo de água na folha, especialmente na maior concentração de NaCl. Fisiologicamente, houve redução nos pigmentos fotossintéticos e no índice de desempenho (PI) das plantas, indicando estresse funcional, embora a eficiência quântica máxima do fotossistema II (Fv/Fm) tenha se mantido estável. Quanto a resposta adaptativa, a planta exibiu alterações anatômicas, como o aumento da espessura da epiderme adaxial e do parênquima paliçádico. Além disso, o estresse salino alterou o conteúdo de poliaminas livres totais, com destaque para a putrescina, sugerindo potenciais limitações nos mecanismos de defesa da planta frente a salinidade. O Experimento II investigou a mitigação do estresse salino (50 mM) pela suplementação exógena de biotina. Observou-se que a salinidade, nesta concentração, causou uma limitação não estomática e bioquímica à fotossíntese, evidenciada pela queda acentuada na taxa de assimilação líquida de CO_2 e na eficiência de carboxilação (A/Ci), sugerindo um comprometimento na capacidade metabólica de fixação de carbono. A suplementação com biotina (16 mg L^{-1}) demonstrou ser um regulador metabólico eficaz, restaurando a taxa de assimilação líquida de CO_2, a eficiência de carboxilação e a eficiência intrínseca do uso da água (A/gs). Essa recuperação funcional ocorreu sem alterações na condutância estomática ou na anatomia foliar. Portanto, a salinidade limita a fotossíntese em *D. riparius* por meio de restrições bioquímicas e induz ajustes morfoanatômicos e metabólicos. Os resultados indicam que a biotina pode atuar como um agente modulador fisiológico na mitigação ao estresse salino em *D. riparius*. Esses dados contribuem para estratégias de conservação e avanço do conhecimento biotecnológico sobre a fisiologia de plantas nativas do Maranhão.

Palavras-chave: Planta medicinal; Melosa; Salinidade; Poliaminas; Biotina; Cultivo *in vitro*.

ABSTRACT

Population growth and climate change are intensifying abiotic stresses, with salinity emerging as one of the main factors limiting agricultural productivity and the survival of native species. In this context, the objectives of this study were to investigate the physiological, morphological, and anatomical responses of Melosa (*Dizygostemon riparius*) to NaCl-induced salt stress under *in vitro* cultivation conditions, and to evaluate the potential of biotin (vitamin B7) in mitigating salt stress. In Experiment I, exposure to 0, 50, and 100 mM NaCl demonstrated that salinity imposed stress on *D. riparius*, as the plants exhibited reductions in the length and dry mass of the aerial parts and in relative water content in the leaves, especially at the highest NaCl concentration. Physiologically, there was a reduction in photosynthetic pigments and in the plants' performance index (PI), indicating functional stress, although the maximum quantum efficiency of photosystem II (Fv/Fm) remained stable. Regarding the adaptive response, the plant exhibited anatomical changes, such as increased thickness of the adaxial epidermis and palisade parenchyma. Furthermore, salt stress altered the content of total free polyamines, particularly putrescine, suggesting potential limitations in the plant's defense mechanisms against salinity. Experiment II investigated the mitigation of salt stress (50 mM) through exogenous biotin supplementation. It was observed that salinity, at this concentration, caused a non-stomatal and biochemical limitation on photosynthesis, evidenced by a marked decrease in the net CO_2 assimilation rate and carboxylation efficiency (A/Ci), suggesting an impairment in the metabolic capacity for carbon fixation. Biotin supplementation (16 mg L^{-1}) proved to be an effective metabolic regulator, restoring the net CO_2 assimilation rate, carboxylation efficiency, and intrinsic water use efficiency (A/gs). This functional recovery occurred without changes in stomatal conductance or leaf anatomy. Therefore, salinity limits photosynthesis in *D. riparius* through biochemical constraints and induces morphoanatomical and metabolic adjustments. The results indicate that biotin can act as a physiological modulator in mitigating salt stress in *D. riparius*. These data contribute to conservation strategies and the advancement of biotechnological knowledge regarding the physiology of plants native to Maranhão.

Keywords: Medicinal plant; Melosa; Salinity; Polyamines; Biotin; *In vitro* cultivation.

CAPÍTULO I

INTRODUÇÃO E REFERENCIAL TEÓRICO

1. INTRODUÇÃO

O crescimento populacional aliado às intensas mudanças climáticas tem ampliado a ocorrência e a severidade de estresses abióticos, e se configura como um dos principais desafios para a sustentabilidade dos ecossistemas naturais e dos sistemas agrícolas (Waseem et al., 2024; Begna et al., 2025; Dubey et al., 2025). Dentre esses estresses, a salinidade destaca-se por sua crescente incidência, especialmente em regiões áridas e semiáridas, em decorrência da irrigação inadequada, da salinização dos solos, da escassez de água doce e das alterações nos regimes hidrológicos (De Souza et al., 2025; Sheikh et al., 2025).

O estresse salino impõe uma cascata de eventos deletérios às plantas, induzindo desequilíbrios iônicos, osmóticos e oxidativos, que resulta em alterações fisiológicas, bioquímicas e morfológicas e comprometem processos essenciais, tais como germinação, crescimento vegetativo, desenvolvimento, floração e frutificação (Yanez-Yazlle et al., 2021; Cao et al., 2023; Arminjon et al., 2025). Esses distúrbios afetam diretamente a produtividade e a sobrevivência das espécies vegetais, podendo levar à redução da diversidade vegetal em ambientes naturais (Kumar et al., 2021; Kumar et al., 2025).

Além dos sistemas agrícolas, espécies nativas de ambientes naturais, especialmente plantas ribeirinhas, também têm sido severamente afetadas pelo avanço da salinidade (Bănăduc et al., 2024; Desta, 2025). Alterações no regime de cheias e vazantes dos rios, a intrusão salina em áreas marginais, o represamento de cursos d'água e a elevação do nível do mar têm promovido mudanças na qualidade da água e do solo, aumentando a concentração de sais em ecossistemas fluviais e ripários (Xin et al., 2022; Sharma et al., 2024; Birati et al., 2025). Plantas ribeirinhas, embora adaptadas a ambientes úmidos, nem sempre apresentam tolerância ao excesso de sais, o que pode comprometer sua plasticidade morfofisiológica, sobrevivência e manutenção das populações naturais (Busoms et al., 2023; Zhang et al., 2025).

Dentre os mecanismos adaptativos acionados pelas plantas em resposta a estresses abióticos, destaca-se a síntese de moléculas de sinalização e proteção celular, como as poliaminas (PAs) (Pagnotta, 2025). Essas moléculas são compostos policatiônicos nitrogenados que contém mais de dois resíduos de amina e desempenham papel fundamental na manutenção da homeostase celular sob condições adversas (Kusano et al., 2014; Maurya et al., 2025). As principais PAs vegetais incluem putrescina (Put),

espermidina (Spd), espermina (Spm) e cadaverina (Cad) (Zhu et al., 2021). O aumento nos níveis de poliaminas constitui um dos indicadores metabólicos mais expressivos em plantas submetidas a diferentes tipos de estresse, como seca, salinidade, frio, calor, hipóxia, radiação UV e metais pesados (Nandy et al., 2022; Alsharafa et al., 2023; Napieraj et al., 2023).

Com o objetivo de mitigar os efeitos da salinidade, diferentes estratégias têm sido investigadas, incluindo o uso de fitoprotetores, biorreguladores e vitaminas, com resultados promissores no aumento da tolerância das plantas ao estresse salino (Albuquerque et al., 2024; Fitzpatrick et al., 2024; Fatahiyan et al., 2025). Entre essas substâncias, a biotina (vitamina B7) destaca-se por ser uma vitamina essencial, hidrossolúvel e amplamente distribuída nas células vivas (Alban et al., 2000). Em plantas, a biotina participa de processos metabólicos fundamentais, incluindo a biossíntese e o acúmulo de ácidos graxos, além de atuar como cofator enzimático, o que sugere seu potencial envolvimento em mecanismos de tolerância a estresses abióticos (Hahn et al., 2015; Wang et al., 2020; Wang et al., 2025).

Apesar dos avanços, ainda são limitadas as informações disponíveis sobre os mecanismos pelos quais a biotina atua na mitigação dos efeitos de estresses abióticos em plantas, especialmente sob condições de salinidade. No caso de *Dizygostemon riparius*, os estudos disponíveis ainda são escassos quanto à caracterização dos efeitos do estresse salino sobre sua morfofisiologia, e, até o presente momento, não há registros na literatura que avaliem o potencial da biotina como agente mitigador desse estresse. Essa espécie foi descrita cientificamente em 2019 no município de São Benedito do Rio Preto, Maranhão, às margens do rio, caracterizando-se como uma espécie ribeirinha adaptada a ambientes sujeitos a variações sazonais no regime hídrico (Scatigna et al., 2019).

Pertencente à família Plantaginaceae, *D. riparius* é um subarbusto aromático, de pequeno porte (até cerca de 50 cm de altura), com caules frequentemente ramificados e presença de tricomas glandulares, responsáveis por sua característica pegajosidade e pela produção de compostos voláteis (Galvão et al., 2023). A espécie ocorre em áreas de transição entre ambientes aquáticos e terrestres, podendo ser submetida a condições de alagamento periódico e, potencialmente, a variações na salinidade, o que a torna um modelo interessante para estudos de respostas a estresses abióticos (Dildar et al., 2025). Além disso, por ser uma espécie endêmica e de distribuição restrita, seu estudo é relevante não apenas do ponto de vista fisiológico, mas também para estratégias de conservação e uso sustentável (Scatigna et al., 2019; Galvão et al., 2023).

A espécie tem despertado interesse científico devido à presença de compostos bioativos identificados em extratos foliares e em seu óleo essencial, aos quais têm sido atribuídas atividades larvicida contra *Aedes aegypti*, fungicida no controle da antracnose (*Colletotrichum gloeosporioides*) em frutos de manga e acaricida no manejo do ácaro-vermelho *Tetranychus neocaledonicus* (Brandão et al., 2020; Galvão et al., 2023; Corrêa et al., 2023; Ferreira et al., 2025). Contudo, até o momento, existe apenas um estudo avaliando a resposta dessa espécie ao estresse salino, em que os autores utilizaram o ácido salicílico como agente mitigador em condições de casa de vegetação (Albuquerque et al., 2024), portanto, ainda é pouco compreendida quanto a sua plasticidade morfofisiológica, anatômica e bioquímica frente a estresses abióticos.

Diante desse cenário, torna-se necessário investigar resposta de *Dizygostemon riparius* sob condições de estresse salino, bem como avaliar o potencial da biotina na atenuação dos efeitos do cloreto de sódio (NaCl) em plantas cultivadas *in vitro*. O presente estudo apresenta caráter inédito ao empregar condições controladas de cultivo *in vitro* para simular o estresse salino em uma espécie ribeirinha endêmica do Maranhão, contribuindo para a elucidação de seus mecanismos fisiológicos e bioquímicos de tolerância. Além disso, os resultados obtidos poderão subsidiar estratégias de conservação, manejo e uso sustentável da espécie, bem como ampliar o conhecimento sobre a aplicação de abordagens biotecnológicas na mitigação de estresses abióticos em plantas medicinais nativas.

2. REFERENCIAL TEÓRICO

2.1 A Melosa (*Dizygostemon riparius*)

A espécie *Dizygostemon riparius* (Figura 1 A) pertence à família Plantaginaceae. Este grupo botânico compreende aproximadamente 25 gêneros e mais de 300 espécies com ampla distribuição mundial, sobretudo em regiões tropicais e subtropicais (Scatigna et al., 2019). O gênero *Dizygostemon* (Benth.) Radlk. ex Wettst. está atualmente inserido na tribo Gratioleae, um grupo predominantemente tropical dentro das Plantaginaceae, e apresenta distribuição restrita ao Nordeste do Brasil, configurando-se como um gênero endêmico dessa região (Souza; Giulietti, 2009).

Figura 1. *Dizygostemon riparius* oriundo de propagação vegetativa cultivada em vaso (A); *D. riparius* cultivado *in vitro* (B).

Historicamente, *Dizygostemon* foi descrito como uma seção monotípica do gênero *Beyrichia* Cham. & Schltdl., entretanto, diferenças morfológicas consistentes levaram à sua segregação e posterior elevação ao status de gênero independente (Scatigna et al., 2019). Até poucos anos atrás, o gênero era representado exclusivamente por *Dizygostemon floribundum* (Benth.) Radlk. ex Wettst. (sin. *Dizygostemon angustifolium* Giul.), espécie com distribuição restrita às formações de Cerrado e Caatinga dos estados do Piauí, Ceará, Pernambuco e Bahia (Giulietti, 1971; Souza; Galvão et al., 2023).

A descrição botânica de *Dizygostemon riparius* é recente, tendo sido publicada em 2019, a partir de populações nativas do leste do estado do Maranhão. Popularmente conhecida como “melosa” pela comunidade local do município de São Benedito do Rio Preto (MA), a espécie apresenta folhas e caules delgados com aroma refrescante característico (Scatigna et al., 2019; Galvão et al., 2023). Morfologicamente, trata-se de

um subarbusto aromático, com caules rastejantes a eretos, quadrangulares, ramificados e frequentemente providos de raízes adventícias, densamente recobertos por tricomas não capitados e glândulas sésseis, cuja densidade aumenta em direção ao ápice (Scatigna et al., 2019).

As folhas são predominantemente opostas, sésseis ou com pecíolos curtos, apresentando indumento diferenciado entre as superfícies adaxial e abaxial, com tricomas capitados e não capitados intercalados com glândulas sésseis. As flores são axilares, solitárias ou geminadas, com pedicelo curto e indumento semelhante ao das folhas. A corola é bilabiada, personada, de coloração branca a lilás, com manchas claras na garganta, característica típica do gênero (Scatigna et al., 2019).

Do ponto de vista ecológico, *D. riparius* apresenta comportamento singular ao ocupar ambientes ribeirinhos sujeitos a variações sazonais acentuadas. Durante a estação seca, a floração ocorre entre os meses de maio e dezembro, enquanto, no período chuvoso, populações naturais já foram registradas parcialmente ou totalmente submersas nas águas do Rio Preto, no município de São Benedito do Rio Preto (MA). Esses registros indicam que a espécie está adaptada a habitats periodicamente alagados, nos quais alternam-se condições de saturação hídrica e escassez de água ao longo do ano, evidenciando elevada plasticidade ecológica frente a variações extremas na disponibilidade hídrica (Scatigna et al., 2019).

Embora se trate de uma espécie recentemente descrita, *D. riparius* tem despertado crescente interesse científico em função de seu potencial biotecnológico. Estudos prévios demonstraram que seus extratos e óleos essenciais apresentam elevada atividade larvicida contra *Aedes aegypti*, evidenciando seu potencial como fonte de compostos naturais para o controle desse vetor de importância sanitária (Brandão et al., 2020; Martins et al., 2023). Além disso, Corrêa et al. (2023) relataram a eficácia antifúngica do óleo essencial da espécie no controle da antracnose em frutos de manga (*Colletotrichum* spp.), enquanto Ferreira et al. (2025) demonstraram efeito acaricida significativo contra *Tetranychus neocaledonicus*, associado a propriedades repelentes e à inibição da oviposição.

Outras abordagens recentes ampliaram a compreensão do potencial biotecnológico da espécie. Santos et al. (2024) evidenciaram o papel de fungos endofíticos associados a *D. riparius* na otimização da produção de metabólitos antifúngicos de interesse farmacêutico. Galvão et al. (2023) demonstraram que o rendimento e a composição química do óleo essencial variam significativamente em

função da sazonalidade e do período do dia, ressaltando a influência de fatores ambientais sobre o metabolismo secundário da espécie. Mais recentemente, Brandão et al. (2025) propuseram a incorporação do óleo essencial de *D. riparius* em sistemas nanoestruturados à base de polissorbato 80, configurando uma estratégia promissora de nanotecnologia verde para aplicações em saúde pública, com valorização da biodiversidade brasileira.

No que se refere ao estresse salino, a literatura disponível sobre *D. riparius* ainda é extremamente limitada. Até o momento, apenas dois estudos abordaram os efeitos da salinidade sobre a espécie. Albuquerque et al. (2024) demonstraram que a aplicação de ácido salicílico em condições de casa de vegetação contribui para a atenuação dos danos fisiológicos induzidos pela salinidade, aumentando a tolerância da planta ao estresse. De forma complementar, Pinheiro et al. (2025) relataram que *D. riparius* apresenta respostas adaptativas restritas ao estresse salino, sendo sua sensibilidade associada principalmente à incapacidade de manter níveis adequados de poliaminas e a eficiência fotossintética sob condições salinas.

Apesar desses avanços iniciais, permanecem lacunas significativas no entendimento do comportamento morfofisiológico, bioquímico e anatômico de *D. riparius* frente ao estresse salino, especialmente em sistemas controlados de cultivo *in vitro*. Ademais, não há registros na literatura sobre o papel de vitaminas, como a biotina, na mitigação dos efeitos deletérios da salinidade nessa espécie. Dessa forma, investigações que explorem o uso de abordagens biotecnológicas, aliadas ao cultivo *in vitro*, são fundamentais para elucidar mecanismos de tolerância ao sal, bem como para subsidiar estratégias de conservação, domesticação e uso sustentável dessa espécie medicinal endêmica do Maranhão.

2.2 Cultivo *in vitro* e estresse salino

Os fundamentos do cultivo *in vitro* de células vegetais e da cultura de tecidos remontam ao início do século XX, quando Gottlieb Haberlandt formulou o conceito de totipotência celular, segundo o qual cada célula vegetal contém toda a informação genética necessária para originar uma planta completa (Haberlandt, 2003; Fehér, 2019; Reddy, 2024). Essa hipótese consolidou a compreensão de que células vegetais diferenciadas são capazes de reentrar no ciclo celular, desdiferenciar-se, proliferar e regenerar tecidos, órgãos e, eventualmente, indivíduos completos, desde que submetidas a condições ambientais e nutricionais adequadas (Bidabadi; Jain, 2020; Boumya et al., 2024; Delgado-Aceves et al., 2025). Tal capacidade regenerativa está diretamente

relacionada à elevada plasticidade do desenvolvimento vegetal, característica que permite às plantas ajustarem seu crescimento e metabolismo em resposta a estímulos endógenos e exógenos (Pulianmackal et al., 2014; Ikeuchi et al., 2019; Zhang, Y et al., 2025).

A cultura de tecidos vegetais pode ser definida como o cultivo asséptico de células, tecidos ou órgãos vegetais isolados, mantidos em meios nutritivos artificiais sob condições ambientais controladas, com o objetivo de promover crescimento, diferenciação e regeneração (Lakshmana et al., 2005; Loyola-Vargas et al., 2018; Mishra et al., 2024;). Esses explantes são previamente desinfestados e cultivados em meios de cultura específicos, contendo macro e micronutrientes, fontes de carbono, vitaminas e reguladores de crescimento, permitindo o controle preciso das variáveis que influenciam o desenvolvimento vegetal (Andrade, 2002; Pasternak et al., 2024). Nesse contexto, a micropropagação destaca-se como uma técnica eficiente para a produção rápida, uniforme e sustentável de plantas, com ampla aplicação na conservação de germoplasma, melhoramento genético e multiplicação de espécies de interesse agronômico, florestal e medicinal (Lakshmana et al., 2005; Shukla et al., 2024; Kavinaya et al., 2025).

Além de seu uso para propagação clonal, o cultivo *in vitro* consolidou-se como uma importante ferramenta biotecnológica para o estudo de respostas das plantas a estresses abióticos, uma vez que permite a simulação de condições ambientais adversas em sistemas altamente controlados e reprodutíveis (Akhtar et al., 2024; Sota et al., 2025). A aplicação de estresses abióticos em condições *in vitro* tem sido amplamente utilizada para investigar alterações fisiológicas, bioquímicas, anatômicas e moleculares, possibilitando a identificação de mecanismos de tolerância e adaptação em nível celular e tecidual (Ritonga et al., 2021; Saharan et al., 2022; Al-Khayri et al., 2023; Kaur et al., 2025).

No contexto do estresse salino, a adição de cloreto de sódio (NaCl) ao meio de cultura é uma estratégia recorrente para simular ambientes salinos e avaliar o comportamento das plantas em condições controladas (Al Kashgry, 2025). Diversos estudos demonstram que essa abordagem permite reproduzir, de forma eficiente, os efeitos osmóticos e iônicos da salinidade observados em condições naturais, sendo amplamente empregada na avaliação da tolerância ao sal em diferentes espécies (Nas et al., 2023; Sahu et al., 2023; Yarte et al., 2023; Mahadik et al., 2024; Raspor et al., 2024; Atrous et al., 2025; Batool; Ma et al., 2025). Dessa forma, o cultivo *in vitro* constitui uma plataforma experimental robusta para a análise dos impactos da salinidade sobre o crescimento e o metabolismo vegetal.

O estresse salino é reconhecido como um dos principais fatores abióticos limitantes ao desenvolvimento das plantas, promovendo uma série de alterações fisiológicas, bioquímicas e morfológicas (Dixit et al., 2024; Admas et al., 2025). Durante as fases iniciais do desenvolvimento, a salinidade compromete a germinação das sementes, interferindo na absorção de água, na mobilização de reservas e na atividade metabólica necessária ao estabelecimento das plântulas (Wu et al., 2019; Manono, 2025). O aumento do potencial osmótico do meio e o acúmulo de íons tóxicos reduzem o potencial hídrico celular, afetam a disponibilidade de reservas energéticas e alteram o metabolismo proteico, comprometendo a viabilidade e o vigor das plantas jovens (Saud et al., 2022; Atta et al., 2024; Crivellaro et al., 2026).

Além disso, a salinidade reduz a taxa de germinação e altera o equilíbrio hormonal, promovendo o acúmulo de açúcares solúveis, ácido abscísico e amido, concomitantemente à redução dos níveis de ácido giberélico, o que resulta em atraso no crescimento e no desenvolvimento vegetal (Zorb et al., 2019; Anwar et al., 2023; Ghosh et al., 2025). Em estágios mais avançados, observa-se diminuição acentuada da biomassa, da área foliar e do crescimento de raízes e caules, refletindo limitações tanto no crescimento celular quanto na expansão dos tecidos (Silvestro et al., 2025; Falcioni et al., 2025; Linger et al., 2025).

A fotossíntese é um dos processos fisiológicos mais sensíveis ao estresse salino (Wang et al., 2024). A salinidade afeta diretamente a transpiração e a condutância estomática, resultando em redução da assimilação de carbono (Barhoumi et al., 2022; Jha, 2024; Dhansu et al., 2026). Essas limitações podem ocorrer tanto por mecanismos difusivos, associados ao fechamento estomático, quanto por danos diretos ao aparato fotoquímico e ao metabolismo do carbono no cloroplasto, incluindo a inibição de enzimas-chave do ciclo de Calvin (Maia et al., 2016; Qiao et al., 2024). Consequentemente, a eficiência fotossintética é comprometida, afetando o crescimento e a produtividade das plantas (Li et al., 2025).

Adicionalmente, o estresse salino interfere em parâmetros fisiológicos fundamentais, como a fluorescência da clorofila, a síntese de carotenoides e a estabilidade dos pigmentos fotossintéticos, favorecendo a degradação da clorofila e prejudicando a funcionalidade do sistema fotossintético (Tsai et al., 2019; Dou et al., 2025). A salinidade também impacta negativamente o potencial hídrico foliar, a pressão de turgor e o potencial osmótico celular, além de induzir toxicidade iônica decorrente do acúmulo

excessivo de Na^+ e Cl^- nos tecidos vegetais (Tripathi et al., 2025; Himami et al., 2025; Dhiman et al., 2026).

Esse acúmulo iônico favorece a geração de espécies reativas de oxigênio (EROs), como oxigênio singlete ($^1O_2$), ânion superóxido (O_2^-), peróxido de hidrogênio (H_2O_2) e radical hidroxila (OH), como resultado da desorganização do fluxo de elétrons nas cadeias transportadoras, estes promovem estresse oxidativo e danos às membranas celulares, proteínas e ácidos nucleicos (Fathi et al., 2025). Essa desorganização (Cloroplasto) promove o "vazamento" de elétrons, que passam a reagir com o oxigênio molecular, intensificando a formação dessas espécies altamente reativas. (Jomova et al., 2023). Paralelamente, ocorre redução na absorção e no transporte de íons essenciais, como potássio (K^+) e cálcio (Ca^{2+}), que agrava o desequilíbrio nutricional comprometendo processos metabólicos vitais (Wani et al., 2019; Wang et al., 2025; Sharma et al., 2025). O desequilíbrio iônico induzido pela salinidade afeta diretamente a arquitetura do sistema radicular, a absorção de nutrientes e a eficiência fisiológica das plantas, culminando em severas restrições ao crescimento e ao desenvolvimento (Sirohi et al., 2016; Khoza et al., 2025).

Nesse cenário, o cultivo *in vitro* destaca-se como uma abordagem estratégica para a compreensão integrada dos efeitos do estresse salino, permitindo a avaliação de respostas específicas em diferentes níveis de organização biológica. Essa abordagem possibilita investigar não apenas os impactos fisiológicos e estruturais, mas também a atuação de moléculas sinalizadoras, como as poliaminas, que participam da estabilização celular e da modulação do estresse oxidativo, e da biotina, uma vitamina essencial envolvida em reações metabólicas de carboxilação e na manutenção da homeostase celular. Dessa forma, o cultivo *in vitro* subsidia o desenvolvimento de estratégias mitigadoras baseadas na modulação bioquímica e metabólica, especialmente em espécies de interesse medicinal e ecológico.

2.3 Poliaminas (PAs)

As poliaminas (PAs) constituem um grupo de compostos alifáticos nitrogenados, de baixo peso molecular, hidrossolúveis e caracterizados pela presença de dois ou mais grupos amino, e amplamente distribuídas em todos os organismos vivos (Dinler et al., 2021; Shoket, 2024; Şengül et al., 2025). Nas células vegetais, podem ocorrer tanto na forma livre quanto conjugadas a ácidos fenólicos, proteínas, fosfolipídios e ácidos nucleicos, o que amplia sua diversidade funcional e seu papel na regulação metabólica e

estrutural (Fazilati et al., 2015; Rakesh et al., 2021; Puente-Moreno et al., 2025). Em plantas, as poliaminas participam ativamente de processos fundamentais do desenvolvimento, incluindo embriogênese somática, organogênese, diferenciação celular, floração, frutificação e senescência, além de desempenharem funções centrais nas respostas a estresses abióticos e bióticos (Rajam et al., 1998; Kumria et al., 2002; Tyagi et al., 2023; Blázquez et al., 2024; Sreepathi et al., 2025).

As poliaminas mais abundantes em plantas superiores são a putrescina (PUT), a espermidina (SPD) e a espermina (SPM), embora termospermina e cadaverina também possam ser detectadas em tecidos específicos ou em determinadas espécies (Minocha et al., 2014; Jancewicz et al., 2016; Burke et al., 2024). A distribuição subcelular dessas moléculas é distinta e funcionalmente relevante: SPD e SPM encontram-se predominantemente associadas à parede celular, interagindo com polissacarídeos e contribuindo para a regulação da lignificação, da rigidez celular e do pH apoplástico, enquanto a PUT é majoritariamente citosólica, e está diretamente envolvida em processos metabólicos dinâmicos (Oelmüller et al., 2023; Kolesnikov et al., 2024; Lavandosque et al., 2025). A cadaverina, por sua vez, ocorre em concentrações reduzidas, é mais frequentemente relatada em leguminosas (Bais et al., 2000; Rakesh et al., 2021). Essas diferenças refletem não apenas a diversidade estrutural das PAs, mas também sua especialização funcional ao longo da evolução vegetal (Munné-Bosch, 2025).

Do ponto de vista fisiológico, as poliaminas exercem papel central na proteção das plantas frente a estresses ambientais, como variações extremas de temperatura, déficit hídrico, salinidade e ataques bióticos (Shao et al., 2022; Amiri et al., 2024). Evidências experimentais indicam que essas moléculas atuam em múltiplos níveis de organização celular, desde a regulação da expressão gênica até a modulação direta da estabilidade de membranas e macromoléculas (Berberich et al., 2014; Schibalski et al., 2024). Sob condições de estresse salino, a biossíntese, o acúmulo e o balanço entre PUT, SPD e SPM são frequentemente alterados, configurando um importante marcador metabólico da capacidade adaptativa das plantas (González-Hernández et al., 2022; Jangra et al., 2023).

Diversos estudos demonstram que as PAs contribuem para a tolerância ao estresse em plantas por meio de mecanismos complementares e interdependentes. Entre esses mecanismos destacam-se:

(i) sua atuação como solutos compatíveis, em conjunto com outros osmólitos, como prolina, glicina betaína e ácido gama-aminobutírico (GABA), auxiliando na manutenção do potencial osmótico celular;

(ii) a interação eletrostática com macromoléculas negativamente carregadas, como DNA, RNA, proteínas e membranas, promovendo estabilização estrutural e proteção contra desnaturação;

(iii) a função antioxidante direta, por meio da neutralização de espécies reativas de oxigênio (EROs), e indireta, via indução da atividade de enzimas antioxidantes e da síntese de metabólitos protetores;

(iv) o papel como moléculas sinalizadoras em vias de resposta ao estresse, frequentemente integradas à sinalização mediada por ácido abscísico (ABA) e pela geração controlada de peróxido de hidrogênio (H_2O_2);

(v) a regulação de canais iônicos, especialmente aqueles relacionados ao transporte de K^+, Ca^{2+} e Na^+, contribuindo para a homeostase iônica;

(vi) a participação em processos de morte celular programada, fundamentais para a adaptação e defesa das plantas (Takahashi et al., 2010; Alet et al., 2011; Hussain et al., 2011; Gupta et al., 2013; Shi et al., 2014; Jangra et al., 2023; Blázquez, 2024; Burke et al., 2024; Lavandosque et al., 2025; Khan et al., 2026).

No contexto do cultivo *in vitro*, as poliaminas assumem relevância adicional, uma vez que seus níveis endógenos e a razão entre PUT, SPD e SPM podem refletir com precisão o estado fisiológico das plantas submetidas a condições controladas de estresse (Mogazy, 2024). Sistemas *in vitro* permitem a avaliação detalhada do metabolismo de PAs em resposta à salinidade, possibilitando identificar padrões metabólicos associados à tolerância ou sensibilidade ao estresse, sem a interferência de variáveis ambientais externas (Talarico et al., 2025).

Para espécies medicinais nativas e pouco estudadas como *D. riparius*, a investigação do metabolismo de poliaminas sob estresse salino torna-se particularmente relevante. Estudos recentes indicam que a espécie apresenta respostas adaptativas limitadas à salinidade, associadas, entre outros fatores, à incapacidade de manter níveis adequados de PAs e à redução da eficiência fotossintética sob estresse (Pinheiro et al., 2025). Esses achados reforçam a hipótese de que o balanço de poliaminas pode desempenhar papel decisivo na sensibilidade da espécie ao NaCl.

Assim, a análise integrada do conteúdo de poliaminas em *D. riparius* cultivada *in vitro*, associada a parâmetros morfofisiológicos, anatômicos e bioquímicos, representa uma abordagem promissora para elucidar os mecanismos subjacentes à resposta da

espécie ao estresse salino. Além disso, esse conhecimento pode subsidiar estratégias de mitigação, incluindo o uso de agentes moduladores do metabolismo, como vitaminas e reguladores de crescimento, contribuindo para a conservação, domesticação e exploração biotecnológica sustentável dessa espécie endêmica de elevado valor medicinal.

2.4 Biotina

A biotina, também conhecida como vitamina H ou vitamina B7, é uma vitamina hidrossolúvel amplamente reconhecida por sua atuação como cofator essencial de uma família restrita, porém metabolicamente estratégica, de carboxilases envolvidas no metabolismo primário (Carling et al., 2019; Malik et al., 2023). Seu papel bioquímico foi elucidado há quase três décadas, quando se estabeleceu que a biotina integra o grupo prostético de enzimas-chave responsáveis por reações de carboxilação, descarboxilação e transcarboxilação, fundamentais para a manutenção da homeostase celular (Alban et al., 2000; Pinon et al., 2005; Mildvan et al., 2025).

Diferentemente dos animais, que dependem exclusivamente da ingestão dietética dessa vitamina, as plantas e a maioria dos microrganismos possuem vias metabólicas próprias para a biossíntese de biotina (Yang et al., 2024). Em plantas superiores, essa biossíntese ocorre de forma compartimentalizada, envolvendo etapas no citosol e nas mitocôndrias, o que reflete a integração funcional da biotina com processos metabólicos energéticos e redox (Alban et al., 2000; Fonseca-Pereira et al., 2023). No nível celular, a biotina pode ser encontrada tanto na forma livre quanto ligada covalentemente a proteínas, especialmente às carboxilases (Shen et al., 2024). Enquanto em células animais e bacterianas a fração livre é extremamente reduzida ou indetectável, células vegetais apresentam quantidades relativamente elevadas de biotina livre, sugerindo funções adicionais além da atuação clássica como cofator enzimático (Xu et al., 2023; Kim et al., 2023).

A descoberta da biotina está historicamente associada à identificação da avidina, uma proteína presente na clara do ovo capaz de se ligar fortemente à biotina, induzindo estados de deficiência em modelos animais. Esse achado foi decisivo para o entendimento de sua função biológica e impulsionou pesquisas sobre sua importância metabólica em diferentes organismos (Hertz, 1946; Lanska, 2012; Balzer et al., 2023). Em plantas, estudos subsequentes revelaram que a biossíntese e a disponibilidade de biotina são rigidamente reguladas, uma vez que seu acúmulo excessivo implica elevado custo

energético, especialmente em tecidos com alta taxa metabólica (Dias et al., 2025; Garneau et al., 2025).

A demanda por biotina é particularmente intensificada em fases de rápido crescimento e diferenciação celular, como o desenvolvimento vegetativo, a formação de sementes e as respostas adaptativas a condições adversas (Fan et al., 2024). Entre as enzimas biotina-dependentes, destaca-se a acetil-CoA carboxilase (ACC), responsável pela etapa inicial e limitante da biossíntese de ácidos graxos (Čavužić et al., 2024). Dessa forma, a biotina desempenha papel central na manutenção da integridade das membranas celulares, na fluidez da bicamada lipídica e na funcionalidade de organelas como cloroplastos e mitocôndrias (Baldet et al., 1997; Alban et al., 2000; Alban, 2011; Gibbs et al., 2021).

Além de sua função estrutural e metabólica, evidências recentes sugerem que a biotina pode atuar indiretamente na modulação das respostas das plantas ao estresse abiótico (Fonseca-Pereira et al., 2023; Ergashev et al., 2024). Alterações na disponibilidade de biotina afetam o metabolismo de lipídios, carboidratos e aminoácidos, impactando a eficiência fotossintética, o balanço redox e a capacidade de adaptação celular frente a condições de salinidade, déficit hídrico e estresse oxidativo (Kumari et al., 2022; Wang et al., 2025). Isso porque a vitamina B7 é indispensável para a atividade da Acetil-CoA Carboxilase (ACCase), uma enzima-chave na biossíntese de ácidos graxos, precursores de lipídios estruturais e de reserva nas plantas (Tong, 2013).

Além disso, é um cofator essencial para a Piruvato Carboxilase, que participa da gliconeogênese, convertendo piruvato em oxaloacetato, um intermediário crucial para a síntese de açúcares a partir de fontes não carboidratos (Lietzan et al., 2014). A sua presença assegura, portanto, a integridade de processos energéticos e estruturais que sustentam o crescimento, desenvolvimento e adaptação das plantas (Alban, 2011). Nesse contexto, a suplementação exógena de biotina tem sido investigada como uma estratégia potencial para mitigar danos metabólicos induzidos por estresses ambientais (Dakshinamurti, 2005; Wal et al., 2023), embora seus mecanismos de ação ainda não estejam completamente elucidados, especialmente em espécies nativas e não modelo.

Para *Dizygostemon riparius*, uma espécie medicinal endêmica e associada a ambientes ribeirinhos sujeitos a flutuações sazonais de disponibilidade hídrica e qualidade da água, a investigação do papel da biotina torna-se relevante. Considerando a sensibilidade previamente relatada da espécie à salinidade e sua limitada capacidade de ajuste bioquímico sob estresse (Pinheiro et al., 2025), a biotina pode representar um fator

modulador do metabolismo, contribuindo para a manutenção da integridade das membranas, da eficiência fotossintética e do crescimento vegetal em condições salinas.

Assim, o estudo da biotina no contexto do cultivo *in vitro* de *D. riparius* oferece uma abordagem inovadora para compreender a integração entre metabolismo primário, estresse abiótico e mecanismos de mitigação, além de fornecer subsídios para estratégias biotecnológicas voltadas à conservação e ao uso sustentável dessa espécie.

Com o intuito de melhor apresentação e a discussão dos resultados, esta tese foi estruturada em capítulos independentes e complementares. O Capítulo II aborda o estudo intitulado "Homeostase de poliaminas e respostas morfofisiológicas à salinidade em *Dizygostemon riparius*: uma espécie endêmica do bioma Cerrado brasileiro", no qual são investigados os mecanismos fisiológicos e bioquímicos associados à resposta da espécie ao estresse salino. Já o Capítulo III contempla o trabalho "A biotina exógena modula a limitação fotossintética não estomática sob salinidade em *Dizygostemon riparius* cultivado *in vitro*", focando no papel da biotina como agente mitigador, com ênfase nas respostas fotossintéticas e metabólicas. Dessa forma, os capítulos se articulam de maneira complementar, proporcionando uma compreensão integrada dos mecanismos de resposta a salinidade na espécie estudada.

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CAPÍTULO II

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Polyamine Homeostasis and Morphophysiological Responses to Salinity in *Dizygostemon riparius*: An Endemic Species from Brazilian 'Cerrado' Biome

Abstract

Dizygostemon riparius is an endemic tropical Brazilian species whose physiological responses to salinity are poorly understood. This study evaluated the effects of NaCl (0, 50, and 100 mM) on *in vitro*-grown *D. riparius* by integrating growth, anatomical, photosynthetic, and biochemical analyses. Salinity significantly reduced biomass and growth parameters, indicating impaired development. Photosystem II efficiency declined, as evidenced by decreases in the performance index and chlorophyll content, while anatomical changes such as epidermal thickening and reduced vascular bundles reflected structural adjustments under stress. At the biochemical level, salinity altered polyamine metabolism, with reductions in total free polyamines, suggesting potential limitations in defense mechanisms. Hyperhydricity observed under high salinity indicated a non-adaptive response. Overall, *D. riparius* displayed limited tolerance to salt stress, with physiological and biochemical impairments outweighing structural plasticity. This study provides the first data-driven characterization of salinity effects in this species and highlights the value of *in vitro* culture as a tool to investigate stress responses.

Keywords: anatomical plasticity; *in vitro* culture; morpho-physiology; polyamines; salinity.

1. INTRODUCTION

Dizygostemon riparius, commonly known as “melosa”, is a tropical aromatic subshrub endemic to the northeastern Brazilian Cerrado, which belongs to the Plantaginaceae family [1]. Although recently cataloged, this species has already demonstrated remarkable potential for medicinal and agrochemical applications [2]. Its essential oils and crude extracts exhibit significant larvicidal, acaricidal, and antifungal activities [2–5], with promising results being obtained in green nanotechnological formulations [2]. Considering the potential of biotechnological techniques for the production of such compounds, it is important to investigate the phenotypic plasticity of *D. riparius* under *in vitro* conditions, decreasing the knowledge gaps regarding *D. riparus* cultivation, and allowing its domestication and sustainable use.

Salinity, a major abiotic stress that strongly impacts plant growth, can also act as an elicitor in the production of secondary metabolites [6,7]. In tropical regions such as Brazil, salinity is an increasingly critical problem due to irregular rainfall distribution, high evapotranspiration rates, and inadequate irrigation management, which promote the accumulation of soluble salts in agricultural soils [6]. These conditions threaten biodiversity and the productivity of endemic and cultivated species adapted to these ecosystems. This stress primarily disrupts the osmotic balance of cells, reducing the uptake of water and nutrients, and causing ionic toxicity due to the accumulation of Na^+ and Cl^-, which compromise membrane integrity, nutrient balance, and DNA stability [6]. Such disturbances stimulate excessive production of reactive oxygen species (ROS), including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH), ultimately reducing cell viability [8–10]. Therefore, understanding the physiological and biochemical responses of endemic tropical species to salinity stress is essential for developing strategies to improve their resilience and conservation under changing environmental conditions.

To counter these effects, plants rely on biochemical defense systems, among which polyamines have emerged as key modulators of developmental and stress-related processes [11]. These small aliphatic amines, such as putrescine (Put), cadaverine (Cad), spermidine (Spd), and spermine (Spm), contribute to membrane stabilization, ion homeostasis, ROS scavenging, and regulation of gene expression [12]. Salinity often reduces Put and Spd, while Cad and Spm show variable responses depending on species and stress intensity [13,14]. Despite their importance, the dynamics of polyamines under salinity remain poorly studied in tropical medicinal plants cultured *in vitro*.

Disturbances at the cellular and biochemical levels often extend to plant structure and function. Anatomical modifications include epidermal thickening, reduction of palisade and spongy parenchyma, and vascular collapse, which impair hydraulic conductivity and tissue functionality [15]. Physiologically, salinity diminishes chlorophylls and carotenoids, impairs chlorophyll *a* fluorescence, and compromises photosystem II, resulting in reduced photosynthetic efficiency [16]. These effects culminate in macroscopic symptoms such as chlorosis, necrosis, premature senescence, and growth inhibition, leading to reduced leaf area, impaired stem and root development, and decreased overall biomass production [17,18]. Hormonal imbalances, including increased abscisic acid and reduced auxins and cytokinins, further aggravate these responses by impairing cell division, promoting turgor loss, and restricting development [19].

In pot-grown plants of *D. riparus*, salinity was reported to impair carbon assimilation and dry mass production, with no reductions in photosynthetic pigment content or photosystem II efficiency [20]. Although *in vitro* culture offers a powerful and controlled platform to investigate plant responses to abiotic stress, no studies to date have characterized the integrated morpho-physiological, anatomical, and biochemical responses of *D. riparius* to salinity under this system. This knowledge gap restricts our understanding of its adaptive capacity and limits its future as a phytotherapeutic and agroindustrial resource derived from tropical vegetation [20].

Here, we hypothesize that salinity impairs growth, tissue structure, and pigment content in *D. riparius* while simultaneously inducing alterations in polyamine metabolism, since these molecules are associated with antioxidant defenses and the regulation of cell division and expansion, which may indicate limited adaptive plasticity under *in vitro* conditions. Based on this hypothesis, this study aimed to evaluate the effects of salinity on growth parameters, water status, photosystem II integrity, pigment content, anatomical traits, and polyamine metabolism in *D. riparius* cultured *in vitro*.

2. MATERIALS AND METHODS

2.1 Plant Cultivation and Experimental Design

The *D. riparius* plants used in this experiment originated from the plant collection maintained at the Tissue Culture Laboratory of the State University of Maranhão (LCT/UEMA), MA, Brazil (2°34′00″ S and 44°12′00″ W), since 2022 and

subcultured every 90 days. These plants were maintained *in vitro* under a photomixotrophic system, in transparent 350 mL glass flasks, each containing 60 mL of Murashige and Skoog (MS) medium with vitamins [21] (PhytoTechnology®, Lenexa, KS, USA), supplemented with 30 g L^{-1} sucrose (w/v; Dinâmica® Química Contemporânea Ltda, São Paulo, SP, Brazil) and solidified with 5.5 g L^{-1} agar (Êxodo Científica®, São Paulo, SP, Brazil). The cultures were maintained at 25 ± 2 °C, under an irradiance of 85 µmol m^{-2} s^{-1} provided by six white tubular LED lamps (T8, 9 W; Avant, São Paulo, SP, Brazil), with a 16 h photoperiod.

Single-node stem explants approximately 1.5 to 2.0 cm in length were excised from the bank plants and inoculated into 350 mL glass flasks containing 60 mL of MS medium [21] (PhytoTechnology®, Lenexa, KS, USA), under the same environmental conditions previously described. The salinity experiment consisted of three treatments with different NaCl concentrations: 0 mM (control), 50 mM, and 100 mM. The flasks were sealed with polypropylene caps containing two 10 mm holes covered with microporous tape membranes, following the methodology described by Saldanha et al. [22]. After inoculation, the flasks were maintained for 45 days in a growth room under an irradiance of 85 µmol m^{-2} s^{-1}, with a 16 h photoperiod and a temperature of 25 ± 2 °C. The experimental design was completely randomized, with three salinity treatments and twelve replicates per treatment, and the experimental unit consisted of five explants per flask.

2.2 Growth Parameters

After 45 days of *in vitro* cultivation, shoot length (cm), stem diameter (mm), and the length of the longest root (cm) were measured. Subsequently, leaf area (cm^2) was deter- mined using ImageJ® software (Version 1.52e). After the measurements, leaves, stems, and roots were sectioned, collected, and placed into pre-labeled paper bags for drying. These plant organs were oven-dried (SolidSteel®, Piracicaba, SP, Brazil) at 45 °C until reaching constant weight, and dry mass was then determined for the aerial part (g) and roots (g).

2.3 Relative Leaf Water Content (RLWC*)*

Five leaf disks (3 mm in diameter) were collected from fully expanded leaves in each treatment. The disks were immediately weighed on an analytical balance to determine fresh mass (FM). They were then transferred to 8 mL plastic cups containing distilled water for 24 h to obtain turgid mass (TM). Afterward, the disks were oven-dried

at 45 °C for 48 h to determine dry mass (DM). The RLWC was calculated using the formula [23]: RLWC (%) = [(FM − DM)/(TM − DM)] × 100.

2.4 Chlorophyll a Fluorescence

Chlorophyll *a* fluorescence measurements were performed on the third pair of fully expanded leaves, counted from the apex toward the base of the stem, at 45 days of *in vitro* cultivation. A non-modulated fluorometer, model Pocket PEA (Plant Efficiency Analyser, Hansatech®, King′s Lynn, UK), was used. Prior to measurement, leaves were dark- adapted for 30 min using leaf clips to ensure that the reaction centers were fully open and heat dissipation was minimized.

The following parameters were evaluated: initial fluorescence (F0), maximum fluorescence (Fm), variable fluorescence (Fv), maximum quantum yield of photosystem II (Fv/Fm), energy absorbed per active reaction center (RC/ABS), variable fluorescence rela- tive to initial fluorescence (Fv/F0), and performance index (PI).

2.5 Extraction and Determination of Photosynthetic Pigment Concentrations

After 45 days of *in vitro* cultivation, pigments were extracted from three leaf disks, each 3 mm in diameter, collected from the third fully expanded leaf pair counted from the apex to the base of the stem. The disks were placed in test tubes containing 3 mL of dimethyl sulfoxide (DMSO) as the organic extracting solvent and kept in the dark for 48 h, as described by Santos et al. [24].

Absorbance readings were performed using a UV/Vis spectrophotometer (model UV-M51; BEL Engineering, Monza, Italy) at wavelengths of 480, 645, and 665 nm in 10 mm cuvettes. The concentrations of chlorophyll a, chlorophyll b, chlorophyll total, and carotenoids were calculated using the equations proposed by Wellburn [25]. In addition, the chlorophyll a/b ratio and the total chlorophyll-to-carotenoid ratio were calculated.

2.6 Leaf Thermography

Thermal images of the leaf surface were captured from the third and fourth leaf pairs, counted from the apex to the base of the stem, using a FLIR E8 WIFI thermal camera (FLIR Systems®, Wilsonville, OR, USA). The camera was positioned vertically 90 cm from the leaves. Image acquisition was conducted between 08:30 and 09:00 AM (Brasília time, Bra- zil). The images were processed using FLIR Thermal Studio Suite software, version 2.0.x (Copyright®, 2024, Thousand Oaks, CA, USA).

2.7 Determination of Polyamine Concentration

Samples of 300 mg of fresh mass were freeze-dried and homogenized with 0.6 mL of 5% (v/v) perchloric acid (Merck®, Darmstadt, Germany), incubated on ice for 1 h, and centrifuged at 16,000× g for 20 min at 4 °C. The free polyamines (PAs) were then dansylated. For this, 40 µL of the extract were mixed with 20 µL of 1.7-diaminoheptane (DAH) at 0.05 mM (used as an internal standard), 50 µL of saturated sodium bicarbonate solution (Na- HCO3), and 100 µL of dansyl chloride (5 mg mL^{-1} in acetone; 1.8 mM) (Merck®).

The samples were incubated in the dark at 70 °C for 50 min. Excess dansyl chloride was removed by adding 25 µL of proline solution (100 mg mL^{-1}), followed by incubation at room temperature in the dark for 30 min. The dansylated PAs were extracted with 200 µL of toluene, and 175 µL of the organic (non-polar) phase containing the PAs were collected, evaporated under a nitrogen stream, and resuspended in 175 µL of absolute acetonitrile.

Identification and quantification of the PAs were performed using high-performance liquid chromatography (HPLC), with a reverse-phase C18 column (Shimadzu Shim-pack CLC ODS, 5 µm). The mobile phase consisted of 10% acetonitrile in water (pH 3.5, adjusted with 1 N HCl) as solvent A, and absolute acetonitrile as solvent B. Peak areas and retention times of the PAs were compared with commercial standards of putrescine (Put), spermidine (Spd), and spermine (Spm) (Sigma-Aldrich®, St. Louis, MO, USA). Polyamines were determined through an estimate of dry mass.

2.8 Anatomy and Micromorphometry of Leaf, Stem, and Root

For anatomical characterization, samples of leaf, stem, and root tissues were fixed in a 50% FAA solution (formalin, acetic acid, and ethyl alcohol) following the protocol de- scribed by Johansen [26] for 48 h. Subsequently, the samples were dehydrated in a graded ethanol series composed of 30%, 40%, 50%, 60%, and 70% ethanol for one hour each, followed by further dehydration in 80%, 90%, 95%, and 100% ethanol for two hours each at 4 °C. After dehydration, the samples were embedded in Historesin® (Leica Instruments, Heidelberg, Germany), according to the manufacturer's recommendations. Transverse sections with a thickness of 6 µm were obtained using a rotary microtome (Lupetec® model MRP2015, São Carlos, Brazil). These sections were mounted on glass slides and stained with toluidine blue solution at 0.05% and pH 4.0, as described by O'Brien and McCully [27], for eight minutes.

Micromorphometric analysis was performed on transverse sections of the leaf mid- rib, stem, and root. From each slide, four anatomical sections were photographed using a light microscope (model B20T; Bioptika, Colombo, PR, Brazil) equipped with a U-photo system and a digital camera (CMOS-5.0; Bioptika, Colombo, PR, Brazil), connected to a computer with Capture V2.1 software. The images were captured at a magnification of 4× and analyzed using the ImageJ® software, with measurements expressed in millimeters. In leaf tissues, the parameters measured included the thickness of the adaxial and abaxial epidermis, the palisade parenchyma, the spongy parenchyma, as well as the diameter of both transverse and longitudinal vascular bundles. In stem tissues, measurements in cluded the thickness of the epidermis, cortical parenchyma, vascular bundles, and medullary parenchyma. In root tissues, the thickness of the epidermis and cortical parenchyma was determined, along with the diameter of the transverse and longitudinal vascular bundles.

2.9 Data Analyses

Growth and physiological analyses were performed with 10 replicates per treatment, while polyamine analyses were performed with 3 replicates per treatment. All evaluated variables were tested for homogeneity using Bartlett's test and for normality with the Shapiro–Wilk test. Subsequently, they were subjected to analysis of variance (ANOVA), and when significant effects were detected, the means were compared using Tukey's test ($p \leq 0.05$). The statistical procedures were performed using the Sisvar software (version 5.0) [28].

3. RESULTS

3.1 Salinity impairs growth and alters morphoanatomy of *Dizygostemon riparius* cultured *in vitro*

Salinity induced pronounced morphological changes in *D. riparius* cultured *in vitro* after 45 days (Figure 1A). A progressive reduction in the length of the aerial part was observed with increasing NaCl concentration, with statistically significant differences among treatments (Figure 1B). Compared to the control, shoot length was decreased by 21% and 49% under 50 and 100 mM NaCl, respectively (Figure 1B). Root length remained unchanged between 0 and 50 mM but was 40% smaller at 100 mM NaCl (Figure 1C). Stem diameter was also negatively affected, decreasing by 28% under 100 mM NaCl compared to the control (Figure 1D).

The dry mass of the aerial part and roots was decreased by 46% and 40%, respectively, at the highest salinity level (Figure 1E,F). In contrast, relative leaf water content was increased under salinity, reaching its maximum at 100 mM NaCl, corresponding to a 257% increase over the control (Figure 1G). The number of leaves declined by 19% under 100 mM NaCl (Figure 1H), and leaf area was similarly reduced by 45% (Figure 1I). Leaf temperature showed only minor variations across treatments, with a 1.1% increase at 100 mM NaCl, which was not statistically significant (Figure 1J).

Salinity also induced significant anatomical changes in leaves and roots of *D. riparius* (Figure 2A,C). The adaxial epidermis thickness increased with rising NaCl concentrations, with increments of approximately 46% at 50 mM and 75% at 100 mM compared to the control (Table 1). In contrast, the abaxial epidermis remained statistically unchanged across treatments. Palisade parenchyma thickness, in turn, was slightly increased under salinity, with values rising by 3% and 6% at 50 and 100 mM NaCl, respectively (Table 1). Spongy parenchyma was also increased by about 4% under both salinity levels, compared to the control. In the vascular system, transverse bundle thickness was progressively decreased with increasing salinity, with a 13% reduction at 100 mM NaCl compared to the control (Table 1). However, longitudinal vascular bundle thickness was not significantly affected.

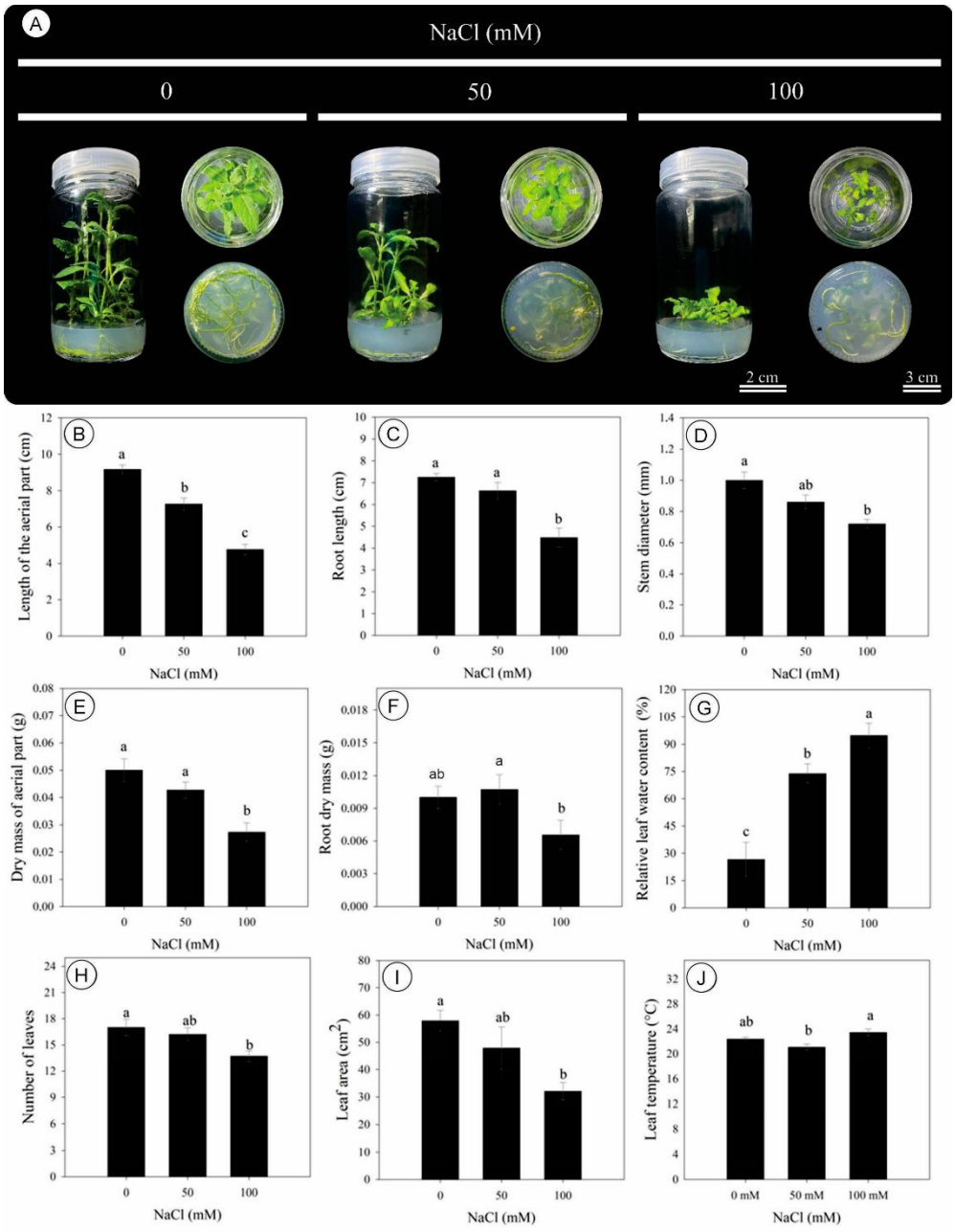

Figure 1. Morphology, growth, and relative leaf water content of *Dizygostemon riparius* under three NaCl concentrations (0, 50, and 100 mM) at 45 days of *in vitro* cultivation. (**A**) Pictures of representa- tive plants; (**B**) shoot length (cm); (**C**) root length (cm); (**D**) stem diameter (mm); (**E**) shoot dry mass (g); (**F**) root dry mass (g); (**G**) relative leaf water content (%); (**H**) number of leaves; (**I**) leaf area (cm^2); (**J**) leaf temperature (°C). Different letters above the bars indicate significant differences ($p \leq 0.05$) according to Tukey's test. Values are means ± standard error (n = 10). Scale for the flasks standing up = 2 cm, and scale for the bottom of the flasks = 3 cm.

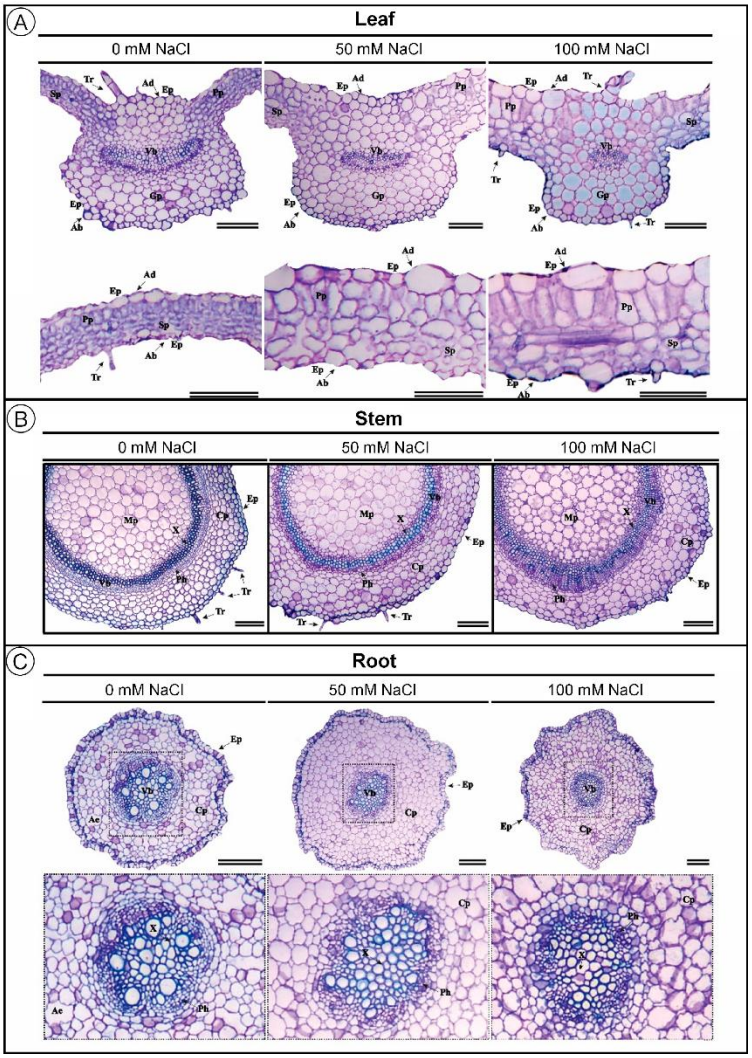

Figure 2. Transverse section micrography of *Dizygostemon riparius* leaves, stems, and roots after 45 days of *in vitro* cultivation under three NaCl concentrations (0, 50, and 100 mM). (**A**) Transverse sections of the leaf midrib (up) and leaf blade (below); (**B**) transverse sections of stems; (**C**) transverse sections of roots. Ep = epidermis, Ad = adaxial epidermis, Ab = abaxial epidermis, Vb = vascular bundle, Pp = palisade parenchyma, Sp = spongy parenchyma, Gp = ground parenchyma, X = xylem, Ph = phloem, Cp = cortical parenchyma, Mp = medullary parenchyma, Tr = trichome, and Ae = aerenchyma. Scale bar = 100 µm.

Unlike the leaf, stem anatomy of *D. riparius* remained structurally stable under salinity (Figure 2B; Table 1). No significant differences were observed in epidermis thickness, cortical parenchyma, vascular bundle thickness, or medullary parenchyma, indicating high anatomical resilience of stem tissues even under moderate to high salinity conditions. In contrast, salinity significantly altered root anatomy, particularly the vascular system (Figure 2C; Table 1). Transverse vascular bundle thickness was progressively decreased with increasing NaCl, showing a 48.57% reduction at 100 mM compared to the control. This reduction was statistically significant, indicating root vascular sensitivity to elevated salinity. Meanwhile, epidermis thickness, cortical

parenchyma, and longitudinal vascular bundle thickness remained statistically unchanged.

Table 1. Micromorphometric parameters of *Dizygostemon riparius* leaves, stems, and roots after 45 days of *in vitro* cultivation under three NaCl concentrations (0, 50, and 100 mM).

Organ	Variables (mm)	0 mM NaCl	50 mM NaCl	100 mM NaCl
Leaf	Adaxial epidermis	0.024 ± 0.002 c	0.035 ± 0.003 b	0.042 ± 0.006 a
	Abaxial epidermis	0.019 ± 0.005 a	0.024 ± 0.005 a	0.025 ± 0.004 a
	Palisade parenchyma	0.032 ± 0.009 c	0.053 ± 0.003 b	0.081 ± 0.017 a
	Spongy parenchyma	0.044 ± 0.004 b	0.088 ± 0.008 a	0.087 ± 0.007 a
	Transv. vascular bundle	0.303 ± 0.052 a	0.218 ± 0.035 ab	0.195 ± 0.014 b
	Long. vascular bundle	0.102 ± 0.021 a	0.076 ± 0.018 a	0.065 ± 0.005 a
Stem	Epidermis	0.027 ± 0.009 a	0.027 ± 0.004 a	0.027 ± 0.003 a
	Cortical parenchyma	0.183 ± 0.011 a	0.186 ± 0.042 a	0.194 ± 0.011 a
	Vascular bundle	0.087 ± 0.012 a	0.074 ± 0.022 a	0.082 ± 0.008 a
	Medullary parenchyma	0.336 ± 0.033 a	0.314 ± 0.019 a	0.308 ± 0.016 a
Root	Epidermis	0.038 ± 0.023 a	0.037 ± 0.008 a	0.032 ± 0.009 a
	Cortical parenchyma	0.229 ± 0.083 a	0.293 ± 0.010 a	0.246 ± 0.022 a
	Transv. vascular bundle	0.355 ± 0.063 a	0.276 ± 0.012 ab	0.182 ± 0.034 b

Values are means ± standard error of 4 individuals. Different letters indicate significant differences ($p \leq 0.05$) among NaCl concentrations (lines) according to Tukey's test. Transv = Tranverse; Long = Longitudinal.

3.2 Salinity decreases photosynthetic pigment content and chlorophyll *a* fluorescence in *Dizygostemon riparius*

The contents of photosynthetic pigments in *D. riparius* were significantly affected by salinity, with chlorophyll a content being decreased by 14% and 36% under 50 and 100 mM NaCl, respectively, compared to the control (Figure 3A). Similarly, the content of total chlorophylls had a marked reduction of 36% at 50 mM and 42% at 100 mM NaCl, com- pared to the control (Figure 3B). Carotenoid content was also negatively affected, with decreases of 26% at 50 mM and 50% at 100 mM, compared to control (Figure 3C). On the other hand, chlorophyll b content, chlorophyll a/b ratio, and total chlorophyll-to-carotenoid ratio were not affected by salinity.

Chlorophyll a fluorescence was significatively impaired by 100 mM NaCl, with the initial fluorescence (F0), maximum fluorescence (Fm), and variable fluorescence (Fv) being reduced by 29%, 23%, and 22%, respectively, compared to the control (Figure 3D–F). The Fv/Fm ratio of salt-stressed plants did not differ from the control, although plants under 50 mM exhibited slightly higher values compared to 100 mM NaCl (Figure 3G). In turn, the number of active reaction centers per absorbed energy unit (RC/ABS) and the performance index (PI) were decreased by 22% and 30% at 100 mM, respectively (Figure 3H,I). The Fv/F0 ratio was not affected by salinity levels.

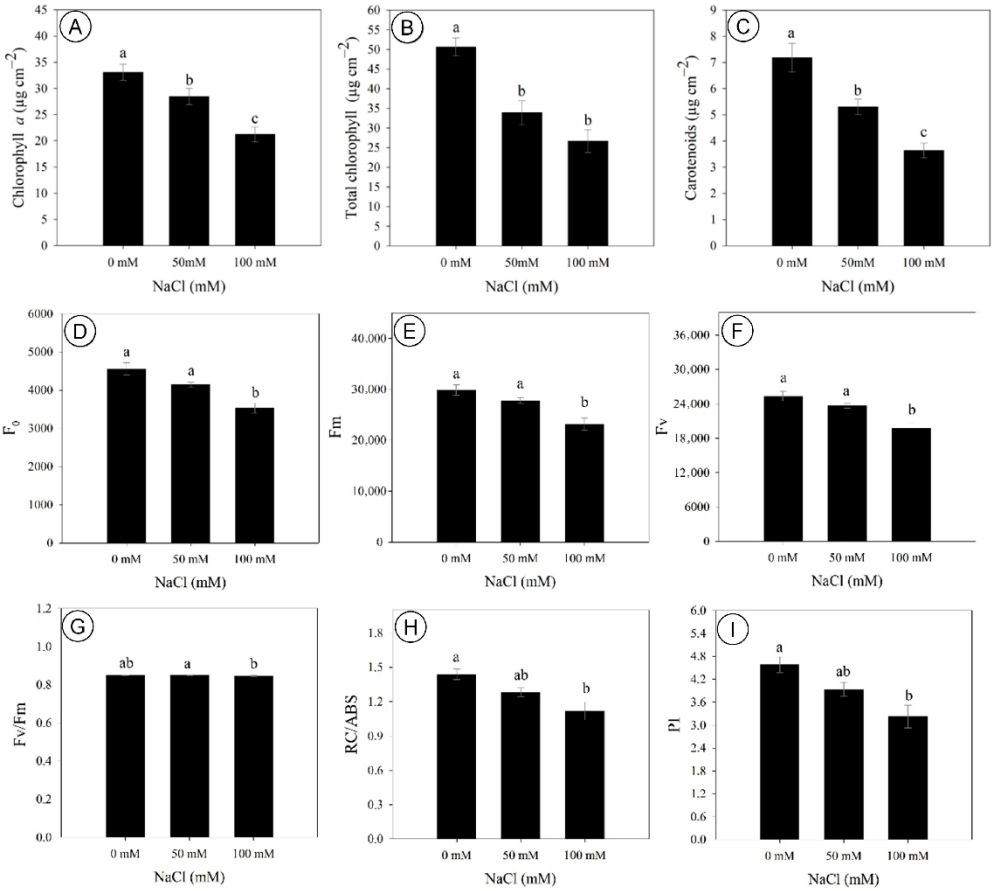

Figure 3. Photosynthetic pigments and chlorophyll *a* fluorescence parameters of Dizygostemon riparius at 45 days of *in vitro* cultivation under three NaCl concentrations (0, 50, and 100 mM). (**A**) Chlorophyll a (µg cm^{-2}); (**B**) total chlorophyll (µg cm^{-2}); (**C**) carotenoids (µg cm^{-2}); (**D**) initial fluorescence (F0); (**E**) maximum fluorescence (Fm); (**F**) variable fluorescence (Fv); (**G**) maximum quantum yield of photosystem II (Fv/Fm); (**H**) absorbed energy per active reaction center (RC/ABS); and (**I**) performance index (PI). Different letters above the bars indicate significant differences ($p \leq 0.05$) according to Tukey's test. Values are means ± standard error (n = 10).

3.3 Salinity modulates endogenous polyamine levels in *Dizygostemon riparius* cultured *in vitro*

Salinity significantly affected the contents of free endogenous polyamines in *D. riparius* cultured *in vitro* (Figure 4). Compared to the control, the contents of putrescine (Put) and cadaverine (Cad) were decreased by 67% and 42% at 100 mM NaCl, respectively, while spermidine (Spd) was decreased by 46% at 50 mM NaCl (Figure 4A–C). In contrast, spermine (Spm) contents remained unchanged across treatments (Figure 4D). Total free polyamines were significantly lower under salinity, with reductions of 59% and 57% under 50 and 100 mM NaCl, respectively (Figure 4E). Similarly, the PAs ratio [Put/(Spd + Spm)], commonly used as a metabolic stress indicator, was decreased by 56% at 100 mM NaCl compared to the control (Figure 4F).

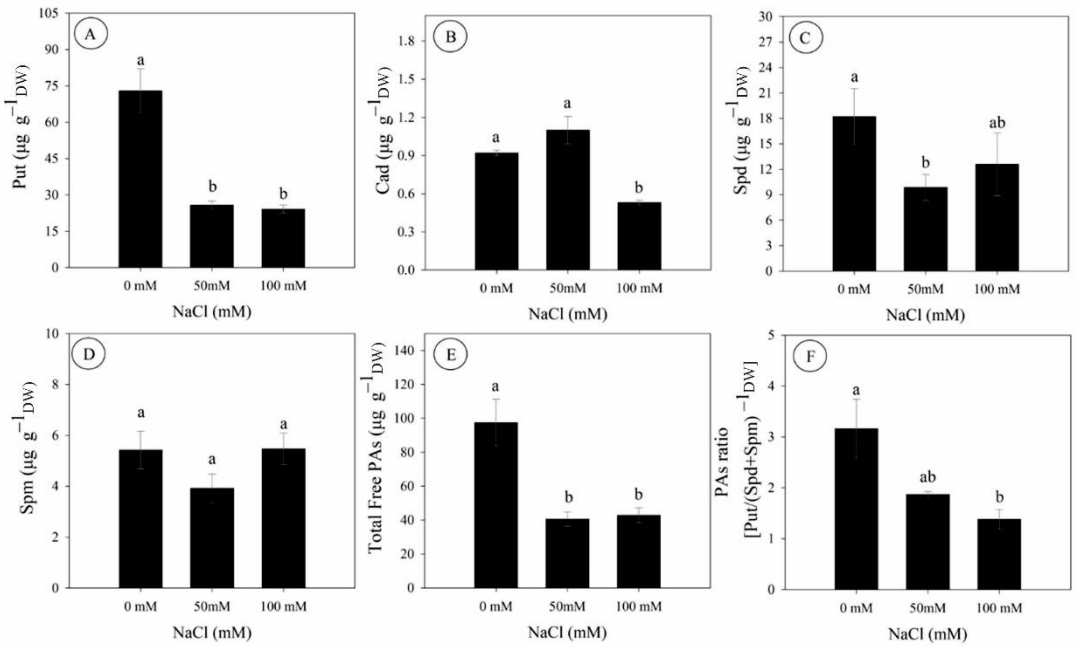

Figure 4. Endogenous polyamine content (determined by dry mass) in *Dizygostemon riparius* at 45 days of *in vitro* cultivation under three NaCl concentrations (0, 50, and 100 mM). (**A**) Putrescine (µg g^{-1}); (**B**) Cadaverine (µg g^{-1}); (**C**) Spermidine (µg g^{-1}); (**D**) Spermine (µg g^{-1}); (**E**) Total free polyamines (µg g^{-1}); and (F) Polyamine ratio [Put/(Spd + Spm)]. Different letters above the bars indicate significant differences ($p \leq 0.05$) according to Tukey's test. Values are means ± standard error (n = 3).

4. DISCUSSION

Salinity compromised the growth of both aerial and root organs, mainly under the highest NaCl concentration (100 mM). Shoot length, leaf number, leaf area, stem diameter, and dry biomass were all decreased under high NaCl concentrations. This growth inhibition is likely related to salt-induced osmotic stress and ionic toxicity, as they are known to inhibit cell division and elongation, decrease meristematic activity, and impair the deposition of cellulose and lignin [6]. The impairment in stem and leaf development also disrupts sap transport and compromises the physiological performance [29,30]. Root growth also declined under 100 mM NaCl, likely due to ionic toxicity and oxidative stress in the root zone [6]. Moreover, root growth inhibition might be related to the salinity-induced reduction in water potential of the medium, which impairs nutrient and water uptake and leads to nutritional imbalances [31–33].

Anatomical responses highlighted both compensatory mechanisms and structural damages. The adaxial epidermis was thickened in response to salinity, which may be a protective barrier against osmotic stress by reducing transpiration and restricting ion entry [34]. Palisade and spongy parenchyma were also thickened, suggesting an attempt to maintain photosynthetic capacity and partially compensate for pigment loss

and reduced PSII efficiency [35,36]. In contrast, the transverse vascular bundles in both leaves and roots became thinner under high salinity, suggesting impaired transport of water and nutrients. This reduction likely resulted from cell retraction, vascular collapse, or stress-induced deposition of lignin and suberin [37,38].

Relative water content was markedly increased under 100 mM NaCl, which could initially suggest osmotic adjustments. In general, plants maintain cellular turgor in saline environments by accumulating compatible solutes such as sugars, amino acids, and inorganic ions, which balance water potential and protect cellular structures [39–41]. In this study, however, the exceptionally high RWC values revealed hyperhydricity induced by salinity. This physiological disorder, commonly observed in *in vitro* conditions, involves excessive water accumulation in tissues, resulting in a translucent appearance, aqueous consistency, and structural abnormalities [42,43]. A high NaCl concentration is likely to trigger osmotic imbalances and altered membrane permeability, thereby favoring this phenomenon. Thus, although the data show water retention, this scenario represents a maladaptive stress response rather than a functional adaptation.

Salinity also altered the content and proportion of photosynthetic pigments. Chlorophyll a and total chlorophyll declined, suggesting pigment degradation probably through ROS that damage thylakoid membranes and pigment-protein complexes [44,45], and salinity-induced inhibition of chlorophyll biosynthesis by targeting key enzymes such as ALA synthase (5-aminolevulinic acid) [46]. As carotenoids dissipate excess light energy and scavenge ROS such as singlet oxygen and peroxyl radicals, their reduction likely increases cellular vulnerability to photoinhibition and lipid peroxidation [47,48]. Thus, low carotenoid contents under salinity indicate impaired non-enzymatic antioxidant defenses. The unchanged chlorophyll a/b and chlorophyll/carotenoid ratios suggest proportional pigment degradation, indicating that both photosystems and their associated light-harvesting complexes were similarly affected. These results differ from those of Albuquerque et al. [20], who reported unexpectedly high pigment levels in pot-grown *D. riparius* under salinity. Differences in stress intensity or exposure duration may explain this discrepancy, since both factors directly influence pigment dynamics.

Chlorophyll *a* fluorescence, a sensitive tool for evaluating the integrity and efficiency of photosystem II (PSII), was also strongly affected by salinity. The decreases in maximum fluorescence (Fm) under 100 mM NaCl indicate damages to reaction centers and impaired energy coupling between antenna pigments and PSII [49]. Initial fluorescence (F0) was also decreased, indicating irreversible inactivation of PSII centers

[50], while the decline in active reaction centers per absorption (RC/ABS) corresponds to decreases in functional PSII density [51]. This likely resulted from inhibited protein synthesis, particularly of D1, a primary target of degradation under stress, as well as increased oxidative damage in thylakoid membranes [45,52]. The drop in performance index (PI) further confirmed reduced PSII vitality and potential collapse of the electron transport chain [53].

Salinity significantly reduced the endogenous levels of putrescine and spermidine, while cadaverine only declined at higher concentrations, resulting in a decrease in the total content of free polyamines and indicating a disruption in nitrogen metabolism. Plants synthesize putrescine from L-ornithine via ODC or from L-arginine through arginine decarboxylase (ADC), followed by agmatine, with both pathways driving the initial stages of PA biosynthesis [54,55]. Thus, the sharp decline in putrescine may reflect the inhibition of ornithine decarboxylase (ODC) or precursor molecules such as L-arginine. The reductions in cadaverine and spermidine may also reflect a metabolic bottleneck or enhanced degradation of polyamines as a stress response [56].

The decreased Put/(Spd + Spm) ratio indicates that *D. riparius* experienced an imbalance in polyamine dynamics under higher salinity levels, potentially due to metabolic diversion of putrescine toward conjugation or oxidation. The pronounced reduction in total free polyamines at both 50 and 100 mM NaCl may have contributed to physiological constraints such as impaired cell elongation, disrupted ion homeostasis, and altered water relations, which are likely related to the hyperhydricity observed under high salinity [57– 60]. While these interpretations are based on species-specific observations, we did not measure oxidative stress markers; thus, the proposed interplay between polyamine reduction and hyperhydricity remains hypothetical and should be further explored in fu- ture studies.

In summary, salinity reduced growth, biomass accumulation, and anatomical integrity of *D. riparius*, impairing both shoot and root development. Photosynthetic efficiency declined due to pigment degradation and reduced PSII activity, particularly through losses of chlorophylls and carotenoids. Salinity also disrupted polyamine metabolism, with marked reductions in putrescine, spermidine, and total free PAs, and a decline in cadaverine under higher stress, all of which indicate nitrogen metabolism imbalance and low adaptive capacity. Anatomical modifications, such as epidermal thickening and expanded parenchyma, indicate partial compensatory responses that may support limited homeostasis under moderate salinity conditions.

Importantly, this study provides the first evidence of salinity-induced alterations in polyamine metabolism and anatomical plasticity in *D. riparius*, an endemic medicinal species of the Brazilian semiarid region. Unlike widely studied model or crop species, *D. riparius* thrives under naturally fluctuating osmotic conditions, making it a valuable model for understanding how polyamine-mediated mechanisms contribute to stress tolerance in plants adapted to seasonally saline tropical ecosystems. By characterizing these responses, our findings broaden the physiological basis for exploring the ecological resilience and potential biotechnological uses of native tropical species under increasing salinity pressures.

5. CONCLUSIONS

Our findings demonstrated that *Dizygostemon riparius*, when cultivated *in vitro*, exhibits limited adaptive responses under increasing salinity. Salinity impaired shoot and root development, reduced biomass accumulation, and altered vascular structure. Physiological evaluations revealed decreased photosynthetic performance, pigment loss, and symptoms of hyperhydricity. Biochemically, the marked decline in endogenous polyamines, particularly putrescine, cadaverine, and spermidine, indicates disruption of polyamine metabolism. Although structural adjustments such as adaxial epidermal thickening and mesophyll expansion occurred, these changes were not sufficient to sustain functional balance under elevated salt concentrations.

These findings enhance our understanding of salinity responses in a poorly studied medicinal tropical species, highlighting its potential use in *in vitro* screening, clonal propagation, and conservation under controlled conditions. Moreover, the generated data may guide future efforts to optimize cultivation protocols in saline-prone regions, such as the Tropics, and support the selection of tolerant genotypes for *ex situ* conservation and biotechnological applications.

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CAPÍTULO III

Exogenous biotin modulates non-stomatal photosynthetic limitation under salinity in *in vitro*–grown *Dizygostemon riparius*

Abstract

This study investigates whether exogenous biotin (vitamin B7) mitigates salinity-induced physiological limitations in *Dizygostemon riparius*, a medicinal species endemic to Maranhão, Brazil, grown *in vitro*. Salinity (50 mM NaCl) significantly restricts shoot growth, reduces shoot dry mass, and decreases leaf number. Gas exchange analyses demonstrate that the salinity-induced decline in photosynthesis is driven predominantly by non-stomatal limitations, as evidenced by reduced carboxylation efficiency and intrinsic water use efficiency, indicating biochemical constraints on carbon fixation. Biotin supplementation (16 mg L^{-1}) partially alleviates these effects, restoring photosynthetic performance and attenuating growth reductions under salinity. In contrast, photosynthetic pigment composition, maximum quantum efficiency of photosystem II (Fv/Fm), and leaf micromorphology remain stable across treatments, indicating that salinity does not induce photochemical impairment or structural damage. These findings demonstrate that biotin modulates salinity responses primarily at the metabolic level, sustaining carbon assimilation and water use efficiency without anatomical or photochemical adjustment. The results highlight the sensitivity of *D. riparius* to salinity and identify biotin as a potential metabolic regulator of photosynthetic resilience *in vitro*.

Keywords: Salinity stress; exogenous biotin; non-stomatal limitation; carbon assimilation; *in vitro* culture.

1. INTRODUCTION

Soil salinity is one of the most restrictive abiotic stresses to plant productivity and species conservation in tropical regions, a condition that has been intensified by climate change and inadequate irrigation practices (Naorem et al., 2023; Sanga et al., 2024; Rahim et al., 2025). Salinity imposes a biphasic challenge to plants, initially through an osmotic component that reduces the water potential of the growth medium and limits water uptake, followed by an ionic toxicity phase resulting from excessive accumulation of Na^+ and Cl^- ions in plant tissues (Khalid et al., 2023; Albaladejo-Marico et al., 2025; Singh et al., 2025). These processes promote nutritional imbalances and stimulate the generation of reactive oxygen species (ROS), which may lead to oxidative damage to cellular membranes, photosynthetic pigments, and the integrity of the photosynthetic apparatus (Zhang et al., 2024; Roosta et al., 2025).

Within this context, photosynthesis is among the physiological processes most sensitive to salinity (Kumari et al., 2025). Reductions in net carbon assimilation may arise from both diffusive limitations associated with stomatal closure and non-stomatal limitations related to biochemical constraints within the chloroplast stroma (Du et al., 2025; Pena et al., 2025). Recent evidence indicates that ionic toxicity can impair the activity of key enzymes, such as ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), and compromise the regeneration of the CO_2 acceptor ribulose-1,5-bisphosphate (RuBP) due to reduced availability of ATP and NADPH (Rajkhowa et al., 2022; Lobo et al., 2024; Gietler et al., 2025). Importantly, such functional photosynthetic limitations may occur before detectable changes in photochemical efficiency or leaf anatomy, particularly under moderate stress conditions. Given the complexity of these responses, *in vitro* culture has become a strategic experimental tool, as it allows controlled and reproducible investigation of salinity effects on plant physiological, anatomical, and biochemical processes at cellular and tissue levels (Ortega-Ante et al., 2025).

Approaches aimed at alleviating salinity effects have increasingly considered the role of B-complex vitamins, with particular emphasis on biotin (vitamin B7) (Salifu et al., 2025). Biotin plays a fundamental role in carboxylation reactions and in the metabolism of fatty acids, amino acids, and carbohydrates, and is indirectly associated with cellular membrane integrity and energy metabolism regulation (Velazquez-Arellano et al., 2020; Wal et al., 2023; Vo et al., 2025). Recent evidence suggests that biotin may contribute to metabolic stability, photosynthetic performance, and plant growth under adverse conditions (Fan et al., 2024). However, the functional nature of biotin-mediated

functional responses under salinity stress, particularly with respect to photosynthetic limitations, remains insufficiently understood, especially in controlled *in vitro* systems.

Experimental evidence supporting this conceptual framework has been provided in *Arabidopsis thaliana* under carbonate stress. Wang et al. (2020) demonstrated that alkaline stress alters endogenous biotin metabolism and induces the expression of BIO2, a gene encoding biotin synthase, and that enhanced biotin availability, either through exogenous supply or genetic overexpression, improves plant performance under stress conditions. Importantly, these effects were primarily associated with metabolic regulation, redox balance, and stress tolerance, rather than with constitutive structural alterations, reinforcing the view that stress exposure may induce functional B vitamin deficiencies, in which modest disruptions in vitamin-derived cofactors constrain metabolic fluxes without necessarily causing overt structural or biochemical damage (Hanson et al., 2016).

Within this physiological framework, *Dizygostemon riparius* (Plantaginaceae), a medicinal species endemic to the state of Maranhão, Brazil, represents a suitable non-model system for evaluating biotin-mediated responses under salinity. The species exhibits recognized bioactive potential associated with the production of essential oils and compounds of pharmacological and biotechnological interest (Scatigna et al., 2019; Brandão et al., 2025) and has attracted scientific attention due to its larvicidal, antifungal, insecticidal, and acaricidal activities (Brandão et al., 2020; Martins et al., 2023; Corrêa et al., 2023; Ferreira et al., 2025). Although recent studies have addressed morphophysiological and biochemical responses of *D. riparius* to salinity (Albuquerque et al., 2024; Pinheiro et al., 2025), its responses to vitamin-mediated modulation under saline conditions remain largely unexplored, particularly *in vitro*.

Based on these gaps, this study tested the hypothesis that exogenous biotin modulates salinity-induced morphophysiological responses in *Dizygostemon riparius* grown *in vitro*. Specifically, we evaluated whether exogenous biotin supply attenuates non-stomatal photosynthetic limitations and partially sustains plant growth under NaCl stress, without necessarily inducing detectable changes in leaf anatomy or photosystem II efficiency. To address this question, growth parameters were integrated with gas exchange measurements, chlorophyll fluorescence, pigment composition, and anatomical analyses *in vitro*-grown plants.

2. MATERIALS AND METHODS

2.1 Cultivation and experimental design

The *Dizygostemon riparius* plants used in this experiment originated from the plant bank maintained at the Tissue Culture Laboratory of the State University of Maranhão (LCT/UEMA), MA, Brazil (2°34′00″ S, 44°12′00″ W). This bank is maintained *in vitro* under a photomixotrophic system in 350 mL transparent glass vials, each containing 60 mL of Murashige and Skoog (MS) medium with vitamins (Murashige and Skoog, 1962) (PhytoTechnology®, Lenexa, KS, USA), supplemented with 30 g L^{-1} sucrose (w/v; Dinâmica® Química Contemporânea Ltda, São Paulo, SP, Brazil) and solidified with 5.5 g L^{-1} agar (Êxodo Científica®, São Paulo, SP, Brazil). Cultures were maintained at 25 ± 2 °C under an irradiance of 85 μmol m^{-2} s^{-1} provided by six white LED tubular lamps (T8, 9 W; Avant, São Paulo, SP, Brazil), with a 16 h photoperiod. Single-node stem explants, approximately 1.5–2.0 cm long, were excised from bank plants and inoculated into 350 mL glass flasks containing 60 mL of MS medium (Murashige and Skoog, 1962) (PhytoTechnology®, Lenexa, KS, USA) under the same environmental conditions described above.

The experiment consisted of four treatments: 0 mM (control), 16 mg L^{-1} biotin, 50 mM NaCl, and 16 mg L^{-1} biotin + 50 mM NaCl. Flasks were sealed with polypropylene caps containing two 10 mm holes covered with microporous tape membranes, following the methodology described by Saldanha et al. (2012). After inoculation, flasks were maintained for 45 days in a growth room under an irradiance of 85 μmol m^{-2} s^{-1}, a 16 h photoperiod, and a temperature of 25 ± 2 °C.

The experimental design was completely randomized, with four treatments and ten replicates per treatment. The flask was considered the experimental unit, containing five explants.

2.2 Growth Parameters

After 45 days of *in vitro* cultivation, shoot length (cm), stem diameter (mm), and the length of the longest root (cm) were measured. Subsequently, leaves, stems, and roots were separated, collected, and placed into pre-labeled paper bags for drying. Plant organs were oven-dried (SolidSteel®, Piracicaba, SP, Brazil) at 45 °C until constant weight, and dry mass was determined for leaves (g), stems (g), and roots (g).

2.3 Chlorophyll a fluorescence and Leaf Gas Exchange

Chlorophyll a fluorescence measurements were performed on the third pair of fully expanded leaves, counted from the apex toward the base of the stem, at 45 days of

in vitro cultivation. A non-modulated chlorophyll fluorimeter (Pocket PEA; Plant Efficiency Analyser, Hansatech®, England) was used. Prior to measurements, leaves were dark-adapted for 30 min using leaf clips to ensure that reaction centers were fully open and heat dissipation was minimized. The following parameters were evaluated: initial fluorescence (F_0), maximum fluorescence (Fm), variable fluorescence (Fv), maximum quantum yield of photosystem II (Fv/Fm), energy absorbed per active reaction center (RC/ABS), variable fluorescence relative to initial fluorescence (Fv/F_0), and performance index (PI).

Gas exchange analyses were performed using an open gas exchange system (LI-6400XT; LI-COR, Lincoln, NE, USA). Net CO_2 assimilation (A), stomatal conductance to water vapor (gs), transpiration rate (E), internal CO_2 concentration (Ci), the intercellular-to-ambient CO_2 concentration ratio (Ci/Ca), carboxylation efficiency (A/Ci), and intrinsic water use efficiency (A/gs) were measured between 06:30 and 08:00 h (local time), under an external CO_2 concentration of 400 μmol mol^{-1} air and a mean air temperature of 32 °C. To optimize stomatal opening, all measurements were conducted under artificial saturating light of 1200 μmol m^{-2} s^{-1} provided by a light-emitting diode with 10% blue light.

2.4 Extraction and determination of photosynthetic pigment concentrations

After 45 days of *in vitro* cultivation, pigments were extracted from three leaf discs (3 mm diameter each) collected from the third fully expanded leaf pair counted from the apex toward the base of the stem. Discs were placed in test tubes containing 3 mL of dimethyl sulfoxide (DMSO) and kept in the dark for 48 h, following the method described by Santos et al. (2008).

Absorbance readings were performed using a UV/Vis spectrophotometer (model UV-M51; BEL Engineering, Monza, Italy) at wavelengths of 480, 645, and 665 nm using 10 mm cuvettes. Chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid concentrations were calculated using the equations proposed by Wellburn (1994). The chlorophyll a/b ratio and the total chlorophyll-to-carotenoid ratio were also calculated.

2.5 Anatomy and Micromorphometry of Leaf

For anatomical characterization, leaf tissue samples were fixed in 50% FAA solution (formaldehyde, acetic acid, and ethyl alcohol) following the protocol described by Johansen (1940) for 48 h. Samples were dehydrated through a graded ethanol series (30%, 40%, 50%, 60%, and 70% for 1 h each), followed by dehydration in 80%, 90%,

95%, and 100% ethanol for 2 h each at 4 °C. Subsequently, samples were embedded in Historesin® (Leica Instruments, Heidelberg, Germany) according to the manufacturer's recommendations.

Cross sections (6 μm thick) were obtained using a rotary microtome (Lupetec® model MRP2015, São Carlos, Brazil), mounted on glass slides, and stained with 0.05% toluidine blue solution at pH 4.0 for 8 min, following O'Brien and McCully (1981). Micromorphometric analyses were performed on cross sections of the leaf midrib. From each slide, five anatomical sections were photographed using an optical microscope (model B20T; Bioptika, Colombo, PR, Brazil) equipped with a U-photo system and a digital camera (CMOS-5.0; Bioptika, Colombo, PR, Brazil), connected to a computer running Capture V2.1 software. Images were captured at 4× magnification and analyzed using ImageJ® software, with measurements expressed in millimeters. Parameters measured included the thickness of the adaxial and abaxial epidermis, palisade parenchyma, spongy parenchyma, and the diameter of transverse and longitudinal vascular bundles.

2.6 Statistical analysis

All variables were subjected to analysis of variance (ANOVA), and when significant effects were detected, means were compared using the Scott–Knott test at a 5% probability level ($p \leq 0.05$). Statistical analyses were performed using the Sisvar software (Ferreira, 2011).

3. RESULTS

3.1 Biotin-mediated modulation of morphological responses of *Dizygostemon riparius* to salinity under *in vitro* conditions

Salinity induced marked morphological changes in *Dizygostemon riparius* grown *in vitro* after 45 days of cultivation (Fig. 1). NaCl exposure significantly reduced shoot length by 46% at 50 mM compared with the control, whereas the combined treatment (B7 + NaCl) resulted in a numerically lower reduction (44%), indicating partial attenuation of salinity effects (Fig. 2A). Root length, stem diameter, and root dry mass did not differ significantly among treatments (Figs. 2B, 2C, and 2E).

Shoot dry mass decreased by approximately 42% under salinity, however, plants grown under the combined B7 + NaCl treatment exhibited values statistically like the control, indicating partial recovery of aboveground biomass (Fig. 2D). The number of leaves was reduced by 27% under NaCl stress, while the presence of biotin reduced this decrease to 19%, suggesting that exogenous biotin modulated the severity of salinity-induced morphological effects rather than fully reversing them (Fig. 2F).

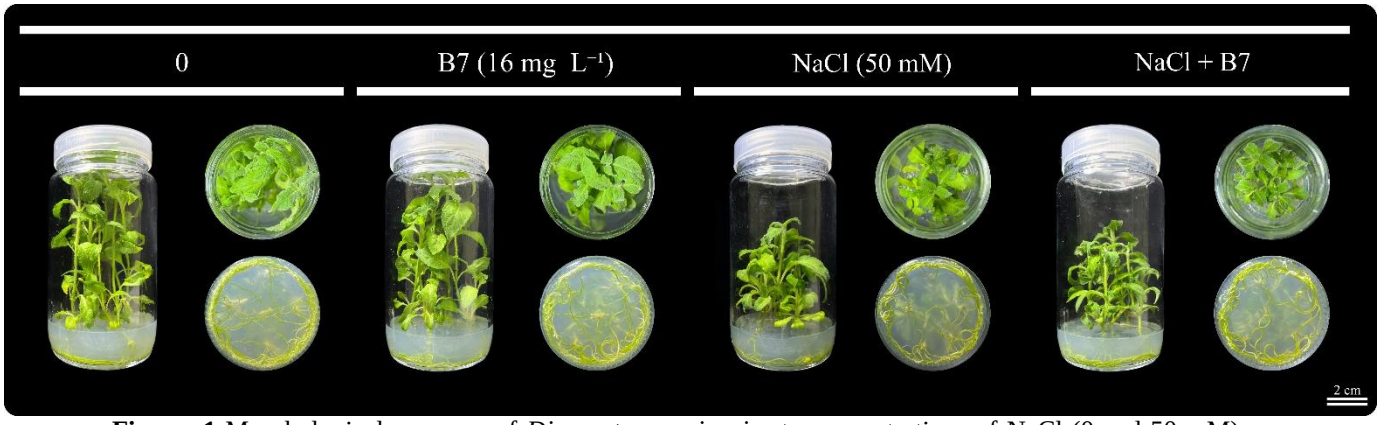

Figure. 1 Morphological response of *Dizygostemon riparius* to concentrations of NaCl (0 and 50 mM), biotin (16 mg L^{-1}), and the combination of both B7 (16 mg L^{-1}) + NaCl (50 mM) after 45 days of *in vitro* culture. Scale bar = 2 cm

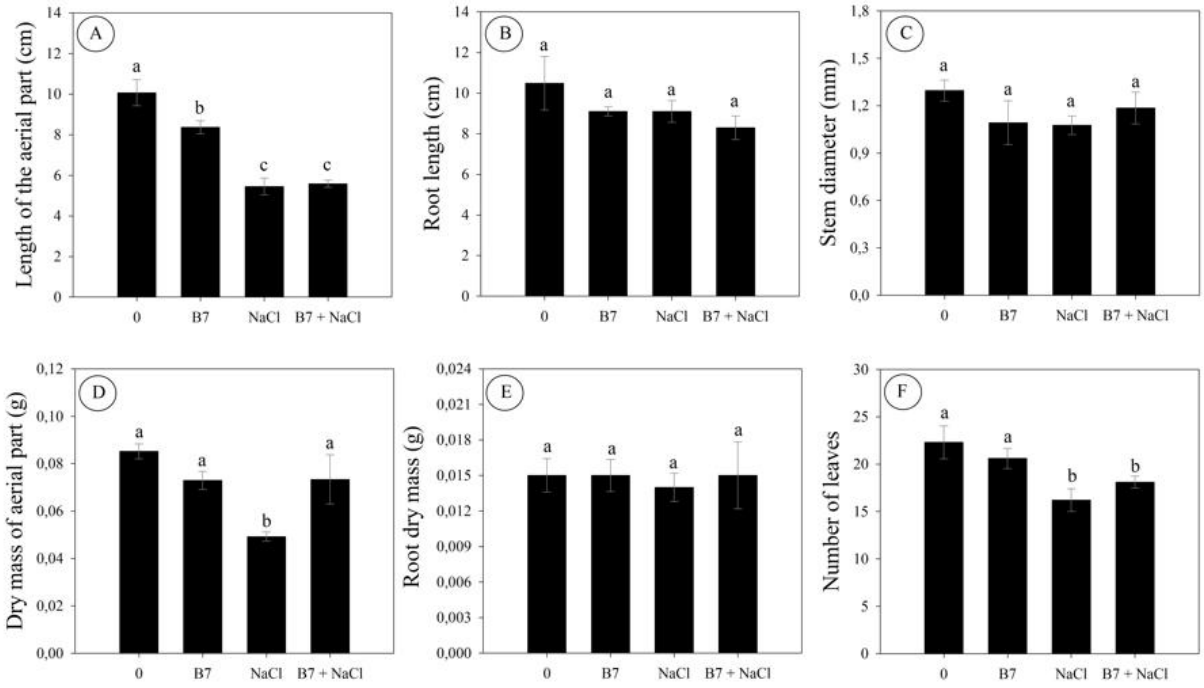

Figure. 2 Parameters related to the growth of *Dizygostemon riparius* at 45 days of *in vitro* culture under concentrations of NaCl (0 and 50 mM), biotin (16 mg L^{-1}), and the combination of both B7 (16 mg L^{-1}) + NaCl (50 mM). (A) Shoot length (cm); (B) Root length (cm); (C) Stem diameter (mm); (D) Shoot dry mass (g); (E) Root dry mass (g); (F) Number of leaves. Different letters above the bars indicate significant differences ($P < 0.05$) according to Skoot-Knot test. Values are means ± standard error (n = 5).

3.2 Biotin effects on chlorophyll fluorescence and photosynthetic pigments of *Dizygostemon riparius* under salinity *in vitro*

The maximum quantum efficiency of photosystem II (Fv/Fm) remained within a narrow and physiologically optimal range across all treatments, indicating the absence of photoinhibitory stress (Fig. 3A). Although plants exposed to NaCl, with or without biotin supplementation, exhibited slightly higher Fv/Fm values compared with the control, these differences were small in magnitude and reflect the overall stability of PSII photochemistry under the experimental conditions. No significant differences were observed among treatments for the performance index (PI), total chlorophyll content, carotenoid concentration, or the total chlorophyll-to-carotenoid ratio (Figs. 3B–E).

These results indicate that neither salinity nor exogenous biotin induced detectable changes in the photochemical efficiency of PSII or in pigment composition,

supporting the interpretation that biotin-mediated responses under salinity occurred independently of alterations in photosynthetic pigments or photochemical processes.

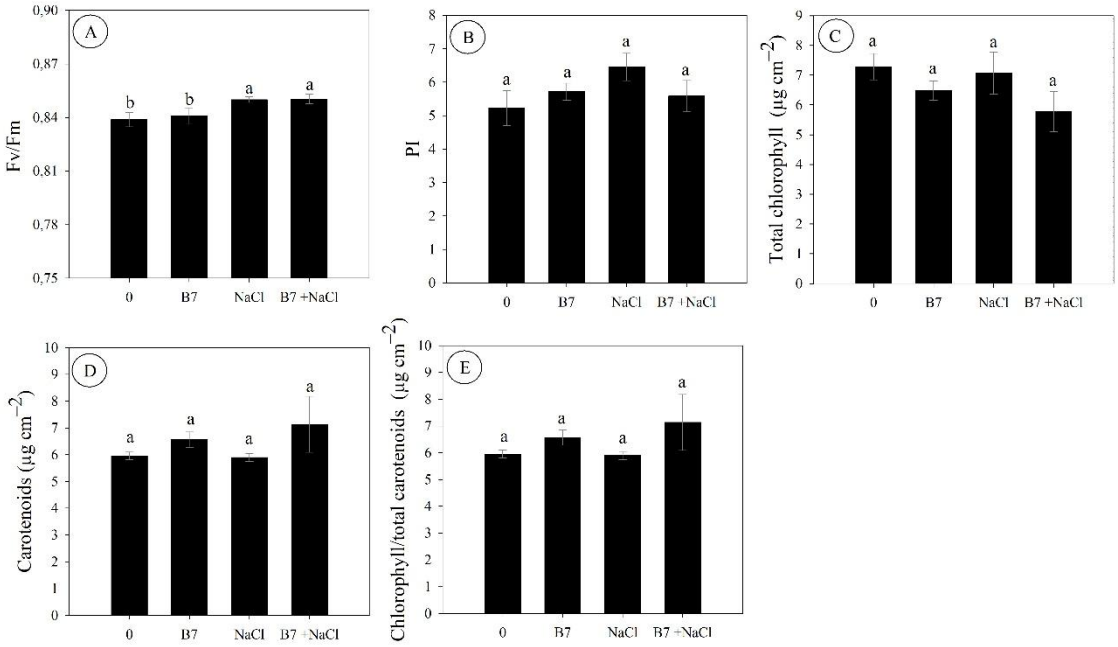

Figure. 3 Chlorophyll *a* fluorescence parameters and Photosynthetic pigments of *Dizygostemon riparius* at 45 days of *in vitro* culture under concentrations of NaCl. (0 and 50 mM), biotin (16 mg L^{-1}), and the combination of both B7 (16 mg L^{-1}) + NaCl (50 mM). (A) Maximum quantum yield of photosystem II (F_v/F_m); (B) Performance index (PI); (C) Total chlorophyll (µg cm^{-2}); (D) Carotenoids (µg cm^{-2}); (E) Total chlorophyll to carotenoid ratio Different letters above the bars indicate significant differences ($P < 0.05$) according to Skoot-Knot test. Values are means ± standard error (n = 5).

3.3 Effects of exogenous biotin on gas exchange of *Dizygostemon riparius* under salinity *in vitro*

Salinity significantly altered gas exchange parameters in *Dizygostemon riparius* grown *in vitro* (Fig. 4). Net photosynthetic CO_2 assimilation (A) decreased by approximately 57% under 50 mM NaCl compared with the control, indicating a strong functional limitation of carbon assimilation under saline conditions (Fig. 4A). In contrast, plants grown under the combined B7 + NaCl treatment exhibited A values statistically like the control, indicating a substantial recovery of photosynthetic performance.

Stomatal conductance (gs) did not differ significantly among treatments (Fig. 4B), indicating that reductions in A under salinity were not primarily driven by stomatal limitation. Consistently, internal CO_2 concentration (Ci) increased by approximately 3% in response to biotin application and by 5% under NaCl stress (Fig. 4C), suggesting

reduced biochemical capacity for CO_2 fixation. A similar pattern was observed for the Ci/Ca ratio (Fig. 4E), further supporting the predominance of non-stomatal limitations to photosynthesis.

Transpiration rate (E) increased under both biotin and NaCl treatments (Fig. 4D), reflecting altered gas exchange dynamics under stress and supplementation conditions. In contrast, the instantaneous carboxylation efficiency (A/Ci) declined sharply under salinity (59% reduction) but was fully restored in the presence of biotin (Fig. 4F). Likewise, intrinsic water use efficiency (A/gs) was severely reduced under NaCl stress (82% reduction), whereas biotin supplementation under salinity restored A/gs to control levels (Fig. 4G).

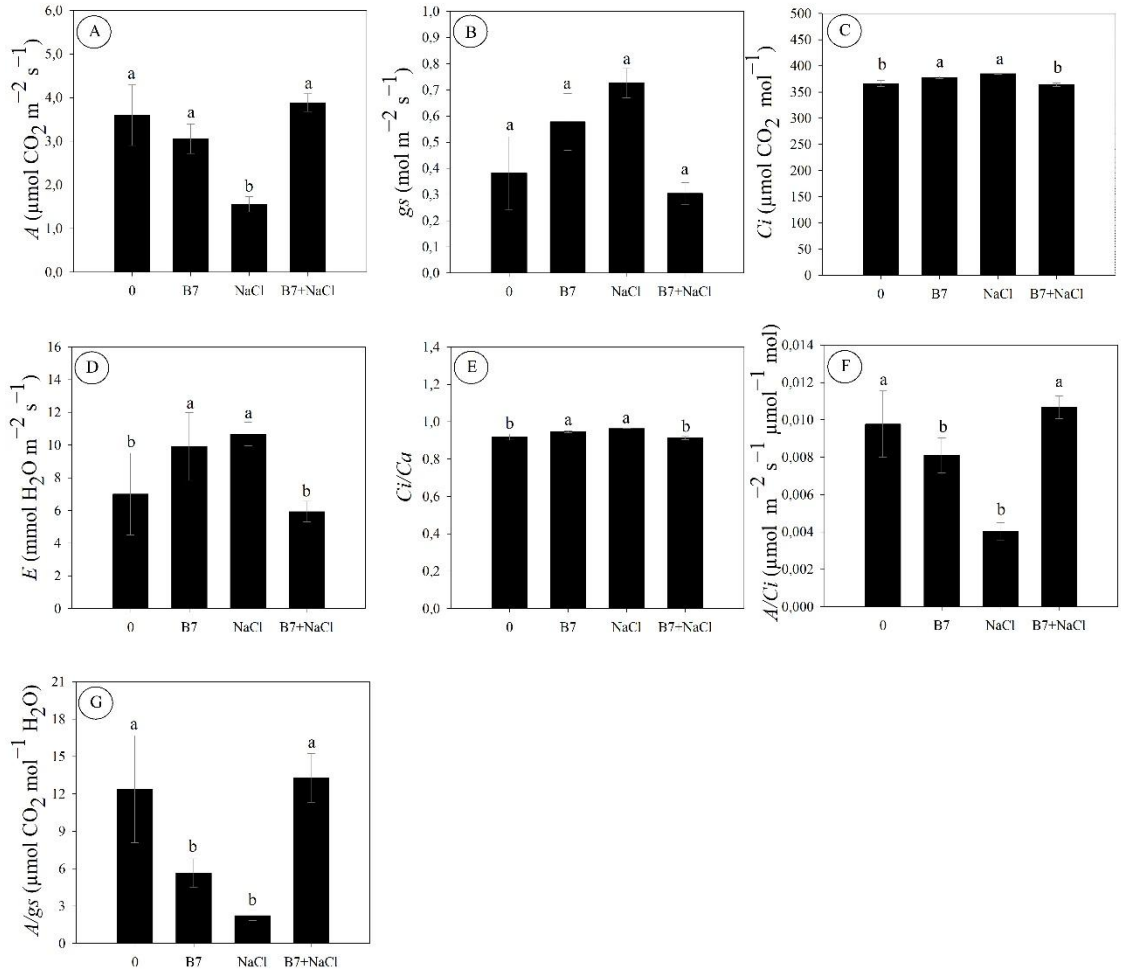

Figure. 4 Gas exchange parameters of *Dizygostemon riparius* at 45 days of *in vitro* culture under concentrations of NaCl (0 and 50 mM), biotin (16 mg L^{-1}), and the combination of both B7 (16 mg L^{-1}) + NaCl (50 mM). (A) Net CO_2 assimilation rate (A); (B) Stomatal conductance (gs); (C) Intercellular CO_2 concentration (Ci); (D) Transpiration rate (E); (E) Ci/Ca ratio; (F) Carboxylation efficiency (A/Ci); and (G) Intrinsic water-use efficiency (A/gs)**.** Different letters above the bars indicate significant differences ($P < 0.05$) according to Skoot-Knot test. Values are means ± standard error (n = 3).

3.4 Leaf micromorphology of *Dizygostemon riparius* under salinity and exogenous biotin *in vitro*

Leaf anatomical analyses revealed a high degree of structural stability in *Dizygostemon riparius* across all treatments (Fig. 5). No significant differences were detected in the thickness of the adaxial and abaxial epidermis, palisade parenchyma, spongy parenchyma, or in the dimensions of transverse and longitudinal vascular bundles among control plants and those subjected to biotin supplementation, salinity, or their combination (Figs. 5C–H).

Consistently, qualitative observations of leaf midrib and blade cross sections showed preserved tissue organization, with no evidence of anatomical disruption or structural rearrangement under salinity or biotin treatments (Figs. 5A and 5B). These results indicate that neither salinity nor exogenous biotin induced detectable changes in leaf micromorphology under experimental conditions, supporting the interpretation that biotin-mediated responses to salinity occurred independently of anatomical adjustments.

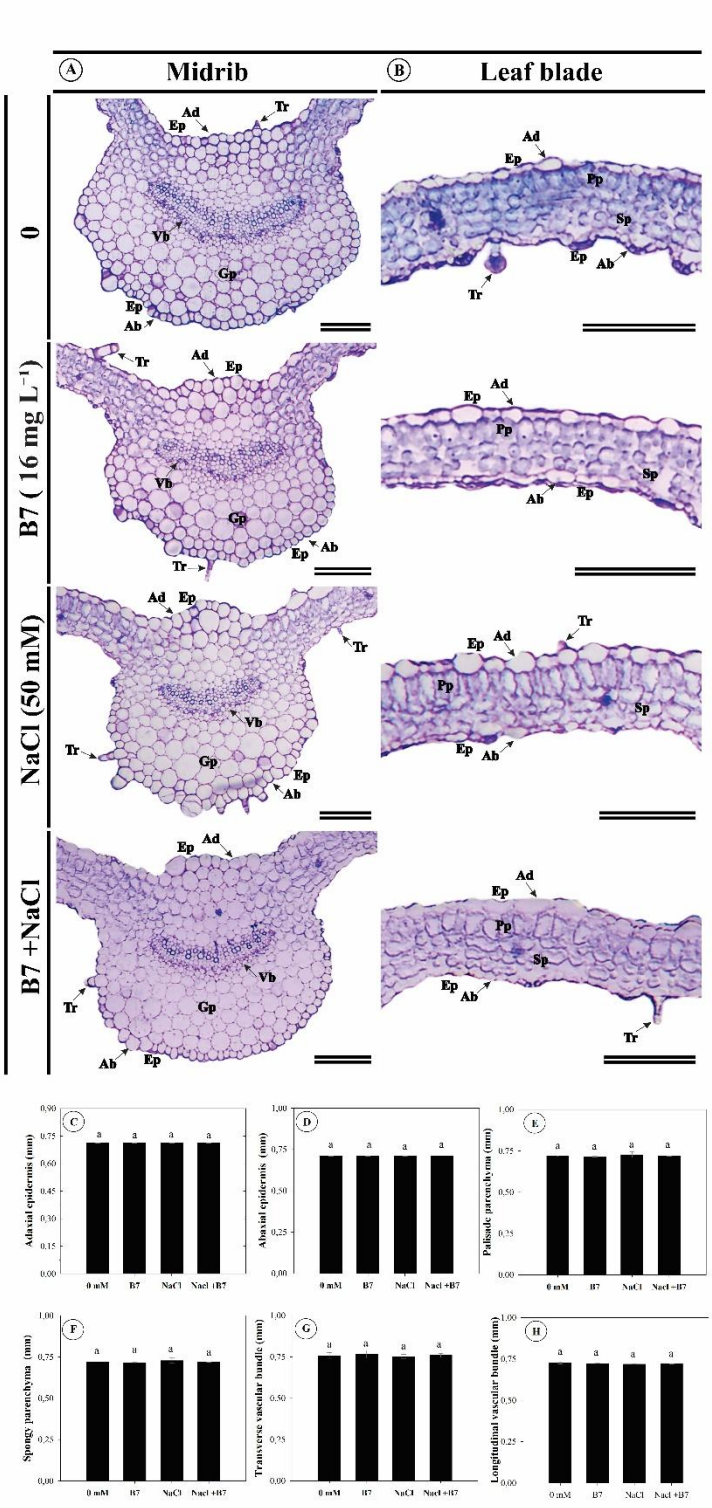

Fig. 5 Micromorphometric parameters of *Dizygostemon riparius* at 45 days of *in vitro* culture under concentrations of NaCl (0 and 50 mM), biotin (16 mg L^{-1}), and the combination of both B7 (16 mg L^{-1}) + NaCl (50 mM). (A) Transverse sections of the leaf midrib; (B) Transverse sections of the leaf blade; (C) Adaxial epidermis thickness (mm); (D) Abaxial epidermis thickness (mm); (E) Palisade parenchyma thickness (mm); (F) Spongy parenchyma thickness (mm); (G) Transverse vascular bundle thickness (mm); and (H) Longitudinal vascular bundle thickness (mm). Different letters above the bars indicate significant differences ($P < 0.05$) according to Skoot-Knot test. Values are means ± standard error (n = 5). Ep = epidermis, Ad = adaxial epidermis, Ab = abaxial epidermis, Vb = vascular bundle, Pp = palisade parenchyma, Sp = spongy parenchyma, Gp = ground parenchyma, Tr = trichome. Scale bar = 100 µm

4. DISCUSSION

Salinity imposed a clear functional constraint on the growth of *Dizygostemon riparius* under *in vitro* conditions, as reflected by the pronounced reduction in shoot length and aboveground biomass. Such inhibition is consistent with the osmotic and ionic components of salt stress, in which excess Na^+ and Cl^- disrupt cellular water relations, nutrient balance, and cell expansion, thereby constraining growth processes (Kaur et al., 2024; Pandit et al., 2024; Dias et al., 2025; Xu et al., 2025). The preferential sensitivity of aerial tissues indicates that salinity primarily affected carbon-dependent growth rather than inducing generalized growth impairment.

In contrast, root length and root dry mass remained unchanged across treatments, suggesting a conservative allocation strategy that preserves root structural integrity and function under saline conditions (Tsogtsaikhan et al., 2025; Vasconcelos et al., 2025). Maintenance of the root system under stress is particularly relevant given its role in water uptake and ion exclusion or compartmentalization, often occurring at the expense of shoot growth (Yetgin, 2024; Gao et al., 2025). This divergence between shoot and root responses supports the view that salinity constrained growth primarily through limitations in assimilating availability rather than through structural damage.

Photosynthetic pigment composition remained stable under salinity, with no detectable changes in chlorophylls or carotenoids. This stability indicates that the applied stress did not induce severe photo-oxidative damage or pigment degradation and that photoprotective capacity was sufficient to preserve thylakoid integrity (Naqi et al., 2025; Abdi et al., 2025; Khan et al., 2025). The absence of carotenoid accumulation further suggests that non-enzymatic photoprotection was not markedly upregulated, consistent with moderate stress intensity (Dash et al., 2025; Lee et al., 2025).

Consistently, the maximum quantum efficiency of photosystem II (F_v/F_m) remained within a narrow optimal range across treatments, with only minor increases under salinity and biotin supplementation. This response reflects stable PSII photochemistry rather than enhanced photosynthetic capacity and confirms that salinity-induced limitations occurred downstream of photochemical energy conversion (Ahmed et al., 2024; Rudikovskii et al., 2025; Wang et al., 2025).

Gas exchange analyses clearly indicate that salinity-induced reductions in net CO_2 assimilation were not driven by stomatal limitation. Despite the strong decline in photosynthetic rate, stomatal conductance and transpiration remained unchanged or increased, while internal CO_2 concentration and Ci/Ca ratio rose. Together with the

marked decrease in instantaneous carboxylation efficiency (A/Ci), these patterns demonstrate a predominance of non-stomatal, biochemical limitations to photosynthesis under NaCl stress (Amaral et al., 2024; Qiao et al., 2025). Such limitations are consistent with impaired RuBisCO activity and reduced RuBP regeneration due to constraints on ATP and NADPH supply (Chen et al., 2025; Yang et al., 2025), explaining the concomitant reduction in shoot biomass.

Exogenous biotin supplementation under salinity effectively restored net photosynthetic assimilation, carboxylation efficiency, and intrinsic water use efficiency, without altering stomatal conductance, pigment composition, or PSII photochemistry. This response indicates that biotin acted at the biochemical level, modulating metabolic capacity rather than diffusive or photochemical processes (Dias et al., 2025; Kunz et al., 2024). Given its role as a cofactor in carboxylation reactions and lipid metabolism, biotin supply likely compensated for stress-induced limitations in vitamin-dependent enzymatic fluxes within the Calvin cycle (Liu et al., 2022).

Importantly, these functional recoveries occurred in the absence of anatomical plasticity. Leaf micromorphological traits remained unchanged across treatments, indicating that salinity-induced photosynthetic constraints preceded any requirement for structural adjustment and that biotin-mediated modulation operated independently of anatomical remodeling (Pinheiro et al., 2025).

Collectively, these findings demonstrate that salinity imposed a predominantly non-stomatal limitation on photosynthesis in *Dizygostemon riparius*, characterized by reduced biochemical capacity for carbon fixation without photochemical or anatomical disruption. Exogenous biotin effectively modulated these functional constraints, restoring carbon assimilation and water use efficiency under salinity. This response pattern aligns with the concept that stress exposure may induce functional B vitamin deficiencies, in which modest disruptions in vitamin-derived cofactors constrain metabolic fluxes without causing overt structural damage (Hanson et al., 2016).

5. CONCLUSION

This study demonstrates that salinity constrains photosynthesis in *Dizygostemon riparius* primarily through non-stomatal, biochemical limitations, occurring in the absence of detectable changes in photochemistry or leaf anatomy. Exogenous biotin restores carbon assimilation and water use efficiency under salinity without affecting stomatal conductance, pigment composition, or structural traits. These findings establish biotin as a metabolic regulator of photosynthetic resilience under salinity and support the concept that stress induces functional B vitamin limitations that restrict metabolic fluxes rather than structural integrity.

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CONSIDERAÇÕES FINAIS

Os resultados desta tese demonstram que *D. riparius* apresenta sensibilidade ao estresse salino, evidenciada principalmente pela redução do crescimento da parte aérea e por limitações fisiológicas associadas à assimilação de carbono. As análises indicaram que essas restrições ocorreram predominantemente em nível bioquímico, caracterizando limitações não estomáticas à fotossíntese, sem comprometimento significativo da fotoquímica do fotossistema II ou da estrutura anatômica foliar, o que sugere que os efeitos do estresse se manifestam inicialmente em nível funcional.

No que diz respeito à homeostase de poliaminas, observou-se uma capacidade limitada de ajuste metabólico frente à salinidade, indicando que, embora essas moléculas desempenhem papel relevante na estabilidade celular e na resposta ao estresse oxidativo, sua regulação não foi suficiente para mitigar os efeitos deletérios do NaCl. Por outro lado, a aplicação exógena de biotina promoveu mitigação parcial dos efeitos do estresse, restaurando a assimilação fotossintética, a eficiência de carboxilação e o uso da água, sem alterações nos parâmetros estomáticos, pigmentares ou anatômicos, evidenciando sua atuação predominante em nível metabólico.

De forma integrada, os achados reforçam que a salinidade impõe limitações iniciais ao metabolismo fotossintético em *D. riparius*, e que a modulação bioquímica, especialmente via biotina exógena, representa uma estratégia promissora de mitigação. Além disso, o cultivo *in vitro* mostrou-se uma ferramenta eficaz para a investigação desses mecanismos, contribuindo para o avanço do conhecimento sobre a ecofisiologia de uma espécie endêmica ainda pouco explorada, fornecendo subsídios para estratégias de conservação, domesticação e uso sustentável. Adicionalmente, abre perspectivas para futuras pesquisas voltadas à integração de abordagens metabolômicas, moleculares e biotecnológicas, com vistas ao aprofundamento dos mecanismos de tolerância ao estresse salino e à aplicação de compostos moduladores, como a biotina, em espécies de interesse agronômico e farmacológico.