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VITÓRIA KARLA DE OLIVEIRA SILVA MORAES

**CO₂ ELEVADO E CONDICIONAMENTO SALINO *IN VITRO*: RESPOSTAS
MORFOFISIOLÓGICAS E ANATÔMICAS DE *Ananas comosus***

São Luís - MA

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Orientadora: Prof^a Dra. Thais Roseli Corrêa

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Lista de siglas e abreviações

μg = micrograma

μM = micromolar

A = photosynthetic CO_2 assimilation

A/Ci = carboxylation efficiency

A/gs = intrinsic water-use efficiency

Ca^{2+} = cálcio

CAM = metabolismo ácido das crassuláceas

CAT = catalase

Ci = internal CO_2 concentration

Ci/Ca = ambient intercellular CO_2
concentration ratio

Cl^- = cloro

Cm = centimeter

CO_2 = dióxido de carbono

DM = dry mass

E = transpiration rate

ECO_2 = elevated CO_2

EROS = espécies reativas de oxigênio

F0 = initial fluorescence

FM = fresh mass

Fm = maximum fluorescence

Fv = variable fluorescence

Fv/F0 = variable fluorescence per initial
fluorescence ratio

Fv/Fm = maximum quantum yield of
photosystem II

g = gram

GPx = glutathione

gs = stomatal conductance to water vapor

H_2O_2 = hydrogen peroxide

IC = Concentração inibitória

K^+ = potássio

Mg^{2+} = magnésio

mL = milliliter

mM = milimolar

mm = millimeter

Na^+ = sódio

NaCl = Cloreto de sódio

O_2 = singlet oxygen

O_2^- = superoxide anion

OH^- = hydroxyl radical P

OTC = câmara de topo aberto

PEPCase = fosfoenolpiruvato carboxilase

PI = performance index

RC/ABS = energy absorbed per active
reaction center

RNA = ácido ribonucleico

ROS = reactive oxygen species

Rubisco = 1,5-ribulose-1,5-bisfosfato
carboxilase/oxigenase

SOD = superoxide dismutase

TM = turgid mass

Resumo

A salinidade e o aumento da concentração de CO₂ atmosférico são fatores que influenciam diretamente o desempenho fisiológico de *Ananas comosus*, especialmente em cultivo *in vitro* e *ex vitro*. De modo geral, a elevação do CO₂ tem sido associada à melhoria do crescimento, da fotossíntese e pode atenuar parcialmente os efeitos negativos de estresses abióticos, enquanto o priming salino *in vitro* surge como uma estratégia para induzir memória e aumentar a tolerância das plantas em condições subsequentes de estresse. O capítulo I corresponde ao referencial teórico. O capítulo II teve como objetivo avaliar os efeitos combinados da elevação do CO₂ e da salinidade no crescimento, fotossíntese e estrutura anatômica foliar em plantas de *Ananas comosus* cultivadas *in vitro*. O experimento seguiu um delineamento inteiramente casualizado em esquema fatorial 2 × 2 (0 e 120 mM de cloreto de sódio × 420 ± 30 e 800 ± 30 µmol mol⁻¹ CO₂). O CO₂ elevado promoveu incrementos expressivos na taxa fotossintética, na eficiência do uso da água e no acúmulo de biomassa, além de reduzir parcialmente os impactos negativos sobre o crescimento e a função estomática. No entanto, os efeitos da salinidade sobre a redução dos pigmentos fotossintéticos, como clorofilas e carotenoides, não foram revertidos pelo aumento de CO₂. O capítulo III avaliou os efeitos do condicionamento *in vitro* à salinidade no crescimento e desempenho fotossintético de *Ananas comosus* (L.) exposto à salinidade *ex vitro*. As plantas foram cultivadas *in vitro* com 0 ou 80 mM de NaCl por 90 dias e, em seguida, transferidas para condições *ex vitro* com 0 ou 500 mM de NaCl em um delineamento fatorial 2 × 2. O priming salino *in vitro* induziu alterações fisiológicas caracterizadas por ajustes fotoprotetores e modificações no desempenho fotoquímico, como reduções nos valores de Fv/Fm e no índice de desempenho fotossintético. Quando transferidas para condições *ex vitro*, a salinidade severa reduziu as trocas gasosas e o acúmulo de biomassa em mudas de abacaxizeiro, independentemente do condicionamento prévio. Embora as plantas condicionadas tenham apresentado alguns sinais de aclimatização fisiológica, como menor redução da eficiência de carboxilação e manutenção dos pigmentos fotossintéticos, esses ajustes não resultaram em maior crescimento. Além disso, o condicionamento esteve associado à redução do porte das plantas, indicando que as adaptações fisiológicas induzidas foram insuficientes para mitigar os efeitos da elevada salinidade sobre a produção de biomassa. Os resultados evidenciam que tanto o CO₂ elevado quanto o priming salino modulam a tolerância ao estresse salino em *A. comosus* por meio de mecanismos predominantemente fisiológicos e bioquímicos, com destaque para a regulação da fotossíntese e da eficiência no uso da água. Entretanto, a dissociação entre melhoria fisiológica e acúmulo de biomassa reforça a complexidade das respostas ao estresse e indica a necessidade de abordagens integrativas que conciliem tolerância e produtividade em condições salinas.

Palavras-chave: Bromeliaceae, enriquecimento de CO₂, mitigação, priming, salinidade.

Abstract

Salinity and the increase in atmospheric CO₂ concentration are factors that directly influence the physiological performance of *Ananas comosus*, particularly under *in vitro* and ex vitro cultivation. In general, elevated CO₂ has been associated with enhanced growth and photosynthesis and may partially alleviate the negative effects of abiotic stresses, whereas *in vitro* salinity priming has emerged as a strategy to induce stress memory and increase plant tolerance under subsequent stress conditions. Chapter I presents the theoretical framework. Chapter II aimed to evaluate the combined effects of elevated CO₂ and salinity on the growth, photosynthesis, and leaf anatomical structure of *in vitro*-grown *Ananas comosus* plants. The experiment was conducted in a completely randomized design using a 2 × 2 factorial arrangement (0 and 120 mM sodium chloride × 420 ± 30 and 800 ± 30 μmol mol⁻¹ CO₂). Elevated CO₂ promoted significant increases in the photosynthetic rate, water-use efficiency, and biomass accumulation, while partially mitigating the negative effects of salinity on plant growth and stomatal function. However, the salinity-induced reductions in photosynthetic pigments, including chlorophylls and carotenoids, were not reversed by elevated CO₂. Chapter III evaluated the effects of *in vitro* salinity priming on the growth and photosynthetic performance of *Ananas comosus* (L.) exposed to salinity under ex vitro conditions. Plants were cultured *in vitro* with either 0 or 80 mM NaCl for 90 days and subsequently transferred to ex vitro conditions with either 0 or 500 mM NaCl in a 2 × 2 factorial design. *In vitro* salinity priming induced physiological changes characterized by photoprotective adjustments and modifications in photochemical performance, including reductions in Fv/Fm values and the photosynthetic performance index. After transfer to ex vitro conditions, severe salinity reduced gas exchange and biomass accumulation in pineapple plantlets regardless of previous priming. Although primed plants exhibited some signs of physiological acclimation, such as a smaller reduction in carboxylation efficiency and maintenance of photosynthetic pigments, these adjustments did not result in enhanced growth. Furthermore, priming was associated with reduced plant size, indicating that the induced physiological adaptations were insufficient to mitigate the effects of severe salinity on biomass production. Overall, the results demonstrate that both elevated CO₂ and salinity priming modulate salt-stress tolerance in *A. comosus* through predominantly physiological and biochemical mechanisms, particularly by regulating photosynthesis and water-use efficiency. However, the dissociation between improved physiological performance and biomass accumulation highlights the complexity of plant responses to stress and emphasizes the need for integrative approaches that reconcile stress tolerance with productivity under saline conditions.

Keywords: Bromeliaceae, CO₂ enrichment, mitigation, priming, salinity.

CAPÍTULO I

Introdução geral e referencial teórico

1. Introdução geral

O abacaxizeiro (*Ananas comosus* (L.) Merr.), pertencente à família Bromeliaceae, é uma espécie de grande relevância econômica, amplamente cultivada em regiões tropicais e subtropicais (Hossain, 2016). Seu fruto apresenta elevada aceitação no mercado global, destacando-se entre as principais frutas tropicais consumidas (Mohd et al., 2020). No entanto, os métodos convencionais de propagação ainda apresentam limitações importantes, como a heterogeneidade entre mudas e a disseminação de patógenos, fatores que comprometem a uniformidade e a produtividade das lavouras (Reinhardt et al., 2018). Nesse sentido, a cultura de tecidos vegetais tem se consolidado como uma ferramenta eficiente, que permite a produção de mudas clonais em condições assépticas e controladas, além de possibilitar a investigação das respostas das plantas a diferentes condições ambientais (Nazatul Asikin et al., 2026).

Entre os fatores que limitam a produção agrícola, a salinidade do solo ocupa posição de destaque, e tem afetado extensas áreas cultiváveis em todo o mundo. Esse problema está associado a múltiplas causas, como o manejo inadequado da irrigação, drenagem ineficiente, uso de águas salinas e alterações climáticas (Eswar, Karuppusamy e Chellamuthu, 2021). Em plantas, o excesso de sais provoca alterações fisiológicas e metabólicas que comprometem o crescimento, a absorção de água e nutrientes e a eficiência fotossintética (Ma et al., 2025). No abacaxizeiro, esses efeitos refletem-se na redução do desenvolvimento vegetativo, no acúmulo de biomassa e na qualidade dos frutos, devido a distúrbios osmóticos, toxidez iônica e danos estruturais às células (Liu e Chen, 2014; Melo et al., 2017).

Paralelamente, o aumento da concentração de dióxido de carbono (CO₂) na atmosfera tem sido intensificado nas últimas décadas, que configura um dos principais componentes das mudanças climáticas (Weisheimer et al., 2025). Embora esse fenômeno esteja relacionado a impactos negativos no clima global, o CO₂ exerce papel central na fotossíntese e pode, em níveis elevados, favorecer o crescimento vegetal ao intensificar a assimilação de carbono e a produção de biomassa (Gamage et al., 2018; Boretti, 2025). No cultivo *in vitro*, o enriquecimento com CO₂ tem sido associado à melhoria do desempenho fotossintético, ao aumento da produção de matéria seca e ao fortalecimento do aparato fisiológico das plantas (Pinheiro et al., 2021; Pan et al., 2025, de Oliveira Silva-Moraes et al., 2025).

Como forma de aumentar a tolerância das plantas ao estresse salino, estratégias como o condicionamento salino (salinity priming) têm sido amplamente investigadas. Essa abordagem consiste na exposição prévia das plantas a níveis moderados de sal no qual desencadeia mecanismos de defesa ativos e favorecem uma resposta mais eficiente em exposições posteriores (Sahoo, Swain e Yadav, 2025). Em condições *in vitro*, esse processo apresenta

vantagens adicionais, uma vez que permite o controle rigoroso dos fatores ambientais e facilita a indução de respostas fisiológicas e moleculares associadas à chamada memória de estresse (Conrath et al., 2015). Evidências indicam que plantas previamente condicionadas apresentam melhor desempenho após a transferência para condições naturais, e demonstram maior estabilidade fisiológica e capacidade adaptativa (He et al., 2022; Turgut-Kara et al., 2020).

Diante desse contexto, torna-se fundamental compreender como fatores ambientais, como o aumento de CO₂, interagem com estresses abióticos clássicos, como a salinidade. Apesar dos avanços na área, ainda são limitadas as investigações que abordam esses fatores em abacaxizeiro, especialmente em sistemas *in vitro*, nos quais é possível avaliar com maior precisão os mecanismos envolvidos nas respostas das plantas.

Nesse sentido, o presente estudo teve como objetivo analisar os efeitos combinados do CO₂ elevado e da salinidade sobre o crescimento, a fisiologia e a anatomia foliar de plantas de *A. comosus* cultivadas *in vitro*, bem como avaliar o crescimento e as respostas fisiológicas dessas plantas quando expostas à salinidade durante a aclimação.

2. Referencial teórico

2.1 Abacaxizeiro [*Ananas comosus* (L.) Merrill]

O abacaxizeiro (*Ananas comosus* var. *comosus* (L.) Merr.) é uma frutífera amplamente cultivada nas regiões tropicais e subtropicais do mundo. O fruto desta espécie apresenta elevada importância comercial sendo considerado o terceiro fruto mais consumido do mundo (Abraham, Joshi, Abdullah, 2023).

Desde a sua descoberta, o abacaxi tem sido uma fruteira de grande importância mundial com produção de cerca de 29,3 milhões de toneladas, com destaque para a Costa Rica com 3 milhões de toneladas, em segundo lugar as Filipinas com aproximadamente 2,9 milhões de toneladas, em terceiro lugar a Indonésia com 2,7 milhões de toneladas e em quarto lugar o Brasil com 2,2 milhões de toneladas (Faostat, 2025).

No Brasil, o abacaxizeiro possui área plantada de 57.317 ha, com produção de 1.482.136.000 bilhões de frutos que correspondem ao valor de produção de R\$ 4.380.071.000 bilhões de reais. Neste cenário, a região Nordeste destaca-se com a maior participação da produção nacional que corresponde a 39% e o estado da Paraíba como maior produtor com cerca de 20% da produção agrícola (Ibge, 2025).

O abacaxizeiro é uma planta monocotiledônea, herbácea e perene, pertencente à família Bromeliaceae, que compreende cerca de 56 gêneros e 2.794 espécies (Crestani et al., 2010).

Entre as diversas cultivares de abacaxizeiro, ‘Smooth Cayenne’ e ‘Pérola’ são as de maior importância comercial nos mercados nacional e internacional, destacando-se entre outras, como Singapore Spanish, Queen, Spañola Roja e Perolera (Reis et al. 2019).

A planta de abacaxi pode atingir aproximadamente 1 m de altura e apresenta caule curto, espesso em formato claviforme. As folhas são estreitas, rígidas e canaliculadas, dispostas em roseta densa e compacta ao longo do caule, podendo variar de 40 a 80 unidades. O sistema radicular é fasciculado, superficial e fibroso, concentrando-se geralmente entre 0 e 30 cm de profundidade (Reinhardt et al., 2018).

O fruto do abacaxizeiro consiste em uma infrutescência carnosa, do tipo baga composta, formada pela fusão de múltiplos frutos individuais que se desenvolvem a partir de flores organizadas ao longo de um eixo central. Cada escama da casca corresponde ao desenvolvimento de uma flor, e a coalescência dessas estruturas origina o fruto característico (Reinhardt et al., 2018).

O metabolismo desta espécie está inserido no grupo do metabolismo ácido das crassuláceas (CAM), no entanto há controvérsias sobre o metabolismo desta planta principalmente porque apresenta adaptações devido às suas características morfológicas, anatômicas e fisiológicas (Aragón et al., 2012). O metabolismo CAM se desenvolve como resposta a um ambiente estressante e resulta em folhas mais suculentas, maior concentração de amido durante o período de luz e taxas fotossintéticas mais elevadas do que o metabolismo C₃ (Aragón et al., 2012).

A propagação da espécie ocorre por meio de estruturas vegetativas como coroa, filhote, filhote-rebentão e rebentão (Cunha e Reinhardt, 2004). A produção é restrita pois demanda da quantidade de material gerada por planta o que dificulta a rápida expansão de áreas cultivadas. Além disso, a variabilidade no tamanho e vigor das mudas resulta em campos desuniformes e colheitas irregulares. Outro entrave é a sanidade pois as mudas carregam patógenos e pragas, exigindo processos de cura e seleção rigorosos para reduzir riscos de fusariose e infestações por cochonilhas, o que demanda tempo e mão de obra especializada que torna o processo mais oneroso (Reinhardt et al., 2018).

Como uma alternativa eficaz para superar as limitações da produção de mudas convencional aplicada a diversas culturas de propagação vegetativa, como o abacaxizeiro, a micropropagação tem se mostrado uma ferramenta capaz de elevar a taxa de multiplicação de plantas a partir de poucos explantes, produção de plantas uniformes em tamanho e vigor, além de garantir mudas saudáveis, livres de doenças e pragas, sem depender das variações sazonais (Ranjan et al., 2026).

Estudos *in vitro* exploram protocolos eficientes de propagação em plantas de abacaxizeiro, como por exemplo, o potencial fotoautotrófico em sistema de biorreatores (Alves et al., 2023), fotomixotrofia e aclimatização de mudas (de Paula Alves et al., 2023), sistemas de cultivo com redução de sacarose e tipos de vedação de frascos e aclimatização *ex vitro* (Pires et al., 2023), regeneração de microbrotos via cluster nodulares com uso de putrescina (Silva-Moraes et al., 2024) e aplicação foliar de ureia na aclimação de mudas (Soares et al., 2026).

Além destes estudos, o cultivo *in vitro* permite entender como as plantas de abacaxizeiro se comportam sob condições de estresse, em situações de baixa temperatura as plantas modificam a expressão gênica para aumentar a tolerância, causando mudanças fisiológicas e bioquímicas (Tarmizi e Dolhaji, 2022), na tolerância à seca o uso de reguladores de crescimento (hexanoato de dietilaminoetila e oligossacarídeo de quitosana) regulam o sistema antioxidante por meio da bromelina e do estresse oxidativo em plantas de abacaxizeiro (Huang et al., 2023) e sob estresse salino, plantas regeneradas *in vitro* apresentam alterações morfofisiológicas decorrentes do efeito tóxico dos elevados níveis de sal no meio de cultivo com redução da biomassa aérea e radicular (Samuel et al., 2018).

2.2 Estresse salino em plantas

A salinização do solo é um dos maiores desafios da agricultura global (Wang et al., 2025). De acordo com o relatório Global Status of Salt-Affected Soils, publicado pela Organização das Nações Unidas para a Alimentação e a Agricultura (FAO) em 2024, aproximadamente 10,7% da área terrestre mundial está afetada pela salinidade, condição que representa um desafio à sustentabilidade ambiental e à produtividade dos sistemas agrícolas.

O processo de salinização ocorre de forma natural, por meio do acúmulo de sais decorrente do intemperismo, e também por ação antrópica, incluindo práticas inadequadas de irrigação, desmatamento, má drenagem, superexploração de recursos hídricos subterrâneos e atividades industriais (Kaushal et al., 2021).

O excesso de sais no solo induz inicialmente o estresse osmótico, com redução do potencial hídrico do solo e, conseqüentemente, restrição da absorção de água e nutrientes pelas raízes (Parihar et al., 2015). Além disso, a planta desencadeia uma série de respostas que levam à restrição da abertura estomática e à redução da assimilação fotossintética de CO₂ (Silveira et al., 2010). Posteriormente, ocorre o estresse iônico, caracterizado pela absorção e acúmulo de íons nos tecidos, principalmente Na⁺ e Cl⁻, além da inibição da absorção de K⁺ e Ca²⁺, resultando em desequilíbrios nutricionais e metabólicos (Isayenkov and Maathuis, 2019).

A salinidade não provoca apenas desequilíbrio osmótico e iônico, mas também desencadeia estresse oxidativo como efeito secundário. Nesse processo, espécies reativas de oxigênio (EROS) acumulam-se nas células em níveis elevados, com oxidação de proteínas e ácidos nucleicos, além de peroxidação de lipídios (Fichman et al., 2023). Entre essas espécies destacam-se o peróxido de hidrogênio (H_2O_2), o radical hidroxila (OH), o ânion superóxido (O_2^-) e o oxigênio singlete ($^1O_2$), os quais afetam o metabolismo vegetal e modulam a expressão gênica associada à adaptação ao estresse (El Moukhtari et al., 2020).

A salinidade afeta as plantas em três níveis principais: (1) nível molecular, causa danos ao DNA e RNA, inativação enzimática e oxidação de biomoléculas; (2) nível celular, promove estresse osmótico e iônico, desequilíbrio nutricional, alterações nas trocas gasosas (O_2/CO_2), perda de eletrólitos, superprodução de ROS e danos ao sistema de endomembranas; e (3) nível fisiológico, reduz a absorção de água e nutrientes, o alongamento celular, a pressão de turgor, a abertura estomática e a taxa fotossintética, como consequência final, redução do crescimento, desenvolvimento e produtividade (Hualpa-Ramirez et al., 2024).

Ao enfrentar esses estresses, as plantas desenvolvem mecanismos de tolerância que permitem seu crescimento e a conclusão do ciclo de vida, como forma de mitigar os efeitos deletérios da salinidade, desenvolvem tolerância osmótica, iônica e tecidual (Rajendran et al., 2009; Roy et al., 2014). A tolerância osmótica constitui a primeira linha de defesa das plantas sob estresse salino. Nesse contexto, ocorre a síntese de osmólitos compatíveis, como prolina, açúcares solúveis e glicina betaína, que contribuem para a manutenção da turgescência celular, da atividade metabólica, da absorção de água, da expansão foliar e da condutância estomática (Alharazi et al., 2025). Dentre esses compostos, a prolina destaca-se como um osmólito crucial na regulação osmótica, pois contribui para o equilíbrio hídrico celular, reduz danos causados por EROS, protege estruturas celulares e participa de vias bioquímicas relacionadas à regeneração de $NADP^+$, além de auxiliar na fotossíntese e na fixação biológica de nitrogênio (Abdi et al., 2025).

A tolerância iônica está relacionada ao controle da entrada, transporte e compartimentalização de Na^+ , que entra nas células via apoplasto por meio de transportadores de alta afinidade para K^+ (HKT), canais catiônicos não seletivos (NSCCs) e canais semelhantes a receptores de glutamato (GLRs), levando ao desequilíbrio iônico (Singh et al., 2022). O Ca^{2+} extracelular pode reduzir a atividade desses canais, limitar a entrada de Na^+ ou promover remoção do xilema antes que esse íon seja translocado para as folhas (Xiao e Zhou, 2023). Além disso, o Na^+ compete com o K^+ pelos sítios de absorção na rizosfera. A proteína SOS1

atua como um antiporte Na^+/H^+ , promovendo a extrusão de Na^+ do citoplasma para o meio externo, utilizando o gradiente de prótons gerado por bombas de H^+ (Ullah et al., 2025).

A tolerância tecidual baseia-se na compartimentalização do Na^+ no vacúolo. Quando a exclusão citoplasmática não é suficiente, transportadores do tipo NHX atuam como antiportes Na^+/H^+ e promovem o sequestro de Na^+ para o vacúolo por meio do gradiente de H^+ gerado pelas bombas V-ATPase e V-PPase, mecanismo que garante a proteção do citoplasma e a manutenção da homeostase celular (Balasubramaniam et al., 2023).

A adaptação das plantas ao ambiente salino está associada a características evolutivas, fisiológicas, anatômicas e moleculares (Rakhmankulova et al., 2026). Plantas halófitas apresentam elevada tolerância ao NaCl, suportam concentrações entre 50 e 500 mM, e ocorrem em ambientes como manguezais, desertos salinos e regiões costeiras. Em contraste, plantas glicófitas evoluíram em solos com baixa concentração de sódio, no qual mantém níveis reduzidos de Na^+ nos tecidos, especialmente nas folhas (Khan et al., 2022).

2.3 Priming salino

A seleção de genótipos tolerantes torna-se fundamental para assegurar a produtividade agrícola em solos afetados pela salinidade, com mitigação dos efeitos adversos desse estresse abiótico (Yassin et al., 2019). Nesse sentido, o priming salino surge como uma estratégia promissora, pois pré-condiciona as plantas a concentrações subletais de sal e ativa mecanismos fisiológicos e moleculares associados à memória de estresse (Pissolato et al., 2024).

Nos estudos de priming em plantas, um estímulo inicial é aplicado com o objetivo de preparar o organismo para responder de forma mais eficiente a estresses futuros (Kambona et al., 2023). Esse processo está associado ao conceito de 'memória de plantas', que se trata de um conjunto de mecanismos moleculares e fisiológicos que permitem às plantas responder melhor a condições adversas recorrentes (Pissolato et al., 2024). Assim, plantas previamente submetidas a condições de estresse podem ativar mecanismos de defesa mais rapidamente e com maior intensidade quando expostas novamente ao mesmo fator adverso, resultando em maior tolerância e estabilidade no desempenho produtivo (Sarma et al., 2023).

Estudos têm demonstrado o potencial do priming salino, inclusive em sistemas *in vitro*, na promoção da tolerância em diferentes espécies. Em *Saccharum* spp., o priming favoreceu o perfilhamento e induziu memória adaptativa após novo ciclo de estresse (Melo et al., 2014). Em *Myracrodruon urundeuva*, promoveu aumento da altura, manutenção do número de folhas e preservação da biomassa sob salinidade (Souza et al., 2022). Em *Arabidopsis thaliana*, plantas condicionadas com 50 mM de NaCl apresentaram menor impacto no crescimento (altura e

folhas), menor perda de clorofila, menor dano celular e uma resposta molecular mais eficiente em comparação às não condicionadas (Arikan et al., 2026). Em *Ananas comosus*, a combinação de NaCl com fitorreguladores (ANA e BAP) resultou em melhorias nos parâmetros de crescimento, incluindo aumento do número de folhas, altura de brotos, produção de gemas e enraizamento (Melo et al., 2011).

Diversos mecanismos estão envolvidos no priming salino, entre eles a ativação antecipada de respostas de defesa. Um dos primeiros sistemas ativados é o antioxidante, com aumento da atividade de enzimas como ascorbato peroxidase (APX), catalase (CAT), superóxido dismutase (SOD) que contribui para a redução do acúmulo de ROS (El-Shazoly, Hamed e El Sayed, 2024). Essa ativação contribui para a manutenção do equilíbrio redox, redução da perda de eletrólitos e menor impacto no crescimento (Azeem et al., 2023). Além disso, baixos níveis de ROS atuam como moléculas sinalizadoras que promovem respostas adaptativas e memória ao estresse (Kesawat et al., 2023).

O priming também favorece a homeostase iônica, pois aumenta a capacidade das plantas de regular os níveis de Na^+ e Cl^- e manter concentrações adequadas de K^+ e Ca^{2+} , por meio da ativação de transportadores e da via SOS (Salt Overly Sensitive), que promovem a extrusão ou compartimentalização de Na^+ e reduzem sua toxicidade (Martínez-Ballesta et al., 2020; Li e Yang, 2023; Rodríguez Coca et al., 2023).

Outro componente essencial na resposta ao estresse salino é a regulação da expressão gênica. A ativação de genes codificadores de enzimas antioxidantes, transportadores de íons e vias de biossíntese de osmoprotetores permite uma adaptação eficiente (Kumari e Parida, 2018; Balasubramaniam et al., 2023). Além disso, mecanismos epigenéticos, como metilação do DNA, modificações de histonas e memória epigenética, contribuem para respostas adaptativas de longo prazo (Turgut-Kara et al., 2020).

A resposta das plantas à salinidade não ocorre de forma isolada, normalmente é modulada por outros fatores abióticos. O dióxido de carbono (CO_2), por exemplo, pode mitigar os efeitos da salinidade, promover maior eficiência no uso da água e redução da condutância estomática (Javaid et al., 2022).

Em síntese, o estresse salino impõe severas limitações ao crescimento e à produtividade das plantas, afeta o equilíbrio hídrico, a homeostase iônica e a estabilidade redox. No entanto, o priming salino destaca-se como uma estratégia eficaz para aumentar a tolerância vegetal, por meio da indução coordenada de respostas fisiológicas, bioquímicas e moleculares.

2.4 CO₂ em plantas

O CO₂ é um dos principais gases de efeito estufa responsáveis pelo aquecimento global (Bajoria, Kanpariya e Bera, 2024). O aumento das concentrações de CO₂ ocorre principalmente devido à queima de combustíveis fósseis, às atividades industriais relacionadas ao carvão e ao petróleo, ao desmatamento e a práticas agrícolas inadequadas (Nunes, 2023).

Em 2024, a concentração de CO₂ atmosférico atingiu aproximadamente 420 ppm (NOAA, 2024). Entretanto, projeções futuras indicam que esse valor poderá alcançar 800 ppm até o final do século XXI (IPCC, 2021). O aumento contínuo dos gases de efeito estufa ao longo das últimas décadas tem resultado em diversos impactos climáticos, incluindo maior frequência e intensidade de eventos extremos, como elevação da temperatura, secas prolongadas e chuvas acima da média (Ayanlade et al., 2018).

Apesar dos efeitos adversos do aumento de CO₂ para o clima global, nas plantas o CO₂ atua como a principal fonte de carbono para a fotossíntese e, em concentrações elevadas, pode estimular a assimilação de carbono em espécies C₃, C₄ e CAM, embora tenha respostas distintas em função do metabolismo fotossintético (Sharma, Sinha e Bhatnagar, 2014).

As respostas ao enriquecimento de CO₂ variam de acordo com o mecanismo de fixação de carbono desenvolvido ao longo da evolução (Tan e Chen, 2023). De modo geral, plantas C₃ são mais responsivas ao aumento de CO₂, pois apresentam maior limitação pela disponibilidade de CO₂, o que resulta em aumento da taxa fotossintética, especialmente sob condições de temperaturas mais baixas. Em contraste, plantas C₄ são mais adaptadas a ambientes com temperaturas elevadas e possuem mecanismos de concentração de CO₂, o que implica em menor resposta ao enriquecimento atmosférico e maior eficiência em condições de baixas concentrações de CO₂ (Tian et al., 2024). Em plantas CAM, o aumento da concentração atmosférica de CO₂ promove maior assimilação de carbono, aumento da produção de biomassa e maior eficiência no uso da água. Esse efeito ocorre devido ao incremento da fixação de CO₂ tanto pela PEPCase durante a noite quanto pela Rubisco durante o dia, que resulta em maior atividade fotossintética e crescimento vegetal (Drennan e Nobel, 2001).

Estudos com enriquecimento de CO₂ têm sido conduzidos em câmaras de topo aberto (OTCs), nas quais plantas de maior porte são cultivadas em estruturas cilíndricas transparentes com injeção controlada de CO₂, com concentrações superiores às do ambiente (Novello et al., 2024). Essa abordagem permite avaliar o efeito de fertilização por CO₂ e compreender como as plantas se comportam diante de cenários futuros de elevação da concentração atmosférica do CO₂ (Kumar et al., 2024; Lamichaney et al., 2022).

Em condições *in vitro*, estudos envolvendo o enriquecimento de CO₂ têm sido conduzidos com o objetivo de promover o crescimento e a autotrofia das plantas cultivadas (Liang et al., 2026). O enriquecimento tem promovido aumento do crescimento, maior acúmulo de massa fresca e seca, elevação da taxa fotossintética e maiores teores de pigmentos fotossintéticos (Louback et al., 2021; Pinheiro et al., 2021; Shi et al., 2024).

Além do desenvolvimento e aclimação das mudas com o enriquecimento de CO₂, o cultivo *in vitro* permite estudar os efeitos combinados de diversos estresses abióticos em condições controladas, por exemplo, radiação UV, estresse hídrico, temperatura, salinidade (Espinosa-Leal et al., 2022; Pungin et al., 2023).

Estudos tem demonstrado que o CO₂ elevado pode mitigar os efeitos negativos da salinidade. Em *Zea mays*, o CO₂ mitigou os efeitos adversos da seca e do estresse salino no crescimento e na eficiência do uso da água (Xu et al., 2026) além de promover reforço nas defesas antioxidantes pela regulação positiva da SOD e GPx (peroxidase) e ajuste do equilíbrio hídrico acumulando açúcares e outros compostos de carbono nas folhas (Ma et al., 2026). O enriquecimento de CO₂ atenuou os efeitos da salinidade em *Salicornia ramosissima* com melhorias na eficiência do uso da água e reduções no estresse oxidativo (Pérez-Romero et al., 2019). Em *Ananas comosus*, o CO₂ mitigou parcialmente os efeitos da salinidade com aumento significativo da biomassa vegetal, da taxa fotossintética líquida e da eficiência intrínseca do uso da água (de Oliveira Silva-Moraes et al., 2025).

Por fim, a integração entre CO₂ elevado e o estresse salino sugere um efeito complementar na tolerância ao estresse, embora ainda existam lacunas importantes, especialmente em espécies CAM e em estudos que integrem múltiplos fatores ambientais.

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CAPÍTULO II

de Oliveira Silva-Moraes, V.K., Felipe, S.H.S., Alves, G.L. *et al.* Combined effects of elevated CO₂ and salinity on photosynthesis, growth, and leaf anatomy of *Ananas comosus* grown *in vitro*. *Plant Cell Tissue Organ Cult* 162, 72 (2025).
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Combined effects of elevated CO₂ and salinity on photosynthesis, growth, and leaf anatomy of *Ananas comosus* grown *in vitro*

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Abstract

Elevated atmospheric CO₂ concentration has been proposed as a positive modulator of plant growth and development, with potential to mitigate the adverse effects of abiotic stresses. This study aimed to evaluate the combined effects of elevated CO₂ and salinity on growth, photosynthesis, and leaf anatomical structure *Ananas comosus* plants cultivated *in vitro*. The experiment followed a completely randomized design under a 2 × 2 factorial scheme (0- and 120-mM sodium chloride × 420 ± 30 and 800 ± 30 μmol CO₂ mol⁻¹), with ten replicates per treatment. Under saline conditions, elevated CO₂ increased net photosynthetic rate by approximately 350%, intrinsic water-use efficiency by 186%, and plant dry mass by 47% compared to plants cultivated under ambient CO₂. Additionally, elevated CO₂ partially reduced the negative effects of salinity on growth and stomatal function. However, salinity markedly reduced chlorophyll and carotenoid concentrations, and elevated CO₂ did not restore them. These findings suggest that elevated CO₂ concentration can partially alleviate salt stress in *A. comosus* plants grown *in vitro*, mainly by improving photosynthetic performance, water-use efficiency, and biomass accumulation.

Keywords: CO₂ enrichment · Salinity · Gas exchange · Pineapple micropropagation

Key message

Elevated CO₂ concentration partially mitigates salinity-induced impairments in growth and photosynthesis of *Ananas comosus* micropropagated *in vitro* by enhancing water-use efficiency and promoting stomatal functional adjustment.

Abbreviations

aCO₂: ambient CO₂, BAP: 6-benzylaminopurine; eCO₂: elevated carbon dioxide, NAA: naphthaleneacetic acid, NaCl: sodium chloride.

1. Introduction

Pineapple [*Ananas comosus* (L.) Merr.] is a monocot from the Bromeliaceae family widely cultivated in tropical and subtropical regions. Its infructescence is one of the most consumed tropical fruits worldwide (Silva-Moraes et al. 2024; Francisco-Rodríguez et al. 2025). Despite its economic and agricultural importance, traditional methods for producing pineapple plantlets face several problems, such as genetic heterogeneity and poor plant health due to pests and diseases (Da Silva et al. 2021; Alves et al. 2024). These issues can hinder productivity and lead to uneven plantations (Da Silva et al. 2021). Plant tissue culture offers a promising alternative, enabling clonal propagation under controlled aseptic conditions (Zuzarte et al. 2024).

Recent advances in plant biotechnology include the use of bioreactor systems and hormonal regulators such as putrescine to regenerate nodular clusters (Zhang et al. 2022; Alves et al. 2024; Silva-Moraes et al. 2024). These strategies aim to enhance the effectiveness of large-scale micropropagation. In addition to supporting large-scale seedling production, plant tissue culture also allows researchers to study plants responses to environmental stress under controlled conditions (see UV-B radiation by Felipe et al., 2019b; salinity by Zarei et al. 2024; drought stress by El-Naggar and Osman 2024; heavy metals by Cadore et al. 2024; and others). Although photomixotrophic and photoautotrophic *in vitro* systems have already been reported for *A. comosus*, no study has addressed the combined effects of elevated CO₂ (eCO₂) and salinity on this species. This integration is relevant because it allows, for the first time, a precise evaluation of how a climate change factor (eCO₂) interacts with a major abiotic stressor (NaCl-induced salinity) in a fully controlled micropropagation environment, which would be difficult to achieve under field conditions due to environmental complexity.

Carbon dioxide (CO₂) is among the primary greenhouse gases contributing to the intensification of the greenhouse effect (Nicholls 2004). Current atmospheric CO₂ concentrations are around 420 ppm (NOAA 2024), and under a high emission scenario (SSP5-8.5), concentrations could reach approximately 550 ppm by 2050 and exceed 800 ppm by 2100 (IPCC 2021). At the same time, soil salinity (electrical conductivity of saturated paste extract – EC_e ≥ 4 dS m⁻¹) has become a major challenge for agriculture (Corwin 2020; WateReuse 2025). High salinity alters plant morphophysiology, reducing growth, development, and ultimately crop yield (Zhou et al. 2024; El-Ramady et al. 2024).

Salt stress reduces osmotic potential, induces stomatal closure, damages membranes, causes sodium (Na⁺) and chloride (Cl⁻) toxicity, disrupts redox balance, and reduces photosynthesis at the whole-plant level (Isayenkov and Maathuis 2019; Hussain et al. 2024;

Felipe et al. 2019a). To cope with these effects, plants activate defense mechanisms that include accumulating compatible osmolytes and activating hormonal and enzymatic pathways to restore homeostasis and reduce oxidative damage (Hussain et al. 2024; Zhou et al. 2024).

In plant tissue culture, elevated CO₂ levels (eCO₂) appears to promote plant growth and development, both for propagation and for inducing secondary metabolites of interest to the chemical industry. For example, in *Pfaffia glomerata* (Spreng.) Pedersen, eCO₂ enhanced the production of metabolic compounds and reinforced cell structure (Louback et al. 2021). In *Etilingera elatior* (Jack) R.M. Smith and *Cannabis sativa* L., it boosted biomass, pigment content, and vegetative growth (Pinheiro et al. 2021; Shi et al. 2024). In *Hevea brasiliensis* (Willd. ex A.Juss.) Müll. Arg., the response varied with the genotype (Tisarum et al. 2018). However, to our knowledge, no studies have examined the combined effect of eCO₂ and salinity on *A. comosus* under ex- or *in vitro* conditions.

Based on the findings above, we hypothesize that eCO₂ can mitigate the harmful effects of salinity in *A. comosus* grown *in vitro* by enhancing physiological performance and anatomical adaptations associated with stress tolerance. To test this, we evaluated the combined effects of eCO₂ and salinity on growth, photosynthesis, and leaf anatomy in micropropagated plants, aiming to elucidate the morphophysiological mechanisms underlying this adaptive response.

2. Materials and methods

‘Pérola’ pineapple shoots, previously established *in vitro*, were obtained from the plant bank of the Tissue Culture Laboratory (LCT) at the State University of Maranhão (UEMA), São Luís, MA, Brazil. The shoots were successively subcultured in 30 mL of MS medium (Murashige and Skoog 1962) (PhytoTechnology, Lenexa, KS, USA), supplemented with 30 g L⁻¹ sucrose (Dinâmica, Jardim da Glória, SP, Brazil), 100 mg L⁻¹ myo-inositol (Sigma-Aldrich, St. Louis, MO, USA), 4 µM 6-benzyladenine (BAP) (Sigma Aldrich, St. Louis, MO, USA), 2 µM naphthaleneacetic acid (NAA) (Sigma-Aldrich, St. Louis, MO, USA), and 2 g L⁻¹ phytigel (Sigma-Aldrich, St. Louis, MO, USA). The shoots were maintained in a growth room at 25 ± 2 °C, 60% relative humidity, with a 16-h photoperiod, and light irradiance of 100 µmol m⁻² s⁻¹ provided by four T8 white LED tubes (9 W, Avant, São Paulo - SP, Brazil). Pineapple shoots measuring 2.5 cm in length were inoculated into 350 mL glass culture vessels containing 60 mL of MS medium, supplemented with 100 mg L⁻¹ myo-inositol, 30 g L⁻¹ sucrose, 4 µM NAA, and two NaCl (sodium chloride) concentrations (0 and 120 mM). The concentration of 120 mM NaCl was chosen based on the *in vitro* responses of *Ananas comosus* reported by Melo et al.

(2011), where 100 mM and 150 mM reduced shoot height and leaf number compared to the control. This intermediate level ensures measurable effects without excessive damage. The medium was solidified with 2.0 g L⁻¹ phytagel, adjusted to pH 5.7 ± 0.1, and autoclaved at 121 °C under 1.5 atm for 15 min. Two plants were inoculated per glass tube. The glass tubes were maintained for 50 days in a plant growth chamber (Tecnal, model TE-4002/3, Uninorte Piracicaba, SP, Brazil) under controlled conditions of temperature (25 ± 2 °C), relative humidity (60%), and 16-h photoperiod with irradiance of 100 µmol m⁻² s⁻¹ provided by three lamps. The atmospheric CO₂ concentration within the chamber was maintained at 420 ± 30 µmol mol⁻¹ for ambient CO₂ (aCO₂) and 800 ± 30 µmol mol⁻¹ for eCO₂ using an external CO₂ injection system connected to a gas cylinder. CO₂ levels were monitored and adjusted via the chamber's integrated control system to ensure consistency throughout the experimental period. The experiment followed a completely randomized 2 × 2 factorial design with two CO₂ concentrations (aCO₂ and eCO₂) and two NaCl concentrations (0 and 120 mM), totaling four treatments with ten replicates each. Each replicate consisted of two plants per glass tube.

2.1 Analyzes

2.1.1 Chlorophyll a fluorescence and leaf gas exchange

Chlorophyll a fluorescence was measured on the fifth fully expanded leaf using a portable non-modulated fluorimeter (Pocket PEA, Hansatech Instruments Ltd., King's Lynn, Norfolk, UK). Prior to measurement, leaves were dark adapted in situ for 30 min using a leaf-clip system (Hansatech Instruments Ltd.) to ensure complete QA oxidation and full PSII reaction center opening while minimizing heat accumulation. The instrument requires no conventional calibration, as its internal optical and electronic circuitry maintains stable light output and accurate signal detection, provided that manufacturer guidelines are strictly followed. The maximum quantum yield of PSII (Fv/Fm) was calculated as the ratio of variable fluorescence, obtained by subtracting the minimal fluorescence yield (Fo) from the maximal fluorescence yield (Fm) to the maximal fluorescence yield. The performance index (PI), derived from the OJIP fluorescence transient, reflects PSII functional efficiency by integrating light absorption (RC/ABS), maximum photochemical efficiency ($\phi P_0 = F_v/F_m$), and the efficiency of initial electron transport from QA⁻ to plastoquinone (ψ_0).

Leaf gas exchange analyses were conducted two hours after light onset in the growth chambers. Measurements were performed under artificial saturated light at 1,000 µmol m⁻² s⁻¹ provided by a red/blue LED system with 10% blue light. External CO₂ concentrations were

maintained at either ambient (aCO₂, 420 µmol mol⁻¹) or elevated (eCO₂, 800 µmol mol⁻¹), according to each treatment, with an average air temperature of 30 °C. Gas exchange parameters were recorded using an open gas exchange system (LI-6400XT, Li-Cor, Lincoln, NE, USA), including net photosynthetic CO₂ assimilation (A), stomatal conductance to water vapor (gs), transpiration rate (E), intercellular CO₂ concentration (Ci), the ratio of intercellular to ambient CO₂ concentration (Ci/Ca), carboxylation efficiency (A/Ci), and intrinsic water-use efficiency (A/gs).

2.1.2 Extraction and quantification of photosynthetic pigments

Pigments were extracted from leaf discs (5 mm in diameter) collected from the fourth fully expanded leaves below the apex. The leaf discs were immersed in 5 mL of dimethyl sulfoxide (DMSO; Êxodo científica®, Sumaré, SP, Brazil) and left in the dark for 48 h. Absorbance measurements were taken at 665 nm, 649 nm, and 480 nm using a UV-visible spectrophotometer (mono-beam) (model UV-M51; BEL Engineering Company, Monza, Italy) in a 10 mm quartz cuvette. The calculation of chlorophyll a, b, and carotenoids followed the method outlined by Wellburn (1994):

$$\text{Chlorophyll } a \text{ (}\mu\text{g mL}^{-1}\text{)} = 12,19A_{665} - 3,45A_{649}$$

$$\text{Chlorophyll } b \text{ (}\mu\text{g mL}^{-1}\text{)} = 21,99A_{649} - 5,32A_{665}$$

$$\text{Total carotenoids (}\mu\text{g mL}^{-1}\text{)} = \frac{(1000A_{480} - 2,14\text{Chlorophyll } a - 70,16\text{Chlorophyll } b)}{220}$$

2.2 Growth analysis

To assess the growth and development of *in vitro* plants after 50 days, the following parameters were evaluated: plantlet length (cm), root length (cm), number of leaves, leaf area (cm²), plantlet dry mass (g), specific leaf mass (g cm⁻²), and root dry mass (g). Leaf area (cm²) was determined using the software Image J®. For dry mass measurements, samples were dried at 60 °C in an oven with forced air circulation (AmbiKontrol, Arujá, SP, Brazil) until reaching a constant weight.

2.3 Relative leaf water content

Five leaf discs (3 mm in diameter) were collected from the fourth fully expanded leaves. The discs were immediately weighed to access the fresh mass (FM), then placed in plastic cups with 8 mL of distilled water for 24 h to obtain the turgid mass (TM). Afterward, the discs were

oven-dried at 45 °C for 48 h to obtain the dry mass (DM) (Barr and Weatherley 1962). The relative water content was calculated using the following formula:

$$\text{RLWC (\%)} = [(\text{FM} - \text{DM}) / (\text{TM} - \text{DM})] \times 100$$

2.4 Stomatal index

To evaluate the stomatal index, impressions of the abaxial epidermis were made using instant adhesive (SuperBonder®) of the fifth leaf from the apex. The impressions were mounted on slides and analyzed under a light microscope (model B20T; Bioptika, Colombo, PR, Brazil) equipped with a U-photo system and a digital camera (model CMOS 5.0; Bioptika, Colombo, PR, Brazil). Images were captured with Capture V2.1 software and processed using the Image J® software. The stomatal index was calculated according to Cutter (1986), using the following formula:

$$\text{Stomatal index (\%)} = \frac{(C_2 \times 100)}{(C_1 + C_2)}$$

where C_1 is the number of epidermal cells and C_2 is the number of stomata.

2.5 Leaf anatomy and micromorphometric analysis

Samples from the fifth fully expanded leaves, taken from the median region, were fixed in a 50% FAA solution (formaldehyde: acetic acid: 50% ethanol at a 1:1:18 ratio) for 48 h, following Johansen (1940). After fixation, samples were dehydrated in an ascending ethanol series (30%, 40%, 50%, 60%, and 70%) for 1 h each, followed by further dehydration in higher concentrations (80%, 90%, 95%, and 100%) for 2 h each at 4 °C. Subsequently, samples were embedded in Historesin® (Leica Instruments, Heidelberg, Germany). Cross-sections of 7 µm thickness were obtained using a semi-automated rotary microtome (Lupetec® model MRP2015, São Carlos, Brazil) and stained with 0.05% toluidine blue (pH 4.0), following O'Brien and McCully (1981).

2.6 Statistical analyzes

Data were first tested for normality and homogeneity using the Shapiro-Wilk and Bartlett tests, respectively. Only the following variables were transformed using the equation $\sqrt{(x + 0.5)}$ to meet ANOVA assumptions: root length, root dry mass, PSII maximum quantum yield (F_v/F_m), stomatal conductance to water vapor (gs), and transpiration rate (E). All variables, including non-transformed data, were subjected to analysis of variance (ANOVA),

and means were compared using Tukey's test at a 5% probability level ($P \leq 0.05$), using the Sisvar statistical software (Ferreira 2019).

3. Results

3.1 eCO₂ mitigates salinity-induced growth Inhibition in *A. comosus* plantlets cultivated *in vitro*

After 50 days of cultivation under two CO₂ concentrations (aCO₂ = 420 $\mu\text{mol mol}^{-1}$ and eCO₂ = 800 $\mu\text{mol mol}^{-1}$) and two salinity levels (0 and 120 mM NaCl), clear morphological differences were observed (Fig. 1). Under saline conditions (120 mM NaCl), plantlets grown under aCO₂ showed stunted growth and poor root development. In contrast, eCO₂ partially mitigated the effects of salt stress, resulting in greener leaves and improved overall growth. Under non-saline conditions, eCO₂ further enhanced vegetative growth and root development compared to aCO₂.

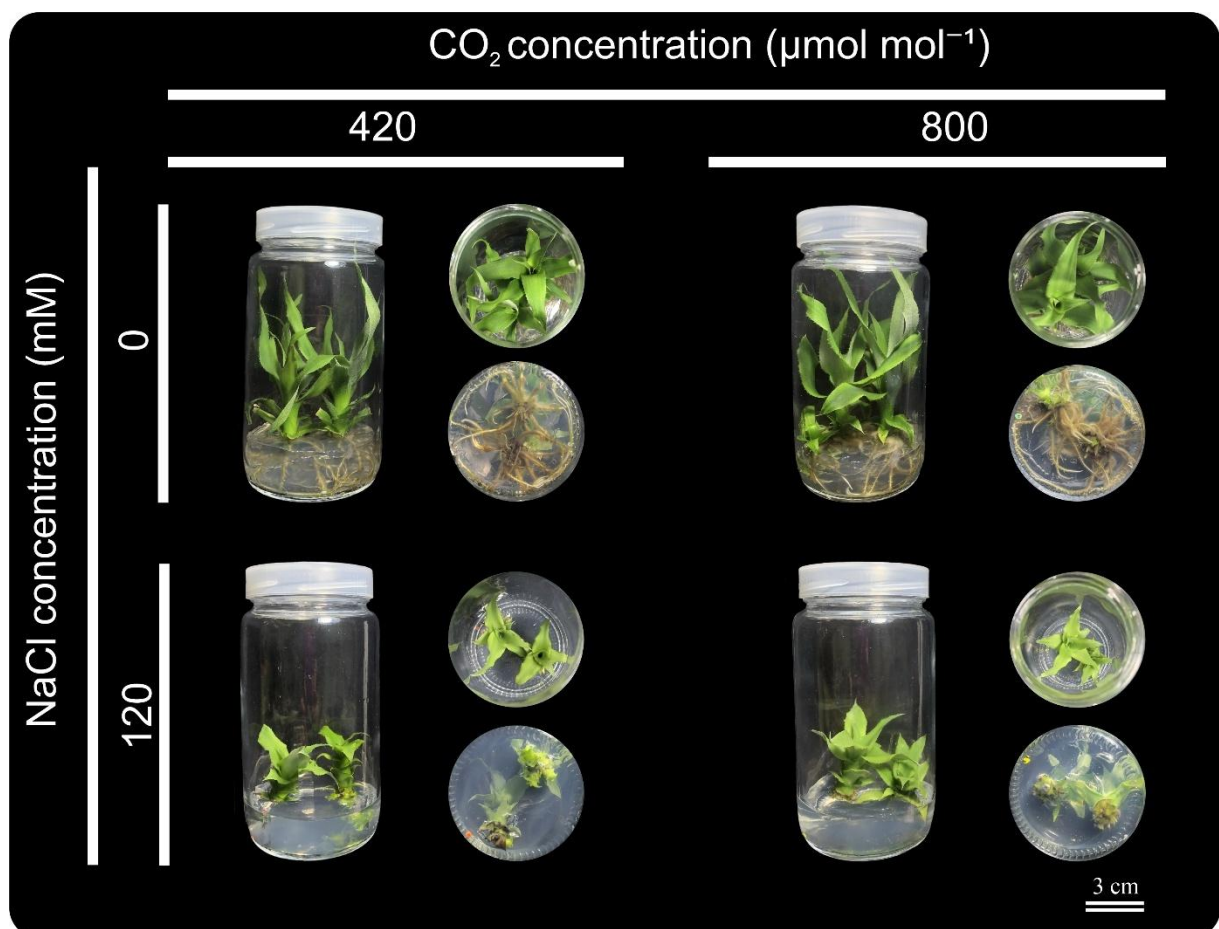


Fig. 1 Morphological response of *Ananas comosus* plantlets to combined salinity (0 and 120 mM NaCl) and CO₂ concentrations (aCO₂ = 420 and eCO₂ = 800 $\mu\text{mol mol}^{-1}$) after 50 days of *in vitro* cultivation. Scale bar = 3 cm

3.2 eCO₂ mitigates salinity-induced reductions in CO₂ assimilation, stomatal conductance, and water-use efficiency

Chlorophyll fluorescence parameters were only slightly affected by the treatments. The maximum quantum yield of PSII (Fv/Fm) remained stable across both CO₂ concentrations, but declined under salinity, from 0.79 to 0.74 (Fig. 2A). A similar trend was observed for the performance index (PI), which was unaffected by CO₂ enrichment but decreased by ~ 66% under salinity (Fig. 2B).

In contrast, gas exchange parameters responded strongly to eCO₂, particularly under saline conditions. Net CO₂ assimilation (A) increased by approximately 90% under eCO₂ without salinity, and was approximately 4.5-fold higher under eCO₂ with salinity compared to aCO₂. These results indicate a clear mitigation of salinity-induced photo synthetic suppression (Fig. 2C).

Stomatal conductance (gs) increased by approximately 122% under eCO₂ without salinity and by ~ 56% under eCO₂ with salinity (Fig. 2D), suggesting that CO₂ enrichment partially restored stomatal function under ionic stress. Similarly, intercellular CO₂ concentration (Ci) rose by ~ 232% under eCO₂ in plants without salinity and by ~ 76% in saline plants (Fig. 2E), reinforcing its positive effect on CO₂ availability in the mesophyll.

Transpiration rate (E) also increased under eCO₂ by ~ 93% without salinity and by ~ 42% under salinity (Fig. 2F). The Ci/Ca ratio was unaffected by salinity, but increased under eCO₂, suggesting improved internal diffusion of CO₂ (Fig. 2G).

Carboxylation efficiency (A/Ci) decreased under eCO₂ in non-saline conditions and remained unchanged under salinity (Fig. 2H), suggesting a possible biochemical limitation downstream of CO₂ assimilation. Importantly, intrinsic water-use efficiency (A/gs) increased significantly (~ 186%) under salinity with eCO₂, further supporting the hypothesis that eCO₂ improves both photosynthetic performance and water-use efficiency under salt stress (Fig. 2I).

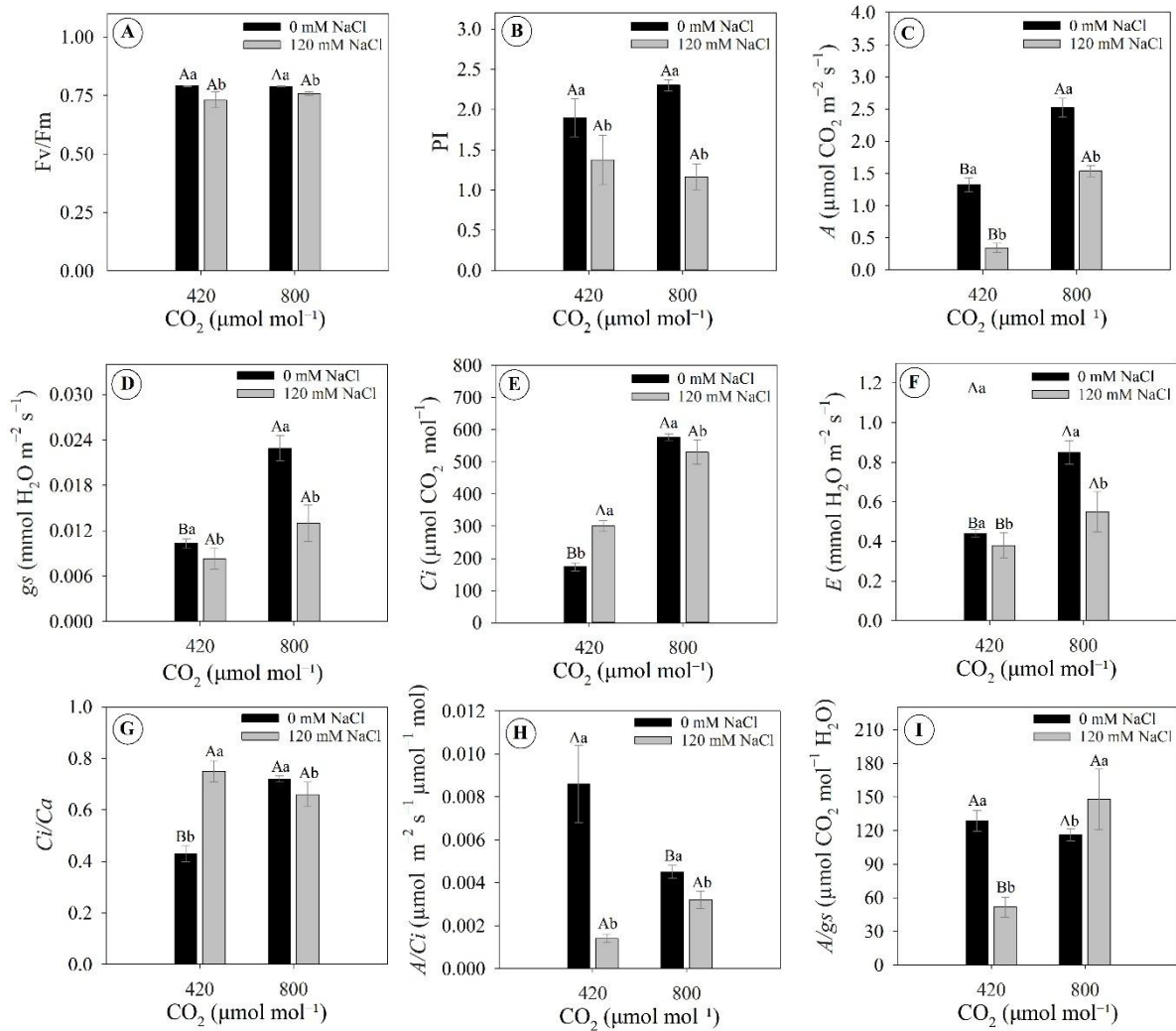


Fig. 2 Chlorophyll a fluorescence and gas exchange parameters of *Ananas comosus* plantlets after 50 days of *in vitro* cultivation under two salinity levels (0 and 120 mM NaCl) and two CO₂ concentrations (aCO₂ = 420 and eCO₂ = 800 μmol mol⁻¹). (A) Maximum quantum yield of photosystem II (Fv/Fm); (B) Performance index (PI); (C) Net CO₂ assimilation rate (A; μmol CO₂ m⁻² s⁻¹); (D) Stomatal conductance (gs; mmol H₂O m⁻² s⁻¹); (E) Intercellular CO₂ concentration (Ci; μmol CO₂ mol⁻¹); (F) Transpiration rate (E; mmol H₂O m⁻² s⁻¹); (G) Ci/Ca ratio; (H) Carboxylation efficiency (A/Ci; μmol m⁻² s⁻¹ μmol⁻¹ mol); and (I) Intrinsic water-use efficiency (A/gs; μmol CO₂ mol⁻¹ H₂O). Uppercase letters indicate significant differences between CO₂ concentrations within the same NaCl level; lowercase letters indicate differences between salinity levels within the same CO₂ concentration (Tukey's test, $P \leq 0.05$). Values are means $\pm$ standard error (n = 8).

3.3 eCO₂ does not mitigate salinity-induced reductions in chlorophyll and carotenoids

Salinity (120 mM NaCl) strongly reduced pigment levels across all treatments. Compared to saline conditions, plants grown without NaCl had ~ 60% higher chlorophyll a and ~ 77% higher chlorophyll b, regardless of CO₂ concentration (Fig. 3A and B). The chlorophyll a/b ratio increased by ~ 11% under salinity, suggesting a shift in pigment composition rather than pigment recovery (Fig. 3C). Total chlorophyll content, carotenoid levels, and the total chlorophyll/ carotenoid ratio were also markedly higher in non-saline plants -by approximately 64%, 43%, and 14%, respectively - under both aCO₂ and eCO₂ (Fig. 3D and E, and 3F). These results indicate that salinity strongly suppresses pigment accumulation and that eCO₂ alone does not counteract this effect.

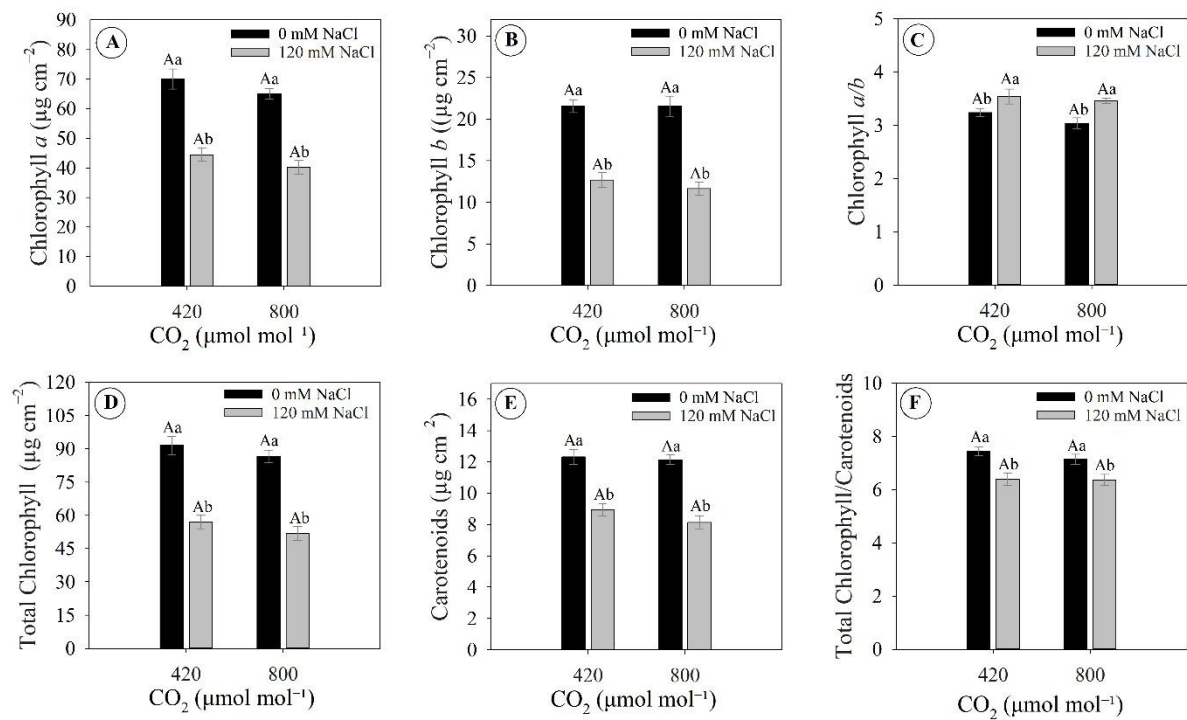


Fig. 3 Photosynthetic pigments of *Ananas comosus* plantlets after 50 days of *in vitro* cultivation under two salinity levels (0 and 120 mM NaCl) and two CO₂ concentrations (aCO₂ = 420 and eCO₂ = 800 $\mu\text{mol mol}^{-1}$). (A) Chlorophyll a ($\mu\text{g cm}^{-2}$); (B) Chlorophyll b ($\mu\text{g cm}^{-2}$); (C) Chlorophyll a/b ratio; (D) Total chlorophyll ($\mu\text{g cm}^{-2}$); (E) Carotenoids ($\mu\text{g cm}^{-2}$); and (F) Total chlorophyll/carotenoid ratio. Uppercase letters indicate significant differences between CO₂ concentrations within the same salinity level; lowercase letters indicate differences between salinity levels within the same CO₂ concentration (Tukey's test, $P \leq 0.05$). Values are means $\pm$ standard error ($n = 6$).

3.4 eCO₂ enhances biomass under salinity but does not fully reverse stress-induced growth inhibition

The eCO₂ did not affect plantlet length at either salinity level (Fig. 4A). However, plantlets grown without salinity (0 mM NaCl) were approximately 74% longer than those under saline conditions (120 mM NaCl), regardless of CO₂ concentration, indicating a strong inhibitory effect of salinity on overall shoot development. A similar trend was observed in root development: neither root length nor root dry mass responded to CO₂ enrichment under either salinity condition (Fig. 4B and G). In the absence of salinity, root length averaged around 3 cm and root dry mass ranged from 0.111 to 0.119 g across CO₂ treatments.

In contrast, eCO₂ increased the number of leaves under both non-saline and saline conditions (Fig. 4C). Under salinity, eCO₂ resulted in a 21% increase in leaf number compared to aCO₂, while in non-saline conditions, the increase was 17%, suggesting a modest positive effect of eCO₂ on shoot development.

Leaf area was unaffected by eCO₂, regardless of salinity level (Fig. 4D). Still, plants grown without salinity displayed ~ 65% greater leaf area than those under salt stress at both CO₂ concentrations, underscoring the detrimental effect of salinity on leaf expansion. Total plantlet dry mass increased under eCO₂, but only in plants subjected to salinity, with a 47% increase compared to aCO₂ (Fig. 4E). No significant change was observed in non-saline plants, suggesting that eCO₂ promoted partial recovery of biomass accumulation under salt stress.

Specific leaf mass was not influenced by eCO₂ but was ~ 78% higher in plants under 120 mM NaCl than in those without salinity, regardless of CO₂ concentration (Fig. 4F). This increase may reflect a compensatory thickening of leaves in response to stress.

Relative leaf water content increased under eCO₂ in both saline and non-saline plants (Fig. 4H). Under salinity, eCO₂ led to an 8% increase compared to aCO₂, with a similar pattern observed under non-saline conditions, suggesting improved water retention capacity associated with CO₂ enrichment.

Stomatal index was not altered by eCO₂ in non-saline plants. However, under salinity, eCO₂ caused a ~ 24% reduction in the stomatal index compared to aCO₂ (Fig. 4I), suggesting a possible anatomical adjustment to reduce transpirational water loss under stress.

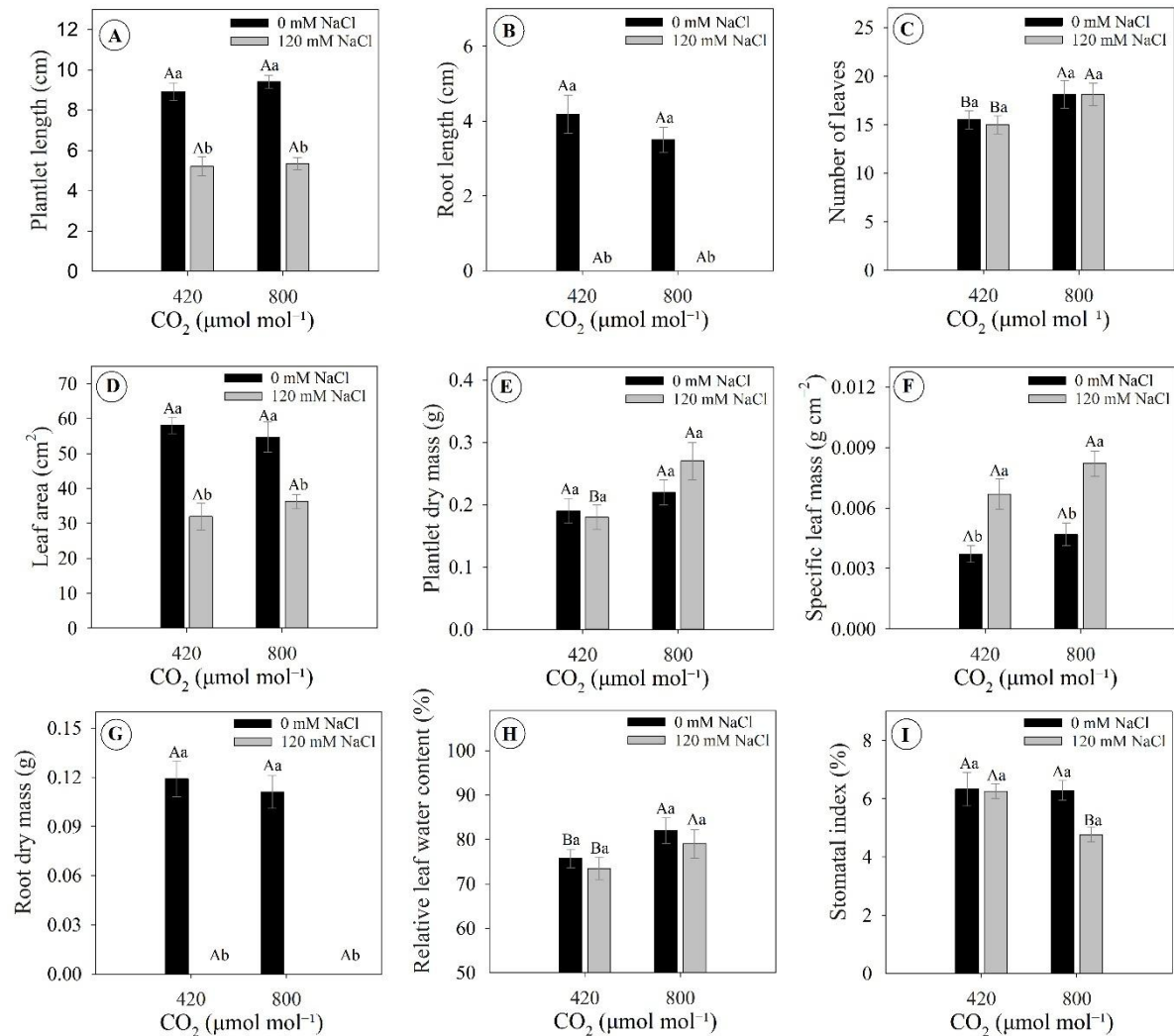


Fig. 4 Growth parameters of *Ananas comosus* plantlets after 50 days of *in vitro* cultivation under two salinity levels (0 and 120 mM NaCl) and two CO₂ concentrations (aCO₂ = 420 and eCO₂ = 800 μmol mol⁻¹). (A) Plantlet length (cm); (B) Root length (cm); (C) Number of leaves; (D) Leaf area (cm²); (E) Plantlet dry mass (g); (F) Specific leaf mass (g cm⁻²); (G) Root dry mass (g); (H) Relative leaf water content (%); and (I) Stomatal index (%). Uppercase letters indicate significant differences between CO₂ concentrations within the same salinity level; lowercase letters indicate differences between salinity levels within the same CO₂ concentration (Tukey's test, $P \leq 0.05$). Values are means $\pm$ standard error ($n = 8$).

3.5 eCO₂ does not alter micromorphometric structure, but salinity induces anatomical thickening

Micromorphometric traits were not affected by eCO₂ under either salinity condition (Fig. 5). However, salinity (120 mM NaCl) significantly increased tissue thickness compared to non-saline conditions (0 mM NaCl) across all CO₂ concentrations. Adaxial and abaxial epidermal thickness increased by ~ 60% and ~ 59%, respectively (Fig. 5A and B). Similarly, aquifer and chlorophyll parenchyma thickness were ~ 79% and ~ 34% higher, respectively, under salinity (Fig. 5C and D). Transverse and longitudinal vascular bundle thickness also increased under saline conditions by ~ 18% and ~ 10%, respectively (Fig. 5E and F). These results indicate that salinity, rather than CO₂ enrichment, triggers anatomical adjustments potentially associated with stress tolerance mechanisms.

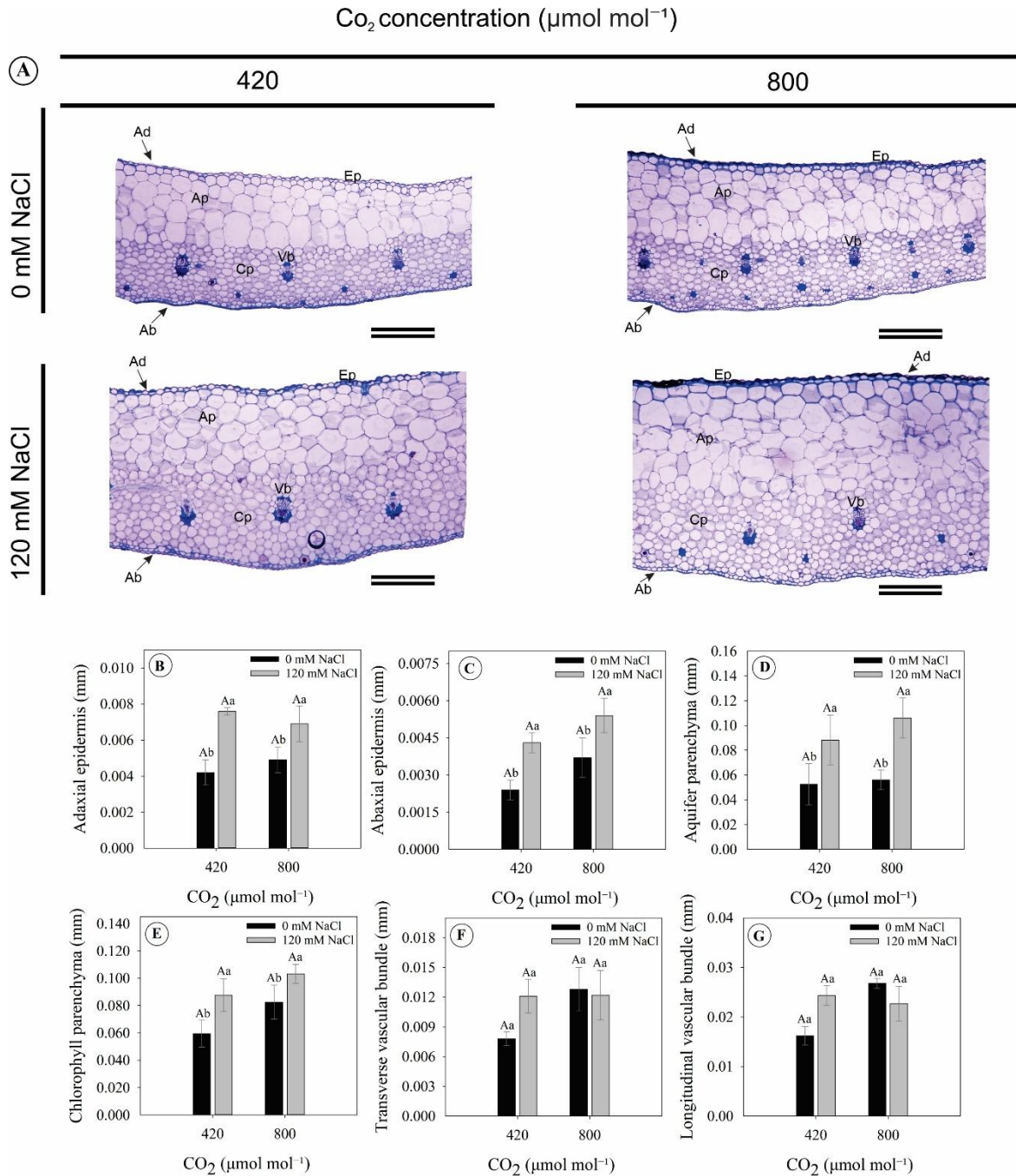


Fig. 5 Leaf micromorphometric parameters of *Ananas comosus* plant lets after 50 days of *in vitro* cultivation under two salinity levels (0 and 120 mM NaCl) and two CO_2 concentrations ($a\text{CO}_2 = 420$ and $e\text{CO}_2 = 800 \mu\text{mol mol}^{-1}$). (A) Leaf cross-section; (B) Adaxial epidermis thickness (mm); (C) Abaxial epidermis thickness (mm); (D) Aquifer parenchyma thickness (mm); (E) Chlorophyll parenchyma thickness (mm); (F) Transverse vascular bundle thickness (mm); and (G) Longitudinal vascular bundle thickness (mm). Uppercase letters indicate significant differences between CO_2 concentrations within the same salinity level; lowercase letters indicate differences between salinity levels within the same CO_2 concentration (Tukey's test, $P \leq 0.05$). Ad = adaxial epidermis, Ab = abaxial epidermis, Ap = Aquifer parenchyma, Cp =

Chlorophyll parenchyma, Ep = epiderms, Vb = vascular bundle. Values are means $\pm$ standard error (n = 3). Scale bar = 200 μ m.

4. Discussion

Understanding how salinity (0 and 120 mM NaCl) and atmospheric CO₂ concentrations (420 and 800 μ mol mol⁻¹) affect growth, survival, and carbon fixation capacity in plants propagated *in vitro* is essential for anticipating the impacts of climate change. This study is among the first to evaluate the combined effects of salinity and eCO₂ on *A. comosus* cultivated *in vitro*, focusing on growth, physiological, and anatomical responses.

Our results partially confirmed the initial hypothesis. eCO₂ significantly improved photosynthetic performance, intrinsic water-use efficiency, dry biomass accumulation, and relative water content under salt stress. However, eCO₂ did not fully reverse salinity-induced reductions in shoot and root elongation or pigment degradation, nor did it induce significant alterations in anatomical traits. These findings suggest that the observed mitigation was primarily due to physiological adjustments rather than anatomical modifications.

Specifically, eCO₂ increased dry biomass accumulation in plants exposed to salinity. This result is consistent with previous studies showing that eCO₂ enhances plant growth and development under adverse conditions (Cruz et al. 2018; Kiba et al. 2019). In *Arabidopsis thaliana*, eCO₂ significantly increased dry mass and leaf number under *in vitro* conditions (Kiba et al. 2019). CAM plants, such as *A. comosus*, are known to exhibit increased biomass production under eCO₂, particularly under water deficit, high temperatures, and intense light. These traits may enhance their resilience to future climate scenarios (Reddy et al. 2010). Plants subjected to salt stress often experience growth inhibition due to osmotic limitations that restrict water availability, leading to reduced stomatal opening and impaired CO₂ assimilation (García-Sánchez and Syvertsen 2006). However, the ability to cope with multiple stressors, such as salinity and eCO₂, may enhance abiotic stress tolerance through the activation of genes related to ionic homeostasis, osmotic regulation, and the biosynthesis of compatible solutes (Sinaga and Marpaung 2024). Our findings align with these observations, as plants cultivated under eCO₂ maintained higher photosynthetic rates and improved water-use efficiency under salt stress. Similar trends have been reported in tomato plants, where eCO₂ ameliorated the negative impacts of salinity and drought by improving biomass accumulation and alleviating water stress (Liang et al. 2024). Consistent with the positive correlation between biomass production and photosynthetic performance, eCO₂ increased net photosynthesis by approximately 4.5-fold

under salinity compared to aCO₂. This result aligns with previous studies demonstrating that eCO₂ enhances photosynthetic efficiency under stress conditions (Louback et al. 2021; Liang et al. 2024). Nevertheless, under severe stress, photosynthesis is typically reduced due to both stomatal and non-stomatal limitations, including impaired Rubisco activity, RuBP regeneration, triose phosphate utilization, electron transport rates, and photosystem II efficiency (Chaves, Flexas, and Pinheiro, 2008; Bellasio et al. 2018).

In vitro studies, such as those conducted with *Phalaenopsis* under varying CO₂ and sucrose conditions, have demonstrated that eCO₂ enhances gas exchange, increases total Rubisco activity, and supports higher transpiration rates (Yoon et al. 2009). Similarly, eCO₂ may improve RuBP carboxylation and regeneration, leading to enhanced photosynthesis even under stress (Sekhar et al. 2017). By promoting higher carbon assimilation, eCO₂ may counteract the osmotic and ionic effects of salinity, as previously suggested (Hussain et al. 2020).

Additionally, *Ananas comosus* exhibited high intrinsic water-use efficiency under combined salinity and eCO₂ conditions. This response is closely associated with optimized stomatal regulation, enabling plants to minimize water loss while maintaining sufficient CO₂ uptake to sustain biomass production and photosynthesis (Li et al. 2024). The eCO₂ environments generally enhance intrinsic water-use efficiency, representing a critical adaptive trait that mitigates stress impacts and promotes growth (Wang and Wen 2022). Furthermore, eCO₂ partially alleviates the photosynthetic decline by reducing stomatal conductance and transpiration rates induced by salinity stress. Studies on *Salicornia ramosissima* under salinity and eCO₂ have demonstrated comparable improvements in water-use efficiency and reductions in oxidative stress (Pérez-Romero et al. 2019). Another investigation reported that eCO₂ enhances water use efficiency and hydraulic conductivity in plants subjected to prolonged salinity (Christy et al. 2018).

Despite improvements in physiological performance, eCO₂ did not reverse the salinity-induced reductions in chlorophyll and carotenoid contents. This suggests that eCO₂ does not eliminate oxidative stress but merely attenuates its impact on the photosynthetic process. Furthermore, under eCO₂ conditions, plants still rely on a variety of enzymatic systems to sustain their metabolic functions; otherwise, pigment levels will not be restored, particularly when exposed to saline stress (Pérez-López, et al. 2009). A similar trend has been reported in *Atriplex* species subjected to salinity, in which photosynthesis improved under eCO₂, yet pigment concentrations remained suppressed (Calone et al. 2021). In the current study, eCO₂

possibly supplied the diffusive limitation imposed by salinity, allowing greater carbon fixation even with a lower content of photosynthetic pigments.

Micromorphometric analysis revealed that anatomical adjustments, such as epidermal and aquifer parenchyma thickening, were induced exclusively by salinity and not modulated by eCO₂. These changes are adaptive responses to reduced water potential, improving internal water storage and sustaining turgor under osmotic stress (Zhang and Ambrose 2024; Tang et al. 2024). This structural plasticity likely contributed to the high intrinsic water-use efficiency observed under combined salinity and eCO₂, where optimized stomatal regulation minimized water loss while maintaining sufficient CO₂ uptake for photosynthesis (Li et al. 2024; Wang and Wen 2022). Similar correlations between anatomical reinforcement, WUE, and stress mitigation have been reported for halophytes such as *Salicornia ramosissima* (Pérez-Romero et al. 2019) and in prolonged salinity studies (Christy et al. 2018).

In summary, the partial mitigation of salt stress in *A. comosus* under eCO₂ was predominantly associated with functional physiological adjustments, including enhanced photosynthesis, improved water-use efficiency, and increased biomass allocation. These responses occurred without significant anatomical remodeling or recovery of pigment levels, highlighting that the primary action of eCO₂ under combined stress conditions is to sustain gas exchange and water balance rather than modulate secondary metabolism or structural traits.

5. Conclusion

The eCO₂ partially mitigated the adverse effects of salinity on *A. comosus* plantlets propagated *in vitro* by significantly increasing plant biomass, net photosynthesis, and intrinsic water-use efficiency. These physiological improvements occurred without complete recovery of photosynthetic pigments or anatomical modifications, suggesting that the primary effect of eCO₂ was to sustain gas exchange and water balance under stress. This study highlights the potential of eCO₂ to alleviate salt-induced limitations during micropropagation and provides a foundation for future research on its broader applications in stress management strategies for *A. comosus*.

Supplementary Information

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Data availability The authors confirm that the data supporting the findings of this study are available in the article.

Declarations

Competing interests The authors declare no conflict of interest.

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CAPÍTULO III

Silva-Moraes, V.K.O., Felipe, S.H.S., Alves, G.L. et al. *In vitro* salinity priming modulates photosynthetic acclimation in *Ananas comosus* with limited growth recovery. *Vegetos* (2026). <https://doi.org/10.1007/s42535-026-01791-8>

***In vitro* salinity priming modulates photosynthetic acclimation in *Ananas comosus* with limited growth recovery**

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Abstract

Salinity is a major abiotic constraint limiting pineapple (*Ananas comosus*) growth and productivity, and strategies that improve stress acclimation during early developmental stages may enhance plant performance under adverse conditions. This study evaluated whether *in vitro* salinity priming could modulate subsequent physiological and growth responses of pineapple seedlings exposed to severe *ex vitro* salt stress. Seedlings were subjected to salinity conditioning during the *in vitro* phase and subsequently acclimatized under greenhouse conditions, with or without exposure to 500 mM NaCl. Growth, gas exchange, chlorophyll fluorescence, and photosynthetic pigment content were assessed. Severe salinity reduced net CO₂ assimilation, stomatal conductance, transpiration, and biomass accumulation in both primed and non-primed plants. However, primed seedlings showed partial physiological acclimation, including a less pronounced decline in apparent carboxylation efficiency and maintenance of pigment concentrations under salinity. Contrasting *Ci/Ca* responses suggested differences in the balance between stomatal and non-stomatal limitations depending on priming history. Despite these physiological adjustments, priming did not improve biomass accumulation and was associated with reduced plant size even under non-saline conditions. These findings indicate that *in vitro* salinity priming can alter subsequent physiological responses in pineapple, but under the severe salinity imposed here, these adjustments were insufficient to promote growth recovery, suggesting a decoupling between selected physiological traits and biomass performance.

Keywords Chlorophyll fluorescence · Gas exchange · *In vitro* culture · Salinity priming

1. Introduction

Soil salinity is among the most important abiotic factors limiting agricultural productivity and currently affects substantial portions of cultivated land worldwide (Murtaza et al. 2025). In *Ananas comosus* (pineapple), salt exposure triggers both osmotic and ionic constraints that impair vegetative development and reduce productivity (Da Silva et al. 2025). These detrimental effects are largely associated with disturbances in cellular ion balance and water relations, which compromise fundamental metabolic processes necessary for plant growth. At the physiological level, salinity disrupts water balance and photosynthetic activity, decreases photochemical efficiency and relative water content, destabilizes membrane integrity, promotes electrolyte leakage and potassium loss, and accelerates the breakdown of total photosynthetic pigments (Zhang et al. 2025; Saleem et al. 2025).

Although substantial progress has recently been made in the development of *in vitro* propagation and regeneration protocols for *A. comosus*, expanding its biotechnological applications (Ranjan et al. 2026), improving salt tolerance in this species remains an important challenge. In this context, *in vitro* culture provides a valuable experimental platform for the precise application of controlled salinity treatments, enabling the investigation of adaptive physiological responses and the induction of stress priming mechanisms that may contribute to improved stress resilience. Within this framework, salinity priming has gained attention as a strategy in which exposure to moderate salt stress induces physiological and molecular adjustments capable of enhancing plant performance during later stress events, a phenomenon linked to stress memory (Pissolato et al. 2024).

The controlled nature of *in vitro* systems allows precise manipulation of stress intensity and exposure duration, facilitating the induction of a primed physiological state that may persist after acclimatization (He et al. 2022). In *A. comosus*, previous studies suggest that physiological conditioning during *in vitro* culture can influence plant performance after transfer to *ex vitro* conditions, highlighting the broader potential of *in vitro* approaches for improving stress resilience (Melo et al. 2011). However, it remains unclear whether *in vitro* salinity priming specifically can confer persistent physiological benefits that translate into integrated improvements in growth and photosynthetic performance during subsequent salinity exposure.

Addressing this issue is critical because enhanced physiological acclimation does not necessarily translate into superior plant growth. Stress tolerance mechanisms require energetic

and metabolic investment, which may redirect resources from biomass accumulation to protective processes under adverse conditions (Zhao et al. 2020; He et al. 2022). Therefore, evaluating whether priming improves stress performance without compromising growth is essential for determining its functional and agronomic relevance.

Accordingly, this study evaluated the effects of *in vitro* salinity priming on growth, photosynthetic performance, and pigment stability in *A. comosus* plants exposed to salt stress after acclimatization. We hypothesize that *in vitro* salinity conditioning promotes improved physiological acclimation during subsequent salt exposure, contributing to greater photosynthetic stability, but that these protective adjustments may not necessarily translate into enhanced biomass accumulation due to the energetic and metabolic costs associated with maintaining stress defense mechanisms.

2. Materials and methods

2.1 Plant material and *in vitro* salinity priming

The experiment was with *Ananas comosus* cv. Pérola shoot previously established *in vitro*, which were successively subcultured 60 mL of Murashige and Skoog (PhytoTechnology®) medium containing 30 g L⁻¹ sucrose (Dinâmica®), 100 mg L⁻¹ myo-inositol (Sigma-Aldrich®), 4 µM 6-benzyladenine, and 2 µM naphthaleneacetic acid. The culture medium was solidified using 2 g L⁻¹ phytagel, its pH adjusted to 5.7 ± 0.1 prior to sterilization, followed by autoclaving at 121 °C under 1.5 atm for 15 min.

Shoots approximately 2 cm long were subjected to two NaCl treatments: 0 mM (control) and 80 mM (saline preconditioning). Every 30 days, the plants were subcultured in a new culture medium supplemented with the respective NaCl concentrations (0 or 80 mM), totaling 90 days of culture (Carretero et al. 2007). Cultures were kept under controlled environmental conditions (25 ± 2 °C, 60% RH, 16-h photoperiod, and irradiance of 100 µmol m⁻² s⁻¹ supplied by white LED lamps).

Each treatment consisted of five replicates, with two explants per flask, and each flask considered as one experimental unit. At the end of the experimental period, growth and chlorophyll fluorescence were evaluated. Data were assessed by comparing control and NaCl-treated plants using a two-tailed t-test ($P \leq 0.05$).

2.2 *Ex vitro* salinity exposure

Following the *in vitro* phase, plantlets were transferred to 500 mL pots containing 450 g of washed sand and acclimatized under greenhouse conditions prior to the onset of salinity treatment. The experiment was arranged in a completely randomized design following a 2 × 2

factorial scheme, combining two *in vitro* conditions (0 and 80 mM NaCl) with two *ex vitro* salinity levels (0 and 500 mM NaCl). This design resulted in four treatment groups: unprimed control (0/0 mM), unprimed salinized (0/500 mM), primed control (80/0 mM), and primed salinized (80/500 mM). Each treatment consisted of 11 replicates, with one plant per pot considered as the experimental unit.

The concentration of 500 mM NaCl was selected to impose a severe salinity stress under controlled conditions. Previous studies indicate that 400 mM NaCl is sufficient to induce severe stress in pineapple without causing immediate lethality (Zhou et al. 2024).

Plants were maintained for 35 days under greenhouse conditions (28 ± 3 °C, 60% relative humidity, 12-h photoperiod, and irradiance of approximately $1075 \mu\text{mol m}^{-2} \text{s}^{-1}$). Salinity treatments were established by daily irrigation with 100 mL of the respective NaCl solution. Irrigation was standardized across treatments, with drainage observed. Fertigation was applied at 7, 14, and 21 days after transplantation, with 100 mL per pot using Plantpar® fertilizer (Indústria e Comércio de Fertilizantes LTDA, Umuarama, PR, Brazil). According to the manufacturer's recommendation, the fertigation solution was prepared with 4.2 g of Plantpar Red Flex and 4.2 g of Plantpar Blue Flex per 10 L of water. Plantpar Red Flex contains N (9.0%), Mg (0.6%), S (3.0%), P_2O_5 (9.0%), K_2O (37.0%), and the micronutrients Mn (0.048%), Cu (0.031%), Co (0.002%), Zn (0.019%), B (0.048%), Mo (0.009%), Fe (0.148%), and Ni (0.006%), while Plantpar Blue Flex contains N (13.0%), Ca (15.5%), and Mg (2.85%). Control plants received the same irrigation volume and fertigation schedule, but without NaCl addition.

At the end of the experimental phase, growth, gas exchange, chlorophyll fluorescence, and pigment content were evaluated. Data were tested for normality using the Shapiro–Wilk test and for homogeneity of variances using Bartlett's test. Since the assumptions were met, no data transformation was required. Data were then subjected to two-way analysis of variance (ANOVA) in a 2×2 factorial design (priming $\times$ salinity), and treatment means were compared using Tukey's test at $P \leq 0.05$. All statistical analyses were performed using the Genes software (Cruz 2016).

2.3 Chlorophyll fluorescence and gas exchange

Chlorophyll fluorescence measurements were performed on leaf D using a portable chlorophyll fluorometer (Pocket PEA, Hansatech Instruments Ltd., King's Lynn, UK), which records fast chlorophyll fluorescence transients (OJIP). Before measurements, leaves were dark-adapted for 30 min using leaf clips to ensure full reoxidation of QA and complete stabilization of PSII reaction centers. The following fluorescence parameters were obtained: initial fluorescence

(F₀), maximum fluorescence (F_m), variable fluorescence (F_v), maximum quantum efficiency of PSII (F_v/F_m), the ratio of variable to initial fluorescence (F_v/F₀), density of active reaction centers per absorption (RC/ABS), and the performance index (PI).

Gas exchange was evaluated between 06:30 and 08:00 h using an open-flow infrared gas analyzer (LI-6400XT, LiCor, Lincoln, NE, USA). Because *A. comosus* is a CAM species, preliminary prescreening measurements were performed to determine a time window in which stomata were consistently open under our experimental conditions, and the early morning period was selected accordingly to allow standardized comparison among treatments. Readings were obtained at a reference CO₂ concentration of 400 µmol mol⁻¹, air temperature maintained at approximately 29 °C, and a saturating photosynthetic photon flux density of 1000 µmol m⁻² s⁻¹ provided by an LED light source containing 10% blue wavelengths to ensure stomatal activation. Airflow, chamber humidity/VPD, inserted leaf area, and instrument correction parameters were maintained according to the manufacturer's standard operating conditions for the LI-6400XT. Although this approach enabled consistent treatment comparisons, it does not represent the full diel pattern of CO₂ assimilation characteristic of CAM metabolism, which should be considered when interpreting the results. The stabilization time inside the IRGA chamber was 3 min for all measurements.

2.4 Photosynthetic pigments

Photosynthetic pigments were determined from leaf discs obtained from the fifth fully expanded leaves. Samples were incubated in 3 mL of dimethyl sulfoxide (DMSO; Êxodo Científica®, Sumaré, SP, Brazil) and maintained in darkness for 48 h to ensure complete pigment extraction. Absorbance was subsequently recorded at 665, 649, and 480 nm using a UV–visible spectrophotometer (UV-M51, BEL Engineering, Monza, Italy), according to the method described by Pinheiro et al. (2025).

2.5 Growth analysis

Plant growth was evaluated based on the following parameters: shoot length, root length, rosette diameter, rosette base diameter, number of leaves, leaf area, and fresh and dry biomass of shoots and roots. Leaf area was determined using ImageJ® software from digital images of detached leaves captured against a contrasting background alongside a reference scale for calibration. The scale was calibrated within the software using the known reference measurement, and only fully expanded leaves without severe necrosis or deformation were included in the analysis to ensure consistent and reliable estimation of leaf area.

2.6 Relative leaf water content

Relative leaf water content (RLWC, %) was measured on the sixth fully expanded leaf. Five leaf discs (3 mm in diameter) were collected and immediately weighed to obtain the fresh mass (FM). Discs were then placed in plastic cups containing 8 mL of distilled water for 24 h to obtain the turgid mass (TM). Afterward, discs were oven-dried at 45 °C for 48 h to obtain the dry mass (DM) (Barr and Weatherley, 1962). Relative water content (RWC) was determined as described by Pinheiro et al. (2025).The relative water content was calculated using the following formula:

RLWC (%) = [(FM – DM) / (TM – DM)] × 100

3. Results

3.1 Salinity reduces growth, photosynthetic efficiency, and pigment content in A. comosus plantlets cultivated in vitro

According to the F-test from the two-way ANOVA, all evaluated variables showed significant interaction effects between priming and salinity factors (P ≤ 0.05).

Salinity-treated plants exhibited reduced growth, yellowing leaves, necrosis, and an atrophied root system. In contrast, control plants displayed higher shoot and root development with greener leaves during the first subculture. However, root length declined during the second and third subcultures even under non-saline conditions, which may reflect limitations inherent to the in vitro culture system, such as hormonal effects of the culture medium or physical constraints imposed by the cultivation flasks (Fig. 1).

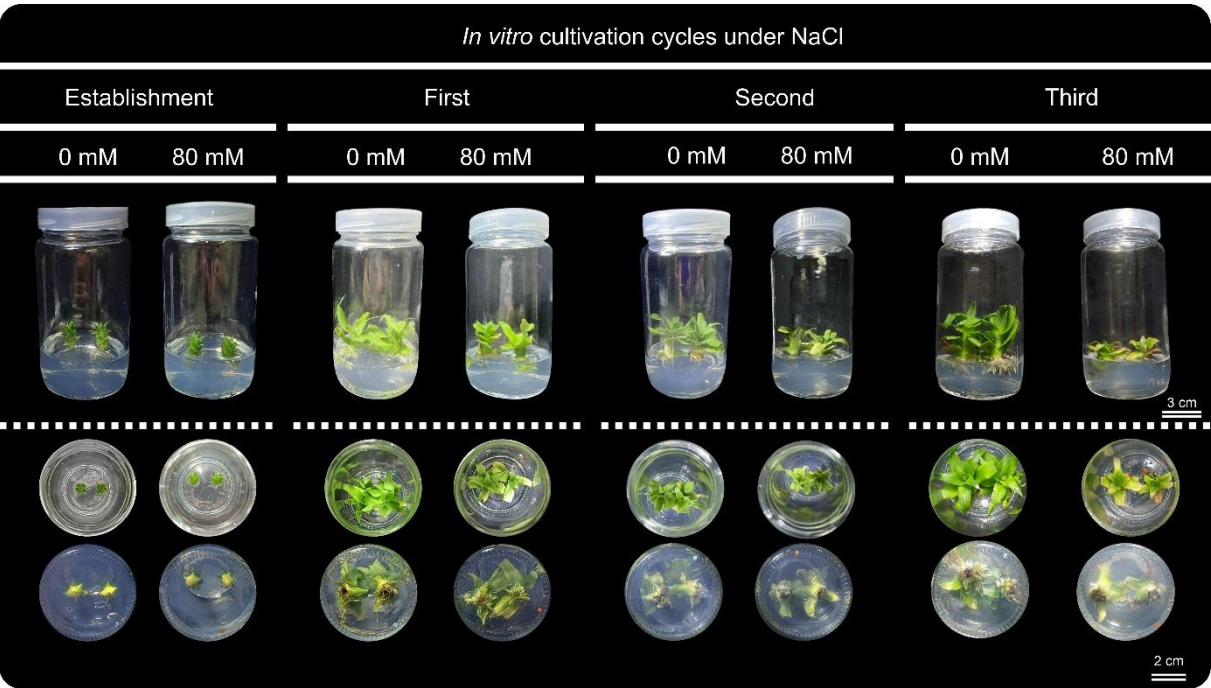


Fig. 1 *Ananas comosus* plantlets during three successive *in vitro* subcultures under 0 and 80 mM NaCl. Representative images correspond to the final subculture.

Salinity affected the length of the aerial part, root length, number of leaves, and leaf area across subcultures. Root fresh and dry mass showed a differential response depending on the cultivation stage, with increases under 80 mM NaCl during the first cultivation, no significant changes in the second cultivation, and significant reductions in the third cultivation (Table 1). Plants grown under 80 mM NaCl showed a 33% reduction in growth and a 70% reduction in root length under salinity. Under 80 mM NaCl, root fresh and dry mass increased by 91% and 54%, respectively, during the first subculture, but decreased by 54% and 40%, respectively, during the third subculture. Salinity did not affect rosette base diameter; however, it reduced leaf number and leaf area, indicating a negative impact on leaf production and expansion. The effects on chlorophyll fluorescence parameters varied across subcultures, with progressively stronger reductions under salinity in later cultivation stages, indicating increasing impairment of PSII photochemical performance over time (Table 2).

Table 1 Growth parameters of *Ananas comosus* during successive *in vitro* subcultures under two salinity concentrations (0 and 80 mM NaCl)

Growth parameters	First cultivation		Second cultivation		Third cultivation	
			NaCl			
	[0mM]	[80mM]	[0mM]	[80mM]	[0mM]	[80mM]
Length of the aerial part (mm)	50.24 ± 4.42	41.62 ± 1.83*	55.00 ± 4.55	36.60 ± 2.25*	65.10 ± 1.69	36.10 ± 2.68*
Root length (mm)	31.95 ± 5.52	8.10 ± 0.96*	13.60 ± 3.18	5.40 ± 1.36*	0.84 ± 0.02	0.27 ± 0.04*
Rosette base diameter (mm)	10.34 ± 1.16	14.66 ± 1.57 ^{NS}	7.14 ± 0.45	8.41 ± 0.85 ^{NS}	7.88 ± 0.39	7.50 ± 0.45 ^{NS}
Number of leaves	16 ± 0.43	13 ± 0.64*	18 ± 2.20	14 ± 0.54 ^{NS}	21 ± 0.99	12 ± 0.68*
Plantlet fresh mass (mg)	1503 ± 141	1383 ± 95 ^{NS}	1630 ± 230	1760 ± 150 ^{NS}	2280 ± 84	1250 ± 138*
Plantlet dry mass (mg)	139 ± 13	142 ± 13 ^{NS}	180 ± 37	223 ± 23 ^{NS}	250 ± 7	211 ± 24 ^{NS}

Root fresh mass (mg)	169 ± 19	323 ± 44*	320 ± 43	313 ± 30 ^{NS}	1260 ± 51	581 ± 68*
Root dry mass (mg)	14 ± 1.7	21.6 ± 0.65*	40 ± 4.6	39 ± 4.3 ^{NS}	112 ± 4.45	67 ± 9.23*
Leaf area (cm ²)	38.91 ± 6.40	22.44 ± 3.19*	30.27 ± 6.29	24.93 ± 4.88 ^{NS}	50.61 ± 2.61	29.01 ± 2.20*

Data are means ± standard error (n=5). Asterisks indicate significant differences between treatments (* $P \leq 0.05$ two-tailed t-test) and NS, non-significant.

Salinity significantly affected chlorophyll *a* fluorescence (Table 2), indicating stress-induced damage to the photosynthetic apparatus. Initial (F_0), maximum (F_m), and variable fluorescence (F_v) decreased by 21%, 45%, 51% and decreased maximum quantum yield of PSII (F_v/F_m) 0.77 to 0.61 under salinity. Salinity also reduced the density of active PSII reaction centers (RC/ABS) and the maximum primary efficiency of PSII (F_v/F_0) by 40% and 44%, respectively. The photosynthetic performance index (PI) decreased sharply from 2.33 to 0.91 (Table 2), indicating substantial loss of photosynthetic efficiency. Pigments were also reduced: chlorophyll *a* (54%), chlorophyll *b* (60%), carotenoids (50%), total chlorophylls (55%), and Chl/carotenoids (10%) (Table 2).

Table 2 Physiological parameters of *Ananas comosus* during successive *in vitro* subcultures under two salinity concentrations (0 and 80 mM NaCl)

Physiological parameters	First cultivation		Second cultivation		Third cultivation	
	NaCl					
	[0mM]	[80mM]	[0mM]	[80mM]	[0mM]	[80mM]
F ₀	6590 ± 216	6839 ± 455 ^{NS}	6717 ± 237	5698 ± 216*	6806 ± 252	3306 ± 499*
F _m	29069 ± 1033	23561 ± 2126*	30423 ± 1148	19926 ± 1952 *	30492 ± 622	5985 ± 977*
F _v	22480 ± 852	16722 ± 1704*	23706 ±1010	14227 ± 1800*	23686 ± 375	2678 ± 635*
F _v /F _m	0.77 ± 0.003	0.70 ± 0.012*	0.77 ± 0.008	0.70 ± 0.01*	0.78 ± 0.003	0.42 ± 0.05*
RC/ABS	1.39 ± 0.02	0.94 ± 0.07*	1.20 ± 0.13	1.1 ± 0.12 ^{NS}	1.42 ± 0.06	0.34 ± 0.08*
F _v /F ₀	3.42 ± 0.07	2.45 ± 0.13*	3.54 ± 0.13	2.48 ± 0.24*	3.49 ± 0.07	0.82 ± 0.20*

PI	2.32 ± 0.10	1.08 ± 0.02*	2.06 ± 0.42	1.48 ± 0.37 ^{NS}	2.62 ± 0.19	0.17 ± 0.09*
Chlorophyll <i>a</i> (µg cm ⁻²)	26.31 ± 1.71	19.23 ± 1.22*	69.44 ± 2.41	40.58 ± 7.14*	69.58 ± 7.90	16.26 ± 1.04*
Chlorophyll <i>b</i> (µg cm ⁻²)	8.46 ± 0.55	5.81 ± 0.37*	20.69 ± 0.90	11.06 ± 2.21*	20.77 ± 3.15	3.31 ± 0.26*
Carotenoids (µg cm ⁻²)	4.67 ± 0.48	3.01 ± 0.16*	13.63 ± 0.57	9.08 ± 1.28*	13.94 ± 1.17	4.07 ± 0.13*
Total chlorophyll (µg cm ⁻²)	34.77 ± 1.42	25.04 ± 1.55*	90.12 ± 3.29	51.64 ± 9.36*	90.35 ± 11.05	19.57 ± 1.30*
Chlorophyll <i>a/b</i>	3.18 ± 0.32	3.31 ± 0.05 ^{NS}	3.36 ± 0.04	3.69 ± 0.08*	3.41 ± 0.10	4.96 ± 0.11*
Total chlorophyll/carotenoids	7.73 ± 0.73	8.41 ± 0.68 ^{NS}	6.61 ± 0.07	5.65 ± 0.20*	6.41 ± 0.21	4.80 ± 0.25*

Data are means ± standard error (n=5). Asterisks indicate significant differences between treatments (* $P \leq 0.05$ two-tailed t-test) and NS, non-significant. Fo: Initial fluorescence, F_m: maximum fluorescence, F_v: variable fluorescence, F_v/F_m: maximum quantum yield of PSII, RC/ABS: density of active reaction centers of photosystem II, F_v/Fo: maximum primary efficiency of photosystem II, PI: photosynthetic performance index

3.2 Salinity priming *in vitro* modulates photosynthetic performance and morphological characteristics under salt stress *ex vitro*

Salinity stress during acclimatization did not affect chlorophyll *a* fluorescence parameters in any priming treatment (Fig. 2). However, non-primed seedlings showed 15% higher F₀ than primed ones in both NaCl concentration (Fig. 2 A). Non-primed plants also maintained relatively stable F_m (Fig. 2B), F_v (Fig. 2C), and RC/ABS (Fig. 2E). The F_v/F_m remained approximately at 0.77 in both control and salinity treatments (Fig. 2D). In contrast, primed seedlings had 6% higher F_v/F₀ compared to non-primed plants regardless of salinity (Fig. 2F). No effect on PI was observed (Fig. 2G).

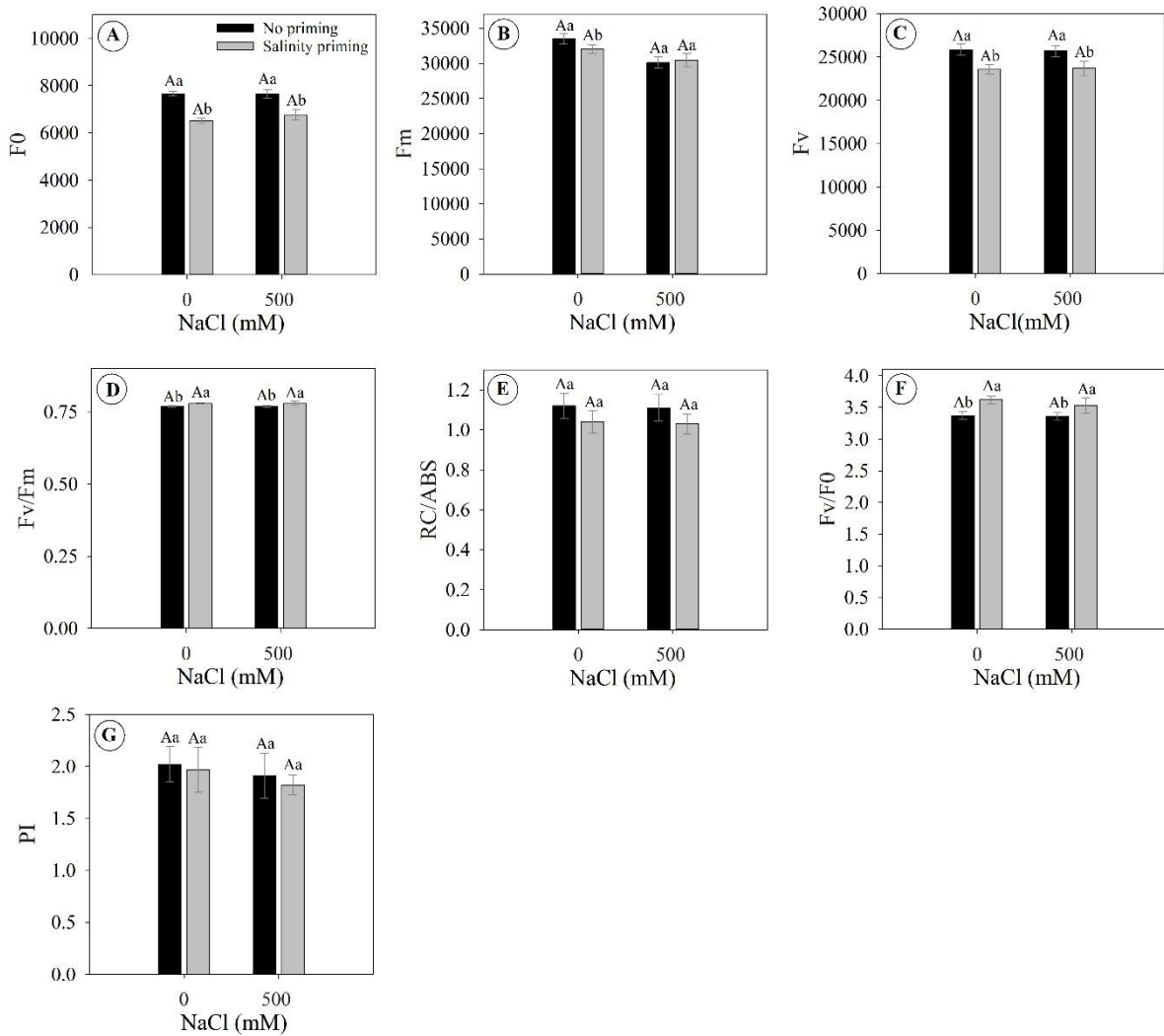


Fig. 2 Chlorophyll a fluorescence parameters of no-primed and salinity-primed *Ananas comosus* seedlings cultivated for 35 days under two salinity concentrations (0 and 500 mM NaCl). Data are means $\pm$ SE ($n = 11$). Uppercase letters indicate significant difference among NaCl concentrations within each priming treatment, whereas lowercase letters indicate differences between priming treatments within each NaCl concentration (Tukey's test, $P \leq 0.05$)

Gas exchange parameters were significantly affected by the interaction between salinity and priming (Fig. 3). In non-primed plants, salinity reduced net CO₂ assimilation rate (A) by 68%, stomatal conductance (g_s) by 60%, and transpiration rate (E) by 55% compared with the control (Fig. 3A, B, D). In primed plants, the reductions were less pronounced for A (48%), g_s (58%), and E (58%). Non-primed plants under salinity showed a 7% increase in intercellular CO₂ concentration (C_i) and C_i/C_a ratio relative to the control, whereas primed plants exhibited

reductions of 13% in both parameters under salinity (Fig. 3C, E). The decrease in g_s , E , and C_i/C_a under salinity is consistent with stomatal limitation to CO_2 assimilation, particularly in primed plants. However, carboxylation efficiency (A/C_i) declined markedly under salinity in both treatment groups, decreasing by 69% in non-primed plants and by 40% in primed plants relative to their respective controls (Fig. 3F). Notably, under 500 mM NaCl, primed plants exhibited 56% higher A/C_i values than non-primed plants, indicating partial preservation of carboxylation efficiency under saline stress. These results suggest that, in addition to stomatal constraints, non-stomatal limitations contributed to the reduction in photosynthetic performance. Intrinsic water-use efficiency (A/g_s) decreased by 45% in non-primed plants under salinity, whereas primed plants maintained values statistically similar to their respective control (Fig. 3G).

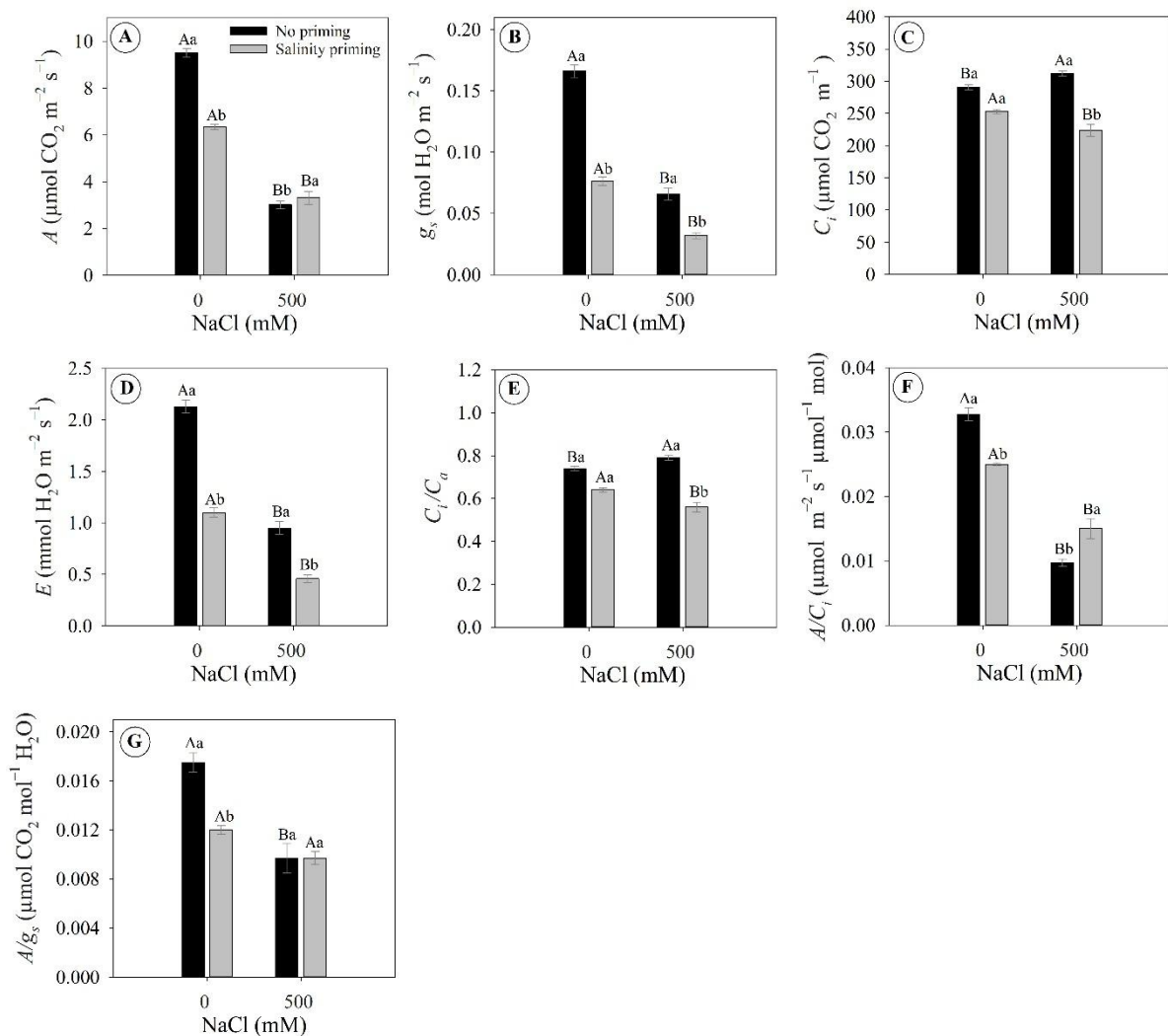


Fig. 3 Gas exchange parameters of non-primed and salinity-primed *Ananas comosus* seedlings cultivated for 35 days under two salinity concentrations (0 and 500 mM NaCl). Data are means

$\pm$ SE ($n = 11$). Uppercase letters indicate significant differences between NaCl concentrations within each priming treatment, whereas lowercase letters indicate differences between priming treatments within each NaCl concentration (Tukey's test; $P \leq 0.05$)

Salinity did not affect the chlorophyll *a/b* ratio (Fig. 4C) or the total chlorophyll to carotenoid ratio (Fig. 4F). However, non-primed plants exposed to salinity showed higher chlorophyll *a* (39%, Fig. 4A), chlorophyll *b* (38%, Fig. 4B), total chlorophyll (39%, Fig. 4D), and total carotenoid (46%, Fig. 4E) levels compared with the control. Rather than indicating enhanced photoprotective capacity, this apparent increase may reflect a concentration effect associated with salt-induced morphological adjustments, such as reduced leaf expansion, increased tissue thickness, or smaller cell size, resulting in higher pigment content when expressed on a tissue basis. Therefore, pigment accumulation alone should be interpreted cautiously and not as direct evidence of improved stress protection. This apparent increase may reflect a concentration effect rather than enhanced pigment biosynthesis, potentially resulting from reduced leaf expansion and/or smaller cell size under salt stress, which would increase pigment concentration on a tissue mass basis.

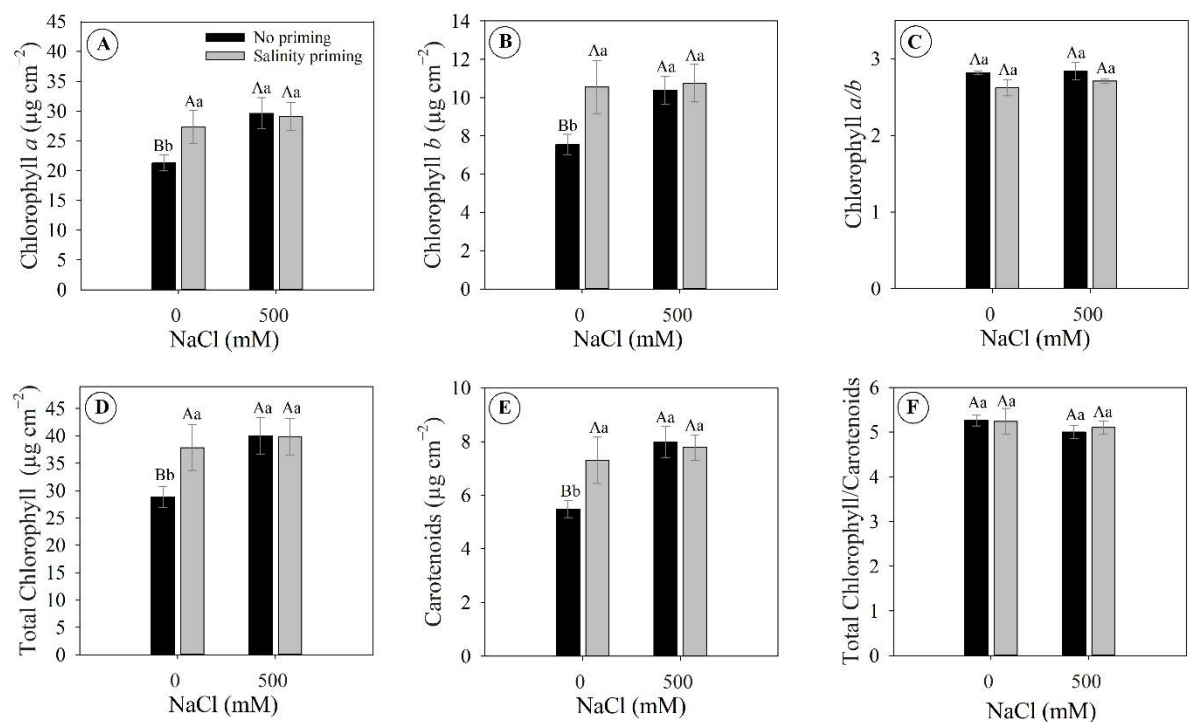


Fig. 4 Photosynthetic pigment contents of non-primed and salinity-primed *Ananas comosus* seedlings cultivated for 35 days under two salinity concentrations (0 and 500 mM NaCl). Data are means $\pm$ SE (n = 4). Uppercase letters indicate significant differences among NaCl concentrations within each priming treatment, whereas lowercase letters indicate differences between priming treatments within each NaCl concentration (Tukey's test, $P \leq 0.05$).

Salinity altered plant morphology depending on priming treatment. Under control conditions, primed plants exhibited reduced overall growth compared with non-primed plants. Under salinity, non-primed plants developed visible leaf necrosis and paler leaves, whereas primed plants also showed reduced growth, although with less pronounced visual damage. The increase in root length observed in non-primed plants under salinity should be interpreted cautiously, as it was accompanied by a reduction in root dry mass, suggesting a shift toward root elongation rather than enhanced root biomass accumulation. Overall, the morphological responses indicate distinct acclimation patterns between treatments rather than unequivocal growth advantages associated with priming (Fig. 5).

Salinity did not affect shoot length or rosette diameter (Fig. 5B and F), but non-primed plants were 106% longer and 126% larger than primed ones. Root length increased 37% under salinity in non-primed plants (Fig. 5C). However, salinity reduced leaf number (18%), rosette base diameter (44%), plantlet dry mass (41%), root dry mass (57%), leaf area (38%), and relative water content (31%) in non-primed plants, while primed plants were less affected (Fig. 5D, E, G, H, I and J).

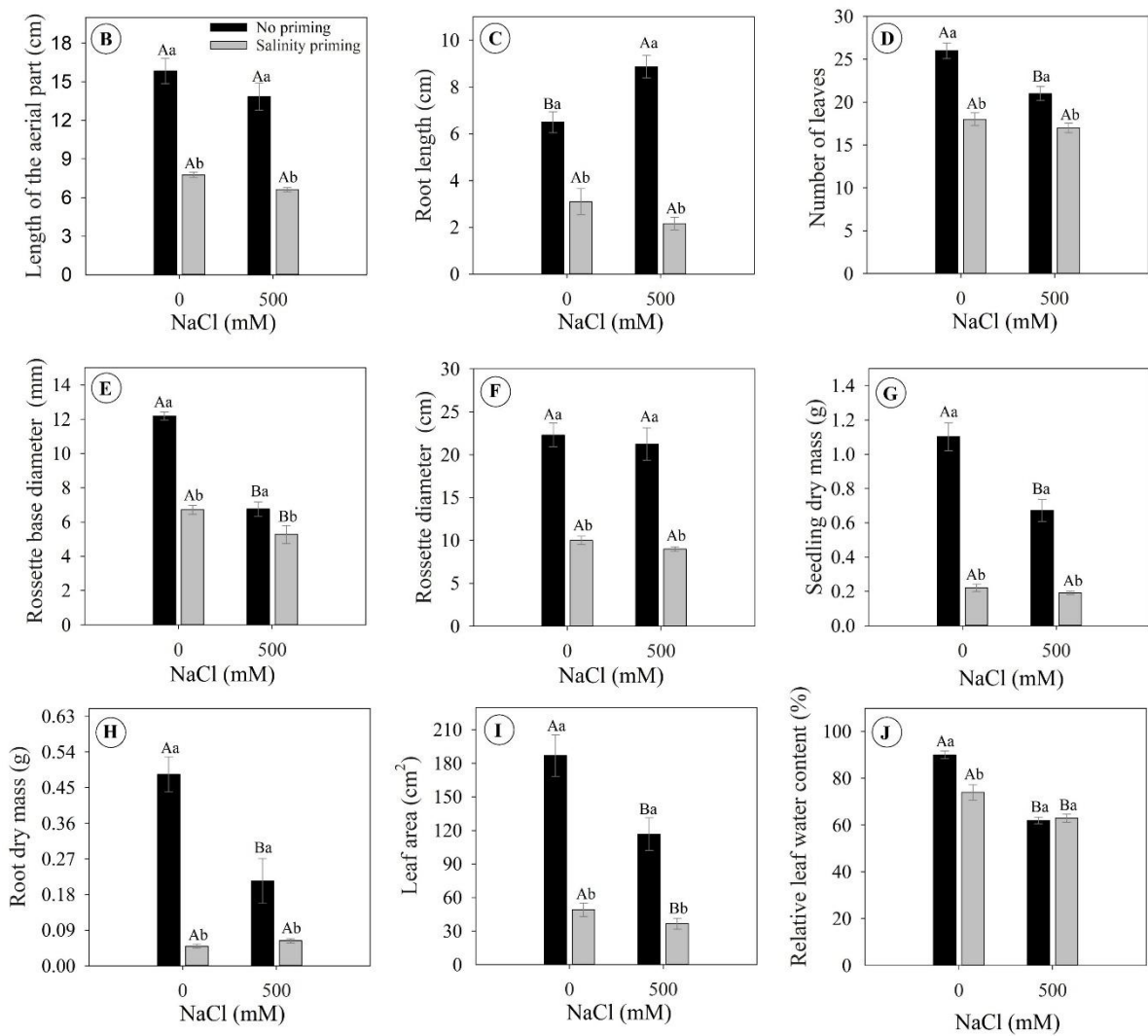
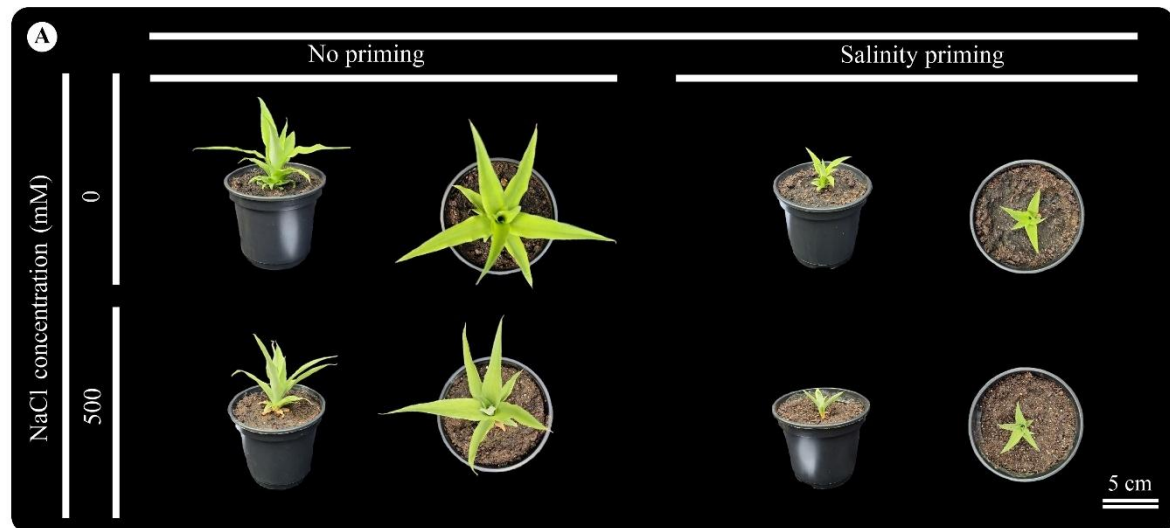


Fig. 5 Growth parameters of non-primed and salinity-primed *Ananas comosus* seedlings cultivated for 35 days under two salinity concentrations (0 and 500 mM NaCl). Data are means $\pm$ SE ($n = 5$). Uppercase letters indicate significant differences between NaCl concentrations

within each priming treatment, whereas lowercase letters indicate differences between priming treatments within each NaCl concentration (Tukey's test; $P \leq 0.05$)

4. Discussion

The present results indicate that *in vitro* salinity priming altered subsequent physiological responses of *A. comosus* under *ex vitro* salt stress, partially attenuating the decline in apparent carboxylation efficiency and modulating pigment responses. However, these physiological adjustments were accompanied by reduced plant growth under both control and saline conditions, indicating that improved acclimation responses did not translate into greater biomass accumulation. Similar priming-induced photosynthetic adjustments have been reported in other species and are consistent with priming-like responses suggestive of stress memory and enhanced physiological plasticity (Karalija et al. 2025; Sghaier-Hammami et al. 2023). However, direct confirmation of stress memory would require targeted molecular or epigenetic analyses, as well as assessment of plant performance following a controlled recovery period. The less pronounced reduction in carboxylation efficiency (A/C_i) in primed plants compared with non-primed plants suggests that salt priming partially preserved the biochemical capacity for carbon assimilation under saline conditions. Since A/C_i reflects the efficiency of CO_2 fixation independent of stomatal limitations, this response indicates that priming may have mitigated non-stomatal constraints associated with salinity, such as reduced Rubisco activity, impaired regeneration of photosynthetic intermediates, or damage to the photosynthetic apparatus, thereby contributing to better photosynthetic performance under stress (Molassiotis et al. 2010; Nasrallah et al. 2022).

Despite these physiological adjustments, the absence of proportional gains in net CO_2 assimilation and biomass indicates that the partial preservation of photosynthetic performance was insufficient to sustain growth under salinity. The reduction in stomatal conductance and transpiration under salinity indicates that stomatal restriction contributed to the limitation of carbon assimilation, particularly in primed plants, where the decline in C_i/C_a suggests reduced CO_2 diffusion into the leaf. In contrast, the increase in C_i/C_a observed in non-primed plants under salinity, despite lower stomatal conductance, indicates that non-stomatal limitations also played an important role, likely reflecting impaired carbon fixation capacity. Together, these contrasting responses suggest that salinity constrained photosynthesis through different combinations of stomatal and non-stomatal limitations depending on the priming history. Simultaneously, maintenance of chlorophyll and carotenoid pools indicates that priming

promoted photoprotective stabilization of the photosynthetic apparatus, likely preserving PSII integrity and limiting oxidative pigment degradation (Zandi and Schnug 2022).

However, the reduced growth observed in primed plants may suggest a potential trade-off associated with maintaining stress-responsive physiological adjustments. One possible explanation is that the sustained activation of protective and repair mechanisms could impose additional energetic demands, potentially reducing carbon allocation to biomass production (Badavath and Saski 2026). Nevertheless, this interpretation remains hypothetical, as the metabolic processes underlying such trade-offs were not directly evaluated in the present study. This potential trade-off may have been further intensified by the severity of the salinity treatment, as 500 mM NaCl represents an extreme stress condition likely to shift plant responses toward survival and maintenance rather than growth. Under such conditions, even partial physiological acclimation may not be sufficient to promote biomass accumulation, as substantial energetic and metabolic resources are likely redirected toward sustaining cellular homeostasis under severe ionic and osmotic stress (Nigam et al. 2026).

In the present study, the observed divergence between partial physiological acclimation and reduced biomass accumulation suggests that improved stress-responsive physiological adjustments do not necessarily translate into enhanced growth under severe salinity. Although priming has frequently been associated with improved biomass production and yield under moderate stress conditions (Sahoo et al. 2025), these benefits appear to be strongly context-dependent. Under extreme stress, such as the 500 mM NaCl imposed here, the capacity of priming-induced acclimation to support growth may be limited, potentially due to the prioritization of maintenance and survival processes over biomass accumulation. However, because the present study evaluated only a single *ex vitro* salinity level, it is not possible to define the threshold at which priming ceases to confer growth benefits. Future studies employing a gradient of salinity levels will be important to determine the range over which priming promotes beneficial acclimation without compromising plant growth.

5. Conclusion

Under the severe *ex vitro* salinity imposed here, *in vitro* salinity priming partially attenuated the decline in apparent carboxylation efficiency and maintained pigment concentrations, but did not improve biomass accumulation in *Ananas comosus*. These results suggest a decoupling between selected physiological adjustments and growth recovery under severe salt stress, in which the maintenance of stress responsive acclimation may impose constraints on carbon allocation to biomass production.

Author contributions Conceptualization and supervision were performed by Felipe, S.H.S. and Corrêa, T.R. Data collection, investigation, and methodology were carried out by Silva-Moraes, V.K.O., Alves, G.L., Albuquerque, I.C., Pinheiro, J.F., Lima, A.S., Rivas, P.M.S. Formal analysis was performed by Silva-Moraes, V.K.O., Corrêa, T.R., Felipe, S.H.S., Batista, D.S., Figueiredo, F.A.M.M.A., Reis, F.O., Ferraz, T.M. The first draft of the manuscript was written by Silva-Moraes, V.K.O. All authors reviewed, edited, and approved the final version of the manuscript for publication.

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Declarations

Conflict of interest D.S.B. serves as an Associate Editor of the journal at the time of submission. However, this did not influence the peer review process or the final decision. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability The authors confirm that the data supporting the findings of this study are available in the article.

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CAPÍTULO IV

Considerações finais

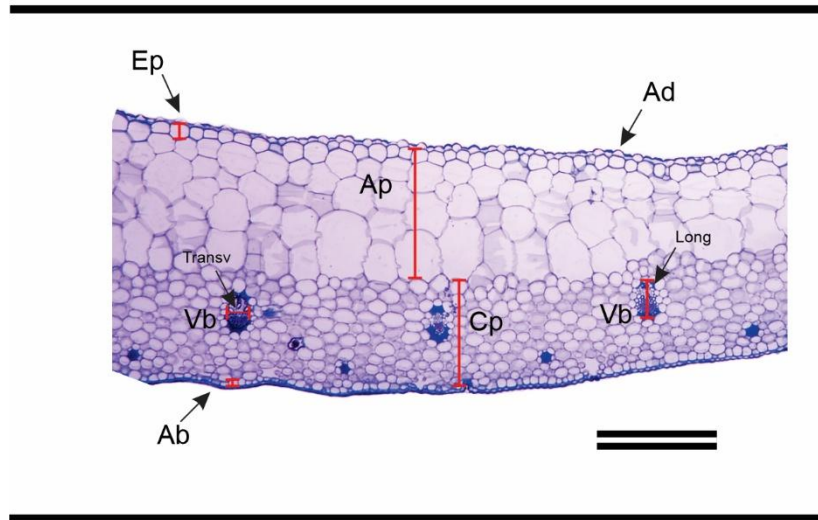
Considerações finais

O eCO₂ mitigou parcialmente os efeitos da salinidade em plantas de *Ananas comosus*, especialmente em salinidade severa (120 mM de NaCl). O enriquecimento de 800 µmol mol⁻¹ de CO₂ aumentou significativamente a biomassa vegetal, a taxa fotossíntese líquida e a eficiência intrínseca do uso da água nas plantas. No entanto, não houve recuperação dos pigmentos e mudanças anatômicas estruturais nas plantas.

A salinidade *in vitro* reduziu o conteúdo dos pigmentos e o crescimento, conforme indicado pelas reduções no rendimento quântico máximo do PSII e nos valores do índice de desempenho fotossintético. Sob condições de salinidade *ex vitro*, as plantas apresentaram aclimação fisiológica parcial, incluindo um declínio menos pronunciado na eficiência aparente de carboxilação e a manutenção das concentrações de pigmentos sob salinidade. No entanto, esses benefícios fisiológicos não se traduziram em aumento de biomassa, sugerindo que o custo metabólico da proteção sustentada limitou o crescimento.

Dessa forma, ambas as abordagens evidenciam potencial para aumentar a tolerância ao estresse salino, porém com limitações distintas. O CO₂ elevado favoreceu o desempenho fisiológico e o crescimento sob estresse e o priming salino contribui para a resiliência funcional, mas com possível custo metabólico que restringe o acúmulo de biomassa. Esses resultados reforçam a complexidade das respostas das plantas ao estresse salino e indicam que a integração de diferentes estratégias pode ser necessária para conciliar tolerância fisiológica e produtividade em *A. comosus*.

Measurements leaf micromorphometric



Supplementary Fig. S1 Cross-section of the median region of an *Ananas comosus* leaf showing the anatomical traits measured in the micromorphometric analysis. Ad = adaxial epidermis; Ab = abaxial epidermis; Ep = epidermis thickness; Ap = aquifer parenchyma thickness; Cp = chlorophyll parenchyma thickness; Vb = vascular bundle. Transverse vascular bundle thickness corresponds to the maximum distance across the vascular bundle in a section where the xylem and phloem are oriented perpendicular to the leaf's longitudinal axis (labelled Transv), measured from the outer edge of the bundle sheath on the adaxial side to the outer edge on the abaxial side. Longitudinal vascular bundle thickness corresponds to the maximum diameter of bundles aligned parallel to the leaf's longitudinal axis (labelled Long), measured perpendicularly to the main vascular elements. Measurements were taken in micrometers (μm) using ImageJ® software on images captured at 4 $\times$ magnification. Scale bar = 200 μm .