



UNIVERSIDADE
ESTADUAL DE LONDRINA

GIOVANNA CAMARGO DO CARMO

**NANOPARTÍCULAS DE QUITOSANA CONTENDO S-
NITROSOGLUTATIONA EM ESPÉCIES ARBÓREAS
NATIVAS DA MATA ATLÂNTICA: RESPOSTAS
FISIOLÓGICAS E POTENCIAL PARA A RESTAURAÇÃO
FLORESTAL**

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Orientador: Prof. Dr. Halley Caixeta de Oliveira

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CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS**ATA DE DEFESA REMOTA DE TESE**

Aos 18 dias do mês de setembro do ano de 2026, em sala remota do Google Classroom, desta Universidade, às 13:30 horas, reuniu-se a Banca Examinadora homologada pelo Programa de Pós-Graduação em Ciências Biológicas, composta por Dr. Yves Rafael Bovolenta como presidente da banca, Dr. Danilo Miralha Franco, Dra. Neidiquele Maria Silveira, Dra. Rosimeri De Oliveira Fragoso e Dra. Amedea Barozzi Seabra. A reunião teve por objetivo julgar o trabalho da estudante GIOVANNA CAMARGO DO CARMO sob o título: "NANOPARTÍCULAS DE QUITOSANA CONTENDO DOADOR DE ÓXIDO NÍTRICO EM ESPÉCIES ARBÓREAS NATIVAS DA MATA ATLÂNTICA: DA RESPOSTA AO DÉFICIT HÍDRICO À APLICAÇÃO NA RESTAURAÇÃO FLORESTAL". Os trabalhos foram abertos pelo professor Dr. Yves Rafael Bovolenta. A seguir, foi dada a palavra à estudante para apresentação do trabalho. Cada examinador arguiu a Doutoranda, com tempos iguais de arguição e resposta. Terminadas as arguições, procedeu-se ao julgamento do trabalho, sendo que os membros remotos Dr. Danilo Miralha Franco, Dra. Neidiquele Maria Silveira, Dra. Rosimeri De Oliveira Fragoso e Dra. Amedea Barozzi Seabra enviaram simultaneamente seus formulários de avaliação os quais foram impressos e anexados à presente ata, concluindo a Banca Examinadora por sua aprovação. Nada mais havendo a tratar, foi lavrada a presente ata, que vai assinada pelo presidente.

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do CARMO, Giovanna Camargo. **Nanopartículas de quitosana contendo S-nitrosoglutationa em espécies arbóreas nativas da Mata Atlântica**: respostas fisiológicas e potencial para a restauração florestal. 2026. 157 f. Tese (Doutorado em Ciências Biológicas) – Universidade Estadual de Londrina, Londrina, 2026.

RESUMO GERAL

As mudanças climáticas têm intensificado a ocorrência de eventos de seca, comprometendo o estabelecimento de mudas de espécies arbóreas nativas em áreas de restauração florestal. O óxido nítrico (NO) é uma molécula sinalizadora central na tolerância das plantas contra estresses abióticos. Além disso, o NO participa da regulação estomática, mas seu papel é paradoxal: dependendo da espécie, da dose e das condições ambientais, o NO pode tanto promover o fechamento quanto a abertura dos estômatos. Sua aplicação direta é dificultada devido às suas características químicas e natureza instável. A nanoencapsulação de moléculas doadoras de NO constitui uma estratégia para promover a liberação lenta e controlada deste gás, podendo aumentar sua atividade biológica. Nesta tese, avaliou-se o uso de nanopartículas de quitosana contendo S-nitrosoglutationa (CS NPs GSNO), molécula doadora de NO, como estratégia para aumentar a tolerância de mudas de *Cecropia pachystachya* (pioneira) e *Cariniana estrellensis* (não pioneira) ao déficit hídrico (DH) e melhorar seu desempenho inicial em campo. No primeiro capítulo, o efeito das CS NPs GSNO foi avaliado em mudas das duas espécies submetidas a DH moderado de longo prazo. Apenas a espécie pioneira respondeu positivamente ao tratamento com 50 μM , apresentando aumento da condutância estomática e fotossíntese, melhora do status hídrico e maior acúmulo de biomassa, além de alterações positivas na arquitetura da raiz. Em contraste, não foram detectados benefícios consistentes em *C. estrellensis* em nenhuma das concentrações testadas. No segundo capítulo, *C. pachystachya* foi avaliada em condições de campo. Na segunda semana após o plantio, as CS NPs GSNO (100 μM) aumentaram a fotossíntese e a eficiência de carboxilação em relação ao controle; na nona semana, esses valores não diferiram do controle. Ao final do experimento, as mudas tratadas apresentaram maior área foliar e maior assimilação total de CO_2 que o controle e uma nanoformulação comercial. A biomassa de caule e de parte aérea foi maior apenas em relação à nanoformulação comercial ($p < 0,10$). Além disso, o custo estimado da formulação foi de cerca de US\$ 0,025 por muda tratada. Essa é uma estimativa preliminar, que não inclui os custos operacionais de aplicação. Por fim, no capítulo 3, uma revisão narrativa da literatura discute os mecanismos moleculares por trás da dualidade do NO na regulação dos movimentos estomáticos e apresenta uma triagem com 36 espécies arbóreas nativas da Mata Atlântica e dois métodos de aplicação, totalizando 46 combinações espécie-método. Destas, em 31 não foi detectada alteração na condutância estomática, enquanto 12 resultaram em aumento e 3 em redução da condutância. Isso indica que, nas condições avaliadas, a resposta estomática à aplicação de CS NPs GSNO variou entre as espécies e com o contexto metodológico. Em conjunto, os resultados indicam que as CS NPs GSNO têm potencial para mitigar os efeitos do DH em algumas espécies arbóreas nativas da Mata Atlântica, como *C. pachystachya*. Esse potencial depende da espécie e das condições avaliadas. Assim, sua aplicação prática deve considerar a alta variabilidade interespecífica na resposta à tecnologia.

Palavras-chave: S-nitrosoglutationa; Nanotecnologia; Seca; Restauração florestal; Mudanças climáticas.

do CARMO, Giovanna Camargo. Chitosan nanoparticles containing S-nitrosogluthathione in native tree species of the Atlantic Forest: physiological responses and potential for forest restoration. 2026. 157 pp. Thesis (Doctorate degree in Biological Sciences) – Universidade Estadual de Londrina, Londrina, 2026.

GENERAL ABSTRACT

Climate change has intensified the occurrence of drought events, compromising the establishment of native tree seedlings in forest restoration areas. Nitric oxide (NO) is a key signaling molecule involved in plant tolerance to abiotic stresses. In addition, NO participates in stomatal regulation, but its role is paradoxical: depending on the species, dose, and environmental conditions, NO can promote either stomatal closure or opening. Its direct application is hindered by its chemical properties and unstable nature. The nanoencapsulation of NO donor molecules represents a strategy to promote the slow and controlled release of this gas, potentially enhancing its biological activity. In this thesis, chitosan nanoparticles containing S-nitrosogluthathione (CS NPs GSNO), an NO donor molecule, were evaluated as a strategy to enhance the tolerance of *Cecropia pachystachya* (pioneer) and *Cariniana estrellensis* (non-pioneer) seedlings to water deficit (WD) and to improve their initial performance under field conditions. In the first chapter, the effects of CS NPs GSNO were evaluated in seedlings of both species subjected to long-term moderate WD. Only the pioneer species responded positively to treatment with 50 μM , showing increased stomatal conductance and photosynthesis, improved water status, greater biomass accumulation, and positive changes in root architecture. In contrast, no consistent benefits were detected in *C. estrellensis* at any of the concentrations tested. In the second chapter, *C. pachystachya* was evaluated under field conditions. Two weeks after planting, CS NPs GSNO (100 μM) increased photosynthesis and carboxylation efficiency compared to the control; after nine weeks, these values did not differ from the control. At the end of the experiment, treated seedlings showed greater leaf area and total CO_2 assimilation than the control and a commercial nanoformulation. Stem and shoot biomass were higher only compared to the commercial nanoformulation ($p < 0.10$). In addition, the estimated cost of the formulation was approximately US\$0.025 per treated seedling. This is a preliminary estimate that does not include operational application costs. Finally, in Chapter 3, a narrative literature review discusses the molecular mechanisms underlying the dual role of NO in the regulation of stomatal movements and presents a screening of 36 native Atlantic Forest tree species using two application methods, totaling 46 species–method combinations. Of these, no change in stomatal conductance was detected in 31, whereas 12 resulted in increased and three in decreased conductance. This indicates that, under the conditions evaluated, the stomatal response to CS NPs GSNO varied among species and with the methodological context. Taken together, the results indicate that CS NPs GSNO have the potential to mitigate the effects of WD in some native Atlantic Forest tree species, such as *C. pachystachya*. This potential depends on the species and on the conditions evaluated. Therefore, their practical application must account for the high interspecific variability in the response to this technology.

Keywords: S-nitrosogluthathione; Nanotechnology; Drought; Forest Restoration; Climate Change.

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nanoparticles containing GSNO at 100 μM ; WD + NP GSNO 200: water deficit + chitosan nanoparticles containing GSNO at 200 μM ; WD + NP GSNO 400: water deficit + chitosan nanoparticles containing GSNO at 400 μM ; WD + NP GSNO 800: water deficit + chitosan nanoparticles containing GSNO at 800 μM . Data are expressed as mean $\pm$ standard error ($n = 8$). Equal letters indicate similar means according to ANOVA followed by Tukey's test ($p < 0.05$). 62

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LISTA DE ABREVIATURAS E SIGLAS

A	Taxa fotossintética líquida
ABA	Ácido abscísico
ANOVA	Análise de variância
ARFs	Fatores de resposta à auxina
A_{total}	Assimilação total de CO ₂
BRL	Reais
cGMP	Monofosfato de guanosina cíclico
C_i	Concentração intercelular de CO ₂
cPTIO	Sequestrador de óxido nítrico
CS	Quitosana
CSNPs	Nanopartículas de quitosana
CS NPs GSNO	Nanopartículas de quitosana contendo S-nitrosoglutationa
DAF-2DA	Sonda fluorescente sensível ao óxido nítrico
DAP	Dias após o plantio
DH	Déficit hídrico
DLS	Dynamic light scattering
E	Taxa de transpiração
EROs	Espécies reativas de oxigênio
FAA	Solução fixadora de ácido acético, formaldeído e etanol
FC	Capacidade de campo
F_v/F_m	Eficiência máxima do fotossistema II
g_s	Condutância estomática
GSH	Glutationa reduzida
GSNO	S-nitrosoglutationa
GSNO-CSNPs	Nanopartículas de quitosana contendo GSNO

GSNOR	GSNO redutase
GSSG	Glutathiona oxidada
H ₂ O ₂	Peróxido de hidrogênio
HRTEM	Microscopia eletrônica de transmissão de alta resolução
IRGA	Analizador de gás por infravermelho
<i>k</i>	Eficiência instantânea de carboxilação
M-NO	Complexos Metal-nitrosyl
NAA	1-naphthyl acetic acid
NADH	Nicotinamida adenina dinucleotídeo reduzida
NADPH	Nicotinamida adenina dinucleotídeo fosfato reduzida
NaNO ₂	Nitrito de sódio
NH ₃	Amônia
NIA1/NIA2	Genes que codificam a nitrato redutase em <i>Arabidopsis thaliana</i>
NMs	Nanomateriais
NO	Óxido nítrico
NO ₂	Dióxido de nitrogênio
NO ₂ ⁻	Nitrito
NO ₃ ⁻	Nitrato
NONOates	N-diazeniumdiolates
NOS	Óxido nítrico sintase
NP	Nanopartícula de quitosana sem doador de óxido nítrico
NP GSNO	Nanopartícula de quitosana contendo GSNO
NPs	Nanopartículas
NR	Nitrato redutase
NTA	Análise de rastreamento de nanopartículas
ONOO ⁻	Peroxinitrito
OST1	Open Stomata 1

PA-ACS	Reagente de grau analítico
PAST	Paleontological Statistics (software)
PCA	Análise de componentes principais
PDI	Índice de polidispersão
PIN	Proteínas PIN-FORMED
PP2Cs	Proteínas fosfatases tipo
PSII	Fotossistema II
PTMs	Modificações pós-traducionais
PYR/PYL/RCAR	Receptor intracelular de ácido abscísico
RBOHs	NADPH oxidases
RNS	Espécies reativas de nitrogênio
ROS	Espécies reativas de oxigênio
RSNOs	S-nitrosotióis
RWC	Conteúdo relativo de água
SNAC	S-nitroso-N-acetilcisteína
SNAP	S-nitroso-N-acetyl-DL-penicillamine
S-nitroso-MSA	Ácido S-nitrosomercaptosuccínico
SNP	Nitroprussiato de sódio
STATISTICA	Software de análise estatística
TPP	Tripolifosfato de sódio
USD	Dólares americanos
WD	Déficit hídrico
WUE	Eficiência no uso da água
$^1\text{O}_2$	Oxigênio singlete
$\bullet\text{O}_2^-$	Ânion superóxido
$\bullet\text{OH}$	Radical hidroxila
Ψ_w	Potencial de água

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1 INTRODUÇÃO GERAL

1.1 RESTAURAÇÃO FLORESTAL E SILVICULTURA DE MUDAS DE ESPÉCIES NATIVAS

A restauração da paisagem florestal é uma agenda estratégica de importância global e um dos maiores desafios atuais, devido ao seu papel na conservação da biodiversidade, recuperação de serviços ecossistêmicos, mitigação dos efeitos das mudanças climáticas e cumprimento de compromissos internacionais de sustentabilidade (Fargione et al., 2021; Yang et al., 2022; Silva et al., 2024). Nas últimas décadas, o assunto tem recebido cada vez mais atenção no âmbito político e científico. Compromissos internacionais já somam a meta de restaurar mais de 160 milhões de hectares ao redor do mundo; desses, aproximadamente 82% têm foco em regiões tropicais, consideradas essenciais para o sequestro de carbono e mitigação do aquecimento global (Brancalion et al., 2019). Não por acaso, a restauração florestal foi identificada enquanto um dos eixos centrais do Acordo de Paris, evidenciando o contexto político favorável à restauração de florestas como estratégia promissora no enfrentamento da atual crise climática (Grassi et al., 2017).

Além de recuperar a biodiversidade, a restauração florestal desempenha um papel crucial na oferta de serviços ecossistêmicos fundamentais à sociedade. Entre os benefícios, estão a regulação do clima por meio do sequestro e armazenamento de carbono, proteção dos recursos hídricos com aumento da infiltração de água no solo e diminuição da erosão, além da melhoria da qualidade do ar e da fertilidade do solo (Silva et al., 2024). Ademais, as florestas tropicais figuram entre os ecossistemas com maior capacidade de produção primária global, estimada em aproximadamente 40,8 bilhões de toneladas de carbono por ano, e constituem um componente fundamental do sumidouro terrestre de carbono (Pan et al., 2011; Ellison et al., 2017). No entanto, áreas degradadas podem levar várias décadas para recuperar seus estoques de biomassa, um ritmo que contrasta com os prazos mais curtos adotados pelo mercado de carbono (Brancalion et al., 2019).

Embora seja importante, o progresso das políticas públicas nacionais de restauração ainda encontra desafios significativos. Os elevados custos de implantação e manutenção das áreas restauradas, aliados à alta taxa de mortalidade

das mudas nos primeiros meses após o plantio constituem os principais desafios para a viabilidade econômica dos projetos (Bustamante et al., 2019; Lopes-Oliveira et al., 2019; Yang et al., 2022; Silva et al., 2024; Turchetto et al., 2025). Diante desse cenário, cresce a busca por estratégias inovadoras que não apenas recuperem o funcionamento ecológico dos ecossistemas, mas que também considerem aspectos econômicos, sociais e culturais, ampliando o conceito de restauração e fortalecendo sua viabilidade em larga escala (Gann et al., 2019).

A silvicultura de espécies nativas do Brasil se apresenta como uma alternativa promissora, ao integrar métodos, práticas e tecnologias que asseguram maior qualidade das mudas produzidas, desempenho superior no campo e maior eficácia nos projetos de restauração (Brancalion et al., 2019; Sartori et al., 2025; Turchetto et al., 2025). Esse enfoque se torna ainda mais relevante quando direcionado a biomas como a Mata Atlântica, reconhecida como um dos hotspots de biodiversidade mais vulneráveis aos efeitos das mudanças climáticas (Ribeiro et al., 2011; Macedo et al., 2021). O passado de intensa urbanização e ampliação das fronteiras agrícolas levou a uma significativa diminuição da cobertura florestal, o que resultou em perda de biodiversidade e na degradação de recursos fundamentais, como solo e água (Sartori et al., 2025; Turchetto et al., 2025). Atualmente, apenas cerca de 24% de toda a cobertura original da Mata Atlântica permanece, tornando esse bioma um dos mais ameaçados do mundo (Amaral et al., 2025).

Diante disso, a escolha de espécies nativas pioneiras mostra-se estratégica por exercer papel fundamental na estruturação inicial dos ecossistemas. Espécies pioneiras contribuem para o sombreamento e ciclagem de nutrientes, criando condições favoráveis ao estabelecimento de outras espécies (Brancalion et al., 2019). Isso reforça a necessidade de criar e adotar soluções inovadoras capazes de aumentar sua taxa de sobrevivência e que acelerem seu crescimento inicial em áreas destinadas à restauração (Adenesky-Filho; Maçaneiro; Vitorino, 2017).

1.2 MUDANÇAS CLIMÁTICAS E DÉFICIT HÍDRICO EM ESPÉCIES ARBÓREAS NATIVAS

O sucesso da restauração de ecossistemas florestais via plantio de mudas depende diretamente do estabelecimento e da sobrevivência delas após o plantio. Porém, em áreas degradadas, as condições ambientais frequentemente são inóspitas

ao desenvolvimento vegetal, sobretudo por conta da baixa cobertura do dossel, da compactação do solo e da redução na capacidade de retenção de água, fatores que facilitam o aumento da temperatura e da evapotranspiração do solo (Axelsson et al., 2020; Souza e Engel, 2024). Além disso, as mudanças climáticas têm intensificado a frequência e duração dos períodos de seca em muitas regiões, tornando o déficit hídrico (DH) um dos principais fatores limitantes à sobrevivência das plantas (Chiang et al., 2021).

Para espécies arbóreas, esse cenário é particularmente preocupante durante a fase inicial de estabelecimento das mudas, quando as raízes ainda são pouco desenvolvidas e a capacidade de explorar o solo ainda é limitada. Sob essas condições de baixa disponibilidade hídrica, o crescimento se torna comprometido, reduz a sobrevivência das plantas e pode comprometer o sucesso de programas de restauração (Ovalle et al., 2015; Grossnickle, 2018).

O DH ocorre quando a oferta de água disponível para a planta é insuficiente para atender à demanda transpiratória, resultando em uma redução no conteúdo de água nos tecidos (Swann et al., 2018). Esta condição é consequência da diminuição do potencial hídrico do solo, que reduz o gradiente de potencial de água responsável pelo fluxo dela no sistema solo-planta-atmosfera (Marusig et al., 2020). Como resultado, tem-se a diminuição da absorção de água pelas raízes, redução do potencial hídrico da planta e comprometimento de diversos processos metabólicos essenciais ao crescimento e à sobrevivência vegetal (Souza e Engel, 2024).

Após a percepção da redução da disponibilidade hídrica, uma rede de respostas fisiológicas, bioquímicas, anatômicas e moleculares é desencadeada a fim de minimizar a perda de água e manter a integridade celular (Fang e Xiong, 2014; Khanna, 2024). Entre as principais respostas ao DH, destaca-se o aumento da síntese de ácido abscísico (ABA), que promove o fechamento estomático, reduzindo a transpiração e a perda de água pelas folhas (Gupta et al., 2020a).

Embora o fechamento estomático seja um importante mecanismo de conservação de água em situações de DH, essa resposta limita a difusão de CO₂ para o interior da folha, reduzindo a atividade da enzima Rubisco e consequentemente a assimilação líquida de carbono (Gupta et al., 2020a). A diminuição da fotossíntese resulta em menor produção de fotoassimilados e reduz o crescimento da planta, afetando negativamente a produção de biomassa e o estabelecimento em campo (Khanna, 2024).

Em paralelo, a redução da fixação de carbono compromete também a etapa fotoquímica da fotossíntese, favorecendo o desvio de elétrons para o oxigênio, gerando espécies reativas de oxigênio (EROs). Como consequência, ocorre o acúmulo de EROs, incluindo ânion superóxido ($\bullet\text{O}_2^-$), peróxido de hidrogênio (H_2O_2), radical hidroxila ($\bullet\text{OH}$) e oxigênio singleto ($^1\text{O}_2$), caracterizando um estresse oxidativo (Anjum et al., 2011; Alam et al., 2026).

Embora pequenas quantidades de EROs desempenhem funções importantes na sinalização celular, sua produção excessiva provoca danos oxidativos a lipídeos, proteínas, pigmentos fotossintéticos e ácidos nucleicos, comprometendo a integridade estrutural e funcional das células vegetais (Anjum et al., 2011; Hussain et al., 2019; Gupta et al., 2020b).

Para limitar esses danos, as plantas ativam mecanismos antioxidantes altamente eficientes, compostos por sistemas enzimáticos e não enzimáticos, que determinam se o dano ocorrerá ou não, e caso ocorra, em que gravidade (Swann et al., 2018). Entre as principais enzimas antioxidantes destacam-se a superóxido dismutase, catalase, ascorbato peroxidase e glutathione redutase (Esposito et al., 2018). Além disso, mecanismos fotoprotetores, como a dissipação do excesso de energia luminosa na forma de calor, fluxo cíclico de elétrons no fotossistema I e a fotorrespiração, também contribuem para diminuir a formação de EROs, preservar a integridade do aparato fotossintético e evitar a fotoinibição crônica (Orabi e Abou-Hussein, 2019; Gupta et al., 2020b).

Além das alterações fisiológicas e bioquímicas, o DH promove importantes modificações morfológicas. A redução da expansão celular, por exemplo, leva à diminuição da área foliar, ao menor crescimento da parte aérea e à menor biomassa da planta toda (Hasanuzzaman et al., 2021). A senescência e a abscisão foliar também são estratégias para reduzir a superfície transpirante, assim como a menor área foliar. Em contrapartida, muitas espécies direcionam recursos energéticos para o desenvolvimento das raízes, estratégia que favorece o aumento da superfície de contato da planta com o solo, melhorando a performance da planta ao explorá-lo e favorecendo a absorção de água (Hasanuzzaman et al., 2021; Gupta et al., 2020a).

Além disso, muitas espécies realizam o chamado ajuste osmótico, promovido pelo acúmulo de solutos osmoticamente ativos, como prolina e glicina-betaína, que auxiliam no ajuste osmótico da planta e favorecem a manutenção da absorção de água mesmo diante do baixo potencial hídrico do solo (Per et al., 2017; Alam et al.,

2026).

A intensidade dessas respostas frente ao DH varia entre espécies. De modo geral, as plantas classificadas como isohídricas são aquelas que fecham rapidamente os estômatos em resposta à mudança na disponibilidade hídrica, enquanto espécies anisohídricas mantêm os estômatos abertos por mais tempo (Chen et al., 2019).

Espécies isohídricas são capazes de manter relativamente constante o potencial hídrico foliar, em detrimento da absorção líquida de CO₂ e da taxa fotossintética, enquanto as anisohídricas adotam a estratégia inversa, sustentando a assimilação de carbono às custas de um maior risco de falha hidráulica, que acontece quando a perda de água pela transpiração é maior do que a absorção pelas raízes, resultando em cavitação (Chen et al., 2019; Li et al., 2020).

Essas duas respostas integram, respectivamente, as hipóteses de inanição de carbono (*carbon starvation*) e de falha hidráulica (*hydraulic failure*), propostas como os dois principais mecanismos fisiológicos que levam plantas à morte quando estão sob DH (McDowell et al., 2008). No entanto, essa classificação representa, na verdade, um contínuo fisiológico, e diferentes estratégias podem coexistir em função da espécie, das condições ambientais e da intensidade do estresse (Tomasella et al., 2019; Li et al., 2020).

1.3 ÓXIDO NÍTRICO COMO MOLÉCULA SINALIZADORA EM PLANTAS

O óxido nítrico (NO) é uma molécula sinalizadora gasosa que desempenha um papel crucial no metabolismo vegetal, regulando processos que vão desde a germinação de sementes até estágios mais avançados, como floração e senescência (Nabi et al., 2019; Kolbert, Lindermayr e Loake, 2021). Essa versatilidade é resultado de sua natureza química, uma vez que o NO é uma espécie reativa de nitrogênio radical livre, caracterizada pela presença de um elétron desemparelhado em seu orbital π^* , que lhe confere alta reatividade e instabilidade (Sahay, Gupta, 2017).

Essas características químicas levaram a uma série de estudos focados em sua caracterização físico-química, essenciais para entender o comportamento do NO no ambiente celular. Sob a perspectiva físico-química, o NO é um gás incolor com uma meia-vida extremamente curta no ambiente celular (geralmente inferior a 6 segundos), altamente difusível em meio aquoso, apresentando coeficiente de difusão de aproximadamente $4,8 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ em H₂O, além de ser lipofílico, o que favorece

sua rápida passagem pelas membranas plasmáticas (Sahay, Gupta, 2017; Singhal et al., 2021; Sedlářová et al., 2025). Essas propriedades permitem que o NO atue como um mensageiro de curta distância, desencadeando respostas rápidas e localizadas (Gupta et al., 2022). Além disso, ele estabelece interações complexas com espécies reativas de oxigênio (EROs) e grupos tióis de proteínas, por meio da formação de compostos como S-nitrosotióis (RSNOs). Essa rede de interações químicas demonstra a versatilidade do NO e explica seu papel como um dos principais reguladores de crescimento vegetal (Campos et al., 2023; Sedlářová et al., 2025).

Os mecanismos envolvidos na síntese endógena de NO em plantas ainda são objeto de discussão. Sabe-se que a produção e sinalização desse gás estão fortemente relacionadas a organelas como mitocôndrias, cloroplastos e peroxissomos, que atuam como centros de regulação de espécies reativas e de sinalização celular (Kolbert, Lindermayr e Loake, 2021). Em plantas, o NO pode ser produzido por diferentes vias, tanto enzimáticas quanto não enzimáticas, variando conforme as condições ambientais e os estímulos fisiológicos.

Dentre essas rotas, a via redutiva, em que o NO é formado a partir da redução do nitrato, é a mais amplamente caracterizada e comprovada (Gupta et al., 2020b; Sedlářová et al., 2025). Nessa rota, a enzima nitrato redutase (NR) catalisa a redução de nitrato a nitrito, utilizando NADH como doador de elétrons, necessitando também de molibdopterina, heme e flavina adenina dinucleotídeo como coenzimas. Sob determinadas condições, como hipóxia, anóxia, baixo pH ou infecção por patógenos, a NR pode atuar como uma redutase alternativa, produzindo o NO a partir do nitrito (Gupta et al., 2022).

Assim, a produção de NO constitui tanto um mecanismo bioquímico relevante quanto a base para sua função como molécula sinalizadora. Nesse contexto, o NO desempenha um papel na regulação do metabolismo dos ácidos graxos nitrados, que participam da sinalização celular e podem desencadear respostas como o fechamento estomático em plantas (Gupta et al., 2022). Além disso, um dos principais mecanismos pelos quais o NO atua como sinalizador é por meio de modificações pós-traducionais de proteínas. A nitração é a adição de um grupo nitro em determinados aminoácidos, como resíduos de tirosina e triptofano, o que regula dinamicamente a atividade proteica e serve como um biomarcador de estresse nitrosativo (Romera et al., 2023).

Outra modificação pós-traducional importante é a S-nitrosação, que configura o mecanismo central de transdução de sinal mediado por essa molécula. A S-

nitrosação é uma modificação de natureza rápida, reversível e seletiva, caracterizada pela formação de uma ligação covalente S-NO no grupo tiol (-SH) de resíduos da cisteína-alvo, resultando na formação de RSNOs (Nabi et al., 2019; Sedlářová et al., 2025). Ela é vista como um processo não enzimático, mediado diretamente pelo NO ou indiretamente por doadores de NO. A especificidade da reação é determinada pela estrutura da proteína-alvo e pela concentração de NO nas proximidades (Romera et al., 2023).

Essa modificação afeta tanto a estrutura quanto a função de proteínas, influenciando desde a atividade enzimática (ativação ou inibição) até interações com outras proteínas, e desempenha papel crucial na regulação de processos fisiológicos e na resposta a estresses, pois permite modulação de inúmeras proteínas em diferentes compartimentos celulares e permite uma sinalização ampla, específica e dinâmica. Isso aumenta a complexidade e a flexibilidade da resposta celular (Astier; Lindermayr, 2012; Kolbert, Lindermayr e Loake, 2021).

Essas alterações moleculares dão ao NO um papel crucial na tolerância de plantas a condições adversas. Em situações de estresse abiótico, como déficit hídrico, salinidade e altas temperaturas, o NO exerce papel central na regulação da tolerância das plantas, contribuindo para minimizar os efeitos deletérios desses fatores ambientais (Seabra et al., 2022; Campos et al., 2023). As alterações mediadas por essa molécula afetam vários processos fisiológicos, incluindo o controle da abertura estomática, contribuindo para a redução da perda de água por transpiração (Nabi et al., 2019); o aumento da eficiência fotossintética, com maior conteúdo de clorofilas e acúmulo de biomassa (Oliveira et al., 2016; Do Carmo et al., 2025); a promoção da formação de raízes laterais, adventícias e pelos absorventes, além do crescimento da raiz principal (Lombardo; Lamattina, 2018; Do Carmo et al., 2021); a redução de danos oxidativos, por meio da eliminação de EROs, maior atividade de enzimas antioxidantes e síntese de compostos protetores (Gan et al., 2015; Wang et al., 2016); e a manutenção do status hídrico, fundamental para a sobrevivência em condições adversas (Silveira et al., 2016; Do Carmo et al., 2025).

No entanto, apesar de seu amplo envolvimento em respostas fisiológicas, as mesmas propriedades físico-químicas que fazem do NO um sinalizador eficiente também constituem um desafio para sua utilização prática. Devido às suas propriedades químicas, a aplicação direta em plantas é dificultada, o que reforça a necessidade do desenvolvimento de estratégias que permitam sua entrega de forma

controlada e eficaz, abrindo espaço para o uso de moléculas doadoras de NO como ferramentas promissoras tanto em pesquisas experimentais quanto em aplicações agrícolas e florestais (Seabra et al., 2022).

1.4 MOLÉCULAS DOADORAS DE NO

Uma estratégia eficiente para aumentar os níveis endógenos de NO e induzir respostas é a aplicação exógena de moléculas doadoras de NO. Além disso, essa abordagem farmacológica tem sido amplamente utilizada para esclarecer os mecanismos de ação envolvidos na sinalização mediada pelo NO (Nabi et al., 2019; Silveira et al., 2021).

Atualmente, são conhecidas diferentes classes de moléculas doadoras de NO, diferenciadas em relação à estrutura química, à cinética de liberação de NO e à meia-vida. No entanto, a eficiência desses compostos pode variar de acordo com fatores ambientais, como exposição à luz e variação de temperatura, além da concentração aplicada, o que afeta diretamente o padrão de liberação de NO (Seabra et al., 2015; Silveira et al., 2021). Além disso, alguns doadores de NO podem gerar subprodutos tóxicos para as células durante a sua decomposição. Por exemplo, um dos doadores de NO amplamente utilizados, o nitroprussiato de sódio, é um complexo inorgânico que gera cianeto durante sua decomposição (Calabrese e Agathokleous, 2023).

Dentre as diferentes classes de doadores de NO, entre as classes mais promissoras estão os S-nitrosotióis (RSNOs), como a S-nitrosoglutathiona (GSNO), S-nitroso-N-acetilcisteína (SNAC) e o ácido S-nitrosomercaptosuccínico (S-nitroso-MSA) (Oliveira et al., 2016). Esses doadores de baixo peso molecular apresentam instabilidade térmica e fotoquímica, características que afetam diretamente sua taxa de decomposição e, por consequência, a liberação de NO. A liberação de NO através dessas moléculas se dá pela quebra da ligação entre o S-NO, sem que haja produção de subprodutos tóxicos durante sua decomposição (Seabra e Oliveira, 2016; Silveira et al., 2021; Seabra et al., 2022).

A GSNO é um dos RSNOs de baixo peso molecular mais abundantes nas células vegetais e é considerada um dos principais reservatórios intracelulares de NO, além de atuar como um intermediário para o transporte de NO a longas distâncias. Em contraste com os outros RSNOs citados anteriormente, a GSNO ocorre de

maneira natural nas células vegetais, enquanto os demais são compostos artificiais (Jahnová, Luhová, Petřivalský, 2019; Romera et al., 2023). A GSNO é formada de forma não enzimática, por meio da reação entre o NO e a glutathiona reduzida (GSH), um dos tióis mais abundantes na célula. A ligação covalente entre o NO e a GSH ocorre de maneira rápida e eficiente, resultando na formação da GSNO (Frunghillo e Spoel, 2017; Jahnová, Luhová, Petřivalský, 2019).

Os níveis intracelulares de GSNO no metabolismo vegetal são controlados pela enzima GSNO redutase (GSNOR), uma desidrogenase alcoólica que converte GSNO em glutathiona oxidada (GSSG) e amônia (NH_3). Além dessa decomposição enzimática catalisada pela GSNOR, a GSNO também pode ser decomposta de forma não enzimática, liberando NO e GSSG na presença de agentes redutores, como a própria GSH (Romera et al., 2023).

Embora os doadores de NO tenham um grande potencial para uso em plantas, sua aplicação prática pode ser dificultada devido à instabilidade dessas moléculas. Elas são sensíveis a fatores ambientais como pH, luminosidade, temperatura e presença de íons metálicos, que podem acelerar sua decomposição e resultar em liberação rápida e excessiva de NO (Seabra et al., 2015). Essa liberação em excesso pode causar toxicidade, pois o NO pode reagir com outras moléculas, com o radical superóxido e formar peroxinitrito (ONOO^-), que pode provocar estresse nitrosativo e danos às membranas celulares (Corpas et al., 2013; Gupta et al., 2020b).

1.5 NANOTECNOLOGIA PARA LIBERAÇÃO CONTROLADA DE COMPOSTOS BIOATIVOS

Embora haja divergências na literatura sobre sua definição, a nanotecnologia é considerada um campo da ciência, tecnologia e engenharia de materiais dedicado à criação, design e uso de estruturas e instrumentos na escala nanométrica (Srii, 2023).

Nos últimos anos, o uso de nanomateriais tem se tornado cada vez mais relevante em vários campos, incluindo medicina, eletrônica, processamento de alimentos e agricultura. Os nanomateriais (NMs) possuem uma grande área superficial em relação ao volume, o que lhes proporciona propriedades únicas, como alta capacidade de interação com células e um grande potencial de uso como bioestimulantes (Gann et al., 2019; Pereira et al., 2021).

De forma simplificada, os NMs podem ser classificados em quatro categorias, sendo elas: 0D, caracterizada por nanopartículas (NPs) com todas as dimensões menores que 100 nm; 1D, com uma dimensão maior que 100 nm, como as nanofibras, nanotubos, nanofios e nanobastões; 2D, com duas dimensões maiores que 100 nm, sendo as nanocamadas, grafeno, nanorrevestimentos e nanofilmes; e 3D, com três dimensões maiores que 100 nm, como as zeólitas, materiais porosos, pós e dispersões (Tortella et al., 2023).

De acordo com Pereira et al. (2021), as NPs podem ser divididas em dois grupos: NPs ativas e sistemas nanocarreadores de liberação controlada. NPs ativas são aquelas que causam efeito biológico, funcionando como bioestimulantes, antipatógenos ou uma combinação de ambos. Os nanocarreadores com propriedades de liberação controlada são sistemas em que as NPs transportam um composto ativo de interesse, oferecendo proteção contra a degradação além de permitir liberação controlada e prolongada.

Nos últimos anos, o uso de nanocarreadores contendo moléculas doadoras de NO em plantas tem recebido destaque, graças ao papel do NO como molécula sinalizadora (Silveira et al., 2021; Seabra et al., 2022). Contudo, a eficácia da utilização de nanocarreadores está diretamente relacionada à cinética de liberação do NO, ou seja, à velocidade e ao tempo em que essa molécula é fornecida às células vegetais (Yang et al., 2021). Essa cinética é afetada por diversos fatores, como o tipo de nanocarreador utilizado, o mecanismo de liberação de NO e as condições ambientais às quais as plantas estão submetidas (Seabra et al., 2022; Preisler et al., 2025).

Diferentes tipos de nanocarreadores podem ser utilizados na entrega de NO, afetando significativamente a cinética de liberação da molécula. Além disso, a encapsulação da molécula de interesse pode ser feita com diferentes tipos de matrizes, o que gera partículas de diversos tamanhos, dependendo da técnica empregada na síntese (Campos et al., 2023). Entre as matrizes mais utilizadas estão os polímeros naturais e sintéticos, a sílica e os metais (Tortella et al., 2023).

Atualmente, há uma tendência a optar por métodos e materiais que estejam alinhados com a síntese verde (Jiménez-Arias et al., 2020). Nesse sentido, os biopolímeros têm sido amplamente empregados em processos de nano e microencapsulação, graças às suas propriedades de biodegradabilidade, biocompatibilidade e baixa toxicidade para os seres vivos (Jiménez-Arias et al., 2020;

Campos et al., 2023). Essa diversidade estrutural amplia as oportunidades de uso da nanotecnologia em plantas, especialmente em casos de restauração florestal, pois permite a liberação controlada de compostos bioativos em condições ambientais complexas.

A quitosana (CS) é o segundo biopolímero mais abundante na natureza, superado apenas pela celulose. Sua obtenção ocorre através da desacetilação da quitina, um componente presente no exoesqueleto de crustáceos, insetos e na parede celular de fungos (Seabra et al., 2014; Yu et al., 2021). As propriedades físico-químicas e biológicas da CS são amplamente reconhecidas, assim como seu baixo custo. Trata-se de um polímero catiônico, formado pela repetição do monômero β -1,4-glucosamina, possuindo carga positiva, sendo biodegradável, biocompatível, de baixa toxicidade, sensível ao pH e apresentando atividade antimicrobiana, antifúngica e capacidade elicitora (Riseh et al., 2024).

Essas propriedades fazem da CS um material altamente relevante para o desenvolvimento de sistemas nanocarreadores. Isso se deve ao fato de que a CS, com sua grande área superficial específica, carga positiva e propriedades mucoadesivas, facilita o transporte de compostos ativos, potencializa a bioatividade e melhora a interação com células vegetais (Riseh et al., 2024; Nandhini et al., 2025). Além disso, as interações eletrostáticas fortes entre a matriz de CS e as moléculas bioativas contribuem para a elevada eficiência de encapsulação de compostos instáveis, como os doadores de NO (Seabra et al., 2014; Pelegrino et al., 2017).

Portanto, o uso da CS como matriz polimérica para a síntese de NPs é estratégico, pois combina relevância, abundância, viabilidade econômica e sustentabilidade. Assim, possibilita a formulação de nanocarreadores seguros e eficientes e com aplicações que vão desde a medicina até a agricultura e restauração florestal (Seabra et al., 2015; Yu et al., 2021; Nandhini et al., 2025).

1.6 NANOPARTÍCULAS DE QUITOSANA COMO CARREADORAS DE DOADORES DE NO

Nos últimos anos, a utilização das CS NPs como sistemas carreadores de moléculas doadoras de NO tem avançado de forma significativa, com resultados consistentes em diferentes espécies e condições experimentais (Lopes-Oliveira et al., 2019; Silveira et al., 2021; Methela et al., 2023). Mais recentemente, a aplicação

prática vem ganhando espaço em trabalhos focados em espécies arbóreas nativas da Mata Atlântica, como *Heliocarpus popayanensis* (jangadeiro), *Cariniana estrellensis* (jequitibá-branco) e *Cecropia pachystachya* (embaúba-do-brejo) (Silveira et al., 2021; Do Carmo et al., 2021; Seabra et al., 2022; Do Carmo et al., 2025).

Em um estudo pioneiro na aplicação das CS NPs como sistemas carreadores e liberadores de NO, Oliveira et al. (2016) verificaram o efeito da aplicação dessas CS NPs contendo o doador S-nitroso-MSA sobre plantas de milho (*Zea mays*) submetidas ao estresse salino. Os resultados demonstraram uma melhora na atividade do fotossistema II, no teor de clorofila e no acúmulo de biomassa, reflexo da liberação lenta e controlada de NO.

Silveira et al. (2021) investigaram os efeitos da aplicação de CS NPs carregadas com diferentes doadores de NO em plantas de cana-de-açúcar (*Saccharum officinarum*) submetidas ao déficit hídrico. Foram avaliadas aplicações foliares de CS NPs contendo S-nitroso-MSA, GSNO, SNAC e nitroprussiato de sódio (SNP). Os resultados mostraram que a pulverização de SNAC foi capaz de atenuar parcialmente os efeitos negativos da falta de água sobre a taxa fotossintética, manteve a eficiência no uso da água em níveis semelhantes às plantas bem hidratadas e promoveu crescimento mesmo sob estresse. Além disso, as pulverizações com SNAC e GSNO melhoraram a fotossíntese durante o período de recuperação das plantas. Já a aplicação de SNP não foi eficaz em nenhum dos parâmetros mensurados e levou a danos oxidativos após a reidratação.

Em seu estudo com soja (*Glycine max*) submetida ao déficit hídrico, Methela et al. (2023) verificaram que a aplicação das CS NPs contendo GSNO promoveu efeitos benéficos. Plantas tratadas via solo e foliar com 50 μM da nanoformulação e submetidas à falta de água permaneceram com a cor verde e maior porte. Em concentrações mais elevadas (75 e 100 μM) as plantas apresentaram efeitos positivos apenas no comprimento das raízes. Em plantas não estressadas, a concentração de 50 μM diminuiu o extravasamento de eletrólitos em relação às plantas não tratadas, indicando efeitos benéficos da nanoformulação na integridade da parede celular. De maneira geral, os dados mostram menor conteúdo de prolina e peroxidação lipídica e maior acúmulo de pigmentos fotossintéticos em plantas que receberam as NPs contendo GSNO sob déficit hídrico.

No estudo de Preisler et al. (2025), sementes de soja foram tratadas com GSNO nanoencapsulada em duas matrizes poliméricas: CS e alginato. As CS NPs

apresentaram potencial zeta positivo, menor tamanho e liberaram o NO mais rapidamente em comparação às NPs de alginato, que possuem potencial zeta negativo. Quanto aos efeitos observados, o tratamento das sementes com as CS NPs aumentou significativamente a porcentagem de germinação, o comprimento da raiz, o número de raízes secundárias, a massa seca das raízes e dos nódulos em relação ao controle não tratado. Já as NPs de alginato contribuíram somente para o aumento da massa seca da parte aérea e do número de raízes secundárias.

Embora a maioria das primeiras pesquisas com CS NPs contendo doadores de NO tenha se concentrado em espécies cultivadas de interesse agrônomo, nos últimos anos essa tecnologia também tem sido avaliada em espécies arbóreas nativas da Mata Atlântica, ampliando as possibilidades de aplicação à conservação e à restauração ecológica (Do Carmo et al., 2021).

Lopes-Oliveira et al. (2019) avaliaram os efeitos de S-nitroso-MSA em mudas de espécies arbóreas nativas da Mata Atlântica cultivadas em viveiro sob sol pleno, tanto em sua forma livre quanto nanoencapsulada. Os resultados mostraram que o doador nanoencapsulado em CS NPs aumentou a estabilidade do doador de NO, diminuindo sua degradação, além de aumentar o crescimento das mudas de *H. popayanensis*, uma espécie pioneira. Para a espécie *C. estrellensis*, não pioneira, a aplicação do S-nitroso-MSA, tanto livre quanto nanoencapsulado, não resultou em diferenças nos parâmetros avaliados. No entanto, foi possível observar aumento no conteúdo de RSNOs foliares, especialmente nas plantas tratadas com CS NPs contendo o doador.

Em um estudo com *H. popayanensis*, Do Carmo et al. (2021) também avaliaram o uso de S-nitroso-MSA livre e nanoencapsulado em condições de déficit hídrico. De acordo com os resultados obtidos, a nanoencapsulação levou à liberação mais lenta e controlada de NO, o que levou a níveis de RSNOs maiores tanto nas folhas quanto nas raízes. Além disso, em comparação com sua forma livre, o doador nanoencapsulado em CS NPs impediu a redução da fotossíntese de forma mais rápida e com um menor número de aplicações. Ainda, o tratamento com CS NPs contendo S-nitroso-MSA elevou o conteúdo relativo de água das folhas das plantas estressadas, estimulou a formação de pelos absorventes e reduziu o estresse oxidativo nas raízes.

Outros estudos avaliaram um maior número de espécies, na fase de aclimação das plantas. Guariz et al. (2025) avaliaram o efeito da GSNO, livre e em

CS NPs, na aclimação de mudas de três espécies arbóreas: *Hymenaea courbaril* (jatobá), *Amburana cearensis* (amburana-de-cheiro) e *Hymenaea stigonocarpa* (jatobá-do-cerrado). De maneira geral, os tratamentos com GSNO, independentemente da forma, beneficiaram o desenvolvimento inicial das mudas, melhorando a fotossíntese, condutância estomática e acúmulo de biomassa, quando comparados ao controle. Porém, embora não tenham sido observadas diferenças significativas entre as formas, o uso de CS NPs contendo GSNO em concentrações mais elevadas (200 μ M) mostrou maior potencial para beneficiar a aclimação das mudas, destacando a aplicabilidade da tecnologia em programas de restauração.

Mais recentemente, a aplicação das CS NPs contendo GSNO foi avaliada em duas espécies submetidas a déficit hídrico de longo e curto prazo (Do Carmo et al., 2025). Esse artigo corresponde ao Capítulo 1 desta tese. A espécie *C. pachystachya* se mostrou responsiva à aplicação de 50 μ M GSNO nanoencapsulada quando submetida a déficit hídrico de curta e longa duração. Isso refletiu em aumento na condutância estomática, taxa fotossintética e potencial hídrico, além de aumentar a biomassa de raízes e folhas e estimular a formação de pelos absorventes. Esses efeitos não foram registrados quando se utilizou GSNO ou CS NPs sem NO. Em contrapartida, não foram detectados benefícios dos tratamentos em *C. estrellensis* em nenhuma das concentrações testadas (25 a 800 μ M), o que corrobora os resultados de Lopes-Oliveira et al. (2019) e indica que a resposta à GSNO nanoencapsulada depende da espécie.

Semelhantemente, a aplicação de CS NPs com GSNO em mudas de *Araucaria angustifolia* (araucária), uma gimnosperma de grande importância cultural para o país e que está criticamente ameaçada de extinção, sob déficit hídrico promoveu uma melhoria significativa na tolerância ao estresse (Silva et al., 2026). Isso ficou evidenciado pela redução da peroxidação lipídica e das EROs, assim como pelo aumento da atividade de enzimas antioxidantes e pela elevação dos níveis de S-nitrosotióis. Além disso, o tratamento favoreceu o crescimento das mudas indicando que a GSNO nanoencapsulada atua como um eficiente modulador da resposta antioxidante e do desempenho morfofisiológico sob condições de escassez hídrica até mesmo em espécies de diferentes divisões.

Embora progressos tenham acontecido na utilização das CS NPs contendo doadores de NO em espécies arbóreas nativas da Mata Atlântica e efeitos promissores tenham sido observados, todos os estudos até o momento foram

conduzidos em casa de vegetação ou viveiro. Portanto, testes em condições de campo são essenciais para validar a aplicabilidade prática dessa tecnologia em programas de restauração e silvicultura de espécies nativas.

2 OBJETIVOS E HIPÓTESES

2.1 OBJETIVO GERAL

Avaliar as respostas fisiológicas e de crescimento de espécies arbóreas nativas da Mata Atlântica à aplicação de nanopartículas de quitosana contendo S-nitrosoglutationa (CS NPs GSNO), considerando a resposta ao déficit hídrico em condições controladas, o desempenho inicial de *Cecropia pachystachya* após o plantio em campo e a variabilidade interespecífica da condutância estomática.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar os efeitos da aplicação das CS NPs GSNO em duas espécies nativas com estratégias ecológicas distintas, *Cecropia pachystachya* (pioneira) e *Cariniana estrellensis* (não pioneira), submetidas a déficit hídrico de longo prazo (Capítulo 1).
- Avaliar o desempenho de *C. pachystachya* tratada com CS NPs GSNO em condições de campo e estimar o custo de produção da formulação e o custo correspondente ao protocolo por muda (Capítulo 2).
- Revisar os mecanismos moleculares envolvidos na dualidade do NO na regulação estomática e caracterizar, por meio de uma triagem, a variabilidade interespecífica da condutância estomática em resposta às CS NPs GSNO em espécies arbóreas nativas da Mata Atlântica (Capítulo 3).

2.3 HIPÓTESES

- Muda de *C. pachystachya* (pioneira) responde de forma mais positiva às CS NPs GSNO sob déficit hídrico do que muda de *C. estrellensis* (não pioneira). Como hipótese interpretativa, essa diferença estaria ligada à maior plasticidade fisiológica das espécies pioneiras, o que deve ser testado com várias espécies por grupo sucessional.

- A aplicação de CS NPs GSNO melhora o desempenho inicial de *C. pachystachya* após o plantio em campo. Além disso, espera-se que o custo de produção da formulação por muda seja baixo.
- A resposta estomática de espécies nativas da Mata Atlântica às CS NPs GSNO é espécie-específica, refletindo a natureza contexto-dependente do NO já documentada na literatura.

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4 CAPÍTULO 1 – EFFECTS OF NANOENCAPSULATED NITRIC OXIDE DONOR ON *Cecropia pachystachya* TRÉCUL AND *Cariniana estrellensis* (RADDI) KUNTZE SEEDLINGS SUBJECTED TO LONG-TERM WATER DEFICIT

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Nota: Este capítulo é baseado no artigo publicado acima. A publicação original apresenta resultados referentes a experimentos conduzidos sob DH de curto e longo prazo. Para atender aos objetivos desta tese, foram mantidos apenas os resultados relativos ao experimento de longo prazo, enquanto as demais informações foram retiradas por não integrarem o período da realização deste trabalho. As adaptações realizadas não alteram as conclusões referentes ao experimento de longo prazo.

Effects of nanoencapsulated nitric oxide donor on *Cecropia pachystachya* Trécul and *Cariniana estrellensis* (Raddi) Kuntze seedlings subjected to long-term water deficit

4.1 ABSTRACT

Nitric oxide (NO), a critical signaling molecule in plants, plays a protective role against water deficit (WD). However, its application is hindered by its relatively unstable chemical nature. To address this, researchers have explored the nanoencapsulation of NO donor molecules. The study aimed to evaluate the effects of treatments using chitosan nanoparticles (NPs) containing the NO donor S-nitrosoglutathione (GSNO) on neotropical tree seedlings (*Cecropia pachystachya* and *Cariniana estrellensis*) exposed to long-term WD in a greenhouse. Seedlings under long-term WD received nanoformulations in the substrate three times at ten-day intervals. The treatment with NPs containing GSNO (50 μ M) increased the stomatal conductance, photosynthetic rate, and plant water potential of *C. pachystachya* submitted to WD, reaching levels similar to those of plants kept at field capacity. The same effects were not induced by free GSNO and NPs without NO. GSNO-loaded NPs also increased root and leaf biomass in comparison to other WD treatments and increased the amount and incidence of root hairs. In contrast, *Cariniana estrellensis* seedlings did not respond to the application of NPs containing GSNO at the tested concentrations (from 25 to 800 μ M). Results suggest that nanoencapsulated GSNO can protect *C. pachystachya* seedlings in WD conditions, highlighting the potential for obtaining drought-tolerant tree seedlings in reforestation programs. However, this action is species-dependent, as no effect was induced in *C. estrellensis*.

Keywords: Nanotechnology; Reforestation; Chitosan; Stomatal conductance; Water potential; Root hairs.

Author Contribution Statement

G.C.C.: conceptualization, formal analysis, investigation, methodology, and Writing – original draft. J.V.S.M.: formal analysis, investigation, methodology. A.C.P.: methodology. J.D.O.L.: methodology. R.H.P.: methodology. J.C.P.: conceptualization, methodology, and Writing – original draft. R.S.M.: conceptualization. J.A.P.: conceptualization, methodology, and supervision. A.B.S.: conceptualization, formal analysis, investigation, methodology, funding acquisition, and Writing – review and editing. H.C.O.: conceptualization, formal analysis, investigation, methodology, supervision, funding acquisition, and Writing – review and editing. All authors read and approved the final version of the manuscript.

Declaration of competing interest

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.

Key Message

Low concentrations of nanoencapsulated NO donor protect tree seedlings from drought, enhancing photosynthesis, stomatal conductance, root hair growth, and stem water potential, but the effects are species-dependent.

4.2 INTRODUCTION

Climate change resulting from global warming directly impacts the environment, leading to extreme weather patterns, such as alterations in the rainfall regime, resulting in increasingly frequent and long droughts. Furthermore, the intensity of drought events is expected to double by the end of this century, resulting in approximately two-thirds of the global population experiencing water scarcity (Zeng et al., 2021). Drought can negatively interfere with the survival of plant species in natural environments, so the ecological relationships of the future will depend on how plants respond to climate change (Ojima et al., 2021; Zandalinas et al., 2021; Zeng et al., 2021).

In this context, forest restoration with native tree species emerges as a critical and pressing measure to mitigate the impact of climate change, particularly in highly biodiverse tropical regions, where there is a growing demand for such initiatives (Calzavara et al., 2017; Philipson et al., 2020). This is because forest ecosystems play a crucial role in regulating carbon and water cycles through biomass accumulation and the formation of water vapor fluxes via evapotranspiration, especially in continental environments (Cunningham et al., 2015; Ellison et al., 2017; Macedo et al., 2021). In this scenario, comprehension of the responses of tree seedlings to abiotic stresses, such as water deficit (WD), and the development of strategies to enhance their tolerance and resilience are crucial for the success of restoration programs (Nolan et al., 2018; Philipson et al., 2020).

Under WD conditions, there is a restriction in gas exchange in plants due to increased levels of abscisic acid (ABA). This leads to stomatal closure, resulting in a reduced transpiration rate and photosynthesis, and consequently leading to impaired plant growth (Hasanuzzaman et al., 2017; Hasanuzzaman et al., 2021). Additionally, decreases in relative water content and water potential are commonly observed in plants due to soil water scarcity (Nolan et al., 2018; Gupta et al., 2020).

In recent years, studies have highlighted the efficiency of nitric oxide (NO) in protecting plants against extreme conditions (Seabra et al., 2022; Campos et al., 2023). NO is a small gaseous reactive nitrogen species that acts as a signaling molecule which is involved in a wide range of signal transduction pathways related to responses against abiotic stresses (Singhal et al., 2021; Farnese et al., 2016). Recent research emphasizes the crucial role of NO in various signaling networks in plants, where it can induce antioxidant system activation, regulate stomatal physiology, enhance photosynthetic efficiency, improve plant water status, and promote root growth and root hair formation (Campos et al., 2023; do Carmo et al., 2021; Iqbal et al., 2022; Singhal et al., 2021).

Due to the chemical nature of NO, exogenous NO donors are widely used to induce metabolic and physiological tolerance responses in plants under stressful conditions (Silveira et al., 2021). Among these donors, S-nitrosothiols (RSNOs), such as S-nitrosoglutathione (GSNO), are particularly important due to their low molecular weight, in spite of their sensitivity to light and heat (Campos et al., 2023; Silveira et al., 2021). One way to reduce the sensitivity of these NO donors is by incorporating them into nanomaterials, a technique developed to protect molecules from rapid degradation (Seabra et al., 2015; Silveira et al., 2021; Seabra et al., 2022). One of the most commonly used techniques is the incorporation of the donor into chitosan nanoparticles (NPs), as chitosan is a biodegradable, biocompatible polymer widely employed as a nanocarrier system (Silveira et al., 2021; Yu et al., 2021).

Several studies have demonstrated positive outcomes from the application of chitosan nanoparticles containing NO donors in plants under abiotic stress, including those subjected to WD conditions (Oliveira et al., 2016; Silveira et al., 2016; Nabi et al., 2019; Silveira et al., 2019; Lopes-Oliveira et al., 2019; do Carmo et al., 2021; Gomes et al., 2022; Methela et al., 2023).

Previous studies have tested the effect of NO-releasing NPs against abiotic stress in cultivated plants. Oliveira et al. (2016), in a pioneering study on the application

of NPs as NO carriers in plants, investigated the effect of chitosan NPs containing S-nitroso-mercaptosuccinic acid (S-nitroso-MSA) on maize plants subjected to saline stress. The results indicated slower and more controlled release of NO, resulting in protection against the negative effects of high salinity, including improved photosystem II activity, chlorophyll content, and plant growth.

More recently, Silveira et al. (2021) conducted a study in which well-watered sugarcane plants were compared with drought-stressed plants previously sprayed with different nanoencapsulated NO donors, namely S-nitroso-MSA, GSNO, S-nitroso-*N*-acetylcysteine (SNAC), and sodium nitroprusside (SNP). SNAC spraying partially mitigated the impact of drought on photosynthesis, maintained water use efficiency (WUE) at levels similar to well-watered plants, and promoted plant growth. SNAC and GSNO were also able to enhance photosynthesis during the plant recovery period from drought. The application of SNP was not effective in mitigating the effects of drought.

Considering neotropical species, studies have been conducted to test the application of nanoencapsulated donors against different stresses. Lopes-Oliveira et al. (2019) found that under light stress conditions, the application of chitosan NPs carrying S-nitroso-MSA favored the development of *Heliocarpus popayanensis* seedlings. However, for *Cariniana estrellensis*, there were no changes in seedling development with the application of NO-releasing NPs.

Do Carmo et al. (2021), in a study with *H. popayanensis*, found that nanoencapsulation in chitosan slowed the release of NO through the S-nitroso-MSA donor, resulting in higher RSNO levels in roots and leaves. Furthermore, even without being nanoencapsulated, the NO donor was able to prevent the drought-induced decline in photosynthesis in this species. However, NPs containing S-nitroso-MSA induced this effect more rapidly, with fewer applications. Additionally, only the nanoencapsulated donor was able to increase leaf relative water content and the formation of root hairs in drought-stressed plants.

The results presented by previous studies highlight the significant role played by NO in plants subjected to stress, underscoring the potential application of NO-releasing NPs in producing more drought-tolerant tree seedlings. However, it is essential to verify the protection against the harmful effects of drought in a larger number of species, from different successional groups. Therefore, the aim of the current study was to assess the protective effect of GSNO-loaded NPs on seedlings of native Atlantic Forest tree species widely used in reforestation programs, the pioneer

Cecropia pachystachya Trécul and the non-pioneer *Cariniana estrellensis* (Raddi) Kuntze, under long-term WD.

4.3 MATERIAL AND METHODS

4.3.1 SYNTHESIS AND CHARACTERIZATION OF THE FORMULATIONS

Chitosan NPs were obtained using the ionotropic gelation method (Oliveira et al., 2016). Initially, chitosan (CS) (2.6 mg mL^{-1}) was dissolved in 1% acetic acid and added to the glutathione (GSH) (40 mM) solution. The mixture was stirred magnetically for 90 min, followed by dropwise addition of sodium tripolyphosphate (TPP) (0.6 mg mL^{-1}), following the 3 CS/GSH:1 TPP volumetric ratio. The final mixture was stirred for an additional 45 min at room temperature, resulting in a suspension of CS NPs containing 40 mM GSH.

Prior to application on the plants, an equimolar amount of sodium nitrite (NaNO_2), related to GSH, was added to the NP suspension to allow the GSH nitrosation and the formation of GSNO. The suspension was kept in the dark at 10°C for 1 h, before being diluted in distilled water to achieve the desired concentrations of GSNO.

Chitosan nanoparticles were characterized by several techniques. Dynamic light scattering (DLS) analysis (Nano ZS Zetasizer, Malvern Instruments Co, Malvern, UK) was employed to measure the hydrodynamic size, polydispersity index (PDI), and zeta potential. Measurements were performed in folded capillary zeta cells with a 10 mm path length and using a fixed angle of 173° (Gomes et al., 2021). Nanoparticle Tracking Analysis (NTA) was obtained using a NanoSight NS300 (Malvern Instruments Co, Malvern, UK). Samples were diluted 1000-fold in water, injected into the equipment, and individually tracked. Results were automatically calculated using NTA 3.4 Build 3.4.4 software. Solid-state size was measured by high-resolution transmission electron microscopy (HRTEM, Talos, Thermo Fisher Scientific, Cleveland, OH, USA). Samples were diluted (1:50) and dropped onto formvar/carbon Ni grids, dried in a vacuum desiccator, and inserted in the sample holder for analysis.

For the synthesis of GSNO in its free form, the required amount of GSH was dissolved in hydrochloric acid (1 mol/L), and an equimolar amount of NaNO_2 , related

to GSH, was added to the GSH solution. The nitrosation reaction was carried out in ice bath conditions for 40 min with magnetic stirring. The obtained GSNO was precipitated by adding acetone, filtered, and washed with cold water. The resulting solid was freeze-dried for 24 h and stored at -20 °C (Pelegriño et al., 2021). Just before the treatments, the powdered GSNO was dissolved in distilled water to achieve the desired concentration.

4.3.2 PLANT MATERIAL AND GROWTH CONDITIONS

Cecropia pachystachya, popularly known as “Embaúba-do-brejo”, is a shade-intolerant, pioneer species. It quickly colonizes forest clearings and edges, reaching heights of four to eight meters. Belonging to the Urticaceae family, it is attractive to fauna, providing food to various animal species (Lorenzi, 2008; Carvalho, 2014).

Cariniana estrellensis, commonly known as “Jequitibá-branco”, is a shade-tolerant, non-pioneer species. Belonging to the Lecythidaceae family, it can grow to between 15 and 35 meters in height (Lorenzi, 2008; Carvalho, 2014).

Seeds of both species were sown in sterilized sand, and, after approximately 60 days, the seedlings were transplanted to 290-mL polypropylene tubes filled with a commercial substrate composed of vermiculite, charcoal, *Pinus/Eucalyptus* bark, and coconut powder (Fertilizare, Toledo, Paraná, Brazil), supplemented with 5 g/L of a controlled-release fertilizer (14% N, 14% P₂O₅ and 14% K₂O). Seedlings were kept well-hydrated in a greenhouse with partial shade (60% light retention) for about 30 days, to optimize growth. After this period, they were transferred to a non-shaded greenhouse and kept well-hydrated for 45 days before initiating treatments, to undergo acclimation. The WD treatments started when the plants reached approximately 10 centimeters in height.

4.3.3 TREATMENTS

A randomized block design was used, with twelve replicates per treatment. Each block contained one replicate of each treatment, and plants were randomly assigned within each block. Randomization was performed both within the blocks and across the greenhouse benches. Although environmental factors such as light, temperature,

and humidity were not controlled artificially, all seedlings were kept under the same greenhouse conditions throughout the experiments.

4.3.4 LONG-TERM DROUGHT

For *Cecropia pachystachya*, the following treatments were applied: field capacity (FC), water deficit (WD), WD plus chitosan nanoparticles containing non-nitrosated glutathione (GSH), which do not release nitric oxide (WD + NP), WD plus free GSNO (WD + GSNO), and WD plus GSNO-loaded chitosan nanoparticles (WD + NP GSNO), all at 50 μM (for WD + NP, this concentration refers to GSH). This concentration was selected based on preliminary dose-response experiments, which showed that 50 μM resulted in the most consistent and beneficial physiological effects under water deficit conditions.

For *C. estrellensis*, FC, WD, and WD + NP GSNO were tested at 25, 50, 100, 200, 400, and 800 μM , based on preliminary experiments where no concentration had a consistent effect.

Thirty milliliters of the formulations were applied to the soil every 10 days, totaling three applications. Plants of the WD treatment received the same volume of distilled water, while plants in the FC treatment received daily irrigation until soil reached maximum water saturation, with excess water draining from the bottom of the polypropylene tubes. After the first application, long-term WD was imposed by suspending irrigation until plants showed wilting symptoms in the early morning, which corresponds to approximately 30% soil moisture. Small amounts of water were added when necessary to avoid reaching permanent wilting. The experiment concluded 30 days after the first application.

4.3.5 PHYSIOLOGICAL ANALYSES

The youngest fully expanded leaf of each seedling was used for physiological analyses. Stomatal conductance (g_s , $\text{mmol m}^{-2} \text{s}^{-1}$) was measured on the abaxial surface of leaves from both species, using a leaf porometer SC-1 (Decagon, Pullman, Washington, USA) at four pre-established times: 8 h, 10 h, 14 h, and 16 h. These times were selected based on preliminary evaluations to capture the diurnal variation in g_s under greenhouse conditions. Measurements at 8 h served as a reference point when

stomata are typically open but not yet at maximum conductance. At 10 h, g_s reached its peak in preliminary trials. The 14 h time point represented the onset of the afternoon decline due to increased vapor pressure deficit and temperature, and 16 h was chosen as a late-afternoon point when g_s was consistently low. These four time points provided a representative profile of diurnal g_s variation, allowing a comprehensive assessment of the treatment effects on stomatal regulation. g_s was measured on the second day after each application (three time points in total).

Gas exchange measurements were conducted on sunny days, starting at 8 am. Measurements were performed using a portable infrared gas analyzer (IRGA), model LI-COR 6400 XT (LI-COR Biosciences, Lincoln, USA), connected to a 6-cm² chamber under saturating photosynthetically active radiation of 1,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and flow rate of 400 $\mu\text{mol s}^{-1}$. Net photosynthetic rate (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$) and transpiration rate (E , $\text{mmol m}^{-2} \text{s}^{-1}$) were evaluated. Water use efficiency (WUE) was calculated as A/E . Measurements were taken three days after treatment application sets.

4.3.6 WATER STATUS ANALYSIS

The plant water potential of both species was measured pre-dawn using a Scholander type pressure chamber (Soil Moisture - SAPSII, model 3115, Santa Barbara, CA, USA). The shoot with leaves was placed in a hermetic container that withstands the pressure of the inert gas, and upon adding the gas, the sap exuded from the stem was observed (Scholander et al., 1965). The value marked on the manometer at the moment when sap exudes represents the xylem sap pressure potential value, and since the osmotic potential in this case can be considered insignificant, this value is considered the water potential in MPa.

4.3.7 BIOMETRICAL ANALYSES

The dry weights of the root, stem, and leaves were measured at the end of the experiment, after the organs were dried at 60°C for 72 h.

For root morphological analysis, 0.1 g of fresh roots were individually fixed in FAA solution (5 mL acetic acid, 5 mL formaldehyde, 90 mL 50% ethanol). Samples were mounted on slides with a series of grids and examined under a Carl Zeiss EL microscope using a 10× objective. The incidence of root hairs was evaluated by

recording their presence or absence in 100 intersections of fine roots per sample. In addition, the density of root hairs per mm² was determined. The length of the root hairs was measured from 100 hairs per root sample. The diameter of fine roots was determined at three points along twenty root segments per sample (Zangaro et al., 2005).

4.3.8 STATISTICAL ANALYSIS

Data were assessed for normality (Shapiro-Wilk W test) and homogeneity of variance (Levene's test), followed by Analysis of Variance (ANOVA) and repeated measures ANOVA (for g_s) using STATISTICA software. When necessary, data were transformed using logarithmic or square root transformations to meet parametric test assumptions. Percentage and proportion data were arcsine square root transformed. When detecting significance by ANOVA, means were compared using Tukey's test ($p < 0.05$). Data for root-hair length and fine-root diameter were assessed by the Kruskal-Wallis test followed by Dunn's test.

4.4 RESULTS

4.4.1 *Cecropia pachystachya*

Regarding g_s in *C. pachystachya* seedlings, significant differences were observed after the first set of treatments (Fig. 1A). At 8 h, plants under WD that received GSNO-containing NPs exhibited the highest g_s average among all treatments. Control plants in FC showed higher averages compared to those in WD subjected to other treatments. Subsequently, at 10 h and 14 h, g_s averages for the FC and WD + NP GSNO treatments were elevated and equivalent, similar to the treatment with direct application of free GSNO (WD + GSNO). However, plants in the WD + GSNO treatment also resembled the averages of other treatments under drought conditions. Interestingly, at 16 h, all experimental conditions showed similar g_s averages, regardless of water availability or formulation (Fig. 1A).

After the second set of treatments (Fig. 1B), at 8 h, plants maintained in FC and those treated with WD + NP GSNO exhibited the highest g_s among all experimental

groups, differing from other treatments under drought conditions. At 10 h, the WD + NP GSNO treatment stood out, maintaining the highest g_s compared to other treatments, followed by FC and WD + GSNO treatments, which also maintained higher values relative to other treatments under water deficit. At 14 h and 16 h, g_s averages for the FC and WD + NP GSNO treatments remained the highest and similar to each other, differing from all other treatments (Fig. 1B).

In the analysis of g_s after the third and final set of treatments (Fig. 1C), once again, at 8 h, the FC and WD + NP GSNO treatments maintained the highest averages among all experimental groups. At 10 h and 14 h, the WD + NP GSNO treatment was the only one capable of increasing g_s averages. Additionally, for the same time points, plants kept in FC maintained the second-highest average among all treatments. Regarding other treatments under drought conditions (WD; WD + NP; WD + GSNO), all of them maintained similar averages at 10 h, but at 14 h, the WD + GSNO treatment stood out. The results observed at 16 h were similar to those measured at 8 h (Fig. 1C).

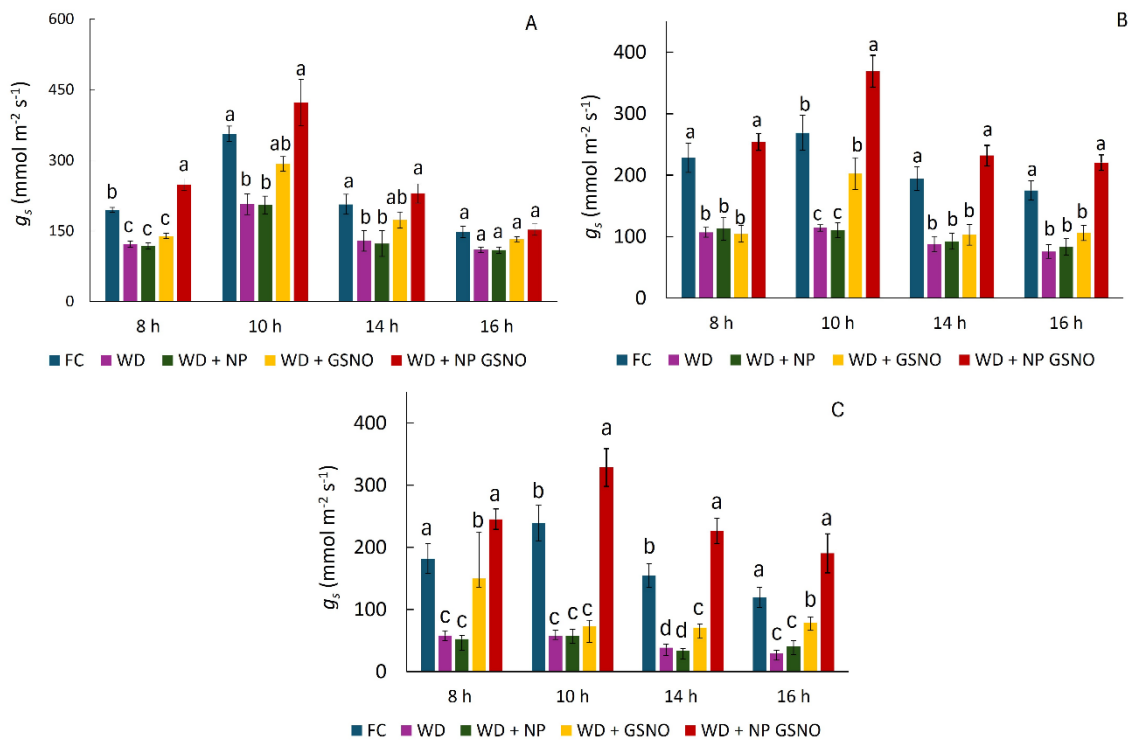


Fig. 1. Stomatal conductance (g_s , mmol m⁻² s⁻¹) of *Cecropia pachystachya* seedlings measured at 8 h, 10 h, 14 h, and 16 h on the second day after each treatment application under long-term water deficit conditions: (A) after the first application, (B) second application and (C) third application. Time points

refer to hours of the day, capturing diurnal variation on g_s . FC: field capacity; WD: water deficit; WD + NP: water deficit + chitosan nanoparticles containing non-nitrosated glutathione at 50 μM ; WD + GSNO: water deficit + free GSNO at 50 μM ; WD + NP GSNO: water deficit + chitosan nanoparticles containing GSNO at 50 μM . Data are expressed as mean $\pm$ standard error ($n = 9$). Bars above the columns correspond to the standard error. Equal letters above the bars indicate similar means according to repeated measures ANOVA followed by Tukey's test ($p < 0.05$).

Following the first set of treatments, as anticipated, WD reduced the A in all treatments, except for the plants treated with nanoencapsulated GSNO. Remarkably, the WD + NP GSNO treatment maintained a photosynthetic rate similar to well-hydrated controls. In the second set of treatments, the application of nanoencapsulated GSNO resulted in the highest A values among all treatments, closely resembling those of the FC treatment. After the third treatment application, all means were similar to the WD + NP GSNO treatment, except for the WD control. Similarly, means were comparable to the WD control, except for the WD + NP GSNO treatment (Table 1).

After the first set of treatments, WD reduced the E in all treatments under drought conditions, except for WD + GSNO, which remained comparable to the well-watered control. After the second set of treatments, all WD conditions maintained similar and higher average values compared to the FC. However, after the third and final set of treatments, there was no significant difference among any of the treatments, regardless of the formulations applied or the water status (Table 1).

Regarding WUE, after the first treatment application, the only significant difference was observed between the WD + NP and WD + NP GSNO treatments. However, after the second and third applications, all means were similar, regardless of water availability or formulation application (Table 1).

Table 1

Net photosynthesis (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$), transpiration (E , $\text{mmol m}^{-2} \text{s}^{-1}$), and water use efficiency (WUE, $\mu\text{mol mmol}^{-1}$) of *Cecropia pachystachya* seedlings after the first, second, and third set of treatments with the formulations in plants under long-term water deficit. FC: field capacity; WD: water deficit; WD + NP: water deficit + chitosan nanoparticles containing non-nitrosated glutathione at 50 μM ; WD + GSNO: water deficit + free GSNO at 50 μM ; WD + NP GSNO: water deficit + chitosan nanoparticles containing GSNO at 50 μM . Data are expressed as mean $\pm$ standard error ($n = 9$). Equal letters indicate similar means according to ANOVA followed by Tukey's test ($p < 0.05$).

		FC	WD	WD + NP	WD + GSNO	WD + NP GSNO
First set of treatments	A	8.98 ± 0.96 ^a	2.12 ± 0.43 ^b	3.28 ± 0.48 ^b	4.17 ± 0.37 ^b	11.14 ± 1.33 ^a
	E	1.89 ± 0.37 ^a	0.38 ± 0.05 ^b	0.38 ± 0.05 ^b	3.39 ± 0.57 ^a	0.62 ± 0.08 ^b
	WUE	5.47 ± 0.50 ^{ab}	7.04 ± 2.34 ^{ab}	10.26 ± 2.13 ^a	8.78 ± 2.23 ^{ab}	3.64 ± 0.31 ^b
Second set of treatments	A	11.34 ± 0.44 ^{ab}	8.03 ± 0.26 ^c	9.86 ± 0.51 ^b	10.65 ± 0.40 ^b	13.38 ± 0.43 ^a
	E	1.27 ± 0.06 ^b	1.94 ± 0.17 ^a	1.69 ± 0.09 ^a	2.38 ± 0.14 ^a	1.96 ± 0.16 ^a
	WUE	6.13 ± 0.48 ^a	6.37 ± 0.21 ^a	5.90 ± 0.27 ^a	5.62 ± 0.39 ^a	5.75 ± 0.32 ^a
Third set of treatments	A	11.23 ± 0.85 ^{ab}	8.23 ± 0.99 ^b	10.87 ± 0.84 ^{ab}	9.93 ± 0.69 ^{ab}	12.11 ± 0.79 ^a
	E	2.23 ± 0.27 ^a	1.28 ± 0.17 ^a	1.9 ± 0.23 ^a	1.73 ± 0.30 ^a	1.70 ± 0.17 ^a
	WUE	5.34 ± 0.47 ^a	6.60 ± 0.51 ^a	6.25 ± 0.57 ^a	6.06 ± 0.43 ^a	9.21 ± 1.96 ^a

C. pachystachya seedlings subjected to WD alone showed lower (more negative) plant water potential than well-hydrated plants. In contrast, seedlings treated with WD + NP GSNO maintained higher values than the remaining drought treatments and did not differ from FC plants (Fig. 2A).

Visually, it is evident that the WD + NP GSNO treatment effectively maintained turgid plants, similar to those in FC (Fig. 2B).

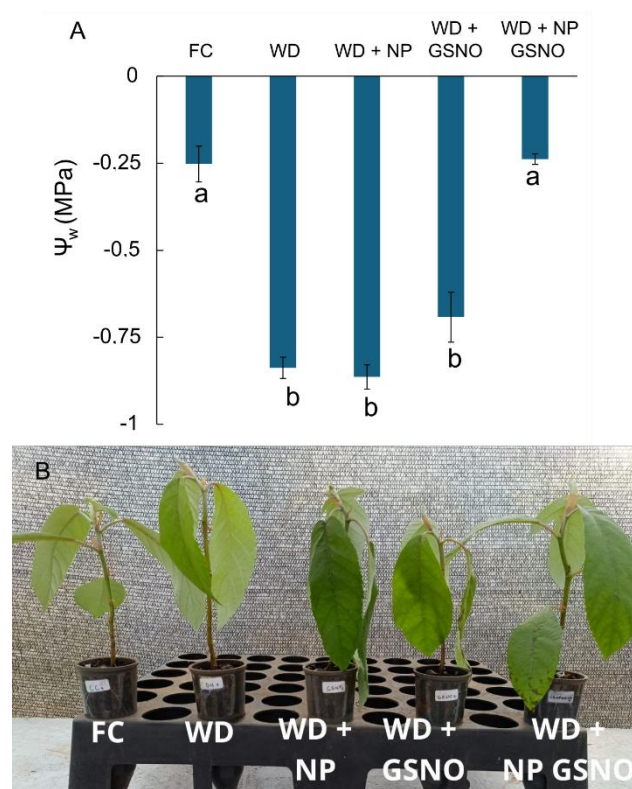


Fig. 2. Plant water potential (Ψ_w , MPa) (A), and representative image of *Cecropia pachystachya* seedlings at the end of the long-term water deficit (B). FC: field capacity; WD: water deficit; WD + NP:

water deficit + chitosan nanoparticles containing non-nitrosated glutathione at 50 μM ; WD + GSNO: water deficit + free GSNO at 50 μM ; WD + NP GSNO: water deficit + chitosan nanoparticles containing GSNO at 50 μM . Data are expressed as mean $\pm$ standard error ($n = 5$). Bars above the columns correspond to the standard error. Equal letters above the bars indicate similar means according to ANOVA followed by Tukey's test ($p < 0.05$).

Under WD conditions, root biomass decreased in all treatments compared to FC, except for the WD + NP GSNO treatment, which effectively maintained similar means to well-hydrated controls. Regarding leaf biomass, the WD + NP GSNO treatment once again stood out, maintaining the highest mean among all treatments. The stem biomass did not differ when comparing the treatments (Fig. 3).

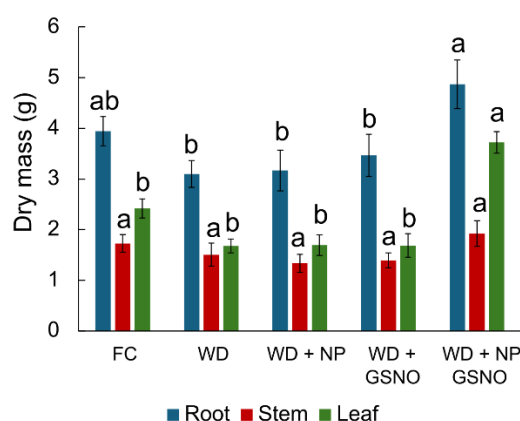


Fig. 3. Dry mass of root, leaf, and stem (g) of *Cecropia pachystachya* seedlings measured at the end of the long-term water deficit. FC: field capacity; WD: water deficit; WD + NP: water deficit + chitosan nanoparticles containing non-nitrosated glutathione at 50 μM ; WD + GSNO: water deficit + free GSNO at 50 μM ; WD + NP GSNO: water deficit + chitosan nanoparticles containing GSNO at 50 μM . Data are expressed as mean $\pm$ standard error ($n = 9$). Bars above the columns correspond to the standard error. Equal letters above the bars indicate similar means according to ANOVA followed by Tukey's test ($p < 0.05$).

The treatment with nanoencapsulated NO donor induced an increase in root-hair incidence in plants under long-term WD, presenting the highest incidence average observed among all treatments, comparable only to FC. Regarding the quantity of root hairs, the WD + NP GSNO treatment again maintained the highest average across all treatments, regardless of water condition. Additionally, the WD + NP treatment was the only one comparable to both the nanoencapsulated GSNO and all other treatments (Table 2).

Regarding root-hair length, all treatments differed from one another, with the highest average observed for the WD + GSNO treatment, followed by WD + NP GSNO, WD + NP, WD, and FC treatments, respectively. All formulations resulted in a decrease in fine-root diameter compared to the FC control, with the WD + NP GSNO treatment showing the smallest decrease, followed by the WD + GSNO, WD, and WD + NP treatments, respectively (Table 2).

Table 2

Root-hair incidence (%), amount (mm²), and length (μm), and fine-root diameter (μm) of *Cecropia pachystachya* seedlings measured at the end of the long-term water deficit. FC: field capacity; WD: water deficit; WD + NP: water deficit + chitosan nanoparticles containing non-nitrosated glutathione at 50 μM; WD + GSNO: water deficit + free GSNO at 50 μM; WD + NP GSNO: water deficit + chitosan nanoparticles containing GSNO at 50 μM. Data are expressed as mean ± standard error (n = 5). For root-hair incidence and amount, equal letters indicate similar means according to ANOVA followed by Tukey's test (p < 0.05). For root-hair length and fine-root diameter, equal letters indicate similar means according to Kruskal-Wallis test followed by Dunn's test (p < 0.05).

	FC	WD	WD + NP	WD + GSNO	WD + NP GSNO
Root-hair incidence	78 ± 1.67 ^{ab}	44.8 ± 1.93 ^c	51.6 ± 5.67 ^c	71.6 ± 2.65 ^b	86 ± 3.27 ^a
Root-hair amount	12.04 ± 0.278 ^b	13.84 ± 0.775 ^b	15.52 ± 0.917 ^{ab}	11.72 ± 1.415 ^b	19.08 ± 0.909 ^a
Root-hair length	135.60 ± 0.37 ^e	143.64 ± 1.66 ^d	170.24 ± 1.89 ^c	199.76 ± 1.19 ^a	186.96 ± 4.63 ^b
Fine-root diameter	266.8 ± 2.66 ^a	216.6 ± 0.502 ^d	213.1 ± 1.29 ^e	231 ± 2.33 ^c	243.24 ± 1.65 ^b

4.4.2 *Cariniana estrellensis*

For *C. estrellensis*, g_s was also measured on the second day following each set of treatments. Figure 4A presents the averages of g_s after the third set of treatments. As expected, WD resulted in lower g_s averages compared to the well-watered control. Notably, none of the concentrations of NO-releasing NPs applied exhibited significant differences relative to the FC.

Regarding the plant water potential, a decrease was observed in WD-treated plants compared to those maintained in FC. However, the application of NO-releasing NPs at any concentration did not significantly affect this parameter (Fig. 4B).

As for biomass accumulation in seedlings, as expected, long-term WD reduced the growth of all evaluated organs compared to well-hydrated controls. Notably, no concentration of nanoencapsulated GSNO was able to reverse this scenario (Fig. 4C).

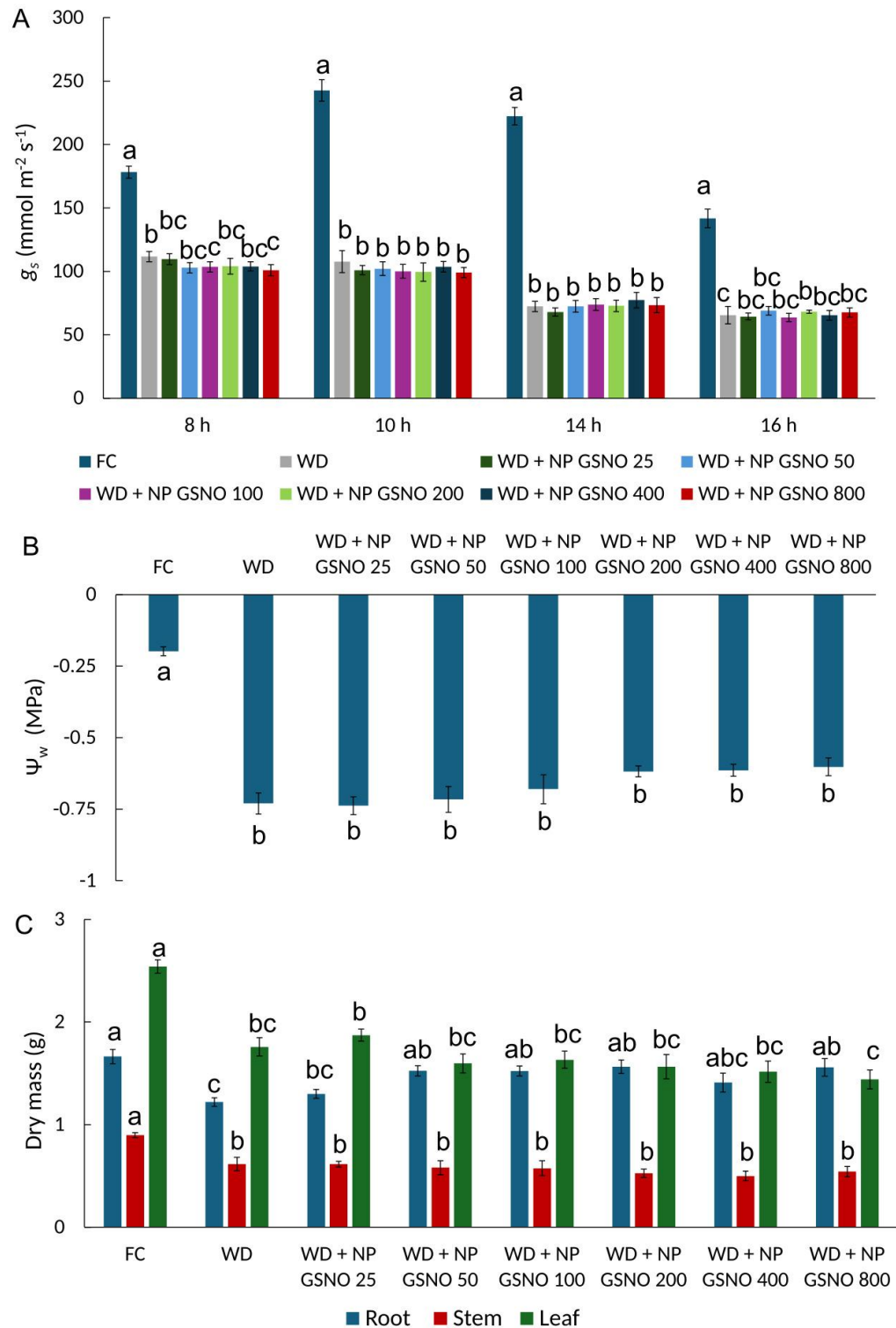


Fig. 4. Stomatal conductance (g_s , $\text{mmol m}^{-2} \text{s}^{-1}$) (A) of *Cariniana estrellensis* seedlings after the third set of treatments. Plant water potential (Ψ_w , MPa) (B) and dry mass of root, leaf, and stem (g) (C) of *Cariniana estrellensis* seedlings at the end of the long-term water deficit. FC: field capacity; WD: water deficit; WD + NP GSNO 25: water deficit + chitosan nanoparticles containing GSNO at 25 μM ; WD + NP GSNO 50: water deficit + chitosan nanoparticles containing GSNO at 50 μM ; WD + NP GSNO 100: water deficit + chitosan nanoparticles containing

GSNO at 100 μM ; WD + NP GSNO 200: water deficit + chitosan nanoparticles containing GSNO at 200 μM ; WD + NP GSNO 400: water deficit + chitosan nanoparticles containing GSNO at 400 μM ; WD + NP GSNO 800: water deficit + chitosan nanoparticles containing GSNO at 800 μM . Data are expressed as mean $\pm$ standard error ($n = 8$ for g_s and dry mass; $n = 5$ for Ψ_w). Bars above the columns correspond to the standard error. Equal letters above the bars indicate similar means according to repeated measures ANOVA for g_s , and ANOVA for Ψ_w and biomass, followed by Tukey's test ($p < 0.05$).

Regarding the A and E of *C. estrellensis*, after all sets of treatments, a decrease in the averages of plants maintained under long-term WD was observed compared to those maintained at FC. Again, no nanoformulation was able to induce an increase in the averages for either parameter. Concerning WUE, the averages of all treatments remained similar, regardless of the application of formulations or water condition (Table 3).

Table 3

Net photosynthesis (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$), transpiration (E , $\text{mmol m}^{-2} \text{s}^{-1}$), and water use efficiency (WUE, $\mu\text{mol mmol}^{-1}$) of *Cariniana estrellensis* seedlings after the first, second, and third set of treatments with the formulations in plants under long-term water deficit. FC: field capacity; WD: water deficit; WD + NP GSNO 25: water deficit + chitosan nanoparticles containing GSNO at 25 μM ; WD + NP GSNO 50: water deficit + chitosan nanoparticles containing GSNO at 50 μM ; WD + NP GSNO 100: water deficit + chitosan nanoparticles containing GSNO at 100 μM ; WD + NP GSNO 200: water deficit + chitosan nanoparticles containing GSNO at 200 μM ; WD + NP GSNO 400: water deficit + chitosan nanoparticles containing GSNO at 400 μM ; WD + NP GSNO 800: water deficit + chitosan nanoparticles containing GSNO at 800 μM . Data are expressed as mean $\pm$ standard error ($n = 8$). Equal letters indicate similar means according to ANOVA followed by Tukey's test ($p < 0.05$).

		FC	WD	WD + NP GSNO 25	WD + NP GSNO 50	WD + NP GSNO 100	WD + NP GSNO 200	WD + NP GSNO 400	WD + NP GSNO 800
First set of treatments	A	8.38 $\pm$ 0.24 ^a	4.13 $\pm$ 0.27 ^b	4.5 $\pm$ 0.28 ^b	4.11 $\pm$ 0.30 ^b	4.26 $\pm$ 0.36 ^b	4.38 $\pm$ 0.035 ^b	3.40 $\pm$ 0.41 ^b	4.45 $\pm$ 0.35 ^b
	E	1.89 $\pm$ 0.03 ^a	1.05 $\pm$ 0.03 ^b	1.04 $\pm$ 0.02 ^b	1.04 $\pm$ 0.02 ^b	1.05 $\pm$ 0.02 ^b	1.04 $\pm$ 0.01 ^b	0.83 $\pm$ 0.04 ^b	1.07 $\pm$ 0.01 ^b
	WUE	4.44 $\pm$ 0.15 ^a	3.97 $\pm$ 0.29 ^a	4.32 $\pm$ 0.25 ^a	3.96 $\pm$ 0.30 ^a	4.12 $\pm$ 0.42 ^a	4.21 $\pm$ 0.32 ^a	4.11 $\pm$ 0.20 ^a	4.16 $\pm$ 0.29 ^a
Second set of treatments	A	7.76 $\pm$ 0.43 ^a	3.43 $\pm$ 0.35 ^b	3.29 $\pm$ 0.34 ^b	3.39 $\pm$ 0.28 ^b	3.65 $\pm$ 0.36 ^b	3.51 $\pm$ 0.030 ^b	4.36 $\pm$ 0.22 ^b	3.52 $\pm$ 0.35 ^b
	E	1.89 $\pm$ 0.02 ^a	0.81 $\pm$ 0.09 ^b	0.84 $\pm$ 0.03 ^b	0.88 $\pm$ 0.03 ^b	0.89 $\pm$ 0.04 ^b	0.83 $\pm$ 0.04 ^b	1.06 $\pm$ 0.01 ^b	0.89 $\pm$ 0.03 ^b
	WUE	4.12 $\pm$ 0.27 ^a	4.79 $\pm$ 0.83 ^a	3.97 $\pm$ 0.43 ^a	3.91 $\pm$ 0.40 ^a	4.10 $\pm$ 0.40 ^a	4.29 $\pm$ 0.44 ^a	4.12 $\pm$ 0.50 ^a	4.01 $\pm$ 0.43 ^a
Third set of treatments	A	7.69 $\pm$ 0.76 ^a	3.54 $\pm$ 0.43 ^b	3.81 $\pm$ 0.33 ^b	3.54 $\pm$ 0.35 ^b	3.35 $\pm$ 0.51 ^b	2.89 $\pm$ 0.27 ^b	3.32 $\pm$ 0.30 ^b	3.09 $\pm$ 0.43 ^b
	E	1.68 $\pm$ 0.06 ^a	0.98 $\pm$ 0.06 ^b	0.81 $\pm$ 0.06 ^b	0.93 $\pm$ 0.09 ^b	0.88 $\pm$ 0.08 ^b	0.77 $\pm$ 0.06 ^b	0.77 $\pm$ 0.06 ^b	0.60 $\pm$ 0.05 ^b
	WUE	4.64 $\pm$ 0.52 ^a	3.75 $\pm$ 0.67 ^a	4.79 $\pm$ 0.46 ^a	3.99 $\pm$ 0.46 ^a	4.14 $\pm$ 0.78 ^a	3.95 $\pm$ 0.50 ^a	4.48 $\pm$ 0.50 ^a	5.24 $\pm$ 0.81 ^a

4.5 DISCUSSION

The results indicated that NO-releasing NPs increased drought tolerance in *C. pachystachya* seedlings under long-term WD conditions. Positive effects were observed on gas exchange parameters, plant water status, root hair characteristics, and seedling growth. However, for the shade-tolerant species *C. estrellensis*, the application of nanoencapsulated NO donors had no effect. These results highlight that the plant response to NO-releasing NPs is species-specific.

The absence of responses to nanoencapsulated GSNO in *C. estrellensis* may be associated with anatomical, physiological, and ecological characteristics specific to the species, which may limit its sensitivity to the treatment. Shade-tolerant species, such as *C. estrellensis*, generally adopt more conservative resource-use strategies and exhibit foliar traits such as high fresh and dry leaf mass, which are associated with drought tolerance and water conservation (Toledo-Aceves et al., 2022). These species also tend to display lower stomatal conductance, which could have reduced the physiological impact of the applied treatments.

Previous studies support the hypothesis that the effects of NO-releasing nanoparticles are species-dependent. For instance, Lopes-Oliveira et al. (2019) observed that seedlings of *C. estrellensis* did not exhibit significant changes in photosynthesis or growth when treated with NO-releasing nanoparticles under light stress, whereas other species, such as *Heliocarpus popayanensis*, showed clearly beneficial physiological effects. Another possible explanation relates to root morphological traits and the capacity for nanoparticle uptake. If *C. estrellensis* possesses a smaller absorptive root surface area, this may limit effective absorption or interaction with the nanoparticles, thereby reducing NO bioavailability at the target tissues (Lombardo & Lamattina, 2018). These findings reinforce the need for NO-releasing nanoparticles to be evaluated on a species-specific basis (Lei et al., 2025), particularly in ecologically diverse systems such as tropical forests.

In contrast, the beneficial effect of nanoencapsulated GSNO in *C. pachystachya* plants subjected to drought is likely related to protection of the NO donor from degradation by the polymeric shell of the NPs, which results in sustained NO release compared to the free GSNO and in the potentiation of the effects of NO on plant growth and physiology (Oliveira et al., 2016; Lopes-Oliveira et al., 2019; do Carmo et al., 2021; Methela et al., 2023). In addition, chitosan has mucoadhesive properties, which could

facilitate NP interaction with the roots (Seabra et al., 2014; Sousa et al., 2024) enhancing NO bioavailability to plant cells both locally and systemically (Seabra et al., 2014; Lopes-Oliveira et al., 2019; do Carmo et al., 2021).

Do Carmo et al. (2021) reported higher levels of RSNOs in *H. popayanensis* plants treated with nanoencapsulated S-nitroso-MSA compared to the same donor in its free form. However, in the present study, the donor used was GSNO, a naturally occurring molecule considered a reservoir of NO in cells and an RSNO. Therefore, GSNO releases NO through the cleavage of the sulfur-NO bond, without producing toxic compounds (Oliveira et al., 2016; Silveira et al., 2021). In this sense, NPs containing GSNO emerge as a sustainable and cost-effective solution to enhance plant drought tolerance due to their biodegradability, biocompatibility, and low synthesis cost (Seabra et al., 2015, 2022).

Regarding the root morphological parameters of *C. pachystachya*, some interesting effects were observed. Notably, free GSNO was the only treatment that surpassed the nanoencapsulated molecule in increasing the root-hair length. NPs without NO showed intermediate values of root-hair amount, which did not differ statistically from the other treatments. Nevertheless, chitosan can also have positive effects on plant physiology. Nanochitosan has attracted interest due to its promising role in agriculture, including plant protection against abiotic stress. In rice plants under salt stress, nanochitosan can increase chlorophyll and proline content, as well as enhancing the activity of antioxidant enzymes (Thuy et al., 2024).

Remarkably, the application of nanoencapsulated GSNO to seedlings subjected to long-term WD was also the only treatment that promoted an increase in the incidence, amount, and length of root hairs compared to control WD plants. Indeed, enhancing the number of root hairs may result in improved root adaptation to low water availability soils, without affecting the total absorption surface (Lombardo and Lamattina, 2018).

NO acts in concert with phytohormones in various signaling cascades, playing a significant role in the regulation of root growth and development (Sun et al., 2021; da Silva et al., 2024). Notably, NO interacts with hormones such as auxin and abscisic acid to modulate multiple physiological processes. For instance, NO directly affects auxin transport by modulating the activity of PIN-FORMED (PIN) proteins and auxin response factors (ARFs). The S-nitrosylation of PIN proteins and ARFs regulates polar auxin transport, directing it towards the root apices, where it can stimulate root growth

and help plants adapt to soils with low water availability (Ergashev et al., 2025). This mechanism enhances root plasticity, optimizing water and nutrient absorption, and thereby improving plant resilience under stress conditions (Sánchez-Vicente et al., 2021; Wang et al., 2024). However, these pathways were not directly assessed in the present study. Their involvement in the responses of *C. pachystachya* remains a hypothesis, which could be tested by quantifying NO and auxin in roots or by using NO scavengers and auxin transport inhibitors.

The interaction between NO and auxin signaling is particularly relevant during stress situations, where NO can adjust the architecture of the root system to facilitate resource acquisition. In 2006, Lombardo et al. demonstrated that NO acts as a non-traditional regulator of root hair growth by promoting the differentiation of trichoblasts into specialized cells that form root hairs during lettuce development. Supporting this, an increase in NO content within root hairs was observed following treatment with 1-naphthyl acetic acid (NAA), suggesting that auxin-induced root hair formation is NO-dependent. Moreover, recent research indicates that NO may cooperate with ABA in cytoskeletal organization processes, further modulating root hair formation (Lombardo and Lamattina, 2018; Sun et al., 2021; da Silva et al., 2024). Importantly, previous studies have indicated that the effect of NO on root hair development is dose-dependent, requiring an optimal concentration range to stimulate growth. This is evidenced by the inhibition of root hair formation in response to applied concentrations of cPTIO, an NO scavenger, demonstrating that both insufficient and excessive NO levels can impair this process (Lombardo et al., 2006; Sun et al., 2021).

In *C. pachystachya*, the enhancement of root hair development induced by the nanoencapsulated donor, particularly the increased incidence and density, may have contributed to water uptake. This interpretation is compatible with the maintenance of plant water potential, the higher g_s and net photosynthesis, and the greater biomass accumulation observed. However, this causal sequence was not directly tested, and other mechanisms, such as direct effects of NO on guard cells and photosynthesis, may also have contributed. Similar results have been reported by Silveira et al. (2019; 2021) and do Carmo et al. (2021).

The treatment with NO-releasing NPs led to increased g_s throughout the day in comparison with other treatments under WD. Indeed, NO can either enhance or reduce g_s , depending on the applied dose (Wang et al., 2016; Casaretto et al., 2021; do Carmo et al., 2021). NO is associated with S-nitrosylation of the Open Stomata 1 (OST1)

protein, which regulates ABA signaling in guard cells. S-nitrosylation of OST1 inhibits its activity, acting as a negative regulator of ABA signaling in plants (Wang et al., 2016; Casaretto et al., 2021; Singhal et al., 2021). This process promotes stomatal opening and CO₂ assimilation even under drought conditions (Wang et al., 2016; Casaretto et al., 2021).

Moreover, the improvement in photosynthesis-related parameters may be associated with the effects of NO on non-stomatal processes, such as the protection of chlorophyll molecules by reducing their degradation, prevention of photoinhibition through enhanced stability of photosystem II (PSII), and protection against oxidative stress by inducing the antioxidant response (Oliveira et al., 2016; Wang et al., 2019). NO also stimulates the activity of key enzymes in photosynthesis, such as Rubisco, thereby enhancing CO₂ assimilation (Gan et al., 2015; Silveira et al., 2021).

Similarly, in soybean, de Sousa et al. (2020) demonstrated that free SNP contributed to maintaining leaf water status. Similar to our results, individuals exposed to drought stress and treated with NO donors exhibited greater stomatal opening compared to untreated plants. This increased stomatal aperture was associated with morphoanatomical changes, including higher stomatal density on the abaxial leaf surface and reduced stomatal size on the adaxial surface, ultimately enhancing hydraulic conductivity and reducing leaf desiccation (Sousa et al., 2020).

Methela et al. (2023) also reported that GSNO-loaded chitosan NPs at 50 µM improved drought tolerance in soybean plants, inducing an increase in plant height, biomass, root length, and root volume. Furthermore, do Carmo et al. (2021) found that S-nitroso-MSA prevented the inhibition of CO₂ assimilation induced by WD, regardless of nanoencapsulation. However, the nanoencapsulated donor induced this effect earlier in *H. popayanensis* seedlings. Similar to the results observed here with *C. pachystachya*, the NO-releasing NPs were capable of increasing leaf and absorptive root hair formation in *H. popayanensis* plants under WD.

Considering the promising results obtained with *C. pachystachya*, it is important to consider the cost of this nanotechnology. GSNO-based treatments can demonstrate a higher cost when compared to other strategies used in agriculture. However, when encapsulated in chitosan NPs, the delivery is more efficient and the NO donor is more stable, so lower concentrations may be sufficient to obtain the targeted effect. The production cost of this formulation is estimated in Chapter 2 of this thesis. However, its

cost-competitiveness relative to other strategies and its operational feasibility at large scale still need to be evaluated.

Finally, our results indicate that only the pioneer species responded to the application of the nanoencapsulated donor, suggesting a species-specific dependency in the treatment efficacy, corroborating the findings of Lopes-Oliveira et al. (2019). To confirm and expand these findings, further studies involving a broader range of species from different successional groups are essential. Such studies will provide a more comprehensive understanding of the mechanisms involved and assess the potential applicability of this approach across diverse ecological contexts.

4.6 CONCLUSIONS

Based on the analyses conducted in this study, it is concluded that the application of chitosan nanoparticles containing the NO donor GSNO has the potential to increase drought tolerance in seedlings of *C. pachystachya* under long-term conditions, whereas no consistent benefits were detected in *C. estrellensis*. This beneficial effect on *C. pachystachya* was achieved with a relatively low concentration of nanoencapsulated GSNO, specifically 50 μ M. These conclusions are restricted to the species, doses, variables, and conditions evaluated. In addition, the comparison between two species does not establish a general rule for successional groups. Depending on the species, this technique may be useful for producing seedlings with higher drought tolerance, which is desirable in reforestation programs.

Although this study demonstrates the effects of NO-releasing NPs in greenhouse conditions, there is a possibility that these results may be replicated in field settings, influenced by variations in specific abiotic and biotic conditions. Therefore, the effects of NO-releasing NPs on native tree species of the Atlantic Forest, within the context of forest restoration, need to be investigated in future studies in order to make the application of this nature-based technology feasible in reforestation programs.

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5 CAPÍTULO 2 – NITRIC OXIDE DELIVERY VIA CHITOSAN NANOPARTICLES IMPROVES FIELD PERFORMANCE OF *Cecropia pachystachya* SEEDLINGS: A COST-EFFECTIVE APPROACH FOR FOREST RESTORATION

Artigo submetido à revista *Restoration Ecology*
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Nota: Este capítulo corresponde ao manuscrito submetido à revista *Restoration Ecology* e é apresentado integralmente, uma vez que todo o seu conteúdo foi desenvolvido durante o período da realização deste trabalho.

Running head: NO-releasing nanoparticles improve *Cecropia pachystachya* seedlings

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Author Contribution

GCC, HCO, JCP, CNL, GCT, ABS conceived and designed the research; GCC, RCS, LFT, IRTT performed the experiments; GCC, HCO, JCP, ABS analyzed the data; GCC wrote the original draft; GCC, HCO, JAP, RCS, LFT, IRTT, JCP, CNL, GCT, ABS reviewed and edited the manuscript.

5.1 ABSTRACT

Introduction: The poor field performance of nursery-produced tree seedlings may limit large-scale forest restoration, leading to costly replanting, impaired carbon storage, and delayed ecosystem recovery. In this sense, nitric oxide (NO) is an important signaling molecule in plants. However, due to its gaseous nature and short half-life, the direct application of NO is difficult. The encapsulation of NO donors, such as S-nitrosoglutathione (GSNO), in chitosan nanoparticles (CSNPs) represents a promising strategy to increase seedling growth.

Objective: This study aimed to evaluate the effects of chitosan nanoparticles containing S-nitrosoglutathione (GSNO-CSNPs) on physiological and growth parameters of *Cecropia pachystachya* seedlings under field conditions, assessing their

potential as a cost-effective strategy.

Methods: We evaluated the effects of GSNO-CSNPs on physiological and growth parameters of *C. pachystachya* seedlings, a pioneer species of the Atlantic Forest. The experiment was conducted in randomized blocks, with three treatments applied to the substrate: control (water), GSNO-CSNPs (100 μ M), and a commercial nanoformulation as a reference.

Results: Two weeks after planting, seedlings treated with GSNO-CSNPs showed higher photosynthetic rate and carboxylation efficiency than the control, but did not differ from the reference; after nine weeks, these parameters did not differ from the control. At the end of the experiment, GSNO-CSNPs increased leaf area and total CO₂ assimilation compared to both the control and the reference. Stem and shoot dry mass were higher only compared to the reference ($p < 0.10$). The estimated cost of the formulation was low, approximately US\$ 0.025 per seedling.

Conclusion: NO-releasing chitosan nanoparticles improved the initial field performance of *C. pachystachya* seedlings at a low estimated cost. These results were obtained with one species, at one site, and over six months. Therefore, they indicate a potential for forest restoration that needs to be confirmed in other species and conditions. To our knowledge, this is the first field study of a NO-releasing nanotechnology applied to native tree seedlings for forest restoration.

Implications for practice: The results indicate that the use of nitric oxide-releasing chitosan nanoparticles (GSNO-CSNPs) is a promising, low-cost option for promoting the physiological performance and growth of *Cecropia pachystachya* seedlings under field conditions. Increases in leaf area and total CO₂ assimilation indicate that this technology can improve seedling performance during the critical post-planting phase. Considering their biodegradable nature, reduced application volume, and low estimated cost per seedling, GSNO-CSNPs could be incorporated into existing nursery production and field planting protocols. Future studies including other species and environmental contexts are needed to assess the applicability of this technology in more complex scenarios.

Keywords: Nanotechnology, Nitric oxide, Reforestation, Plant biomass, Photosynthesis.

5.2 INTRODUCTION

In recent decades, forest restoration has emerged as a key strategy to address global issues, including ecosystem degradation, biodiversity loss, and the climate emergency (Cunningham et al. 2015; Brancalion et al. 2019; Macedo et al. 2021). In addition to repairing the structure and functionality of degraded environments, forest

restoration helps to provide vital ecosystem services, like soil conservation, carbon sequestration and storage, and regulation of the global hydrological regime, all of which have social and economic positive effects (Fargione et al. 2021; Silva et al. 2024).

However, there are many challenges related to large-scale forest restoration. The effectiveness of these efforts is seriously hampered by the high implementation and maintenance costs, as well as the high seedling death rate in the first few months following planting (Bustamante et al. 2019; Souza & Engel 2023; Silva et al. 2026). Furthermore, in contrast to the urgency of globally set climate and biodiversity targets, ecosystems undergoing restoration may take decades to achieve maturity and ecological resilience at levels capable of providing ecosystem services in a self-sufficient way (Brancalion et al. 2019). This disparity highlights the necessity of field planting techniques that promote early growth, improve physiological performance, and lower seedling losses, particularly in extremely sensitive biomes like the Atlantic Forest (Sartori et al. 2025; Turchetto et al. 2025).

In this context, the use of pioneer species as facilitators, such as *Cecropia pachystachya* Trécul (Urticaceae), can accelerate the natural succession of the ecosystem, creating favorable conditions for later successional species to occupy the environment, and thus ensuring long-term structural and functional diversity (Adenesky-Filho et al. 2017).

At the same time, the development of nature-based solutions that increase the survival, tolerance, and growth of seedlings in the field is becoming increasingly relevant. In this sense, the use of nitric oxide (NO), a gaseous signaling molecule naturally present in plants, emerges as a promising strategy, as it is involved in a lot of physiological processes related to growth, development, and stress responses (Nabi

et al. 2019; do Carmo et al. 2021; 2025).

However, due to its physicochemical properties, the direct application of NO to plants is difficult. Thus, NO donor molecules, such as S-nitrosoglutathione (GSNO), are commonly used as an alternative for the exogenous supply of NO to plants (Seabra et al. 2022). These donor molecules are also unstable when exposed to light, heat, and pH variation, leading to rapid and uncontrolled release of NO (Silveira et al. 2021; Seabra et al. 2022).

In this scenario, nanotechnology emerges as a solution to overcome the limitations of NO donor molecules, enabling their incorporation into controlled release systems (Silveira et al. 2021; Seabra et al. 2022). Among these, chitosan nanoparticles (CSNPs) stand out for combining low cost, biodegradability, and biocompatibility, in addition to being able to protect NO donors against premature degradation (Seabra et al. 2015; Riseh et al. 2024). Research conducted under greenhouse and nursery conditions has already demonstrated that the application of CSNPs containing different NO donors improves physiological and growth parameters of different plant species, including tree seedlings (Lopes-Oliveira et al. 2019; do Carmo et al. 2025; Guariz et al. 2025). However, the lack of field tests still constitutes a key bottleneck in proving the effectiveness of this technology in forest restoration programs.

Given this context, the current study aimed to evaluate the effects of applying CSNPs containing GSNO (GSNO-CSNPs) on physiological and growth parameters of *C. pachystachya* seedlings under field conditions, in addition to discussing the cost-effectiveness of this nanotechnology in the context of restoration programs. The hypothesis was that treatment with GSNO-CSNPs would promote greater CO₂ assimilation and biomass accumulation by seedlings after planting in the field, proving it to be a physiologically efficient and economically viable strategy for large-scale forest

restoration.

5.3 METHODS

5.3.1 PLANT MATERIAL AND GROWTH CONDITIONS

Seedlings of *Cecropia pachystachya* Trécul (Urticaceae), commonly known as Embaúba-do-brejo, were used. This tree is a typical shade-intolerant, pioneer species, establishing itself quickly in clearings and forest edges, and reaching four to eight meters in height. In addition to its rapid growth, it is considered very attractive to wildlife, providing food for several species of animals (Lorenzi 2008; Carvalho 2014).

Considering these ecological characteristics and its potential use in forest restoration programs, *C. pachystachya* seedlings were purchased from the Flora Londrina nursery (Londrina, Paraná, Brazil), in 55 mL tubes filled with the reference substrate Florestal 3 (Mecplant, Telêmaco Borba, Paraná, Brazil). This substrate is composed of *Pinus* bark, vermiculite, NPK, and simple superphosphate, with a particle size of 6 mm, mean moisture content ranging from 54 to 58%, and bulk density between 360 g L⁻¹ and 400 g L⁻¹.

At the beginning of the experiment, the seedlings, which had already been hardened off in full sun, were four months old (approximately 120 days after sowing), with a mean height of 27.5 cm and two to three fully expanded leaves. To reduce variability, homogeneous individuals were selected before starting the treatments.

5.3.2 SYNTHESIS AND CHARACTERIZATION OF THE NANOFORMULATION

The CSNPs were obtained using the ionotropic gelation method (Oliveira et al.

2016; Pelegrino et al. 2017; do Carmo et al. 2025). Chitosan (Sigma, PA-ACS grade, 2.6 mg mL⁻¹) was dissolved in 1% acetic acid (Sigma, PA-ACS grade) and added to a solution of 53 mM reduced glutathione (GSH, Sigma, PA-ACS grade). The mixture was kept under magnetic stirring for 90 minutes, followed by the dropwise addition of sodium tripolyphosphate (TPP, Sigma, PA-ACS grade, 0.6 mg mL⁻¹), in a volumetric ratio of 3:1 (v/v) CS/GSH:TPP. The suspension obtained was kept under stirring for another 45 minutes at room temperature, resulting in CSNPs containing 40 mM GSH as the final concentration.

The CSNPs obtained were characterized using the dynamic light scattering technique (Nano ZS Zetasizer, Malvern Instruments Co., Malvern, UK), to provide the hydrodynamic size, polydispersity index (PDI), and zeta potential. The measurements were performed in folded capillary cells with an optical path of 10 mm and using a fixed angle of 173° (Gomes et al. 2021).

Just before applying the treatments, sodium nitrite (NaNO₂, Sigma, PA-ACS grade) was added in an equimolar amount to the GSH present in the suspension, *i.e.*, 40 mM, promoting the S-nitrosation and consequent formation of GSNO in the CSNP suspensions. The suspension was kept at 10 °C for 1 h, protected from light, before being diluted in distilled water to obtain a final concentration of 100 µM GSNO.

5.3.3 ESTIMATED COST OF THE NANOFORMULATION

The production costs of the formulation were assessed based on a 100-liter batch of the product. The calculations were structured in a spreadsheet, taking into account the unit price (R\$/kg) and the quantity of each input used. Based on these values, the total and individual costs of the process were calculated.

The values of the raw materials used in the formulation were the main factors

taken into consideration, obtained from market quotes. Costs associated with equipment and infrastructure were also considered, using the mean values and assumptions presented in Table S1. Based on this information, it was possible to estimate the total cost of the batch and determine the percentage share of each component in the overall cost of the process.

All costs were converted from Brazilian reais (BRL) to U.S. dollars (USD) using the mean monthly exchange rate for the period in which the treatments were applied (USD 1 = BRL 5.55), based on data from the Central Bank of Brazil.

5.3.4 TREATMENTS

The experiment was distributed into randomized blocks, with 10 replicates per treatment, as follows: Control, receiving only water irrigation; GSNO-CSNPs, application of CSNP suspension containing GSNO at 100 μM ; and a commercial nanoformulation, used as a comparative reference for GSNO-CSNPs (Arbolina®, Krilltech Nanotecnologia, Brasília, DF, Brazil), which is a carbon NP-based nanofertilizer (Vieira et al. 2024). The commercially available reference nanoformulation was purchased and diluted in distilled water to the dose recommended by the manufacturer (0.2 mg/mL), according to the package insert instructions.

Each plant received 15 mL of the freshly prepared GSNO-CSNP formulation, applied directly to the substrate, following previous results (do Carmo et al. 2025). The reference formulation (5 mL) was applied via foliar spray, as recommended by the manufacturer. The applications were carried out on three alternate days (do Carmo et al. 2025).

The day after the applications were completed, the seedlings were transplanted

to the area designated for conducting the field experiment, located at the Biological Sciences Center of the State University of Londrina, Paraná, Brazil (23°19'29" S 51°11'53" W). The soil at the site is classified as very clayey (76.0% clay, 20.3% silt, and 3.8% sand), with 26.16 g dm⁻³ of organic matter (report provided by the Soil Analysis Laboratory – Institute of Technology and Laboratories, Londrina, 2025).

The soil was prepared for planting manually, with the removal of weeds and superficial tilling of the soil to avoid compaction. The holes were dug with a hoe, maintaining a spacing of 50 cm between plants in the row and 50 cm between rows. Weed control was performed manually, whenever necessary, throughout the experimental period, in order to avoid competition for light, water, and nutrients.

Approximately four months after setting up the field experiment, a reinforcement application of the formulations was carried out, with 100 mL of each formulation applied directly to the base of the seedling stem in the field, totaling four applications throughout the experimental period. The field trial lasted approximately six months (August 2024 to January 2025), at the end of which destructive analyses were conducted for the final evaluation of the seedlings. Meteorological data were obtained from the weather station of the State University of Londrina Experimental Farm, available through the university's official website.

5.3.5 PHYSIOLOGICAL ANALYSIS

The maximum photochemical activity of photosystem II (F_v/F_m) was determined in intact leaves using an OS1p fluorometer (Opti-Sciences, Hudson, USA), according to the methodology described by Calzavara et al. (2017). Readings were taken early in the morning, between 6 and 8 a.m., after the leaves had been acclimated to darkness.

Stomatal conductance (g_s , $\text{mmol m}^{-2} \text{s}^{-1}$) was monitored on the abaxial surface of the leaves using an SC-1 porometer (Decagon, Pullman, USA), starting at 8 a.m. Measurements of g_s and F_v/F_m were performed at three time points: before planting (pre-planting), one day after planting (1 DAP), and five days after planting (5 DAP).

Leaf gas exchange was evaluated at two time points (second and ninth weeks after planting), using a portable infrared gas analyzer (IRGA, model LI-6400 XT; LI-COR Biosciences, Lincoln, USA). The net photosynthetic rate (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$), transpiration (E , $\text{mmol m}^{-2} \text{s}^{-1}$), and intercellular CO_2 concentration (C_i) were measured in fully expanded leaves using a 6-cm^2 chamber under saturating photosynthetically active radiation ($1,500 \mu\text{mol m}^{-2} \text{s}^{-1}$) and an air flow of $400 \mu\text{mol s}^{-1}$. Measurements were taken on sunny days, starting at 8 a.m. Instantaneous carboxylation efficiency (k , $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$) was calculated as $A/C_i \times 10.75$.

Total CO_2 assimilation per plant (A_{total} , $\mu\text{mol CO}_2 \text{s}^{-1}$) was estimated by multiplying A (measured just before the end of the experiment) by the total leaf area of each plant.

5.3.6 GROWTH ANALYSIS

Seedling growth was assessed by determining the leaf area and dry biomass of leaves and stems. The leaf area (m^2) of fully expanded leaves was measured using a LI-3000CAP leaf area meter coupled with a LI-3050 mat (LI-COR Biosciences, Lincoln, USA).

To determine dry biomass, the leaves and stems were separated, placed in paper bags, and dried in an oven at 60°C for 240 hours. The materials were then weighed on an analytical scale. The total shoot dry mass was determined from the sum of the leaf and stem dry mass.

5.3.7 STATISTICAL ANALYSIS

The data were analyzed for normality (Shapiro-Wilk W test) and homogeneity of variance (Levene's test), followed by one-way analysis of variance (ANOVA) using STATISTICA software ($p < 0.10$). When necessary, the data were transformed to meet the assumptions of the parametric test. When significance was detected by ANOVA, the means were compared using Tukey's test ($p < 0.10$). A significance level of 10% was adopted considering the greater environmental variability of field conditions, and results with $0.05 \leq p < 0.10$ were interpreted with caution. Principal component analysis (PCA) was performed using PAST software.

5.4 RESULTS

GSNO-CSNPs were successfully prepared by ionotropic gelation and had a mean hydrodynamic size of 177 ± 3 nm, PDI of 0.368 ± 0.04 , and zeta potential of 22.9 ± 1 mV, indicating nanoparticles with good homogeneity and colloidal stability.

The cost calculations for the nanoformulation indicated that the largest portion of the total investment is related to raw materials (US\$ 6,509.29 per 100 L batch), representing approximately 98% of the total cost of the batch. Labor costs (US\$ 86.48 per batch), equipment (US\$ 20.79 per batch), and energy/services (US\$ 1.65 per batch) represented a small portion of the total value. Table 1 presents a summary of the estimated production costs of the nanoformulation. A detailed breakdown of the components can be found in Table S1. For one liter of the formulation (40 mM), the cost is US\$ 66.18. Considering the scheme of the treatment used in the present study, in which the formulation was diluted to 100 μ M and applied at a total volume of 145 mL per seedling (15 mL per application, totaling three applications, plus a 100 mL reinforcement applied in the field), one liter of the stock solution is sufficient to treat

approximately 2,760 seedlings, resulting in an estimated cost of approximately US\$ 0.025 per seedling.

Table 1. Summary estimate of the production costs of the GSNO-CSNP nanoformulation used in the experiment, detailing the main cost components and the final value per batch and liter of the formulation.

TOTAL COST (USD)	
Raw materials	US\$ 6,509.29
Equipment	US\$ 20.79
Energy/Services	US\$ 1.65
Labor	US\$ 86.48
TOTAL COST PER BATCH (100 L)	US\$ 6,618.21
TOTAL COST PER LITER	US\$ 66.18

During the experimental period, seasonal variations in weather patterns were observed in Londrina (PR) (Table 2). Monthly precipitation ranged from 28 mm in August to 206.8 mm in December, marking the beginning of the rainy season. Mean minimum temperatures ranged from 9.28 °C in August to 22.6 °C in December, while maximum temperatures ranged from 28.8 °C in August to 33.05 °C in January. Mean relative humidity followed a similar pattern, with lower values in August and higher values in December.

Table 2. Meteorological data recorded by the weather station at the State University of Londrina (UEL) during the experimental period. Month/Year: reference months; Total Rainfall: total monthly precipitation (mm); Min. Temperature: mean monthly minimum temperatures (°C); Max. Temperature: mean monthly maximum temperatures (°C); Mean relative humidity: monthly mean relative humidity (%).

Month/Year	Total Rainfall (mm)	Min. Temperature (°C)	Max. Temperature (°C)	Mean relative humidity (%)
08/2024	28	9.28	28.81	54.51
09/2024	49.8	14.6	32.8	53.27
10/2024	71.3	18.57	32.38	63.23

11/2024	176.72	19.13	31.12	66.37
12/2024	206.8	22.6	30.85	77.47
01/2025	140.23	20.02	33.05	74.00

Source: Meteorological station of the School Farm, State University of Londrina (UEL), Brazil.

Concerning stomatal conductance, no significant differences were observed between treatments at the three evaluation times: pre-planting ($p = 0.926$), one day after planting ($p = 0.941$), and five days after planting ($p = 0.868$). At all times analyzed, stomatal conductance values (Figure 1A) remained similar between groups. Nevertheless, there was a tendency for an increase in the mean values of stomatal conductance over the period (from approximately $320 \text{ mmol m}^{-2} \text{ s}^{-1}$ before planting to $600 \text{ mmol m}^{-2} \text{ s}^{-1}$ five days after planting).

Similarly, the maximum activity of photosystem II also showed no significant differences between treatments (Figure 1B), with values of $p = 0.953$, $p = 0.758$, and $p = 0.893$ for pre-planting, and one day and five days after planting, respectively. Despite this, it was possible to verify a reduction in the mean values of maximum photosystem II efficiency one day after planting (from 0.7 to 0.45), followed by recovery on the fifth day after planting (0.7).

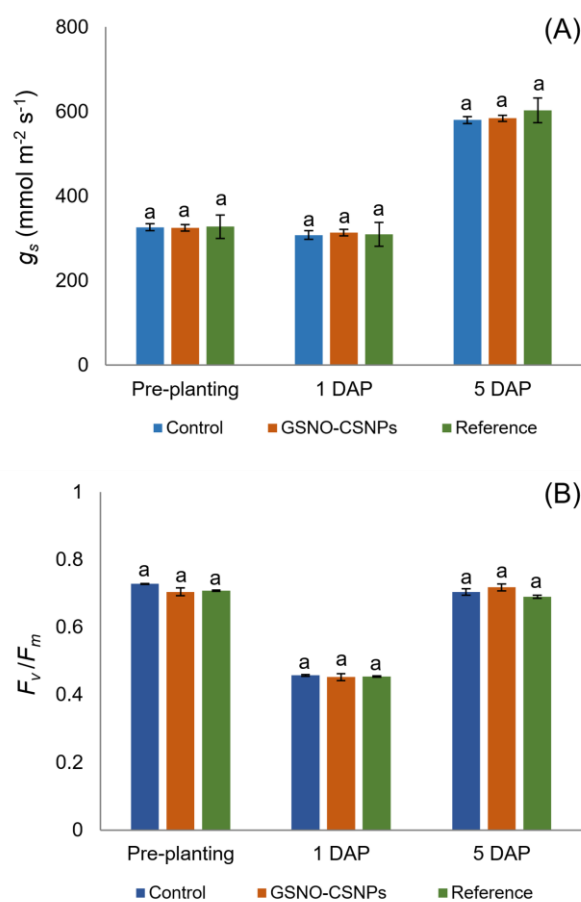


Figure 1. Stomatal conductance (A, g_s) and maximum quantum efficiency of photosystem II (B, F_v/F_m) of *Cecropia pachystachya* seedlings evaluated at pre-planting, one day after planting (1 DAP), and five days after planting (5 DAP). Treatments: Control (water); GSNO-CSNPs (chitosan nanoparticles containing GSNO); Reference (commercial nanoformulation). Values are expressed as mean $\pm$ standard error ($n = 10$). Different letters indicate significant differences among means according to ANOVA followed by Tukey's test ($p < 0.10$).

Regarding the first assessment of leaf gas exchange parameters (Table 3), the net photosynthetic rate was 46.49% higher in seedlings treated with GSNO-CSNPs compared to the control ($p = 0.0384$). The reference showed means similar to both the control and GSNO-CSNPs. Regarding transpiration, no significant differences were detected among treatments ($p = 0.351$). The intercellular concentration of CO_2 also did not vary significantly among treatments ($p = 0.935$). Instantaneous carboxylation efficiency was significantly higher in plants treated with GSNO-CSNPs than in the control ($p = 0.035$; 43.90% higher). The reference did not differ from either treatment, although its mean was 13.46% lower than that of GSNO-CSNPs.

In the second assessment of leaf gas exchange parameters (Table 3), the net photosynthetic rate values remained higher in GSNO-CSNPs and in the control, compared to the reference ($p = 0.001$). Similarly, the GSNO-CSNPs and control treatments showed higher instantaneous carboxylation efficiency than the reference ($p = 0.021$). At this time point, GSNO-CSNPs and the control did not differ from each other. In contrast, the three treatments showed no significant differences in transpiration and intercellular CO_2 concentration ($p = 0.139$ and $p = 0.506$, respectively).

Table 3. Net photosynthetic rate (A , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$); Transpiration (E , $\text{mmol m}^{-2} \text{ s}^{-1}$); Intercellular CO_2 concentration (C_i); and instantaneous carboxylation efficiency (k , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ Pa}^{-1}$) in *Cecropia pachystachya* seedlings in the second and ninth weeks after planting. Treatments: Control (water); GSNO-CSNPs (chitosan nanoparticles containing GSNO at $100 \mu\text{M}$); Reference (commercial nanoformulation Arbolina®). Values expressed as mean $\pm$ standard error ($n = 10$). Different letters indicate statistical differences between means, according to ANOVA followed by Tukey's test ($p < 0.10$).

		Control	GSNO-CSNPs	Reference
Two weeks after planting	A	11.4 ± 1.49^b	16.7 ± 0.67^a	14.8 ± 1.34^{ab}
	E	5.716 ± 0.668^a	5.395 ± 0.717^a	5.704 ± 0.880^a
	C_i	299.7 ± 4.80^a	302.4 ± 4.74^a	300.8 ± 4.87^a
	k	0.41 ± 0.052^b	0.59 ± 0.027^a	0.52 ± 0.043^{ab}
Nine weeks after planting	A	15.9 ± 0.98^a	16.1 ± 0.61^a	12.2 ± 1.16^b
	E	1.817 ± 0.304^a	2.58 ± 0.236^a	2.335 ± 0.180^a
	C_i	282.1 ± 3.32^a	289.0 ± 6.61^a	275.23 ± 14.80^a
	k	0.60 ± 0.048^a	0.61 ± 0.035^a	0.48 ± 0.050^b

Regarding the leaf area of the plants at the end of the experimental period, the GSNO-CSNP treatment again stood out, presenting the highest mean among all treatments, while the control and reference did not differ from each other ($p = 0.001$) (Figure 2A). The mean values for the GSNO-CSNP treatment showed an increase of 80.24% compared to the control and 93.40% compared to the reference.

With respect to total CO_2 assimilation, the same pattern observed for leaf area

was repeated, with the GSNO-CSNP treatment standing out and maintaining the highest mean among all treatments ($p < 0.001$, an increase of 74.75% and 96.70% compared to the control and reference, respectively) (Figure 2B).

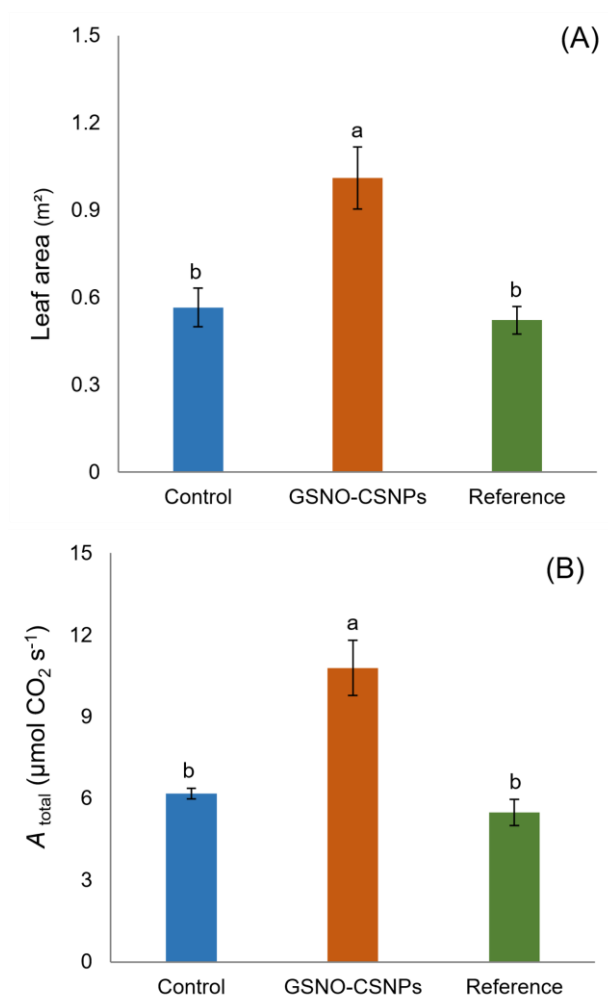


Figure 2. Leaf area (A) and total CO_2 assimilation (B, A_{total}) of *Cecropia pachystachya* seedlings evaluated at the end of the field experiment. Treatments: Control (water); GSNO-CSNPs (chitosan nanoparticles containing GSNO); Reference (commercial nanoformulation). Values are expressed as mean $\pm$ standard error ($n = 10$). Different letters indicate significant differences among means according to ANOVA followed by Tukey's test ($p < 0.10$).

No significant differences were observed in leaf dry mass among treatments ($p = 0.221$) (Figure 3A). For stem dry mass, the treatment with GSNO-CSNPs showed the highest mean, 86.40% higher than the reference ($p = 0.093$) (Figure 3B), but did not differ from the control. Similarly, the GSNO-CSNP treatment stood out with the highest mean of shoot dry mass, which differed from the reference but not from the

control ($p = 0.079$, Figure 3C). These differences were significant only at the 10% level.

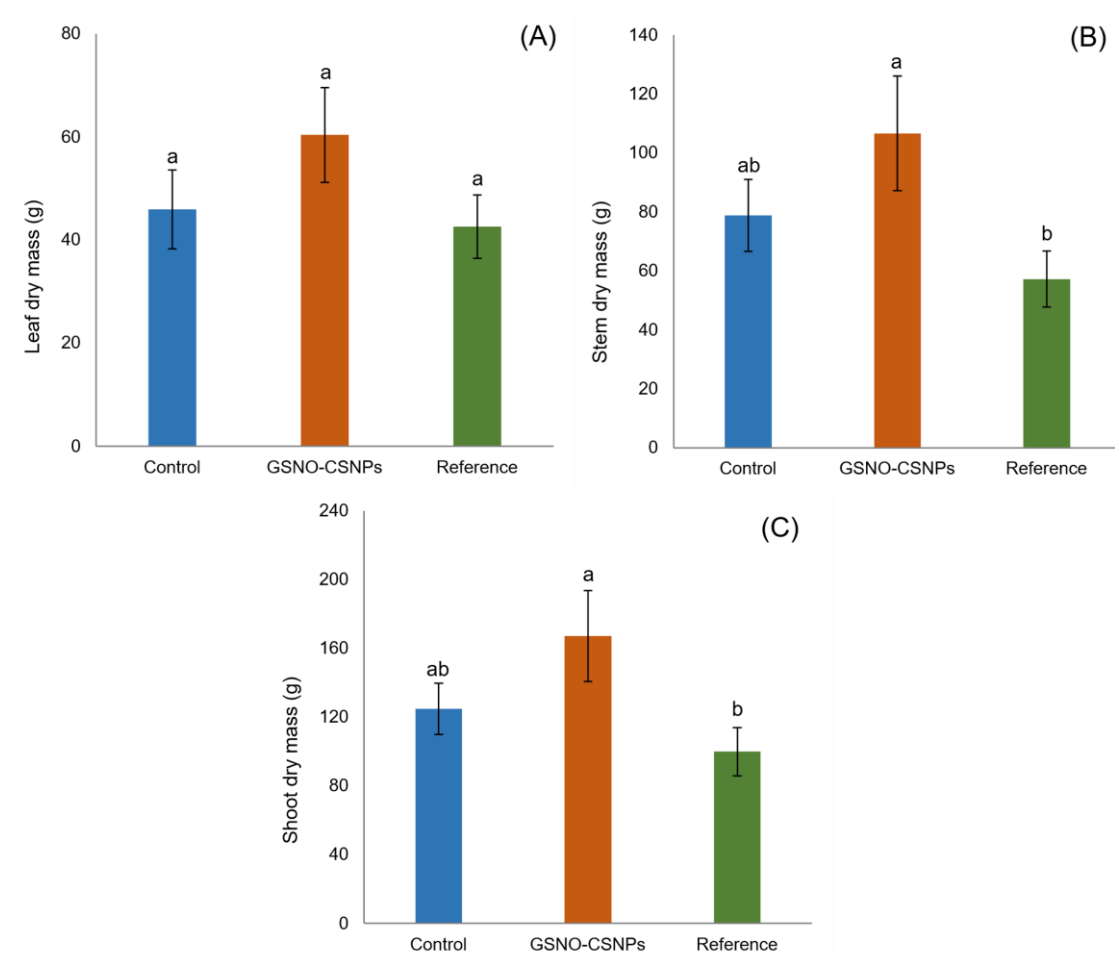


Figure 3. Dry mass of leaves (A), stem (B), and shoot parts (C) of *Cecropia pachystachya* seedlings evaluated at the end of the field experiment. Treatments: Control (water); GSNO-CSNPs (chitosan nanoparticles containing GSNO); Reference (commercial nanoformulation). Values are expressed as mean $\pm$ standard error ($n = 10$). Different letters indicate significant differences among means according to ANOVA followed by Tukey's test ($p < 0.10$).

Principal component analysis (Figure 4) showed a clear separation of the treatments along both principal components (PC1 and PC2, which explained 65.10 and 34.90% of the total variation in the data). The GSNO-CSNP treatment was positioned on the right side of the graph, while the control and reference treatments were grouped on the left, indicating similar responses among themselves and contrasting responses in relation to the GSNO-CSNPs.

The separation of the GSNO-CSNP treatment was influenced by variables associated with photosynthetic performance and biomass accumulation, such as the

photosynthetic rate and intercellular CO₂ concentration two and nine weeks after planting, total CO₂ assimilation, instantaneous carboxylation efficiency in the ninth week after planting, transpiration and stomatal conductance two weeks after planting, leaf area, and dry biomass.

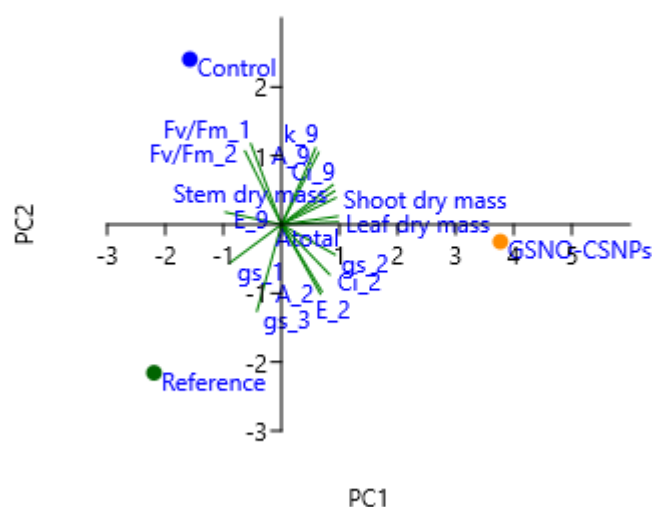


Figure 4. Principal component analysis (PCA) of data obtained from *Cecropia pachystachya* seedlings evaluated in the field. Treatments: Control (water); GSNO-CSNPs (chitosan nanoparticles containing GSNO); Reference (commercial nanoformulation). Parameters indicated: Pre-planting stomatal conductance (g_{s_1}); one day after planting (g_{s_2}) and five days after planting (g_{s_3}); Maximum photochemical efficiency of photosystem II pre-planting (F_v/F_{m_1}), one day post-planting (F_v/F_{m_2}); Total CO₂ assimilation (A_{total}); Net photosynthetic rate (A_2 and A_9), transpiration (E_2 and E_9), intercellular CO₂ concentration (C_{i_2} and C_{i_9}), instantaneous carboxylation efficiency (k_2 and k_9) after two and nine weeks of planting; dry mass of stem, leaf, and shoot.

5.5 DISCUSSION

The results obtained in this field study partially support the proposed hypothesis that treatment with GSNO-CSNPs promotes higher CO₂ assimilation and biomass accumulation in *C. pachystachya* seedlings after planting in the field. In general, the nanoformulation favored some physiological and growth parameters of the seedlings, and its estimated cost was low. Shortly after planting, the seedlings showed rapid recovery of stomatal conductance and maximum photosystem II activity, with no

differences among treatments. This reflects the ability of the species to acclimatize to transplanting stress and cannot be attributed to the nanoformulation. In the second week, GSNO-CSNPs increased net photosynthesis and carboxylation efficiency compared to the control, but not compared to the reference; in the ninth week, they did not differ from the control. At the end of the experiment, GSNO-CSNPs increased leaf area and total CO₂ assimilation compared to both the control and the reference, while stem and shoot dry mass were higher only compared to the reference.

The findings of the current field study corroborate and expand on the evidence obtained by do Carmo et al. (2025) under greenhouse conditions, in which GSNO-CSNPs increased stomatal conductance, net photosynthetic rate, and water potential of *C. pachystachya* seedlings subjected to short- and long-term water deficit. The authors also observed additional effects of NO release, including significant increases in root and leaf biomass, as well as improved formation of root absorbent hairs. A comparison between the present study and the previous one suggests that some positive effects of GSNO-CSNPs on *C. pachystachya* observed in the greenhouse may also occur under the variable environmental conditions of the field.

The consistency of the results observed under different experimental conditions can be explained by the protection provided by the chitosan polymeric matrix, which is capable of reducing the premature degradation of the NO donor and promoting its slow and gradual release, maximizing its positive effects (Oliveira et al. 2016; Kolbert, Lindermayr & Loake 2021; Preisler et al. 2025). In fact, the parameters obtained through the characterization of the nanoformulation indicate the formation of nanoscale particles expected for a polymeric system, with good particle size homogeneity and a positive surface charge larger than 20 mV, ensuring colloidal stability by electrostatic repulsion. In addition, chitosan has mucoadhesive properties that promote the

interaction of NPs with roots, increasing the availability of NO, both locally and systemically (Seabra et al. 2015; Lopes-Oliveira et al. 2019). This combination of greater stability and availability allows NO to perform its role as a signaling molecule more efficiently in the long term (Silveira et al. 2021).

An interesting effect observed in the present study was the increase in leaf area without a proportional increase in leaf dry mass, which can be related to the role of NO in stimulating cell division and expansion (Khan et al. 2023). This could result in larger and thinner leaves. However, leaf thickness was not measured, and this hypothesis remains to be tested, for example, by measuring leaf thickness or specific leaf area. These characteristics may increase the light absorption area and consequently the photosynthetic efficiency, without requiring a greater investment in leaf biomass. Concomitantly, NO improves stomatal regulation, photosystem II activity, photosynthetic carbon fixation, and antioxidant response, allowing efficient photosynthesis even under stressful conditions (Wang et al. 2019; Singhal et al. 2021; Lee et al. 2025).

Therefore, the gradual release of NO by GSNO-CSNPs may have favored both the direct increase in photosynthetic activity and carboxylation efficiency, as well as expansion of the leaf area, which in turn increases solar energy capture. These two effects may have acted in a complementary manner, enabling biomass accumulation, particularly in the stem. Future research should include measurements of root biomass and carbon accumulated in the soil.

The observed physiological benefits also highlight the potential of GSNO-CSNPs as a tool capable of increasing atmospheric carbon capture, thus contributing to mitigating the climate crisis (Gelaye & Getahun 2023). In addition to acting as a natural carbon sink, *C. pachystachya* is a pioneer species, capable of improving

microclimatic conditions and nutrient cycling in degraded areas, reinforcing its potential as a key pioneer species in forest landscape restoration strategies (Carvalho 2014; Brancalion et al. 2019). Furthermore, species of the genus *Cecropia* play fundamental ecological roles, facilitating forest regeneration and providing habitat and food for a wide diversity of fauna (Reis et al. 2022; Al Shammari 2024).

In addition to the ecophysiological effects, the current study also provides a preliminary cost estimate of the technology. A relevant aspect is the low estimated production cost of GSNO-CSNPs. At 40 mM GSNO, the estimated cost for the production of the nanoformulation is US\$ 66.18 per liter. Considering that the application was made at 100 μ M (a 400-fold dilution of the stock suspension), and a total volume of 145 mL of the working solution was applied per seedling, the cost per seedling was approximately US\$ 0.025. This amount is low compared to the total cost of implementing and maintaining restoration projects, which are often very expensive and limited by high mortality rates (Brancalion et al. 2019). However, this estimate does not include operational costs, such as labor and transport for application, and a complete cost-benefit analysis was not performed.

It should be noted that the costs estimated in this study were calculated based on the use of analytical grade reagents (PA-ACS, Sigma), which are highly pure and therefore more expensive. Considering the possibility of applying this technology on a large scale, it is entirely feasible to test cheaper technical-grade reagents, which would significantly reduce the final cost per liter. In addition, the synthesis can be optimized and scaled up for production in even larger volumes, enabling increases in yield and reductions in input and energy consumption, which could also lead to a reduction in the final cost per liter.

Furthermore, the formulation is also environmentally sustainable, which is

another important aspect. All components used are biodegradable and biocompatible. GSNO is naturally found as an endogenous NO donor in living organisms (Romera et al. 2023). Chitosan is biodegradable and biocompatible, and is derived from fishing industry waste, further contributing to the circular economy (Riseh et al. 2024). In addition, the GSNO-CSNP synthesis process has a low carbon footprint and, when associated with the increased CO₂ assimilation and biomass accumulation observed in the treated seedlings, demonstrates the potential of this technology as a nature-based solution to enhance carbon sequestration and contribute to mitigating the current climate crisis.

Therefore, the results of this study indicate that GSNO-CSNPs can improve some performance parameters of *C. pachystachya* at a low estimated cost. However, their economic viability at large scale still depends on operational analyses. The gains in photosynthesis and biomass accumulation observed in *C. pachystachya* suggest that this technology has the potential to increase the competitiveness of pioneer species, while the low estimated cost per plant supports further tests in restoration programs. It is also worth noting that no *C. pachystachya* seedlings died during the experiment, regardless of the treatment applied, which is in accordance with the rapid recovery of physiological parameters. Because no mortality occurred, this study could not demonstrate benefits on survival or reductions in replanting. It remains to be evaluated whether this technology could improve the survival of seedlings of species with poor establishment in the field. In addition, the field trial evaluated initial seedling performance and was not a controlled drought tolerance test, as in the greenhouse study (do Carmo et al. 2025).

Future studies that consider different species and conditions of natural competition are essential to assess the efficiency of the technology in more complex

and representative contexts at the landscape scale. Another relevant point is that the experiment was conducted with only one species, under manual weed control, which limits the generalization of the results to more diverse communities.

In summary, GSNO-CSNPs increased leaf area and total CO₂ assimilation of *C. pachystachya* seedlings in the field, and increased photosynthesis and carboxylation efficiency compared to the control in the second week after planting. These results were obtained with one species, at one site, over six months, and under manual weed control. Therefore, they should not be extrapolated to other sites, species, or competition conditions without further tests. Future studies should include other species and sites, natural competition, and longer evaluations of survival and growth.

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5.7 SUPPLEMENTARY INFORMATION

Table S1. Detailed presentation of the production costs of the GSNO-CSNPs nanoformulation used in the experiment, including capital and operational components and the economic assumptions adopted.

PROCESSING COSTS				
Raw materials				
Material	Price (USD/kg)	Quantity (kg)	Cost	Cost Contribution (%)
Chitosan (low molecular weight)	1,558.56	0.1	155.86	2.39%
L-Glutathione reduced (GSH)	5,153.15	1.23	6,338.38	97.37%
Sodium tripolyphosphate (TPP)	60.61	0.06	3.64	0.06%
Sodium nitrite (NaNO ₂)	2.70	0.276	0.75	0.01%
Acetic Acid	9.91	0.75	7.43	0.11%
Water	0.03	120	3.24	0.05%
Total cost per batch (100 L)			US\$ 6,509.28	
			Cost per liter US\$ 65.08	
Equipment				
	Capacity	COST (USD)		
Precision balance (2.5 kg)	2.5kg	1,351.34		
Reactor/Mixer	150L	17,117.12		
Deionized water distiller	50L/h	2,702.69		
Laminar flow hood	150L	900.91		
Pump	5 m3/h	450.45		
Peristaltic pump	500ml/min	900.91		
Total equipment acquisition cost			23,423.42	
Total installation cost (TIC) - Lang=5			117,117.12	
Annualized cost (7% interest / 10-year useful life)			16,630.63	
Annual maintenance (10% of TIC)			1,663.05	
Fixed OPEX costs (15% of TIC)			2,494.58	
Cost per batch (1000 batches/year)			161,328.80	
Energy/Utilities				
	Unit price (USD)	Consumption rate	Cost (USD)	
Reactor/Mixer	0.14 (USD/kwh)	10 kwh	1.40	
Remaining equipment	0.14 (USD/kwh)	1 kwh	0.78	
Waste treatment	1.04 (USD/m3)	0,1 m3	0.10	
Operating cost per batch				US\$ 2.28
Labor		Cost		
Labor cost		5.40 US\$/h		
Labor hours		8 h		
Supervision		+ 100%		
		CUST LC/BATCH US\$ 86.49		

ASSUMPTIONS TABLE

COST	Contribution to cost	Notes
CAPEX		
	Equipment cost	-
	Total installed cost (TIC)	LANG factor = 5 (typical value for fluid processing)
	Interest rate	From equation 2: $k=0.142$ (considering $i=7\%$ and $t=10$ years)
OPEX		
	Raw materials	-
	Electricity	0.78 BRL/kwh (Brazilian average)
	Waste treatment	5.75 BRL/m ³ (Brazilian average)
	Labor	-
	Supervision and indirect OPEX cost	100% of labor cost
	Annual maintenance	10% TIC
	Fixed OPEX costs	15% TIC

6 CAPÍTULO 3 – THE DUAL ROLE OF NITRIC OXIDE IN STOMATAL MOVEMENT: MECHANISMS, CONTRADICTIONS AND NEW EVIDENCE FROM ATLANTIC FOREST NATIVE TREE SPECIES

Artigo a ser submetido à revista Plant Science

<https://www.sciencedirect.com/journal/plant-science>

Nota: Este capítulo corresponde ao manuscrito que será submetido à revista *Plant Science* e é apresentado integralmente, uma vez que todo seu conteúdo foi desenvolvido durante o período da realização deste trabalho.

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6.1 ABSTRACT

Stomata regulate gas exchange between the plant and the atmosphere, balancing CO₂ uptake and water loss. Among the signaling molecules involved in stomatal movement, nitric oxide (NO) plays a central but still ambiguous role: in many studies it promotes stomatal closure, while in others it favors opening, depending on the dose, the NO source, environmental factors, and the species studied. This duality motivated the present review, which addresses NO biosynthesis pathways, the molecular mechanisms underlying stomatal closure and opening, and discusses the main sources of variability that explain this paradox. It then presents emerging strategies for controlled NO delivery, highlighting chitosan nanoparticles (CS NPs) as carriers for NO donor molecules such as S-nitrosoglutathione (GSNO). Finally, the chapter presents a screening conducted with 36 native Atlantic Forest tree species, treated with GSNO-loaded CS NPs, evaluating the stomatal conductance response via foliar or soil application. The results indicate that, under the conditions evaluated, the response to NO varied widely among species, even under the same donor, which highlights the need for caution when extrapolating results from model species to the different crops and the broad biodiversity.

Keywords: Stomatal conductance; S-nitrosoglutathione; Chitosan nanoparticles;

Species-specific responses.

6.2 INTRODUCTION

Most land plants have stomata, which are specialized, tiny pores in their epidermis that are surrounded by guard cells that control their opening by altering turgor (Clark et al., 2022; Rhaman et al., 2025). Stomata are essential for photosynthesis, respiration, and transpiration because they regulate the flow of gases between the plant and the atmosphere, balancing the uptake of carbon dioxide with the loss of water (Buckley, 2005; Guo et al., 2023). Stomata have also influenced the hydrological cycle and plant adaptation to a variety of climatic situations since they first appeared more than 400 million years ago (Berry et al., 2010; Giannoutsou et al., 2025).

As dynamic regulators of gas exchange, stomata play a crucial role in determining plant water-use efficiency (WUE). To balance carbon assimilation and water conservation, their aperture must be tightly regulated (Giannoutsou et al., 2025). Long-term stomatal closure can significantly restrict photosynthesis; therefore, plants must precisely regulate their stomatal movements to optimize the trade-off between CO₂ uptake and water loss, a process largely governed by an abscisic acid (ABA)-dependent signaling pathway (Huber et al., 2019; Rhaman et al., 2025).

In addition to ABA, nitric oxide (NO) has emerged as a crucial regulator of the network of interacting signals involved in stomatal movement. NO is a small, redox-active gaseous molecule that participates in many physiological processes in plants, such as growth, development, and responses to biotic and abiotic stresses (Sun et al., 2019; Sarma et al., 2025). In guard cells, NO has been linked with the regulation of ion channels, cytosolic Ca²⁺ levels, and reactive oxygen species (ROS), placing it at the core of the signaling pathways governing stomatal movements (Courtois et al., 2008; Santisree et al., 2019).

Nevertheless, the function of NO in stomatal responses is still ambiguous. Numerous studies show that NO functions as a downstream element of ABA signaling, leading to stomatal closure in stress conditions. On the other hand, additional research has demonstrated that, depending on the doses, experimental and environmental conditions, NO can either negatively influence ABA responses or even contribute to

stomatal opening (Mata-Pérez et al., 2023; Perlikowski et al., 2025). These conflicting results imply that NO's role in guard cells is highly context-dependent and cannot be explained by a simple linear pathway (Sun et al., 2019).

This dual function is consistent with the idea that NO is a “Janus-faced” signaling molecule that, depending on its concentration and cellular context, can exert both positive and negative effects (Correa-Aragunde et al., 2016). At controlled physiological levels, NO acts as a versatile signaling molecule, primarily through post-translational modifications such as S-nitrosylation, which modulates protein activity, stability, and interactions in a rapid and reversible way (Gupta et al., 2020). In contrast, higher or unregulated levels of NO can promote processes such as protein nitration and the formation of reactive nitrogen species (RNS), which may alter cellular function and contribute to nitrosative stress (Mata-Pérez et al., 2023).

Variations in experimental techniques, such as the use of different NO donors, concentrations, exposure times, and plant systems, may contribute to some of this discrepancy (León and Costa, 2019). Stomatal behavior may also be influenced by environmental factors, species-specific responses, and interactions with other signaling molecules, necessitating a more nuanced understanding of NO's multifaceted roles in stomatal physiology (Gahir et al., 2020).

This review aims to explore the complex and often dual roles of NO in stomatal movement, going beyond a straightforward open-close paradigm underlying its diverse effects. We summarize what is now known about NO-mediated stomatal responses, emphasizing both inhibitory and promotive functions, and investigate the possible mechanisms behind these divergent results. We offer some hypotheses to explain the apparent paradox in NO signaling. Additionally, we address new approaches to regulate NO delivery, focusing on nanotechnology-based systems as potential instruments to enhance our comprehension and control of NO-mediated stomatal regulation. Finally, this chapter presents an interspecific screening of 36 native Atlantic Forest species, evaluating stomatal responses to a nanoencapsulated NO donor. The goal is to characterize the variability of stomatal conductance responses under the conditions evaluated.

6.3 NO IN PLANT SYSTEMS: FUNCTIONS AND SOURCES

6.3.1 BIOLOGICAL ROLES OF NO IN PLANTS

NO signaling in plants relies on multiple, sometimes interconnected mechanisms, including post-translational modifications (PTMs) of proteins; direct nitrosylation of metal centers such as copper and iron to form metal-nitrosyl complexes (M-NO), as well as protein nitration mediated by the reactive NO-derived species peroxynitrite; and NO can also trigger alterations in gene expression by regulating different transcription factors and mitogen-activated protein kinases (Gupta et al., 2021; Khator et al., 2024).

Among these mechanisms, PTMs, and S-nitrosylation in particular, are the most extensively characterized in the context of guard cell signaling and are the focus of the remainder of this section. S-nitrosylation, which is the covalent binding of the nitrosyl group to cysteine thiol residues to generate S-nitrosothiols, is a key mechanism of NO-mediated signal transduction. Depending on the local NO availability and protein structure, this fast, reversible, and highly selective alteration enables dynamic modulation of protein activity, stability, and interactions (Kovács and Lindermayr, 2013; Nabi et al., 2019; Sedlářová et al., 2025; Azzawi et al., 2026).

Apart from S-nitrosylation, NO can also promote protein nitration, especially on tyrosine and tryptophan residues. This is frequently used as a biomarker of nitrosative stress and is associated with changes in protein function (Corpas et al., 2021; Romera et al., 2023). When combined, these changes increase NO's regulatory ability, allowing it to flexibly and context-dependently influence several signaling pathways in various cellular compartments (Perlikowski et al., 2025).

Through these molecular mechanisms, NO plays a central role in plant responses. By controlling several physiological and biochemical processes, NO promotes stress tolerance in situations like drought, salt, and high temperatures (Santisree et al., 2019). These include maintaining cellular redox equilibrium, improving antioxidant defenses through enhanced activity of ROS-scavenging enzymes, and modifying stomatal movement to regulate water loss. Furthermore, NO affects plant growth and development by supporting photosynthetic performance by maintaining carbon uptake and chlorophyll content, as well as promoting root system

architecture, including lateral and root hair formation and seed germination (Campos et al., 2023; Mata-Pérez et al., 2023). When taken as a whole, these results demonstrate the complex function of NO as an integrative regulator of plant responses.

6.3.2 BIOSYNTHESIS PATHWAYS AND NO SOURCES

There is ongoing discussion over the mechanisms of NO biosynthesis in plants, with evidence suggesting both enzymatic and non-enzymatic pathways contributing to its cellular pool (Simontacchi et al., 2015). Organelles such as mitochondria, chloroplasts, and peroxisomes, which serve as important centers of reactive species metabolism and cellular signaling, are known to be intimately linked to NO biosynthesis (Kolbert et al., 2021).

The most thoroughly studied of these NO biosyntheses is the reductive pathway. This route requires cofactors such as molybdopterin, heme, and flavin adenine dinucleotide. Nitrate reductase (NR) uses NADH as an electron donor to catalyze the reduction of nitrate to nitrite. As an alternative reductase, NR can also convert nitrite to NO under certain situations, such as hypoxia, anoxia, low pH, or pathogen infection (Gupta et al., 2022; Rhaman et al., 2025; Sedlářová et al., 2025).

NO can also be formed from nitrite through non-enzymatic mechanisms, particularly in low pH or highly reducing environments, as well as by the action of the mitochondrial electron transport chain (López-Gómez et al., 2023). In parallel, a lot of research has been done on the existence of an oxidative pathway for NO synthesis in plants, which is comparable to that seen in mammals via the enzyme NO synthase (NOS). These oxidative pathways are oxygen-dependent and involve L-arginine, polyamines, and hydroxylamine (Timilsina et al., 2022).

Nevertheless, despite their existence in cyanobacteria and algae, no analogous NOS genes have been found in embryophyte plants so far. However, scientific evidence suggests that plants have NOS-like activity, indicating the presence of alternate NO generation pathways. All of this data supports the idea that NO biosynthesis in plants is intricate, varied, and heavily influenced by physiological and environmental factors (Kolupaev et al., 2024; Perlikowski et al., 2025).

6.4 MECHANISMS OF NO-MEDIATED STOMATAL REGULATION

6.4.1 EVIDENCE FOR NO-INDUCED STOMATAL CLOSURE

Several studies have shown that NO has an important role in stomatal closure, particularly during ABA signaling and under abiotic stress. Early research demonstrated the significance of NO in guard cell signaling by demonstrating that the application of exogenous NO donors could promote stomatal closure in some species (van Meeteren et al., 2020; Perlikowski et al., 2025).

Since then, NO has gained widespread recognition as a crucial element of ABA-induced stomatal responses, including studies demonstrating that ABA-induced stomatal closure is reversed in the presence of NO scavengers, highlighting that this process is highly complex and involves multiple signaling cascades in guard cells (García-Mata and Lamattina, 2002; Xu et al., 2024).

The pathway begins when ABA binds to its intracellular receptor (PYR/PYL/RCAR), promoting a conformational change that allows the receptor complex to inhibit type 2C protein phosphatases (PP2Cs), which normally act as negative regulators of the pathway (Rhaman et al., 2025). With PP2C inhibition, the protein kinase open stomata 1 (OST1) becomes activated. Active OST1 is then able to phosphorylate NADPH oxidases (RBOHs), triggering the production of reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2), which subsequently promotes NO production in guard cells (Postiglione and Muday, 2020; Rhaman et al., 2025).

Once produced, NO acts through downstream messengers such as cyclic guanosine monophosphate (cGMP) to promote the mobilization of Ca^{2+} from intracellular stores into the cytosol. The increase in cytosolic Ca^{2+} concentration leads to the inactivation of K^+ channels and activation of slow-type anion efflux channels (Gayatri et al., 2013; Gahir et al., 2020).

In addition, NO stimulates phospholipases C and D to produce phosphatidic acid, which reinforces NADPH oxidases activity and helps maintain PP2Cs inhibition, sustaining the signaling pathway (Bharath et al., 2021). The massive efflux of solutes increases guard cell osmotic potential, leading to water loss and reduced turgor pressure, ultimately causing guard cells to shrink and the stomatal pore to close.

(Correa-Aragunde et al., 2016).

However, many studies have revealed conflicting effects of NO on guard cell behavior under particular physiological and experimental settings, so this well-established model cannot account for all observed responses. These findings imply that stomatal movement mediated by NO is very context-dependent.

6.4.2 EVIDENCE FOR NO-INDUCED STOMATAL OPENING

Although the role of NO in stomatal closure is well documented, in certain situations the opposite effect has been observed, in which NO acts to promote stomatal opening or even inhibit its closure.

The stimulation of stomatal opening by NO depends on its concentration, as its effect on guard cells is highly dose-dependent. While low concentrations of NO can promote stomatal opening or inhibit stress-induced closure in certain species, higher concentrations consistently act as essential components in the ABA signaling cascade for closure (Daszkowska-Golec and Szarejko, 2013; Gayatri et al., 2013).

A central mechanism of NO-mediated stomatal opening is the *S*-nitrosylation of the OST1 protein, in which it is covalently bound to NO at cysteine residue 137, thereby inhibiting its kinase activity (Xu et al., 2024). Since OST1 is a positive regulatory protein essential for ABA-induced closure, its inactivation by NO acts as a negative feedback mechanism that shuts down signaling for stomatal closure, thereby promoting the maintenance of stomatal opening (Wang et al., 2014; Chong et al., 2022).

In addition, NO can induce the nitration of tyrosine residues on ABA receptors (PYR/PYL/RCAR). This post-translational modification leads to the alteration and subsequent inactivation of the receptor, which is then recognized by the cell and targeted for degradation via the proteasome. Since this degradation removes the components that would initiate ABA-mediated stomatal closure, it ultimately promotes the maintenance of stomatal opening or prevents new closure signals from being detected, resulting in insensitivity to ABA signaling (Mata-Pérez et al., 2023).

In addition to acting directly on the ABA-signaling components, NO can also promote stomatal opening indirectly by its effects on cellular redox homeostasis. Depending on the concentration and physiological context, NO can alter ROS accumulation and stimulate the antioxidant system, causing these signals, necessary for ABA-induced closure, to be attenuated (Gahir et al., 2020; Wani et al., 2021). This

suggests that NO may function not only as a signaling molecule but also as a regulator of the redox state that modulates guard cell responses.

These closing and opening-associated pathways are also modulated by several contextual factors such as species, dose and donor, environmental conditions, and methodological choices, which are examined in detail in the following sections (Figure 1).

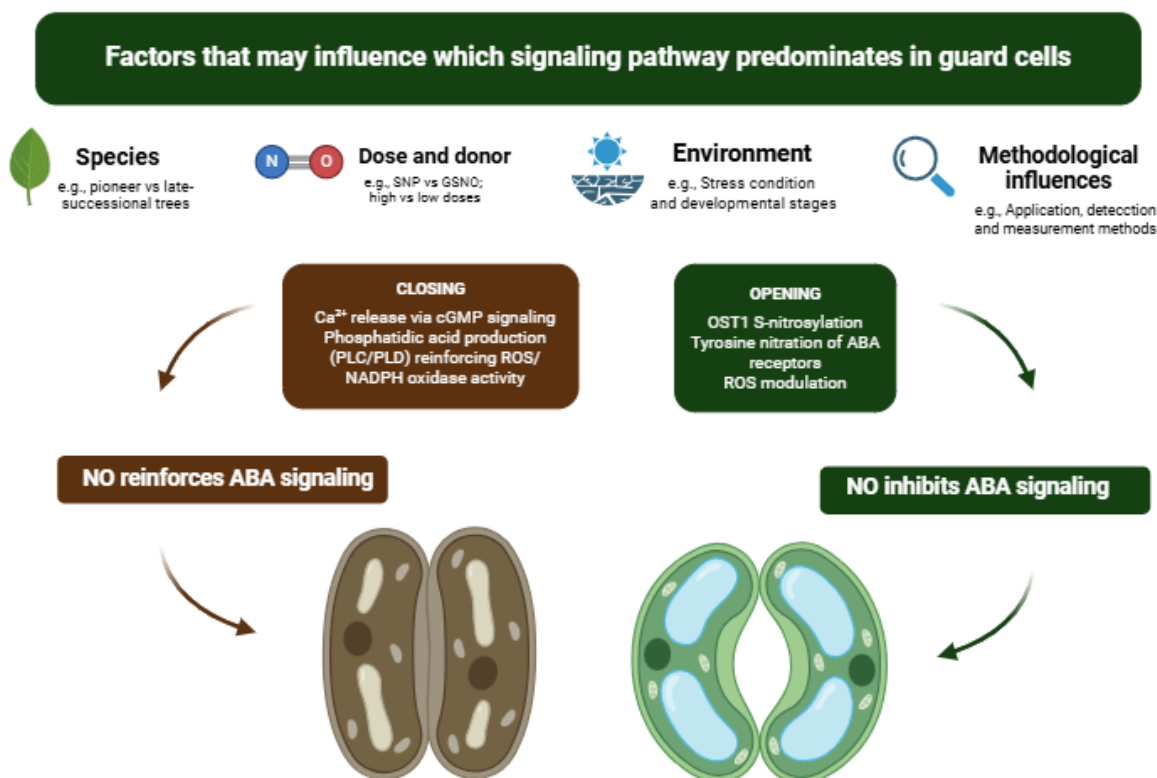


Fig. 1. Contextual factors (species, dose and donor, environment, and methodological influences) that may determine which signaling pathway predominates in the guard cell, and the main molecular events associated with each outcome.

6.4.3 GENETIC AND OMICS INSIGHTS INTO NO-MEDIATED STOMATAL REGULATION

Advances in the fields of genetics and the omics sciences have shown that NO does not act as a passive messenger, but rather as a central and sophisticated regulator that uses PTMs and the control of protein function regulation to modulate stomatal movement.

For example, studies using mutants were crucial for identifying the genetic origin of NO in guard cells. In *Arabidopsis*, NR is encoded by two genes, *NIA1* and *NIA2*,

with *NIA1* isoform being primarily responsible for NO synthesis during ABA-induced stomatal closure. On the other hand, *nia1nia2* double mutants exhibit reduced levels of L-arginine, which also negatively affects NO production (Modolo et al., 2006; Neill et al., 2008; Santisree et al., 2015).

Beyond genetic studies, proteomics also played a key role by identifying that NO acts as a biochemical “brake” through specific PTMs in components of the ABA-mediated signaling pathway. For example, through the S-nitrosylation of the OST1 protein and nitration of the PYR/PYL/RCAR, it was found that NO can act as a negative regulator of ABA-mediated stomatal closure (Wang et al., 2014; Laxalt et al., 2016).

In addition, the transcriptome has also provided significant new insights into the versatility of NO signaling. For example, in tomatoes, the transcription factor SIWRKY81 has been identified as an inhibitor of stomatal closure by regulating NO levels in guard cells during periods of drought (Ahammed et al., 2020).

Previous studies have also shown that NO is capable of altering gene expression in response to drought. In *Arabidopsis* plants expressing rat neuronal NO-synthase, significant changes were observed compared to wild-type plants, with approximately 184 genes upregulated and 263 downregulated. Among the main transcriptional changes observed were those in genes involved in redox and sugar metabolism, which were followed by high survival and biomass rates, indicating a significant effect of NO (Farnese et al., 2016).

Taken together, these findings show that NO controls stomatal behavior at several levels, including gene expression and PTMs of important signaling proteins. NO affects a complex regulatory network rather than operating through a single pathway. This complexity may contribute to the explanation of why, depending on the physiological and environmental conditions, NO can either promote or suppress stomatal opening.

6.5 LITERATURE OVERVIEW OF NO-INDUCED STOMATAL RESPONSES

As discussed in the sections above, NO can act to induce both stomatal closure and opening, depending on the signaling pathway activated. Table 1 synthesizes this overview, gathering information on the species, donor, application method, presence of stress, and the stomatal effect observed in each study. The following sections discuss the main factors that may explain this variability, as synthesized in Table 1. As

this chapter is a narrative review, Table 1 presents a selection of representative studies rather than an exhaustive survey of the literature.

Table 1

Reported stomatal responses (opening, closing, or no change) to NO across plant species, application methods, and stress conditions. Note: SNP, sodium nitroprusside; SNAP, S-nitroso-N-acetyl-DL-penicillamine; GSNO, S-nitrosoglutathione; CS NPs, chitosan nanoparticles; NR, nitrate reductase; MSA, mercaptosuccinic acid (as in S-nitroso-MSA); N/A, not applicable.

Species	Family	Donor/ NO source	Concentration/ dose range	Application method	Stress condition	Observed effect	Reference
<i>Triticum aestivum</i> L.	Poaceae	SNP/SNAP	150 μ M	Detached leaves	Drought	(-) Closing	García-Mata and Lamattina, 2001
<i>Tradescantia</i> sp.	Commelinaceae	SNP	200 μ M	Detached leaves	Drought	(-) Closing	García-Mata and Lamattina, 2001
<i>Salpichroa origanifolia</i> (Lam.) Baill.	Solanaceae	SNP	200 μ M	Detached leaves	Drought	(-) Closing	García-Mata and Lamattina, 2001
<i>Vicia faba</i> L.	Fabaceae	SNP	200 μ M	Detached leaves	Drought	(-) Closing	García-Mata and Lamattina, 2001
<i>Pisum sativum</i> L.	Fabaceae	SNP and GSNO	100 μ M (SNP) and 500 μ M (GSNO)	Epidermal peel	No stress	(-) Closing	Neill et al., 2002
<i>Arabidopsis thaliana</i> (L.) Heynh.	Brassicaceae	SNP/endogenous NO (via NR)	50 μ M (SNP)	Epidermal peel	No stress	(-) Closing	Desikan et al., 2002
<i>Vicia faba</i> L.	Fabaceae	SNP	150 μ M	Epidermal peel	No stress	(-) Closing	García-Mata and Lamattina, 2002
<i>Vicia faba</i> L.	Fabaceae	SNAP	5000 μ M	Epidermal peel	No stress	(+) Opening	Sakihama et al., 2003
<i>Vicia faba</i> L.	Fabaceae	Endogenous NO	N/A	Epidermal strip bioassay	UV-B radiation	(-) Closing	He et al., 2005
<i>Arabidopsis thaliana</i> (L.) Heynh.	Brassicaceae	Endogenous NO (via NR)	N/A	Intact leaf	No stress	(-) Closing	Bright et al., 2006

Species	Family	Donor/ NO source	Concentration/ dose range	Application method	Stress condition	Observed effect	Reference
<i>Poncirus trifoliata</i> (L.) Raf.	Rutaceae	SNP	150 µM	Hydroponics and pots	Drought	(-) Closing	Fan and Liu, 2012
<i>Arabidopsis thaliana</i> (L.) Heynh.	Brassicaceae	GSNO	50 to 500 µM (in vitro); 250 µM (in vivo)	In vitro and in vivo incubation	No stress	(+) Opening	Wang et al., 2014
<i>Brassica juncea</i> (L.) Czem.	Brassicaceae	SNP	100 µM	Foliar spray	No stress and salt stress	(+) Opening	Fatma et al., 2016
<i>Crambe abyssinica</i> Hochst. ex R.E.Fr.	Brassicaceae	SNP	75 and 150 µM	Foliar spray	Drought	(+) Opening	Batista et al., 2018
<i>Glycine max</i> (L.) Merr.	Fabaceae	SNP	100 µM	Foliar spray	Drought	(+) Opening	Sousa et al., 2019
<i>Saccharum</i> sp.	Poaceae	Endogenous NO (via nitrate)	N/A	Nutrient solution	Drought	(+) Opening	Pissolato et al., 2020
<i>Vicia faba</i> L.	Fabaceae	Gaseous NO/SNP	50 to 1000 µM	Leaf disc/intact leaf	No stress	(+) Opening at low doses; (-) closing at high doses	van Meeteren et al., 2020
<i>Solanum lycopersicum</i> L.	Solanaceae	Endogenous NO (via NR/SIWRKY81)	N/A	Intact plant	Drought	(-) Closing	Ahammed et al., 2020
<i>Glycine max</i> (L.) Merr.	Fabaceae	Endogenous NO	N/A	Epidermal peel	Drought	(-) Closing during the early stages of stress	Casaretto et al., 2021
<i>Kandelia obovata</i> Sheue, H.Y.Liu & J.W.H.Yong	Rhizophoraceae	SNP	100 µM	Hydroponics	Salt stress	(+) Opening	Hasanuzzaman et al., 2021
<i>Vitis vinifera</i> L.	Vitaceae	SNP	≈ 200 µM	Foliar spray	No stress	(-) Partial closing	Pontin et al., 2021
<i>Vitis vinifera</i> L.	Vitaceae	SNP	≈ 200 µM	Foliar spray	Drought	(=) No change	Pontin et al., 2021

Species	Family	Donor/ NO source	Concentration/ dose range	Application method	Stress condition	Observed effect	Reference
<i>Heliocarpus popayanensis</i> Kunth	Malvaceae	CS NPs containing S-nitroso-MSA	200 µM	Soil application	Drought	(+) Opening	do Carmo et al., 2021
<i>Glycine max</i> (L.) Merr.	Fabaceae	CS NPs containing S-nitroso-MSA	1000 µM	Soil application	Copper stress	(=) No change under moderate stress and (+)	Gomes et al., 2022
<i>Hymenaea stigonocarpa</i> Mart. ex Hayne	Fabaceae	CS NPs containing GSNO and free GSNO	200 µM	Soil application	Hardening-off process	(+) Opening	Guariz et al., 2025
<i>Hymenaea courbaril</i> L.	Fabaceae	CS NPs containing GSNO	200 µM	Soil application	Hardening-off process	(+) Opening	Guariz et al., 2025
<i>Amburana cearensis</i> (Allemão) A.C.Sm.	Fabaceae	CS NPs containing GSNO	200 µM	Soil application	Hardening-off process	(+) Opening	Guariz et al., 2025
<i>Cecropia pachystachya</i> Trécul	Urticaceae	CS NPs containing GSNO	50 µM	Soil application	Drought	(+) Opening	do Carmo et al., 2025
<i>Cariniana estrellensis</i> (Raddi) Kuntze	Lecythidaceae	CS NPs containing GSNO	25 to 800 µM	Soil application	Drought	(=) No change	do Carmo et al., 2025
<i>Glycine max</i> (L.) Merr.	Fabaceae	GSNO	1000 µM	Seed treatment	No stress	(-) Closing	Preisler et al., 2025
<i>Glycine max</i> (L.) Merr.	Fabaceae	CS NPs containing GSNO	1000 µM	Seed treatment	No stress	(=) No change	Preisler et al., 2025
<i>Glycine max</i> (L.) Merr.	Fabaceae	Alginate NPs containing GSNO	1000 µM	Seed treatment	No stress	(=) No change	Preisler et al., 2025

Table 1 summarizes 32 experimental records on NO-induced stomatal responses compiled from the literature. The reported effects were nearly evenly split between stomatal closure (14 records) and opening (13 records), with the remaining five records reporting no significant change; two records described opposite responses within the same study, depending on the NO dose applied

or the severity of the imposed stress. Nine of the records in Table 1, from five studies, used a nanoencapsulated NO donor, and these nanoencapsulated treatments were associated with stomatal opening in most cases. Because the compiled studies differ in species, donor chemistry, application method, and stress condition, this heterogeneity itself illustrates the central argument of this chapter: that the apparently contradictory role of NO in stomatal movement is better explained by the interaction of these contextual factors than by a single generalizable mechanism.

6.6 THE NO PARADOX IN STOMATAL BIOLOGY

6.6.1 SPECIES-DEPENDENT VARIABILITY

One of the factors frequently associated with differences in NO-mediated stomatal movement responses is interspecies variability. Although NO is widely recognized as an important molecule in stomatal closure, the effects observed following its application are not always consistent across different plant species. While some species show a high sensitivity and clear benefits from NO administration, others remain unresponsive or exhibit unexpected effects.

One reason for this variation may be linked to factors intrinsic to each plant, such as the successional group to which it belongs, since plants exhibit contrasting strategies regarding resource use, growth, and stress tolerance. For example, fast-growing, pioneer plants, adapted to open environments with high light availability, tend to exhibit higher growth and photosynthesis rates, in addition to being more responsive to stress signals and signaling molecules such as NO (Zabotto et al., 2024; Lopes-Oliveira et al., 2019).

In contrast, slow-growing, late-successional species, with more conservative strategies, may exhibit less pronounced responses or rely on different signaling pathways for the modulation of stomatal conductance. In these species, the sensitivity of guard cells to signals such as ABA, ROS, and NO may differ substantially, resulting in less pronounced stomatal responses (Zabotto et al., 2024; do Carmo et al., 2025).

Another possible explanation for the interspecific variability in responses to NO may be related to differences in nitrogen acquisition and assimilation strategies. Since NO is closely linked to nitrogen metabolism, particularly through the activity of NR enzyme, species that differ in their primary nitrogen source may exhibit different capacities for NO production and signaling (Frunghillo et al., 2014).

Pioneer species tend to exhibit greater uptake and assimilation of nitrate (NO_3^-) (Silva et al., 2021). Since NR is one of the main sources of NO in plants, these species may have a greater potential for endogenous NO production, especially under stress conditions (Farnese et al., 2016). On the other hand, late-successional species exhibit greater capacity for ammonium (NH_4^+) uptake and assimilation, reducing their

dependence on the NR pathway (Zabotto et al., 2024), and NO production may differ. In this context, it is possible that pioneer species rely more on NO-mediated signaling pathways and, consequently, exhibit greater responsiveness to it, although this hypothesis still requires experimental validation.

Furthermore, much of our current understanding of NO signaling has been discovered from a limited number of model species, such as *Arabidopsis thaliana* and *Vicia faba*. Although studies with these species are fundamental to understanding the mechanisms involved in the effects of NO on plant metabolism, extrapolating these results to other species, especially tree species, can be complex. This issue is particularly relevant in megadiverse biomes, such as the Atlantic Forest, where species with different ecological strategies coexist. Given the great functional diversity within this biome, it is unlikely that a single response exists capable of describing the patterns of response to NO observed among species, further reinforcing the need for approaches that take into account the ecological and physiological context of each group.

6.6.2 DOSE-DEPENDENT VARIABILITY

NO concentration is one of the factors frequently cited to explain the contrasting effects observed in stomatal movement. Unlike other plant regulators, whose responses follow more predictable patterns, NO can trigger different and even opposite effects depending on its concentration, as is the case with stomatal opening versus closure (Aboul-Soud et al., 2009; Gomes et al., 2024).

Studies conducted in various experimental systems support the premise that low concentrations of NO may promote stomatal opening, while high concentrations tend to induce closure. Furthermore, the response appears to be different over time, suggesting that, in addition to the dose, the dynamics of NO bioavailability also influence stomatal movement (van Meeteren et al., 2020; Sousa et al., 2019; do Carmo et al., 2021).

These dose-dependent differences occur because, at low concentrations, NO is often involved as a negative feedback mechanism in ABA signaling, as it mediates the S-nitrosylation of OST1, which promotes stomatal opening even in the presence of ABA (van Meeteren et al., 2020). In contrast, higher concentrations tend to stimulate

processes associated with closure, such as changes in cellular redox status and modulation of ion channel activity (van Meeteren et al., 2020).

Taken together, this evidence shows that NO concentration is one of the factors responsible for directing the stomatal response; however, dose-dependent effects do not occur in isolation. The effects can also be strongly influenced by the physiological and environmental context, where factors such as abiotic stresses and the plant's developmental stage can alter the sensitivity and the response to NO (Simontacchi et al., 2015). Therefore, the effects of NO on stomatal movement must be interpreted within a broader context, where environmental conditions and plant characteristics contribute to the variations found in the literature.

6.6.3 ENVIRONMENTAL AND DEVELOPMENTAL INFLUENCES

In addition to the differences mentioned above, environmental and developmental factors can also influence NO-mediated stomatal responses, since NO acts as an integral part of a complex signaling network. Therefore, the final effect appears to depend not only on factors such as concentration but also on the environmental and physiological context in which it is produced or detected by guard cells.

Among the environmental factors that can modulate responses to NO, abiotic stress conditions, such as water deficit, can alter the interaction between NO, ABA, ROS and Ca^{2+} , resulting in divergent responses in stomatal closure (Khan et al., 2023). Consequently, the same NO signal may trigger distinct physiological responses depending on the prevailing environmental conditions. During drought, for example, NO is often associated with stomatal closure as part of a water-conservation strategy. However, the intensity and duration of the stress may alter how these signaling pathways are integrated, resulting in different physiological outcomes (Sun et al., 2019; do Carmo et al., 2025).

Additionally, factors related to plant development may contribute to this variability. Characteristics such as leaf age, guard cell maturity, and phenological stage influence metabolic processes, hormonal signaling, and redox homeostasis, which are closely related to NO production and perception. Therefore, tissues at different stages of development may exhibit different sensitivities to the same stimulus (Simontacchi et

al., 2015; Stefanov et al., 2023).

Taken together, these observations indicate that the effects of NO on stomatal movement depend not only on the molecule itself but also on the interaction between multiple physiological and environmental factors. This context dependency reinforces the idea that NO should not be regarded as an intrinsic promoter of either stomatal opening or closure, but rather as a versatile messenger whose effects emerge from the integration of multiple signaling pathways (Laxalt et al., 2016; Prakash et al., 2018).

6.7 DECIPHERING THE NO PARADOX: TOWARD A CONTEXT-DEPENDENT FRAMEWORK

6.7.1 NO-RELEASE KINETICS AND DONOR CHEMISTRY

Much of the interpretation regarding the effects of NO on stomatal movement is based on the premise that its physiological effects are related solely to the concentration of the molecules available in the tissues. However, this approach simplifies a system whose response depends not only on the amount of NO produced or applied, but also on the form, location, and speed at which this signal is made available to guard cells. In this context, the chemistry of NO donors and their release kinetics also emerge as fundamental factors for understanding the wide range of different results found in the literature (Procházková et al., 2013; Perlikowski et al., 2025).

Although different compounds are classified as NO donors, they exhibit distinct physicochemical properties. Molecules such as sodium nitroprusside, nitrosothiols, N-diazeniumdiolates, and others release NO through different mechanisms, resulting in release rates that vary drastically from minutes to hours, which directly impacts the transience of the signal perceived by guard cells (Pieretti et al., 2023). Thus, even when different donors are utilized at the same concentration, it does not necessarily mean that guard cells receive the same amount of NO or for the same period.

Beyond the total amount released, the release rate seems to play an equally important role. Some donors lead to a rapid and transient increase in NO concentration, producing a short-term signaling spike, while others can maintain a slow and controlled release over hours (Yang et al., 2021; Pieretti et al., 2023). From a

signaling biology perspective, these two scenarios are completely distinct. As is the case with plant hormones, cells can respond differently to short- and long-term stimuli (Caumon and Vernoux, 2023).

This hypothesis is particularly interesting when considering the complexity of stomatal movement signaling pathways. In this sense, transient NO production may act as a component of the signaling cascade for ABA-induced stomatal closure (Bharath et al., 2021). In contrast, prolonged exposure to NO may favor PTMs of key signaling pathway proteins, such as S-nitrosylation of OST1 or nitration of PYR/PYL/RCAR receptors, thereby promoting an attenuation of the ABA response and favoring the maintenance of stomatal opening (Wang et al., 2014; Mata-Pérez et al., 2023).

Another important and often neglected aspect concerns toxic secondary byproducts that can be generated during the decomposition of the donor molecule. SNP, for instance, can release cyanide during its degradation. In turn, some NONOates exhibit decomposition profiles highly dependent on pH and temperature (Pieretti et al., 2023).

Furthermore, the effective availability of NO in guard cells rarely corresponds to the amount initially applied. This occurs because, after its release, NO exhibits high diffusibility and an extremely short half-life, reacting rapidly with other molecules, such as ROS, glutathione, proteins, and other targets (Singhal et al., 2021; Corpas et al., 2023). Consequently, the biologically active concentration of NO results from a balance between production, diffusion, consumption, and storage in the form of nitrosylated compounds, rather than just the nominal dose used in the experiment (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2007). Table 2 summarizes the main characteristics of NO donors commonly used in plant research, highlighting differences in their release mechanisms and factors that may influence their performance.

Taken together, these facts suggest that part of the so-called “NO paradox” may reflect different factors inherent to donor chemistry and the kinetics of molecule availability, rather than necessarily solely the contradictory biological responses available in the literature. From this perspective, understanding how different release profiles modulate signal intensity, duration, and localization represents an essential step toward building a more integrated and context-dependent model for NO action in guard cells.

Table 2

Main characteristics of nitric oxide (NO) donors and delivery systems commonly used in plant research. Differences in donor release mechanisms, major determinants, and main advantages and limitations associated with each approach.

Characteristic	NONOates	S-Nitrosothiols (RSNOs)	Nitrate/Nitrite (NO precursors)	Sodium nitroprusside (SNP)	Controlled-release systems
How NO is released	Decomposition of the NONOate group	Cleavage/decomposition of the S-NO bond	Biological reduction of nitrate/nitrite leading to NO formation	NO liberation from the metal complex; mechanism depends on the compound	Carrier protects the donor and modulates its release, followed by NO liberation
Main triggers	Strongly influenced by chemical structure, temperature and pH conditions	Light, temperature, reducing agents and metal ions can influence decomposition	Plant physiological and environmental conditions; enzymatic and non-enzymatic pathways	Premature decomposition, releasing NO faster than intended	Carrier properties, donor chemistry and environmental conditions
Advantages	Defined NO-donor chemistry; release profile can be tuned according to molecular structure	Biologically relevant NO carriers; GSNO is widely used in plant studies	Naturally occurring precursors that can contribute to endogenous NO production	Simple experimental application	Protection of donor molecules; potential for gradual/sustained release and prolonged NO availability
Limitations	Release can be highly condition-dependent	Limited stability; release depends strongly on environmental conditions	NO formation is indirect and strongly dependent on plant metabolism and environmental context	Toxic decomposition byproducts; release profile can vary with experimental conditions	Interaction with plant tissues still not fully understood; performance depends on carrier properties and release behavior

Sources: Sodium nitroprusside: Pieretti et al. (2023); S-Nitrosothiols (RSNOs): Pieretti et al. (2023), Silveira et al. (2021), Seabra et al. (2022); Nitrate/Nitrite: Farnese et al. (2016), Khan et al. (2023); Controlled-release systems: Seabra et al. (2022), Pieretti et al. (2023).

6.7.2 METHODOLOGICAL INFLUENCES

In addition to factors related to the chemistry of NO donors, another important aspect concerns the methodology applied in experiments, which may contribute significantly to the diversity of responses observed in the literature. Although many studies compare the effects of different NO concentrations, few consider that apparently secondary experimental factors can profoundly alter the amount of NO effectively perceived by guard cells.

Among these factors, the application method deserves special attention. NO can be supplied to plants via donors or NO gas, and in various ways, such as foliar spraying, leaf disc incubation, fumigation, root application, supplementation in nutrient solutions, and even seed priming (Ullah et al., 2024). Each of these strategies results in different barriers to the diffusion of the molecule, such as the cuticle and the stomatal pore itself, and determines distinct patterns of absorption, distribution, and retention in the tissues (van Meeteren et al., 2020). Thus, two experiments using the same donors and the same concentration, for instance, will rarely result in equivalent biological levels.

Biological variability also represents an important component of this scenario. Plant species differ in anatomical, physiological, and metabolic aspects that influence NO perception. Even within the same species, factors such as developmental stage, nutritional status, and stress history can modify the capacity of guard cells to respond to the signal (León et al., 2013; Buet et al., 2019; Khan et al., 2023).

NO research in plant systems is fraught with methodological difficulties that have led to inconsistent and sometimes contradictory results in the literature. The fundamental characteristics of NO, such as its short half-life, high reactivity, and quick diffusion, provide a significant obstacle to its detection and quantification in plant systems (Corpas et al., 2023).

To overcome these challenges, numerous experimental techniques, such as fluorescent probes, electrochemical sensors, and indirect biochemical assays, have been developed, each with its own advantages and limitations related to specificity and sensitivity (Gupta et al., 2019; Vishwakarma et al., 2019). For instance, while fluorescent probes offer spatial resolution, their specificity can be compromised by interactions with other reactive nitrogen species (Kalyanaraman et al., 2011).

In addition, exogenous application of NO donors has been a popular strategy to increase NO levels and study its signaling functions (Campos et al., 2023); however, these methods add extra variability that needs to be carefully taken into account in experimental studies.

NO scavengers, such as cPTIO, are also widely used. Together with NO donors, such as S-nitrosothiols, these tools have been crucial for understanding NO-related processes, but they also have significant drawbacks (D'Alessandro et al., 2013; Santisree et al., 2019). For example, NO scavengers are not always fully specific and may interfere with other signaling pathways (D'Alessandro et al., 2013). Also, the release kinetics, stability and byproducts of NO donors can vary significantly, and these differences may have an impact on plant physiology. Additionally, because NO donors are extremely susceptible to environmental conditions, their intrinsic instability frequently limits their practical applications (Silveira et al., 2019).

Another factor is the biological system used in the experiment. Epidermal peels, detached leaves, and guard cell protoplasts allow for good control of the concentration and duration of exposure to donors (Zhu et al., 2015). However, cutting the tissue is a stress stimulus itself. It is possible that part of the response attributed to exogenous NO is actually the sum of this signal with a wounding response, something that does not occur in intact leaves or whole plants (Doğramacı et al., 2024).

The methodology used to measure stomatal response also matters in this discussion. Microscopic measurements generally capture a single moment, whereas gas exchange measurements track the response over time (Lawson and Vialet-Chabrand, 2018; Lawson and Leakey, 2024). As the effect of NO appears to depend significantly on exposure time, a point measurement may record as closure or opening what is a transient stage of the same response.

6.7.3 IMPLICATIONS FOR FUTURE RESEARCH AND NO-BASED TECHNOLOGIES

The incorporation of a context-dependent view for NO action, bringing together the factors discussed in this review, holds important implications for both basic research and the development of new technologies. In this context, the apparent divergence found in the literature may reflect, more than opposing biological mechanisms, differences in the way NO is made available to and perceived by guard

cells.

In practice, this means that, much more than comparing concentrations or different NO sources, it becomes essential to consider the dynamics of its availability to the cells and the context in which its action was detected. To this end, a detailed description of experimental protocols, including the species used, characteristics of the plant material, environmental conditions, type of donor, and release mechanism, is fundamental to allow more consistent comparisons between different studies (Al-Hakeem and Khan, 2025; Sedlářová et al., 2025).

In this scenario, there is growing interest in strategies capable of controlling the spatial and temporal availability of NO in plant tissues. More than representing new forms of application, these technologies offer the possibility of minimizing, at the source, one of the main sources of experimental variability discussed in this chapter. Systems capable of protecting NO donors and promoting a more gradual and predictable release can contribute to increasing both the reproducibility of studies and the efficiency of future applications (Seabra et al., 2022).

6.8 STRATEGIES FOR NO DELIVERY: FROM CONVENTIONAL DONORS TO NANOCARRIERS

6.8.1 FROM NO GAS TO CONTROLLED-RELEASE SYSTEMS

The same physicochemical characteristics that make NO an effective signaling molecule also provide difficulties for its practical application, despite its extensive involvement in physiological processes (Pelegriño et al., 2017). Direct application in plants is hampered by its high reactivity and instability, which emphasizes the need for methods that allow for regulated and efficient NO delivery (Seabra et al., 2022). According to Seabra et al. (2022), NO donor molecules have become a potential tool for experimental research as well as agricultural and forestry applications.

Numerous techniques ranging from direct approaches to ways based on precursors and controlled-release systems have been developed to permit the application of NO in plant systems, due to the limits associated with its instability (Pande et al., 2022). These strategies differ in terms of capacity to control NO

availability, distribution, and persistence within plant tissues.

Using fumigation or controlled chambers to expose plants to NO in its gaseous state is one of the most direct methods. This method allows for precise dosage control but suffers from rapid dissipation and limited penetration depth into tissues, as well as the risk of toxicity at large doses (Yang and Liu, 2018). Furthermore, NO fumigation may result in the atmospheric release of nitrogen dioxide (NO₂) as a pollutant, although these emissions are typically minimal (Kasten et al., 2017).

Another possible strategy involves using NO precursors, like nitrate and nitrite, which plants can convert into NO. However, this method relies heavily on various physiological and environmental factors and on regulatory mechanisms that are still not fully understood, which can make the NO production a bit unpredictable (Farnese et al., 2016; Khan et al., 2023).

The use of NO donor molecules is the most common method in experimental studies. Compounds like sodium nitroprusside (SNP) and S-nitrosothiols (RSNOs) provide a more controlled NO release, but they do come with some issues, usually related to low stability, release rates, and, in the case of SNP, the generation of toxic byproducts. Additionally, factors like concentration, application method, and environmental conditions can significantly affect the results (Silveira et al., 2021; Seabra et al., 2022).

More recently, controlled-release systems have emerged as significant alternatives to overcome the limitations of conventional NO donors. These types of approaches use materials such as polymers, natural and non-natural, silica, and metals to create delivery systems that protect the donor molecules and promote a gradual and sustained NO-release. These systems may help reduce toxicity and prolong the NO biological effects (Seabra et al., 2022; Campos et al., 2023; Tortella et al., 2023). Figure 2 summarizes these strategies for NO delivery in plants.

STRATEGIES FOR NO DELIVERY IN PLANT SYSTEMS

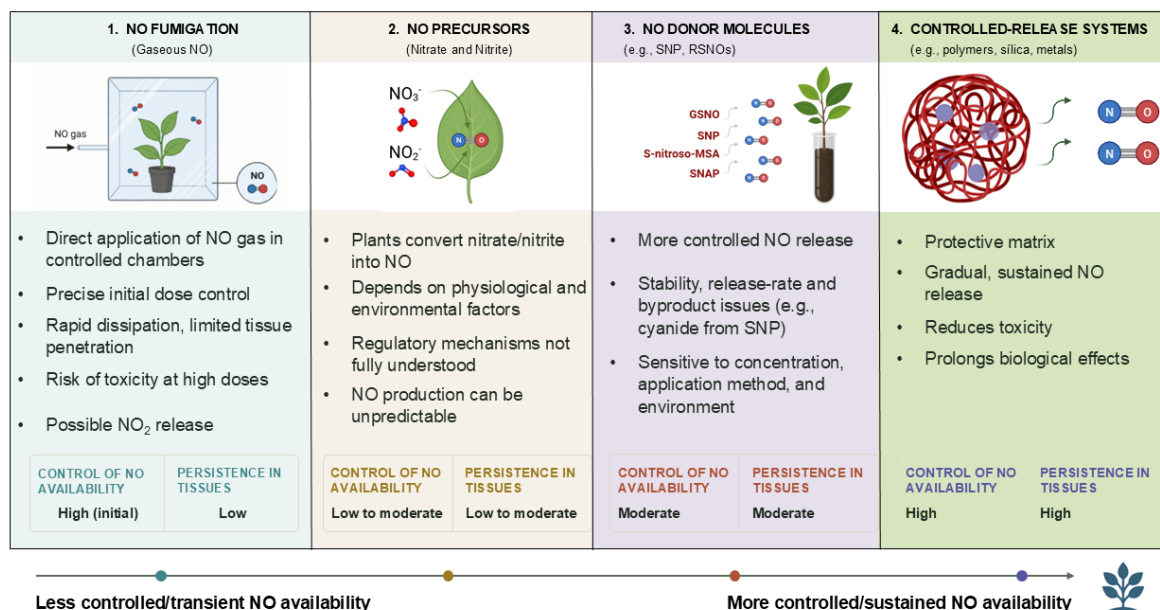


Fig. 2. Strategies for nitric oxide (NO) delivery in plant systems, arranged according to increasing control over NO release and persistence in plant tissues. (1) NO fumigation: direct exposure to gaseous NO in controlled chambers. (2) NO precursors: nitrate (NO_3^-) and nitrite (NO_2^-) are reduced to NO through the plant's endogenous pathways. (3) NO donor molecules: compounds that release NO in a more controlled manner, though they remain sensitive to concentration, application method, and environmental conditions. (4) Controlled-release systems: encapsulation of NO donors in nanocarriers protects the donor molecule and provides gradual, sustained NO release, reducing toxicity and prolonging biological effects. The horizontal axis at the bottom summarizes this gradient, from less controlled/transient (gas) to more controlled/sustained (encapsulated systems) NO availability.

6.8.2 NANOTECHNOLOGY-BASED APPROACHES

As discussed above, the chemical instability of NO donors is one of the main limitations to their experimental and practical use. Allied to this, the use of nanotechnology has grown, driven by the search for more efficient systems for the delivery of nutrients, growth regulators, agricultural pesticides, and other biologically active molecules (Kumari et al., 2023).

In the context of NO, this strategy becomes particularly relevant, as the main limitation of using the molecule lies not only in its physicochemical characteristics, but also in the difficulty of controlling its availability to the biological system (Seabra et al., 2022). In this approach, the NO donor is encapsulated in a nanomaterial, which acts as a physical protective barrier against premature degradation and as a controlled-release system (Ali et al., 2021).

Different types of nanomaterials have already been tested for this purpose,

including matrices derived from natural and synthetic polymers, silica, and metals (Tortella et al., 2023). Each of these systems presents its own limitations and advantages, but they all share the same objective: to protect the donor against premature degradation and allow for a prolonged release of NO, which circumvents the rapid degradation characteristic of conventional formulations (Pande et al., 2022; Campos et al., 2023).

In addition to the physical protection of the donors, nanocarriers can also enhance leaf adhesion, increase the residence time of the formulation on the plant, facilitate transport across cellular barriers depending on their physicochemical properties, and reduce the concentration of the working solution and the number of repeated applications (Pieretti et al., 2023; do Carmo et al., 2021). Although the use of these systems in plant physiology is still relatively recent, the results already obtained indicate that nanoencapsulation is a viable strategy to overcome the physicochemical limitations of conventional NO donors. One important limitation of these systems, however, is the difficulty in experimentally isolating the specific contribution of NO from potential physiological effects of the nanocarrier itself, which reinforces the importance of adequate controls, such as blank nanoparticles without the donor, already adopted in several of the studies, and recommended as standard practice.

6.8.3 CONTROLLED AND SUSTAINED NO RELEASE

Although nanoencapsulation represents a promising strategy to protect NO donor molecules, its main advantage lies not only in the protection conferred to the molecule but also in the modulation of its release kinetics. Studies demonstrate that, rather than promoting a rapid release of NO immediately after application, controlled-release systems allow the molecule to be made available gradually over time, prolonging its biological activity and reducing premature degradation (Seabra et al., 2022; Campos et al., 2023; Gomes et al., 2024).

This function is particularly important for studies involving NO due to its high reactivity and short half-life, as in conventional formulations, a large part of the released NO can be rapidly consumed by reactions with other molecules. As a result, only a fraction of the applied NO remains biologically available to trigger the physiological signaling cascade (Riseh et al., 2024).

Therefore, by controlling the donor release rate, nanostructured systems help

maintain more stable NO concentrations in plant tissues, reducing abrupt release peaks. This not only increases the molecule's physiological efficiency but can also mitigate adverse effects associated with rapid release or potentially toxic byproducts resulting from donor decomposition (Pande et al., 2022; Tortella et al., 2024).

From this perspective, sustained and controlled release can also be an important tool for physiological studies (Seabra et al., 2022). As previously discussed, different NO release profiles can result in different responses in guard cells. Therefore, systems capable of modulating release kinetics offer an opportunity to more precisely study how signal intensity, duration, and dynamics influence NO-modulated processes, such as stomatal movement.

This mechanism has recently been addressed directly in the case of chitosan-based nanocarriers. Preisler et al. (2026) investigated the interaction of rhodamine-labeled chitosan nanoparticles (CS NPs) loaded with GSNO in soybean roots in a hydroponic system, combining confocal microscopy with the NO-sensitive fluorescent probe (DAF-2DA) over two exposure times (30 minutes and 24 hours). The fluorescence associated with the NPs showed extensive colocalization with the NO-derived signal in the roots, and the origin of the specific NO signal was confirmed using the cPTIO scavenger. The root S-nitrosothiol content was significantly higher in roots treated with the nanoencapsulated donor than with free GSNO, being approximately 30% higher after 24 hours. These results provide evidence that nanoencapsulation increases the NO bioavailability compared to the free donor, corroborating the controlled-release mechanisms discussed above.

Although there are still challenges related to understanding their interaction with plant tissues, especially given the great diversity of species in regions such as the Atlantic Forest and their physiological differences, recent advances show that controlled-release systems represent a promising path to overcome limitations associated with conventional donors.

6.8.4 CHITOSAN-BASED NANOCARRIERS

Among the different materials that can be used for controlled NO release, chitosan stands out as one of the most studied for plant systems. It is a biodegradable, biocompatible, and low-toxicity polymer, obtained from the deacetylation of chitin, with a good capacity to form efficient matrices for the encapsulation of donors such as

GSNO (Methela et al., 2023; Seabra et al., 2015). Furthermore, chitosan has a relatively low cost and is mainly derived from fishing industry waste, which makes its use quite attractive for large-scale applications (Yu et al., 2021).

The physicochemical properties of CS make it highly relevant in the formulation of nanocarriers, such as nanoparticles. CS exhibits a high specific surface area, positive charge, and bioadhesive characteristics, features that optimize the transport of active compounds, enhancing their bioactivity and facilitating interaction with plant cells (Riseh et al., 2024; Nandhini et al., 2025).

Experiments using chitosan nanoparticles as NO donor carriers have been conducted in different species and stress conditions. In sugarcane, CS NPs containing GSNO sprayed on the leaves favored plant drought tolerance (Silveira et al., 2019). The same NO delivery system, but applied to the substrate, triggered a dose-dependent effect in maize plants subjected to light stress (Gomes et al., 2024).

In Atlantic Forest tree species, the application of CS NPs containing the S-nitroso-MSA donor to the substrate favored the acclimation of *Heliocarpus popayanensis* seedlings to full sun in the nursery (Lopes-Oliveira et al., 2019) and increased protection against water deficit in the same species (do Carmo et al., 2021). This same strategy and method of application was also effective with GSNO-loaded CS NPs, which promote adaptive responses to drought in *Araucaria angustifolia* (Silva et al., 2026). In contrast, *Cariniana estrellensis*, evaluated in the same full-sun acclimation study, did not benefit from the application of the nanoencapsulated donor (Lopes-Oliveira et al., 2019).

This same trend was repeated subsequently under drought. While *Cecropia pachystachya* had its drought tolerance increased by the application via substrate of GSNO-loaded CS NPs, *C. estrellensis* again showed no improvement with the treatment (do Carmo et al., 2025). The repetition of the non-response pattern in *C. estrellensis*, observed with different donors under different stresses, reinforces the hypothesis that the species-dependent variability discussed throughout this review also manifests even when NO delivery is achieved via nanoencapsulated systems.

Importantly, most of the studies discussed above included controls with CS NPs without NO, which help to attribute the reported effects to NO rather than to the chitosan carrier itself. In these comparisons, the blank CS NPs did not cause any significant change in stomatal conductance. This suggests that the stomatal responses described above are associated with the released NO rather than with a nonspecific

effect of the nanocarrier. However, this pharmacological evidence does not demonstrate the underlying mechanism. The same caution applies to the screening presented below, which did not include carrier or free-donor controls.

Therefore, it is also worth noting that the results presented above were obtained from species with different life histories, ecology, and physiological characteristics, ranging from agricultural crops to native species of different successional stages. Furthermore, cultivation conditions and the type of stress applied also vary among the studies. This diversity of responses reinforces the hypothesis discussed throughout this review that the effect of NO, even when delivered by a controlled-release system, still depends on the biological and ecological context, as well as the experimental conditions under which it was evaluated.

6.9 SCREENING OF STOMATAL RESPONSES TO CHITOSAN NANOPARTICLES CONTAINING GSNO IN NATIVE ATLANTIC FOREST TREE SPECIES

To investigate the variability of stomatal response to the application of GSNO-loaded chitosan nanoparticles among different tree species, a screening was conducted with seedlings of 36 native Atlantic Forest species. The seedlings were provided by the Flora Londrina nursery, were approximately 15 cm in height, and were maintained in 55 mL tubes containing the commercial substrate MecPlant Florestal.

The plants were transferred to a greenhouse, without light restriction, and maintained at field capacity using a capillary self-irrigation system. This system consisted of floral foam blocks placed inside 15 L plastic containers filled with water, into which the bases of the seedling tubes were inserted. The water was replenished whenever the level decreased. This protocol has previously been described in the literature (Marchin et al., 2020).

The treatment with chitosan nanoparticles containing GSNO followed an increasing dose scheme applied via two methods: soil application or foliar spraying. The initial application consisted of 50 μM of the nanoformulation, applied directly to the soil (for 34 species) or sprayed onto the leaves (for 12 species). The following day, stomatal conductance was measured using an SC-1 leaf porometer, always on sunny days, starting at 8 AM. When no perceptible change was observed, the dose was doubled and reapplied, repeating this cycle until a stomatal effect was detected or the maximum dose of 800 μM was reached. The tested doses were, therefore, 50, 100, 200, 400, and 800 μM .

The stomatal response was defined based on the statistical significance between the stomatal conductance measured in the group that received the nanoformulation and the control group. Prior to statistical analysis, data normality was assessed using the Shapiro–Wilk test, and homogeneity of variances was evaluated using Levene's test ($p > 0.05$ for both). Control and treated stomatal conductance were compared using Student's t-test when both assumptions were met. When Levene's test indicated unequal variances, data were $\log_{10}$ -transformed before the t-test (*Guazuma ulmifolia* via foliar application and *Tabebuia heptaphylla* via soil). When non-normality persisted even after transformation, the non-parametric Mann–Whitney U test was used instead (Application via soil: *Rhamnidium elaeocarpum*,

Pseudobombax grandiflorum, *Inga uraguensis*, *Vitex montevidensis*; Foliar application: *Pseudobombax grandiflorum*). Differences were considered significant at $p < 0.05$. Statistical analyses were performed using Paleontological Statistics (PAST). Figure 3 summarizes the experimental setup and the overall distribution of responses across species; the complete results are presented in Table 3.

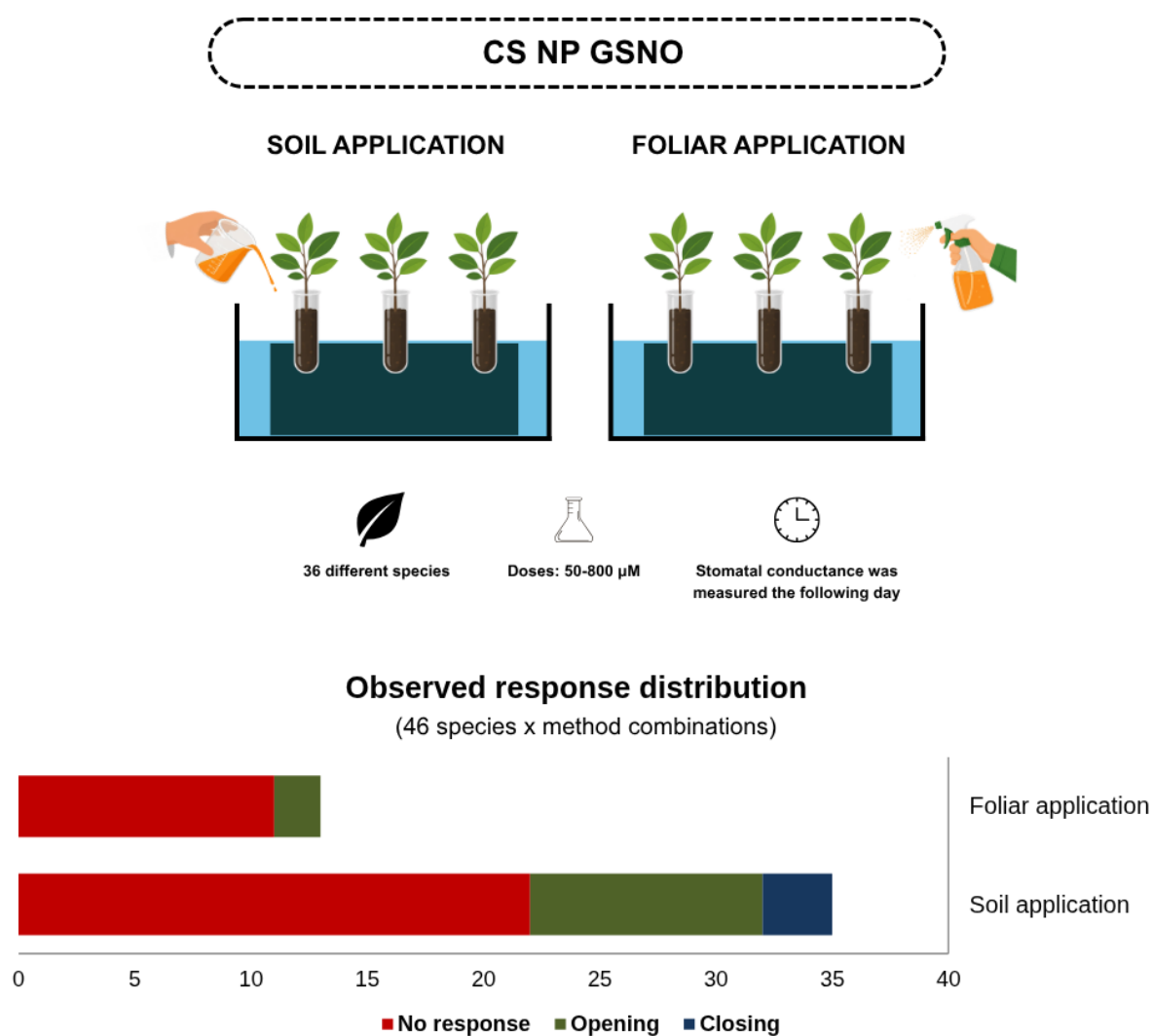


Fig. 3. Overview of the interspecific screening using GSNO-loaded chitosan nanoparticles (CS NP GSNO), applied via soil or foliar to native Atlantic Forest seedlings. A total of 36 species were tested, totaling 46 species versus method combinations; the value is reflected in the response distribution chart.

Table 3

Stomatal responses of native Atlantic Forest tree species to chitosan nanoparticles loaded with GSNO, screened via soil and foliar application. g_s = stomatal conductance, measured on clear days starting at 8:00 h. g_s is expressed in $\text{mmol m}^{-2} \text{s}^{-1}$. SE = standard error, n = number of biological replicates per group. Increases and decreases in g_s are interpreted as stomatal opening and closing, although pore aperture was not directly measured. Reported dose corresponds to the first dose at which a significant difference (t-test or Mann–Whitney test, $p < 0.05$) was detected between treated and control plants; where no dose led to a significant difference, the highest dose tested (800 μM) is reported. Data normality (Shapiro–Wilk test) and variance homogeneity (Levene's test) were assessed prior to each comparison. Student's t-test was used when both assumptions were met; when only variance homogeneity required correction, data were $\log_{10}$ -transformed before the t-test. Where normality could not be achieved even after transformation, the non-parametric Mann–Whitney test was used instead (p-values marked with *).

Species	Common name	Family	Application method	Dose (μM)	Control g_s , mean $\pm$ SE (n)	Treated g_s , mean $\pm$ SE (n)	p-Value	Response
<i>Schinus terebinthifolia</i>	Aroeira-pimenteira	Anacardiaceae	Foliar	800	155.6 $\pm$ 12.0 (n=9)	162.1 $\pm$ 11.4 (n=9)	0.6991	(=) No difference detected
<i>Luehea divaricata</i>	Açoita-cavalo	Malvaceae	Foliar	800	320.5 $\pm$ 9.3 (n=9)	320.0 $\pm$ 10.3 (n=9)	0.9717	(=) No difference detected
<i>Cedrela</i> sp.	Cedro	Meliaceae	Foliar	800	210.4 $\pm$ 11.2 (n=9)	209.5 $\pm$ 11.7 (n=9)	0.9607	(=) No difference detected
<i>Pseudobombax grandiflorum</i>	Embiruçu	Malvaceae	Foliar	50	140.5 $\pm$ 17.3 (n=9)	202.5 $\pm$ 16.6 (n=9)	0.0134*	(+) Increase
<i>Achistus arborescens</i>	Fruto-de-sabiá	Solanaceae	Foliar	800	226.8 $\pm$ 7.5 (n=9)	225.6 $\pm$ 7.6 (n=9)	0.9051	(=) No difference detected
<i>Eugenia</i> sp.	Grumixama	Myrtaceae	Foliar	800	223.6 $\pm$ 5.9 (n=9)	223.3 $\pm$ 6.1 (n=9)	0.9794	(=) No difference detected
<i>Cariniana estrellensis</i>	Jequitibá	Lecythidaceae	Foliar	800	165.9 $\pm$ 9.7 (n=9)	168.8 $\pm$ 9.9 (n=9)	0.8353	(=) No difference detected
<i>Guazuma ulmifolia</i>	Mutambo	Malvaceae	Foliar	50	110.1 $\pm$ 4.8 (n=9)	212.5 $\pm$ 22.0 (n=9)	0.0002	(+) Increase
<i>Gallesia integrifolia</i>	Pau-d'alho	Phytolaccaceae	Foliar	800	340.4 $\pm$ 16.6 (n=9)	330.3 $\pm$ 16.3 (n=9)	0.6708	(=) No difference detected

Species	Common name	Family	Application method	Dose (μ M)	Control g_s , mean $\pm$ SE (n)	Treated g_s , mean $\pm$ SE (n)	p-Value	Response
<i>Aspidosperma polyneuron</i>	Peroba-rosa	Apocynaceae	Foliar	800	155.5 $\pm$ 10.7 (n=9)	162.7 $\pm$ 10.5 (n=9)	0.6371	(=) No difference detected
<i>Croton urucurana</i>	Sangra-d'água	Euphorbiaceae	Foliar	800	170.1 $\pm$ 8.0 (n=9)	171.2 $\pm$ 7.9 (n=9)	0.9235	(=) No difference detected
<i>Senna alata</i>	<i>Senna</i>	Fabaceae	Foliar	800	115.6 $\pm$ 8.7 (n=9)	121.4 $\pm$ 8.8 (n=9)	0.6451	(=) No difference detected
<i>Chrysophyllum marginatum</i>	Aguaí-preto	Sapotaceae	Soil	800	163.6 $\pm$ 9.8 (n=9)	166.1 $\pm$ 9.2 (n=9)	0.8581	(=) No difference detected
<i>Schinus terebinthifolia</i>	Aroeira-pimenteira	Anacardiaceae	Soil	800	243.8 $\pm$ 11.9 (n=9)	251.2 $\pm$ 10.8 (n=9)	0.6515	(=) No difference detected
<i>Vernonia polysphaera</i>	Assa-peixe	Asteraceae	Soil	50	216.9 $\pm$ 6.2 (n=9)	238.3 $\pm$ 5.3 (n=9)	0.0188	(+) Increase
<i>Luehea divaricata</i>	Açoita-cavalo	Malvaceae	Soil	800	245.4 $\pm$ 13.6 (n=8)	260.5 $\pm$ 16.2 (n=8)	0.4864	(=) No difference detected
<i>Rhamnidium elaeocarpum</i>	Cafezinho	Rhamnaceae	Soil	50	129.5 $\pm$ 20.1 (n=8)	248.2 $\pm$ 28.3 (n=8)	0.0070*	(+) Increase
<i>Myrsine gardneriana</i>	Capororoca	Primulaceae	Soil	800	150.8 $\pm$ 7.9 (n=9)	159.2 $\pm$ 8.2 (n=9)	0.4709	(=) No difference detected
<i>Trichilia catigua</i>	Catuaba	Meliaceae	Soil	800	326.7 $\pm$ 16.1 (n=9)	328.8 $\pm$ 16.6 (n=9)	0.9280	(=) No difference detected
<i>Cedrela</i> sp.	Cedro	Meliaceae	Soil	800	255.9 $\pm$ 14.4 (n=9)	272.8 $\pm$ 10.8 (n=9)	0.3606	(=) No difference detected
<i>Cestrum</i> sp.	Coerana	Solanaceae	Soil	800	126.9 $\pm$ 6.2 (n=9)	127.3 $\pm$ 4.3 (n=9)	0.9630	(=) No difference detected
<i>Poecilanthe parviflora</i>	Coração-de-negro	Fabaceae	Soil	800	159.5 $\pm$ 5.6 (n=9)	154.8 $\pm$ 6.5 (n=9)	0.5899	(=) No difference detected
<i>Trema micrantha</i>	Crindiúva	Cannabaceae	Soil	200	180.0 $\pm$ 5.2 (n=7)	165.1 $\pm$ 3.2 (n=7)	0.0323	(-) Decrease

Species	Common name	Family	Application method	Dose (μ M)	Control g_s , mean $\pm$ SE (n)	Treated g_s , mean $\pm$ SE (n)	p-Value	Response
<i>Pseudobombax grandiflorum</i>	Embiruçu	Malvaceae	Soil	800	200.1 $\pm$ 56.7 (n=8)	211.4 $\pm$ 53.7 (n=8)	0.6058*	(=) No difference detected
<i>Ficus</i> sp.	Figueira	Moraceae	Soil	100	132.1 $\pm$ 12.1 (n=9)	229.6 $\pm$ 20.9 (n=9)	0.0010	(+) Increase
<i>Solanum mauritianum</i>	Fumo-bravo	Solanaceae	Soil	800	697.9 $\pm$ 33.5 (n=6)	708.6 $\pm$ 48.2 (n=6)	0.8558	(=) No difference detected
<i>Eugenia</i> sp.	Grumixama	Myrtaceae	Soil	800	165.6 $\pm$ 8.6 (n=9)	139.4 $\pm$ 9.0 (n=9)	0.0513	(=) No difference detected
<i>Patagonula americana</i>	Guajuvira	Boraginaceae	Soil	50	234.9 $\pm$ 15.0 (n=9)	314.5 $\pm$ 12.9 (n=9)	0.0010	(+) Increase
<i>Calophyllum brasiliense</i>	Guanandi	Calophyllaceae	Soil	50	220.2 $\pm$ 13.4 (n=8)	335.3 $\pm$ 15.1 (n=8)	0.0001	(+) Increase
<i>Casearia decandra</i>	Guaçatonga	Salicaceae	Soil	50	92.0 $\pm$ 6.9 (n=9)	162.0 $\pm$ 6.6 (n=9)	<0.0001	(+) Increase
<i>Inga uraguensis</i>	Ingá-do-rio	Fabaceae	Soil	800	211.5 $\pm$ 11.3 (n=9)	187.4 $\pm$ 9.0 (n=9)	0.0701*	(=) No difference detected
<i>Tabebuia heptaphylla</i>	Ipê-rosa	Bignoniaceae	Soil	50	93.1 $\pm$ 1.5 (n=9)	151.0 $\pm$ 5.3 (n=9)	<0.0001	(+) Increase
<i>Miconia</i> sp.	Jacatirão	Melastomataceae	Soil	800	167.7 $\pm$ 10.1 (n=9)	139.0 $\pm$ 8.1 (n=9)	0.0423	(-) Decrease
<i>Lonchocarpus</i> sp.	<i>Lonchocarpus</i>	Fabaceae	Soil	800	124.3 $\pm$ 9.2 (n=9)	126.7 $\pm$ 8.0 (n=9)	0.8512	(=) No difference detected
<i>Guazuma ulmifolia</i>	Mutambo	Malvaceae	Soil	400	278.8 $\pm$ 40.8 (n=8)	182.0 $\pm$ 16.3 (n=8)	0.0362	(-) Decrease
<i>Gallesia integrifolia</i>	Pau-d'alho	Phytolaccaceae	Soil	800	157.9 $\pm$ 15.6 (n=9)	149.4 $\pm$ 9.4 (n=9)	0.6468	(=) No difference detected
<i>Triplaris americana</i>	Pau-formiga	Polygonaceae	Soil	50	320.2 $\pm$ 45.3 (n=9)	469.9 $\pm$ 20.4 (n=9)	0.0082	(+) Increase

Species	Common name	Family	Application method	Dose (µM)	Control g_s , mean $\pm$ SE (n)	Treated g_s , mean $\pm$ SE (n)	p-Value	Response
<i>Citharexylum myrianthum</i>	Pau-viola	Verbenaceae	Soil	100	169.2 $\pm$ 11.0 (n=9)	260.1 $\pm$ 26.8 (n=9)	0.0064	(+) Increase
<i>Aspidosperma polyneuron</i>	Peroba-rosa	Apocynaceae	Soil	800	170.0 $\pm$ 15.7 (n=9)	173.9 $\pm$ 16.3 (n=9)	0.8634	(=) No difference detected
<i>Prunus sellowii</i>	<i>Prunus</i>	Rosaceae	Soil	800	192.3 $\pm$ 21.6 (n=9)	183.0 $\pm$ 16.1 (n=9)	0.7339	(=) No difference detected
<i>Croton urucurana</i>	Sangra-d'água	Euphorbiaceae	Soil	800	229.5 $\pm$ 16.9 (n=9)	214.8 $\pm$ 11.4 (n=9)	0.4815	(=) No difference detected
<i>Psychotria carthagenensis</i>	Sanhaçaíba	Rubiaceae	Soil	800	151.0 $\pm$ 10.3 (n=9)	153.1 $\pm$ 10.7 (n=9)	0.8907	(=) No difference detected
<i>Senna alata</i>	<i>Senna</i>	Fabaceae	Soil	800	251.6 $\pm$ 12.7 (n=9)	250.1 $\pm$ 11.7 (n=9)	0.9323	(=) No difference detected
<i>Alchornea triplinervia</i>	Tapiá	Euphorbiaceae	Soil	800	156.4 $\pm$ 9.6 (n=9)	149.5 $\pm$ 9.6 (n=9)	0.6202	(=) No difference detected
<i>Vitex montevidensis</i>	Tarumã	Lamiaceae	Soil	50	184.3 $\pm$ 9.9 (n=8)	274.1 $\pm$ 12.6 (n=8)	0.0001*	(+) Increase
<i>Allophylus edulis</i>	Vacum	Sapindaceae	Soil	800	194.7 $\pm$ 10.0 (n=9)	201.9 $\pm$ 7.7 (n=9)	0.5793	(=) No difference detected

Among the species tested, no significant change in stomatal conductance was detected in 31 of the 46 species-method combinations evaluated. An increase in g_s (interpreted as stomatal opening) was the most frequent response, recorded in 12 combinations; 10 via soil application, including *Vernonia polysphaera* (Assa-peixe), *Rhamnidium elaeocarpum* (Cafezinho), *Ficus* sp. (Figueira), *Patagonula americana* (Guajuvira), *Calophyllum brasiliense* (Guanandi), *Casearia decandra* (Guaçatonga), *Tabebuia heptaphylla* (Ipê-rosa), *Triplaris americana* (Pau-formiga), *Citharexylum myrianthum* (Pau-viola), and *Vitex montevidensis* (Tarumã); and 2 via foliar application, in *Pseudobombax grandiflorum* (Embiruçu) and *Guazuma ulmifolia* (Mutambo). A decrease in g_s (interpreted as stomatal closure) was the least frequent response, occurring in only 3 combinations, all via soil application: *Trema micrantha* (Crindiúva), *Miconia* sp. (Jacatirão), and *Guazuma ulmifolia* (Mutambo). This last case is particularly illustrative of the paradox discussed throughout this review because the same species showed opposite responses in different combinations of dose and application method: g_s decreased with 400 μM applied to the soil and increased with 50 μM applied to the leaves. Therefore, this comparison does not show that the application method alone reverses the response. In addition, not all species were tested with both methods, which limits aggregate comparisons between methods.

Regardless, it is important to highlight that this screening has some limitations. The assays were conducted in a single controlled environment (greenhouse), without testing the response under field conditions or combined environmental stress. Stomatal conductance was measured at a single time point, the day after application, which does not capture the full temporal dynamics of the NO response. If the same plants received successive doses, dose and cumulative exposure may be confounded. Other limitations include the number of comparisons performed without correction for multiplicity, the incomplete botanical identification of some taxa, the absence of carrier and free-donor controls, and the different doses between application methods. Finally, the 46 combinations describe the set evaluated and are not a representative estimate of all Atlantic Forest species.

Also, these stomatal responses were observed for these specific species under field capacity conditions, using nanoencapsulated GSNO as a NO donor, and at doses pre-established based on previous assays conducted with other species. Therefore, under different conditions, such as other types of NO donors, application vehicles, or tested dose ranges, new responses might be observed, including in species classified

here as non-responsive.

6.10 CONCLUSIONS AND FUTURE PERSPECTIVES

NO plays an important role in the regulation of stomatal movement, integrating different signaling pathways involved in plant responses to the environment. However, the evidence gathered in this review demonstrates that its effects cannot be interpreted in a generalized manner, as we were able to discuss that the duality found in the literature may reflect the dependence of NO on the context, the donor used, the route of application, the dose, and the intrinsic characteristics of each species.

This complexity reinforces the need for caution when extrapolating results obtained in model species to the wide biodiversity found in natural ecosystems, especially in tropical regions. The interspecific screening presented in this review reinforces this point directly, as, using the same nanoencapsulated donor and the same protocol, native species of the Atlantic Forest showed opposite responses or no detectable change in g_s .

It is expected that the integration of the factors discussed here will contribute to a more comprehensive understanding of NO signaling and to the development of more efficient strategies for its application in plants.

The body of evidence discussed in this study points to applications that extend beyond a mechanistic understanding of NO involvement in stomatal movement. Given the worsening climate crisis and the growing demand for strategies for ecological restoration and agricultural production that are more efficient in water use, the management of NO signaling emerges as a promising tool, still little explored on a large scale.

In the context of reforestation, the acclimation of seedlings before final planting is a critical step, especially when planting is carried out in degraded areas with water restriction. Therefore, NO controlled-release nanosystems may become a useful tool in nursery practices to improve physiological resilience and post-planting establishment (do Carmo et al., 2025; Guariz et al., 2025). However, the interspecific variability of the response, evidenced both in the literature and in the screening presented in this review, reinforces that application in forest nurseries should not follow a single protocol. Screening schemes, as used here, testing multiple factors, can serve as a methodological basis for the exploratory selection of responsive species and

doses. However, these protocols need independent confirmation, predefined doses, and evaluation of growth and water status before being recommended for nurseries, since an increase in g_s alone does not demonstrate a benefit under drought.

Ultimately, transforming knowledge about the role of NO into an applied tool requires exactly the type of systematic and species-specific characterization discussed in this review, especially in regions recognized as biodiversity hotspots, such as the Atlantic Forest. In this sense, the reforestation of degraded areas emerges as a possible field of application, but also as an opportunity to test, under real-world conditions, the limits and potential of NO signaling as a management strategy.

Declaration of competing interests

The authors declare no competing interests.

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7 CONSIDERAÇÕES FINAIS

Este trabalho avaliou o potencial das CS NPs GSNO em aumentar a tolerância de espécies arbóreas nativas da Mata Atlântica ao DH e melhorar seu desempenho pós-plantio em campo, além de discutir as possíveis limitações desta tecnologia em um cenário de grande biodiversidade.

No capítulo 1, a aplicação das CS NPs GSNO aumentou a tolerância de *C. pachystachya* ao DH em casa de vegetação, na concentração de 50 μM , mas não foram detectados benefícios consistentes em *C. estrellensis* em nenhuma das concentrações testadas. No capítulo 2, o tratamento com CS NPs GSNO a 100 μM melhorou parâmetros do desempenho inicial de *C. pachystachya* em campo, como a área foliar e a assimilação total de CO_2 , com baixo custo estimado da formulação. Isso indica que parte dos resultados positivos observados em condições controladas pode se estender à fase inicial de estabelecimento das mudas em campo. No capítulo 3, foram discutidas as divergências encontradas na literatura sobre o papel do NO na regulação estomática, evidenciando que as respostas são variáveis entre espécies e dependentes do método de aplicação e do contexto biológico.

Em conjunto, os capítulos convergem para a mesma conclusão: a resposta às CS NPs GSNO depende da espécie. Isso é reforçado pela ausência de benefícios detectados em *C. estrellensis* em experimentos independentes e pelo padrão responsivo de *C. pachystachya* da casa de vegetação ao campo. Ao mesmo tempo, os resultados e discussões do capítulo 3 demonstram que essa especificidade não pode ser dissociada das condições experimentais utilizadas para avaliar a resposta.

Em relação às hipóteses, a primeira foi apoiada para as duas espécies estudadas: *C. pachystachya* respondeu de forma mais positiva que *C. estrellensis*. No entanto, a comparação entre duas espécies não permite generalizar esse resultado para os grupos sucessionais. A segunda hipótese foi parcialmente apoiada: as CS NPs GSNO aumentaram a área foliar e a assimilação total de CO_2 em campo, mas o ganho em fotossíntese dependeu do momento e do tratamento de comparação, e a biomassa não diferiu do controle. A terceira hipótese foi apoiada, pois a condutância estomática variou entre as espécies da triagem. Ainda assim, os experimentos avaliaram o efeito da formulação e não demonstraram diretamente os mecanismos do NO. Além disso, a ausência de diferença detectada não significa ausência definitiva de resposta.

Isso reforça que as CS NPs GSNO são uma ferramenta de baixo custo estimado e com potencial de aplicação, demonstrado em *C. pachystachya*. No entanto, a sobrevivência das mudas, a relação custo-benefício, os mecanismos envolvidos e o desempenho em diferentes ambientes ainda não foram demonstrados. Assim, seu uso depende de uma triagem específica das espécies e da definição de protocolos adequados antes de qualquer aplicação em larga escala. Portanto, pesquisas futuras devem compreender os mecanismos responsáveis pelas respostas, ou ausência delas, aumentar os testes para um maior número de espécies em condições de campo, avaliar diferentes concentrações e estratégias de aplicação e acompanhar a sobrevivência das mudas por mais tempo. Além disso, a validação em diferentes condições ambientais será importante para determinar em que nível os benefícios observados se mantêm diante da variabilidade ambiental, característica de áreas destinadas à restauração.

Assim, superar esses gargalos exigirá integrar estudos mecanísticos, triagens interespecíficas e validações em campo. Essa integração permitirá, no futuro, a definição de protocolos espécie-específicos que podem ser adotados pelos viveiros produtores de mudas de espécies destinadas ao reflorestamento, bem como a transformação dessa tecnologia em uma ferramenta aplicável à restauração florestal.