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EM AGRICULTURA TROPICAL E SUBTROPICAL

NANOTECNOLOGIA PARA POTENCIALIZAR A TOLERÂNCIA À SECA EM PLANTAS DE CITROS

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Orientadora: Dra. Neidiquele Maria Silveira

Dissertação apresentada como requisito para a obtenção do **título de Mestre** em Agricultura Tropical e Subtropical, Área de Concentração em Sistemas de Manejo e Qualidade (SMQA).

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I. Título.

GOVERNO DO ESTADO DE SÃO PAULO
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ATA DE DEFESA DE DISSERTAÇÃO DE MESTRADO

Aos 30 de março de 2026, às 09h00, reuniu-se a banca examinadora homologada pelo Programa de Pós- Graduação em Agricultura Tropical e Subtropical, composta pelos membros abaixo listados visando à defesa de dissertação de mestrado de Ana Caroline dos Santos de Almeida, para obtenção do título de "**MESTRE**", conforme Processo SAA nº PRT4446/2024-00. A sessão de defesa foi realizada em formato híbrido, sob a presidência da Profª. Drª. Neidiquele Maria Silveira, orientadora da aluna, em sessão pública aberta. Iniciados os trabalhos, a candidata submeteu-se ao exame de sua dissertação, intitulada “Nanotecnologia para potencializar a tolerância à seca em plantas de citros”. Terminado o exame, procedeu-se ao julgamento, cujo resultado foi o seguinte:

Profª. Drª. Neidiquele Maria Silveira - UNESP	**APROVADA (x) REPROVADA ( )**
Prof. Dr. Fernando Alves de Azevedo - IAC	**APROVADA (x) REPROVADA ( )**
Profª. Drª. Brenda Mistral de Oliveira Carvalho - CENA	**APROVADA (x) REPROVADA ( )**

Apurados os resultados, constatou-se que a candidata foi habilitada, fazendo jus, portanto, ao título de **“MESTRE EM AGRICULTURA TROPICAL E SUBTROPICAL”**, na área de concentração: Sistema de Manejo e Qualidade Ambiental, do que, para constar, lavrou-se a presente ata, assinada pelos membros da comissão examinadora:

Profª. Drª. Neidiquele Maria Silveira - UNESP

Prof. Dr. Fernando Alves de Azevedo – IAC (participação remota)

Profª. Drª. Brenda Mistral de Oliveira Carvalho – CENA (participação remota)

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Esta dissertação não representa apenas uma etapa acadêmica concluída. Representa superação, renúncias, amadurecimento e a concretização de um sonho que começou muito antes de mim. Mãe, essa conquista é a prova de que o seu sonho atravessou o tempo, venceu a ausência e floresceu em mim. Eu consegui. Por nós.

SUMÁRIO

1. INTRODUÇÃO GERAL

Tem-se observado um aumento na frequência e intensidade de extremos climáticos (Silva Dias, 2014), acarretando impactos significativos na sociedade, na agricultura e na biodiversidade (Unesco, 2020). As variações climáticas globais têm alterado o ambiente em que as plantas se desenvolvem, representando uma ameaça a sua sobrevivência, produtividade e consequente segurança alimentar (Espeland et al., 2018; Piao et al., 2019). Nesse cenário, as projeções climáticas para o século, indicam não apenas maior variabilidade nos regimes de precipitação, mas a intensificação e maior recorrência de eventos de seca, configurando um dos principais desafios para a sustentabilidade dos sistemas agrícolas, conforme destacado pelo Painel Intergovernamental sobre Mudanças Climáticas (IPCC, 2023).

O déficit hídrico afeta negativamente a citricultura ao comprometer o metabolismo e a fisiologia das plantas, prejudicando a absorção e transporte de nutrientes, o equilíbrio hídrico e a fotossíntese. Também interfere no desenvolvimento reprodutivo, reduzindo a viabilidade do pólen, a fertilização e a formação de frutos, o que leva à queda na produtividade e na qualidade (Newman, 1968). Além disso, o estresse hídrico pode provocar estresse oxidativo devido ao aumento da produção de espécies reativas de oxigênio (EROs), que, em excesso, danificam estruturas celulares e limitam o crescimento vegetal (Sharma et al., 2012; Mittler, 2002; Miller et al., 2010; Carvalho, 2008; Gill & Tuteja, 2010).

Dada a relevância da citricultura para o agronegócio brasileiro, especialmente como líder mundial na exportação de suco de laranja (FAO, 2024), torna-se essencial buscar soluções para minimizar os impactos do déficit hídrico, que ameaça tanto a produtividade quanto a qualidade dos frutos. Com grande peso econômico e social (Azevedo et al., 2020), o setor pode se beneficiar da aplicação de nanopartículas, uma alternativa promissora para aumentar a tolerância das plantas ao estresse hídrico.

Nesse cenário, o uso de nanomateriais ganha destaque, oferecendo propriedades únicas que os tornam especialmente eficazes na agricultura sob condições de estresse. Esses materiais são caracterizados por apresentarem dimensões entre 1 e 100 nm em pelo menos uma de suas dimensões (comprimento, altura ou largura). Dentre as abordagens mais promissoras, destaca-se a nanoencapsulação, que consiste na incorporação de compostos ativos em sistemas carreadores, visando sua proteção e liberação controlada. Essa estratégia, aliada à elevada área de superfície e maior interação com o ativo de interesse, favorece a absorção pelos tecidos vegetais e reduz impactos ambientais, resultando em maior eficiência nos processos agrícolas (Takeshita et al., 2021).

A nanotecnologia tem sido amplamente aplicada na agricultura, com destaque para o desenvolvimento de nanofertilizantes, nanoherbicidas, nanofungicidas e nanossensores (Paramo et al., 2020). Embora sua aplicação no enfrentamento de estresses abióticos ainda seja relativamente recente, o crescente número de estudos evidencia seu potencial tanto na ciência básica quanto aplicada. Essas inovações contribuem para o aumento da produtividade ao promover um uso mais eficiente e direcionado de agroquímicos, além de maior proteção das culturas frente a condições adversas. Ademais, intervenções nanotecnológicas têm demonstrado eficácia na regulação de diversos processos fisiológicos, como germinação, crescimento, fotossíntese, nutrição e resistência a doenças (El-Shetehy et al., 2021; Jiang et al., 2021; Ahmad et al., 2022).

O uso de sistemas de liberação de compostos ativos baseados em polissacarídeos oferece vantagens como biocompatibilidade, não toxicidade e liberação controlada (Elsoud & El Kady, 2019), contribuindo para resolver desafios como perdas nutricionais, baixa eficiência de fertilizantes e impacto de pragas e doenças (Hamed et al., 2016). Nesse contexto, materiais biodegradáveis e nanomateriais com liberação controlada de compostos ativos, despontam como ferramentas promissoras para uma agricultura mais eficiente e sustentável (Kumar et al., 2019; Arruda et al., 2015).

Entre as estratégias associadas a esses materiais, destaca-se a utilização de compostos sinalizadores como o óxido nítrico (NO), uma molécula-chave na regulação de respostas metabólicas e fisiológicas das plantas sob condições de estresse. Para explorar seus efeitos benéficos, diferentes doadores exógenos de NO têm sido empregados, variando quanto à composição, meia-vida e taxa de liberação (Silveira et al., 2019a). Dentre eles, os *S*-nitrosotióis (RSNOs), como a *S*-nitrosoglutationa (GSNO), a *S*-nitroso-N-acetilcisteína (SNAC) e o ácido *S*-nitroso-mercaptossuccínico (*S*-nitroso-MSA), têm recebido atenção, apesar de sua instabilidade química e suscetibilidade à degradação fotoquímica e térmica (Shishido et al., 2003).

Neste contexto, o aprisionamento desses doadores de NO em nanomateriais surge como uma alternativa eficaz para aumentar sua estabilidade e controlar sua liberação. Incorporar os doadores em matrizes poliméricas, como nanopartículas à base de quitosana, permite proteger as moléculas da degradação precoce e prolongar seus efeitos fisiológicos (Seabra et al., 2015; Cardozo et al., 2014). A quitosana, por sua vez, é um polissacarídeo biodegradável e biocompatível amplamente explorado como sistema nanocarreador, especialmente em aplicações de liberação controlada de fármacos e compostos bioativos (Pelegrino et al., 2017a, b).

Assim, o uso de nanopartículas na agricultura tem ganhado destaque como uma abordagem emergente, especialmente na última década (Savithramma et al., 2012). Entre essas estratégias, o encapsulamento se mostra particularmente eficaz, aumentando a estabilidade física e a bioatividade de compostos durante o armazenamento (Mohammadi et al., 2015). No contexto do fornecimento controlado de óxido nítrico (NO), estudos recentes demonstram que a encapsulação da *S*-nitrosoglutationa (GSNO), um doador de NO, promoveu maior assimilação de CO_2 e aumentou a razão raiz/parte aérea em plantas de cana-de-açúcar, evidenciando que a liberação prolongada de NO favorece a resposta ao déficit hídrico, quando comparada à aplicação do composto na forma livre (Silveira et al., 2019b). Resultados semelhantes foram observados com o uso do *S*-nitroso-MSA encapsulado, que reduziu significativamente os danos à atividade fotoquímica em plantas de milho, reforçando a superioridade da formulação nanoestruturada em relação à convencional (Oliveira et al., 2016). Além disso, a aplicação foliar de GSNO e SNAC encapsulados em cana-de-açúcar também se mostrou eficaz durante a fase de recuperação pós-estresse hídrico, melhorando a eficiência fotossintética e o crescimento das plantas (Silveira et al., 2021). Esses compostos atuaram mitigando limitações bioquímicas da fotossíntese, promovendo a *S*-nitrosação de proteínas associadas ao metabolismo fotossintético e reduzindo o estresse oxidativo, com destaque para o aumento da atividade antioxidante nas raízes e a desintoxicação parcial do peróxido de hidrogênio (H_2O_2).

Esses achados reforçam o potencial das nanopartículas como sistemas eficientes de liberação de NO, promovendo impactos positivos na regulação metabólica e na resposta das plantas ao estresse hídrico. Embora promissora, essa abordagem ainda requer estudos adicionais para ampliar sua aplicação em larga escala e consolidar seu papel como ferramenta estratégica no manejo agrícola sustentável. Diante desse contexto, esta dissertação foi estruturada no formato de artigo científico, conforme apresentado a seguir.

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Encapsulated nitric oxide donors enhance physiological performance, antioxidant defense and post-drought recovery in citrus

Abstract

Nanobiotechnology has emerged as a promising strategy to mitigate water deficit, a challenge intensified by climate change and associated with negative impacts on crops such as citrus. In this context, the encapsulation of nitric oxide (NO) represents an innovative approach to enhance drought tolerance. However, the use of encapsulated NO donors has not yet been investigated in citrus plants, especially under water deficit and recovery conditions, representing an important knowledge gap. Citrus is a perennial woody crop sensitive to water limitation and of significant economic importance in semi-arid and vulnerable regions. This study aimed to test the hypothesis that encapsulated NO donors would differentially alleviate water deficit–induced oxidative stress, thereby promoting improvements in plant growth and photosynthetic performance. 'Valencia' sweet orange plants grafted onto 'Rangpur' lime rootstock were assigned to different treatments: well-watered plants (control) and plants subjected to water deficit, which were previously sprayed either with water (WD) or with encapsulated NO donors (100 μM), namely *S*-nitrosoglutathione (GSNO), *S*-nitroso-mercaptosuccinic acid (MSA), and *S*-nitroso-N-acetylcysteine (SNAC). An empty nanoparticle formulation (ENP) was used as an additional control. Our results demonstrated that the nanoencapsulated NO donors GSNO and MSA were the most effective in mitigating the effects of water deficit, particularly during the post-drought recovery period. GSNO was especially effective in maintaining plant water status and chlorophyll content during recovery. Both NO donors enhanced the antioxidant defense system by increasing the activities of superoxide dismutase (SOD) and ascorbate peroxidase (APX). MSA was particularly effective in reducing root hydrogen peroxide (H_2O_2) accumulation under water deficit, indicating improved oxidative stress control. Furthermore, GSNO sustained elevated antioxidant activity during the recovery phase. These results suggest that GSNO and MSA are promising strategies for improving the physiological performance of citrus plants under and after water deficit.

Keywords: Water deficit; Climate change; Oxidative stress; Nanotechnology; Signaling.

1. Introduction

Climate projections indicate an increase in precipitation variability and a higher frequency of extreme events, such as prolonged droughts. These events negatively affect plant survival and, consequently, food security (IPCC, 2023; Zhao et al., 2020). Water deficit affects crucial physiological processes, including water and nutrient uptake and transport, photosynthesis, and reproductive development. Additionally, water deficit can trigger oxidative stress by increasing the production of reactive oxygen species (ROS), which damage cellular structures and inhibit plant growth (Sharma et al., 2012).

This physiological disruption has been particularly evident in citrus plants, where, in addition to the occurrence of diseases such as huanglongbing (HLB), water deficit has significantly impaired growth and development (Fundecitrus, 2024). In this context, there is a growing demand for strategies aimed at mitigating the negative effects of drought on citrus crops. Accordingly, nanobiotechnology emerges as a promising approach to alleviate drought stress, optimize input use, and reduce associated losses.

Among the approaches, the exogenous application of chemical signaling molecules such as nitric oxide (NO) has been explored to enhance plant tolerance to water deficit (Silveira et al., 2016, 2017a, 2020). Several classes of NO donors differ in chemical structure, half-life, and NO release rate, with their activity being influenced by factors such as concentration, pH, temperature, and light (Silveira et al., 2019a; De Souza et al., 2019). An important class of NO donors comprises *S*-nitrosothiols (RSNOs), including *S*-nitrosoglutathione (GSNO), *S*-nitroso-N-acetylcysteine (SNAC), and *S*-nitroso-mercaptosuccinic acid (SN-MSA). These low-molecular-weight compounds are photochemically and thermally unstable (Shishido et al., 2003), releasing NO through homolytic cleavage of the *S*–NO bond without generating toxic by-products. However, their limited stability restricts their practical effectiveness. In this context, encapsulation in nanomaterials has emerged as a promising strategy to enhance stability and enable controlled and sustained NO release (Seabra et al., 2015, 2022).

Although the use of nanomaterials to mitigate abiotic stresses is still an emerging field, recent studies have demonstrated their effectiveness in improving essential physiological processes such as germination, growth, photosynthesis, nutrient uptake, and disease resistance (Singh et al., 2021; Silveira et al. 2020). Among the various nanotechnological strategies, controlled-release systems based on polysaccharides, particularly chitosan, stand out due to their biocompatibility, low toxicity, and ability to promote the gradual release of active compounds (De Oliveira et al., 2021). Studies involving nanoencapsulated NO donors have reported promising results, including increased CO_2 assimilation, improved root/shoot ratio, and enhanced photochemical protection in crops such as sugarcane and maize under water restriction. These compounds also promoted *S*-nitrosation of photosynthesis-related proteins, contributing to reduced oxidative damage and enhanced antioxidant enzyme activity, even during post-stress recovery (Oliveira et al., 2016; Silveira et al., 2019a; Silveira et al., 2021).

Encapsulated NO donors supply have not yet been investigated in citrus plants; thus, this study provides the first evaluation under water deficit and recovery conditions. Beyond addressing this knowledge gap, citrus is a perennial woody crop sensitive to water limitation, with significant economic importance in semi-arid and vulnerable regions. Therefore, developing controlled-release strategies capable of enhancing drought tolerance may contribute not only to improving physiological performance but also to increasing water-use efficiency and long-term orchard sustainability. Moreover, understanding how encapsulated NO donors modulate redox balance and photosynthetic performance in citrus may provide mechanistic insights into NO-mediated stress tolerance, supporting the development of innovative and environmentally compatible tools for sustainable agricultural management.

Herein, our aim was to evaluate whether foliar spraying of chitosan-encapsulated NO donors (MSA, GSNO, and SNAC) can mitigate the effects of water deficit in citrus plants and improve post-drought recovery. An empty nanoparticle (ENP) formulation was used as the control. We hypothesized that encapsulated NO donors would differentially alleviate water deficit induced oxidative stress, thereby promoting improvements in plant growth and photosynthetic performance.

2. Material and methods

2.1 Plant material and growth conditions

Ten-month-old ‘Valencia’ sweet orange [*Citrus sinensis* (L.) Osbeck] plants grafted onto ‘Rangpur’ lime, also known as ‘Mandarin’ lime (*Citrus limonia* Osbeck), were used in this study. ‘Rangpur’ lime is widely employed as a citrus rootstock due to its tolerance to citrus tristeza virus (Muller et al., 2005). It also exhibits high compatibility with different scion varieties, strong vegetative vigor, a well-developed root system, and promotes earlier fruiting in grafted plants (Pompeu Junior, 2005; Roncatto, 2021). Plants with similar height and leaf area were selected, maintaining three shoots per plant. They were cultivated in 4.5-L plastic bags containing a commercial organic substrate composed of pine bark (Tropstrato V8 Citrus, Vida Verde, Mogi Mirim, SP, Brazil) under greenhouse conditions. The experiment was conducted in a completely randomized design, with six treatments and three replicates per treatment, with each experimental unit consisting of one plant. During the experimental period, the average maximum temperature was 35.7 ± 4.8 °C, the average minimum temperature was 26.9 ± 3.0 °C, and relative humidity ranged from 42.3% to 72.1%. Environmental conditions were continuously monitored using a data logger (B-Max, model BM-HTC2 Biomax®, São Paulo, Brazil).

2.2 Synthesis of chitosan nanoparticles containing NO donors

Chitosan nanoparticles (CSNPs) were prepared using the ionotropic gelation method, as previously reported (Silveira et al., 2019b). Briefly, chitosan was dissolved in 1% acetic acid to obtain a final concentration of 1 mg mL^{-1}. Subsequently, 50 mmol L^{-1} of reduced L-glutathione (GSH), mercaptosuccinic acid (MSA), or N-acetyl cysteine (NAC) was incorporated into the solution, which was maintained under magnetic stirring for 90 min. Nanoparticle formation was induced by the dropwise addition of sodium tripolyphosphate (TPP; 0.6 mg mL^{-1}). Empty CSNPs were produced following the same procedure, but without the addition of GSH, MSA, or NAC. For the preparation of *S*-nitrosothiol-containing nanoparticles, GSH, MSA, and NAC were nitrosated to generate *S*-nitrosoglutathione (GSNO)-, *S*-nitroso-mercaptosuccinic acid (MSA), or *S*-nitroso-N-acetyl cysteine (SNAC)-

loaded CSNPs, respectively. Nitrosation was achieved by adding sodium nitrite ($NaNO_2$; 50 mmol L^{-1}) to the final solutions, which were incubated for 90 min under light-protected conditions. The successful formation of *S*-nitrosothiol-loaded CSNPs was confirmed by detecting the characteristic *S*–NO absorption bands at 336 and 545 nm using a UV–visible spectrophotometer (Agilent 8454, Palo Alto, CA, USA).

2.3 Water deficit and spraying of NO donors

The plants were divided into two groups, one group remained irrigated (control), with substrate moisture around 90% of its maximum water storage capacity (MWC) throughout the experimental period. The other group was subjected to water deficit (WD) by water withholding until ~25% MWC of substrate moisture. Substrate moisture was monitored by weighing the bags (substrate + plant) with an electronic scale and water supply was done to keep moisture at the desired levels (~90% and ~25% MWC), following Silva et al. (2023).

Citrus leaves were sprayed (35 mL per plant) with freshly prepared solutions containing encapsulated NO donors at 100 μM, a concentration previously used in other studies (Silveira et al., 2016, 2017, 2019b). Spraying was performed once daily for three consecutive days. During application, plants were temporarily removed from the greenhouse to prevent nanoparticle dispersion among treatments. Plants were then subjected to the following treatments: (i) Control (well-watered) + water spraying; (ii) Water deficit (WD) + water spraying; (iii) WD + MSA nanoparticles (MSA); (iv) WD + GSNO nanoparticles (GSNO); (v) WD + SNAC nanoparticles (SNAC); and (vi) WD + empty nanoparticles (ENP, without NO, used as control).

Measurements and sampling were performed at maximum water deficit (MWD), i.e., after 10 days after water withholding, and on the second day of the recovery period (REC), i.e., two days after irrigation was restored to control conditions (Fig. S1). Leaf and root samples were collected, immediately frozen in liquid nitrogen, and stored at −80 °C for further analyses.

2.4 Leaf water potential and chlorophyll content

Leaf water potential (Ψ_w) was evaluated in fully expanded leaves between 11:00 and 12:00 h using a pressure chamber (model 3005F01, Plant Water Status Console, Soil Moisture Equipment Corp., Santa Barbara, CA, USA). For estimating chlorophyll content, a portable chlorophyll meter (CFL 1030, Falker, Porto Alegre RS, Brazil) was used to provide chlorophyll *a* and *b* values. Total chlorophyll content was calculated as the sum of chlorophyll *a* + *b*.

2.5 Leaf gas exchange and photochemistry

Leaf CO_2 assimilation (A) and stomatal conductance (g_s) were measured using an infrared gas analyzer (LI-6400, LI-COR, Lincoln, NE, USA) under a photosynthetically active radiation of 1000 μmol m^{-2} s^{-1} and a reference CO_2 partial pressure of 400 μbar. Measurements were performed between 10:00 and 12:00 h, following Miranda et al. (2020).

Instantaneous carboxylation efficiency (K = A/C_i) was calculated according to Machado et al. (2009). Chlorophyll fluorescence was assessed simultaneously with gas exchange, and the effective quantum efficiency of photosystem II (Φ_{PSII}) was estimated using the saturation pulse method (Edwards and Baker, 1993). All measurements were conducted at maximum water deficit (MWD) and on the second day of the recovery period (REC), except for *K*, which was calculated only in REC period.

2.6 Biometry

Leaves and roots were harvested, and the dry matter quantified after drying samples in an oven (60 ◦C) with forced-air circulation until constant weight. The leaf area of each plant was evaluated with a portable leaf area meter (LI-3100C Area Meter, Biosciences, Lincoln, NE, USA), following the manufacturer's instructions.

2.7 Quantification of hydrogen peroxide and lipid peroxidation

Hydrogen peroxide (H_2O_2) content and lipid peroxidation were determined from the same leaf and root extracts. Fresh tissue samples (0.02 g) were homogenized in 2 mL of 0.1%

(w/v) trichloroacetic acid (TCA) and centrifuged at 12,000 × g for 20 min. H_2O_2 was quantified by adding 30 µL of the supernatant to 10 mM potassium phosphate buffer (pH 7.0) and 1 M potassium iodide, reaching a total volume of 170 µL per well, followed by incubation at 30 °C for 10 min and measurement at 390 nm, according to Gay and Gebicki (2000), using a standard curve.

Lipid peroxidation was estimated by malondialdehyde (MDA) content. For this, 300 µL of the supernatant was incubated with 1.7 mL of 0.5% (w/v) thiobarbituric acid (TBA) at 90 °C for 30 min, followed by cooling in an ice bath for 10 min. Absorbance readings were taken at 535 and 600 nm, and results were calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}, expressed as nmol MDA g^{-1} fresh weight (Heath and Packer, 1968).

2.8 Antioxidant activity

Leaves (0.02 g) were homogenized in 1.5 mL of extraction buffer containing 400 mM potassium phosphate buffer (pH 7.8), 10 mM EDTA, 200 mM ascorbic acid, and distilled water. The homogenate was centrifuged at 12,000 × g for 15 min at 4 °C, and the resulting supernatants were used as crude enzyme extracts. Total soluble protein content was quantified using the Bradford method (1976).

Catalase (CAT; EC 1.11.1.6) and superoxide dismutase (SOD; EC 1.15.1.1) activities were determined according to Peixoto et al. (1999), while ascorbate peroxidase (APX; EC 1.11.1.11) activity was assessed following Nakano and Asada (1981). SOD activity was determined by adding 10 µL of enzyme extract to 190 µL of reaction medium containing 400 mM potassium phosphate buffer (pH 7.8), methionine, 10 µM EDTA, 1 mM nitroblue tetrazolium (NBT), 0.2 mM riboflavin, and distilled water, according to Del Longo et al. (1993). The reaction was conducted under illumination from a 15 W fluorescent lamp for 10 min, followed by dark incubation. The formation of blue formazan, resulting from NBT photoreduction, was measured at 560 nm, with control samples kept in the dark. One unit of SOD was defined as the amount of enzyme required to inhibit 50% of NBT photoreduction (Beauchamp & Fridovich, 1971).

CAT activity was assessed by adding 10 µL of crude enzyme extract to 170 µL of reaction medium containing 200 mM potassium phosphate buffer (pH 7.0) and distilled water

(Havir and Mchale, 1987). The reaction was initiated by adding 20 µL of hydrogen peroxide (H_2O_2), and H_2O_2 consumption was monitored by the decrease in absorbance at 240 nm over 3 min at 25 °C. Enzyme activity was calculated using a molar extinction coefficient of 36 mM^{-1} cm^{-1} (Anderson et al., 1995) and expressed as µmol per unit of protein.

APX activity was determined by adding 10 µL of crude enzyme extract to 170 µL of reaction medium containing 50 mM potassium phosphate buffer (pH 7.0) and 2.5 mM ascorbic acid. The reaction was initiated by adding 20 µL of H_2O_2 and monitored by the decrease in absorbance at 290 nm over 3 min at 25 °C. Enzyme activity was calculated based on the absorbance change (ΔA) using a molar extinction coefficient of 2.8 mM^{-1} cm^{-1} and expressed as µmol per unit of protein.

2.9 Data analysis

Data were analyzed using Bayesian statistics (JASP software, https://jaspstats.org/). When significant differences were detected, the mean values were compared using Bayes Factor (BF_{10}): $1 < BF_{10} < 3$, there is a weak support for the alternative hypothesis (H1); $3 < BF_{10} < 20$ indicates positive support for H1; and $BF_{10} > 20$ indicates strong support to H1 (Miranda et al., 2021). The results presented are the mean ± standard error, and the number of replicates is indicated in each figure caption. Each plant represents one biological replicate.

3. Results

3.1 Leaf water potential and chlorophyll content

Leaf water potential decreased in plants subjected to water deficit (WD) compared to the control. However, WD plants treated with MSA, SNAC, and empty nanoparticles (ENP) showed water potential values similar to the control. Interestingly, WD plants that received GSNO exhibited a higher hydration status, with water potential values even higher than those of the control plants (Fig. 1A).

Regarding total chlorophyll content, a reduction was observed under WD conditions compared with the control. However, plants treated with encapsulated NO donors exhibited higher chlorophyll levels even under restrictive conditions. After the recovery period, only GSNO-treated plants maintained chlorophyll content similar to the control (Fig. 1B).

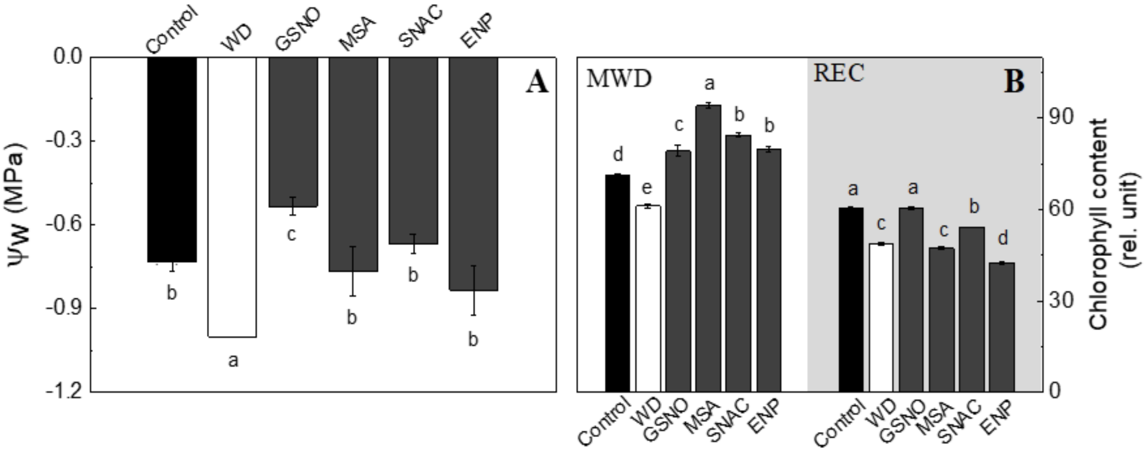

Fig 1. Leaf water potential (Ψ_w, in A) and total chlorophyll content (in B) of 'Valencia' sweet orange scions grafted on 'Rangpur' lime, well hydrated (control), subjected to water deficit and sprayed with water (WD), or subjected to water deficit and sprayed with chitosan-encapsulated NO donors (GSNO, MSA, SNAC) and empty chitosan nanoparticles (ENP). MWD indicates the maximum water deficit and REC indicates the second day of recovery. The bars represent the mean value of three replications ± standard error. Letters indicate statistical difference ($BF_{10} > 3$) among treatments in each evaluation.

3.2 Plant biomass

Water deficit caused a significant reduction in root dry mass in citrus plants, independent of encapsulated NO donor application. A more pronounced reduction was observed in MSA-treated plants (Fig. 2B). No significant changes were detected in shoot dry mass in response to water deficit, regardless of the treatment (Fig. 2A). Leaf area decreased under water deficit conditions, and this effect was attenuated only in plants sprayed with GSNO (Fig. 2C).

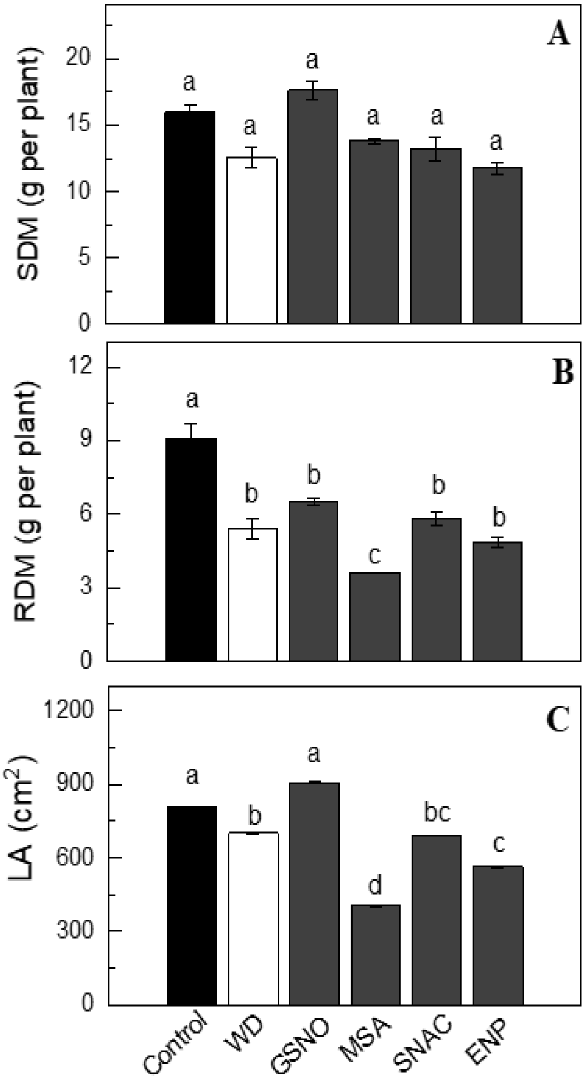

Fig 2. Shoot (SDM, in A) and root dry mass (RDM, in B) and leaf area (LA, in C) of 'Valencia' sweet orange scions grafted on 'Rangpur' lime, well hydrated (control), subjected to water deficit and sprayed with water (WD), or subjected to water deficit and sprayed with chitosan-encapsulated NO donors (GSNO, MSA, SNAC) and empty chitosan nanoparticles (ENP). Evaluations were carried out at the end of the experiment (2nd day of recovery). The bars represent the mean value of three replications ± standard error. Letters indicate statistical difference ($BF_{10} > 3$) among treatments in each evaluation.

3.3 Leaf gas exchange and photochemistry

Water deficit induced a marked reduction in leaf CO_2 assimilation in all plants, regardless of encapsulated NO donor application (Fig. 3A). During the recovery period, citrus plants treated with SNAC exhibited a recovery of photosynthesis compared with plants treated with other NO donors as well as untreated WD plants (Fig. 3A). Stomatal conductance decreased under WD conditions. However, plants treated with encapsulated NO donors and empty nanoparticles showed higher stomatal conductance than WD plants at both maximum water deficit (MWD) and during recovery (REC) (Fig. 3B). At maximum stress, the effective quantum efficiency of PSII (Φ_{PSII}) was increased by MSA and SNAC application compared with WD alone. During the recovery period, only plants treated with empty nanoparticles exhibited higher Φ_{PSII} than WD plants (Fig. 3C). Instantaneous carboxylation efficiency (*K*) was significantly reduced by water deficit, and this negative effect was partially attenuated by SNAC supply. In addition, at the end of the recovery period, GSNO and empty nanoparticle-treated plants displayed higher *K* values than WD plants (Fig. 3D).

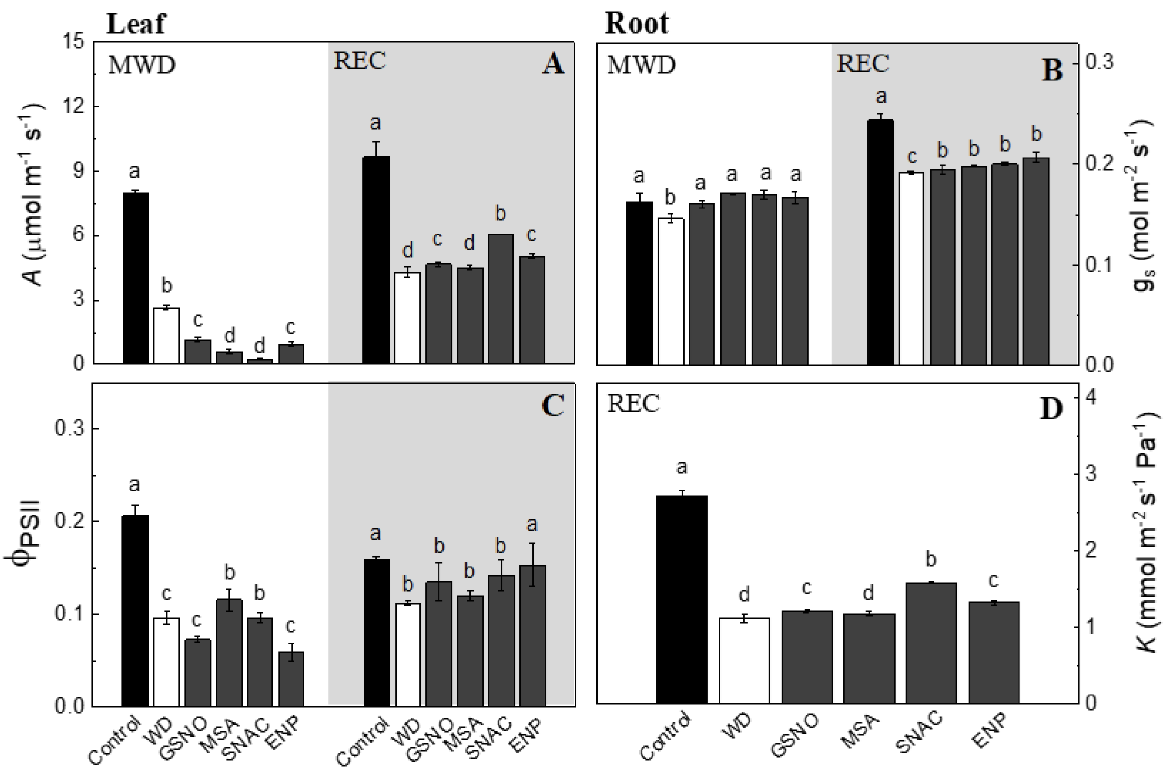

Fig 3. Leaf CO_2 assimilation (*A*, in A), stomatal conductance (g_s, in B), effective quantum efficiency of photosystem II (Φ_{PSII}, in C) and instantaneous carboxylation efficiency (*K*, in D) of 'Valencia' sweet orange scions grafted on 'Rangpur' lime, well hydrated (control), subjected to water deficit and sprayed with water (WD), or subjected to water deficit and sprayed with chitosan-encapsulated NO donors (GSNO, MSA, SNAC) and empty chitosan nanoparticles (ENP). MWD indicates the maximum water deficit and REC indicates the second day of recovery. The bars represent the mean value of three replications ± standard error. Letters indicate statistical difference ($BF_{10} > 3$) among treatments in each evaluation.

3.4 Oxidative damage and antioxidant metabolism

MDA and H_2O_2 concentrations were higher in leaves than roots, however, no significant differences among treatments were observed in leaves at either evaluation period (MWD and REC) (Fig. 4A, C). In contrast, root H_2O_2 concentration increased in plants subjected to WD alone, and this effect was attenuated by the application of encapsulated NO donors, particularly MSA (Fig. 4D).

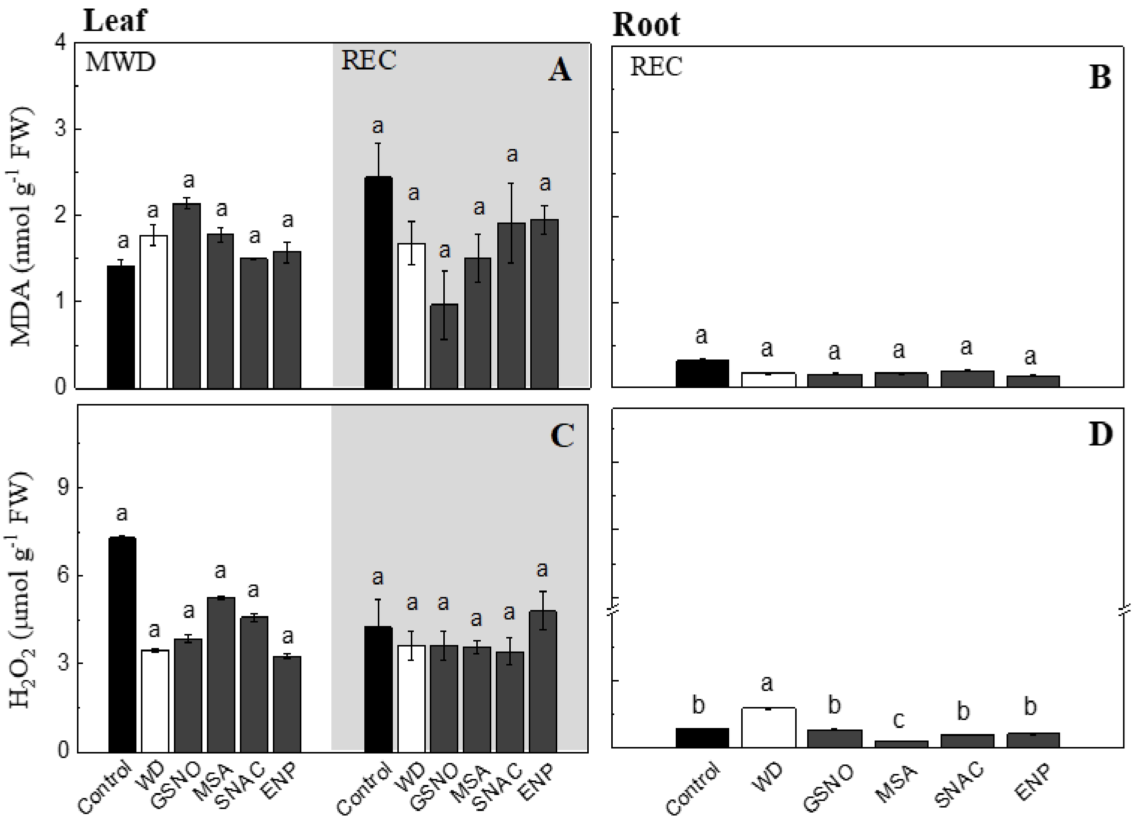

Fig 4. Concentration of malondialdehyde (MDA, in A and B) and hydrogen peroxide (H_2O_2, in C and D) in leaves (A, C) and roots (B, D) of 'Valencia' sweet orange scions grafted on 'Rangpur' lime, well hydrated (control), subjected to water deficit and sprayed with water (WD), or subjected to water deficit and sprayed with chitosan-encapsulated NO donors (GSNO, MSA, SNAC) and empty chitosan nanoparticles (ENP). MWD indicates the maximum water deficit and REC indicates the second day of recovery. The bars represent the mean value of three replications ± standard error. Letters indicate statistical difference ($BF_{10} > 3$) among treatments in each evaluation.

Regarding antioxidant enzymes, leaf SOD activity increased in plants exposed to water deficit, with the highest activity found in plants sprayed with GSNO. A similar trend was observed during the recovery period (Fig. 5A). Leaf APX activity decreased under water deficit conditions. However, plants sprayed with GSNO and MSA showed increased APX activity compared with WD alone. During the recovery period, GSNO-treated plants maintained high APX activity (Fig. 5E). No significant changes in CAT activity were observed among treatments in either evaluation period (MWD and REC) and organ analyzed (Fig. 5C, D). In roots, SOD and APX activities increased in plants previously subjected to water deficit, with the highest activities observed in plants sprayed with GSNO and MSA (Fig. 5B, F).

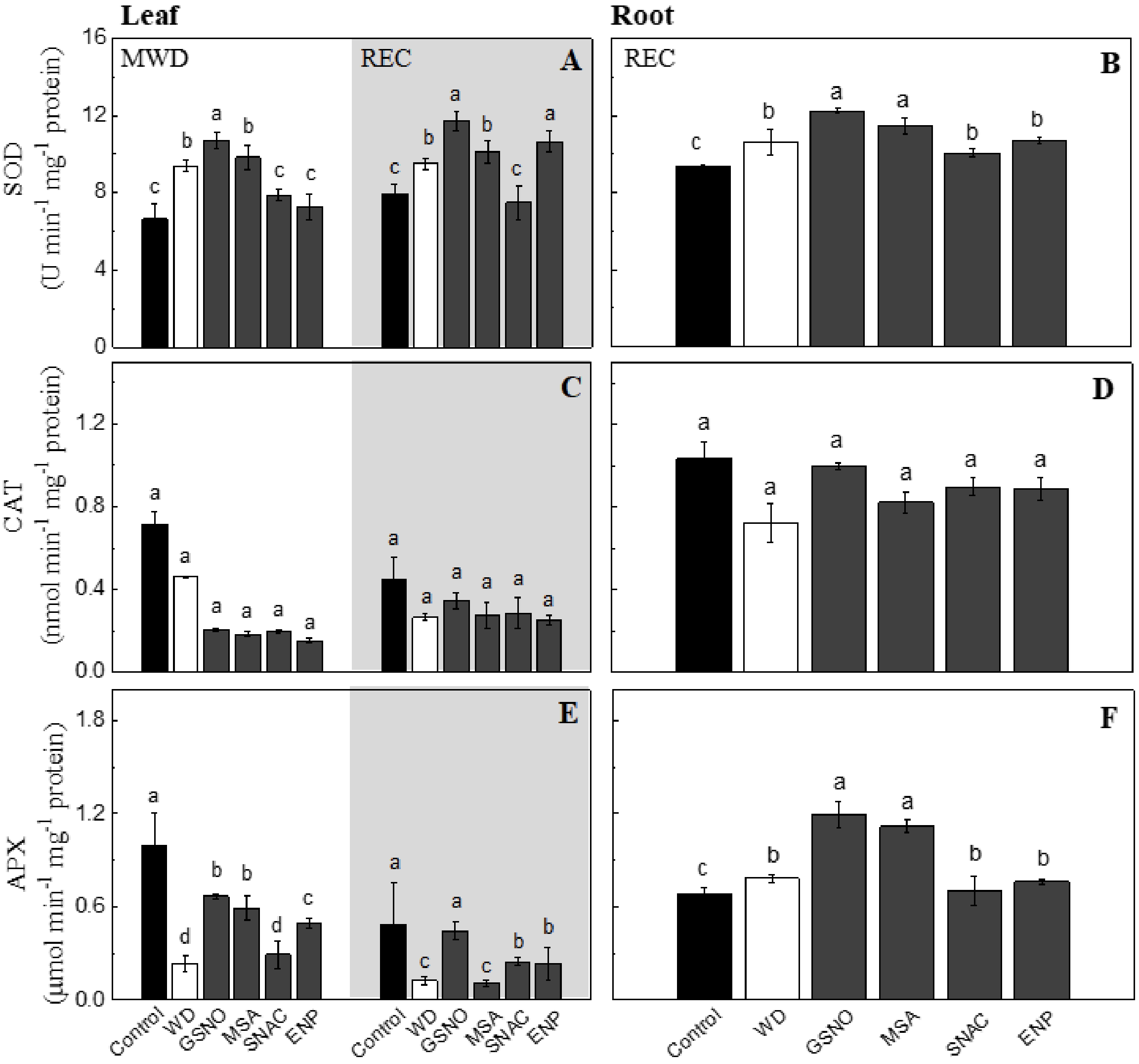

Fig 5. Activity of superoxide dismutase (SOD, in A and B), catalase (CAT, in C and D) and ascorbate peroxidase (APX, in E and F) in leaves (A, C, E) and roots (B, D, F) of 'Valencia' sweet orange scions grafted on 'Rangpur' lime, well hydrated (control), subjected to water deficit and sprayed with water (WD), or subjected to water deficit and sprayed with chitosan-encapsulated NO donors (GSNO, MSA, SNAC) and empty chitosan nanoparticles (ENP). MWD indicates the maximum water deficit and REC indicates the second day of recovery. The bars represent the mean value of three replications ± standard error. Letters indicate statistical difference ($BF_{10} > 3$) among treatments in each evaluation.

4. Discussion

4.1 Encapsulated GSNO enhances water status and photosynthetic recovery in citrus

Our findings demonstrate that foliar encapsulated GSNO supply was more effective in maintaining plant water status during post-drought recovery (Fig. 1A). Although root biomass in these plants remained similar to WD treatment (Fig. 2B), NO is recognized as an important intermediate in auxin-regulated signaling cascades, influencing root morphology and physiology (Correa-Aragunde et al., 2007; Silveira et al., 2020). Several studies indicate that NO plays a regulatory role in auxin homeostasis and signaling pathways, affecting its synthesis, transport, and cellular response. For example, NO has been associated with increased levels of indole-3-acetic acid in alfalfa seedlings (Sanz et al., 2014), increased primary root growth (Gouvea et al., 1997), stimulation of adventitious root formation (Pagnussat et al., 2002) and lateral root formation (Correa-Aragunde et al., 2004).

Under water deficit conditions, the reconfiguration of the root system becomes essential to optimize water uptake from deeper soil layers. In this context, auxin acts as a key regulator of apical dominance and lateral root formation (Carmo et al., 2022; Gomes et al., 2025). Therefore, even in the absence of detectable differences in root dry mass, NO-induced modifications in root architecture or functional activity may have enhanced water acquisition and contributed to the maintenance of plant water status. Indeed, Silveira et al. (2024) reported that NO production in ‘Rangpur’ lime roots was associated with modulation of root morphology, including increased root length, surface area, diameter, and volume under water deficit. Fine roots are known to expand the root–soil interface, thereby enhancing the plant’s capacity to absorb water and nutrients (McCormack et al., 2015).

Another explanation for the improved water status in plants treated with GSNO is the enhanced osmotic adjustment through the accumulation of osmoprotectants, such as proline and glycine betaine. Proline acts as an osmoprotectant and antioxidant, contributing to the stabilization of cellular structures, especially membranes, under stress (Sadak et al., 2019). Glycine betaine, in turn, participates in osmotic adjustment and protein stabilization, including Rubisco, protecting the photosynthetic apparatus and assisting in the mitigation of reactive oxygen species (ROS) (Wani et al., 2019; Ilyas et al., 2021). Recent study reinforces this mechanism. In common bean (*Phaseolus vulgaris*) subjected to water stress simulated by PEG, the exogenous application of NO promoted physiological and biochemical improvements associated with the modulation of proline metabolism, resulting in greater drought tolerance (Rehaman et al., 2025). These results corroborate that NO can act as a regulator of osmolyte biosynthesis, favoring cellular osmotic adjustment under water deficit.

Furthermore, foliar encapsulated GSNO supply was more effective in maintaining leaf area and chlorophyll content during post-drought recovery (Fig. 2C and 1B). The preservation of these photosynthetic pigments during recovery likely contributed, at least in part, to the maintenance of the photosynthetic rate observed in these plants (Fig. 3A). Plants sprayed with SNAC also showed improved photosynthetic recovery. Notably, during the post-drought recovery period, the superior performance of plants treated with GSNO and SNAC appears to be associated with the maintenance of instantaneous carboxylation efficiency (*K*) (Fig. 3D), indicating enhanced biochemical capacity for CO_2 fixation. Similar results were reported by Silveira et al. (2021), who demonstrated that NO donor application favored the recovery of CO_2 assimilation after rehydration in sugarcane plants. Perlikowski et al. (2025) also reported that NO plays a central role in regulating photosynthesis during rehydration, sustaining photosynthetic activity and photochemical efficiency during this phase. However, the improved photosynthetic performance of GSNO and SNAC treated plants during post-drought recovery does not appear to be restricted solely to the maintenance of instantaneous carboxylation efficiency (*K*). During recovery, foliar supply of all nanoparticle formulations increased stomatal conductance compared with WD treatment (Fig. 3B). Indeed, previous studies have demonstrated that exogenous NO can partially maintain stomatal opening and CO_2 assimilation under restrictive conditions (Silveira et al., 2016, 2017, 2021a, b).

4.2 Encapsulated NO donors modulate oxidative stress and antioxidant defenses

Although no lipid peroxidation was detected based on MDA concentration in leaves or roots in either evaluated period (Fig. 4A, B), the occurrence of other types of oxidative damage, such as to proteins and DNA, cannot be ruled out (Oliveira et al., 2010). Moreover, leaf H_2O_2 concentration was also similar among treatments (Fig. 4C). However, previous studies by Silveira et al. (2017) reported localized H_2O_2 accumulation in mesophyll cells adjacent to stomatal guard cells under water deficit, indicating localized oxidative stress. This accumulation was detected through histochemical analyses, as biochemical quantification may mask localized changes due to a dilution effect. In contrast, root H_2O_2 concentration increased under water deficit, and this effect was attenuated by nanoparticle treatments, particularly MSA (Fig. 4D). This response suggests that MSA supply may have enhanced ROS detoxification capacity in roots, promoting a more efficient redox buffering system under post-drought conditions.

Indeed, the increased root SOD and APX activities observed in plants sprayed with MSA and GSNO (Fig. 5B, F), may have contributed to enhanced ROS detoxification capacity. SOD acts as the first enzymatic line of defense by dismutation the superoxide anion into H_2O_2, which is subsequently detoxified by enzymes such as APX and CAT. Thus, the coordinated increase in SOD and APX activities may have promoted more efficient control of ROS dynamics, particularly under MSA treatment.

Additionally, leaf SOD activity increased in plants supplied with encapsulated GSNO, a pattern that persisted during the recovery period (Fig. 5A). In addition, APX activity decreased under WD, however, plants trated with GSNO and MSA enhanced its activity, with GSNO maintaining elevated APX activity even during post-drought recovery. These differences may be associated with the distinct NO-release kinetics of the encapsulated donors. Silveira et al. (2021) demonstrated that MSA exhibits a rapid and nearly complete NO release (reaching approximately 100% within 11 h), whereas GSNO shows a more sustained release pattern over time (approximately 30 mmol NO L^{-1} after 24 h from an initial concentration of 50 mmol L^{-1}). Such differences in NO availability and temporal dynamics may influence the magnitude and persistence of antioxidant defense system activation.

5 Conclusion

Foliar application of encapsulated GSNO and MSA alleviated water deficit induced damage in citrus plants, especially during the post-drought recovery phase. GSNO contributed to the maintenance of plant water status and chlorophyll content throughout recovery. Both donors enhanced the antioxidant defense system, as evidenced by increased activities of SOD and APX in leaves and roots. MSA was especially effective in reducing root H_2O_2 levels under water deficit conditions, indicating improved oxidative stress control. Overall, the effectiveness of NO in promoting drought mitigation and recovery was donor-dependent. These findings highlight the potential of GSNO and MSA as promising strategies to improve the physiological performance of citrus plants under water-limited conditions. Importantly, the selection of encapsulated NO donors for agricultural applications should consider NO release dynamics, as well as the potential bioactivity and toxicity of associated by-products.

Supplementary material

Figure S1

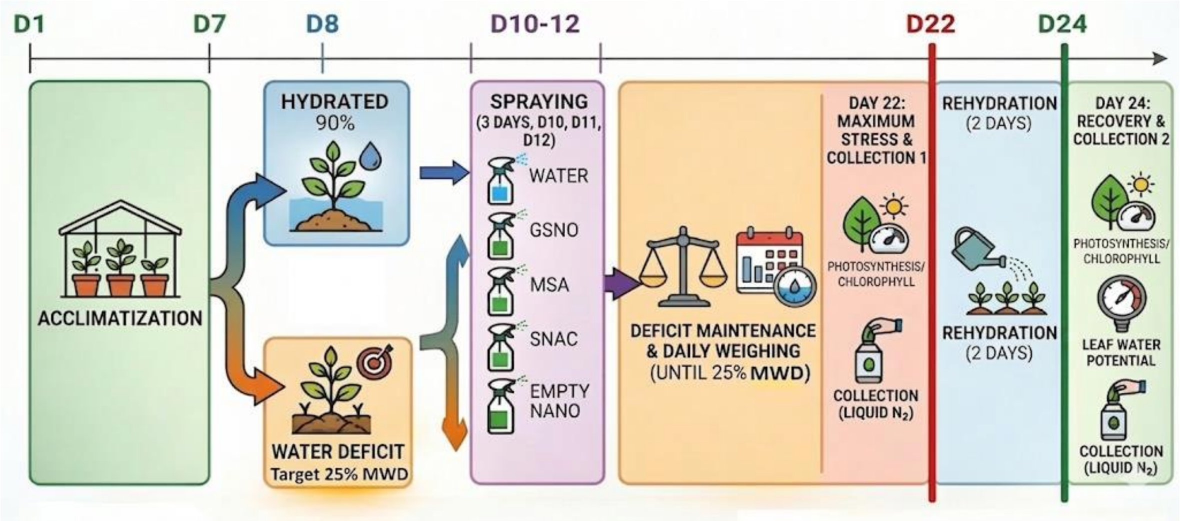

Fig. S1 Simplified schematic diagram illustrating the NO donors supply, the imposition of water deficit, the recovery period, and sampling days. Abbreviation: MWD, maximum water storage capacity. Source: Gemini artificial intelligence (Google, 2026).

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CONSIDERAÇÕES FINAIS

O aumento na frequência e intensidade de eventos de seca associados às mudanças climáticas impõe desafios significativos à citricultura, especialmente em regiões caracterizadas por irregularidade na distribuição das chuvas e períodos prolongados de estiagem. A cultura dos citros é altamente dependente da disponibilidade hídrica, e a limitação de água pode comprometer a produtividade a longo prazo dos pomares. Nesse contexto, estratégias que promovam maior tolerância ao déficit hídrico tornam-se essenciais para a sustentabilidade da produção citrícola frente aos cenários climáticos futuros. O uso de doadores de NO nanoencapsulados representa uma abordagem inovadora que integra conhecimentos de fisiologia vegetal e nanotecnologia, permitindo a liberação controlada e sustentada de moléculas sinalizadoras.

No presente estudo, os resultados demonstram que os doadores de NO encapsulados GSNO e MSA foram os mais eficientes na mitigação dos efeitos do déficit hídrico em plantas de citros, especialmente durante o período de recuperação pós-seca. O GSNO destacou-se na manutenção do status hídrico e do conteúdo de clorofila durante a recuperação. Ambos os doadores promoveram maior ativação do sistema antioxidante, especialmente das enzimas SOD e APX. O MSA foi particularmente eficiente na redução do acúmulo radicular de H_2O_2 sob déficit hídrico, indicando maior controle do estresse oxidativo. Além disso, o GSNO manteve a atividade antioxidante elevada durante a fase de recuperação. Esses resultados sugerem que a eficácia observada está associada à dinâmica de liberação de NO, reforçando o potencial de GSNO e MSA como estratégias promissoras para aumentar a resiliência dos citros ao déficit hídrico.

Apesar dos resultados promissores, ainda são necessários estudos adicionais para validar a relevância agronômica desses sistemas em condições de campo e em diferentes combinações copa/porta-enxerto. Investigações futuras devem explorar com maior profundidade outros mecanismos envolvidos nas respostas mediadas pelo NO, incluindo aspectos moleculares, interações com vias hormonais e modificações pós-traducionais, como a *S*-nitrosilação de proteínas. A definição de doses, frequência e estratégias de aplicação também será fundamental para viabilizar a adoção prática dessa tecnologia em condições reais de cultivo.

Entretanto, alguns desafios ainda precisam ser considerados. A elevada reatividade e a curta meia-vida do NO exigem cuidado no desenvolvimento de formulações que garantam liberação adequada e efeitos fisiológicos consistentes. Nesse sentido, sistemas nanoencapsulados representam um avanço importante, pois permitem maior controle sobre a dinâmica de disponibilização do sinalizador. O aprimoramento dessas tecnologias, aliado à definição de protocolos de aplicação bem estabelecidos, poderá ampliar a previsibilidade das respostas em condições de campo e fortalecer o uso do NO como ferramenta complementar no manejo sustentável da citricultura frente às mudanças climáticas.

Adicionalmente, frente aos cenários de maior frequência de seca e à expansão do cinturão citrícola para regiões com maior limitação hídrica, como áreas do semiárido brasileiro e outras regiões com distribuição irregular de chuvas, a escolha de porta-enxertos com características adaptativas torna-se um fator estratégico para a sustentabilidade dos pomares. Porta-enxertos que apresentam sistemas radiculares vigorosos, maior eficiência na absorção de água e maior tolerância ao déficit hídrico tendem a conferir maior resiliência às plantas. Nesse contexto, materiais como o limoeiro ‘Cravo’ (Citrus limonia), amplamente reconhecido por sua elevada tolerância à seca e adaptação a condições ambientais mais restritivas, mostram-se particularmente promissores para essas regiões em expansão. Assim, a integração entre a escolha de porta-enxertos mais tolerantes e o uso de abordagens nanotecnológicas, como a aplicação de doadores de NO nanoencapsulados, representa uma estratégia promissora para mitigar os impactos das mudanças climáticas na citricultura. Nesse sentido, a combinação de materiais genéticos adaptados e tecnologias inovadoras desponta como um dos principais caminhos para o desenvolvimento de sistemas de produção mais resilientes, eficientes e sustentáveis.

APÊNDICE

Figura 1. Visão geral do experimento em casa-de-vegetação (em A), imagem representativa da aplicação foliar das nanopartículas (em B) e pesagem dos vasos para a determinação da máxima capacidade de armazenamento do substrato (em C).