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

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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 short and long-term WD in a greenhouse. Seedlings under long-term WD received nanoformulations in the substrate three times at ten-day intervals. Under short-term WD, seedlings were treated for three alternate days before initiating the WD. The treatment with NPs containing GSNO (50 μM) increased the stomatal conductance, photosynthetic rate, and plant water potential of *C. pachystachya* submitted to short and long-term 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. Under long-term WD, 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 μM), in any WD condition. In summary, results suggest that nanoencapsulated GSNO can protect *C. pachystachya* seedlings in both 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*.

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.

1. 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 resistance 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 drought 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 (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, and with different durations of drought. 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 short and long-term WD.

2. Material and methods

2.1 Synthesis and characterization of the formulations

Chitosan NPs were obtained using the ionotropic gelation method (Oliveira et al., 2016). Initially, chitosan (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 famvar/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 ºC (Pelegrino et al., 2021). Just before the treatments, the powdered GSNO was dissolved in distilled water to achieve the desired concentration.

2.2 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 to 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% P_2O_5 and 14% K_2O). 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 acclimatation. The WD treatments started when the plants reached approximately 10 centimeters in height.

2.3 Treatments

2.3.1 Short-term drought

A randomized block design was employed with twelve replicates for each treatment. For *C. pachystachya*, the following treatments were used: field capacity (FC), water deficit (WD), water deficit plus application of nanoparticles without NO (WD + CS NP), water deficit plus application of GSNO in its free form (WD + GSNO), and water deficit plus application of chitosan nanoparticles containing the GSNO donor (WD + NP GSNO), all at 50 µM. This concentration was selected based on a pilot experiment, where WD-stressed *C. pachystachya* seedlings were treated with different concentrations of NO-releasing NPs.

For *C. estrellensis*, the treatments applied were FC, WD, and WD + NPs containing GSNO at 200, 400, and 600 µM. To instigate the treatments, 30 mL of recently prepared formulations were applied to the soil, on three alternate days, totaling three applications. Plants in the WD treatment received the same volume of distilled water, while plants in the FC treatment received daily irrigation until the soil reached maximum water saturation. Between treatment days, all plants received normal irrigation. After the third application of NP suspensions, WD was imposed by halting irrigation. The experiment concluded after four days of WD, which was the day after the seedlings reached permanent wilt, and the destructive analyses were conducted.

2.3.2 Long-term drought

A randomized block design was employed with twelve replicates for each treatment. For *C. pachystachya*, the following treatments were used: FC; WD; WD + CS NP; WD + GSNO; and WD + NP GSNO, all at 50 µM. This concentration was selected based on a pilot experiment, where WD-stressed *C. pachystachya* seedlings were treated with different concentrations of NO-releasing NPs.

For *C. estrellensis*, the following treatments were applied: FC, WD, WD + NPs containing GSNO at 25, 50, 100, 200, 400, 600, and 800 µM. For this species, no single dose stood out in the pilot experiment; hence, a broader range of concentrations was selected to test their effects.

After initiating the treatments, 30 mL of freshly prepared formulations were applied directly to the soil, once 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 application of the first set of treatments, the long-term WD was imposed by suspending the irrigation until plants showed wilting symptoms in the early morning (which corresponds to approximately 30% soil moisture). Whenever necessary, 15 mL of water were added to the polypropylene tubes to maintain the pre-established wilting level. The experiments were concluded 30 days after the first set of treatments and the imposition of long-term WD, when destructive analyses were conducted.

2.4 Physiological analyses

The youngest fully expanded leaf of each seedling was used for physiological analyses. For both WD conditions, stomatal conductance (g_s, mmol m^{-2} 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. For long-term WD, g_s was evaluated on the second day after each treatment application set, whereas for short-term WD, it was measured two days after the last treatment application.

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

2.5 Water status analyses

The plant water potential of both species was measured pre-dawn using a Schölander 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. 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.

2.6 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 10x 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).

2.7 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.

3. Results

3.1 Characterization of chitosan nanoparticles

Chitosan nanoparticles were successfully synthesized by ionotropic gelation. Fig.1A presents the HRTEM micrograph for the obtained chitosan NPs, evidencing a spherical morphology, with a slight distortion promoted by the sample preparation process. The solid-state size showed a mean of 108.1 ± 9.8 nm. Hydrodynamic analysis evidenced chitosan nanoparticles with 177.8 ± 3.6 nm, which is in accordance with the solid-state size, presenting a size increase due to the presence of the solvation layer. The polydispersity index measured was 0.368 ± 0.04, and the zeta potential was positive (22.9 ± 1.1 mV), as a result of the amino groups present in the chitosan molecules, conferring electrostatic stability for the synthesized nanoparticles. NTA analysis (Fig.1B and Fig.1C) also corroborated the previous data, indicating a mean size of 182.9 ± 7.3 nm and a mode of 138.3 ± 13.1 nm.

3.2 Short-term drought

3.2.1 Cecropia pachystachya

Considering *C. pachystachya* plants under short-term WD, it was possible to observe a decrease in g_s compared to FC, except for the first measurement at 8 h, when there was no difference between treatments (Fig. 2). At both 10 h and 16 h, the lowest g_s was observed in the WD + GSNO treatment. The WD and WD + NP treatments were similar to both the GSNO and WD + NP GSNO treatments, while the WD + NP GSNO treatment was also comparable to FC. However, at 14 h, the lowest g_s values were observed in plants under WD without any formulation, treated with NPs without NO, and treated with free GSNO. In contrast, WD plants treated with the nanoencapsulated NO donor showed g_s values similar to FC at 14 h (Fig. 2).

The photosynthetic and transpiration rates decreased due to the imposition of short-term WD (Table 1). However, the application of NPs GSNO not only mitigated the effect of short-term WD on *A* and *E*, but also maintained averages similar to those of plants under well-watered conditions. In contrast, the *A* and *E* values obtained in the WD + NP and WD + GSNO treatments did not differ from the WD without any treatment. Regarding WUE, all treatments exhibited similar averages, irrespective of the application of nanoformulations and the water condition.

Table 1 Net photosynthesis (*A*, μmol m^{-2} s^{-1}), transpiration (*E*, mmol m^{-2} s^{-1}), and water use efficiency (WUE, μmol $mmol^{-1}$) in *Cecropia pachystachya* seedlings measured at the end of the short-term water deficit FC: field capacity; WD: water deficit; WD + NP: water deficit + chitosan nanoparticles 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 = 9). Equal letters above the bars indicate similar means according to ANOVA followed by Tukey's test ($p < 0.05$).

	FC	WD	WD + NP	WD + GSNO	WD + NP GSNO
A	12.37 ± 1.30^{a}	2.67 ± 1.20^{b}	7.81 ± 1.98^{ab}	6.07 ± 0.169^{b}	11.65 ± 1.80^{a}
E	2.32 ± 0.361^{a}	0.54 ± 0.132^{c}	1.28 ± 0.325^{bc}	1.11 ± 0.315^{bc}	2.09 ± 0.363^{ab}
WUE	5.72 ± 0.33^{a}	3.07 ± 1.27^{a}	5.67 ± 0.89^{a}	4.86 ± 0.916^{a}	5.76 ± 0.42^{a}

Regarding plant water potential, seedlings subjected to WD exhibited more negative values compared to seedlings in FC (Fig. 3A). The only exception occurred for seedlings treated with nanoencapsulated NO donor, which kept the water potential values equal to those of FC plants (Fig. 3A). Additionally, it was visually evident that NPs containing GSNO could prevent the wilting symptoms under short-term WD conditions (Fig. 3B).

3.2.2 Cariniana estrellensis

For *Cariniana estrellensis* seedlings subjected to short-term WD, g_s decreased in the seedlings compared to those kept in well-watered conditions at all measurement times. Interestingly, none of the treatments involving the addition of NO-releasing NPs at any concentration significantly differed from the control WD treatment (Supplementary Fig. S1A).

The same pattern of response was observed for the plant water potential (Supplementary Fig. S1B), where differences were only evident between FC plants and those maintained under drought stress. Once again, none of the applied concentrations of NPs GSNO altered the results.

At the end of the experiment, decreases in *A* and *E* was observed in WD plants compared to those kept at FC, and no concentration of the nanoformulation was able to induce an increase in these parameters. Regarding WUE, no differences were detected among the treatments, regardless of the application of formulations and water condition (Supplementary Table S1).

3.3 Long-term drought

3.2.1 *Cecropia pachystachya*

Regarding g_s in *C. pachystachya* seedlings, significant differences were observed after the first set of treatments (Fig. 4A). 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. 4A).

After the second set of treatments (Fig. 4B), 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. 4B).

In the analysis of g_s after the third and final set of treatments (Fig. 4C), 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. 4C).

Following the first set of treatments, as anticipated, long-term 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 2).

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 2).

Regarding WUE, after the first treatment application, the only significant difference was observed between the WD + NP CS and WD + NP GSNO treatments. However, after the second and third applications, all means were similar, regardless of water availability or formulation application (Table 2).

Table 2 Net photosynthesis (*A*, μmol m^{-2} s^{-1}), transpiration (*E*, mmol m^{-2} s^{-1}), and water use efficiency (WUE, μmol $mmol^{-1}$) 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 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 = 9). Equal letters above the bars 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}

Regarding the plant water potential, the observed results mirrored those of leaf RWC. Specifically, *C. pachystachya* plants exposed solely to WD exhibited lower water potential (more negative) compared to well-hydrated seedlings. Remarkably, the water potential of seedlings treated with WD + NP GSNO surpassed that of other drought treatments, not differing from FC plants (Fig. 5A).

Visually, it is evident that the WD + NP GSNO treatment effectively maintained turgid plants, similar to those in FC (Fig. 5B).

Under long-term 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. 6).

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 3).

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 3).

Table 3 Root-hair incidence (%), amount (mm^{-2}), 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 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 above the bars indicate similar means according to ANOVA followed by Tukey's test ($p < 0.05$). For root-hair length and fine-root diameter, equal letters above the bars 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}

3.2.2 *Cariniana estrellensis*

For *C. estrellensis*, g_s was also measured on the second day following each set of treatments. Supplementary Figure S2A 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 (Supplementary Fig. S2B).

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 (Supplementary Fig. S2C).

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 (Supplementary Table S2).

4. Discussion

The results indicated that NO-releasing NPs increased drought tolerance in *C. pachystachya* seedlings under both long-term and short-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 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; Souza et al., 2024).

Thus, nanoencapsulation plays a crucial role in improving 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).

For *C. pachystachya* plants, 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., 2020; 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., 2020; Singhal et al., 2021). This process promotes stomatal opening and CO_2 assimilation even under drought conditions (Wang et al., 2016; Casaretto et al., 2020).

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. Additionally, NPs without NO were also able to increase the root-hair amount compared to other treatments, resembling the effect of nanoencapsulated GSNO. Indeed, 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).

Interestingly, 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). 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 forming root hairs during lettuce plant development. The increase in NO content in root hairs following treatment with 1-naphthyl acetic acid (NAA) suggests that auxin-induced root hair formation is NO-dependent. Recent research indicates that NO may cooperate with abscisic acid in cytoskeletal organization processes, thereby modulating root hair formation (Lombardo and Lamattina, 2018; Sun et al., 2021; da Silva et al., 2024).

Previous studies have indicated that the effect of NO on root hair formation is dose-dependent, as it needs to be within a specific range to stimulate growth. This is evidenced by the inhibition of root hair growth in response to applied concentrations of cPTIO, a NO chelating agent (Lombardo et al., 2006; Sun et al., 2021).

In *C. pachystachya*, the nanoencapsulated donor increased the g_s of the plants even under short-term drought conditions, which likely led to enhanced carbon assimilation, without adversely affecting the water status, as the plant water potential remained high. In addition, the adjustment of g_s allied to improved root hair traits in plants kept under long-term WD, may have favored the maintenance of plant water potential, *A* and *E*, leading to improved biomass accumulation.

Furthermore, 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 (Misra et al., 2014; Oliveira et al., 2016; Wang et al., 2019). NO also stimulates the activity of key enzymes in photosynthesis, such as Rubisco, thereby enhancing CO_2 assimilation (Gan et al., 2015; Silveira et al., 2021).

In a study with soybean plants, 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).

According to Methela et al. (2023), the application of GSNO-loaded chitosan NPs at 50 µM also 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 CO_2 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.

The results obtained in the current study indicate that only the pioneer species responded to the application of the nanoencapsulated donor. This observation suggests a species-specific dependency in the efficacy of the treatment, corroborating the findings of Lopes-Oliveira et al. (2019). To confirm and expand upon these results, it is crucial to conduct additional studies with a larger number of species, from different successional groups. Testing a broader range of species could provide a more comprehensive understanding of the mechanisms involved and the potential applicability of this approach across different ecological contexts.

5. 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 both short-term and long-term conditions, but not in *C. estrellensis*. This beneficial effect on *C. pachystachya* was achieved with a relatively low concentration of nanoencapsulated GSNO, specifically 50 µM. Therefore, it is concluded that depending on the species, this technique may be efficient 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.

Declarations

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.

Supplementary Information

The supplementary material can be found online.

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Data availability

The dataset generated during and analyzed during the current study are available in the Supplementary Material.

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Figures

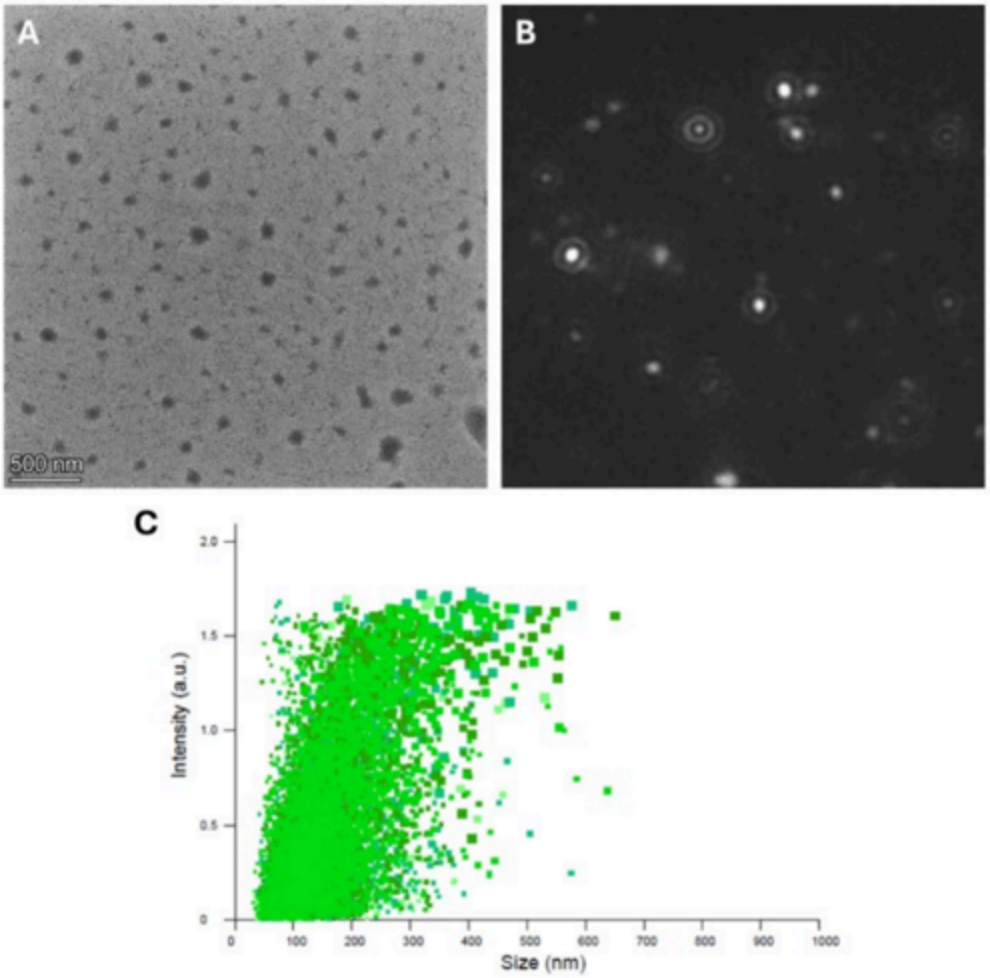

Figure 1

(A) HRTEM micrograph and (B) NTA image of Chitosan nanoparticles, and (C) size distribution of individually measured nanoparticles by NTA.

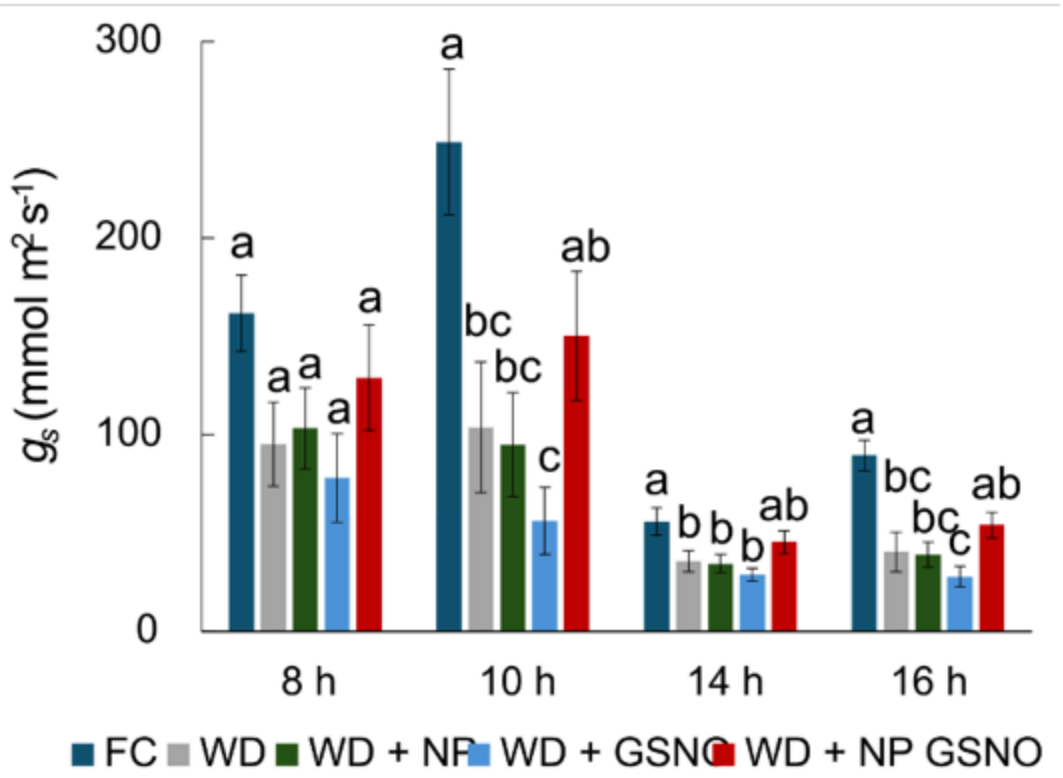

Figure 2

Stomatal conductance (g_s, mmol m^{-2} s^{-1}) of *Cecropia pachystachya* seedlings after two days of short-term water deficit. FC: field capacity; WD: water deficit; WD + NP: water deficit + chitosan nanoparticles 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 = 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 ($p < 0.05$).

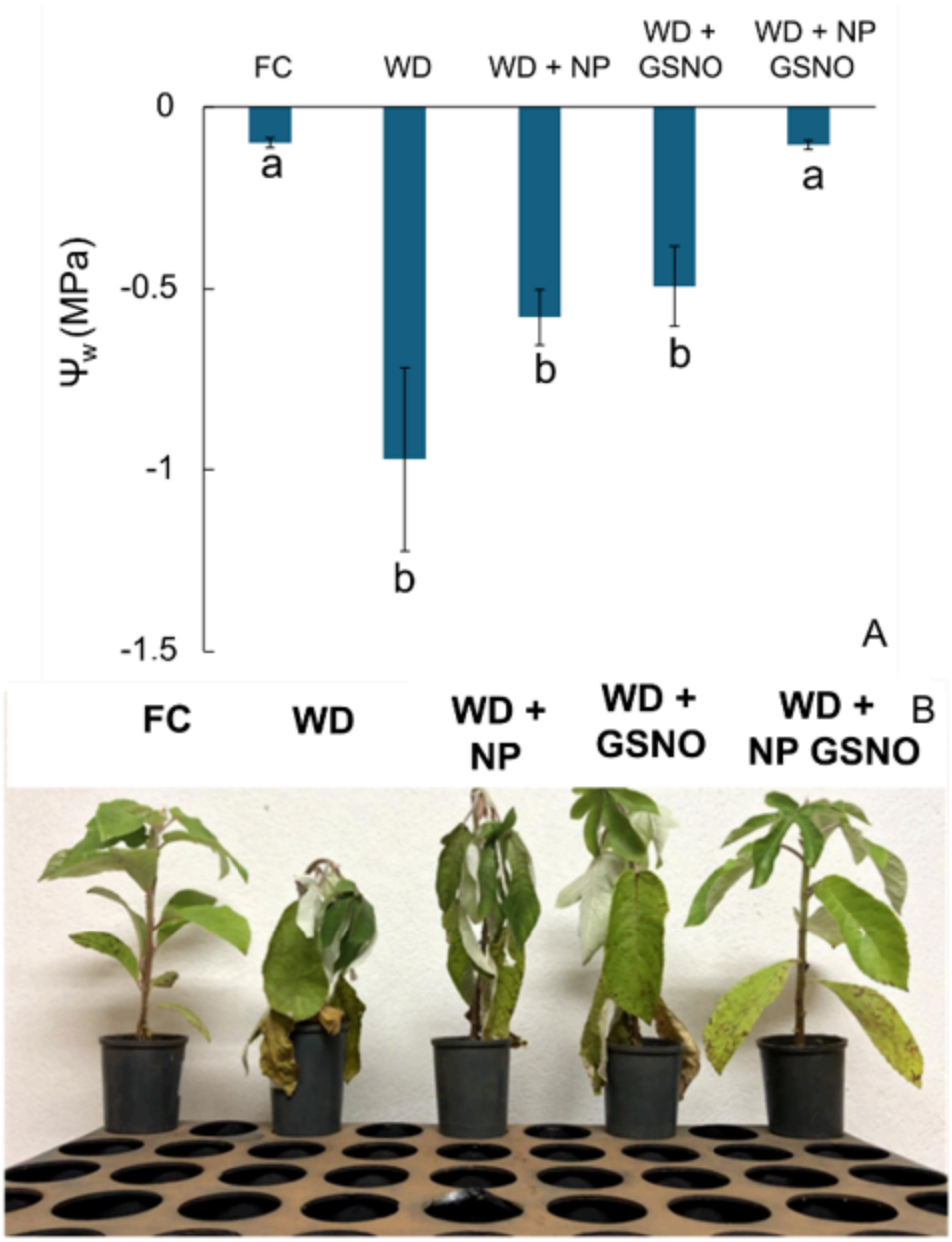

Figure 3

Plant water potential (Ψw, MPa) (A) and a photo representing the water status (B) of *Cecropia pachystachya* at the end of the short-term water deficit experiment FC: field capacity; WD: water deficit; WD + NP: water deficit + chitosan nanoparticles 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). 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 ($p < 0.05$).

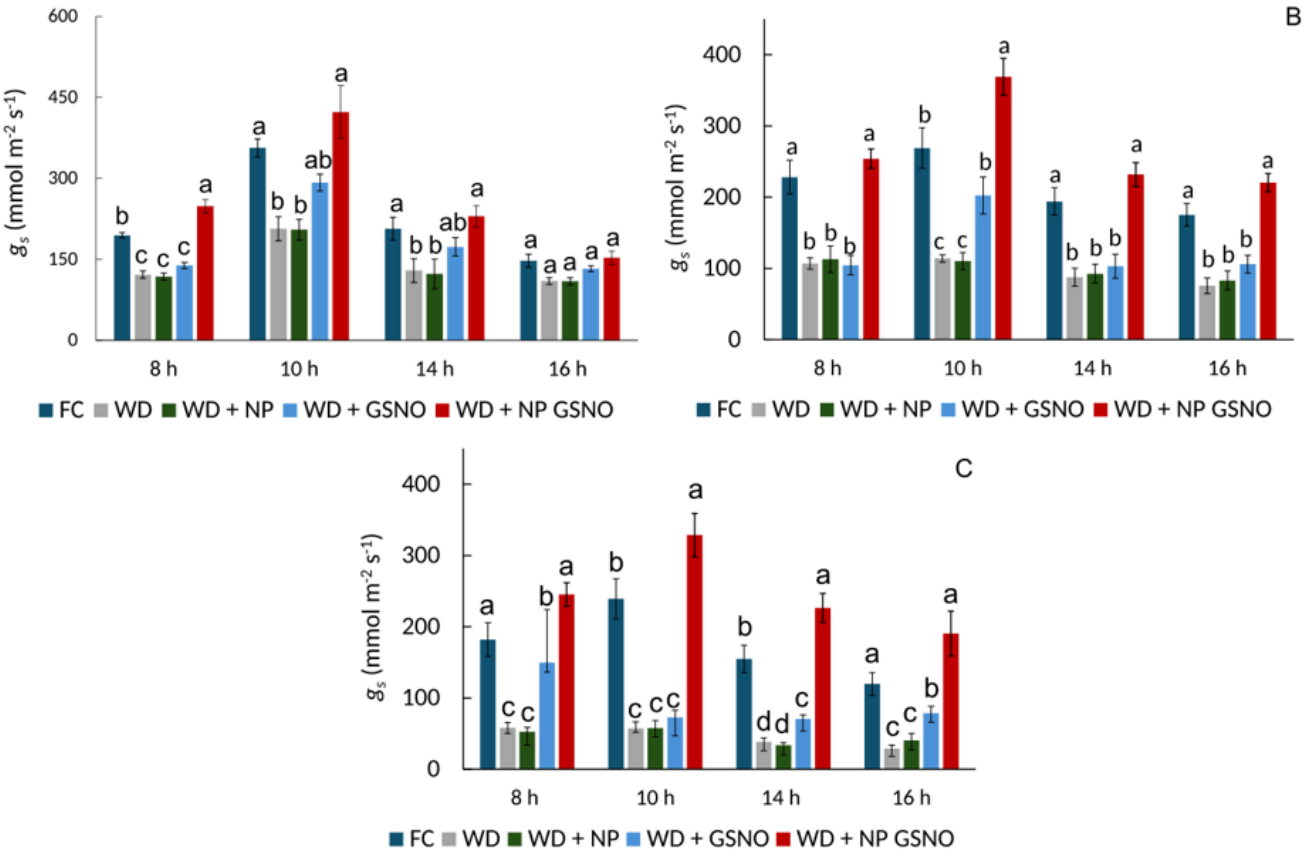

Figure 4

Stomatal conductance (g_s, mmol m^{-2} s^{-1}) of *Cecropia pachystachya* seedlings at 8 h, 10 h, 14 h, and 16 h PM after the first (A), second (B), and third (C) 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 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 = 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 ($p < 0.05$).

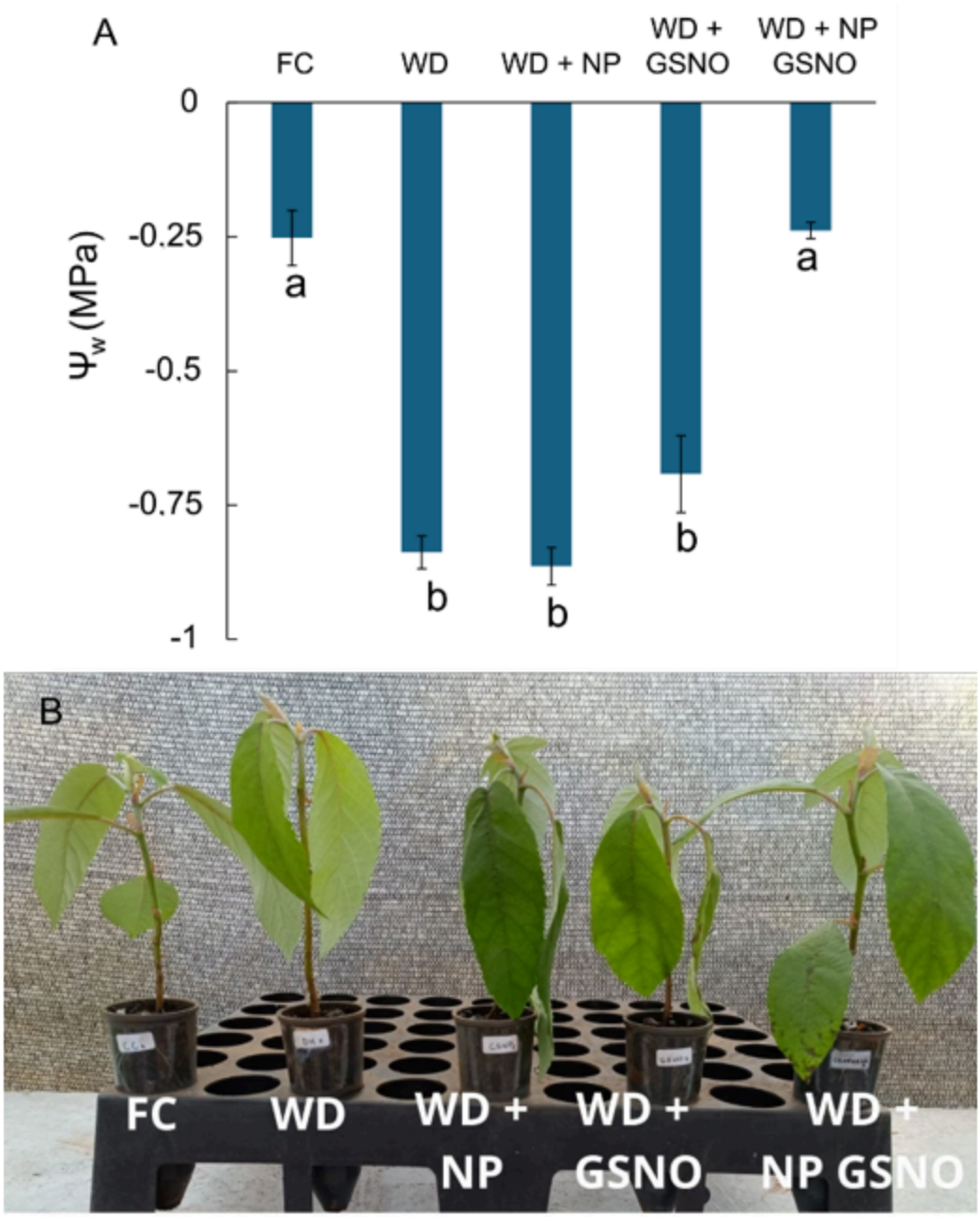

Figure 5

Plant water potential (Ψw, 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 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). 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 ($p < 0.05$).

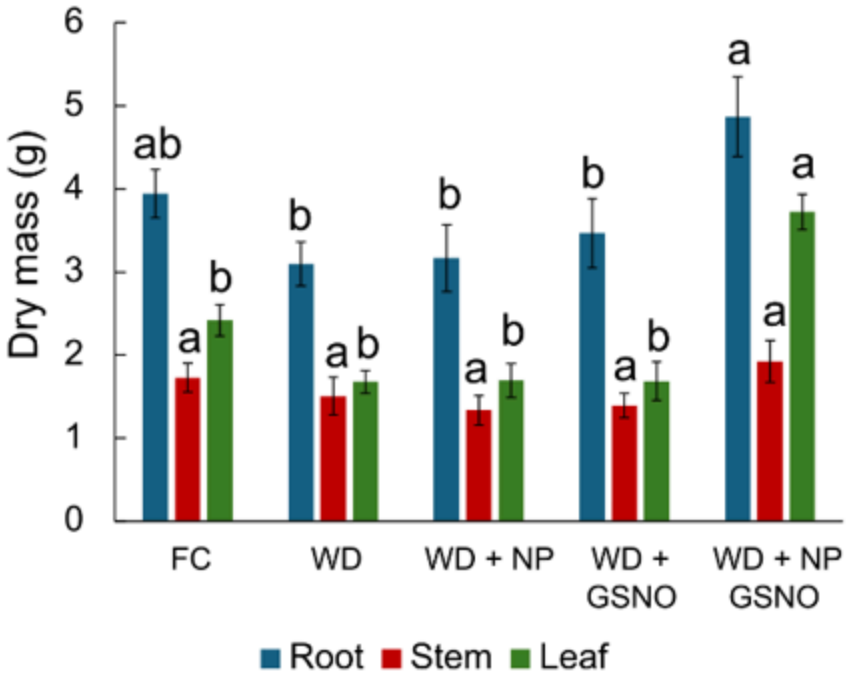

Figure 6

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 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 = 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 ($p < 0.05$).

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