Physiological and biochemical responses of *Neltuma ruscifolia* under Na_2SO_4 stress

Respuestas fisiológicas y bioquímicas de *Neltuma ruscifolia* bajo estrés con Na_2SO_4

Diego Ariel Meloni [1*], José Aliçandro Bezerra da Silva [2], María Judith Ochoa [1], Julia Andrea Lescano [1]

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Abstract

Salt stress limits plant production in arid and semi-arid zones. Although Na_2SO_4 is frequent in saline soils, most studies on plant physiological responses to salt stress were conducted using NaCl. This study aimed to determine the effect of Na_2SO_4 salt stress on physiological and biochemical responses in *Neltuma ruscifolia*. Increasing concentrations of Na_2SO_4 were added to 6-month-old plants grown hydroponically in 25% Hoagland nutrient solution. After 60 days of saline treatments, biomass, cysteine concentration, gas exchange, mineral composition, abscisic acid and salicylic acid concentrations, and antioxidant enzyme activity were determined. It is concluded that 200 mmol L^{-1} Na_2SO_4 is the threshold for *N. ruscifolia* seedling growth. Growth inhibition can be attributed to altered ionic homeostasis and photosynthesis inhibition after stomatal closure. Nevertheless, the species shows adaptive responses to this salt. Stomatal closure and increased foliar concentrations of abscisic acid contribute to water economy, while cysteine synthesis reduces sulfate toxicity. In parallel, salt stress induces salicylic acid accumulation in leaves, increasing the activity of antioxidant enzymes that prevent oxidative stress.

Keywords
ion homeostasis • phytohormones • antioxidant enzymes • photosynthesis • salinity stress

1 Universidad Nacional de Santiago del Estero. Facultad de Agronomía y Agroindustrias. INDEAS. Av. Belgrano (S) 1912. 4200- Santiago del Estero. Argentina. * dmeloniunse@gmail.com
2 Universidade Federal do Vale São Francisco. Av. Antônio Carlos Magalhães 510. Juazeiro. BA. Brazil.

Resumen

El estrés salino limita la producción vegetal en zonas áridas y semiáridas. Aunque el Na_2SO_4 es muy frecuente en suelos salinos, la mayoría de los estudios sobre las respuestas fisiológicas de las plantas al estrés salino se realizaron utilizando NaCl. El objetivo de este trabajo fue determinar el efecto del estrés salino con Na_2SO_4, sobre las respuestas fisiológicas y bioquímicas en *Neltuma ruscifolia*. Se adicionaron concentraciones crecientes de Na_2SO_4 a plantas de 6 meses, cultivadas hidropónicamente en solución nutritiva de Hoagland al 25%. Después de 60 días de tratamientos salinos, se determinó la biomasa, la concentración de cisteína, el intercambio gaseoso, la composición mineral, las concentraciones de ácido abscísico y salicílico, y la actividad de enzimas antioxidantes. Se concluye que el umbral para el crecimiento de plántulas de *N. ruscifolia* es 200 mmol L^{-1} Na_2SO_4. La inhibición del crecimiento puede atribuirse a alteraciones en la homeostasis iónica y a la inhibición de la fotosíntesis, debido al cierre de los estomas. Sin embargo, la especie muestra respuestas adaptativas a esta sal. Por lo tanto, el cierre de los estomas, asociado con mayores concentraciones foliares de ácido abscísico, contribuye a la economía del agua. La síntesis de cisteína reduce la toxicidad del ion sulfato absorbido por las raíces. Por otro lado, el estrés salino induce la acumulación de ácido salicílico en las hojas y un aumento de la actividad de enzimas antioxidantes, que pueden prevenir el estrés oxidativo.

Palabras clave
homeostasis iónica • fitohormonas • enzimas antioxidantes • fotosíntesis • estrés salino

Introduction

Approximately 7% of world land surface (7 million hectares) is affected by salinization (16). This situation worsens in arid and semi-arid areas due to global climate change, inadequate irrigation practices, and deforestation (9, 22, 26). Arid and semi-arid areas with sodic saline soils present high concentrations of NaCl, Na_2SO_4, or Na_2CO_3 (30). Given that NaCl is the most abundant salt in these soils, it has starred numerous studies on plant physiological responses to salt stress (31). However, sulfates strongly affect several countries like Canada, USA, Mexico, Australia and central Argentina (10, 15).

Neltuma ruscifolia (ex *Prosopis ruscifolia*) is a colonizing species with many ecotypes, from shrubby forms to 16m tall trees. It is distributed in the southeast of Bolivia, west of Paraguay, north and center of Argentina, and the extreme south of the State of Mato Grosso do Sul in Brazil (14, 34). Its wood is used to manufacture poles and tool handles, and its fruits are suitable as fodder and for human consumption (14).

N. ruscifolia tolerance to NaCl has been previously studied (18). *N. ruscifolia* seedlings can develop up to concentrations of 400 mmol L^{-1} NaCl (equivalent to seawater), showing higher aerial and root biomass than the control. Higher concentrations caused seedling death after seven days. This high tolerance to NaCl was attributed to canopy exclusion abilities, compartmentalizing the salt in the roots, and to the activity of antioxidant enzymes (17). However, the species tolerance to Na_2SO_4 and its associated physiological responses are unknown.

Salinity can inhibit plant growth through osmotic or ion-specific effects. It can produce nutrient imbalance, alterations in endogenous levels of phytohormones and oxidative damage (37). Greater sensitivity to Na_2SO_4 than to NaCl has been reported in *Prosopis strombulifera*, a species phylogenetically related to *N. ruscifolia* (27). This response was correlated with decreased K, Ca, P, and Mg leaf concentrations and damaged photosynthetic apparatus (27, 30). SO_4^{-2} increased the concentrations of abscisic and salicylic acids and the activity of antioxidant enzymes (10). The accumulation of abscisic acid would have a protective role against dehydration, whereas salicylic acid would signal SO_4^{-2} damage.

This study aimed to determine the effect of Na_2SO_4 salt stress on physiological and biochemical responses in *Neltuma ruscifolia*. We hypothesize that *N. ruscifolia* tolerates high Na_2SO_4 concentrations. Affected ion homeostasis and photosynthesis determine growth thresholds. Hormonal (increased abscisic and salicylic acid concentrations) and antioxidant responses seem to contribute to Na_2SO_4 tolerance in this species.

Materials and methods

Study site

N. ruscifolia seeds were harvested in January 2022 in Maco village, Santiago del Estero (27°51'20" S - 64°13'27" W). The region has a subtropical climate with a dry season and absolute maximum and minimum temperatures of 45°C and -10°C, respectively (5). In this region, *N. ruscifolia* forms secondary forests (vinalares), naturally distributed in flooding areas of the Dulce and Salado rivers and on the margins of salt marshes (7). Soils originate from loessial silts and are saline-sodic, with a higher proportion of sodium chlorides and sulfates (12).

Plant material

Seeds were scarified in concentrated sulfuric acid for 10 min and rinsed with running water for 30 min. Then, they were sown with paper towels, watered with 25% Hoagland nutrient solution, and incubated in a growth chamber at 26°C and 12 h photoperiod. The seedlings were grown hydroponically in 15 L containers with 25% Hoagland nutrient solution (12 seedlings per container). The pH was adjusted daily to 6.5 by adding HCl or KOH 1N. The trial was conducted under greenhouse conditions, with 26°C and 6 MJ m^{-2} solar irradiance. After six months of age, Na_2SO_4 was added, initiated by pulses of 50 mmol L^{-1} every 24 h, until concentrations of 50, 100, 150, 200, 250, or 300 mmol L^{-1} Na_2SO_4 were achieved. The control consisted of a 25% Hoagland nutrient solution. After 60 days of cultivation, gas exchange was measured, and roots and aerial parts were separated and dried at 60°C in a forced ventilation oven for biomass and mineral composition determinations. Cysteine, antioxidant enzymes, and phytohormones were determined with leaf samples.

Mineral composition

Plant material was ground in a Wiley-type mill and sieved through a 40-mesh screen. Subsequently, digestion was carried out with nitric acid and perchloric acid (87:13 v/v). Mineral composition was determined by inductively coupled plasma mass spectrometry. Results were expressed as mg g DW^{-1} (8).

Cysteine determinations

Leaves were homogenized in a mortar with liquid nitrogen and HCl 0.1 N. The homogenate was centrifuged at 15,000 g for 30 min at 5°C. Cysteine concentration was quantified in the supernatant according to Riemenschneider *et al.* (2005) and expressed as nmol g FW^{-1}.

Gas exchange

Gas exchange measurements were performed using an infrared gas analyzer in an open system (IRGA-LCpro+ System ADC, BioScientific Ltd.), with an Arabidopsis leaf chamber, under conditions of saturating artificial light (1000 µmol m^{-2} s^{-1}) and ambient CO_2 concentration. We measured net photosynthesis (A), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and transpiration (E).

Enzymatic determinations

Leaves were homogenized in a mortar with liquid nitrogen and 25 mM HEPES buffer pH 7.8, containing 0.2 mM Na_2EDTA, 2 mM ascorbate, and 2% (m/v) polyvinylpyrrolidone. The homogenate was centrifuged at 12,000 g and 4°C for 20 min. Subsequently, the supernatant was separated, and the soluble protein concentration was determined according to Bradford (1976). In this supernatant, enzymatic activities were also quantified.

Superoxide dismutase activity (SOD, EC1.15.11) was quantified according to Giannopolitis and Ries (1977). The reaction mixture had 100 mM phosphate buffer pH 7.4, 1 mM EDTA, 10 mM methionine, 50 µM riboflavin, and 75µM NBT. After inciting 15 min under a 15-W fluorescent tube, absorbance was read at 560 nm in spectrophotometer. One unit of SOD consisted of the amount of enzyme required to inhibit half the photoreduction of nitro blue tetrazolium chloride. SOD activity was expressed as U mg^{-1} protein min^{-1}.

Ascorbate peroxidase activity (APX, EC1.11.11) was determined according to Nakano and Asada (1981). The reaction mixture contained 50 mM phosphate buffer pH 7.5, 100 µl

of each EDTA, ascorbate, enzyme, and H_2O_2. Absorbance was recorded at 290 nm for 2 min, and an extinction coefficient of 2.8 mM^{-1} cm^{-1} was used for calculation. APX activity was expressed as µmol ascorbate mg^{-1} protein min^{-1}.

Abscisic (ABA) and Salicylic Acids (SA)

Phytohormones were extracted and quantified according to Durgbanshi *et al.* (2005). Lyophilized leaves (50 mg DW) were homogenized in a mortar with liquid nitrogen and 3 mL ultrapure water. Next, 25 µL of a mixture of standards containing 100 ng [$^2H_6$] ABA and 100 ng [$^{13}C_6$] SA was added and centrifuged at 8,000 g for 15 min. The supernatant was partitioned with diethyl ether, and the organic phase was evaporated in a vacuum at 37°C. Dried extracts were resuspended in 1 mL methanol.

Thirty µL of this solution were directly injected in the Ultra Performance Liquid Chromatography (UPLC) system coupled to a Triple Quadrupole Mass Spectrometer (TQD Mass Spectrometer coupled to an Acquity LC, Waters Milford, MA, USA) through an orthogonal Z-spray electrospray interface. Separation was performed with a reverse phase C18 column (Gravity, 50 × 2.1 mm 1.8 µm particle size, Macherey-Nagel GmbH, Germany), using a methanol: water gradient, both supplemented with 0.1% acetic acid at a flow rate of 0.3 mL min^{-1} (11). Calibration curves were constructed using known amounts of pure standard samples to determine phytohormone concentrations. ABA and SA concentrations were expressed in µg g DW^{-1}.

Experimental design and statistical analysis

A completely randomized experimental design with five replications was used. The experimental unit was represented by a 15 L container with 12 seedlings. After checking homoscedasticity and normality, the results were analyzed using ANOVA and the Tukey test.

Results and discussion

Seedlings could grow up to salt concentrations of 200 mmol L^{-1} Na_2SO_4 (figure 1). Higher salt concentrations resulted in chlorosis and seedling death before the end of the trial. Aerial growth was more sensitive than root growth. Aerial growth was reduced at 100 mmol L^{-1} Na_2SO_4, while root growth was reduced at 150 mmol L^{-1} Na_2SO_4. These results differ from those reported for the species in NaCl, in which growth threshold was 400 mmol L^{-1} NaCl (18).

Vertical bars represent mean standard deviation (n=5). For each organ, different letters indicate significant differences according to the Tukey test at 5%.

Las barras verticales representan la desviación estándar de la media (n=5). Para cada órgano, letras diferentes indican diferencias significativas según el test de Tukey al 5%.

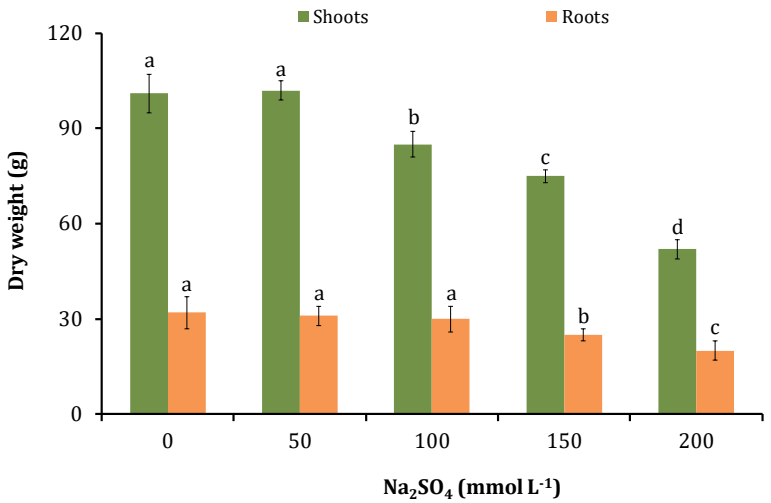


Figure 1. Dry matter of aerial part and roots of *Neltuma ruscifolia* seedlings grown hydroponically in increasing concentrations of Na_2SO_4.

Figura 1. Materia seca de parte aérea y raíces de plántulas de *Neltuma ruscifolia* cultivadas hidropónicamente en concentraciones crecientes de Na_2SO_4.

Neltuma ruscifolia excluded Na from the aerial part up to 100 mmol L^{-1} Na_2SO_4, compartmentalizing in the roots (table 1). However, SO_4 accumulated in leaves in all saline treatments.

Table 1. Mineral composition (mg g DW^{-1}) in leaves and roots of *N. ruscifolia* seedlings grown in increasing concentrations of Na_2SO_4.

Tabla 1. Composición mineral (mg g MS^{-1}) en hojas y raíces de plántulas de *N. ruscifolia* cultivadas en concentraciones crecientes de Na_2SO_4.

Na_2SO_4	Na	S	Mg	Ca	K
Leaves					
0	2.93 ± 0.51 a	2.62 ± 0.15 a	0.71 ± 0.23 a	9.21 ± 2.10 a	23.42 ± 5.32 a
50	3.45 ± 0.63 a	5.48 ± 0.57 b	0.64 ± 0.15 a	8.50 ± 1.43 a	24.11 ± 6.20 a
100	3.15 ± 0.71 a	8.11 ± 1.19 c	0.68 ± 0.09 a	8.92 ± 2.67 a	21.85 ± 4.73 a
150	5.01 ± 2.24 b	10.23 ± 1.82 d	0.47 ± 0.11 b	6.34 ± 1.93 b	14.35 ± 3.89 b
200	11.92 ± 3.40 c	11.54 ± 2.30 e	0.39 ± 0.07 c	4.11 ± 0.59 c	11.68 ± 1.54 c
Roots					
0	4.18 ± 0.37 a	1.71 ± 0.21 a	0.91 ± 0.12 a	8.73 ± 0.53 a	29.31 ± 0.98 a
50	6.17 ± 0.42 b	2.58 ± 0.17 b	0.61 ± 0.09 b	5.36 ± 1.08 b	26.83 ± 0.53 b
100	9.08 ± 0.25 c	3.75 ± 0.43 c	0.54 ± 0.02 c	4.04 ± 0.36 c	22.12 ± 0.73 c
150	15.69 ± 1.98 d	4.99 ± 0.33 d	0.39 ± 0.09 d	2.99 ± 0.66 d	14.56 ± 1.08 d
200	20.51 ± 3.10 e	6.84 ± 0.52 e	0.21 ± 0.07 e	1.27 ± 0.24 e	11.87 ± 0.69 e

Different letters indicate significant differences among treatments according to the Tukey test at 5%.

Letras diferentes indican diferencias significativas entre tratamientos según el test de Tukey al 5%.

Plant tolerance to salt stress depends on the species and the type of salt. In *Brassica rapa* (31) and *Aeluropus littoralis* (4), Na_2SO_4 was more toxic than NaCl. The opposite was observed in *Oryza sativa* (15) and *Chenopodium quinoa* (25). In *Allium cepa*, both salts had the same inhibitory effect on growth (1). Reginato *et al.* (2014) reported that *Strombocarpa strombulifera* (ex *Prosopis strombulifera*), a shrub species distributed from the Arizona desert to Argentine Patagonia, behaved as a halophyte to NaCl but was quite sensitive to Na_2SO_4.

It has been reported that Neltuma species respond to salinity through anatomical modifications that allow minimizing detrimental effects. Bravo *et al.* (2016) reported that stress increases the number of root cortex cell strata and decreases the diameter of xylem vessels in *N. ruscifolia*. The increased number of cortex cells suggests a greater capacity for toxic ion storage. Decreasing diameter of xylem vessels increase cavitation resistance at low water potentials in saline soils. In *Strombocarpa strombulifera*, salt stress produces suberization and early endodermis lignification, contributing to ionic entrance control in the root (26).

The increase in Na concentrations in the aerial part and roots was accompanied by a reduction in Mg, Ca, and K concentrations (table 1). These results agree with Reginato *et al.* (2019) in *S. strombulifera,* in which Na_2SO_4 produced a significant decrease in foliar Ca and Mg concentrations, correlating with growth inhibition and senescence. According to Ahmadizadeh *et al.* (2016), the accumulation of Na and SO_4^{2-} causes ionic imbalance and compromises uptake of other essential nutrients, such as K and Ca. The antagonism between Ca and Na ions has been attributed to the nonselective cation channels in cell membranes (NSCC), allowing the entry of both cations without discriminating one from the other (27, 32).

All salt treatments increased foliar cysteine concentrations (figure 2, page XXX). Thus, in the 200 mmol L^{-1} Na_2SO_4 treatment, cysteine concentrations were six times higher than in the control.

Vertical bars represent mean standard deviation (n=5).

Different letters indicate significant differences according to the Tukey test at 5%.

Las barras verticales representan la desviación estándar de la media (n=5).

Letras diferentes indican diferencias significativas según el test de Tukey al 5%.

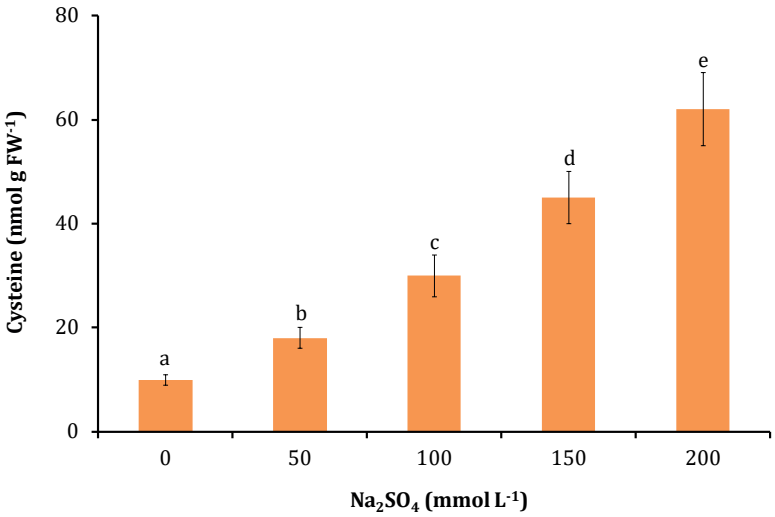


Figure 2. Cysteine concentration in leaves of *N. ruscifolia* seedlings grown in increasing concentrations of Na_2SO_4.

Figura 2. Concentración de cisteína en hojas de plántulas de *N. ruscifolia* cultivadas en concentraciones crecientes de Na_2SO_4.

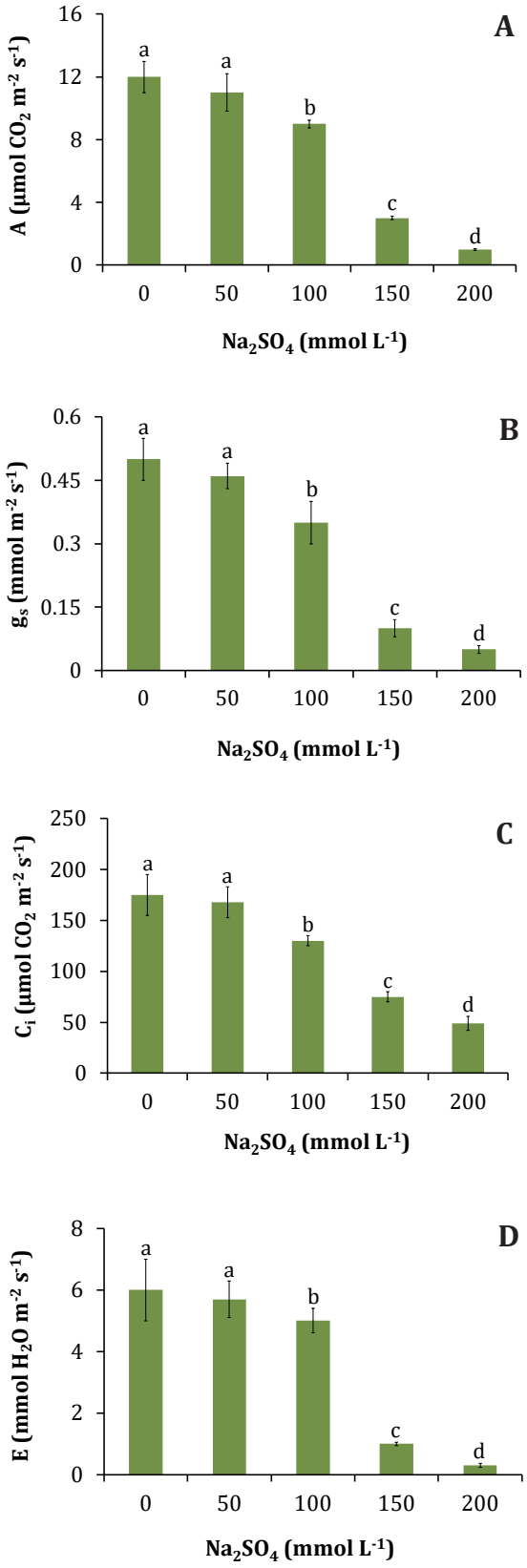
Sulfate is stored in the vacuoles of root and xylem parenchyma cells or transported via xylem to the aerial part (Takahashi *et al.*, 2011). Once in leaves, sulfate is again stored in vacuoles or reduced to sulfite in chloroplasts. Sulfite can be involved in cysteine synthesis in a reaction catalyzed by cysteine synthase. This mechanism incorporates sulfite into organic compounds, avoiding its inhibitory effect on mitochondrial respiration. Cysteine is a highly reactive thiol and helps maintain redox homeostasis in plants subjected to different abiotic stresses (39). Reginato *et al.* (2019) also reported increased foliar cysteine concentrations in *S. strobulifera* under Na_2SO_4 stress.

Salinity inhibited photosynthesis and stomatal conductance at 100 mmol L^{-1} Na_2SO_4 (figure 3A, B, page XXX), causing internal CO_2 concentration (figure 3C) and transpiration (figure 3D, page XXX) to decrease.

Photosynthesis can be inhibited by salinity due to stomatal and nonstomatal limitations. The latter include inhibition of Rubisco activity, decreased photosynthetic pigment concentration, and alterations at photochemical level (17). In the present case, salt stress simultaneously reduced net photosynthesis, stomatal conductance, and internal CO_2 concentration. Therefore, in this study, photosynthesis inhibition can be attributed to stomatal closure. In agreement with these results, in *Neltuma alba* the photochemical stage of photosynthesis was only inhibited by concentrations over 400 mM NaCl (20). In *S. strombulifera*, photosynthesis response to salt stress depends on the type of salt. Thus, whereas NaCl did not affect maximum quantum yield of photosystem II, Na_2SO_4 produced a significant reduction of this variable, indicating photoinhibition (29).

Concentrations over 50 mmol L^{-1} Na_2SO_4 produced a significant increase in foliar ABA (figure 4A, page XXX). In the 200 mmol L^{-1} Na_2SO_4 treatment, ABA concentration was 100% higher than the control.

Phytohormones play a key role in plant physiological responses under stress conditions. In saline stress, ABA gains particular ecophysiological importance as it causes stomatal closure and reduces transpiration (35). Under water and salt stress, ABA works as a signal from the root to the aerial part, increasing water economy through stomatal closure and reduced leaf expansion (38). In *S. strombulifera*, Na_2SO_4 significantly increases foliar ABA concentrations (10), suggesting that ABA accumulation in leaves would be protective against dehydration.



Vertical bars represent mean standard deviation (n=5). Different letters indicate significant differences according to the Tukey test at 5%.

Las barras verticales representan la desviación estándar de la media (n=5). Letras diferentes indican diferencias significativas según el test de Tukey al 5%.

Figure 3. Net photosynthesis (A), stomatal conductance (B), internal CO_2 concentration (C), and transpiration (D) in *N. ruscifolia* seedlings grown in increasing concentrations of Na_2SO_4.

Figura 3. Fotosíntesis neta (A), conductancia estomática (B), concentración interna de CO_2 (C) y transpiración (D) en plántulas de *N. ruscifolia* cultivadas en concentraciones crecientes de Na_2SO_4.

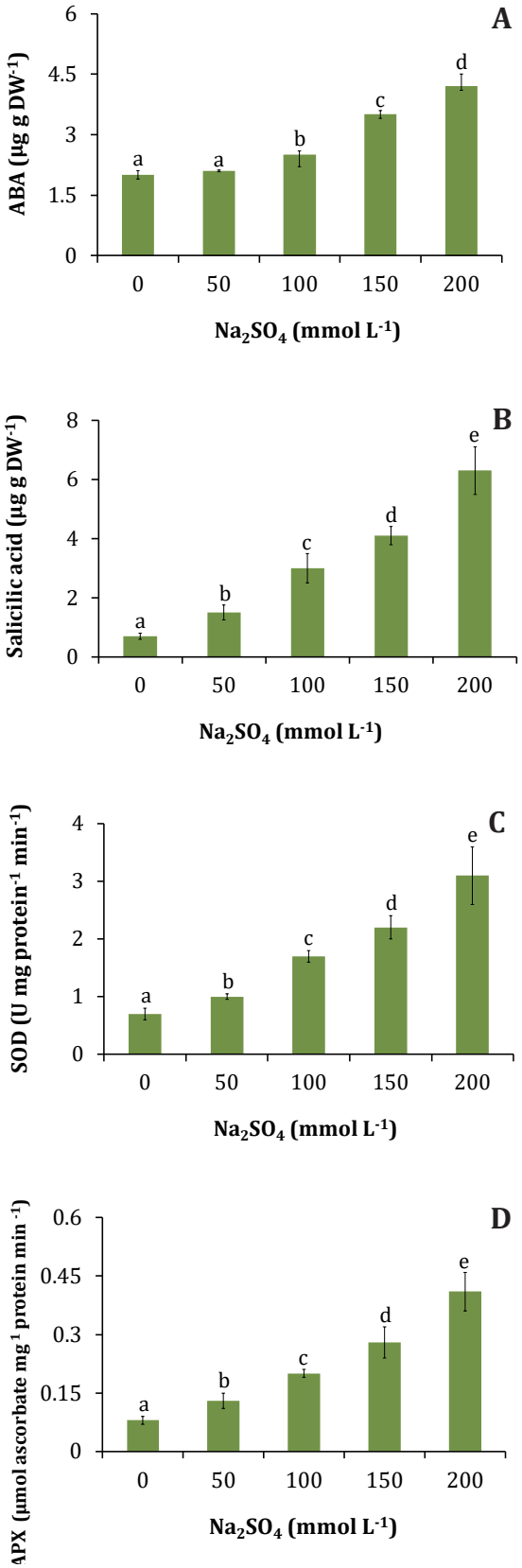


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Figure 4. Abscisic acid (A), salicylic acid (B), superoxide dismutase activity (C), and ascorbate peroxidase activity (D) in *N. ruscifolia* seedlings grown in increasing concentrations of Na_2SO_4.

Figura 4. Concentración de ácido abscísico (A), concentración de ácido salicílico (B), actividad superóxido dismutasa (C) y actividad peroxidasa (D) en plántulas de *N. ruscifolia* cultivadas en concentraciones crecientes de Na_2SO_4.

All salt concentrations significantly increased foliar SA concentrations (figure 4B, page XXX). Discrepancies exist regarding the role of SA in regulating plant tolerance to salt stress. In *Vigna angularis* seedlings, SA mitigated the inhibitory effect of salt stress on photosynthesis and growth. This response was associated with increased tissue antioxidant activity (2). In contrast, SA was not involved in NaCl tolerance of the halophytes *Lycium humile* and *S. strombulifera*, nor was it identified as a stress signal (10, 24).

It has been suggested that SA induce antioxidant enzymes or alter the expression of their genes (21). In agreement with that observation, all salt concentrations increased the activities of SOD and APX enzymes (figure 4 C, D, page XXX). However, according to Miura *et al.* (2013), low SA concentrations improve antioxidant capacity, whereas high concentrations produce oxidative stress and cell death. In agreement with these results, the high tolerance of *N. ruscifolia* to NaCl was attributed in part to its high antioxidant capacity due to the activities of SOD and peroxidase enzymes and polyphenols (19). Reginato *et al.* (2021) also reported significant increases in SOD and APX activities in leaves of *S. strombulifera* seedlings subjected to salt stress with Na_2SO_4.

Conclusions

N. ruscifolia growth threshold is 200 mmol L^{-1} Na_2SO_4. Growth inhibition can be attributed to affected ionic homeostasis and inhibition of photosynthesis due to stomatal closure. Nevertheless, the species shows adaptive responses to this salt. Thus, stomatal closure associated with increased foliar ABA concentrations contributes to water economy. Cysteine synthesis reduces sulfate toxicity when absorbed by the roots. On the other hand, salt stress induces SA accumulation in leaves and increases antioxidant activity, preventing oxidative stress. These characteristics demonstrate the high potential of the species for afforestation schemes on sodic saline soils.

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