Effects of salt stress on germination, seedling growth, osmotic adjustment, and chlorophyll fluorescence in *Prosopis alba* G.

Efectos del estrés salino sobre la germinación, crecimiento de plántulas, ajuste osmótico y fluorescencia de la clorofila en *Prosopis alba* G.

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Abstract

Prosopis alba G. is a species of high forest importance in the phytogeographical region of Western Chaco. Although *P. alba* has been considered as salinity tolerant, its salinity thresholds for germination and seedling growth are unknown, as well as the physiological mechanisms involved in them. The aim of this study was to elucidate the mechanisms of tolerance of *P. alba* to salt stress. Seed germination, seedling growth, osmotic adjustment, and chlorophyll fluorescence were analyzed. Germination was more tolerant to salinity than seedling growth, with thresholds of 600 mM and 500 mM, respectively. The species showed a high capability of osmotic adjustment, with values near to those observed in halophytes. The photochemical phase of photosynthesis was highly tolerant to saline stress, showing photoinhibition from 400 mM NaCl, as indicated by the fluorescence variables of chlorophyll. This behavior was associated to an increase in anthocyanin concentrations in the leaves.

Keywords
Germination • seedling growth • osmotic adjustment • chlorophyll fluorescence

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Resumen

Prosopis alba G es una especie de importancia forestal en la Región fitogeográfica del Chaco Occidental. Aunque *P. alba* ha sido mencionada como tolerante a la salinidad, no se conocen los umbrales de salinidad para la germinación y el crecimiento de plántulas, ni los procesos fisiológicos involucrados. El objetivo de este trabajo fue dilucidar los mecanismos de tolerancia de *P. alba* al estrés salino. Se estudió la germinación de semillas, el crecimiento de plántulas, ajuste osmótico y fluorescencia de la clorofila. La germinación fue más tolerante a la salinidad que el crecimiento de plántulas, con umbrales de 600 y 500 mM, respectivamente. La especie mostró una alta capacidad de ajuste osmótico, con valores cercanos a los observados en halófitas. La etapa fotoquímica de la fotosíntesis fue altamente tolerante al estrés salino, mostrando fotoinhibición a partir de 400 mM de NaCl, tal como lo indicaron las variables de fluorescencia de la clorofila. Este comportamiento estuvo asociado a un incremento en la concentración de antocianinas en hojas.

Palabras clave

Germinación • crecimiento de plántulas • ajuste osmótico • fluorescencia de la clorofila

Introduction

Prosopis alba G. is a tree species of forest importance in the phytogeographical region of Western Chaco. It is used for furniture and carpentry, and its fruits are consumed by people and cattle. It inhabits the humid savanna and the most humid low lands with sandy soils, forming belts around saline depressions (7).

Germination and seedling establishment constitute the most critical periods among the physiological processes in the life cycle of the plants that grow in arid regions; therefore, the presence of adaptations at these ontogenetic stages can determine their natural distribution (10, 32). Salinity can inhibit germination and seedling growth by preventing water absorption due to either the low water potential of the soil solution or the toxic effect of ions (31).

Among the terrestrial nitrogen-fixing plants, the genus *Prosopis* presents high tolerance to salinity. *P. juliflora* (5), *P. alpataco,* and *P. Argentina* (33) have shown the capability to grow in salinities at about 500 mM NaCl. Velarde *et al*. (2003) compared the survival of 27 *Prosopis* families (seed from individual mother trees) as a function of salinity from 10 to 45 dSm^{-1}. The *P. pallida* families had significantly greater mean survival than *P. alba* at seawater salinities of 45 dSm-1 (61.1% *vs.* 41.7%).

Osmotic adjustment (OA) has been considered an important physiological adaptation associated with tolerance to salt stress and has received much attention in recent years (11). It is the accumulation of osmotically active solutes (proline, glycine betaine, sugar alcohols, etc.) that reduce the water potential of tissues, maintaining a gradient with the soil solution to allow water absorption. This strategy enables the maintenance of cell turgor and the physiological processes that depend on it, such as growth (19).

Stored solutes and its contribution to OA vary widely among species. For instance, in *Prosopis strombulifera* the degree of tolerance to salt stress correlates with the concentration of proline, polyols, and ions, which also contribute to OA (17).

Saline stress affects photosynthesis either directly or indirectly. In order to understand the physiological status in higher plants and determine photosynthetic damage affected by environmental stresses, chlorophyll fluorescence assay is a rapid and sensitive measure of photosynthetic competence (1).

The aim of this study was to contribute with information on the mechanisms of tolerance of *P. alba* to salt stress. The working hypotheses were that *P. alba* tolerates saline concentrations close to seawater (500 mM NaCl), performs osmotic adjustment, and exhibits a photochemical stage of stable photosynthesis at high saline concentrations.

Materials and Methods

Plant Material

The fruits were harvested randomly within a *P. alba* forest developed outside temporary pools in the town of Maco (27° 51'20 "S, 64° 13'27" W), Santiago del Estero Province, Argentina. The seeds were manually extracted from the fruit and scarified by removing a small portion of the coat at the cotyledon end with nail clippers.

Germination

Four lots of twenty-five seeds were placed to germinate between paper towels moistened with 10 ml of NaCl solutions containing 0 (control), 100, 200, 300, 400, 500, 600, and 700 mM. Germination towels were rolled, covered with polythene bags to minimize water loss by evaporation, and placed vertically in a growth chamber at 25 °C with a 12- hour photoperiod (22). The seeds that emerged in the cotyledons were considered germinated. Both the percentage of germination and the mean germination time (MGT) were calculated according to Nichols and Heidecker (1996).

Growth

Seeds were placed to germinate under the conditions described in the previous item. Subsequently, 2-week-old seedlings were hydroponically cultivated in 5-liter containers with 25% Hoagland nutritive solution with the addition of 0 (control), 100, 200, 300, 400, 500, or 600 mM NaCl (20 seedlings per container).

The trial was conducted in a growth chamber (25°C, with a 12-hour photoperiod). Transpiration losses were offset by the addition of distilled water, keeping the level of solution in the containers constant.

After reaching the highest salinity, seedlings were kept under the same conditions for seven days. In that material, dry matter was determined, and the variables associated with water relations and chemical determinations were measured.

Water relations and quantification of inorganic and organic solutes

Water potential (Ψ_W), relative water content (RCW), and osmotic potential (Ψ_S), were determined using the methodology described by Silva *et al*. (2010). Pressure potential (Ψ_P) was calculated using the difference between Ψ_W and Ψ_S.

It was determined OA as the difference in osmotic potential at full turgor between control ($\Psi_{SC}{}^{100}$) and salt stress ($\Psi_{SS}{}^{100}$) conditions (3). It was quantified Na^+ and K^+ concentrations by flame spectrophotometry and determined Cl^- concentration bytitration with $AgNO_3$ (18).

Proline, soluble sugars, glycine betaine, and anthocyanin were extracted and spectrophotometrically quantified according to techniques described by Bates *et al*. (1973), Dubois *et al*. (1956), Grieve and Grattan (1983), and Krizek *et al.* (2016), respectively.

Each solute concentration was expressed as mmol of solute per kg of water in the tissue. Relative contribution (RC) of every solute to Ψ_S was estimated as a percentage of osmolarity and calculated by the following rate: RC=solute concentration (mmol kg^{-1} water)/solvent osmolarity (mmol kg^{-1} solvent), according to Silva *et al.* (2010).

Chlorophyll fluorescence parameters

Chlorophyll fluorescence parameters were assessed using a portable photosynthesis meter. Minimal fluorescence (F_o), was measured in 30-min dark-adapted leaves, whereas maximal fluorescence (F_m), was measured in the same leaves in full light-adapted conditions. Maximal variable fluorescence ($F_v = F_m - F_0$) and the photochemical efficiency of PSII (F_v/F_m) for dark-adapted leaves were also calculated from the measured parameters (21). In light-adapted leaves (for 15 min), steady state fluorescence yield (F_s'), maximal fluorescence (F_m') after 0.8 s saturating white light pulse, and minimal fluorescence (F_0') were measured when actinic light was turned off; further calculation was made by using the equation $F_0' = F_0/(F_v/F_m + F_0/F_m')$ (28).

Non-photochemical quenching (NPQ) value due to the dissipation of absorbed light energy was determined at each saturating pulse in accordance with the equation $NPQ = (F_m - F_m')/F_m'$. The coefficient for photochemical quenching, qP, which represents the fraction of open PSII reaction centre, was calculated as $qP = (F_m' - F_s')/(F_m' - F_0')$ (21).

Photochemical efficiency of photosystem II (Φ_{PSII}) was calculated as followed (11): $\Phi_{PSII} = (F_m' - F_s')/F_m'$.

Experimental design and statistical analysis

Each experiment was repeated at least twice. An experimental completely randomized design with four repetitions was used. For the germination tests, the experimental unit consisted of paper towels containing twenty-five seeds (four rolls of twenty-five seeds per treatment). As regards growth, water relations, and chlorophyll fluorescence tests, the experimental unit consisted of a 5-liter container accomodating twenty seedlings (four recipients per treatment). The plant physiological parameters were recorded from fully expanded second or third youngest leaves.

The germination and growth results did not meet the assumptions of ANOVA, which were analyzed with the nonparametric test Kruskal-Wallis at 0.05 confidence level. The results of water relations and chlorophyll fluorescence parameters were subjected to ANOVA, and means were compared using the Tukey test al 0.05 confidence level.

Results

P. alba showed great tolerance to salinity during the germination stage (table 1, page 73). The percentage of germination was not affected by concentrations of 100, 200, 300, and 400 mM NaCl, compared with the control maintained in the order of 98%. At 500 mM NaCl, there was a small but significant decrease, reaching an average of 86%. At 600 mM, a significant inhibition of germination with an average of 12% was recorded.

Table 1. Percentage of germination, mean germination time (MGT), root and shoot dry weight, and root/shoot ratio in seeds and seedlings of *P. alba* incubated in NaCl solutions[1].

Tabla 1. Porcentaje de germinación, tiempo medio de germinación (MGT), peso seco de raíces y parte aérea, y relación raíces/parte aérea en semillas y plántulas de *P. alba* incubadas en soluciones de NaCl[1].

NaCl (mM)	Germination (%)	MGT	Dry Weight (mg)		Root/Shoot
			Root	Shoot	
0	98.12 a	4.07 a	22.71 a	76.23 a	0.29 a
100	95.08 a	4.14 a	21.53 a	74.90 a	0.28 a
200	97.14 a	4.19 a	22.10 a	75.31 a	0.29 a
300	94.32 a	4.02 a	21.44 a	76.27 a	0.28 a
400	91.25 a	5,13 b	18.32 b	45.18 b	0.40 b
500	86.07 b	5.06 b	17.47 c	34.60 c	0.50 c
600	12.17 c	9.01 c	--	--	--

[1] For each variable, different letters indicate significant differences by Kruskal-Wallis test ($P < 0.05$).

[1] Para cada variable, letras diferentes indican diferencias significativas por el test de Kruskal-Wallis ($P < 0,05$).

At higher concentrations of NaCl, germination was completely inhibited and, though radicles emerged, cotyledons did not. Therefore, the threshold of the species in the germination stage was 600 mM NaCl.

The average germination time (TMG) was more sensitive to NaCl than the final percentage of germination, registering an increase from 400 mM NaCl, probably due to a slower imbibition of seeds as a result of the low water potential in the incubation solution.

The growth of hydroponically grown seedlings was more sensitive to salt stress than the germination process (table 1), and a different behavior was observed in shoots and roots, respectively. At 400 mM NaCl, aboveground biomass decreased 20% compared with the control, and 46% at 500 mM. On the other hand, at 400 mM NaCl, root biomass decreased 24 % compared with the control.

As a result, a significant increase was observed in the root/shoot ratio from 400 mM NaCl.

Seedlings grown at 600 mM NaCl showed chlorosis and necrosis, dying before concluding the test. Therefore, the threshold for seedling growth white carob is 500 mM NaCl.

The water potential of seedlings decreased gradually as the salt concentration in the nutrient solution increased, reaching values of -0.6 MPa in the control and -3 MPa in plants grown at 500 mM NaCl (table 2, page 74). A similar trend was observed in the osmotic potential, with maximum values of -1.5 MPa in the control and -3.9 MPa at 500 mM NaCl. As a result, the seedlings were able to maintain cell turgor, with potential values of 0.9 MPa pressure.

The maintenance of cell turgor and WRC values (table 2, page 74) reinforces the hypothesis of the presence of OA mechanism as a response to salt stress. In agreement with these results, OA values calculated as the difference between Ψ_{SC}^{100} and Ψ_{SS}^{100} increased as the WNaCl concentration increased (table 2, page 74). It was observed OA high values of 1.42 MPa at the highest level of salinity.

Table 2. Water potential (Ψ_w), osmotic potential (Ψ_S), pressure potential (Ψ_p), water relative content (WRC), and osmotic adjustment (OA) in seedlings of *P. alba* incubated in NaCl solutions[1].

Tabla 2. Potencial hídrico (Ψ_w), potencial osmótico (Ψ_S), potencial de presión (Ψ_p), contenido relativo de agua (WRC) y ajuste osmótico (OA) en plántulas de *P. alba* incubadas en soluciones de NaCl[1].

NaCl (mM)	Ψ_W (MPa)	Ψ_S (MPa)	Ψ_p (MPa)	WRC (%)	OA (MPa)
0	- 0.6 a	- 1.5 a	0.9 a	74.2 a	--
100	- 0.9 b	- 1.9 b	1.0 a	72.1 a	0.16 a
200	- 1.4 c	- 2.3 c	0.9 a	73.4 a	0.42 b
300	-1.8 d	- 2.7 d	0.9 a	75.6 a	0.82 c
400	- 2.3 e	- 3.2 e	0.9 a	75.3 a	1.22 d
500	- 3.0 f	-3.9 f	0.9 a	72,7 a	1.42 e

[1] For each variable, different letters indicate significant differences according to Tukey's test ($P < 0.05$).

[1] Para cada variable, letras diferentes indican diferencias significativas de acuerdo con el test de Tukey ($P < 0,05$).

The contribution of K^+ to OA was declining as a result of salt stress, but it was always high, which shows a significant selectivity of this cation relative to sodium (table 3). As the contribution of K^+ decreased, Na^+, Cl^- increased, but the contribution of the cation relative to the anion was always greater (table 3).

As regards organic solutes, the contribution of proline to osmolarity was very low, 0.94% in the control and 0.33% at the highest level of salinity (table 3).

The contribution of soluble sugars to osmolarity decreased as the NaCl concentration in the medium increased. In control, its contribution was 26.78% and, at 500 mM NaCl, it was 10.75%. The opposite was observed with glycine betaine (GB); its contribution tripled at 500 mM NaCl, compared with the control (table 3).

Table 3. Contribution of K^+, Na^+, Cl^-, proline, total soluble sugars (TSS), and glycine betaine (GB) to osmolarity in seedlings of *P. alba* incubated in NaCl solutions[1].

Tabla 3. Contribución de K^+, Na^+, Cl^-, prolina, azúcares solubles totales (TSS) y glicina betaína (GB) a la osmolaridad, en plántulas de *P. alba* incubadas en soluciones de NaCl[1].

NaCl (mM)	K^+ (%)	Na^+ (%)	Cl^- (%)	Proline (%)	TSS (%)	GB (%)
0	40.41 a	1.67 a	0.93 a	0.94 a	26.78 a	1.98 a
100	33.19 b	9.93 b	3.45 b	0.71 a	22.50 b	2.83 a
200	22.48 c	23.15 c	4.27 b	0,59 b	17.48 c	5.26 b
300	16.92 d	28.10 d	4.24 b	0.47 b	14.43 d	6.16 c
400	13.63 e	33.95 e	4.33 b	0.42 b	12.37 e	6.11 c
500	11.60 e	33.08 e	5.63 c	0.33 c	10.75 f	6.80 cd

[1] For each variable, different letters indicate significant differences according to Tukey's test ($P < 0.05$).

[1] Para cada variable, letras diferentes indican diferencias significativas de acuerdo con eltest de Tukey ($P < 0,05$).

Fluorescence variables did not show significant differences in the range from 0 to 300 mM NaCl (table 4). Higher concentrations produced a decrease in F_v/F_m, Φ_{PSII} and qP values, whereas NPQ increased notoriously. The anthocyanin content in leaves was very sensitive to stress, increasing in all the saline concentrations (table 4).

Discussion

P. alba was more tolerant to salinity in the germination stage tha in the seedling growth stage (table 1, page 73). The threshold for germination was similar to the one reported for *Prosopis ruscifolia*, a pioneer halophyte in the phytogeographic region of Western Chaco (22) and higher than *Schinopsis lorentzii,* a glycophyte part of the climax community together with *P. alba*. In *S. lorenzii,* the threshold was 300 mM, with an increase in the MGT from 100 mM NaCl (22).

Concentrations of 400 and 500 mM NaCl not only inhibited biomass production (table 1, page 73), but also increased the root/shoot ratio, resulting in more efficient water and nutrient uptake under saline stress (31).

In Western Chaco, the germination of *P. alba* occurs in summer, when precipitation reduces the salinity levels in the upper soil layer (23). In autumn, when precipitation ceases and the soil becomes dry, *P. alba* seedlings already possess a profound root system reaching the less saline soil layers, with more water available.

Salt stress can alter the physiology of plants either by an osmotic effect, reducing the water potential of the soil solution and thus limiting the water uptake by the roots, or by a specific toxic effect of ions (25).

Cellular adaptive mechanisms, such as OA, and compartmentalization of toxic ions are vital for plant tolerance to salinity (19). Under severe salt stress, *P. alba* was able to reduce its osmotic potential, and thus its water potential, keeping turgor constant (table 2, page 74).

Hence, the growth was not affected as a result of the water deficit caused by the low osmotic potential of the external solution.

The results of this work show that *P. alba* has an efficient adaptive mechanism that allows it to tolerate high levels of salinity through the maintenance of a good water status of the leaves and an effective osmotic adjustment. Table 3 (page 74) shows that he K^+ involvement in osmolarity decreased with salt stress as the Na^+ contribution increased, but it was always higher than the Cl^- contribution.

Table 4. Effects of saline stress on F_v/F_m, NPQ, $\Phi_{PSII,}$ and qP in *P. alba*[1].
Tabla 4. Efectos del estrés salino sobre F_v/F_m, NPQ, Φ_{PSII} and qP en *P. alba*[1].

NaCl (mM)	F_v/F_m	qP	Φ_{PSII}	NPQ	Anthocyanins (mg g^{-1})
0	0.710 a	0.227 a	0.154 a	0.180 a	0.190 a
100	0.709 a	0.210 a	0.148 a	0.184 a	0.220 b
200	0.698 a	0.219 a	0.152 a	0.183 a	0.259 c
300	0.703 a	0.215 a	0.149 a	0.182 a	0.308 d
400	0.651 b	0.163 b	0.112 b	0.229 b	0,341 e
500	0.643 b	0.157 b	0.093 b	0.231 b	0.394 f

[1] Means within a column followed by different letters are different according to Tukey's test ($P < 0.05$).

[1] En cada columna, medias seguidas por letras diferentes difieren de acuerdo con el test de Tukey ($P < 0,05$).

In species that accumulate high concentrations of ions in cells, such as *P. alba* and halophytes, vacuoles play generally a central role in osmotic adjustment (22), allowing the compartmentalization of toxic ions such as Na^+ and Cl^-. Similar results were found by Villagra and Cavagnaro (2005) for *P. alpataco*, another salt-tolerant species.

In the cytoplasm, salinity can induce the accumulation of organic solutes compatible with cellular metabolism that, in addition to participating in the OA, act as protectors of macromolecules (22). Thus, the accumulation of ions in both vacuole and cytoplasm organic molecules allows a balance in the water relations within the cell itself.

From the organic solutes studied in this work, GB was the only one that increased its concentration as a response to increased salinity, reinforcing its contribution to osmolarity (table 3, page 74). If stored in the cytoplasm, it is normally around 10% of the cell volume.

These results differ from those observed in *Prosopis strombulifera* (17), which tolerates up to 700 mM NaCl; it had a significant increase in foliar concentrations of proline and soluble sugars, whereas the concentration of GB remained constant. Moreover, whereas Na^+ played a key role in the OA in *P. alba* when compared with Cl^-, in *P. strombulifera* the opposite was observed (17). These results suggest that *Prosopis* species have different levels of tolerance to salinity, and they have also developed differential physiological adaptations during the speciation process.

The Chlorophyll fluorescence analysis shows that in *P. alba* the photochemical stage of photosynthesis is highly tolerant to salinity, because it was only disturbed at concentrations higher than 300 mM NaCl (table 4, page 75). The F_v/F_m value is the ratio of variable fluorescence to maximal fluorescence and measures the maximum efficiency of PSII (26). This value can be used to estimate the potential efficiency of PSII by considering dark-adapted measurements (8). An increase in F_v/F_m reflects more light use efficiency in plants (16). In addition, an increase in F_v/F_m is also correlated with reduced energy loss as heat. These results are in agreement with Lauer and Ross (2016), who reported that an increase in salinity caused a reduction in F_v/F_m,, indicating that there was more energy dissipated as heat (table 4, page 75).

The qP value approximates the proportion of open PSII reaction centers. In other words, qP represents the energy consumed in the photosynthesis. On the other hand, NPQ is the amount of dissipated excessive irradiation into heat. It represents the effective way in which photosynthetic organisms can accomplish the process of dissipating excessive irradiation into heat (29).

The Φ_{PSII} value gives an estimation of the efficiency. It represents the photochemistry at different photon fluxes. There is an inverse relationship between qP and NPQ as well as between Φ_{PSII} and NPQ (6). In this study, both Φ_{PSII} and qP decreased with an increasing value of NPQ and were in line with the results reported by Hazrati *et al.* (2016). Under favourable conditions, almost all the absorbed light is consumed in photochemical reactions (15). There is a negative correlation between ΦPSII and NPQ (6). In addition, it has been reported that there is a linear relationship between Φ_{PSII} and CO_2 absorption (20). In a study on *Aloe vera*, water deficit stress decreased Φ_{PSII} and qP but increased NPQ (12). In this study, the NPQ value increased with increasing saline stress severity. The higher value of NPQ indicates the ability to mitigate the negative effects of environ-

mental stress at the chloroplast level, as these organelles can dissipate the excess of excitation energy (16). Similar results were found in *P. alba* plants exposed to NaCl stress for four days (24).

It has been shown that in photosynthetic tissues, anthocyanin acts as a "sunscreen", protecting cells from high-light damage by absorbing ultraviolet light, thereby protecting the tissues from photoinhibition. It has also been reported that there is a positive correlation between water stress and anthocyanin biosynthesis (12). All treatments in *P. alba* increased the anthocyanin content in leaves, which is in agreement with the high stability of the photochemical stage of photosynthesis at high salt concentrations (less than 400 mM NaCl).

Conclusions

P. alba tolerates up to 600 mM NaCl during the germination stage and 500mM NaCl for seedling growth. Its high tolerance to salt stress is determined by its ability to adjust osmotically maintaining cell turgor through the accumulation of osmolytes, such as Na^+ and GB.

The growth inhibition of the aerial part observed at the highest salt concentrations (400 and 500 mM NaCl) matches the photoinhibition of photosynthesis, as indicated by a reduced quantum yield of PSII, Φ_{PSII}, and qP, as well as higher NPQ. The high concentration of anthocyanins constitutes a protection mechanism against adverse effects of saline stress.

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