



Research article

Differential effects of NaCl and Na₂SO₄ on the halophyte *Prosopis strombulifera* are explained by different responses of photosynthesis and metabolism

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ABSTRACT

Prosopis strombulifera (Lam.) Benth. is a halophytic shrub found in highly saline soils in Argentina, with high tolerance against NaCl but strong growth inhibition by Na₂SO₄. In the present study, the differences in the physiological responses caused by these salts and an iso-osmotic combination thereof on photosynthesis, mineral composition and metabolism were analyzed. Na₂SO₄ treated plants were the most affected by salinity, showing a significant decrease in several photosynthetic parameters. Proline and cysteine accumulated significantly in the plants in response to salt stress. These results show by the first time that the SO₄²⁻ anion is triggering damage in the photosynthetic apparatus and consequently affecting the photosynthetic process, which may explain the strong growth inhibition in these plants at high salinity. Moreover, the SO₄²⁻ anion provoke challenges in the incorporation of nutrients, decreasing the levels of K, Ca, P and Mg, and inducing a strong antioxidant activity in *P. strombulifera*.

1. Introduction

Soil salinity can reduce crop, horticulture and forage production in arid and semiarid regions. Salinity is becoming more extensive as a result of land clearing, unsustainable irrigation practices and through pressures for bringing marginal land into production (Munns and Gilliam, 2015). Salinity causes hyperosmotic and hyperionic stress in plants, causing disorders in the metabolism. Photosynthesis is the most important metabolic processes in plants and is fundamental for the survival and growth of plants. Its study provides interpretation about the general “health” of plants. Generally, photosynthesis was shown to be adversely affected by salinity and such impairment is mostly due to limitations in photosynthetic capacity, stomatal conductance, uptake of carbon dioxide, and chlorophyll content (Reinoso et al., 2005). However, in some halophytes, photosynthesis has been shown to be unaffected by salinity, or even stimulated at low salt concentrations (Rabhi et al., 2012).

The genus *Prosopis* (Leguminosae, Mimosoideae) involves about 44 woody species distributed in arid and semi-arid regions of America, Africa and Asia. *Prosopis strombulifera* (Lam.) Benth. is a halophytic spiny shrub of less than 1.5 m in height which grows in areas that

spread in salty soils of Peru, north of Chile and Argentina (Burkart, 1976). This species is particularly abundant in high-salinized areas in central Argentina, in which proportions of NaCl and Na₂SO₄ salts are generally similar (Sosa et al., 2005). Although NaCl is one of the most abundant salts in soils, others salts may also be present in high concentrations in some soil types, as Na₂SO₄. However, salt stress studies involving Na₂SO₄ are still scarce and the mechanism of its toxicity in plants is yet poorly understood.

In previous studies, we clearly demonstrated that plant response in *P. strombulifera* varies significantly depending on the anion associated with sodium. We found that *P. strombulifera* presented a contrasting growth response under NaCl, Na₂SO₄ and their iso-osmotic mixture. NaCl treatment (500 mM) caused shoot growth stimulation. On the contrary, Na₂SO₄ salt caused an immediate and significant reduction of shoot height and leaf number per plant. This general growth inhibition was accompanied by senescence symptoms such as chlorosis, necrosis and finally leaf abscission (Reginato et al., 2013, 2014a; 2014b). In addition, an important oxidative damage was induced in tissues when the SO₄²⁻ anion was present in the medium, revealed by a significant increase in H₂O₂ and MDA content. Oxidative damage in *P. strombulifera* was accompanied by accumulation of polyphenols (mainly

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flavonoids) in Na₂SO₄-treated plants, with an antioxidant activity directly correlated with their concentration in leaves and roots. Additionally, the photosynthetic pigment profile was analyzed, showing that *P. strombulifera* has a good ability to tolerate elevated NaCl concentrations with chlorophyll concentration remaining unchanged, while Na₂SO₄ stress significantly reduced chlorophyll concentration, both without changes in carotenoids. In that study, we showed that one of the most effective mechanisms of excess energy dissipation, the de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin through the xanthophyll cycle (VAZ) (Demmig-Adams and Adams, 1992) was carried out by salinized plants of *P. strombulifera*, principally those grown with Na₂SO₄, showing maximal de-epoxidation (DEPS index) (Reginato et al., 2014b). However, the impact of NaCl and Na₂SO₄ on the photosynthetic process in *P. strombulifera* was not analyzed.

The aim of this study was to investigate if the putative mechanisms involved in Na₂SO₄ toxicity in this species are related to damage in the photosynthetic apparatus and alterations in sulfur metabolism.

2. Materials and methods

2.1. Plant materials and growth conditions

Pods of *P. strombulifera* were randomly collected from 100 individual plants within the same population, in south-western San Luis province, Argentina. Peeled seeds were scarified with 98% sulphuric acid for 10 min, washed overnight under running water, rinsed in distilled water, and germinated in a Petri dish over two layers of water-saturated filter paper at 37 °C for 24 h. The germinated seedlings with 20 mm-long radicles were grown in hydroponic conditions in black trays (200 seedlings per each tray of 28 × 22 × 10 cm) with 25% of full-strength Hoagland's solution (Hoagland and Arnon, 1950) (osmotic potential (Ψ_0) = -0.11 MPa) containing: 1 mM MgSO₄, 0.5 mM KH₂PO₄, 2.5 mM KNO₃, 2.5 mM Ca(NO₃)₂, 0.5 μM MnCl₂, 0.04 μM CuSO₄, 0.05 μM ZnSO₄, 0.05 μM H₃BO₃, 0.02 μM (NH₄)₆Mo₇O₂₄, 45 μM EDTA-Fe. The seedlings were self-supported in small holes on the tray cover; the trays were placed in a growth chamber (GroBanks, CLF PlantClimatics GmbH, Wertingen, Germany) under a 16 h light (360 μmol m⁻² s⁻¹) (28 °C): 8 h dark (20 °C) cycle and 70% relative humidity. The pH of the medium was 6 in all cases, and, to provide aeration, an aquarium tubing system with a peristaltic pump was used. The complete experiment was performed consecutively two times (3 trays per treatment each time). Plants were grown hydroponically for 7 weeks (48 days) and allowed to acclimate to the different salt regimes.

2.2. Salt treatment

Salt treatments were initiated after 21 d of seedling growth by adding NaCl and Na₂SO₄ pulses of 50 mmol l⁻¹ and 38 mmol l⁻¹, respectively, for the single-salt treatments, or iso-osmotic mixture for the two-salt treatment, every 48 h until reaching final Ψ_0 values of -1, -1.9, and -2.6 MPa (verified with a vapor pressure osmometer Model 5500, Wescor Inc., Logan, UT, USA), as shown in Table 1. These Ψ_0 values corresponded to plants aged 29, 40, and 48 d, respectively. Control plants remained in 25% Hoagland's solution (Ψ_0 = -0.11 MPa). At each of these time points, roots and leaves of 30 control plants and 30 salt-treated plants were randomly collected from each tray, frozen with liquid N₂, and stored at -80 °C.

2.3. Elemental analysis of plant material

Mineral analysis of leaves and roots was done according to Weese et al. (2015). Lyophilized plant material was ground to fine powder (MM 400 grinder, Retsch GmbH, Haan, Germany). Thirty-eight milligrams dry weight of the ground powder was incinerated for 8 h in a muffle furnace at 480 °C (M104, Thermo Fisher Scientific Corporation, Waltham Massachusetts, USA). After cooling the samples to room

temperature, 1.5 mL 66% aqueous (v/v) nitric acid was added. After 10 min, 13.5 mL of ultra-pure water was added. The solutions were filtered (0.45 μm pore size, Carl Roth, Karlsruhe, Germany) and stored in vials at 4 °C before final analysis. The samples were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) (iCAP 6000 ICP spectrometer, Thermo Fisher Scientific) and the following elements were quantified: Al, B, Ba, Ca, Cr, Fe, K, Mg, Mn, Na, P, S, Sr and Zn.

2.4. Proline quantification

For the determination of proline, the protocol of Carillo and Gibbon (2011) was followed. 50 mg of frozen ground plant material were mixed with 1 mL of 40% ethanol and slowly shaken overnight on an overhead shaker at 4 °C. Samples were centrifuged for 5 min at 18,400 g. Then 125 μL of supernatant were mixed with 250 μL of reaction mix. The reaction mix consisted of ninhydrin 1% (w/v) in glacial acetic acid 60% (v/v) and ethanol 20% (v/v). The samples were incubated in a water bath at 95 °C for 20 min. After cooling to room temperature, 100 μL were transferred into a microtiter plate and read at 520 nm in a microplate reader (Synergy Mx). The results were compared to proline standards ranging from 0.1 to 2 mM.

2.5. Effects of salt stress on photosynthesis

Salt stress impact on photosynthesis was analyzed by chlorophyll fluorescence measurements using an Imaging PAM M series device and ImagingWin v2.32 software (Heinz Walz, Effeltrich, Germany). Measurements (n = 3) were performed when the plants reached -1, -1.9, and -2.6 MPa (these Ψ_0 values corresponded to plants aged 29, 40, and 48 d, respectively) in the morning, 2 h after the beginning of the light period. Light curves using different photosynthetically active radiations (PAR) as recommended in the manufacturer's handbook were examined. The effective PAR values were about 15% lower due to the utilization of the filter plate IMAG-MAX/F. Before the measurement, plants were dark adapted for 25 min. The parameters *Fv/Fm* (maximal PSII quantum yield), *Y (II)* (effective PSII quantum yield), *Y(NPQ)* (quantum yield of regulated energy dissipation), *Y(NO)* (quantum yield of non-regulated energy dissipation), NPQ/4 (nonphotochemical quenching/4) and ETR (electron transport rate) were analyzed (Baker, 2008; Sperdouli and Moustakas, 2012). *Fv/Fm* values were obtained from the false-color images created by Imaging-Win software. ETR values were determined using a mean value of PAR 396–801 μmol quanta m⁻² s⁻¹. The other parameters were analyzed based on the PAR 396 (approximate growth light intensity) and 801 (approximately twice the growth light intensity) results.

2.6. LMW thiols determination

For the determination of GSH, cysteine, the frozen plant material was grinded to a fine powder with a pestle and mortar using liquid nitrogen. Five replicates of 10–20 mg sample were extracted in 1 mL of 0.1 M HCl and centrifuged at 15,000 g for 40 min at 4 °C, where the pellet were re-suspended in a further 1 mL of 0.1 M HCl, centrifuged as before and the two supernatants combined. The supernatant was used in the procedure as detailed by Riemenschneider et al. (2005) to determine the concentration of LMW thiols.

2.7. Oxygen radical absorbance capacity (ORAC) assay

The oxygen radical absorbance capacity (ORAC) assay was based on Huang et al. (2002) and Gillespie et al. (2007) with some modifications. A black 96-well microplate was kept on ice, 120 μL of 112 nM fluorescein in 75 mM phosphate buffer (pH 7.4) was pipetted into each well, followed by 20 μL of standards, sample or blanks. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) standard (0.25–50 mM)

Table 1

Mineral composition of *P. strombulifera* leaves at low (–1 MPa/29 days of culture), moderate (–1.9 MPa/40 days of culture) and high salinity (–2.6 MPa/48 days of culture) that were measured by ICP-OES. Different letters indicate significant differences between treatments according to Tukey test ($P < 0.05$). DM, dry mass.

Days of culture/Osmotic potential	Elements (mg g ⁻¹ DM ± SE)	Control	Salt treatment		
			Na ₂ SO ₄	NaCl	NaCl + Na ₂ SO ₄
29 days/-1 MPa	Ca	9.841 ± 3.511 cd	5.031 ± 2.207 a	8.763 ± 1.252 abcd	10.333 ± 0.365 d
	Fe	0.084 ± 0.036 a	0.050 ± 0.024 a	0.069 ± 0.015 a	0.085 ± 0.010 a
	K	23.556 ± 6.498 a	12.614 ± 5.321 a	11.828 ± 2.968 a	12.462 ± 5.863 a
	Mg	0.726 ± 0.199 cd	0.415 ± 0.012 ab	0.538 ± 0.013 abcd	0.576 ± 0.055 bcd
	Na	3.423 ± 1.823 a	20.372 ± 3.200 cd	15.569 ± 1.772 bc	16.899 ± 2.836 c
	P	5.607 ± 2.755 bc	4.517 ± 1.937 bc	4.426 ± 0.435 bc	5.490 ± 0.226 c
40 days/-1.9 MPa	S	2.596 ± 0.776 b	11.102 ± 3.053 d	2.022 ± 0.290 ab	6.015 ± 0.3177 c
	Ca	18.344 ± 1.144 e	7.165 ± 0.457 abcd	8.515 ± 2.411 bcd	9.002 ± 1.577 bcd
	Fe	0.107 ± 0.014 a	0.052 ± 0.025 a	0.095 ± 0.014 a	0.097 ± 0.044 a
	K	15.413 ± 3.201 a	14.948 ± 2.735 a	7.759 ± 4.421 a	8.524 ± 3.649 a
	Mg	1.025 ± 0.411 d	0.485 ± 0.105 abcd	0.494 ± 0.239 abc	0.580 ± 0.091 bcd
	Na	3.983 ± 1.203 ab	37.149 ± 0.841 f	32.713 ± 11.283 ef	25.883 ± 3.014 cdef
48 days/-2.6 MPa	P	4.072 ± 0.883 bc	2.564 ± 0.111 a	3.197 ± 0.223 ab	3.097 ± 0.319 ab
	S	3.006 ± 0.652 b	27.909 ± 0.557 e	1.246 ± 0.308 a	9.928 ± 1.963 cd
	Ca	16.988 ± 0.305 e	7.038 ± 0.478 abc	6.298 ± 0.451 ab	8.213 ± 0.376 abcd
	Fe	0.072 ± 0.015 a	0.061 ± 0.001 a	0.059 ± 0.011 a	0.076 ± 0.004 a
	K	16.615 ± 4.637 a	9.973 ± 2.580 a	8.526 ± 0.247 a	11.236 ± 1.202 a
	Mg	0.806 ± 0.069 d	0.381 ± 0.038 ab	0.347 ± 0.021 a	0.423 ± 0.037 abc
	Na	1.812 ± 2.135 a	20.383 ± 0.047 cd	30.863 ± 4.017 def	24.391 ± 0.463 cde
	P	3.438 ± 0.205 abc	3.286 ± 0.421 ab	2.123 ± 0.099 a	3.231 ± 0.334 ab
	S	2.423 ± 0.363 b	11.253 ± 0.793 d	1.217 ± 0.019 a	6.921 ± 0.491 cd

was diluted in the identical phosphate buffer. Samples were diluted 1200-fold with phosphate buffer to be within the range of the standard curve. The plate was incubated for 15 min at 37 °C and the fluorescence 485/520 was measured at time point 0. Then, 80 µL of 62 mM 2,2-azobis(2-amidino-propane) dihydrochloride were added to each well and the fluorescence was measured every minute for 80 min in a microplate reader (Synergy Mx, BioTek, Winooski, USA). The difference in absorbance at time point 0 and after 80 min was calculated and quantified using a standard curve.

2.8. Statistical analysis

Data were analyzed using InfoStat software (2011 v Universidad Nacional de Córdoba, Argentina). Two-way general linear model ANOVA was used to determine the effect of each treatment at each osmotic potential. Thus, the factors considered for two-way ANOVA were osmotic potential (Co) (21.0, 21.9 or 22.6 MPa) and salt treatment (control, NaCl, Na₂SO₄ and salt mixture). The data were tested for normality by the Shapiro-Wilk test and for homogeneity of variance by Levene's test. If the ANOVA assumptions were not correct (homogeneity of variance and normal distribution of the residuals), a natural logarithm transformation was applied. Tukey's test was used for comparisons of the means, and the differences were considered significant if p value < 0.05. For cases in which normality and homogeneity of variance were not verified, the non-parametric Kruskal–Wallis test was used.

3. Results

3.1. Elemental analysis of plant material

The elemental composition in leaves (Table 1) and roots (Table 2) was measured at low (–1 MPa/29 d), moderate (–1.9 MPa/40 d) and high salinity (–2.6 MPa/48 d). As expected, there was an increase in Na concentration over time in both leaves and roots correlating with increasing salinity. Although the decrease in K concentration in *P. strombulifera* leaves was not statistically significant in salt treated plants, even at high salinity ($\Psi_o = -2.6$ MPa), the K/Na ratio was certainly reduced in all salt treatments, mainly in NaCl treated plants,

ranging from 0.76 at –1 MPa to 0.27 at –2.6 MPa (Table 3). K root concentration decreased significantly in Na₂SO₄ treated plants at –1.9 MPa. K/Na ratio in roots was also reduced in all salt treatments. Ca leaf concentration decreased significantly in all salt treatments at moderate and high salinity in relation to controls. In roots, Ca concentration were significantly affected in Na₂SO₄ treated plants at –1.9 MPa. Low Na₂SO₄ concentration decreased content in leaves significantly, while at high salinity there was a significant Mg content decrease under all the salt treatments. In roots, Mg concentration was not affected. Respect to sulfur accumulation in leaves of Na₂SO₄ treated plants, values increased significantly from low salinity, reaching a maximal value of 11.253 ± 0.793 mg⁻¹ DW at –2.6 MPa Na₂SO₄ + NaCl treated plants showed lower increases in S concentration in leaves but still significant, reaching 6.921 ± 0.491 mg⁻¹ DW at –2.6 MPa. In roots, S concentrations increased significantly only under those treatments containing Na₂SO₄ at high concentration. On the contrary, S content decreased in NaCl treated plants in relation to control plants. P concentration in leaves decreased significantly in Na₂SO₄ treated plants at moderate salinity. In roots, P concentrations were not affected by any treatment. Fe content in leaves was not affected but there was a significant decrease in Fe concentration in roots at high salinity with all salt treatments (Table 1, Table 2). Concentrations of the micronutrients Al, B, Ba, Cu, Mn, Sr, and Zn were not affected by the different salt treatments (data not shown).

3.2. Effect of salt stress on proline accumulation

In the halophyte *P. strombulifera*, a significant accumulation of proline in leaves and roots was observed in all salt treatments, as salinity increased (Fig. 1). Proline accumulation was much higher in leaves than in roots (42.280 ± 0.357 nmol mg⁻¹ FW as maximal value in leaves vs. 27.309 ± 0.405 nmol mg⁻¹ FW in roots). In both, leaves and roots, proline content was significantly higher in those plants treated with low salt concentration being greater with Na₂SO₄. At moderate salinity, NaCl treated plants and Na₂SO₄ + NaCl treated plants had the highest proline content. At high salinity there was a significant increase in proline content (more than 50% in relation to moderate salinity) in leaves in all salt treatments, with values 8 times higher than controls (Fig. 1A). In roots, proline content was significantly increased in all salt

Table 2

Mineral composition of *P. strombulifera* roots at low (−1 MPa/29 days of culture), moderate (−1.9 MPa/40 days of culture) and high salinity (−2.6 MPa/48 days of culture) that were measured by ICP-OES. Different letters indicate significant differences between treatments according to Tukey test ($P < 0.05$). DM, dry mass.

Days of culture/Osmotic potential	Elements (mg g ⁻¹ DM ± SE)	Control	Salt treatment		
			Na ₂ SO ₄	NaCl	NaCl + Na ₂ SO ₄
29 days/-1 MPa	Ca	8.033 ± 0.770 d	6.810 ± 6.201 cd	3.395 ± 0.117 abc	2.426 ± 0.736 ab
	Fe	0.883 ± 0.454 d	0.949 ± 0.587 d	0.648 ± 0.140 abc	0.427 ± 0.008 ab
	K	25.814 ± 9.051 ab	12.874 ± 3.680 ab	27.775 ± 6.672 b	20.985 ± 9.565 ab
	Mg	0.752 ± 0.231 a	0.429 ± 0.169 a	0.716 ± 0.049 a	0.714 ± 0.147 a
	Na	9.863 ± 7.833 ab	20.102 ± 6.477 abc	22.951 ± 4.695 bc	28.513 ± 10.311 c
	P	6.890 ± 2.892 a	6.247 ± 0.643 a	6.309 ± 0.513 a	6.253 ± 0.814 a
40 days/-1.9 MPa	S	2.737 ± 0.241 bc	5.120 ± 2.331 bc	2.438 ± 0.287 abc	6.459 ± 2.488 c
	Ca	5.888 ± 1.560 bcd	1.559 ± 0.710 a	1.957 ± 1.224 ab	2.118 ± 0.350 ab
	Fe	0.947 ± 0.103 bcd	0.746 ± 0.344 abcd	0.394 ± 0.173 a	0.456 ± 0.252 ab
	K	25.728 ± 7.415 b	5.691 ± 4.270 a	12.605 ± 7.207 ab	20.157 ± 3.121 ab
	Mg	0.981 ± 0.143 a	0.225 ± 0.062 a	0.348 ± 0.043 a	0.500 ± 0.048 a
	Na	7.768 ± 6.024 ab	26.148 ± 6.721 c	18.022 ± 2.232 bc	28.506 ± 7.982 c
48 days/-2.6 MPa	P	6.881 ± 1.060 a	4.932 ± 0.554 a	4.189 ± 1.984 a	5.203 ± 0.362 a
	S	3.083 ± 0.580 abc	8.712 ± 2.833 c	1.282 ± 0.254 a	6.297 ± 1.527 c
	Ca	5.213 ± 2.163 abcd	2.247 ± 0.024 ab	2.109 ± 0.031 ab	2.323 ± 0.015 a
	Fe	0.918 ± 0.298 cd	0.360 ± 0.038 a	0.296 ± 0.046 a	0.335 ± 0.0252 ab
	K	23.631 ± 7.241 ab	12.907 ± 1.480 ab	24.666 ± 2.928 ab	12.714 ± 4.624 ab
	Mg	0.644 ± 0.076 a	0.296 ± 0.123 a	0.585 ± 0.081 a	0.374 ± 0.0174 a
	Na	3.783 ± 4.247 a	25.892 ± 6.195 c	22.456 ± 0.881 bc	26.720 ± 1.475 c
	P	5.841 ± 1.183 a	4.553 ± 0.645 a	5.636 ± 0.979 a	4.099 ± 1.008 a
	S	1.727 ± 0.329 ab	8.412 ± 2.672 c	1.442 ± 0.196 ab	5.557 ± 0.234 c

Table 3

K/Na rate in *P. strombulifera* leaves and roots at low (−1 MPa/29 days of culture), moderate (−1.9 MPa/40 days of culture) and high salinity (−2.6 MPa/48 days of culture).

Osmotic potential/Days of culture	Treatment			
Leaves	Control	Na ₂ SO ₄	NaCl	Na ₂ SO ₄ + NaCl
−1 MPa/29 days	6.88	0.62	0.76	0.73
−1.9 MPa/40 days	3.86	0.49	0.24	0.33
−2.6 MPa/48 days	9.16	0.49	0.27	0.46
Roots				
−1 MPa/29 days	2.62	0.64	1.21	0.73
−1.9 MPa/40 days	3.31	0.21	0.69	0.70
−2.6 MPa/48 days	6.24	0.49	1.09	0.47

treatments being greater in Na₂SO₄ treated plants, with values 10 times higher than controls (Fig. 1B).

3.3. Analysis of photosynthetic parameters by chlorophyll fluorescence measurements

Chlorophyll fluorescence was examined by the factors ETR, *Fv/Fm*, NPQ/4, Y(II), Y(NPQ) and Y(NO) using the light curves obtained (Fig. 2A, B, C, D). All parameters were affected by salt treatments in *P. strombulifera* mainly at high salinity. In general, salinity caused decreases in *Fv/Fm* and ETR and increases in NPQ. Na₂SO₄ treated plants were the most affected showing a significant decrease in *Fv/Fm*, ETR and Y(II), and increases in Y(NPQ) and NPQ. *P. strombulifera* plant in its natural habit and the general aspect of plants growing under control conditions and different salt treatments (Na₂SO₄, NaCl and their iso-osmotic mixture at 40 days of culture/−1.9 MPa) are showed in Fig. 3.

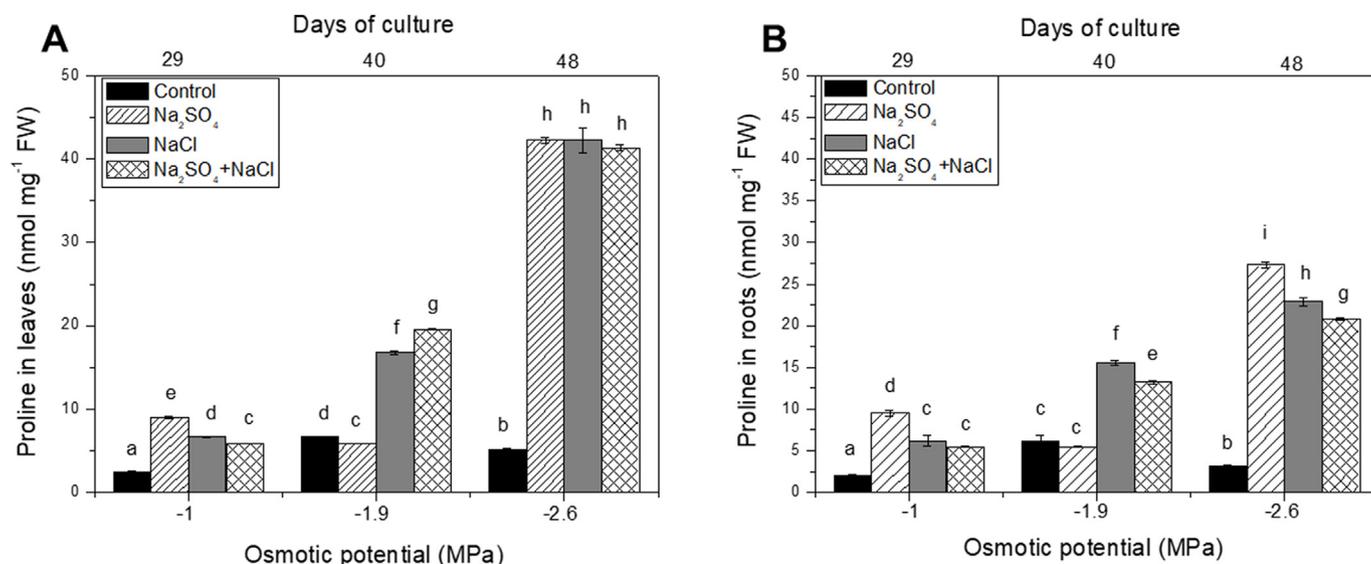


Fig. 1. Effects of NaCl, Na₂SO₄, and their iso-osmotic mixture on proline content in leaves (A) and roots (B) of *P. strombulifera* plants. Mean values (± S.E) followed by different letters above bars are significantly different at $P < 0.05$ ($n = 3$).

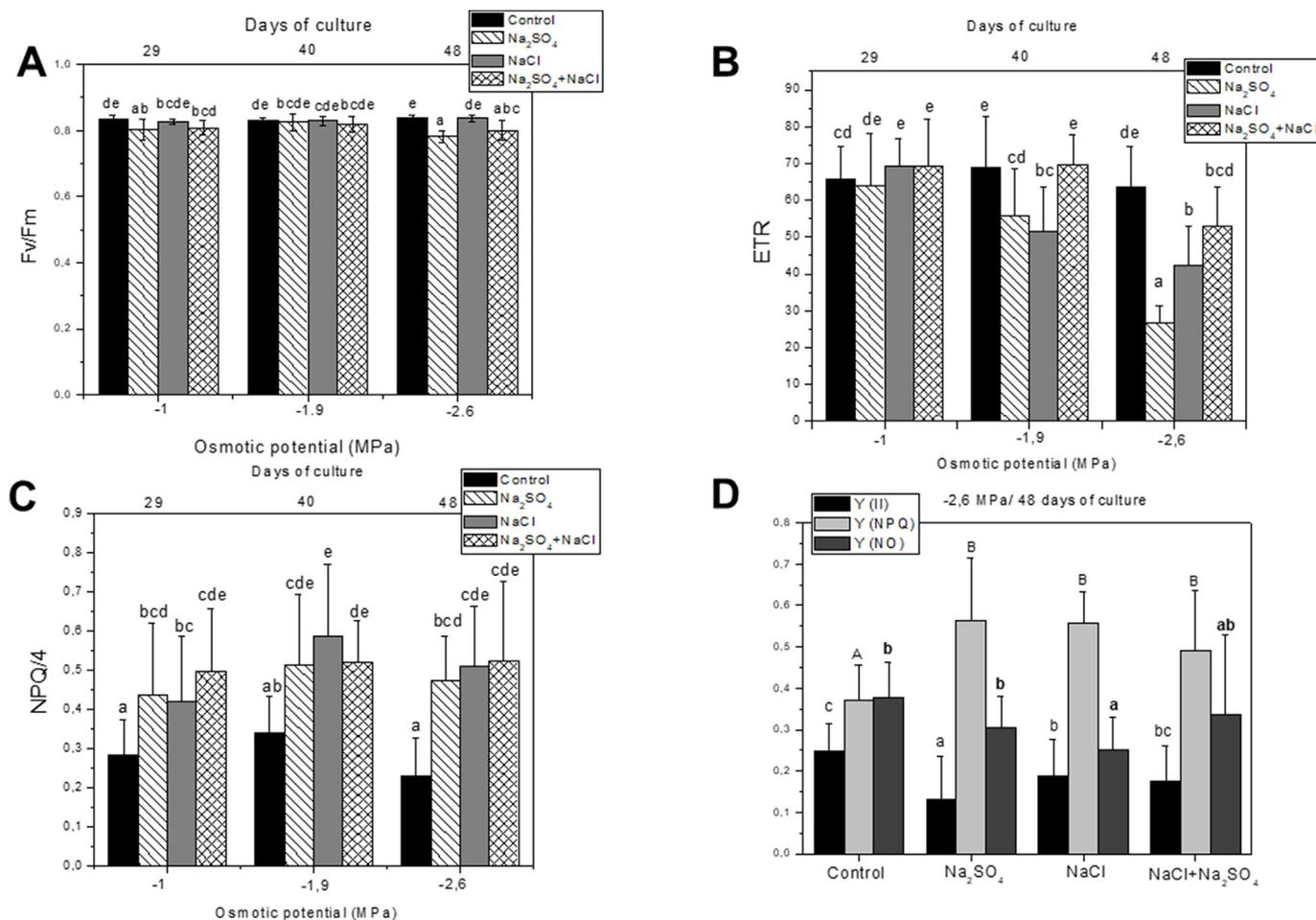


Fig. 2. Effects of NaCl, Na₂SO₄, and their iso-osmotic mixture on (A) *F_v/F_m*, (B) ETR, (C) NPQ/4, and (D) Y(II) Y(NO) Y(NPQ) through chlorophyll fluorescence measurement at 396 μmol quanta m⁻² s⁻¹; Mean values (± S.E) followed by different letters above bars are significantly different at *P* < 0.05 (n = 5).

3.4. LMW thiols under salt stress

Glutathione concentration in *P. strombulifera* was 3 times higher in leaves than in roots in control and treated plants, without differences

(Fig. 4A and B). In roots, it decreased significantly in all saline treatments at moderate and high salinity (Fig. 4B). Cysteine concentration in leaves increased significantly in Na₂SO₄ treated plants at moderate and high salinity, as well as in bisaline-treated plants at high salinity

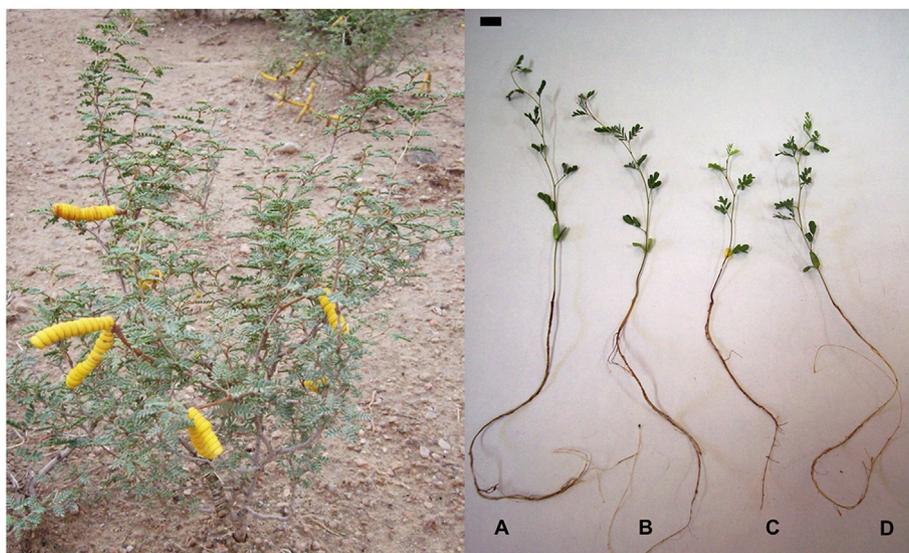


Fig. 3. Left: *P. strombulifera* in its natural habit. Right: General aspect of *P. strombulifera* plants at 40 days of culture under controlled conditions with the different treatments. A, control; B NaCl (-1.9 MPa); C Na₂SO₄ (-1.9 MPa); D Na₂SO₄ + NaCl (-1.9 MPa). Scale: 1 cm.

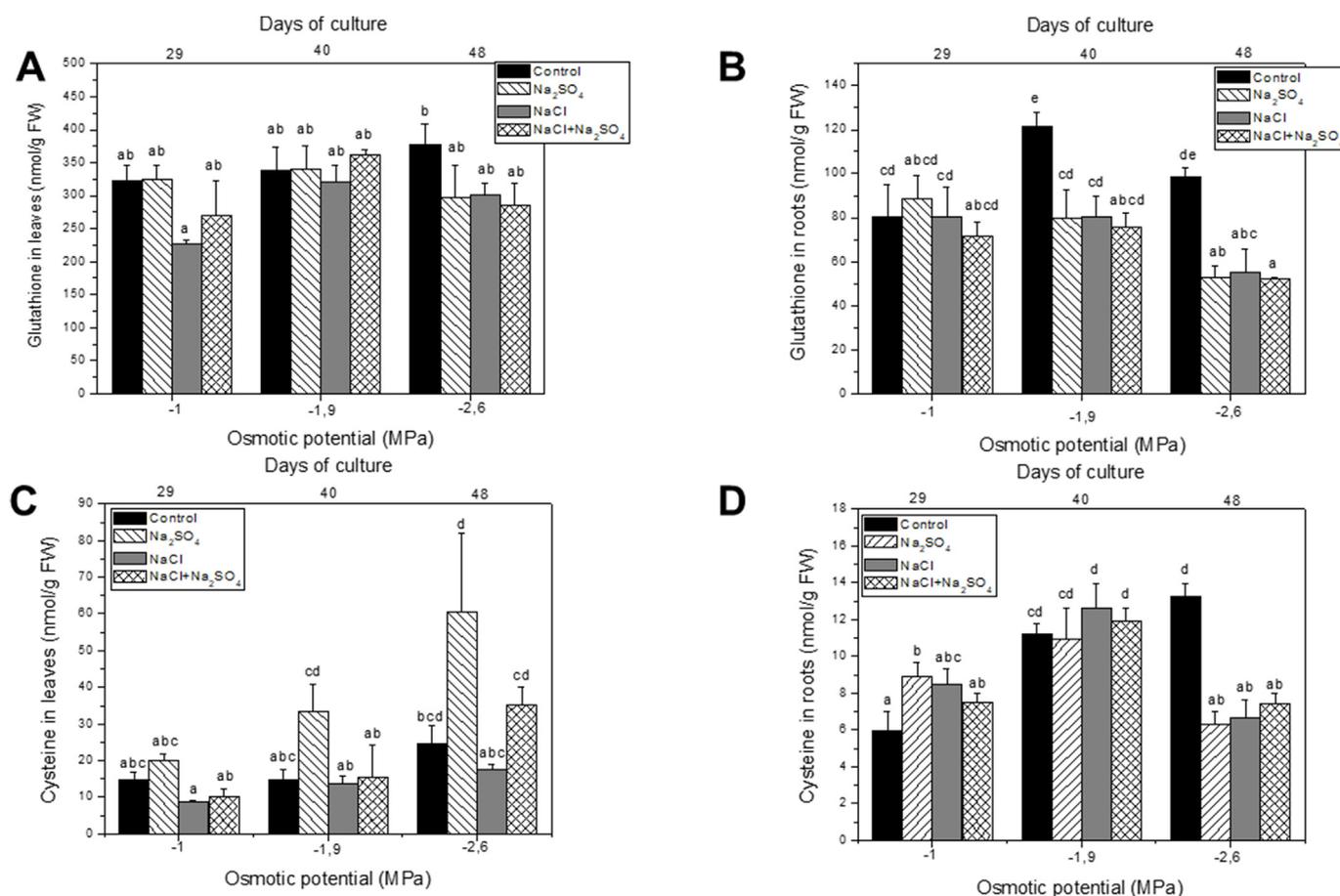


Fig. 4. Effects of NaCl, Na₂SO₄, and their iso-osmotic mixture on glutathione (A,B) and cysteine (C,D) contents in leaves and roots of *P. strombulifera* plants. Mean values (\pm S.E) followed by different letters above bars are significantly different at $P < 0.05$ ($n = 3$).

(Fig. 4C). In roots, cysteine increased under Na₂SO₄ treatment at low concentration and decreased at high salinity in all saline treatments. In control roots, cysteine increased with aging (Fig. 4D).

3.5. Determination of the oxygen radical absorption capacity (ORAC)

As the result of the abundance and action of many antioxidants, ORAC was evaluated in *P. strombulifera*. In leaves, there was a significant increase in ORAC in Na₂SO₄ treated plants a high salinity in relation to control and treatment with NaCl, with a maximal ORAC value of 350 $\mu\text{mol TE g}^{-1}$ FM (Fig. 5A). In roots, ORAC values did not vary with any salt treatment (Fig. 5B).

4. Discussion

Plants deploy a variety of traits to combat salt in soil solution. The most essential one is osmotic adjustment. Halophytes survive salinity by sequestering salts in vacuoles and accumulating organic osmolytes in their cytoplasm (Flowers and Colmer, 2008; Nedjimi, 2014), thus reducing ion toxicity while maintaining osmo-balance. *P. strombulifera* possess specialized characteristics in the root system, e.g. precocious lignification and suberisation of the endodermis (Reinoso et al., 2004, 2005; Reginato et al., 2015), to control the entrance of ions into its tissues and prevent excess salts at the root level under salinity conditions, similar to several other economically important *Prosopis* species (Felker et al., 2008). To understand the effects of the different salt treatments on *P. strombulifera* plants, we analyzed the elemental composition in leaves and roots. As expected, there was an increase in the Na concentration in *P. strombulifera* over time correlating with increasing salinity, reaching concentrations 6 times higher at -2.6 MPa

in leaves. This high Na concentration suggests that when the exclusion mechanisms at root level are surpassed and Na enters the root, it is exported to the shoot and accumulated in leaves. Accumulation of ions, such as Na⁺, Cl⁻ and SO₄²⁻ cause ionic imbalance and further hinders the uptake of minerals such as K⁺, Ca²⁺, and Mn²⁺ (Ahmadzadeh et al., 2016). Although the decrease in K concentration in *P. strombulifera* leaves was not statistically significant in salt treated plants even at high salinity ($\Psi_0 = -2.6$ MPa), the K/Na ratio was certainly reduced, mainly in NaCl treated plants. The maintenance of an optimal K/Na ratio inside the cytosol under saline conditions is critical for normal functioning of the cytoplasm (Bose et al., 2015). *P. strombulifera* NaCl treated plants seems to be able to replace K by Na as osmoticum in cells, because their growth remained unaffected even when K/Na ratio was severely diminished. Na₂SO₄ treated plants were the most affected by salt stress showing significant decreases in Ca and Mg concentration in leaves and K, Ca and Fe concentration in roots at high salinity, in correlation with growth inhibition and senescence symptoms (significant decrease in total photosynthetic pigment concentration, chlorosis, necrosis and finally leaf abscission (Reginato et al., 2013, 2014a; 2014b)). It seems that the SO₄²⁻ anion provokes challenges to several nutrients uptake. Decrease in Ca²⁺ concentration may be explained by the interaction and cation antagonism with Na⁺, by the presence of membrane non-selective cation channels (NSCC) that allow passage of both monovalent and divalent cations without distinction. This could provide the primary route for Ca²⁺ influx to cells, together with hyperpolarisation-activated Ca²⁺ channels (HACC; Demidchik et al., 2002).

The highest levels of S in leaves were found in Na₂SO₄ treated plants. Similarly, exposure of Chinese cabbage plants to Na₂SO₄ salinity resulted in an increase in the total sulfur content of both roots and

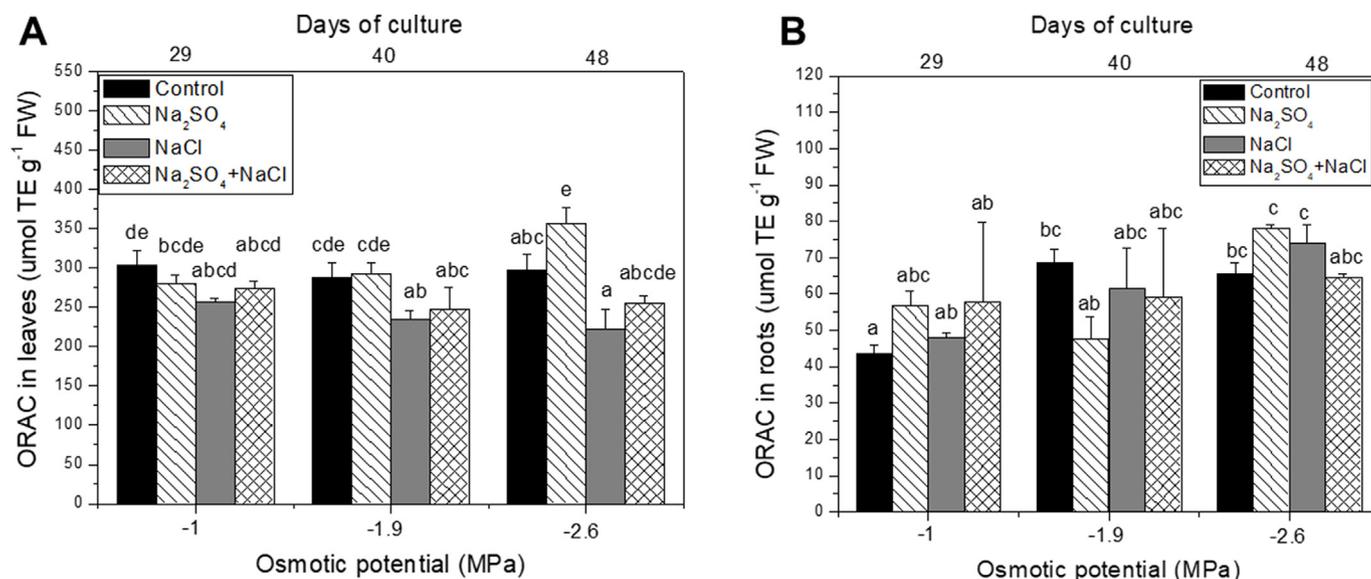


Fig. 5. Effects of NaCl, Na₂SO₄, and their iso-osmotic mixture on ORAC in leaves (A) and roots (B) of *P. strombulifera* plants. Mean values (\pm S.E) followed by different letters above bars are significantly different at $P < 0.05$ ($n = 5$).

shoots (Reich et al., 2015). Certain plants of semi-arid or desert habitats, called “thiophores” are capable of accumulating high sulfur concentrations in their tissues (Duvigneaud and De Smet, 1968). These behavior is considered an adaptation to phytotoxic soil sulfate concentrations, affording these species an ecological niche and the opportunity to avoid competition (Ruiz et al., 2003). Sulfur is primarily taken up by the roots as dissolved sulfate, then stored in the vacuoles of root and xylem parenchymal cells, and transported via the xylem to leaves, where the sulfate (S^{6+}) is again stored in the vacuoles or reduced to sulfide (S^{2-}) in the chloroplast, wherein sulfite reductase catalyzes the reduction of sulfite to sulfide using reduced ferredoxin as electron donor (De Kok et al., 2005). Then, sulfide is incorporated into L-cysteine through cysteine synthase, which is the most efficient way to keep its concentration low in order to avoid inhibitory effects (Takahashi et al., 2011). However, if all free sulfide is not consumed by this assimilatory step, it could be released to the environment or could bind to cytochromes, thereby inhibiting mitochondrial respiration and causing deleterious effects on leaf development, and on root and shoot elongation (Schmidt, 2005).

The proposed tolerance mechanisms for toxic cytosolic sulfur concentrations include vacuolar sulfate compartmentalization (Hawkesford and De Kok, 2006), enhanced protein assimilation (Ruiz et al., 2003) and salt gland excretion (Storey and Thomson, 1994). In *P. strombulifera*, cysteine (Cys) concentration was sharply increased in leaves of Na₂SO₄ treated plants at moderate and high salinity, related to the highest levels of S found in leaves of these plants, as an efficient way to keep S concentration low. An increase in free Cys levels in response to various abiotic stress factors has been reported (Harms et al., 2000; Ruiz and Blumwald, 2002). Cys and glutathione (GSH) are the main cellular Low-molecular-weight (LMW) thiols, highly reactive compounds involved in the maintenance of cellular redox homeostasis that are implicated in plant responses to almost all stress factors, as well as in the regulation of cellular metabolism. Cys is the main product of sulfur assimilation and has a core role in sulfur metabolism, occupying a key position on numerous metabolic pathways; it is generally found at low concentrations because it is rapidly converted into other compounds or incorporated into proteins (Pilon-Smits and Pilon, 2006). Cys is also the precursor of a number of non-thiol sulfur compounds, including vitamin H (biotin), vitamin B1 (thiamine), coenzyme A, the molybdenum cofactor, certain phytoalexins, Fe-S clusters (Hell and Wirtz, 2011). In general, the majority of plant tissue sulfur (80–90%)

can be attributed to amino acids as Cys, methionine and the tripeptide reduced glutathione (Khan et al., 2008). In most studies, Cys increase was reported together with increased GSH concentrations, leading to the conclusion that Cys is mainly needed for the biosynthesis of sulfur-rich compounds with anti-stress activity, such as GSH and stress-related proteins (Zagorchev et al., 2013). GSH is considered the most important LMW thiol in plants because of its pivotal role in sulfur metabolism as the preferred molecule for storing reduced sulfur. It can move through xylem and phloem fluids, so it is involved in long-distance sulfur transport between organs and it is used to protect the cell from oxidative stress, detoxify xenobiotics, and regulate protein function. However, glutathione concentration in *P. strombulifera* leaves did not show variations in our experiments, possibly because it can be rapidly converted again into Cys or other compounds.

Accumulation of compatible solutes helps to maintain water balance in plants growing under salt stress. Many studies have suggested that proline accumulation in plants is an adaptation to high salinity (Tipirdamaz et al., 2006). In the halophyte *P. strombulifera* a significant and marked accumulation of proline in roots and leaves resulted from increased salinity in all salt treatments (NaCl, Na₂SO₄ or bi-saline). Proline accumulation was much higher in leaves than in roots. This observation is consistent with previous reports that described a linear relationship of proline content with conductivity and salinity of the medium (Demiral and Turkan, 2006). Proline is an osmoprotectant and a low-molecular-weight chaperone, and can reduce the inhibitory effects of ions on enzyme activity, increase the thermal stability of enzymes and prevent the dissociation of enzyme complexes such as the oxygen-evolving complex of PSII (Hasegawa et al., 2000). Moreover, proline accumulated during salt stress may be involved in recycling of NADPH (de Ronde et al., 2004) or in signaling pathways that regulate translation of ‘dehydrin’ genes. In the halophyte *Pancratium maritimum* under NaCl stress, proline was shown to induce dehydrin synthesis in roots and stems (Khedr et al., 2003). However, proline accumulation in *P. strombulifera* does not appear to be related to the enhanced expression of LEA proteins (Llanes et al., 2012). Ghars et al. (2008) showed that the accumulation of proline itself does not confer higher tolerance to salt stress in *Thellungiella halophila*. Similarly, proline content in *P. strombulifera* increased under high salinity regardless of salt composition (NaCl, Na₂SO₄ or bi-saline mixture). Thus the proline level may be more of a stress intensity signal rather than a tolerance indicator.

Photosynthesis can be used as a global stress sensor in plants, and

many attempts have been made to detect differences in salinity tolerance by measuring photosynthetic parameters (Koyro, 2006; Cambrollé et al., 2011). The analysis of chlorophyll fluorescence allows assessment of excitation energy flux at PSII in three fundamentally different pathways, namely photochemical utilization, heat dissipation and fluorescence, respectively. When plants are exposed to abiotic and biotic stresses in the light, increases in nonphotochemical quenching processes are frequently observed, which decreases Fv/Fm (Baker, 2008). In many stress situations, increases in nonphotochemical quenching can often be accompanied by photoinactivation of PSII reaction centers, which then dissipate excitation energy as heat rather than as photochemistry (Melis, 1999). Photoinactivation can lead to oxidative damage and loss of PSII reaction centers (Aro et al., 1994). With respect to the analysis of chlorophyll fluorescence in *P. strombulifera*, several parameters were affected by salt treatments at high salinity. In general, salinity caused decreases in Fv/Fm and in ETR and increases in non-photochemical quenching (NPQ). However, Fv/Fm remained unaffected in NaCl treated plants, even at high salinity. Reductions in the Fv/Fm ratio represent a decline in the ability of PSII to reduce the primary electron acceptor, QA (the first stable quinone electron acceptor of PSII) (Calatayud and Barreno, 2001). On the other hand, increased NPQ functions as a protection mechanism under stressful conditions, which dissipates light energy and decreases the efficiency of photochemical reactions of photosynthesis (Graßes et al., 2002). The NPQ deactivates excited Chl (Havaux et al. 2007) avoiding singlet oxygen (1O_2) production and preventing photo-oxidation. Na_2SO_4 treated plants were the most affected by salinity, showing a significant decrease in several photosynthetic parameters as ETR and in the effective yield of PSII (Y (II) that estimates the efficiency at which light absorbed by PSII is used for photochemistry), accompanied by increases in Y (NPQ) and NPQ at high salinity. A slight but significant decrease in Fv/Fm in Na_2SO_4 treated plants was also observed, although may be not different enough to have biological significance. These results confirm that the SO_4^{2-} anion, that causes a strong oxidative damage in the seedlings, is triggering damage in the photosynthetic apparatus and consequently affecting the photosynthetic process, which may explain the strong growth inhibition in these plants. At 48 days of culture ETR was severely affected in Na_2SO_4 treated plants, however, there is no difference between ETR values of NaCl and NaCl + Na_2SO_4 treated plants, indicating a partial alleviation of SO_4^{2-} toxicity when Cl⁻ is present in the medium. Similar observations were made and reported in our previous papers Llanes et al. (2005); Llanes et al. (2012); Sosa et al. (2005), Reginato et al. (2014a, 2015). In agreement with our results, Na_2SO_4 -salt exhibited the most harmful effects on the halophyte *Aeluropus litoralis*, more than KCl and NaCl, and this appeared clearly on all measured growth parameters. Its highest deleterious effects appeared also on the photosynthetic process, reducing drastically the net photosynthetic rate and the intrinsic water use efficiency, and increased the internal CO_2 accumulation by two-folds more than do KCl and NaCl-salts (Barhoumi, 2018).

P. strombulifera Na_2SO_4 treated plants, which showed a slight decrease in Fv/Fm at the end of the experiment, concomitantly displayed higher xanthophyll de-epoxidation state (Reginato et al., 2014b). In our study we did not attribute photoinhibition of PSII to photo damage in the growth chamber. Instead, considering the proposal of Schmidt (2005) that if all free sulfide is not consumed by the assimilatory step to L-cysteine it could be released to the environment or could bind to cytochromes, there may be inhibition of cytb559 in PSII by SH_2 production from sulfate reduction. If there are inactive units in PSII, there is great potential for ROS formation.

The oxygen radical absorbance capacity (ORAC) determines mainly chain-breaking antioxidants such as (poly)phenols, vitamin C, vitamin E, uric acid and bilirubin (Ou et al., 2002). In the halophyte *P. strombulifera* the ORAC increased in leaves of Na_2SO_4 treated plants at high salinity, with a maximal value of 350 $\mu\text{mol TE g}^{-1}$ FM. This ORAC value resulted higher than maximal ORAC values in other halophytes,

like *Tripolium pannonicum* (160 $\mu\text{mol TE g}^{-1}$ FM (Boestfleisch et al., 2014) *Crithmum maritimum* (80 $\mu\text{mol TE g}^{-1}$ FM) and *Halimione portulacoides* (45 $\mu\text{mol TE g}^{-1}$ FM) (Boestfleisch and Papenbrock, 2017), indicating a strong antioxidant activity in *P. strombulifera*. This antioxidant activity would be closely related to the sharp increase in total phenols and flavonoid compounds previously found in these plants under Na_2SO_4 treatment (Reginato et al., 2014b). These polyphenol accumulation was also evidenced by microscopic analysis, where leaflets of Na_2SO_4 -treated plants showed a highly increased condensed tannins accumulation in mesophyll and epidermal cells (Reinoso et al., 2005; Reginato et al., 2014b). This very high antioxidant activity found in *P. strombulifera* highlight this species as a valuable source of bioactive compounds.

Summarizing, a general analysis of different mechanisms of tolerance/non-tolerance to salinity of the halophyte *P. strombulifera* was carried out with special emphasis on the effects of Na_2SO_4 , being able to demonstrate that the presence of the SO_4^{2-} anion in the culture medium was the determinant of the toxicity observed in plants. The metabolism and carbon partitioning was seriously affected, having to divert the energetic resources towards the synthesis of secondary metabolites such as condensed tannins and precursors of lignins and polyphenols to face the great oxidative stress. Ca and Mg concentration in leaves are also reduced. As a consequence, there is strong inhibition of growth with chlorosis, necrosis and foliar abscission.

As a possible explanation for its great toxicity, it is proposed that the SH_2 that has not been metabolized to cysteine would be left in excess, and could be binding to PSII cytochrome b559 blocking its partial or complete functioning and thus inhibiting the photosynthesis process, which opens a new interesting topic for research.

5. Contributions

Experiments and sample collection was carried out by M.R. Chlorophyll fluorescence measurements and all biochemical analyses were conducted by M.R., aided by A.T. Statistical analyses were conducted by M.R. M.R wrote the manuscript; A.T., J.P. and V.L. edited the manuscript. J.P. was the tutor of M.R. at the Institute of Botany in Hannover, Germany. V.L. is the Head of the Plant Physiology Laboratory in Argentina where the project is currently being carried on. All authors approved the final version of this article.

Declaration of interest statement

No conflict of interest.

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