plant biology

Plant Biology ISSN 1435-8603

RESEARCH PAPER

Different sodium salts cause different solute accumulation in the halophyte *Prosopis strombulifera*

A. Llanes[1], G. Bertazza[2], G. Palacio[3] & V. Luna[1]

1 Laboratorio de Fisiología Vegetal, Departamento de Ciencias Naturales, Universidad Nacional de Río Cuarto, Río Cuarto-Córdoba, Argentina

2 Instituto di Biometeorología-Sezione di Bologna, Bologna, Italy

3 Departamento de Matemáticas, Facultad de Ciencias, Universidad Nacional de Río Cuarto, Río Cuarto-Córdoba, Argentina

Keywords
Amino acids; carbohydrates; NaCl; Na_2SO_4; osmotic potential; proteins.

Correspondence
V. Luna, Laboratorio de Fisiología Vegetal, Departamento de Ciencias Naturales, Universidad Nacional de Río Cuarto, Route 36 Km. 601, (CP X5804BYA) Río Cuarto-Córdoba, Argentina.
E-mail: vluna@exa.unrc.edu.ar

Editor
M.Günthardt-Goerg

Received: 17 February 2012; Accepted: 16 April 2012

doi:10.1111/j.1438-8677.2012.00626.x

ABSTRACT

The success of *Prosopis strombulifera* in growing under high NaCl concentrations involves a carefully controlled balance among different processes, including compartmentation of Cl^- and Na^+ in leaf vacuoles, exclusion of Na^+ in roots, osmotic adjustment and low transpiration. In contrast, Na_2SO_4 causes growth inhibition and toxicity. We propose that protection of the cytoplasm can be achieved through production of high endogenous levels of specific compatible solutes. To test our hypothesis, we examined endogenous levels of compatible solutes in roots and leaves of 29-, 40- and 48-day-old *P. strombulifera* plants grown in media containing various concentrations of NaCl, Na_2SO_4 or in mixtures of both, with osmotic potentials of −1.0,−1.9 and −2.6 MPa, as correlated with changes in hydric parameters. At 24 h after the last pulse plants grown in high NaCl concentrations had higher relative water content and relatively higher osmotic potential than plants grown in Na_2SO_4 (at 49 days). These plants also had increased synthesis of proline, pinitol and mannitol in the cytoplasm, accompanied by normal carbon metabolism. When the sulphate anion is present in the medium, the capacities for ion compartmentalisation and osmotic adjustment are reduced, resulting in water imbalance and symptoms of toxicity due to altered carbon metabolism, *e.g.* synthesis of sorbitol instead of mannitol, reduced sucrose production and protein content. This inhibition was partially mitigated when both anions were present together in the solution, demonstrating a detrimental effect of the sulphate ion on plant growth.

INTRODUCTION

Salt stress is a major cause of reduced crop productivity worldwide. Like other common abiotic stresses (*e.g.* drought, temperature), salt stress induces a variety of biochemical and physiological plant responses, and the phenomenon of tolerance is therefore quite complex (Munns & Tester 2008). Development of crop plants with enhanced tolerance to environmental stresses is an increasingly important strategy to meet the growing food demands of developing and underdeveloped countries. However, successful implementation of this strategy requires detailed knowledge of the physiological mechanisms underlying the relevant traits in engineered and parental genotypes at various developmental stages. Such knowledge remains fragmentary, in spite of significant advances achieved during the past decade.

Salt stress in plant cells results from a combination of osmotic, ionic and oxidative factors (Chen *et al.* 2007). Leaf tissues of halophytes (plants adapted to high-salinity environments) are able to accumulate high concentrations of salt ions, which is necessary to generate a water potential gradient from roots to shoots, and to maintain water flux throughout the plant (Silveira *et al.* 2009).

Accumulation of compatible solutes is a common, basic strategy for protection and survival of plants under salt stress (Türkan & Demiral 2009). The presence of such solutes protects plants against stress through cellular osmotic adjustment, detoxification of reactive oxygen species, maintenance of membrane integrity and stabilisation of enzymes/proteins (Ashraf & Foolad 2007; Miller *et al.* 2010). Compatible solutes can be divided into three major groups: amino acids (*e.g.* proline), quaternary amines (*e.g.* glycine betaine, dimethylsulphoniopropionate) and polyalcohols (*e.g.* mannitol, pinitol). Some of these solutes (*e.g.* methylated sulphonio compounds in *Spartina* spp.; methylated proline compounds in *Melaleuca* spp.) are accumulated in just a few halophyte species, while others (*e.g.* glycine betaine, proline) are found in numerous species across many genera (Naidu *et al.* 2000). Gorham *et al.* (1980) described amounts of the quaternary ammonium compounds, proline, inositol and pinitol, in 27 species collected from a salt marsh in north Wales. More recently, Tipirdamaz *et al.* (2006) analysed organic solutes in leaves of 51 species found near a salt marsh in Turkey. Studies such as these have confirmed the existence of species-specific compatible solutes. The specific functions of these solutes in plant stress adaptation are still not understood. Although several roles have been attributed to the accumulation of proline upon stress, its role in plant stress adaptation is still a subject of debate (Lehmann *et al.* 2010). Few studies have focused on changes in polyalcohol levels in halophyte responses to salt stress.

Regions in which the halophytic shrub *Prosopis strombulifera* (Lam.) Benth. (Burkart 1976) grow range from the Arizona Desert (USA) to Patagonia (Argentina), being particularly abundant in high-salinity areas in central Argentina (Cantero *et al.* 1996). Proportions of NaCl and Na_2SO_4 in soils are generally similar in these areas, although the Na_2SO_4 concentration may be up to threefold higher (mainly near the surface). The rooting profile of this shrub varies in depth from 10 to 35 cm due to its vegetative reproduction habit, with horizontal budding roots, which allow this species to colonise saline areas. Since Na_2SO_4 and NaCl are typically the major salts present in high-salinity soils worldwide, it is important to quantify and compare their effects on plant growth at various concentrations (Sosa *et al.* 2005; Manivannan *et al.* 2008).

Our previous studies have shown that *P. strombulifera* has higher NaCl tolerance limits than most halophytes, but is much less tolerant to Na_2SO_4 than to NaCl (Reginato 2009; Reginato *et al.* 2012). Plants grown in the presence of Na_2SO_4 show immediate and sustained reduction of shoot height and leaf number, and exhibit senescence symptoms (chlorosis, necrosis, leaf abscission; Reinoso *et al.* 2005). The toxic effects of the SO_4^{2-} ion are partially mitigated by growth in bi-saline solution (combined NaCl and Na_2SO_4; Sosa *et al.* 2005; Llanes *et al.* 2005). Stimulation of root growth was observed in NaCl-treated plants, suggesting a generalised adaptive response favouring water and nutrient absorption. The SO_4^{2-} anions had a larger detrimental effect on root growth than Cl^- anions, which was partially mitigated when the two anions were present together in a bi-saline solution.

Our previous studies also showed that *P. strombulifera* accumulates Na^+ preferentially over Cl^-, resulting in high Na^+ levels in roots as well as in leaf tissues, without showing symptoms of toxicity during optimal growth of NaCl-treated plants. In leaves of Na_2SO_4-treated plants, SO_4^{2-} levels were lower than those of Cl^- but were sufficient to cause senescence symptoms (chlorosis, necrosis, leaf abscission). The Na^+ concentration in leaves was two to threefold higher than the K^+ concentration (0.48 *versus* 0.185 mM g^{-1} DW) during optimal growth of NaCl-treated plants (ψ_o = −1.9 MPa; 500 mM), suggesting that Na^+ is transported to the shoot after entering the root and thereby helps to establish a water potential gradient (Reginato *et al.* 2012). In addition, the highest levels of absicic acid (ABA) and ABA–glucose ester (GE), and the highest ABA–GE glucosidase activity were observed in Na_2SO_4-treated plants at moderate and high salinity. However, the stress imposed by this salt blocked activity of ABA, hence stomata remained open and high transpiration (water loss) values were recorded (Llanes 2010). Taken together, our findings suggest that the adaptive success of *P. strombulifera* growing under salt stress (high NaCl concentration) involves a delicate and carefully controlled balance that includes compartmentation of Cl^- and Na^+ in leaf vacuoles, exclusion of Na^+ in roots, osmotic adjustment and low transpiration. We hypothesise that cytoplasm protection can be achieved through production of high endogenous levels of specific compatible solutes.

In the present study, we grew *P. strombulifera* seedlings in iso-osmotic solutions of NaCl, Na_2SO_4 or their bi-saline mixture, analysed endogenous levels of total amino acids, proline, glycine betaine, total proteins, total sugars, glucose, fructose, sucrose and polyalcohols in roots and leaves, and correlated these levels with hydric parameters in treated *versus* control plants.

MATERIAL AND METHODS

Plant material

Prosopis strombulifera seeds were collected from an area in southwest San Luis province, Argentina. This area belongs to the mesquite tree forest located in a saline depression between the annual 300 and 400 mm isohyets in the Monte phytogeographic region (Anderson *et al.* 1970; Carosio *et al.* 2009). The soil has a franc sandy texture, with abundant calcareous material and moderate salinity; chemical composition was determined according to Peña Zubiate *et al.* (1998). The profile from depths of 0 to 35 cm is presented in Table 1.

Pods were collected at random from 100 plants within the same population. Seeds were selected visually for uniform size and healthy appearance, scarified with sulphuric acid for 10 min, washed overnight under running water, rinsed in distilled water and placed in Petri dishes with two layers of water-saturated filter paper for 24 h at 37 °C before sowing (Reinoso *et al.* 2005). Germinated seeds with roots 20-mm long were grown under hydroponic conditions, in two black trays per treatment per experiment (200 seedlings per tray), with 10% full-strength Hoagland's solution. Seedlings were grown for 1 week in a chamber with a cycle of 16-h light (28 °C)/8-h dark (20 °C), 70% relative humidity, then transferred to 25% full-strength Hoagland's solution (osmotic potential −0.11 MPa) (Hoagland & Arnon 1950). Aeration was provided with an aquarium tubing system and a peristaltic pump; pH of all media was 6. Each experiment was performed three times.

Salt treatments

Salt treatments were applied after plants had grown for 15 days, using a simple randomised design. Pulses of NaCl alone (50 mM), Na_2SO_4 alone (38 mM) or an iso-osmotic mixture of the two salts (bi-saline treatment) were applied every 48 h until reaching a final osmotic potential (ψ_o) of −1.0,−1.9 or −2.6 MPa, respectively (measured with a vapour pressure osmometer Model 5500; Wescor Inc., Logan, UT, USA; Table S1). These ψ_o values corresponded to age 29, 40

Table 1. Chemical composition of the soil profile in the sampling area (Sosa 2005).

depth (cm)	0–10	10–25	25–35
water pH	7.6	7.5	7.5
cations			
Ca^{2+}	38	30	30
Mg^{2+}	4	2	4
Na^+	40	155	210
K^+	2.9	2.5	2.9
anions			
CO_3^{2-}	–	–	–
CO_3H^-	2	2	2
Cl^-	6	10	36
SO_4^{2-}	28	33	33
ESP	11	33	43
SAR	9	34	51
EC (dS m^{-1})	8.4	10.5	11

and 48 days, respectively. Iso-osmotic bi-saline solutions were obtained by mixing equal volumes of the respective monosaline solutions at each osmotic potential, as shown in Table S1. For each biochemical determination, 70 untreated plants (no salt added; $\psi_o = -0.03$ MPa) and 70 treated plants were collected at random after 24 h from each tray after the respective medium reached the final osmotic potential described above. Roots and leaves from each plant were separated and pooled per treatment, frozen in liquid nitrogen and stored at −80 °C for *a posteriori* analysis.

Amino acid, total protein and total soluble carbohydrate analyses

A total of 200 mg dry tissue from roots and leaves of treated and non-treated plants were used for biochemical studies. The ninhydrin method was used for determination of total amino acids (Rosen 1957). Proline content was determined as described in Magne & Larher (1992). Quaternary ammonium compounds (QACs) were extracted and measured as glycine betaine (GB) equivalents, as described in Grieve & Grattan (1993). Total proteins were analysed using the method of Bradford (1976). Soluble, non-structural carbohydrate concentration was determined with the phenol-sulphuric acid method (Ciha & Brun 1978).

Glucose, fructose, sucrose and polyalcohol analyses

Levels of these compounds were analysed using gas chromatography with flame ionisation detection (GC-FID). About 1 g dried tissue of roots and leaves was treated with 50 ml of a 1:1 solution of imidazole buffer (0.05 M, pH 7.2) and ethanol, to which phenyl-gluco-pyranoside as internal standard was added, stirred for 24 h and centrifuged at 10,000 *g*. The pellet was extracted with 48 ml imidazole solution, stirred for 24 h and centrifuged. The two supernatants were combined and the volume brought to 100 ml with ethanol. For derivatisation prior to GC, fractions of 2 ml (equivalent to 100 mg sample) were added to 400 µl anhydrous pyridine, and placed in an oven for 1 h at 55 °C. The solution was mixed with 400 µl derivatisation solution (pyridine/hexamethyldisilazane/trimethylchlorosilane 1/2/1), placed in an oven for 1.5 h at 55 °C, and cooled for 24 h at room temperature (28 °C). For GC-FID analysis, 0.5 ml derivatised sample was injected in a capillary column (20 m × 0.25 mm) with SGE BPX-30 polar medium, using helium as carrier gas. Gas combustion: air 250 ml min^{-1}; hydrogen 30 ml min^{-1}; nitrogen 30 ml min^{-1}. Compounds were identified from their retention time and quantified by comparison of peak areas with those of pure standards.

Osmotic potential and relative water content

For measurement of osmotic potential, leaves and roots (300 mg each) were placed in tubes and frozen at −80 °C for 24 h, thawed and centrifuged for 30 min at 8000 *g*. A 150-µl aliquot of each sample was placed in a capsule and osmotic potential was measured with a freezing point osmometer (Semi Micro K-7400; Knauer, Berlin, Germany) (Sayar *et al.* 2008). Note, roots were previously washed three times with abundant deionised water, checking that no ions were detected in the final wash.

Relative water content (RWC) was calculated as described in Silveira *et al.* (2001). Fresh weight was determined in ten plants per treatment. Roots and whole leaves were then placed in Petri dishes containing distilled water for 4 h (the time determined previously as required to reach turgid weight). Dry weight (DW) was obtained after placing the tissues in an oven at 80 °C for 48 h.

Statistical analysis

Data were analysed using InfoStat (v. 2011; InfoStat, National University of Córdoba, Argentina) program. The 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 (i) osmotic potential (ψ_o; −1.0, −1.9 or −2.6 MPa) and (ii) treatment (control, NaCl, Na_2SO_4 or bi-saline). Normality was verified with the Shapiro–Wilks test. Homogeneity of variance was verified with Levene test. When necessary, data were transformed to meet the assumptions of ANOVA. The Tukey test was used for *post-hoc* analysis to determine differences between means. Differences were considered significant at $P < 0.05$.

RESULTS

Effect of salt stress on amino acid content

Accumulation of compatible solutes helps to maintain water balance in growing plants under salt stress. Total amino acid content in roots and leaves of *P. strombulifera* seedlings was not altered as a result of high salinity (−2.6 MPa; Fig. 1A and B). However, a significant and marked accumulation of proline in roots and leaves (∼twofold increase in concentration) resulted from increased salinity in all salt treatments (NaCl, Na_2SO_4 or bi-saline). This observation is consistent with previous reports that described a linear relationship of proline content with conductivity and salinity of the medium (Demiral & Turkan 2006). Proline accumulation was much higher in leaves than in roots. We did not observe accumulation of glycine betaine in leaves or roots of salt-treated plants (Fig. 2A and B).

Effect of salt stress on total soluble protein and carbohydrate content

Treatment of seedlings with moderate salt concentrations (ψ_o: −1.9 MPa) caused changes in total soluble protein content of both roots and leaves, with no appreciable difference in results for the three salt treatments (Fig. 1C). At high salinity (−2.6 MPa), total protein content was increased in NaCl-treated plants, but decreased in Na_2SO_4-treated plants and was not altered in leaves of plants treated with bi-saline solution (Fig. 1C and D).

Total soluble carbohydrate content increased as salt concentration increased. The increase was higher in roots than in leaves for all three salt treatments. For low (−1.0 MPa) and moderate (−1.9 MPa) salinity glucose content in roots of treated *versus* untreated plants was similar, whereas glucose content was significantly higher (1.6 mg/100 g DW; $P < 0.05$) in high (−2.6 MPa) Na_2SO_4-treated plants. Total carbohydrate content in roots of untreated plants increased gradually as the plants grew (Fig. 1E and F).

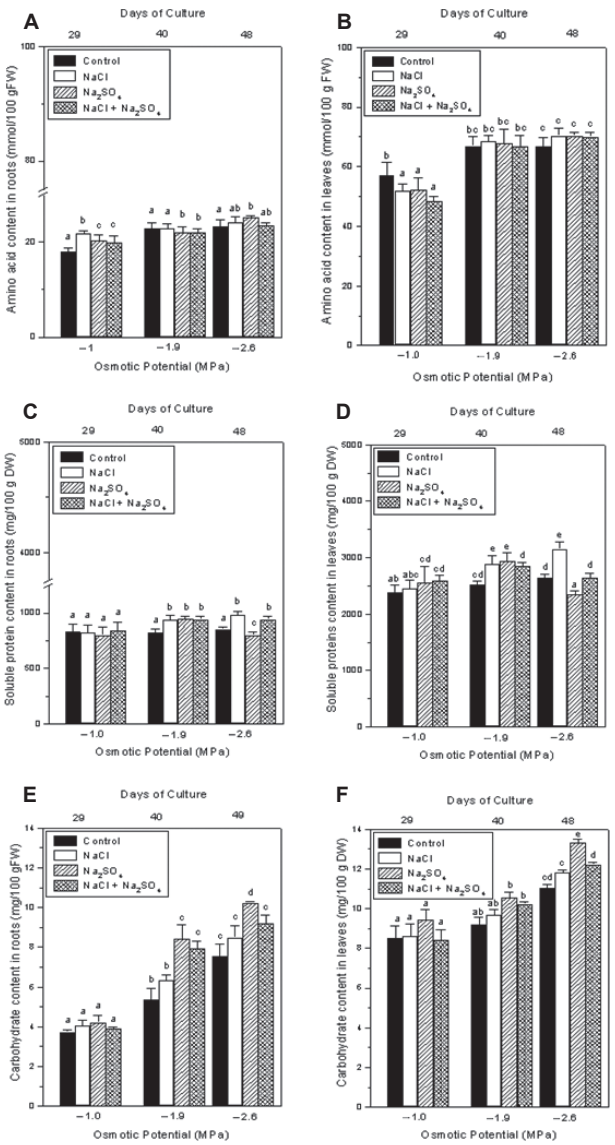

Fig. 1. Effects of NaCl, Na_2SO_4 and their iso-osmotic mixture on amino acid, carbohydrate and protein content of *Prosopis strombulifera*. A, C, E: Roots. B, D, F: Leaves. Data are mean ± SE. Different letters above bars indicate significant differences among treatments ($P < 0.05$).

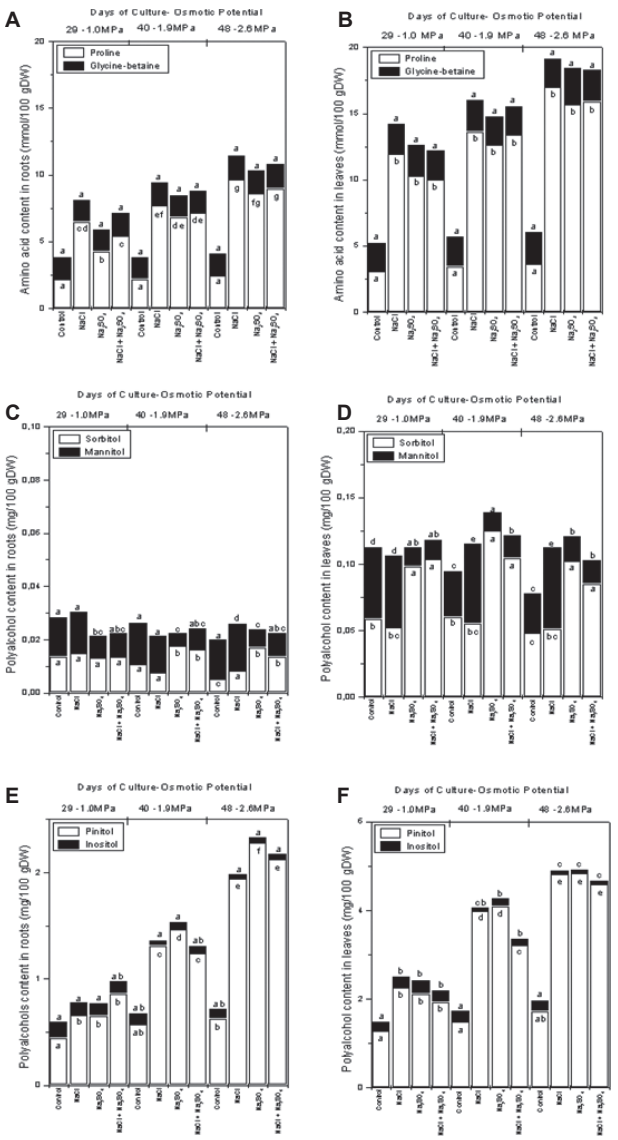

Fig. 2. Effects of NaCl, Na_2SO_4 and their iso-osmotic mixture on proline, glycine betaine, sorbitol, manitol, pinitol and inositol content of *Prosopis strombulifera*. A, C, E: Roots. B, D, F: Leaves. Data are mean ± SE. Different letters above bars indicate significant differences among treatments ($P < 0.05$).

Effect of salt stress on polyalcohol content

Mannitol content in roots of untreated plants was consistently low (∼0.015 mg/100 g DW; Fig. 2C and D). This content decreased significantly ($P < 0.05$) in Na_2SO_4- and bi-saline-treated plants, but was higher in plants treated with −2.6 MPa NaCl compared to the other treatments ($P < 0.05$; Fig. 2C).

Mannitol content in leaves was 0.053 mg/100 g DW at time zero of salt treatments, and decreased to 0.03 mg 100 g^{-1} DW at the end of experiments. This was similar to results in untreated plants for −1.0 MPa NaCl-treated plants, but was 75% lower ($P < 0.05$) in Na_2SO_4- and bi-saline-treated plants. In plants treated with NaCl at lower osmotic potentials (−1.9 or −2.6 MPa), the accumulation of mannitol in leaves was 40% higher than in untreated plants ($P < 0.05$).

Root sorbitol content was low in untreated plants, but was significantly higher in −1.9 and −2.6 MPa Na_2SO_4-treated plants (Fig. 2C). Leaf sorbitol content was twofold higher in Na_2SO_4- and bi-saline-treated plants than in NaCl-treated or untreated plants ($P < 0.05$), reaching a peak of 0.12 mg/100 g DW in the −1.9 MPa treatment (Fig. 2D).

Content of cyclic alcohols was higher than that of non-cyclic alcohols (Fig. 2E and F). Root inositol content was not altered as a result of salinity. However, in leaves, the inositol content of salt-treated plants was significantly lower than that of untreated plants ($P < 0.05$), with no appreciable difference among the three salt treatments (Fig. 2F). Root pinitol content was up to threefold higher and leaf pinitol content was up to 2.8-fold higher (at −2.6 MPa) in salt-treated *versus* untreated plants (Fig. 2E and F).

Effect of salt stress on glucose, fructose and sucrose content

Root sucrose content of plants grown at low (−1.0 MPa) salinity was significantly higher ($P < 0.05$) than in untreated plants. The value for NaCl-treated plants was highest, although differences from the other two salt treatments were not significant. At moderate salinity (−1.9 MPa) there was no significant difference from values of untreated plants; however, the Na_2SO_4-treated plants (−2.6 MPa) had a significantly lower sucrose content compared to the controls ($P < 0.05$; Fig. 3A).

Leaf sucrose content was significantly higher in −1.9 and −2.6 MPa salt-treated plants than in controls. This parameter was higher in NaCl-treated plants than in Na_2SO_4- and bi-saline-treated plants (Fig. 3B). Leaf glucose content did not differ from control values in any of the three salt treatments (data not shown). Root glucose and fructose content was insignificantly higher than the corresponding leaf content in all three salt treatments.

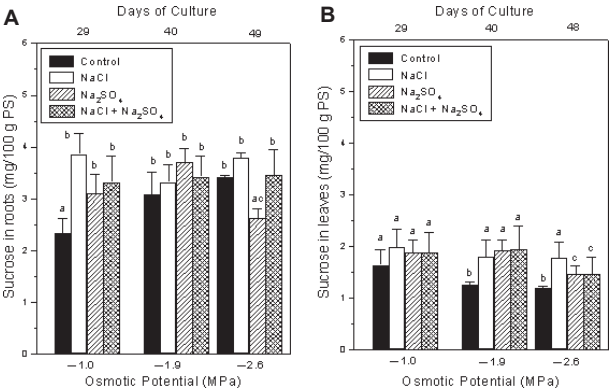

Fig. 3. Effects of NaCl, Na_2SO_4 and their iso-osmotic mixture on sucrose content of *P. strombulifera*. A: Roots. B: Leaves. Data shown are mean ± SE. Different letters above bars indicate significant differences among treatments ($P < 0.05$).

Hydric parameters during salt stress

In roots, RWC did not change with saline treatments of low and moderate salinity (−1.0 and −1.8 MPa), whereas RWC increased at higher salt concentrations (−2.6 MPa). In leaves from salt-treated plants at −1.0 MPa a decrease in RWC was observed compared to untreated plants. At −2.6 MPa, NaCl-treated seedlings showed an increase in water content compared to the other treatments (Fig. 4A and B).

Osmotic potential was not altered in roots of treated *versus* untreated seedlings (data no shown). Leaves of seedlings at high salt concentrations (−2.6 MPa) showed a significant decrease in osmotic potential. The Na_2SO_4-treated plants had the lowest osmotic potential (Fig. 5).

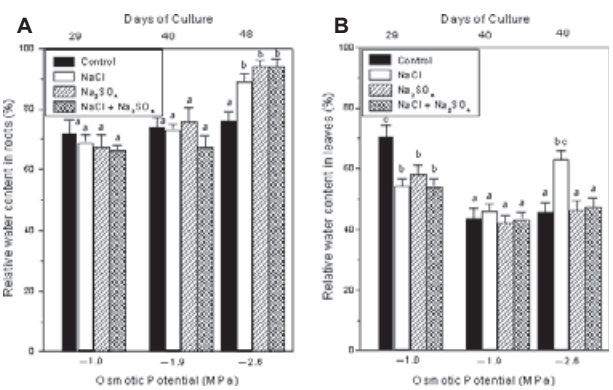

Fig. 4. Effects of NaCl, Na_2SO_4 and their iso-osmotic mixture on relative water content (A) in roots and (B) in leaves of *Prosopis strombulifera*. Data are mean ± SE. Different letters above bars indicate significant differences among treatments ($P < 0.05$).

DISCUSSION

The degree of resistance to abiotic stress in plants is associated with their relative ability to regulate water content and leaf water potential under stressful conditions. In particular, resistance to water stress depends on the plant's ability to maintain water content as soil water potential decreases. Halophyte plants are characterised by this ability; their growth and survival depends on resistance to water stress through accumulation of ions and compatible solutes for maintenance of turgor and for osmotic adjustment (Khan *et al.* 2000).

In the halophyte *P. strombulifera*, increased water content in leaves helps mitigate the detrimental effect of excessive ion accumulation in the tissues, which is promoted by such anatomical adaptations as precocious lignification of the endodermis in the root system (Reinoso *et al.* 2005) and changes in number and size of stomata (Reginato 2009). These adaptations allow more efficient use of water under NaCl stress in this species. In contrast, Na_2SO_4-treated plants have much reduced osmotic potential and lower RWC, reflecting a failure of this osmotic adjustment.

Total soluble protein and amino acid accumulation

Total soluble protein content in roots and leaves of *P. strombulifera* was not affected by moderate (−1.9 MPa) salinity. At high (−2.6 MPa) salinity, proteins accumulated mainly in the NaCl-treated plants. The decrease of protein content in Na_2SO_4-treated plants may reflect the growth inhibitory effect induced by this salt, which was mitigated in the bi-saline mixture.

Salt-treated *P. strombulifera* did not show an appreciable change in total amino acid content, but changes in individual amino acids were observed. Total amino acid content in response to salt treatment has been reported to increase in several plant species (Verbruggen & Hermans 2008) and to decrease in others, *e.g.*, *Aegiceras corniculatum* (black mangrove; Parida *et al.* 2004). In particular, proline content was increased (up to 200%) in all three salt treatments. Consistent with this finding, Demiral & Turkan (2006) reported that proline content in plants is directly correlated with conductivity and salinity of the medium. In NaCl-treated *Medicago truncatula*, proline content increased 13-fold in leaves and eightfold in roots compared to controls as a result of salt stress (Parida *et al.* 2004). In *P. strombulifera*, proline also accumulated more in leaves than in roots.

Many studies have suggested that proline accumulation in plants is an adaptation to high salinity and plays an impor-

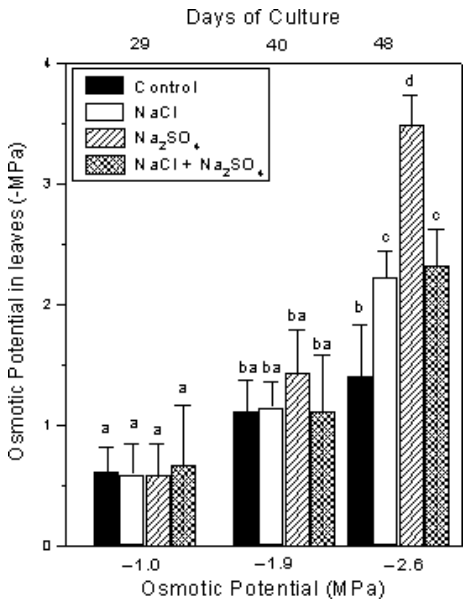

Fig. 5. Effects of NaCl, Na_2SO_4 and their iso-osmotic mixture on osmotic potential in leaves of *Prosopis strombulifera*. Data are mean ± SE. Different letters above bars indicate significant differences among treatments ($P < 0.05$).

tant role in osmotic adjustment (Naidu *et al.* 2000; Tipirdamaz *et al.* 2006). Besides functioning as a compatible solute, proline accumulated during salt stress may be involved in recycling of NADPH (De Ronde *et al.* 2004) or in signalling 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). In rice, proline, NaCl, drought and ABA all induced translation of the dehydrin-encoding *salT* gene (Garcia *et al.* 1997). Using Northern blot analysis, we found that expression of a gene encoding Late Embryogenesis Abundant (LEA) proteins in roots of *P. strombulifera* was inhibited in mild (−1.0 MPa) NaCl treatment but increased in −1.9 or −2.6 MPa Na_2SO_4 treatments (data not shown). We also observed that ABA and ABA–GE accumulated in Na_2SO_4-treated plants. This response suggests that ABA is involved in regulation of the *LEA* gene, and that the expression of this gene reflects stress levels as sensed by the root system (Llanes 2010). However, proline accumulation in *P. strombulifera* does not appear to be related to the enhanced expression of LEA proteins. Ghars *et al.* (2007) 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, Na_2SO_4 or bi-saline mixture). Thus the proline level may be more of a stress intensity signal rather than a tolerance indicator.

Studies of several other plant species have shown a linear relationship between leaf osmotic potential and glycine betaine content under salt stress (Waditee *et al.* 2005). However, glycine betaine did not accumulate in salt-stressed *P. strombulifera* in the present study and therefore is not related to salt tolerance in this species.

Total carbohydrate content

Total content of carbohydrates, like other cellular constituents in plants, is often affected by environmental stress. In the present study, total soluble carbohydrate content increased under all three salt treatments. Similarly, Prado *et al.* (2000) reported that NaCl treatment of the grain food crop quinoa (*Chenopodium quinoa*) resulted in an increase of soluble carbohydrates and reduced osmotic potential of shoots. Total soluble carbohydrate content of *P. strombulifera* increased in direct correlation with salt concentration, and this effect was stronger in roots than in leaves. This observation is consistent with our previous work on maintaining or inducing root elongation at low osmotic potentials and the increase of fibrous or lignified tissues as an adaptive response to salt stress (Reinoso *et al.* 2004, 2005), suggesting an alteration in carbon partitioning within the plant.

Leaf content of glucose and fructose was not affected by salt treatment in *P. strombulifera*. Root content of these monosaccharides increased at moderate or high salinity, particularly in Na_2SO_4-treated plants, indicating translocation of monosaccharides from leaves to roots. This process may reflect the need for a substrate for polyalcohol synthesis, or for lignin synthesis in the case of Na_2SO_4-treated plants, whose primary roots are characterised by precocious secondary vascular tissue differentiation with highly lignified elements (Reinoso *et al.* 2005).

Accumulation of the disaccharide sucrose plays a role in osmotic adjustment and may result from increased starch hydrolysis and/or sucrose synthesis. Sucrose may act as an osmoprotectant, maintaining cell turgidity and stabilising membranes against osmotic stress factors such as increased salinity (Cooper & Farrant 2002). The decreased disaccharide level in roots of Na_2SO_4-treated *P. strombulifera* at −2.6 MPa (present study) and general inhibition of growth at −1.9 MPa (Reinoso *et al.* 2005) illustrate the detrimental effect of the sulphate ion on sucrose synthesis and transport.

Polyalcohol accumulation

In the present study, synthesis of polyalcohols appeared to be regulated by the concentration of salts in the medium. Root mannitol and sorbitol levels were unaffected by salt treatments, whereas leaf mannitol content was increased in −1.9 and −2.6 MPa NaCl, and leaf sorbitol content was increased after Na_2SO_4 treatment. One possible interpretation of these findings is that *P. strombulifera* uses mannitol for osmotic adjustment under NaCl stress, but synthesises sorbitol in the presence of Na_2SO_4, perhaps because of a disorder in carbon metabolism related to the toxic effect of sulphate. Mannitol is synthesised in many plant species and may accumulate under low water potentials. Transgenic *Arabidopsis* plants that accumulated mannitol under salt stress showed increased growth (Chen & Murata 2002). Salt-stressed *Apium graveolens* (celery) accumulated mannitol in the same proportion as sucrose, and the two together comprised >50% of translocated photosynthesised molecules (Abebe *et al.* 2003). Although the function of mannitol accumulation has not been well studied in halophytes, a possible role of this compound at moderate or high NaCl concentrations in this study may be to increase salt tolerance through protective mechanisms, *e.g.*

stabilisation of macromolecular structures, and promotion of scavenging systems for reactive oxygen species, as proposed by Noiraud *et al.* (2001).

Transgenic tobacco plants, in comparison to the wild type, accumulated sorbitol and showed increased salt tolerance. When sorbitol accumulation was excessive, however, plants had necrotic spots on young leaves, reduced chlorophyll levels and reduced rates of growth and development. Leaf sucrose synthesis was reduced in salt-stressed transgenic plants, perhaps because of competition from sorbitol production for glucose-6-phosphate required for activation of sucrose-phosphate synthase (Shelevela *et al.* 1998). The function of sorbitol accumulation in halophytes is not well studied. In the present study, accumulated sorbitol may have acted as an osmolyte in −1.0 and −1.9 MPa Na_2SO_4- and in bi-saline-treated plants, but had detrimental effects in −2.6 MPa Na_2SO_4-treated plants, possibly through reducing the synthesis and transport of carbohydrates essential for growth.

In the halophyte *Mesembryanthemum crystallinum* (ice plant), myo-inositol is converted to D-pinitol and D-ononitol, which are accumulated under salt stress. *P. strombulifera* does not accumulate inositol compounds; rather, inositol is rapidly converted to pinitol, which accumulates under salt stress. Salt-stressed *M. crystallinum* showed increased transcription of genes encoding a Na^+/myo-inositol co-transporter, which may enhance transport of myo-inositol from leaf to root in the phloem, and of Na^+ from the root to shoot in the xylem. In *P. strombulifera* myo-inositol may not be involved in root-to-shoot transport of Na^+, since its level is reduced in both organs under salt stress. Perhaps the majority of myo-inositol is used to form methylated pinitol, and a small amount is used as a precursor compound in production of signalling molecules, cell wall components, membrane lipids, ceramides, storage products and phosphate conjugates of hormones.

Leaf and root pinitol levels in *P. strombulifera* increased under not only the NaCl, but also Na_2SO_4 and bi-saline treatment, suggesting that pinitol is the main carbohydrate produced in response to salt stress. In a study of drought-tolerant and sensitive lines of white clover (*Trifolium repens*), McManus *et al.* (2000) observed pinitol accumulation under drought stress. Since the sensitive genotype accumulated pinitol but did not survive drought, these authors suggested that successful adaptation to stress in the tolerant genotype resulted from other plant characteristics acting synergistically with the increase in pinitol. This concept is consistent with our findings and indicates that pinitol would not be a marker for salt tolerance *per se* in *P. strombulifera*.

In conclusion, we propose that the high NaCl tolerance of *P. strombulifera* results from efficient ion compartmentalisation in leaf vacuoles (as demonstrated in our previous results) and increased synthesis of proline, pinitol and mannitol in the cytoplasm, accompanied by normal carbon metabolism to provide the plant skeleton for growth. The results, however, are still inconclusive with respect to the inhibitory effect of sulphate and some alternative interpretations should be considered. When the sulphate anion is present in the medium, the capacities for ion compartmentalisation and osmotic adjustment are reduced, resulting in water imbalance and symptoms of toxicity due to altered carbon metabolism, *e.g.* synthesis of sorbitol instead of mannitol, reduced sucrose production and protein content. Studies are in progress to test the hypothesis that proline + pinitol + mannitol in combination constitute a 'tolerance formula' in this species after NaCl treatment.

Additionally, the complexity of the salt tolerance mechanisms described here compounds the complexity of the ionic interactions that take place among the various salts present in soil. To add to this complexity, each region in each country has its own specific soil salt profile.

Previous results (Llanes 2010) showed that individuals randomly collected from the same native population of *P. strombulifera* used in this work can be defined using a hierarchical approach as belonging to five different genetic groups. Maintenance of high levels of genetic diversity has adaptive significance for several species, and in this case, it may be absolutely necessary for *P. strombulifera* to withstand extreme environmental conditions, such as lack of water, high temperatures and salt toxicity, mainly sulphate toxicity. The present results contribute to better understanding of physiological responses of woody halophytic plants to soil salinity (NaCl + Na_2SO_4) and provide the basis for future molecular studies for generating salt-tolerant, economically important species for salinised soils throughout the world (Shi & Sheng 2005; Manivannan *et al.* 2008).

ACKNOWLEDGEMENTS

This study was supported by funds from CONICET PIP No 5628, PICT-ANPCYT Nº 02232/07, SECYT-Universidad Nacional de Río Cuarto and Ministerio de Ciencia y Tecnología de la Provincia de Córdoba, Argentina to V.L. We thank Dr. Steve Anderson for English editing.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Increasing salt concentrations obtained by sequential addition of pulses every 48 h; i.e. four pulses means 4 × 37.9 ml aliquot of Na2SO4 l^{-1} Hoagland solution.

REFERENCES

Abebe T., Guenzi A., Martin B., Cushman J. (2003) Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. *Plant Physiology*, **131**, 1748–1755.

Anderson D., Del Aguila J., Bernardon E. (1970) *Las formaciones vegetales en la provincia de San Luis*. Revista de Investigación Agropecuaria, INSTA. Biología y Producción Vegetal Serie 2, Volumen 7, Numero 3. Buenos Aires, Argentina.

Ashraf M., Foolad M. (2007) Improving plant abiotic-stress resistance by exogenous application of osmoprotectants glycine betaine and proline. *Environmental and Experimental Botany*, **59**, 206–216.

Bradford M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry*, **72**, 248–254.

Burkart A. (1976) A monograph of the genus *Prosopis* (Leguminosae subfam Mimosoideae). Catalog of the recognized species of Prosopis. *Journal of the Arnold Arboretum*, **57**, 450–525.

Cantero J., Cantero A., Cisneros J. (1996) *La vegetación de los paisajes hidro-halomórficos del centro de Argentina*. Editorial Fundación Universidad Nacional de Río Cuarto, Córdoba, Argentina.

Carosio M., Junqueras M., Endersen A., Fernandez Belmonte M., Martinez Carretero E., Dalmasso A. (2009). *Flora de las Salinas del Bebedero*. Sociedad de Biología de Cuyo, Argentina.

Chen T., Murata N. (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Current Opinion in Plant Biology*, **5**, 250–257.

Chen Z., Cuin T., Zhou M., Twomey A., Naidu B., Shabala S. (2007) Compatible solute accumulation and stress-mitigating effects in barley genotypes contrasting in their salt tolerance. *Journal of Experimental Botany*, **58**, 4245–4255.

Ciha A., Brun W. (1978) Effect of pod removal on nonstructural carbohydrate concentration in soybean tissue. *Crop Science*, **18**, 773–776.

Cooper K., Farrant J. (2002) Recovery of the resurrection plant *Craterostigma wilmsii* from desiccation: protection versus repair. *Journal of Experimental Botany*, **53**, 1805–1813.

De Ronde W., Cress G., Krüger K., Strasser R., Van Staden J. (2004) Photosynthetic response of transgenic soybean plants, containing an Arabidopsis P5CR gene, during heat and drought stress. *Journal of Plant Physiology*, **161**, 1211–1224.

Demiral T., Turkan I. (2006) Exogenous glycine betaine affects growth and proline accumulation and retards senescence in two rice cultivars under NaCl stress. *Environmental and Experimental Botany*, **56**, 72–79.

Garcia A., Engler J., Iyer S., Gerats T., Van Montague M., Caplan A. (1997) Effects of osmoprotectants upon NaCl stress in rice. *Plant Physiology*, **155**, 159–169.

Ghars M., Parre E., Debez A., Bordenave M., Richard L., Leport L. (2007) Comparative salt tolerance analysis between *Arabidopsis thaliana* and *Thellungiella halophila*, with special emphasis on K+/Na+ selectivity and proline accumulation. *Journal of Plant Physiology*, **165**, 588–599.

Gorham J., Hughes L., Wyn-Jones R. (1980) Chemical composition of salt marsh plants from Ynys Môn (Anglesey): the concept of physiotypes. *Plant, Cell and Environment*, **3**, 309–318.

Grieve C., Grattan S. (1993) Rapid assay for determination of water-soluble quaternary-amino compounds. *Plant and Soil*, **70**, 303–307.

Hoagland D., Arnon D. (1950) The water-culture method for growing plants without soil. *California Agricultural Experiment Station Circular*, **347**, 1–32.

Khan A., Kuek C., Chaudhry T., Khoo C., Hayes W. (2000) The role of plants, mycorrhizae, and phytochelators in heavy metal contaminated land remediation. *Chemosphere*, **41**, 197–207.

Khedr A., Abbas M., Wahid A., Quick W., Abogadallah G. (2003) Proline induces the expression of salt-stress-responsive proteins and may improve the adaptation of *Pancratium maritimum* L. to salt-stress. *Journal of Experimental Botany*, **54**, 2553–2562.

Lehmann S., Funck D., Szabados L., Rentsch D. (2010) Proline metabolism and transport in plant development. *Amino Acids*, **39**, 949–962.

Llanes A. (2010) Mecanismos fisiológicos y moleculares de la tolerancia a salinidad en *Prosopis strombulifera*, su correlación con los niveles endógenos de ABA. Ph.D. thesis, Universidad Nacional de Río Cuarto, Córdoba, Argentina.

Llanes A., Reinoso H., Luna V. (2005) Germination and early growth of *Prosopis strombulifera* seedlings in different saline solutions. *World Journal of Agricultural Science*, **1**, 120–128.

Magne C., Larher F. (1992) High sugar content of extracts interferes with colorimetric determination of amino acids and free proline. *Analytical Biochemistry*, **200**, 115–118.

Manivannan P., Jaleel C., Somasundaram R., Panneerselvam R. (2008) Osmoregulation and antioxidant metabolism in drought stressed *Helianthus annuus* under triadimefon drenching. *Comptes Rendus Biologies*, **331**, 418–425.

McManus M., Bieleski R., Carodus J., Barker D. (2000) Pinitol accumulation in mature leaves of white clover in response to water deficit. *Environmental and Experimental Botany*, **43**, 11–18.

Miller G., Suzuki N., Cifti-Yilmaz S., Mittler R. (2010) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant, Cell and Environment*, **33**, 453–467.

Munns R., Tester M. (2008) Mechanisms of salinity tolerance. *Annual Review of Plant Biology*, **59**, 651–681.

Naidu B., Paleg L., Jones G. (2000) Accumulation of proline analogues and adaptation of *Melaleuca* species to diverse environments in Australia. *Australian Journal of Botany*, **48**, 611–620.

Noiraud N., Delrot S., Lemoine R. (2001) The sucrose transporter of celery. Identification and expression during salt stress. *Plant Physiology*, **122**, 1447–1456.

Parida A., Das A., Mittra B., Mohanty P. (2004) Salt-stress induced alterations in protein profile and protease activity in the mangrove *Bruguiera parviflora*. *Zeitschrift für Naturforschung*, **59**, 408–414.

Peña Zubiate C., Anderson D., Demmi M., Saenz J., D'hiriart A. (1998) *Carta de suelos y vegetación de la Provincia de San Luis. Secretaria de Agricultura, Ganadería, Pesca y alimentación*. Instituto Nacional de Tecnología Agrícola, Estación Experimental Agropecuaria San Luis. Gobierno de la Provincia de San Luis, Subsecretaría de Estado de Producción y Desarrollo Agropecuario, Argentina.

Prado F., Boero C., Gallardo M., Gonzalez J. (2000) Effect of NaCl on germination, growth, and soluble sugar content in *Chenopodium quinoa* (Willd.) seeds. *Botanical Bulletin of Academia Sinica*, **41**, 27–34.

Reginato M. (2009) Respuesta de la halófita *Prosopis strombulifera* a diferentes medios salinos. Modificaciones de los parámetros morfofisiológicos y su regulación hormonal. Ph.D. thesis, Universidad Nacional de Río Cuarto, Córdoba, Argentina.

Reginato M., Sgroy M., Llanes A., Cassán F., Luna V. (2012) The American halophyte *Prosopis strombulifera,* a new potential tool to confer salt tolerance to crops. In: Ashraf M., Öztürk M., Ahmad M.S.A., Aksoy A. (Eds), *Crop production for agricultural improvement*. Springer, Berlin, pp 136–167.

Reinoso H., Sosa L., Ramírez L., Luna V. (2004) Salt-induced changes in the vegetative anatomy of *Prosopis strombulifera* (Leguminosae). *Canadian Journal of Botany*, **82**, 618–628.

Reinoso H., Sosa L., Reginato M., Luna V. (2005) Histological alterations induced by sodium sulfate in the vegetative anatomy of *Prosopis strombulifera* (Lam.) Benth. *World Journal of Agricultural Science*, **1**, 109–119.

Rosen H. (1957) A modified ninhydrin colorimetric analysis for amino acid. *Archives of Biochemistry and Biophysics*, **67**, 10–15.

Sayar R., Khemira H., Kameli A., Mosbahi M. (2008) Physiological tests as predictive appreciation for drought tolerance in durum wheat (*Triticum durum* Desf.). *Agronomy Research*, **6**, 70–90.

Shelevela E., Marquez S., Chmara W., Zegeer A., Jensen R., Bohnert H. (1998) Sorbitol-6-phosphate dehydrogenase expression in transgenic tobacco: high amounts of sorbitol lead to necrotic lesions. *Plant Physiology*, **117**, 831–839.

Shi D., Sheng Y. (2005) Effect of various salt-alkaline mixed stress conditions on sunflower seedlings and analysis of their stress factors. *Environmental and Experimental Botany*, **54**, 8–21.

Silveira J., Melo A., Viégas R., Oliveira J. (2001) Salinity-induced effects on nitrogen assimilation related to growth in cowpea plants. *Environmental and Experimental Botany*, **46**, 171–179.

Silveira J., Araújo S., Santos Lima J., Almeida Viégas R. (2009) Roots and leaves display contrasting osmotic adjustment mechanisms in response to NaCl-salinity in *Atriplex nummularia*. *Environmental and Experimental Botany*, **66**, 1–8.

Sosa L. (2005). Adaptaciones fisiológicas de *Prosopis strombulifera* a condiciones de salinidad por cloruros y sulfatos. Ph.D. thesis, Universidad Nacional de Río Cuarto, Córdoba, Argentina.

Sosa L., Llanes A., Reinoso H., Reginato M., Luna V. (2005) Osmotic and specific ion effects on the germination of *Prosopis strombulifera*. *Annals of Botany*, **96**, 261–267.

Tipirdamaz R., Gagneul D., Duhaze C., Aınouche A., Monnier C., Ozkum D., Larher F. (2006) Clustering of halophytes from an inland salt marsh in Turkey according to their ability to accumulate sodium and nitrogenous osmolytes. *Environmental and Experimental Botany*, **57**, 139–153.

Türkan L., Demiral T. (2009) Recent developments in understanding salinity tolerance. *Environmental and Experimental Botany*, **67**, 2–9.

Verbruggen N., Hermans C. (2008) Proline accumulation in plants: a review. *Amino Acids*, **35**, 753–759.

Waditee R., Bhuiyan N., Rai V., Aoki K., Tanaka Y., Hibino T., Suzuki S., Takano J., Jagendorf A., Takabe T. (2005) Genes for direct methylation of glycine provide high levels of glycine betaine and abiotic-stress tolerance in *Synechococcus* and *Arabidopsis*. *Proceedings of the National Academy of Sciences USA*, **102**, 1319–1323.