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RESEARCH PAPER

Growth responses and ion accumulation in the halophytic legume *Prosopis strombulifera* are determined by Na_2SO_4 and NaCl

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ABSTRACT

Halophytes are potential gene sources for genetic manipulation of economically important crop species. This study addresses the physiological responses of a widespread halophyte, *Prosopis strombulifera* (Lam.) Benth to salinity. We hypothesised that increasing concentrations of the two major salts present in soils of central Argentina (Na_2SO_4, NaCl, or their iso-osmotic mixture) would produce distinct physiological responses. We used hydroponically grown *P. strombulifera* to test this hypothesis, analysing growth parameters, water relations, photosynthetic pigments, cations and anions. These plants showed a halophytic response to NaCl, but strong general inhibition of growth in response to iso-osmotic solutions containing Na_2SO_4. The explanation for the adaptive success of *P. strombulifera* in high NaCl conditions seems to be related to a delicate balance between Na^+ accumulation (and its use for osmotic adjustment) and efficient compartmentalisation in vacuoles, the ability of the whole plant to ensure sufficient K^+ supply by maintaining high K^+/Na^+ discrimination, and maintenance of normal Ca^{2+} levels in leaves. The three salt treatments had different effects on the accumulation of ions. Findings in bi-saline-treated plants were of particular interest, where most of the physiological parameters studied showed partial alleviation of SO_4^{2-}-induced toxicity by Cl^-. Thus, discussions on physiological responses to salinity could be further expanded in a way that more closely mimics natural salt environments.

INTRODUCTION

Salinity is an expanding problem, and has posed a threat to agricultural production in certain parts of the world for over 3000 years. As the world population continues to expand, so does the need to grow more food. This requires an increase in land area under cultivation, as well as in land productivity (yield ha^{-1}; Flowers & Flowers 2005). Arid and semiarid regions constitute roughly one-third of the world's land surface. In view of ongoing human population growth and land degradation, genetic improvement of salt tolerance in crop plants has become a critical need for the future of agriculture (Munns 2007).

The physiology and genetics of salt tolerance are highly complex. Various approaches have been used to generate salt-tolerant crops, but no single approach offers a universal solution (Flowers & Flowers 2005; Munns 2007). Hence, functional analysis of the specialised physiology and biochemistry of halophytes (*i.e.* plant species that have evolved a high degree of salt tolerance) requires increasing research attention because our knowledge in this area remains quite limited.

Several genera of the Fabaceae subfamily Mimosoideae are found in arid and semiarid regions. The genus *Prosopis* includes many important arboreal and shrub-like species that are major components of various ecosystems and high salinity areas of North and South America, having the unique ability to fix nitrogen and grow in such habitats (Burkart 1976). This genus includes about 44 species grouped in five sections and eight series (Burkart 1976). Many of the species, particularly those in the Algarobia section, have economic and ecological importance, providing shade, firewood, food and forage for wildlife and livestock (Felker 2007). Populations of some species, particularly *P. pallida*, *P. juliflora*, *P. tamarugo* and *P. alba*, include individuals that display rapid growth at seawater salinity (approximately 45 $dS \cdot m^{-1}$). This is nearly 20 times the maximum salinity that can be tolerated by temperate annual legumes (Felker 2007). The spiny shrub *P. strombulifera* (Lam.) Benth. (Burkart 1976) occurs from the Arizona Desert (USA) to Patagonia (Argentina), and is particularly abundant in high salinity areas of central Argentina (Cordoba and southwest San Luis provinces). In these high salinity soils, the proportions of NaCl and Na_2SO_4 salts are generally similar, although Na_2SO_4 is up to three times more abundant in certain samples (Sosa *et al.* 2005). Similarly, in many countries, NaCl and Na_2SO_4 are the most abundant salts (Iqbal 2003; Shi & Sheng 2005; Manivannan *et al.* 2008). Hence, it is important to

compare the effects of these two salts on plant growth in order to better understand the physiological responses of plants.

Comparative studies have shown that SO_4^{2-} -based solutions have a considerably stronger inhibitory effect on *P. strombulifera* germination than Cl^--based solutions at iso-osmotic concentrations (Llanes *et al.* 2005; Sosa *et al.* 2005). *Prosopis strombulifera* grown in an increasing gradient of NaCl (250–700 $mM \cdot l^{-1}$) do not develop salt glands in the leaves. Some tissues display vacuolisation, and the root system undergoes precocious lignification and/or suberisation of endodermal cells, with Casparian strips found much closer to the root tip than in glycophytes. These plants can therefore more efficiently filter soil solution to prevent passage of excess ions into the xylem (Reinoso *et al.* 2004). Na_2SO_4 treatment of *P. strombulifera* induced structural alterations in cells and tissues, with consequent changes in growth patterns at various levels of organisation, and anatomical and histological differences in roots, stems and leaflets, compared to control plants or plants grown in high NaCl (Reinoso *et al.* 2005). These results demonstrate that plant responses may vary depending on the anion associated with sodium. In others species, including *Hordeum vulgare* (Huang & Redmann 1995), *Triticum aestivum* (Hampson & Simpson 1990) and *Pinus banksiana* (Croser *et al.* 2001), Na_2SO_4 had a stronger inhibitory effect than NaCl. On the other hand, species including *Brassica napus* (Huang & Redmann 1995) and *Kochia scoparia* (Curtin *et al.* 1993) showed the opposite effect. Yeo & Flowers (1980) reported that growth in terms of organic dry matter production in the halophyte *Suaeda maritima* at high (340 $mM \cdot l^{-1}$) salt concentrations differed in relation to the salt supplied, indicating some degree of ion specificity at such concentrations, with maximum production of organic dry matter occurring with NaCl, in the sequence $Cl^- > SO_4^{2-} > NO_3^-$. These seemingly contradictory findings may reflect a difference in ability to maintain charge balance and sufficient Ca^{2+} in tissues, which are important factors in salt tolerance (Curtin *et al.* 1993).

In view of our previous findings on *P. strombulifera* seed germination (Sosa *et al.* 2005), and taking into account that this species is a useful model for elucidation of salt tolerance mechanisms in halophytes, we hypothesised that different salt compositions (Na_2SO_4, NaCl or their iso-osmotic mixture) would produce distinct physiological responses. We used hydroponically grown *P. strombulifera* to test this hypothesis.

MATERIAL AND METHODS

Plant material and growth conditions

Prosopis strombulifera seeds were collected from an area in southwest San Luis province, Argentina (33°43′ S, 66°37′ W, 400–500 m.a.s.l.), with a temperate climate (average annual temperature 15–20 °C). This is predominantly a *Prosopis alba* forest located in a saline depression between annual 300–400 mm isohyets in the El Monte phytogeographic region. The soil is saline–sodic with abundant calcareous material and moderate salinity (8 $dS \cdot m^{-1}$ electrical conductivity at the surface and 10 $dS \cdot m^{-1}$ at 25–35 cm depth), with a sandy-loam texture and pH 7.5. The major cation in the soil is Na^+ and the major anion is SO_4^{2-}.

Pods were collected randomly from 100 plants in the population. Seeds were visually selected on the basis of uniform size and generally good health, scarified with 98% H_2SO_4 for 10 min, washed overnight under running water, rinsed in distilled water, and germinated in Petri dishes with two layers of water-saturated filter paper at 37 °C for 24 h (Reinoso *et al.* 2004). Germinated seedlings with roots approximately 20-mm long were grown in hydroponic conditions in black trays (28 × 22 × 10 cm; 200 seedlings per tray) with 10% full-strength Hoagland's solution. Seedlings were self-supported in small holes on the tray cover until the end of the experiment. The trays were placed in a growth chamber (Conviron E15; Controlled Environments Ltd., Winnipeg, Manitoba, Canada) with a cycle of 16-h light (200 $\mu M \cdot m^{-2} \cdot s^{-1}$; 28 °C): 8-h dark (20 °C), relative humidity 70%. After 1 week, the nutrient solution was changed to 25% Hoagland's solution (osmotic potential (Ψo = −0.11 MPa). After this, the nutrient solution was changed each week to maintain adequate nutrient availability. Aeration was provided by an aquarium tubing system with a peristaltic pump. The medium was maintained at pH 6. The experiment was performed four times (two trays per treatment each time).

Salt treatment

A simple randomised design with four treatments was used (Control, NaCl, Na_2SO_4, NaCl + Na_2SO_4). After 21 days growth in Hoagland's solution, salt treatment was initiated by adding NaCl and/or Na_2SO_4 (50 $mM \cdot l^{-1}$ and 38 $mM \cdot l^{-1}$, respectively) every 48 h until reaching final Ψo values of −1,−1.9 and−2.6 MPa for monosaline and salt mixture treatments (confirmed using a vapour pressure osmometer, Model 5500; Wescor Inc., Logan, UT, USA). The salt mixture solution was made by mixing equal volumes of the monosaline solutions of the corresponding osmotic potentials (Table 1). At each interval, 70 control plants (no salt added, Ψo =

Table 1. Increasing salt concentrations obtained by sequential addition of pulses every 48 h. *i.e.* four pulses are 4 × 37.9 ml aliquots of Na_2SO_4 l^{-1} Hoagland solution. Bold numbers indicate the sampling date/collection of plants.

salt pulse	ml Na_2SO_4 l^{-1} Hoagland	ml NaCl l^{-1} Hoagland	salt mixture ml Na_2SO_4 + ml NaCl l^{-1} Hoagland	ψ_o
1°pulse	37.9	50	18.9/25	−0.3
2°pulse	75.8	100	37.9/50	−0.47
3°pulse	113.7	150	56.8/75	−0.65
4°pulse	151.7	200	75.9/100	−0.82
5°pulse (sampling)	189.7	**250**	94.8/125	**−1.0**
6°pulse	227.5	300	113.8/150	−1.18
7°pulse	265.4	350	132.7/175	−1.35
8°pulse	303.3	400	151.7/200	−1.53
9°pulse	341.2	450	170.6/225	−1.71
10°pulse (sampling)	379.2	**500**	189.6/250	**−1.9**
11°pulse	417.1	550	208.5/275	−2.06
12°pulse	455.0	600	227.5/300	−2.24
13°pulse	492.9	650	246.4/325	−2.42
Last pulse (sampling)	530.8	**700**	265.4/350	**−2.6**

−0.11 MPa) and 70 treated plants were collected at random from each tray (sufficient to determine fresh weight (FW) and dry weight (DW) and to perform all the biochemical analyses), frozen in liquid nitrogen and stored at −80 °C for *a posteriori* analysis. Upon collection, roots were washed three times with deionised water to eliminate excess salts, until no ions were detected in the final wash.

Measurements of growth and water potential

Leaf number, root length and shoot height were measured each week in 30 plants from each treatment, from the time that salt pulses were started (−0.3, −0.65, −1.18, −1.71, −2.24, −2.6 MPa). Twenty-four hours after the desired Ψo was reached shoot and root FW were determined. DW was determined after 48 h of sample lyophilisation. DW percentage was calculated as: % $W_d = (W_d/W_f) \times 100$, where W_d is sample DW and W_f is sample FW (mg).

Total leaf area was determined from three plants of each treatment. Individual leaf area was determined from the fourth and fifth leaves of the same three plants. A Hewlett Packard flat scanner was used to scan each leaf, and images were processed using the Image-Pro Plus 4.1 Software (SDK, version 6; Media Cybernetics, Rockville, MD, USA).

The shoot water potential was measured according to the method described in Scholander *et al.* (1965) using a pressure chamber (Model 10; Bio-Control, Buenos Aires, Argentina), for which 12 plants per treatment were collected at the same time (afternoon) from the growth chamber.

Photosynthetic pigment analysis

Pigment concentrations were determined according to the method of Castagna *et al.* (2001). Frozen samples were homogenised in the dark in 100% HPLC-grade acetone with 1 mM sodium ascorbate then filtered through 0.2-μm filters. The analysis was performed with HPLC (HPLC P200; Thermo Fisher Scientific, Waltham, MA, USA) using a non-end-capped column (Zorbax ODS column; Chrompack, Raritan, NJ, USA) for pigment separation. Two solvents were used: A (acetonitrile/methanol, 75/25, v/v) and B (methanol/ethylacetate, 68/32, v/v). The separation cycle was 1920 s with a flow rate of 16.67 $mm^3 \cdot s^{-1}$. Pigments were eluted using 100% A for the first 900 s, followed by a 150-s linear gradient to 100% B, which continued isocratically until the end of the cycle. The column was allowed to re-equilibrate in 100% solvent A for 600 s before the next injection. Pigments were detected from their absorbance at 445 nm, and quantification was realised by injection of known amounts of pure standard into the HPLC system and the equation correlating peak area to pigment concentration; the latter was expressed as $nM \cdot g^{-1}$ DW.

Determination of inorganic ions

Samples containing 200 mg DW were ground in a mortar with liquid nitrogen, digested with concentrated HNO_3 at 200 °C, and dissolved in deionised water to a final volume appropriate for the standard curve. Contents of Na^+ and K^+ were determined by flame photometry and confirmed with atomic absorption spectrometry (Skoog *et al.* 1995); Ca^{2+} by atomic absorption spectrometry; SO_4^{2-} by the turbidimetric method (Patnaik 1997); and NO_3^- using a colorimetric method (Yadav 1997). The Cl^- content was determined by Volhard's titration following digestion in 0.1 M HNO_3/10% acetic acid for 24 h (Skoog *et al.* 1995).

Statistical analysis

Data were analysed using the InfoStat program (2011). 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 (o) (−1.0, −1.9 or −2.6 MPa); and (ii) treatment (control, NaCl, Na_2SO_4, salt mixture). Normality was verified with the Shapiro–Wilk test. Homogeneity of variance was verified with Levene test. When necessary, data were transformed to meet the assumptions of ANOVA. For cases in which normality and homogeneity of variance were not verified, the non-parametric Kruskall–Wallis test was used. Bonferroni test was used as *post-hoc* analysis to determine differences between means. P values < 0.05 were considered statistically significant.

RESULTS

Growth parameters and water potential

The NaCl treatment resulted in a significant increase ($P < 0.05$) in shoot height (25%) at low and moderate concentrations (Ψo = −0.7, 1.18 MPa; 150, 300 $mM \cdot l^{-1}$), but decreased shoot height at higher concentration (Ψo = −2.24 MPa; 600 $mM \cdot l^{-1}$; Fig. 1A). Compared to control plants, NaCl-treated plants were more vigorous, had more leaves and harder and darker spines. At the highest NaCl concentration tested (Ψo = −2.6 MPa; 700 $mM \cdot l^{-1}$), a 15% reduction in shoot height was observed (Fig. 1A).

The Na_2SO_4 treatment significantly inhibited shoot height relative to control and NaCl−treated plants at moderate concentration (Ψo = −1.71 MPa). This effect became more pronounced as the concentration increased (55% inhibition at the end of the experiment) and caused visible toxicity symptoms: chlorosis, necrosis and, finally, leaf abscission in surviving plants (90% at end of experiment; Fig. 1A). In the experiment with iso-osmotic bi-saline solution (NaCl and Na_2SO_4 in combination; Fig. 1A), the growth curve was intermediate between those with the two mono-saline solutions (25% shoot height inhibition at the end of the experiment).

Leaf number increased 10% with NaCl treatment up to Ψo = −1.18 MPa, and then abruptly declined at higher concentrations (20% decrease at the end of the experiment). In the Na_2SO_4-treated plants, leaf number was reduced even at Ψo = −1.18 MPa, and the reduction reached 65% at the highest concentration (Ψo = −2.6 MPa). Application of bi-saline solution partially alleviated this effect, with a final reduction similar to that after NaCl alone (20%; Fig. 1B).

Root length in NaCl-treated plants showed a 20% increase between Ψo = −1.18 MPa and Ψo = −2.24 MPa, and then declined slightly at Ψo = −2.6 MPa, although it remained higher that in controls. In the Na_2SO_4-treated plants, root length underwent a significant reduction at Ψo = −1.71 MPa and lower, reaching 40% at Ψo = −2.6 MPa. With bi-saline

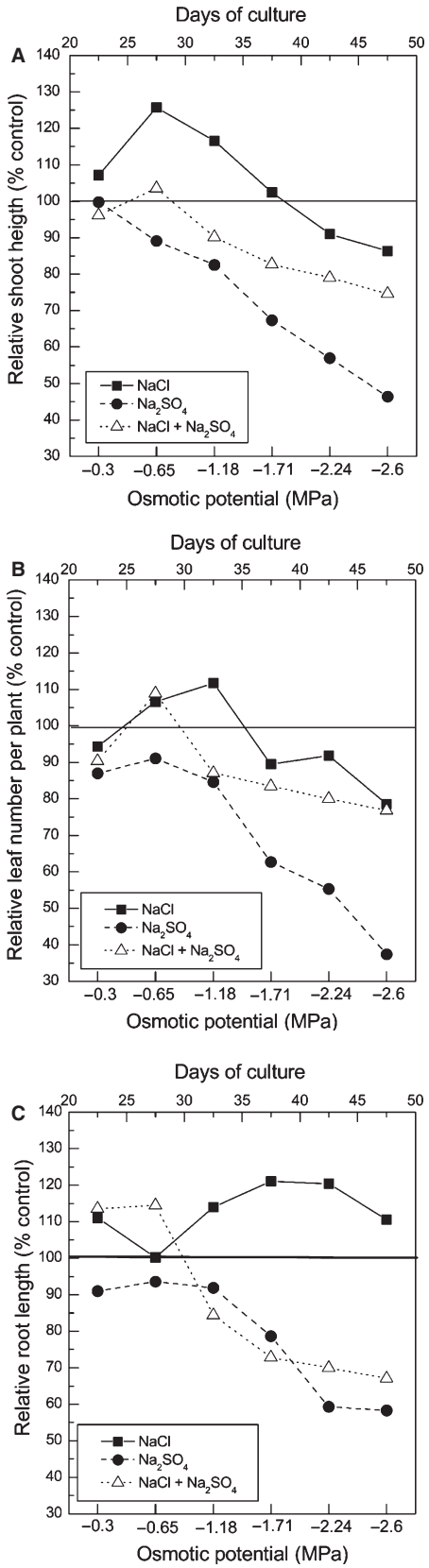

Fig. 1. Effects of NaCl, Na_2SO_4 and their iso-osmotic mixture on growth of *Prosopis strombulifera*. A: relative shoot height; B: relative leaf number; C: relative root length; Horizontal line at 100% represents growth of control plants (n = 6).

solution, root length increased slightly between Ψo = −0.3 and −0.65 MPa, and then steadily declined (30% reduction at Ψo = −2.6 MPa; Fig. 1C).

Shoot DW accumulation was not significantly affected by any salt treatment at Ψo = −1.0 MPa, but increased at Ψo = −1.9 MPa and below, particularly at high concentrations (Ψo = −2.6 MPa) in NaCl and Na_2SO_4-treated plants (Fig. 2A). Root DW accumulation increased only at −2.6 MPa in all salt treatments (Fig. 2B).

Total leaf area was affected at moderate salinity (Ψo = −1.9 MPa), being significantly lower in Na_2SO_4-treated plants, with a 60% decrease at Ψo = −2.6 MPa in relation to controls (Fig. 3A). Individual leaf area showed a significant decrease (30%) only after Na_2SO_4 treatment at Ψo = −2.6 MPa (Fig. 3B).

Shoot water potential was severely decreased in all salt treatments at all osmotic potentials tested. At Ψo = −1.9 MPa, NaCl-treated plants had a water potential more negative than

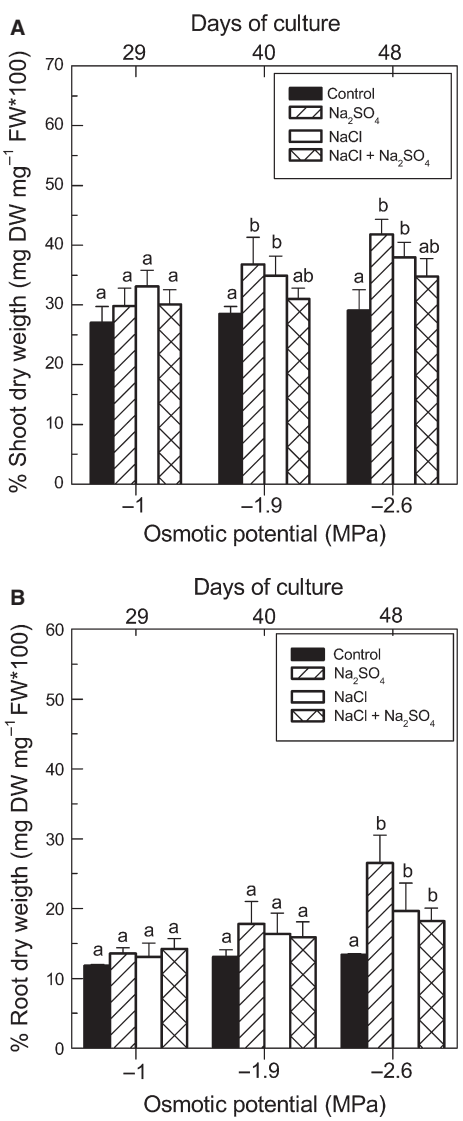

Fig. 2. Effects of NaCl, Na_2SO_4 and their iso-osmotic mixture on DW of *Prosopis strombulifera*. A: Shoot DW; B: root DW. Means (± SE) followed by different letters above bars are significantly different at $P < 0.05$ (n = 4).

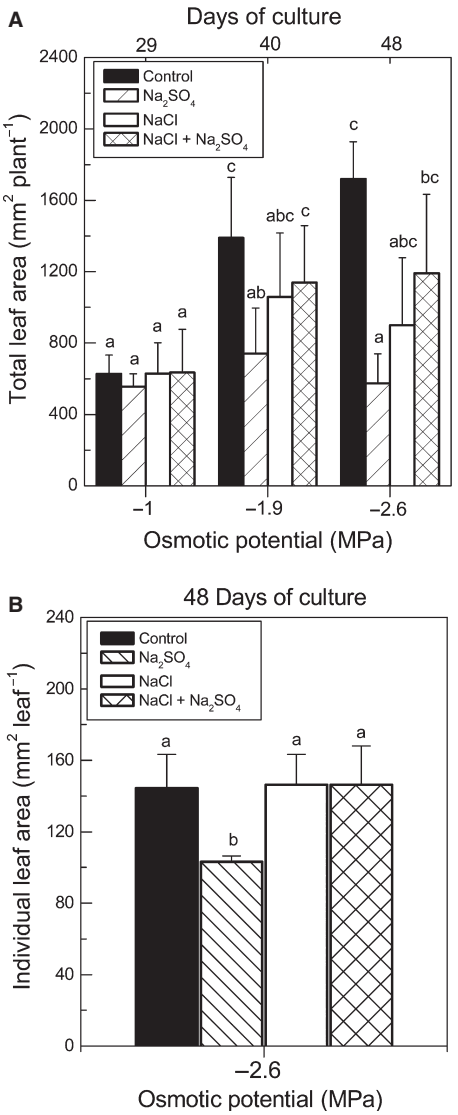

Fig. 3. Effects of NaCl, Na_2SO_4 and their iso-osmotic mixture on leaf area of *Prosopis strombulifera*. A: total leaf area; B: individual leaf area. Means (± SE) followed by different letters above bars are significantly different at $P < 0.05$ (n = 4).

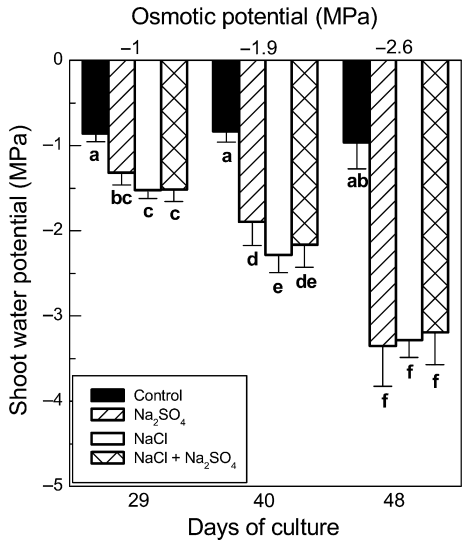

Fig. 4. Effects of NaCl, Na_2SO_4 and their iso-osmotic mixture on shoot water potential of *Prosopis strombulifera*. Means (± SE) followed by different letters above bars are significantly different at $P < 0.05$ (n = 10).

Na_2SO_4-treated plants. At higher salinity (Ψo = −2.6 MPa), shoot water potential continued to decrease up to −3.35 MPa in all salt treatments (Fig. 4).

Photosynthetic pigments

Chlorophyll *a* and *b* concentrations were not significantly affected by any of the salt treatments at Ψo = −1.0 and −1.9 MPa. However, both chlorophyll *a* and *b* decreased significantly in Na_2SO_4-treated plants at Ψo = −2.6 MPa (Fig. 5A,B). Carotenoid concentrations were not significantly affected by any salt treatment (data not shown).

Inorganic ions

Sodium (Na^+) concentrations in leaves of salt-treated plants were consistently higher than in control plants at −1.9 MPa, increasing from 0.22 $mM \cdot g^{-1}$ DW at Ψo = −1.0 MPa to 0.6 $mM \cdot g^{-1}$ DW at Ψo = −2.6 MPa (Fig. 6A). The Na^+ concentration in roots was higher for Na_2SO_4- and bi-saline-treated plants than for NaCl-treated plants at Ψo = −1.0 MPa (Fig. 6B). At Ψo = −2.6 MPa, no difference was observed among the three salt treatments, but they each had Na^+ concentration that were higher than in the control roots, reaching a final concentration of 0.7 $mM \cdot g^{-1}$ DW in Na_2SO_4-treated plants.

Potassium (K^+) concentration in leaves was not altered by any of the three salt treatments at Ψo = −1.0 or −1.9 MPa, but was consistently reduced in Na_2SO_4- and bi-saline-treated plants at Ψo = −2.6 MPa (Fig. 6C). The K^+ concentration in roots was significantly reduced only in Na_2SO_4-treated plants at Ψo = −1.9 and −2.6 MPa (Fig. 6D).

Calcium (Ca^{2+}) concentration in leaves was significantly reduced, compared to controls, in all three salt treatments at Ψo = −1.0 MPa. At Ψo = −1.9 and −2.6 MPa, this reduction was maintained for Na_2SO_4- and bi-saline-treated plants, but disappeared in NaCl-treated plants, which maintained similar concentrations of this cation to control plants (Fig. 6E). In contrast, the Ca^{2+} concentration in roots was more affected by salinity, with significant reductions for all salt treatments at all Ψo values (Fig. 6F).

Chloride (Cl^-) concentration in both leaves and roots consistently increased in NaCl- and bi-saline-treated plants, particularly at Ψo = −2.6 MPa, reaching a maximum concentration of 0.023 $mM \cdot g^{-1}$ DW in leaves of NaCl-treated plants. In Na_2SO_4-treated plants, this parameter was not significantly different from control plants (Fig. 7A, B).

Sulphate (SO_4^{2-}) concentration strongly increased in Na_2SO_4-treated plants, principally in leaves, even at low salt concentrations (Ψo = −1.0 MPa). In bi-saline-treated plants, SO_4^{2-} also increased in relation to controls at Ψo = −1.9 and −2.6 MPa, but to a far lesser extent than in Na_2SO_4-treated plants (Fig. 7C, D). The SO_4^{2-} concentration in roots also increased in Na_2SO_4-and bi-saline-treated plants from Ψo = −1.9 MPa (Fig. 7D).

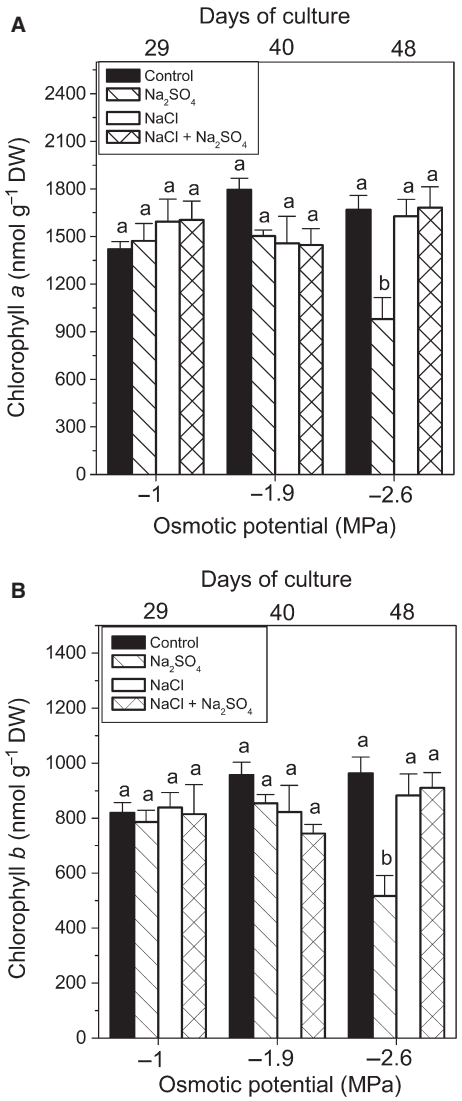

Fig. 5. Effects of NaCl, Na_2SO_4 and their iso-osmotic mixture on content of photosynthetic pigments of *Prosopis strombulifera*. A: chlorophyll *a*; B: chlorophyll *b*. Means (± SE) followed by different letters above bars are significantly different at $P < 0.05$ (n = 4).

Nitrate (NO_3^-) concentrations in leaves and roots were not affected by any of the salt treatments or at any Ψo value (data not shown).

DISCUSSION

In this study, we observed considerable variability in the response of *Prosopis strombulifera* to salinity, depending on the type of salt(s) used and osmotic potential (Ψo) of the culture medium. Stimulation of shoot growth at Ψo values up to −1.9 MPa (500 mM) NaCl is an interesting halophytic response, distinct from findings in other *Prosopis* species (Felker 2007). Several studies indicate that the NaCl tolerance of *P. strombulifera* exceeds the limits described for most other halophytes (Catalán *et al.* 1994; Almeida Viégas *et al.* 2004). However, *P. strombulifera* was much less tolerant to Na_2SO_4 than NaCl. Plants grown in the presence of Na_2SO_4 showed an immediate and sustained reduction in shoot height and leaf number per plant, accompanied by senescence symptoms, such as chlorosis, necrosis and leaf abscission (Reinoso *et al.* 2005). This salt at Ψo = −2.6 MPa also caused a significant reduction in chlorophyll *a* and *b*, while in the other salt treatments no photosynthetic pigments were altered. In contrast, Egan & Ungar (1998) observed an increase in shoot growth after Na_2SO_4 treatment in *Atriplex prostata* seedlings, and inhibition of growth in the presence of iso-osmotic NaCl solutions. In a study of the halophyte *Chenopodium rubrum*, Warne *et al.* (1990) reported growth inhibition at Ψo = −1.6 MPa with SO_4^{2-} -containing solutions, and at Ψo < −2.0 MPa with Cl^--containing solutions. In the halophyte *Suaeda maritima*, Yeo & Flowers (1980) reported that growth at high concentrations (340 mM·l^{-1}) was maximal in NaCl, decreased with other salts, and led to toxicity in KCl.

An important finding of the present study is the partial alleviation of SO_4^{2-} toxicity when Cl^- is present in the medium, as shown in the growth parameters for *P. strombulifera* cultured in the bi-saline solution, and consistent with our previous studies on germination (Llanes *et al.* 2005; Sosa *et al.* 2005). Stimulation of root growth was also observed in NaCl-treated plants; this is considered an important feature of plant adaptation to water-limited conditions that helps to maintain an adequate plant water supply (Sharp & Davies 1989; Ober & Sharp 2007). The SO_4^{2-} anion had a more detrimental effect on root growth than the Cl^- anion, and this inhibitory effect was partially alleviated when both anions were present in the bi-saline solution.

We observed that inhibition of shoot and root growth in Na_2SO_4-treated plants was not correlated with dry matter accumulation, since DW increased at Ψo = −1.9 and −2.6 MPa. This suggests that although low water potential and ionic toxicity strongly affected cell growth in this species, the photosynthetic apparatus did not lose its efficiency and was still able to provide carbon skeletons necessary for formation of new parenchyma cell layers in the stem, as well as fibres and new synthesis of lignin, suberin and tannins in stems and roots, where concentrations increased in proportion to increases in salinity (Reinoso *et al.* 2005). Presence of these complex high-molecular weight compounds would lead to increases in total DW of treated seedlings, particularly those treated with Na_2SO_4. The influence in total ion concentrations on dry matter accumulation was negligible in our experiments, since its magnitude was similar in NaCl− and Na_2SO_4-treated plants. Thus, an increase of DW is not a useful experimental or field parameter for measuring growth in plants of this type.

Prosopis strombulifera controls the entrance of ions into its tissues, as expected from the specialised characteristics of the root system, *e.g.* precocious lignification and suberisation of the endodermis (Reinoso *et al.* 2004, 2005), to prevent excess salts from entering tissues at the root level, similar to several other economically important *Prosopis* species (Felker 2007). This mechanism allows more control of apoplastic salt delivery to the xylem in mature roots, but does not preclude the possibility of entry *via* a symplastic route or through the root tip. Reduced net Na^+ uptake by roots and increased Na^+ sequestration in shoots work in parallel in *P. strombulifera*.

Halophytes must be able to select K^+ from a mixture dominated by Na^+ and yet still accumulate sufficient Na^+ for osmotic adjustment (Flowers & Colmer 2008). The Na^+ con-

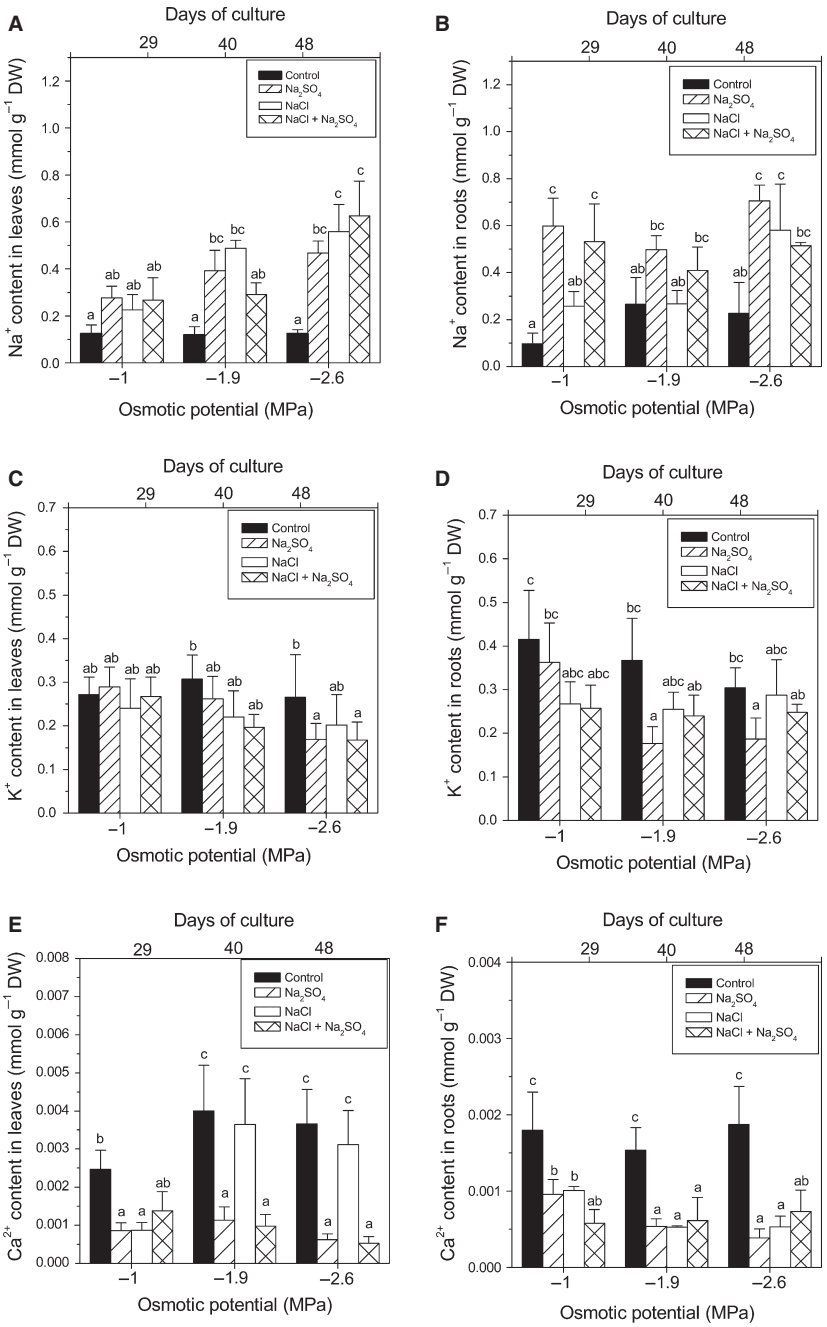

Fig. 6. Effects of NaCl, Na_2SO_4 and their iso-osmotic mixture on cation levels in leaves and roots of *Prosopis strombulifera*. A: sodium (Na^+) in leaves; B: Na^+ in roots; C: potassium (K^+) in leaves; D: K^+ in roots; E: calcium (Ca^{2+}) in leaves; F: Ca^{2+} in roots. Means (± SE) followed by different letters above bars are significantly different at $P < 0.05$ (n = 6).

centration in leaves of *P. strombulifera* was *ca*. two-fold higher than the K^+ concentration (0.48 *versus* 0.22 mM·g^{-1} DW, respectively) during optimal growth of NaCl-treated plants ($\Psi o = -1.9$ MPa; 500 mM). 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. However, the K^+ concentration in these plants remained unaffected, with similar levels to controls, even at high salinity ($\Psi o = -2.6$ MPa). Long-distance Na^+ transport, particularly the mechanism of Na^+ loading into the xylem, plays a major role in establishing a water potential gradient in *Suaeda altissima* (Balnokin *et al.* 2005). Likewise, efficient long-distance Na^+ transport to stems and leaves is essential for salt tolerance in many halophytes. Although most plants require K^+, and Na^+ has a toxic effect on many biological reactions in the cytoplasm, the situation differs for vacuolar processes. Na^+ accumulates preferentially in vacuoles, and is more suitable than K^+ for osmotic adjustment in several species, where replacement of K^+ by Na^+ in vacuoles does not produce toxicity (Subbarao *et al.* 2003). Raven (1985) suggested that the 'ATP cost' for active uptake and compartmentation of inorganic ions, *versus* synthesis of compatible solutes, differs by a factor of about ten. The use of inorganic ions is therefore more efficient and economical for plants under salt stress than synthesis of compatible organic solutes (Shabala & Lew 2002). However, the real extent of the energetic demands of processes essential for salt tolerance in halophytes remains unknown (Flowers & Colmer 2008). We propose that

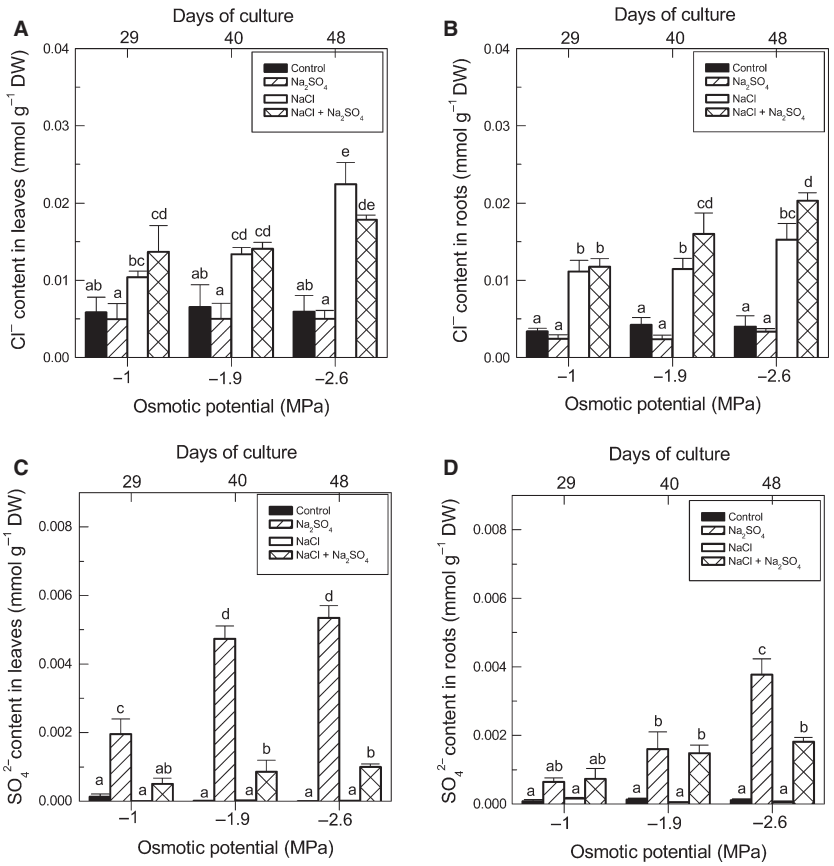

Fig. 7. Effects of NaCl, Na_2SO_4 and their iso-osmotic mixture on anion levels in leaves and roots of *Prosopis strombulifera*. A: chloride (Cl^-) in leaves; B: Cl^- in roots; C: sulphate SO_4^{2-} S in leaves; D: SO_4^{2-} in roots. Means (± SE) followed by different letters above bars are significantly different at $P < 0.05$ (n = 6).

the unusual salt tolerance of *P. strombulifera* is due to a combination of Na^+ exclusion mechanisms in roots and efficient accumulation/compartmentation in leaves.

Prosopis strombulifera preferentially accumulated Na^+ over Cl^- (which values were ten-fold lower) during optimal growth of NaCl-treated plants. Flowers *et al.* (1986) noted that the Cl^-:(Na^+ + K^+) ratios were generally above one in cells of halophytes, suggesting that this might be the result of the higher toxicity of Cl^- on protein synthesis than that of organic anions. Control of Cl^- transport and Cl^- 'exclusion' from shoots is correlated with salt tolerance in many species, particularly legumes, *e.g. Trifolium* (Rogers *et al.* 1997), *Medicago* (Sibole *et al.* 2003), *Glycine* (Luo *et al.* 2005) and *Lotus* (Teakle *et al.* 2006, 2007).

In contrast, SO_4^{2-} concentrations in *P. strombulifera* were much lower than those of Cl^- in leaves of Na_2SO_4-treated plants, but were sufficient to cause metabolic disorders manifested as chlorosis, necrosis and leaf abscission. In bi-saline-treated plants, preferential accumulation of Cl^- over SO_4^{2-} was observed; consistent with previous observations that the SO_4^{2-} anion is absorbed slowly by roots of higher plants (Bie *et al.* 2004). The deleterious effects of SO_4^{2-} on leaf development, and on root and shoot elongation, are presumably a consequence of several metabolic reactions, *e.g.* sulphide formation in the process of sulphate assimilation in the chloroplast, wherein sulphite reductase catalyses the reduction of sulphite to sulphide using reduced ferredoxin as electron donor (De Kok *et al.* 2005). Free sulphide can be incorporated into L-cysteine through cysteine synthase, which is the most efficient way to keep its concentration low in order to avoid inhibitory effects. If all free sulphide is not consumed by this assimilatory step, it could be released to the environment or could bind to cytochromes, thereby inhibiting mitochondrial respiration (Schmidt 2005).

In the present study, leaves of Na_2SO_4- and bi-saline-treated plants showed a significant reduction of Ca^{2+} concentration, whereas leaves of NaCl-treated plants showed normal Ca^{2+} levels at moderate and high salinities, suggesting distinct effects of the different anions, *i.e.* Cl^- is not toxic for this species. Similarly, Curtin *et al.* (1993) found that selectivity for Ca^{2+} in species of *Kochia* and *Hordeum* was higher when plants were stressed with Cl^- salts. In *P. strombulifera* roots a different response was found: there was a distinct decrease in Ca^{2+} concentration in all salt treatments. This important decrease in Ca^{2+} concentration in roots of salt-treated plants may be explained 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 Ca^{2+} influx to cells, together with hyperpolarisation-activated Ca^{2+} channels (HACC; Demidchik *et al.* 2002).

In certain halophytes, *e.g. Suaeda salsa*, NO_3^- accumulation plays an important role in osmotic adjustment under high salinity (Martínez-Ballesta *et al.* 2004). In *P. strombulifera* the NO_3^- anion does not seem to have such a role, since its concentrations remained unaffected under salt stress. We previously found that *P. strombulifera* cells synthesise high concentrations of compatible solutes such as proline and pinitol for charge balance and osmotic adjustment (Llanes *et al.* 2012). It appears that the high NaCl tolerance of this species is due to its capacity for osmotic adjustment, which enables seedlings to make efficient use of water under our experimental

conditions. Shoot water potential dramatically decreased in salt-treated plants, reaching values below −3 MPa at higher salinity (Ψo = −2.6 MPa). The sharp decrease in water potential under salt stress, with few associated changes in tissue hydration, is considered an adaptive strategy of evergreen sclerophyllous trees and shrubs to stress (Noitsakis & Tsiouvaras 1990). Moreover, our results demonstrate that the salt-induced decrease in shoot water potential was accompanied by an adaptive decrease in osmotic potential (Llanes *et al.* 2012).

A reduction in total leaf area at Ψo = −1.9 and −2.6 MPa, accompanied by an increase in stomatal density and epidermal cell density with smaller stomata (Reginato 2009) was observed in Na_2SO_4-treated plants and may be considered additional anatomical adaptations to extreme environmental conditions, and comprise additional features of these plants for survival.

In view of the results presented in this study and the anatomical modifications reported previously (Reinoso *et al.* 2004, 2005), the explanation for the adaptive success of *P. strombulifera* growing under high NaCl conditions seems to be related to: (i) a delicate balance among Na^+ accumulation (and its use for osmotic adjustment) and efficient compartmentation in vacuoles; (ii) the ability of the whole plant to ensure a sufficient K^+ supply by maintaining a high degree of K^+/Na^+ discrimination in spite of large amount of Na^+ in the medium; (iii) maintenance of normal Ca^{2+} levels in leaves; and (iv) osmotic balance and protection by compatible solutes such as proline, polyols (Llanes *et al.* 2012) and polycations such as polyamines under salt stress (Reginato *et al.* 2012). Increasing concentrations of bi-saline solution, compared to increasing concentrations of Na_2SO_4 solution, allowed higher salt tolerance based of various cellular phenomena, including ionic antagonism and/or mutual competence, which indicate important specific effects of anions on membrane permeability.

Bearing in mind limitations of experimental designs of this type (when testing different salt compositions at different osmotic potentials), it would be equally valid to compare effects at equal concentrations of ions. The difference in valences of these salts/mixtures (NaCl, Na_2SO_4, NaCl + Na_2SO_4) means that Na^+ and anion concentrations are never the same in iso-osmotic solutions. Notwithstanding, those treatments in which the Na^+ concentration was equal (day 48, Ψo = −2.6 MPa for NaCl; day 36, Ψo = −1.71 MPa for Na_2SO_4; day 42, Ψo = −2.06 MPa for NaCl + Na_2SO_4) showed different growth responses, mainly attributable to the presence of the SO_4^{2-} anion and regardless of the 'aging factor'. The same response occurred when the highest concentrations of Cl^- and SO_4^{2-} in the mixture were experienced at day 48, Ψo = −2.6 MPa, but similar levels were present on day 33, Ψo = −1.35 MPa, for plants exposed to the single salt. Also in this case, different growth responses are mainly attributable to the presence of the SO_4^{2-} anion in the solution.

In conclusion, we have demonstrated that salt tolerance of *P. strombulifera* mostly depends on the chemical composition of the salts in the soil solution. 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. Hence, the common practice of studying salt tolerance by growing plants in nutrient solutions with a range of NaCl concentrations may not be a completely valid representation of all situations. On the other hand, halophytes have been considered a useful approach for generating salt-tolerant crops in the future, as alternative cash crops or as gene donors for genetic manipulation of economically important crop species (Flowers & Colmer 2008; Mittler & Blumwald 2010). Exploration of highly salt-tolerant *Prosopis* species has important implications in both theoretical and applied agricultural biology. Almost all the important species of annual legume in the subfamily Papilonoideae (family Fabaceae), including soybeans, common beans and peas, are highly salt sensitive. Among the commercial legumes, only alfalfa has a moderate degree of salt tolerance. Elucidation of mechanisms of salt tolerance in *Prosopis* (also a member of the Fabaceae) is therefore relevant to commercially important legumes. Along this line, we are currently engaged in a search for cDNAs and differentially expressed RNA sequences in *P. strombulifera*.

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