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Differential growth responses to sodium salts involve different abscisic acid metabolism and transport in *Prosopis strombulifera*

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Abstract

In this work, the response of the halophytic shrub *Prosopis strombulifera* to lowering an osmotic potential (Ψo) to -1.0, -1.9, and -2.6 MPa generated by NaCl, Na_2SO_4, and the iso-osmotic combination of them was studied at 6, 12, and 24 h after reaching such values in the growing media. By analyzing the content of abscisic acid (ABA) and related metabolites and transpiration rates, we observed that ABA content varied depending on type of salt, salt concentration, organ analyzed, and age of a plant. ABA content in leaves was much higher than in roots, presumably because of rapid biosynthesis and transport from roots. Leaves of Na_2SO_4-treated plants had the highest ABA content at Ψo -2.6 MPa (24 h) associated with sulfate toxicity symptoms. Significant content of ABA-glucose ester (ABA-GE) was found in both the roots and leaves, whereas only low content of phaseic acid (PA) and dihydrophaseic acid (DPA). The roots showed high ABA-GE accumulation in all treatments. The highest content of free ABA was correlated with ABA-GE glucosidase activity. The results show that ABA-GE and free ABA work together to create a specific stress signal.

Additional key words: abscisic acid glucose ester, dihydrophaseic acid, halophyte, NaCl, Na_2SO_4, osmotic potential, phaseic acid, salinity.

Introduction

Plant growth is greatly affected by environmental stresses such as temperature extremes, drought, high salinity, or combination of them. From an agricultural point of view, such stresses are the most significant causes of losses in crop production that can be both substantial and unpredictable. The physiological mechanisms governing plant responses to salinity and drought are similar suggesting that both stresses are perceived by the plant cell as water deprivation (Mantri *et al*. 2007). High salt concentrations in the soil reduce water potential and consequently decrease water availability (Hasegawa *et al*. 2000, Munns and Tester 2008, Jenkins *et al*. 2012).

Flowers and Colmer (2008) in a recent review noted the general paucity of information on regulation of tolerance mechanisms in halophytes, despite their widespread occurrence, and recommended focusing the research on certain model species representing the various mechanisms involved in salt tolerance. The halophytic shrub *Prosopis strombulifera* (Lam.) Benth. ranges from the Arizona desert to Patagonia and is particularly abundant in the salinized areas of Central Argentina (Burkart 1976, Cantero *et al*. 1996). In these areas, proportions of NaCl and Na_2SO_4 are generally similar, although Na_2SO_4 was as much as three times more abundant in some samples (Sosa *et al*. 2005). It is important to compare effects of Na_2SO_4 and NaCl on plant growth to better understand plant responses to the major salts found in salinized soils in various countries (Shi and Sheng 2005, Sosa *et al*. 2005, Manivannan *et al*. 2008). In previous studies we observed considerable variability in the response of *Prosopis strombulifera* to salinity depending on the type of salt(s) used and osmotic potential (Ψo) in the culture medium. Stimulation of shoot growth at Ψo values up to -1.9 MPa (500 mM)

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Abbreviations: ABA - abscisic acid; ABA-GE - abscisic acid glucose ester, DPA - dihydrophaseic acid; PA - phaseic acid; Ψo - osmotic potential.
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NaCl is an interesting response of this halophyte, distinct from findings in other woody *Prosopis* species (Felker 2007). Several studies indicate that the NaCl tolerance of *P. strombulifera* exceeds the limits described for most halophytic plants (Catalán *et al*. 1994). However, *P. strombulifera* is much less tolerant to Na_2SO_4 than to NaCl. Plants grown in the presence of Na_2SO_4 showed immediate and sustained reduction of shoot height and leaf number per plant accompanied by senescence symptoms, such as chlorosis, necrosis, and leaf abscission (Reinoso *et al*. 2005, Reginato *et al*. 2012). Furthermore, treatment of *P. strombulifera* seedlings with Na_2SO_4 induced structural alterations in cells and tissues and modification of growth patterns. These alterations included anatomical and histological differences in roots, stems, and leaves of plants grown under high NaCl concentrations as compared to control plants (Reinoso *et al*. 2004, 2005). These anatomical modifications are consistent with our previous physiological studies which demonstrate that the adaptive success of *P. strombulifera* grown under high NaCl salinity involves a delicate balance among Na^+ and Cl^- accumulation and compartmentation in vacuoles (Reginato *et al*. 2012), osmotic balance with compatible solutes such as proline, polyol synthesis (Llanes *et al*. 2012), Cl^- charge balanced by polycations such as polyamines, and a near normal photosynthetic rate (Reginato *et al*. 2012).

Many plants, when grown under salinity or drought stress, accumulate the abscisic acid (ABA) that controls stress-induced stomatal closure, induction of gene expression for drought tolerance, and many other adaptive responses (Zhang *et al*. 2006). ABA is classified as a stress hormone because of its rapid production in response to salt stress, and its equally rapid catabolism when salt stress is relieved (Umezawa *et al*. 2006). Because so many stress responses are mediated by ABA, the most important signal transduction pathway among plant responses to stresses is the initial perception of dehydration and consequent changes in gene expression leading to rapid ABA biosynthesis (Umezawa *et al*. 2010). In glycophytes, stress tolerance increases as ABA concentration increases up to a certain point. In halophytes, the roles of ABA and its metabolites are essentially unexplored.

ABA plays fundamental roles in plant growth, development, and adaptation to abiotic and biotic stresses (De Torres-Zabala *et al*. 2007). ABA limits water loss by regulating the opening of stomata and modifying activity of ion channels in guard cells (Sirichandra *et al*. 2009). These functions of ABA overlap with several signaling pathways in plants including hormonal responses, developmental responses, sugar-signaling pathways, and stress-response pathways, reflecting a complex network of interactions.

ABA catabolism is governed by two pathways: an oxidative pathway and sugar conjugation (Nambara and Marion-Poll 2005). In higher plants, ABA catabolism is initiated by oxidation of ABA by ABA 8'-hydroxylase to form 8'-hydroxy-ABA. 8'-hydroxy-ABA is then spontaneously isomerized to phaseic acid (PA) that is reduced to dihydrophaseic acid (DPA; Cutler *et al*. 2010). ABA is inactivated by conjugation with glucose (Lim *et al*. 2005, Priest *et al*. 2006). ABA-glucose ester (ABA-GE), a predominant ABA conjugate, may function as a storage form of releasable ABA. Field crops contain high content of ABA-GE (Sauter *et al*. 2000, 2002). ABA-GE cannot migrate passively through plasma membranes and the mechanisms underlying transport of ABA and its conjugates remain not completely clear as well as the functions of conjugated forms of ABA (Schachtman and Goodger 2008).

We hypothesize that the differential growth responses observed in the halophytic shrub *P. strombulifera* to Na_2SO_4, NaCl, and their iso-osmotic mixture involve different ABA signaling and regulation of transpiration. The aim of this work was to test this hypothesis.

Materials and methods

Plants and treatments: *P. strombulifera* seeds were collected from southwestern San Luis province, Argentina, located at 33º 43' S, 66º 37' W, altitude 400 - 500 m a.s.l., with a temperate climate (average annual temperature of 15 - 20 ºC). The soil was saline-sodic with abundant calcareous material, moderate salinity (electrical conductivity of 8 dS m^{-1} at the surface and 10 dS m^{-1} at 25 - 35 cm depth), and a sandy-loam texture. Pods were collected at random from 100 plants within the same population. Seeds were selected visually for uniform size and health, scarified with sulfuric 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 at 37 ºC for 24 h before sowing (Reinoso *et al*. 2004). Germinated seeds with roots 20 mm long were transferred to hydroponic cultures (two black trays per treatment, 200 seedlings per tray, 10 % (m/v) Hoagland's solution for the first week and 25 % for further growth). Seedlings were grown in a chamber with a 16-h photoperiod, irradiance of 200 µmol m^{-2} s^{-1}, day/night temperatures of 28/20 ºC, and relative humidity of 70 %. Aeration was provided by an aquarium pump and pH was 6 for all media.

Salt treatments were applied after 21 d of plant growth at osmotic potential (Ψo) of -0.03 MPa using a simple randomized design (Steel and Torrie 1995). Pulses of NaCl alone (50 mM), Na_2SO_4 alone (38 mM), or an iso-osmotic mixture of the two salts ("bisaline treatment") were applied every 48 h until reaching final Ψo of -1.0, -1.9, or -2.6 MPa, respectively (measured by a vapor pressure osmometer *Model 5500*, *Wescor*, Logan, UT, USA). These Ψo values were reached at age of 29, 40, and 48 d, respectively. Iso-osmotic bisaline solutions were obtained by mixing equal volumes of the respective

monosaline solutions at each osmotic potential as shown in Table 1. For each sampling, 25 treated plants were collected at random 6, 12, and 24 h after the medium reached the final osmotic potential, and also 25 control plants (no salt added; Ψo of medium -0.03 MPa) were collected for each treatment. The plants were frozen in liquid nitrogen and stored at -80 ºC for further analyses. Each experiment was performed four times.

Table 1. Increasing salt concentrations obtained by sequential addition of pulses every 48 h. Sampling was done after 5th, 10th, and 14th pulse.

Salt pulses	Na_2SO_4 [mM]	NaCl [mM]	Bisaline [mM]	Ψo [MPa]
1	37.9	50	18.95 + 25	-0.30
2	75.8	100	37.90 + 50	-0.47
3	113.7	150	56.80 + 75	-0.65
4	151.7	200	75.85 + 100	-0.82
5	189.7	250	94.80 + 125	-1.00
6	227.5	300	113.75 + 150	-1.18
7	265.4	350	132.70 + 175	-1.35
8	303.3	400	151.70 + 200	-1.53
9	341.2	450	170.60 + 225	-1.71
10	379.2	500	189.60 + 250	-1.88
11	417.1	550	208.50 + 300	-2.06
12	455.0	600	227.50 + 300	-2.24
13	492.9	650	246.40 + 325	-2.42
14	530.8	700	265.40 + 350	-2.60

Abscisic acid extraction: ABA was extracted and purified according to a modified protocol of Luna *et al*. (1993). Leaf or root dry mass (150 mg) was ground in a mortar with liquid nitrogen and 20 cm^3 of imidazole buffer (pH 7) and 2,6-di-tert-butyl-*p*-cresol as antioxidant. [2H6] ABA (*OlChemIm*, Olomouc, Czech Republic; 50 ng) was added as an internal standard and each sample was incubated at 4 ºC overnight, centrifuged, and evaporated with isopropanol. Aqueous fractions were loaded onto a conditioned amino anion exchange minicolumn [*Bakerbond speTM Amino (NH2)*, *Q1 Mallinckrodt Baker*, Phillipsburg, USA] and washed sequentially with hexane, ethyl acetate, and acetonitrile (6 cm^3 each). These solvents were discarded and ABA was eluted with methanol + acetic acid (95:5, v/v) and evaporated to dryness.

Dried extracts were dissolved in 100 cm^3 of elution solvent (methanol + water + acetic acid, 70:30:0.1, v/v/v) and separated on a preparative high-performance liquid chromatography (HPLC) system (*KNK-500*, *Q2 Konic Instruments*, Barcelona, Spain) equipped with *RP C18* column (*m-Bondapack*, a 3.9 mm internal diameter and 5 mm particle size; *Waters Associates*, Milford, MA, USA) coupled to a spectrometry system (UV-Vis) with a diode array detector (*Konic Instruments*). Samples were subsequently eluted at a flow rate of 1 cm^3 min^{-1} with an isocratic mixture of methanol + water + acetic acid (70:30:0.1, v/v/v). Fractions corresponding to ABA, determined by spectrophotometry at 262 nm and by previous HPLC, were pooled and dried. Samples containing ABA were dissolved in 100 cm^3 of derivatization compound NO-bis-trimethylsilyltrifluoroacetamide (BSTFA) and then converted to methylester-trimethylsilylether (MeTMSi) derivatives. Samples were placed in an oven with temperature increasing from 70 to 90 ºC during 30 min. Samples (0.001 cm^3) were then injected split-splitless in a gas chromatograph-mass spectrometer system with selected ion monitoring (GC-MS-SIM) (*5890 Series II GC*, *Hewlett Packard*, town? USA), with capillary direct interface to a *5972* mass selective detector equipped with a 25-m *Chrompack CPSil 19* capillary column (an internal diameter of 0.25 mm and film thickness of 0.22 mm). Carrier gas was He, a flow rate of 1 cm^3 min^{-1}, GC injector temperature of 280 ºC, and oven temperature initially maintained at 100 ºC for 1 min, then increased from 100 to 195 ºC at a rate of 15 ºC min^{-1} and then 4 ºC min^{-1} up to 280 ºC. For ABA quantification, ions 196 (deuteron) and 190 (proton) were monitored for 9 - 10 min.

PA, DPA, and ABA-GE were extracted and purified as described by Zhou *et al*. (2003) with modification. Dry mass (150 mg) of leaves or roots was ground in a mortar with liquid nitrogen and extracted with 3 cm^3 of acetone + water + acetic acid (80:19:1, v/v/v). Internal standards, 100 ng each of [2H3] PA, [2H3]DPA, and [2H5]ABA-GE (NRC-Plant Biotechnology Institute, Saskatoon, Canada) were added. Extracts were transferred to 50-cm^3 tubes, centrifuged for 15 min, and supernatants were collected and evaporated at 35 ºC under vacuum in a *SpeedVac*. Dried extracts were dissolved in 100 cm^3 of methanol + acetic acid (99:1, v/v) and then mixed with 900 cm^3 of 1 % acetic acid. Samples were filtered through a syringe filter tip and purified with 3 cm^3 *Q3 BondElut-C18* cartridges (*Varian*, Palo Alto, CA, USA) on a vacuum manifold at a flow rate < 1 cm^3 min^{-1}. Cartridges were conditioned with 1.5 cm^3 of methanol and equilibrated with 1.5 cm^3 of methanol + water + acetic acid (10:89:1, v/v/v). Samples (1.5 cm^3) were loaded onto cartridges and washed with 1.5 cm^3 of the same mixture. ABA metabolites were eluted with 1.5 cm^3 of methanol + water + acetic acid (80:19:1), and collected in a 2-cm^3 flat-bottom Eppendorf tube. The eluate was dried under vacuum by centrifugation (1 000 *g*, 30 min) at 35 ºC. Extracts were resuspended in 0.1 cm^3 of methanol (100 %), and placed in vials. Samples (0.001 cm^3) were injected, and PA, DPA, and ABA-GE were determined by liquid chromatography with electronspray ionization (LC; *Waters Corp*., New York, USA) coupled to a tandem mass spectrometer (MS-MS) (*Micromass*, Manchester, UK), monitored using *Masslink v. 4.1* software.

ABA-GE glucosidase activity assay: ABA-GE glucosidase activity was determined by the procedure of Kato-Noguchi and Tanaka (2008). Lyophilized tissues of leaves and roots (4.8 g and 2.1 g, respectively) were extracted and homogenized with 70 cm^3 of sodium

phosphate buffer, pH 7.3, with 10 mM mercaptoethanol for 30 min. Samples were centrifuged at 8 000 *g* for 20 min and supernatants (40 cm^3) were collected and subjected to saline precipitation with solid ammonium sulfate to 100 % saturation. After centrifugation (8 000 *g* for 20 min), the supernatant was resuspended in sodium acetate buffer, pH 5.5, with 10 mM mercaptoethanol, and dialyzed against the same buffer for 2 h. The reaction mixture containing 50 mM MES (pH 6), 4 mM magnesium acetate, 2 mM pure ABA-GE (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Canada), and 0.05 - 0.150 cm^3 of the plant extract was incubated at 35 °C for 30 min. The reaction was stopped by 20 mM Na_2CO_3 and absorption was read at 275 nm with a spectrophotometer *Beckman DU 650* (*Analytical Instruments*, Golden Valley, MN, USA). A standard curve was established for free ABA.

Transpiration: Transpiration was determined indirectly by recording changes in volume of culture solutions at defined Ψo values (Burguess 1983). Plants (three per treatment) were inserted in the perforated rubber stopper of a transparent graduated cylinder, containing a defined volume of solution, which was then sealed with silicone. Plants were maintained at the same conditions as mentioned for the hydroponic cultures for 4, 8, 12, 24, and 28 h, and the volume of the solution consumed was measured. To calculate the leaf area, leaves of seedlings were cut, digitally scanned (*Hewlett Packard* scanner *PSC 1410*), and their area was determined using *Image-Pro Plus* (*Ipwin32*) program.

Statistical analysis: Data were analyzed using *InfoStat v. 2011* program (National University of Córdoba, Cordóba, Argentina). The two-way *ANOVA* was used to determine the effect of each treatment (osmotic potential and type of sodium salt). 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

ABA content was determined in leaves and roots of NaCl, Na_2SO_4, and bisaline-treated plants. ABA content was higher in leaves that in roots regardless of the salt treatment (Table 2). In the roots, at Ψo -1.0 and -1.9 MPa, ABA content was higher in the control than in the salt-treated plants 6 h after treatment initiation. In the salt-treated plants, ABA content was high at 6 h after the last salt pulse (necessary to reach desired Ψo value) and subsequently decreased. At Ψo -2.6 MPa, the NaCl treatment caused maximal ABA content at 12 and 24 h after the salt pulse. In the leaves at Ψo -1.0 MPa, no significant differences in ABA content were observed between the salt treatments. For all Ψo values, a significant reduction in ABA level was observed 12 h after the salt pulse followed by a new peak at 24 h. From the 29-d culture, the plants showed maximum ABA content after 6 and 24 h with a sharp decrease at 12 h. ABA content in the leaves was the highest for the Na_2SO_4-treated plants at -2.6 MPa after 6 and 24 h, followed in descending order by bisaline-treated, control, and NaCl-treated plants.

ABA-GE was the predominant metabolite found in *P. strombulifera* under the all treatments. Its content was consistently higher than that of PA or DPA in the both leaves and roots, except for the leaves from the Na_2SO_4-treated plants at Ψo -1.0 and -1.9 MPa (Table 3). At low salinity (Ψo -1.0 MPa) in roots of the NaCl-treated and control plants, there was significant ABA-GE accumulation. In contrast, the roots of the Na_2SO_4- and bisaline-treated plants showed lower ABA-GE accumulation, mainly at 12 and 24 h after the salt pulse. In the leaves of the NaCl-treated plants, there was a significant increase in ABA-GE content at 6 h after the salt pulse. At moderate salinity (Ψo -1.9 MPa), ABA metabolism in the roots was unaffected by salt treatments. In contrast, the leaves showed the highest content of ABA-GE at 12 h for Na_2SO_4-treated plants, whereas a slight increase was observed in the NaCl-treated plants at 24 h post the pulse. At high salinity (Ψo -2.6 MPa) in roots, content of ABA metabolites at 6 h was unaffected by the salt treatment. At 12 h and 24 h, ABA-GE level was the highest for the Na_2SO_4-treated plants, followed in descending order by the NaCl-treated, bisaline-treated, and control plants. In the leaves, the Na_2SO_4-treated plants showed a progressive increase in ABA-GE content that was 12-fold higher than for the other treatments (Table 3).

Activity of ABA-glucosidase at Ψo -2.6 MPa in the Na_2SO_4-treated plants was higher in the leaves than in the roots (Table 4) reflecting the highest content of ABA-GE in the leaves; this was 5.8 fold higher than in the NaCl-treated and control plants.

Up to 12 h after the salt pulse, none of the salt treatments affected transpiration but at 24 h, water loss sharply increased in all the treatments (Table 5). However, transpiration increased significantly in the control plants at 29 d. At 40 or more days, the Na_2SO_4-treated plants showed the highest transpiration exceeding the level in the control plants. Transpiration was the lowest in the NaCl- and bisaline-treated plants, and non significant differences were observed between these treatments.

Discussion

Abscisic acid (ABA) is a key hormone regulating adaptive responses of plants to various stresses. ABA affects stomatal conductance, growth, accumulation of osmolytes, and expression of specific genes (Zhang *et al*. 2006, Cutler *et al*. 2010). Results of the present study show that the ABA content in the halophyte *P. strombulifera* under salt stress varied widely depending on the salt type (Na_2SO_4, NaCl, or their mixture), salt concentration, and plant organ (roots *vs*. leaves).

Table 2. Effects of NaCl, Na_2SO_4, and their iso-osmotic mixture on ABA content in roots and leaves of *Prosopis strombulifera*. Ψo was -1.0 MPa at 29 d of culture, -1.9 MPa at 40 d of culture, and -2.6 MPa at 48 d. The ABA content was measured 6, 12, and 24 h after the last pulse. Means ± SE, $n = 4$; different letters in the columns indicate significant differences among treatments ($P < 0.05$) according to Tukey test.

Treat-ments	Ψo [MPa]	Culture [d]	Time [h]	ABA in roots [μg g^{-1}(d.m.)]	ABA in leaves [μg g^{-1}(d.m.)]
Control	-0.03	29	6	236.1 ± 5.0 ^{a}	411 ± 4.0 ^{a}
			12	128.0 ± 2.0 ^{b}	285 ± 4.6 ^{b}
			24	49.0 ± 1.5 ^{b}	381 ± 12.6 ^{a}
	-0.03	40	6	213.0 ± 11.2 ^{a}	422 ± 12.5 ^{a}
			12	63.9 ± 5.0 ^{b}	182 ± 11.0 ^{b}
			24	42.0 ± 2.3 ^{b}	398 ± 12.9 ^{ab}
	-0.03	48	6	88.0 ± 12.0 ^{a}	390 ± 14.0 ^{b}
			12	71.0 ± 4.5 ^{b}	377 ± 13.2 ^{b}
			24	40.8 ± 13.0 ^{b}	457 ± 11.6 ^{a}
NaCl	-1.0	29	6	147.0 ± 5.0 ^{b}	393 ± 13.2 ^{a}
			12	126.0 ± 11.0 ^{b}	269 ± 4.0 ^{b}
			24	75.0 ± 12.0 ^{b}	316 ± 11.6 ^{b}
	-1.9	40	6	69.1 ± 5.0 ^{b}	350 ± 12.9 ^{a}
			12	35.0 ± 5.0 ^{b}	172 ± 14.2 ^{b}
			24	35.0 ± 9.0 ^{b}	350 ± 13.5 ^{ab}
	-2.6	48	6	36.0 ± 4.6 ^{b}	388 ± 13.5 ^{b}
			12	106.5 ± 4.2 ^{a}	337 ± 15.5 ^{b}
			24	100.7 ± 6.6 ^{a}	363 ± 16.4 ^{b}
Na_2SO_4	-1.0	29	6	164.0 ± 8.0 ^{b}	436 ± 5.5 ^{a}
			12	102.0 ± 4.5 ^{b}	304 ± 7.7 ^{b}
			24	50.0 ± 7.0 ^{b}	374 ± 8.1 ^{a}
	-1.9	40	6	69.1 ± 11.0 ^{b}	375 ± 14.9 ^{a}
			12	46.8 ± 5.0 ^{b}	268 ± 11.0 ^{ab}
			24	31.0 ± 11.0 ^{b}	328 ± 11.0 ^{ab}
	-2.6	48	6	61.0 ± 4.5 ^{ab}	500 ± 16.2 ^{a}
			12	85.3 ± 4.4 ^{b}	373 ± 13.0 ^{b}
			24	31.3 ± 4.0 ^{b}	539 ± 16.0 ^{a}
NaCl + Na_2SO_4	-1.0	29	6	140.0 ± 5.0 ^{b}	363 ± 10.0 ^{a}
			12	135.0 ± 7.0 ^{b}	251 ± 6.1 ^{b}
			24	50.0 ± 8.0 ^{b}	370 ± 2.0 ^{a}
	-1.9	40	6	107.0 ± 27.0 ^{b}	317 ± 11.0 ^{ab}
			12	72.0 ± 25.0 ^{b}	260 ± 15.0 ^{ab}
			24	35.0 ± 10.0 ^{b}	293 ± 11.0 ^{b}
	-2.6	48	6	44.0 ± 6.0 ^{b}	385 ± 13.0 ^{b}
			12	71.0 ± 5.0 ^{b}	365 ± 12.6 ^{b}
			24	50.0 ± 5.0 ^{b}	509 ± 16.7 ^{a}

One interesting finding was the high free ABA content in the control plants, particularly those grown for 40 d at Ψo -0.03 MPa. At this age, optimal growth is observed in plants grown in 400 - 500 mM NaCl (Ψo -1.9 MPa) (Reinoso *et al*. 2004, Reginato *et al*. 2010). Content of free ABA in these plants is significantly lower than that of control plants. These results can indicate that both high and low salt concentrations are perceived as stressful by this species. It is remarkable that similar free ABA content is observed in all salt-treated plants, although those treated with Na_2SO_4 begin to show growth inhibition and toxicity symptoms from Ψo -1.9 MPa (Reinoso *et al*. 2005).

ABA synthesis can occur in both roots and leaves. When intact *Arabidopsis* plants are subjected to drought stress, ABA content increases in both leaves and roots (Ikegami *et al*. 2009), whereas in experiments with detached leaves and roots, only leaves show an ABA increase. These authors proposed that ABA acting on guard cells is produced in leaves, other chemical signals are produced in roots at an early stage of stress, and these signals initiate ABA biosynthesis in leaves. The highest ABA content in salt treated *P. strombulifera* at Ψo -2.6 MPa correlates with sharply reduced carotenoid content in leaves (Reginato *et al*. 2012) since carotenoids are precursors of ABA.

The peak of free ABA in the leaves at 24 h might result from hydrolysis of ABA-GE by a leaf specific β-glucosidase as well as due to active transport to the leaves of ABA synthesized in the roots. This response is very important to control stomatal aperture under high transpiration.

Recent studies confirm that catabolism is also important for control of ABA content during stress. Preferential occurrence of one or the other pathway depends on the plant species, organ, developmental stage, and biological process involved (Oritani and Kyoto 2003).

P. strombulifera shows low DPA content and usually has no detectable PA, indicating that PA is rapidly metabolized. Rapid metabolism of ABA is essential to prevent accumulation of PA, and to regulate precisely stomatal conductance (Nambara and Marion-Poll 2005). This is consistent with our findings of the relatively high content of DPA in the leaves of plants that also showed active production of free ABA and ABA-GE (Na_2SO_4-treated plants at -2.6 MPa). The biological activity of PA and DPA appears to be very low. Previous studies show that different ABA-binding proteins in apple and in aleurone cells of barley are unable to bind to PA, suggesting that this metabolite is inactive in at least some physiological processes (Zhang *et al*. 2001).

In the past decade, it can be seen controversy in opinions whether accumulated ABA-GE is merely a sign of stress or a stored form of releasable ABA. Sauter *et al*. (2002) proposed ABA-GE as a good candidate for hormonal stress signal. Lee *et al*. (2006) showed that

Table 3. Effects of NaCl, Na_2SO_4, and their iso-osmotic mixture on ABA catabolism in roots and leaves of *Prosopis strombulifera*. Ψo was -1.0 MPa at 29 d of culture, -1.9 MPa at 40 d of culture, and -2.6 MPa at 48 d. ABA content was measured 6, 12, and 24 h after the last pulse. Means of 4 independent samples; different letters in the columns indicate significant differences among treatments ($P < 0.05$) according to Tukey test (nd - not detectable).

Treat-ments	Ψo [MPa]	Culture [d]	Time [h]	PA in roots [μg g^{-1}(d.m.)]	PA in leaves [μg g^{-1}(d.m.)]	DPA in roots [μg g^{-1}(d.m.)]	DPA in leaves [μg g^{-1}(d.m.)]	ABA-GE in roots [μg g^{-1}(d.m.)]	ABA-GE in leaves [μg g^{-1}(d.m.)]
Control	-0.03	29	6	nd	0.22 ^{a}	0.16 ^{a}	0.19 ^{a}	3.90 ^{b}	0.42 ^{a}
			12	nd	0.21 ^{a}	0.17 ^{a}	0.19 ^{a}	4.09 ^{b}	0.42 ^{a}
			24	nd	0.20 ^{a}	0.09 ^{a}	0.19 ^{a}	3.73 ^{b}	0.43 ^{a}
	-0.03	40	6	nd	0.01 ^{a}	0.05 ^{a}	0.25 ^{a}	1.80 ^{a}	0.46 ^{a}
			12	nd	0.06 ^{a}	0.04 ^{a}	0.25 ^{a}	0.80 ^{a}	0.45 ^{a}
			24	nd	0.06 ^{a}	0.05 ^{a}	0.30 ^{a}	1.20 ^{a}	0.50 ^{ab}
	-0.03	48	6	nd	nd	0.07 ^{a}	0.15 ^{a}	0.73 ^{a}	1.56 ^{a}
			12	nd	nd	0.10 ^{a}	0.20 ^{a}	1.90 ^{ab}	0.98 ^{a}
			24	nd	nd	0.10 ^{a}	0.16 ^{a}	0.89 ^{a}	0.97 ^{a}
NaCl	-1.0	29	6	nd	0.09 ^{a}	0.03 ^{a}	0.20 ^{a}	3.65 ^{b}	0.68 ^{b}
			12	nd	0.09 ^{a}	0.08 ^{a}	0.29 ^{a}	4.96 ^{a}	0.43 ^{a}
			24	nd	0.11 ^{a}	0.09 ^{a}	0.33 ^{a}	2.73 ^{b}	0.42 ^{a}
	-1.9	40	6	nd	0.01 ^{a}	0.03 ^{a}	0.34 ^{a}	0.90 ^{a}	0.41 ^{a}
			12	nd	0.06 ^{a}	0.03^{a}	0.64 ^{ab}	0.95 ^{a}	0.50 ^{b}
			24	nd	0.06 ^{a}	0.04 ^{a}	0.30 ^{a}	0.99 ^{a}	0.98 ^{ab}
	-2.6	48	6	nd	nd	0.08 ^{a}	0.20 ^{a}	0.87 ^{a}	1.26 ^{a}
			12	nd	nd	0.06 ^{a}	0.70 ^{b}	5.20 ^{c}	1.10 ^{a}
			24	nd	nd	0.14 ^{a}	0.80 ^{b}	7.10 ^{cd}	1.02 ^{a}
Na_2SO_4	-1.0	29	6	nd	0.04 ^{a}	0.18 ^{a}	0.74 ^{a}	3.05 ^{b}	0.43 ^{a}
			12	nd	0.01 ^{a}	0.03 ^{a}	0.32 ^{a}	1.38 ^{c}	0.41 ^{a}
			24	nd	0.90 ^{b}	0.12 ^{a}	0.22 ^{a}	1.37 ^{c}	0.43 ^{a}
	-1.9	40	6	nd	0.01 ^{a}	0.06 ^{a}	0.59 ^{ab}	1.00 ^{a}	0.65 ^{a}
			12	nd	0.05 ^{a}	0.06 ^{a}	0.64 ^{ab}	1.20 ^{a}	1.56 ^{b}
			24	nd	0.05 ^{a}	0.07 ^{a}	1.12 ^{b}	1.30 ^{a}	0.85 ^{ab}
	-2.6	48	6	nd	nd	0.09 ^{a}	0.20 ^{a}	0.90 ^{a}	2.45 ^{a}
			12	nd	nd	0.06 ^{a}	0.41 ^{ab}	8.00 ^{d}	5.23 ^{b}
			24	nd	nd	0.12 ^{a}	0.91 ^{b}	9.00 ^{d}	12.46 ^{c}
NaCl + Na_2SO_4	-1.0	29	6	nd	0.10 ^{a}	0.19 ^{a}	0.23 ^{a}	2.72 ^{b}	0.39 ^{a}
			12	nd	0.03 ^{a}	0.02 ^{a}	0.18 ^{a}	1.36 ^{c}	0.51 ^{a}
			24	nd	0.06 ^{a}	0.06 ^{a}	0.19 ^{a}	1.56 ^{c}	0.38 ^{a}
	-1.9	40	6	nd	0.04 ^{a}	0.05 ^{a}	0.59 ^{ab}	0.90 ^{a}	0.43 ^{a}
			12	nd	0.04 ^{a}	0.10 ^{a}	0.63 ^{ab}	0.81 ^{a}	0.50 ^{b}
			24	nd	0.06 ^{a}	0.04 ^{a}	0.70 ^{ab}	1.40 ^{a}	0.93 ^{ab}
	-2.6	48	6	nd	nd	0.08 ^{a}	0.16 ^{a}	0.80 ^{a}	0.60 ^{a}
			12	nd	nd	0.06 ^{a}	0.21 ^{a}	3.00 ^{b}	0.70 ^{a}
			24	nd	nd	0.11 ^{a}	0.21 ^{a}	3.00 ^{b}	0.97 ^{a}

Table 4. β-glucosidase activity [U mg^{-1}(f.m.)] at Ψo -2.6 MPa in roots and leaves of *P. strombulifera*. Means from three independent samples. Different letters above data indicate significant differences among treatments ($P < 0.05$).

Treatments	Roots	Leaves
Control	undetected	0.007 ^{b}
NaCl	0.0027 ^{c}	0.016 ^{b}
Na_2SO_4	0.0073 ^{b}	0.093 ^{a}

ABA-GE can be hydrolyzed by β-glucosidases in response to stress, leading to increased content of active ABA. Loss of the β-glucosidase *AtBG1* gene expression in *Arabidopsis* causes defective stomatal movement, early germination, abiotic stress-sensitive phenotypes, and reduced ABA content, whereas plants with ectopic *AtBG1* expression display higher ABA content and enhanced tolerance to abiotic stress (Lee *et al.* 2006). Dehydration induced rapid polymerization of the AtBG1 protein and consequently 4-fold increase in the enzymatic activity. These findings support a model in which ABA is released rapidly and locally by AtBG1, through post-translational polymerization of AtBG1 protein localized in endoplasmic reticulum, and that yet-uncharacterized transport systems are involved in the movements of ABA-GE and ABA (Lee *et al.* 2006). ABA conjugation and deconjugation are clearly dynamic processes which play a key role in controlling the amount of biologically active ABA under both non-stressful and stressful conditions (Verslues and Zhu 2007).

Table 5. Effects of NaCl, Na_2SO_4, and their iso-osmotic mixture on transpiration (water loss) in *Prosopis strombulifera*. Ψo was -1.0 MPa at 29 d of culture, -1.9 MPa at 40 d of culture, and -2.6 MPa at 48 d. ABA content was measured 6, 12, and 24 h after the last pulse. Means of 4 independent samples; different letters in the columns indicate significant differences among treatments ($P < 0.05$) according to Tukey test.

Treatments	Ψo [MPa]	Culture [d]	Time [h]	Transpiration [mg (H_2O) m^{-2}]
Control	-0.03	29	4	58.1 ± 18 [a]
			8	58.0 ± 22 [a]
			12	77.5 ± 25 [a]
			24	118.7 ± 18 [b]
	-0.03	40	4	43.7 ± 12 [a]
			8	55.4 ± 15 [a]
			12	43.7 ± 14 [a]
			24	86.6 ± 15 [c]
	-0.03	48	4	30.5 ± 13 [a]
			8	30.5 ± 12 [a]
			12	51.0 ± 14 [a]
			24	81.6 ± 7 [c]
NaCl	-1.0	29	4	31.3 ± 5 [a]
			8	31.4 ± 5 [a]
			12	31.4 ± 5 [a]
			24	25.5 ± 5 [a]
	-1.9	40	4	25.4 ± 4 [a]
			8	24.7 ± 4 [a]
			12	35.4 ± 1 [a]
			24	65.0 ± 5 [b]
	-2.6	48	4	29.0 ± 5 [a]
			8	29.0 ± 7 [a]
			12	29:0 ± 5 [a]
			24	72.0 ± 11 [b]
Na_2SO_4	-1.0	29	4	37.9 ± 5 [a]
			8	27.8 ± 7 [a]
			12	28.9 ± 6 [a]
			24	59.0 ± 14 [a]
	-1.9	40	4	42.5 ± 12 [a]
			8	42.5 ± 13 [a]
			12	31.9 ± 15 [a]
			24	128.0 ± 6 [d]
	-2.6	48	4	57.0 ± 12 [a]
			8	57.0 ± 10 [a]
			12	60.0 ± 12 [a]
			24	148.0 ± 6 [d]
NaCl + Na_2SO_4	-1.0	29	4	21.9 ± 7 [a]
			8	29.2 ± 5 [a]
			12	34.0 ± 10 [a]
			24	36.0 ± 4 [a]
	-1.9	40	4	54.0 ± 10 [a]
			8	44.0 ± 4 [a]
			12	41.0 ± 5 [a]
			24	63.0 ± 8 [ab]
	-2.6	48	4	58.0 ± 14 [a]
			8	58.0 ± 5 [a]
			12	67.0 ± 10 [a]
			24	79.0 ± 13 [b]

Remarkably, in the present study, ABA synthesis in the roots seems to be immediately followed by conjugation since ABA-GE is the major metabolite found in the roots from the beginning of salinization. Both the roots and leaves of the Na_2SO_4-treated plants at Ψo -1.9 and -2.6 MPa showed high amounts of ABA-GE and free ABA, suggesting the presence of a system for transport of ABA-GE from roots to leaves. The Na_2SO_4-treated plants showed a high β-glucosidase activity in the roots and mainly in the leaves, suggesting that ABA-GE would be a reservoir of free ABA in roots as well as an ABA-transport form from roots to leaves in this species. In the NaCl-treated plants, ABA-GE peaked in the roots at 12 and 24 h, whereas in the leaves, there were no differences from the controls. Thus, roots would act as a reservoir of free ABA. These plants had no need to transport additional ABA to the leaves because their growth was healthy, as evidenced by a low water loss. Water balance in these plants seemed to be favored in part due to their ability to compartimentalize ions for osmoregulation. On the contrary, the Na_2SO_4-treated plants showed a poor osmotic adjustment, more negative osmotic potential in the leaves, and consequently water imbalance, correlated with a major reduction in individual and total leaf area. In spite that sulfate ion accumulation was not very high in these plants [up to 0.55 mmol g^{-1}(DM) in the leaves and 0.35 mmol g^{-1}(DM) in the roots], it was enough to cause metabolic disorder at membrane level disturbing appropriate ion compartmentation (Reginato 2009, Reginato *et al*. 2012).

A well-known ABA-regulated response to water stress is reduction of transpiration (Cutler et al. 2010, Kholová *et al*. 2010). Although ABA content did not vary significantly between the salt treatments in the present study, transpiration of the plants grown at low salinity (Ψo -1.0 MPa) was lower than that of the control plants. At the higher Ψo values, the NaCl-treated plants had lower transpiration than the control or Na_2SO_4-treated plants, indicating superior adaptive response. This finding is consistent with the proposal by Lovelock and Ball (2002) that decreased stomatal opening under salt stress reduces water loss and movement of ions into the xylem.

The control plants showed high transpiration compared to the plants grown at low salinity (Ψo -1.0 MPa) and at high salinity (Ψo -2.6 MPa), only the Na_2SO_4-treated plants showed greater transpiration. This high transpiration might be due to several factors. Sharp and Davies (2009) hypothesized that alkalinization of xylem sap allowed quick, precise control of stomatal conductance through its effect on ABA redistribution**;** *i.e.*, proton pumps (ATPases) control apoplastic pH by driving H^+ ions into the sap when water is available, leaving stomata open. A similar mechanism could take place in *P. strombulifera* control plants; *i.e.*, non-salinized Hoagland solution allowed good water availability causing sap acidification. Under this situation, stomata remain open regardless of ABA concentration.

In the Na_2SO_4-treated plants, transpiration was the highest at moderate and high Ψo values. In the presence

of this salt, high ABA and ABA-GE content in leaves do not inhibit stomatal opening, resulting in increased water loss, growth inhibition, and acceleration of senescence processes (Reinoso *et al*. 2005, Reginato *et al*. 2012). Earns *et al*. (2012) suggested that sulfate ion has an interactive effect on ABA, resulting in greater reduction of transpiration rate in maize and of stomatal opening in *Vicia faba* compared to ABA alone. The anti-transpiratory effect of ABA, reaching stomata at early stage, is enhanced by increased content of sulfate ion, which is also transported within the xylem, and effectively downregulates transpiration in these systems. In contrast, in *P. strombulifera,* sulfate ion and ABA content were much higher in the leaves and roots of the Na_2SO_4-treated plants, particularly at Ψo -2.6, and had no effect on closing stomata. Sulfate ion accumulation in these tissues seemed to be interfering at some point with the ABA signaling pathway.

Conclusions

Our results show that ABA and ABA-GE from roots and leaves worked together to create and intensify a specific stress signal. This mechanism had not been previously reported for halophytes. The highest content of ABA and ABA-GE and the highest ABA-GE glucosidase activity were observed in the Na_2SO_4-treated plants. However, the stress imposed by the presence of sulfate anion in the culture medium blocked activity of ABA, stomata remained open, and high transpiration values were recorded. Responses to the bisaline (Na_2SO_4 plus NaCl) treatment were similar to those elicited by NaCl alone, suggesting that the adverse effect of SO_4^{2-} is reduced by interaction with Cl^- at the membrane level. The mechanism of SO_4^{2-} effect in this species is currently under study in our laboratory.

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