

REGULAR ARTICLE

Growth responses to sulfate and chloride are related to different phytohormone profiles in the halophyte *Prosopis strombulifera*

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Abstract

In this study, the profile of plant phytohormones traditionally considered as plant growth promoters (GA_1 , GA_3 , GA_4 , IAA and Z) was determined in roots and leaves of the halophyte *Prosopis strombulifera* grown hydroponically in iso-osmotic solutions of NaCl, Na_2SO_4 and their mixture. Hormonal levels were correlated with growth parameters and ABA levels. Different sodium salts in the medium differentially affected the synthesis of these compounds. NaCl treated plants showed the greatest accumulation of GA_4 and GA_1 and lower levels of their metabolites GA_8 and GA_{34} both in root and leaves, in coincidence with the optimum growth observed at Ψ_0 -1.9 Mpa this response was also observed at Ψ_0 -2.6 MPa. IAA level was unaltered in these plants while Z content was higher in roots than in leaves. In contrast, both leaves and roots from Na_2SO_4 treated plants showed lower GA_1 and GA_4 and increased GA_8 and GA_{34} levels at high salinity. High levels of IAA were found in roots of these plants, but the ABA/IAA ratio increased with increasing salt concentrations in the culture medium. This pattern was also observed in the high ABA/GAs ratios. Z levels were very low in all salt treatments. The results of this study show that the halophytic growth under NaCl treatment is mainly regulated by GA_1 and GA_4 and low relative levels of ABA and IAA. The toxicity of sulfate alters this pattern reversing the ratio ABA/GAs and increasing the synthesis of IAA in conjunction with increased lateral root formation. In bisaline-treated plants intermediate values were observed in the levels of these phytohormones as compared to monosaline treatment.

Key words: halophytes, NaCl, Na_2SO_4 , phytohormones, *Prosopis strombulifera*

Introduction

Excess of sodium in the soil is an extended and usual stress in natural and agricultural ecosystems (FAO, 2008). High soil salinity is one of the most deleterious abiotic stresses, and induces important perturbations at both the cell and whole plant levels (Shabala and Cuin, 2008; Munns and Tester, 2008). Plant's behavioral response to salinity is complex, and different mechanisms are adopted by plants when they encounter salinity. However, plant species have different degrees of sensitivity or tolerance to salinity (Jamil et al., 2007; Duan et al., 2008). Plants that are adapted to live at high

concentrations of salt in the soil and grow well under this condition are called halophytes. Remarkably, halophytes can survive and reproduce in environments with salt concentrations similar to or higher than 200 mM NaCl. Usually, these plants can tolerate salt concentrations that die out 99% of other plant species (Flowers and Colmer, 2008). There is a wide range of physiological, morphological and biochemical adaptation mechanisms in these plants, which vary widely according to their degree of salt tolerance.

The shrub *Prosopis strombulifera* (Lam.) Benth. (Burkart, 1976) grows in areas from the Arizona desert (U.S.A.) to Patagonia (Argentina), and is abundant in high-salinized areas of central Argentina. The major cations present in saline environments are typically Na^+ , Ca^{2+} , and Mg^{2+} ; the main anions are Cl^- , SO_4^{2-} , and HCO_3^- (Grattan and Grieve, 1999). In many countries, NaCl and Na_2SO_4 are the most abundant salts (Manivannan et al., 2008). In high-salinized soils of southern Cordoba and southwestern San Luis provinces, Argentina, proportions of these two salts are

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generally similar, although Sosa et al. (2005) found that Na_2SO_4 was up to three times more abundant in some soil samples. It is important to compare the effects of both salts on plant growth for a better understanding of plant physiological responses to these environments.

Previous results of our group showed a halophytic response of *P. strombulifera* to NaCl surviving up to 1M NaCl in in vitro experiments, but a strong growth inhibition was found at much lower Na_2SO_4 concentrations. Seedlings grown in increasing concentrations of NaCl (250 up to 700 mM) did not develop salt glands in the leaves. Some tissues showed greater vacuolization, and the root system underwent precocious lignification and/or suberization of endodermal cells, with Casparian strips found much closer to the root tip than in glycophytic plants. These plants are able to filter more efficiently the salts of the soil and to prevent passage of excess of ions to the xylem (Reinoso et al., 2004). Na_2SO_4 treated *P. strombulifera* plants underwent structural alterations in cells and tissues, resulting in changes in growth patterns at different levels of organization. Anatomical and histological differences in leaves stem and roots were observed in Na_2SO_4 treated plants when compared to non-salinized plants or plants grown in high NaCl concentrations (Reinoso et al., 2005). These differential responses to the most abundant salts present in the soils of several countries (Manivannan et al., 2008) make this species a good model to study salt-tolerance mechanisms in halophytes.

The integrated plasticity in plant development involves phytohormones playing an essential role in the long-distance communication between different organs (Sachs, 2005). Phytohormones have been reported to be involved in stress responses and adaptation (Shaterian et al., 2005; Peleg and Blumwald, 2011) mainly abscisic acid (ABA), ethylene and salicylic acid (SA). However, much less is known about the role of cytokinins, gibberellins and auxins, traditionally not related to stress responses except for some few examples.

The homeostasis of these hormones is tightly controlled between the biosynthetic and metabolic pathways. A small variation in the concentration of a phytohormone may change its physiological activity, although their specific roles in different biological processes still require being elucidated (Pieterse et al., 2009).

One of the principal topics studied in plant responses to abiotic stress, especially drought and salinity, is ABA accumulation. The biosynthesis and redistribution of this hormone is one of the

fastest plant responses to abiotic stresses, causing stomatal closure to reduce water loss via transpiration and eventually limiting cellular growth (Peleg and Blumwald, 2011).

Previous studies from our group analyzed the concentration of ABA and related metabolites in leaves and roots of *P. strombulifera* NaCl and Na_2SO_4 -treated plants in correlation with transpiration levels, and demonstrated that ABA levels varied depending on type of salt, salt concentration, organ analyzed and age of plants. ABA levels were much higher in leaves than in roots, probably because of rapid biosynthesis in leaves and transport from roots. Leaves of Na_2SO_4 -treated plants had the highest ABA levels, associated with sulfate toxicity symptoms (Reginato et al., 2014). In this species, ABA metabolism occurred mainly through conjugation; high levels of ABA-glucose ester (ABA-GE) were accumulated in both roots and leaves in all treatments, whereas levels of hydroxylated metabolites (PA and DPA) were low. The highest levels of free ABA in leaves were correlated with high ABA-GE glucosidase activity in these organs, demonstrating ABA-GE transport from roots to leaves. Plants treated with Na_2SO_4 showed the highest levels of ABA-GE and free ABA in roots and leaves; thus, both compounds work together to create and intensify the salt-specific stress signal (Llanes et al., 2013). It is worthy to note that in these plants stomata remained opened and high transpiration occurred, suggesting that sulfate toxicity interfered at some point with ABA signaling.

The synthesis of salicylic acid by *P. strombulifera* seedlings in response to both salts was also analyzed. The high SA levels observed in Na_2SO_4 -treated plants at 48 days of culture were correlated with a failure in ion compartmentalization by these plants when compared with NaCl-treated plants. In fact, NaCl-treated plants accumulated similar levels of Na^+ than Na_2SO_4 -treated plants in their leaves, but they succeeded in ion compartmentalization and osmoregulation with direct consequences on their growth; these plants showed the lowest levels of SA. This response suggests that SA production is not a protective hormonal signal like ABA but a signal of injury in *P. strombulifera* under these adverse conditions (Devinar et al., 2013).

Both auxins and cytokinins act as endogenous regulators whose levels can be environmentally modulated to regulate root and shoot morphogenesis and their relative growth (Sachs, 2005). It has been proposed that a decrease in CK

flux from the root to the shoot could inhibit leaf growth while enhancing root growth and thus modifying the root/shoot ratio (Rahayu et al., 2005). Auxin cooperates with cytokinins in the regulation of cell cycle and plays a fundamental role as regulator of cell expansion (Rechenmann, 2009; Jurado et al., 2010). Auxin homeostasis, including its metabolism and distribution, is altered by various types of abiotic stresses. Indeed, auxin metabolism is modulated by oxidative degradation of IAA catalyzed by peroxidases, which in turn are induced by different stresses (Jain and Khurana, 2009). Additionally, two putative molecular mechanisms have been proposed for altered distribution of auxin under stress: an altered expression of PIN genes, which govern polar auxin transport, and inhibition of this transport by phenolic compounds produced by several tissues in response to stress (Kovtun et al., 2000; Potter et al., 2009).

Therefore, cytokinins and auxins may act as stress hormones directly or indirectly altering the expression of several stress-responsive genes, although this assumption requires further validation.

Gibberellins are generally involved in growth and development. However, the vast majority of the different GA structures were identified from immature seeds, whereas vegetative and young reproductive tissues contain a relatively restricted and conserved array of structures, representing members of the biosynthetic pathways to GA₁, GA₃ and GA₄ and their metabolites. GA deactivation is essential for plants to be able to regulate precisely their GA content rapidly in response to environmental changes, providing a means to regulate homeostasis. Several mechanisms for GA deactivation have been identified, the most prevalent being 2 β -hydroxylation. The enzymes responsible for this activity are giberellin 2-oxidases (GA₂oxs) which, for example, inactivate GA₄ and GA₁ forming GA₃₄ and GA₈, respectively (Hedden and Thomas, 2012).

Few authors have demonstrated the capacity of GAs to overcome adverse effects of salinity (Chakraborti and Mukherji, 2003). Although a great deal of work has confirmed the potential of GA₃ exogenous applications to improve plant performance under unstressed conditions, very little is known on the influence of GA₃ during salt stress. One of these reports has shown alleviation of salt stress on photosynthesis, pigment level, and water use efficiency by GA₃ exogenous applications (Aldesuquy and Ibrahim, 2001).

Considering the antecedents described above, it can be stated that studies on the role of endogenous phytohormones in plant responses to salinity are very scarce, and most of these studies have analyzed only one or two of the major groups of phytohormones following a step-change in salinity or other stresses. Therefore, interpretation of global changes in phytohormone biosynthesis and metabolism under stress has conducted to several divergent hypotheses on their putative roles (Sachs, 2005). Additionally, the roles of phytohormones in halophytes under salinity conditions remain relatively obscure.

Kappusamy et al. (2009) reported that one of the classic paradoxes of phytohormones is how a small number of simple organic molecules produce a large variety of developmental, metabolic and environmental changes throughout the plants. Moreover, the subsets of these phytohormones apparently carry redundant signals; although, in most cases, they cannot substitute for one another. They can be interpreted as nodes in a growth network, instead of simple pathways acting in isolation. Combining information from multiple input pathways, the strength, accuracy and nature of the biological outcome can be fine-tuned, amplified, or attenuated (Bardwell et al., 2007). Thus, the importance of phytohormones balance and the cross-talk between their signal pathways is being recognized as central to the outcome of plant-stress responses.

Therefore, the aim of this study was to analyze the profile of endogenous gibberellins, auxins and cytoquinins in correlation with growth parameters in the halophyte *P. strombulifera*, in order to contribute to the understanding of the differential responses of this species to increasing concentrations of NaCl and Na₂SO₄ and their iso-osmotic mixture.

Materials and methods

Plant Materials

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). Pods were collected randomly 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 with 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 and 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 21 d, using a simple randomized design (Steel and Torrie, 1996). As shown in Table 1, pulses of NaCl alone (50 mM) and Na₂SO₄ alone (38 mM) were applied every 48 h until reaching final osmotic potentials (Ψ_0) = -1.0, -1.9, or -2.6 MPa respectively (measured by a vapor pressure osmometer Model 5500, Wescor Inc., Logan, UT, USA). These Ψ_0 values corresponded to age 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. For each sampling, 25 treated plants were collected randomly 24 h after the medium reached a final osmotic potential as indicated above; 25 control plants (no salt added; Ψ_0 of medium = -0.11 MPa) were collected for each treatment. Plants were frozen with liquid nitrogen, and stored at -80°C for a posteriori analysis. Each experiment was performed three times.

Determination of growth parameters

Root length, shoot height and number of leaves were measured weekly in 20 plants from each treatment, from the time that salt pulses were started from 21, 29, 40 and 48 days of culture.

Extraction of abscisic acid, gibberellin, auxin and zeatin

Phytohormones were extracted and purified as described by Zhou et al. (2003), with modifications. 150 mg dry weight equivalent of leaves or roots were ground in a mortar with liquid nitrogen and ABA, GAs (GA₁, GA₃, GA₄, GA₈ and GA₃₄) and auxin (IAA) were extracted with 3 ml extraction buffer pH 2.8. Zeatin (Z) was extracted with 3 ml extraction buffer pH 7.

Determination of phytohormones

50 ng of 2H6-ABA, 2H2-GA₁, 2H2-GA₃, 2H2-GA₄, 2H2-GA₈, 2H2-GA₃₄ and 2H5-AIA (OlChemIm Ltd, Olomouc, Czech Republic) were added as internal standards. Z was determined by using a standard curve. Extracts were transferred to 50 ml tubes, centrifuged at 8000 rpm for 15 min, and supernatants were collected and mixed with ethyl acetate. Then, the organic phase was extracted and evaporated at 37°C. Dried extracts were dissolved in 1500 μ l methanol and evaporated and immediately resuspended in 50 μ l methanol (100%). 10 μ l of each sample were injected onto a Liquid Chromatograph (LC) with Electrospray Ionization (ESI) (Waters Corp., New York, NY, USA).

Liquid chromatography

Analyses were performed using an Alliance 2695 (LC Separation Module, Waters, USA) quaternary pump equipped with auto-sampler. A Restek C18 (Restek, USA) column (2.19 x100 mm, 5 μ m) was used at 28°C. The binary solvent system used for elution gradient consisted of 0.2% acetic acid in H₂O (solvent B) and MeOH (solvent A), at a constant flow-rate of 200 μ l min⁻¹. A linear gradient profile with the following proportions (v/v) of solvent A was applied [t (min), % A]: (0, 40), (25, 80), with 7 min for re-equilibration.

MS/MS experiments were performed on a Micromass Quattro UltimaTM PT double quadrupole mass spectrometer (Micromass, Manchester City, UK). All analyses were performed using turbo ion spray source in negative ion mode with the following settings for each hormone: capillary voltage -3000 V, energy cone 35 V, RF Lens1 (20), RF Lens2 (0.3), source temperature 100°C, solvation temperature 380°C, gas cone 100 l h⁻¹, collision (50), and multiplier (650).

Mass spectrometry

MS/MS parameters were optimized in infusion experiments using individual standard solutions of each hormone at a concentration of 50 ng μ l⁻¹ diluted in mobile phase A/B (40:60, v/v). MS/MS product ions were produced by collision-activated dissociation of selected precursor ions in the collision cell of the double quadrupole mass spectrometer, and mass was analyzed using the second analyzer of the instrument. In negative mode, the spectrum for each hormone gave deprotonated molecules [M-H]⁻. Quantitation was performed by injection of samples in multiple reaction monitoring (MRM) modes, since many compounds could present the same nominal

molecular mass. The combination of parent mass and unique fragment ions was used to selectively monitor hormones in plants extracts. MRM acquisition was performed by monitoring the ABA and 2H6ABA: 263>153 and 269>159, GA₁ and 2H2-GA₁: 348>242 and 350>244; GA₃ and 2H2-GA₃: 345>221 and 347>223; GA₄ and 2H2-GA₄: 332>244 and 334>246; GA₈ and 2H2-GA₈: 364>276 y 366>278; GA₃₄ and 2H2-GA₃₄: 347>244 and 349>246; while for AIA and 2H5-AIA: 175>130 y 180>135 respectively, with dwell 1000 ms for each transition. Zeatin was identified and quantified by comparison with the standard curve establishing parent and transition ions respectively.

Data were acquired and analyzed using MassLynxTM 4.1 and QuanLynxTM 4.1 (Micromass, Manchester, UK) software. For quantification, values were obtained from a calibration curve previously constructed using each hormone (ABA, GAs, AIA) and their hormone pure standard/ deuterated internal standards (Sigma, St. Louis, MO, USA).

Statistical analysis

Data were analyzed using INFOSTAT (v 2011). A two factorial experiment: osmotic potential (Ψ_o) (-1.0, -1.9, or -2.6 MPa) and salt treatment (control, NaCl, Na₂SO₄) was set up in a completely randomized design. Two way ANOVA was performed and significant differences among treatments were calculated by the use of pair-wise comparisons using Duncan significant difference test ($p < 0.05$).

Results

Growth parameters

Root growth increased from the beginning of the experience in all treatments and no significant differences were detected between them (Figure 1). Shoot growth also increased during the whole experience and was not affected by the salt treatments at low osmotic potentials (-0.3 and -1 MPa). From -1.9 MPa and lower, Na₂SO₄-treated plants showed growth inhibition, while NaCl treated plants showed maximum growth although no significant differences with controls were detected. The number of leaves showed no significant differences between treated and control plants at 40 days of culture. However, at the end of

the experiment Na₂SO₄-treated plants showed the lowest number of leaves as compared to controls at 48 days of culture.

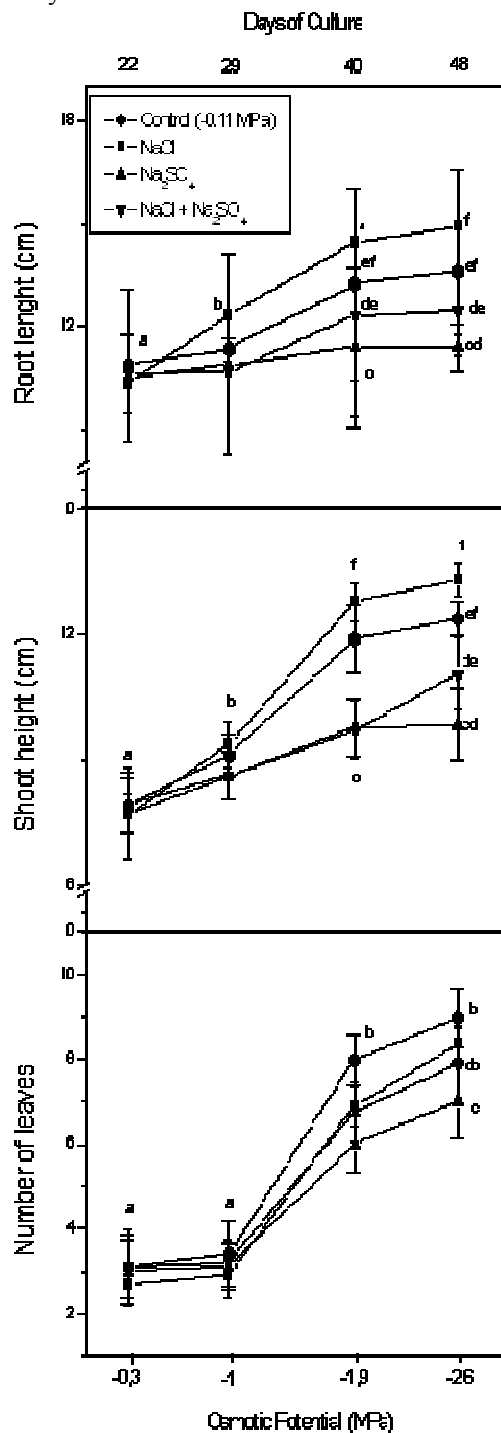


Figure 1. Effects of NaCl, Na₂SO₄ and NaCl + Na₂SO₄ on root length shoot height and number of leaves of *Prosopis strombulifera* at different osmotic potentials (MPa).

Different letters above data indicate significant differences among treatments ($p <$

0.05). Bars are means \pm S.E.

**Phytohormones content
In NaCl-treated plants**

As shown in Figure 2, at Ψ_o -1.0 and -1.9 MPa, ABA level in roots was higher in non-treated plants than in salt-treated plants. Instead, at Ψ_o -2.6 MPa, NaCl treatment caused maximal ABA level, while in leaves no significant differences in ABA levels were observed in relation with non-treated plants.

Gibberellins content followed a different profile according to the salt treatment applied. However, GA₄ was the predominant gibberellin while GA₃ was the less abundant (and in some samples not detected, data not shown), both in roots and leaves from all salt-treated plants. NaCl treated plants showed the greatest accumulation of GA₄ and GA₁ from the beginning of the assay, especially at -1.9 and -2.6 MPa when compared to other salt treatments.

In roots, the highest content in GA₃₄ was observed at -1 MPa which decreased when salt concentration increased while in leaves this inactive gibberellin maintained high levels during the whole experiment. GA₈ levels in leaves and roots of NaCl treated plants increased at -1 and -1.9 MPa and decreased at -2.6 MPa.

Contrary to GAs profile, IAA levels in roots and leaves of NaCl treated plants were not

significantly modified as compared to non treated plants (Figure 4).

Remarkably, Zeatin levels increased significantly in roots of these plants along the experiment, with a sharp peak at -1.9MPa. (ca. 4-fold increase in relation to non-treated plants) (Figure 5), while in leaves of NaCl treated plants Z content increased significantly only at low salinity (-1 MPa).

In Na₂SO₄-treated plants

Hormonal profile was different in Na₂SO₄-treated plants as compared to NaCl treated plants. In roots, no significant differences in ABA levels were observed between these plants and non-treated plants. In leaves, a sharp increase in ABA levels was observed in Na₂SO₄-treated plants from -1.9 MPa and lower.

In roots of Na₂SO₄-treated plants levels of GA₁ and GA₄ were higher at the beginning of salinization; GA₄ diminished abruptly at -1.9MPa while GA₁ did it at -2.6MPa.

In leaves, GA₁ levels showed the same tendency that in roots, while GA₄ levels there were not significant differences with non-treated plants.

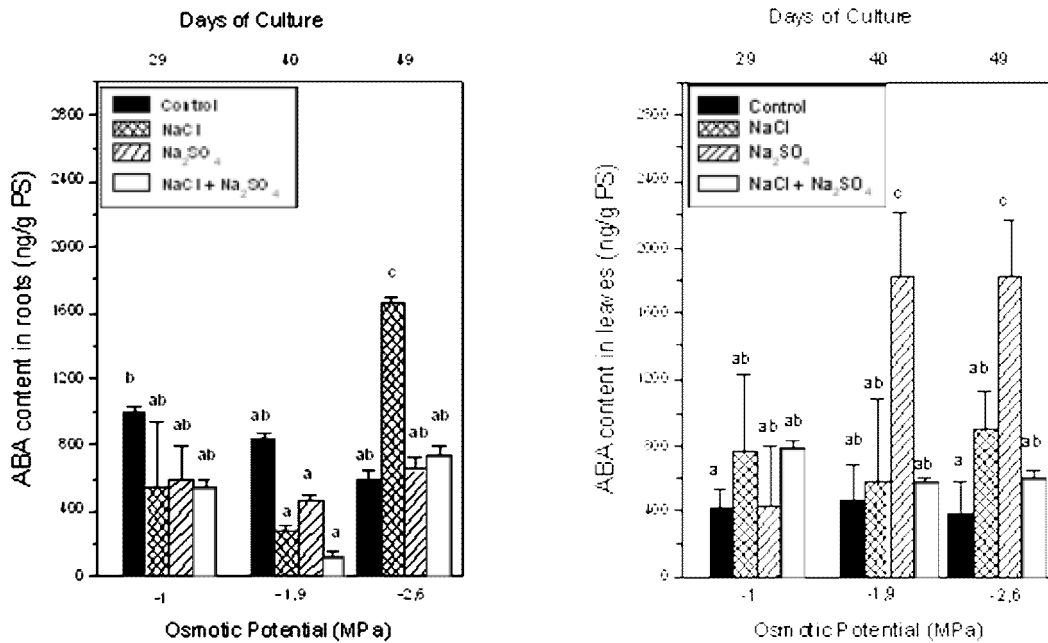


Figure 2. Effects of NaCl, Na₂SO₄ and NaCl + Na₂SO₄ on abscisic acid (ABA) content in roots and leaves of *Prosopis strombulifera* at different osmotic potentials (MPa). Different letters above data indicate significant differences among treatments (p < 0.05). Bars are means ± S.E.

GA₃₄ and GA₈ levels in roots of Na₂SO₄-treated plants decreased when the salinity increased in the medium (Figure 2). In leaves, GA₈ was increased at low and moderate salinity (-1 and -1.9MPa) with no difference with controls at -2.6MPa. GA₃₄ showed very high levels (similar to those found in NaCl treated plants) at low and moderate salinity, but decreased sharply at -2.6MPa.

As shown in Figure 3, highest levels of IAA were observed in roots of Na₂SO₄-treated plants at high salinity (-2.6 MPa), while in leaves only an increase at the beginning of the assay was observed (Figure 3).

Z accumulation was observed in roots of Na₂SO₄-treated plants at low and high salinity, while in leaves, an increase was observed only at high salinity (Figure 4).

The hormonal profile found in NaCl + Na₂SO₄ treated plants showed intermediate responses between those in monosaline solutions. The pattern of both active and inactive GAs was similar to that in NaCl treated plants (Figure 2). No significant differences were observed in IAA levels between bisaline-treated and non treated plants. Z levels were no modified in these plants as compared to non-treated plants (Figures 3 and 4).

As shown in Figure 5, highest levels of ABA/IAA rate were observed in roots of Na₂SO₄-treated plants at low salinity (-1 MPa), while highest levels of ABA/GA were observed in these plants at high salinity (-2.6 MPa). A high IAA/Z rate was observed in roots when sulfate salt is present in the culture medium.

ABA/Z rate is higher in roots of Na₂SO₄ and bisaline treated plants at moderate salinity (-1.9 MPa). In leaves, remarkable highest levels of ABA/GA and ABA/IAA rates and the lowest IAA/Z rate in Na₂SO₄-treated plants were observed.

Discussion

Phytohormones play an essential role in the regulation of plant developmental responses and counteracting adverse biotic and abiotic conditions. In some plants, stress tolerance has been related to morphological adaptations probably regulated by specific phytohormones (Arbona and Gómez-Cadenas, 2008).

Our previous studies demonstrated that *P. strombulifera* is much less tolerant to Na₂SO₄ than

to NaCl. Plants grown in the presence of Na₂SO₄ showed immediate and sustained reduction of growth parameters, accompanied by senescence symptoms such as chlorosis, necrosis, and leaf abscission (Reinoso et al., 2005; Reginato et al. 2014). In this study, root length, shoot height and number of leaves of Na₂SO₄-treated plants were also more affected than those in NaCl-treated plants, confirming previous observations on the inhibition and toxicity induced when sulfate anion is present in the medium.

As expected, results of this study indicate that exposure to different concentrations of Na₂SO₄ and NaCl, the most abundant salts in Central Argentina and also in several other countries (Iqbal, 2003; Shi and Sheng, 2005; Manivannan et al., 2008) altered endogenous hormonal balance both in leaves and roots of *P. strombulifera*.

Gibberellins are known to regulate several aspects of plant development, such as seed germination, stem elongation, and fruit development. Among the complex plant responses to stress, growth decrease is a common strategy that allows resources to be focused on withstanding the stress. Many hormone signaling pathways have been implicated in stress responses, including the accumulation of DELLA proteins which restrain growth and promote stress resistance (Achard et al., 2006); a sublethal level of salt stress usually delays leaf expansion and plant growth. Also, gibberellin concentrations in salt stress-treated plants are lower than those in unstressed plants. Therefore, the limitation of growth by salinity is at least partly because of a reduction in gibberellin concentration. Along this line, studies have revealed that a quadruple DELLA mutant lacking GAI, RGA, RGL1 and RGL2 is more sensitive to salt stress as compared to the wild type. Thus, it has been proposed that stress reduces gibberellin levels and leads to DELLA proteins accumulation, which put down plant growth and confers stress tolerance (Achard et al., 2006). An emerging theme is stress-induced up-regulation of GA2ox gene expression as a means of reducing GA content and allowing DELLA accumulation (Hedden and Thomas, 2012).

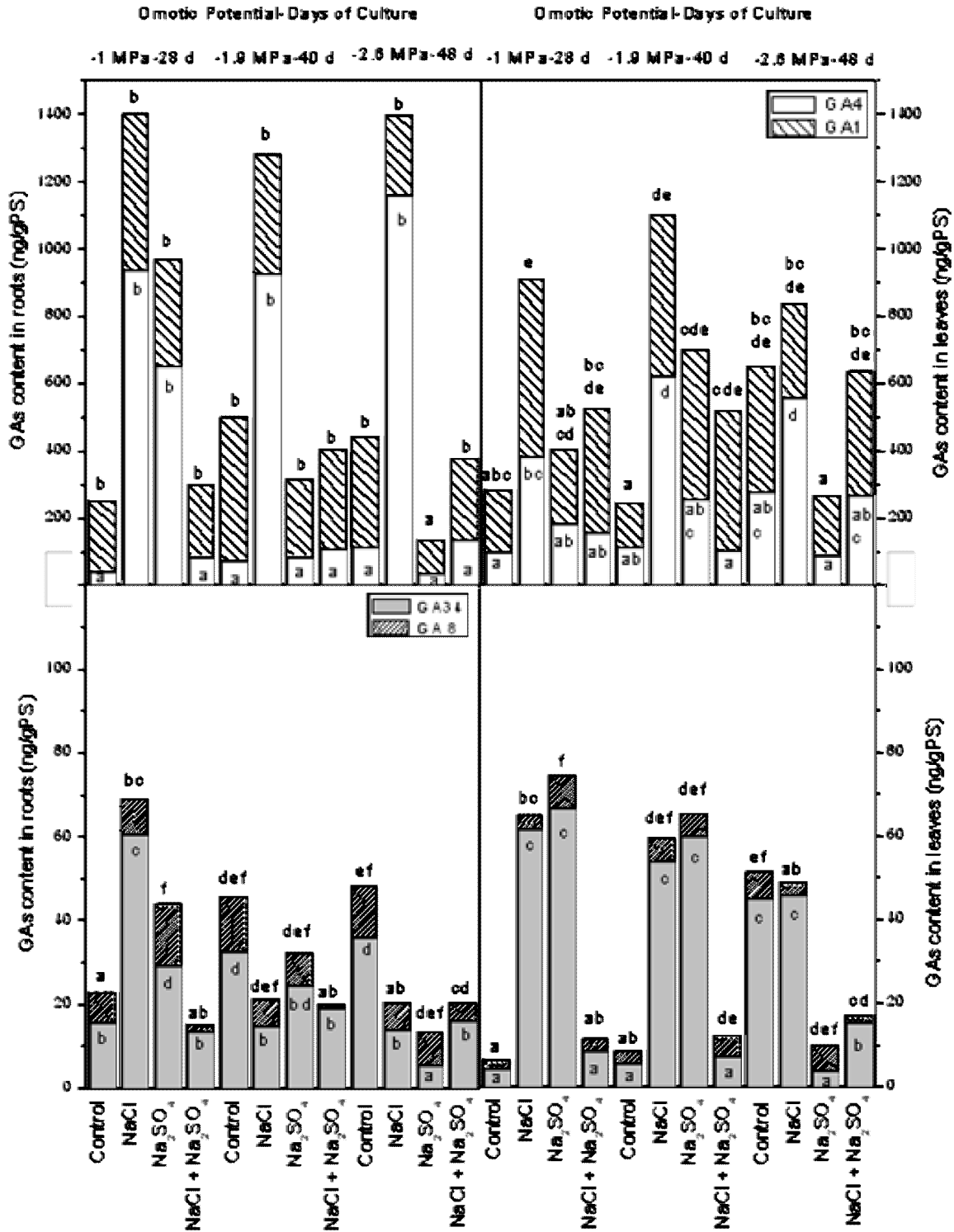


Figure 3: Effects of NaCl, Na₂SO₄ and NaCl + Na₂SO₄ on gibberellins (GAs) GA₁, GA₄, GA₃₄ and GA₈ content in roots and leaves of *Prosopis strombulifera* at different osmotic potentials (MPa). Different letters above data indicate significant differences among treatments (p < 0.05).

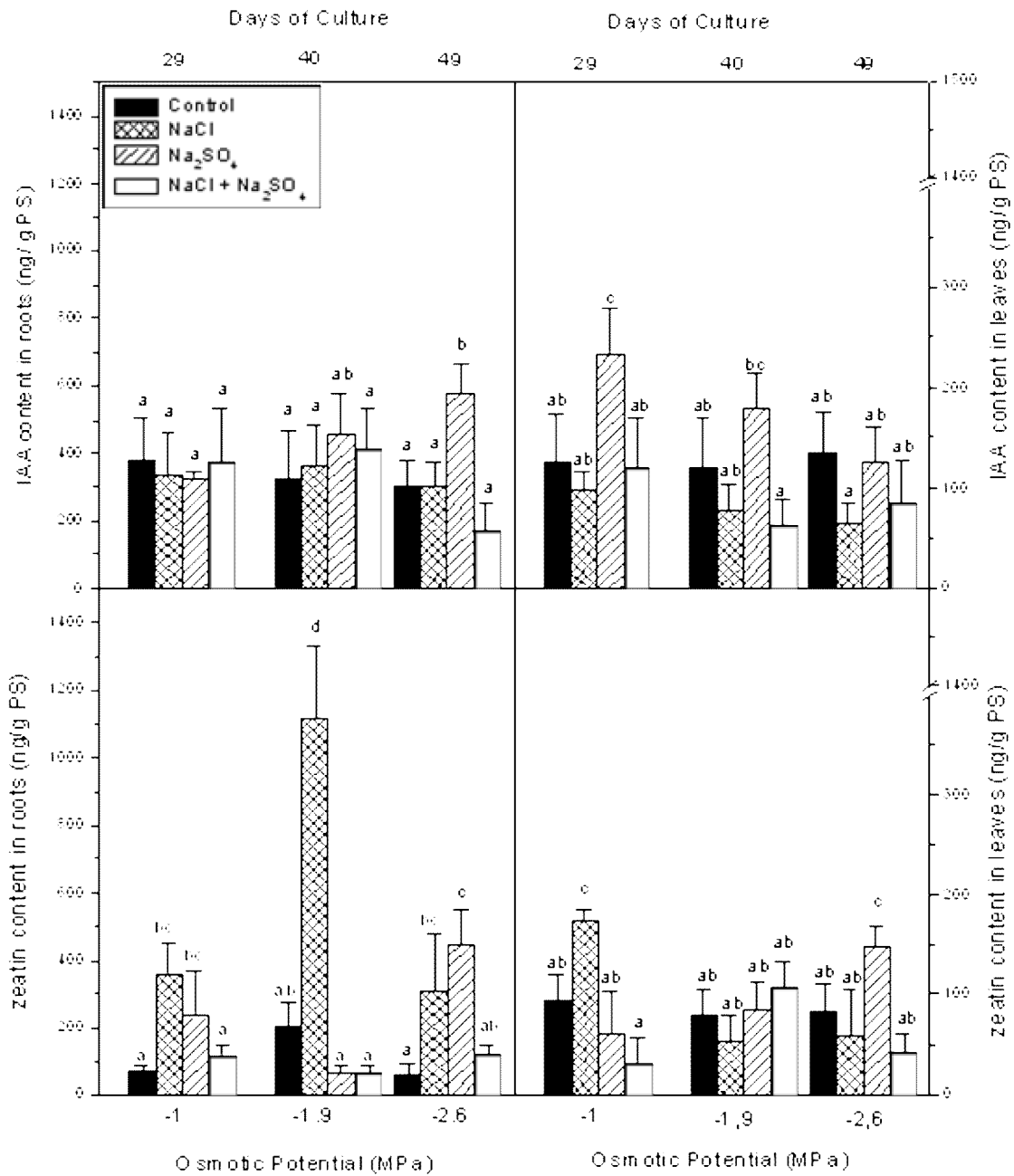


Figure 4: Effects of NaCl, Na₂SO₄ and NaCl + Na₂SO₄ on indole-3-acetic acid (IAA) and zeatin (Z) content in roots and leaves of *Prosopis strombulifera* at different osmotic potentials (MPa). Different letters above data indicate significant differences among treatments (p < 0.05). Bars are means ± S.E.

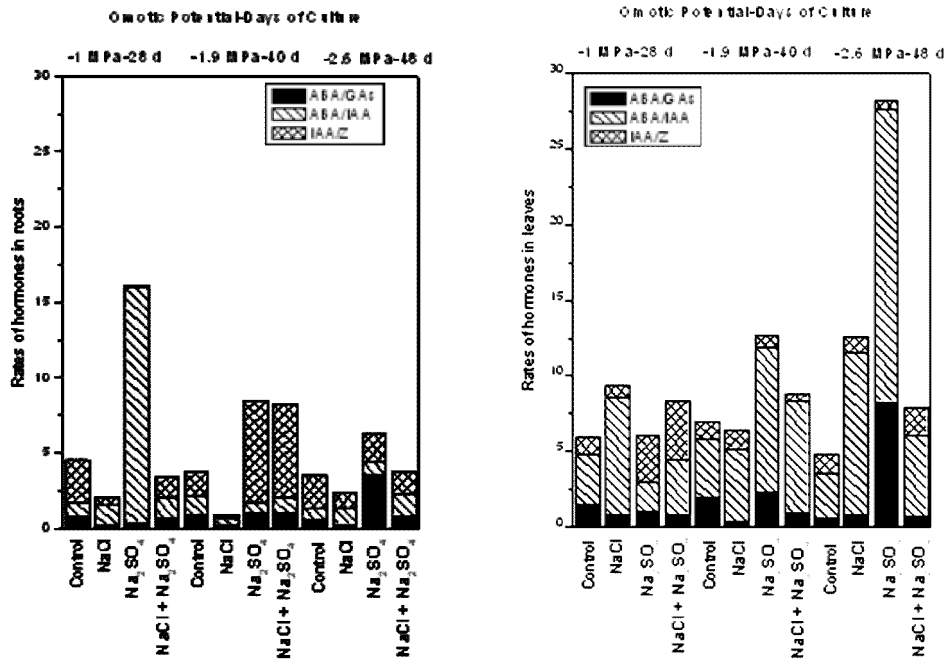


Figure 5: Effects of NaCl, Na₂SO₄ and NaCl + Na₂SO₄ on ABA/GAs, ABA/IAA and IAA/Z rates in roots and leaves of *Prosopis strombulifera* at different osmotic potentials (MPa).

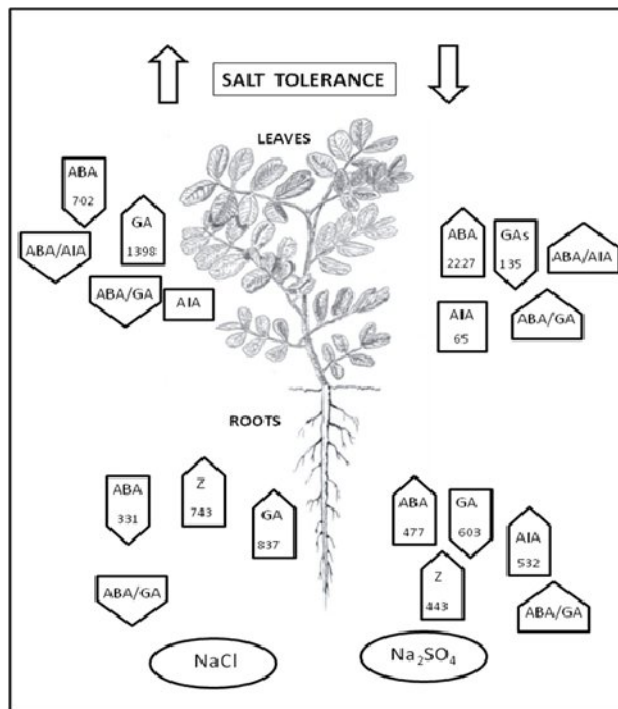


Figure 6. Hormonal patterns in leaves and roots of *P. strombulifera* plants differing in salt tolerance to NaCl (left) and Na₂SO₄ (right) at -2.6 MPa. Arrows indicate the relative increase (arrow up), decrease (arrow down) or not change (rectangle) in each phytohormone at the end of the experiment. The numbers inside the arrows indicate the corresponding absolute concentration of each hormone.

Several reports have indicated that GA application on halophytic species increased growth. In *Suaeda maritima* var. *macrocarpa* and *S. depressa* GA₃ was found to stimulate growth under salinity levels up to 360 mM NaCl and this was interpreted as an indication that there might be some interaction between salinity and induction of GA activity (Boucaud and Ungar, 1976). At high salinity (800 mM NaCl) shoot growth of *Suaea fruticosa* was stimulated by application of GA₃, but non salinized plants and those growing at lower salinities were not promoted by this hormone (Khan et al., 2000). This type of experiments led to the conclusion that GA₃ application significantly stimulated growth of halophytes treated with NaCl (Ungar, 1978; Wochok and Sluis, 1980; Ke-Fu et al., 1986; Khan et al., 2000).

However, it should be noted that in all these reports, the relationship between salinity and gibberellins has been analyzed solely in experiments with exogenous applications, and by using the most commercially available gibberellin, GA₃, which is not a common endogenous gibberellin in most species. Notwithstanding, a possible explanation for the shoot growth promotion observed in the papers cited above, is that the same GA-3 β -hydroxylase that catalyzes the conversion of GA₂₀ to GA₃ in some species (Hedden and Phillips, 2000), also catalyzes exogenously applied GA₃ to GA₁, as proposed by Chen et al. (2008).

Our results show that in *P. strombulifera*, the concentration and chemical composition of the salts present in the medium modulated GA synthesis and metabolism. GA₄ is an active gibberellin present in several species and would be the most important shoot growth regulator in *Arabidopsis* and some members of genus *Cucurbita* (Lange et al., 2011).

Notably, our results show that GA₄ is the predominant active form found in *P. strombulifera* especially in roots, where GA₄ accumulation in NaCl-treated plants was very high during the whole experiment. In leaves, a 5 fold increase respect to controls was determined at -1.9 MPa in correlation with optimum growth for this species. High GA₄ levels were maintained at -2.6 MPa. In these organs, also GA₁ levels were significantly increased in relation to controls (3.5 fold at -1.9 MPa) indicating that both gibberellins play a role in controlling shoot growth in this species under NaCl treatment. The high levels of active GAs in *P. strombulifera* NaCl-treated plants are correlated with their low ABA levels compared with Na₂SO₄-

treated plants, probably due to competition for the common intermediate for the synthesis of both carotenoids and GA from the precursor geranyl-geranyl diphosphate (Lefsrud et al., 2006).

In Na₂SO₄-treated plants at high salinity (-2.6 MPa) the lowest levels of GA₄ and GA₁ were detected, in coincidence with the maximum growth inhibition related to sulfate toxic effect which was accentuated with increasing salinity. Furthermore, bisaline-treated plants showed intermediate levels of GA₄ and GA₁ in concordance with an intermediate shoot length, showing a partial reversion of sulfate toxicity when both anions (chloride and sulfate) are present in the medium. Therefore, these results suggest that differential growth responses to both salts would be mediated, at least in part, by GA₁ and GA₄.

This pattern was correlated with the inactive GAs profile observed in our experiments. In fact, in roots of NaCl-treated plants the diminution of GA₃₄ levels at -2.6MPa corresponds to an increment in GA₄ levels, probably due to the need of maintaining growth rate under high salt concentration; as mentioned above, under 450-550 mM NaCl *P. strombulifera* seedlings showed growth stimulation. Inversely, Na₂SO₄-treated plants had much lower levels of GA₄ together with high levels of its metabolite GA₃₄ suggesting that GA₄ inactivation may be a consequence of sulfate toxicity; the highest concentration of this salt causes very low levels of all GAs, in their active or inactive forms. Lower levels of GA₈ in relation to GA₃₄ in most salt treatments would indicate that, for some reason, there was less GA₁ oxidation in our experiments.

Recently, it has been demonstrated that GA₁ has a role in cell expansion of the endodermis in the elongation zone of the primary root of *Arabidopsis thaliana* (Uehara et al., 2008). In our study, less content of GA₁ in roots of Na₂SO₄-treated plants might be related with their scarce root development as compared to controls (Reinoso et al., 2005). An interesting topic to investigate is the putative involvement of DELLA proteins in these responses. Achard et al. (2009) established that GAs control both cell proliferation and expansion rates, processes that depend on DELLAs destruction. Therefore, throughout plant development, DELLAs slow down growth of leaves and primary root, by decreasing the rate of division of proliferating cells, and by altering the rate of elongation once cells are differentiated. Additionally, Peres et al. (2007) demonstrated that DELLAs restrain cell cycle by stimulating the accumulation of cell cycle

inhibitors, particularly members of the plant-specific SIM gene family. Since it has been shown that expression of the SIM gene family responds to various biotic and abiotic stresses it was suggested that this class of cyclin-dependent kinase inhibitors couples the cell cycle to environmental conditions. Recent researches revealed that DELLA restraint provides a common mechanism allowing flexible and appropriate growth in response to changes in natural environment (Achard et al., 2006, 2008). Overall, these studies suggest that DELLA-mediated control of SIM expression provides such a mechanism, enabling plants to modulate their cell proliferation rate according to environmental conditions (Achard et al., 2009)

Auxins also influence plant growth responses under environmental stresses.

Among the most important functions of auxin in plants is the formation of lateral roots, which is of particular significance to plant growth under different adverse conditions. The initiation of lateral roots plays a vital role in plant development, since it determines the root system architecture, and thus, provides physical support and ensures water and nutrient uptake potential for the whole plant. Lateral root formation is a good example of a canalized developmental process (i.e., buffered against perturbation) (Siegal and Bergman, 2002). Roots have the capacity to adapt to changes in the environmental conditions to maximize the acquisition of nutrients and water from the soil. Recently, it has been known that the initiation of lateral roots proceeds from a small number of pericycle cells which differentiate into primordia that grows out of the primary root (De Smet et al., 2006). Auxin is a trigger for lateral root morphogenesis and its local maximum acts as the signal for initiation of these organs (Dubrovsky et al., 2008). Several roles of auxins are mediated by transcription factors of the auxin response factor family (ARF), and some of these ARFs play essential roles in the development of lateral roots (Wilmoth et al., 2005). Recently, Marin et al. (2010) have showed that auxin, the micro RNAs miR390 and TAS3, and their ARFs targets determinate a regulatory network that quantitatively controls lateral root growth. This complex network fine-tunes local auxin responses and thus provides robustness and flexibility to lateral root growth. Thus, modifications in auxin homeostasis can lead to impaired growth and development as a component of a strategy of general acclimation that avoids or reduces the deleterious effects of adverse environmental conditions (Potters et al., 2010; Tognetti et al., 2012). Our results show that in roots

of Na₂SO₄-treated plants the high level of IAA is in relation with increased lateral root formation (Reinoso et al., 2005). However, under NaCl treatment plant roots showed low IAA content, no lateral roots formation and no anatomical and histological differences when compared to controls (Reinoso et al., 2004). These results suggest involvement of IAA signaling in the physiological responses of *P. strombulifera* roots to salt stress, related to the induction of lateral roots by ionic stress caused by the presence of sulfate anion in the medium.

It has also been reported that halophytic plants as *Atriplex leucoclada*, *Suaeda fruticosa* and *Salicornia virginica* showed higher concentration of ABA as compared to IAA. In these plants there was a strong interaction between ABA/IAA ratio, K⁺/Na⁺ ratio, and proline content. *Atriplex leucoclada* had maximum ABA/IAA ratio as well as higher K⁺/Na⁺ ratio and higher proline and protein contents, while *Haloxylon salicornicum* and *Salicornia virginica* had lower K⁺/Na⁺ ratio, lower ABA/IAA ratio as well as lower proline and protein content (Samiullah and Bano, 2011). In *P. strombulifera* higher ABA/IAA ratio was observed only in Na₂SO₄ treated plants, however, no increased K⁺/Na⁺ ratio or proline and protein content was observed (Reginato et al., 2014; Llanes et al., 2012).

On the other hand, it has been proposed that the ratio between ABA and CKs in the xylem sap could potentially modulate several stresses and/or developmental processes as a long-distance signal (Schachtman and Goodger, 2008). CKs are known to induce several processes such as cell division, initiation of lateral buds, promotion of leaf expansion and delay of senescence. CKs endogenous concentrations decrease under stress conditions. This response is accompanied by an increase in ABA levels, which results in stomatal closure and ABA-dependent gene expression. These changes induce leaf senescence and abscission, which cause a small canopy and limited water loss. Therefore, resources are employed to ensure plant survival.

Recent studies have proposed that CK signaling is indeed involved in several plant responses to abiotic stress. Double mutations in two CK receptor genes, AHK2 and AHK3 (*Arabidopsis* histidine kinase 2 and 3), improve the tolerance to osmotic stress. Under lethal salt conditions (200 mM NaCl), the survival rate of this mutant is significantly higher than that of the wild type. A similar response was observed when the plants were compared under severe water stress. The higher survival rate

indicates that these two CK receptors play negative roles in plant stress responses (Tran et al., 2007b). Other studies have demonstrated that the content and transport of CKs were reduced by drought and/or salinity in several plant species (Argueso et al., 2009; Nishiyama et al., 2011). Cytokinin reduction in Arabidopsis shoots under stress may be due to IPT gene repression and/or cytokinin oxidase/dehydrogenase (CKX) activation, as well as decreased transport in the xylem of cytokinins produced by roots (Nishiyama et al., 2011). Tobacco plants showing root specifically increased CKX gene expression, constitutive CKX overexpression, and the corresponding WT, indicated that constitutive overexpression of CKX1, linked to greater root system and dwarf shoots, confers enhanced tolerance to both drought and heat stresses (Macková et al., 2013). Also, Vyroubalová et al. (2009) demonstrated that O-glucosylation takes part in the rapid homeostasis of cytokinin metabolites under different physiological stimuli.

Altogether, these studies support the idea that CKs are negative regulators of salinity tolerance.

Cytokinins are commonly discussed as antagonists of ABA action on stomata. Exogenous application of cytokinins has been shown to keep stomata open, although the importance of these hormones in plant growth and development under salinity remains unresolved. The effects of exogenous cytokinins on stomatal conductance are less clear than those of ABA and very severe stresses are needed to significantly reduce cytokinin delivery to shoots. Certainly, increased levels of leaf cytokinins have been shown to correlate with stomatal opening in some plants (Vysotskaya et al., 2004); transgenic tobacco plants overproducing cytokinins, are characterized by increased transpiration and even wilting (Teplova et al., 2000).

In our study, the accumulation of Z in leaves of Na₂SO₄-treated plants at high salinity may explain the high transpiration previously observed in these plants, in spite of the high ABA levels (Llanes et al., 2013). Similarly, studies with the halophytes *Suaeda depressa* and *S. maritima* under water stress and treated with cytokinins suggest that this hormone may increase the symptoms associated with water stress (Boucaud and Ungar, 1976). This could be the result of the influence of cytokinin on stimulating stomatal opening, thus facilitating transpiration and therefore magnifying the effect of salt stress. On the contrary, the low transpiration observed in leaves of *P. strombulifera*

plants treated with NaCl correlated with their low Z content probably as a result of less synthesis or decreased transport of Z from the roots. In the halophyte *Mesembryanthemum crystallinum* exogenous treatment with 6-Bencyl-amino-purine (BAP) was shown to inhibit the activity of CAM-metabolism key enzyme phosphoenolpyruvate carboxylase, and plant transition to CAM-metabolism under salt stress. That is why the increase of zeatin, zeatin riboside and isopentenyladenine levels observed in these plants may be considered as a mechanism preventing the metabolism-type changes under salinity (Schmitt et al., 1996). However, BAP addition to nutrient solution in hydroponic culture of *M. crystallinum* resulted in the same quantitative phosphoenolpyruvate carboxylase increase as the NaCl addition; exogenous cytokinin could mimic salt-induced responses, greatly increasing PEPCase, proline, ononitol, pinitol, and osmotin (Thomas and Bohnert, 1993).

In *P. strombulifera*, pinitol or proline levels are increased under NaCl as well as Na₂SO₄ and bisaline treatment (Llanes et al., 2012), while Z levels in roots and leaves of plants under these treatments were different.

In this species endogenous Z seems to be involved in the cross regulation with IAA to induce or inhibit lateral root formation. Effectively, cytokinin (Z) is primarily synthesized in the root cap (Miyawaki et al., 2006) and has a strong antagonistic effect to auxin on root architecture where cytokinins have an inhibitory effect on lateral root branching and increased numbers of lateral roots are found in mutants for cytokinin biosynthesis and sensitivity (Laplaze et al., 2007). Auxin is synthesized essentially in young aerial tissues and undergoes polar transport towards the root tissues. Then, it is distributed in the root tissues through strategic positioning of auxin carriers, especially the auxin efflux carrier PINFORMED (PIN). This carrier directs the auxin flux allowing IAA accumulation in specific regions (Bohn-Courseau, 2010). The inhibitory effect of cytokinin on lateral root branching seems to be caused by cytokinin-mediated down regulation of PIN gene expression. These PIN family auxin efflux facilitators are needed to produce the targeted auxin maxima for priming pericycle cells to form correctly patterned lateral root primordial (Laplaze et al., 2007). In coincidence, our results show that in roots of *P. strombulifera* plants, the Z levels are negatively correlated with IAA content; the lower Z content was observed in roots of Na₂SO₄ treated

plants where the highest IAA levels and increased lateral root formation were observed (Reinoso et al., 2005) as mentioned above. Inversely, roots of NaCl treated plant showed highest Z levels at moderate salinity associated with an enlarged root system.

Conclusions

As the plant hormone network becomes clearer and local concentrations of key components are being accurately measured, models are required to evaluate which steps are most likely acting to control specific aspects of plant growth and development.

As previously mentioned, numerous authors have demonstrated that plant hormones are involved in the regulation of plant responses to salinity conditions, mainly in glycophytes.

This is the first study reporting the hormonal profile in *Prosopis strombulifera*, a halophytic plant which is differentially affected by NaCl and Na₂SO₄ providing a very useful experimental system which allows to compare two different phenotypes, a tolerant and a non-tolerant one.

The attribute of salinity tolerance (under NaCl treatment) was correlated with high CK and GA₄ levels and lower ABA/GA and ABA/IAA ratios. In the absence of secretory glands, anatomical adaptations of this halophytic plant are related to metabolic changes that result from a complex and accurately tuned physiological regulation involving an improved water uptake, ion compartmentalization and compatible solute synthesis to protect the cytoplasm in high NaCl salinity (Llanes et al., 2013; Reginato et al., 2014), all of which allowed optimum growth at -1.9MPa (500 mM NaCl). These plants showed anatomical and histological differences between control, tolerant, and non-tolerant-treated seedlings, mainly in the speed of tissue differentiation, tissue organization and tannin accumulation (Reinoso et al., 2004).

On the other hand, the anatomical modifications imposed by the SO₄²⁻ salt treatment, tended to ensure the survival of the plant under an extremely stressing condition. They were: greater number of cortical layers in primary root and earlier development of endodermis, induction of lateral root formation, reduced cambial activity in older root zones and hypocotyls, one to three layers of highly vacuolated cortical parenchyma in young stems, thin leaflets with dense chlorenchyma and abundant small stomata, and strongly increased polyphenols accumulation in the whole plant (Reinoso et al., 2005). The hormonal profile of

these plants showed very low levels of bioactive gibberellins, high ABA levels in leaves and roots, high IAA/CK levels in roots and high CK levels in leaves.

These different patterns of phytohormones elicited by different sodium salts indicate that primary signaling pathways were modified by the chemical composition of the salts present in the medium (summarized in Figure 5). The understanding of the interaction between hormonal changes and stress tolerance is fundamental for identifying key steps involved in stress signaling and plant stress adaptation. However, the chain of events leading to these metabolic differences caused by chloride and sulfate sodium salts is far from being elucidated, and adds complexity to the already known wide range of ionic factors involved in plant responses to salinity. Indeed, in bisaline-treated plants intermediate values were observed in the levels of these phytohormones as compared to monosaline treatment, resembling previous observations on ion content, compatible solutes and quantity and quality of polyphenols (Llanes et al., 2013; Reginato et al., 2013, 2014).

Finding mutants that enhance or limit cross-regulation between pathways may finally answer the old question of how hormones manage to do so many different things in so many different contexts in such an accurate manner.

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