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Research article

Metabolomic profiling of the halophyte *Prosopis strombulifera* shows sodium salt- specific response

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ABSTRACT

Primary and secondary metabolite profiles were analyzed in roots and leaves of the halophytic shrub *Prosopis strombulifera* in response to control plants (no salt added in the growing media) and to lowering the osmotic potential to −1.0, −1.9, and −2.6 MPa generated by NaCl, Na_2SO_4, and the iso-osmotic combination of them at 24 h after reaching such potential. A rapid production of metabolites in response to sodium salt was found, which was correlated with modifications in growth parameters. Analysis of polar metabolite profiles by GC-MS rendered a total of 108 significantly altered compounds including 18 amino acids, 19 secondary metabolites, 23 carbohydrates, 13 organic acids, 4 indole acids, among others. Primary metabolites showed a differential response under the salt treatments, which was dependent on salt type and concentration, organ and age of plants. Most of identified compounds showed the strongest accumulation at the highest salt concentration assayed for Na_2SO_4-treated plants, which was correlated with damaging effects of sulfate anion on plant growth. Roots of NaCl-treated plants showed a higher number of altered metabolites (analyzed by UPLC-ESI-QqTOF-MS) compared to other treatments, while leaves of Na_2SO_4-treated plants showed the highest number of altered signals. A low degree of overlapping between secondary metabolites altered in roots and leaves of NaCl and Na_2SO_4-treated plants was found. However, when both NaCl and Na_2SO_4 salts were present plants always showed a lower number of altered metabolites. Three compounds were tentatively identified: tryptophan, lysophosphatidylcoline and 13-hydroxyoctadecadienoic acid. Increasing knowledge on *P. strombulifera* metabolism will contribute to unravel the underlying biochemical mechanism of salt tolerance.

1. Introduction

Soil salinity is one of the main environmental conditions affecting the crop production. High salts contents are increasing due to many irrigation practices and the global climate changes, thus it was estimated that 45 million hectares of irrigated are affected by soil salinity (Rengasamy, 2010). The detrimental effects of salinity on agricultural yield are very important, mainly because the deleterious effect of salinity can limit plant growth and development (Munns and Tester, 2008). High salt contents on soils can provoke a water deficit in plants and an ion toxicity and deficiency in some nutrients leading to molecular damage and consequently the death of plants (Maggio et al., 2010). Several viable management options have been developed to improve plant productivity in saline soils, for example different methods of irrigation and drainage, but these options have not been successful to date. Thus, improving salt tolerance of crops by molecular and plant breeding approaches is the most attractive and sustainable option to support crop production in salt-affected soils (Ondrasek et al., 2011).

The generation of salt-tolerant crops may be aided by a clear understanding of the complex mechanism of abiotic stress

Abbreviations: ESI mode, electrospray ionization mode; GC-MS, gas chromatography-mass spectrometry; HCA, hierarchical cluster analysis; PCA, principal component analysis; UPLC-ESI-QqTOF-MS, ultra pressure liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry.

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tolerance in key species. The process of evolution under saline environments gave rise to halophytes which have developed adaptive characteristics to cope with soil salinity (Flowers et al., 2010).

One halophyte model is *Prosopis strombulifera* (Lam.) Benth (Burkart, 1976). This plant species is a spiny shrub which grows in lands from the Arizona desert (U.S.A.) to Patagonia (Argentina), being particularly abundant in highly salinized soils from central areas of Argentina. The most abundant ions present in salinized soils are Na^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}, and HCO^{3-}. NaCl and Na_2SO_4 are the most commonly found salts in soils of many countries (Manivannan et al., 2008). In saline regions of southern Córdoba and southwestern San Luis provinces from Argentina, these two salts are found at similar concentrations. However, Sosa et al. (2005) found that Na_2SO_4 was up to three times more abundant than NaCl in some soil samples. Thus, it is important to research and compare both NaCl and Na_2SO_4 effects on plant growth, for a better understanding of the physiological and biochemical responses that plants develop to cope with soil salinity in natural environments. Previous results from our laboratory demonstrated that *P. strombulifera* is able to survive up to 1M NaCl in hydroponic culture medium, which shows a halophytic plant response (Sosa et al., 2005). On the contrary, *P. strombulifera* plants grown in the presence of Na_2SO_4 in the culture medium showed damaging effects on roots and shoots not reported for NaCl-growing plants, confirming previous reports on the growth-inhibitory effect of sulfate anion and the induction of senescence symptoms such as chlorosis, necrosis, and leaf abscission (Reinoso et al., 2005). *P. strombulifera* seedlings grown in increasing concentrations of NaCl in the culture medium (250 up to 700 mmol L^{-1}) did not develop salt glands in the leaves. The ability of this plant to survive to high NaCl concentrations depends on the efficient exclusion of toxic ions preventing their entrance to the root xylem, evidenced by their precocious lignification and suberization of the endodermis (Reinoso et al., 2004), an efficient compartmentation of Cl^- and Na^+ in leaf vacuoles and an increase of synthesis of compatible solutes such as pinitol, mannitol and proline, in the cytoplasm. The presence of Na_2SO_4 in the medium resulted in plants with a limited capacity of ion compartmentalization and osmotic adjustment, triggering water imbalances and symptoms of ion toxicity caused by an altered carbon metabolism (Llanes et al., 2013; Reginato et al., 2014a).

These differential physiological responses to the most commonly found salts in soils and the exceptional degree of tolerance to NaCl makes this species a good model to expand our knowledge about salt tolerance mechanisms.

The high throughput omics analyses, including transcriptomics, proteomics and metabolomics, enable a clear understanding of alterations in gene-protein-metabolite networks in plants growing in adverse environmental conditions (Urano et al., 2010).

In the last decades, metabolomic studies have developed mainly in the field of crop breeding and phytochemical genomics. It has been also used in the research of different plant responses related to environmental and/or genetic factors (Matsuda et al., 2012; Quanbeck et al., 2012; Saito, 2013). The end-products of the cellular functions are the metabolites; therefore the presence and relative concentrations of metabolites could be regarded as the best descriptors of the organism's phenotype. The metabolome of plants involve a wide variety of compounds with diverse biological functions and chemical characteristics such as amino acids, carbohydrates, lipids and other hydrophobic compounds. The complexity of compounds constitutes a huge challenge to the analytical technologies employed in current plant metabolomics programs. Thus, powerful analytical tools are necessary for suitable extraction, separation, purification and characterization of the wide diversity of compounds present in biological samples (Jorge et al., 2015). In addition, several plant species can produce their own unique array of metabolites, some of them still unknown, leading to a thorough analysis of the plant metabolome that exceeds the capabilities of existing analytical techniques (Saito and Matsuda, 2010). However, the information on plant metabolomic profiling may allow the identification of compounds playing a key role in plant stress tolerance and reveal novel pathways involved in salinity responses.

Therefore, the aim of this study was to analyze primary and secondary metabolite profiles in the halophyte *P. strombulifera* in response to increasing concentrations of NaCl and Na_2SO_4 and their iso-osmotic mixture and to correlate the differential metabolite expression with growth parameters, in order to establish their potential contribution to salinity tolerance.

2. Materials and methods

2.1. Biological material

P. strombulifera pods were collected from southwestern San Luis province, Argentina, located at 33° 43′ S, 66° 37′ W, altitude 400–500 m a.s.l., which has a temperate climate and an average annual temperature of 15–20 °C). The soil was sodium saline with abundant calcareous material, a 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 uniformity in size and healthy appearance. After collection, seeds were 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 °C for 24 h before sowing. Germinated seeds with roots 20 mm long were transferred to hydroponic cultures (two black trays per treatment, 200 seedlings per tray) using 10% (w/v) Hoagland's solution for the first week and 25% subsequently. Seedlings were grown in a growth chamber under a 16 h photoperiod, irradiance of 200 μmol m^{-2} s^{-1}, day/night temperatures of 28/20 °C, and relative humidity of 70%. Aeration was provided by an aquarium pump and pH was 6 for all media.

2.2. Salt treatments

Salt treatments were applied after 21 days of plant growth when media had osmotic potentials (Ψo) of −0.03 MPa using a simple randomized design (Steel and Torrie, 1995). Pulses of NaCl (50 mM) or Na_2SO_4 (38 mM) alone, and an iso-osmotic mixture of the two salts ("bisaline treatment") were applied every 48 h until reaching final Ψo of −1.0, −1.9, and −2.6 MPa, respectively (measured by a vapor pressure osmometer Model 5500, Wescor, Logan, UT, USA). These Ψo values were reached at plant 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. On each sampling date, 25 treated plants were collected at random 24 h after the medium reached the final osmotic potential, and also 25 control plants (no salt added; Ψo of medium −0.11 MPa) were collected for each treatment. The plants were frozen in liquid nitrogen and stored at −80 °C for further analyses.

2.3. Determination of growth parameters and Na^+ and K^+ ions

Root length and shoot height were measured weekly in 20 plants from each treatment from the time that salt pulses were started (21, 29, 40 and 48 days of culture). Samples containing 150 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 a Zeltec ZF250 IND flame photometer (Skoog et al., 2000).

2.4. Extraction, derivatization, and analysis of P. strombulifera polar and semipolar metabolites using gas chromatography–mass spectrometry

For GC-MS metabolite profiling analyses, 5 mg of lyophilized plant material (leaves or roots) was weighed and extracted in 300 μL of pure methanol (LC-MS grade, Panreac, Barcelona, Spain) spiked to 0.2 $mgml^{-1}$ribitol (IS) as described by Roessner et al. (2001). Extractions were performed by ultra-sonication for 10 min at room temperature. After centrifugation and recovery of supernatants, these were mixed vigorously with 200 μL of chloroform and 400 μL of water followed by centrifugation at 13.000 rpm and 4° C for 10 min. The upper water layer was recovered and evaporated to dryness using a SpeedVac (Jouan, Saint Herblain in Cedex, France). Dry residues were redissolved 50 μL of 20 mg mL^{-1}methoxyamine in pyridine followed by incubation at 30 °C in a water bath for 90 min. Afterwards, 70 μL of methylsilyltrifluoroacetamide (Macherey-Nagel, Germany) were added and subsequently incubated at 37 °C for 30 min. Finally, the solution was mixed with 10 μL of a commercial mixture of fatty acid methyl esters (C8–C24 FAME mix, Sigma-Aldrich, Madrid, Spain) as retention index (RI) markers.

Derivatized polar extracts were independently injected into a gas chromatography–mass spectrometry (GC-MS) system composed of an Agilent 7683 autosampler, an Agilent 6890 gas chromatograph and TOF-MS analyzer (GCT, Micromass Ltd., UK) equipped with an electron impact ion source. The GC separation was performed on a 30-m 5% polydimethylsiloxane capillary column (0.250 mm id, 0.25 μm film thicknesses, Agilent Technologies,

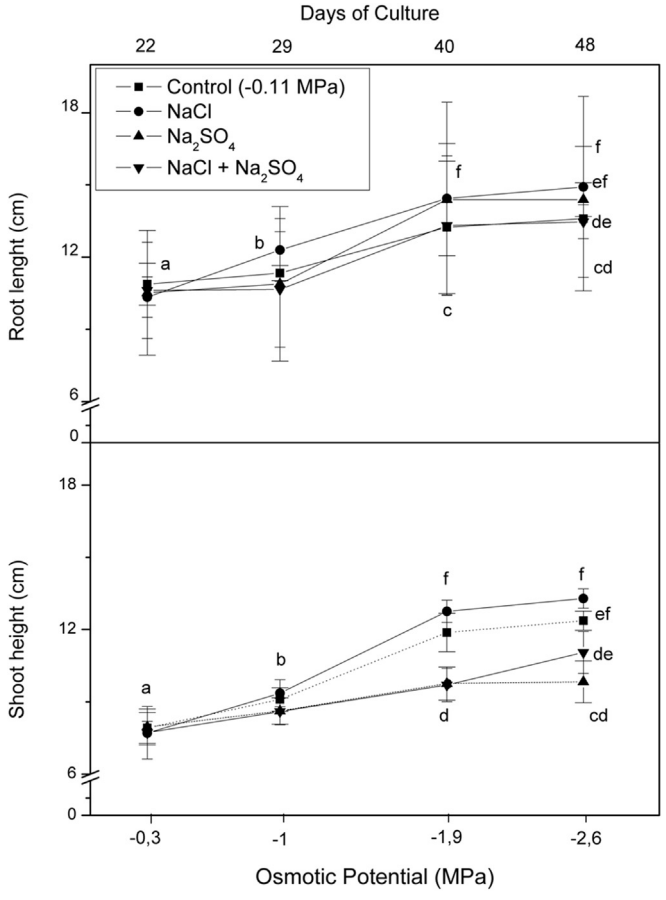


Fig. 1. Effects of NaCl, Na_2SO_4 and NaCl + Na_2SO_4 on root length and shoot height of *Prosopis strombulifera* at different osmotic potentials (MPa). Different letters above data indicate significant differences among treatments ($P < 0.05$). Data represent means ± S.E.

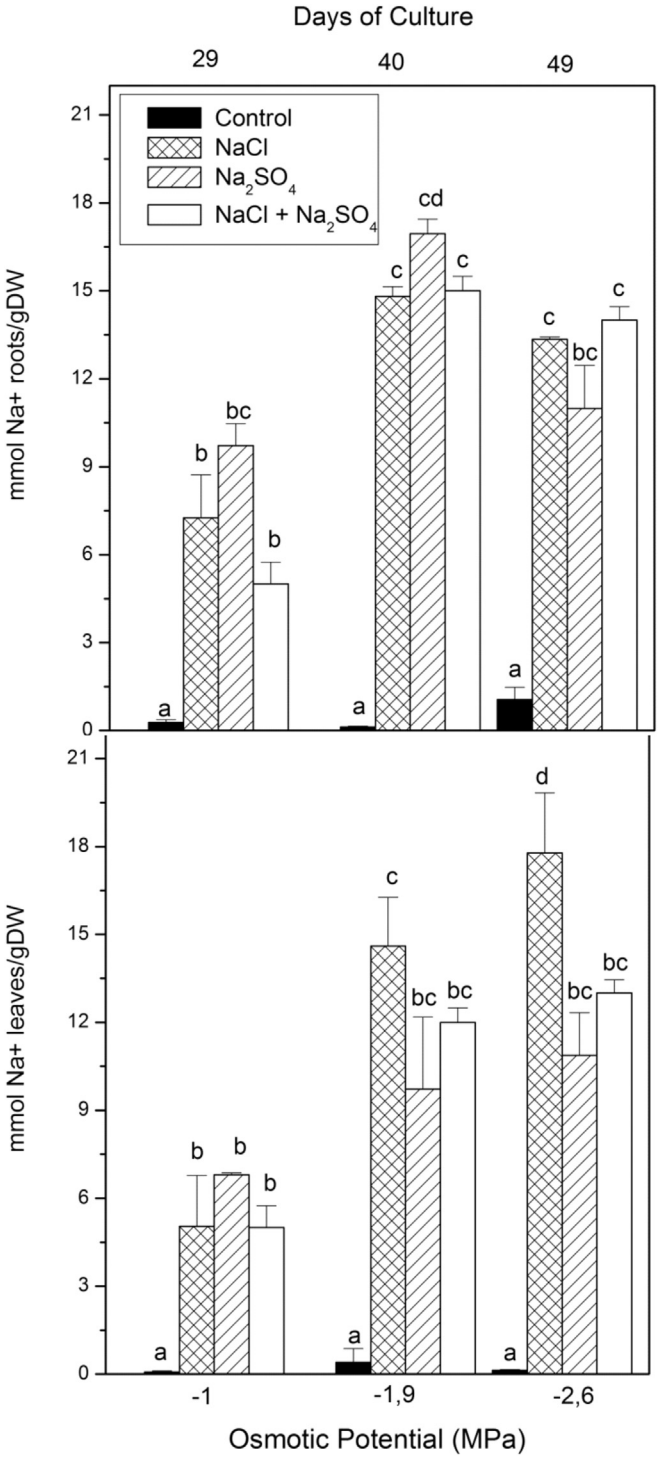
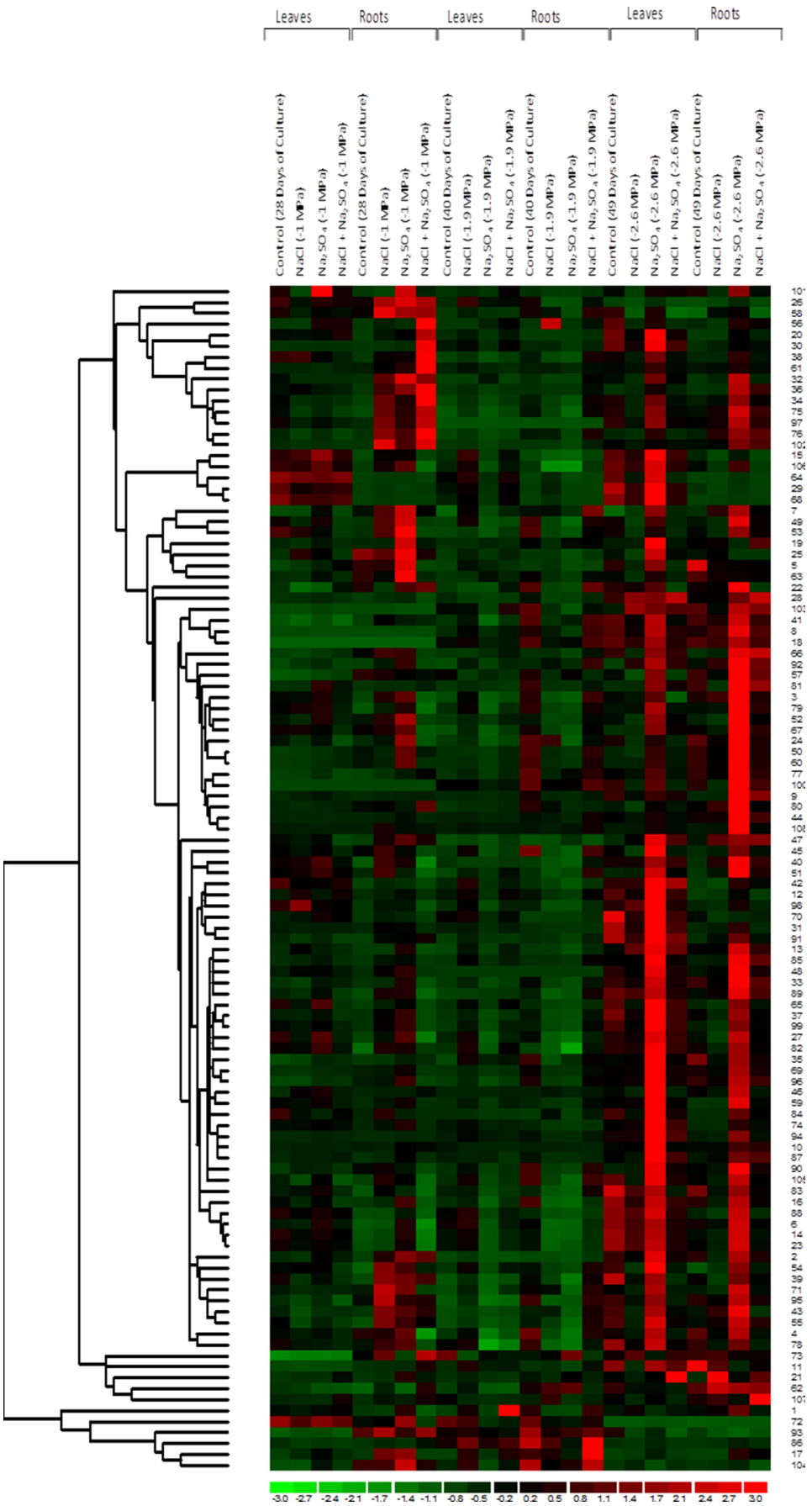


Fig. 2. Effects of NaCl, Na_2SO_4 and NaCl + Na_2SO_4 on Na^+ content in root and shoot of *Prosopis strombulifera* at −1.0, −1.9 and −2.9 MPa. Different letters above bars indicate significant differences among treatments ($P < 0.05$). Data represent means ± S.E.


Leaves
Roots
Leaves
Roots
Leaves
Roots
Control (28 Days of Culture)
NaCl (-1 MPa)
Na2SO4 (-1 MPa)
NaCl + Na2SO4 (-1 MPa)
Control (28 Days of Culture)
NaCl (-1 MPa)
Na2SO4 (-1 MPa)
NaCl + Na2SO4 (-1 MPa)
Control (40 Days of Culture)
NaCl (-1.9 MPa)
Na2SO4 (-1.9 MPa)
NaCl + Na2SO4 (-1.9 MPa)
Control (40 Days of Culture)
NaCl (-1.9 MPa)
Na2SO4 (-1.9 MPa)
NaCl + Na2SO4 (-1.9 MPa)
Control (49 Days of Culture)
NaCl (-2.6 MPa)
Na2SO4 (-2.6 MPa)
NaCl + Na2SO4 (-2.6 MPa)
Control (49 Days of Culture)
NaCl (-2.6 MPa)
Na2SO4 (-2.6 MPa)
NaCl + Na2SO4 (-2.6 MPa)
-3.0 -2.7 -2.4 -2.1 -1.7 -1.4 -1.1 -0.8 -0.5 -0.2 0.2 0.5 0.8 1.1 1.4 1.7 2.1 2.4 2.7 3.0

Palo Alto, USA). Separations were carried out using He as carrier gas at a constant pressure of 1 KPa and a temperature gradient of 10 ®C/min starting from 80 °C up to 350 in 30 min. Inlet and source temperatures were 250 °C and injection was performed in split mode (1:10 of 1 μL injection volume). Scan rate was set at 10 scan/s within 50–850 amu mass range. During acquisitions, mass chromatograms were centroided using the perfluorobutylamine predominant ion at mz^{-1}218.98. Chromatogram files were subsequently converted to NetCDF before xcms processing. Before analyses, system was calibrated using mass spectrum of perfluorobutylamine.

Mass chromatographic features were extracted with xcms (Smith et al., 2006) and subsequently processed with Target Search software (Cuadros-Inostroza et al., 2009) using Golm Metabolite Databases (available from http://www.mpimp-golm.mpg.de/). Briefly, this software calculates RI for all compounds in chromatograms based on tabulated RI values for the constituents of the FAME mix, and subsequently matches RI and mass fragments of compounds in samples with those found in databases. Identified metabolites were cross-referenced in chromatograms and peaks integrated using Masslynx 4.1 software (Micromass Ltd.). Peak areas of identified metabolites were normalized to the IS (ribitol) area and to actual sample weight before statistical analyses.

2.5. Extraction and analysis of P. strombulifera metabolites using liquid chromatography–mass spectrometry (UPLC-QqTOF-MS)

For LC/MS analyses, 5 mg of powdered leaf or root dry tissue were extracted in 500 μL of 70% methanol (LC/MS grade, Panreac) spiked with biochanin (1 mg/L) by ultrasonication for 10 min at room temperature. Afterwards, samples were incubated at 80 °C for 15 min in a water bath to ensure complete enzyme denaturalization. Extracts were centrifuged at 10,000 rpm for 10 min at 4 °C and supernatants recovered and filtered through disposable PTFE (0.20 μm) membrane syringe filters. The extracts were then collected in disposable screw cap amber glass HPLC vials fitted with 300 μL-glass inserts.

Chromatographic separations were performed on a 100 mm × 2.1 mm i.d., 2.1 μm, ProntoSIL C18SH (Bischoff Chromatography, Leonberg, Germany) using an Acquity SDS system (Waters Corp. Ltd., Milford, MA) interfaced to a QTOF Premier from Micromass Ltd. through an ESI source. Samples (10 μL) were injected onto the UPLC system and separated using gradient separation (acetonitrile and ultrapure water, both supplemented with formic acid at a 0.1% concentration) at a flow rate of 300 μL min^{-1} as in Zandalinas et al. (2012). During analyses, column temperature was maintained at 40 °C, and samples were maintained at 10 °C to slow down degradation. Samples were analyzed in both negative and positive ionization modes in the 50–1000 amu scan range using a capillary and cone voltages of 3.5 KV and 30 V, respectively. Desolvation and nebulization gas was nitrogen at 800 and 60 l h^{-1}, respectively. Source block temperature was set at 120 °C and desolvation gas temperature at 350 °C. Mass chromatograms were centroided using the accurate mass of the molecular ion of leucine-enkephalin (ESI+ 556.2771 and ESI- 554.2625). After acquisition, files were converted to NetCDF for subsequent xcms processing. Chromatographic peak detection was performed using the matched filter algorithm and retention time correction was achieved in three consecutive iterations reducing bandwidth. Ion type identification, adduct annotation and grouping of related mass chromatographic features was achieved with the CAMERA package (Kuhl et al., 2012). Normalization of peak areas was achieved by taking into account the area of the internal standard biochanin and the actual sample weight. Significantly-altered mass chromatographic features were identified after maSigPro analysis (Argamasilla et al., 2014) using osmotic pressure of the medium and saline treatment as factors.

Annotation of metabolites was achieved by comparison of mass spectra and retention time with an in house-built database. Additionally, unknown metabolites were identified by manual curation of mass pseudospectra (generated by CAMERA), database search and comparison of actual and *in silico*-generated fragmentation patterns (MetFrag) with those measured (Wolf et al., 2010).

2.6. Statistical analysis

Growth parameters and ions data and the identified metabolite intensity were analyzed using Di Rienzo et al., 2011. A two factorial experiment: osmotic potential (Ψo) (−1.0, −1.9, or −2.6 MPa) and treatment (Control, NaCl, Na_2SO_4 and bisaline) 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 ($p < 0.05$).

The spreadsheets generated were subjected to hierarchical cluster analysis (HCA) using the publicly available microarray analysis software DChip. Distances between samples and signals in data sets were calculated as 1-correlation; subsequently, a centroid clustering method was performed. Clusters were ordered by tightness. P value thresholds of 0.001 for signal enrichment function and 0.01 for sample were set. The distance cutoff value used to establish the different sample clusters was 0.01.

GC-MS peak areas of identified metabolites and LC-MS data were subjected to principal component analysis (PCA) using SIMCA-P+ (Umetrics, Umeå,Sweden). The exported data included the sample information and variables. The integrated peak area of a specific mass-to-charge ratio (m/z) at a specific retention time in the extracted ion chromatogram (EIC) was taken as variable name for analysis.

Fig. 3. Hierarchical cluster analysis of GC/MS metabolite profiling in leaves and roots of *Prosopis strombulifera* under non-salt treatment (controls), NaCl, Na_2SO_4 and NaCl + Na_2SO_4 treatments at −1.0, −1.9 and −2.9 MPa. Compounds: 3-Indoleacetic acid (1), 4-Hydroxyphenyl-beta-glucopyranoside (2), 6-epi-castanospermine (3), acetamide (4), adenosine-5-monophosphate (5), adipic acid (6), anabasine (7), androst-4-en-3,17-dione, 19-hydroxy (8), androst-5-ene, 3beta, 17beta-dihydroxy (9), aphidicolin (10), artemisinic acid (11), aspartic acid (12), barbituric acid (13), benzaldehyde (14), biotin (15), cadaverine (16), calystegine (17), canavanine (18), catechin (19), cellobiose (20), cholesterol (21), cinnamaldehyde (22), cinnamic acid (23), cinnamic acid, 3-hydroxy (24), corticosterone (25), cystathionine (26), cysteine (27), cysteinyl-glycine (28), D176099 (29), D192781 (30), D230150 (31), D283309 (32), D304560 (33), docosenoic acid (34), estriol (35), fructofuranosyl (36), fructose-1-phosphate (37), galactonic acid (38), galactopyranosyl (39), galactosamine (40), glucaric acid (41), glucopyranose (42), glutamine (43), glycerol-2-phosphate (44), glycine (45), guanosine-5-monophosphate (46), hesperetin (47), histidinol (48), homoserine lactone (49), hydantoin- 5-propionate (50), indole-3-acetamide (51), indole-3-acetic acid 5 hydroxy (52), indole-3-pyruvic acid (53), jasmonic acid methyl ester (54), kynurenine (55), lactic acid (56), lactitol (57), leucine (58), levulinic acid (59), lysine (60), maltitol (61), maltotriitol (62), maltotriose (63), mannopyranoside (64), mannose-6-phosphate (65), melezitose (66), methionine (67), methionine sulfoxide (68), morin (69), muramic acid (70), NA161009 (71), NA21820316 (72), NA272011 (73), NA692307 (74), neuraminic acid (75), nonacosanoic acid (76), nonadecane (77), octanoic acid (78), ornithine (79), panthenol (80), pentacosanoic acid (81), phenoxyacetic acid (82), phytol (83), pregnane-3alpha, 21-diol-11,20-dione, 5-beta (84), proline (85), prostaglandin D2 (86), quinic acid (87), rhamnose (88), ribose-5-phosphate (89), serine (90), shikimic acid-3-phosphate (91), sorbitol (92), spermine (93), sphingosine (94), tartronic acid semialdehyde (95), taxifolin (96), tetracosenoic acid (97), threonic acid (98), thymidine (99), trehalose (100), trehalose-6-phosphate (101), tryptophan (102), tryptophan-5 -ydroxy (103), turanose (104), tyramine (105), tyrosine (106), uridine (107), uridine 5'-monophosphate (108).

3. Results

Root length increased in all treatments from the beginning of the experiments and no significant differences were detected among them (Fig. 1A). Shoot growth also increased during the whole experimental period being affected by the salt treatments at −0.3 and −1.0 MPa. Nevertheless, at −1.9 MPa and lower, Na_2SO_4-treated plants showed growth inhibition, while NaCl-treated plants showed maximum growth although no significant differences were detected respect to non-salt-treated plants (control). No significant differences in growth parameters were observed between plants treated with the bisaline solution and plants subjected to monosaline treatments (Fig. 1B). The Na^+ content in both roots and leaves of salt-treated plants was consistently higher than in non-salt-treated plants throughout the whole experiment, with no significant differences between them. Leaves of NaCl-treated plants showed the highest Na^+ content at −2.6 MPa (Fig. 2). The K^+ content was not altered by any of the salt treatments in roots and leaves along the experience (Fig. S1 available as Supplementary Data).

The analysis of polar and semipolar metabolite profiles by GC-MS revealed more than 100 significantly altered compounds, including 18 amino acids, 19 secondary metabolites, 23 carbohydrates, 13 organic acids, 4 indole acids, 5 nucleotides, among others (Fig. 3, Tables S1 and S2 available as Supplementary Data). The metabolite profiling of roots and leaves showed a significant up−regulation (above 1.5-fold, respect to control or no-salt treatments values) in the majority of compounds in salt-treated plants compared to control plants (Tables S1 and S2). Roots of salt-treated plants accumulated higher amounts of compounds than leaves at low osmotic potential, Na_2SO_4-treated plants showing the highest accumulation of polar metabolites. Certain amino acids could only be observed in NaCl-treated plant roots, such as leucine and the peptidecysteinyl-glycine. However, when both salts were present in the medium, changes to more polar compounds in plant tissues were less dramatic than those observed in monosaline treatments. In roots, Na_2SO_4 concentrations at −1.0 MPa and −2.6 MPa induced an up-regulation in most of the identified compounds. The main amino acids found in roots of all salt treated plants were proline, tryptophan and aspartic acid while in leaves proline and serine were the most abundant. The up-regulation of indole acids and carbohydrates was similar in both organs in all salt treatments. Remarkably, the intensity of both polar and semipolar compounds such as sorbitol and catechin were higher in leaves and roots of Na_2SO_4-treated plants. The polyamine cadaverine accumulated only in leaves of Na_2SO_4-treated plants at the lowest osmotic potential (−2.6 MPa). Jasmonic acid methyl ester was also identified by GC-MS and it was up-regulated in NaCl treated plant roots at −1.0 MPa, while in leaves accumulated at −1.9 MPa, Na_2SO_4 treated plant roots accumulated more jasmonate at −2.9 MPa. Two compounds related to ascorbic acid metabolism, galactonic acid and tartronic acid semialdehyde, were identified by GC-MS. An up-regulation of these compounds was found in all salt treated *P. strombulifera* plants at the beginning of the experiments, but at moderate and lowest osmotic potentials these compounds accumulated only in Na_2SO_4-treated plants.

Hierarchical cluster analysis was used to group metabolites based on similar accumulation patterns and specific metabolite relative levels which are displayed as a heatmap (Fig. 3). Separation of sample classes into non-overlapping clusters suggests large metabolic differences between them, while the separation of similar classes of samples into clusters suggests high biological variability. The production of metabolites increased in roots and leaves in concordance with concentration increases of all salts added in the culture medium.

Roots and leaves of *P. strombulifera* from treated and non-treated plants were analyzed by LC/ESI-QTOF-MS. Principal component analysis provided an evaluation of the data variance structure. As can be observed in Fig. 4, the major sources of variance within data sets allowed clustering of biological samples into three groups differentiated by their osmotic potential: −1, −1.9 and −2.6 MPa. In addition, control plants were separated by the age of culture at 29, 40, and 48 days respectively. As an example, component 1 of PCA of positive and negative LC-MS mode resolved well leaves samples at −1.9 MPa from those at −1.0 and −2.6 MPa, while component 2 and 3 resolved samples at −1.0 MPa from those at −2.6 MPa. Similar results were observed in root samples. As shown in Fig. 5, roots showed a higher number of altered mass chromatographic features than leaves (3968 mass species in roots vs. 1994 in leaves for positive mode, and 2237 mass species in roots vs. 2789 in leaves for negative mode). When metabolite profiles from control and salt-treated plants were compared, a high degree of overlapping was found in both organs, mainly in roots. For example, 801and 702 mass chromatographic features were common to NaCl, Na_2SO_4 and bisaline treatments in roots. In leaves, a total of 444 and 708 overlapping mass chromatographic features were found among treatments. Both monosaline treatments were compared and few mass chromatographic features were found to be common in both organs (383 and 289 in roots and only 124 and 145 in leaves in positive and negative electrospray mode, respectively). Finally, roots of NaCl-treated plants showed the highest number of altered chromatographic mass features while in leaves the higher number of altered mass species was found in Na_2SO_4 treated plants. When both NaCl and Na_2SO_4 salts are present in the medium, roots and leaves showed the lowest altered mass chromatographic features in both ESI modes.

Each experiment was analyzed separately by using control treatment (no salt added) vs. salinization, and time of culture (age of plants) as the two main factors to take into account. Significantly altered mass chromatographic features of roots and leaves were grouped into four clusters of tendency for each ESI mode (Fig. S2 and Fig. S3). After analyzing data of *P. strombulifera* roots subjected to salt treatments, three of four tendency clusters obtained for each ESI mode were considered biologically meaningful: cluster1 in positive ESI mode was equivalent to cluster 4 in negative ESI mode, clusters 2 in both modes had similar behavior, and cluster 4 in positive ESI was equivalent to cluster 3 in negative ESI. Only clusters 3 (positive mode) and 1 (negative mode) were discarded as biologically meaningful due to their odd behavior that was attributed to biological variance and not to increasing salinity in the culture medium. Cluster 1 (positive) and 4 (negative) grouped mass chromatographic features in which controls (non-salt treated plants of 29 days of culture) increased as compared to salt treated plants at −1.0 MPa and masses were not affected by the salt treatments at −1.9 and −2.6 MPa. Contrarily, cluster 2 contained mass chromatographic features (353 and 338 respectively) which levels exhibited a transient increase in controls of 48 day of culture as compared to salt treatments at −2.6 MPa. Cluster 4 (positive) and 3 (negative) grouped mass chromatographic features in which NaCl-treated plants showed the highest levels of mass species. Likewise, data from leaves showed one cluster for each ESI mode considered biologically meaningful (cluster 1 in positive mode and cluster 2 negative modes). These clusters had similar behavior, in which significantly altered mass chromatographic features were related to an increasing salinity in the culture medium, being significantly affected at the highest salt concentration.

Tentative annotations were achieved by searching the most plausible structures in databases. As shown in Fig. 6, three metabolites, tryptophan, lysophosphatidyl coline and 13-hydroxyoctadecadienoic, were putatively identified and analyzed in roots and leaves, and extracted from clusters with mass

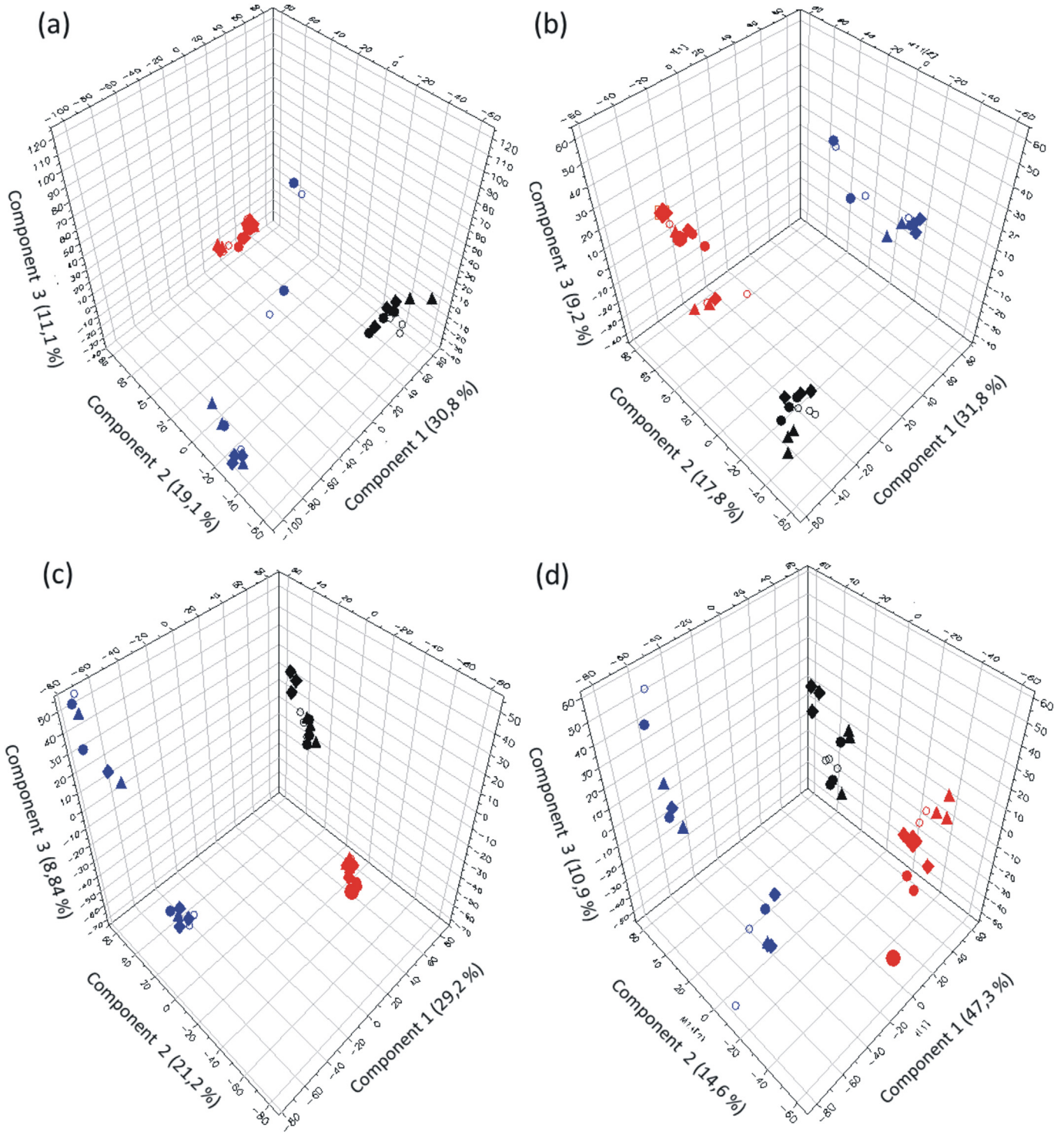


Fig. 4. PCA plot showing the three major source of variability among in leaves (a and b) and roots (c and d) in *P. strombulifera* plants under non-stressed (control) and salinity conditions (NaCl, Na_2SO_4 and NaCl + Na_2SO_4) after UPLC-MS-ESI in positive (a and c) and negative (b and d). Percentage of variability explained is given on each axis. Red symbols represent the osmotic potential −1 MPa. Blue symbols: −1.9. MPa. Black symbols: −2.6 MPa. Treatments: Control: Triangle; NaCl: Dot; Na_2SO_4: Diamond; NaCl + Na_2SO_4: Circle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

chromatographic features considered biologically meaningful. In roots of stressed *P. strombulifera* plants, normalized peak intensity of tryptophan followed a different profile depending on the salt treatment applied. The lowest intensity was observed in roots of Na_2SO_4-treated plants at −1.9 MPa. However, when osmotic potential due to the presence of Na_2SO_4 increased in the medium (up to−2.6 MPa) no significant differences in tryptophan intensity was observed compared to control plants. In leaves, NaCl treatment at −2.6 MPa induced the maximum tryptophan accumulation. At this osmotic potential, the leaves of NaCl + Na_2SO_4 treated plants showed intermediate levels between those in monosaline treatments.

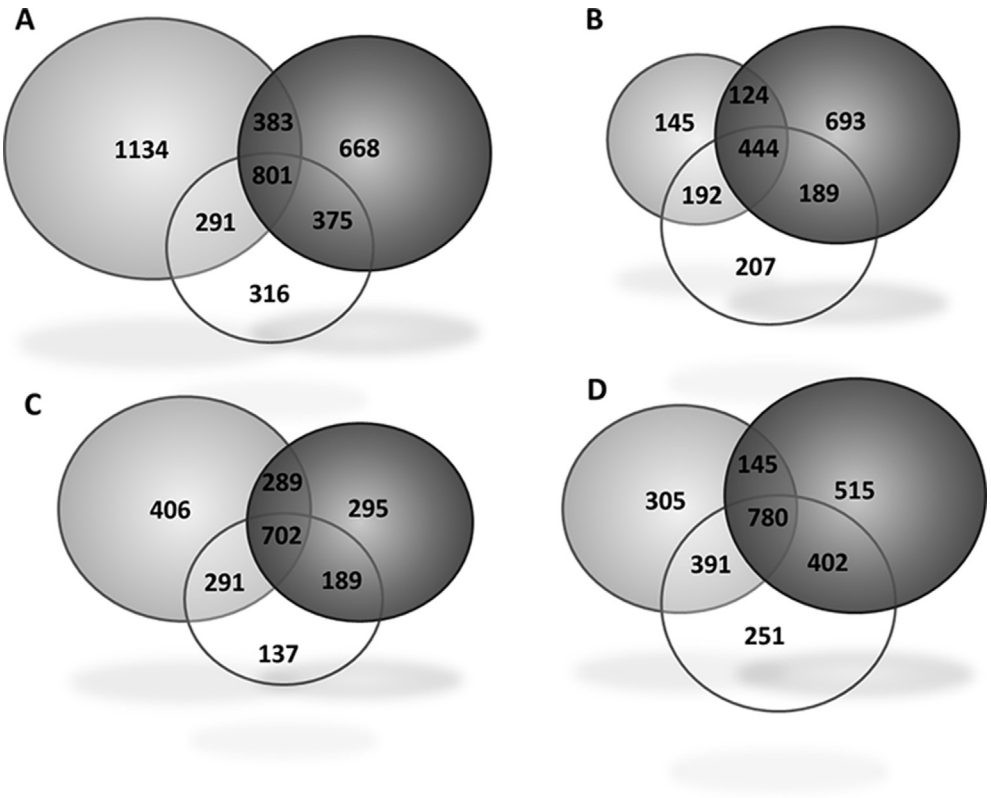


Fig. 5. Venn diagrams depicting overlapping mass chromatographic features in roots (A and C) and leaves (B and D) in non-salt treated (controls) and salt-treated *Prosopis-strombulifera* plants. Salt treatments: NaCl (gray circles), Na_2SO_4 (black circles) and bisaline (NaCl + Na_2SO_4; white circles). A and B represent orthogonal electrospray source (ESI) operated in positive mode. Cand D represent orthogonal electrospray source (ESI) operated in negative mode. The size of the circle is proportional to the amount of altered mass chromatographic features.

Lysophosphatidyl choline intensity increased with osmotic potential decrease although to a higher extent in salt-treated plants, being particularly evident at the lowest osmotic potential. Similarly, intensity of 13-hydroxyoctadecadienoic increased in roots and leaves of non-salt treated plants throughout the experimental period. However, levels of this compound in roots of NaCl-treated plants increased until −1.9 MPa but did not progress further, showing values 55% lower than controls at the same date. In leaves, no significant differences were observed in the intensity of this compound at the beginning of the experiment, but a significant decrease was observed in Na_2SO_4-treated plants at −2.6 MPa.

4. Discussion

This is the first study in which a general overview of the metabolomic profiling in a salt treated halophyte native from Argentina is reported. The metabolite profile was evaluated in plants growing under the most commonly found salts in soils of many countries, NaCl and Na_2SO_4, and their combination in an iso-osmotic mixture. Previous studies from our laboratory demonstrated that *P. strombulifera* is less tolerant to Na_2SO_4 than to NaCl or the mixture of both salts. In this study, shoot height was also more affected by Na_2SO_4 than by NaCl treatment although great variability was found as usually occurs in native species. Classic reports on plant responses to salinity have generally attributed ion toxicity of salt in the soils to the presence of excess of Na^+ and consequently, poor K^+ incorporation by roots. However, in our experiments plants growing in the presence of NaCl and/or Na_2SO_4 in the culture medium accumulated similar proportions of both cations in leaves and roots. Indeed, during the last years it could be demonstrated through determinations such as relative water content, osmotic potential and osmotic adjustment, transpiration rate, oxidative damage, soluble carbohydrates content, organic acids, chlorophyll fluorescence, photosynthetic electron flow, respiration, phytohormones and polyphenols levels and metabolism (Llanes et al., 2013; Devinar et al., 2013; Reginato et al., 2014a, 2014b; Llanes et al., 2014; Reginato et al., 2014b) that SO_4^{2-} presence in the culture medium was the determinant of salt toxicity in *P. strombulifera* plants. They showed a reduced capacity for ion compartmentalization and osmotic adjustment, water imbalance and toxicity symptoms due to a disturbance in the carbon metabolism. Moreover, Na_2SO_4 treatment accentuated the decrease of NPQ (Non Photochemical Quenching) values caused by plant aging contributing to photoinhibition, as determined by the low values of Fv/Fm found in these plants (Devinar, 2015). These results are coincident with the strong inhibition of the xanthophyll cycle by SO_4^{2-} observed in a previous work.

Plant responses to salinity also involve changes on genes and protein activities, which invariably lead to modifications in plant metabolism. The identification of metabolite markers potentially involved in salt stress tolerance can be performed through non-targeted metabolite analysis approaches; thus, to provide a general overview of the metabolome in the halophyte *P. strombulifera*, alterations in polar and semipolar metabolites resulting from the presence of Na_2SO_4 and NaCl in the medium were investigated. Primary and secondary metabolite profiles were analyzed by means of GC-MS and LC-MS, respectively. Integrating metabolomic profiling with global gene expression data such as microarrays or RNASeq could allow the elaboration of gene-to-metabolite correlations improving the knowledge of how plant metabolism is

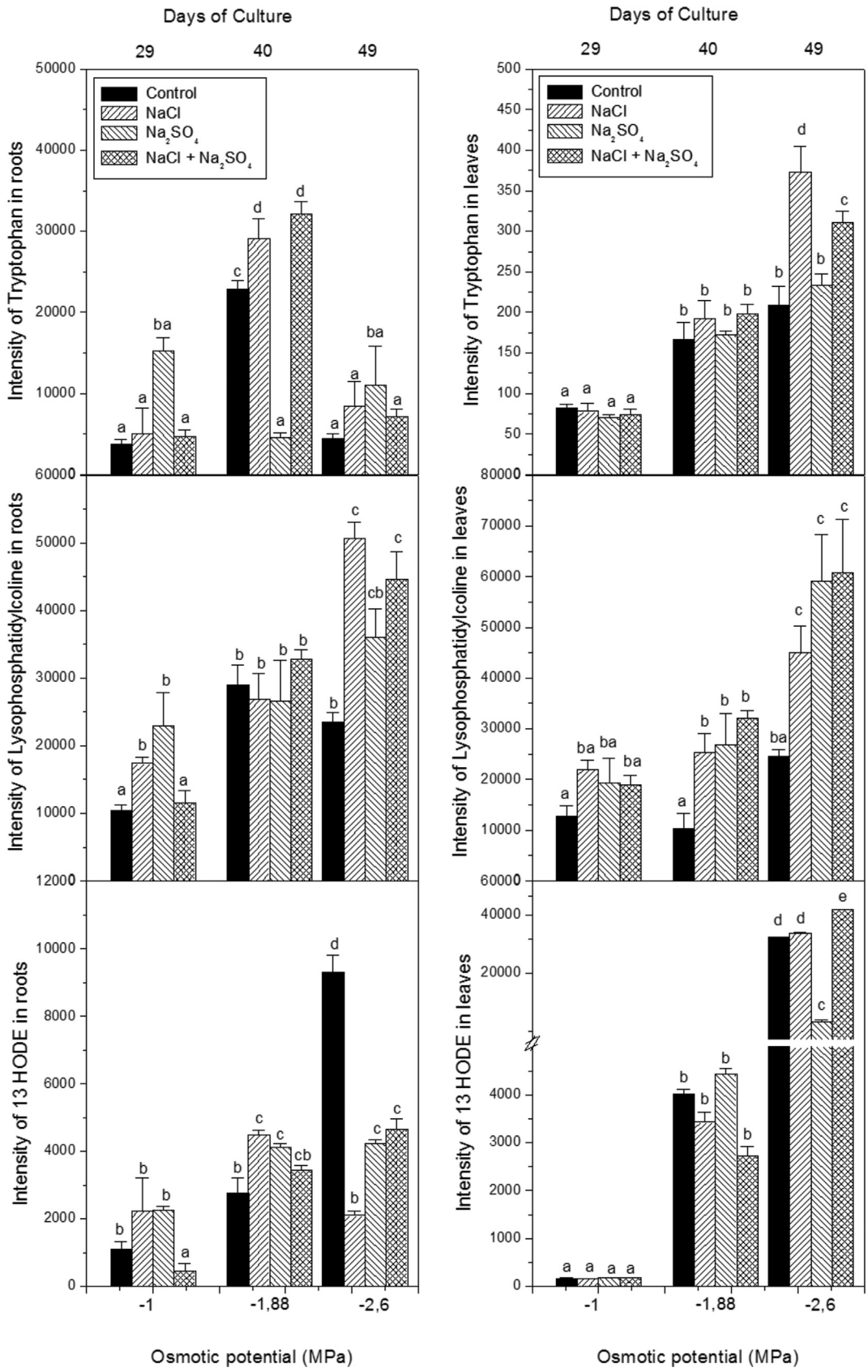


Fig. 6. Effects of NaCl, Na_2SO_4 and NaCl + Na_2SO_4 on metabolites intensity in roots and leaves of *Prosopis strombulifera* plants at different osmotic potentials (MPa). Different letters above data indicate significant differences among treatments ($P < 0.05$). Bars are means ± S.E.

regulated under stress environmental conditions (Saito, 2013). In this work, *P. strombulifera* rapidly induced the synthesis of diverse metabolites under salt treatments and the intensity of each compound varied depending on the type and content of salts in the culture medium, organ analyzed and age of plants. In this sense, it has been reported that the chemical composition of plants is highly specific and similar compounds in some plants could involve different protective and/or signaling functions (Arbona et al., 2010).

Accumulation of amino acids has been reported in many researches about plants exposed to salt stress (Lugan et al., 2010). In this regard, an up-regulation of amino acids was observed in metabolome studies reports in the *Aeluropus lagopoides* halophyte (Sobhanian et al., 2010). The increase in these compounds may be derived of the production of amino acids and/or from increased of protein degradation induced by stress. Although the increased levels of specific amino acids could have a beneficial effect and be an adaptation in plants under salt stress, the global accumulation of amino acids under stress could mean cell damage for some species (Widodo et al., 2009). Indeed, an accumulation of amino acids have been previously reported in plants under several stress treatments, which was associated with senescence and cell damage (VanDoorn and Woltering, 2008). In this study, the accumulation of amino acids in Na_2SO_4 treated plants is in line with its association with cell damage, presumably related with an increased respiration. On the contrary, certain amino acids such as leucine in roots or peptides such as cysteinyl-glycine in leaves, observed only in NaCl-treated plants, could be a response induced to maintain metabolic and osmotic homeostasis during stress. These results highlight the variability of plant metabolism responses in different organs to different salt treatments as well as the role of some amino acids under salinity conditions. In this regard, from the beginning of salinization all treated plants accumulated proline. Different functions have been reported to proline accumulation in plants growing in adverse conditions, but the proline role as a mechanism of plant stress adaptation is still controversial (Lehmann et al., 2010). In *P. strombulifera*, proline content increased sharply in plants under high salinity independently of the salt composition of the culture medium (NaCl, Na_2SO_4 or monosaline mixture). Thus, it was proposed that proline accumulation could be considered a signal of stress intensity rather than a tolerance indicator (Llanes et al., 2013).

It has been reported that accumulation of carbohydrates may be a common response to salt stress, but there is little agreement about the changes of specific carbohydrates in plants under adverse conditions, which implies the existence of differential metabolic rearrangements between species. Carbohydrates act not only as osmoprotectants that help to maintain osmotic balance and stabilize macromolecules under stress conditions, but could also be a source of readily available energy for plants to resume growth after a period of stress. An increase in carbohydrates content under salinity has also been reported in the salt-tolerant *Thellungiella halophila* (Gong et al., 2005), the halophytic species *Limonium latifolium* (Gagneul et al., 2007), and the salt tolerant tree *Populus euphratica* (Brosche et al., 2005). In our study, a high regulation of several metabolites from glycolysis/gluconeogenesis pathways was observed in salt-treated plants. This response suggests that metabolic flux through these pathways is essential for the production of downstream products important for the acquisition of salt tolerance. However, the highest concentration of some carbohydrates found in Na_2SO_4-treated plants could be indicating injury. Sorbitol accumulation found in Na_2SO_4-treated plants confirms previous results in which accumulation of this sugar alcohol took place in injured *P. strombulifera* plants reflecting a disorder in the carbon metabolism, *e.g.* reduced sucrose production and protein content (Llanes et al., 2013). We also found very high levels of a sorbitol derivative, maltitol (4-O-α-glucopyranosyl-D-sorbitol) which role in plants is unknown.

Interestingly, maltotriitol, a C18 polyalcohol synthesized from maltose, was found to be produced only in NaCl and bisaline treated plants. In the last decade, an important role for maltose in cold tolerance is emerging, and Kaplan and Guy (2005) found that in Arabidopsis, increased maltose content by itself or together with a maltose-dependent increase in other soluble sugars, contribute to the protection of the photosynthetic electron transport chain during freezing stress. Drought, salinity, and low temperature stress share common features and all of them impose an osmotic stress that can lead to turgor loss. Hence, it might be thought that maltose and metabolites could be involved in the NaCl tolerance of *P. strombulifera*.

Other relevant carbohydrates were accumulated in roots in this species when $Na_2SO_4$was present in the medium, such as fructofuranosyl residues and cellobiose. This is consistent with the decrease of sucrose content and transport observed in roots of Na_2SO_4-treated plants which could explain their general growth inhibition showed previously (Llanes et al., 2013). For cellobiose, the main function suggested is carbohydrate transport and metabolism emphasizing the fundamental role that carbohydrates play in an interconnected-self regulated network to keep metabolic balance according demand.

GC-MS metabolomic profile allowed the identification of compounds related to ascorbic acid metabolism (galactonic acid and tartronic acid semialdehyde). The increase of these low molecular-weight antioxidant compounds has been proposed to have a protective role against oxidative stress in several plants (Gils and Tuteja, 2010). Therefore, their accumulation in salt-treated *P. strombulifera* seedlings at the beginning of the experiments could be a quick response to the presence of salt in the medium; their accumulation in Na_2SO_4-treated plants at intermediate osmotic potentials would be related to a pronounced oxidative stress, confirmed by a higher malondialdehyde (MDA) content than those under NaCl treatment previously observed (Reginato et al., 2014b). This response is similar to that of catechin, a phenolic compound with antioxidant properties (Rice-Evans et al., 1997) that was accumulated in our experiments and up-regulated mainly in roots of Na_2SO_4-treated plants at −1.0 MPa (16.4-fold) as well as in plants under the bisaline treatment at −1.9 and −2.6 MPa (maybe because of the dilution of sulfate). The presence of the uncommon diamine cadaverine in leaves of Na_2SO_4-treated plants has been associated with the capacity of some species to grow under stressful conditions (Flores, 1991) and confirms previous results in *P. strombulifera*. All together, these results support the idea that both compounds (catechin and cadaverine) are metabolic responses to prevent the adverse effects of oxidative stress associated with salinity, by scavenging free radicals thereby contributing to maintain cell and tissue integrity.

An interesting finding in this work is that related to cholesterol content. Cholesterol is found in plant membranes sometimes being the major sterol as part of the surface lipids of leaves, but quantitatively scarce if expressed as a percent of total lipids (Berhman and Gopalan, 2005). In our experiments, a sharp increase in cholesterol was observed in Na_2SO_4 treated plants at −1.0 MPa (lower salt concentration) then decreasing. Instead, NaCl treated plants showed the cholesterol peak at the highest salt concentration (−2.6 MPa). Since cholesterol contributes to membrane firmness and maintains both membrane structural fluidity and integrity, these findings may be interpreted as an adaptive response upon sensing the ionic stress caused by each salt.

Secondary metabolite analysis by LC/ESI-QTOF-MS showed a higher number of altered mass chromatographic features in roots than in leaves in response to salt stress. This stronger alteration in

root metabolism of *P. strombulifera* is in correlation with previous results from our laboratory showing that H_2O_2 content remained unaltered in leaves from all treatments while it was significantly higher in roots under Na_2SO_4 treatment, similarly to what happened with MDA levels (Reginato et al., 2014b). Thus, the number of altered mass signals on each salt treatment reflects the impact of different salt concentrations on plant metabolism.

Three semipolar metabolites, tryptophan, lysophosphatidylcoline and 13-hydroxyoctadecadienoic, were putatively identified in *P. strombulifera* plants. The amino acid tryptophan is the main precursor of auxin biosynthesis in plants (Mashiguchi et al., 2011; Zhao, 2012). Auxins play a pivotal role in nearly every aspect of growth and development and in several adaptations to adverse environmental conditions. It has been reported that salinity modulates the root system architecture by altering auxin distribution in root cells setting the direction of lateral roots growth and development (Galvan-Ampudia and Testerink, 2011; Petricka et al., 2012). In this work, *P. strombulifera* roots treated with Na_2SO_4 at −1.9 MPa showed the lowest tryptophan content as compared to the rest of treatments, which could be associated to its depletion caused by a high rate of auxin biosynthesis. Increased levels of indol-3-acetic acid, 5-hydroxy and indol-3-pyruvic acid were determined previously in our laboratory (Llanes et al., 2014) as well as an increased lateral root formation (Reinoso et al., 2005). Conversely, NaCl treatment induced the highest tryptophan accumulation and the lowest auxin content, subsequently, no lateral root formation and no anatomical and histological differences were observed compared to controls plants (Reinoso et al., 2004; Llanes et al., 2014). Besides production of many indole-containing substances in plants which could be protective against stresses such as indol-acetic-acid, indol-glucosinolates, some phytoalexins (such as camalexin) and tryptamine derivatives (Rao et al., 2012) it has been recently reported that foliar sprays of tryptophan appear to mitigate the detrimental effects of salt stress supporting the idea that tryptophan mitigates the deleterious effects of salt stress (Hussein et al., 2014).

Plants can modify membrane phospholipid composition to maintain the suitable fluidity and integrity in response to adverse environmental conditions (Li et al., 2008; Jia et al., 2013). For example, an accumulation of phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine were registered in some desiccation tolerant plants (Testerink and Munnik, 2011; Gasulla et al., 2013). Similarly, a rapid increase in phospholipid content was showed in *Arabidopsis* grown under salt stress conditions (McLoughlin et al., 2013). In *P. strombulifera* plants, the high salt concentration in the culture medium increased lysophosphatidylcoline content in leaves and roots. Lysophospholipids such as lysophosphatidylcoline, have been related to cell signaling implicated with plant growth, development and several stress responses, (Munnik and Testerink, 2009). Its increase observed in roots of salt treated of *P. strombulifera* plants could be related to the lysophospholipids role associated with maintaining root system architecture during water limiting environmental conditions (McLoughlin and Testerink, 2013).

Oxygenated fatty acids (oxylipins), such as hydroxyl octadecadienoic acid, are a secondary metabolites family which is produced by oxidative metabolism of polyunsaturated fatty acids. An increased oxylipin concentration is a characteristic response to biotic stress such as pathogenesis, wounding and/or herbivory with an important role as signaling compounds in plant response to these stresses (Prost et al., 2005; Christensen and Kolomiets, 2011). Diverse studies on oxylipins have been focused mainly on the plant signaling molecule jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA). Jasmonic acid (JA) derives from the 13-LOX pathway and it is one of the main oxylipins with a signaling role in growth and development of plants growing under stress conditions (Wu and Baldwin, 2010). In our study in *P. strombulifera* the intensity of the oxylipin 13 hydroxyoctadecadienoic acid decreased in roots of salt treated plants at −2.6 MPa, being the lowest value found with NaCl-treatment. On the contrary, in Na_2SO_4-treated plants at −2,6 MPa, the lowest concentration of this compound was found in leaves. Similarly, jasmonic acid methyl ester identified by GC-MS varied with organ and salt treatment. Considering that high levels of JA were previously reported in both roots and leaves of *P. strombulifera* plants under salt treatments, the low levels of 13 hydroxyoctadecadienoic acid in plants under high salinity could be associated with an increase in the activity of the β-oxidation pathway to produce JA.

In summary, in this study we analyzed primary and secondary metabolite profiles in the halophyte *P. strombulifera* in response to different salt treatments and established a correlation between the differential expression of metabolites and growth parameters. The results indicate that salinity provoked disturbs in amino acids, organic acids, indoles, phospholipids, and the overall metabolism and signaling of carbohydrates. We have demonstrated that ionic interactions between the salts commonly present in most soils modify the biochemical and morpho-physiological responses of this species to salinity. Previous studies showed that this species is highly tolerant to NaCl and less tolerant to Na_2SO_4 in the medium. Notwithstanding, it is interesting to note that metabolites identified by GC-MS shown in Tables S1 and S2, mostly appeared at the lower osmotic potential in both monosaline solutions, and mostly in roots. This would indicate that the whole metabolic machinery starts on at the beginning of salt treatments independently of the ions involved, maybe upon sensing osmotic changes. After 29 days of culture (−1.0 MPa) the greatest differences in the expression of each cluster of compounds can be observed, which in general decrease their intensity at −1.9 MPa and many of them cannot be detected. When salinity reaches the highest values, their expression accentuates, especially under Na_2SO_4 treatment. Remarkably, when both NaCl and Na_2SO_4 salts are present in the medium, both roots and leaves showed the lowest altered mass chromatographic features in both ESI modes. A good question to answer in a next future is why this happens, and the answer might probably be obtained through transcriptomics analysis and knowing the biosynthetic pathways.

Metabolomics approaches combined with genomics, transcriptomics and proteomics methodologies allow the understanding of the relationship between an organism's phenotype and its genome. This information associated with plants growing in adverse environmental conditions will generate varieties of crops or forest trees capable to efficiently cope with adverse environmental conditions. In this sense, the woody shrub *P. strombulifera* constitute a good model plant as source of salt stress tolerance genes. Additionally, the understanding of the interplay between up and down regulation of different metabolites and the stress tolerance mechanisms in model systems that tend to mimic real field conditions is crucial to provide the basis for generating salt-tolerant plants.

Authors' contributions

A.L. and V.A. designed and performed the experiments, analyzed data and wrote the manuscript. A.G.C. and V. L assisted in analysis of data and edited the manuscript. All authors have read and approved the final manuscript.

Contribution

This manuscript represents the first study in which a general

overview of the metabolome in a salt treated halophyte native shrub from Argentina is reported. The primary and secondary metabolite profiles were evaluated in plants growing under the most commonly found salts in soils of many countries, such as NaCl and Na_2SO_4 and their combination in an iso-osmotic mixture. We established a correlation between the differential expression of metabolites and plant growth parameters. Results indicate that ionic interactions between different salts provoke disturbs in aminoacids, organic acids, indoles, phospholipids, and the overall metabolism and signaling of carbohydrates. This study highlights about the interplay between regulation of different metabolites and stress tolerance mechanisms in a model system that tend to mimic real field conditions being crucial to provide the basis for future molecular studies for generating salt-tolerant plants.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.07.010.

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