Salt tolerance mechanisms in three Irano-Turanian Brassicaceae halophytes relatives of *Arabidopsis thaliana*

Roghieh Hajiboland¹ · Sara Bahrami-Rad¹ · Hossein Akhani² · Charlotte Poschenrieder³

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Abstract

Salt tolerance mechanisms were studied in three Irano-Turanian halophytic species from the Brassicaceae (*Lepidium latifolium*, *L. perfoliatum* and *Schrenkiella parvula*) and compared with the glycophyte *Arabidopsis thaliana*. According to seed germination under salt stress, *L. perfoliatum* was the most tolerant species, while *L. latifolium* and *S. parvula* were rather susceptible. Contrastingly, based on biomass production *L. perfoliatum* was more salt sensitive than the other two species. In *S. parvula* biomass was increased up to 2.8-fold by 100 mM NaCl; no significant growth reduction was observed even when exposed to 400 mM NaCl. Stable activities of antioxidative defense enzymes, nil or negligible accumulation of superoxide anion and hydrogen peroxide, as well as stable membrane integrity in the three halophytes revealed that no oxidative stress occurred in these tolerant species under salt stress. Proline levels increased in response to salt treatment. However, it contributed only by 0.3–2.0% to the total osmolyte concentration in the three halophytes (at 400 mM NaCl) and even less (0.04%) in the glycophyte, *A. thaliana* (at 100 mM NaCl). Soluble sugars in all three halophytes and free amino acids pool in *S. parvula* decreased under salt treatment in contrast to the glycophyte, *A. thaliana*. The contribution of organic osmolytes to the total osmolyte pool increased by salt treatment in the roots, while decreased in halophyte and glycophyte, *A. thaliana* leaves. Interestingly, this reduction was compensated by a higher relative contribution of K in the leaves of the halophytes, but of Na in *A. thaliana*. Taken together, biomass data and biochemical indicators show that *S. parvula* is more salt tolerant than the two *Lepidium* species. Our data indicate that *L. latifolium*, as a perennial halophyte with a large biomass, is highly suitable for both restoration of saline habitats and saline agriculture.

Keywords Antioxidative defense system · Brassicaceae · *Lepidium latifolium* L. · *Lepidium perfoliatum* L. · Organic osmolytes · *Schrenkiella parvula*

Introduction

Soil salinity is one of the most important abiotic constraints for crop production, especially under semi-arid and arid climate conditions (Cabot et al. 2014). At the beginning of the century, 831 million hectares of world soils were considered affected by salinity. This area increases at a rate of 10% annually (FAO 2000). Plant tolerance to soil salinity varies greatly among species (Munns and Tester 2008). Only about 2% of higher plant species are highly salt tolerant halophytes, while the remaining 98%, including most crop plants, are salt sensitive glycophytes (Flowers and Colmer 2008).

The physiological determinants of the superior salt tolerance in halophytes are poorly known (Flowers and Colmer 2008). It is often considered that halophytes and glycophytes basically utilize the same mechanisms to cope
Salt tolerance in halophytes is a complex trait. It involves multiple physiological changes at the level of ion uptake and transport, compartmentalization of Na and K, and the synthesis and transport of compatible solutes (Flowers and Colmer 2008). It has been suggested that halophytes exhibit enhanced capacities for compatible solute accumulation, but there is no hard evidence either in favor or against this hypothesis. In general, glycophytes also tend to accumulate such compounds when under exposure to a broad variety of stresses, including salt, drought, frost, or heavy metal toxicity (Munns and Tester 2008).

Production of reactive oxygen species (ROS) is an unavoidable consequence of aerobic metabolism and occurs in the apoplast, cytosol and within organelles (Foyer and Noctor 2003). The most abundant types of ROS in plants are hydrogen peroxide (H$_2$O$_2$) and the two free oxygen radicals, namely superoxide radical (O$_2$·−) and hydroxyl radical (OH·). Reactive oxygen species play a dual role: At low levels they are part of a signaling mechanism. At high levels they are toxic molecules and are responsible for various stress-induced damages to macromolecules ultimately injuring the cell structure (Foyer and Noctor 2003). Thus, stress-specific modulation of ROS production and scavenging is crucial for plant responses to stress (Zepeda-Jazo et al. 2011). A complex antioxidative defense system including enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and peroxidases (POD) are responsible for scavenging ROS and modulate ROS levels and mitigate stress-induced ROS damage (Foyer and Noctor 2003). A high antioxidant capacity of halophytes has been suggested as one of the important reasons for their superior ability to tolerate high levels of salinity stress (Jithesh et al. 2006; Kosová et al. 2013; Ozgur et al. 2013). However, only a few studies have made a direct comparison between glycophytes and halophytes to elucidate the difference in regulation of the antioxidant machinery (Bose et al. 2014).

Halophytes with a broad variety of salt tolerance mechanisms are widely, but unevenly spread over higher plant families and orders (Flowers and Colmer 2008). Among vascular plants, the chenopods are the group with most halophytes, followed by Poaceae, Plumbaginaceae and Aizoaceae (Aronson 1989). The outdated checklist of Aronson listed only 19 salt tolerant Brassicaceae species. However, based on our own unpublished data (H. Akhani), there are at least 26 salt tolerant species in this family in Iran alone. The fact that some halophyte Brassicaceae are close relatives to the model plant Arabidopsis thaliana (L.) Heynh has intensified the research addressing salt tolerance mechanisms in halophytes from this botanical family. Halophytic Brassicaceae may be an important source of information for physiological, molecular and genetic mechanisms allowing adaptation to saline soils.

Iran can be considered a hotspot for halophytes which are still poorly explored from the functional point of view. Iran, with a total surface area of 1.6 million km$^2$, is a typical country of large “sabkhas”, littoral and inland salt marshes, and diverse brackish and salty river ecosystems (Akhani 2006). According to our present knowledge a total of 365 species within 151 genera and 44 families of Iranian vascular plants are known to be true halophytes, or species successfully performing on salty soils (Akhani 2006). So far, 528 Species belonging to 230 genera and 56 families of halophytic and salt tolerant plants have been identified in Iran (Akhani 2016). The largest number of halophytes in the flora of Iran belong to the Chenopodiaceae (151 species) followed by the Poaceae (50 species), Asteraceae (47 species), Tamaricaceae and Brassicaceae (each with 26 species) and Plumbaginaceae (15 species) (Akhani 2016). The family Brassicaceae with several halophytic species in the Irano-Turanian region deserves more attention particularly considering rapid expansion of the salt-affected areas world-wide.

Among the Brassicaceae, Eutrema halophilum (Alemán et al. 2009; Inan et al. 2004; M’rah et al. 2006; Taji et al. 2004) and Cakile maritima (Debez et al. 2004) are the most extensively studied halophytes. There is also physiological evidence on the halophytic properties of Cochlearia anglica, Cochlearia officinalis, Crambe maritima and Diplotaxis tenuifolia (De Vos et al. 2013). The physiology of salt tolerance is greatly unknown in other halophytic species within this family. In order to characterize halophyte-specific mechanisms, it is necessary to establish an Arabidopsis Relative Model System (ARMS) for halophytes (Inan et al. 2004). A species in this category is E. halophilum (Bressan et al. 2001) that is a close relative of Arabidopsis and genetic and genomic resources exist for this species. Comparative studies between salinity stress adaptation in Arabidopsis and its relatives could provide insights into the genetic bases of the halophytism. Recent molecular phylogenetic studies on the family Brassicaceae added new insights into the phylogeny of this complex family and showed surprisingly closer affinity of the genus Lepidium to Arabidopsis than the genera Schrenkella and Eutrema (Huang et al. 2016).

In the present study, we compared salt stress in three halophytic Irano-Turanian Brassicaceae species collected from a saline playa in Central Iran with Arabidopsis thaliana and investigated the effects of salt stress on germination, growth, osmolytes accumulation and antioxidative enzyme activity. The results explain the mechanisms for different salt tolerance in these species and contribute to our knowledge of some ecologically important halophytic species.
Materials and methods

Collection of seeds

Seeds were collected from northern and eastern parts of Meyghan Playa (known as Meyghan wetland), Kavire-Meyghan or Meyghan Salt Lake (located at 15 km N of Arak, North-Central Iran) (Fig. S1). The seeds of Schrenkiella parvula were collected in saline soils of the northern edge of the Lake in association with Halocnemum strobilaceum community in 2006. Those of the Lepidium latifolium were collected during 2016 from a dried runoff in association with Petrosimonia glauca. The seeds of Lepidium perfoliatum were collected in a high saline, dried plain close to the shore of the lake. Phytogeographically, the area belongs to the Irano-Turan region with typical continental climate. Using Global Bioclimatic Classification, this region belongs to the Mediterranean Xeric Continental, with average precipitation of 341 mm based on Arak Climatic Station (Djamali et al. 2011). Soil samples from the top 0–20 cm were collected along with the seeds of S. parvula and analyzed for some chemical properties and total concentrations of selected elements (Benton Jones 1999). Soil texture was sandy loam (Table S1).

The collected seeds of Lepidium latifolium L. and Lepidium perfoliatum L. were germinated and used directly for physiological studies in this work. Plants of Schrenkiella parvula (Schrenk) D.A. German & Al-Shehbaz [= Eutrema parvulum (Schrenk) Al-Shehbaz & Warwick), Thellungiella parvula (Schrenk) Al-Shehbaz & O’Kane and Arabidopsis parvula (Schrenk) O.E. Schulz, were raised from field-collected seeds and were used to provide further seeds, which were used in the experiment. Seeds of A. thaliana (accession Columbia) were produced under growth chamber condition from the seed stock kindly provided by Sherryl R. Bisgrove (Simon Fraser University, Canada).

Study of seed germination

Seeds were surface-sterilized using the vapor phase method (Clough and Bent 1998). Seeds in an open container were placed into a 2 L jar in a fume hood. Prior to sealing the jar, a 250 mL beaker containing 100 mL commercial bleach was placed and 3 mL concentrated HCl was carefully added into the bleach. The jar with chlorine fumes remained sealed 45–60 min.

Seeds were sown on 90 mm Petri dishes containing autoclaved GM (germination medium)-agar (0.7%) medium (pH 5.6) with the following composition: macronutrients Ca(NO$_3$)$_2$·4H$_2$O (0.25 mM), CaCl$_2$ (0.75 mM), KCl (1.0 mM), MgSO$_4$·7H$_2$O (1.0 mM), KH$_2$PO$_4$ (0.2 mM) and micronutrients Fe(III)EDTA (50 µM), H$_2$BO$_3$ (50 µM), MnCl$_2$·4H$_2$O (5 µM), ZnSO$_4$·7H$_2$O (10 µM), CuSO$_4$·5H$_2$O (0.5 µM), Na$_2$MoO$_4$ (0.1 µM) (Conn et al. 2013). GM medium was supplemented with different NaCl concentrations including 0, 50, 100, 150, 200, 250 and 300 mM. Petri dishes were sealed with parafilm strips. Four replicated Petri dishes each containing 30 seeds were considered for each treatment. Seeds were stratified at 4 ºC for 4 days, and then transferred to a growth chamber (16/8 h of light/dark period and 22/18 ºC).

Germinated seeds were counted daily for 7 days. Non-germinated seeds were transferred to Petri dishes containing autoclaved GM agar (0.7%) medium without NaCl for another 7 days in order to assess the germination recovery as described below. The criterion for germinated seed was the emergence of a radicle of 1 mm length.

Germination percentage (%) was calculated according to the following equation in that NG is number of total germinated seed and NT is total number of seeds (Scott et al. 1984):

\[
\text{Germination} = \frac{\text{NG}}{\text{NT}} \times 100.
\]

Rate of germination was calculated with a Timson’s germination velocity index (% day$^{-1}$) according to the following equation where G is the percentage of seed germinated each day and T is the total germination period (Scott et al. 1984):

\[
\text{Timson’s germination velocity index} = \sum \frac{G}{T} \times 100.
\]

Non germinated seeds were placed in distilled water for another 7 days and the rate of recovery (%) of germination was calculated using the following equation, where a is the total number of seeds germinated after being transferred to distilled water, b is total number of seeds germinated in saline solution and c is total number of seeds (Scott et al. 1984):

\[
\text{Recovery} = \frac{(a - b)}{(c - b)} \times 100.
\]

Plant culture for study of salt tolerance

Seeds were surface sterilized as described above and sown in 1.5 mL (conical bottom) microfuge tubes filled with GM agar (0.7%) medium. The seeds were stratified at 4 ºC for 4 days and then transferred to a growth chamber (16/8 h of light/dark period and 22/18 ºC). After emergence of the roots and when roots had grown approximately two-thirds down the length of the tube, the conical base of the microfuge tubes were removed and transferred to 500 mL
plastic containers filled with aerated BNS (Basal Nutrient Solution) (Arabidopsis nutrient solution medium, pH 5.6). The solution composition was as follows: macronutrients, NH4NO3 (2 mM), KNO3 (3 mM), CaCl2 (0.1 mM), KCl (2 mM), Ca(NO3)2·4H2O (2 mM), MgSO4·7H2O (2 mM), KH2PO4 (0.6 mM), NaCl (1.5 mM) and micronutrients, Fe(III)EDTA (50 µM), H3BO3 (50 µM), MnCl2·4H2O (5 µM), ZnSO4·7H2O (10 µM), CuSO4·5H2O (0.5 µM), Na2MoO3 (0.1 µM) (Conn et al. 2013). The initial concentration of the BNS medium was 25%; it was increased to 50% after 1 week and to 100% for 2-week-old seedlings.

4 weeks (for L. latifolium, L. perfoliatum and A. thaliana) or 6 weeks (for S. parvula) after sowing, plants were subjected to treatments of 0, 100, 200, 300 and 400 mM NaCl. Salt was applied to the nutrient solution step-wise by increasing the NaCl concentration by 50 mM in each up to the respective concentration, so that the final NaCl concentration of all treatments was reached simultaneously (4 days after starting treatment) (Table S2). 1 week after reaching the final NaCl concentration, plants of all treatments were harvested.

At harvest, whole roots and shoots of control and salt-treated plants were excised separately. Shoots were washed with deionized water, blotted dry on filter paper, and their fresh weights (FW) were recorded. In order to remove salt from the root free space, roots were rinsed for 2–3 min with deionized water, blotted dry on filter paper and FW was determined. The plant materials were dried at 65 °C for 2 days and dry weights (DW) were determined. For A. thaliana only plants exposed to 0, 50 and 100 mM NaCl could be analyzed because plants treated with 200 mM NaCl or higher concentrations died. Biochemical analyses could not be performed in roots of S. parvula because of low biomass.

### Analysis of organic and inorganic osmolytes

For K and Na analysis, oven dried samples were weighed and ashed in a muffle furnace at 550 °C for 8 h, resolved in 0.5 M HCl and made up to fixed volume by double-distilled water. Concentration of K and Na was determined by flame photometry (PFP7, Jenway, UK).

Concentrations of organic osmolytes were determined according to the optimized protocols described elsewhere (Bahrami-Rad and Hajiboland 2017). For the analysis of soluble carbohydrates, leaf and root samples were homogenized in ethanol at 4 °C. After centrifugation at 12,000×g for 15 min, an aliquot of the supernatant was mixed with anthrone-sulfuric acid reagent and incubated for 10 min at 100 °C. After cooling, the absorbance was determined at 625 nm. Glucose (Merck) was used to construct a standard curve. Total soluble protein was determined using a commercial Bradford reagent (Sigma) and bovine serum albumin (BSA, Merck) as standard. Total free α-amino acid content was assayed using a ninhydrin colorimetric method. Glycine (Merck) was used to construct a standard curve. For the assessment of proline concentrations, samples were homogenized with 3% sulfosalicylic acid and the homogenate was centrifuged at 3000×g for 20 min. The supernatant was reacted with acid ninhydrin and glacial acetic acid for 1 h at 100 °C. The reaction was terminated in an ice bath then the mixture was extracted with toluene by vigorous shaking. The chromophore-containing toluene layer was aspirated from the aqueous phase and the absorbance read at 520 nm using toluene as blank. Proline (Sigma) was used to construct a standard curve (Bahrami-Rad and Hajiboland 2017).

In order to estimate the relative contribution of each organic and inorganic osmolyte to the total osmolyte pool, the molar concentration (mmol kg DW−1) of five major osmolytes (total soluble sugars, total free α-amino acids, proline, K+, Na+) (Table S3) were used in the following equation (Bahrami-Rad and Hajiboland 2017):

\[
\text{Relative contribution} = \frac{\text{Concentration of each osmolyte}}{\text{Total concentration of osmolytes}} \times 100.
\]

### Antioxidative defense system

Activity of antioxidative enzymes and concentrations of related metabolites were determined in the leaf and root samples according to the optimized protocols described elsewhere (Hajiboland et al. 2010). Total superoxide dismutase (SOD, EC 1.15.1.1) activity was determined using mono-formazan formation. One unit of SOD was defined as the amount of enzyme required to induce a 50% inhibition of NBT (p-nitro blue tetrazolium chloride) reduction as measured at 560 nm, compared with control samples without enzyme aliquot. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured by determining the oxidation of ascorbic acid as a decrease in absorbance at 290 nm using an extinction coefficient of 2.8 mM⁻¹ cm⁻¹. Peroxidase (POD, EC 1.11.1.7) activity was assayed using the guaiacol test in that the tetra-guaiacol formed in the reaction medium was determined at 470 nm (Hajiboland et al. 2010).

For H2O2 analysis, leaf and root samples (0.1 mg) were homogenized in an ice bath with 1 ml 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged 12,000 × g for 15 min and 0.5 ml of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1.0 M KI. The absorbance of the supernatant was measured at 390 nm. A calibration curve was constructed with H2O2 standard solutions prepared in 0.1% TCA (Hajiboland et al. 2010). The concentration of superoxide radical (O2·−) was determined using the method describe by Chaitanya and Naithani (1994). Leaf and root samples were homogenized in cold (0–4 °C) sodium phosphate buffer (200 mM, pH 7.2) containing diethyldithiocarbamate.
(1 mM) to inhibit SOD activity. The homogenate was immediately centrifuged for 1 min at 3000×g. In the supernatant, superoxide anion was measured by its capacity to reduce nitroblue tetrazolium (250 µM). The absorbance of the end product was measured at 540 nm. Superoxide anion formation was expressed as ΔA min⁻¹ g FW⁻¹ of the sample (Chaitanya and Naithani 1994). Lipid peroxidation was estimated from the amount of malondialdehyde (MDA) formed in a reaction mixture. Leaf and root material (0.1 g) was homogenized in 5 mL 0.1% (w/v) TCA, the homogenate was centrifuged at 10,000×g for 20 min and 0.5 mL of the supernatant was added to 1 mL 0.5% (w/v) thiobarbituric acid in 20% TCA. The mixture was incubated in boiling water for 30 min, and then quickly cooled in ice. The mixture was centrifuged at 10,000×g for 5 min and the absorbance of supernatant was read at 532 nm. MDA levels were calculated from a 1,1,3,3-tetraethoxyx propane (Sigma) standard curve (Hajiboland et al. 2010).

Experimental design and statistical analyses

This experiment was undertaken using complete randomized block design with four independent replications. Data were presented as mean ± standard deviation. Statistical analyses were carried out using Sigma Stat 3.5 (Systat Software Inc., USA) with Tukey test (P < 0.05).

Results

Seed germination

Seed germination decreased under salinity depending on the species (Fig. 1). A considerable reduction of seed germination percentage was observed in L. latifolium exposed to the lower NaCl concentrations (50–100 mM), while these concentrations did not influence seed germination in L. perfoliatum (Fig. 1). S. parvula showed similar or even higher susceptibility to low NaCl concentrations than A. thaliana; but S. parvula was less salt-sensitive than L. latifolium. Higher NaCl concentrations (150–300 mM) decreased seed germination in L. latifolium, S. parvula and A. thaliana to a similar extent, while in L. perfoliatum a considerable germination percentage (20%) was still observed in the 250 mM NaCl treatment (Fig. 1). Salt-induced changes in the germination rate were similar to those observed for the germination percentage. The germination rate in L. latifolium was much lower than in the other species. Recovery of germination increased by increasing salinity treatments in all three halophytes, while it decreased in A. thaliana. Again, the recovery rate was lower (30–50%) in L. latifolium than that in L. perfoliatum and S. parvula. The recovery rate in the latter two species reached 100% in the seeds with a previous salt treatment of 100–150 mM (Fig. 1).

Plant growth and biomass production

Salinity decreased dry biomass of plants to different extents depending on the species (Fig. 2). The three halophytes remained alive under salinity as high as 400 mM NaCl, while A. thaliana died at salt concentrations higher than 100 mM. The three halophytes considerably differed in their biomass production both under low (100–200 mM) and high (300–400 mM) salt concentrations. While a significant growth stimulation was observed for S. parvula under 100 and 200 mM NaCl, growth of L. perfoliatum decreased under these salinity levels. The biomass of L. latifolium was only slightly increased at 100 mM NaCl. Under higher NaCl concentrations, there was still considerable biomass production in L. latifolium and S. parvula while up to 92% biomass reduction was observed in L. perfoliatum under 400 mM NaCl (Fig. 2).

Function of antioxidative defense system

Activities of SOD and APX either remained unchanged (leaves of L. perfoliatum) or decreased under salinity conditions in the three halophytes (Fig. 3). Activity of POD remained unchanged in the roots while increased in the leaves of the three halophytes. In A. thaliana activity of all three enzymes increased under salt treatment both in the leaves and the roots (Fig. 3).

Concentrations of superoxide anion increased in the leaves and roots of L. perfoliatum by exposure to 300 mM NaCl or higher (Fig. 4). No salt-induced changes were observed in the leaves of S. parvula and in the roots of L. latifolium. In the latter species, superoxide anion concentrations were significantly decreased under 100–200 mM NaCl. In A. thaliana, exposure to 100 mM NaCl induced superoxide radical accumulation in the leaves but not in the roots (Fig. 4).

The H₂O₂ concentrations in leaves were not influenced by salt treatment in the Lepidium species. In the roots, higher H₂O₂ concentrations were observed under 400 mM salt in L. latifolium, while in L. perfoliatum exposure to 200 mM NaCl or higher significantly increased the H₂O₂ concentrations (Fig. 4). In S. parvula the H₂O₂ concentrations decreased under 100 and 200 mM NaCl and increased under higher salt concentration reaching the level of that in control plants. In A. thaliana salt exposure caused accumulation of H₂O₂ in both leaves and roots (Fig. 4).

The salt treatments did not cause membrane damage as judged by stable or rather lower MDA concentrations in the leaves of L. perfoliatum and S. parvula and in the roots of L. latifolium (Fig. 4). However, MDA concentration
increased by 300 mM NaCl or higher in the leaves of *L. latifolium* and by 200 mM or higher in the roots of *L. perfoliatum*. MDA concentration increased in the 100 mM salt treatment in the leaves of *A. thaliana* (Fig. 4).

**Concentrations of Na and K**

Under salt exposure, Na accumulated in the leaves of all species (Fig. 5). Less Na accumulation was observed in
The concentrations of K decreased in the roots of all species (Fig. 5). Contrastingly, salinity decreased the leaf K concentrations only in L. latifolium and to a much lesser extent in S. parvula. Leaf K concentrations did not change significantly in L. perfoliatum. A strong reduction of K concentrations up to 75–79% was observed in the leaves and roots of A. thaliana, respectively (Fig. 5). K concentrations in L. latifolium under control conditions (160 mg g DW⁻¹) were higher than that in the other two halophytes (70–80 mg g DW⁻¹). This difference disappeared under salinity treatment equal or higher than 200 mM.

The K⁺/Na⁺ selectivity was expectedly decreased under saline conditions in all species (Fig. 5). Significant differences in K⁺/Na⁺ selectivity were observed between low (100–200 mM) and higher (300–400 mM) salt treatments. At a comparable salt stress level (100 mM) the K⁺/Na⁺ selectivity was much lower in A. thaliana (0.08) than in the three halophytes (0.59–0.78) both in the leaves and roots (Fig. 5).

**Concentrations of organic osmolytes**

Under salt treatment, the concentrations of soluble sugars and proteins in the leaves decreased in the three halophytes. This reduction was more pronounced in S. parvula (Table 1). In the roots, soluble sugars and protein concentrations decreased by salt in L. latifolium, while increased in L. perfoliatum. Salt induced increases of proline concentrations in the three halophytes. Free amino acids accumulated in both leaves and roots in the Lepidium species, while decreasing in the leaves of S. parvula. Among the analyzed organic osmolytes, soluble sugars and proline accumulated in response to salt treatments in both leaves and roots in the glycophyte A. thaliana. In contrast, soluble proteins decreased in the leaves and roots under salt treatment. The levels of free amino acids other than proline also decreased in the A. thaliana leaves, but increased in the roots under salt treatment (Table 1).

**Contribution of each osmolyte to the total osmolyte pool**

Under control conditions, K was the major osmolyte contributing to 70–90% of the osmotic potential of plants (Table 2). Under increasing salinity levels, however, the contribution of Na increased gradually, while that of K decreased in all species. Nonetheless, in the glycophyte A. thaliana the contribution of K was much lower and that of Na was much higher than in the three halophytes grown at the same NaCl concentration (100 mM). Also, the relative contribution of organic osmolytes changed with increasing NaCl concentrations. Among the individual osmolytes, the contribution of soluble sugars decreased by salt treatment in both leaves and roots in L. latifolium, while the contribution of proline
and free amino acids to the total osmolytes concentration increased. The sum of contributions of organic osmolytes to the total osmolyte pool decreased from 4.29 to 3.87 in the leaves, while it increased slightly from 18.11 to 19.38 in the roots of *L. latifolium* exposed to 400 mM NaCl. In *L. perfoliatum*, the contribution of organic osmolytes did not respond consistently to increasing salt levels. An exception was the consistent reduction of the contribution of soluble sugars in the leaves and an increase of it for free amino acids in the roots. Nevertheless, like in *L. latifolium*, the sum of contributions of organic osmolytes to the total osmolyte pool decreased (from 20.84 to 9.3) in the leaves while...
increased (from 12.3 to 20.6) in the roots in the 400 mM NaCl treatment. In *S. parvula*, the contribution of all organic osmolytes in the leaves decreased consistently under salinity. Similar to the *Lepidium* species, the sum contribution of these osmolytes in the leaves decreased from 11.59 to 1.22 under exposure to 400 mM NaCl. In the glycophyte *A. thaliana* grown in hydroponics and treated with different concentrations of NaCl for 1 week. Data are mean±SD, n=4. Bars indicated by the same letters are not statistically different (P<0.05). *nd* not determined.
*thaliana*, the contribution of soluble sugars and free amino acids decreased in the leaves, while it increased in the roots; only proline, increased in both leaves and roots. Also in *A. thaliana* the contributions of organic osmolytes, decreased in the leaves (from 6.751 to 2.784) while it increased in the roots (from 3.94 to 5.59) (Table 2).

**Fig. 5** Concentrations (mg g DW$^{-1}$) of Na and K and the ratio of K/(K + Na) in the leaves (above x-axis) and roots (below x-axis) in three halophytes from the Brassicaceae in comparison with *Arabidopsis thaliana* grown in hydroponics and treated with different concentrations of NaCl for 1 week. Data are mean ± SD, $n = 4$. Bars indicated by the same letters are not statistically different ($P < 0.05$). *nd* not determined.
Discussion

Salt tolerance at the halophyte level has independently evolved in different subclasses, orders and families of higher plants. Therefore, it can be expected that the nature of salt tolerance mechanisms in halophytes is variable, depending on a species’ phylogenetic origin (Flowers et al. 2010). The high diversity of Brassicaceae in the Irano-Turanian region with large areas of saline ecosystems provides excellent opportunities to investigate the salt tolerance mechanisms and salt tolerance evolution in different lineages of Brassicaceae (Koornneef and Meinke 2010). Of particular interest is the fact that the Brassicaceae species *A. thaliana* which is the best-studied genetic model plant, grows naturally in the Irano-Turanian floristic region (Hedge 1968).

Seed germination of three studied halophytes and the glycophyte *A. thaliana* under salinity

Salinity differently affected seed germination of the three studied halophytes. The differences in salt sensitivity during the germination phase did not correspond to the response to salinity of the adult plants. Salt hypersensitivity of the germination process has previously been reported for seeds of other halophytes with high salt tolerance in the adult stage (Inan et al. 2004; Orsini et al. 2010). This behavior is mediated by an increased dormancy under salt conditions (Inan et al. 2004) and is likely a protective strategy for halophytes to ensure maximal seedling survival (Inan et al. 2004; Orsini et al. 2010). In our work, germination of *L. latifolium* showed higher susceptibility even to low salt concentrations and less recovery capability than the other

Table 1 Concentrations of various organic osmolytes including soluble sugars (mg g DW\(^{-1}\)), soluble proteins (mg g DW\(^{-1}\)), proline (µmol g DW\(^{-1}\)) and total free amino acids (µmol g DW\(^{-1}\)) in the leaves and roots of three halophytes from the Brassicaceae in comparison with *Arabidopsis thaliana* grown in hydroponics and treated with different concentrations of NaCl for 1 week

<table>
<thead>
<tr>
<th>NaCl (mM)</th>
<th>Soluble sugars</th>
<th>Soluble proteins</th>
<th>Proline</th>
<th>Free amino acids</th>
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<td>Lepidium latifolium</td>
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<td>25.28 ± 0.84(^a)</td>
<td>41.00 ± 4.70(^a)</td>
<td>10.91 ± 1.83(^a)</td>
<td>10.03 ± 2.58(^ab)</td>
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<td>100</td>
<td>21.56 ± 0.77(^ab)</td>
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Data are mean ± SD, n = 4. Data of each parameter within each species indicated by the same letters are not statistically different (P < 0.05). nd not determined.
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Data are mean ± SD, n = 4. Data of each parameter within each species indicated by the same letters are not statistically different (P < 0.05)

nd not determined
two species. Since *L. latifolium* is a perennial stoloniferous plant, its germination behavior under saline conditions does not seem to be a critical factor for the population and distribution of this species in its habitats. Perennial halophytes are not under the adaptive pressure to develop suitable germination strategies under extreme environmental conditions and rely primarily on clonal growth. Contrastingly, in annual halophytes germination strategies are important for survival of plants (Gul et al. 2013). Comparing two annual halophytes, germination of *S. parvula* was more susceptible than *L. perfoliatum* to both low and high salt treatments. This behavior is opposite to the salt response in the adult plants. The limited distribution area of *S. parvula*, which is in the verge of extinction in Iran, may be the consequence of this higher susceptibility to salt during germination period. Similar to other halophyte species (Gul et al. 2013; Khan and Gul 2006) a high percentage of ungerminated seeds of *S. parvula* recovered and germinated when they were transferred to distilled water (Fig. 1). In the last 3 decades both drought and human exploitation is causing reduced water flow into the lake. In consequence, large parts of the area are desiccating and the existence of *S. parvula* is critically threatened (Akhani 2016). The high salt accumulation in the soil even during the spring months might prevent the recovering of the germination rate and the establishment of *S. parvula* seedlings under prevailing stressed condition.

**Biomass production of three studied halophytes and the glycophyte *A. thaliana* under salinity**

According to biomass data, the salt tolerance of *L. latifolium* (~40% reduction at 400 mM) was in the range of data reported for other halophytes from Brassicaceae such as *E. halophilum* (Alemán et al. 2009) and *Cakile maritima* (Amor et al. 2007) with about 20–50% growth reduction at 300–400 mM salt after 1–2 weeks growth. For *L. perfoliatum*, however, salt tolerance was much less than the reported data for *E. halophilum* and *C. maritima*.

A significant stimulation of growth (by about 2.9-fold) under salt treatments up to 200 mM was observed in *S. parvula*. Such a stimulation has not previously been reported neither in the few former studies undertaken on this species (Oh et al. 2014; Orsini et al. 2010; Uzilday et al. 2015) nor in the experiments performed with its close relative, *E. halophilum*. No stimulation has been reported even after a short-term (72 h) salt exposure (Ellouzi et al. 2014) or under much lower NaCl treatments (25–50 mM) applied for a longer time period (3 weeks) (Alemán et al. 2009). According to Flowers and Colmer (2008) halophytes grow best at concentrations around 50 mM NaCl for monocots, and between 100 and 200 mM for dicots (Flowers and Colmer 2008; Glenn et al. 1999). Growth stimulation by NaCl concentrations in the range of 50–200 mM is not rare in halophytes and has been reported for the dicots *Suameda maritima* (Amaranthaceae/Chenopodiaceae) and *Disphyma australe* (Aizoaceae) (Flowers and Colmer 2008) and the monocot *Aeluropus littoralis* (Poaceae) (Hajiboland et al. 2015). Growth stimulation in halophytes of the Brassicaceae was observed under salt treatment of 50–100 mM for *Cochlearia anglica*, *Cakile maritima*, *Cochlearia officinalis*, and *Diplotaxis tenuifolia* (de Vos et al. 2013). Under higher salt treatments (400 mM), however, growth of these species was reduced up to 40–80% (de Vos et al. 2013). It is noteworthy that biomass production in *S. parvula* in this work was not influenced by salt treatment as high as 400 mM.

Considering a wide range of resistance to salt found among different halophytic plants and growth stimulation by salt in several of these species, some authors have further classified halophytes as ‘facultative halophytes’ and ‘obligate halophytes’ (Caldwell 1974). ‘Facultative halophytes’ are species occurring both in saline and non-saline habitats and usually exhibit moderate degrees of salt tolerance. Contrastingly, ‘obligate halophytes’ are confined to saline habitats and exhibit high degrees of salt tolerance combined with a physiological requirement of salt for optimal growth (Caldwell 1974; Rozema and Schat 2013). Accordingly, our data confirm *S. parvula* as an obligate halophyte, while the two *Lepidium* species are facultative halophytes. In fact, *S. parvula* is characterized as a succulent and only occurs on saline soils in association with *Halocnemum strobilaceum*. Contrarily, the two *Lepidium* species occur both in saline and non-saline habitats (Akhani 1988; Hedge 1968).

**Species differences in Na accumulation under salinity**

Leaf and root Na accumulation data revealed clear differences between the three halophytes and the glycophyte, *A. thaliana* under comparable salt treatments (100 mM). The halophytes accumulated considerably lower Na concentrations when exposed to 100 mM NaCl than *A. thaliana*. The ability to selectively exclude Na+ from the leaf blades is an important property of salt tolerant species (Sibole et al. 2005). Our results indicate that under salinity levels higher than 100 mM, *A. thaliana* is unable to efficiently restrict Na transport to the shoots and the accumulation of high Na in the leaves causes plant death. Using electrophysiological approaches applied to *E. halophilum* along with *A. thaliana*, this difference has been attributed to a lower unidirectional Na+ influx (Wang et al. 2006) and more effective mechanisms to restrict Na+ transport to the shoot (Alemán et al. 2009) in *E. halophilum* compared to *A. thaliana*.

Although the three halophytes excluded Na+ more efficiently than the glycophyte *A. thaliana*, considerable Na concentrations accumulated in their leaves. Leaf Na concentrations observed in our *L. latifolium* and *S. parvula* plants
were higher (120–130 mg g DW⁻¹) than those reported by others, who observed Na leaf concentrations around 70 mg g DW⁻¹ for *E. halophilum* (M’rah et al. 2006; Orsini et al. 2010), *S. parvula* (Orsini et al. 2010) and *Cakile maritima* (Debez et al. 2004) when grown with 500 mM NaCl for 4–6 weeks.

The K⁺/Na⁺ selectivity is an important determinant for salt tolerance (Shabala and Mackay 2011) and depends on the characteristics of the transporters that mediate K⁺ and Na⁺ absorption. Differences in the regulation and the function of these transporters among species may lead to a different control of K⁺/Na⁺ homeostasis that result in different salt tolerance (Alemán et al. 2009). In our work, a steep reduction of K/(K + Na) ratio as an indicator of K⁺/Na⁺ selectivity was observed under salt treatment in all species. However, this ratio was considerably higher in the two *Lepidium* species (0.59–0.61) and particularly in *S. parvula* (0.78) than in *A. thaliana* (0.08) at a comparable salt treatment (100 mM). Comparative studies showed that a much higher K⁺/Na⁺ selectivity in *E. halophilum* roots than *A. thaliana* is related to the higher K⁺/Na⁺ selectivity of inward-rectifier K⁺ channels and voltage-independent non-selective cation channels (Wang et al. 2006).

### Role of antioxidative defense system in the response of three studied halophytes to salinity

The superior salinity tolerance in halophytes has been attributed by some authors to constitutive or salt-inducible higher antioxidative activity and a larger pool of non-enzymatic antioxidants compared with glycophytes (Ozgur et al. 2013). In this work, however, SOD and APX activity either remained unchanged or even decreased by salinity treatments. Considering the dual role of ROS in signaling events and acting as toxic molecules depending on the concentration and sub-cellular compartmentation (Foyer and Noctor 2003; Zepeda-Jazo et al. 2011), function of antioxidative defense system in the modification of ROS levels could be highly important for the ultimate plant response to salt. A detailed literature review on the effect of salt on the antioxidative enzymes using different methods including transcriptomics and proteomics approaches revealed that, in a range of halophytes, increasing the salt concentration has yielded all the possible scenarios (increase, decrease, and no change) in the activity of antioxidative enzymes (for a review see Bose et al. 2014; Jithesh et al. 2006; Kosová et al. 2013). These discrepancies seem mainly due to differences in both the intensity of the applied salt stress and the analyzed fractions (whole plant, whole tissue or cell fractions). Even under low salinity ROS induce a substantial remodeling of cation and anion conductance and affect Ca²⁺ signaling. These effects are dependent on the type of ROS, the plant species, and the type of tissue (Pottosin et al. 2014). Only under high salt stress ROS may cause extensive damage to macromolecules and cell ultrastructure. Bose et al. (2014) has argued that truly salt-tolerant species possess efficient mechanisms for Na⁺ exclusion from the cytosol. Therefore, they may not require a high level of antioxidant activity, as they simply do not allow excessive ROS production in the first instance (Bose et al. 2014). In our work, activity of SOD and APX and accumulation of O₂⁻ and concentration of MDA all revealed that the three studied halophytes avoided oxidative damage under salinity treatment more effectively than *A. thaliana*. Among the three halophytes, *S. parvula* was more efficient than the two *Lepidium* species as indicated by both the lack of O₂⁻ accumulation and a low and stable MDA concentration. Further confirmation is provided by the corresponding biomass data (Fig. 2).

Among the three analyzed antioxidative enzymes in this work, only the activity of POD was increased by salt treatment at different NaCl concentrations depending on species. Plants contain abundant amounts of POD that are involved in different responses from lignin synthesis and defense against insects to H₂O₂ scavenging (Kawano 2003). Excretion of PODs can produce apoplastic ROS that play a role in active extracellular signal transduction (Kawano 2003). Considering the enhanced POD activity in halophytes in comparison to glycophytes (Jithesh et al. 2006), a signaling versus scavenging role has been suggested for PODs in halophytes (Bose et al. 2014). In this work, the activity of POD showed a significant positive correlation (*r* = 0.82, *P* < 0.05) with H₂O₂ concentrations in *S. parvula* that could partly confirm a role for POD in the production of H₂O₂ and activation of plant defense against salt stress. Hydrogen peroxide as a signaling molecule elevates the level of cytosolic free Ca²⁺ via activation of plasma membrane Ca²⁺-permeable influx channels and the Ca²⁺ transport protein Annexin I. This is involved in the H₂O₂-induced cytosolic free Ca²⁺ signature and the downstream signaling in the stressed roots (Richards et al. 2014). In addition to H₂O₂, O₂⁻ could also participate in signaling events in our plants. The small variations in O₂⁻ concentrations observed in the halophytes exposed to salinity indicate a signaling role for O₂⁻ rather than a salt-induced membrane damage. Superoxide is converted to H₂O₂ and OH⁻ at the plasma-membrane (Demidchik 2018). The plasma-membrane OH⁻-activated K⁺ channel, that is responsible for K⁺ efflux from root cells during stress, is involved in stress-induced cell death (Demidchik 2018). The hydroxyl radical is also able to activate plasma membrane Ca²⁺-ATPase and affect H⁺ pumping with a synergistic effect with polyamines particularly in salt-sensitive plants (Pottosin et al. 2014). Direct role of O₂⁻ in the post-transcriptional regulation of the outward-rectifying K⁺ (GORK) channels has also been demonstrated (Tran et al. 2013).
**Osmotic adjustment mechanism in three studied halophytes**

Plants grown under saline conditions meet the challenge of osmotic adjustment in order to maintain water uptake. The three major inorganic ions, Na⁺, Cl⁻ and K⁺, account for 80–95% of the cell sap osmotic pressure in both halophyte grasses and dicots (Glenn et al. 1999; Shabala and Mackay 2011). Since Na⁺ is toxic at the concentrations required for osmotic adjustment, Na⁺ and Cl⁻ are compartmentalized predominately in vacuoles. Thus, halophytes rely also on compatible solutes in the cytosol to balance the osmotic pressure of the inorganic ions in vacuole (Slama et al. 2015). Various compatible solutes occur in halophytes (polyols, amino acids, betaines and a variety of sugars) (Slama et al. 2015). Although trace amounts of glycine betaine have been detected in several Brassicaceae species, this compound has never been found to play any osmotic role in this family (Slama et al. 2015). It is widely accepted that proline is the main important compatible osmolyte under salinity conditions in Arabidopsis and its halophytic relatives (Slama et al. 2015). The concentrations of the three-major organic osmolytes in our experimental plants were in the range of those reported by others for Arabidopsis and in Brassicaceae halophytes (M’rah et al. 2006; Uzilday et al. 2015). The amount of proline relative to the total amino acids in three studied halophytes was in the range of 7–23% under control conditions and increased to 31–98% at 400 mM salt (Table S3). In the halophytes Suaeda nudiflora and Pyunkofia (Salsola) brachiata (Chenopodiaceae/Amaranthaceae),[ and Sesuvium portulacastrum (Aizoaceae) proline comprised 20–83% of total free amino acids under salinity conditions (Joshi 1981). In a metabolomic analysis, Lugan et al. (2010) observed that in *E. halophilum* in contrast to *A. thaliana* a notable increase under salt stress occurred for only a few compounds, such as proline, γ-aminobutyric acid and aliphatic and aromatic compounds.

The relative contribution of each individual osmolyte to the total osmolyte pool, observed here indicates that organic osmolytes may play only a partial role in the osmotic adjustment in these halophytes. Each organic osmolyte contributed only about 0.05–12% to the plant total osmolyte concentration under control conditions and between 0.3–14% under exposure to 400 mM NaCl. An interpretation of the contribution of organic osmolytes to the osmotic adjustment of plants is lacking in the literature and the majority of researchers confined only to reporting an increase of organic osmolyte concentrations upon salinity treatment. Among three analyzed organic solutes, soluble sugars and free amino acids pool were superior than proline in the contribution to total osmolytes pool in this work. Authors have frequently demonstrated that higher proline accumulation is one of the mechanisms for higher salt tolerance in *E. halophilum* compared to the glycophyte *A. thaliana* (Gong et al. 2005) and studies have been undertaken to show an activation/upregulation of proline synthetizing enzymes and/or downregulation of catabolizing ones upon salt treatment (Taji et al. 2004). However, a very low contribution of proline to the total osmotic potential of plants has been largely neglected. Nevertheless, osmotic adjustment within the cell may not be the only essential function of compatible solutes. Even when present at osmotically insignificant concentrations, such solutes may function to scavenge ROS and stabilize the tertiary structure of proteins (Szabados and Savoure 2010). Interestingly, the constitutive and salt-inducible accumulation of proline was higher in the three halophytes than the glycophyte, *A. thaliana* which may confirm an irreplaceable role of proline beyond its osmotic role for salt tolerance in these halophytes. In contrast, Ghars et al. (2008) showed that proline accumulated to similar extent in *A. thaliana* and *E. halophilum*.

Lower levels of organic osmolytes as observed here for soluble sugars and free amino acids under salinity in the three halophytes particularly in *S. parvula* in contrast to the glycophyte *A. thaliana*, may be considered an adaptive value because of the cost associated with their synthesis (Raven 1985; Shabala and Mackay 2011). In addition to the high energy requirement in halophytes for the selective ion acquisition and transport (Alemán et al. 2009), a considerable amount of ATP is required for the synthesis and accumulation of organic solutes. On a molar basis this cost has been estimated as 34 for mannitol, 41 for proline, 50 for glycine betaine, and approximately 52 for sucrose (Raven 1985). A small variation in total organic solutes observed in the leaves of *E. halophilum* (Lugan et al. 2010) led to the conclusion that, in contrast to *A. thaliana*, the leaves of *E. halophilum* are able to lose more water under osmotic stress without losing turgor. Such a strategy probably based on more elastic cell walls (Barcelo et al. 1986) would be less expensive than a strategy of osmo-adjustment (Lugan et al. 2010). Interestingly, salt treatment caused a decrease in the sum of organic osmolytes that contribute to the total osmolyte pool both in the halophytes and in the glycophyte. However, this was compensated by higher contribution of K under salt treatment in the halophytes, but not in *A. thaliana*. This emphasizes again the relevance of high K⁺/Na⁺ selectivity in the salt tolerance of halophytes.

**Schrenkiella parvula as a model halophyte**

Our results indicated that *S. parvula* an Irano-Turanian species, is even more salt tolerant than its well-studied close relative, *E. halophilum*. In contrast to our observations, exposure to 300 mM NaCl of *S. parvula* whose seeds had been collected from salt flats in Tuz Lake in Central Anatolia (Turkey), caused a significant (31%) growth...
reduction after 2 weeks growth, without any growth stimulation even under salt concentrations as low as 50 mM (Uzilday et al. 2015). We suggest here an ecotype difference between the two populations collected from Meyghan Salt Lake, Iran and Tuz Lake, Turkey. It will be interesting to compare the physiology of salt tolerance in both populations in a common experiment for unraveling the mechanisms involving in the superior salt tolerance in the population collected in Iran. Unfortunately, *S. parvula* is becoming critically endangered due to the lack of an integrated strategy for management of salt habitats in the country. The genome of this species has been sequenced recently (Dassanayake et al. 2011). This will contribute to further use of this species a model plant for the investigation of the mechanisms of extreme halophytism.

*Lepidium latifolium* as an ecologically and economically important halophyte

The two *Lepidium* species studied in this work have different life forms and different phenology. While *L. perfoliatum* is an annual with two different ecotypes occurring in high saline and non-saline soils, *L. latifolium* is a tall-growing perennial herb with ecological and economic importance. The latter species occurs in a wide range of habitats mostly as ruderal and weed plants near coastal wetlands, inland waterways, irrigation ditches, rangeland, hay meadows and waste areas, and tolerates saline/alkaline environments (Francis and Warwick 2007). Although this species was shown here to be more salt susceptible at the seed germination stage than the other studied species, its life form and large biomass production makes it a good candidate for restoration of saline habitats. Planting *L. latifolium* may prevent soil erosion and reduce the generation and spreading of saline micro-aerosols around the abandoned saline areas of the country. *L. latifolium* has already demonstrated its potential as a tool in soil restoration by improving chemical properties of mesic, silty-clay calcareous soils rich in Na (Renz and Blank 2004). Prolific seed production, rapid and substantial vegetative growth, creeping rhizomes with high salt storage capacity, potential for bud production at each node, and deep rhizome penetration, all contribute to the widespread establishment of *L. latifolium* (Francis and Warwick 2007). In addition, *L. latifolium* has potential as a vegetable crop for saline agriculture. Domestication of halophytes is an approach towards large-scale saline agriculture (Rozema and Schat 2013). Finally, the closer affinity of *A. thaliana* with the diverse genus *Lepidium* (Huang et al. 2016) in comparison to its relationship with other halophytic genera like *Schrenkniella*, *Eutrema* and *Thellungiella* is compelling for future studies on the mechanisms and evolution of halophytism in Brassicaceae.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this paper.

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