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Multiple mechanisms mediate growth and survival in young seedlings of two populations of the halophyte *Atriplex halimus* (L.) subjected to long single step salinity treatments.

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Abstract

Understanding how halophytes survive high soil salinity in realistic long-term experiments is important for strategies to mitigate effects of increasing soil salinity world-wide. Protective mechanisms in halophytes enabling survival, include sequestration of salt via Na^+/H^+ antiporters, synthesis and accumulation of osmolytes, and activation of protective mechanisms against reactive oxygen species (ROS). Protective mechanisms elicited by a single step-up to a range of NaCl treatments (34-256 mM) in two populations of the halophyte *Atriplex halimus* L. from contrasting environments (arid steppe and saline coastline) were compared over six weeks. The coastal population survived significantly better at high salinity compared to the steppe population although in both populations salinity inhibited growth. Increased Na^+ and K^+ concentration was accompanied by higher induction of Na^+/H^+ antiporter gene expression in coastal compared to steppe population leaves. Osmolytes increased more significantly in the coastal compared to the steppe population with greater induction of choline mono-oxygenase gene expression. Activation of ROS scavenging mechanisms was greater in coastal compared to steppe plants. Differential responses found through time, salt concentrations and between leaves and roots indicate a finely tuned response. Sharp changes in responses at 171 mM NaCl indicate that different mechanisms may be invoked at different stress levels.

Key words: *Atriplex halimus* L., halophyte, Na+/H+ antiporter, CMO gene expression, osmolytes, reactive oxygen species.

Introduction

Increases in irrigated agriculture and intense utilization of water resources in hot and dry countries lead to inevitable increases in soil and water salinity. In Algeria long periods of dryness have resulted in soil salinization affecting 3.2 million hectares (Belkhodja and Bidai 2004). Faced with likely increases in aridity due to climate change, species adapted to local conditions such as *Atriplex halimus* are being identified and selected to mitigate desertification (Benderradji *et al*., 2006). Re-establishment programmes for these species require the identification of genotypes that are salt-tolerant at early seedling stage. This is important, to minimize the use of costly fresh water for their irrigation in nurseries since more readily available ground water used for irrigation is highly saline.

The genus *Atriplex* (Amaranthaceae) comprises about 200 species in temperate and subtropical regions and is associated with saline and alkaline soils in arid, desert or semi-desert environments (Mulas and Mulas 2004). These shrubs constitute an important forage reserve in times of shortage. *Atriplex halimus* L. (Haddioui and Baaziz 2001) is a perennial C4 native shrub native to the Mediterranean Basin which shows an excellent tolerance to salinity and drought (Ortiz-Dorda *et al.* 2005). This species is genetically variable and populations from different areas of the Mediterranean Basin were clearly separated using RAPD markers (Ortiz-Dorda *et al.* 2005).

Plants exposed to salt stress face two key constraints: firstly osmotic stress from the rise in external osmotic pressure, resulting in a rapid reduction in plant growth rate. In a second phase, toxic ions (Na^+ and Cl^-) accumulate, which can lead to premature leaf senescence and ultimately death of the whole plant (Munns and Tester 2008). Mechanisms for achieving salt tolerance vary amongst species. Some halophytes exclude salts from the leaves by accumulating them in salt glands on their leaf surface (Sangam *et al.* 2005). Others are internal accumulators, accumulating salt by sequestering it into the

cell vacuole and controlling cellular K^+/Na^+ ratio through a family of Na^+/H^+ antiporters (Flowers and Colmer 2008). NHX Na^+/H^+ antiporter genes have been isolated from several *Atriplex* species including *A. halimus*, and at least in *A. gmelini,* the antiporter localises to the tonoplast membrane (Hamada *et al.* 2001). In *A. gmelini* the *AgNHX* transporter gene was rapidly up-regulated by salt treatments of 100-400 mM NaCl in both roots and leaves although expression was much higher in leaves (Hamada *et al.* 2001).

Another mechanism evolved by plants to combat stress is the biosynthesis and accumulation of osmolytes (that act as osmoprotectants) such as soluble sugars, proline, and glycine betaine (Peel *et al.* 2010). The Chenopodiaceae and Amaranthaceae produce large amounts of this quaternary ammonium compound (Brouquisse *et al.* 1989) which stabilizes the quaternary structures of complex proteins such as PSII (Papageorgiou and Murata 1995) and protects membranes from high Na^+ and Cl^- concentrations (Rhodes and Hanson 1993). The concentration of glycine betaine accumulated usually correlates with the level of salt tolerance (Rhodes and Hanson 1993). Choline mono-oxygenase (CMO) oxidises choline to betaine aldehyde, which is then converted by BADH into glycine betaine. CMO expression increased dramatically in *A. prostrata* stems, leaves and roots following a 3 day treatment with 1-2% NaCl (Wang and Showalter 2004). The major site of synthesis of glycine betaine in plant species studied to date is in the leaves (Rhodes and Hanson 1993) with CMO being chloroplast localised. However in some species such as barley, glycine betaine is likely synthesised also in roots (Fujiwara *et al.* 2008). An *A. nummularia* CMO gene was expressed at low levels in roots and was salt-inducible (Tabuchi *et al.* 2005). Proline is also accumulated very rapidly in *A. halimus* under saline treatments (Ben Hassine *et al.* 2008) and contributes to the osmotic adjustment.

Salt stress induces increased levels of reactive oxygen species (ROS) that disrupt redox homeostasis leading to lipid peroxidation and other cellular damage (Noctor and Foyer 1998). Salt treatment elevates ROS levels in both

halophytes and glycophytes. However in halophytes, the rise can be transient, lasting only a few hours (Ellouzi *et al.* 2011) due to the activation of antioxidant mechanisms. Ascorbic acid is a key antioxidant and ROS scavenger (Smirnoff 2000). Key ROS moieties include superoxide, hydroxyl radicals and singlet oxygen and their cellular levels are regulated within narrow tolerable ranges (Foyer and Noctor 2003). Antioxidant enzymes are also a central to the ROS scavenging system activated under salt stress. These include superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) (Noctor and Foyer, 1998). Activities of these enzymes are frequently elevated in salt-tolerant species including *A. halimus* and are induced by salt exposure (Boughalleb *et al.* 2010).

The degree of tolerance and mechanisms for resisting salt stress varies within and amongst plant species. For example *Atriplex halimus* plants originating from coastal saline sites were more tolerant of high salinity and produced higher levels of glycine betaine, whereas plants from a semi-arid non-saline site were more tolerant to water-stress and produced more proline (Ben Hassine *et al.* 2008). In other *A. halimus* populations, (Bouchenak *et al.* 2012) plants from a more saline origin did contain more proline as well as quaternary ammonium compounds. However, both these experiments were performed on 4-6 week old plants over a relatively short 10-18 day treatment period, therefore effects on early plant growth were not studied. Many studies on salt stress tolerance are performed by gradually increasing the salinity over a period of time to enable the plants to adapt, study only germination, or treat older plants that are already well-established. For example Boughalleb *et al.* (2009) exposed *Atriplex halimus* to up to 800 mM NaCl but this stress was imposed in increments of 100 mM NaCl at 2 day intervals until the maximum salinity concentration tested was reached. We were therefore interested to know how very young plants respond to a sudden increase in salinity and whether mechanisms differ between populations derived from areas differing in salinity. Using a single-step up approach, we exposed young plants directly

to a range of environmentally-relevant salinities and compared two Algerian *Atriplex halimus* L. populations from differing environments: semi-arid steppe and saline coastline over a six week treatment period. We hypothesized that the populations from the more saline environment would be more tolerant to higher saline treatments and display different or more efficient mechanisms for salt tolerance.

Materials and methods

Plant material and growth

Atriplex halimus seeds were from wild plants growing in two distinct regions of Algeria. Population 1 (steppe) is from the Algerian steppe, a semi-arid area located in Northern Algeria (Chott Zahrez in the province of Djelfa, 3°03'E longitude, 34°36'N latitude). The geology in this area is mainly cretaceous, with some quaternary deposits. Soil salinity is between 1.99 and 4.47 dSm^{-1} depending on the season, at a depth of 15-20 cm (Nedjimi 2012), corresponding to the rooting zone of *A. halimus*. Soil texture encompasses silt-clays and silt-sands (Pouget 1973) and the water table is between 1-3 m below the soil surface. In this region groundwater is in the form of semi-captive and unconfined aquifers, surrounded by the presence of a more or less saline and unequally deep groundwater that contributes to the formation of saline soils (Pouget 1973). Chott Zahrez is essentially Mediterranean, with wet winters and hot dry summers (the minimum average is 5˚C in January and average maximum is 26 °C in July) and a mean annual precipitation of 250 mm year-¹ (Nedjimi *et al.* 2012). Population 2 (coastal) is from the Algerian coastline also in Northern Algeria (in the province of Tipasa, 36° 35' 22" N, 2° 26' 50" E), in a sub-humid area with an average annual rainfall of 600 mm (1978-2004) (Boudjelal 2007). Temperatures are mild with an annual average of 17-18 ° C (absolute minimum on record of -2 ° C). This area is characterized by sedimentary cliffs and rocky areas (Grimes 2010) with a salinity of 9 dSm^{-1} (Tifour 2000). The plants are also subjected to frequent sea

water spray (55.38 dSm^{-1}), but not total submersion, due to high winds in this area, making it a highly saline environment.

Seeds (15-20 per pot, ten pots per treatment) were sown in washed and dried medium coarse sand irrigated with distilled water, and grown in a Phytotron at a constant 25°C, with 16:8 hours light: dark at 90 µmol $m^{-2}s^{-1}$ from warm white fluorescent tubes and 40 % relative humidity, until cotyledons appeared. Then irrigation continued with a nutrient solution (pH 5.6; Morard 1995; Supplementary Tables 1 and 2). Salt stress was applied just after the appearance of the first leaf pair, 10 days after sowing (NaCl concentrations: 0, 34, 85, 171 and 256 mM). Electrical conductivity was constant throughout the experiment (Supplementary Table 3). Plants were grown for six weeks. Leaves and roots for analysis were randomly selected from more than one plant at each analysis time point and material was pooled into three biological replicates; roots were used directly as there was no soil to wash off.

Percentage survival (for each of the 10 pots) was recorded after 6 weeks and plant height over 6 weeks (for all surviving plants; average height per pot was calculated). Relative growth rate (RGR) relating to plant height was calculated from plant height data at 1, 2, and 6 weeks (Wang 2011). To determine relative water content (RWC), leaf and root tissue was dried at 105 °C to a constant dry weight. The relative water content was determined by the relationship: RWC (%) = FW – DW/ FW * 100.

Metabolite analyses and enzyme activity measurements

Analyses of Na^+, K^+, proline and soluble sugars were carried out on fresh leaves and roots (in triplicate) after 1, 2, and 6 weeks growth under salt stress. For analysis of Na^+ and K^+, samples were dried at 105 °C for 1 h followed by 520°C for 2 h, digested in HNO_3 (0.5N) and assayed by flame photometry (using a Cecil 6000 series spectrophotometer). Total chlorophyll was extracted from fresh leaves with 80% acetone and absorption measured at 652

nm. Concentrations of chlorophyll were determined according to Plummer (1989), then converted to µg/g FW. Proline concentration was measured spectrophotometrically at 528 nm according to Troll and Lindsley (1955) from 100 mg of leaf tissue. Soluble sugars were analyzed using the anthrone method (Plummer 1989) from 100 mg of fresh plant material. Absorbance was read spectrophotometrically at 585 nm, and calibrated using a standard curve. Glycine betaine was measured according to Grieve and Grattan (1983) from 150 mg of fresh plant tissue in triplicate. Absorbance was measured at 365 nm using glycine betaine (Sigma Aldrich Poole. UK) as standard, and expressed as mg g^{-1} DW. The concentration of total solutes in roots and leaves over time was calculated by dividing the sum of K+, proline, soluble sugars and glycine betaine concentrations by the amount of water present in the plant tissue, based on the % water content.

Ascorbic acid was extracted by a freezing procedure (Nojavan *et al.* 2008). from 100 mg of fresh tissue, in triplicate HPLC analysis was carried out using an isocratic elution procedure with a UV Detector at 240 nm. Separation was carried out on a 5µm RP C18 column of 250 mm × 4.6 mm (Kinetex-Phenomenex). The mobile phase consisted of 0.5% NaH_2PO_4 (pH 2.25 with H_3PO_4) - acetonitrile (2% of final volume). An injection volume of 20 µL was used in quantitative analyses.

An Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Invitrogen) was used to measure H_2O_2 concentrations in fresh leaves after 6 weeks under saline conditions. The absorbance (at 560 nm) was measured using an Infinite 200 PRO microplate reader (Tecan, Switzerland). Catalase activity was measured by spectrophotometry at 240 nm. Leaves (250 mg in triplicate) according to Aebi (1984).

RNA extraction and Real time PCR

RNA was extracted and purified from contaminating genomic DNA using an RNeasy Mini Kit (Qiagen) from two independent biological replicates of

tissue that was flash frozen in liquid nitrogen and stored at -80 ^{o}C until used. Retrotranscription and real-time PCR were carried out essentially as in ElMaghrabi *et al.* (2013) using 2 µg of RNA an Ambion kit (RETROscript ® Reverse transcription for RT-PCR) and an Absolute TM QPCR SYBR ® Green Mix (Thermo Scientific) kit. Reactions were cycled in an MJ Research OPTICON TM 2. Relative quantification of gene expression data used the 2^{-DDCT} method (Livak and Schmittgen 2001). Mt18S rRNA primers were used to normalise the results (mean of three technical and two biological replicates). Primers for the *Atriplex halimus* CMO gene were derived from an alignment of CMO genes from *A. nummularia* (AB112481), *A. prostrata* (AY082068) and *A. hortensis* (AF270651). Primers for the antiporter gene were derived from alignment of sequences from *A. dimorphostegia* (AY211397) and *A. gmelini* (AB038492). The *A. halimus* PCR products were fully sequenced to verify their homology. All primers are listed in Supplementary Table 4.

Statistical analyses

Data were analyzed using StatBox6 and R software (R version 2.15.3, R Foundation for Statistical Computing). A 2-way ANOVA test was performed on % survival and antioxidant data; all other data were analysed using a 3-way ANOVA. Where significant ($P < 0.05$) interactions or mean effects were found, comparisons were made using a Newman-Keuls test and consolidated by Tukey's test.

Results

Seedling survival and chlorophyll content with increasing NaCl concentration in coastal and steppe Atriplex halimus seedlings

Atriplex halimus seedlings germinated equally in the two populations but survival fell significantly ($P < 0.05$) at the highest two salt concentrations, compared to non-stressed controls in both populations, thus a sudden step-up to 85mM NaCl did not affect greatly seedling survival of either population.

At 256 mM NaCl, coastal region (P2) seedlings survived significantly better ($P < 0.05$) than steppe region (P1) seedlings (Fig. 1A). P2 seedlings were also significantly taller than P1 at all time-points (Fig 1B) and grew significantly faster in the first two weeks at NaCl concentrations >34 mM, with an RGR that was significantly higher than the control at all salt concentrations while the P1 RGR was reduced at the highest salt concentration but unaffected at lower salinity (Fig. 1C). The RGR after 6 weeks (relative to 1 week) was reduced equally in P1 and P2 with increasing salt. P2 seedlings also retained significantly greater relative water content at all salt concentrations than P1 in both leaves and roots at all time-points (Fig. 2A, B).

Chlorophyll concentration rose significantly between week 1 and week 6 at all salt concentrations ($P< 0.05$) and was significantly higher in no salt control coastal plants (P2) compared to steppe (P1) especially after 6 weeks (Fig. 2C). With increasing NaCl, chlorophyll concentration fell slightly at all time-points, although remained > 80% of control even at the highest salt treatment after 6 weeks.

Differential ion accumulation in seedling roots and leaves with increasing salt concentration

For the first two weeks, Na^+ concentration increases were similar between P1 and P2 leaves (Fig. 3A). However, at each salt treatment at 34 mM - 171 mM, Na^+ concentration was significantly higher ($P < 0.05$) in P2 leaves, while at 256 mM, there was no difference between them. After six weeks there was a significantly greater concentration of Na^+ in all the salt treated seedlings compared to the control, but P2 seedling leaves accumulated more Na^+ at all concentrations of NaCl reaching a maximum of (334.3 ± 4.8) $\mu molg^{-1}$ FW at 171 mM NaCl, and the highest differential in Na^+ between the two populations.

Changes in Na^+ in roots was different to those in leaves (Fig. 3B), and concentrations were much lower, reaching only one third those of leaves in

P2 (71.2 ± 2.7 μmolg^{-1} FW) after 6 weeks. After 6 weeks at 34 mM NaCl, Na^+concentration was higher in P2 roots than P1 roots, and higher than control roots of either population ($P < 0.05$). At 85 mM there was significant ($P < 0.05$) NaCl accumulation in both P1 and P2 seedling roots, both after 2 weeks and 6 weeks of treatment, but no significant difference between the two populations. Na^+ was however higher in coastal (P2) seedling roots ($P < 0.05$) at 171 mM NaCl after both two and six weeks of treatment compared to P1. At 256 mM NaCl, Na^+ was significantly higher in P2 than P1 roots after 2 weeks (35.7 ± 3.1 μmolg^{-1} FW and 31.5 ± 1.2 μmolg^{-1} FW respectively), but after 6 weeks this difference was abolished.

Leaf K^+ levels were not affected by the first two weeks of salt treatment (Fig. 3C). However, after 6 weeks, K^+ concentration was almost four-fold higher and was significantly greater ($P < 0.05$) in coastal P2 leaves compared to P1 in all but the highest NaCl treatment. In roots, K^+ levels showed few changes between P2 and P1 or between salt concentrations at 2 or 6 weeks (Supplementary Figure 1).

The K^+/Na^+ ratio fell with increasing NaCl at all time-points in both P1 and P2 leaves (Fig. 4A). After 1 week, in no salt controls, the K^+/Na^+ ratio was significantly higher ($P < 0.05$) in coastal (P2) compared to steppe (P1) leaves, however at all other time-points and salt treatments the K^+/Na^+ ratio was the same or higher in P1 leaves. The pattern was essentially the same in roots after 6 weeks, although at 2 weeks there were no significant differences between the two populations or amongst salt treatments (Supplementary Figure 2).

Changes in K^+ and Na^+ concentration were reflected in the induction of the *A. halimus AhNXX1* Na^+/H^+ antiporter gene expression in leaves under salt treatments (Fig. 4B). At 85- 256 mM NaCl, P2 Na^+/H^+ antiporter expression was significantly up-regulated compared to the control peaking at 171 mM

NaCl. In contrast, expression in P1 leaves was only induced at 171but remained high at 256 mM NaCl. In roots both P1 and P2 antiporter expression was above control at 85-256 mM NaCl, but P2 expression was only significantly higher than P1 at 256 mM NaCl. As in leaves, expression of the antiporter in P1 roots remained constant at171- 256 mM.

Osmolyte accumulation and expression of the glycine betaine biosynthesis related gene CMO were induced differentially by salt treatments in P1 and P2.

Proline concentration increased slightly even in control leaves after 6 weeks, reaching 20.9±0.01 and 27.8 ± 0.02 $\mu molg^{-1}$ FW respectively for P1 and P2 (Fig. 5A). However salt treatment induced an almost 3-fold increase in maximal proline concentration. Proline rose in both P1 and P2 from 85 to 171 mM NaCl at all three time points, but fell back at 256 mM in P2 whereas in P1 it reached a plateau at 171 mM NaCl after 2 and 6 weeks. The greatest difference in proline concentration between the two populations was at 171 mM NaCl at all time points although after 6 weeks proline was significantly higher ($P < 0.05$) in P2 compared to P1 leaves at all concentrations including the control.

The pattern was similar in roots (Fig. 5B) although proline concentration remained more similar over time with less than a 2 fold difference in maximal accumulation, and was much lower than in leaves. Again, proline rose between 34-171 mM NaCl in both populations and at both time-points compared to the control, and P2 roots accumulated significantly higher levels of proline than P1 roots at >34 mM NaCl. However, P2 roots accumulated significantly less proline than P1 at 256 mM NaCl at both week 2 and week 6.

Soluble sugars increased with time in leaves at all salt concentrations, but also increased in response to the salt (Fig. 5C). After 1 week, maximal levels were at 171 mM NaCl, but at later time points concentrations continued to rise to

256 mM NaCl. Soluble sugar levels were significantly higher in P2 compared to P1 leaves at all salt concentrations after 2 weeks. In roots the pattern was similar but there were no significant differences at any salt treatment or time point between the two populations although there was a rise in soluble sugars with increasing salt concentration from 0- 171 mM at both time points and then a fall at 256 mM NaCl (Supplementary Figure 3).

Glycine betaine concentration was significantly higher in coastal (P2) compared to steppe (P1) leaves and roots at all time points and NaCl concentrations including the control (Fig. 6). In both the P1 and P2 leaves glycine betaine concentration increased with increasing NaCl and with time. However in roots, whereas glycine betaine rose with salt in P2 at > 34 mM NaCl, in P1 it remained constant up to 171 mM and only rose at 256 mM. Although the glycine betaine concentrations in roots only reached one quarter of that in leaves at 256 mM NaCl, the fold induction at the salt concentration compared to the non-saline control was similar in the two tissues and for the two populations.

The increase of glycine betaine in leaves with salinity was at least in part transcriptional since CMO expression rose with increasing salt in leaves at $\geq$85 mM NaCl (Fig. 6C) and was significantly higher in P2 than P1. CMO expression was much lower in roots, and was not significantly induced by salt.

Antioxidant capacity

H_2O_2 concentration was lower at all salt concentrations compared to the control, but there was no significant difference between the two populations (Supplementary Figure 4). In leaves of both populations, ascorbic acid increased linearly with NaCl from 34-256 mM (Fig. 7A). At each salt concentration P2 accumulated significantly more ($P < 0.05$) ascorbic acid than P1 leaves, and the rate of accumulation was also significantly faster. Catalase activity was also induced by salt in both P1 and P2 leaves (Fig. 7B),

however in P1, activity was only greater than control at $\geq$ 170 mM NaCl. In contrast catalase increased linearly in P2 leaves from 85-256 mM NaCl (R^2 = 0.994).

Discussion

Despite the application of NaCl in a single step-up, *Atriplex halimus* seedlings challenged at the first leaf stage were remarkably resilient, with over 70% survival at 256 mM NaCl after 6 weeks of treatment. Much higher salt concentrations of 600 mM have been previously tested on *A. halimus* (Bouchenak *et al.* 2012) but only on much more mature, 4 week old, plants with 10-12 leaves, and only for a much shorter period of 18 days. As noted in other species (e.g. *Tecticornia* spp.; English and Colmer 2013) young seedlings are much more sensitive to high salt than even slightly older plantlets. Given the widespread use of saline irrigation water in arid Mediterranean areas, and growth of this species very close to the sea, the data presented here are of direct relevance to the semi-natural environment where salt stress is imposed early in development and over long periods and the natural environment where saline stress can be imposed soon after germination through sea spray.

Survival of coastal population (P2) plants was significantly higher than the P1 steppe plants, at 256 mM NaCl. P2 plants also grew taller than P1 plants at all salt concentrations including the control and over time, indicating a difference between the two populations in their growth, irrespective of the salt treatment. In fact there were significant differences between the two populations in the no salt control for many of the characters including ion ratio, glycine betaine and proline concentrations and catalase activity indicating differences in normal metabolism as well as salt responses. Interestingly the RGR of the coastal population between one and two weeks

of salt treatment was greater at all the salt treatments compared to the no salt control, indicating that this population may grow optimally in the presence of short periods of salinity. In many *Atriplex* spp. salinity stimulates growth, including *A. halimus* (Belkheiri and Mulas 2013), and a single-step salt treatment of 150 mM for 10 days increased shoot RGR in a Tunisian *A. halimus* population, although the RGR decreased progressively at higher stress intensities (300, 450 and 600 mM; Bajji *et al.* 1998). However experiments are usually conducted with more mature plants than those used here. In contrast, the 1-2 week RGR of the steppe population (P1) was not stimulated by salt, and was reduced at 256 mM NaCl, indicating an important difference in early salt responses between the two populations. After 6 weeks, the RGR and shoot height were reduced by the saline treatment in both populations even at 34 mM NaCl. This suggests that prolonged salt treatments are inducing some stress.

It is not possible from these data to unequivocally determine whether the stress was osmotic or due to ion toxicity which would require more detailed measurements of leaf growth and senescence. However the small reductions in chlorophyll at the lower salt concentrations suggest that the effects here may be primarily osmotic whereas at higher concentrations, more significant chlorophyll reductions suggest also an ion toxicity effect.

Differences between coastal and steppe populations are in agreement with previous work using coastal and semi-arid populations from Tunisia (Ben Hassine *et al.* 2008) where at 160 mM NaCl for 10 days, dry weight of semi-arid derived plants was reduced but was in not coastal derived plants. However, loss of chlorophyll in the first 1-2 weeks contrasts with experiments on a coastal population of Tunisian *A. halimus* where there was no loss of chlorophyll over 10 days of treatment at 160 mM NaCl (Ben Hassine and Lutts 2010). The difference is likely due to the age of the plants, which were already 6 weeks old in the Ben Hassine and Lutts (2010) experiments when treated.

Both populations of *A. halimus* studied here maintained relative water content which increased over the 6 weeks of treatments. This may be at least in part due to the ability of C4 plants like *A. halimus* to regulate stomatal closure which is the main cause of reduced photosynthesis and therefore reduced growth in other species under mild to moderate drought stress and high salinity (Chaves *et al.* 2009).

In both populations Na^+ concentration increased in both leaves and roots in response to the saline treatments, but the level of Na^+ in the leaves was almost 10 fold higher than in roots, suggesting that in these populations salt is being accumulated rather than excluded in the leaves as was previously found in some *A. halimus* populations (Belkheiri and Mulas 2013). As found by Ben Hassine *et al.* (2008) the coastal population accumulated significantly more Na^+. However after two weeks at 256 mM NaCl, the differential between the two populations was lost, suggesting a threshold level between 171 and 256 mM NaCl for salt accumulation in the both populations. Notably, after 6 weeks, the differential was restored, suggesting that at a later stage of development (as indicated by increasing chlorophyll levels throughout the experiment) additional mechanisms for Na^+ accumulation may become available. The greater inducibility of the Na^+/H^+ antiporter gene at 6 weeks in the coastal (P2) leaves and roots, which is particularly dramatic in leaves at 171 mM NaCl and in roots at 256 mM NaCl, may be a factor in the higher Na^+ accumulation in the coastal plants. Roots of the P2 plants were still able to up- regulate the Na^+/H^+ antiporter gene expression at the highest salt level, while the steppe region plants were not. Bajji *et al.* (1998) found that roots responded less than leaves in this species to high salt concentrations but was unable to explain this effect mechanistically. Here results suggest that this effect might be due at least in part to a greater inducibility of the Na^+/H^+ antiporter in roots at high salt concentrations compared to leaves, thus excluding salt from the cytoplasm more effectively.

458 The slight fall in leaf but not root K^+ levels in the steppe (P1) plants between
459 0 and 85 mM NaCl at later time-points is in agreement with previous reports
460 in *A. halimus* (Bajji *et al.* 1998; Boughalleb *et al.* 2010). The differential
461 between the coastal and steppe populations in leaf K^+ accumulation after 6
462 weeks at most salt concentrations, however, suggests a greater ability of the
463 P2 plants to retain K^+ in leaves (but not in roots) under saline conditions. The
464 finding that the K^+/Na^+ ratio remains >1 at all concentrations of external salt
465 throughout the experiment in leaves fits with the requirement to balance these
466 two ions to protect protein synthesis (Flowers *et al.* 2015).

467 As shown by Ben Hassine *et al.* (2008) mechanisms other than glycine
468 betaine accumulation are involved in *Atriplex halimus* salt tolerance, and their
469 relative importance varies with different populations. Induction of glycine
470 betaine accumulation in both leaves and roots was higher in P2 plants
471 indicating that this may be a more important protection mechanism in P2
472 compared to P1 plants against long term salt stress. The higher levels of CMO
473 expression in leaves compared to roots agrees with expression in *A.*
474 *nummularia* (Tabuchi *et al.* 2005), as is the salt-induction in both leaves and
475 roots. We show here that higher glycine betaine levels in both tissues of the
476 coastal population are matched by higher CMO expression levels.
477 Accumulation of glycine betaine in the roots of the coastal *A. halimus* plants
478 may therefore derive from synthesis in the roots as well as more efficient
479 phloem loading or transport, from the leaves.

480 In the populations studied by Ben Hassine *et al.* (2008) the coastal
481 populations preferentially accumulated glycine betaine while the inland
482 populations accumulated more proline. However this differential mechanism
483 was not supported by the study of the two Algerian populations of contrasting
484 origins (Bouchenak *et al.* 2012) where both quaternary ammonium
485 compounds and proline were higher in populations from saline areas when
486 challenged with salt treatments but not with drought. Results from the two

populations studied here support a role for both proline and glycine betaine in salt tolerance in *A. halimus* and highlight a difference between leaves and roots and across time. Notably after 6 weeks >2-fold more of proline accumulated compared to earlier time points at all levels of salt treatment and in both plant populations. This agrees with Martinez *et al.* (2005) where older leaves accumulated more proline in response to salt treatment than young leaves. Even after 2 weeks, however, coastal population leaves here accumulated significantly more proline than the steppe leaves even at a lower salt concentration (85 mM) than that tested by Ben Hassine *et al.* (2008). At 171 and 256 mM the enhanced proline accumulation by the coastal population P2 after six weeks was striking. In roots the pattern was different: although at 85 and 171 mM NaCl which span the 160 mM NaCl used by Ben Hassine *et al.* (2008) the coastal plants accumulated significantly more proline in roots than the steppe plants, the ratio was indeed reversed at 256 mM NaCl. Thus it would seem that after six weeks at high salt concentrations both proline and glycine betaine accumulation in leaves are important for salt tolerance of coastal population plants, while in roots the glycine betaine accumulation may be more important.

A third type of osmolite, soluble sugars, also appears to be involved in the protective mechanisms of both populations. Accumulation of soluble sugars in leaves but not roots may be relevant to longer term salt tolerance of the coastal population since accumulation was more highly induced in this population after 6 weeks at all salt concentrations. This contrasts with a previous study on *A. halimus* where there was no difference in sugar accumulation between saline and non-saline environment derived populations (Bouchenak *et al.* 2012). In contrast also to Bajji *et al.* (1998) and Martinez *et al.* (2005), here soluble sugars were induced by low salt concentrations (< 50 mM) in leaves after two weeks as well as at higher concentration as reported before (Bajji *et al.* 1998; Boughalleb *et al.* 2010; Bouchenak *et al.*

2012), suggesting that leaf soluble sugars may be more relevant as a protective mechanism at low salinity in the very young leaves and the different populations studied here. In agreement with Bajji *et al.* (1998) though, root soluble sugars increased with increasing external salt concentrations and then fell back or remained constant. Here the upper limit was 171 mM NaCl whereas for Bajji *et al.* (1998) it was 300 mM NaCl again suggesting differences between the plants tested and growth stage. The fall in soluble sugar levels is interpreted by Bajji *et al.* (1998) as an inhibition of phloem transport which would also inhibit transport of glycine betaine from the leaves, thought to be via the phloem (Chen and Murata 2011). However, the continued increase in glycine betaine concentration even at 256 mM NaCl together with the up-regulation of the CMO gene expression in roots, suggests that at least some of the glycine betaine may be synthesised directly in the roots rather than translocated.

A comparison of the total concentration of internal solutes with the external solute concentration (Supplementary Fig. 5) indicates that at 1-2 weeks osmotic adjustment in leaves may have occurred in the 34 mM NaCl when the 15.8 mM of nutrient solutes is included in the calculation. However, after 6 weeks, leaves may be able to adjust osmotically up to the combined external solute concentration of 15.8 mM from the population. In contrast, osmotic adjustment does not appear to occur in roots at any concentration. This difference between roots and leaves has been noted previously in *Atriplex mummularia* (Silveira *et al.* 2009). However, the calculation here needs to be interpreted with caution and may be a significant under-estimate. Although the concentration of many of the major organic osmolytes normally considered to be important for osmotic adjustment (proline, soluble sugars and glycine betaine) as well as K^+ (Singh *et al.* 2015) have been included, other cellular solutes such as other amino acids and ions will contribute to the internal solute concentration and hence may alter the threshold.

The very similar changes in H_2O_2 concentration in the two populations under salt treatment, and the reduction in H_2O_2 compared to the control no-salt treatment suggests that the antioxidant mechanisms are not being compromised at the NaCl concentrations tested here, consistent with other studies (e.g. Boughalleb *et al.* (2010). A greater activation of antioxidant mechanisms may be a component of differential salt tolerance mechanisms of the two populations under high salt treatment since there was a significantly higher ascorbic acid concentration in coastal P2 leaves compared to steppe P1 leaves at all salt concentrations. The differences in the catalase activity between the two populations were most evident at 85 mM NaCl suggesting that at this intermediate salt concentration catalase plays a more important differential role between the two populations. In contrast to previous work showing no increase (Boughalleb *et al.* 2010; *A. halimus*) or a reduction (Sai Kachout *et al.* 2013; *A. hortensis*) in catalase activity in response to salt, here there was a small increase from 85–256 mM external NaCl. In the steppe population catalase activity rose between 85 and 171 mM treatments but did not rise further at 256 mM whereas in the coastal population activity rose up to 256 mM external salt. This higher catalase activity in the coastal population is in broad agreement with Bouchenak *et al.* (2012). However, here there was a small but significant induction of the catalase activity by all salinity treatments of $\geq$ 85 mM NaCl in both populations whereas in the populations described in Bouchenak *et al.* (2012) catalase activity dropped in the non-saline population. Differences may also again be related to the age of the plants indicating that in young plants catalase plays a more important role in protection against the salt-induced ROS changes. Note also that in addition to ROS scavenging enzymes and non-enzymatic antioxidants, soluble sugars can also play an antioxidant role against ROS under biotic and abiotic stress (Keunen *et al*. 2013), acting in concert with other protective mechanisms. Hence the increase in soluble sugars seen here in both populations, and the relatively higher accumulation in the coastal population may be contributing

to maintain ROS homeostasis under salinity stress.

In conclusion, it emerges that young *A. halimus* seedlings are able to cope with relatively high saline environments which may be important in their survival after germination in their natural ecosystems in seasons where rainfall is sporadic and or reduced. Furthermore, different mechanisms are invoked at different salt concentrations in different tissues of the plant and at different times during a long single-step salt treatment of young seedlings and some responses differ to those in older plants. Na^+, proline and glycine betaine accumulation seem to be greater contributors at high salt concentrations, while soluble sugars and antioxidant mechanisms are involved throughout. In roots glycine betaine biosynthesis and Na^+/H^+ antiporter inducibility may also contribute to salt tolerance at 256 mM NaCl, while actual Na^+ accumulation, proline and soluble sugars may be less relevant. Differences were noted between the coastal population, where plants would naturally be exposed to higher salt concentrations, and the inland population. However, as there are also differences in the annual rainfall between the two environments, further work would be required to assess whether mechanisms that have evolved to adapt to drought are also contributing to the differences noted in response to salt treatments. However, from a practical perspective, given the greater induction of many of the salt tolerance mechanisms and more rapid growth of the coastal population seedlings, this population may be better suited for re-establishment of this species in areas where increased aridity is affecting its survival.

Acknowledgments

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FIGURES

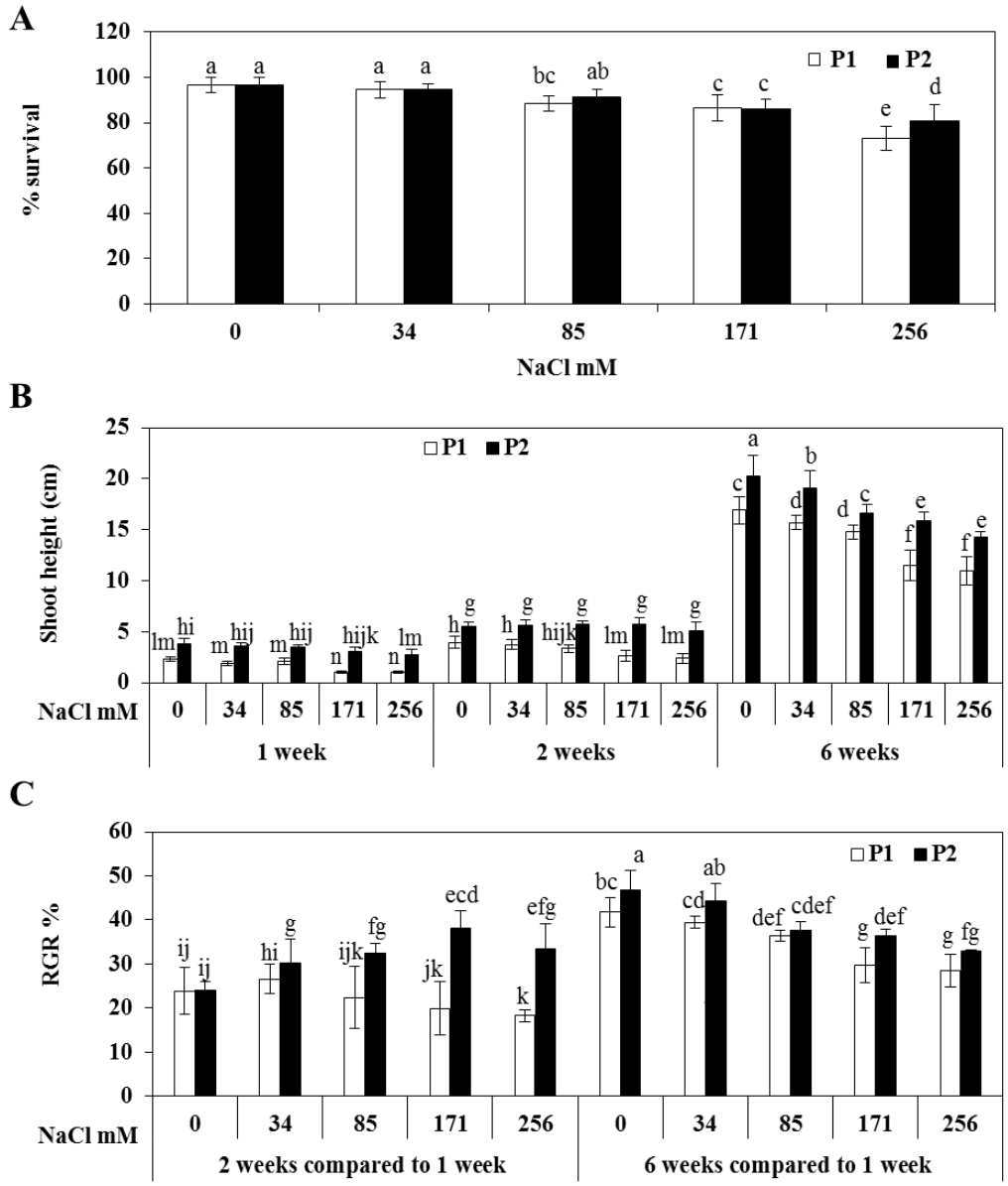


Fig. 1. Mean percentage survival per pot (A), shoot height (B) and relative growth rate (C) of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions after 6 weeks of salt stress (A); and over time (B, C). Mean ± S.D; different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples (n =10).

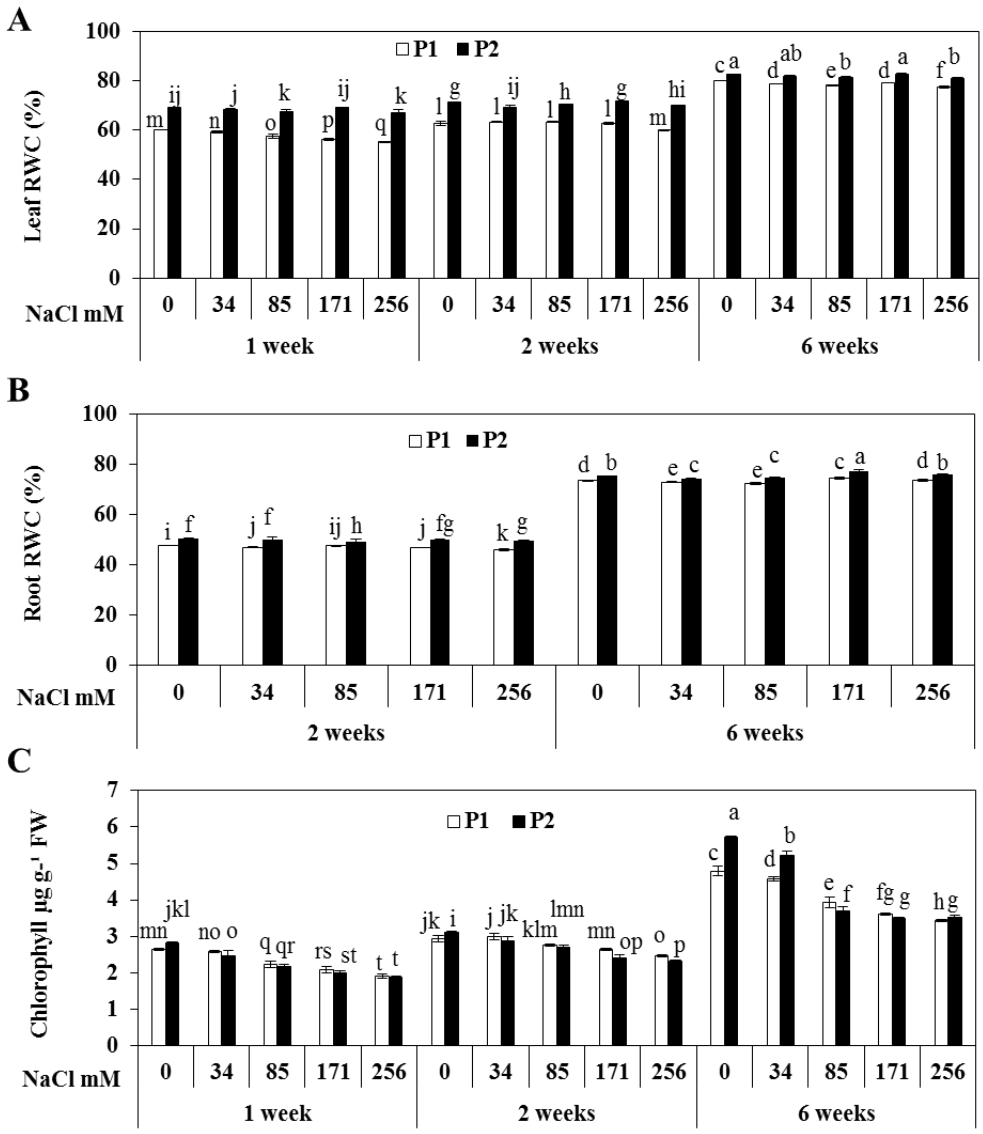


Fig. 2. Relative water content (RWC) in leaves (A), roots (B) and chlorophyll concentration in leaves (C) of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions over time and across salt stress treatments (mean ± S.D; n = 3; different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples.

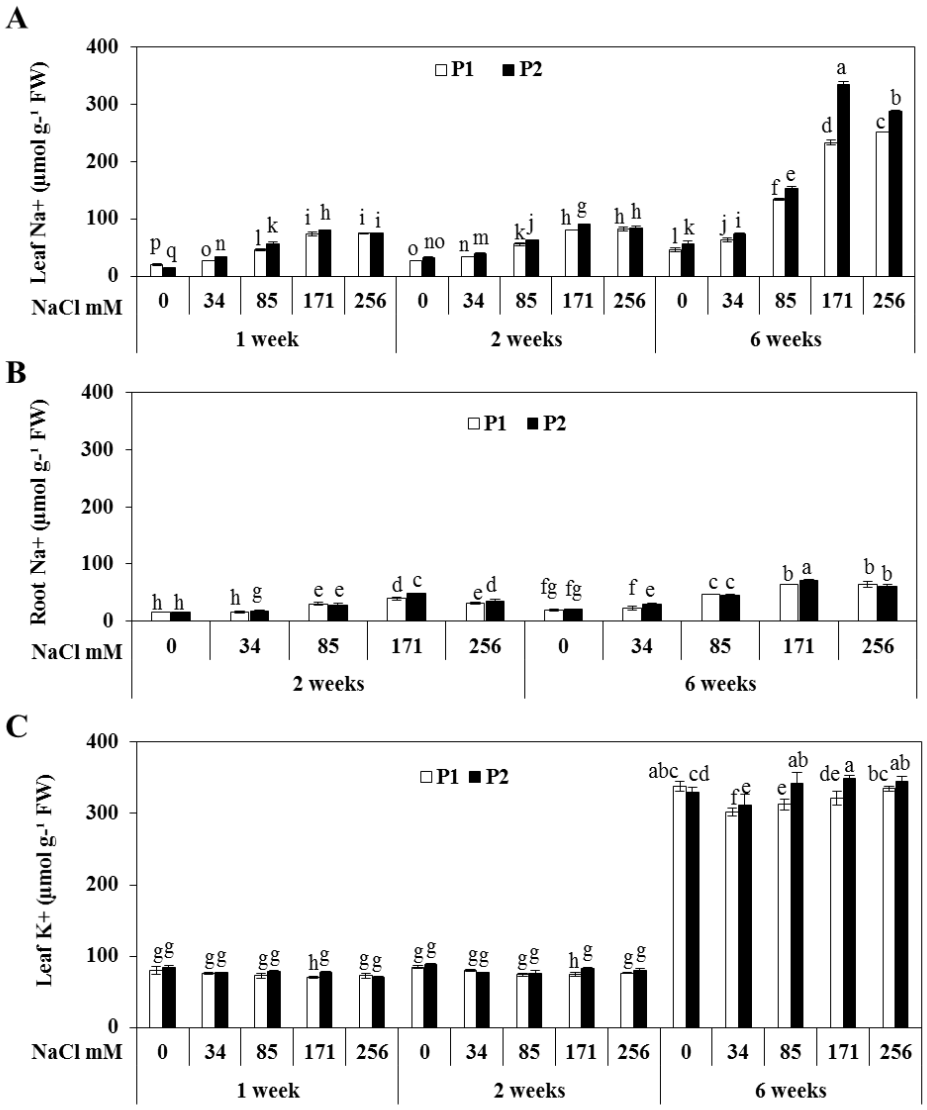


Fig. 3. Na+ accumulation in leaves (A) and roots (B); K+ accumulation in leaves (C) over time in *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions across salt stress treatments (mean ± S.D; n = 3; different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples.

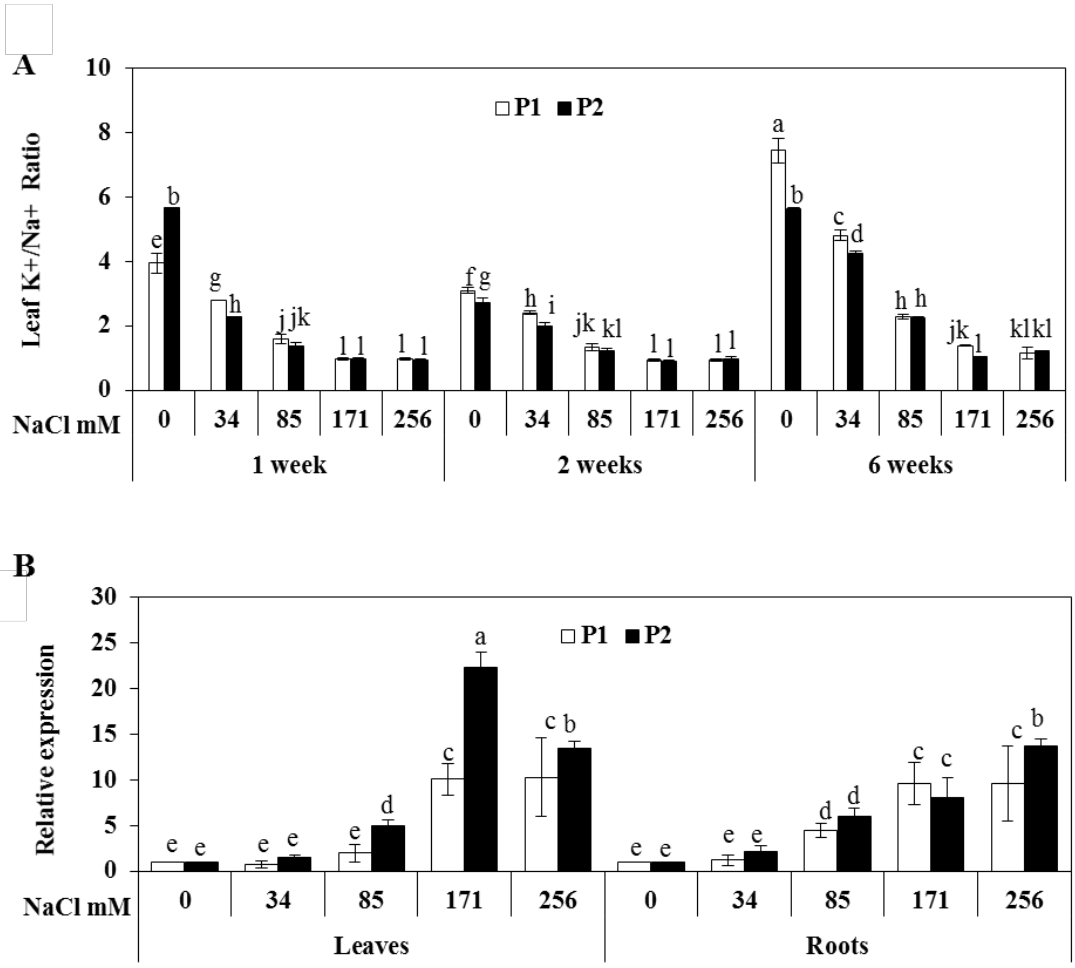


Fig. 4. K+/Na+ ratio in leaves (A) over time and relative Na+/H+ antiporter gene expression after 6 weeks compared to the no salt control (B) of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions across salt stress treatments (mean ± S.D; n = 3 (A); n = 6 (B); different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples.

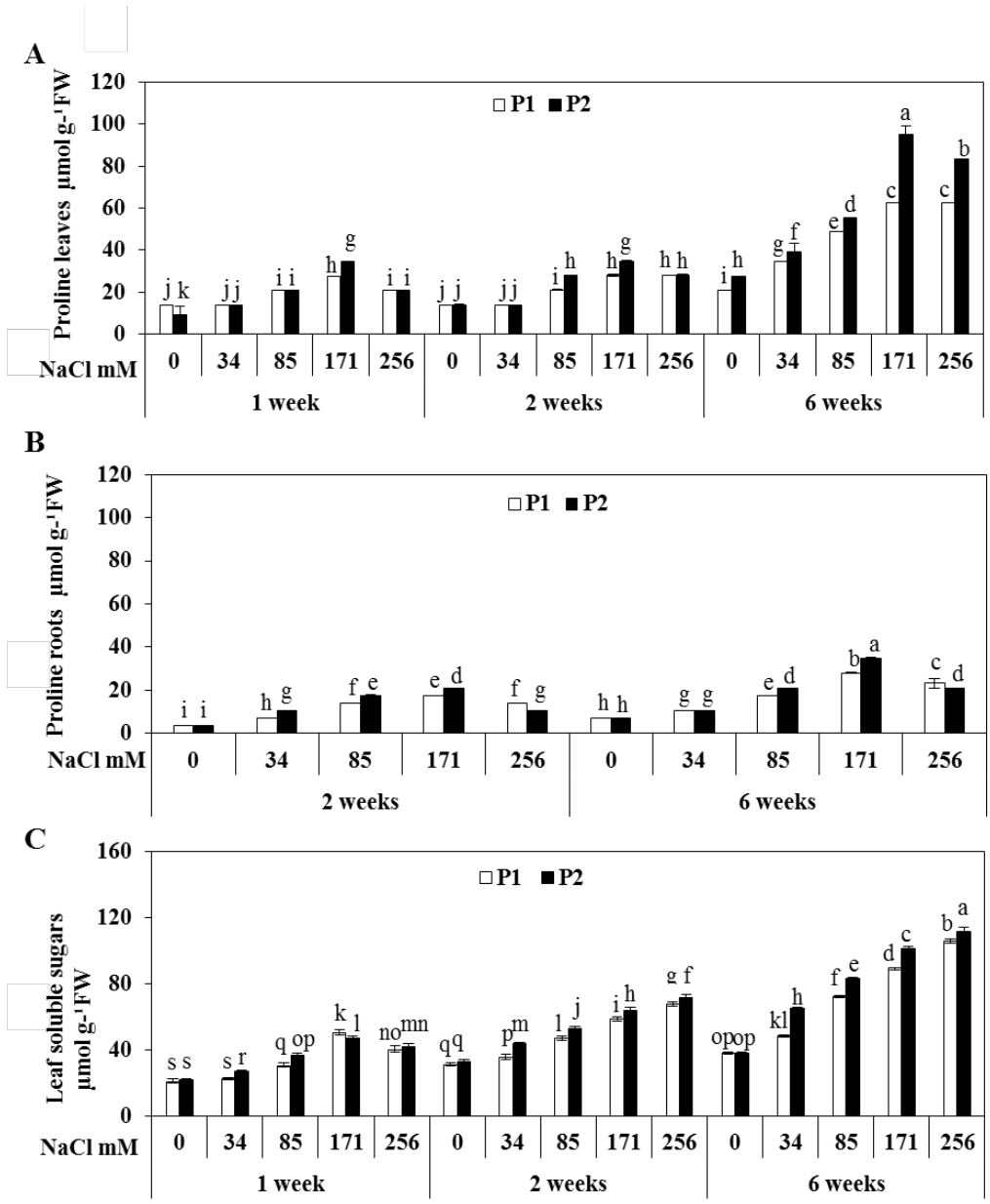


Fig. 5. Proline concentration in leaves (A) and roots (B); soluble sugar concentration in leaves (C) of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions over time and across salt stress treatments (mean ± S.D; n = 3 ; different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples.

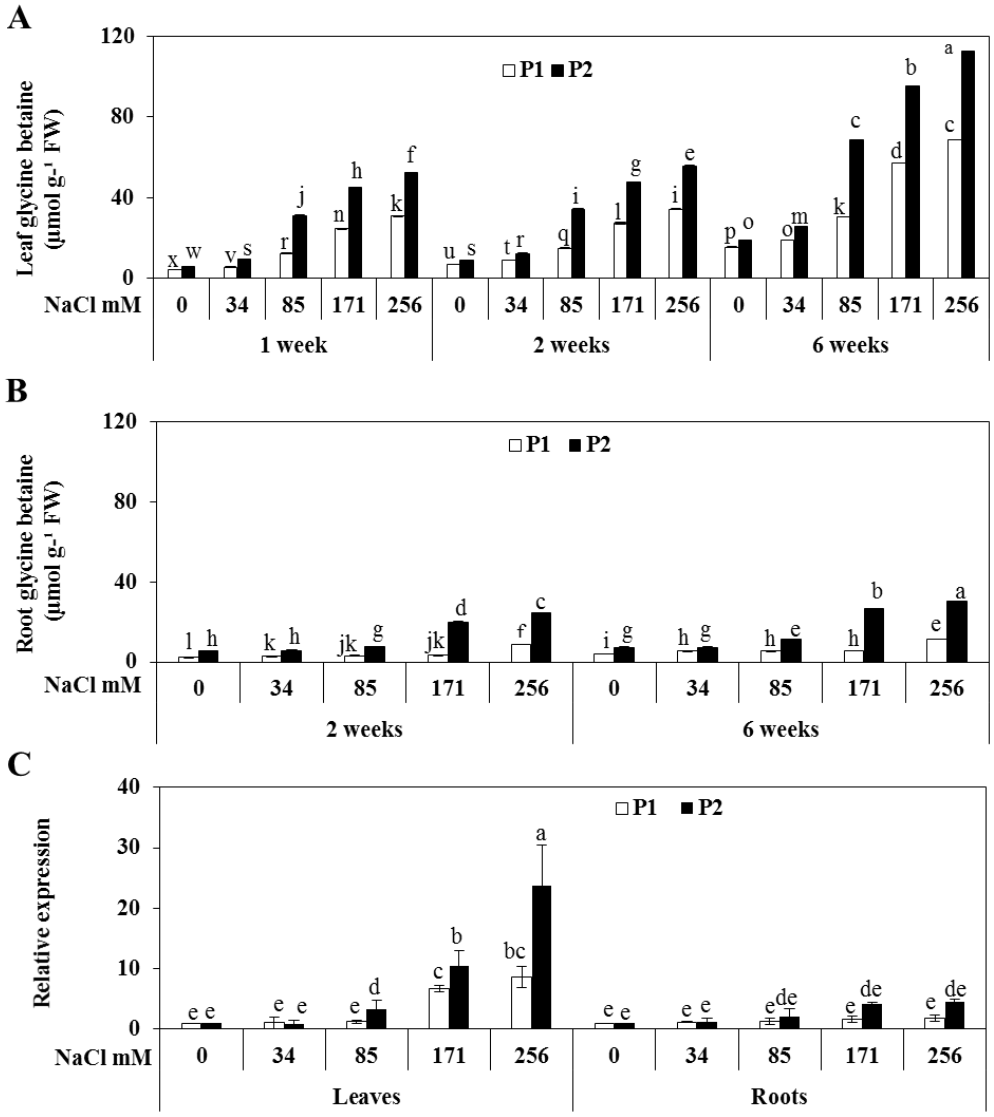


Fig. 6. Glycine betaine concentration in leaves (A), roots (B) and CMO gene expression relative to the no salt control (C) in *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions over time and across salt stress treatments (mean ± S.D; n = 3 (A,B); n = 6 (C); different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples.

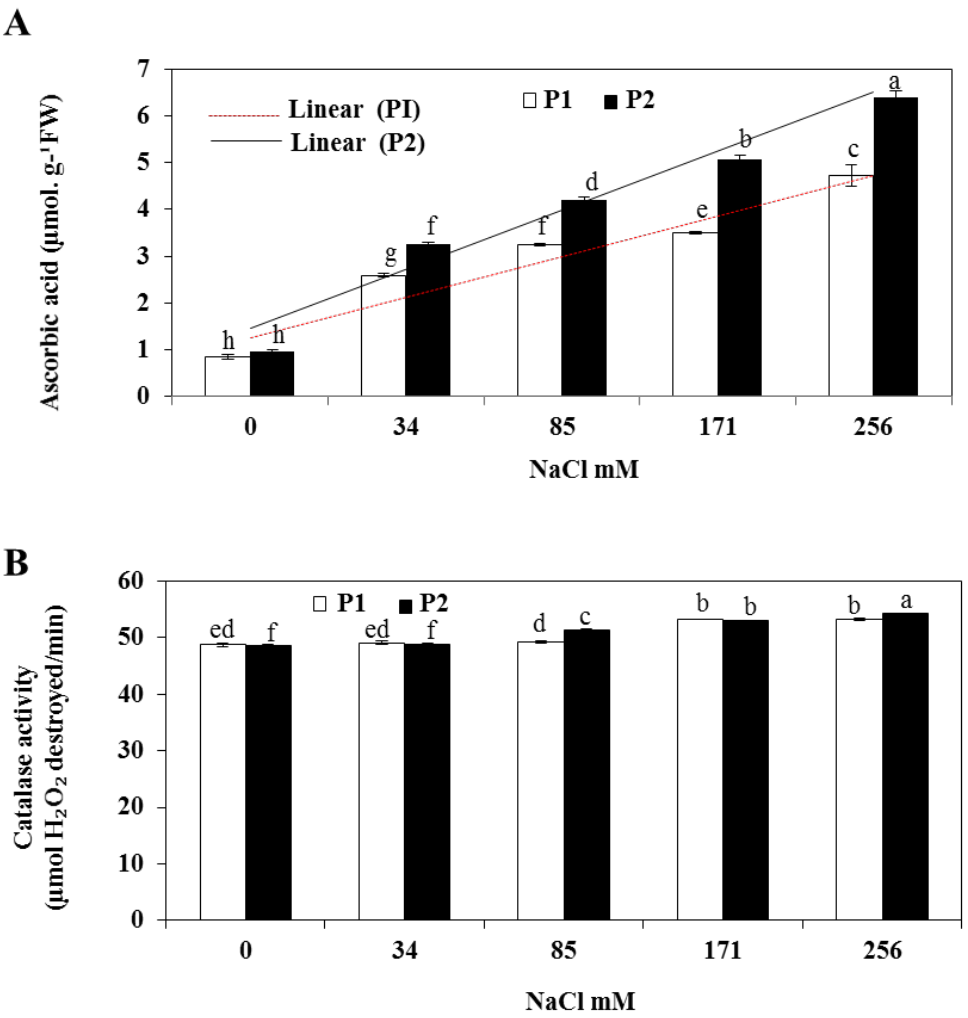


Fig. 7. Ascorbic acid concentration (A) and catalase activity (B) in leaves of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions after 6 weeks of salt stress (mean ± S.D; n = 3 ; different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples.

SUPPLEMENTARY INFORMATION

Supplementary Table 1. Nutrient solution, Macroelements (Morard 1995)

Macroelements	K	Ca	Mg	Na	N	P	S	Cl
	K^+	Ca^{++}	Mg^{++}	Na^+	$NO3^-$	$H2PO4^-$	$SO4^{--}$	Cl^-
Concentration (mM)	7	5	1.5	-	15	2	1.5	-

Supplementary Table 2. Nutrient solution, Microelements (Morard 1995)

Microelements	Fe	Mn	Cu	Zn	B	Mo
Concentration (mM)	0.089	0.008	0.0009	0.001	0.024	0.0001

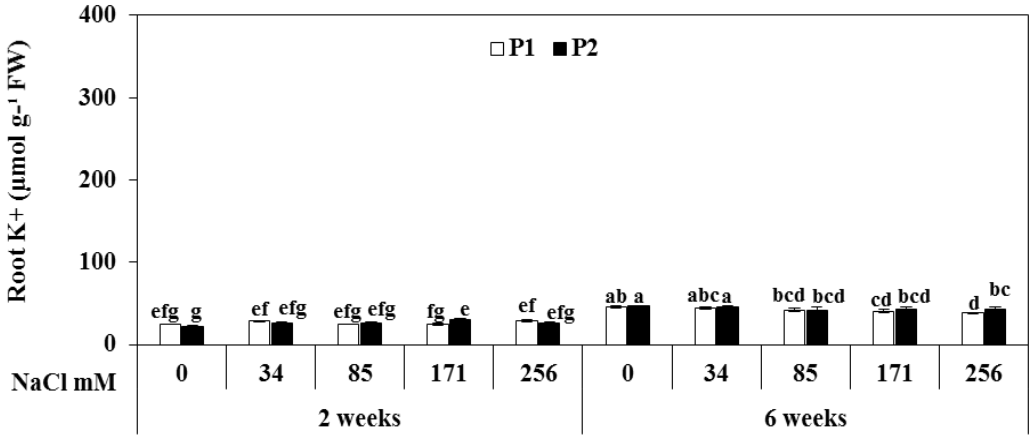
Supplementary Table 3: Electrical Conductivity of different treatments

Treatment	T0	T1	T2	T3	T4
NaCl concentration (mM)	0	34	85	171	256
Electrical Conductivity (ms) at 25°C	2.34	5.78	10.93	18.75	25

Supplementary Table 4. PCR primers used for real-time PCR

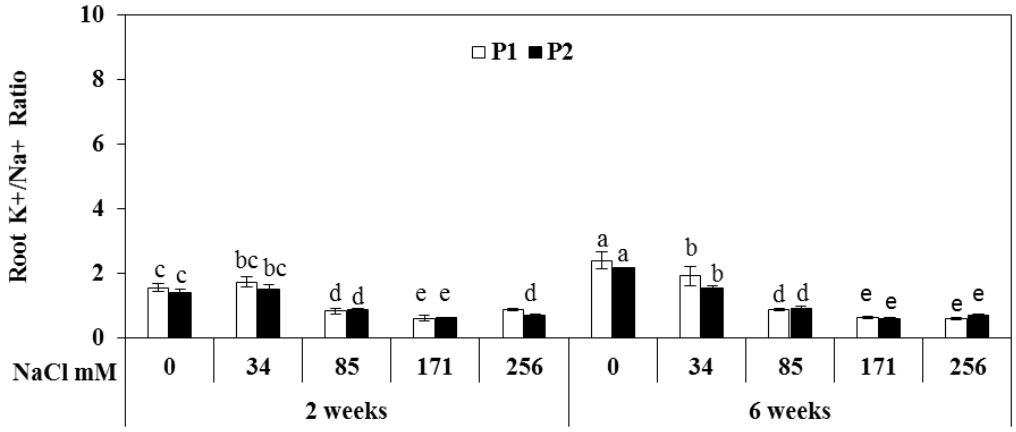
Oligo Name	Sequence (5´-3´)
CMOatriplexF	CGAACCTGCCTTCTATGCTC
CMOatriplexR	AAGGGCATACGAAACAYGAC
Na-HF	GATGTGGGAAACGGAAACC
Na-HR	CAAATTGTTGGTGCTTTGTT
Mt 18S-F	TGACGGAGAATTAGGGTTCG

Supplementary Figure 1



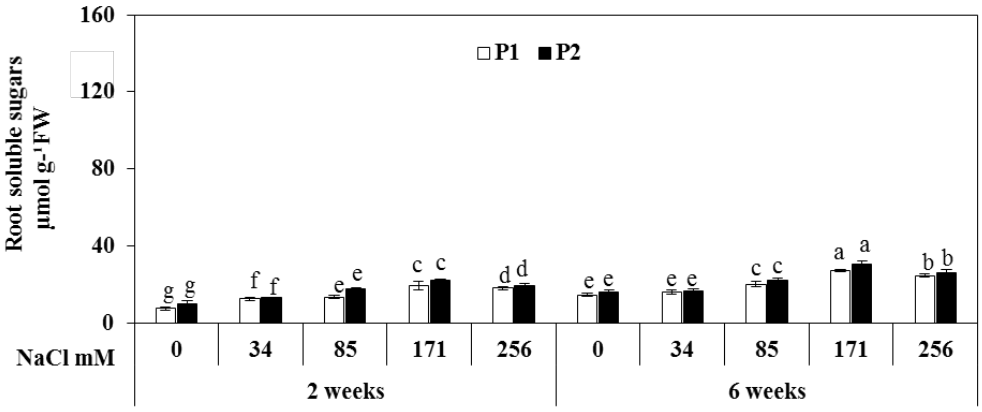
K+ accumulation in roots of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions over time and across salt stress treatments (mean ± S.D; .; n = 3 ; different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples.

Supplementary Figure 2



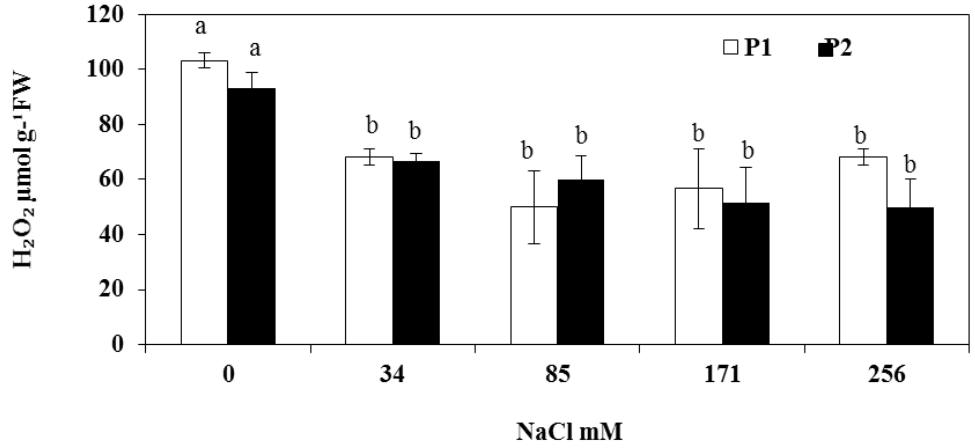
K+/Na+ ratio in roots of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions. Over time and across salt stress treatments (mean ± S.D; n = 3; different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples.

Supplementary Figure 3



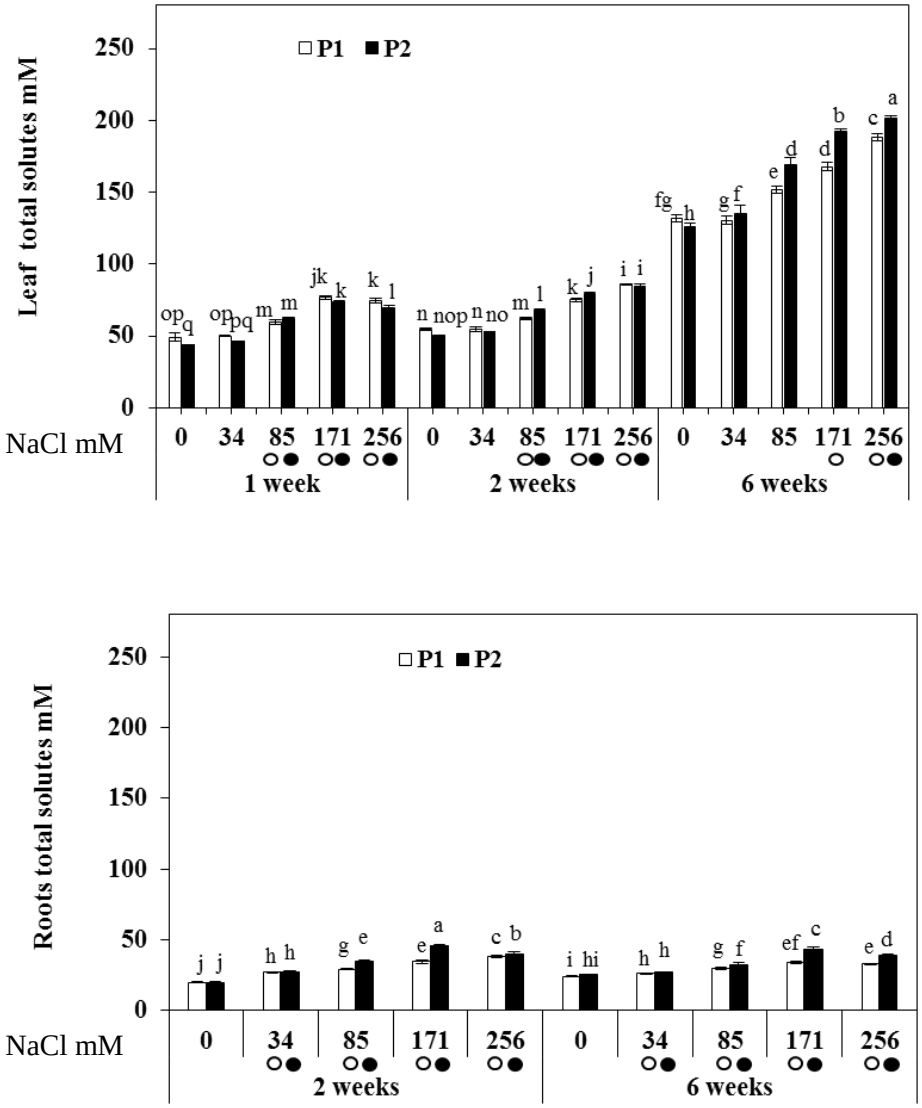
Soluble sugar concentration in roots of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions over time and across salt stress treatments (mean ± S.D; n = 3 ; different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples.

Supplementary Figure 4



H_2O_2 concentration in leaves of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions after 6 weeks of salt stress (mean ± S.D; n = 3 ; different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples.

Supplementary Figure 5.



Total internal solutes (expressed in mM) in leaves (A) and roots (B) of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions over time against total external solutes comprising NaCl (0, 34, 85, 171, 256 mM) and total nutrient solutes of 15.8 mM (mean ± S.D; n = 3; different letters above the bars indicate significant differences based on a Newman- Keuls test ($P < 0.05$) across all samples. Open or closed circles indicate that the internal solute concentration is below the external concentration for P1 and P2 respectively.