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1 **ORIGINAL FULL PAPER**

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

5

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19 **Abridged title:** Effects of salinity on *Atriplex halimus*

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28 **Abstract**

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

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

51 **Introduction**

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

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

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

80 cell vacuole and controlling cellular K^+/Na^+ ratio through a family of Na^+/H^+
81 antiporters (Flowers and Colmer 2008). NHX Na^+/H^+ antiporter genes have
82 been isolated from several *Atriplex* species including *A. halimus*, and at least
83 in *A. gmelini*, the antiporter localises to the tonoplast membrane (Hamada *et al.*
84 *2001*). In *A. gmelini* the *AgNHX* transporter gene was rapidly up-regulated
85 by salt treatments of 100-400 mM NaCl in both roots and leaves although
86 expression was much higher in leaves (Hamada *et al.* 2001).

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

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

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

121 The degree of tolerance and mechanisms for resisting salt stress varies within
122 and amongst plant species. For example *Atriplex halimus* plants originating
123 from coastal saline sites were more tolerant of high salinity and produced
124 higher levels of glycine betaine, whereas plants from a semi-arid non-saline
125 site were more tolerant to water-stress and produced more proline (Ben
126 Hassine *et al.* 2008). In other *A. halimus* populations, (Bouchenak *et al.* 2012)
127 plants from a more saline origin did contain more proline as well as
128 quaternary ammonium compounds. However, both these experiments were
129 performed on 4-6 week old plants over a relatively short 10-18 day treatment
130 period, therefore effects on early plant growth were not studied. Many studies
131 on salt stress tolerance are performed by gradually increasing the salinity over
132 a period of time to enable the plants to adapt, study only germination, or treat
133 older plants that are already well-established. For example Boughalleb *et al.*
134 (2009) exposed *Atriplex halimus* to up to 800 mM NaCl but this stress was
135 imposed in increments of 100 mM NaCl at 2 day intervals until the maximum
136 salinity concentration tested was reached. We were therefore interested to
137 know how very young plants respond to a sudden increase in salinity and
138 whether mechanisms differ between populations derived from areas differing
139 in salinity. Using a single-step up approach, we exposed young plants directly

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

146 **Materials and methods**

147 *Plant material and growth*

148 *Atriplex halimus* seeds were from wild plants growing in two distinct regions
149 of Algeria. Population 1 (steppe) is from the Algerian steppe, a semi-arid area
150 located in Northern Algeria (Chott Zahrez in the province of Djelfa, 3°03'E
151 longitude, 34°36'N latitude). The geology in this area is mainly cretaceous,
152 with some quaternary deposits. Soil salinity is between 1.99 and 4.47 dSm⁻¹
153 depending on the season, at a depth of 15-20 cm (Nedjimi 2012),
154 corresponding to the rooting zone of *A. halimus*. Soil texture encompasses
155 silt-clays and silt-sands (Pouget 1973) and the water table is between 1-3 m
156 below the soil surface. In this region groundwater is in the form of semi-
157 captive and unconfined aquifers, surrounded by the presence of a more or less
158 saline and unequally deep groundwater that contributes to the formation of
159 saline soils (Pouget 1973). Chott Zahrez is essentially Mediterranean, with
160 wet winters and hot dry summers (the minimum average is 5°C in January
161 and average maximum is 26 °C in July) and a mean annual precipitation of
162 250 mm year⁻¹ (Nedjimi *et al.* 2012). Population 2 (coastal) is from the
163 Algerian coastline also in Northern Algeria (in the province of Tipasa, 36° 35'
164 22" N, 2° 26' 50" E), in a sub-humid area with an average annual rainfall of
165 600 mm (1978-2004) (Boudjelal 2007). Temperatures are mild with an annual
166 average of 17-18 ° C (absolute minimum on record of -2 ° C). This area is
167 characterized by sedimentary cliffs and rocky areas (Grimes 2010) with a
168 salinity of 9 dSm⁻¹ (Tifour 2000). The plants are also subjected to frequent sea

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

171 Seeds (15-20 per pot, ten pots per treatment) were sown in washed and dried
172 medium coarse sand irrigated with distilled water, and grown in a Phytotron
173 at a constant 25°C , with 16:8 hours light: dark at $90 \mu\text{mol m}^{-2}\text{s}^{-1}$ from warm
174 white fluorescent tubes and 40 % relative humidity, until cotyledons
175 appeared. Then irrigation continued with a nutrient solution (pH 5.6; Morard
176 1995; Supplementary Tables 1 and 2). Salt stress was applied just after the
177 appearance of the first leaf pair, 10 days after sowing (NaCl concentrations:
178 0, 34, 85, 171 and 256 mM). Electrical conductivity was constant throughout
179 the experiment (Supplementary Table 3). Plants were grown for six weeks.
180 Leaves and roots for analysis were randomly selected from more than one
181 plant at each analysis time point and material was pooled into three biological
182 replicates; roots were used directly as there was no soil to wash off.

183 Percentage survival (for each of the 10 pots) was recorded after 6 weeks and
184 plant height over 6 weeks (for all surviving plants; average height per pot was
185 calculated). Relative growth rate (RGR) relating to plant height was
186 calculated from plant height data at 1, 2, and 6 weeks (Wang 2011). To
187 determine relative water content (RWC), leaf and root tissue was dried at
188 105°C to a constant dry weight. The relative water content was determined
189 by the relationship: $\text{RWC (\%)} = \text{FW} - \text{DW} / \text{FW} * 100$.

190

191 *Metabolite analyses and enzyme activity measurements*

192 Analyses of Na^+ , K^+ , proline and soluble sugars were carried out on fresh
193 leaves and roots (in triplicate) after 1, 2, and 6 weeks growth under salt stress.
194 For analysis of Na^+ and K^+ , samples were dried at 105°C for 1 h followed by
195 520°C for 2 h, digested in HNO_3 (0.5N) and assayed by flame photometry
196 (using a Cecil 6000 series spectrophotometer). Total chlorophyll was
197 extracted from fresh leaves with 80% acetone and absorption measured at 652

198 nm. Concentrations of chlorophyll were determined according to Plummer
199 (1989), then converted to $\mu\text{g/g}$ FW. Proline concentration was measured
200 spectrophotometrically at 528 nm according to Troll and Lindsley (1955)
201 from 100 mg of leaf tissue. Soluble sugars were analyzed using the anthrone
202 method (Plummer 1989) from 100 mg of fresh plant material. Absorbance
203 was read spectrophotometrically at 585 nm, and calibrated using a standard
204 curve. Glycine betaine was measured according to Grieve and Grattan (1983)
205 from 150 mg of fresh plant tissue in triplicate. Absorbance was measured at
206 365 nm using glycine betaine (Sigma Aldrich Poole, UK) as standard, and
207 expressed as mg g^{-1} DW. The concentration of total solutes in roots and leaves
208 over time was calculated by dividing the sum of K^+ , proline, soluble sugars
209 and glycine betaine concentrations by the amount of water present in the plant
210 tissue, based on the % water content.

211 Ascorbic acid was extracted by a freezing procedure (Nojavan *et al.* 2008).
212 from 100 mg of fresh tissue, in triplicate HPLC analysis was carried out using
213 an isocratic elution procedure with a UV Detector at 240 nm. Separation was
214 carried out on a $5\mu\text{m}$ RP C18 column of $250\text{ mm} \times 4.6\text{ mm}$ (Kinetex-
215 Phenomenex). The mobile phase consisted of 0.5% NaH_2PO_4 (pH 2.25 with
216 H_3PO_4) - acetonitrile (2% of final volume). An injection volume of $20\ \mu\text{L}$ was
217 used in quantitative analyses.

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

224

225 *RNA extraction and Real time PCR*

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

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

242 *Statistical analyses*

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

249

250 **Results**

251 *Seedling survival and chlorophyll content with increasing NaCl* 252 *concentration in coastal and steppe Atriplex halimus seedlings*

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

257 At 256 mM NaCl, coastal region (P2) seedlings survived significantly better
258 ($P < 0.05$) than steppe region (P1) seedlings (Fig. 1A). P2 seedlings were also
259 significantly taller than P1 at all time-points (Fig 1B) and grew significantly
260 faster in the first two weeks at NaCl concentrations >34 mM, with an RGR
261 that was significantly higher than the control at all salt concentrations while
262 the P1 RGR was reduced at the highest salt concentration but unaffected at
263 lower salinity (Fig. 1C). The RGR after 6 weeks (relative to 1 week) was
264 reduced equally in P1 and P2 with increasing salt. P2 seedlings also retained
265 significantly greater relative water content at all salt concentrations than P1
266 in both leaves and roots at all time-points (Fig. 2A, B).
267 Chlorophyll concentration rose significantly between week 1 and week 6 at
268 all salt concentrations ($P < 0.05$) and was significantly higher in no salt control
269 coastal plants (P2) compared to steppe (P1) especially after 6 weeks (Fig. 2C).
270 With increasing NaCl, chlorophyll concentration fell slightly at all time-
271 points, although remained $> 80\%$ of control even at the highest salt treatment
272 after 6 weeks.

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

275 For the first two weeks, Na^+ concentration increases were similar between P1
276 and P2 leaves (Fig. 3A). However, at each salt treatment at 34 mM - 171 mM,
277 Na^+ concentration was significantly higher ($P < 0.05$) in P2 leaves, while at
278 256 mM, there was no difference between them. After six weeks there was a
279 significantly greater concentration of Na^+ in all the salt treated seedlings
280 compared to the control, but P2 seedling leaves accumulated more Na^+ at all
281 concentrations of NaCl reaching a maximum of $(334.3 \pm 4.8) \mu\text{molg}^{-1}$ FW at
282 171 mM NaCl, and the highest differential in Na^+ between the two
283 populations.

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

286 P2 ($71.2 \pm 2.7 \mu\text{molg}^{-1}$ FW) after 6 weeks. After 6 weeks at 34 mM NaCl,
287 Na^+ concentration was higher in P2 roots than P1 roots, and higher than
288 control roots of either population ($P < 0.05$). At 85 mM there was significant
289 ($P < 0.05$) NaCl accumulation in both P1 and P2 seedling roots, both after 2
290 weeks and 6 weeks of treatment, but no significant difference between the
291 two populations. Na^+ was however higher in coastal (P2) seedling roots ($P <$
292 0.05) at 171 mM NaCl after both two and six weeks of treatment compared
293 to P1. At 256 mM NaCl, Na^+ was significantly higher in P2 than P1 roots after
294 2 weeks ($35.7 \pm 3.1 \mu\text{molg}^{-1}$ FW and $31.5 \pm 1.2 \mu\text{molg}^{-1}$ FW respectively),
295 but after 6 weeks this difference was abolished.

296 Leaf K^+ levels were not affected by the first two weeks of salt treatment (Fig.
297 3C). However, after 6 weeks, K^+ concentration was almost four-fold higher
298 and was significantly greater ($P < 0.05$) in coastal P2 leaves compared to P1
299 in all but the highest NaCl treatment. In roots, K^+ levels showed few changes
300 between P2 and P1 or between salt concentrations at 2 or 6 weeks
301 (Supplementary Figure 1).

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

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

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

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

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

332 The pattern was similar in roots (Fig. 5B) although proline concentration
333 remained more similar over time with less than a 2 fold difference in maximal
334 accumulation, and was much lower than in leaves. Again, proline rose
335 between 34-171 mM NaCl in both populations and at both time-points
336 compared to the control, and P2 roots accumulated significantly higher levels
337 of proline than P1 roots at >34 mM NaCl. However, P2 roots accumulated
338 significantly less proline than P1 at 256 mM NaCl at both week 2 and week
339 6.

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

343 256 mM NaCl. Soluble sugar levels were significantly higher in P2 compared
344 to P1 leaves at all salt concentrations after 2 weeks. In roots the pattern was
345 similar but there were no significant differences at any salt treatment or time
346 point between the two populations although there was a rise in soluble sugars
347 with increasing salt concentration from 0- 171 mM at both time points and
348 then a fall at 256 mM NaCl (Supplementary Figure 3).

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

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

363 *Antioxidant capacity*

364 H₂O₂ concentration was lower at all salt concentrations compared to the
365 control, but there was no significant difference between the two populations
366 (Supplementary Figure 4). In leaves of both populations, ascorbic acid
367 increased linearly with NaCl from 34-256 mM (Fig. 7A). At each salt
368 concentration P2 accumulated significantly more ($P < 0.05$) ascorbic acid
369 than P1 leaves, and the rate of accumulation was also significantly faster.
370 Catalase activity was also induced by salt in both P1 and P2 leaves (Fig. 7B),

371 however in P1, activity was only greater than control at ≥ 170 mM NaCl. In
372 contrast catalase increased linearly in P2 leaves from 85-256 mM NaCl ($R^2 =$
373 0.994).

374

375 **Discussion**

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

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

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

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

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

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

435 In both populations Na^+ concentration increased in both leaves and roots in
436 response to the saline treatments, but the level of Na^+ in the leaves was almost
437 10 fold higher than in roots, suggesting that in these populations salt is being
438 accumulated rather than excluded in the leaves as was previously found in
439 some *A. halimus* populations (Belkheiri and Mulas 2013). As found by Ben
440 Hassine *et al.* (2008) the coastal population accumulated significantly more
441 Na^+ . However after two weeks at 256 mM NaCl, the differential between the
442 two populations was lost, suggesting a threshold level between 171 and 256
443 mM NaCl for salt accumulation in the both populations. Notably, after 6
444 weeks, the differential was restored, suggesting that at a later stage of
445 development (as indicated by increasing chlorophyll levels throughout the
446 experiment) additional mechanisms for Na^+ accumulation may become
447 available. The greater inducibility of the Na^+/H^+ antiporter gene at 6 weeks in
448 the coastal (P2) leaves and roots, which is particularly dramatic in leaves at
449 171 mM NaCl and in roots at 256 mM NaCl, may be a factor in the higher
450 Na^+ accumulation in the coastal plants. Roots of the P2 plants were still able
451 to up- regulate the Na^+/H^+ antiporter gene expression at the highest salt level,
452 while the steppe region plants were not. Bajji *et al.* (1998) found that roots
453 responded less than leaves in this species to high salt concentrations but was
454 unable to explain this effect mechanistically. Here results suggest that this
455 effect might be due at least in part to a greater inducibility of the Na^+/H^+
456 antiporter in roots at high salt concentrations compared to leaves, thus
457 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

487 populations studied here support a role for both proline and glycine betaine
488 in salt tolerance in *A. halimus* and highlight a difference between leaves and
489 roots and across time. Notably after 6 weeks >2-fold more of proline
490 accumulated compared to earlier time points at all levels of salt treatment and
491 in both plant populations. This agrees with Martinez *et al.* (2005) where older
492 leaves accumulated more proline in response to salt treatment than young
493 leaves. Even after 2 weeks, however, coastal population leaves here
494 accumulated significantly more proline than the steppe leaves even at a lower
495 salt concentration (85 mM) than that tested by Ben Hassine *et al.* (2008). At
496 171 and 256 mM the enhanced proline accumulation by the coastal population
497 P2 after six weeks was striking. In roots the pattern was different: although at
498 85 and 171 mM NaCl which span the 160 mM NaCl used by Ben Hassine *et al.*
499 *et al.* (2008) the coastal plants accumulated significantly more proline in roots
500 than the steppe plants, the ratio was indeed reversed at 256 mM NaCl. Thus
501 it would seem that after six weeks at high salt concentrations both proline and
502 glycine betaine accumulation in leaves are important for salt tolerance of
503 coastal population plants, while in roots the glycine betaine accumulation
504 may be more important.

505 A third type of osmolite, soluble sugars, also appears to be involved in the
506 protective mechanisms of both populations. Accumulation of soluble sugars
507 in leaves but not roots may be relevant to longer term salt tolerance of the
508 coastal population since accumulation was more highly induced in this
509 population after 6 weeks at all salt concentrations. This contrasts with a
510 previous study on *A. halimus* where there was no difference in sugar
511 accumulation between saline and non-saline environment derived populations
512 (Bouchenak *et al.* 2012). In contrast also to Bajji *et al.* (1998) and Martinez
513 *et al.* (2005), here soluble sugars were induced by low salt concentrations (<
514 50 mM) in leaves after two weeks as well as at higher concentration as
515 reported before (Bajji *et al.* 1998; Boughalleb *et al.* 2010; Bouchenak *et al.*

516 2012), suggesting that leaf soluble sugars may be more relevant as a
517 protective mechanism at low salinity in the very young leaves and the
518 different populations studied here. In agreement with Bajji *et al.* (1998)
519 though, root soluble sugars increased with increasing external salt
520 concentrations and then fell back or remained constant. Here the upper limit
521 was 171 mM NaCl whereas for Bajji *et al.* (1998) it was 300 mM NaCl again
522 suggesting differences between the plants tested and growth stage. The fall in
523 soluble sugar levels is interpreted by Bajji *et al.* (1998) as an inhibition of
524 phloem transport which would also inhibit transport of glycine betaine from
525 the leaves, thought to be via the phloem (Chen and Murata 2011). However,
526 the continued increase in glycine betaine concentration even at 256 mM NaCl
527 together with the up-regulation of the CMO gene expression in roots, suggests
528 that at least some of the glycine betaine may be synthesised directly in the
529 roots rather than translocated.

530 A comparison of the total concentration of internal solutes with the external
531 solute concentration (Supplementary Fig. 5) indicates that at 1-2 weeks
532 osmotic adjustment in leaves may have occurred in the 34 mM NaCl when
533 the 15.8 mM of nutrient solutes is included in the calculation. However, after
534 6 weeks, leaves may be able to adjust osmotically up to the combined external
535 solute concentration of 15.8 mM from the population. In contrast, osmotic
536 adjustment does not appear to occur in roots at any concentration. This
537 difference between roots and leaves has been noted previously in *Atriplex*
538 *mummularia* (Silveira *et al.* 2009). However, the calculation here needs to be
539 interpreted with caution and may be a significant under-estimate. Although
540 the concentration of many of the major organic osmolytes normally
541 considered to be important for osmotic adjustment (proline, soluble sugars
542 and glycine betaine) as well as K⁺ (Singh *et al.* 2015) have been included,
543 other cellular solutes such as other amino acids and ions will contribute to the
544 internal solute concentration and hence may alter the threshold.

545 The very similar changes in H₂O₂ concentration in the two populations under
546 salt treatment, and the reduction in H₂O₂ compared to the control no-salt
547 treatment suggests that the antioxidant mechanisms are not being
548 compromised at the NaCl concentrations tested here, consistent with other
549 studies (e.g. Boughalleb *et al.* (2010). A greater activation of antioxidant
550 mechanisms may be a component of differential salt tolerance mechanisms of
551 the two populations under high salt treatment since there was a significantly
552 higher ascorbic acid concentration in coastal P2 leaves compared to steppe
553 P1 leaves at all salt concentrations. The differences in the catalase activity
554 between the two populations were most evident at 85 mM NaCl suggesting
555 that at this intermediate salt concentration catalase plays a more important
556 differential role between the two populations. In contrast to previous work
557 showing no increase (Boughalleb *et al.* 2010; *A. halimus*) or a reduction (Sai
558 Kachout *et al.* 2013; *A. hortensis*) in catalase activity in response to salt, here
559 there was a small increase from 85–256 mM external NaCl. In the steppe
560 population catalase activity rose between 85 and 171 mM treatments but did
561 not rise further at 256 mM whereas in the coastal population activity rose up
562 to 256 mM external salt. This higher catalase activity in the coastal population
563 is in broad agreement with Bouchenak *et al.* (2012). However, here there was
564 a small but significant induction of the catalase activity by all salinity
565 treatments of \geq 85 mM NaCl in both populations whereas in the populations
566 described in Bouchenak *et al.* (2012) catalase activity dropped in the non-
567 saline population. Differences may also again be related to the age of the
568 plants indicating that in young plants catalase plays a more important role in
569 protection against the salt-induced ROS changes. Note also that in addition to
570 ROS scavenging enzymes and non-enzymatic antioxidants, soluble sugars
571 can also play an antioxidant role against ROS under biotic and abiotic stress
572 (Keunen *et al.* 2013), acting in concert with other protective mechanisms.
573 Hence the increase in soluble sugars seen here in both populations, and the
574 relatively higher accumulation in the coastal population may be contributing

575 to maintain ROS homeostasis under salinity stress.

576

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

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FIGURES

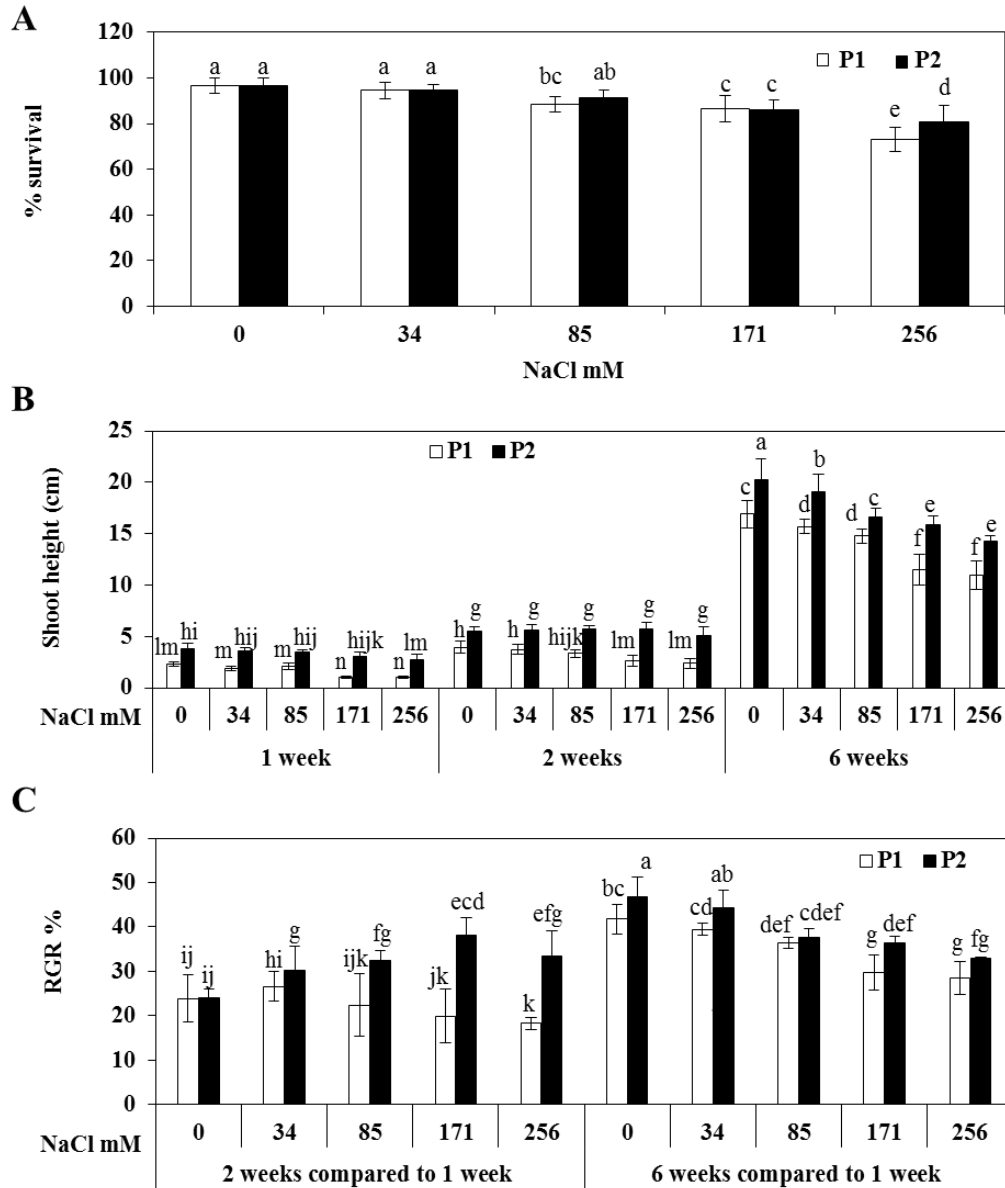


Fig. 1. Mean percentage survival per pot (A), shoot height (B) and relative growth rate (C) of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions after 6 weeks of salt stress (A); and over time (B, C). Mean \pm S.D; different letters above the bars indicate significant differences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

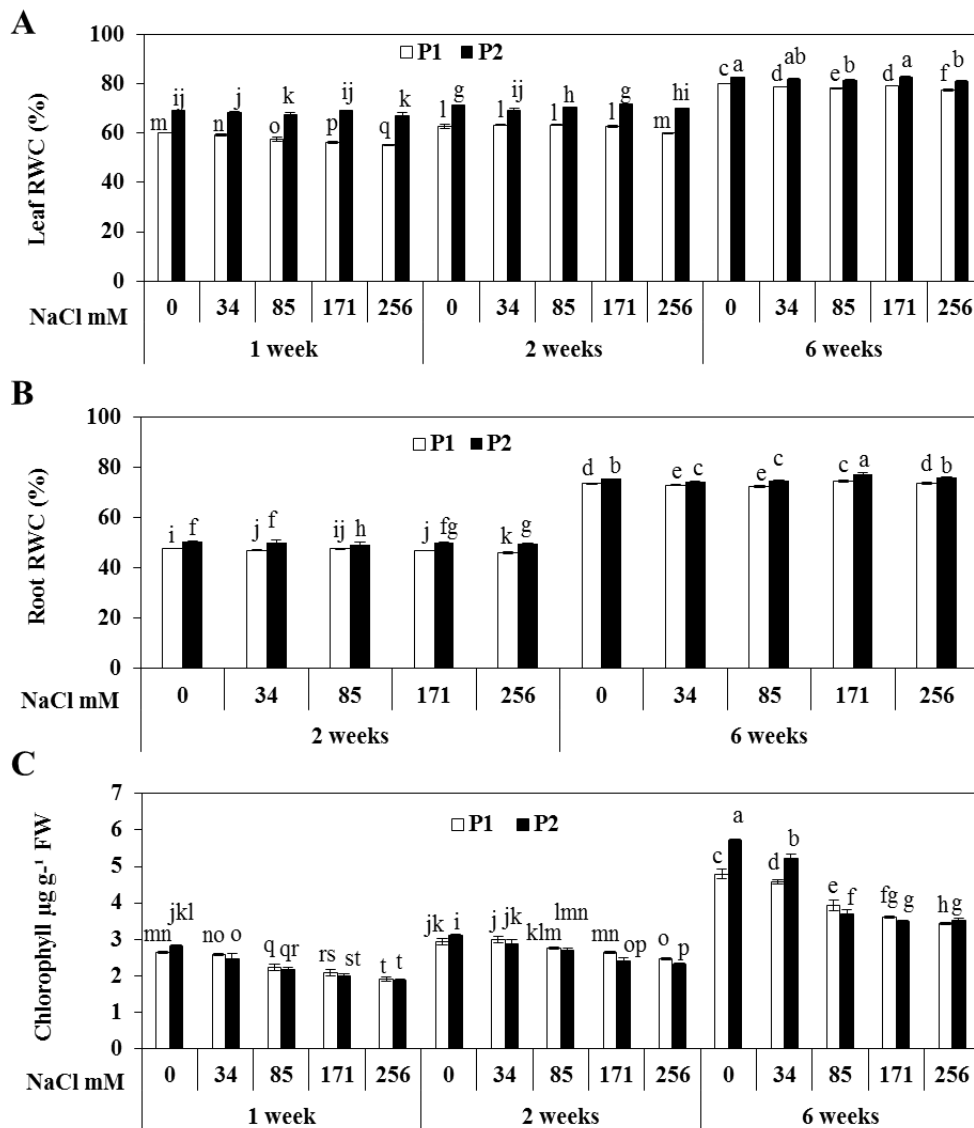


Fig. 2. Relative water content (RWC) in leaves (A), roots (B) and chlorophyll concentration in leaves (C) of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions over time and across salt stress treatments (mean \pm S.D; $n = 3$; different letters above the bars indicate significant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

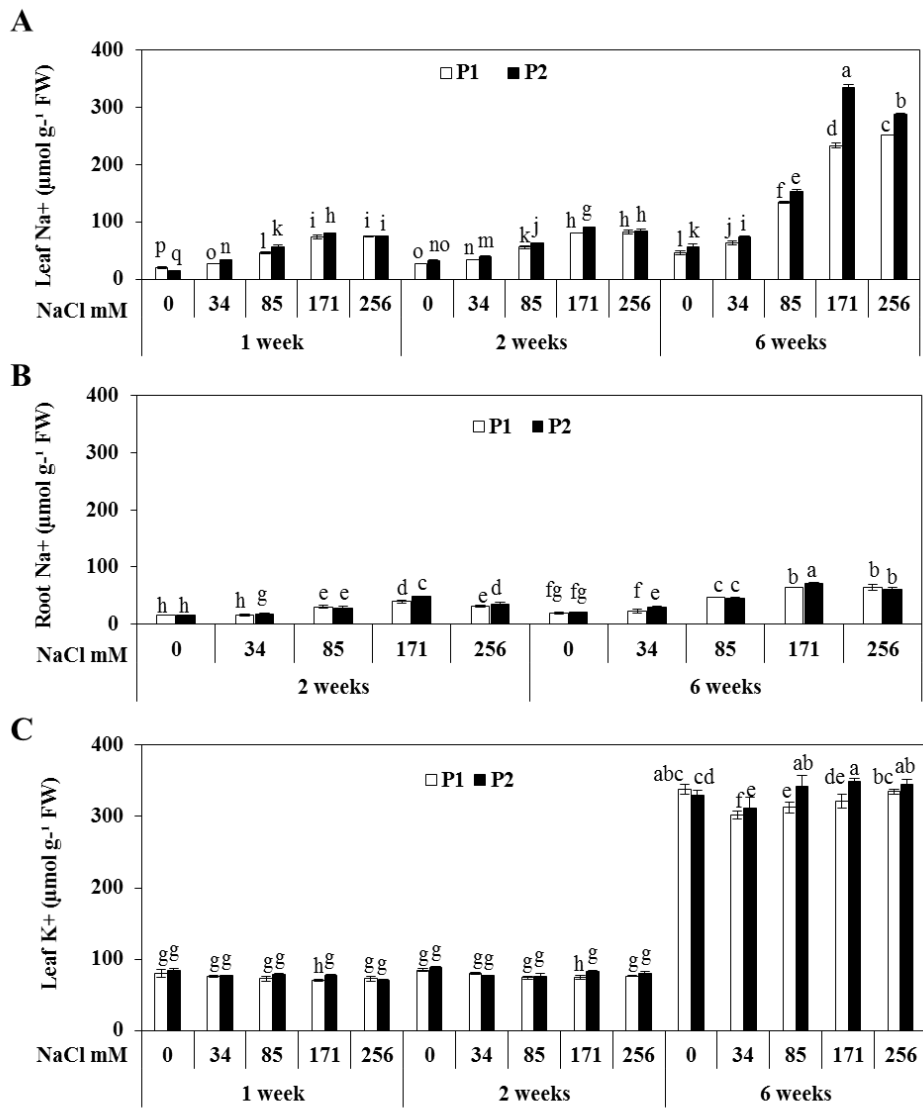


Fig. 3. Na⁺ accumulation in leaves (A) and roots (B); K⁺ accumulation in leaves (C) over time in *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions across salt stress treatments (mean ± S.D; n = 3; different letters above the bars indicate significant differences based on a Tukey's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

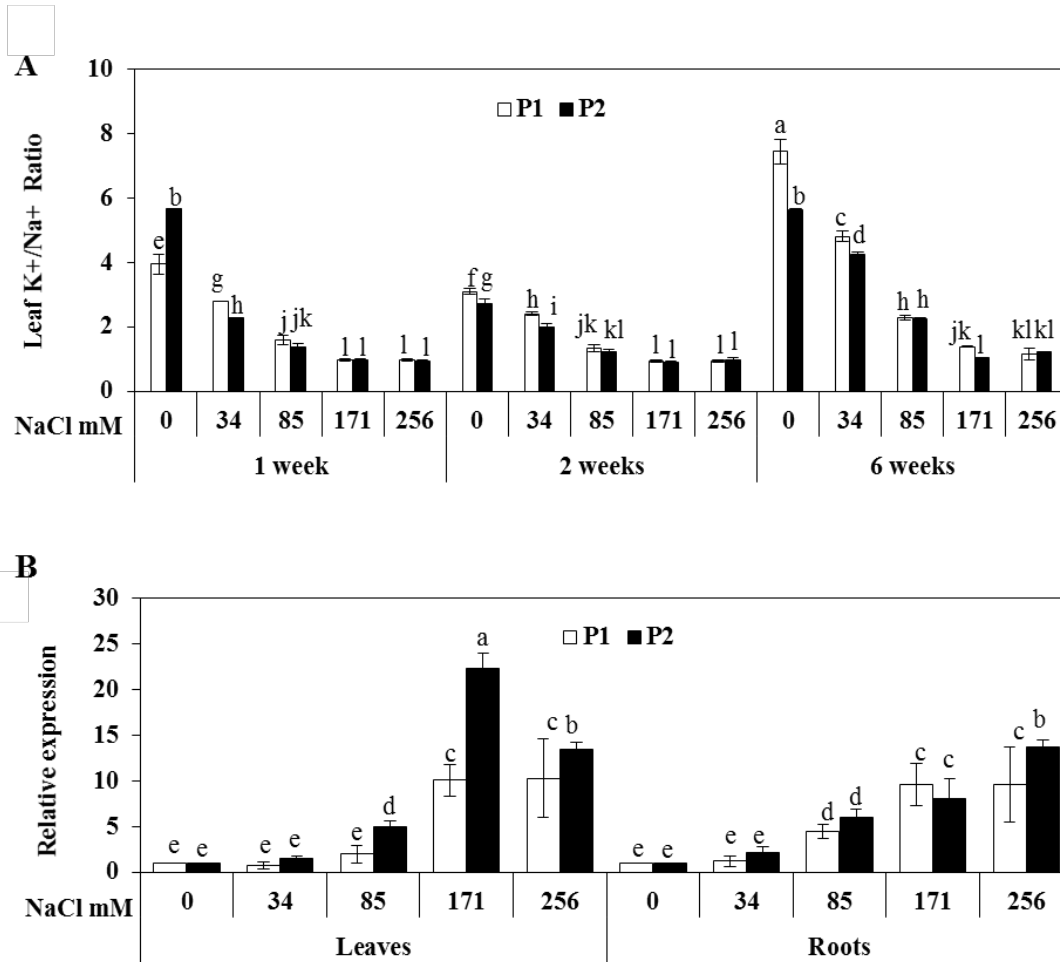


Fig. 4. K⁺/Na⁺ ratio in leaves (A) over time and relative Na⁺/H⁺ antiporter gene expression after 6 weeks compared to the no salt control (B) of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions across salt stress treatments (mean ± S.D; n = 3 (A); n = 6 (B); different letters above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

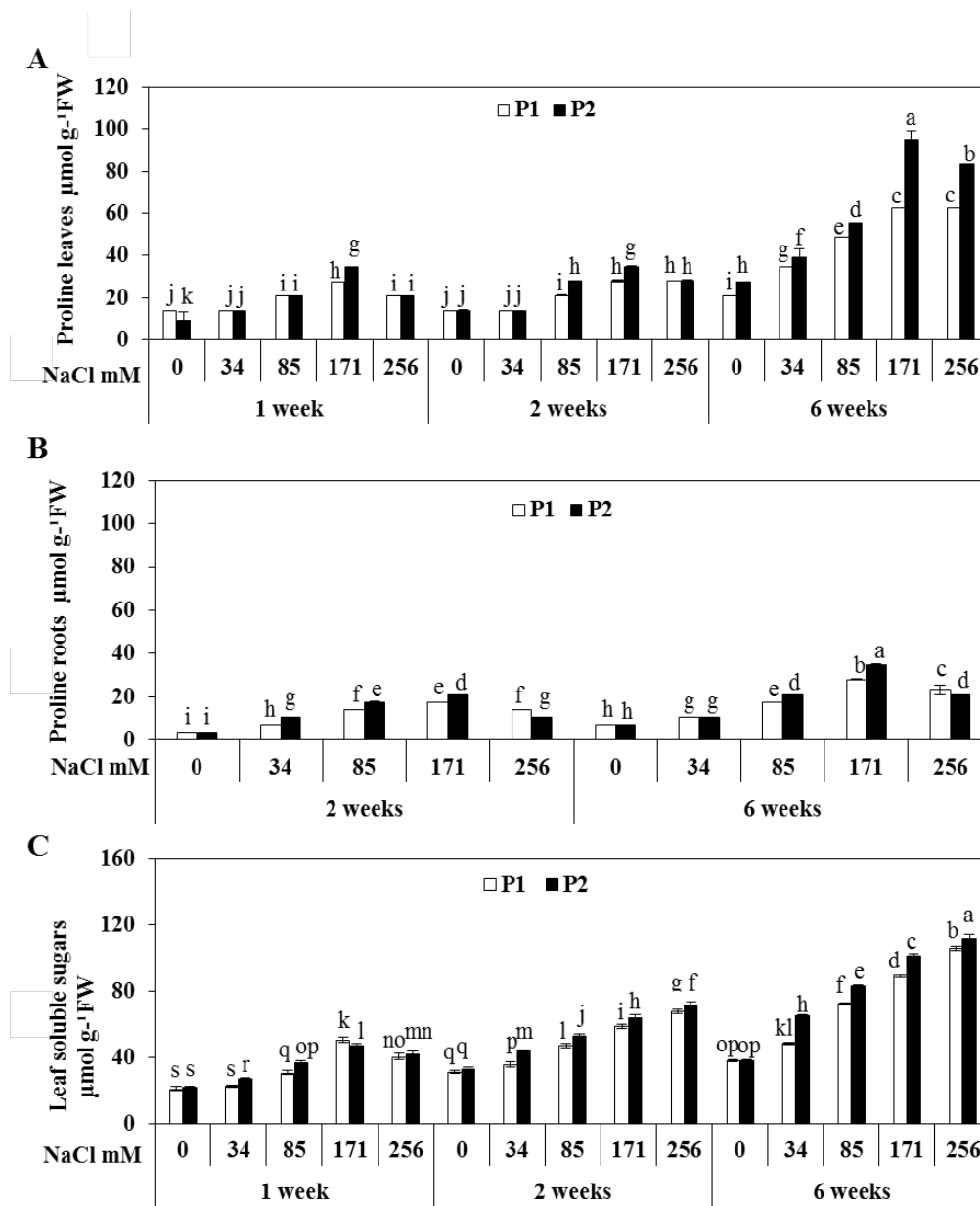


Fig. 5. Proline concentration in leaves (A) and roots (B); soluble sugar concentration in leaves (C) of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions over time and across salt stress treatments (mean \pm S.D; n = 3 ; different letters above the bars indicate significant differences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

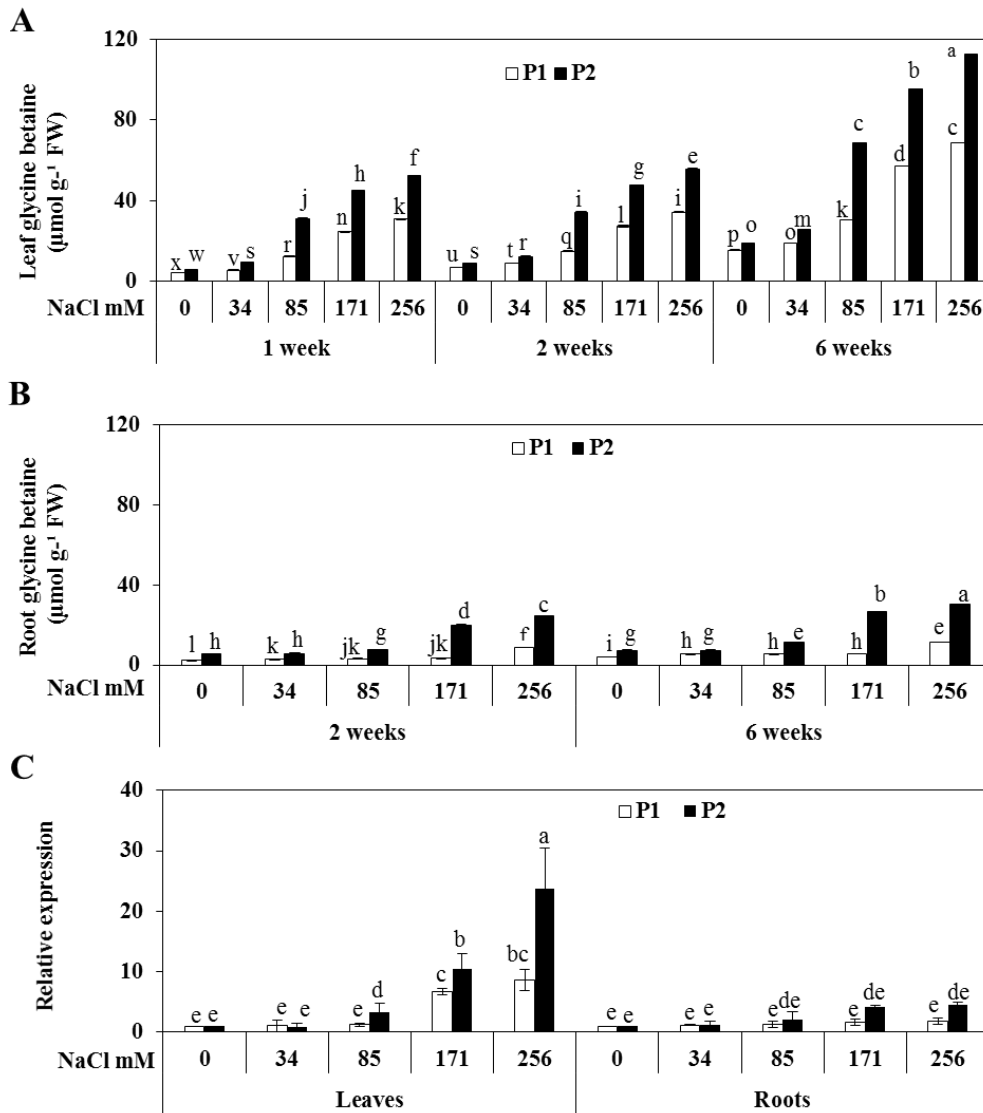


Fig. 6. Glycine betaine concentration in leaves (A), roots (B) and CMO gene expression relative to the no salt control (C) in *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions over time and across salt stress treatments (mean \pm S.D; n = 3 (A,B); n = 6 (C)); different letters above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

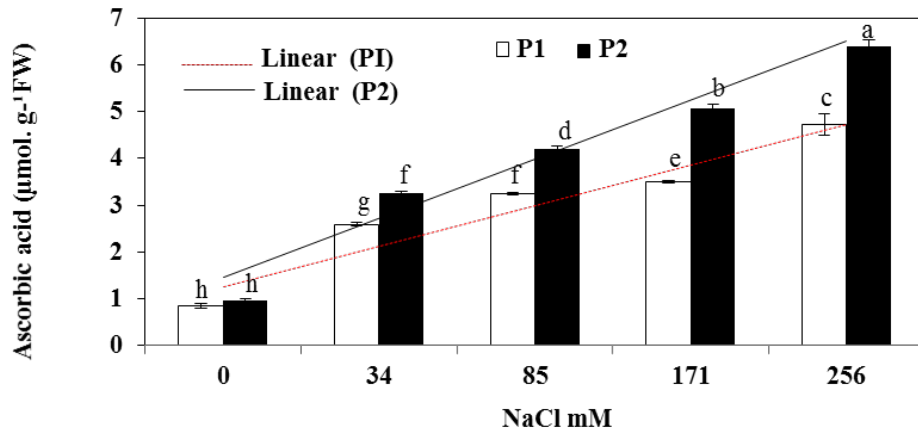
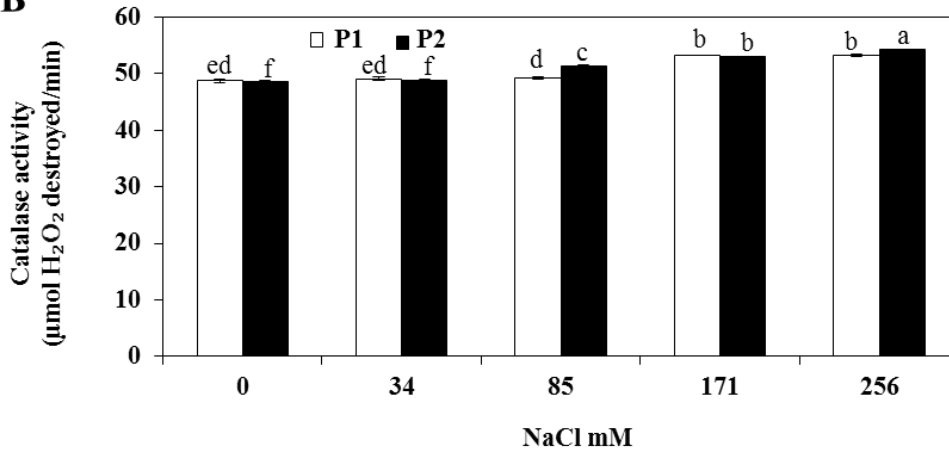
A**B**

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 \pm 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)

	K	Ca	Mg	Na	N	P	S	Cl
Macroelements	K ⁺	Ca ⁺⁺	Mg ⁺⁺	Na ⁺	NO ₃ ⁻	H ₂ PO ₄ ⁻	SO ₄ ⁻	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

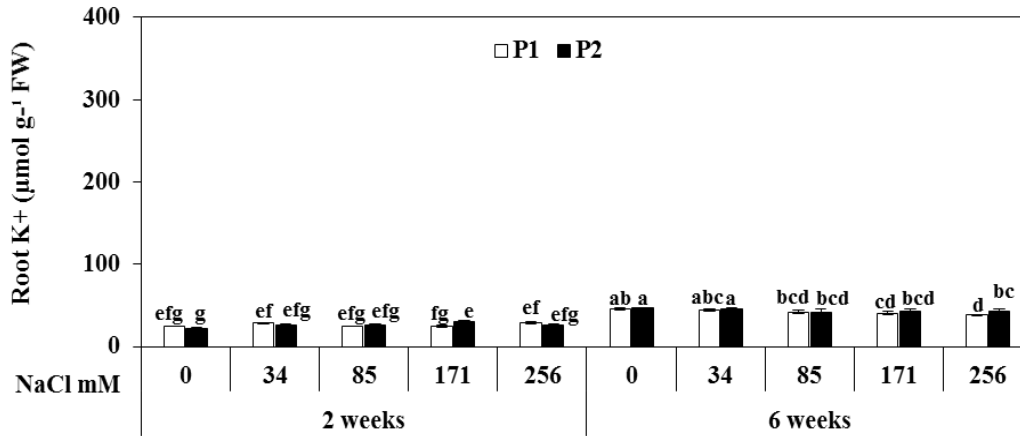
Supplementary Table 3: Electrical Conductivity of different treatments

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

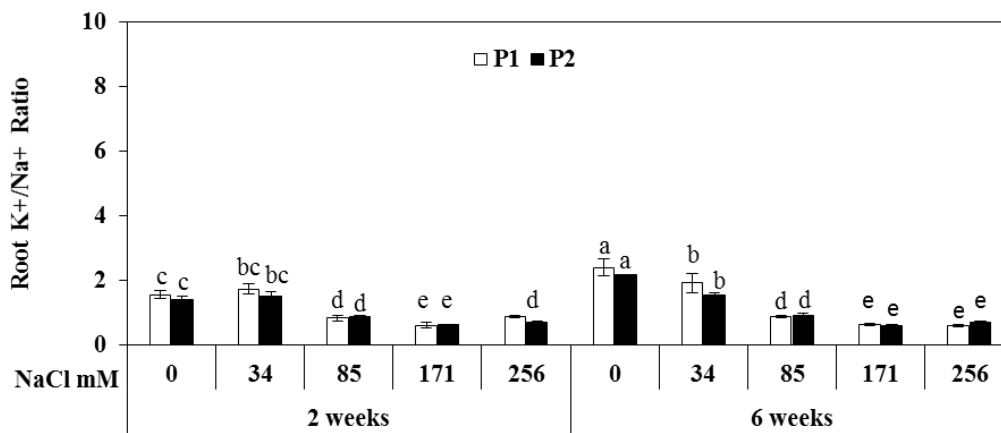
Oligo Name	Sequence (5'-3')
CMOatriplexF	CGAACCTGCCTTCTATGCTC
CMOatriplexR	AAGGGCATACGAAACAYGAC
Na-HF	GATGTGGGAAACGGAAACC
Na-HR	CAAATTGTTGGTGCTTTGTT
Mt 18S-F	TGACGGAGAATTAGGGTTCG

Supplementary Figure 1



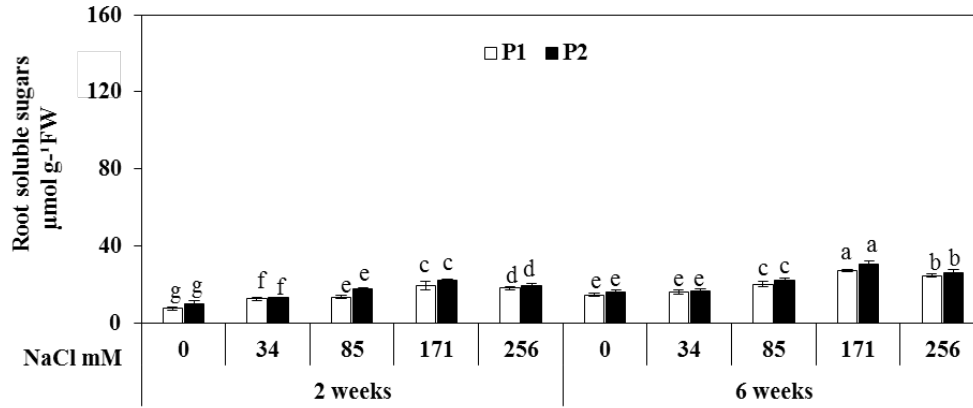
K⁺ accumulation in roots of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions over time and across salt stress treatments (mean ± S.D; .; n = 3 ; different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples).

Supplementary Figure 2



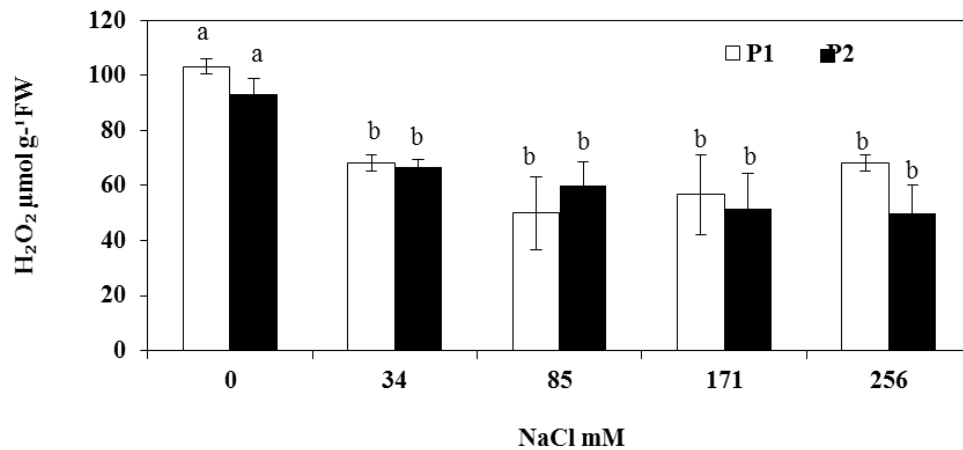
K⁺/Na⁺ ratio in roots of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions. Over time and across salt stress treatments (mean ± S.D; n = 3; different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples).

Supplementary Figure 3



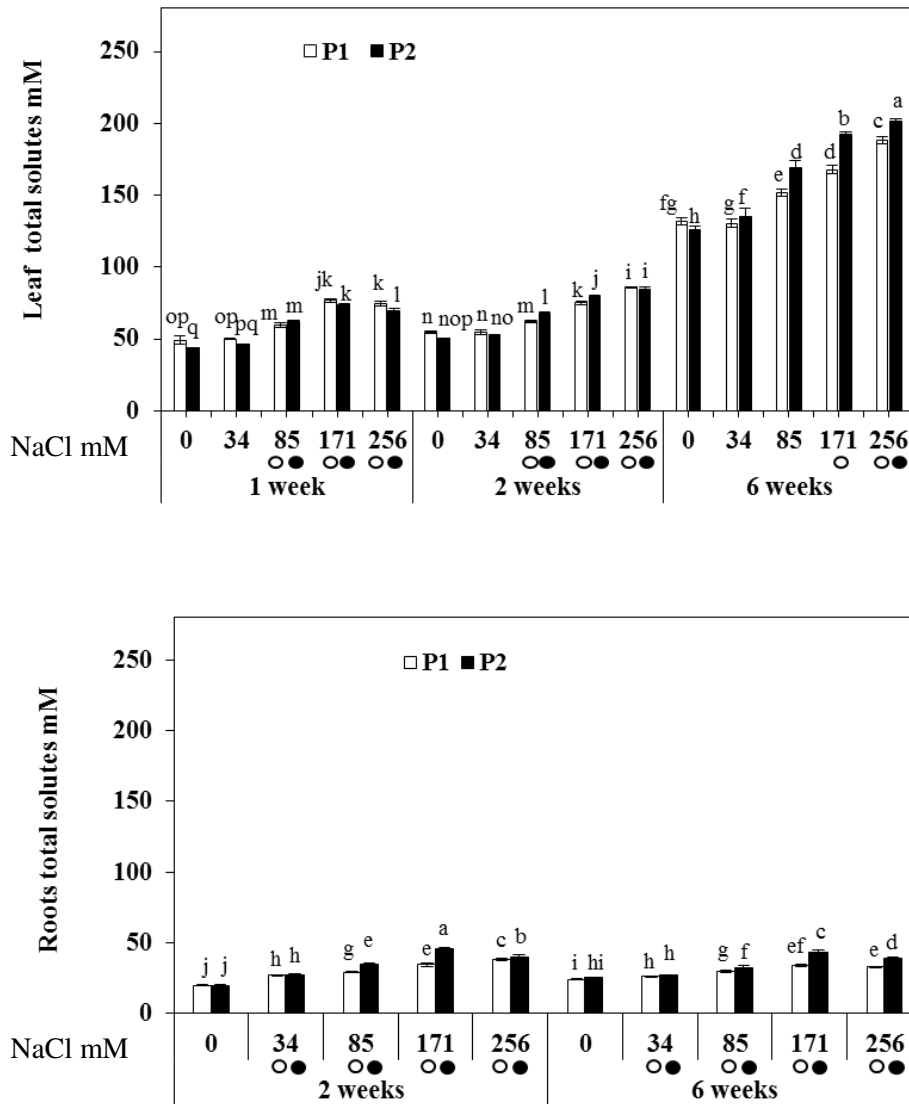
Soluble sugar concentration in roots of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions over time and across salt stress treatments (mean \pm S.D; $n = 3$; different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples.

Supplementary Figure 4



H₂O₂ concentration in leaves of *Atriplex halimus* seedlings from Steppe (P1) and coastal (P2) regions after 6 weeks of salt stress (mean \pm 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 \pm 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.