Diverse salinity responses in *Crithmum maritimum* tissues at different salinities over time

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Abstract

Crithmum maritimum (sea fennel) withstands high salinity, and to better understand how different protective mechanisms against salinity are activated, young seedlings were exposed to increasing concentrations of NaCl (0 to 512 mM) over six weeks. Plant survival and chlorophyll content were reduced at >85 mM NaCl and growth was affected at ≥ 341 mM NaCl. Relative water content fell and Na^+ accumulated more in leaves than in roots. Induction of Na^+/H^+ antiporter expression reached a maximum at 427 mM NaCl in both tissues. Salinity induced the accumulation of proline, soluble sugars and glycine betaine. All three accumulated to higher levels in leaves than roots and greatest accumulation was after 6 weeks and the highest salt concentrations. Hydrogen peroxide levels fell with increasing salinity in leaves, while ascorbic acid and catalase activity rose. Overall, the most dramatic changes occurred after six weeks of saline stress but different mechanisms were activated at different salinity thresholds and in the two tissues. Key salinity thresholds in the response of *Crithmum maritimum* to salinity stress are identified activating different mechanisms. At 85 mM NaCl roots reach osmotic adjustment, at 171 mM further osmolyte protection mechanisms are activated, at 256 mM NaCl leaves reach osmotic adjustment, at 341 mM plant growth is affected and at the highest salinity tested, 512 mM, protective mechanisms are affected in leaves but not in roots.

Keywords: *Crithmum maritimum* L., halophyte, Na^+/K^+ antiporter, reactive oxygen species, salt stress.

1. Introduction

Over 6% of land and one third of world-wide irrigated land are considered saline (FAO, 2008). Algeria is no exception: long periods of dryness have resulted in outward signs of soil salinization affecting 3.2 million hectares (Szabolcs, 1989). Faced with the prospect of further dryness due to climate change, urgent measures are being taken to identify, select and introduce species adapted to local conditions. *Crithmum maritimum* L. (sea fennel) is a halophyte typical of coastal ecosystems that shows high salt tolerance during its vegetative growth (Ben Amor *et al.*, 2005). In fact it has been suggested that it may be of value as an oil seed crop in semi-arid saline soils where other oil crops cannot be grown (Atia *et al.*, 2010). Furthermore, *Crithmum maritimum* L. is an aromatic plant that is rich in antioxidant compounds and is already used in the Mediterranean diet (Siracusa *et al.*, 2011).

Salt stress results in water deficit, which in turn results in osmotic stress and ionic imbalance (Flowers *et al*., 2015). At the cellular level one of the major effects of salinity is an accumulation of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot -}$) and hydroxyl radical ($OH^{\cdot}$) which are damaging to cellular macromolecules (Hossain and Dietz, 2016). These cellular changes produce adverse effects on plant growth and developmentin salt-sensitive plants (e.g. in rice; Mahdieh *et al.*, 2015). Several mechanisms contribute to halophyte salt tolerance. One mechanism involves sequestering salts in the vacuole to control the cellular K^+/Na^+ ratio through a family of Na^+/H^+ antiporters (Flowers and Colmer, 2008). Many plants also accumulate organic osmolytes in their cytoplasm (Negrão *et al*., 2017; Dong *et al.*, 2015). These increase cellular water retention without affecting normal metabolic processes and include soluble sugars, proline, and glycine betaine.

Proline has a protective effect on membranes, proteins and enzymes against damage from a range of abiotic stresses (Ashraf and Foolad, 2007). Glycine betaine is a quaternary ammonium compound that protects the photosynthetic machinery. It does this by accelerating the recovery of PSII from photoinactivation (Hölmstrom *et al.*, 2000). Furthermore, it may directly or indirectly induce H_2O_2-mediated signalling that increase both the expression and the activity of antioxidant enzymes (Gill and Tuteja, 2010). Plants have evolved both non-enzymatic and enzymatic antioxidative defence mechanisms in order to mitigate the effects of increased ROS during salinity stress. Ascorbic acid is a primary substrate in the cyclic enzymatic pathway that detoxifies hydrogen peroxide (Akram *et al*., 2017). The enzymatic defence system consists of a battery of enzymes that interconvert ROS moieties into the less

damaging H_2O_2, and then remove it. These include superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Hossain and Dietz, 2016). Catalases (CAT) degrade the H_2O_2 by its dismutation to water and oxygen.

Given the interplay of these multiple detoxification and tolerance mechanisms, the current study aimed to resolve the deployment of these strategies in an Algerian population of the halophyte *Crithmum maritimum.* As salt concentrations were ramped up over a six week treatment period, deployment of different strategies came into play indicating a complex regulation of salt tolerance in this species.

2. Materials and methods

2.1. Plant material and growth

Crithmum maritimum seeds were collected from the Northern Algerian coastline (province of Tipaza: 36° 35' 22" N, 2° 26' 50" E). This area is characterized by an annual precipitation of 600 mm (1978-2004) (Boudjelal, 2007). The annual average temperature is around 18 ° C (1990-2005) with average temperatures around 11°C in winter (December-February) and 25°C in summer (June -August). Crithmum maritimum grows on rocky headlands and sandstone slabs from the Pleistocene and Early Pliocene overlooking the sea. Because of the exposure of this area to salty sea winds, the plants growing here are frequently subjected to sea water (55.38 dSm^{-1}; Khelifi et al., 2013).

Seeds were sown in pots (10 -15 seedlings per pot with six replicate pots) containing medium coarse sand which had been washed and then dried, acting as an inert support for the plants. Following sowing, seeds were watered with distilled water. Once cotyledons appeared, plants were irrigated with a nutrient solution at pH 5.6 (Morard, 1995). Just after the first leaf pair appeared (10 days after sowing), salt treatments were applied (NaCl concentrations: 0, 34, 85, 171, 256, 341, 427 and 512 mM) for six weeks. Six replicate pots were set up for each salt concentration in a homogeneous completely randomized design. Electrical conductivity was checked (Table 1) throughout the 6 week growing period and was constant. All plant growth was in a Phytotron growth cabinet at 25°C, with a photoperiod of: 16 hours light / 8 hours darkness and at 90 $\mu mol\ m^{-2}\ s^{-1}$ using warm white fluorescent tubes. After 1, 2 and 6 weeks under saline conditions, leaves and roots were sampled randomly from more than one plant for each analysis. Roots were not washed prior to analyses as the sandy medium was easy to remove.

The % survival and plant growth were determined on the seedlings after six weeks for each of the six replicate pots. Relative growth rate (RGR) was calculated from the shoot height data over time (Wang, 2011). Relative water content (RWC) in leaves and roots, was determined on three replicate samples that were dried in an oven at 105 °C to a constant dry weight and was calculated using the following formula: RWC (%) = (FW – DW)/ FW X 100 where FW indicates fresh weight and DW indicates dry weight.

2.2. Metabolite analyses and enzyme activity measurements

Ion (Na^+, K^+) and osmolyte (proline and soluble sugars) concentration, and chlorophyll content were measured in leaves and roots over time (after 1, 2 and 6 weeks for leaves; 2 and 6 weeks for roots). Na^+ and K^+ were analysed by flame photometry (using a Cecil 6000 series spectrophotometer). After drying for 1 h at 105°C, 100 mg of each triplicate dry sample were placed in a cold muffle furnace, raising the temperature gradually to 520°C (for 2 h) and then left overnight to cool. Following 15 mins in a desiccator, samples were dissolved in 2 mL HNO_3 (0.5N) and were then boiled for 20 min. They were then filtered, and the volume was adjusted to 50 mL with distilled water. The K^+/Na^+ ratio was calculated following analysis by flame photometry.

Chlorophyll extraction was performed as described by Plummer (1989): 100 mg of leaf tissue (in triplicate) was ground in 80% acetone. Extracts were then centrifuged at 3000 rpm for 10 min in an Eppendorf minispin microcentrifuge. Absorption of the supernatant was measured at 652 nm. To calculate the amount of chlorophyll the formula: chlorophyll mg/mL = absorbance at 652 nm × 5.8, was used then converted to $\mu g\ g^{-1}$ FW.

Proline was determined by spectrophotometry (Troll and Lindsley, 1955). Plant material (triplicate samples of 100 mg) was suspended in 2 mL of 40% ethanol and heated to 85°C in a water bath for 60 min. After cooling, 1 mL of ninhydrin reagent was added (ninhydrin reagent consisted of 120 mL distilled water, 300 mL of acetic acid, 80 mL acetic orthophosphoric acid, at a density of 1.7, and 25 mg of ninhydrin). After boiling the mixture for 30 min and then cooling, benzene (5 mL) was added to each tube. The upper phase was used for the measurement of proline concentration by spectrophotometry at 528 nm.

Soluble sugar content was measured according to the anthrone method (Plummer, 1989). Triplicate, 100 mg plant material samples were macerated in 3 mL 80% ethanol and stored for 48 hours. Samples were heated to 70°C for 30 minutes and then diluted 10 fold with 80% ethanol. Two mL of the sample were then added to 4 mL of anthrone reagent (0.2 g

of anthrone in 100 mL H_2SO_4). The samples were heated for 8 min to 92°C, and cooled for 30 min on ice in the dark. Absorbance was measured at 585 nm.

Glycine betaine was measured by spectrophotometry (Grieve and Grattan, 1983). Triplicates of plant tissue (150 mg) were dried to constant weight at 80 °C. After grinding to a fine powder, they were shaken at 25 °C with 20 mL deionised water for 24 h. Samples were filtered through Whatman filter paper and filtrates were diluted to 1:1 with 2N H_2SO_4. Samples were cooled in ice/water for 1 h and then cold KI-I2 reagent (15.7 g iodine and potassium iodide (20g) dissolved in 100 mL of water) was added and they were vortexed rapidly. Following storage of the samples at 4 °C for 16 h, they were centrifuged at 0 °C and 10,000 g for 15 min using an Allegra Beckman Coulter microcentrifuge. The supernatant was then carefully removed with a fine glass tube. The periodide crystals were dissolved in 9 mL of 1,2-dichloroethane. Following storage for 2 h at room temperature, absorbance at 365 nm was measured. Glycine betaine (Sigma Aldrich Poole. UK) was used as a standard, and glycine betaine content of the plant samples was expressed as mg g^{-1} DW.

Total solute concentration in roots and leaves over time was calculated by dividing the sum of proline, glycine betaine, soluble sugars and K^+ concentrations by the amount of water present in the plant tissue, based on % relative water content of the tissue.

Ascorbic acid was analyzed by HPLC following extraction using a freezing procedure (Nojavan *et al.*, 2008). Triplicate 100 mg samples of plant material were snap frozen in liquid nitrogen. Samples were then mixed with 5 mL of 5% metaphosphoric acid (MPA). The mixture was homogenized for 5 min and then was centrifuged for 10 min at 2000 rpm in an Allegra Beckman Coulter microcentrifuge. All extractions were conducted at 4°C in reduced light. HPLC analysis was performed using an isocratic elution procedure and a UV Detector at 240 nm. Separation was on a 5µm RP C18 column of 250 mm × 4.6 mm (Kinetex-Phenomenex) with a mobile phase of 0.5% NaH_2PO_4 (pH 2.25 with H_3PO_4) - acetonitrile (2% of final volume). Quantitative analyses were performed using an injection volume of 20 µl. Amber flasks were used for all standard solutions and samples to avoid loss of the ascorbic acid, due to light exposure.

After six weeks of salt stress, the H_2O_2 concentration in leaves was measured using an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Invitrogen). Leaf tissue (in triplicate) was ground in liquid nitrogen, then to each 50 mg of ground frozen tissue, 500 µl of phosphate buffer (20 mM K_2HPO_4, pH 6.5) were added and samples were centrifuged for 5 min at 13 000 rpm in an Eppendorf minispin microcentrifuge. Horseradish peroxidase (0.2 U mL^{-1}) and 100 mM Amplex Red reagent (10-acetyl-3,7-

dihydrophenoxazine) were added to 50 µl of the supernatant and the reaction was incubated at room temperature in the dark for 30 min. Absorbance at 560 nm was measured using an Infinite 200 PRO microplate reader (Tecan, Switzerland).

Catalase activity was determined following the method of Aebi (1984). Leaf tissue (250 mg in triplicate) was ground with polyvinylpolypyrrolidone (10% w/w) and 10 mL of 100 mM K-phosphate buffer (pH 7.0), and the homogenate filtered through four layers of cheesecloth. The filtrate was then centrifuged at 27 000 x g at 4°C for 20 min and the supernatant was used for the enzyme activity assay. The enzyme extraction procedure was carried out at 0-4°C throughout. To determine catalase activity, the reaction mixture (1 mL) comprised 0.05 mL of enzyme extract, 50 mM K-phosphate buffer (pH 7.0), 0.1% (v/v) Triton X-100, and 0.1 mL of 10.5 mM H_2O_2 solution. The reaction was at 25°C for 2.5 min. The amount of enzyme which breaks down 1 µmol of H_2O_2 min^{-1} under the assay conditions described is defined as one unit of catalase activity.

2.3. RNA extraction and Real time PCR

RNA extractions were performed using an RNeasy Mini Kit (Qiagen) and contaminating genomic DNA was removed on the column. cDNA was synthesized using an Ambion kit (RETROscript ® Reverse transcription for RT-PCR) and 2 µg of extracted RNA. Real time PCR was performed using an Absolute TM QPCR SYBR ® Green Mix (Thermo Scientific) kit. Reactions, in a total volume of 25 µl, contained: 5µl of cDNA (diluted 1:20), 12.5µl of Absolute TM QPCR SYBR ® Green Mix, 1.75µl of each primer (10µM) and 4µl H_2O. Triplicate reactions were cycled in an MJ Research OPTICON TM 2, at 95˚ C for 10 min, followed by 40 cycles of 95˚C for 15 s, 60˚C for 30 s and 72˚C for 30 s and one cycle of 72˚C for 30 s. For verifying primer specificity, melting curve analysis (from 60 °C to 98 °C with an increasing heat rate of 0.5 °C s^{-1}) was carried out after amplification. The relative quantification of the gene expression data was calculated using the 2^{-DDCT} or comparative CT method (Livak and Schmittgen, 2001). Mt18S rRNA primers (Mt 18S-F: TGACGGAGAATTAGGGTTCG and Mt 18S-R: CCTCCAATGGATCCTCGTTA) (El Maghrabi *et al.,* 2013) were used for 6ormalization. Primers for the *Crithmum maritimum* antiporter gene (Na-HF: GATGTGGGAAACGGAAACC and Na-HR:

CAAATTGTTGGTGCTTTGTT),were derived from alignment of sequences from *Atriplex dimorphostegia* (AY211397) and *Atriplex gmelini* (AB038492).The *C. maritimum* PCR products were sequenced fully to verify their homology to the target gene (data not shown).

2.4. Statistical analyses

Statistical analyses were performed using StatBox6 and R software (R version 2.15.3, R Foundation for Statistical Computing). One –way or 2-way ANOVA tests were used, as appropriate, to analyse all the data sets, and where significant ($P < 0.05$) interactions or mean effect were found, a Newman-Keuls test was used, consolidated by a Tukey's test.

3. Results

3.1. Seedling survival, plant growth and chlorophyll content under saline stress.

Six weeks growth under saline stress resulted in significantly reduced seedling survival above 85 mM NaCl which was reduced again at 341 mM NaCl ($P < 0.05$). At the highest concentration tested (512 mM NaCl), survival was reduced to 58% (± 3.0) of the non-stressed controls where survival was 99 ± 1.7% (Figure 1A). This was accompanied by a significant reduction in shoot height at $\geq$ 341 mM NaCl with a maximum reduction at 512 mM of 18% compared to the controls after 6 weeks (Figure 1B).The relative growth rate (RGR) of shoot height was significantly lower between the first and second week at NaCl concentrations >341 mM (Figure 1C). After 6 weeks (relative to growth after 1 week), a significant effect of salt stress on RGR was recorded at NaCl concentrations > 256 mM with a further significant reduction at 427 and 512 mM NaCl. Although chlorophyll levels rose during the 6 week period as the leaves developed, the effect of the salinity treatment became more pronounced with time (Figure 2A). After 2 weeks chlorophyll levels were slightly but significantly reduced at all salinity concentrations; at 6 weeks the effects of salinity treatments could be divided into three tiers. The first tier is represented by the control plants which had the highest chlorophyll content. At 34 mM NaCl concentration and above, chlorophyll levels were reduced, and the effect was significantly greater at 85 to 512 mM NaCl.

3.2. Ion accumulation Na^+/H^+ antiporter gene expression and relative water content in seedling roots and leaves under saline stress.

In both leaves and roots, relative water content (RWC) fell significantly with increasing NaCl concentration, but rose with time (Figure 2B). After 6 weeks relative water content was affected differentially in roots and leaves. Leaf relative water content fell significantly at NaCl levels of just 34 mM whereas in roots, it was in fact higher at 34 mM NaCl compared to no salt controls but then remained stable until 427 mM NaCl. The magnitude of the effect also differed significantly ($P < 0.05$): while leaves lost only 6% of their relative water content, roots lost 9%.

The increase in Na^+ was significantly higher ($P < 0.05$) in leaves compared to the roots (Figure 2C). The difference became more pronounced over time with about a 3-fold difference after 2 weeks and a 6-fold difference after 6 weeks at the highest salt concentration tested. Effects of salinity treatments also differed between the two organs. In leaves the effect of salinity became much more pronounced after 6 weeks of treatment with a sudden almost 6-fold increase between 85 mM and 171 mM treatments. In the roots, however, the sudden increase in Na^+ occurred at a higher concentration of Na^+ (256 mM) and was less dramatic with only a 1.4-fold increase, although a further 1.4 increase was noted between 341 and 427 mM NaCl.

In control seedlings, K^+ levels increased in both leaves and roots over time, to a similar extent (1.6-fold increase between 2 and 6 weeks; Figure 3A). Following 2 weeks of treatment, K^+ levels in seedling leaves were relatively constant at most salt treatment concentrations compared to the control. However, after 6 weeks K^+ concentration fell significantly, by 19%, at 512 mM (607 ± 8 compared to the control at 747 ± 16 $\mu molg^{-1}FW$). This pattern different from that in roots: here K^+ accumulation fell substantially (by 24%) even after only two weeks at 512 mM NaCl compared to the control (57 ± 2 compared to the control at 75 ± 3$\mu molg^{-1}FW$; Figure 4A). After six weeks of salt treatment at 512 mM NaCl, root K^+ seemed to have recovered a bit as it was only 18% lower than the control 99 ± 2 compared to the control at 120 ± 4$\mu molg^{-1}FW$.

Changes in relative K^+ and Na^+ concentrations resulted in a fall of the K^+/Na^+ ratio with NaCl increases at all three time points in both seedling leaves and roots (Figure 3B). However, the pattern of change differed markedly between time points and between the two organs. Whereas the decrease was gradual in the earlier time points in both organs, by 6 weeks there was a dramatic decrease in K^+/Na^+ ratio in leaves between 85 and 171 mM NaCl treatments. In roots however, there seemed to be a more gradual decline in the ratio with increasing salinity.

The changes in K^+ and Na^+ accumulation were reflected in an induction of the

Crithmum maritimum Na^+/H^+ antiporter gene expression in leaves and roots under salt treatment (Figure 3 C). Expression increased significantly at $\geq$ 256 mM NaCl in leaves and rose to a maximum expression at 427 mM NaCl before falling back at 512 mM NaCl. In roots the pattern of induction was similar: expression was induced at a lower salinity of 171 mM NaCl but again reached a maximum at 427 mM NaCl before falling back at the highest salt concentration tested.

3.3 Induction of osmolyte accumulation in leaves and roots

There was a significant increase in proline in both the leaves and the roots with increasing NaCl concentrations. However after 1 week the maximal concentration of proline in the leaves was recorded at the highest two NaCl concentrations tested (427 mM and 512 mM; Figure 4A) while after 2 and 6 weeks, proline concentration peaked at 427 mM (162 ± 2 µmol g^{-1} FW) and then fell back at 512 mM NaCl. In contrast, root proline concentration continued to increase with increasing salt concentration up to and including 512 mM NaCl even after 6 weeks of treatment. After 6 weeks of treatment there was a marked rise in proline between 256 and 341 mM NaCl in leaves, whereas this threshold effect was not seen in the roots.

Soluble sugar concentration in leaves and roots also rose significantly with increasing time and NaCl concentration (Figure 4B). However, the 256 to 341 mM threshold effect noted with proline concentration in leaves, was seen with leaf soluble sugars after only 2 weeks as well as after 6 weeks and again was not evident in the roots. In leaves, soluble sugar concentration reached a maximum at 427 mM NaCl with only a slight reduction in level at the highest salt concentration. In contrast soluble sugar accumulation in roots after 6 weeks clearly peaked at 427 mM NaCl and fell significantly at 512 mM NaCl.

Glycine betaine accumulation was also induced with increasing salinity. In leaves again a threshold effect between 256 to 341 mM NaCl was evident after 6 weeks and concentrations fell at the highest salinity tested (Figure 4C). In roots however, after 6 weeks, there were two notable rises in glycine betaine concentration: between 85 and 171 mM and between 256 and 341 mM NaCl. Furthermore, in roots glycine betaine concentrations remained high even at the highest salt concentration tested whereas in leaves they fell significantly at 512 mM NaCl. Over time, glycine betaine concentrations increased significantly in both tissues at most salt concentrations, however the most dramatic increases were seen at the higher NaCl concentration between 2 and 6 weeks in leaves.

In leaves the total internal solutes were greater than external solute concentration up to about 256 mM NaCl, while in roots this threshold was reached at 85 mM NaCl, indicating that this is the point at which osmotic adjustment occurs. However, this is clearly an estimate, as other cellular solutes which were not measured here will contribute to the total internal solute concentration (Figure 5).

3.4 Antioxidant capacity and catalase activity rose with increasing salinity, while ROS levels fell.

Leaf H_2O_2 concentration fell significantly between control (no salt) and plants exposed to the highest two salt concentrations (Figure 6A). In contrast seedling leaves accumulated ascorbic acid in response to saline treatment with a linear increase with increasing NaCl concentration from 34-512 mM NaCl (Figure 6B) and the highest accumulation at 512 mM. Catalase activity was also induced by the saline treatment in leaves of *C. maritimum.* However the activity was only consistently greater than control levels at 256-427 mM NaCl, falling back again at 512 mM NaCl (Figure 6C).

4. Discussion

The reduced growth of *C. maritimum* under elevated salt treatments was in agreement with previous reports (Ben Hamed *et al.,* 2004; Ben Hamed *et al.,* 2007; Ben Amor *et al.,* 2005). However here effects on shoot height were only significant at $\geq$ 341mM NaCl whereas in the Tunisian population studied by Ben Hamed *et al*. (2004), biomass after 5 weeks as well as leaf number and total leaf area were affected even at 150 mM NaCl. This suggests that the Algerian population studied here was substantially more salt tolerant. Ben Hamed *et al*. (2004) saw no chlorosis even at 300 mM NaCl. The more sensitive chlorophyll measurements made here indicate some loss of chlorophyll even at low salt concentrations of $\geq$ 34 mM after similar periods of treatment, although losses were relatively small even at the highest salt concentrations applied. This suggests that the salt stress imposed was not inducing premature senescence as it does on less salt-tolerant species (e.g. pomegranate; Mastrogiannidou *et al.,* 2016), although it may be contributing to the reduced growth even in

halophytes such as *C. maritimum* through reduced carbon fixation as previously suggested (Flowers and Colmer, 2008). The apparently better growth of the plants in their natural habitat was ascribed by Ben Hamed *et al*. (2004) to small differences in growth conditions or, more likely, a protection of the root system from extremes of salt due to their growth in rock crevices. The Algerian population used here also grew vigorously very close to the sea indicating a similar protection of the roots from the saline water.

Maintaining a high K^+/Na^+ ratio is likely to be important to avoid effects of ion toxicity under salt stress (Flowers *et al.,* 2015). Although this ratio dropped dramatically with increasing salinity, even after 6 weeks it remained relatively constant between 171 and 512 mM NaCl. A clear difference was seen, however, in the accumulation of Na^+ between roots and shoots, consistent with the studies of Tunisian *C. maritimum* populations (Ben Hamed *et al.,* 2004; Ben Amor *et al.,* 2005). In both the Tunisian *C. maritimum* (Ben Hamed *et al.,* 2004), and the Algerian population reported here, there was a sudden increase in shoot Na^+ accumulation at > 100 mM NaCl. However, whereas in the Tunisian *C. maritimum* roots, Na^+ accumulation plateaued after 100 mM NaCl, the Algerian *C. maritimum* roots continued to accumulate increasing Na^+ concentrations with increasing salinity up to the maximal level of 512 mM tested. Thus, the increased tolerance to salinity seen in the Algerian population here is not due to better exclusion of the Na^+ from the roots. Leaf relative water content changes also differed between the Algerian *C. maritimum* presented here and the Tunisian populations previously studied (Ben Hamed *et al.,* 2004; Ben Amor *et al.,* 2005). Whereas the relative water content remained stable in the Tunisian *C. maritimum* populations, here a significant loss in relative water content was seen at concentrations of > 85 mM NaCl. In contrast, root relative water content remained much more stable up to 427 mM NaCl, linked presumably to the lower accumulation of Na^+. In both tissues, loss of relative water content seems to be linked to Na^+ accumulation of > 100 $\mu molg^{-1}FW$, which may therefore be a threshold level of Na^+ before water retention is affected. Differences between the Algerian and Tunisian *C. maritimum* response may also be due to the maturity of the plants used since the Tunisian *C. maritimum* plants were already 1 month (Ben Amor *et al.,* 2005) or 2 months (Ben Hamed *et al.,* 2004) old when the saline treatments were applied. Alternatively the method of treatment may have affected the outcome: here salinity was imposed directly to simulate natural conditions where changes in salinity can occur rapidly; the previous studies (Ben Hamed *et al.,* 2004; Ben Amor *et al.,* 2005) applied the salinity treatments gradually.

Despite the increases in Na^+ accumulation, K^+ concentrations remained remarkably stable up to 427 mM NaCl in leaves, while there was a gradual decline in root K^+

concentration from 171 mM NaCl. This again contrasts with the previous studies (Ben Hamed *et al.*, 2004; Ben Amor *et al.*, 2005) where both leaf and root K^+ concentrations fell dramatically between the no salt control and a 50mM NaCl treatment. Again, this suggests a better resilience of the Algerian population to the salt stress. Part of this resilience may be due to the induction of the Na^+/H^+ antiporter gene expression, which rises significantly at $\geq$ 256mM and 171 mM NaCl in leaves and roots respectively. It is assumed, based on experimental evidence from other halophytes (Flowers and Colmer, 2008) that the activation of the antiporter in *C. maritimum* is important for Na^+ sequestration into the vacuole. Its activation in both organs contrasts with *Mesembryanthemum crystallinum* L where expression of subunit E of the vacuolar H^+-ATPase, required for Na^+ transport into the vacuole, was only upregulated in leaves but not roots in response to salt (Golldack and Dietz, 2001). However the *M. crystallinum* gene expression was only studied following a very short salt exposure of 72 h so it may be that both root and leaf Na^+ transport into the vacuole only become important after prolonged salt exposure. The reduction in expression of the *C. maritimum* Na^+/H^+ antiporter at 512 mM NaCl in both organs is presumably linked to a failure of the salt tolerance mechanisms which may be down-regulating transcription of many genes and which is also seen in the significant reduction of shoot height and K^+ concentration at this level of salinity.

Despite the importance of osmolyte production in halophytes, known to mitigate effects of saline stress by providing cytoplasmic osmotic adjustment (Flowers *et al.*, 2015), to our knowledge this is the first analysis of these metabolites in *C. maritimum*. All three osmolytes analysed here: proline, glycine betaine and soluble sugars accumulated with increasing salt concentrations as has been found for other halophytes (Flowers and Colmer, 2008). Increase in proline was not as high as is some halophytes such as *Mesembryanthemum crystallinum* L., where the increase is > 10-fold (Sanada *et al.*, 1995) but its concentration was still significantly (3 to 4-fold) induced. It was proposed (Tipirdamaz *et al.*, 2006), that there was an inverse relationship between proline and glycine betaine accumulation amongst halophyte species. However, *C. maritimum* does not appear to follow this trend since both osmolytes accumulated to similar extents in both roots and leaves. All three osmolyte concentrations increased substantially between 256 and 341 mM NaCl suggesting that as salinity rises above 300 mM osmolytes become particularly important in *C. maritimum.* Concentrations of all three osmolytes also fell in leaves at 512 mM NaCl supporting the hypothesis that at this high salt level transcription and/ or biosynthetic pathways are being affected. At this salt level, the

concentration of soluble sugars fell back also in roots, however concentrations of proline and glycine betaine remained stable suggesting that their biosynthesis was not being inhibited, and that at this very high salt concentration their action as osmolytes may be important for cellular protection. By considering the total concentration of solutes measured here, which are considered to be the major contributors to osmotic adjustment (Singh *et al.*, 2015) it was possible to estimate the concentration at which osmotic adjustment occurs. Unlike some other halophytes such as *Atriplex mummularia* (Silveira *et al.*, 2009) osmotic adjustment appeared to occur in both leaves and roots of *C.maritimum.* However, this occurred at very different concentrations of external salts: with roots the osmotic adjustment occurred at 85 mM NaCl maximally, while in leaves that maximum was reached at 171 mM.

One reason proposed for a growth reduction at high salinity is an increase in reactive oxygen species causing cellular damage. A rise in markers for lipid peroxidation has been seen in several halophytes at >300 mM NaCl even over short treatments (Ben Hamed *et al.*, 2007). However an increase in ROS seems an unlikely explanation for the growth effects in *C. maritimum.* H_2O_2 concentrations in fact decreased with increasing salinity while ascorbic acid concentrations rose, indicating that redox status was not adversely affected. Catalase activity also increased with salinity indicating an induction of protective antioxidant mechanisms which may be resulting in the maintenance of redox balance. This contrasts with a previous report on a Tunisian population of *C. maritimum* (Ben Hamed *et al.*, 2007) where H_2O_2 concentrations rose while ascorbic acid concentrations and catalase activity fell as salinity was increased to 300 mM NaCl. This further suggests that the Algerian population studied here is less affected by the salinity. A fall in H_2O_2 and induction of CAT activity in response to increased salinity has been reported for other halophytes (e.g. *Atriplex halimus* and *Nitraria retusa*, Boughalleb *et al.*, 2010) indicating that the response of the *C. maritimum* population reported here is not entirely anomalous.

5. Conclusions

Overall the response to increased salinity in this population of *Crithmum maritimum* seems to involve concentration thresholds which activate different tolerance mechanisms (Figure 7). Already at 34 mM NaCl concentration, some osmolyte production is upregulated, but protective mechanisms in leaves, and some signals of stress are evident. At 85 mM NaCl roots reach osmotic adjustment, and at 171mM further osmolyte protection mechanisms are

upregulated in both, leaves and roots but % survival has been compromised. At 256 mM NaCl leaves reach osmotic adjustment and at 341 mM plant growth is affected suggesting that tolerance mechanisms are struggling to overcome the stress. Finally at 512 mM all leaf osmolyte levels but only soluble sugars fall in roots, and catalase activity is reduced, indicating that at this very high salinity concentration, most protective mechanisms in leaves are unable to be sustained. In roots however, where Na^+ concentrations are still increasing, stress is less severe enabling some growth to continue.

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Figure and Table Legends

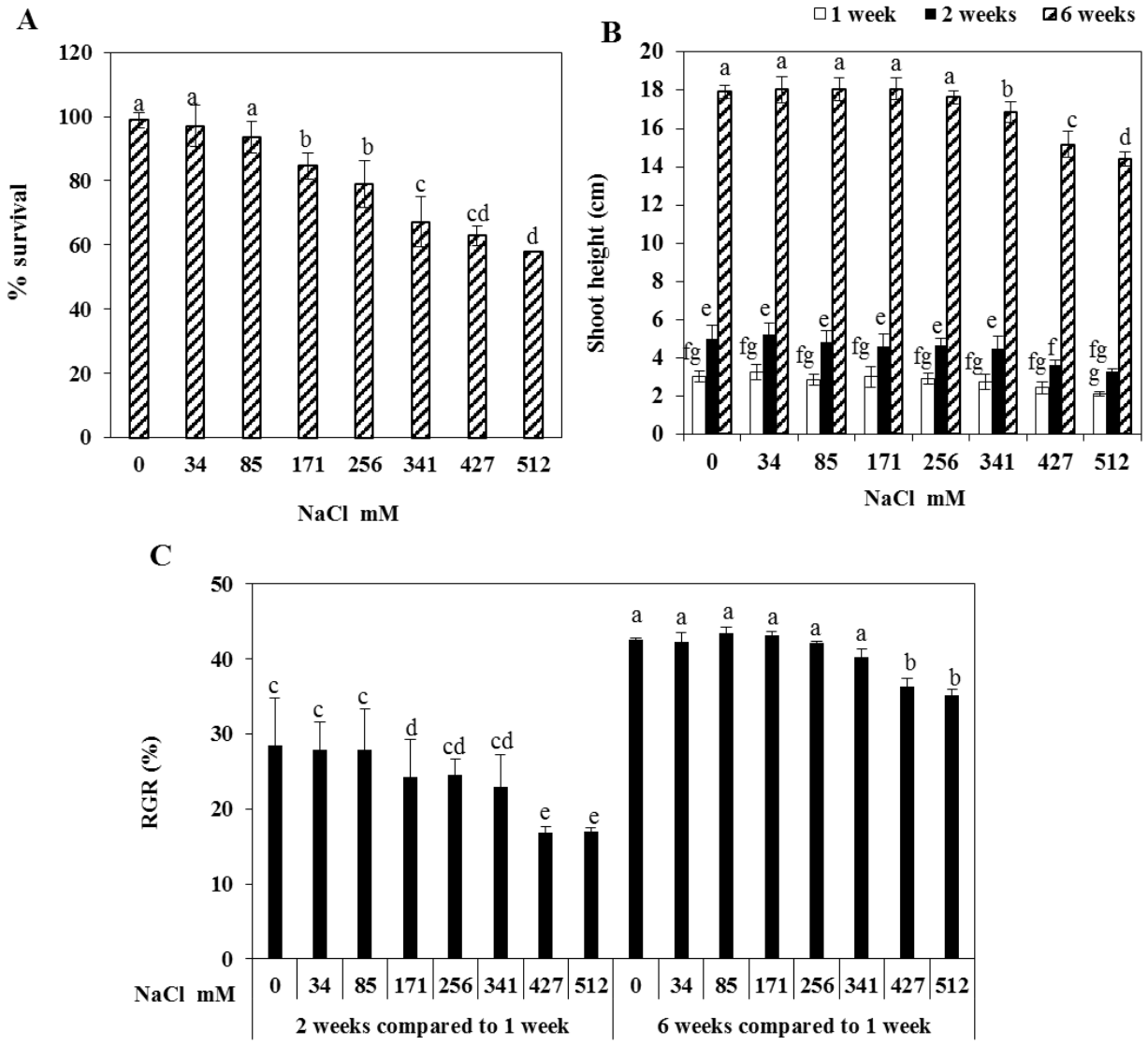

Figure 1. Growth and survival of *Crithmum maritimum* seedlings under salt stress. Mean percentage survival per pot after 6 weeks (A), shoot height over time per pot (B), RGR (% shoot height) after 2 weeks compared to 1 week and 6 weeks compared to 1 week (C). Mean ± S.D; different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples (n = 6).

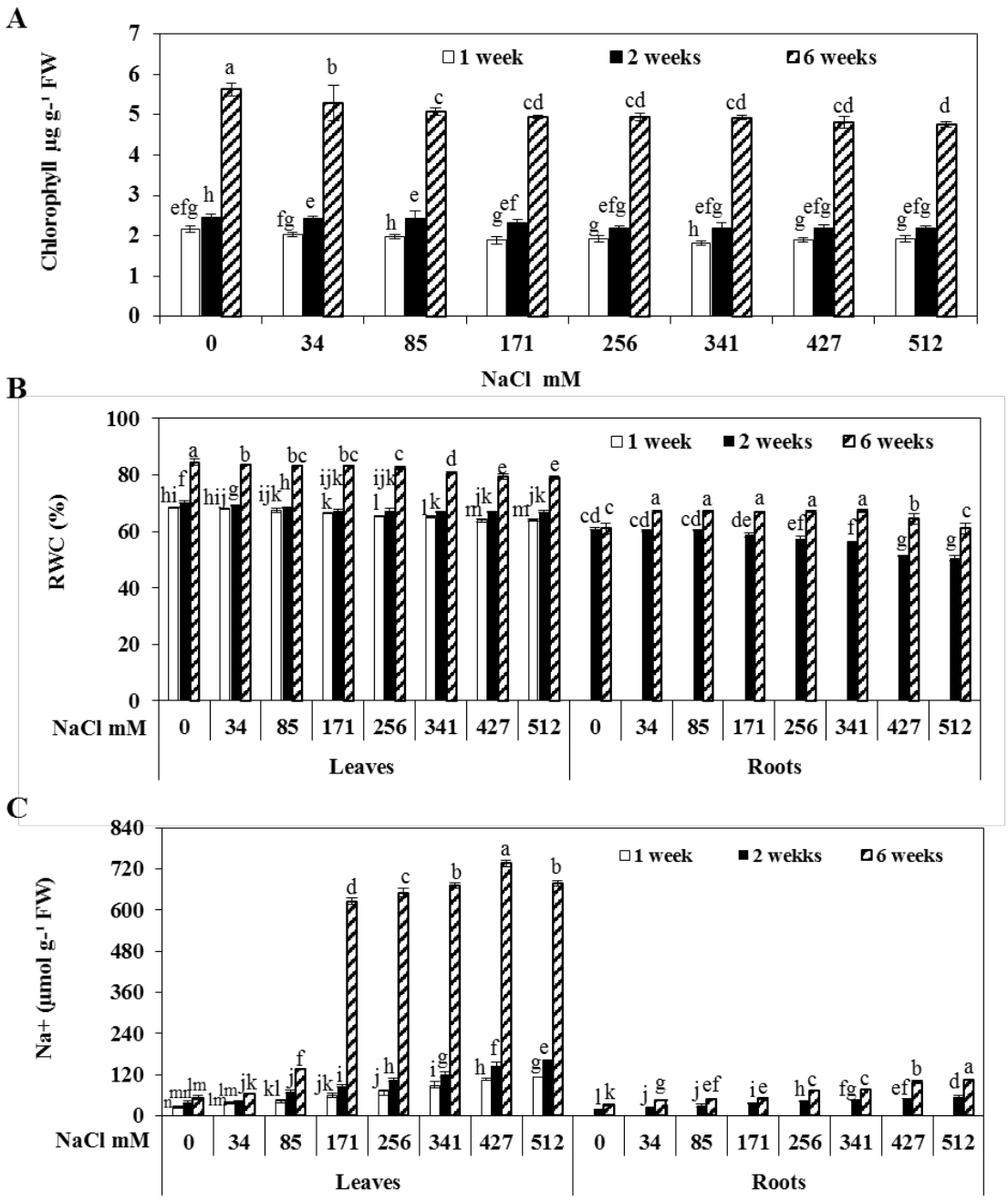

Figure 2. Chlorophyll content (A), relative water content (RWC) (B) and Na^+ accumulation (C) of *Crithmum maritimum* seedlings over time under salt stress. Mean ± S.D; n = 3; different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples.

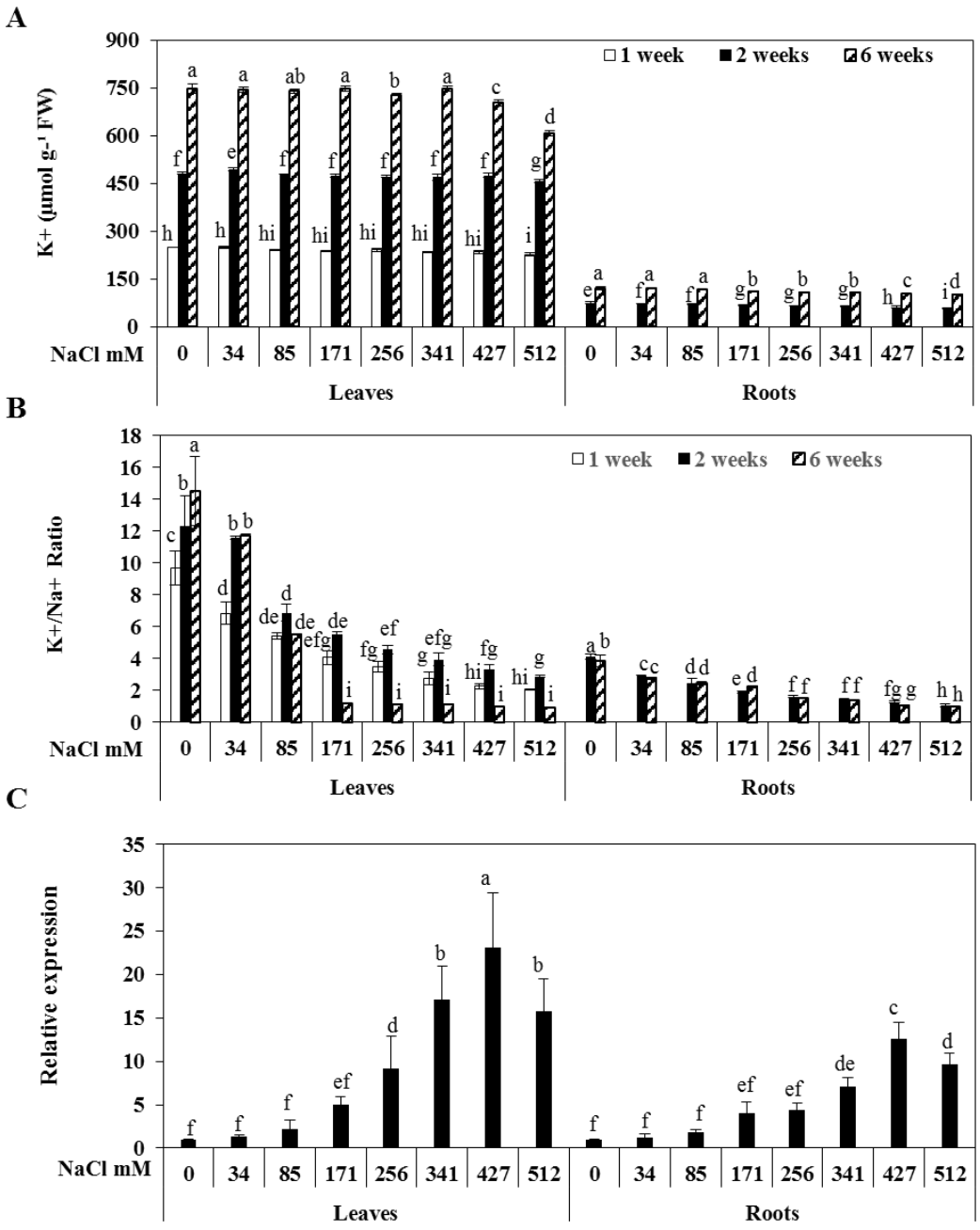

Figure 3. K^+ accumulation (A), K^+/Na^+ ratio (B) of *Crithmum maritimum* seedlings over time under salt stress . Relative Na^+/H^+ antiporter expression (C) after 6 weeks under salt treatment. Mean ± S.D; n = 3 (A and B); n= 6 (C); different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples.

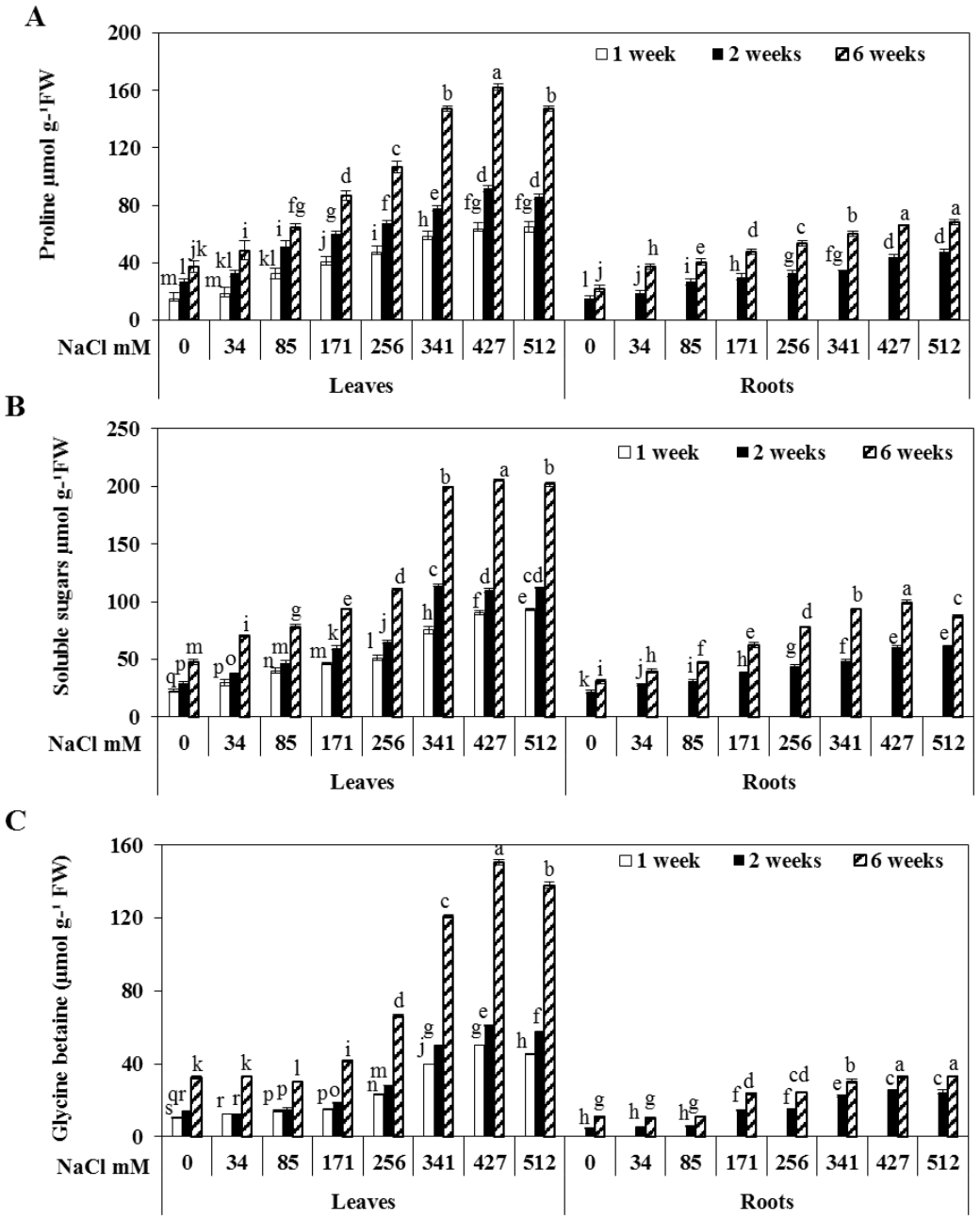

Figure 4. Proline concentration (A), soluble sugars concentration (B) and glycine betaine concentration of *Crithmum maritimum* seedlings over time under salt stress. Mean ± S.D; n = 3 (A and B); n= 6 (C); different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples.

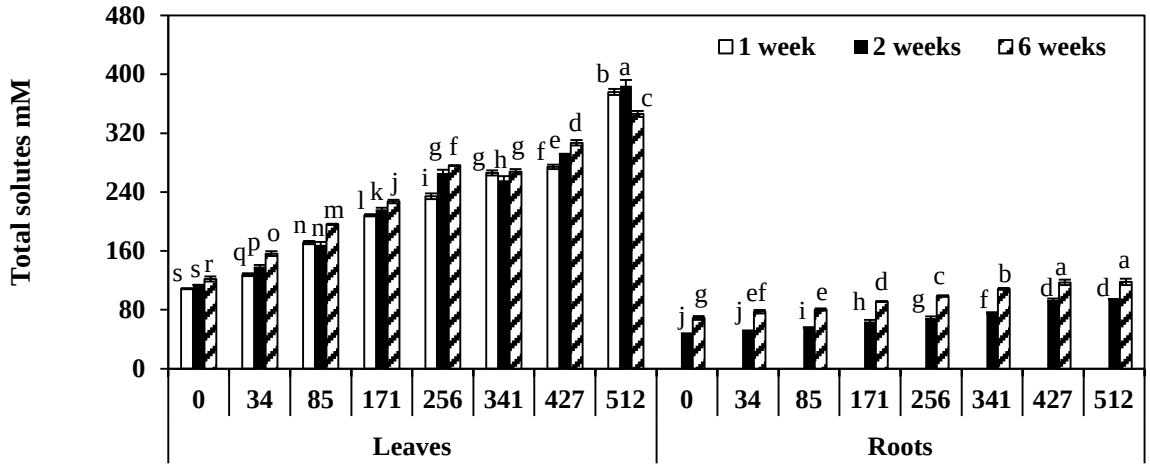

Figure 5. Total internal solutes (expressed in mM) of *Crithmum maritimum* seedlings over time against total external solutes comprising NaCl (0, 34, 85, 171, 256, 341, 427, 512 mM) and total nutrient solutes of 32.1 mM. Mean ± S.D; n = 3; different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples.

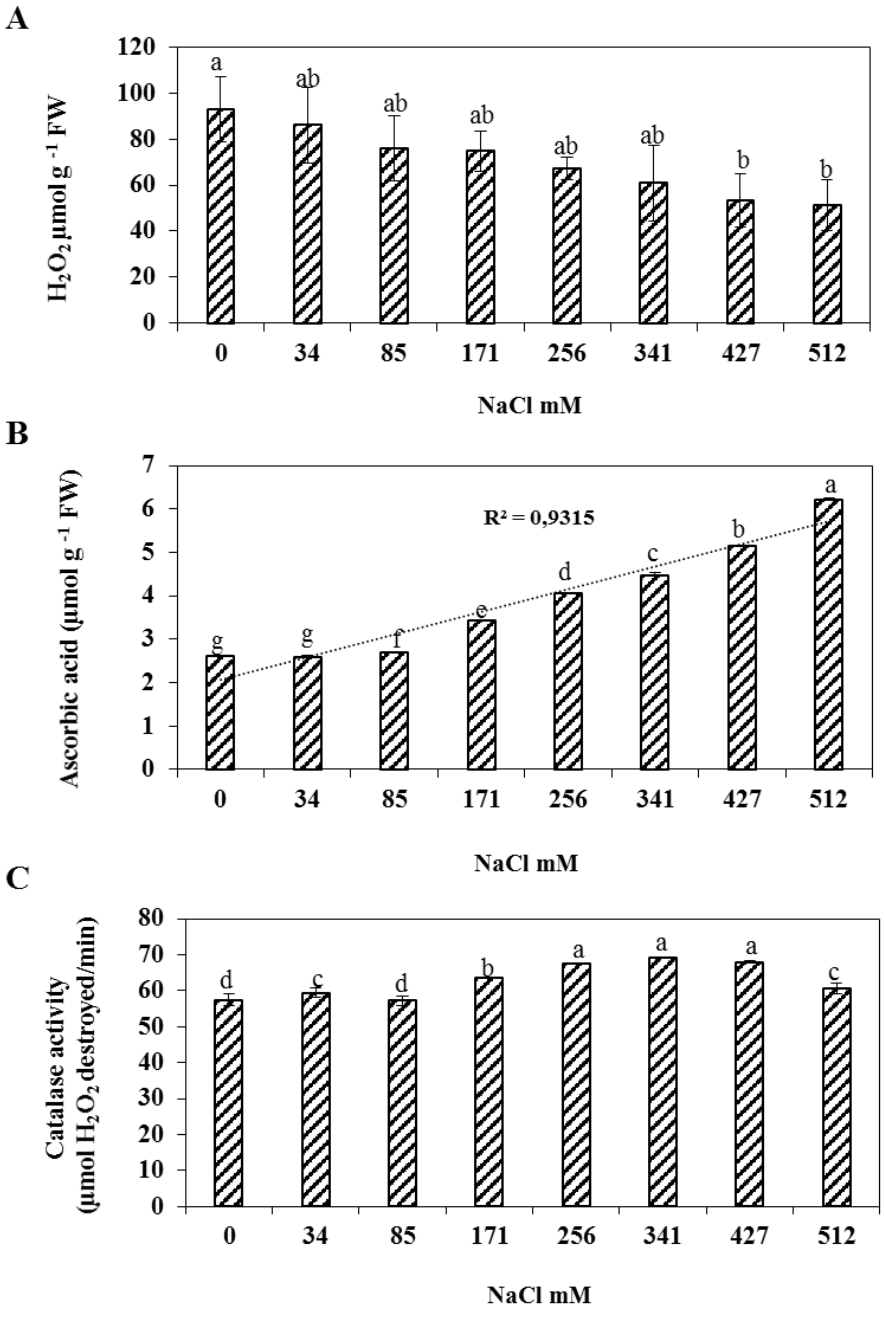

Figure 6. H_2O_2 concentration (A), Ascorbic acid concentration (B) and catalase activity (C) in leaves of *Crithmum maritimum* seedlings. After 6 weeks (means ± S.D ; n = 3 ; different letters above the bars indicate significant differences based on a Tukey's test ($P < 0.05$) across all samples for B and C, while for A, letters indicate differences based only on the salt concentration.

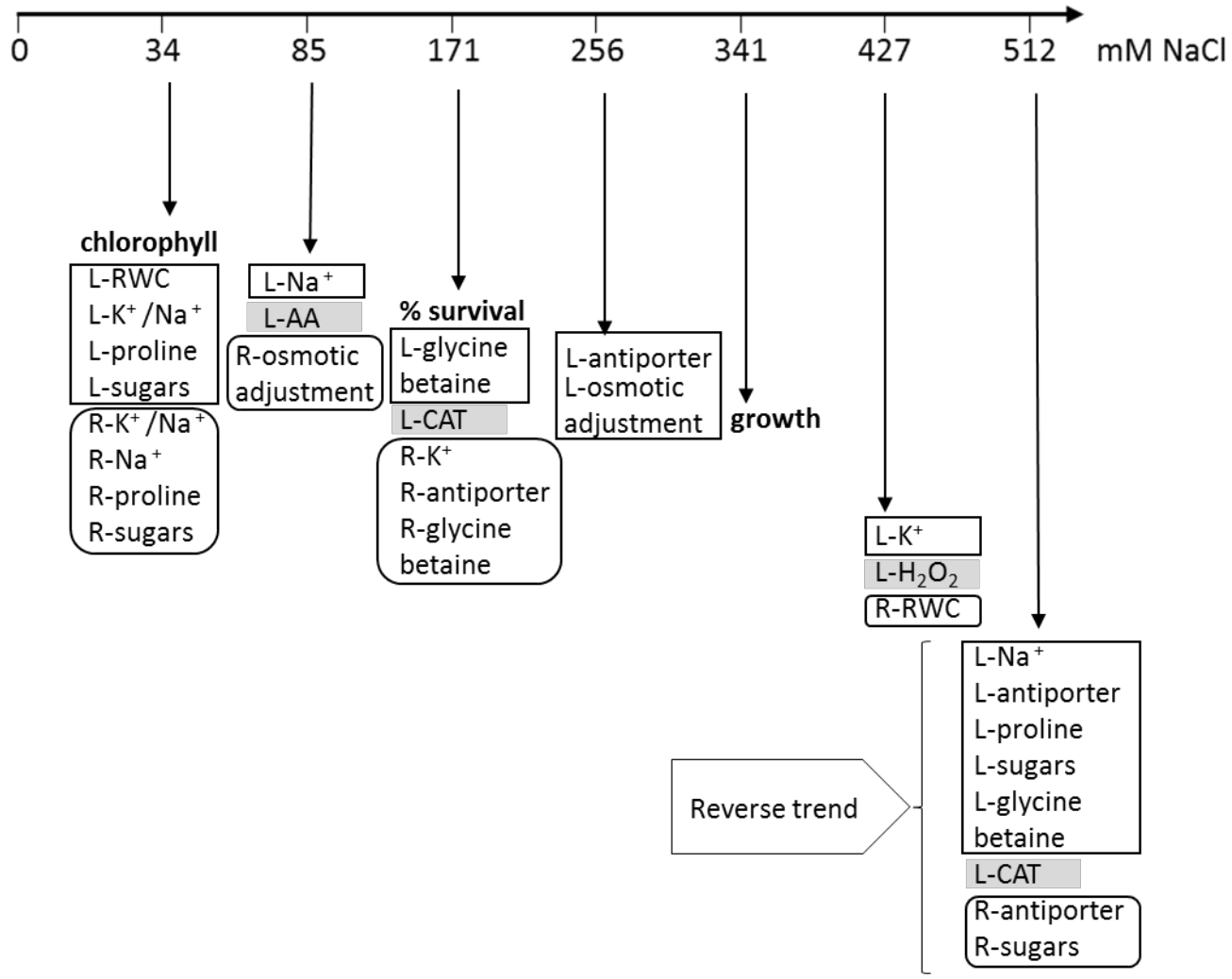

Figure 7. Diagram of changes in physiological (bold), metabolite, enzyme activity and gene expression changes with increasing salinity after 6 weeks growth of *Crithmum maritimum*. L= leaf (square box); R = root (rounded box); RWC (relative water content); shaded box = ROS regulation.

NaCl concentration (mM)	0	34	85	171	256	341	427	512
Electrical Conductivity (ms) at 25°C	2.34	5.78	10.93	18.75	25	34.25	43	54.14

Table 1. Electrical Conductivity of different treatments.