

Ion Content, Antioxidant Enzyme Activity and Transcriptional Response Under Salt Stress and Recovery Condition in the Halophyte Grass *Aeluropus Littoralis*

Seyyed Hamidreza Hashemi-Petroudi (✉ irahamidreza@yahoo.com)

Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT) <https://orcid.org/0000-0002-0870-1691>

Gholamreza Ahmadian

National Institute of Genetics Engineering and Biotechnology (NIGEB)

Farzaneh Fatemi

Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT)

Ghorbanali Nematzadeh

Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT)

Ahad Yamchi

Department of Plant Breeding and Biotechnology

Markus Kuhlmann

RG Heterosis, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK)

Research

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Abstract

Background: The production of reactive oxygen species (ROS) is a common feature of various plant cells in dealing with abiotic stresses. Plants have developed an effective enzymatic and non-enzymatic antioxidant defense systems to scavenge ROS and cope with oxidative stress. Halophyte antioxidant defense mechanisms represent one of the best examples for this trait. Several of salt-responsive promoters and genes originated here during evolution. The present work aimed to investigate a set of physiological, elemental, biochemical and molecular responses involved in tolerance to salt stress in the halophyte grass *Aeluropus litoralis*.

Results: The content of total Chlorophyll (Chl), Chl a and Cars were increased under salinity condition, while the Chl b content was reduced. Sodium ion (Na^+) in three time-points of salinity condition (S1, S2 and S3) significantly increased, and reduction was observed in three time-points of recovery condition (R1, R2 and R3). The amount of potassium ions (K^+) in leaf and stem was decreased during salt stress, and increased during recovery condition. K^+ accumulations in root significantly increased in S2 and S3. Calcium (Ca^{2+}) and magnesium (Mg^{2+}) content significantly declined in leaf, root and stem during stress treatment, whereas it increased significantly during recovery condition in the leaves. The amount of the amino acid proline, associated with drought and salt stress, as well as the activity of ROS related enzyme showed an increase during salt treatment. The APX, POD and SOD maximum activities were reported at S3 in roots while decreased at R. RT-qPCR analysis of antioxidant related genes showed up-regulation at S1 and S3 in root, but the down-regulation was observed in R. The highest transcription levels were observed in *CAT* and *pAPX* at S1 in leaf, while the maximum levels were seen in *SOD* and *cAPX* at S2.

Conclusions: The halophyte mechanisms in *A. litoralis*, contribute to overcome ROS in oxidative stress. SOD activity was more responsive in S, indicating the importance of SOD in oxidative damage. Increasing proline content may be considered as a stress-induced marker to identify oxidative damage. During the salt stress phase an increase of mRNA abundance from genes encoding enzymes associated with antioxidant activities was found. The positive correlation between the transcript level of *CAT* and *CAT* activities in leaf indicated the important regulatory function during salt stress for detoxifying ROS. Based on the transcript abundance and activity *CAT* is proposed as the main H_2O_2 -scavenging enzyme to keep the balance of redox reaction in *A. litoralis*. The salt stress tolerance is associated with high Na^+ absorption for osmotic balance and a corresponding reduction in K^+ , Mg^{2+} , and Ca^{2+} ions. Further it was found that plants under S3 and R2 had more chlorophyll b content as compared to chlorophyll a that demonstrated optimization of leaf function under high salt condition.

Introduction

The overproduction and accumulation of reactive oxygen species (ROS) are a common feature of different plant cell in dealing with various abiotic stresses. Different types of ROS are highly reactive and toxic and cause oxidative damage to proteins, DNA, and lipids and carbohydrates [1, 2]. Stress-induced ROS, such as superoxide ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($\text{HO}\cdot$), and singlet oxygen ($^1\text{O}_2$) are also generated continuously as common by-products of various metabolic pathways [3, 4]. In contrast, plants have developed a strong enzymatic and non-enzymatic antioxidant defense systems which act in concert to control

the cascades of uncontrolled oxidation by scavenging of ROS [5]. Enzymatic scavenging of ROS could be efficiently achieved by a variety of scavengers, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX).

The first line of antioxidant defense systems dealing with ROS effects is SOD. The SODs remove O_2^- by catalyzing its dismutation into H_2O_2 and oxygen (O_2) [5, 6]. The SOD isoenzymes are classified into three distinct groups based on their metal cofactors: the iron (Fe-SOD), the manganese (Mn-SOD) and copper/zinc (Cu/Zn-SOD) by different cellular localization [7]. Different isoforms of SOD were isolated, cloned and characterized in various plant species. For example in *Arabidopsis thaliana* as model plant, three isoform of Fe-SOD genes (AT4G25100, AT5G51100 and AT5G23310), three isoform of Cu/Zn-SOD genes (AT1G08830, AT2G28190 and AT5G18100) and one Mn-SOD gene (AT3G10920) have been annotated [8]. The catalase is another antioxidant enzyme, scavenging H_2O_2 into H_2O and O_2 . In plants, CAT is considered as a primary enzymatic defense against oxidative condition induced by metals-toxicity, salt and osmotic stress, wounding, high and low temperature [9–12]. To date, three isoforms of catalase have been detected in angiosperm species including monocots and dicots [13]. The APX enzyme, specialized for ascorbate (ASC) utilization, plays a key role in ROS metabolism by scavenging harmful hydrogen peroxide to water [14, 15]. The APX has a higher affinity of for H_2O_2 than CAT and confers a crucial role for management of ROS during stress [5].

The initial role of ROS as defined as toxic byproduct, but lately there is increasing evidence, that they function as important regulators in the complex signaling network of cells [16, 17]. Redox balance, the ratio between oxidizing and reducing species within the cell, plays an important role in the regulation of signaling pathways, including kinase and phosphatase activity and gene expression through modulation of transcription factor function [18]. The harmful or useful effects of ROS in cells and tissues are dependent on the type of the ROS and their quantity. At physiological low levels, ROS function as “redox messengers” involved in gene regulation and intracellular signaling, whereas high level of ROS cause various oxidative modification of proteins, induce protein degradation, and promote cell death [19]. Redox balance is achieved by various cellular enzymatic and non-enzymatic mechanisms [1].

Halophyte antioxidant defense mechanisms to cope with oxidative stress represent the best example of stress-tolerant plant during evolution. The use of wild plant species or their halophytic relatives were attended in plant breeding program for developing salt and drought tolerant plants [20]. The some morphological and anatomical characteristics, life style features and genetic background allow the halophytes to inhabit various environmental restrictions from very high to low salinity soils, and salty water of seas [21]. Thus the genetic potential of halophytic *Poaceae* can be used for improving glycophyte crops by identification of novel salt-responsive promoters and/or genes base on these resources [22, 23]. There are currently several reports dealing with the genetic engineering using halophytic gene sequences in order to confer tolerance to salt stress. This addresses the alterations of various cellular, physiological, and metabolic mechanisms using different categories of genes [24]. In this view, *Aeluropus littoralis* as a halophyte model for identification and isolation of novel adaptation genes, became a focus. *A. littoralis* is a perennial monocotyledonous diploid grass ($2n = 2X = 14$), with the small haploid genome of 349 Mb and C_4 photosynthesis [25, 26] that growing in dry salty areas or marshes [27]. The *A. littoralis* can survive where the water salinity is periodically high [28] and tolerate up to 1,100 mM NaCl [29]. Thus, *A. littoralis* has the potential to become a precious genetic

resource, not only for understanding the molecular mechanisms of stress-responses in monocots but also for improving tolerance to abiotic stresses in economically important crops [30].

In previous studies, some genes such as Cu/Zn(superoxide dismutase and catalase and APX which are involved in antioxidant pathway have been isolated at salt stress [31, 32]. In the present study, to better understand the adaptability of *A. littoralis* plants to salt stress, we evaluated the stress tolerance of *Aeluropus* plants under 600 mM NaCl treatment and recovery condition by assessing ion content (Na⁺, K⁺, Ca²⁺ and Mg²⁺), some of antioxidant enzyme activity (SOD, CAT, POD and APX) and non-enzymatic antioxidants (proline). The transcriptional responses of *SOD*, *CAT* and *APXs* genes during different time-points of salt stress and recovery condition as well as their enzyme activity changes were also addressed in this research.

Material And Methods

Plant material and treatments

To ensure plant uniformity, the cuttings of one mother plants of *Aeluropus littoralis* collected from Isfahan province (32°33'03.5"N 52°29'31.3"E) were used in this study. After rooting in non-potable water at darkness condition, one week old seedlings were transferred to pots containing half-strength Hoagland's solution [33] and after two weeks cultivation the solution was replaced with full- strength Hoagland. The growth chamber conditions were 25 ± 2 °C with 16 h light/8 h dark photoperiod at 600 μmolm⁻²s⁻¹ photon flux density using cool-white fluorescent light. For salt stress treatment, 600 mM of sodium chloride were added to solution in six passage (received 100 mM sodium chloride per two day). The end of the sixth passage, the both shoot and root of samples were collected at 6h (S1), 48h (S2) and after one week (S3). The stressed plants remaining were transfer to a sodium chlorides-free Hoagland's solution as recovery condition. The recovered samples were collected after 6h (S1), 24h (S2) and one week (S3). All samples as well as control were immediately frozen in liquid nitrogen and stored at -70°C for RNA extraction and other experiments.

Chlorophyll and carotenoids measurement

Chlorophyll (Chl) a, Chl b and the total carotenoids (Cars) were extracted from aerial tissue using Methanol, and the absorbance of the extract was measured at 665.2 and 652.4 and 470 nm [34] using Biochrom WPA Biowave II spectrophotometer. The contents of Chl a, Chl b, total Chl (Chls a+b) and Cars (the sum of the xanthophylls and β-carotene) were calculated using the following formulae.

$$\text{Chl a } (\mu\text{g}/\text{m}^2) = 16.72 A_{665.2} - 9.16 A_{652.4}$$

$$\text{Chl b } (\mu\text{g}/\text{m}^2) = 34.09 A_{652.4} - 15.28 A_{665.2}$$

$$\text{Chls a+b } (\mu\text{g}/\text{m}^2) = 1.44 A_{665.2} + 24.93 A_{652.4}$$

$$\text{Cars } (\mu\text{g}/\text{m}^2) = (1000A_{470} - 1.63\text{Chl a} - 104.96\text{Chl b})/221$$

Proline content measurements

Proline content was extracted and measured according to the method of Bates et al (1973) [35]. Fresh plant material (500 mg) were homogenized in 10 ml of 3 % sulfosalicylic acid and centrifuged at 14 000 x g for 10 min at 4 °C. The 2 ml of acid-ninhydrin and 2 ml of glacial acetic acid were added to 2 ml of supernatant solution. The reaction mixture was incubated at 100 °C for 1 h and then terminated on ice. The red chromogen produced color separated by 4 ml of toluene and the absorbance of aqueous phase were measured at 520 nm in a spectrophotometer. The proline content was calculated using the standard curve drawn from known concentrations of proline, and the results are expressed in $\mu\text{moles per gram tissue (fresh weight)}$ by the below formula.

$$\mu\text{moles per gram tissue} = [(\mu\text{g proline/ml}) \times \text{ml toluene} \times \text{ml salicylic acid}] / (115.5 \mu\text{g } \mu\text{mole} \times \text{sample (g)})$$

Determination of calcium (Ca), potassium (K), iron (Fe), magnesium (Mg) Using ICP–OES Analysis

The stressed plant samples were collected from in root, leaf and internode (stem) as aforementioned time-points. Plant material was dried at 65 °C for 48 h and dry weight was recorded. Samples were digested in 70% nitric acid and 30% hydrogen peroxide for 2.5 h at 120 °C and then the element of Ca, K, Fe and Mg were measured by an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). The plant material samples were analyzed in three replicates. The data quality was assessed using 3 replicates.

Determination of total protein and ROS scavenging enzyme

Root and leaf samples (200 mg) were powdered in liquid nitrogen, and homogenized in 1 ml of 100 mM sodium phosphate buffer (pH 7) [36]. The extract was centrifuged at 14,000 g for 30 min at 4 °C and supernatant was collected for further analysis. Total soluble proteins were measured by Bradford assay (1976) and bovine serum albumin as standard [37]. The activity of SOD (EC 1.15.1.1) was measured using the reduction of nitro blue tetrazolium (NBT) [38]. The reaction mixture (4 ml) contained 63 μM NBT, 13 mM methionine, 0.1 mM EDTA, 13 μM riboflavin, 0.05 M sodium carbonate and 50-100 μl enzyme extract (0.5 ml distilled water in the case of the control). Samples were irradiated under fluorescent lamp for 20 minutes and then transferred to the dark for 20 minutes. The absorbance was determined at 560 nm and activity was expressed as enzyme unit mg^{-1} . One unit of the enzyme activity was defined as the enzyme required for 50 % inhibition of the reduction of NBT in comparison with the tubes lacking the enzyme.

CAT (EC 1.11.1.6) activity was assayed based on decomposition of H_2O_2 [39]. One mL reaction mixture containing 950 μL of 50 mM potassium phosphate buffer (pH 7.0, containing 10 mM H_2O_2) and 50 μL of enzyme extract. The activity was determined by measuring the rate of H_2O_2 reduction ($E = 39.4 \text{ M}^{-1} \text{ cm}^{-1}$) at 240 nm for 1 minute (and expressed as enzyme units g^{-1} fresh weight). One enzyme unit was defined as $\mu\text{mol H}_2\text{O}_2$ destroyed per minute at $25 \pm 2 \text{ }^\circ\text{C}$.

APX (EC 1.11.1.11) was assayed according to the method described by Nakano and Asada [40]. The reaction mixture (2 ml) consisted of 25 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbate, 1 mM H_2O_2 and 100 μl enzyme extract. The enzyme activity was described as decrease in A290 due to ascorbate oxidation ($E = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$). The enzyme activity was calculated in terms of μmol of ascorbate oxidized per minute at $25 \pm 2 \text{ }^\circ\text{C}$. Assays of POD (EC 1.11.1.7) activity were carried out using guaiacol as the hydrogen donor [41]. The reaction mixture consisted of 2.9 ml of a mixture containing 0.25 % (v/v) guaiacol in 0.01 M

sodium phosphate buffer (pH 6.0), 0.1 M H₂O₂ and 100 µl Enzyme extract. Activity of enzyme was monitored by the rate of guaiacol oxidation at 470 nm ($E = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$). Activity was expressed as the increase in absorbance at 470 nm $\text{mm}^{-1} \text{ mg}^{-1}$ protein.

Reverse transcription–qPCR (RT-qPCR)

Total RNAs were extracted using TRIzol reagent (Invitrogen Life Technologies). The quality and quantity of RNA samples were checked by measuring absorbance at OD 260/280 using a Nanodrop spectrophotometer (Biochrom WPA Biowave II, UK). The integrity of RNA was tested by running on 1.2% agarose gel electrophoresis. Contamination by genomic DNA was removed by *DNase* treatment (*DNase* I RNase-free, Thermo Scientific). gDNA contamination in RNA samples was checked by using ribosomal DNA (rDNA)-based primers. The sequences of *CAT*, *SOD* and *cAPX* and *pAPX* as target genes and *actin* as reference gene were obtained from nucleotide database at National Center for Biotechnology Information (NCBI). Primer of target and reference genes (except for APXs) were designed using Primer 3 software [42] and were synthesized by Metabion GmbH (Martinsried, Germany). The gene-specific primers of two isoform of cytosolic and peroxisomal APX were designed with the AlleleID v7.0 software (Premier Biosoft International, Palo Alto, CA). Taxa specific /cross-species assay was conducted with aligned sequences of various APX (different isoform) which able to detect *cAPX* or *pAPX* only. All designed primers have a length of 18-21 nts, GC content >40%, < 55% and primer pairs have similar melting temperatures (59-63°C). The length of the amplicons ranged from 78 to 175 bp. Primer sequences and GenBank accession numbers of these genes are presented in Table 1. The specificity of primers was tested by melting curve analysis. The cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen, Germany) according to the manufacturer's instructions. The Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) were used for measurement of the transcript levels of selected genes in CFX96 real-time PCR instrument (Bio-Rad). After amplification, all PCR reactions were subjected to a thermal melt with continuous fluorescence measurement from 55 to 95 °C for dissociation curve analysis. At least one non-template control (NTC) was used for each primer master mix. The threshold cycles (Ct) were automatically calculated for all reactions in the plate using the CFX manager software (Bio-Rad). All assays were carried out in two biological replications, as well two technical repetitions (two technical repetitions for each biological replicate). The mean values for each assay were obtained, and used for further analysis. The Livak ($2^{-\Delta\Delta C_t}$) method [43] were used for calculation of relative gene expression ratio.

Table 1. The list of primer information was used in this study.

Accession no.	Gene name	abbreviation	organism	Primer sequence	Amplicon length (bp)
HQ389206.1	Catalase	CAT	<i>Aeluropus littoralis</i>	CAACTTCCCCGTCTTCTTCA TGCGACAGAAAGTCGAACAC	119
HM107007.2	copper/zinc superoxide dismutase	Cu/Zn -SOD	<i>Aeluropus littoralis</i>	CAATGGCTGCATGTCAACT TGCTCCAGCTGTCACATTTC	113
JF907687.1	peroxisomal ascorbate peroxidase	pAPX	<i>Aeluropus littoralis</i>	ACGATGCTGGAACCTTACGA GGCTGTGCTCTTCCTCAA	78
JF819725.1	cytosolic ascorbate peroxidase	cAPX	<i>Aeluropus littoralis</i>	CTCCTACGCCGACCTCTA CATCTGCTTGACGAAGACTTG	175
FJ603097.1	beta actin like	actin	<i>Aeluropus laggocoides</i>	GGATCTTTACGGCAATGTC GGCGCAACTACCTTCACCT	123

Results

Changes in Chlorophyll and carotenoids content

The content of Chlorophyll (Chl) a showed a strong increase at 48 h during the salt stress treatment and then significantly decrease in the end of the salt stress treatments (Fig. 1a). During the recovery condition the Chl a abundance tends to go back to control levels, without reaching this prior value. It appears that the Chl a content is stable during the recovery phase. In contrast to this a significantly increase in Chl b content (Fig.1b) and subsequent total Chl/Cars ratio (Fig 1c) were observed at S3 and R2 time-points ($p < 0.01$). In the Chl a/Chl b ratio, the significant difference was observed among R1 time-point and others.

The detectable amount of |Cars showed a strong dynamic in response to the change of condition. After an increase at the time-point S2 during stress a strong decrease at the later stress time-pint was detectable. During the regeneration phase the opposite pattern was detectable. A reduction at R2 was shown, which returned back to the level similar to control condition.

The amount of total Chl content was increasing over the entire stress treatment compared to control (Fig. 1F). This increase is based on the early accumulation of Chl a (S1 and S2) and late accumulation of Chl b (S3). During the regeneration phase R1 and R3 showed a decrease in their contents and in the middle of the regeneration (R2) a strong peak of total Chl based on Chl b abundance was detected.

ICP–OES Analysis

ICP-OES technique was done to determine the composition of elements in treated samples using plasma absorption emission spectroscopy technology. Sodium ion (Na^+) in salt stressed plant significantly increased in S2 and S3 time-points, and were decreased in R2 and R3 condition as expected (Fig. 2a). The

most variation in Na^+ concentration were observed in root tissue by highest and lowest level of Na^+ in salt stress and recovery condition, respectively. No significant differences were observed in Na^+ concentrations between leaf and stem in recovery condition. In contrast, the accumulation of potassium ion (K^+) in leaf and stem were decreased in salt stress, and were increased in recovery condition (Fig. 2b). K^+ accumulations in root significantly increased after 48 h treatment with 600 mM NaCl in compare to control, and then back to control level after one week. By transferring the plant to control solution, the K^+ concentration was declined significantly compare to control. Increasing in ratio of Na^+/K^+ were observed in S2 and S3 in while the decreasing trend were seen in recovery conditions (Fig. 2e). Despite the decreasing trend which was observed, their ratio was still higher than to control.

The calcium ion (Ca^{2+}) content significantly declined in leaf, root and stem during salt stress compare to control, whereas it increased significantly in leaf and stem during the recovery condition (Fig. 2c). It should be noted that, the no significant difference was observed in root Ca^{2+} content in salt stress compared to recovery condition. The trend of $\text{Na}^+/\text{Ca}^{2+}$ ratio was similar to Na^+/K^+ ratio with minor difference (Fig. 3f).

The magnesium (Mg^{2+}) concentration in the leaf gradually declined in salt stress, and after transferring to recovery condition it was gradually increasing (Fig. 2d). In the analyzed stem tissue, a sharp decrease was observed during salt stress (with no significant difference among stress time-points), and then the gradually increased were observed during recovery condition. Although the accumulation of Mg^{2+} in root were significant among control and other samples, but its fluctuation was not high except the R1 that the maximum level of Mg^{2+} accumulation has been observed in this time-point.

Proline content, Total protein and ROS enzyme activities

Proline as a stress-responsive amino acid plays a crucial role in plants, by protecting the plants from various kinds of abiotic stresses. The proline content was significantly lower in leaf than root during salt stress and recovery condition (Fig. 3a). The proline content in salt stressed plant significantly increased rather than control, whereas a reduction was observed in recovery condition in both leaves and roots. In leaf tissue, the proline content increased at S1 time-point, then it decreased gradually in S2 and S3 stressed time-points and recovery condition. In roots, a significant enhancement was observed at S1 time-point, then decreased suddenly at S2 time-point (Fig. 3a).

Total protein in salt stressed plant and recovery condition significantly decreased rather than control in both leaves and roots (Fig. 3b). An increase was observed in leaf tissue at R1 condition in comparison to the stressed time-points, while the lowest amount of protein was obtained at R2 condition. As the results showed in Fig. 3b, the protein content in leaves was higher than roots. The same pattern was observed in roots in stressed plants, however the difference was in R1 and R2 condition.

In the recovery condition, the CAT activity was higher than control and stressed condition in leaf (Fig. 3c). The highest and the lowest CAT activity occurred at R1 and S2 condition, respectively. In roots, the CAT activity increased at S1, S2 and R1, R2 condition, but suddenly decreased significantly in S3 and R3 time-points. The SOD activity increased in both leaf and root compare to the control (Fig. 3d). Stressed condition caused a profound increase in SOD activity to maximum level at S3 time-point, however it reduced suddenly in recovery

condition in leaf. In recovery condition, there was no significant difference from R1 to R3 for SOD activity in root, but an increase was observed in stressed plant.

The increasing changes of APX activity in leaf during stressed condition showed that the highest APX activity occurred at S3 time-point (Fig. 2e). APX activities decreased significantly in recovery condition. In root tissues, the highest APX activity was observed at S2 time-point, while the lowest was at R1 condition. In stressed plant, the POD activity significantly enhanced up to the highest level at S3 time-point, while it decreased in recovery condition in both leaf and root (Fig. 2f). In recovery condition, the POD activity gradually decreased in leaf from R1 to R3, but in roots it did not follow the specific pattern.

RT-qPCR analysis of Antioxidant related genes

The levels of mRNA encoded by genes with a related function to antioxidant activity were tested by real-time qPCR. The four genes *CAT*, *SOD*, *cAPX* and *pAPX* were tested for their relative mRNA abundance under salt and recovery conditions. As in our experimental system the salt is taken up via the root system and extruded via the leaves, the analysis was performed in these two tissues. A strong decrease in *CAT* mRNA level was detectable in leaf tissue at time-point S2 (Fig. 4 a). Even during stress conditions the *CAT* mRNA level returned to control niveau at S3 and stayed mainly unchanged during the recovering phase. In roots an early increase of *CAT* mRNA is detectable in line with the early root response and uptake of salt via the root system. The mRNA level remained on a higher niveau, compared to control, but does not show further modulations.

According to the results of transcript levels in leaf, the *SOD* transcript level in R3 condition was the lowest and the highest *SOD* transcript level presented in S2 time-point (Fig. 4b). In roots, the *SOD* transcript level were up-regulated significantly compare to the control, but in recovery condition it gradually decreased rather than stressed one. The pattern of *cAPX* transcript level was highly similar to *SOD* mRNA: both in leaf and roots. The *cAPX* transcript levels of leaf tended to decrease in duration of recovery condition rather than stressed plant (Fig. 4c). The pattern of *pAPX* transcript level in roots and leaves indicated an increase during salt application and a strong reduction during the recoveryphase (Fig. 4d)

Discussion

The response of antioxidant enzyme activity in *Aeluropus* during high salt concentration and recovery phase

Plants are able to cope with various extreme environmental conditions. One of them is salinity of the soil. *Aeluropus littoralis* represents one outstanding example of a well adapted poacea, able to survive and life in high salt containing soil. Thus, understanding the potential salinity tolerance may cause to develop new salt tolerant varieties. The result of this experiment showed that the antioxidant enzyme and non-enzymatic antioxidants of *A. littoralis*, including SOD, CAT, APX, POD and proline, total protein contribute to overcome and reduction of ROS concentrations. The response of investigated enzymes was variable at different times-courses of salinity and recovery condition. ROS are known as cytotoxic compound that have important roles in homeostasis and cell signaling, with in which activates antioxidant defense mechanisms [44]. SOD is one of the most important front line enzymes in cellular defense against ROS attack, since it could scavenge rapidly the $O_2^{\cdot-}$ into H_2O_2 [45]. It has been demonstrated that CAT and APX scavenge the H_2O_2 which is produced by interacting under oxidative stress [46].

CAT catabolizes H_2O_2 to water and dioxygen [47], and APX reduces H_2O_2 to water and generates Malondialdehyde, a biomarker of oxidative damage [48]. It was showed that APX activity increased under salt stress by applying paclobutrazol treatment [49]. The NaCl increase promotes oxidation of enzymes and leads to rapid cellular damage [5]. In this study, the changing of SOD activity was related with the level and duration of salinity. There were no significant changes in recovery condition in root, however, SOD activity increased with salinity increasing in both leaf and root (Fig. 3d).

There was different between CAT and SOD activity with salinity increasing and duration. They could act to scavenge H_2O_2 under stress, rapidly. SOD activity was more responsive to the variation in higher duration of salinity compared to CAT. The highest SOD activity in leaf occurred after one week of storage, which proposed that H_2O_2 content reached the lowest level. Furthermore, the different changing for CAT and SOD activity indicated the importance of SOD rather than CAT in oxidative damage as the stress-time duration extended. These two enzymes may interact with each other and have complementary roles. The activities of SOD and POD in leaf showed similar trends. The reductions in CAT, APX, SOD and activities could be due to accumulation of ROS to toxic levels [50]. Minimizing the ROS damages through scavenging ROS by antioxidant enzymes was reported [3].

The response of proline

The accumulation of proline, as one of the major metabolic responses to abiotic stress[51], was shown to scavenge ROS [52]. In our study, proline content tended to increase during salt treatment. It was found that proline accumulation could induce adaptive advantages under stress. However, the increasing of proline content may also be considered as a stress-induced marker to identify oxidative damage [53]. As the proline content was significantly increased during salt stress, but decreased in recovery condition (Fig. 3a). Meanwhile, antioxidant enzyme activity also tended to increase in salt stress during different time courses, but decrease in those recovery condition (Fig. 3c-f). This result suggested that both proline and antioxidant enzymes played a more important role in adapting to oxidative stress caused by salinity.

The response of antioxidant related gene

To illustrate the role of the ROS scavenging system during stress and recovery condition, we analyzed the transcript levels of antioxidant enzyme related genes of *A. littoralis* by real-time PCR. As expected from other studies these genes are responding to salt stress treatment. Here we could show that the root response is stronger or farster, in line with the earlier exposition of the salt. The changes in transcript levels of APX were not consistent with the APX activity neither in leaf nor root in stress and recovery condition (Fig. 4c, d). It indicated that APX was not the main enzyme contributing to total APX activity. The transcript levels of *CAT* in leaf were consistent with CAT activities at different time courses, suggesting that CAT might be the main H_2O_2 -scavenging enzyme to keep the balance of redox reaction in *A. littoralis*. From these results, the expressions of *APX* and *SOD* were suppressed during salinity caused to accumulation of H_2O_2 . Similar results have been reported in rice [46]. Interestingly SOD and cAPX show a very similar pattern of mRNA abundance. This indicates a similar regulation via a common regulatory hub or transcriptionfactor. Overexpression of the investigated antioxidant genes such as SOD, APX, and CAT was reported in wheat [54], Arabidopsis [55] and oat [56].

The response of ions

High concentrations of Na^+ decreases the amounts of available K^+ , Mg^{2+} and Ca^{2+} by displacing membrane-bound Ca^{2+} [57]. Moreover, Na^+ may interfere with the function of K^+ , as a cofactor, and then have a direct toxic effect [58]. In our experiments, absorption of Na^+ and K^+ seemed to be competitive processes. The Na^+ concentrations increased mainly in stressed condition, while K^+ concentrations reduced specially in roots and stem. This phenomenon occurred because halophytes need less K^+ than glycophytes for growth [59], confirming the capability to substitute K^+ with Na^+ and finally salt tolerance. This is also the case that was reported by Belkheiri and Mulas (2013) in *Atriplex halimus* [60].

The Ca^{2+} and Mg^{2+} contents were extremely low in the leaves, stem and roots of both stressed and recovery condition, agreeing with results found for other halophytes [58, 60]. The toxic solutes raised the cytoplasmic K^+/Na^+ ratio by increasing the salt concentration in the culture medium. So, the trend is towards a higher increase in Na^+ concentration and a corresponding reduction in K^+ , Mg^{2+} , and Ca^{2+} ions. As a result, the tolerance to salt stress is bound through a common mechanism of Na^+ absorption for osmotic balance.

The response in Chlorophyll abundance

The plants of *A. littoralis* exposure with high concentration of salt (600 mM), and then the remained-stress seedlings had been returned to the control solution (7 days) for recovery. In this study, the light-harvesting pigments (chlorophylls) levels were not equally sensitive to salt stress and recovery condition (Fig 1). The reduction of Chl a one week after the salt stress could be attributed to the inhibition of chlorophyll biosynthesis and/or the stimulation of chlorophyll degradation [61]. On the other hand, plants under salt stress (S3) and recovery condition (R2) had more chlorophyll b content as compared to chlorophyll a. Also, the time-point of S3 and R3 had a significantly higher chl b as well as in Chl a/Chl b ratio than the other time-points. In chlorophyll cycle is known that inter-conversion of chlorophyll a and chlorophyll b levels occurs via 7-hydroxymethyl chlorophyll [62]. Generally, optimization of leaf function in salt stress calls for greater investment of leaf resources in light harvesting rather than energy processing.

Conclusion

The findings of this study point to some specific characteristics in *A. littoralis* by decreasing oxidative stress caused by ROS accumulation. It can be concluded that SOD activity was more responsive in higher duration of salinity to compensate the damages of ROS, indicating the importance of SOD in oxidative damage. The transcription levels of antioxidant genes emphasize the existence of metabolic and genetic capacities in a wide range to activate signaling and transcription pathways as a response to oxidative stress. Overexpression of antioxidant genes such as SOD, APX, and CAT was clear in salinity stress condition. The relation between the transcript levels of *CAT* and *CAT* activities in leaf suggested that *CAT* might be the main H_2O_2 -scavenging enzyme to keep the balance of redox reaction in *A. littoralis*. Otherwise, increasing the proline content may also be considered as a stress-induced marker to identify oxidative damage, suggesting more important role of proline to resolve the imbalance created by oxidative stress. The obtained results indicated the method of discharge ions which would be suitable for monitoring the membrane damage in roots and leaf. The salt stress tolerance is bound through a common mechanism of Na^+ absorption for osmotic balance due to a higher

increase in Na⁺ concentration and a corresponding reduction in K⁺, Mg²⁺, and Ca²⁺ ions. Plants under salt stress (S3) and recovery condition (R2) had more chlorophyll b content as compared to chlorophyll a that demonstrated optimization of leaf function in salt stress. We suggest that the higher antioxidant capacity and less damage indices may be specific for salinity sensitive species like rice to survive or promote their recovery phase. These results demonstrated important indices of halophyte describing salinity tolerance mechanisms in *A. littoralis*. Nevertheless, new research should be performed on the other components of salinity response to have better understanding of tolerance processes.

Abbreviations

ROS: reactive oxygen species; Chl: Chlorophyll; K⁺: potassium; Ca²⁺: Calcium; Mg²⁺: magnesium; CAT: Catalase; SOD: Superoxide dismutase; APX: ascorbate peroxidase; O₂^{•-}: superoxide; H₂O₂: hydrogen peroxide, HO•: hydroxyl radicals; ¹O₂: singlet oxygen; Cars: carotenoids; RT-qPCR: Reverse transcription–qPCR; NCBI: National Center for Biotechnology Information.

Declarations

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Authors' contributions

SH conducted all experiments, lab work, data analyses and interpretation of results. GA, GN and AY conceived and designed the study. SHH and FF wrote the manuscript. MK revised the manuscript, and had all critical supervision. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets measured and analyzed during the study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All procedure related to plant material used here were in compliance with national and international guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹ Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT), Sari Agricultural Sciences and Natural Resources University, PO Box 578, Sari, Iran. ² National Institute of Genetics Engineering and Biotechnology (NIGEB), Tehran, Iran. ³ Department of Plant Breeding and Biotechnology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran. ⁴ RG Heterosis, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany.

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Figures

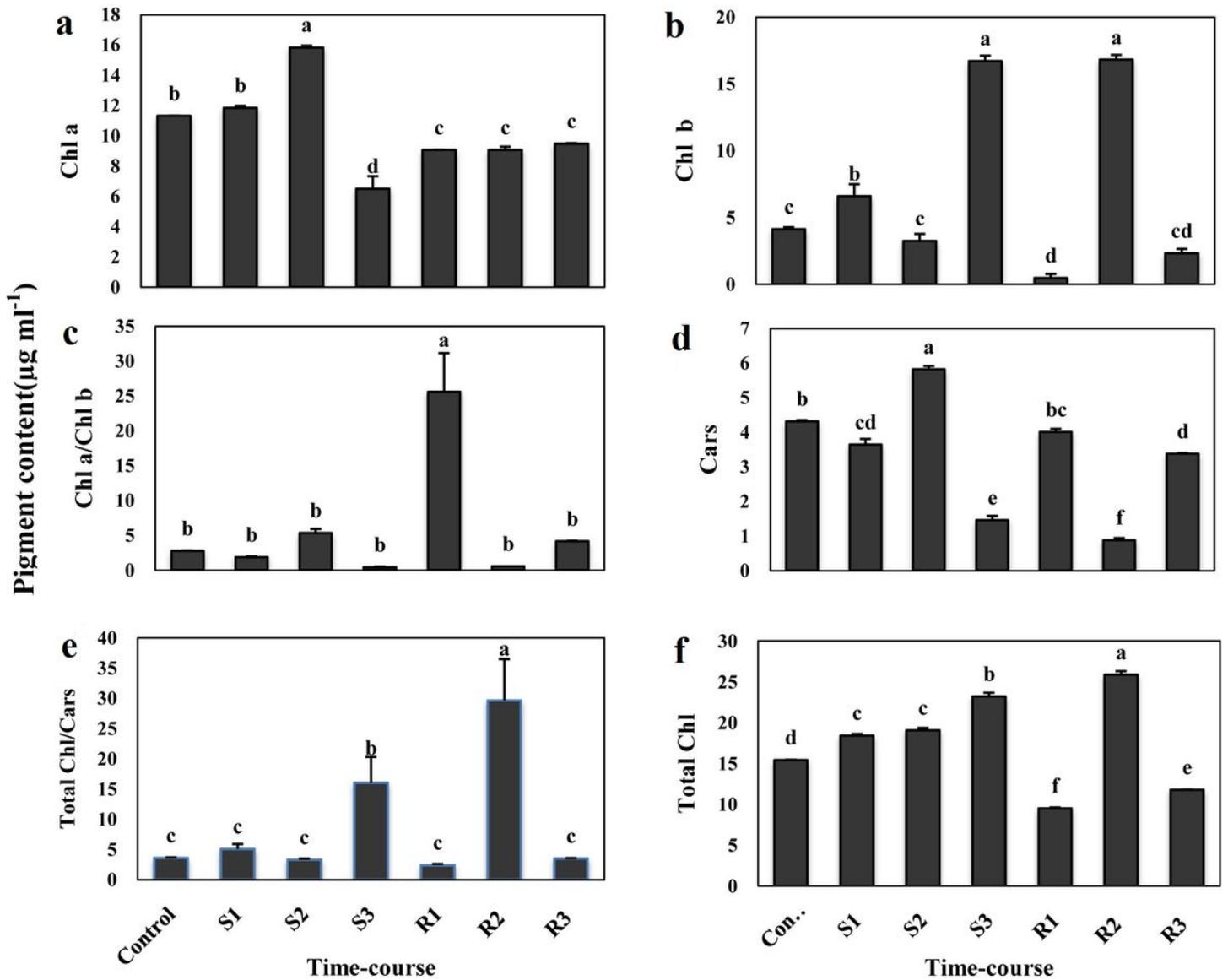


Figure 1

Chlorophyll a (Chla), chlorophyll b (Chlb), chlorophyll a to chlorophyll b ratio (Chla/Chlb), carotenoids (Cars), total chlorophyll to carotenoids ratio and total chlorophyll content of *Aeluropus littoralis*, time-course treated in salinity and recovery conditions. The values are mean (\pm SD) of three replicates.

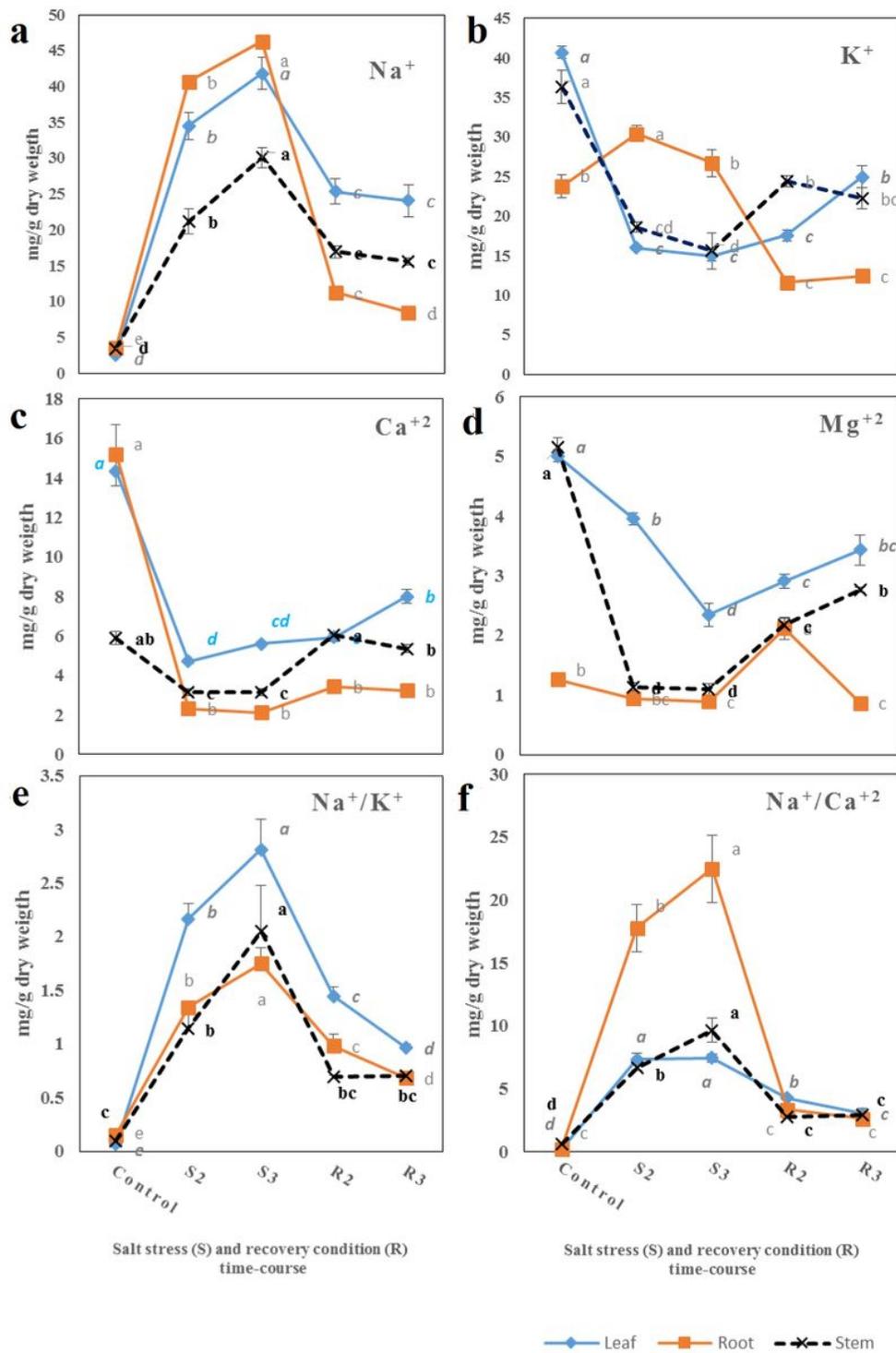


Figure 2

Sodium (Na^+) (a), potassium (K^+) (b), calcium (Ca^{2+}) (c) and magnesium (Mg^{2+}) (d), sodium to potassium ratio (Na^+ / K^+) (e) and sodium to calcium ratio ($\text{Na}^+ / \text{Ca}^{2+}$) (f) content in mg/gr dry weight of the leaf, root and stem of *Aeluropus littoralis*, time-course treated in salinity and recovery conditions.

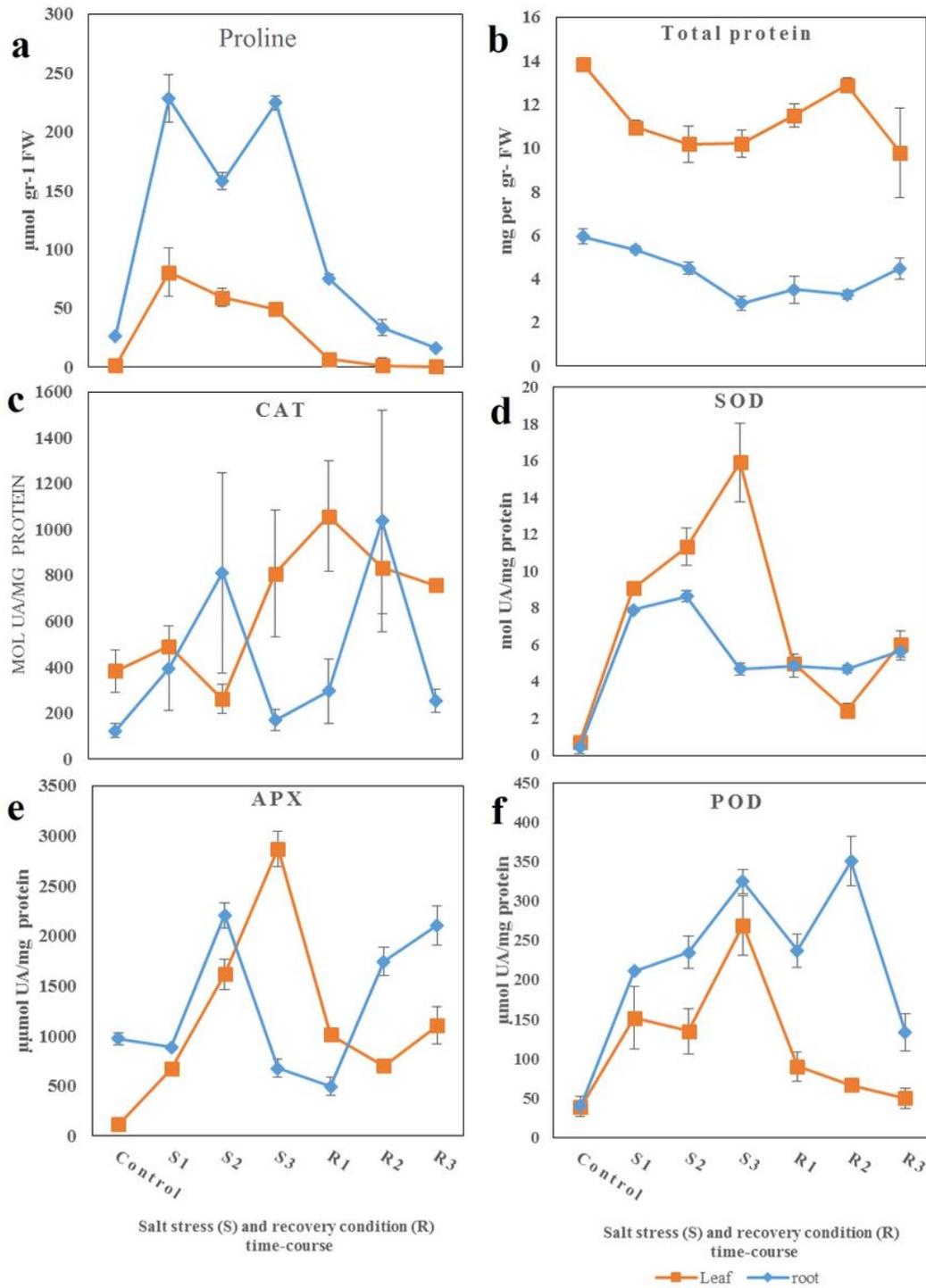


Figure 3

Effect of time-course salinity treatments and recovery conditions on the proline content (a), total protein (b), activity of catalase (CAT) (c), superoxide dismutase (SOD) (d) ascorbate peroxidase (APX) (e), and peroxidase (f) in leaf and root of *Aeluropus littoralis*. The error bars represent the standard deviation (\pm SD) for replicates.

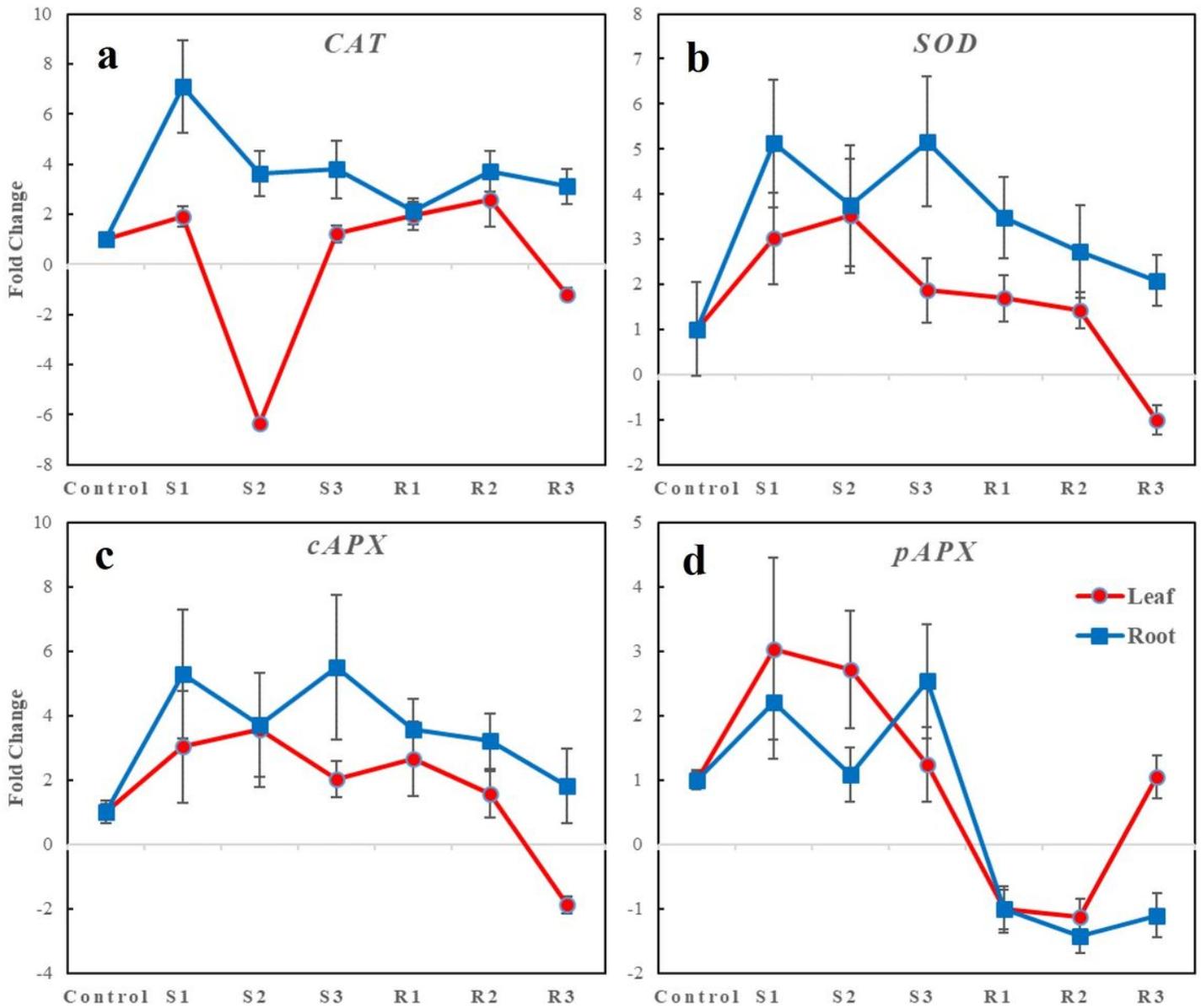


Figure 4

Effect of time-course salinity treatments and recovery conditions on the relative expression of catalase (CAT) (a), superoxide dismutase (SOD) (b), ascorbate peroxidase (cAPX) (c) and pAPX (d) in leaf and root of *Aeluropus littoralis*. The error bars represent the standard deviation (\pm SD) for replicates.