

# Physiological and molecular comparison of oxidative stress responses in *Oryza sativa* and *Aeluropus littoralis* under salt stress condition

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## Research Article

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# Abstract

The present study aimed to investigate the effects of different concentrations of NaCl on membrane stability, potassium ( $K^+$ ) to sodium ( $Na^+$ ) ratio, and the expression of stress-responsive genes in aerial and underground organs of *O. sativa* cv. IR64 and *Aeluropus littoralis* to shed more light on the salt stress adaptive mechanisms in the grass species towards the improvement of salt tolerance in rice. The expression of stress-responsive genes signified the importance of tissue-specific isoforms to circumvent the harmful effects of salinity. Among the antioxidant enzymes, catalase (CAT) showed a complete differential expression pattern in two species and its expression was induced in both organs of halophyte but was suppressed in IR64, suggesting it may function as a determining factor in salt adaptability differences in these two species. The cytoplasmic APX (cAPX) seems to be more effective than its peroxisomal counterpart (pAPX), confirming the specialized functions of subcellular isoforms in association with conferring salt tolerance in each species. In general, *A. littoralis* displayed a more coordinated function among different antioxidant genes, leading to a more elaborated adaptive mechanism to salinity stress in this halophyte, as evidenced by a higher  $K^+/Na^+$  ratio and lower membrane permeability. The finding of this study provide relevant information regarding the molecular mechanisms underlying salt adaptability in grasses and highlight the importance of some of the antioxidant enzymes in reducing the detrimental effects of high salt concentrations, which would be highly beneficial for utilization in crop improvement programs and incorporation of salt hardiness into elite rice cultivars.

## Introduction

Salinity stress is a major growth-limiting factors in most glycophyte plants, negatively affecting agricultural productivity and posing a significant challenge to sustainable agriculture and food production worldwide. More than 25% of the world's cultivated lands are salt-affected (Shahid et al. 2018) with this amount projected to increase to 50% of total irrigated lands worldwide by 2050 (Qureshi et al. 2007; Gupta and Huang, 2014). The situation is particularly critical in arid and semi-arid regions, such as Iran, where about 50% of the arable soils, representing 4.1 million hectares of irrigated croplands, are salt affected (Qureshi et al. 2007; Qadir et al. 2008). Anthropogenic activities, poor irrigation practices, and climate change-related events are the primary reasons for accelerating soil salinization. Given the rate of population growth, ensuring sufficient food production is one of the major challenges facing humanity.

Rice (*Oryza sativa* L.) is one of the staple cereal food crops that feeds approximately half of the global population and is expected to play a crucial role in meeting future food requirement (Bevilacqua et al. 2015). Estimates suggest that rice production needs to double by 2050 to keep up with the increasing global demand for food (Muthurajan et al. 2018). Although rice can grow successfully in a wide range of soils, its yield and quality are significantly influenced by various environmental stresses. Soil salinity is one of the most detrimental stresses that reduce growth, development and ultimately the yield of rice plants (Lokeshkumar et al. 2023). Rice is considered as the most sensitive cereal to salinity stress, with even moderate levels of soil and water salinity ( $30 \text{ mmol L}^{-1} \text{ NaCl}$ ) having a significant impact on its yield (Chen et al. 2021).

*Aeluropus littoralis*, an Iranian endemic halophyte grass of the Poaceae family, is highly tolerant to salt stress and can tolerate more than 600 mmol L<sup>-1</sup> NaCl concentration (Zouari et al. 2007). Genome sequence analysis shows a high level of homology between *A. littoralis* and *O. sativa* (Zouari et al 2007). Due to its high tolerance to salinity, *A. littoralis* is suggested to be highly beneficial for understanding the salt tolerance mechanism in halophytes and a valuable genetic resource for improving the salt tolerance characteristic of glycophytes (Kakeshpour et al. 2017).

Plants have evolved various adaptive mechanisms to cope with multicomponent salinity stress. Accumulation of compatible solutes and induction of antioxidant systems capacity are among the main modulating mechanisms that plants use to reduce the detrimental effects of salts stress (Kibria et al. 2017; Faghih et al. 2019). Accumulation of reactive oxygen species (ROS) is one of the main responses of salt-treated plants and functions as an important signaling component leading to rearrangement of plant defensive systems in response to the salt stress. The major ROS produced by different intracellular compartments of plants in response to salinity include superoxide anion (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radical (OH<sup>-</sup>), and singlet oxygen (<sup>1</sup>O<sub>2</sub>) (Chen et al. 2021). High levels of ROS can damage plant cell constituents, accelerate membrane lipid peroxidation, increase electrolyte leakage, and disrupt chloroplast function, leading to a substantial reduction in carbon fixation and subsequent cell death (Faghih et al. 2017).

Plants use both enzymatic and non-enzymatic antioxidant defense system to minimize the detrimental effects of ROS (Azemi-Ardakani et al. 2020). Enzymatic antioxidant defense systems, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (POX) and catalase (CAT), are considered as the first line of defense against oxidative stresses in plants. These enzymes play a pivotal role in plant adaptability to salt stress by scavenging ROS and protecting plant cells from oxidative damage.

The adaptation capacity to salt stress in halophytes is generally more efficient than in glycophytes. This may be due to the differences in the expression levels of antioxidant genes and/or other physiological adaptive systems. As the antioxidant enzyme activities act as the fastest plant response to deal with oxidative burst, it is likely that the ROS-scavenging system in *A. littoralis* functions more efficiently than in *O. sativa*.

The development of salt-tolerant rice varieties is considered a promising approach to boost rice yields in salt-affected areas, in line with the increasing demand for rice production to keep pace with the food requirements of growing global population (Lokeshkumar et al. 2023). Comparative analyses between taxonomically related plants can help clarify underlying mechanisms of stress response (Al Hassan et al. 2016). In fact, elucidating the contrasting features of a physiological responses between plant species can be highly beneficial in uncovering the underlying mechanisms of stress tolerance. Such understanding can inform the development of crop improvement strategies. Therefore, a comparative study of the contents of monovalent ions, membrane stability, and transcript expression of antioxidant-related genes of *O. sativa* and *A. littoralis* under salt stress conditions can shed more light on the relevant mechanisms of salt stress tolerance/sensitivity between these two species displaying distinct reactions in response to salinity stress. The results of such investigations could be a highly beneficial resource for elucidating the mechanisms participating in higher salt stress tolerance in halophytes than glycophytes and can subsequently be utilized to improve salt stress hardness in rice and other glycophytes.

# Materials and methods

## Plant materials

The seeds of *A. littoralis* were purchased from the Center of Agricultural Research and Natural Resources of Isfahan and were planted in sterilized bed under controlled condition (photoperiod of 12 h light and 12 h dark and temperature 22/18°C day/night) in growth chamber of laboratory of biotechnology institute, Shiraz University. After sprouting, a pretest was conducted to determine the most suitable nutrient solutions for *A. littoralis*, and it was found that Yoshida was the most suitable. The pH of nutrient solution was adjusted to 5.5 daily, and the nutrient solution was completely refreshed on a weekly basis.

The seeds of *O. sativa* cv. IR64, a sensitive variety to salinity (Priya et al. 2015), were provided by the rice research institute of Amol, Iran. After surface sterilization, the seeds were planted under the same condition as *A. littoralis*.

## Salinity treatment and sampling

Three weeks after seedling transplantation, the salinity treatment was initiated. In *A. littoralis*, the NaCl concentration in the hydroponic solution was gradually increased to 100 mM every 48 hours until reaching a final concentration of 300 mM. Similarly, the NaCl concentration in the hydroponic solution of *O. sativa* was increased in three stages with 48-hour intervals (30, 60 and 100 mM) to a final concentration of 100 mM. The electrical conductivity (EC) of the nutrient solution was measured throughout the experiment to monitor the salt concentration.

In both *A. littoralis* and *O. sativa*, the sampling was conducted at specific time intervals after each salt treatment to evaluate the physiological and biochemical responses to salinity stress. In *A. littoralis*, the sampling was conducted from the leaves and roots at 2, 4, 8, 24, and 48 hours after each treatment of 100, 200, and 300 mM NaCl. In *O. sativa*, the sampling was conducted from the leaves and roots at 2, 4, 8, 24, and 48 hours after treatments of 30, 60, and 100 mM NaCl.

## Plant growth parameters

To determine the effects of salinity stress on plant water status, the fresh weight of the samples was measured immediately after sampling, and the weight of the dried samples was determined after 48 hours of oven drying at 75°C. To estimate the membrane stability index (MSI), electrolyte leakage was measured following the method described by Sairam et al (2002). The samples, both treated and control, were immersed in 10 ml of distilled water for 24 hours at room temperature. The electrical conductivity (EC) of the solution was then recorded as an index of initial leakage ( $EC_{1c}$  as initial leakage of control samples and  $EC_{1t}$  as initial leakage of treated samples). The final leakage index was measured after heating the samples at 121°C for 30 min ( $EC_{2c}$  as final leakage of control samples and  $EC_{2t}$  as final leakage of treated samples). The following equations were used to calculate the MSI of the treated ( $MSI_t$ ) and control ( $MSI_c$ ) samples:

$$MSI_t = 1 - \left( \frac{EC_{1t}}{EC_{2t}} \right) \times 100$$

$$MSI_c = 1 - \left( \frac{EC_{1c}}{EC_{2c}} \right) \times 100$$

The Na<sup>+</sup> and K<sup>+</sup> content of the plant samples was also determined to assess the effects of salinity stress on ion homeostasis. The wet ashing method, as described by Enders and Lehmann (2012) was used to prepare the sample for analysis. The ashed samples were then analyzed using a flame photometer (PFP7, Jenway, Dunmow, UK) to quantify the Na<sup>+</sup> and K<sup>+</sup> content.

## RNA extraction and cDNA synthesis

Total RNA was extracted from the plant samples using the DENAzist Column RNA Isolation Kit (DENAzist Asia Co., Mashhad, Iran). The purity and concentration of the RNA were determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The integrity of the RNA was checked by running it on an agarose gel electrophoresis. To eliminate the possibility of genomic DNA contamination, the extracted RNA was treated with DNase I (Thermo®) according to manufacturer's instruction. The RNA was then checked for the absence of genomic DNA contamination by running it on an agarose gel electrophoresis.

One microgram of DNase I-treated RNA was used as a template for cDNA synthesis using the Thermo Scientific Revert Aid first strand cDNA synthesis Kit according to the manufacturer's instruction. To enhance the efficiency of cDNA synthesis, a combination of oligo (dT) and random primers were used. The resulting cDNA samples were diluted and stored at -20°C for qPCR analysis.

## Primer designing and qPCR analysis

To investigate the expression of antioxidant genes in response to salinity stress, specific primers were designed based on the conserved sequences of the respective genes in NCBI and used for PCR amplification using Supreme HF DNA Polymerase (GenetBio, South Korea). The resulting amplicons were purified from 1.5% agarose gel using a DNA recover kit (Vivantis, Malaysia) according to the manufacturer's instructions. The purified fragments were then cloned in *Escherichia coli* strain DH5α using pTZ57R vector. The amplified plasmids were purified from the bacteria cultures and sequenced in both the forward and reverse directions using universal M13 primers by Macrogen (South Korea). Specific primers for qRT-PCR of each antioxidant genes were designed based on the sequencing results, using Vector NTI v.10 (Lu and Moriyama, 2004) and Primer-Premier v.5 (Premier Biosoft Interpairs, Palo Alto, CA) (Table 1). The specificity of the designed primers was confirmed using the Primer BLAST tool of NCBI and the primers were synthesized by Macrogen (Seoul, South Korea).

Table 1

Specific primer used for expression analysis of some stress-related genes in *Oryza sativa* and *Aeluropus littoralis*.

| Plant species        | Gene             | Accession number | Primer sequences (5'–3')                                                                   | Annealing temperature (C) | Product size (bp) |
|----------------------|------------------|------------------|--------------------------------------------------------------------------------------------|---------------------------|-------------------|
| <i>O. sativa</i>     | <i>Cu/Zn SOD</i> | XM_015777123.2.2 | Forward Primer:<br>GTCACCGCTGGAGAAGATGG<br><br>Reverse Primer:<br>GATGATCCCGCAAGCAACAC     | 63                        | 189               |
|                      | <i>CAT</i>       | NM_001401748.1   | Forward Primer:<br>CGGCGTCAACACCTACACC<br><br>Reverse Primer:<br>GAAGTTTCCTGCTGCGATGG      | 61                        | 178               |
|                      | <i>cAPX</i>      | NM_001402136.1   | Forward Primer:<br>GCCAGTGTGAACCAGCAGAC<br><br>Reverse Primer:<br>AACGCAATACCGCATTTCATACC  | 62                        | 175               |
|                      | <i>pAPX</i>      | XM_015778949.2   | Forward Primer:<br>GGAGTTGTTGCCGTTGAAGTC<br><br>Reverse Primer:<br>CGCCTGAGAGTGCTACTATGTC  | 61                        | 181               |
|                      | <i>GR</i>        | NM_001401997.1   | Forward Primer:<br>CCAAACGGCAGGAGAAGAC<br><br>Reverse Primer:<br>AGCAGCAGACGGGTGAATAC      | 61                        | 182               |
|                      | <i>ubq</i>       | XM_015766305.2   | Forward Primer:<br>GCGGAAGTAAGGAAGGAGGAG<br><br>Reverse Primer:<br>CAGAGGTGATGCTAAGGTGTTC  | 61                        | 116               |
|                      | <i>elf</i>       | XM_015774317.2   | Forward Primer:<br>ACGGTGATGCTGGTATGG<br><br>Reverse Primer:<br>GGTTGGGTCCTTCTTCTCC        | 60                        | 155               |
| <i>A. littoralis</i> | <i>Cu/Zn SOD</i> | HM107007.2       | Forward Primer:<br>GGTGTC AAGGGCTCCATCTTC<br><br>Reverse Primer:<br>CGGATCTCATCTTCTGGTGCTC | 63                        | 197               |
|                      | <i>CAT</i>       | HQ389206.1       | Forward Primer:<br>CGCACCACAACAACCACTAC                                                    | 61                        | 184               |

| Plant species | Gene        | Accession number | Primer sequences (5'–3')                    | Annealing temperature (C) | Product size (bp) |
|---------------|-------------|------------------|---------------------------------------------|---------------------------|-------------------|
|               | <i>cAPX</i> | JF907687.1       | Reverse Primer:<br>GGCTGCTTGAAGTCGTTCTG     | 60                        | 147               |
|               |             |                  | Forward Primer:<br>CTCCTACGCCGACCTCTACC     |                           |                   |
|               | <i>pAPX</i> | JF819725.1       | Reverse Primer:<br>CATCTGCTTGACGAAGACTTGC   | 62                        | 175               |
|               |             |                  | Forward Primer:<br>CACGATGCTGGAACCTTACGAC   |                           |                   |
|               | <i>GR</i>   | XM_012849171.3   | Reverse Primer:<br>CCTTGGATTCTTTGCTTTGATGG  | 57                        | 131               |
|               |             |                  | Forward Primer:<br>GGATGCTGCTCTGGATTTG      |                           |                   |
|               | <i>βact</i> | FJ603097.1       | Reverse Primer:<br>TCTTCTGCCGAATAAACAC      | 60                        | 113               |
|               |             |                  | Forward Primer:<br>TTGCTGGCCGAGACCTTAC      |                           |                   |
|               | <i>elf</i>  | XM_004962214.4   | Reverse Primer:<br>GGCGAGCTTTTCCTTGATG      | 63                        | 90                |
|               |             |                  | Forward Primer:<br>ACCTTCTCTGAATACCCTCCTCTG |                           |                   |
|               |             |                  | Reverse Primer:<br>CTTCTCCACACTCTTGATGACTCC |                           |                   |

The qRT-PCR was conducted in triplicates for each sample using a Real-Time PCR thermocycler (Applied Bioer, LineGeneK, China) and RealQ Plus master mix green (Ampliqon, Denmark). The amplification was performed as follows: 5 minutes at 95°C for initial denaturing, followed by 40 cycles of 95°C for 10 seconds, a specific annealing temperature for each primer for 20 seconds, and 72°C for 20 seconds. To normalize the calculation of gene expression, specific elongation factor  $\alpha$  (*ef $\alpha$* ) and Ubiquitin 5 (UBQ5) of rice, as well as  $\alpha$  and Beta Actin ( $\beta$ Act) of *A. littoralis*, were used as reference genes. The mean Ct values of technical and biological replicates were normalized against the mean values of the two housekeeping genes. The relative quantitative expression was determined using the  $2^{(-\Delta\Delta Ct)}$  method (Livak and Schmittgen, 2001).

## Experimental design and statistical analysis

The experiment was conducted based on the completely randomize design, with three replications. After data collection, analysis of variance (ANOVA) was performed using Minitab software (v.16) and the means were compared according to t-Student Test at a significant level of 5%.

## Results

The preliminary experiment revealed that the membrane stability index (MSI) of control rice plants increased during growth in both aerial and underground organs. However, the MSI was significantly reduced in the salt-treated plants from the lowest NaCl concentration (30 mM) and continued during higher concentrations (Supplementary Fig. 1). Although the MSI reduction was lower in the aerial parts compared to the underground parts, the 60 mM salinity stress significantly affected this index. Therefore, a gradual increase in concentrations of 30–100 mM was applied during the complementary experiment.

The MSI of NaCl-treated *A. littoralis* was significantly reduced in salt concentrations above 200 mM, indicating that the plants were suffering from salinity and were in critical stressful conditions. Therefore, NaCl concentrations up to 300 mM were selected for *A. littoralis* treatment. Similarly, the aerial parts of this plant had higher MSI than root tissues.

## **K<sup>+</sup>/Na<sup>+</sup> ratio**

Preliminary studies also showed that roots of *A. littoralis* had a higher K<sup>+</sup>/Na<sup>+</sup> ratio at the beginning of the experiment and a gentler decreasing trend for this ratio during salt addition than *O. sativa* (Supplementary Fig. 2). The K<sup>+</sup> content of IR64 was nearly half of the *A. littoralis* and showed a more intense decreasing trend under salinity treatments (Fig. 1). In the root of *A. littoralis*, the accumulation rate of Na<sup>+</sup> was higher than the rate of K<sup>+</sup> reduction, and the content of K<sup>+</sup> was not tremendously affected even at very high NaCl concentrations. In contrast, in the root of *O. sativa*, a significant reduction in K<sup>+</sup> content was accompanied by an increase in Na<sup>+</sup> content, indicating that K<sup>+</sup> uptake was extremely impacted by Na<sup>+</sup> accumulation.

Similarly, the areal parts of *A. littoralis* also had a higher ratio of K<sup>+</sup>/Na<sup>+</sup> and a slower decreasing trend than *O. sativa*. Although showing an overall decreasing trend during salt treatment, the K<sup>+</sup> content had higher fluctuation in the aerial parts of *A. littoralis* than in their underground parts. The K<sup>+</sup> and Na<sup>+</sup> contents of aerial parts of *O. sativa* remained unchanged even at 100 mM NaCl but were significantly affected by NaCl concentrations above 100 mM, indicating lower susceptibility of the aerial parts compared with the root tissues at moderate salt stress (Fig. 1). However, the K<sup>+</sup>/Na<sup>+</sup> ratio of aerial parts fluctuated greatly at low and moderate salt concentrations but had a decreasing trend during the experiment and reached the lowest value at 200 mM NaCl concentration.

## **Expression analysis of antioxidant genes**

qRT-PCR analyses were conducted for six NaCl concentrations/time in three biological and two technical treatments for both aerial and underground parts of the two species to investigate the probable association between the expression of these genes and sensitivity/tolerance to salt stress.

### **Cu/Zn SOD**

The expression trend of Cu/Zn SOD was different in the roots of *A. littoralis* and *O. sativa* (Fig. 2a). In *A. littoralis* the relative expression of Cu/Zn SOD was significantly increased at 100 mM NaCl and retained this high expression for 6 h at 200 mM, then decrease in the three next time points (6 h, 48 h and 144 h) at 300 mM NaCl, before increasing again and recording the highest expression at the last stage (264 h at 300 mM).

However, in *O. sativa*, the highest expression was recorded at 30 mM NaCl treatment, and the expression significantly decreased over time, reaching the lowest level at the last stage (264 h at 100 mM).

A completely different trend of expression was recorded in the aerial parts of these two species compared with the underground tissues (Fig. 2b). In *A. littoralis*, the expression of Cu/Zn SOD gradually increased, reaching the highest value at 48 h treatment in 300 mM and then significantly decreased in two last stages below the control plants. The similar trend was observed in *O. sativa* but the highest expression was recorded after 144 h in 100 mM NaCl treatment.

## CAT

The CAT expression was also different in the two species. *A. littoralis* had significantly higher expression in the root of salt-treated plants than control, while *O. sativa* showed a lower expression than control plants in most sampling times (Fig. 2c). After an initial increase at 6 h in 100 mM NaCl concentration, the expression of CAT decreased in both species, but again increased in halophyte species and a second peak was detected during the salinity increment. In *A. littoralis*, the highest expression of CAT (three-fold than control) was recorded after 144 h exposure to 300 mM NaCl, while in *O. sativa*, the highest expression (1.8-fold increase than control) was recorded after 6 h exposure to 30 mM NaCl.

The expression of CAT was higher than untreated plants in most sampling times in the aerial parts of *A. littoralis*, with the highest expression (3.56-fold increase than control) was recorded after 48 h exposure to 300 mM NaCl (Fig. 2d). Similarly, the expression of CAT was lower in the aerial parts of the salt-treated *O. sativa* plants than control and *A. littoralis* plants in most sampling times. However, a gradual increasing followed by a gradual decreasing expression forming a bell-shape trend during the experiment, with the highest expression recorded after 6 h exposure to 100 mM salinity.

## Ascorbate peroxidase (APX)

The study found that in the root tissues of both *A. littoralis* and *O. sativa*, the expression of cytosolic APX showed a similar increasing trend during the experiment with an increment in the salt concentration (Fig. 2e). The highest expression of cytosolic APX was observed after 144 h exposure to 300 mM NaCl for *A. littoralis* and after 48 h exposure to 100 mM NaCl for *O. sativa*. Compared to control plants, the expression of cytosolic APX was significantly increased by 2.59 and 2.57-fold in *A. littoralis* and *O. sativa*, respectively. In contrast, there were no significant differences in the expressions of peroxisomal APX in the roots of treated and control *A. littoralis* in most stages, although a significant higher expression (3.14-fold increase) was recorded after 48 h in 300 mM NaCl treatment (Fig. 2g). Similarly, in the roots of salt-treated *O. sativa*, only 30 mM NaCl treatment induced a significant increase (1.6-fold increase) in the expression of peroxisomal APX compared to control plants, and no significant differences were detected between the expression of salt-treated and control plants afterward.

In the aerial parts of *A. littoralis*, the expression of cytosolic APX initially increased in response to salinity, but then decreased to a lower expression than control plants from 48 h in 300 mM NaCl to the end of experiment (Fig. 2f). This is in contrast to the expression of cytosolic APX in the aerial parts of *O. sativa*, which showed an increasing trend during salt treatment and recorded the highest expression (2.34-fold increase than control) after 144 h exposure to 100 mM NaCl concentration.

Regarding peroxisomal APX expression, only one of the sampling points (48 h exposure to 300 mM NaCl) had significantly higher expression (3.7-fold increase) of peroxisomal APX in the aerial parts of salt-treated *A. littoralis*, while the remaining treatments showed lower expression in the salt-treated plants than control ones (Fig. 2h). In the aerial parts of *O. sativa*, the peroxisomal APX showed no significant differences until 6 h exposure to 100 mM NaCl, after which a significant higher expression was recorded at the three last stages than control plants.

## Glutathione reductase (GR)

In the roots of salt-treated *A. littoralis*, the expression of GR showed an increasing trend from the initiation of salt treatment, reaching the highest values (2.94-fold increase than control) after 6 h exposure to 300 mM NaCl (Fig. 2i). However, it decreased significantly to a lower level than control for the two next stages, and then increased again at the final sampling time. In the roots of *O. sativa*, GR was significantly induced after 6 h exposure to 30 mM NaCl (7.29-fold increase than control), but then reduced to a lower level than control plants for the two next stages. It increased slightly after 48 and 144 h exposure to 100 mM NaCl.

In contrast, the aerial parts of salt-treated *A. littoralis* and *O. sativa* showed an upward trend for GR during the experiment, with higher expression than control plants in all sampling times (Fig. 2j). The relative expression of GR in the aerial parts of *O. sativa* was incremented during the experiment, recording the highest expression (5.18-fold increase than control) after 48 h exposure to 100 mM NaCl. However, it decreased afterward and recorded a lower expression (about half of the control plants) at the final stage.

## Discussion

### Membrane stability and $K^+/Na^+$ ratio

To unravel the salt stress responsive differences in *O. sativa* cv. IR64 and the halophyte grass (*A. littoralis*), we optimized the intensity and duration of NaCl stress by evaluating the membrane stability index. Our results indicated that NaCl concentrations above 100 mM and 300 mM caused severe damages to the MSI in IR64 and *A. littoralis*, respectively. Reduction in MSI in response to salinity stress has been reported in previous studies (Azizpour et al. 2010; Kumar et al. 2017). Salt stress leads to lipid peroxidation, resulting in membrane instability and subsequent secondary damage, including an imbalanced ionic situation, low antioxidant capacity, and malfunction of various intracellular organelles (Pandey et al. 2017; Kumar et al. 2017).

The effects of salt stress on the absorption and transportation of monovalent cations, particularly  $Na^+$  and  $K^+$ , are important criteria for evaluating salt tolerance in plant species (Kumar et al. 2017). Comparative analysis of monovalent ions revealed that accumulation and distribution of  $Na^+$  ions differed in the shoots and roots of both species. Roots, being in direct contact with the nutrient medium, had a higher  $Na^+$  content than shoot tissues in both species. However, *A. littoralis* had a higher concentration of  $Na^+$  in shoots compared with IR64, indicating a more efficient distribution system. Additionally, the  $K^+$  content of *A. littoralis* was significantly higher than that of *O. sativa* in both tissues from the beginning of the experiment, while the  $Na^+$  content was not substantially different in these two species. Considering the  $K^+$  and  $Na^+$

content of *O. sativa* tissues, the elevated content of internal  $\text{Na}^+$  was the main reason for the decreasing trend of  $\text{K}^+/\text{Na}^+$ . Our observations suggest that *A. littoralis* had a more efficient controlling system for  $\text{K}^+$  uptake than *O. sativa*. Besides its probable efficient  $\text{K}^+$  uptake and maintenance system, *A. littoralis* as one of the species in the Poaceae family, is able to exclude excess  $\text{Na}^+$  through its aerial tissues (Barhoumi et al. 2007). These abilities result in maintaining a high  $\text{K}^+/\text{Na}^+$  ratio and ameliorating the detrimental effects of high  $\text{NaCl}$  in this halophyte plant species. Moreover, halophytes are reported to use  $\text{Na}^+$  as osmosis pressure regulator, compensating to some extent for the lower uptake of other cations under salinity stress and minimizing the toxicity of  $\text{Na}^+$  by retaining its level below noxious concentrations (Ben Hamed et al. 2014).

Our results indicated that *A. littoralis* more efficiently retained a higher content of  $\text{K}^+$  ions than *O. sativa* when subjected to salt stress, especially in the root tissues that were directly exposed to  $\text{NaCl}$ . Although high concentrations of  $\text{Na}^+$  are toxic to plants and have been suggested as the main contributing factor to salinity (Assaha et al. 2017), it is well documented that  $\text{K}^+/\text{Na}^+$  ratio is a more determining factor than  $\text{Na}^+$  concentration for adaptability and tolerance to salt stress (Shabala and Pottosin, 2010; Wang et al. 2013). In fact,  $\text{Na}^+$  and  $\text{K}^+$  homeostasis is essential for proper plant growth and development, and maintaining a high tissue  $\text{K}^+/\text{Na}^+$  ratio is considered a key salinity tolerance factor in plants (Assaha et al. 2017). On the other hand, in susceptible plants, high external concentrations of  $\text{Na}^+$  competitively reduce the uptake of  $\text{K}^+$ , an essential macronutrient contributing to numerous physiological processes, and subsequent  $\text{K}^+$  deficiency in tissues can result in plant growth impairment. Therefore, salt sensitivity was exclusively attributed to the  $\text{K}^+$  deficiency in plant tissues and not  $\text{Na}^+$  toxicity; hence, adaptability to low  $\text{K}^+$  concentrations was suggested as one of the main breeding objectives for improving salt tolerance in plants (Assaha et al. 2017).

## Antioxidant enzyme expression

The roots of plants, being in direct contact with the culture medium, are the first tissues to sense the presence of  $\text{NaCl}$  salinity condition. This was reflected by rapid increase in the SOD expression in root tissues. However, the induction of antioxidant system in aerial parts was delayed due to the time required for transportation and accumulation of  $\text{Na}^+$  ions in these parts. *A. littoralis* showed a more efficient transport system and reached the highest SOD expression in the aerial parts more quickly than *O. sativa*. This indicates that *A. littoralis* has a better antioxidant activity preparation as a halophyte species.

Similarly, the salt tolerant cultivars of *O. sativa* and other plant species including *Licopersicum sp.*, and *Zea mays*, have shown a rapid induction of Cu/Zn SOD expression and enzymatic activities in response to salinity (Aydin et al. 2014; Gill et al. 2015; Kibria et al. 2017). Overexpression of Cu/Zn SOD in rice cv. IR20 has been reported to double its tolerance to heat shock and salt (150 mM) stresses, and improve rice yield by 25% (Prashanth et al. 2008) and this enzyme has been suggested as a good candidate for engineering rice tolerance to various stresses, including salinity (Singh et al. 2008). SOD acts as the first line of enzymatic antioxidant defense against salt stress (Gill et al. 2015). Cu/Zn SOD is the most widespread form of SODs in cytosol, peroxisome, chloroplast, and apoplast regions of plant cells, which convert  $\text{O}^{\bullet-}$  to  $\text{H}_2\text{O}_2$  and  $\text{O}_2$  (Alscher et al. 2002). The lower durability of Cu/Zn SOD and the delayed start of its expression in aerial tissues may have contributed to the high susceptibility of IR64 rice to the saline conditions. Delayed SOD

expression can lead to the accumulation of  $O^{\bullet-}$  and subsequent  $OH^{\bullet}$  radicals, which are highly reactive and destructive to the plant cells (Prashanth et al. 2008). Inactivity of SOD can also result in a lower production of  $H_2O_2$ , an important redox signaling molecule that plays a pivotal role in activating different antioxidant pathways (Sies, 2017). This could be due to the detrimental effects of  $Na^+$  ions on intracellular compartmentation and or lack of regulatory sequences for continuous expression Cu/Zn SOD in the controlling regions of IR64 rice.

CAT is the main enzyme responsible for the decomposition of  $H_2O_2$ , and its lower activity could lead to the accumulation of  $H_2O_2$  and its subsequent conversion to hydroxyl free radicals (Iannone et al. 2010). Our results showed that low salt concentration induced the expression of CAT in the roots of IR64, but this increase was not long-lasting under moderate and severe salt stresses, indicating lower efficiency of this enzyme in durable salt stress. In addition, the expression of CAT in the aerial parts of salt-treated IR64 was significantly lower than in untreated plants, indicating that NaCl significantly repressed CAT expression. Our observations are in line with the previous reports, which have shown that an increase in salt concentration decreased CAT activity in salt-sensitive rice cultivars but increased in salt-tolerant ones (El-Shabrawi et al. 2010; Hasanuzzaman et al. 2014; Kibria et al. 2017). Therefore, the accumulation of  $H_2O_2$  owing to the lower CAT expression could be one of the main reasons contributing to the lower tolerance of IR64 to salt stress compared to *A. littoralis*. In fact comparing the pattern of  $H_2O_2$  between halophytes and glycophytes provides valuable information about signal transduction and ROS-induced damages (Bose et al. 2013). It has been reported that  $H_2O_2$  concentration in *Cakile maritime* (a halophyte species) significantly increased during the initial periods of salt treatment and then decreases, while in *Arabidopsis thaliana* (a glycophyte species), a steady increase trend is observed until the end of the experiment (Ellouzi et al. 2011). This suggests that halophyte plants are more efficient in rapidly conducting stress messages through  $H_2O_2$  accumulation, followed by an effective antioxidant system for detoxifying ROS. Although, previous studies have indicated an increase, decrease, or unchanged CAT activity or expression in response to salt stress, halophytes generally have higher CAT activity than glycophytes in all cases, supporting the critical role of CAT in salt tolerance (Bose et al. 2013).

Excessive accumulation of  $H_2O_2$  can cause damage to subcellular compartments, which is why multiple mechanisms exist to control  $H_2O_2$  concentration in the plant cells. While CAT is highly efficient in decomposing  $H_2O_2$ , it has a low affinity for low concentrations of  $H_2O_2$ , which makes it less effective in situations where  $H_2O_2$  concentrations are low (Sofa et al. 2015). The ascorbic acid–glutathione (AsA-GSH) recycling pathway, which is the heart of the redox homeostasis, is one of the most important components of  $H_2O_2$  detoxifying mechanisms, and APX functions as a key player in triggering the cycle (Pandey et al. 2017; Chen et al. 2021). The high prevalence of APX in plant cell, the existence of multiple isoforms, and a higher affinity for  $H_2O_2$  than CAT signify the importance of APX in different plant developmental processes (Pandey et al. 2017). On the other hand, APX has a high affinity for  $H_2O_2$  and can efficiently reduce it to water using ascorbate as a specific electron donor, making it more important than CAT at low  $H_2O_2$  concentrations, hence it is an important player in the plant's response to salinity at the beginning of stress (Pessarakli et al. 2014). Due to the transmembrane diffusion of  $H_2O_2$ , organelle-specific APXs are suggested to control the

intra-organelle  $H_2O_2$ , while cytosolic APXs can control both cytosolic and diffused  $H_2O_2$  in organelles (Pessarakli et al. 2014). There are several reports indicating high activities of different isoforms of APX in response to salt stress, and the ability of ectopic or overexpression of APX to mitigate oxidative stress and enhance plant performance (Diaz-Vivancos et al. 2013; Liu et al. 2013; Zhang et al. 2013; Pessarakli et al. 2014; Yan et al. 2016; Pandey et al. 2017).

The multigenic APX family consists of eight genes in *O. sativa* encoding two peroxisomal, two cytosolic, three stromal and one thylakoid isoforms, which differ in their kinetic properties, substrate affinity, and induced stress (Pandey et al. 2017). Out of four isoforms of APX that were reported in plant species, we compared the expression levels of cytosolic (cAPX) and peroxisomal (pAPX) in the root and shoot of *A. littoralis* and *O. sativa* in response to salt stress. It has been reported that the cytosolic isoforms are mainly induced in response to oxidative stresses, salinity, and pathogen attack, while the peroxisomal isoforms respond to salinity and drought stresses (Pandey et al. 2017). However, it appears that the extent of expression of each isoform is a species and cultivar-dependent factor. Therefore, the low expression of pAPX in the roots of *A. littoralis* could be attributed to the involvement of this gene in other stresses in this plant species. Moreover, our results indicated that the expression of cAPX isoform increased in the root of *A. littoralis* in response to an increase in salt concentrations, while the highest expression of this gene was recorded at the initial stage of salt treatment in the root of *O. sativa*. Similarly, an early increase in two APX isoforms was observed in the salt-sensitive tomato cv. M82 compared to its wild relative tolerant species in response to salt treatment (Najami et al. 2008). Our findings suggest that the expression of cytosolic APX is more responsive to salt stress than peroxisomal APX in the root tissues of *A. littoralis* and *O. sativa*. The higher expression of cytosolic APX in response to salt stress may be attributed to its role in detoxifying the  $H_2O_2$  generated in the cytosol under salt stress conditions.

Furthermore, APX activity is highly associated with the intensity, duration, and multiplicity of the imposed stress (Pandey et al. 2017). As reported in a similar experiment (Babgohari et al. 2013), differences in the upstream regulatory sequences, which induce the gene expression in low salt concentrations in *O. sativa* and high salt concentrations in *A. littoralis* may be one of the reasons for this temporally distinct expression. Tissue-specific isoforms could also be another probable explanation for this distinguished expression, with the examined cAPX being induced by both low and high salt concentrations in the aerial parts of *A. littoralis* and *O. sativa* cv. IR64, respectively. However, these observations require further supplementary investigations.

Comparing the expression of CAT and cAPX in IR64 cv. derive us to the conclusion that the lower expression of CAT in the root of rice may be compensated to some extent by higher expression of cAPX. However, the lower capacity of APX to detoxify  $H_2O_2$  can lead to the accumulation of excess  $H_2O_2$ .

Interestingly, during long-term exposure to salt stress, two  $H_2O_2$  detoxifying genes had high expression in *A. littoralis* and low expression in *O. sativa*, which can result in the accumulation of  $H_2O_2$  and subsequent higher susceptibility of the latter to salt stress.

A failure in the AsA-GSH cycle may be the reason for the distinct expression of cAPX and GR in the roots of *A. littoralis* and *O. sativa* cv. IR64, resulting in the retention of high expression of these genes in the salt-

tolerant species and significant suppression in the salt-sensitive ones. Proper function of APX is highly dependent on AsA, and low concentrations of this substance severely repress enzyme activity. Although all enzymes in the AsA-GSH pathway are influenced by salt stress, sensitive and tolerant cultivars respond differently to the stimuli. For example, in the salt-tolerant *O. sativa* cv. Pokkali, all genes in this pathway were overexpressed, while in the salt-sensitive cv. BRRI, only the expression of DHR was increased, and no changes were recorded in the MDHR and GR, while the expression of APX was reduced (Pandey et al. 2015). This signifies the importance of coordinated expression of all enzymes in a pathway to achieve physiological response. Downregulation of chloroplastic GR in tomato using RNAi technique led to higher susceptibility of the transformed plants to cold stress, while overexpression of GR in poplar and tobacco resulted in higher accumulation of AsA and tolerance to oxidative stresses (Pessarakli, 2014). Therefore, it may be deduced that defects in this important cycle may lead to the low expression of cAPX and GR in the roots of IR64 under prolonged exposure to salt stress, subsequently leading to the higher susceptibility of this cultivar to salt stress. However, it is well documented that the presence of other antioxidant enzymes such as GR and SOD enhances the activity of APX, suggesting a cross-talk among different antioxidants enzymes (Pandey et al. 2017). Nevertheless, it is reported that the substantial increase in the activity of GR in response to oxidative stresses is due to the regulation at the translation levels of preexisting GR mRNAs (Pessarakli, 2014). Therefore, in addition to the transcription rate, a more stable enzyme structure could result in more efficient antioxidant activity, suggesting the importance of sequence analysis at the DNA and amino acid levels.

The same type of stress may provoke differential expression of isozymes in each subcellular organelle (Pandey et al. 2017). Comparing the transcript abundance of two APX isoforms in this study leads us to the conclusion that cAPX is more important for confronting salinity stress than pAPX in both species, and its constant expression in both aerial and root tissues plays a crucial role under salt stress. However, pAPX was only induced at the stage of 48 hours exposure to 300 mM salt concentration in both shoot and root tissues, indicating the transient involvement of peroxisome in oxidative stress at this stage.

In general, the threshold level of toxic ROS for triggering oxidative stress highly differs between halophytes and glycophytes. On the other hand, the first significant lipid peroxidation in the aerial parts of halophytes (as an indicator of oxidative stress) is detectable at salt treatment above 150 mM, a concentration that is fatal for the majority of glycophytes. In this regard, it is presumable that halophytes have a higher antioxidant systems capacity compared to glycophytes. In the present study, the relative higher expression of some antioxidant genes in *O. sativa* than *A. littoralis* may represent the higher background expression of those genes in the control *A. littoralis*.

## Conclusion

In this study, the salt stress tolerance of *A. littoralis* and *O. sativa* cv. IR64 were compared, revealing that proper cationic homeostasis and efficient enzymatic antioxidant systems contributed to the higher tolerance of *A. littoralis*. Specifically, the ability of *A. littoralis* to maintain a higher content of K<sup>+</sup> cation and a more efficient system for retaining a high K<sup>+</sup>/Na<sup>+</sup> ratio during salt stress was a fundamental difference between these two species. Furthermore, effective preparation of plants in response to salt stress requires efficient

crosstalk and subsequent harmonic expression of different antioxidant enzymes, resulting in adequate rearrangement of plant defensive systems and ameliorating the detrimental effects of salt stress. Our study found that the lower expression and stability of some antioxidant enzymes, particularly SOD, CAT and cAPX, played key roles in the high susceptibility of *O. sativa* to salt stress. The suppression of these genes in the roots of salt-treated *O. sativa* suggests they may be critical determinant of salt stress adaptability. Therefore, ectopic expression or overexpression of these genes in the roots of salt-sensitive rice cultivars may help plants cope with salt stress more effectively, resulting in improved plant performance in the presence of salt stress. Notably, the expression of two other antioxidant enzymes, pAPX and GR, were not suppressed in *O. sativa* in response to salt stress, indicating their lower involvement in salt hardiness. Overall, our results suggest that the extent of expression of antioxidant genes highly contributes to the salt tolerance of halophytes and glycophytes, and is considered one of the main differences between these two groups of plants. This finding could be beneficial for engineering salinity tolerance in rice cultivars.

## Declarations

**Competing Interests:** The authors declare that they have no competing interests

**Compliance with ethics requirements:** This article does not contain any studies with human or animal subjects.

**Data availability:** All data included in this manuscript are available upon request by contacting with the corresponding authors

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## Author contribution statement

Farajollah Shahriari Ahmadi, Ali Niazi and Nasrin Moshtaghi contributed to the study conception and design and provided the laboratory services. Materials preparation, data collection and analysis were performed by Masoud Fakhrfeshani, Mohammad Zare-Mehrjerdi and Fatemeh Keykha Akhar. Data visualization was performed by Masoud Fakhrfeshani, Abdolkarim Zarei and Fatemeh Keykha Akhar. Abdolkarim Zarei drafted the manuscript and interpreted the data. All authors commented on previous versions of the manuscript and approved the final manuscript.

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## Figures

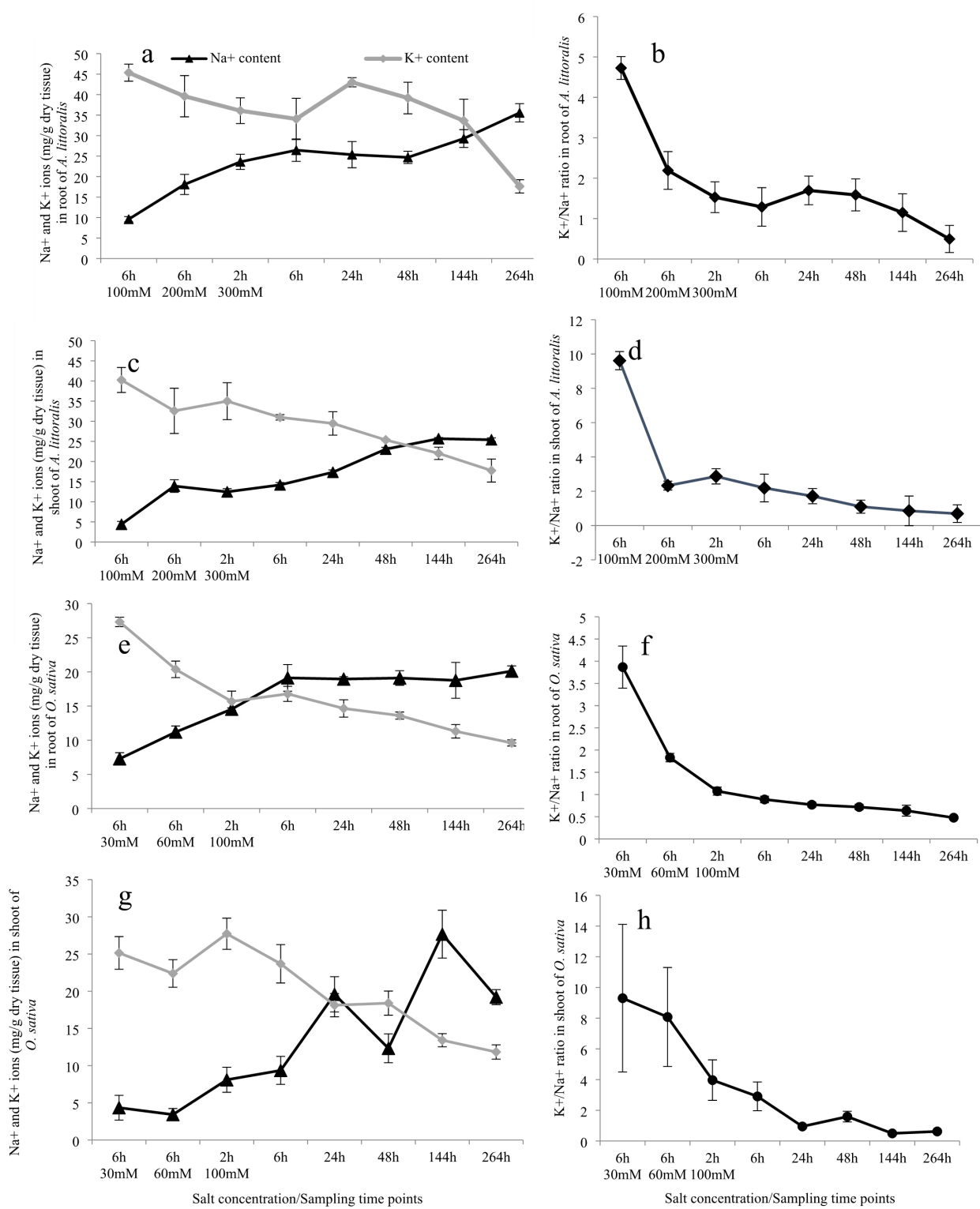


**Figure 1**

The content of Na<sup>+</sup> and K<sup>+</sup> ions as well as the ratio of K<sup>+</sup>/Na<sup>+</sup> in the root and shoot of *A. littoralis* and *O. sativa* under different salt concentrations during the experiment. Na<sup>+</sup> and K<sup>+</sup> content and its ratio in the root (a and b) and shoot (c and d) of *A. littoralis*; Na<sup>+</sup> and K<sup>+</sup> content and its ratio in the root (e and f) and shoot (g and h) of *O. sativa*.

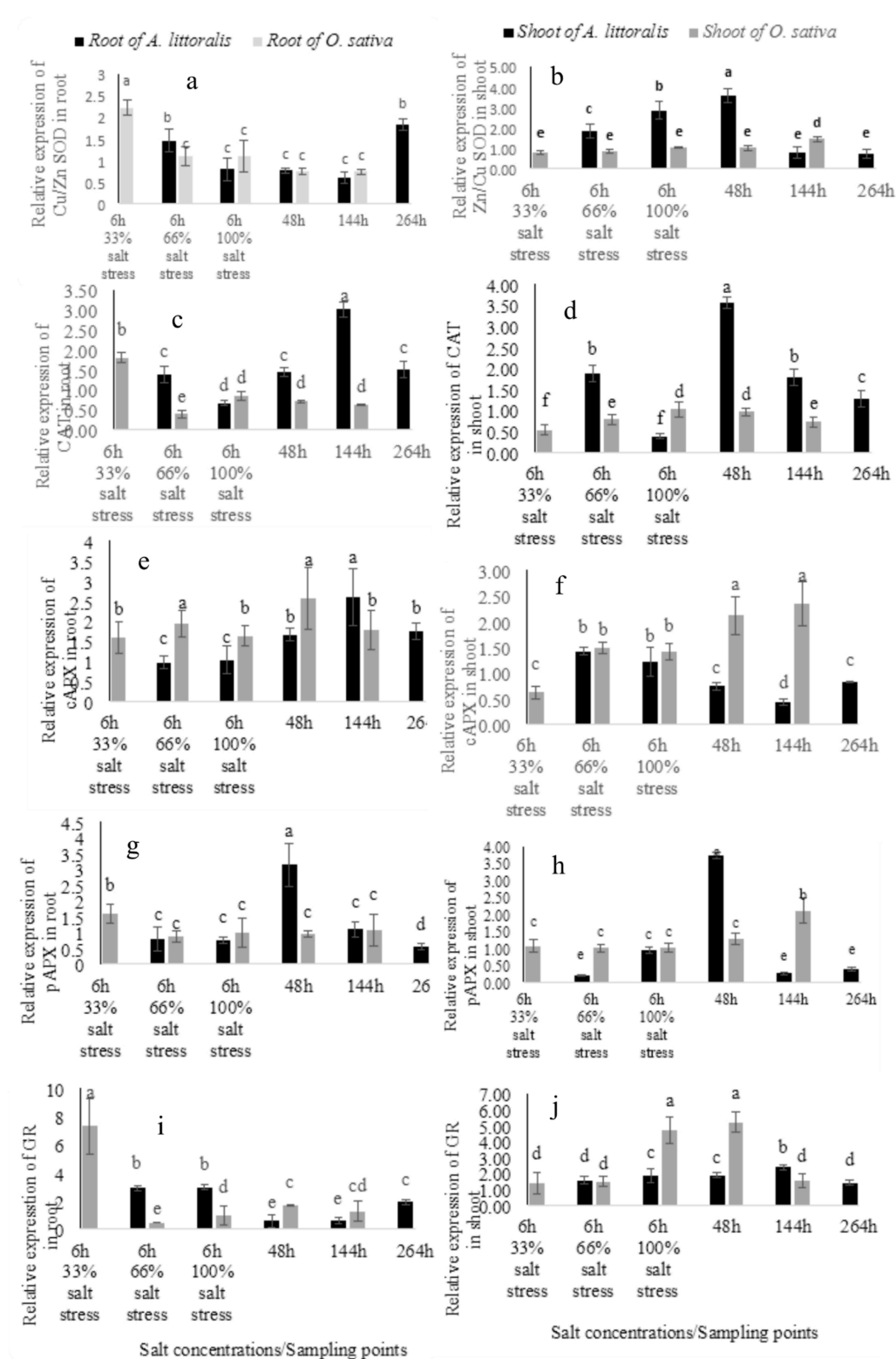


**Figure 2**

Relative expression (RX) of oxidative stress responsive genes in the shoot and root of *A. littoralis* and *O. sativa* after exposure to different concentration of NaCl. RX of Cu/Zn SOD in the root (a) and shoot (b). RX of CAT in the root (c) and shoot (d). RX of cAPX in the root (e) and shoot (f). RX of pAPX in the root (g) and shoot (h). RX of GR in the root (i) and shoot (j).

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