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# Short-term changes in antioxidant response of *Eutrema salsugineum* exposed to severe salinity

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## Abstract

Salinity is one of the major constraints limiting plant productivity. Understanding the mechanism of salinity tolerance is a necessary step for improving crops yield in saline conditions. *Eutrema salsugineum* is a halophyte species and plant model for salinity tolerance mechanism study. Therefore, we studied the ability of *E. salsugineum* plants for their antioxidant response to 400 mM NaCl treatment. Changes in hydrogen peroxide ($H_2O_2$), malondialdehyde (MDA) and tocopherol contents, and kinetics of catalase, superoxide dismutase and peroxidase enzyme activities were investigated. Results shows that, although increasing in the beginning of the salt treatment, the fast decrease in $H_2O_2$ and MDA levels afterwhile indicates the ability of *E. salsugineum* to alleviate oxidative damage and to maintain membrane integrity. Together, with the increase of tocopherol contents, the high enzymatic activity of CAT, SOD and POD reflect the highly efficient antioxidant response of *E. salsugineum.*

***Keywords:*** *Eutrema salsugineum*; halophytes; hydrogen peroxide; salt stress; signal molecule; oxidative stress

## Introduction

Nowadays, agriculture is encountering serious climatic threatening, and crops production is severely limited by abiotic constraints. Particularly, salinity (with drought) is one of the most limiting factors for agricultural activities. According to the global map of salt affected soils, saline or sodic soils are distributed in all continents at various levels of problem intensity, and the total area of salt affected lands is around 1 billion hectares, with an increasing trend (Ivushkine *et al.,* 2019; Zhou *et al.,* 2022). Salinity may cause stress to plants either by an osmotic effect or by an ionic component (Ellouzi *et al.,* 2014). In the first condition, salinity decreases water potential in the substrate so that preventing water uptake by roots. Thus, by causing an osmotic stress salinity provokes stomatal limitation of photosynthesis thereby enhancing formation of reactive oxygen species (ROS) (Chaves *et al*., 2009). The ionic component of salt stress is a consequence of the direct toxic effect of $Na^+$ or $Cl^-$ and of imbalances caused in the homeostasis of other ions (for instance $K^+$ or $Ca^{2+}$) (Munns and Tester, 2008). As mentioned before, ROS formation is enhanced during plant responses to salt stress

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resulting in oxidative damages in plants; however, these compounds are also greatly described as signal molecules in many biological processes. Among all ROS, Hydrogen peroxide ($H_2O_2$) is the most important molecule that plays a dual and paradoxical function in stress perception and plant tolerance to abiotic stresses (Wojtyla *et al*., 2016). To overcome oxidative stress, plants activate a plethora of antioxidant systems enzymatic such as superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) and non-enzymatic systems like glutathione, tocopherols and ascorbic acid (Moller *et al*., 2007). Interestingly, these mechanisms have been proposed as relevant tools in salt stress signaling pathway (Ellouzi *et al.,* 2021). In this context, we previously reported, in a comparative study between *Cakile maritima* (a halophyte) and *Arabidopsis thaliana* (glycophyte plant model), that halophytes, generally can rapidly evolve physiological and antioxidant mechanisms to adapt to salt stress (Ellouzi *et al.,* 2011). This response is due to the ability of halophytes to rapidly convert salt stress to a positive signal through which can manage the oxidative stress damages. From this investigation, we concluded that studying the effect of short-term exposure to salt stress is greatly decisive to determine the tolerant or sensitive criteria of plants to salinity. In the present study, we investigated the effect of short-term salt stress (400 mM NaCl) in a plant model *Eutrema salsugineum*. A special interest is granted to evaluate the dynamics of oxidative stress biomarkers ($H_2O_2$ and MDA) and the antioxidant systems, enzymatic and non-enzymatic ones.

## Materials and Methods

### *Plant material and treatment*

Mature seeds of *E. salsugineum* were sown in plastic pots filled with inert sand and watered daily with distilled water until germination. The seedlings were irrigated for 3 weeks with Hoagland's solution. Four-week-old plants were subjected to salt treatments by adding 400 mM NaCl to the nutrient solution, and samples were collected after 4, 16, 24 and 72 h. A second group of plants was used as control (0 mM NaCl). The experiment was performed in a glasshouse under controlled conditions; a light/dark regime of 16/8 h; an air temperature range of 20 °C - 25 °C and a relative humidity range of 70%-80%.

### *Quantitative measurement of $H_2O_2$*

Leaf samples were ground with acetone for 30 min at 4 °C and filtered through eight layers of gauze. After the addition of active carbon, the samples were centrifuged twice at 3,000 g for 20 min at 4 °C. 20% of $TiCl_4$ were next added to 1 ml of the supernatant. The post-reacted compound was centrifuged at 3,000 g for 10 min. After discarding the supernatant, the pellet was dissolved 1 M $H_2SO_4$, and the absorbance was determined at 410 nm (Ellouzi *et al*., 2011). A standard curve was established using hydrogen peroxide, and leaf contents calculated by comparing absorbance with the standard curve.

### *Estimation of lipid peroxidation*

To assess the lipid peroxidation level, the amount of MDA was estimated spectrophotometrically as described by Ellouzi *et al.* (2011). In this case, fresh shoots and roots of *Eutrema* seedlings from each treatment were extracted with 5% trichloroacetic acid (TCA). MDA content was determined according to the thiobarbituric acid (TBA) test. To calculate MDA content, an extinction coefficient of 155 $mM^{-1}cm^{-1}$ was used

### *Antioxidant enzyme extraction and assay*

Antioxidant enzymes were extracted from frozen leaf samples with polyvinylpyrrolidone (PVP) in 50 mM K-phosphate buffer (pH 7.8), containing 10 mM ethylenediaminetetra-acetic acid (EDTA), 1 mM dithiothreitol, 0.1 mM phenylmethyl-sulfonyl-fluoride (PMSF) acid and 1 mM dithiothreitol (DTT). After centrifugation at 12,000 g for 30 min, supernatant fractions were collected and used for different enzyme assays.

The total protein content was determined by Bradford's reaction (1976) using bovine serum albumin (BSA) as standard.

The superoxide dismutase (SOD, EC.1.15.1.1) activity was carried out by the NBT assay (Scebba *et al*., 1999). The reaction mixture consisted of plant extract, potassium phosphate buffer (pH 7.8), 13 mM methionine, 10 mM NBT, 2 mM riboflavin, and 0.1 mM EDTA. The reaction mixture was incubated in a chamber under the illumination of 15 W at 25 °C for 10 min. SOD activity was determined spectrophotometrically at 560 nm and expressed as U SOD $mg^{-1}$ proteins. Total catalase (CAT, EC 1.11.1.6) activity was determined spectrophotometrically by monitoring the decrease in absorbance at 240 nm (Lück, 1965). The reaction mixture consisted of plant extract, potassium phosphate buffer (pH 6), and $H_2O_2$ solution (10 mM). CAT activity was expressed as U CAT $mg^{-1}$ proteins.

Guaiacol peroxidase (GPX, EC.1.11.1.7) activity was estimated using a reaction medium containing potassium phosphate buffer (pH 6), 5 mM $H_2O_2$, and 18 mM guaiacol (Hall, 1978). The kinetics of absorbance at 470 nm was measured during 2 min. Peroxidase activity was defined as the amount of enzyme that caused the formation of 1 mM of tetraguaicol per min and expressed as U GPX $mg^{-1}$ proteins.

*5. Native gel electrophoresis and relative density of antioxidant enzymes*

Fresh leaf samples (0.5 g) were rapidly extracted in a pre-chilled mortar with 10% (w/w) PVP in 50 mM potassium phosphate buffer (pH 8), containing 0.1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF. After centrifugation, the supernatant was collected and the protein content was determined following Bradford's protocol (1976), using bovine serum albumin as a standard. SOD activity was stained following Beauchamp and Fidovich (1971) after electrophoresis in 10% (w/v) polyacrylamide slab gel, at pH 8.9 under non-denaturating conditions. Activity bands were revealed by first incubating the gel in 50 mL of phosphate buffer (50 mM, pH 7.8) containing 1 mM EDTA, 250 μM nitroblue tetrazolium (NBT), 2.8 mM TEMED and 22 μM riboflavin for 25 min in darkness. Then the gel was illuminated under white light until clear bands appeared.

CAT activity was detected on native 6% (w/v) polyacrylamide gel at pH 8.9 under non-denaturating conditions as described by Woodbury *et al*. (1971). The gel was soaked in 5 mM $H_2O_2$ for 15 min, rinsed in distilled water, and incubated in 1% (w:v) ferric chloride and 1% (w/v) potassium ferricyanide until achromatic bands appeared.

Total peroxydase (POD) activity was determined on native 8% (w/v) acrylamide gel at pH 8.9 under non-denaturating conditions. After electrophoresis, the gel was first incubated for 30 min in 0.1 M sodium acetate buffer (pH 4.0) containing 1% (v/v) guaiacol. Then, revelation was achieved in a solution containing 4.7 mM 3-amino-9-ethylcarbazole, 38 mM N,N-dimethyl formamide, 0.1 M sodium acetate buffer (pH 5.0), 0.1 M $CaCl_2$ and 30% $H_2O_2$ (Vallejos, 1983). After band staining each enzyme, the gels were scanned and converted to images then analyzed with ImageJ software. The images were rectified and transformed into binary images to calculate the relative density (RD) of the bands for individual lanes (Gassmann *et al*., 2009).

*Tocopherol analysis*

Tocopherol levels were determined by HPLC as described by Cela *et al*. (2011).

*Statistical analyses*

Statistical differences between measurements at different times and of different genotypes were analyzed via analysis of variance (ANOVA) using SPSS (Chicago, IL., USA). Differences were considered significant at a probability level of $P \leq 0.05$.

## Results

*Kinetics of $H_2O_2$ and MDA contents*

When treated with 400 mM NaCl and At 16 h of salt treatment, the accumulation of $H_2O_2$ peaked with values around 26-fold before declining thereafter to reach control levels (Figure 1-A). MDA levels were used as an estimation of lipid peroxidation. In plants treated with 400 mM NaCl, the MDA concentration increased by 44% during the first 24 h of salt treatment and returned to basal levels by the end of the treatment (Figure 1-B).

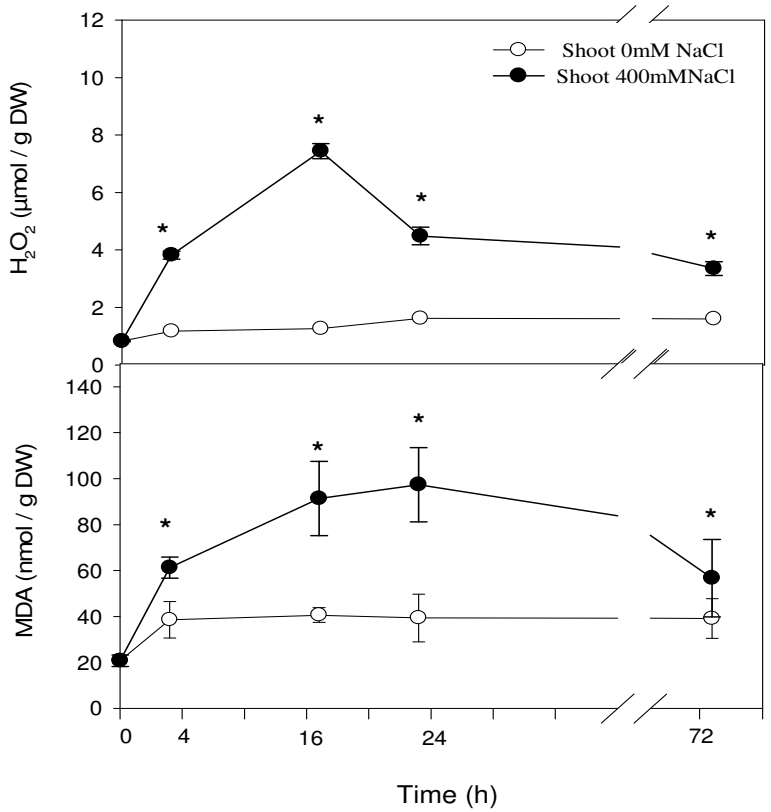


**Figure 1.** Effect of short term NaCl treatment on $H_2O_2$ (A) and MDA (B) levels in leaves of *Eutrema salsugineum* exposed to control or salt stress conditions for 72h

Data are the mean ± SE of $n$=4 replicates. The asterisks indicate significant differences (Student's $t$-test, $P \leq 0.05$) at each time point between salt-stressed and control plants.

*Kinetics of antioxidant enzyme activities*

The changes in the antioxidant enzymes SOD, CAT and GPX were measured spectrophotometrically and illustrated in Figure 2. Almost all antioxidant systems varied significantly, under salt stress conditions as compared to control state (0 mM NaCl). SOD activity showed similar trends as $H_2O_2$, reaching its maximum (200%) just after 4h of salt application and declined at the end of the experiment (Figure 2-A). CAT activity peaked after 24h to attain 120% of increase, relatively to their opposites of control. After that, CAT decreased reaching values of non-stressed plants (Figure 2-B). POD showed opposite profile as compared to CAT and SOD. We found that salt stress caused a continuous and progressive induction of POD activity resulting in an increase of 212% at the end of the experiment, comparatively to non-saline conditions (Figure 2-C).

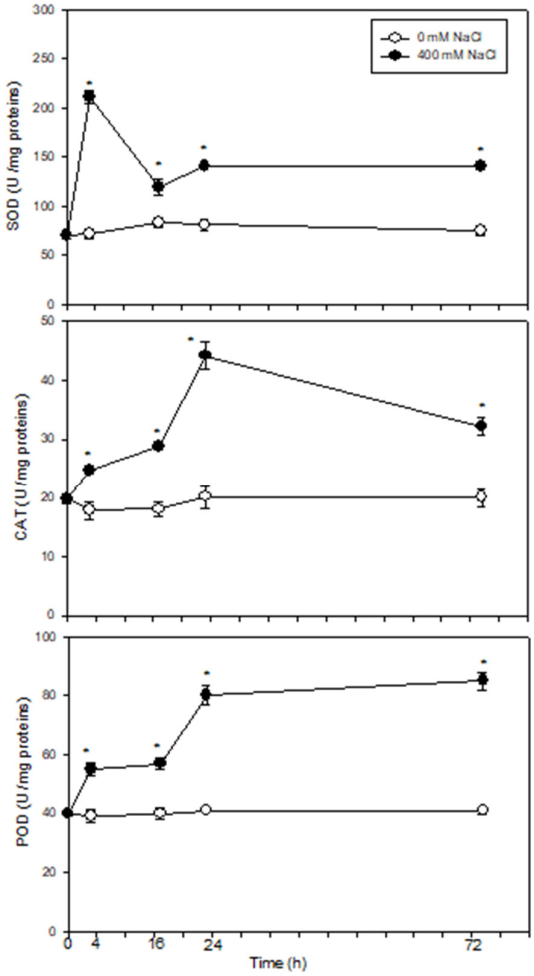


**Figure 2.** Effect of short term NaCl treatment on SOD, POD and CAT activities in leaves of *Eutrema salsugineum* exposed to control or salt stress conditions for 72h

Data are the mean ± SE of $n$=4 replicates. The asterisks indicate significant differences (Student's $t$-test, $P \leq 0.05$) at each time point between salt-stressed and control plants.

*Kinetics of antioxidant enzyme activities in native gel electrophoresis*

Total SOD, CAT and POD activities in the leaves of *E. salsugineum* was determined on native PAGE gels and expressed as relative density (RD). At 400 mM NaCl, the RD of SOD bands had increased 8-fold after the first 4 h and decreased to their lowest values by the end of the treatment (Figure 3-A). CAT bands increased considerably in the stressed leaves of *E. salsugineum* plants and the RD peaked at 24 h by 3.5-fold, relatively to control state. At the end of salt treatment CAT activity declined to reach values of 0 mM NaCl (Figure 3-B). Similarly, to SOD and CAT, POD activity increased continuously to attain the highest value (30-fold) after 72 h of salt stress (Figure 3-C).

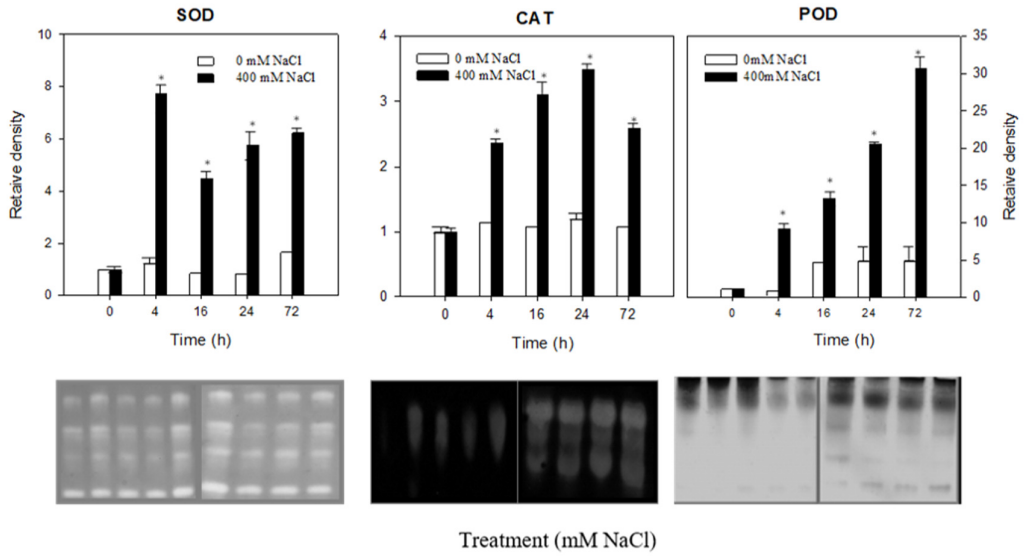


**Figure 3.** Effect of short term NaCl treatment on SOD, POD and CAT activities *in native gel electrophoresis* and relative densities in leaves of *Eutrema salsugineum* exposed to control or salt stress conditions for 72h

*Kinetics of α- and γ-tocopherol contents*

When subjected to 400 mM NaCl, the leaves of *E. salsugineum* showed a 60% of increase after 4 hours of salt treatment and reached maximum levels at the end of the salt treatment (Figure 4). γ-tocopherol levels increased after 4 h of salt treatment, then continued to rise and reached maximum levels at the end of the experiment (72 h).

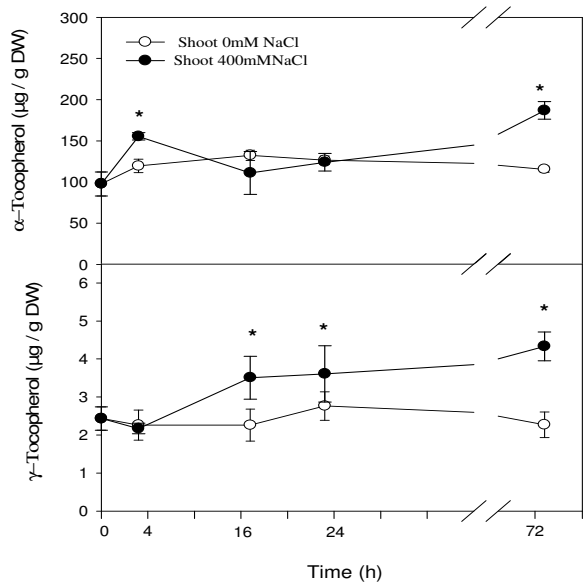


**Figure 4.** Effect of short term NaCl treatment on α- and γ-tocopherol levels in leaves of *Eutrema salsugineum* exposed to control or salt stress conditions for 72h

Data are the mean ± SE of $n$=4 replicates. The asterisks indicate significant differences (Student's $t$-test, $P \leq 0.05$) at each time point between salt-stressed and control plants.

## Discussion

Besides being able to regulate ion and water movements, salt-tolerant plants are also expected to have more effective antioxidant systems for the efficient removal of ROS, which are a common consequence of most abiotic stresses (Mansour *et al.,* 2019).

ROS are continuously produced under normal conditions as byproducts of various metabolic pathways in the apoplast as well as in intracellular compartments such as chloroplasts, mitochondria and peroxisomes (cellular compartments with highly oxidizing metabolic activity or with an intense level of electron flow). Therefore, plants are well equipped with antioxidants and enzymes that scavenge ROS to keep their levels low under favorable growth conditions. However, under unfavorable conditions, ROS production may increase and lead to oxidative stress in many plant species (Mullineaux and Karpinski, 2002; Miller *et al*., 2010). To avoid the accumulation of these compounds to toxic levels, plants have developed several detoxifying systems such as lipid-soluble and membrane-associated compounds (including tocopherols) and enzymatic constituents (which include SOD, CAT and POD) (Mittler *et al*., 2011).

The oxidative stress that accompanies ROS accumulation should not only be considered to be a symptom of cellular dysfunction; it can also represent a signal for plants to induce acclimation mechanisms (Peng *et al*., 2023). Among ROS, $H_2O_2$, a small and relatively mobile molecule that has the potential to carry information between different cellular compartments, has been shown to be a signal molecule in plant response to salinity (Singh, 2022). In agreement with these observations, our results show that short-term salinity induced transient oxidative stress in the leaves of *E. salsugineum* as indicated by the increase of $H_2O_2$ and MDA levels. MDA levels, frequently described as a biomarker of lipid peroxidation, are used to evaluate the extent of oxidative stress in plants, and they usually increase under salt stress, particularly in sensitive plants (Liu *et al.,* 2009). Here, the rapid decline of $H_2O_2$ followed by a decrease in MDA levels reveals the ability of *E. salsugineum* to alleviate oxidative damage and to maintain membrane integrity, which is considered one of the most important biochemical mechanisms of salt tolerance (Stevens *et al.,* 2009). This is in good agreement with the increase in $\alpha$- and $\gamma$-tocopherol contents. The accumulation of these antioxidants, at the same time as MDA and $H_2O_2$ levels decrease, is an important sign of the increased capacity of *E. salsugineum* to avoid potential oxidative damage early on. This behavior may also reflect an efficient antioxidative system, as evidenced by higher activity of SOD, CAT and POD enzymes. After 4 h of saline treatment, SOD activity showed a spectacular increase which reflects the high antioxidant capacity of *E. salsugineum* under 400 mM NaCl if coupled with the activities of enzymes that scavenge hydrogen peroxide. Indeed, elevated SOD activity was accompanied by an increase in CAT activity, 12 h after treatment, and with an extended increase in POD activity until the end of the experiment, especially under the higher salt concentration treatment. Similar results have been obtained in salt-tolerant cotton cultivars (Gossett *et al.,* 1994). This confirms the important role of POD in salt tolerant species where POD activities were found to be higher than in sensitive ones; affording plants protection against oxidative stress (Scalet *et al*., 1995).

Several studies based on long-term effects of salt stress have used the salt-tolerant model plant, *E. salsugineum* (Volcov *et al*., 2003; Ghars *et al*., 2008; Lugan *et al*., 2010). In our study, the response to salt of this species was studied in the short term; from a few hours to 3 days. As described by Munns (2002), the effects of salt stress may occur within minutes or hours of exposure and it is possible that they appear after days or even weeks to months, depending on the species and salt treatment. Very early responses such as those reported in the present study have also been reported in the halophyte *C. maritima* (Ellouzi *et al*., 2011). However, here, *E. salsugineum* showed a special salt stress response which can be characterized as two different kinds of response. One is an extended response that did not change over the long term and which consists of the continuous enhancement of POD activity and $\gamma$-tocopherol levels; this response was proportional to the progressive accumulation of salt, over the period of this study. The other is a set of transient responses that appeared at one time point, even with continuous uptake of $Na^+$. The first of these peaked after 4 h and was

associated with an increase in SOD activity. After 16 h of treatment, there were transient increases in $H_2O_2$ and MDA levels; then, from 16 h until the end of the treatment (72 h) the levels of these two parameters returned to control values. This was concomitant with an increase in $\alpha$-tocopherol levels. A third transient response occurred after 24 h: an increase of CAT activity.

## Conclusions

In conclusion, the study of events that happen early under salt stress seems to be crucial to learn how to improve plant tolerance to saline conditions. *E. salsugineum* appears to be able to tolerate high salinity rapidly, particularly with the activation of oxidative defense system; indeed, antioxidative enzyme activities play a protective role against salinity. Antioxidative defense mechanisms are effective in providing resistance to high level of salinity in *E. salsugineum* plants. The use of this plant as a model may therefore help improve salt intolerance in sensitive plants.

## Authors' Contributions

**HE:** Designing analysis and writing original draft. **MR**: final review and editing. **MH**: final manuscript revision. All authors read and approved the final manuscript.

**Ethical approval** (for researches involving animals or humans)

Not applicable.

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## Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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