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# Modulation of osmotic adjustment and enzymatic antioxidant profiling in *Apera intermedia* exposed to salt stress

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**Abstract:** The effects of salinity on growth, osmotic adjustment, and antioxidative responses were evaluated in *Apera intermedia*. For this purpose, 30-day-old plants were irrigated every other day with Hoagland nutrient solution containing 0, 150, 300, or 600 mM NaCl for 7 and 14 days. The results showed inhibition of growth, relative growth rate, relative water content, and osmotic potential with increasing NaCl concentration. Increased $Na^+$, $Cl^-$, and $Na^+/K^+$ ratio and decreased $K^+$ and $Ca^{2+}$ were determined with increasing NaCl concentrations. The activities of superoxide dismutase and ascorbate peroxidase were conspicuously enhanced at 150 mM NaCl, but activities of catalase, peroxidase, and NADPH oxidase were reduced in a concentration/time-dependent manner. The highest proline, choline, and glycine betaine accumulation assisted higher osmotic adjustment and maintenance of water status at 150 mM. However, the destructive effects of 300–600 mM were more severe in comparison to lower salinity, depending on the increase of hydrogen peroxide and thiobarbituric acid reactive substances for 14 days. After exposure to 300 and 600 mM, only ascorbate peroxidase and glutathione reductase were induced, but they were not sufficient to scavenge $H_2O_2$.

**Key words:** Antioxidant defense system, *Apera*, osmotic adjustment, salt stress

## 1. Introduction

One of the environmental stresses leading to the reduction of plant growth and productivity is soil salinity, a problem that has been increased by improper irrigation practices (Munns and Tester, 2008). The survival of plants under stress conditions may vary depending on cultivars, developmental stage of the plant, application period, and severity of salinity. In general, plants can be grouped as halophytes and glycophytes in terms of salt tolerance. The plants known as glycophytes cannot tolerate extreme salt concentrations (25 mM NaCl can be toxic), but halophytes have adapted to grow in saline soils and can tolerate salt concentrations as high as 500–1000 mM NaCl (Flowers et al., 2010). However, high salt concentrations negatively affect plant metabolism even in halophytes. Understanding the salinity tolerance mechanisms of halophytes can provide useful information about their adaptation strategies. Some protection mechanisms have evolved at the cellular, tissue, or whole plant level. Most of the halophytes utilize certain basic adaptation strategies for reestablishing cellular homeostasis under salinity: 1) preferring high ratios of $K^+/Na^+$ and $Ca^{2+}/Na^+$ in stomatal response of plants to salinity to achieve normal turgor regulation; 2) exclusion of salt from the inner tissues by means of a permeable membrane or salt glands, known as avoidance; and 3) accumulating various compatible osmolytes so as to continue water absorption from saline soil and to maintain osmotic balance under high salinity conditions (Mittler et al., 2011). As these processes are not entirely sufficient or effective against the stress, reactive oxygen species (ROS) generated by the disturbance to the electron transport system leads to the reduction of $O_2$ (Meloni et al., 2003). On the other hand, recent reports show that nontoxic ROS content can play a role in signaling pathways under stress (Jiang and Zhang, 2002) and can also be produced in plants growing under nonstressed conditions. The increasing number of salt-induced free radicals decomposes protein, lipids, and nucleic acids (Mittler et al., 2011). In order to cope with this problem, the most effective alternative pathway in scavenging/eliminating of excess ROS is enzymatic or nonenzymatic antioxidant resistance mechanisms (Munns, 2005; Saeidnejad et al., 2013). Enzymatic antioxidants include superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), peroxidase (POX; EC 1.11.1.7), ascorbate peroxidase (APX; EC 1.11.1.1), and glutathione reductase

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(GR; EC 1.6.4.2) (Meloni et al., 2003; Zhang et al., 2013). A positive relationship between the scavenging potential of radicals and salt tolerance has been reported in *Suaeda salsa* Pall. (Wang et al., 2008). On the other hand, apart from antioxidants, osmolytes are also employed to adjust osmotic potential of the cells and to protect subcellular structure (Munns, 2005). Proline (Pro) has several roles in osmotic adjustment, scavenging of ROS, and maintaining cell redox status under stress. Glycine betaine (GB) effectively stabilizes the quaternary structures of enzymes and maintains the integrity of membranes under salinity (Meloni et al., 2004).

*Apera* Adans. is a small genus of annual grasses known commonly as silky bent grass or wind grass and is native to Europe and West Asia (Davis, 1985). The habitat of *Apera intermedia* Hack., belonging to the family Poaceae, is severely salty soil. It is still unknown if *A. intermedia* can survive via tolerance mechanisms under salt conditions. The survival under high salinity may depend on an antioxidant system; however, there is no report on the defense responses of *A. intermedia* under salinity.

This study was designed to show the possible relationships among the antioxidant enzyme activity, osmotic adjustment, and water status in *Apera intermedia* under salinity. In this light, the study demonstrated the variations in antioxidant enzyme/isozyme activities, NADPH oxidase (NOX; EC 1.6.3.1) enzyme/isozyme activity, and ROS accumulation. Furthermore, these parameters were correlated with some physiological and biochemical parameters including growth, relative growth rate (RGR), leaf relative water content (RWC), osmotic potential ($\Psi_{\Pi}$), osmoprotectant contents, photosynthetic parameters, and the levels of lipid peroxidation in *A. intermedia* exposed to 0, 150, 300, and 600 mM NaCl for 7 and 14 days.

## 2. Materials and methods

### 2.1. Plant material and experimental design

Seeds of *Apera intermedia* were sterilized with 1% dichlon for 20 min and washed with ultrapure water. The seeds were germinated in perlite under controlled conditions. The seedlings of *A. intermedia* were grown in Hoagland's solution for 30 days (Hoagland and Arnon, 1950). On day 30 of normal growth (applied to seedlings with 5 leaves), a salt stress treatment was initiated by giving Hoagland solution containing 0, 150, 300, and 600 mM NaCl. Plants were sampled at 7 and 14 days of salt treatment.

### 2.2. Growth and water status in *Apera intermedia* under salinity

Six plants were used for the control group and for each stress group. The length of shoots was measured with a ruler. Shoot fresh weight (FW) was measured. After the samples were dried, dry weight (DW) was measured. RGR values were calculated according to the following formula by Hunt et al. (2002):

$$RGR = [\ln (DW_2) - \ln (DW_1)] / (t_2 - t_1),$$

where $DW_1$ = dry weight (g) at $t_1$, $DW_2$ = dry weight (g) at $t_2$, $t_1$ is initial harvest, and $t_2$ is final harvest.

After the stress exposure period, 6 leaves were weighed and their FW was recorded. The samples were kept in ultrapure water for 8 h and then the weight of the turgid leaves (TW) was measured again. After oven drying at 75 °C for 72 h, DW was reported. RWC was calculated by the formula given by Smart and Bingham (1974):

$$RWC\ (\%) = [(FW - DW) / (TW - DW)] \times 100.$$

Samples were homogenized with a glass rod. After centrifugation (12,000 × *g*) for 10 min, the extraction was directly used for $\Psi_{\Pi}$ determination. Leaf $\Psi_{\Pi}$ was measured with a Vapro Vapor Pressure Osmometer 5600.

Determination of Pro and Cho content and GB content was done according to the procedures defined by Bates et al. (1973) and Grieve and Grattan (1983), respectively.

### 2.3. Determination of effects of salinity on photosynthetic parameters

The Fluorescence Monitoring System FMS 2 was used for determination of chlorophyll fluorescence. The maximum quantum yield in the dark-adapted state ($F_v/F_m$), the actual quantum yield in the light-adapted steady state ($\Phi_{PSII}$), the coefficients of photochemical quenching (qP), and the nonphotochemical quenching (NPQ) values were measured.

### 2.4. Determination of effects on ion accumulation in salt-treated plants

Finely ground samples (0.2 g) of dried plant material were hydrolyzed with concentrated nitric acid in a microwave reaction system (MARS5; CEM). The $Na^+$, $K^+$, and $Ca^{2+}$ contents in extracts were analyzed with a Varian Vista-MPX simultaneous inductively coupled plasma optical emission spectrometer (Nyomora et al., 1997). $Cl^-$ content was determined by the $AgNO_3$ titration method as previously described (Johnson and Ulrich, 1959).

### 2.5. Determination of total enzyme activities in *Apera intermedia* under salinity

The samples were homogenized and centrifuged at 14,000 × *g* for 30 min. Total protein content measurements were performed (Bradford, 1976). Total SOD, CAT, POX, APX, and GR activities were determined by the methods described by Beauchamp and Fridovich (1971), Bergmeyer (1970), Herzog and Fahimi (1973), Nakano and Asada (1981), and Foyer and Halliwell (1976), respectively.

### 2.6. Identification of isozymes

Samples containing 100 µg of protein were subjected to nondenaturing polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). The different SOD isozymes were identified by incubating gels in inhibitors of SOD

such as 2 mM KCN to inhibit Cu/Zn-SOD activity and 3 mM $H_2O_2$ to inhibit Cu/Zn-SOD and Fe-SOD activities, as described by Vitória et al. (2001).

CAT isozymes were identified as described by Woodbury et al. (1971). The samples including 40 µg of protein were loaded and subjected to PAGE. The gel was transferred to 0.01% $H_2O_2$ for 15 min and incubated for 20 min in staining solution containing 1% $FeCl_3$ and 1% $K_3Fe(CN_6)$.

POX isozymes were identified according to Seevers et al. (1971). The gels loaded with 40 µg of protein were incubated in a tampon including Na-acetate (pH 5.0), benzidine, and $H_2O_2$ for 30 min. The gels were transferred to buffer containing acetic acid for storage. Gels stained for SOD, CAT, and POX isozymes were photographed with the Gel Doc XR+ system and were then analyzed with the Image Lab software package (v.4.0.1).

### 2.7. NOX enzyme/isozyme activity

Total NOX activity was measured according to methods reported previously by Jiang and Zhang (2002). The reduction in XTT was determined at 470 nm. NOX isozymes were determined as given by Sagi and Fluhr (2006). Gels containing 40 µg of protein were stained in buffer including Tris–HCl, NBT, $MgCl_2$, and $CaCl_2$. After adding $NADPH.Na_4$ to the mixture, the resulting blue bands were photographed.

### 2.8. Determination of ROS accumulation

$O_2^-$ content was determined using the process described by Xu et al. (2006) with a slight modification. The specific absorbance was determined at 530 nm. Determination of $H_2O_2$ content was estimated according to the method of Liu et al. (2010). The absorbance of the sample was read at 410 nm. Determination of $OH^-$ scavenging activity was performed according to Chung et al. (1997) with minor changes.

### 2.9. Determination of lipid peroxidation levels

Thiobarbituric acid reactive substances (TBARS) measurement was done according to the method of Madhava Rao and Sresty (2000).

### 2.10. Statistical analysis

Each of the 4 treatment groups included 6 replicates (n = 6) and the standard error was calculated for each measurement. Statistical analysis of the values was performed by using SPSS 20.0 and differences among groups were compared using Tukey's posttest with significant differences at the 5% level. Vertical bars in figures and values in tables indicate ±SE.

## 3. Results

### 3.1. Effects of salinity on growth and water status in *Apera intermedia*

As shown in Table 1, the shoot length of *Apera intermedia* treated with NaCl was significantly inhibited at 600 mM (35% and 65% for 7 and 14 days, respectively). While FW and DW were similar to those of the control group at 150 mM during the experiment, these parameters both decreased after exposure to 300 and 600 mM NaCl at 14 days.

Changes in RGR in response to different salt concentrations are given in Table 2. RGR significantly increased at 150 mM NaCl, but gradually decreased in plants at 600 mM NaCl as compared to the control group throughout the experiment. At 300 mM NaCl, RGR remained the same only in the 7-day group. The highest decrease in RGR was recorded at 600 mM NaCl at 14 days (51%).

RWC in the control plants was 81%. Minimum water content was observed in the 150 and 300 mM NaCl-treated plants (approximately 79% and 67%, respectively) at 14 days (Table 2). RWC was unaltered at 150 mM on

**Table 1.** Effects on length (cm), fresh weight (FW; g plant$^{-1}$), and dry weight (DW; g plant$^{-1}$) in *Apera intermedia* exposed to 0, 150, 300, and 600 mM NaCl for 7 and 14 days. Values followed by the same letter in a column are not significantly different.

| | Groups | Length | FW | DW |
|---|---|---|---|---|
| 7 days | 0 mM | $10 \pm 0.73^a$ | $0.061 \pm 0.002^a$ | $0.0091 \pm 0.0004^a$ |
| | 150 mM | $7.88 \pm 0.14^c$ | $0.061 \pm 0.002^a$ | $0.0097 \pm 0.001^a$ |
| | 300 mM | $7.25 \pm 0.16^c$ | $0.055 \pm 0.005^c$ | $0.0081 \pm 0.001^a$ |
| | 600 mM | $6.5 \pm 0.21^b$ | $0.021 \pm 0.001^b$ | $0.007 \pm 0.001^b$ |
| 14 days | 0 mM | $16.14 \pm 0.51^a$ | $0.088 \pm 0.004^a$ | $0.0101 \pm 0.001^a$ |
| | 150 mM | $8.81 \pm 0.21^d$ | $0.081 \pm 0.001^a$ | $0.0116 \pm 0.001^a$ |
| | 300 mM | $7.37 \pm 0.14^c$ | $0.042 \pm 0.003^d$ | $0.0081 \pm 0.001^c$ |
| | 600 mM | $6.92 \pm 0.78^b$ | $0.019 \pm 0.002^c$ | $0.0071 \pm 0.0002^b$ |

**Table 2.** Effects on relative growth rate (RGR; mg mg$^{-1}$ day$^{-1}$), relative water content (RWC; %), and leaf osmotic potential ($\Psi_{\Pi}$, MPa) in *Apera intermedia* exposed to 0, 150, 300, and 600 mM NaCl for 7 and 14 days. Values followed by the same letter in a column are not significantly different.

| | Groups | RGR | RWC | $\Psi_{\Pi}$ |
|---|---|---|---|---|
| 7 days | 0 mM | 0.075 ± 0.009$^{a}$ | 80.88 ± 0.25$^{a}$ | –1.05 ± 0.026$^{a}$ |
| | 150 mM | 0.084 ± 0.01$^{c}$ | 81.51 ± 1.15$^{a}$ | –1.44 ± 0.039$^{b}$ |
| | 300 mM | 0.071 ± 0.01$^{a}$ | 79.39 ± 0.76$^{a}$ | –1.83 ± 0.051$^{c}$ |
| | 600 mM | 0.046 ± 0.01$^{b}$ | 78.36 ± 1.8$^{b}$ | –3.11 ± 0.017$^{d}$ |
| 14 days | 0 mM | 0.082 ± 0.007$^{a}$ | 80.65 ± 1.36$^{a}$ | –1.3 ± 0.02$^{a}$ |
| | 150 mM | 0.11 ± 0.009$^{d}$ | 79.31 ± 1.33$^{a}$ | –1.74 ± 0.082$^{b}$ |
| | 300 mM | 0.067 ± 0.008$^{c}$ | 66.87 ± 1.55$^{c}$ | –2.79 ± 0.061$^{c}$ |
| | 600 mM | 0.04 ± 0.004$^{b}$ | 58.33 ± 0.37$^{b}$ | –4.19 ± 0.23$^{d}$ |

both sampling days. However, after exposure to 600 mM, a significant reduction in RWC began on the first day of treatment compared with the control group. RWC decreased to 58% when plants were treated with an excessively high NaCl concentration (600 mM).

Table 2 showed the changes in the $\Psi_{\Pi}$ of *Apera intermedia* under various salinity levels. The leaf $\Psi_{\Pi}$ decreased as salt level increased. At 600 mM NaCl, *A. intermedia* exhibited the lowest leaf $\Psi_{\Pi}$, which was 3.22-fold lower than that of the control at 14 days.

The changes in leaf Pro, Cho, and GB contents are shown in Figure 1A-1C. Pro levels increased under salinity and were maintained at high levels until the end of the sampling period (Figure 1A). For example, the highest

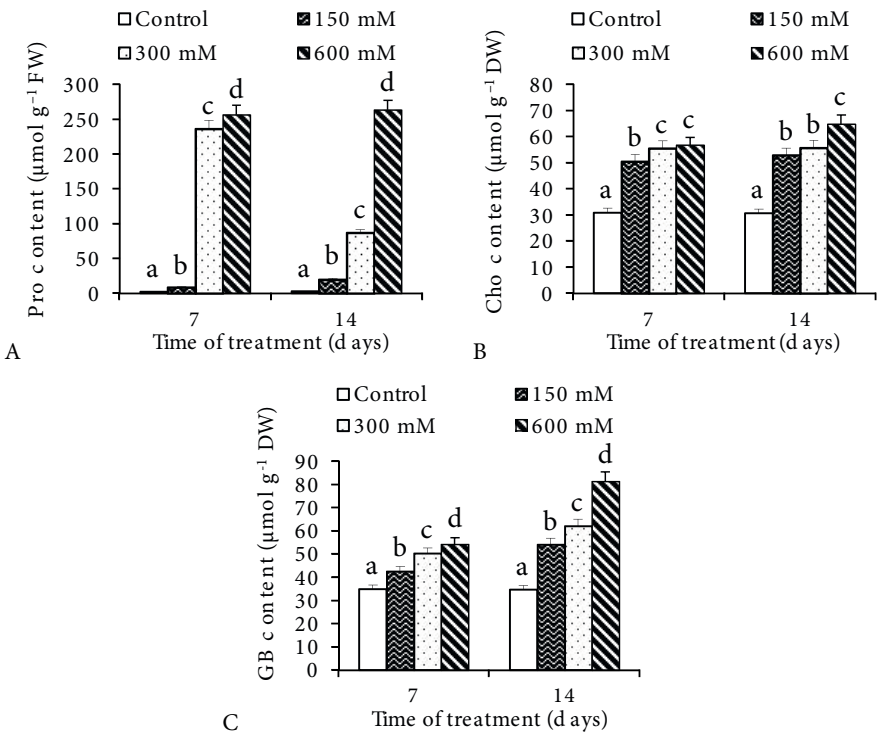


**Figure 1.** Effects on proline (Pro; µmol g$^{-1}$ FW) (A), choline (Cho; µmol g$^{-1}$ DW) (B), and glycine betaine (GB; µmol g$^{-1}$ DW) (C) in *Apera intermedia* subjected to 0, 150, 300, or 600 mM NaCl for 7 and 14 days.

levels of Pro were observed at 600 mM NaCl at 107-fold higher than the control at 7 days. Moreover, salinity caused significant increases in GB accumulation at 7 days of stress. For example, while GB content was 34.73 nmol $g^{-1}$ DW under control conditions at 14 days, after exposure to 600 mM, it was enhanced to 81.45 nmol $g^{-1}$ DW (Figure 1C). Like GB content, there was a steady increase in Cho content with increasing salinity up to 600 mM as compared to the control group (Figure 1B). The progressive enhancement Cho content reached maximum levels in 600 mM NaCl-treated plants (112%) after 14 days of stress.

### 3.2. Effects of salinity on photosynthetic parameters in *Apera intermedia*

Table 3 shows the variations in the maximal efficiency of PSII photochemistry from dark-adapted leaves ($F_v/F_m$), the actual efficiency of PSII in the light-adapted steady state ($\Phi_{PSII}$), the coefficients of photochemical quenching (qP), and nonphotochemical quenching (NPQ) in salt-stressed *Apera intermedia*. After 150 and 300 mM NaCl treatment throughout the experiment, $F_v/F_m$, $\Phi_{PSII}$, and qP did not change, but an increase in NPQ began after 300 mM NaCl treatment. A significant drop in $F_v/F_m$, $\Phi_{PSII}$, and qP was only observed in 600 mM-treated plants at 14 days.

### 3.3. Effects of salinity on ion accumulation in *Apera intermedia*

As shown in Figures 2A–2E, accumulation of $Na^+$, $Cl^-$, or $Na^+/K^+$ in the leaves of plants was significantly increased due to salt stress (Figures 2A, 2B, and 2E), whereas leaf $Ca^{2+}$ and $K^+$ contents were decreased (Figures 2C and 2D). The highest reduction/induction in leaf ion accumulation was observed when 600 mM NaCl was applied for 14 days.

### 3.4. Effects of salinity on antioxidant enzyme/isozyme activities in *Apera intermedia*

As shown in Figure 3A, after the PAGE analysis, 4 SOD isozymes were identified. SOD isozymes in salt-treated *Apera intermedia* were identified as 2 Mn-SODs, 1 Fe-SOD, and 1 Cu/Zn-SOD. Total SOD activity increased under salt stress and the increase in total SOD activity was similar to changes in its isozymes during the experimental period. Intensities of all SOD isozymes showed a resemblance to the increase in total enzyme activity at 600 mM NaCl (Figure 3B). For example, electrophoretic analyses of gels in 600 mM-treated plants showed that Fe-SOD and Cu/Zn-SOD accounted for 52% and 82% in comparison to the control at 14 days, respectively (Figures 3B and 3C).

Isozyme profiles of CAT activity are presented in Figure 4A. CAT was revealed as one major isozyme. The intensity of CAT isozyme in salt-stressed *Apera intermedia* decreased or remained constant (Figure 4B). Moreover, total CAT activity exhibited a similar isozyme staining pattern in all the salt treatments (Figure 4C). At 14 days, the intensity of CAT isozyme showed a concomitant decrease that paralleled total CAT activity. The density of CAT isozyme dropped from 1.3 units to 0.92 units after exposure to 600 mM; total activity decreased by 35% as compared to the control group.

Five isozymes were observed in the evaluation of POX isozyme profiles under salinity (Figure 5A). The POX5 isozyme was not identified in the control group on the first sampling day of stress (Figure 5B). The decline in total POX activity coincided with a decrease in intensity of the POX4 isozyme at all salt concentrations (Figures 5B and 5C). At 7 days, the highest decline in total POX was 13% after 300 mM treatment. Moreover, at 14 days, no alteration was observed in total POX activity after exposure to 150 and

**Table 3.** The changes in the maximal efficiency of PSII photochemistry ($F_v/F_m$), the actual efficiency of PSII in the light-adapted steady state ($\Phi_{PSII}$), the coefficients of photochemical quenching (qP), and nonphotochemical quenching (NPQ) in *Apera intermedia* exposed to 0, 150, 300, and 600 mM NaCl for 7 and 14 days. Values followed by the same letter in a column are not significantly different.

| | Groups | $F_v/F_m$ | $\Phi_{PSII}$ | qP | NPQ |
|---|---|---|---|---|---|
| 7 days | 0 mM | $0.853 \pm 0.03^a$ | $0.844 \pm 0.02^a$ | $0.977 \pm 0.02^a$ | $0.021 \pm 0.01^a$ |
| | 150 mM | $0.852 \pm 0.01^a$ | $0.841 \pm 0.01^a$ | $0.979 \pm 0.02^a$ | $0.023 \pm 0.01^a$ |
| | 300 mM | $0.855 \pm 0.01^a$ | $0.844 \pm 0.02^a$ | $0.977 \pm 0.01^a$ | $0.033 \pm 0.03^b$ |
| | 600 mM | $0.851 \pm 0.02^a$ | $0.826 \pm 0.01^b$ | $0.967 \pm 0.01^a$ | $0.04 \pm 0.01^c$ |
| 14 days | 0 mM | $0.854 \pm 0.01^a$ | $0.848 \pm 0.01^a$ | $0.978 \pm 0.01^a$ | $0.023 \pm 0.01^a$ |
| | 150 mM | $0.851 \pm 0.01^a$ | $0.847 \pm 0.01^a$ | $0.975 \pm 0.03^a$ | $0.027 \pm 0.01^a$ |
| | 300 mM | $0.86 \pm 0.01^a$ | $0.842 \pm 0.01^a$ | $0.967 \pm 0.02^a$ | $0.034 \pm 0.01^b$ |
| | 600 mM | $0.761 \pm 0.1^b$ | $0.787 \pm 0.03^b$ | $0.943 \pm 0.03^b$ | $0.058 \pm 0.04^b$ |

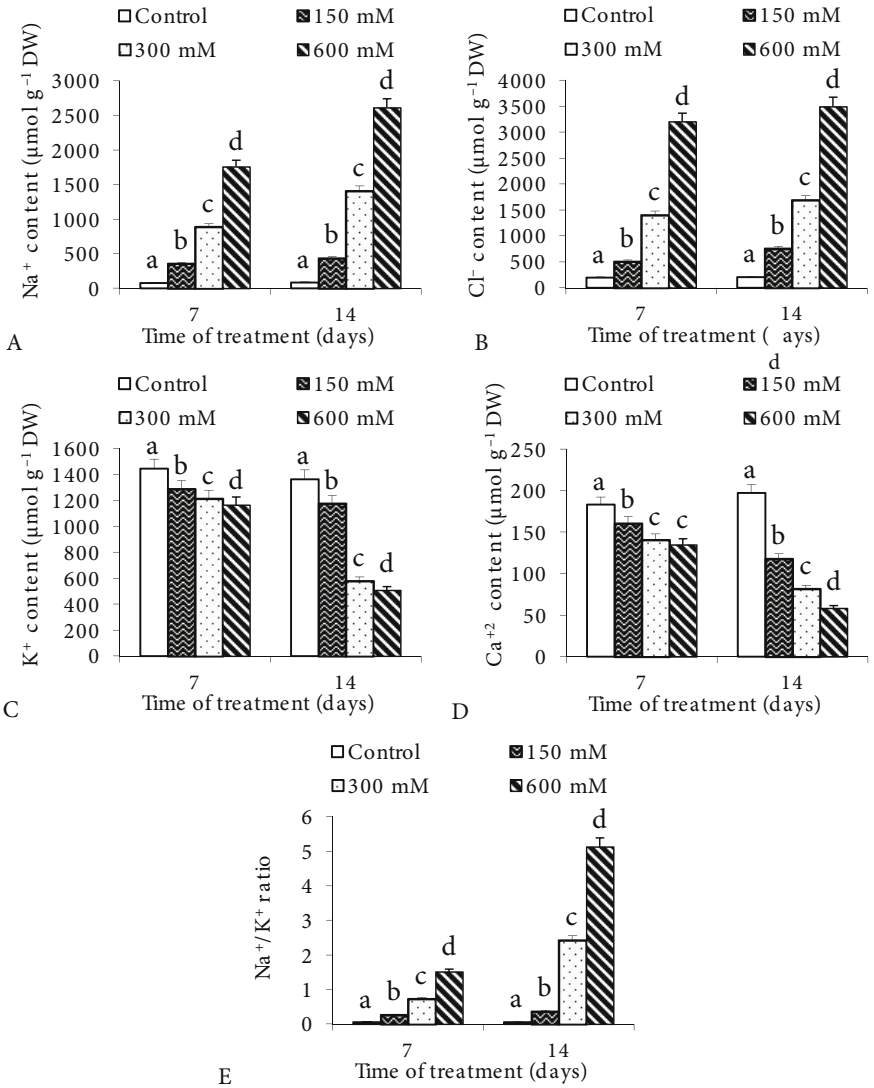


**Figure 2.** Effects on $Na^+$ (A), $Cl^-$ (B), $K^+$ (C), and $Ca^{2+}$ (D) contents (µmol $g^{-1}$ DW) and $Na^+/K^+$ ratio (E) in *Apera intermedia* plants exposed to 0, 150, 300, or 600 mM NaCl for 7 and 14 days.

300 mM (Figure 5C), judging by the expression of POX1, 2, and 3. However, 600 mM caused a decrease in total POX (6%), suggesting a decline in the relative contributions of POX1, 3, and 4.

At 7 and 14 days of stress, APX increased with salinity. At the latter, a gradual induction in APX activity was noticed (Figure 6A). The highest increase was recorded at 600 mM NaCl at 7 and 14 days (31% and 40%, respectively). Except for at 7 and 14 days in 150 mM-treated plants, at which GR did not change or decrease, the stress significantly caused a rise in GR when compared with the control group (Figure 6B).

### 3.5. Effects of salinity on NOX activity in *Apera intermedia*

The decline in total NOX activity was parallel to the densities of NOX isozymes under salt stress (Figure 7A). Regarding NOX isozymes, 6 NOX isozymes in salt-stressed *Apera intermedia* were visualized in native PAGE and 5 of the isozymes, excluding NOX2, were identified in all groups under both control and salt conditions (Figure 7B). The NOX2 isozyme was identified only in the control group at the first day of stress. NOX6 was highly expressed at 7 days of all salt treatments, in spite of the decrease in total NOX activity (Figure 7B). In parallel to the NOX isozyme levels, total NOX activity gradually decreased

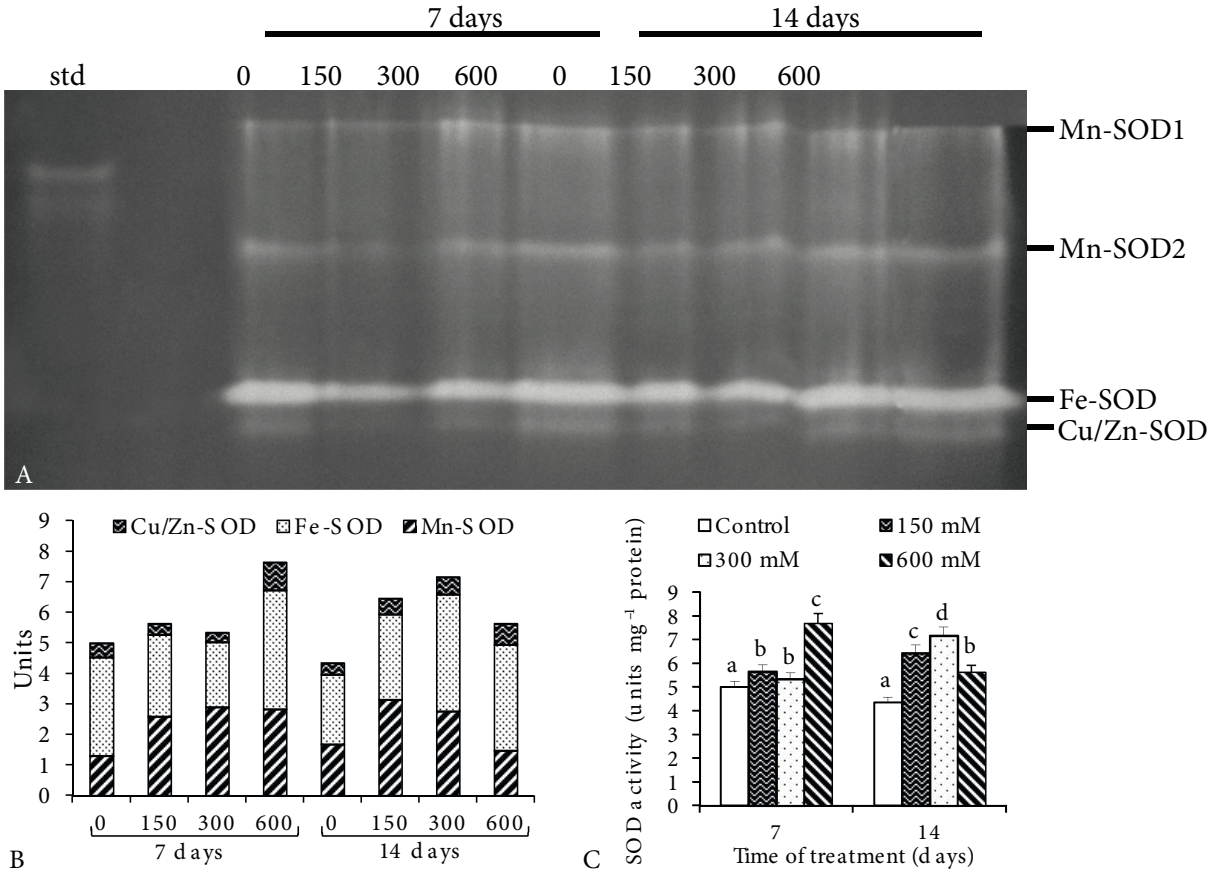


**Figure 3.** Activity staining (A), intensity of each isozymes (unit) (B), and total activity (units $mg^{-1}$ protein) (C) of SOD in the crude extract of *Apera intermedia* subjected to 0, 150, 300, or 600 mM NaCl for 7 and 14 days. Samples applied to the gels contained 100 µg of protein (std. 0.5 units SOD).

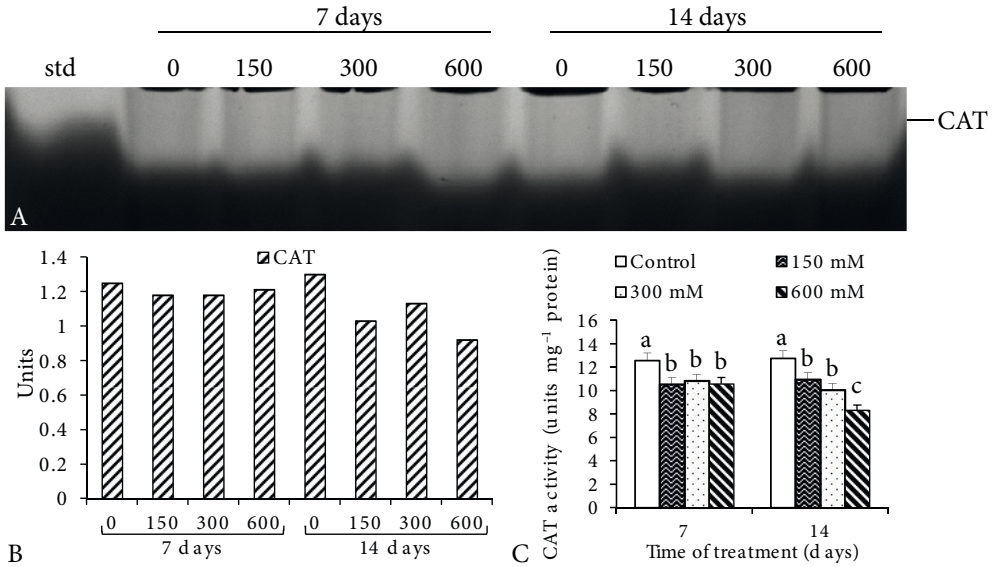


**Figure 4.** Activity staining (A), intensity of each isozymes (unit) (B), and total activity (units $mg^{-1}$ protein) (C) of CAT in the crude extract of *Apera intermedia* subjected to 0, 150, 300, or 600 mM NaCl for 7 and 14 days. Samples applied to the gels contained 40 µg of protein (std. 0.5 units CAT).

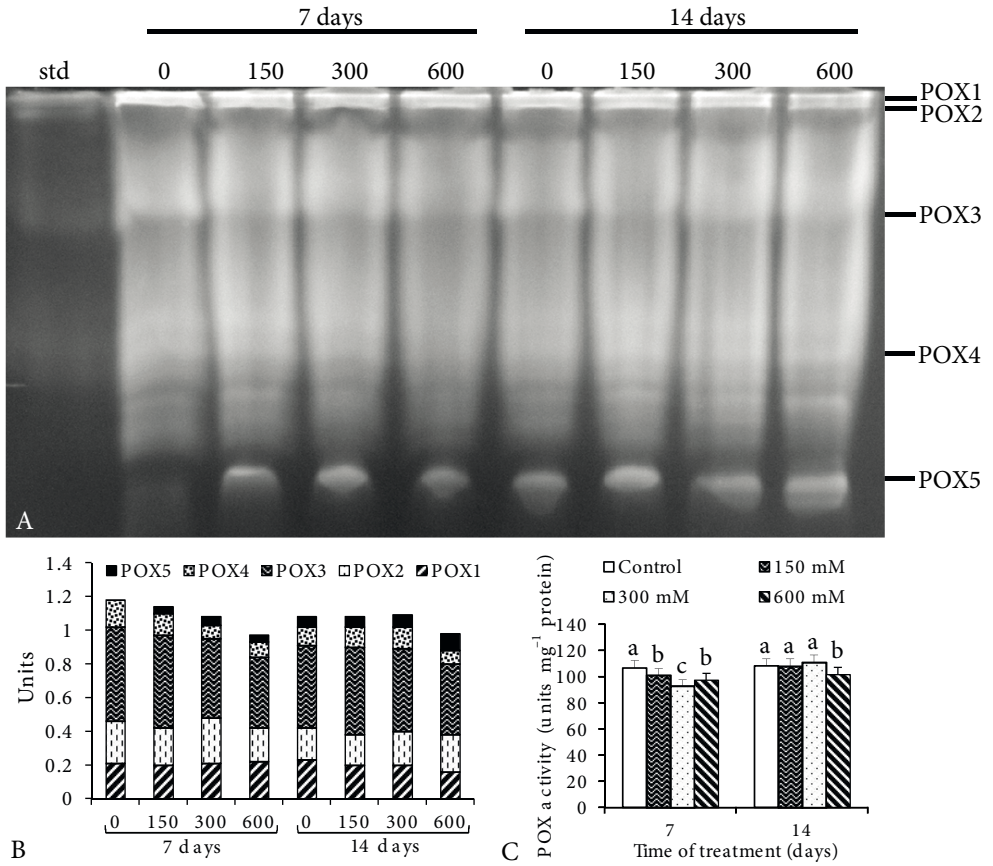


**Figure 5.** Activity staining (A), intensity of each isozymes (unit) (B), and total activity (units $mg^{-1}$ protein) (C) of POX in the crude extract of *Apera intermedia* subjected to 0, 150, 300, or 600 mM NaCl for 7 and 14 days. Samples applied to the gels contained 40 µg of protein (std. 0.2 units POX).

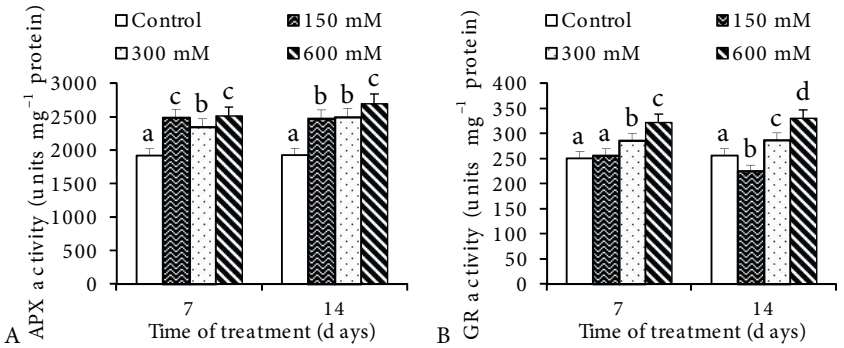


**Figure 6.** Total enzyme activity (units $mg^{-1}$ protein) of APX (A) and GR (B) in the crude extract of *Apera intermedia* subjected to 0, 150, 300, or 600 mM NaCl for 7 and 14 days.

throughout the experimental period. The lowest level of total NOX activity was detected in 600 mM NaCl-treated plants (50%) at 14 days (Figure 7C).

### 3.6. Effects of salinity on ROS accumulation in *Apera intermedia*

$O_2^-$ content progressively increased with NaCl treatments (Figure 8A). $O_2^-$ levels in 600 mM-treated plants were 3 times higher than in the control after 14 days of stress. Salinity-induced $H_2O_2$ content also gradually increased, with a more pronounced induction observed after 14 days of stress (Figure 8B). $H_2O_2$ content increased by 53% in plants exposed to 600 mM on day 7 in comparison with the control group. While significant increases in activity of $OH^-$ scavenging occurred at 150 mM NaCl, 300 mM

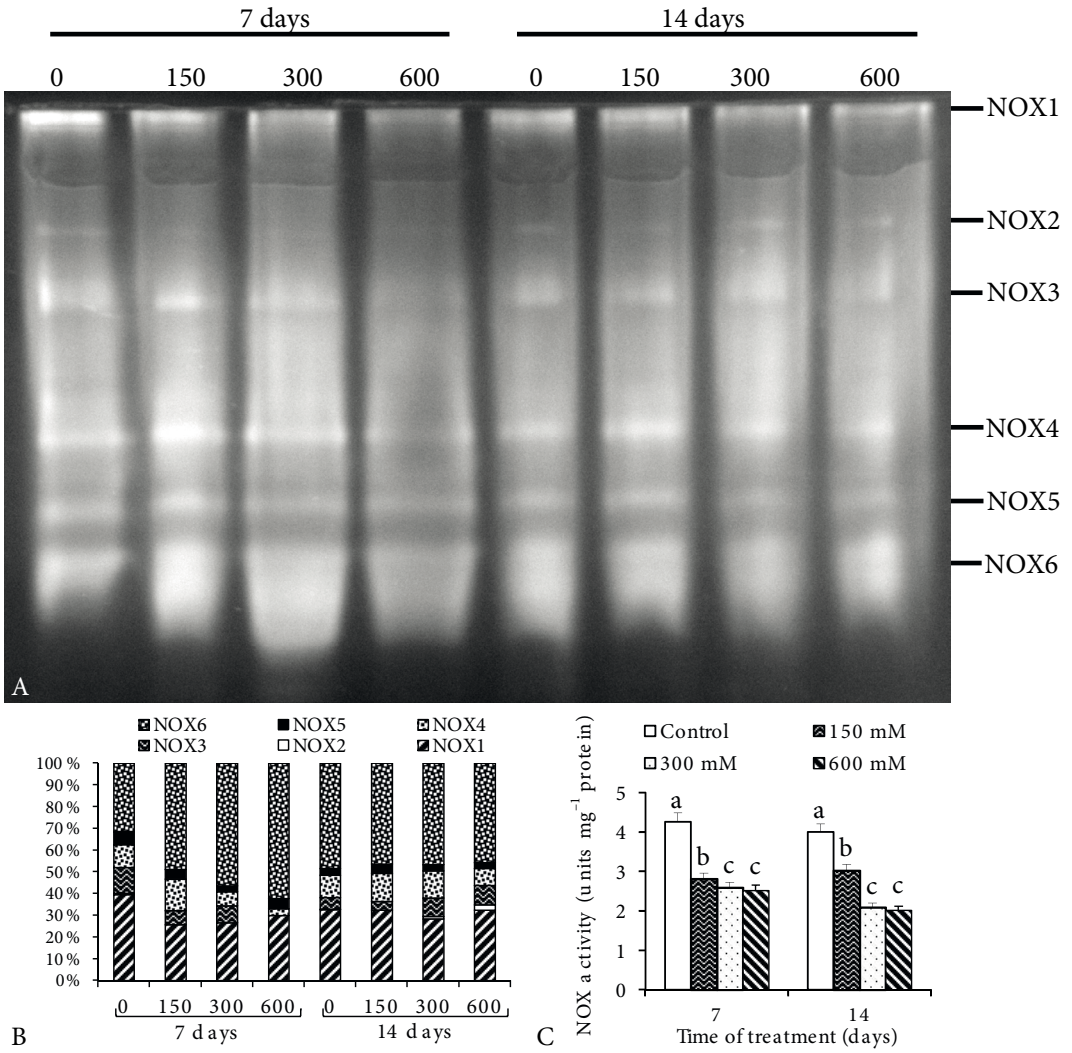


**Figure 7.** Activity staining (A), intensity of each isozymes (unit) (B), and total activity (units $mg^{-1}$ protein) (C) of NOX in the crude extract of *Apera intermedia* subjected to 0, 150, 300, or 600 mM NaCl for 7 and 14 days. Samples applied to the gels contained 40 µg of protein.

did not change this scavenging activity level (Figure 8C). However, it was reduced after 600 mM application for 14 days (7%).

### 3.7. Effects of salinity on TBARS in *Apera intermedia*

To specify the oxidative damage induced by salinity, we obtained variations in TBARS content in the leaves of *Apera intermedia* (Figure 9). It was seen that 150 mM NaCl did not alter TBARS as compared to the control group. TBARS increased at 300 and 600 mM and was more pronounced at 600 mM (176%) at 14 days.

## 4. Discussion

Although the responses of plants have been reported under salinity in many articles, the main adaptations and the mode of action in plants exposed to stress still remain unclear (Hasegawa et al., 2000). The decline in plant metabolic processes at supraoptimal salt concentrations, even in halophytes, is also still awaiting clarification (Flowers and Colmer, 2008). Therefore, this present study could help understand how *Apera intermedia* adapts to extreme salt stress conditions during the vegetative stage. The preliminary study revealed that salinity caused a reduction in plant development via the drop in leaf water potential, enzyme inhibition, ionic instability, and variations in solute content (Munns, 2005; Mittler et al., 2011). As expected, the biomass of *A. intermedia* under saline conditions was considerably decreased and was indicative of severe growth limitations; in this regard, our data agreed with that of Gama et al. (2007). The decrease in RGR at 600 mM was consistent with previously reported

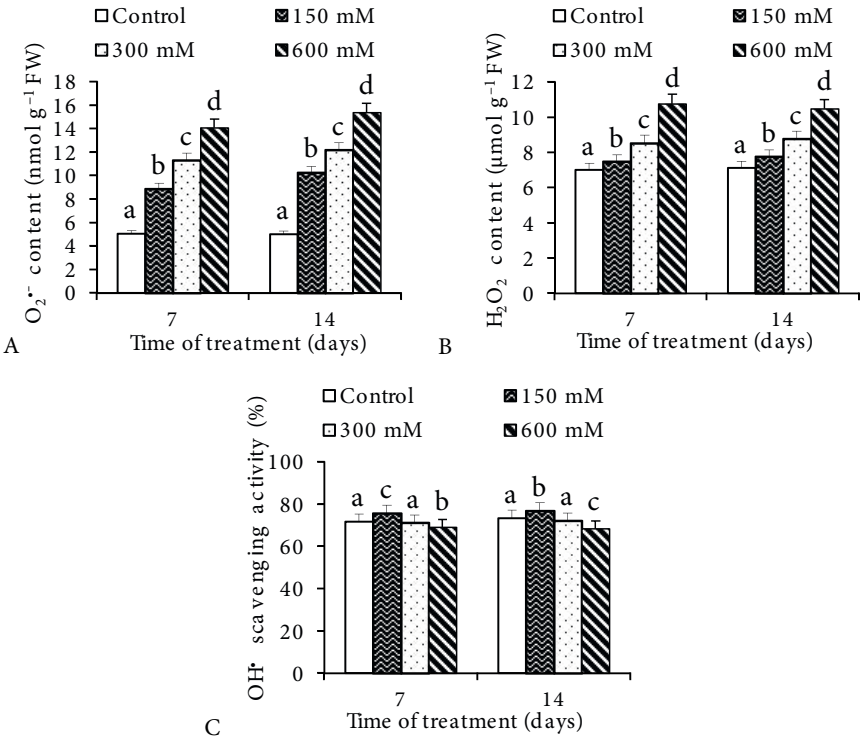


**Figure 8.** Effects on $O_2^-$ content (nmol $g^{-1}$ FW) (A), $H_2O_2$ content (µmol $g^{-1}$ FW) (B), and scavenging activity of $OH^-$ (%) (C) in the crude extract of *Apera intermedia* subjected to 0, 150, 300, or 600 mM NaCl for 7 and 14 days.

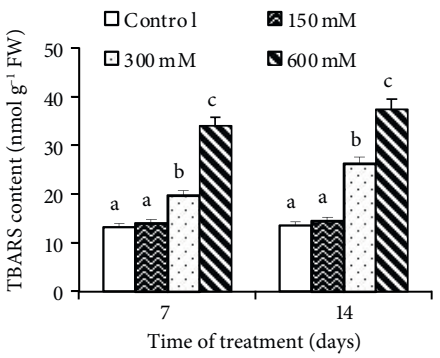


**Figure 9.** Effects on thiobarbituric acid-reactive substances (TBARS; nmol $g^{-1}$ FW) in the crude extract of *Apera intermedia* subjected to 0, 150, 300, or 600 mM NaCl for 7 and 14 days.

data from Ben Hamed et al. (2007). It is reported by Munns (2005) that subjection to short-term salinity causes osmotic effects, while exposure to long-term salinity results in ionic unbalance. The reduction in RWC in the salt-treated plants could be associated with a drop in the transpiration rate and/or the increase in stomatal resistance. This result was consistent with results of a previous study (Kholova et al., 2010). A decline in $\Psi_\Pi$ is an alternative strategy for taking water into cells from the soil and keeping turgor in cells under salt stress (Zhang et al., 2010). The decrease in $\Psi_\Pi$ in salt-treated *A. intermedia* might have originated from osmolyte accumulation. As demonstrated in a previous study (Misra and Gupta, 2005), there is a negative correlation between leaf $\Psi_\Pi$ and proline accumulation in salt treated *A. intermedia*. The increase in osmoprotectant contents has been related to adaptation to salinity (Meloni et al., 2004). The accumulation of these compounds is not only important for cell osmoregulation by stabilizing the $\Psi_\Pi$ in cytosol, but also for the maintenance of subcellular structure and cellular macromolecules (Munns, 2005). The levels of Pro, Cho, and GB in response to salt stress have already been reported in *Prosopis alba* Griseb. (Meloni et al., 2004). Pro, Cho, and GB contents increased in the leaves of salt-stressed *A. intermedia*. The protection of water status in 150 mM NaCl-treated *A. intermedia* could be attributed to significant osmolyte induction. However, the increased osmolyte accumulation in the highest salt treatment was not enough to adjust the osmoregulation in *A. intermedia*, as suggested by the decline in RWC and growth parameters. It is possible that the protective effect of Pro might be weakened with increases in salt concentration and exposure time.

The effects of salinity on photosynthetic parameters have been used to scan for perturbations in the

photosynthetic apparatus (Khan et al., 2006). In this study, $F_v/F_m$, $\Phi_{PSII}$, qP, and NPQ were close to control levels at a low NaCl concentration, but significant changes were observed at 600 mM. Meanwhile, 150 mM NaCl did not have a significant effect on photosynthetic activity on any days of treatment. These results are in accordance with those of Cha-um et al. (2010). On the other hand, 600 mM might be caused by 1) the loss of photosynthetic pigment function, 2) damages in the reaction center of PSII by the excessive excitation energy, or 3) the inducing of photoinhibition.

The increase in $Na^+$ content and decrease in $K^+$ content disturbs ionic imbalance (Kholova et al., 2010), as observed in *Apera intermedia* subjected to salt stress. Ion toxicity leads to cell dehydration and membrane dysfunction. Such accumulation of inorganic ions induced by salt stress may also inactivate both photosynthetic and respiratory electron transport. From these results, we concluded that the reduction in growth, RWC, and chlorophyll fluorescence of 600 mM NaCl-treated *A. intermedia* for 14 days might not be due to the tissue $Na^+$ accumulation. These results are similar to those of Mandhania et al. (2006). On the other hand, a low $Na^+/K^+$ ratio in plants under saline conditions has been suggested as one of the important selection criteria for salt tolerance (Wenxue et al., 2003). However, the $Na^+/K^+$ ratio did not prove to be an effective selection criterion for discriminating *A. intermedia*.

Superoxide ($O_2^-$) production mainly takes place during the reduction of $O_2$ (respiration) and oxidation of $H_2O$ (photosynthesis) in a plant under stress conditions. The utilization of multiple antioxidant enzyme/isozymes is one of the mechanisms involved in preventing the damage caused by ROS (Mittler et al., 2011). SOD plays a crucial role in the detoxification of $O_2^-$ to $H_2O_2$ and $O_2$ (Noctor et al., 2000). SOD activity in NaCl-stressed *Apera intermedia* significantly increased throughout the experiment when compared to the control group. This was mainly due to increase in Mn-SODs and Cu/Zn-SOD. Improvement in SOD activity has mostly been related to salt tolerance (Parida et al., 2004). The data obtained in our paper suggest that, depending on SOD activity, $H_2O_2$ content increased in all the experimental periods. The toxic $H_2O_2$ levels could be scavenged via CAT and/or POX activity by conversion to $H_2O$ and $O_2$. However, CAT enzyme/isozyme activities decreased with salinity, as reported before by Mhadhbi et al. (2004). Similar to CAT, POX activities were similar to control levels or decreased in salt-treated plants. Decreased POX activity and/or inactivation of synthesized enzyme are widely established in many plants, including *Medicago sativa* L., under salinity, as demonstrated by Wang et al. (2009). It appeared in the present study that POX is downregulated in the growth of stressed plants, rather than being upregulated to prevent the damage from excess $H_2O_2$ content (Lin and Kao, 2002). Recent studies have reported that possible sources of $H_2O_2$ generation are xanthine oxidase, oxalate oxidases, amine oxidases, and/or NOX (Gill and Tuteja, 2010). However, in the present study, no detectable increase in NOX enzyme/isozyme activity in salt-treated *A. intermedia* was observed throughout the experiment. Therefore, NOX does not play a role in the production of this radical. Such an inhibition concerning NOX under salt stress was in line with the previous results reported by Rodriguez et al. (2009). The other alternative pathway for scavenging of high $H_2O_2$ production is the ascorbate-glutathione pathway catalyzed by APX, monodehydroascorbate reductase, dehydroascorbate reductase, and GR. In this study, the salt-induced increase in APX might especially suggest a further effective removing mechanism rather than CAT and POX in eliminating $H_2O_2$. However, GR appeared to be inadequate for scavenging the toxic $H_2O_2$ in 150 mM-treated plants. $H_2O_2$ production induced by 150 mM could not cause damage in leaves because of unchanged lipid peroxidation levels. $H_2O_2$ content could play a role in early signaling pathways rather than causing an oxidative damage at 150 mM. However, the drastic enhancement in $H_2O_2$ content at supraoptimal salt concentrations (300 and 600 mM) led to more damage to membrane lipids. After exposure to 300 and 600 mM, APX and GR might have tried to keep $H_2O_2$ at a low level, but they could not succeed in reducing the oxidative damage triggered by $H_2O_2$. $OH^-$ generated from $H_2O_2$ (Haber–Weiss reaction) was more destructive in enhanced cytotoxicity. The enhancement in the activity of $OH^-$ scavenging was only observed with 150 mM NaCl. After 14 days of 600 mM NaCl treatment, the highest level of TBARS (176%) might have stemmed from a decline in scavenging of $OH^-$.

Lipid peroxidation was determined by TBARS accumulation. While 150 mM NaCl did not induce TBARS, after exposure to 300 and 600 mM, toxic levels were quickly reached. The increased TBARS under the higher salt concentrations (300 or 600 mM) pointed to oxidative damage, while it appears that 150 mM is the most appropriate NaCl concentration for the development of *Apera intermedia*. Increase in TBARS in stressed *A. intermedia* might be caused by a disturbance in membrane integrity and an influx of ions to toxic levels. A similar result was obtained during exposure to NaCl by Chang et al. (2012) in *Spirodela polyrhiza* (L.) Schleid.

In conclusion, our data revealed that despite the fact that development of plants was limited by stress, they were able to survive in 150 mM NaCl. It seemed that 150 mM had a moderate effect on TBARS and $H_2O_2$ content at 14 days. *Apera intermedia* did not increase CAT, POX, and GR activities at 150 mM NaCl and 150 mM should not be considered a toxic level. This could be due to: 1) the ability

to keep better subcellular water status as judged by RWC levels close to control levels; 2) better osmotic regulation to salt stress via higher Pro, Cho, and GB contents; 3) lower $\Psi_{\Pi}$ in order to take water into cells from the soil; 4) enhancement in FW, DW, and RGR; 5) more protection of the photosynthetic apparatus; 6) the lower accumulation of TBARS and $H_2O_2$; and 7) increased activity of $OH^-$ scavenging. On the other hand, APX and GR were not sufficient in scavenging of $H_2O_2$ generated by exposure to 300 and 600 mM NaCl for 14 days, as demonstrated by the acute damage to membrane stability.

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