

# BIOCHEMICAL RESPONSES AND SALT REMOVAL POTENTIAL OF *PEGANUM HARMALA* L. (WILD RUE) UNDER DIFFERENT NaCl CONDITIONS

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(Received 6<sup>th</sup> Dec 2019; accepted 6<sup>th</sup> May 2020)

**Abstract.** In this study, biochemical and molecular responses of *Peganum harmala* L. (wild rue) were investigated following NaCl treatment (0, control-, 150- and 300 mmol L<sup>-1</sup>) under growth room conditions. *P. harmala* seeds were placed in pots to germinate the seeds. NaCl application to the seedlings was done at the 4<sup>th</sup> week of seedling growth. The plants were then respectively irrigated with NaCl solution for a period of 5-week. Following harvest, it was noted that fresh and dry weights of *P. harmala* increased with salinity levels. Chlorophyll-*a* and *b*, proline, and malondialdehyde and hydrogen peroxide contents as well as the activity of antioxidant enzymes such as catalase and peroxidase significantly increased as a result of treatments at concentrations between 150 and 300 mmol L<sup>-1</sup> NaCl when compared to those of control plants ( $P \leq 0.05$ ). The highest Na<sup>+</sup> and Cl<sup>-</sup> ions were 78.8 and 68.1 mg g<sup>-1</sup> DW at 300 mmol L<sup>-1</sup> NaCl, respectively. On the other hand, ion contents such as K<sup>+</sup>, Ca<sup>++</sup> and Mg<sup>++</sup> decreased under 150-300 mmol L<sup>-1</sup> NaCl when compared to the control plants ( $P \leq 0.05$ ). Removal of Na<sup>+</sup> as the adjusted removal capacity per hectare under field conditions were 304.6 and 404.2 kg at 150- and 300 mmol L<sup>-1</sup> NaCl concentrations. DNA damage measurement showed that DNA integrity of the halophyte preserved its uniform shape at 150- and 300 mmol L<sup>-1</sup> NaCl according to measurements of the results of the comet assay. This study showed that *P. harmala* could well used to clean-up highly saline soils to regain them for agricultural purposes.

**Keywords:** *phytoremediation, salt stress, ameliorate, comet assay, DNA damage*

## Introduction

Salt stress in soil layers or in irrigation waters is a major threat to modern agriculture via causing inhibition and impairment of plant growth and productivity, especially in arid and semi-arid regions of the world (Hussain et al., 2009; Isayenkov and Maathuis, 2019). More than 20% of cultivated land worldwide, which is about 45 million hectares is affected by salt stress and the amount is increasing day by day (Gupta and Huang, 2014; Shrivastava et al., 2015). A soil is considered to be saline when the electric conductivity (EC) of the soil solution reaches 4 dS m<sup>-1</sup> (equivalent to approximately 40 mmol L<sup>-1</sup> NaCl) and it generates an osmotic pressure of about -0.2 MPa. Under these circumstances, yields of most of the crops are significantly reduced. As a consequence, ion toxicity lead to chlorosis and necrosis mainly due to Na<sup>+</sup> accumulation that interferes with many physiological processes in plants (Munns and Tester, 2008; Roupheal et al., 2018). Under saline conditions, plants have to activate different physiological and biochemical mechanisms in order to cope with the resulting stress. Such mechanisms include changes in toxic ion uptake, ion compartmentation and/or exclusion, osmotic regulation, photosynthesis, chlorophyll content, toxic ion distribution, homeostasis, reactive oxygen species (ROS), and antioxidant metabolites and enzymes (Ashraf and Haris, 2013; Acosta-Motos et al., 2017; Carillo, 2019).

Plants on the basis of adaptive evolution can be classified as either glycophytes or halophytes (Gupta and Huang, 2014; Cheeseman, 2015). Glycophytes are not

salt-tolerant plants, which include most of the crop plants. The survival of these plants is seriously retarded after 100-200 mmol L<sup>-1</sup> NaCl concentrations (Zakharin and Panichkin, 2009; Carillo et al., 2011). Halophytic plants are known to accumulate excess salts in tissues, they remove salt from the immediate environment (Simpson et al., 2018). They are naturally adapted to live in soil characterized by high salt concentrations. They do not have problems with salinity exceeding 300-400 mmol L<sup>-1</sup> NaCl. They have developed better salt resistance mechanisms as described above (Stuart et al., 2012; Cheeseman, 2015).

*P. harmala* belongs to Zygophyllaceae family, which is a perennial plant growing spontaneously in semi-arid conditions, stepp areas and sandy soils. It is native to eastern Mediterranean region. It is a shrub, 0.3-0.8 m tall with short creeping roots, white flowers and round seed capsules carrying more than 50 seeds. The plant is widely distributed and used as a medicinal plant in Central Asia, North Africa and Middle East (Frison et al., 2008; Wanntorp and Ronse De Craene, 2011). In this study, we aimed to determine the performance of *P. harmala* in terms of biochemical responses and Na<sup>+</sup> ion removal capacity under different NaCl conditions (0, control-, 150- and 300 mmol L<sup>-1</sup>) for phytoremediation purposes.

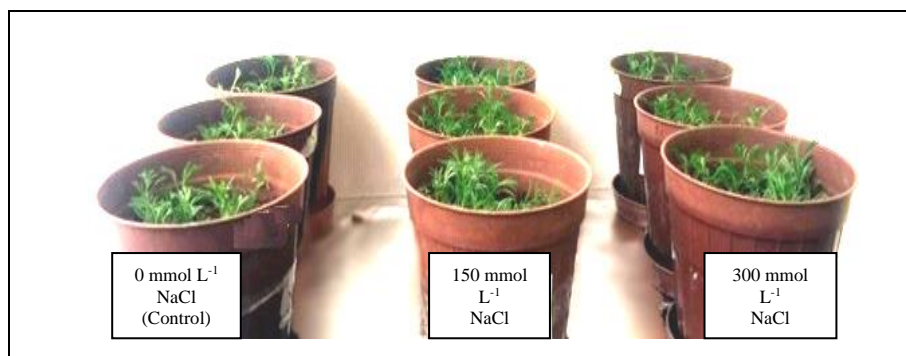
## Material and Methods

### *Experimental set up and plant growth*

Experiment was conducted at the University of Harran, Sanliurfa, Turkey. The halophyte species of *P. harmala* seeds were initially sterilized with 70% ethanol for 30 s and then with 0.1% (w/v) HgCl<sub>2</sub> for five min following three washes with sterile distilled water. Trials were performed in a randomized block design with three replicates. Air-dry soil was passed through 4-mm sieve for the pot experiment and 2-mm sieve for the analysis of physicochemical properties. Soil saturated paste was prepared and electrical conductivity (EC), pH were measured (Richards, 1954). Texture (clay, silt and sand) was determined by using Bouyoucos hydrometer method (Bouyoucos, 1953). Physicochemical properties are reported in *Table 1*. The seeds at batches of 10 were sown into the 1.5 L plastic pots containing 1 kg of air-dry in a growth room at 35/25°C of day/night temperature. After germination, the seedlings were irrigated with full pot capacity during this period; salt treatments were started with the irrigation water containing various concentrations of NaCl (0 (control)-, 150- and 300 mmol L<sup>-1</sup>) solutions (*Fig. 1*). The treatment continued for further 5 weeks, then the plants were harvested for the evaluation of salinity responses. For this, the harvested leaves of *P. harmala* were stored at -22°C until analyses.

**Table 1.** Physicochemical properties of the soil

| Soil parameter           | Values |
|--------------------------|--------|
| EC (dS m <sup>-1</sup> ) | 1.2    |
| pH                       | 7.8    |
| Clay (%)                 | 56.7   |
| Silt (%)                 | 32.5   |
| Sand (%)                 | 10.8   |



**Figure 1.** Growth of *P. harmala* at different NaCl conditions

### **Growth parameters**

For the growth analysis, the fresh weight (FW) was determined right after the harvest. The dry weight (DW) of plants was determined after drying of samples at 70°C until they reached a constant weight.

### **Determination of biochemical responses**

Chlorophyll (Chl-*a* and Chl-*b*) contents of *P. harmala* were determined based on the method of Arnon (1949) with slight modifications (Dikilitas, 2003). Leaf samples (0.5 g) were homogenized in a 10 mL acetone:water (80:20, v:v) mixture and filtered through Whatman No.2 filter paper then placed in dark tubes. Chl-*a* and Chl-*b* of the plant samples were read at a UV microplate spectrophotometer (Epoch, SN: 1611187, made in USA) at 663-645 nm respectively against 80% acetone blank. The results were calculated as mg L<sup>-1</sup> and expressed as mg g<sup>-1</sup> fresh weight.

The proline (pro) measurement was conducted according to the method of Bates et al. (1973) with slight modifications (Karakas et al., 2019a). Leaf material (0.5 g) was homogenized in 3% w/v sulphosalicylic acid using a mortar and a pestle. The homogenate was filtered through Whatman No. 2 filter paper. Then, 2 mL of filtrate was mixed in a test tube with 2 mL of acid-ninhydrin reagent (1.25 g of ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6 M phosphoric acid) and boiled at 100°C for one hour. The reaction was terminated in an ice bath. The reaction mixture was then extracted using 5 mL of toluene. The tubes were thoroughly shaken for 15-20 seconds and left for 20 min at room temperature in order to achieve separation for two layers. The chromophore containing toluene was removed and allowed to warm to room temperature, and the absorbance of the solution was measured at 515 nm using a toluene blank. Proline concentration was determined using calibration curve made with L-proline (Sigma-Aldrich 81202-06-4) as µmol g<sup>-1</sup> fresh tissue.

The malondialdehyde (MDA) content was determined according to the method of Sairam and Saxena (2000) with slight modifications (Karakas et al., 2019a). Leaf material (0.5 g) was homogenized in 10 ml of 0.1% (w/v) TCA solution. The homogenate was centrifuged at 10,000g for five minutes. Four mL of 20% v/v TCA containing 0.5% v/v thiobarbituric acid (TBA) was added to one milliliter of the supernatant. The mixture was incubated in boiling water for 30 min, and the reaction was stopped by placing the reaction tubes in an ice bath. The mixture was centrifuged again at 10,000g for 5 min and the absorbance of the supernatant was read at 532 and 600 nm. Here, the MDA content of leaves is expressed as nmol g<sup>-1</sup> fresh tissue (*Eq. 1*).

$$MDA (nmol g^{-1}) = \frac{\text{Extract volume (ml)} \times [(A_{532} - A_{600}) / (155 \text{ mM}^{-1} \text{ cm}^{-1})]}{\text{Sample amount (g)}} \times 10^3 \quad (\text{Eq.1})$$

Catalase enzyme activity (CAT, E.C. 1.11.1.6) was determined by monitoring the decomposition of H<sub>2</sub>O<sub>2</sub> according to the method of Milosevic and Slusarenko (1996) with slight modifications (Karakas et al., 2019a). Fresh leaf tissue (0.5 g) was homogenized in 10 mL of 50 mmol L<sup>-1</sup> Na-phosphate buffer solution, then 50 µL of plant extract was added to a 2.95 mL (10 mmol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>, 50 mmol L<sup>-1</sup> Na-phosphate buffer and 4 mmol L<sup>-1</sup> Na<sub>2</sub>EDTA) reaction mixture and measured for 30 seconds at 240 nm with a UV microplate spectrophotometer (Epoch, SN: 1611187, made in USA). One CAT activity unit (U) is defined as a change of 0.1 absorbance unit per minute. Activity is expressed as enzyme units per gram FW.

Peroxidase enzyme activity (E.C.1.11.1.7) was determined according to the method by Cvikrova et al. (1994) with slight modifications (Karakas et al., 2019a). For the analysis, 100 µL of extract (obtained as above) was added to 3 mL of the reaction mixture (13 mmol L<sup>-1</sup> guaiacol, 5 mmol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>, and 50 mmol L<sup>-1</sup> Na-phosphate, pH 6.5). The reaction was initiated with a H<sub>2</sub>O<sub>2</sub> addition and was measured at 470 nm using a UV microplate spectrophotometer (Epoch, SN: 1611187, made in USA) at one-minute interval until 3<sup>rd</sup> minute. One unit of POX activity was defined as a change of 0.1 absorbance unit per minute at 470 nm. Activity is expressed as enzyme units per gram of FW.

Hydrogen peroxide levels (H<sub>2</sub>O<sub>2</sub>) were determined according to Sergiev et al. (1997) with slight modifications (Karakas et al., 2019a). Fresh plant tissue (0.5 g) was homogenized in an ice bath with 5 ml 0.1% (w:v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000g for 15 min at 4°C and 0.5 mL of the supernatant were added to 0.5 ml 10 mmol L<sup>-1</sup> potassium phosphate buffer (pH 7.0) and 1 mL 1 M potassium iodide. The absorbance was read at 390 nm using a UV microplate spectrophotometer (Epoch, SN: 1611187, made in USA). The H<sub>2</sub>O<sub>2</sub> content was calculated as µmol g<sup>-1</sup> FW.

### **Determination of ion contents**

The ion contents (Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>) of leaves were determined according to the procedure of Chapman and Pratt (1961) with slight modifications (Karakas, 2013). Samples burned at 500°C were homogenized in 5 mL of a 2N HCl. The homogenate obtained following filtration was analyzed by Inductively Coupled Plasma (ICP, Perkin Elmer). Chloride was determined by ion chromatography (IC) after the filtration through filter paper.

### **Determination of Sodium ion Removal**

The concentration of Na<sup>+</sup> ion removed by harvested *P. harmala* was calculated according to the equation made by Qadir et al. (2003) with slight modifications (Karakas et al., 2017) (Eq. 2).

$$S_{Na-removal} = [(S_{Na-conc})(S_{DW}) / (10^3)] / (MW_{Na}) \quad (\text{Eq.2})$$

where  $S_{\text{Na-removal}}$  is the  $\text{Na}^+$  removal through harvest ( $\text{mmol pot}^{-1}$ ),  $S_{\text{Na-conc}}$  is the ion concentration in the harvested plant ( $\text{mg kg}^{-1}$ ),  $S_{\text{DW}}$  is the plant dry weight ( $\text{g pot}^{-1}$ ), and  $\text{MW}_{\text{Na}}$  is the molecular weight of  $\text{Na}^+$ .

### ***Determination of DNA damage***

Assessment of DNA damage caused by NaCl was made via the comet assay method developed for plants (Gichner et al., 2004; Pourrut et al., 2015). *P. harmala* leaves were placed in a 60-mm Petri dish kept on ice and spread with 250  $\mu\text{l}$  of cold 400  $\text{mmol L}^{-1}$  Tris buffer, pH 7.5. The plate was kept tilted on ice so that the isolated leaves nuclei would collect in the buffer. Frosted-end microscope slides were dipped into a solution of 1% NMP agarose prepared with water at 50°C, dried overnight at room temperature and kept dry in slide boxes until use. Onto each slide, nuclear suspension (50  $\mu\text{l}$ ) and 1% low melting point (LMP) agarose (50  $\mu\text{l}$ ) prepared with phosphate-buffered saline (PBS) were added at 40°C. The nuclei and the LMP agarose were gently mixed and 80  $\mu\text{L}$  aliquots were placed on microscope slides which were pre-coated with 1% normal melting point (NMP) agarose. The drops were then covered with a coverslip and the slides were placed on ice for 5 min. Then, the coverslips were removed and the slides were placed in a horizontal gel electrophoresis tank containing freshly prepared cold electrophoresis buffer (1  $\text{mmol L}^{-1}$   $\text{Na}_2$  EDTA and 300  $\text{mmol L}^{-1}$  NaOH, pH > 13). The nuclei were incubated for 15 min to allow the DNA to unwind prior to electrophoresis at 0.72 V/cm (26V, 300 mA) for 5 min at 4°C. DNA damage was examined after the assay protocol in both control and NaCl-exposed groups. The damaged DNA resembled comets when checked under a fluorescence microscopy. The intensity of the comet tail relative to the head reflected the number of DNA breaks (Collins, 2004). Tail length was assessed using a software called comet assay software program (CASP) (Konca et al., 2003).

### ***Data analysis***

Data were statistically analyzed by one-way analysis of variance (ANOVA) using the SPSS software program (Version 22.0). To separate treatment means for each measured parameter, Duncan's Multiple Range Test was performed at a significance level of  $P \leq 0.05$ .

## **Results**

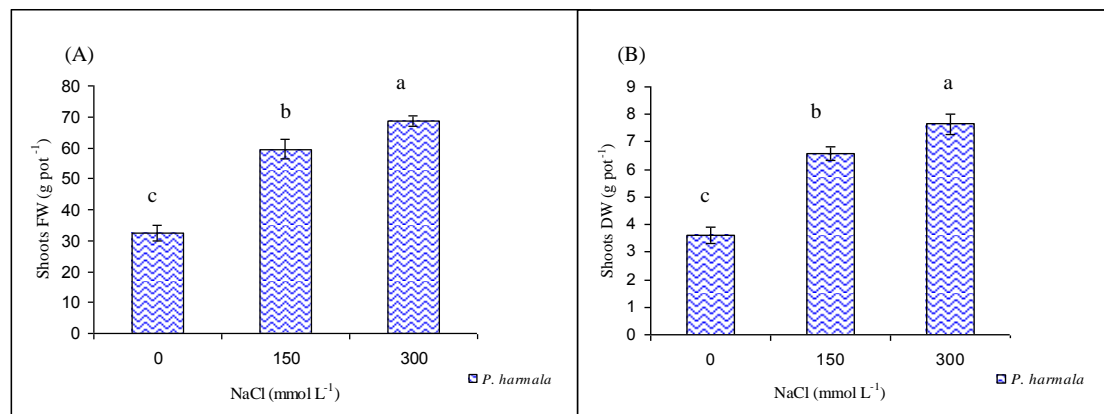
### ***Soil properties***

Soil physicochemical properties were measured before the start the experiment. The soil properties were shown in *Table 1*. According to our findings, soil electrical conductivity of soil paste extract (EC) and pH were 1.2  $\text{dS m}^{-1}$  and 7.8, respectively. The soil texture was determined as clay.

With this table, we observed that the soil was suitable to accumulate excess ions due to its "clay" structure. Therefore, any improvements on this soil through phytoremediation of *P. harmala* would be a promising approach to clean up such soils. Since EC level of the soil was 1.2  $\text{dS m}^{-1}$ , which is quite suitable for agricultural purposes, it is important to determine build up toxic salt ions and their salt level is reduced.

### Plant growth of *P. harmala* under high NaCl stress

Growth parameters were determined as shoot FW and DW. At control, 0 mmol L<sup>-1</sup> NaCl conditions, shoot FW and DW of the plants significantly decreased while at 150- and 300 mmol L<sup>-1</sup> NaCl conditions, shoot FW and DW of the plants significantly increased. *P. harmala* produced almost twice as much shoot DW yield as that of 0 mmol L<sup>-1</sup> NaCl (control) condition (Fig. 2A and 2B, Table 2).

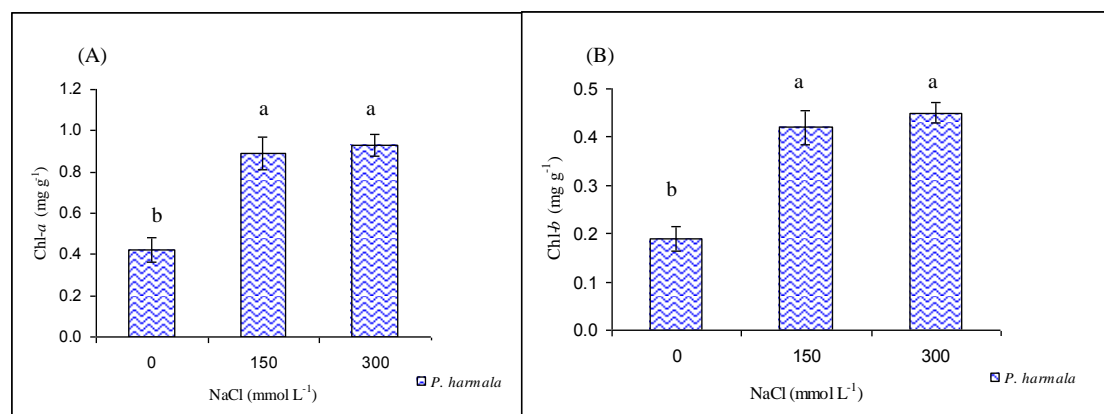


**Figure 2.** A) Shoots FW; B) Shoots DW of *P. harmala* plants at different NaCl concentrations. Bars indicate the means of threes replicates  $\pm$  standard error. Bars with different letters indicate the significant differences from one another according to Duncan's Multiple Range Test at  $P \leq 0.05$

### Determination of biochemical responses of *P. harmala* under high NaCl stress

To determine the biochemical responses of *P. harmala*, measured from the leaves of sampled plants in terms of some parameters were of chlorophyll contents (Chl-*a*, Chl-*b*), proline, MDA, CAT, POX and H<sub>2</sub>O<sub>2</sub> levels.

Chlorophyll contents (Chl-*a* and Chl-*b*) were twice as much under 150-300 mmol L<sup>-1</sup> NaCl as the control plants growing in no salt conditions (Fig. 3A, 3B, Table 2).



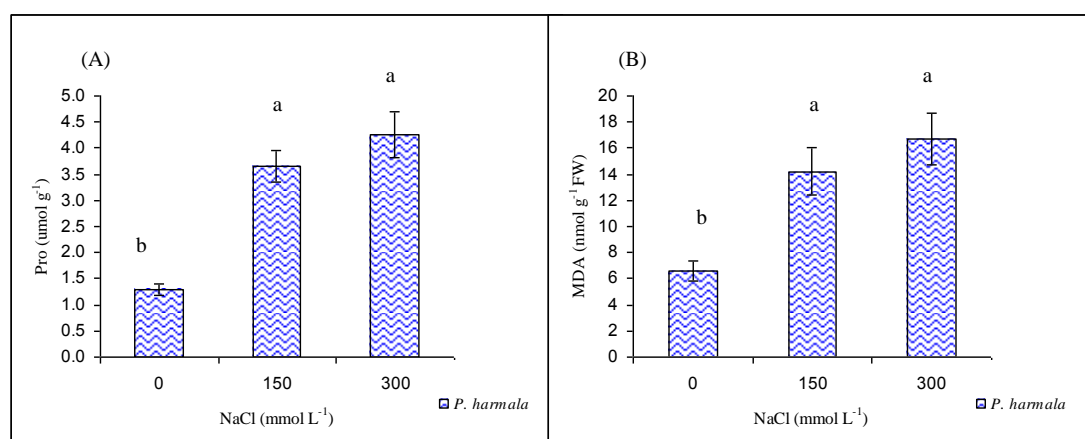
**Figure 3.** A) Chl-*a*; B) Chl-*b* of *P. harmala* plants at different NaCl concentrations. Bars indicate the means of threes replicates  $\pm$  standard error. Bars with different letters indicate significant differences from one another according to Duncan's Multiple Range Test at  $P \leq 0.05$

**Table 2.** Analyses of plant parameters using ANOVA

| Plant parameters              | Salt levels | n | Mean±S.E    | F      | Sig. |
|-------------------------------|-------------|---|-------------|--------|------|
| FW                            | 0           | 3 | 32.45±2.54  | 54.33  | 0.00 |
|                               | 150         | 3 | 59.51±3.19  |        |      |
|                               | 300         | 3 | 68.21±1.67  |        |      |
|                               | Total       | 9 | 53.50±5.56  |        |      |
| DW                            | 0           | 3 | 3.62±0.30   | 43.17  | 0.00 |
|                               | 150         | 3 | 6.60±0.25   |        |      |
|                               | 300         | 3 | 7.65±0.39   |        |      |
|                               | Total       | 9 | 5.95±0.62   |        |      |
| Chl- <i>a</i>                 | 0           | 3 | 0.42±0.07   | 16.10  | 0.00 |
|                               | 150         | 3 | 0.89±0.09   |        |      |
|                               | 300         | 3 | 0.93±0.05   |        |      |
|                               | Total       | 9 | 0.75±0.09   |        |      |
| Chl- <i>b</i>                 | 0           | 3 | 0.19±0.03   | 26.56  | 0.00 |
|                               | 150         | 3 | 0.42±0.04   |        |      |
|                               | 300         | 3 | 0.45±0.02   |        |      |
|                               | Total       | 9 | 0.35±0.04   |        |      |
| pro                           | 0           | 3 | 1.29±0.13   | 24.24  | 0.00 |
|                               | 150         | 3 | 3.66±0.31   |        |      |
|                               | 300         | 3 | 4.25±0.44   |        |      |
|                               | Total       | 9 | 3.07±0.48   |        |      |
| MDA                           | 0           | 3 | 6.58±0.79   | 10.36  | 0.01 |
|                               | 150         | 3 | 14.23±1.86  |        |      |
|                               | 300         | 3 | 16.72±2.00  |        |      |
|                               | Total       | 9 | 12.51±1.73  |        |      |
| CAT                           | 0           | 3 | 0.67±0.08   | 5.70   | 0.04 |
|                               | 150         | 3 | 1.14±0.10   |        |      |
|                               | 300         | 3 | 1.13±0.14   |        |      |
|                               | Total       | 9 | 0.98±0.10   |        |      |
| POX                           | 0           | 3 | 2.39±0.24   | 9.89   | 0.01 |
|                               | 150         | 3 | 4.38±0.37   |        |      |
|                               | 300         | 3 | 3.71±0.34   |        |      |
|                               | Total       | 9 | 3.49±0.33   |        |      |
| H <sub>2</sub> O <sub>2</sub> | 0           | 3 | 3.37±0.20   | 9.18   | 0.02 |
|                               | 150         | 3 | 6.24±0.63   |        |      |
|                               | 300         | 3 | 6.31±0.69   |        |      |
|                               | Total       | 9 | 5.31±0.56   |        |      |
| Leaf Na <sup>+</sup>          | 0           | 3 | 3.53±0.60   | 558.22 | 0.00 |
|                               | 150         | 3 | 65.75±1.59  |        |      |
|                               | 300         | 3 | 78.80±2.41  |        |      |
|                               | Total       | 9 | 49.36±11.64 |        |      |
| Leaf K <sup>+</sup>           | 0           | 3 | 2.58±0.09   | 6.26   | 0.03 |
|                               | 150         | 3 | 2.21±0.12   |        |      |
|                               | 300         | 3 | 2.19±0.04   |        |      |
|                               | Total       | 9 | 2.32±0.08   |        |      |
| Leaf Ca <sup>++</sup>         | 0           | 3 | 1.16±0.06   | 5.20   | 0.05 |
|                               | 150         | 3 | 0.93±0.07   |        |      |
|                               | 300         | 3 | 0.78±0.12   |        |      |
|                               | Total       | 9 | 0.96±0.07   |        |      |

| Plant parameters                                     | Salt levels | n | Mean±S.E       | F     | Sig. |
|--|-------------|---|----------------|-------|------|
| Leaf Mg <sup>++</sup>                                | 0           | 3 | 0.27±0.01      | 13.68 | 0.01 |
|  | 150         | 3 | 0.22±0.01      |       |      |
|  | 300         | 3 | 0.21±0.01      |       |      |
|  | Total       | 9 | 0.23±0.01      |       |      |
| Leaf Cl <sup>-</sup>                                 | 0           | 3 | 7.43±1.03 c    | 54.78 | 0.00 |
|  | 150         | 3 | 45.25±2.96 b   |       |      |
|  | 300         | 3 | 68.14±6.45 a   |       |      |
|  | Total       | 9 | 40.28±9.09     |       |      |
| Removal Na <sup>+</sup><br>(mmol pot <sup>-1</sup> ) | 0           | 3 | 0.57±0.13 c    | 70.05 | 0.00 |
|  | 150         | 3 | 21.56±1.38 b   |       |      |
|  | 300         | 3 | 28.60±2.68 a   |       |      |
|  | Total       | 9 | 16.91±4.30     |       |      |
| Removal Na <sup>+</sup><br>(kg ha <sup>-1</sup> )    | 0           | 3 | 8.10±1.86 c    | 69.90 | 0.00 |
|  | 150         | 3 | 304.57±19.58 b |       |      |
|  | 300         | 3 | 404.17±37.88 a |       |      |
|  | Total       | 9 | 238.94±60.74   |       |      |
| DNA tail length                                      | 0           | 3 | 29.00±2.89 a   | 0.58  | 0.59 |
|  | 150         | 3 | 31.00±3.79 a   |       |      |
|  | 300         | 3 | 34.00±3.21 a   |       |      |
|  | Total       | 9 | 31.331.81      |       |      |
| DNA damage   | 0           | 3 | 4.50±0.58 a    | 0.28  | 0.77 |
|  | 150         | 3 | 4.60±0.67 a    |       |      |
|  | 300         | 3 | 5.13±0.70 a    |       |      |
|  | Total       | 9 | 4.74±0.34      |       |      |

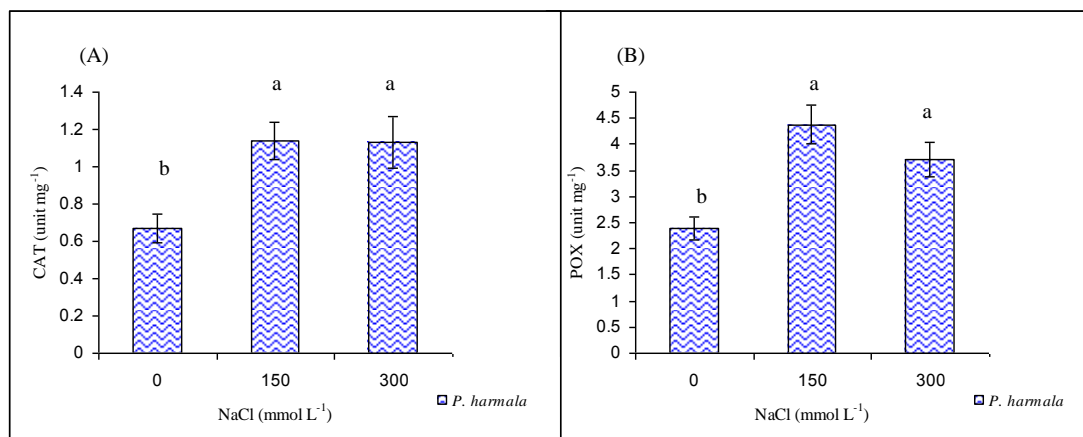
Both proline and MDA contents of *P. harmala* increased at 150-300 mmol L<sup>-1</sup> NaCl stress conditions. At the highest NaCl level (300 mmol L<sup>-1</sup>), proline and MDA contents were 3.3 and 2.5 times higher, respectively than those of leaves in the control group (Fig. 4A, 4B, Table 2).



**Figure 4.** A) Proline (Pro); B) MDA of *P. harmala* plants at different NaCl concentrations. Bars indicate the means of threes replicates ± standard error. Bars with different letters indicate the significant differences from one another according to Duncan's Multiple Range Test at  $P \leq 0.05$

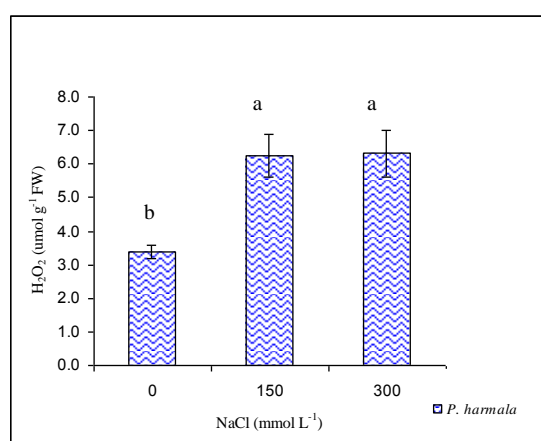


CAT and POX enzymes showed also increasing trend as the concentration of NaCl increased. At 150- and 300 mmol L<sup>-1</sup> NaCl concentrations, both enzymes were remarkably expressed. At 300 mmol L<sup>-1</sup> NaCl, the expression of both enzymes were still high although slightly decreases were evident. However, this was not statistically significant (Fig. 5A and 5B, Table 2).



**Figure 5.** A) CAT; B) POX of *P. harmala* plants at different NaCl concentrations. Bars indicate the means of threes replicates  $\pm$  standard error. Bars with different letters indicate the significant differences from one another according to Duncan's Multiple Range Test at  $P \leq 0.05$

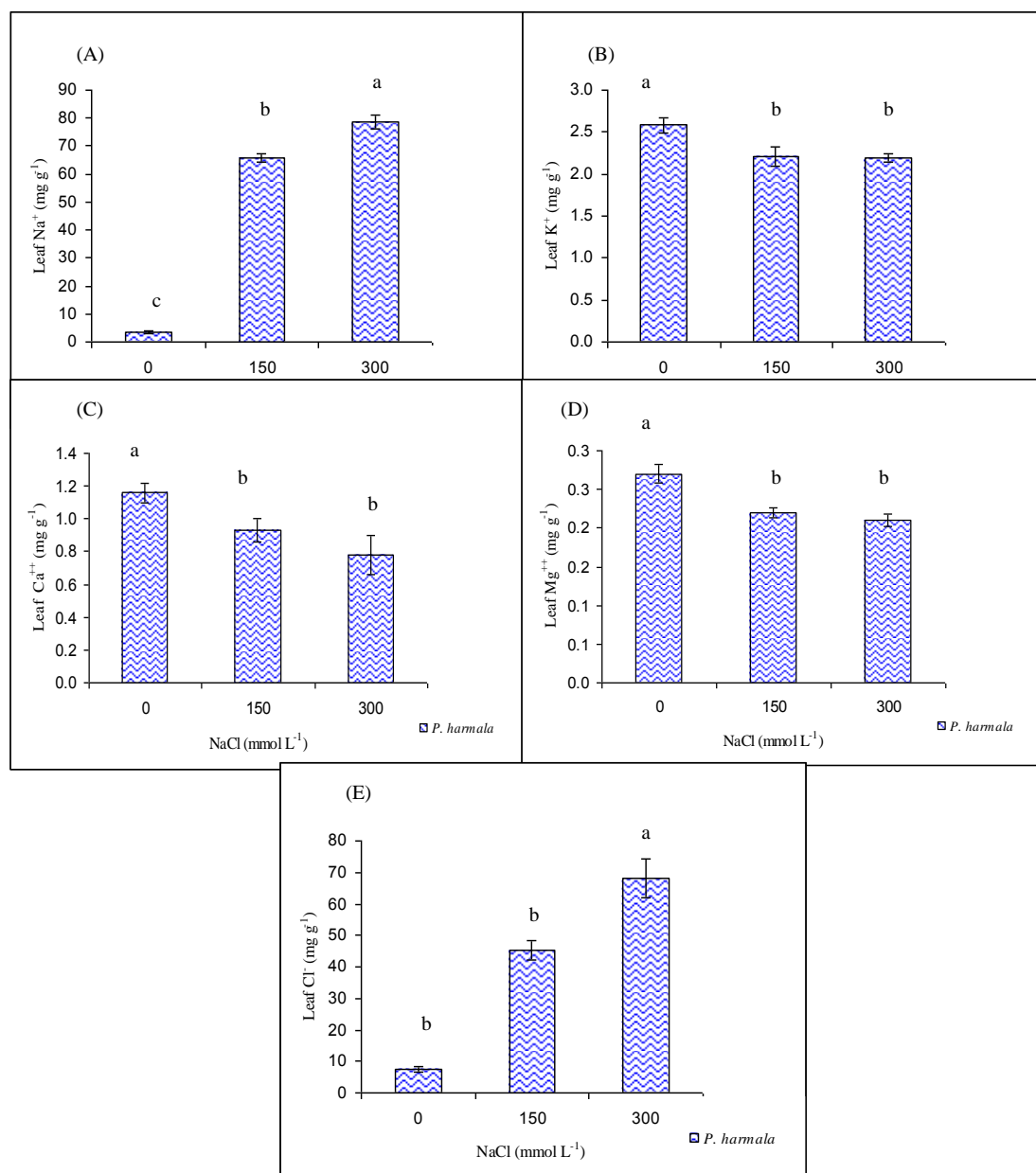
Along with the other enzymes and metabolites, level of oxidant molecules (H<sub>2</sub>O<sub>2</sub>) showed an increasing trend from 3.4  $\mu\text{mol g}^{-1}$  FW at control conditions to 6.3  $\mu\text{mol g}^{-1}$  FW at 300 mmol L<sup>-1</sup> NaCl conditions. The important issue here is that there was no significant difference between 150- and 300 mmol L<sup>-1</sup> NaCl conditions in that the difference between them in terms of H<sub>2</sub>O<sub>2</sub> accumulation was negligible indicating that *P. harmala* was able to stabilize the toxic ions till 300 mmol L<sup>-1</sup> NaCl level after 150 mmol L<sup>-1</sup> NaCl concentration (Fig. 6, Table 2).



**Figure 6.** H<sub>2</sub>O<sub>2</sub> of *P. harmala* plants at different NaCl concentrations. Bars indicate the means of threes replicates  $\pm$  standard error. Bars with different letters indicate the significant differences from one another according to Duncan's Multiple Range Test at  $P \leq 0.05$

### Determination of ion contents of *P. harmala* under high NaCl stress

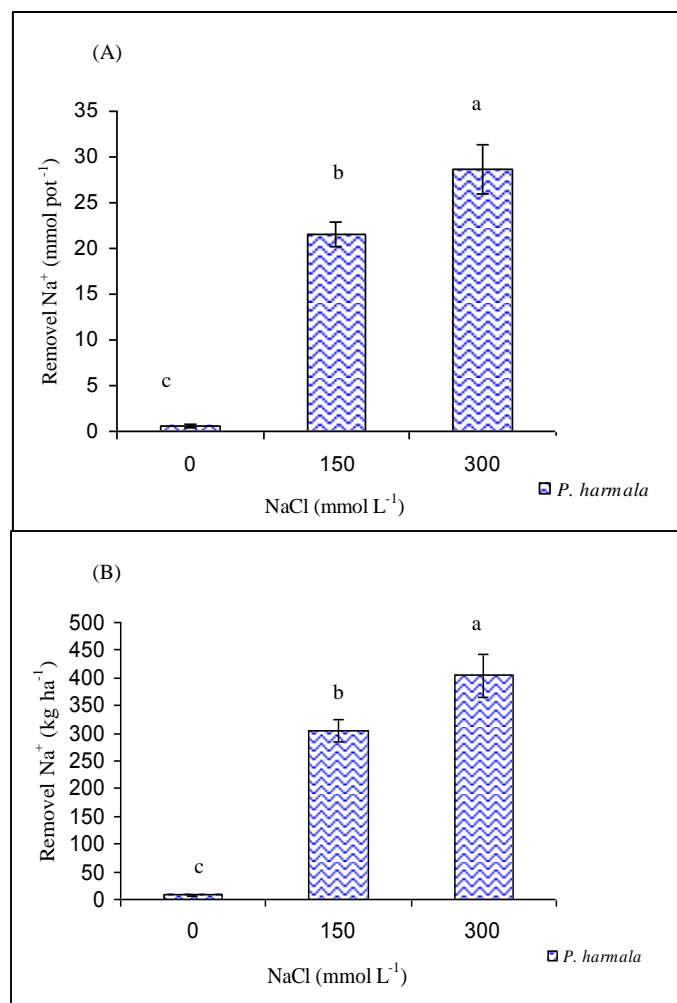
The accumulation of Na<sup>+</sup> and Cl<sup>-</sup> ions showed an increasing trend along with the decrease of K<sup>+</sup>, Ca<sup>++</sup> and Mg<sup>++</sup> ions in the leaves of *P. harmala* plants. In *P. harmala* leaves, Na<sup>+</sup> ion contents increased 19 and 22 times at 150- and 300 mmol L<sup>-1</sup> NaCl conditions, respectively, when compared to those of control plants. K<sup>+</sup>, Ca<sup>++</sup> and Mg<sup>++</sup> ions contents were 1.2, 1.5 and 1.3 times were decreases, respectively than those of leaves in the control group. The plants also accumulated Cl<sup>-</sup> ions 9 times higher under at 300 mmol L<sup>-1</sup> NaCl conditions than those of control plants (Fig. 7A-E, Table 2).



**Figure 7.** A) Na<sup>+</sup>; B) K<sup>+</sup>; C) Ca<sup>++</sup>; D) Mg and E) Cl<sup>-</sup> ions contents of *P. harmala* plants at different NaCl concentrations. Bars indicate the means of threes replicates ± standard error. Bars with different letters indicate the significant differences from one another according to Duncan's Multiple Range Test at P ≤ 0.05

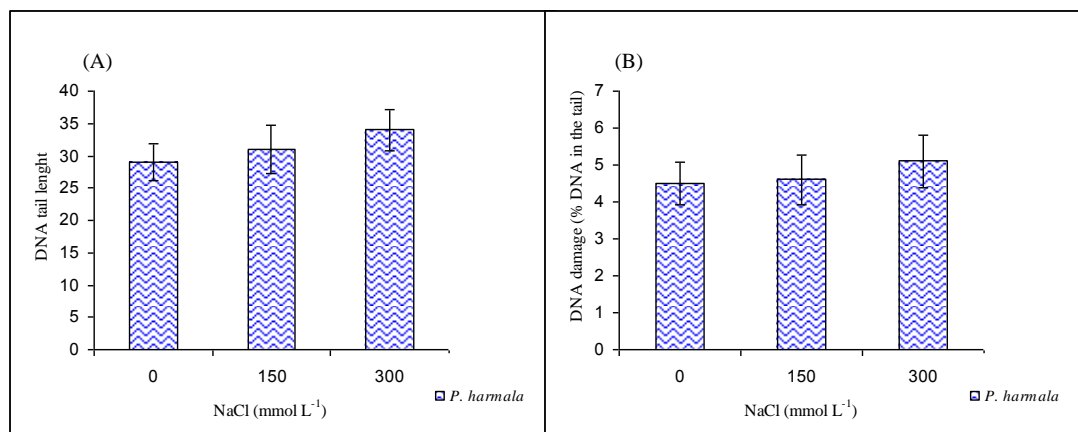
### Determination of sodium ion removal of *P. harmala* under high NaCl stress

*P. harmala* was determined to be quite effective in removing salt from the soils. It was capable of removing 0.6-, 21.6-, and 28.6 mmol pot<sup>-1</sup> Na<sup>+</sup> ion, respectively at 0-, 150-, and 300 mmol L<sup>-1</sup> in NaCl conditions. With regard to the mass removal of ions, we estimated that *P. harmala* was capable of removing 404.2 kg ha<sup>-1</sup> at 300 mmol L<sup>-1</sup> NaCl conditions (Fig. 8A and 8B, Table 2).



**Figure 8.** A) The removal of Na<sup>+</sup> from pots; B) The adjusted removal capacity under field conditions. *P. harmala* plants at different NaCl concentrations. Bars indicate the means of threes replicates ± standard error. Bars with different letters indicate the significant differences from one another according to Duncan's Multiple Range Test at P ≤ 0.05

Assessment of DNA damage caused by NaCl showed that *P. harmala* did not show any dose response to NaCl stress up to 300 mmol L<sup>-1</sup> NaCl level. DNA integrity measurement showed that DNA of the halophyte preserved its uniform shape and was not affected by the toxicity of NaCl as the other components of cell material. DNA tail length and the percentage of tail DNA did not differ from each other (Fig. 9A, 9B, Table 2).



**Figure 9.** A) DNA tail length B) Percentage of DNA damage of *P. harmala* at different NaCl concentrations. Bars indicate the means of three replicates  $\pm$  standard error. Bars with different letters indicate the significant differences from one another according to Duncan's Multiple Range Test at  $P \leq 0.05$

## Discussion

Salinity is one of the most important abiotic stress factors reducing crop yields as well as their quality significantly (Okorogbona et al., 2015). Salinity tolerance of plants is achieved by a series of complex and diverse mechanisms; these involve physiological, biochemical and molecular adaptational traits. Halophyte plants, in general, exhibit high salt tolerance, allowing them to survive and thrive under extremely saline conditions (Meng and Sui, 2018). They can tolerate high levels of salt concentrations between from 200 and 1000 mmol L<sup>-1</sup> of NaCl (Flowers and Colmer, 2008). For example, the halophyte *Arthrocnemum macrostachyum* survives up to 1000 mmol L<sup>-1</sup> NaCl (Khan et al., 2005). Similarly, Debez et al. (2010) applied the *Batis maritima*, a promising halophyte for sand-dune stabilization and saline-soil reclamation, into saline areas. The plant tolerated high salinity stress up to two-fold of seawater concentration (1000 mmol L<sup>-1</sup> NaCl). Plant biomass production was maximal at 200 mmol L<sup>-1</sup> NaCl.

On the other hand, halophyte plant *Carpobrotus acinaciformis* grow rapidly at moderate salt concentrations and is able to survive at extreme saline conditions almost close to seawater salt concentrations (Karakas et al., 2019a). In this study, biochemical responses and salt removal capacity of *P. harmala* under moderate and high NaCl stress were assessed. Evaluation of *P. harmala* was made in terms of growth, chlorophyll (Chl-*a* and Chl-*b*), proline, malondialdehyde (MDA) contents along with the activities of, CAT and POX antioxidant enzymes and H<sub>2</sub>O<sub>2</sub> changes as well as with the determination of mineral contents (Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>++</sup>, Mg<sup>++</sup>, Cl<sup>-</sup>). In our findings, the growth of *P. harmala* under NaCl (150-300 mmol L<sup>-1</sup>) conditions showed great performance when FW and DW of the plants were measured. Increases in salt concentrations of the soil positively correlated with the increases of FW and DW of the plant. Similar approaches were made by Suaire et al. (2016) who studied *Atriplex halimus* and *A. hortensis* exposed to a solution containing 2 g L<sup>-1</sup> of NaCl in a pot experiment for 60 days. The plants were able to accumulate >50 mg g<sup>-1</sup> of DW for Na<sup>+</sup> ions within the aerial parts of the plants. They concluded that *Atriplex halimus* and *A. hortensis* had promising characteristics for phytodesalination of road runoff polluted by deicing salts.

Chl-*a* and chl-*b* contents significantly increased in *P. harmala* leaves with the high NaCl levels when compared to the control plants growing at no-salt conditions.

Our study showed that *P. harmala* significantly accumulated proline contents to adapt to saline conditions when compared to that of control group. The ability of halophytes to accumulate osmolytes such as proline, glycine betaine, sorbitol, choline-O-sulphate or sugar have been intensively demonstrated in various studies (Tipirdamaz et al., 2006; Arbona et al., 2010; Lugan et al., 2010; Slama et al., 2015). Many physiological measurements of different species emphasize an elevated proline level as a response to salt stress (Szabados and Savoure, 2010; Zhang et al., 2019). In these studies, researchers stated that accumulation of proline prevented the loss of water through evaporation via increasing osmotic pressure. By this way, tolerant plants could survive longer in harsh conditions. In the current experiment, MDA and H<sub>2</sub>O<sub>2</sub> contents increased along with the increase of NaCl concentrations as a response to NaCl toxicity. However, increases in MDA and H<sub>2</sub>O<sub>2</sub> contents were stabilized after 150 mmol L<sup>-1</sup> NaCl concentration. Accumulations of MDA and H<sub>2</sub>O<sub>2</sub> are both considered toxic molecules to be determined under stress conditions. They are also positive responses to stress. Therefore, their increases under stress conditions are inevitable, however, the most important issue here is the stabilization of toxic level of molecules after some stages that characterize the plants as tolerant. In our case, this was succeeded after 150 mmol L<sup>-1</sup> NaCl conditions. This made *P. harmala* as a good candidate to be employed in saline-affected areas. For example, Amjad et al. (2015) observed an increase of MDA in leaves of *Chenopodium quinoa* under salt stress. MDA is the measure of damage to membranes and is sometimes regarded as the single most important characteristic to assess the destruction of membranes (Esfandiari et al., 2007). MDA not only directly damages the membranes, but also indirectly causes damage to cell by generating lipid derived radicals that aggravate the oxidative stress (Montillet et al., 2005; AbdElgawad et al., 2016).

Antioxidants play an important role in adapting plants to abiotic stress by detoxifying reactive oxygen species (ROS). Plants evolved different antioxidative mechanisms, among them several enzymatic defense systems are available. The plant defence system includes enzymatic and non-enzymatic antioxidants such as superoxide dismutase (SOD), POX, CAT, glutathione peroxidase (GPX) and ascorbate peroxidase (APX). They act in a sequential concept to scavenge the ROS (Cavalcanti et al., 2007; You and Chan, 2015). In the present study, increased enzyme activities (POX and CAT) played significant roles under NaCl conditions to detoxify and stabilize the toxic molecules. Similar results have recently been documented by Amjad et al. (2015) in *Chenopodium quinoa*, and by Podar et al. (2019) in the halophyte *Petrosimonia triandra* and by Karakas et al. (2019a) in the *Carpobrotus acinaciformis*.

Apart from the accumulation of antioxidant molecules and osmoprotectants to compensate the toxic effects of NaCl, halophyte plants have remarkably high capacity to accumulate toxic ions in their structures. They can accumulate salts in their various parts such as leaves, stems, roots, and they can force them (Na<sup>+</sup> and Cl<sup>-</sup>) across the tonoplast with highly Na<sup>+</sup>/K<sup>+</sup> selective protein transporters (Radyukina et al., 2007; Guo et al., 2019). This and similar other attributes led researchers to suggest that halophyte plants could be grown in salt-affected soils to remove significant amounts of Na<sup>+</sup> and Cl<sup>-</sup> ions through their aerial parts (Qadir et al., 2002; Karakas et al., 2017, 2019b). We noticed that, accumulation of Na<sup>+</sup> and Cl<sup>-</sup> ions were remarkably high as compared to the plants in the control group. Decreases in K<sup>+</sup>, Ca<sup>++</sup> and Mg<sup>++</sup> ions were also noticed in leaves, however, this decrease was stabilized after 150 mmol L<sup>-1</sup> NaCl condition. Similar

findings were also reported by other workers on *Bruguiera parviflora*, *Ceriops tagal*, *Halopyrum mocoronatum*, *Haloxylon recurvum*, *Suaeda fruticose* various plants (Parida and Das, 2005; Pan et al., 2016).

We demonstrated that *P. harmala* is quite effective in removing salt from the soils. Similarly, Karakas et al. (2017) observed *Salsola soda* and *Portulaca oleracea* removing of salt under soil salinity. This study showed that planting saline soils with *P. harmala* in moderate and high salt conditions might be an effective phytoremediation technique. Since we noticed that *P. harmala* showed great performances up to 300 mmol L<sup>-1</sup> NaCl as it showed at 150 mmol L<sup>-1</sup> NaCl level. We should see the performances at much higher NaCl concentrations to desalinate heavily salinized soils. This would increase the characteristics of *P. harmala* if pesticide-polluted soils are to be remediated via the use of this plant. Similarly, Yucel et al. (2017) stated that *Salicornia europaea* removed 426 to 475 kg salt/ha from the saline area. They stated that *Salicornia* biomass also provided sufficient amounts of salt to the animals. Otherwise, the senesced litter-fall of *Salicornia* would contribute to accelerated secondary soil salinization. Availability of these species help us clean up soils characterized with moderate or high salinity. Easy germination and fast growth have been considered as one of the good properties since the fast vegetative growth and accumulation of toxic ions help removing great amount of salt ions from the soil. Management of these species could be one of the novel and holistic approaches for sustainable phytoremediation of saline-affected soils.

Since accumulation of toxic molecules such as H<sub>2</sub>O<sub>2</sub> was stabilized after 150 mmol L<sup>-1</sup> NaCl conditions up to 300 mmol L<sup>-1</sup> NaCl conditions, the integrity of DNA was noticed to be preserved. It is very well known that high ROS including H<sub>2</sub>O<sub>2</sub> can damage to DNA via breaking single or double strands (Sharma et al., 2012). At this case, plants may not be able to recover due to damaged or not functional DNA. In our case, DNA preserved its uniform shape up to 300 mmol L<sup>-1</sup> NaCl conditions. With this measurement system, we could also test and measure the capacity of any plants aimed for cleaning up the soils contaminated with salt, pesitices, heavy metals etc.

## Conclusion

This study suggests that *P. harmala* is a salt-tolerant plant. The plant not only tolerates, but needs salt for optimal growth and physiological processes in its natural habitat. Its adaptation mechanisms include physiological, biochemical and molecular adaptation mechanisms. Chlorophyll contents, accumulation of osmolyte proline, lipid peroxidation, activation of an efficient antioxidative system are good sources for antioxidant mechanisms. Understanding of the mechanisms regarding salt adaptation of *P. harmala* could be of great importance, possibly leading to the extension of the arable land by exploiting the phytoremediation capability of this halophyte in the salt area.

In future studies, these characteristics should be well evaluated by measuring toxic components such as superoxide (O<sub>2</sub><sup>•-</sup>) and hydroxyl (OH<sup>•</sup>) ions. The salt tolerance characteristics of *P. harmala* should also be evaluated by increasing NaCl salinity further above 300 mmol L<sup>-1</sup> NaCl conditions to see if much higher salinity is to be tolerated by stabilizing the toxic ion levels. Since Na<sup>+</sup> and Cl<sup>-</sup> ions are accumulated in *P. harmala* leaves, salt ions removed from the soil due to the uptake should be measured if further accumulation capacity is achieved.

**Acknowledgments.** Biochemical and molecular analyses were performed in the laboratory of Dr. Murat Dikilitas. I thank Dr. Murat Dikilitas for the biochemical and and molecular analyses.

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