

## Effects of long-term salt stress on antioxidant system, chlorophyll and proline contents in pea leaves

Received for publication, January 10, 2010  
Accepted, June 11, 2011

LOKMAN OZTURK<sup>1</sup>, YAVUZ DEMIR\*<sup>2</sup>, ALI UNLUKARA<sup>3</sup>, ILHAMI KARATAS<sup>1</sup>, AHMET KURUNC<sup>4</sup> AND ORAL DUZDEMIR<sup>5</sup>

<sup>1</sup> Department of Biology, Faculty of Arts and Sciences, Gaziosmanpasa University, 60250-Tokat, Turkey

<sup>2</sup> Department of Biology, Faculty of Education, Atatürk University, 25240-Erzurum, Turkey.

<sup>3</sup> Department of Agricultural Structures and Irrigation, Faculty of Agriculture, Erciyes University, 38039-Kayseri, Turkey

<sup>4</sup> Department of Agricultural Structures and Irrigation, Faculty of Agriculture, Akdeniz University, 07058-Antalya, Turkey

<sup>5</sup> Vocational School, Karatekin University, 18100-Çankırı, Turkey

### Abstract

The effects of long-term salt stress on the contents of chlorophyll, proline, protein, hydrogen peroxide ( $H_2O_2$ ), and malondialdehyde (MDA) in terms of lipid peroxidation, and on the changes in activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POD) in leaves of pea (*Pisum sativum* cv Rona) were investigated in field conditions. Salinity and irrigation water regime experiments were set up in pots as randomized plot experimental designs with five replications. The experiment focused on pea responses to irrigation water salinity by irrigating the crops using 6 different levels of saline water (0.7, 2.0, 3.0, 4.0, 5.0 and 7.0  $dS\ m^{-1}$ ) with a constant leaching fraction ( $LF = 0.30$ ). The pots were located in the experimental area under a polyethylene cover which was about 1.8 m height from the surface for the purpose of eliminating rainfall effect on the experiments. Chlorophyll and protein contents were significantly decreased while accumulation of proline was enhanced with increased electrical conductivity (EC). Both MDA and  $H_2O_2$  contents were reduced in the result of high salts application. All of the salt treatments increased total SOD activity significantly, as a remarkable increase in POD activity was observed especially at 5.0 and 7.0  $dS/m$  EC. CAT and APX activities generally decreased in salt stressed seedlings. Our study indicates that its acquisition of salt tolerance may be a consequence of improved resistance to oxidative stress via increased activities of peroxidase and the superoxide dismutase/ascorbate-glutathione cycle.

**Key words:** Antioxidant Enzyme, Pea, Proline, Salinity, MDA,  $H_2O_2$ .

**Abbreviations:** EDTA, Ethylenediaminetetraacetic acid; PVPP, polyvinyl pyrrolidone,

### Introduction

Salinity in the soil and irrigation water is an environmental problem and a major constraint for crop production. Salt stress (NaCl) in plants influences some basic plant metabolic processes such as, photosynthesis, protein synthesis, and energy and lipid metabolism [1]. Salt stress (NaCl) has both osmotic (cell dehydration) and toxic (ion accumulation) effects on plant cells, impairing growth, ion homeostasis, photosynthesis and nitrogen fixation among other key physiological processes. Nowadays, several markers of stress have been measured to assess the physiological status of the plants [1]. These include proline and soluble protein contents, photosynthetic pigments and metabolic products of oxidative damage. In addition to other environmental stresses, salt stress also causes oxidative damage, thereby affecting cellular membrane integrity, enzyme activities, and functioning of

plant photosynthetic apparatus [2]. Such situations enhance the generation of reactive oxygen species (ROS) such as singlet oxygen ( $^1\text{O}_2$ ), superoxide anion radical ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and others, as both extra- and intra-cellular. ROS are extremely reactive, and can interact with a number of cellular macromolecules, e.g., DNA, proteins, lipids and pigments, which can induce destructive processes in plant cells when the generation of ROS is not kept under control [3]. Therefore, excessive ROS are considered as an indicator of stress conditions, and it is crucial for plants to balance the generation and elimination of ROS during exposure to salt environments, especially long-term salt stress [4]. On the other hand, recent evidence shows that at lower concentrations, ROS are not harmful for plant cells, and they can act as signalling molecules, resulting in down-stream response under oxidative stress [5],[6]. The antioxidant enzymes such as SOD, CAT and APX are efficiently involved in scavenging of ROS produced during salt stress, and act as one of the main tolerance mechanisms against oxidative stress in plants. Development of oxidative stress is a result of the imbalance between the formation of ROS and their detoxification [3],[7]. A regulated balance between oxygen radical production and destruction is required, if metabolic efficiency and function are to be maintained either in normal or stress conditions. A constitutively high antioxidant capacity under stress conditions can prevent damage and correlate with plants resistance to that particular stress. Hence, the mechanisms that reduce oxidative stress are expected to play an important role in imparting tolerance in plants under saline conditions [8].

Despite a great deal of research into salinity tolerance of plants, mainly on water relations, photosynthesis, and accumulation of various inorganic ions and organic metabolites, the metabolic sites at which salt stress damages plants and, conversely, the adaptive mechanisms utilized by plants to survive saline stress are still not well understood [9]. Thus, detailed understanding of the salt-tolerance mechanisms and consequently developing salt tolerant crops are essential to maintain the world's food security. Pea is sensitive to salinity. Therefore, the objective of the present investigation was to study potential effects of long-term salt stress under field conditions on the contents of chlorophyll, proline, soluble protein,  $\text{H}_2\text{O}_2$  and MDA being a criteria of lipid peroxidation level, and on the important components of antioxidant system (SOD, CAT, APX and POD) in pea leaves (*Pisum sativum* cv Rona) in order to evaluate the relative significance of these parameters in imparting tolerance. In addition, a potential effect of different salinity levels on EC and pH values of soil solution was also determined.

## Materials and Methods

### Plant material and growth conditions

The pot experiments were set up as completely randomized design with five replications per treatment. The soil was collected from a nearby field and sieved through a 4 mm screen to get rid of both large particles and dried soil lumps. The soil used as pot content has sandy loam coarse texture (15.7% clay, 17.5% silt and 66.8% sand). A 20 kg of air-dried soil was placed in pots. Diammonium phosphates (DAP) as 0.36 g per pot was applied before the transplanting. (Fertilizer needs were decided as 20-40 and 40-60 kg ha<sup>-1</sup> for N and P, respectively. Therefore, 2.0 g diammonium phosphates (DAP) were applied to each pot at the beginning of the experiments). The pots were located in the experimental area under a polyethylene cover which was about 1.8 m height from the surface for the purpose of eliminating rainfall effect on the experiments. The geographic coordinate of the experimental area is 40° 20' 07" N latitude and 36° 28' 26" E longitude [10].

Surface sterilized pea (*Pisum sativum* cv. Rona) seeds were sown into pots and they were started to germinate in a week. Until the plants established, they were irrigated with tap water that was used as control treatment. Saline water treatments were initiated when the plants reach 8-10 cm at height at the third week of growth. Including control group (tap water as a control treatment) with an electrical conductivity of 0.70 dS/m, five irrigation waters with different electrical conductivities (EC) were used (2.0, 3.0, 4.0, 5.0 and 7.0 dS/m). During the preparation of saline waters, sodium adsorption ratio ( $SAR = Na / ((Ca + Mg) / 2) \cdot 0.5$ ) values for each treatment was maintained around 1.0 in order to eliminate possible misinterpretations led by values of SAR on the out-comings of the experiment. Therefore, calculated amounts of  $CaCl_2$ ,  $MgSO_4$  and NaCl were mixed to prepare irrigation water with given salinity for each treatment.

Each pot was weighted before each irrigation practices. The amount of applied water (AW) for each pot was determined by calculating the difference between the field capacity and the weight of each pot before irrigation. AW was determined by  $AW = (W_{fc} - W_a \text{ weight just before irrigation}) / 1 - LF$  [10]. During irrigation, the leaching fraction (LF) was assumed to be 0.20 and including LF calculated amount of irrigation water was applied. Throughout the experiment, the plants were irrigated at 3 to 5 day intervals. The field capacity weight of each pot was determined before the experiments started. To do this, soil in pots were saturated with tap water and then, soil surface were covered to prevent evaporation.

The leaves of pea were harvested at the 7<sup>th</sup> week (Seeds of pea were sown to each pot on April 7 and harvested on July 5.) after salt application. Leaves similar in size, colour, and position on the plant were selected for the assay. At the harvest, soil samples taken from each pot were air dried and crushed to pass through a 2-mm screen. Saturated soil pastes were prepared, kept in laboratory for 24 hours, and then EC and pH of saturated soil extracts were measured.

### **Chlorophyll content**

Total chlorophyll contents were determined following the method of Arnon (1949) [11].

### **Proline content**

Free proline content was measured spectrophotometrically according to the method of Bates et al. (1973) [12].

### **Protein determination**

The protein content in the leaves was determined as described by Bradford (1976) method using bovine serum albumin as the standard [13].

### **Lipid peroxidation**

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) reacted with thiobarbituric acid (TBA) to form TBA-MDA complex. The MDA content was determined using the extinction coefficient as  $155 \text{ mM}^{-1} \text{ cm}^{-1}$  [14].

### **Hydrogen peroxide ( $H_2O_2$ ) content**

$H_2O_2$  content was measured according to Velikova et al (2000) [14].

### **Enzyme assay**

Frozen leaf segments (0.5 g) were crushed into fine powder in a mortar and pestle under liquid nitrogen. The leaf powder was homogenized in 50 mM potassium phosphate ( $K_2HPO_4$ ) buffer (pH 7.0) containing 1 mM EDTA and 1% PVPP. The homogenate was centrifuged at  $20000 \times g$  for 20 min at 4°C and the supernatant was used for the following enzyme assay.

The total SOD (EC 1.15.1.1) was assayed according to modified method of Beyer and Fridovich (1987) [15] by inhibition of the photochemical reduction of nitroblue tetrazolium (NBT).

CAT (EC 1.11.1.6) activity was determined by following the consumption of H<sub>2</sub>O<sub>2</sub> at 240 nm [16].

APX (EC 1.11.1.11) activity was determined according to the method of Wang et al. (1991) [17].

POD (EC 1.11.1.7) activity was assayed according to the method the modified method of Angelini et al. (1990) [18].

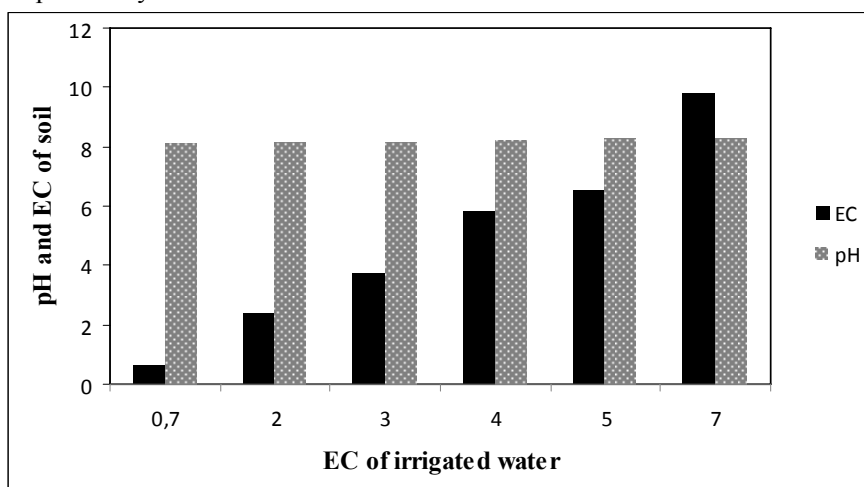
### Statistical analysis

Differences between the values for control and salt stressed seedlings were analyzed by one-way ANOVA, taking  $P < 0.05$  as significance level, according to Duncan's multiple range tests. Data are shown as the mean  $\pm$  standard error (SE).

## Results and Discussion

### Effect of irrigation water salinity on salinity and pH of soil

Salinity is a soil condition characterized by a high concentration of soluble salts. Soils are classified as saline when the EC is 4 dS/m or more (131), which is equivalent to approximately 40 mM NaCl and generates an osmotic pressure of approximately 0.2 MPa. This definition of salinity derives from the EC that significantly reduces the yield of most crops. The hyperosmolarity decreases the osmotic potential of soil solution and restricts water uptake by plant roots; also, this frequently causes a significant increase in the stomatal resistance and reduction of CO<sub>2</sub> photosynthetic assimilation. Soil water was depleted by plant and evaporation from soil surface, leaving salts that escalate the salinity level of soil. We applied greater amount of water than required by plant to prevent excess salt accumulation and to maintain salinity differences between treatments [10]. This provided leaching of some salts in the soil, resulting in different soil salinity levels for each treatment. EC and pH of soil saturation extract in each pot were determined (Figure 1). Soil electrical conductivity increased significantly with respect to increasing salinity level in irrigation water but increase in the soil pH is very little.



**Figure 1.** The effects of different salinity levels of irrigation water on soil pH and electrical conductivity (EC). Data represent the mean value  $\pm$  SE,  $n=5$   $p < 0.05$

### Effect of long-term salt stress on chlorophyll content

Salt stress causes reduction in leaf surface expansion ratio, leading to cessation of expansion as salt concentrations keep up with increasing [19]. Srivastava et al. (1988) [20] reported chlorophyll (Chl) content as one of the parameters of salt tolerance in crop plants. In our study, the salt treatments decreased significantly ( $P < 0.05$ ) Chl content in the salt-treated plants comparing with control plants (Table 1). Hernandez et al. (1995) [21] observed higher Chl degradation in NaCl sensitive pea cultivar as compared to tolerant one. It has been also reported that NaCl stress decreased total Chl, Chl<sub>a</sub> and  $\beta$ -carotene in leaves of tomato [22].

**Table 1:** Effects of long-term salt stress on physiological and biochemical parameters of leaf tissues of *Pisum sativum* L.

| Electrical conductivity of irrigation water    | 0.70 dS/m (Control) | 2.0 dS/m   | 3.0 dS/m   | 4.0 dS/m   | 5.0 dS/m   | 7.0 dS/m    |
|--|---------------------|------------|------------|------------|------------|-------------|
| Chlorophyll (mg/g FW)                          | 2.37±0.05a          | 2.11±0.06b | 2.13±0.07b | 1.49±0.02d | 1.77±0.04c | 1.63±0.07cd |
| Proline ( $\mu$ mol/gFW)                       | 0.61±0.01e          | 0.72±0.04e | 1.04±0.01d | 1.43±0.03c | 2.1±0.13b  | 4.42±0.15a  |
| Protein (mg/g FW)                              | 13.6±0.34a          | 11.6±0.38b | 9.3±0.21c  | 6.11±0.17d | 4.12±0.07e | 3.67±0.19e  |
| H <sub>2</sub> O <sub>2</sub> ( $\mu$ mol/gFW) | 481±25a             | 512±22a    | 356±13b    | 390±32b    | 372±27b    | 335±20b     |
| MDA (nmol/gFW)                                 | 28±0.57a            | 26.3±0.33a | 20.6±0.88b | 16.3±0.88c | 17.6±0.66c | 16.3±1.45c  |

Data represent the mean value  $\pm$  SE, n=5  $p < 0.05$ , FW; fresh weight

### Effect of long-term salt stress on proline and protein contents

Proline is an important parameter to measure the stress tolerance capacity of the plants. In many plants, free proline accumulates in response to the imposition of a wide range of biotic and abiotic stresses [23]. The ability of proline to mediate osmotic adjustment, stabilise subcellular structures, and scavenge free radicals are well known. However, a controversy exists on the extent to which proline accumulation is a consequence of stress or benefit plants under adverse environmental conditions [24]. In the present study, proline content increased with increase in NaCl concentration (Table 1). Similar approaches have been reported in salt stressed barley, soybean and maize seedlings [25]. Lutts et al. (1996) [26] observed an increase in proline accumulation in rice cultivars caused by salinity stress. It was shown that proline accumulates in spinach [27], durum wheat [1] as a consequence of salt stress. The ability of proline to mediate osmotic adjustment, stabilise subcellular structures, and scavenge free radicals are well known. Many plants accumulate proline as a nontoxic and protective osmolyte under salt conditions. In addition, compatible solutes such as proline have the capacity to preserve the activity of enzymes that are in saline solutions [28]. Accumulation of proline, glycine betaine and soluble sugars under stress protect the cell by balancing the osmotic strength of cytosol with that of vacuole and apoplast [29],[30]. Proline and glycine betaine also stabilize the structure and function of various macromolecules [31]. On the other hand, proline accumulation may reduce stress-induced cellular acidification [32]. It seems that increased level of proline has an important role in protecting enzymes involved in the antioxidant system against damaging effects of salt stress. Proline was found to be such a marker here, as its concentration increased [33].

In our study, compared with control, soluble protein content declined at all the treatments considerably. It has been reported that soluble protein was increased by low salinity and decreased by high salinity in mulberry [1]. In another work, salt stress decreased soluble protein content in the leaves of tomato [34].

### **Effect of long-term salt stress on H<sub>2</sub>O<sub>2</sub> content and lipid peroxidation**

Salt stress is known to result in extensive lipid peroxidation [35]. The level of MDA, produced during peroxidation of membrane lipids, is often used as an indicator of oxidative damage. In our study, the salt treatments decreased significantly H<sub>2</sub>O<sub>2</sub> and MDA content of pea leaves except for 2 dS/m (Table 1). Decrease in H<sub>2</sub>O<sub>2</sub> content may be resulted from the increase in antioxidant enzyme activities. APX and other antioxidant enzymes play a role in maintaining low levels of H<sub>2</sub>O<sub>2</sub> in pea under salt stress. Some researchers also reported the sharp decline in H<sub>2</sub>O<sub>2</sub> level to the efficient removal of H<sub>2</sub>O<sub>2</sub> by increased activity of antioxidant enzymes as well as certain non-enzymatic reactions working efficiently in the stressed plants [36]. SOD activity in plant leaves of barley was increased by salinity whereas MDA concentration was decreased [37]. Similarly, the levels of MDA significantly declined as a result of salt stress in *Grewia ilicifolia* [38]. The lipid content in peanut (*A. hypogaea* L.) increased at low concentrations of NaCl (up to 45 mM) as it decreased at higher concentrations [1]. The changes in quantity and content of membrane lipids affect the degree of lipid peroxidation. Peroxidation of lipids can greatly alter the physicochemical properties of membrane lipid bilayers, resulting in severe cellular dysfunction. The greater increase in total peroxidase activity and low malonaldehyde content in NaCl stressed tolerant cultivar (cv Prasad), indicates involvement of peroxidases in cell membrane integrity [8]. In another study, researchers suggested that the better NaCl stress tolerance in salt-tolerant cultivars as compared to salt-sensitive cultivars observed during present investigation may be due to the restriction of damage of cellular membranes with lower MDA and H<sub>2</sub>O<sub>2</sub> content [39],[40]. The lower level of lipid peroxidation in salt resistance than of salt sensitive cultivars suggests that it may have better protection against oxidative damage under salt stress. A lower lipid peroxidation resulting from elevated activities of antioxidants under salt stress was also reported on salt-tolerant wild tomato species [41], wheat [42] and cotton [43]. A lower level of lipid peroxidation, hence a lower degree of membrane damage in *L. pennellii* than in *L. esculentum* leaves might be resulted from the higher SOD and POX activities in *L. pennellii*. Our results are also in a good agreement with the results of Shalata and Tal (1998) [41] who found a lower level of lipid peroxidation and higher constitutive antioxidant enzyme activities in leaves of *L. Pennellii* under salt stress. Similarly, lesser degree of membrane damage and higher activity of SOD and POX in NaCl-treated cotton and in senna (*Cassia angustifolia* Vahl.) plants, respectively, were correlated with higher salinity tolerance [39],[44].

### **Effect of long-term salt stress on enzyme activities**

A regulated balance between oxygen radical production and destruction is required, if metabolic efficiency and function are to be maintained either in normal or stress conditions. A constitutively high anti-oxidant capacity under stress conditions can prevent damage and correlate with plants resistance to that particular stress. Hence, the mechanisms that reduce oxidative stress are expected to play an important role in imparting tolerance in plants under saline conditions. The activities of antioxidant enzymes (SOD, CAT, APX and POD) were varied significantly with medium salt strength (Table 2).

**Table 2:** Effects of long-term salt stress on superoxide dismutase (SOD (EU/g fw)), catalase (CAT (EU/g fw)) ascorbate peroxidase (APX (EU/g fw)), and peroxidase (POD (EU/g fw)) enzyme activities in leaves of *Pisum sativum* cv Rona.

| EC  | 0.70 dS/m<br>(Control) | 2.0 dS/m    | 3.0 dS/m    | 4.0 dS/m    | 5.0 dS/m    | 7.0 dS/m    |
|-----|------------------------|-------------|-------------|-------------|-------------|-------------|
| SOD | 14.38±0.61d            | 17.83±0.75d | 25.97±0.85c | 36.83±1.3b  | 51.5±1.7a   | 55.6±3.18a  |
| CAT | 211±7.9ab              | 242±13.9a   | 183±13.7bc  | 165.6±6.17c | 242±8.68a   | 166±15.67c  |
| APX | 0.80±0.03a             | 0.81±0.01a  | 0.70±0.02bc | 0.46±0.008d | 0.76±0.03ab | 0.68±0.01c  |
| POD | 6.82±0.7b              | 6.10±0.31b  | 7.11±0.53b  | 7.94±0.37b  | 12.85±0.65a | 12.89±0.69a |

Data represent the mean value ± SE, n=5 p<0.05

SODs are the main scavengers of superoxide radical ( $O_2^{\cdot-}$ ) [45]. In our study, SOD activity significantly elevated in plants under the salt stress comparing with control plants (Table 2). The increased SOD activity in stressed pea suggested the view that the superoxide radicals were detoxified efficiently. Salt treatments also caused a significant ( $P<0.05$ ) increase in SOD activity in genotypes of mulberry [46]. Fadzilla et al. (1997) [47] reported that the activities of Mn-SOD and Cu/Zn-SOD of salt-sensitive *Oryza sativa* were significantly increased by NaCl stress. Van-Camp et al. (1996) [48] reported that NaCl stress induced 2-3 fold increase in activities of Fe-SOD, Cu/Zn-SOD in tobacco plant. The increase in SOD activity is an adaptation to remove the excess superoxide anions generated under excess salt stress [49].

The expression of enzymes that detoxify  $H_2O_2$  varies under control and saline conditions [46]. CAT and APX catalyze the breakdown of  $H_2O_2$ . The peroxisomal enzyme CAT decomposes  $H_2O_2$  to water and  $O_2$ , whereas the enzymes of the ascorbate-glutathione cycle, which are localized in the cytosol, chloroplasts, mitochondria and peroxisomes, reduce  $H_2O_2$  to water using ascorbate and glutathione [50]. In the present study, CAT activity significantly ( $P<0.05$ ) decreased in 4.0 and 7.0 dS/m while slightly ( $P>0.05$ ) increased 2.0 and 5.0 dS/m (Table 2). The decrease in catalase activity measured under salt stress has been reported previously [51]. In addition, the decrease in CAT activity under increasing salinity has also been reported in callus cultures of *Suaeda nudiflora* [52] and *Crithmum maritimum* [53]. APX uses ascorbate as a reductant to remove the excess  $H_2O_2$  generated under salt stress. It has a higher affinity for  $H_2O_2$  than CAT and POD and it may have a more crucial role in combating toxic effects of ROS during stress [54]. Salt stress caused a gradual decrease in APX activity and the maximum decrease in APX activity was recorded for 4.0 dS/m (Table 2). In another study, APX activity was found to be low in the control calli as well as low levels of salt (100 and 200 mM NaCl) in the medium [49]. It was reported that CAT, APX and glutathione reductase activities decreased under salt stress, as SOD and reduced glutathione increased in soybean root nodules [55]. Also Gosset et al. (1994) [43] reported that NaCl stress increases the activities of SOD, guaiacol peroxidase, and glutathione reductase, on the other hand, decreases the activities of CAT and APX in cotton (*Gossypium hirsutum* L.). In another study, salt stress decreased SOD and CAT activities and soluble protein content in the leaves [34]. That POD and glutathione peroxidase also use  $H_2O_2$  as a substrate and CAT has low  $K_m$  for  $H_2O_2$  can be cause decrease in CAT and APX activities.

Plant peroxidases are widely distributed in all higher plants and one of their main function is detoxification of the activated oxygen forms occurred by biotic and abiotic stress. It has been reported that elevated antioxidant levels could be associated with salt tolerance mechanism of plants [46]. An increase in total peroxidase activity under saline conditions was reported [56]. An increase in total peroxidase activity is a common response to various

oxidative stress factors [57]. Enhanced productions of oxygen free radicals are responsible for stress-dependent peroxidation of membrane lipids [58]. Increased peroxidation of membrane lipids is known to occur during salinity stress [8]. In the present study, a significant elevation in the activity of POD was recorded at 5.0 and 7.0 dS/m. Further, the degree of increase was found to be dependent on severity and duration of stress (Table 2). In salt-tolerant plant species, POD activity was found to be higher, assuming that POD enables the plants to protect themselves against the oxidative stress. In another study, *Kosteletzky virginica* seedlings were treated with 100, 200, 300 and 400 mM NaCl, CAT activity in leaves showed significant decrease at 200 mM NaCl while decrease roots at 100 mM NaCl in comparison with control. In contrast, plants grown at 200 mM NaCl showed significant increases in SOD, POD activities of roots and leaves compared to that grown at control [59]. High peroxidase isoenzymic activities in the salt-adapted cells reflect the changed mechanical properties of the cell wall which, in turn, could be related to the salt adaptation process [8].

In conclusion, this study showed that the difference of SOD, CAT, APX and POD activities and MDA content in the pea could be ascribed to the difference in mechanisms underlying oxidative stress injury and subsequent tolerance to salinity. In this pea cultivar under our experimental condition, POD played more active roles than CAT in plant cells from oxidative stress. Higher free radical-scavenging capacity and protection mechanism of pea against salt stress was also revealed by resulting in lower H<sub>2</sub>O<sub>2</sub> production and lower level of lipid peroxidation. A perusal of the results show that the lesser degree of membrane damage based on the low rate of lipid peroxidation, and salt induced enhancement of antioxidative enzymes indicate that pea had a higher capacity for the scavenging of ROS generated by salt stress. Thus, the present study confirms a correlation between antioxidant defence system and tolerance to NaCl induced oxidative stress. In contrast, CAT activity with increasing salinity was not correlated with hydrogen peroxide content. The results suggest that under increasing salinity, the primarily prominent peroxidase activity appears to play an active role in scavenging reactive oxygen species in this cultivar, whereas the superoxide dismutase/ascorbate-glutathione cycle seem to be important consequently.

## References

1. PARIDA, A.K., DAS, A.B. Salt tolerance and salinity effects on plants: a review. *Ecotoxicology and Environmental Safety*. 60: 324–349 (2005)
2. JITESH, M.N., PRASHANT, S.R. SIVAPRAKASH, K.R., PARIDA, A.K. Antioxidative response mechanism in halophytes: their role in stress defense. *Journal of Genetics*. 85: 237-254 (2006)
3. MITTLER, R. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science*. 7: 405–410 (2002)
4. LIGHT, G.G., MAHAN, J.R., ROXAS, V.P., ALLEN, R.D. Transgenic cotton (*Gossypium hirsutum* L.) seedlings expressing a tobacco glutathione S-transferase fail to provide improved stress tolerance. *Planta*. 222: 346–354 (2005)
5. JIANG, M., ZHANG, J. Involvement of plasma-membrane NADPH oxidase in abscisic acid- and water stress-induced antioxidant defence in leaves of maize seedlings. *Planta*. 215: 1022–1030 (2002)
6. SKOPELITIS, D.S., PARANYCHIANAKIS, N.V., PASCHALIDIS, K.A., PLIAKONIS, E.D., YAKOUMAKIS, D., DELIS, I.D., KOUVARAKIS, A., PAPADAKIS, A.K., STEPHANOOU, E., ROUBELAKIS ANGELAKIS, K.A. Abiotic stress generated ROS signal expression of anionic glutamate dehydrogenases to form glutamate for proline synthesis. *Plant Cell*. 18: 2767–2781 (2006)
7. ZIMMERMANN, P., ZENTGRAF, U. The correlation between oxidative stress and leaf senescence during plant development. *Cellular and Molecular Biology Letters*. 10: 515–534 (2005)
8. SREENIVASULU, N., RAMANJULU, S., RAMACHANDRA-KINI, K., PRAKASH, H.S., SHEKAR-SHETTY, H., SAVITHRI, H.S., SUDHAKAR, C. Total peroxidase activity and peroxidase isoforms as modified by salt stress in two cultivars of fox-tail millet with differential salt tolerance. *Plant Science*. 141: 1–9 (1999)
9. ASHRAF, M., HARRIS, J.C. Potential biochemical indicators of salinity tolerance in plants. *Plant Science*. 166: 3–16 (2004)



10. DUZDEMIR, O., KURUNC, A., UNLUKARA, A. Response of Pea (*Pisum Sativum*) to Salinity and Irrigation Water Regime. *Bulgarian Journal of Agricultural Science*, 15 (5): 400-409 (2009)
11. ARNON, D.I. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. *Plant Physiology*. 24: 1-15 (1949)
12. BATES, L.S., WALDREN, R.P., TEARE, I.D. Rapid determination of free proline for water stress studies. *Plant Soil*. 39: 205-207 (1973)
13. BRADFORD, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. 72: 248-251 (1976)
14. VELIKOVA, V., YORDANOV, I., EDRAVA, A. Oxidative stress and some antioxidant systems in acid rain-treated bean plants. Protective role of exogenous polyamines. *Plant Science*. 151: 59-66 (2000)
15. BEYER, W.F.JR., FRIDOVICH, I. Assaying for superoxide dismutase activity: Some large consequences of minor changes in conditions. *Analytical Biochemistry*. 161: 559-566 (1987)
16. AEBI, H. Catalase. In: Bergmeyer H (ed.) *Methods of enzymatic analysis*, Weinheim: Verlag Chemie. 1983, p.p. 273-277.
17. WANG, S.Y., JIAO, M., FAUST, M. Changes in ascorbate, glutathione and related enzyme activities during thiodiazuran-induced bud break of apple. *Plant Physiology*. 82: 231-236 (1991)
18. ANGELINI, R., MANES, F., FEDERICO, R. Spatial and functional correlation between diamine-oxidase and peroxidase activities and their dependence upon de-etiolation and wounding in chick-pea stems. *Planta*. 182: 89-96 (1990)
19. WANG, Y., NIL, N. Changes in chlorophyll, ribulose biphosphate carboxylase-oxygenase, glycine betaine content photosynthesis and transpiration in *Amaranthus tricolor* leaves during salt stress. *Journal of Horticultural Science and Biotechnology*. 75: 623-627 (2000)
20. SRIVASTAVA, T.P., GUPTA, S.C., LAL, P., MURALIA, P.N., KUMAR, A. Effect of salt stress on physiological and biochemical parameters of wheat. *Annals of Arid Zone*. 27: 197-204 (1988)
21. HERNANDEZ, J.A., OLMOS, E., CORPAS, F.Z., SEVILLA, F., DEL RIO, R.A. Salt-induced oxidative stress in chloroplasts of pea plants. *Plant Science*. 105: 151-167 (1995)
22. KHAVARINEJAD, R.A., MOSTAFI, Y. Effects of NaCl on photosynthetic pigments, saccharides, and chloroplast ultra structure in leaves of tomato cultivars. *Photosynthetica*. 35: 151-154 (1998)
23. HARE, P.D., CRESS, W.A. Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regulation*. 21: 79-102 (1997)
24. ASHRAF, M., FOOLAD, M.R. Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany*. 59: 206-216 (2007)
25. DASH, M., PANDA, S.K. Salt stress induced changes in growth and enzyme activities in germinating *Phaseolus mungo* seeds. *Biologia Plantarum*. 44: 587-589 (2001)
26. LUTTS, S., KINET, J.M., BOUHARMOUNT, J. NaCl-induced senescence in leaves of rice (*Oryza sativa* L.) cultivars differing in salinity resistance. *Annals of Botany*. 78: 389-398 (1996)
27. OZTURK, L., DEMIR, Y. Effects of putrescine and ethephon on some oxidative stress enzyme activities and proline content in salt stressed spinach leaves. *Plant Growth Regulation*. 40: 89-95 (2003)
28. KISHOR, P.B.K., SANGAM, S., AMRUTHA, R.N., LAXMI, P.S., NAIDU, K.R., RAO, K.R.S.S., RAO, S., REDDY, K.J., THERIAPPAN, P., SREENIVASULU, N. Regulation of proline biosynthesis, degradation, uptake and transport in higher plants. Its implications in plant growth and abiotic stress tolerance. *Current Science*. 88: 424-438 (2005)
29. GADALLAH, M.A.A. Effects of proline and glycinebetaine on *Vicia faba* response to salt stress. *Biologia Plantarum*. 42: 249-257 (1999)
30. H. GREENWAY, R. MUNNS, Mechanism of salt tolerance in nonhalophytes. *Annual Review of Plant Physiology*. 31, 149-190 (1980).
31. N. SMIRNOFF, Q.J. CUMBERS, Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry*. 28, 1057-1060 (1989).
32. AZIZ, A., MARTIN-TANGUY, J., LARHER, F. Salt stress induced proline accumulation and changes in tyramine and polyamine levels are linked to ionic adjustment in tomato leaf discs. *Plant Science*, 145: 83-91 (1999)
33. RUBIO, M.C., SANMAMED, P.B., CLEMENTE, M.R., BECANA, M. Effects of salt stress on the expression of antioxidant genes and proteins in the model legume *Lotus japonicas*. *New Phytologist*, 181: 851-859 (2009)
34. AL-AGHABARY, K., ZHU, Z., SHI, Q. Influence of silicon supply on chlorophyll content, chlorophyll fluorescence, and antioxidative enzyme activities in tomato plants under salt stress. *Journal of Plant Nutrition*. 27: 2101-2115 (2004)
35. NETO, A.D.A., PRISCO, J.T., FILHO, J.E., ABREU, C.E.B., FILHO, E.G. Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. *Environmental and Experimental Botany*, 56: 87-94 (2006)

36. SHARMA, P., DUBEY, R.S. Drought induces oxidative stress and enhances the activities of antioxidant enzymes in growing rice seedlings. *Plant Growth Regulation*. 46: 209–221 (2005)
37. LIANG, Y.C. Effects of silicon on enzyme activity and sodium, potassium and calcium concentration in barley under salt stress. *Plant and Soil*. 209: 217–224 (1999)
38. KENNEDY, B.F., DE FILLIPPIS, L.F. Physiological and oxidative response to NaCl of the salt tolerant *Grevillea ilicifolia* and the salt sensitive *Grevillea arenaria*. *Journal of Plant Physiology*. 155: 746–754 (1999)
39. MELONI, D.A., OLIVA, M.A., MARTINEZ, C. A., CAMBRAIA J. Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. *Environmental and Experimental Botany*. 49: 69–76 (2003)
40. DOGAN, M., TIPIRDAMAZ, R., DEMIR, Y. Salt resistance of tomato species grown in sand culture. *Plant Soil Environ*, 56 (11): 499–507 (2010)
41. SHALATA, A., TAL, M. The effect of salt stress on lipid peroxidation and antioxidants in the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennclii*. *Physiolgia Plantarum*. 104: 169–174 (1998)
42. AGARWAL, S., PANDEY V. Antioxidant enzyme responses to NaCl stress in *Cassia angustifolia*. *Biol. Plant*, 48: 555–560 (2004)
43. DING, M., HOU, P., SHEN, X., WANG, M., DENG, S., SUN, J., XIAO, F., WANG, R., ZHOU, X., LU, C., ZHANG, D., ZHENG, X., HU, Z., CHEN, S. Salt-induced expression of genes related to Na<sup>+</sup>/K<sup>+</sup> and ROS homeostasis in leaves of salt-resistant and salt-sensitive poplar species. *Plant Mol Biol*, 73: 251–269 (2010)
44. DIONISIO-SESE, M.L., TOBITA, S. Antioxidative responses of shoots and roots of wheat to increasing NaCl concentration. *Journal of Plant Physiol*. 155: 274–280 (1999)
45. GOSSET, D.R., MILLHOLLON, E.P., LUCAS, M.C. Antioxidant response to NaCl stress in salt tolerant and salt sensitive cultivars of cotton. *Crop Science*. 34: 706–714 (1994)
46. SUDHAKAR, C., LAKSHMI, A., GIRIDARAKUMAR, S. Changes in the antioxidant enzyme efficacy in two high yielding genotypes of mulberry (*Morus alba* L.) under NaCl salinity. *Plant Science*. 161: 613–619 (2001)
47. FADZILLA, N.M., FINCH, R.P., BURDON, R.H. Salinity, oxidative stress and antioxidant responses in shoot cultures of rice. *Journal of Experimental Botany*. 48: 325–331 (1997)
48. VAN CAMP, W., CAPIAU, K., VAN MONTAGU, M., INZE, D., SLOOTEN, L. Enhancement of oxidative stress tolerance in transgenic tobacco plants overproducing Fe-superoxide dismutase in chloroplasts. *Plant Physiology*. 112: 1703–1714 (1996)
49. LOKHANDE, V.H., NIKAM, T.D., PENNA, S. Biochemical, physiological and growth changes in response to salinity in callus cultures of *Sesuvium portulacastrum* L. *Plant Cell, Tissue and Organ Culture*. 102: 17–25 (2010)
50. JIMENEZ, A., HERNANDEZ, J.A., DELRIO, L.A., SEVILLA, F. Ascorbate-Glutathione Cycle in Mitochondria and Peroxisomes of Pea Leaves: Changes Induced by Leaf Senescence. *Phyton (Austria)*. 37(3): 101–108 (1997)
51. KHEDR, A.H.A., ABBAS, M.A., WAHID, A.A.A., QUICK, W.P., ABOGADALLAH, G.M. Proline induces the expression of salt-stress responsive proteins and may improve the adaptation of *Pancreaticum maritimum* L., to salt-stress. *Journal of Experimental Botany*. 54: 2553–62 (2003)
52. CHERIAN, S., REDDY, M.P. Evaluation of NaCl tolerance in the callus cultures of *Suaeda nudiflora* Moq. *Biologia Plantarum*. 46: 193–198 (2003)
53. BEN AMOR, N., BEN HAMED, K., DEBEZ, A., GRIGNON, G., ABDELLY, C. Physiological and antioxidant responses of the perennial halophyte *Crithmum maritimum* to salinity. *Plant Science*. 168: 889–899 (2005)
54. AHMAD, S., HAHN, E.J., PAEK, K.Y. Aeration volume and photosynthetic photon flux affect cell growth and secondary metabolite contents in bioreactor cultures of *M. citrifolia*. *Journal of Plant Biology*. 51: 209–212 (2008)
55. COMBA, M.E., BENAVIDES, M.P., TOMARO, M.L. Effect of salt stress on antioxidant defence system in soybean root nodules. *Australian Journal of Plant Physiology*. 25: 665–671 (1998)
56. MANDHANIA, S., MADAN, S., SAWHNEY, V. Antioxidant defense mechanism under salt stress in wheat seedlings. *Biologia Plantarum*. 50: 227–231 (2006)
57. GASPAR, T., PENEL, C., HAGAGE, D.H. Peroxidases in plant growth, differentiation and developmental processes, in: J.H. Lobarzewsky, H. Greppin, C. Penel, T. Gaspar (eds.), *Biochemical, Molecular, and Physiological aspects of Plant Peroxidases*, University de Geneve, Geneve, 1991: pp. 249–280.
58. HARINASUTA, P., POONSOPAA, D., ROENGMONGKOLB, K., CHAROENSATAPORN, R. Salinity effects on antioxidant enzymes in Mulberry cultivar. *ScienceAsia*. 29: 109–113 (2003)
59. GUO, Y., TIAN, Z., YAN, D., ZHANG, J., QIN, P. Effects of nitric oxide on salt stress tolerance in *Kosteletzkya virginica*. *Life Science Journal*. 6: 67–75 (2009).