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

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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.

Abbrevations: EDTA, Ethylenediaminetetraacetic acid; PVPP, polyvinyl polypirrolidone,

Introduction

Salinity in the soil and irrigation water is an environmental problem and a major constraint for crop production. Salt stress (NaCI) 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 ($^1O_2$), superoxide anion radical (O_2^{-}), hydrogen peroxide (H_2O_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, H_2O_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^{-1} 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)0.5) values for each treatment was maintained around 1.0 in order to eliminate possible misinterprets 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= (Wfc–Wa 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 7th 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 mM^{-1} 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 × *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_2O_2 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 ± 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_2 photosynthetic assimilation. Soil water was depleted by plant and evaporation from soil surface, leaving salts that escalate the salinity level of soil. We applied grater 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.
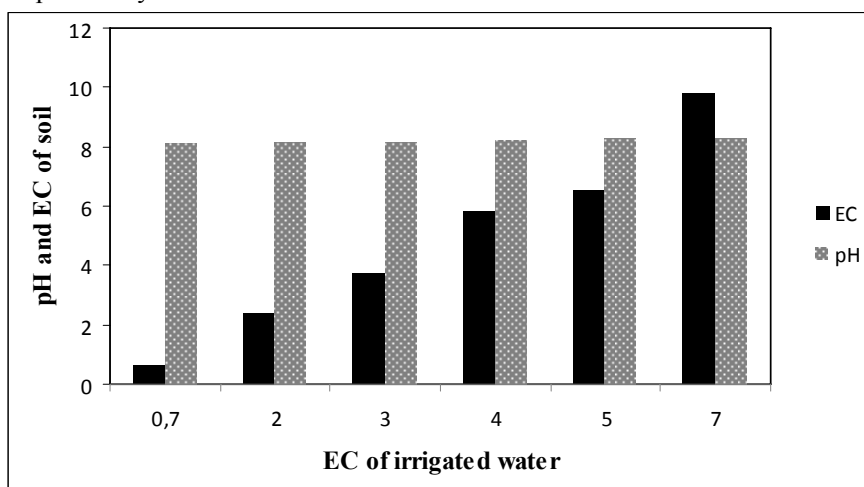

Figure 1. The effects of different salinity levels of irrigation water on soil pH and electrical conductivity (EC). Data represent the mean value ± SE, n=5 p<0.05

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_a and β-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 (µmol/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_2O_2 (µmol/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 ± 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_2O_2 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_2O_2 and MDA content of pea leaves except for 2 dS/m (Table 1). Decrease in H_2O_2 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_2O_2 in pea under salt stress. Some researchers also reported the sharp decline in H_2O_2 level to the efficient removal of H_2O_2 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 *Grevia 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_2O_2 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 (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^{-}) [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 peroxidise also use H_2O_2 as a substrate and CAT has low Km 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, *Kosteletzkya 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_2O_2 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.

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