International Journal of Plant Production 2 (4), October 2008
ISSN: 1735-6814 (Print), 1735-8043 (Online)
This is a refereed journal and all articles are professionally screened and reviewed.

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Responses of proline, lipid peroxidation and antioxidative enzymes in two varieties of *Pisum sativum* L. under salt stress

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Received 28 march. 2008; Accepted after revision 6 Aug 2008; Published online September 2008

Abstract

The possible involvement of activated oxygen species in the mechanism of damage by NaCl stress was studied in leaves of two varieties of pea (*Pisum sativum* L.) cv. EC 33866 and Puget. The level of lipid peroxidation, enzyme activity of superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC 1.6.4.2), dihydroascorbate reductase (DHAR, 1.8.5.1) were studied. High chlorophyll stability was observed in EC 33866 varieties than Puget. Significant accumulation in the levels of Na^+ and Cl^- was observed in Puget as the concentration of salt increased. Decrease in levels of K^+, Ca^{2+} and Mg^{2+} was recorded in both varieties but Puget showed more decrease as compared to EC 33866. Salt stress increased the rate of lipid peroxidation in both the varieties of pea but more decrease was observed in Puget. Salt stress induced significant increase in the activities of the antioxidant enzymes. The SOD and APX increased in both varieties at all concentrations but the increase was more in EC 33866 variety than Puget. GR and DHAR were increased in both the varieties at 150 mM NaCl, however at 200 mM NaCl decline was observed. Decline of DHAR and GR was more pronounced in Puget as compared to the EC 33866 variety.

Keywords: Antioxidant enzymes; inorganic nutrients; Lipid peroxidation; Oxidative stress; *Pisum sativum*; Proline; Salt stress

Introduction

Drought and salinity are two major environmental factors determining plant productivity and plant distribution. Drought and salinity affect more than 10 percent of arable land and desertification and salinization are rapidly increasing on a global scale, declining average yield for most major crop plants by more than 50 percent (Bray et al.,

2000). Salt stress may also occur in areas where soils are naturally high in salt and/or where irrigation, hydraulic lifting of salty underground water, or invasion of sea water in coastal areas brings salt to the surface soil that inhabit plants. Globally 20% of irrigated land and 2.1% of dry land agriculture suffers from the salt problem (FAO, 2000).

Plants have evolved mechanisms that allow them to perceive the incoming stresses and rapidly regulate their physiology and metabolism to cope with them (Zhang et al., 2006). Adaptation of the plant cells to high salinity involves osmotic adjustment and the compartmentation of toxic ions. Whereas an increasing body of evidence suggests that high salinity also induces oxidative stress (Gomez et al., 1999). Therefore, antioxidant resistance mechanisms may provide an strategy to enhance salt tolerance. Salt stress increases the rate of reactive oxygen species (ROS), via enhanced leakage of electron to oxygen in the chloroplasts and mitochondria (El-baky et al., 2003). Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to this oxidative damage (Young and Jung, 1999). Antioxidants are (1) lipid soluble and membrane-associated (α-tocopherol and β-carotene) (2) water soluble reductants, (ascorbate (AsA) and glutathione), (3) enzymes such as superoxide dismutase, catalase, peroxidase, ascorbate peroxidase and glutathione reductase (Foyer, 1993). Superoxide dismutase is a major scavenger of $O_2^{\bullet-}$ and its enzymatic action results in the formation of H_2O_2 and O_2. In the absence of natural scavengers such as catalase and peroxidase, H_2O_2 accumulates in tissues high levels. Ascorbate peroxidase (APX), together with monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase, and glutathione reductase (GR), removes H_2O_2 through the Halliwell–Asada pathway (Halliwell, 1987).

APX reduces H_2O_2 to water at the expense of oxidizing ascorbate to monodehydroascorbate (MDHA) (Asada, 1987) and plays a key role in the ascorbate–glutathione cycle. MDHA is then reduced to ascorbate by the action of MDHAR. Nonetheless, two molecules of MDHA can also be converted non-enzymatically to MDHA and dehydroascorbate, which in turn is reduced to ascorbate via the dehydroascorbate reductase and GR cycle (Noctor et al., 2002).

Salinity results in higher levels of antioxidants like glutathione reductase (GR), catalase (CAT) activities in different plants (Dionisio-Sese and Tobita, 1998; Hernandez et al., 2000; Bor et al., 2003).

Pea (*Pisum sativum* L.), is a member of family Leguminosae, mainly cultivated for its edible seeds and also for cattle fodder. Pea has a wide variety of uses and is grown as a cheap source of protein. It is commonly used in the form of fresh peas and edible podded type. It has been economically grown for the canning industry and traditionally for dry grain or fresh fruit. The crop is generally grown on residual moisture under rainfed conditions and experiences frequent abiotic stress of varying intensity and duration during growth period.

The objective of this study was to quantify the effect of NaCl treatment in two varieties of pea differing in salinity tolerance. The results showed the changes in inorganic nutrients, lipid peroxidation, accumulation of proline content and antioxidative enzymes in both the varieties but the accumulation was more in salt tolerant EC 33866 than susceptible variety Puget.

Materials and methods

Plant material and culture

Seeds of *Pisum sativum* L. cv. EC 33866 and Puget were provided by the Indian Council of Agricultural Research (ICAR), New Delhi, India. Seeds were surface sterilized and germinated in moist sterile vermiculite at 28 °C for 1 week. Seedlings were selected and grown in pots with aerated nutrient solution (KNO_3 3 mM, $Ca(NO_3)_2$ 1 mM, KH_2PO_4 2 mM, $MgSO_4$ 0.5 mM, Fe-K-EDTA 32.9 mM, and micronutrients: H_3BO_4 30 mM, $MnSO_4$ 5 mM, $CuSO_4$ 1 mM, $ZnSO_4$ 1 mM, $(NH_4)_6Mo_7O_{24}$ 1 mM) in growth chamber under optimum conditions for 7 days. The growth chamber was set to 28/18 °C, 80% RH and 16-h photoperiod. Irradiation intensity was in the range of 100 $\mu Em^{-2} s^{-1}$ at the plant level. After 7 days, plants were transplanted to similar media supplemented with 50, 100, 150 and 200 mM NaCl and were grown for 14 days. Pots containing 4 plants were arranged in blocks at random and each treatment (NaCl level and cultivar) was replicated 3 times.

Estimation of nutrients

The leaves of control and salt treated were analysed for sodium, potassium, calcium and magnesium by atomic absortion spectrometry. Chloride content were estimated by potentiometric method (Chapman and Pratt, 1961).

Determination of proline

The proline content was determined using the method of Bates et al. (1973). Fresh material (300 mg each sample) was homogenized in 10 ml of 3% aqueous sulfosalicylic acid. The homogenate was centrifuged at 9000 × g for 15 min. A 2 ml aliquot of the supernatant was mixed with an equal volume of acetic acid and acid ninhydrin and incubated for 1 h at 100 ^{0}C. The reaction was terminated in an ice bath and extracted with 4 ml of toluene. The extract was vortexed for 20 s. The chromatophore-containing toluene was then aspirated from the aqueous phase, and its absorbance determined spectrophotometerically at 520 nm (Beckman 640 D, USA) using toluene for a blank.

Lipid peroxidation

Lipid peroxidation rates were determined by measuring the malondialdehyde equivalents according to Hodges et al. (1999). The leaf tissue (0.5 g) was homogenized in a mortar with 80% ethanol. The homogenate was centrifuged at 3000×*g* for 10 min at 4 °C. The pellet was extracted twice with the same solvent. The supernatants were pooled and 1 ml of this sample was added to a test tube with an equal volume of either the solution comprised of 20% TCA and 0.01% butylated hydroxy toluene (BHT) or solution of 20% TCA, 0.01% BHT and 0.65% TBA. Samples were heated at 95 °C for 25 min and cooled to room temperature. Absorbances were read at 440, 532 and 600 nm. Lipid peroxidation rate equivalents (nmol malondialdehyde ml^{-1}) were calculated by using the formulae given by Hodges et al. (1999).

Enzyme extractions and assays

Superoxide dismutase

Plant material was homogenised in 50 mM phosphate buffer (pH 7.0) containing 1% polyvinyl pyrrolidone. The homogenate was filtered and then centrifuged in a refrigerated centrifuge at 15,000×*g* for 15 min, and the supernatant obtained was used as source of enzyme. Whole extraction procedure was carried out at 4 °C. The activity of superoxide dismutase was assayed by measuring its ability to inhibit to the photochemical reduction of nitroblue tetrazolium as described by Beauchamp and Fridovich (1971). The reaction mixture (3.0 ml) consisting of 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM nitroblue tetrazolium, 0.1 mM EDTA, 2 µM riboflavin and 0.1 ml of enzyme extract. The test tubes containing reaction mixture were shaken and kept for 25 min under 30 cm below a light source (30 W fluroscent lamps). The reaction was then stopped by switching-off the lights. The tubes were covered with black cloth. A non-irradiated reaction mixture did not develop colour and served as control. The absorbance was measured at 560 nm in a Shimadzu 1601 UV-Vis spectrophotometer. The reduction of NBT was inversely proportional to the SOD activity. One unit of SOD was defined as the amount of enzyme that inhibits 50% NBT photoreduction and the activity was expressed in Enzyme Unit (EU) mg^{-1} protein h^{-1}.

Estimation of ascorbate peroxidase activity

Ascorbate peroxidase activity was measured according to Aono et al. (1995). Plants (100 mg) were homogenized in 1ml of 50 mM phosphate buffer (pH 7.8) containing 5 mM ascorbate, 5 mM DTT, 5 mM EDTA, 100 mM NaCl and 2% (w/v) polyvinyl pyrrolidone (PVP). The homogenized material was centrifuged at 15,000×*g* for 15 min at 4 ^{o}C. The reaction was initiated by adding H_2O_2 to a final concentration of 44 µM as described by Nakano and Asada (1981). The reaction rate was monitored by the decrease in absorbance at 290 nm. The rate constant was calculated using the extinction constant 2.8 mM^{-1} cm^{-1} and corrected for the rate obtained prior to the addition of H_2O_2. The enzyme activity was expressed as change in absorbance units, mg^{-1} protein min^{-1}.

Dehydroascorbate reductase

100 to 200 mg of leaf tissue was powdered in 1.5 mL of 50 mM K_2PO_4 (pH 7.8). Containing 0.2 mM EDTA, 10 mm β-mercaptoethanol, and 2.5% PVP. The extracts were centrifuged at 15,000x*g* for 15 min, and the supernatant was used for the assays. DHAR activity was determined by monitoring the formation of AsA at 265 nm (Hossain et al., 1984).

Glutathione reductase

The plant material was extracted in 100 mM potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 10,000×*g* for 10 min. The supernatant was passed through

Sephadex G-25 column; active fractions were collected and used as enzyme source for the assay of glutathione reductase. Glutathione reductase activity was assayed as per the method of Foster and Hess (1980). The reaction mixture consists of enzyme extract, 100 mM potassium phosphate buffer, (pH 7.0) containing 1.0 mM EDTA, 150 µM NADPH and 500 µM oxidized glutathione. The enzyme activity was measured at 340 nm. Activity was calculated using the extinction coefficient for NADPH of 6.22 mM^{-1} cm^{-1} and expressed as umol NADPH oxidized mg^{-1} protein min^{-1}.

Chlorophyll stability

Fresh leaves of pea were taken, washed with distilled water and blot dried. Leaf discs of 1 cm diameter were cut and placed in petridishes containing 50, 100, 150 and 200 mM NaCl solution. The leaf discs in petridishes containing distilled water served as control. After 5 days, the chlorophyll content was estimated by the method of Arnon (1949).

Data analysis

Statistical analysis of the results was carried out according to Duncan's multiple range test.

Results

Nutrients

Table 1 deals with the effect of different concentrations of NaCl on the nutrients of salt stressed leaves of pea cultivar. Na^+ and Cl^- increases as the concentration of NaCl increases in both the cultivars, however Puget accumulates more Na^+ and Cl^- than EC 33866 variety.

K, Ca and Mg decreases by elevated concentration of NaCl. The decrease was more pronounced in Puget than EC 33866 cultivar.

Proline content

It is clear from the table 2 that sharp increase in the accumulation of proline was observed in both the varieties at all stress regimes, however increase of 13.8 fold and 9.9 fold was observed in EC 33866 and Puget variety respectively at the concentration of 200 mM NaCl.

Lipid Peroxidation

Salt stress caused a significant increase in levels of MDA content in salt susceptible variety at all stress levels (table 2). The degree of accumulation was 77.8% in Puget and 47.6% in EC 33866 variety, indicating a high rate of lipid peroxidation in Puget due to salt stress.

Superoxide dismutase

The results pertaining to the effect of different concentrations of salt on SOD activity is summerised in Figure 1. The SOD level increases in both the cultivars at all stress levels. The percent increase in the enzyme activity was more in EC 33866 (69%) than in Puget (58.3%) cultivar at the concentration of 200 mM NaCl.

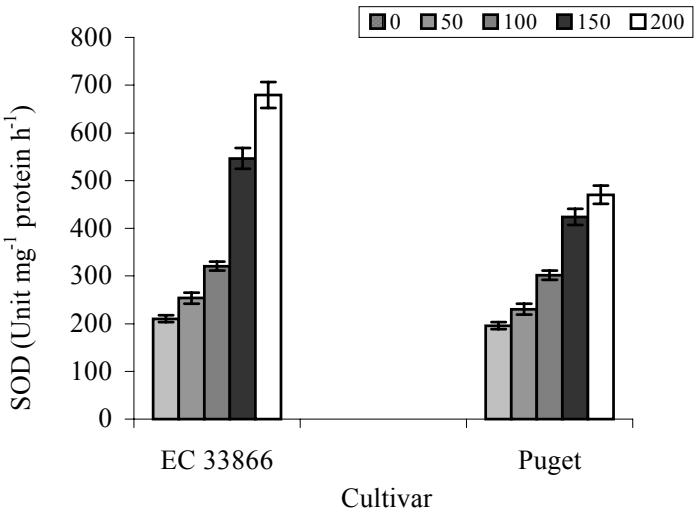

Figure 1. Effect of different concentration of NaCl on SOD (unit mg^{-1} protein h^{-1})
Different letters indicate significant difference between means at $P<0.05$ (DMRT).
Values are means ± S.E (n=3).

Ascorbate peroxidase

Figure 2 deals with the results of effect of different concentrations of NaCl on the activity of APX. In the leaves of salt stressed cultivars of pea the APX increased significantly over control in both the varieties. At 200 mM NaCl stress the increase in the enzyme activity in EC 33866 was 43.3% and in Puget it was 27.9%.

Dehydroascorbate reductase

It is clear from Figure 4 that DHAR was significantly increased in the leaves of salt stressed plants. DHAR continued to increase along the concentration of NaCl and maximum increase in DHAR was 45.4% and 32.4% in EC 33866 and Puget respectively at concentration 150 mM NaCl, however at the higher concentration of 200 mM NaCl it declined.

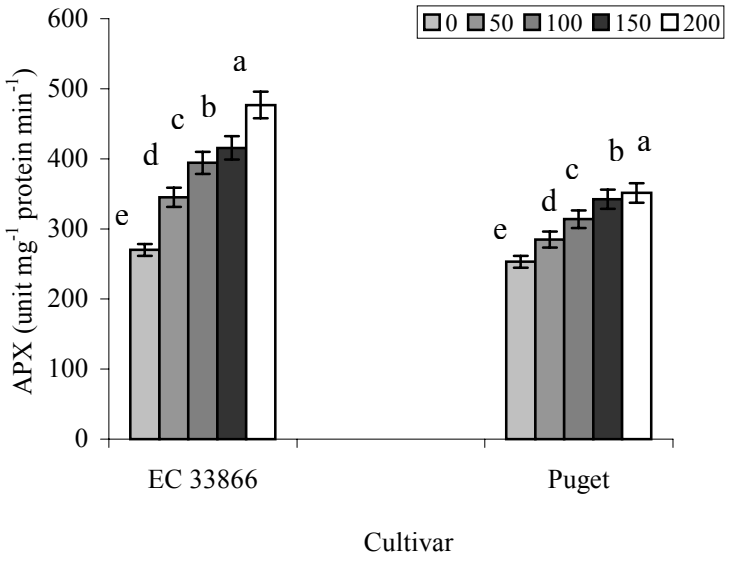

Figure 2. Effect of different concentration of NaCl on APX (unit mg^{-1} protein min^{-1})
Different letters indicate significant difference between means at $P<0.05$ (DMRT).
Values are means ± S.E (n=3).

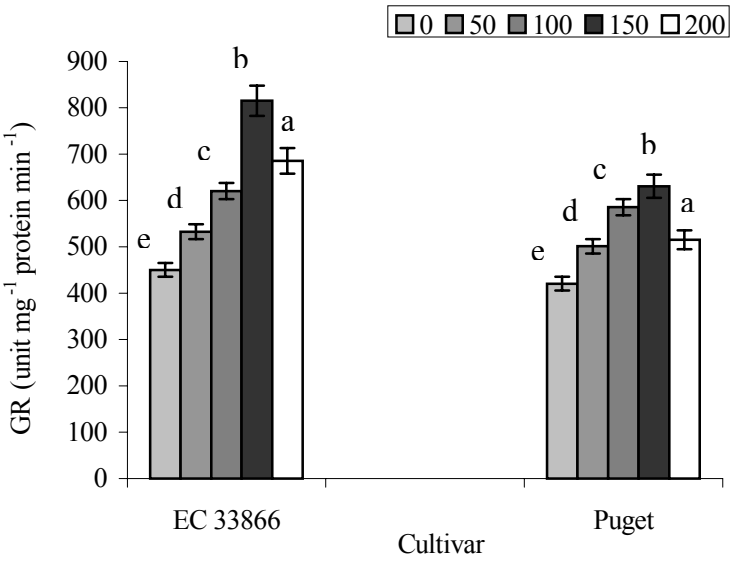

Figure 3. Effect of different concentration of NaCl on GR (unit mg^{-1} protein min^{-1})
Different letters indicate significant difference between means at $P<0.05$ (DMRT).
Values are means ± S.E (n= 3).

Glutathione reductase

The results related to the effect of NaCl on GR activity are depicted in Figure 3. An increase of 44.8% and 33.3% was observed in EC 33866 and Puget respectively at the concentration of 150 mM NaCl. At higher concentration of NaCl (200 mM) a decrease of 15.9% was observed in EC 33866 and 18.3% in Puget.

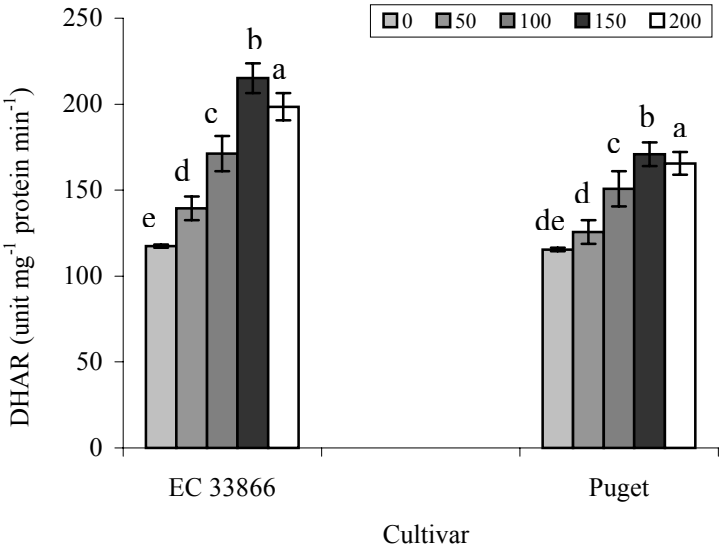

Figure 4. Effect of different concentration of NaCl on DHAR (unit mg^{-1} protein min^{-1})
Different letters indicate significant difference between means at $P<0.05$ (DMRT).
Values are means ± S.E (n = 3).

Chlorophyll stability (%)

The chlorophyll stability was measured in control and salt stressed leaves of both pea varieties and results are depicted in Figure 5. Chlorophyll stability decreases by increasing the concentration osf NaCl in both the cultivars of pea. The better chlorophyll stability was found in EC 33866 (46.3%) than Puget (70.8%).

Discussion

It has been demonstrated that many environmental stresses, including drought and salinity, because oxidative damage to membranes as a result of accumulated ROS in plant tissues. Determining the MDA content and hence, the extent of membrane lipid peroxidation, has often been used as a tool to assess the degree of plant sensitivity to

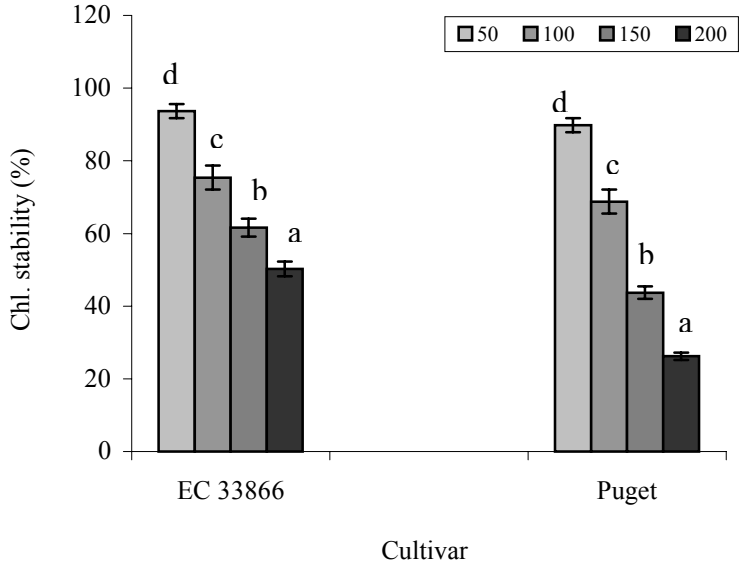

Figure 5. Effect of different concentration of NaCl on Chlorophyll stability (%)
Different letters indicate significant difference between means at $P<0.05$ (DMRT).
Values are means ± S.E (n=3).

oxidative damage (Blokhina et al., 2003). Vendruscolo et al., (2007) reported that MDA content in non transgenic plants were 65% higher when campared to transgenic plants of wheat during water stress. Koca et al., (2007) also showed that lipid peroxidation was higher at 100 mM NaCl treatment in salt sensitive cultivar of *Sesamum indicum* than salt tolerant one. Our data showed marked increase of shoot MDA content in Puget at different concentration of NaCl than EC33866. Lipid peroxidation was shown to remain unchanged in plants tolerant towards salinity or drought (Egert and Tevini, 2002).

Rapid accumulation of free proline is a typical response to salt stress. In organisms ranging from bacteria to higher plants there is a strong correlation between increased cellular proline levels and the capacity to survive both water deficit and the effects of high environmental salinity (Ahmad and Jhon, 2005). Proline content have been reported to increase under NaCl stress in *Phaseolus aureus* (Misra and Gupta, 2005), *Morus alba* (Ahmad et al., 2007), *Sesamum indicum* (Koca et al., 2007). Harinasut et al., (2000) showed that proline content in leaves of mulberry increases 11 folds with 150 mM NaCl conditions over control. Apart from protection of macromolecules from denaturation and carbon and nitrogen reserve for stress relief, proline has several other functions during stress: e.g. osmotic adjustment (Voetberg and Sharp, 1991), osmoprotection (Kishor et al., 2005), free radical scavenger and antioxidant activity (Sharma and Dietz, 2006). A positive correlation between magnitude of free proline accumulation and salt tolerance has been suggested as an index for determining salt tolerance potentials between mulberry cultivars (Ramanjula and Sudhakar, 2001).

Our results of increase in SOD are in accordance with Gómez et al., (2004), who found an increase in all SOD isoenzymes of pea chloroplasts following a long-term NaCl treatment. Koca et al., (2007) also demonstrated that salinity leads to a decrease in SOD activity in salt sensitive plants of *Sesamum indicum* than salt tolerant ones. There is a possibility that high amount of Na^+ and Cl^- ions in leaves directly inhibit chloroplastic SOD catalysis *in vivo*, because in plant most of the SOD is present in chloroplasts (Asada, 1999). The increase in SOD activity under NaCl-salinity stress suggests better oxidative stress tolerance (Panda and Khan, 2004). Under salt and water deficit stresses, Rubio et al., (2002) reported that MnSOD and FeSODs were activated in the alfalfa transgenic chloroplast, and nodular MnSOD isoforms were over-expressed as was FeSOD of chloroplasts. Lee et al., (2007) have reported that CuZn SOD in chloroplast showed enhanced tolerance to oxidative stress.

The role of APX and GR in the H_2O_2 scavenging in plant cells has been well established in ascorbate–glutathione cycle. APX and GR activities have been shown to be enhanced by NaCl stress in rice leaves (Lee et al., 2001) *Sesamum indicum* (Koca et al., 2007). Exogenous ascorbate supplementation of non-transgenic and transgenic plants resulted in a decrease in the toxicity of methyl viologen mediated oxidative stress (Kim et al., 2005).

DHA can be accumulated from disproportion of monodehydroascorbate (MDHA) or AsA oxidation by superoxide radical, a-chromoxyl radical of oxidised a-tocopherol, violoxanthin de-epoxidase reactions in chloroplast, and some other enzymes that require AsA as a cofactor (Smirnoff and Wheeler, 2000). DHAR and MDHAR are involved in enzymatic regeneration of AsA from DHA and MDHA, respectively. Under salinity stress, AsA is mainly regenerated from MDHA or DHA (Hernandez et al., 2000). Overexpression of chloroplast targeted DHAR catalyse the reduction of DHA to AsA which also enhance tolerance to the oxidative and salt induced stress. Plants with large ascorbate pools, accumulated by either ascorbate recycling or exogenous ascorbate supplementation, are able to maintain APX activity even under conditions of oxidative stress (Lee et al., 2007). They also reported that transgenic plants expressing three transgenes, such as those encoing CuZnSOD, APX and DHAR in their chloroplast might be efficiently protected against a variety of environmental stresses.

GR is found in chloroplasts as well as in mitochondria and cytoplasm (Edwards et el., 1990) and has a role to scavenge H_2O_2 in plant cells. GR catalyzes the rate limiting step of ascorbate-glutathione pathway. Our results indicated an increase in GR activity with salt stress in both cultivars of pea corroborates with the findings of Koca et al., (2007) in *Sesamum indicum*. The elevated levels of GR activity perhaps could increase the ratio of $NADP^+$/NADPH, thereby ensuring the availability of $NADP^+$ to accept electrons from the photosynthetic electron transport chain. Under such a situation, the flow of electrons to O_2 and therefore, the formation of O_2^- can be minimized. Baisak et al., (1994) showed that GR would involve in maintaining high ratios of GSH/GSSG which is required for the regeneration of ascorbate and for the activation of several chloroplastic CO_2 fixing enzymes. Increase in the glutathione reductase activity in plants resulted in the accumulation of glutathione (GSH) levels and ultimately confers the tolerance in plants. This is well correlated in the present study with the greater increase in the activity of glutathione reductase in EC-33866 compared to Puget cultivar.

By increasing NaCl concentration the chlorophyll stability indices decreased in both cultivars of pea. EC 33866 showed the better Chl. stability than Puget. CSI is a method for estimating resistance to environmental stress. Ramanjulu and Sudhakar (2000) have reported relatively better chlorophyll stability in drought resistant mulberry line compared to drought sensitive one. In the present study CSI was less affected (a better chlorophyll stability) in the cultivar EC 33866 compared to Puget, further supports the salt tolerant nature of the cultivar EC 33866.

Damage from salinity has been attributed principally to an excess of Cl^- and Na^+ accumulation in the leaves, provoking a nutritional imbalance, as these ions reduce the concentration of Ca, Mg and K. High concentration of Cl^- in the aerial parts of citrus can be prime cause of physiological disturbance and eventual visible damage to the foliage. High foliar concentration of Na^+ interferes with photosynthesis and transpiration. Reduction in the potassium content in the leaves and roots as a result of salt stress has been observed previously and it has been interpreted as resulting from competition between this ion and Na^+ (Azevedo Neto and Tabosa, 2000). The reduction in K^+ content of the most salt-sensitive genotypes suggests that salt induced shoot growth inhibition is mainly due to metabolic changes resulting from ion imbalance or ion toxicity occurring in root system (Munns, 2002). In such conditions, additional application of potassium reduces the impact of abiotic stresses (Umar and Bansal, 1995; Umar and Moinuddin, 2002).

The present study reveals substantial differences in the cellular response between pea cultivars EC-33866 and Puget in response to salinity. In conclusion it has been demonstrated that both osmotic and ionic effects involved in NaCl salinity can limit photosynthesis and respiration leading to an increase in ROS generation, which are responsible for a secondary oxidative stress that can damage cellular structure and metabolism. It is also known that plant responses to salt stress are multigenic, involving both osmotic and ionic homeostasis, as well as cell detoxification. The efficiency of the latter process is dependent upon the plant antioxidant defense mechanisms. The scavenging system forms the primary defense line in protecting the pea cultivars against superoxide radicals. Differences among species in leaf accumulation of Na and K might be responsible for the differential sensitivity to NaCl treatments. A lower Na^+ level and lipid per oxidation combined with higher capacity for oxygen radical scavenging could probably explain the ability of EC 33866 cultivar to grow at higher NaCl concentrations than Puget cultivar which appears to be sensitive.

Acknowledgements

We gratefully acknowledge Council of Scientific and Industrial Research (CSIR), New Delhi, India for providing financial assistance to PA in the form of a Senior Research Fellowship.

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