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Effect of Salinity Stress on Growth, Mineral Composition, Proline Content, Antioxidant Enzymes of Soybean

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Abstract: Present study has explored the effect of salinity stress on growth, mineral composition, proline content, antioxidant enzymes of soybean. Soil salinity is a major limitation to legume production in many areas of the world. The salinity sensitivity of soybean was studied. Soybean plants were exposed to 0, 50, 100 and 200 mM NaCl. The effect of salinity on length and fresh weight of seedling were determined. Increasing salinity level to 50, 100 and 200 mM resulted in a reduction of plant height of 30, 47 and 76% and a reduction of fresh weight of 32, 54 and 76%, respectively. The activity of nitrogenase and Ammonium content of nodules were measured by analyzing ethylene in the gas samples by chromatography and the phenol-hypochlorite method, respectively. Nitrogenase activity had a decrease of 60% and ammonium content a significant increase (100%) at 200 mM salt concentration. Proline accumulation and diamine oxidase, DAO, were studied by reading the absorption of chromophore at 520 nm using spectrophotometer. Seedlings subjected to salt stress in the presence. Both DAO activity and proline content were increased in soybean under 50 to 200 mM NaCl. Using atomic absorption spectrophotometer, ion uptake of for Na^+, K^+, Ca^{2+} and Mg^{2+} were determined. The Na^+ content significantly increased but the contents of K^+, Ca^{2+} and Mg^{2+} decreased significantly as salinity treatment concentrations increased. The behaviour of antioxidant enzymes was analyzed. A significant decrease in superoxide dismutase, catalase and peroxidase activities under 100 and 200 mM salt were found.

Key words: Antioxidant enzyme, *Glycine max*, mineral composition, salinity stress, soybean

INTRODUCTION

The world population is continuing to increase and the amount of the arable land to decrease. Agricultural productivity worldwide is subjected to increasing environmental constraints, particularly to salinity due to its high magnitude of impact and wide distribution. Cultivation of agricultural crops in soil is limited by salt stress, which arises from the excessive uptake of salt by plants and it is an unavoidable consequence of high ion concentrations. Excessive amounts of salt in the soil, most commonly NaCl, has detrimental effects on plant growth and productivity (Reynolds *et al*., 2005; Zilli *et al*., 2008; Sobhanian *et al*., 2010). Greater emphasis must therefore be placed on bringing marginally productive and presently non-arable land under production. Large areas of formerly arable land are being removed from crop production every year due to increasing soil salinity. Use of saline irrigation water and application of fertilizer are the main factors responsible for increasing soil salinity (Epstein *et al*., 1980). The salinity of the soil and irrigated water is a problem that restricts yield on almost 40 million hectares of irrigated land, which is approximately one-third of the irrigated land on earth (Norlyn and Epstein, 1984).

Plants exposed to stresses undergo changes in their metabolism in order to adapt with changes in their environment. Salt stress changes the morphological, physiological and biochemical responses of plants. salinity affects adversely plant growth and development. An excess of salts leads to both osmotic and ionic stress (Munns, 2002; Benlloch-Gonzalez *et al*., 2005). The detrimental effect of salt is generally observed at the whole plant level. On the molecular level these responses are manifested as changes in the pattern of gene expression (Fabre and Planchon, 2000; Maggio *et al*., 2002). Suppression of growth occurs in all plants, but their tolerance levels at high salt concentrations vary widely among different plants (Rabie and Almadini, 2005). Production and accumulation of Free Amino Acids (FAA), especially proline by plant tissue during drought, salt and water stress is an adaptive response. Proline has been considered to play an important role in plant responses to salt stress (Gaspar *et al*., 2002) and proposed to act as a compatible solute that adjusts the osmotic potential in the cytoplasm (Arshi *et al*., 2005; Bartels and Sunkar, 2005). Diamine oxidase (DAO, EC: 1.4.3.6) activity is promoted by high salt stress (Xing *et al*., 2007). Free polyamines degraded via DAO and polyamine oxidase (PAO, EC: 1.5.3.11), can contribute to proline accumulation through γ-aminobutyric acid production (Bouchereau *et al*., 1999; Gaspar *et al*., 2002). In some species such as soybean submitted to salt stress, free polyamines degradation is promoted (Aziz *et al*., 1998; Xing *et al*., 2007) and proline content significantly increased (Tonon *et al*., 2004; Sotiropoulos, 2007). Thus, proline can be used as a metabolic marker in relation to stress. Proline produces immediately after encounter of cells with salt stress and protects the plasma membrane and proteins against stress (Santoro *et al*., 1992). Understanding of plant ability in fight to stresses open a way for crops manipulations for their ability in tolerance, adaptation or resistant to stresses (Kaviani, 2008).

Soybean is one of the main sources of edible vegetable oil and high-protein livestock feed. It is the most important dicot crop due to the high content of oil and protein in its seeds and has been considered as a salt sensitive to moderately salt-tolerant crop (Umezawa *et al*., 2000; Banzai *et al*., 2002; Luo *et al*., 2005).

Oxidative stress is also a factor in abiotic and biotic stress phenomena that occurs when there is a serious imbalance between the production of Reactive Oxygen Species (ROS) and antioxidant defense. ROS have been considered mainly as dangerous molecules and their concentrations must be maintained as low as possible. This concept has changed because activated oxygen has multiple functions. For example $O_2^{\bullet -}$ and H_2O_2 are required for lignification and function as signals in the defense response to pathogen infection (Gratao *et al*., 2005). Negative effect of environmental stresses may be partially due to the generation of ROS. During the reduction of O_2 to H_2O, one, two or three electrons transfer to O_2 can occur to form superoxide ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). These molecules are highly damaging to lipids, nucleic acids and proteins (Gratao *et al*., 2005). ROS result in a variety of injuries to plant metabolism. They damage photosynthetic components, inactivate proteins and enzymes and permeabilize membranes by causing lipid peroxidation (Meloni *et al*., 2003). Moreover, lipid peroxidation induced by ROS is considered to be an important mechanism of membrane deterioration Cell oxidative stress levels are determined by the amounts of $O_2^{\bullet -}$, H_2O_2 and $^{\bullet}OH$ radicals (Foyer and Noctor, 2003). H_2O_2 can be directly metabolized by peroxidase, particularly those in apoplast and by CAT in the peroxizome (Gratao *et al*., 2005).

The major ROS-scavenging mechamisms of plants include enzymes such as superoxide dismutase (SOD; EC 1.15.1.1) catalase (CAT; EC 1.11.1.6) and glutation peroxidase (GPX, EC 1.11.1.9). The primary scavenger is SOD, which converts $O_2^{\bullet -}$ to H_2O_2 which is eliminated by peroxidase (POD; EC 1.11.1.11). When these defenses fail to halt the self-propagating autooxidation with ROS, cell death ultimately results (Li, 2009).

MATERIALS AND METHODS

Plant Materials and Treatment

This project was done at University of Arak, during 2009. Seeds of soybean (*Glycin max* L.) were surface-sterilized with 30% sodium hypochlorite for 10 min and were thoroughly washed with redistilled water. Surface-sterilized seeds were grown in the pots filled with vermiculite saturated with Hoagland nutrient solution (Hoagland and Arnon, 1950) and treated by nutrient solution supplemented with 0, 50, 100 and 200 mM NaCl and grown in growth chamber under white fluorescent light (600 μmol/m^2/sec; 16 h light/8 h dark) at 25/20°C and 70% relative humidity and were simultaneously inoculated with Bradyrhizobium japonicum. Length and fresh weight of hypocotyls and roots of seedlings were measured 4 days after germination.

Some seedlings were watered in Hoagland nutrient solution during the first 5 days and then with a N-free nutrient solution. After 4 weeks, plants were treated with nutrient solution supplemented with 0, 50, 100 and 200 mM NaCl. After 10 days of treatment, nodules were isolated and used for the determinations.

Nitrogen Fixation Assay

Nitrogen fixation was measured as acetylene reduction activity (Hardy *et al.*, 1968). Nodules were enclosed in 100 mL bottles sealed with rubber stoppers containing C_2H_2 (10%, v/v) in air. Gas samples (0.5 mL) were taken 60 min later and analyzed for ethylene in a Konik 3000 HRGC chromatograph equipped with a hydrogen flame ionisation detector (Hewlett Packard fused silica capillary HP-Plot Al_2O_3 column; oven temperature: 120°C; carrier gas: N_2 at a rate of 30 mL min^{-1}).

Ammonium Determination

0.3 grams of nodule were homogenized in 3 mL of 0.3 mM H_2SO_4 and centrifuged at 15,000x g for 15 min. Ammonium content was measured in the supernatant by the phenol-hypochlorite method (Weatherburn, 1967). A calibration curve with NH_4Cl was used as the standard.

Estimation of Activity of DAO

DAO activity in soybean estimated using a Perkin Elmer Lambda 900 UV/VIS spectrophotometer (MC USA). The method described previously by Su *et al.* (2005) were used. Plant material (0.5 g) was ground with the aid of mortar and pestle at 4°C in 1.6 mL 0.1 M sodium phosphate buffer (pH 6.5) containing 5% (m/v) polyvinyl pyrrolidone (PVP). Homogenate was centrifuged at 10 000 g for 20 min at 4°C. Supernatants were used for the determination of DAO activity. Three milliliters of reaction solutions contained 2.5 mL 0.1 M sodium phosphate buffer (pH 6.5), 0.1 mL crude enzyme extracts, 0.1 mL peroxidase (250 U mL^{-1}) and 0.2 mL 4-aminoantipyrine/N, N-dimethylaniline solution. The reaction was initiated by the addition of 0.1 mL 20 mM putrescine as a substrate. A change in absorbance at 555 nm of 0.01 was regarded as unit of the enzyme activity.

Determination of Leaf Proline Content

Numbers of youngest fully expanded leaves (0.5 g) from plants of each of the treatments were harvested. Leaves were ground in liquid nitrogen to a fine powder for the determination of proline content. Proline was extracted and the content assayed spectrophotometrically according to the method of Bates *et al.* (1973). The powder were homogenized with 10 mL of 3% (w/v) sulphosalicylic acid and passed through whatman No. 2 filter paper. Two milliliter of ninhydrin reagent and 2 mL of glacial acetic acid were added to 2 mL of the filtered extract.

The mixture was incubated in 100°C water bath for 1 h. The reaction mixture was placed on ice and extracted with 4 mL toluene. Absorption of chromophore was read at 520 nm using a Perkin Elmer Lambda 900 UV/VIS spectrophotometer (MC USA). Toluene was used as blank. The proline concentration was calculated using L-proline corresponding on the standard curve.

Determination of Na^+ and K^+ Contents

Plant samples were oven dried at 70°C for 24 h and were ground using a pestle and mortar for determination of mineral composition. Ash of plant samples was dissolved in 5.1% HNO_3 and used to determine of Na^+, K^+ and Ca^{2+} Mg^{2+} contents using an atomic absorption spectrophotometer.

Determination of Antioxidative Enzymes

Protein extracts of plant material prepared by a method slightly modified from the one described by Boddi *et al*. (1996). The leaves were ground at 95°C in an extraction buffer of 10% (v/v) glycerol, 4% (w/v) sodium dodecyl sulphate (SDS), 0.3 M dithiothreitol, 0.001% bromophenol blue and 250 mM Tris-HCl, pH 6.8. The proteins were quantified by a colorimetric assay for protein determination using the Bio-Rad DC Protein Assay kit based on the well-documented Lowry assay (Bio-Rad, Richmond, CA). The absorption values were read at 750 nm with a Perkin Elmer Lambda 900 UV/VIS spectrophotometer.

SOD was assayed on the basis of its ability to inhibit the photochemical reduction of Nitro Blue Tetrazolium (NBT), according to the methods of Beauchamp and Fridovich (1971) and Beyer and Fridovich (1987). The reaction mixture contained 50 mM phosphate buffer (pH = 7.8), 13 mM methionine, 75 mM nitro blue tetrazolium, 100 nM EDTA, 200 mL of enzyme extract and 2 mM riboflavin. The reaction mixture was read at 560 nm. One unit of SOD activity (U) was defined as the amount of enzyme that caused 50% inhibition of the initial rate of the reaction in the absence of enzyme. Total SOD activity was expressed as U mg^{-1} protein.

Peroxidase POD, (EC 1.11.1.7) activity was measured on the basis of determination of guaiacol oxidation at 470 nm (Bergmeyer, 1974; Lagrimini, 1991). In the presence of H_2O_2, POD catalyzes the transformation of guaiacol to tetraguaiacol. This reaction can be recorded at 470 nm. The reaction mixture contained 100 mM phosphate buffer (pH 6.0), 33 mM guaiacol and 0.3 mM H_2O_2. Enzyme specific activity was expressed as OD_{470} min^{-1} g^{-1} FW (OD: optical density).

The activity of CAT was measured by the method of Aebi (1984) and was determined by monitorin g the disappearance of H_2O_2 at 240 nm. One unit of the enzyme (U) is the amount necessary to decompose 1 μmol of H_2O_2 at 25°C and CAT activity was expressed as U mg^{-1} FW.

RESULTS

Effect of Salt Stress on Length and Fresh Weight

The effects of salt stress on the morphological characteristics of the treated soybean seedlings were evaluated. Plant heights and fresh weight recorded 4 days after treatment. Plant elongation and fresh weight of soybean were significantly reduced by increasing salinity level (Fig. 1a, b). Increasing salinity level to 50, 100 and 200 mM resulted in a reduction of plant height of 30, 47 and 76% and a reduction of fresh weight of 32, 54 and 76%, respectively. The extent of length and fresh weight lowering was similar under all treatment conditions.

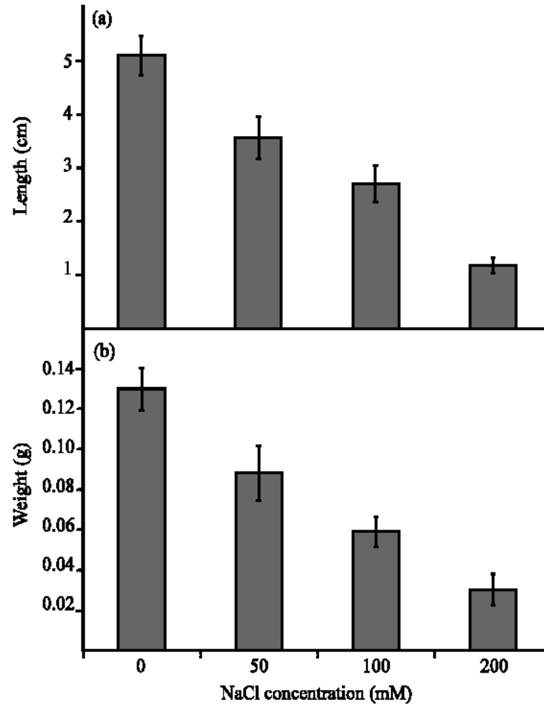

Fig. 1: (a) Effect of NaCl concentrations on the length and (b) fresh weight of soybean seedlings. Seeds were sown and treated with 0, 50, 100 and 200 mM NaCl. The results are presented as Means±SD from five experiments

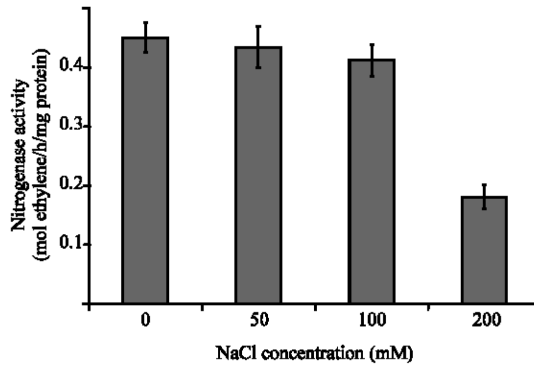

Fig. 2: Effect of different salt concentrations on nodules nitrogenaseactivity. The results are presented as Means±SD from five experiments

Nitrogen Fixation

To indicate the effectiveness of nodules the activity of nitrogenase was measured. In 50 100 mM NaCl concentration no changes were observed in nitrogenase activity. Nitrogenase activity, however, had a decrease of 60% at 200 mM salt concentration (Fig. 2).

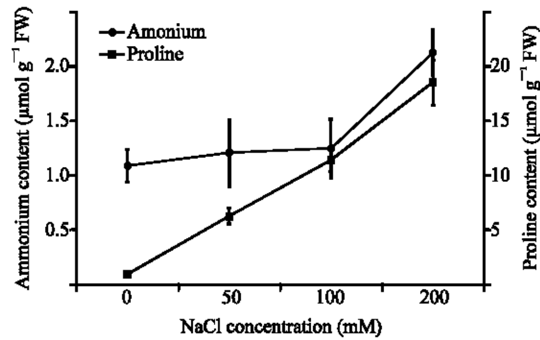

Fig. 3: Effect of different salt concentrations on nodules ammonium content. Values are the mean of five independent experiments and presented as Means±SD

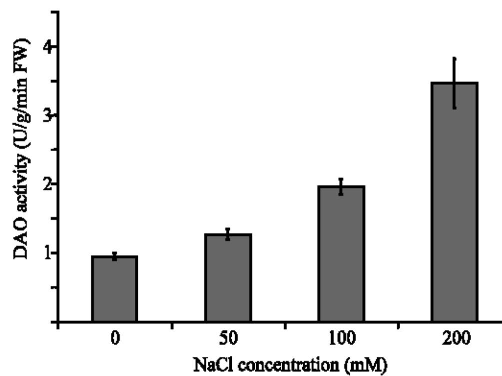

Fig. 4: Effects of different NaCl concentrations on DAO activity in soybean seedlings. Values are the mean of five independent experiments and presented as Means±SD

Ammonium Content

Ammonium contents of soybean nodules were not significantly increased when the salt concentrations were 50 and 100 mM. The 200 mM salt concentration, however, resulted in a significant increase (100%) of ammonium content (Fig. 3).

Effect of NaCl on Proline Accumulation

To determine whether proline accumulates in response to salinity the content of free proline was measured. The result showed an increase of 7, 12 and 20 fold in proline content was observed when treated with 50, 100 and 200 mM NaCl stress, respectively (Fig. 3).

Effect of NaCl on Activity of DAO

To evaluate polyamine catabolism under salinity, DAO activity was determined. The results showed that DAO activity increased with increasing NaCl concentrations. The activity of DAO increased 34 and 107 and 265% of the control (Fig. 4).

Determination of K^+, Na^+, Ca^{2+} and Mg^{2+} Contents

To investigate the effect of salt stress on the K^+, Na^+, Ca^{2+} and Mg^{2+} contents in soybean, the concentrations of these ions at 0, 50, 100 and 200 mM NaCl. Salinity affected the K^+ content of seedling and the K^+ content decreased by increasing the salinity. Results

Table 1: K^+, Na^+, Ca^{2+}, Mg^{2+} concentrations and K^+/Na^+ ratio of soybean seedlings

K^+ (mg g^{-1} FW)	Na^+ (mg g^{-1} FW)	K^+/Na^+ ratio	Ca^{2+} (mg g^{-1} FW)	Mg^{2+} (mg g^{-1} FW)
2.71±0.29	0,18±0,03	14,59±2,03	0,44±0.04	0,23±0,03
2,25±0,20	0,44±0,03	5,57±0,36	0,28±0.04	0,15±0,01
1,88±0,21	0,59±0,04	2,90±0,48	0,23±0,02	0,14±0,02
1,64±0,20	0,85±0,06	2,18±0,34	0,19±0,03	0,15±0,02

Values are the mean of five independent experiments and presented as Means±SD

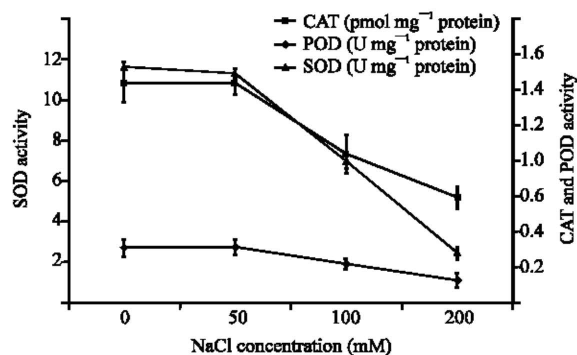

Fig. 5: Effect of different salt concentrations on nodules antioxident enzyme activities. Values are the mean of five independent experiments and presented as Means±SD

showed that K^+ content reduced 19, 31 and 40% when treated by 50, 100 and 200 mM NaCl, respectively (Table 1).

The Na^+ content of seedling significantly increased with increasing salinity level (Table 1). The trend of accumulation Na^+ was different from that of K^+ and increased 2, 3 and 5 times more than the control when treated by 50, 100 and 200 mM NaCl, respectively.

The ratio of K^+/Na^+ was influenced significantly by salinity level. Increase of salinity level resulted in decreasing K^+/Na^+ ratios. The K^+/Na^+ ratio of seedlings at 50, 100 and 200 mM NaCl were 33, 21 and 13% of that of control.

The contents of Ca^{2+} and Mg^{2+} decreased significantly as salinity treatment concentrations increased (Table 1). Ca^{2+} decreased by 36, 46 and 57% when treated with 50, 100 and 200 mM NaCl, respectively. The Mg^{2+} content showed a similar response with increasing salinity. Mg^{2+} contents of seedlings treated with 50, 100 and 200 mM NaCl were 36, 38 and 33% of the control.

Oxidative Stress Generation

In order to evaluate the oxidative stress generated by saline conditions, antioxidative enzymes were determined in soybean plants subjected to 0, 50, 100 or 200 mM NaCl. Taking into account the fact that oxidative stress could be produced by a decrease in antioxidant defences, the activities of the main antioxidant enzymes, such as SOD, CAT and POD were analysed. The activities of SOD, CAT and POD were not significantly decrease with 50 mM NaCl (Fig. 5). Higher level of salinity, however, resulted in decrease of SOD, CAT and POD activities. As shown in Fig. 5, SOD, CAT and POD activities decreased with 100 and 200 mM NaCl to 60 and 21, 67 and 47 and 71 and 40% of control, respectively.

DISCUSSION

Growth significantly decreased in the salt-treated seedlings (Fig. 1). The inhibitory effect on seedling growth was more effective when treated by 200 mM NaCl. Kao *et al.* (2006)

has previously reported similar results on reduction of soybean biomass due to salt stress soybean is, however, reported to be a relatively salt-sensitive crop (Katerji *et al*., 2000; Kao *et al*., 2006). Findings reported by Sobhanian *et al*. (2010) support our results.

Present results show the reduction of nitrogenase activity due to increasing salinity. Reports have shown that an enhancement of heme oxygenase activity contributes to counteract oxidative stress generation (Noriega *et al*., 2004; Balestrasse *et al*., 2005; Yannarelli *et al*., 2006). This antioxidant response may lead to nitrogenase activity diminution (Fig. 2).

Present study shows that ammonium content is increased with salinity especially with high amount of NaCl (200 mM, Fig. 3). Enhancement of ammonium content found at 200 mM salt treatment can be explained by significant reduction in GOGAT and GDH activity (Zilli *et al*., 2008). These data also demonstrated that enzyme responses (induction or repression) often differ among species, cultivars and analysed tissues (Popova *et al*., 2002; Gu *et al*., 2004).

Plants usually accumulate some compatible solutes with low molecular mass such as proline (Ashraf and Harris, 2004; Tripathi *et al*., 2007). It has been shown that accumulation of proline is a common response to a wide range of biotic and abiotic stresses such as salt (Aghaei *et al*., 2009). The results of present research showed that increasing NaCl concentrations lead to increase of proline content of soybean (Fig. 3). These results confirms the previously observation of Su and Bai (2008).

It was found that DAO activity increased with increasing NaCl concentrations (Fig. 4). Its tought that DAO activity is related to endogenous polyamine contents. In plant exposed to salt stress, this response was also found in other species, such as tomato (Aziz *et al*., 1998), *Fraxinus angustifolia* callus (Tonon *et al*., 2004).

The K^+ content is the main cation in plant and is an important component of the cell osmotic potential (Reggiani *et al*., 1995). In present study K^+ concentrations were lower at higher salinity levels. The results are supported by reports of Essa (2002) and Sobhanian *et al*. (2010). Accumulation of inorganic ions for osmotic adjustment is an energy-effective way for higher plants to combine productivity with salt tolerance (Yeo and Flowers, 1982).

Salt-tolerant species maintain high concentrations of Ca^{2+} and K^+ and low concentrations of Na^+ and Cl^-. Sodium is not an essential element for plants and plants accumulate Na^+ at the expense of Ca^{2+} and K^+ in saline conditions. The growth could be inhibited by reduction in K^+ concentration which reducing the capacity for osmotic adjustment and turgor maintenance or adversely affecting metabolic functions (Helal and Mengel, 1979; Greenway and Munns, 1980). The reduced contribution of K^+ ions could probably be offset by an enhanced accumulation of Na^+ ions (Table 1). It has been reported that the main response of plant to salt stress is a change in Ca^{2+} homeostasis (Rengal, 1992).

Salt tolerance of plants is attributed to their ability to avoid Na^+ toxicity and to maintain Ca^{2+} and K^+ concentrations. Turgor may be maintained by help of Na^+. Na^+, however, is unable to substitute for specific functions of Ca^{2+} and K^+, e.g., enzyme activation and protein synthesis to produce adequate growth. Increasing Na^+ contents and decreasing K^+ contents and K^+/Na^+ ratios in plant leaves can be attributed to the effect of competition between Na^+ and K^+ ions on the absorptive sites of the plant roots (Bhivare and Nimbalkar, 1984).

Oxidative stress may be defined as an increment of oxidant species or a depletion of antioxidant defences. Present results have shown that, under 50 mM NaCl, antioxidant enzyme activities remained unchanged respect to controls, indicating that t his salt concentration did not produce oxidative damage (Fig. 5). A significant decreasein the classical antioxidant enzymes (SOD, CAT and POD) activities were observed when plants were treated with 100 and 200 mM NaCl (Fig. 5).

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