The Journal of Animal & Plant Sciences, 23(1): 2013, Page: 203-209
ISSN: 1018-7081

CHANGES IN ACTIVITIES OF ANTIOXIDANT ENZYMES IN RESPONSE TO NaCl STRESS IN CALLUS CULTURES AND REGENERATED PLANTS OF SUGARCANE

N. Munir and F. Aftab*

Department of Biotechnology and Microbiology, Lahore College for Women University, Lahore
*Department of Botany, University of the Punjab, Q. A. Campus Lahore-54590, Pakistan
Corresponding Author e-mail: neelma.munir@yahoo.com

ABSTRACT

Plants have a number of biochemical mechanisms to cope with salt stress. Peroxidases, Catalases and Superoxide dismutases are important antioxidant enzymes in the metabolism of reactive oxygen species (ROS) produced under salt stress. To analyze the possible role of these enzymes in developing resistance to salt stress, 60-day-old callus cultures of sugarcane (cvs. SPF 234 and HSF 240) were subjected to various NaCl levels (0-160 mM; 9 treatments). The activities of antioxidant enzymes (Peroxidase, Catalase and Superoxide dismutase) were later recorded at day 30 after ascorbic acid pretreatment. It was observed that ascorbic acid pretreatment to callus cultures as well as *in vitro* grown plants has a positive effect on different growth parameters tested during the study. Antioxidant enzyme activities underwent an increasing trend in response to increasing concentration of NaCl. Healthy sugarcane plants were also successfully regenerated from 120-days-old NaCl-treated callus cultures treated with various NaCl levels. It was observed that the plants regenerated from salt-treated callus cultures generally had elevated levels of antioxidant enzymes as compared to the control. Hence, it is concluded that high antioxidant enzyme activities in sugarcane cultivars induced salt stress in them which may have a better protection against reactive oxygen species. This hypothesis however needs further testing both under *in vitro* as well as greenhouse conditions.

Key words: Antioxidant enzymes, Callus culture, NaCl stress, Sugarcane.

INTRODUCTION

Soil salinity is an important constraint that affects plant agriculture worldwide (Mahajan and Tutea, 2005). The capability of crops to grow on saline soils varies among species and depends on the concentration of salts present in the root zone as well as on various environmental and cultural conditions (Maas, 1990). It has been reported that many plant species are adversely affected by salinity level greater than 1.38 milli-ohms (Alam *et al.,* 2000).

Sugarcane is considered as moderately sensitive to salinity, while exhibited reduction in biomass and juice quality with an increasing in salinity (Lingle and Weigand, 1996). Salinity affects the plant growth due to osmotic stress, specific ion (Na^+) toxicity, nutritional imbalance, hormonal imbalance or production of reactive oxygen species (Bartels and Sunkar, 2005). Reactive oxygen species (ROS) are molecules like hydrogen peroxide (H_2O_2), ions like the hypochlorite ion, radicals like the hydroxyl radical (OH°) and the superoxide anion (O_2^{-}°). . Reactive oxygen species participate in multiple processes in plants and can damage other molecules and the cell structures of which they are a part (Mittler, 2002).

The precise mechanisms underlying the effects of salt stress on plants are not fully understood because plant resistance to salt stress is a multigenic trait (Parida and Das, 2005). Plant cells have evolved several biochemical and physiological pathways against salt stress. So far, an important strategy of the plants to overcome oxidative damage is to produce antioxidant enzymes including peroxidase, catalase and superoxide dismutase (Bartels and Sunkar, 2005). Peroxidase enzyme decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants (Gaspar *et al*., 1991). Catalase enzyme plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells (Abassi *et al*., 1998). Superoxide dismutase (SOD enzyme converts superoxide ion produced under stressed conditions to hydrogen peroxide and molecular oxygen thus playing important role in defense mechanisms (Harinasut *et al*., 2003).

Tissue culture techniques have been applied to plant species not only to select tolerant clones from non-tolerant plants but also to study the physiological and biochemical mechanisms that operate at the cellular level in response to stress conditions (Lerner, 1985). Changes in activities of various antioxidant enzymes under salinity stress have been reported but none of these focuses on sugarcane callus cultures. The present study therefore was conducted to investigate the role of three antioxidant enzymes i.e., Catalase, Peroxidase and Superoxide dismutase in biochemical adaptive mechanism against salt tolerance in sugarcane under *in vitro* conditions.

MATERIALS AND METHODS

Establishment of callus cultures: Callus cultures were established from the explants ranging from 5-8 mm in diameter (2-3 mm thick) derived from young inner 2-3 whorls of leaves of two sugarcane (*Saccharum* spp. Hybrid) cultivars (SPF 234 and HSF 240) on MS (Murashige and Skoog, 1962) medium supplemented with 13.5 μM 2,4-D. For callus induction cultures were placed at 27 ± 2ºC under dark conditions. All the cultures after callus formation were maintained at 27 ± 2ºC under 16-h photoperiod (35 μmol m^{-2} s^{-1}).

Salt treatments to callus cultures: To observe the effect of salt stress on sugarcane callus cultures, 60-days-old callus cultures were transferred to MS medium supplemented with 9 different NaCl levels (0-160 mM). The amount of soluble proteins, peroxidase, catalase and superoxide dismutase activities were calculated by using UV-visible range Spectrophotometer (HITACHI U1100) before every subculture at day 90, 120 and 150.

Plant regeneration from salt treated callus cultures: Callus cultures after treating with various NaCl concentrations were transferred to already standardized regeneration medium (MS + 8.87 mM BAP + 0.5 μM TDZ) at day 120. At day 30 from the initial culture on regeneration medium, the regenerated shoots were shifted to the MS basal medium for 30 days for further development of shoots. These regenerated shoots were then analyzed for soluble protein contents, peroxidase, catalase and superoxide dismutase activities.

Biochemical Analysis for antioxidant enzyme activities: To analyze the activities of antioxidant enzymes, extraction of the tissue was done with 0.1 M phosphate buffer (pH 7.2) in 1:2 ratio. Peroxidase (E.C 1.11.1.7) activity was measured with the help of a method proposed by Racusen and Foote (1965). To estimate the activity of catalase (E.C 1.11.1.6), method of Beers and Sizer (1952) was employed with some modifications. The total SOD (E.C 1.11.1.6) activity was estimated according to Maral *et al.* (1977) The details of above mentioned methods and modifications were the same as reported earlier (Munir and Aftab, 2009).

Statistical analysis: Statistical analysis was performed using Univariate analysis of variance (SPSS Version 11). To apply the F test, data were transformed where required. P values ≤0.05 were considered as significant.

RESULTS

Peroxidase activities (mg/g tissue) of the callus cultures of both the cultivars (SPF 234 and HSF 240) maintained on nine different salt stress treatments are shown in Fig. 1 and 2. There was an overall enhancement in peroxidase activity with increasing concentration of NaCl up till 120 mM, enzyme activity decreased at 140 mM NaCl and approached zero at 160 mM level. For Cv. SPF 234 peroxidase activity of the callus cultures on the control medium (MS containing 0 mM NaCl) was 0.017 mg/g tissue at 120 day, whereas general increasing trend in enzyme activity was recorded with increase NaCl concentration up till 80 mM NaCl and afterwards a decrease was observed. At day 150 an increase in peroxidase activity was recorded upto 60 m Na Cl level after which a decrease in peroxidase activity was recorded.

It was also interesting to note that in both the cultivars, the respective value of peroxidase activity at 0 or 20 mM NaCl were same at day 90. However, peroxidase activity increased to 0.054 mg/g tissue at 100 mM NaCl level after which its value remained unchanged at 120 mM NaCl level and approached zero at higher concentrations. At day 150, all the NaCl-treated callus cultures had less values of peroxidase activity as compared to the peroxidase activity in control callus cultures at 0 mM salt concentration (0.022 mg/g tissue).

Catalase Activity (units/ml enzyme): Values of catalase activity of sugarcane cultivar SPF 234 have generally shown an increasing trend from 5.75 units/ml enzyme at 0 mM NaCl level to 20.98 units/ml enzyme at 140 mM NaCl concentration (Fig. 3). Almost the same general increasing trend was observed in callus cultures at day 120. However, deviation from this general trend in this cultivar was observed at 80 mM NaCl level where the value of catalase activity was slightly lower (9.31 units/ml enzyme) as compared to catalase activity at 60 mM NaCl (11.37 units/ml enzyme). At day 150, the values of catalase activity were generally less as compared to the catalase activities both at day 90 and 120.

For cv. HSF 240, an increasing trend in the catalase activity at day 90 up to 100 mM NaCl level remained almost the same at the next tested NaCl level, i.e., 18.03 units/ml enzyme before approaching zero at higher concentrations (Fig. 4). It was also observed that callus cultures of cv. HSF 240 at 0 mM had almost similar catalase activities at day 90 and 120 (5.46 and 5.86 units/ml enzyme, respectively). At day 120, a statistically significant effect of different salt concentrations on catalase activity was recorded for callus cultures of cv. HSF 240. Fig 4 also indicates that the maximum value of catalase activity (11.14 units/ml enzyme) at day 120 was observed at 60 mM salt level. At day 150, no significant effect of different salt concentrations on catalase activities of cv. HSF 240 was observed.

Superoxide Dismutase Activity (units/mg protein): It is evident from Fig. 5 that the values of superoxide dismutase activities of both the cultivars were

significantly influenced by the addition of salt in MS medium. The SOD activities of the salt-treated callus cultures at day 90 or 120 were generally greater as compared to control callus cultures. At day 90, there was a general tendency towards an increase in superoxide dismutase activity that reached its peak at 120 mM NaCl level (58.02 units/mg protein). Then a decrease (42.69 units/mg protein) in SOD activity was observed at 140 mM NaCl level. Superoxide dismutase contents approached zero at still higher NaCl level, i.e., 160 mM NaCl treatment. With some minor variations, the SOD activity generally increased in cv. SPF 234 under increasing salt levels at day 120. However, at day 150, SOD activities in NaCl-treated callus cultures were statistically non-significant.

The SOD activity of the callus cultures of cv. HSF 240 maintained on the medium without salt (0 mM salt level) was found to be less (2.63 units/mg protein) as compared to control callus cultures of cv. SPF 234 (Fig. 6). The maximum value (25.45 units/mg protein) was observed at 120 mM NaCl, which was again less than superoxide dismutase activity of callus cultures of cv. SPF 234 at the same salt levels. At day 120, superoxide dismutase activity at all the salt levels was statistically different as compared to the control. It is also evident from figure that generally an increase was recorded in SOD activities at day 120 with increasing salt level with the maximum value at 120 mM NaCl level (17.45 units/mg protein). At day 150, once again a significant effect of various NaCl levels was recorded on the values of SOD activities.

Peroxidase, Catalase and Superoxide Dismutase Activities of the Regenerated Plants of Sugarcane: For cv. SPF 234, regeneration of the callus cultures was recorded up till 120 mM NaCl whereas for cv. HSF 240, the maximum level of salt treatment still allowing some regeneration was 100 mM (data not shown). Peroxidase activities of regenerated plants of SPF 234 are given in Fig. 7. Plants regenerated from callus cultures of cv. SPF 234 maintained on medium supplemented with 0 or 20 mM NaCl had the same peroxidase activity (0.016 mg/g tissue). Plants regenerated from callus cultures treated with 40 mM NaCl showed a slight increase (though non-significant) in peroxidase activity to 0.017 mg/g tissue. The maximum value for peroxidase activity (0.04 mg/g tissue) was observed in the plants regenerated from 80 mM NaCl-treated callus cultures. For HSF 240, the peroxidase activities of plants regenerated from callus cultures were found to be significantly affected with various salt concentrations.

For cv. SPF 234, the control plants had catalase activity of 2.16 units /ml enzyme (Fig. 8). The maximum value of catalase activity for this cultivar (4.38 units /ml enzyme), like peroxidase, was also observed in plants regenerated from calluses treated with 80 mM NaCl. The overall effect of various NaCl levels on catalase activity of regenerated plants (derived from 0-160 mM NaCl-treated callus cultures), however was non-significant. The plants regenerated from callus cultures of cv. HSF 240 treated with different NaCl concentrations, however, did not show significant difference in catalase (Fig 8).

It is evident from Fig 9 that the values of superoxide dismutase activity of the plants regenerated from 0 mM was 3.19 units/mg protein which increased to 7.44 units/mg protein at 40mM NaCl level and afterwards remained almost the same at 60 mM NaCl (7.38 units/mg protein). The maximum value of superoxide dismutase activity (8.74 units/mg protein), was however, observed in the plants regenerated from callus cultures treated with 120 mM NaCl. However, Fig 9 indicates that like catalase activities no significant difference in superoxide dismutase activities was recorded for HSF 240.

Fig 1: Peroxidase activity of sugarcane callus cultures (SPF 234) treated with different NaCl concentrations (0-160 mM) at day 90, 120 and 150

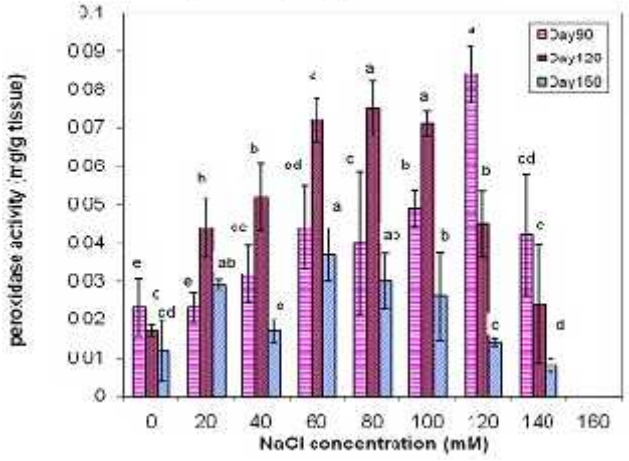


Fig 2 Peroxidase activity of sugarcane callus cultures (HSF 240) treated with different NaCl concentrations (0-160 mM) at day 90, 120 and 150

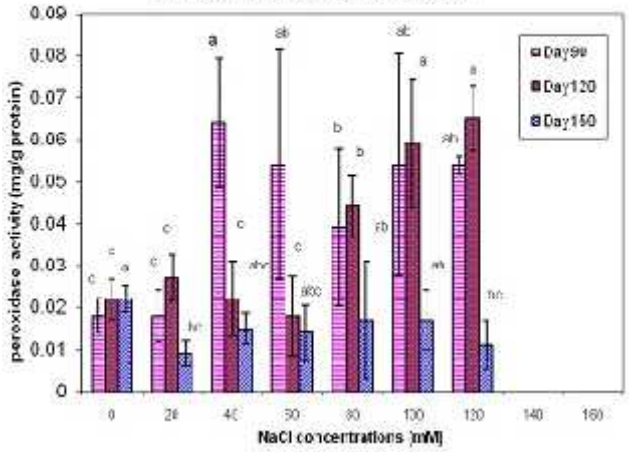


Fig 3: Catalase activity of sugarcane callus cultures (SPF 234) treated with different NaCl concentrations (0-160 mM) at day 90, 120 and 150

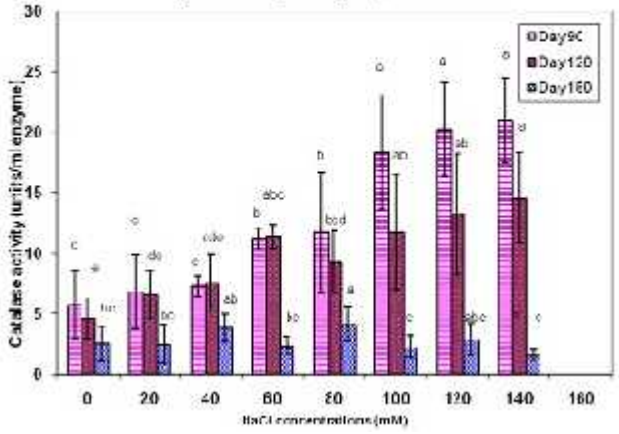


Fig 4. Catalase activity of sugarcane callus cultures (HSF 240) treated with different NaCl concentrations (0-160 mM) at day 90, 120 and 150

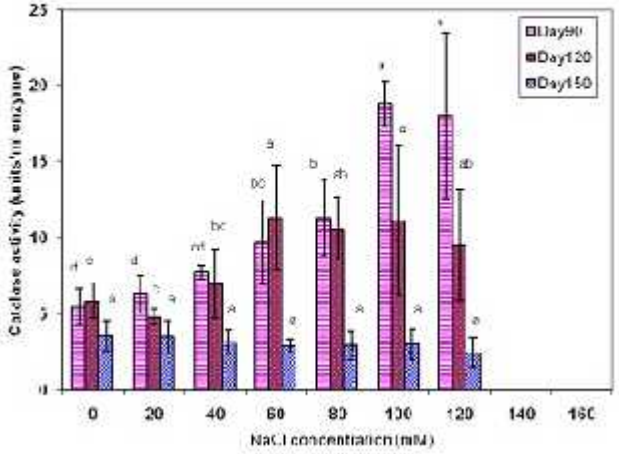


Fig 5: Superoxide dismutase activity of sugarcane callus cultures(SPF 234) treated with different NaCl concentrations (0-160 mM) at day 90, 120 and 150

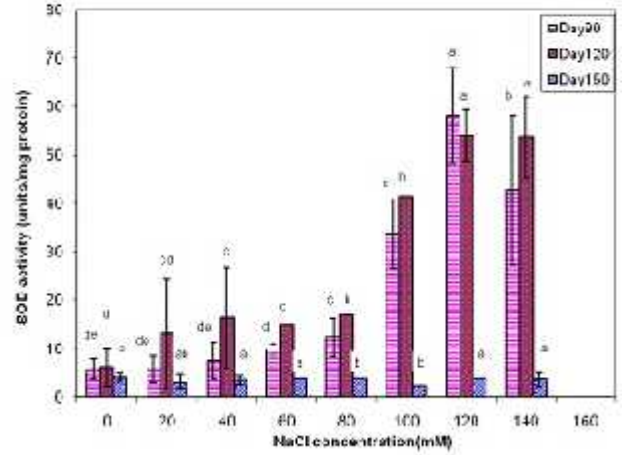


Fig 6: Superoxide dismutase activity of sugarcane callus cultures (HSF 240) treated with different NaCl concentrations (0-160 mM) at day 90, 120 and 150

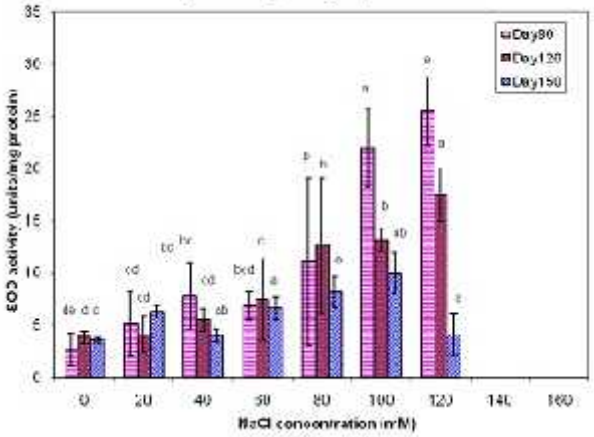


Fig 7: Peroxidase activities of regenerated plants from 0-160mM NaCl-treated callus cultures of sugarcane (cvs. SPF 234 and HSF 240)

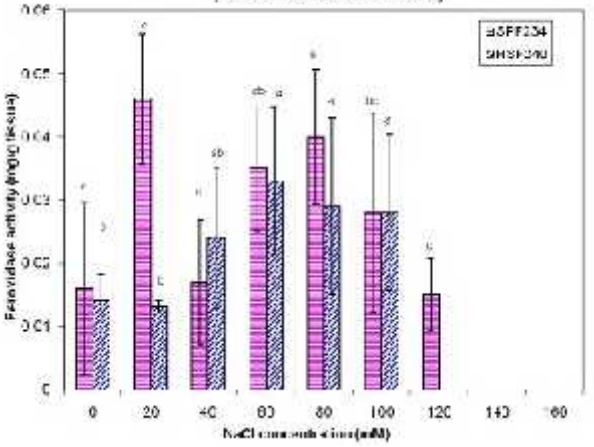
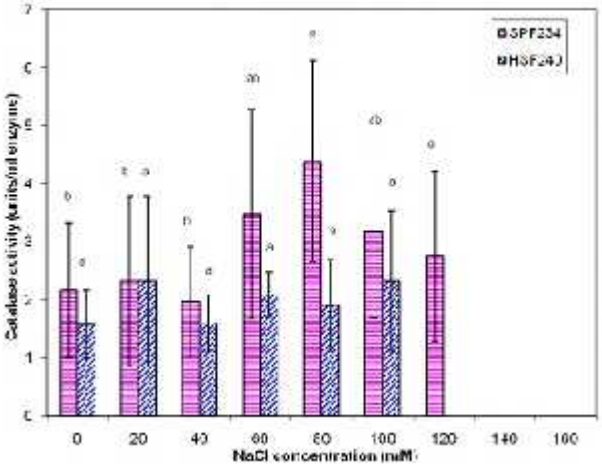
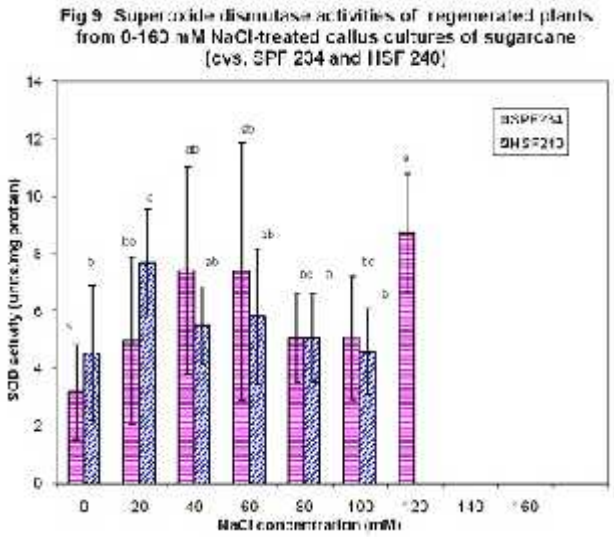


Fig 8: Catalase activities of regenerated plants from 0-160 mM NaCl-treated callus cultures of sugarcane (cvs. SPF 234 and HSF 240)





DISCUSSION

Results of the present study indicated that when salt was supplied to the growth media, generally there was a significant increase in peroxidase activity of the callus cultures as compared to the control treatments. Thus the peroxidase activity increased up to 3-3.6 times in both sugarcane cultivars (SPF 234 and HSF 240) at day 90 in 120 mM NaCl-treated, 4 times in cv. SPF 234 at day 120 in 80 mM NaCl level and relatively less (3 times) in cv. HSF 240 at 100 mM NaCl level. Interestingly, a similar increasing pattern with a maximum of 2.5 or 3.0 times increase of peroxidase activity at 50 or 100 mM NaCl concentrations, respectively was observed in chickpea by Sheokand *et al.* (1995). The increased total peroxidase activities in response to salinity have also been reported in other plants (Lee *et al.,* 2001, Sairam *et al.*, 2005). It is a well documented fact that in plants there exists a relationship between total peroxidase activity and changes in cell wall as well as oxidative stress under salt stress (Chen *et al.*, 1993). This high activity of peroxidase may, therefore, be correlated with the capability of the cells to quash oxygen-free radicals, which can damage the cell compartment.

Catalase is quite important for the maintenance of normal cellular processes because it plays an important role in the removal of electrons that can lead to the production of O^{-2} free radical (Abassi *et al*., 1998). The present investigation indicated that catalase activity of both the sugarcane cultivars showed an increasing trend with a correspondingly increasing salt level in the medium. Our findings are contradicted to previous works where decreased catalase activity was noticed in response to salinity (Neto *et al*.., 2006). The results of our experiments are thus consistent with many previous observations indicating an enhanced catalase activity under different environmental stresses including salt (Gossett *et al*., 1994), heat (El-Shintinawy *et al*., 2004) or cold (Scandalios *et al*., 1984). Therefore, greater catalase activities in salt-treated callus cultures of sugarcane as compared to the control can be correlated to the ability of this enzyme to detoxify the damaging effects of hydrogen peroxide produced under stressed conditions (Willekens *et al.*, 1997).

Superoxide dismutase enzyme also plays a key role in the antioxidative defense mechanism. SOD enzyme converts superoxide to hydrogen peroxide and molecular oxygen. Therefore, SOD activity has been reported to be negatively correlated to the concentration of O_2^- and H_2O_2. An increase in superoxide dismutase activity in response to salt stress was observed during the current study. So our results support previous reports indicating increased SOD activity in plants exposed to different environmental stresses, including salinity (Lee *et al*., 2001; Harinasut *et al.*, 2003). It has been demonstrated that salt-tolerant varieties exhibit higher constitutive and induced levels of SOD as compared to their salt-sensitive counterparts (Bor *et al*., 2003). On the basis of our results, we may also suggest that superoxide dismutases are important enzymes within the antioxidant defense system of sugarcane to detoxify ROS and confer salt tolerance.

It was interesting to note during the present study that the plants regenerated from salt-treated callus cultures had generally more antioxidant enzyme activities as compared to the control plants (regenerated from non-treated callus cultures). Thus it can be suggested that NaCl treatment at the callus level probably triggered the synthesis of antioxidant enzymes which was retained in the regenerated plants from these callus cultures. Although there is no prior study regarding the antioxidant enzymes in sugarcane plants regenerated from NaCl-treated calluses but it is well documented that plants with high levels of antioxidant enzymes have more salinity tolerance because of resistance to oxidative damage (Sehmer *et al.*, 1995). Therefore, it can be suggested that higher antioxidant enzyme activities of the regenerated plants from NaCl-treated callus cultures could result in the increased ability of these plants to withstand NaCl stress.

As mentioned before, there might be the production of reactive oxygen species in sugarcane callus cultures such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) in response to NaCl stress as confirmed by previous study on other plants (Bartels and Sunkar, 2005). In order to quench these ROS, elevation was observed in the activities of antioxidant enzymes peroxidase, catalase and superoxide dismutase in NaCl-treated sugarcane callus cultures as compared to the control. In conclusion, this investigation has shown that salt tolerance is correlated with higher levels of antioxidant enzyme activities and these enzymes can act

as a defense team in protecting the cells from oxidative damage imposed by salt stress. Hence, soluble protein, peroxidase, catalase and superoxide dismutase contents of callus cultures seem quite useful as biochemical parameters of salt tolerance in plants including sugarcane. This study has also shown that healthy and vigorously growing sugarcane were successfully regenerated from calluses maintained under various NaCl levels. Because such higher NaCl levels generally prove lethal for most sugarcane tissues maintained under *in vitro* conditions, the successful regeneration of sugarcane plants from callus cultures maintained at higher NaCl levels (120 mM in cv. SPF 234 and 100 mM in cv. HSF 240) seems quite a significant step forward. Though not tested till the compilation of present work, it is optimistically assumed that regenerated salt-tolerant plants retain salt-tolerance characteristics under greenhouse or field conditions. Further studies on salt tolerance characteristics of regenerated plants from this study under either *in vitro* or greenhouse/field conditions are highly recommended.

Acknowledgement: We thank Higher Education Commission, Islamabad, Pakistan, for the financial support under 'Merit Scholarship Scheme for PhD studies in Science and Technology' to Neelma Munir.

REFERENCES

Abassi, N. A., M. M. Kushad and A. G. Endress (1998). Active oxygen-scavenging enzymes activities in developing apple flowers and fruits. Sci. Hort. 74: 183-194.

Alam, S. M., R. Ansari and M. A. Khan (2000). Saline agriculture and Pakistan. www. pakistaneconomist.com/issue2000/issue19 and 20/iande3.htm

Bartels, D. and R. Sunkar (2005). Drought and salt tolerance in plants. Crit. Rev. Plant Sci. 24: 23-58.

Beers, R. and I. Sizer (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxidase by catalase. J. Biol Chem. 195:133-140.

Bor, M., F. Ozdemir and I. Turkan (2003). The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet (*Beta vulgaris* L.) and wild beet (*Beta maritima* L.). Plant Sci. 16: 77-84.

Chen, Z, H. Silva and D. F. Klessig (1993). Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Sci 262: 1883-1886

El-Shintinawy, F, M. K. H. Ebrahim, N. Sewelam and M. N El-Shourbagy (2004). Activity of photosystem 2, lipid peroxidation, and the enzymatic antioxidant protective system in heat shocked barley seedlings. Photosynthetica 42: 15-21.

Gaspar, T., C. Penel, D. Hagega and H. Greppin (1991). Peroxidases in plant growth, differentiation and development processes. In: *Biochemical, molecular and physiological aspects of plant peroxidases* (eds J. Lobarzewsky, H. Greppin, C. Penel and Th. Gaspar), pp. 249-280. University of Geneve, Switzerland.

Gossett, D. G., E. P. Millhollon and M. C. Lucas (1994). Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivars of cotton. Crop Sci. 34: 706-714.

Harinasut, P., D. Poonsopa, K. Roengmongkol and R. Charoensataporn (2003). Salinity effects on antioxidant enzymes in mulberry cultivar. Sci. Asia 29: 109-113.

Lee, D. H, T. S. Kim and C. B. Lee (2001). The inductive responses of the antioxidant enzymes by salt stress in the rice *(Oryza sativa* L.). J. Plant Physiol. 158: 737-745.

Lerner, H. R. (1985). Adaptation to salinity at the plant cell level. Plant Soil. 89:3-14.

Lingle, S. and C. L. Weigand (1996). Growth and yield responses of sugarcane: II. Sucrose biochemistry in individual internodes. Proc. International American Sugarcane Seminars. pp. 93-102.

Mahajan, S. and N. Tutea (2005). Cold, salinity and drought stresses: An overview. Arch. Biochem. Biophys. 444: 139-158.

Maral, J. K. Puget, and A. M. Micheson (1977). Comparative study of superoxide dismutase, catalase, and glutathione peroxidase levels in erythrocytes of different animals. Biochem. Biophys. Res. Comm. 77: 1525-1535.

Munir, N. and F. Aftab (2009). Role of Polyethylene Glycol (PEG) pre-treatment in improving salt (NaCl) tolerance of sugarcane. Turk J. Bot. 33:407-415

Murashige, T. and F. Skoog (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Neto, A. D. A., J. T. Prisco, J. Eneas-Filho, C. E. B Abreu and E. G Filho (2006). Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. Environ. Exp. Bot. 56: 87-94.

Parida, A. K. and A. B. Das (2005). Salt tolerance and salinity effects on plants: a review. Ecotoxicol. Environ. Saf. 6: 324-349.

Racusen, D. and M. Foote (1965). Protein synthesis in dark grown bean leaves. Can. J. Bot. 43: 817-824.

Sairam, R. K., G. C. Srivastava, S. Agarwal and R. C. Meena (2005). Differences in antioxidant

activity in response to salinity stress in tolerant and susceptible wheat genotypes. Biol. Plant. 49: 85-91.

Scandalios, J. G., L. Guan and A. N. Polidoros (1997). Catalases in plants: gene structure, properties, regulation, and expression. In: *Oxidative Stress and the Molecular Biology of Antioxidant Defenses* (ed. J.G. Scandalios), pp. 343-406. Cold Spring Harbor Laboratory Press, New York.

Sehmer, L., B. Alaoui-Sosse, and P. Dizengremel (1995). Effect of salt stress on growth and on the detoxifying pathway of pedunculate oak seedlings (*Quercus robur* L.). J. Plant Physiol. 147: 144-151.

Sheokand, S, S. Dhandi and K. Swaraj (1995). Studies on nodule functioning and hydrogen peroxide scavenging enzymes under salt stress in chickpea nodule. Plant Physiol. Biochem. 33: 561-566.

Willekens, H., S. Chamnongpol, M. Davey, M. Schraudner, C. Langebartels, M. C. Montagu, D. Inze and W. V. Camp (1997). Catalase is a sink for H_2O_2 and is indispensable for stress defense in C3 plants. Europ. Mol. Biol. Org. J. 16: 4806-4816.