

Effect of Salinity Stress on Antioxidant Defense System of Two Finger Millet Cultivars (*Eleusine coracana* (L.) Gaertn) Differing in Their Sensitivity

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Abstract: Soil salinity is one of the major abiotic stresses limiting plant growth and productivity across the globe. In our work we studied the physiological and biochemical alterations in two finger millet cultivars (PBR 2700 and Saphthagiri) differing in their sensitivity to salinity stress. Seedlings were grown in plastic pots supplemented with ¼ strength Hoagland nutrient solution containing NaCl (100mM and 200mM) for various time periods (0,5,10,15 and 20 days). Under salinity stress, with increase in concentration and time period, the activities of antioxidative enzymes increased significantly when compared to their respective control plants. SOD activity increased 0.2 -1.5 fold, CAT activity increased 0.2-0.7 fold, APX activity increased 0.5-1.8 fold and the activity of GR increased 0.5-1.5fold. Higher Malondialdehyde (MDA) content (0.2-1.4 fold) indicating occurrence of greater lipid peroxidation was observed in seedlings grown under salinity stress than their respective controls. Enhanced Proline accumulation (1-3.5 fold) was noted in plants grown under NaCl stress when compared to control plants. When compared to Saphthagiri, plants of PBR 2700 showed more elevated activities of antioxidant enzymes and higher proline accumulation but lesser MDA levels under salinity stress. The above results clearly revealed that when compared to Saphthagiri, PBR 2700 was found to be relatively resistant to NaCl stress.

Key words: Salinity stress • Finger millet • Proline content • Antioxidative enzymes • Lipid peroxidation

INTRODUCTION

Salinity is one of the major abiotic stresses affecting plant growth, development and productivity across the world. High concentrations of salts in soils account for large decreases in the yield of a wide variety of crops. The problem is huge; increased salinization of arable land is expected to have devastating global effects, resulting in 30% land loss within next 25 years and up to 50% by the middle of 21st century [1]. This constraint effects are through osmotic inhibition, ionic toxicity and disturbance in the uptake and translocation of nutritional ions leading to alterations in physiological and biochemical functions of the plant cells [2, 3]. It is well known that proline is a major organic molecule which accumulates in many plants when exposed to environmental stresses such as drought and salinity [4,5]. Proline accumulation may be a part of the stress signal influencing adaptive responses [6].

When plants are subjected to salinity stress, reactive oxygen species (ROS) (superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH) are generated in response to stress condition. ROS can cause

oxidative damage to many cellular components, including membrane lipids, proteins, nucleic acids and chlorophyll [7]. The possible ROS involvement in the mechanism of damage by salt stress was already confirmed on cotton [8], egg plant genotypes and melon [7].

However, this homeostasis could be disturbed by a number of adverse environmental factors. Plants protect themselves from oxidative damage due to ROS through both enzymatic and nonenzymatic defense mechanisms [9]. Enzymatic ROS-scavenging mechanisms in plants include production of superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), catalase (CAT) and glutathione reductase (GR). The extent of oxidative stress experienced in a cell is determined by the levels of superoxide, H_2O_2 and hydroxyl radicals generated. Additionally, a balance among SOD, APX and CAT activities is crucial for suppressing toxic ROS levels within cells [10, 11]. GR activity regulates the redox potential of cells and is important for the physiological needs of cells under oxidative stress. The role of GR is to protect the cell against oxidative stress effects by maintaining a high reduced glutathione-

to-oxidized glutathione (GSH/GSSG) ratio [12,13]. Previous reports on antioxidative response of plants in response to salt stress indicated increased activities of antioxidative enzymes and lipid peroxidation [14-16].

MATERIALS AND METHODS

Plant Material and Plant Growth Conditions: Seeds of twenty varieties of finger millet were obtained from Regional Agricultural Research Station, Tirupati and ICRISAT, Hyderabad andhra Pradesh, India. After preliminary screening, two cultivars, viz, PBR 2700 (salt resistance) and Saphagiri (salt sensitive) were selected for further studies. Seeds were surface-sterilized and germinated in petriplates containing double layered autoclaved filter paper with distilled water at $27\pm 2^\circ\text{C}$ with 14-h photoperiod. 7-day old seedlings with uniform length were transferred to plastic pots and supplemented with $\frac{1}{4}$ strength Hoagland nutrient solution. Now, the test plants were exposed to 100mM or 200mM NaCl in nutrient solution, while the control plants were continued to grow on nutrient solution alone in green house. Then, to carryout further experiments, plants were harvested on 0, 5, 10, 15 and 20 days after NaCl treatment as designed.

Antioxidant Enzyme Assays: Fresh leaves (0.5 g) were homogenized with a mortar and pestle under chilled conditions with phosphate buffer (0.1 M, pH 7.5) and ethylene diamine tetra acetic acid (EDTA, 0.5mM). The homogenate was filtered through muslin cloth and centrifuged at 12,000 g for 10 min at 4°C . The resulting supernatant was used for the assay of different enzymes.

Superoxide Dismutase (SOD) (EC 1.15.1.1): SOD activity was determined as described by Bayer and Fridovich [17] by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT). The assay mixture contained 1 ml of enzyme extract, 0.1 mM phosphate buffer (pH 7.5), 3 mM NBT and 60 mM riboflavin. The tubes were thoroughly shaken and placed under 15W fluorescent lamp for 10 min, then the lights were switched off and the tubes were covered with a black cloth. For the purpose of blank, the non-illuminated reaction mixture was used. Absorbance of the reaction mixture was read at 560 nm and one unit of SOD activity (EU) was defined as the amount of enzyme required to cause 50% inhibition of the NBT photoreduction rate. The results were expressed as units mg^{-1} F.W.

Catalase (CAT) (EC 1.11.1.6) Activity: CAT activity was measured following the method of Aebi [18]. The assay mixture contained 0.1 ml of enzyme extract, 0.1 mM phosphate buffer (pH 7.5), 0.1 M EDTA and 0.3% H_2O_2 and the absorbance was measured at 240 nm. CAT activity was expressed as $\mu\text{mol H}_2\text{O}_2 \text{ g}^{-1}$ F.W. For the calculation of CAT activity, the extinction coefficient of $0.036 \text{ mM}^{-1}\text{cm}^{-1}$ was used.

Ascorbate Peroxidase (APX) (EC 1.11.1.1) Activity: APX activity was determined according to the method of Nakano and Asada [19] followed by Urbanek *et al.* [20]. The reaction mixture (1.5ml) contained 50mM phosphate buffer (pH 6.0), $0.1\mu\text{M}$ EDTA, 0.5mM ascorbate, 1.0mM H_2O_2 and 50 μL enzyme extract. The reaction was started by the addition of H_2O_2 and ascorbate oxidation was measured at 290nm for 1 min. Enzyme activity was quantified using the molar extension coefficient for ascorbate (2.8mM^{-1}) and the results were expressed in $\mu\text{mol H}_2\text{O}_2 \text{ g}^{-1}$ F.W, taking in to consideration that 2mol ascorbate was required for reduction of 1mol H_2O_2 .

Glutathione Reductase (GR) (EC 1.6.4.2) Activity: GR was assayed as described by Foyer and Halliwell [21], with minor modifications. The reaction mixture (1.0ml) consisted of 100mM phosphate buffer (pH 7.8), $0.1\mu\text{M}$ EDTA, 0.05mM NADPH, 3.0mM GSSG and 50 μL enzyme extract. The reaction was started by the addition of GSSG and the NADPH oxidation rate was monitored at 340nm for 1.0 min. Enzyme activity was expressed as $\mu\text{mol NADPH oxidation g}^{-1}$ F.W.

Lipid Peroxidation: Lipid peroxidation was determined as per Heath and Packer [22] by measuring the amount of Malondialdehyde (MDA) formed by the thiobarbituric acid reaction. Leaves were ground with a pestle and mortar in 1% TCA (10 ml/g F.W) and centrifuged at 10,000 rpm for 5 min. To 1.0 ml of supernatant in a separate test tube, 4.0 ml of 0.5% TBA was added. The mixture was heated at 95°C for 30 min, then cooled in ice-cold water and later centrifuged at 5,000 rpm for 5 min. Absorbance was measured at 532nm and corrected for unspecific turbidity by subtracting the value at 600 nm. The blank contained 1% TBA in 20% TCA. MDA content was calculated using an extinction coefficient of $155 \text{ mM}^{-1}\text{cm}^{-1}$ and the results expressed as $\mu\text{mol MDA g}^{-1}$ F.W.

Determination of Proline Content: The free proline content was determined according to Bates *et al.* [23]. Leaf tissue (0.5g) was homogenized with 10ml of 3% sulfosalicylic acid at 4°C. The extract was filtered with whatman No.2 filter paper. In test tube, 2ml of filtrate, 2ml of acid-ninhydrin and 2ml of glacial acetic acid were mixed and incubated at 100°C for 1h. The reaction was terminated on ice and the reaction mixture was then extracted with 4ml of toluene. The chromophore-containing toluene was separated from the hydrated phase. The absorbance at 520nm was spectrophotometrically determined with toluene as the blank. The proline concentration was calculated based on a standard curve and was expressed as $\mu\text{g proline g}^{-1}$ F.W.

Statistical Analysis: The results are based on four replicates from two independent experiments. The data were subjected to analysis of variance (ANOVA) by using SPSS 16.0 for Windows. Duncan's test was used for multiple comparison at $p < 0.05$ level between treatments. Each value represents the average of five replicates ($n=4$) \pm standard deviation (SD).

RESULTS

Enzyme Activities: In the present study SOD activity increased significantly in both the cultivars in a concentration and time depended manner. Plants of PBR 2700 showed 1.5 fold SOD activity whereas Spathagiri showed 0.6 fold SOD activity at 20th day when compared

to their respective controls (Fig. 1 A&B). Fig. 2 showed 0.8 fold and 0.4 fold enhancement of CAT activity for PBR 2700 and Spathagiri respectively from 10th onwards when compared to control plants under salinity stress. An increase of 2.0 fold and 1.3 fold in the activity of APX was observed in PBR 2700 and spathagiri respectively from 10th day onwards under salinity stress than their control plants (Fig. 3 A&B). Our experiments showed elevated GR activity (1.5 fold in PBR 2700 and 1.0 fold in Spathagiri) at 20th day with salt treatment (Fig. 4 A&B). Together, our results clearly demonstrated that antioxidant system gets elevated to overcome NaCl induced oxidative stress in finger millet. Relatively higher resistance showed by plants of cultivar PBR 2700 than Spathagiri could be owing to more elevated levels of SOD, CAT, APX and GR than Spathagiri cultivar under salinity stress conditions.

Lipid Peroxidation: Lipid peroxidation (LPO) is a measure of the extent of membrane damage occurred due to various stresses. In our study, LPO was measured in terms of MDA content in control and test plants. MDA content increased markedly in both the cultivars under salinity stress. A raise of 1.4 fold and 1.0 fold in MDA content was noted respectively in plants of Spathagiri and PBR 2700 at 20th day under salt treatment than their controls (Fig. 5 A&B). Relatively less MDA levels shown by PBR 2700 than Spathagiri reveals that lesser lipid peroxidation has occurred in this cultivar because it has more efficient antioxidant system and it is relatively resistant to salinity stress.

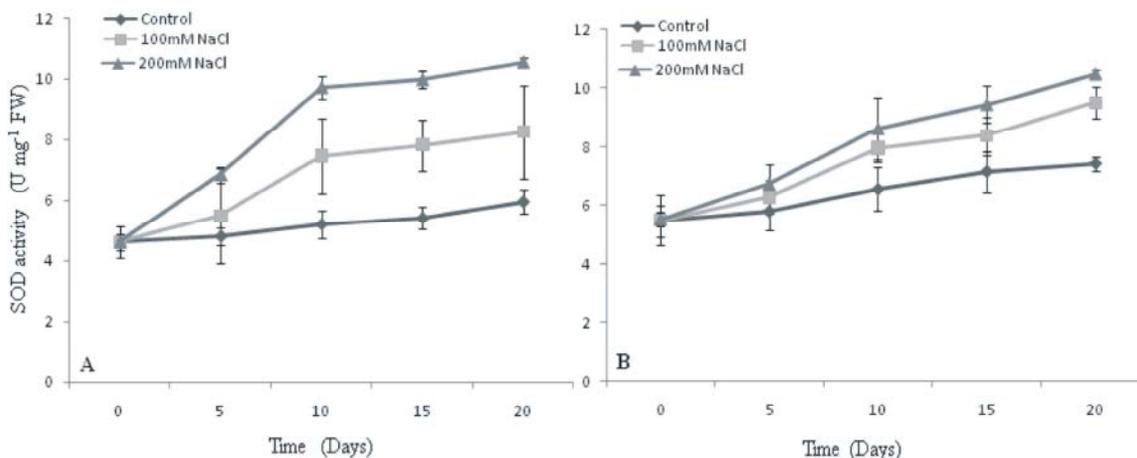


Fig. 1: Effect of NaCl stress on SOD activity in two finger millet cultivars grown under various (0, 100 & 200mM) salt stress conditions. (A) PBR 2700 (B) Spathagiri. Data are means \pm SD ($n=4$). Error bars are significantly different at $p < 0.05$.

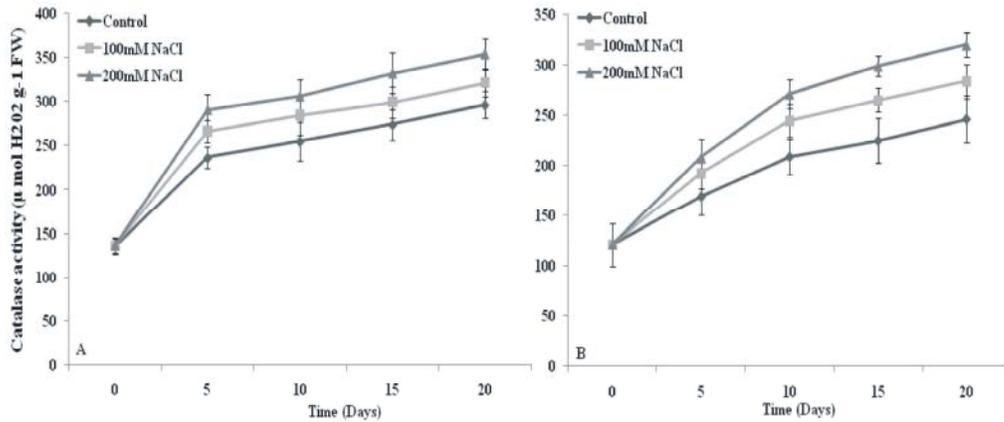


Fig. 2: Effect of salinity stress on CAT activity in two finger millet cultivars grown under various (0, 100 & 200mM) NaCl concentration. (A) PBR 2700 (B) Saphthagiri. Data are means \pm SD (n=4). Error bars are significantly different at $p < 0.05$.

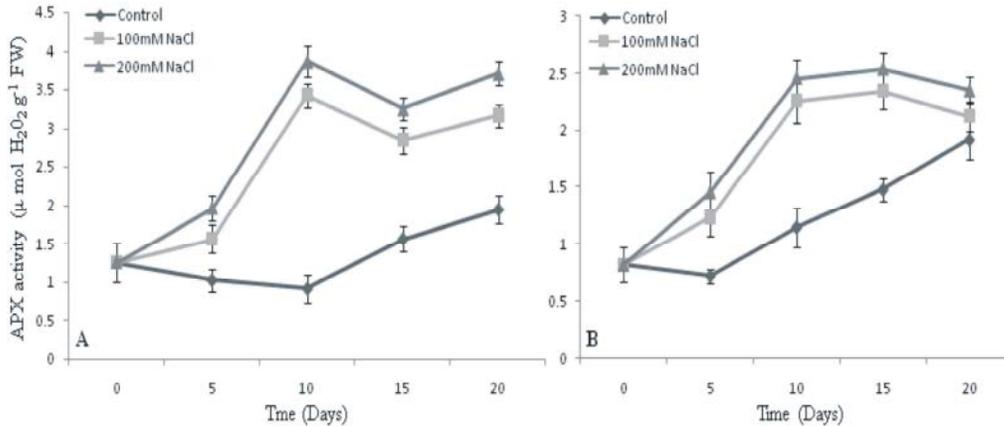


Fig. 3: Effect of NaCl stress on APX activity in two finger millet cultivars grown under various (0, 100 & 200mM) salt stress conditions. (A) PBR 2700 (B) Saphthagiri. Data are means \pm SD (n=4). Error bars are significantly different at $p < 0.05$.

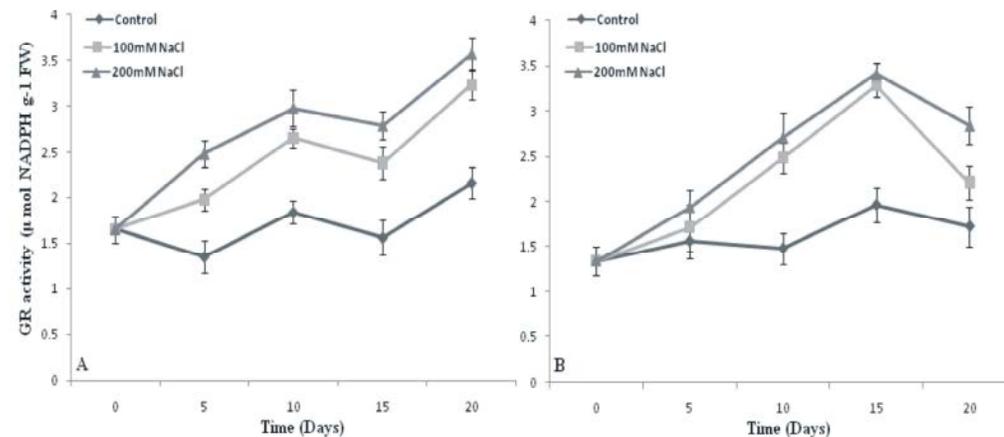


Fig. 4: Effect of NaCl on GR activity in two finger millet cultivars grown under various (0, 100 & 200mM) salt stress conditions. (A) PBR 2700 (B) Saphthagiri. Data are means \pm SD (n=4). Error bars are significantly different at $p < 0.05$.

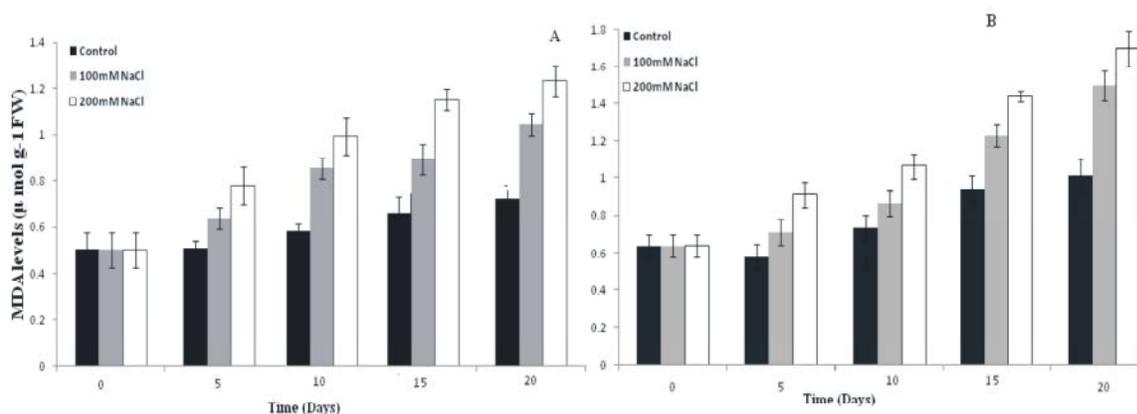


Fig. 5: Effect of NaCl on MDA content in two finger millet cultivars grown under various (0, 100 & 200mM) salt stress conditions. (A) PBR 2700 (B) Saphthagiri. Data are means \pm SD (n=4). Error bars are significantly different at $p < 0.05$.

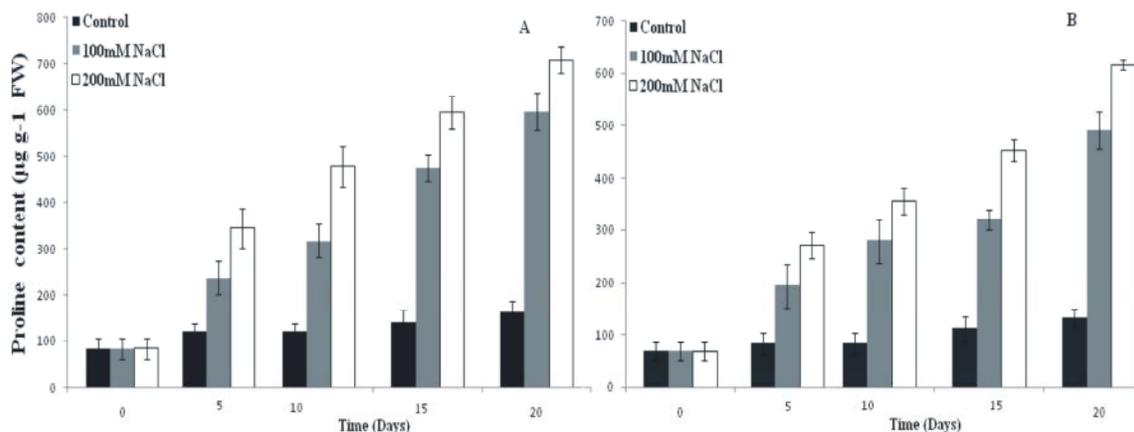


Fig. 6: Effect of NaCl on proline content in two finger millet cultivars grown under various (0, 100 & 200mM) salt stress conditions. (A) PBR 2700 (B) Saphthagiri. Data are means \pm SD (n=4). Error bars are significantly different at $p < 0.05$.

Proline Estimation: Proline is an important osmoprotectant and a biomarker of abiotic stress. It is involved in reduction of adverse effects caused by higher salinity or drought stress in plants by virtue of storage of carbon and nitrogen and by facilitating scavenging of reactive oxygen species (ROS) through enhanced production of antioxidant enzymes. In our study, higher amount of proline accumulation (3.5 fold in PBR 2700) and (2.2 fold Saphthagiri) was noted at 20th day under salinity stress conditions (Fig. 6 A&B).

DISCUSSION AND CONCLUSION

Salinity stress is one of the decisive factors of plant growth and yield world over. In addition to natural factors, intensive agricultural practices and unscrupulous use of

chemicals and fertilizers are aggravating the problem of soil salinity. High NaCl content in soil induces oxidative stress in plant cells which leads to enhanced production of reactive oxygen species such as hydroxyl radical (OH \cdot), hydrogen peroxide (H $_2$ O $_2$), singlet oxygen (1 O $_2$) and superoxide radical (O $_2^{\cdot-}$). These ROS are highly reactive and causes denaturation, mutagenesis, or lipid peroxidation by reacting with macromolecules such as proteins, nucleic acids and lipids resulting in metabolic disturbances [24,25]. In the present work we studied the effect of salinity stress on the activity of antioxidative enzymes (SOD, CAT, APX and GR), lipid peroxidation and proline accumulation at various time boundaries.

SOD is one of the major antioxidative enzymes present in all aerobic organisms and mostly in sub cellular components which generate activated oxygen and this

enzyme is involved in dismutation of superoxide radicals to hydrogen peroxide and oxygen [26]. In the present study SOD activity increased in both the cultivars with increasing NaCl concentration and treatment period (Fig. 1A&B). Plants of PBR 2700 genotype exhibited much more elevated SOD activity than Saphthagiri and therefore could possess better oxygen free radicals scavenging capacity minimizing the oxidative damage. Published reports also showed that overexpression of SOD resulted in efficient stress protection against NaCl stress in plants such as *B. maritima* and *B. vulgaris* [27], cotton [28], maize [29] and rice [30].

SOD action leads to the production of H₂O₂, a highly toxic molecule to living cells which needs to be eliminated from plant cells in subsequent reactions. Under such stress conditions enzymes such as CAT, APX and GPX are generally activated and involved in the elimination of H₂O₂ in the form of H₂O. CAT is an important H₂O₂ scavenging enzyme in plant leaves [29]. Our results showed that enzyme activities of CAT, APX increased significantly in both cultivars under salt stress conditions over their controls. However, PBR 2700 showed much more elevated levels of CAT than Saphthagiri. High catalase activity increases the cell membrane stability by decreasing H₂O₂ content under NaCl stress conditions [31]. We also showed that, increase in CAT, APX activities follows increased SOD activity under salt stress. Our results support the hypothesis that antioxidative enzymes play a central protective role in the detoxification of O₂⁻ and H₂O₂ by scavenging process with coordination of SOD [32, 33].

GR is an important enzyme involved in converting of GSSG to GSH under environmental stress conditions [13]. In our study GR activity increased along with NaCl concentration in both the cultivars, with more prominent raise being observed with salt tolerant cultivar than either salt sensitive cultivar or control plants. Raise in GR activity under Salinity stress was also shown in previous reports on barely [32] and cucumber [34].

Salt stress is a major factor that enhances peroxidation of important macromolecules through oxidative damage of membrane components [35]. In our study MDA content increased significantly in both the cultivars of finger millet. However, salt tolerant cultivar, PBR 2700 showed lesser MDA levels than Saphthagiri, indicating that plants of PBR 700 are relatively resistant to NaCl-induced peroxidation of membrane lipids and other components. The significant raise in MDA content in salt sensitive Saphthagiri genotype may be due to lack of salt

dependant up regulation of its antioxidant enzyme system under salinity stress. Earlier studies also reported altered MDA levels in *P. maritime*, *P. sativum*, *O. basilicum* and *B. maritima* and *Glycine max* under salinity stress conditions [14-16, 27, 36].

Proline plays protective osmolyte function under certain stress conditions. It is a storage of nitrogen and carbon and it is implicated in facilitating scavenging of free radicals in plants under salinity stress conditions, including mangroves [37], tobacco [38], sorghum [39] and wheat [40]. In the present study proline accumulation increased in both salt tolerant PBR 2700 and salt sensitive Saphthagiri genotypes under salt stress than their controls. However, higher proline accumulation was observed in PBR 2700 genotype than Saphthagiri genotype. The accumulation of higher content of proline in salt tolerant PBR 2700 genotype may probably better protect these plants from salt stress by osmotic adjustment and facilitating scavenging of free radicals.

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