

Salinity induced oxidative stress and antioxidant system in salt-tolerant and salt-sensitive cultivars of rice (*Oryza sativa* L.)

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Abstract Indices of oxidative stress viz., superoxide radical and H₂O₂ content increased in leaves of all the cultivars with the rise in salinity level, the increase was more pronounced and significant in salt-sensitive varieties and non-significant in resistant cultivars. Except for glutathione reductase (GR), basal activities of all other antioxidative enzymes viz. superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR) were significantly higher in leaves of all the resistant cultivars as compared to the sensitive ones. A differential response of salinity was observed on various enzymatic and non-enzymatic components of antioxidant system in leaves of salt-tolerant and salt-sensitive cultivars of rice (*Oryza sativa* L.). Activities of superoxide dismutase and glutathione reductase enhanced in all the tolerant cultivar while declined in the sensitive cultivars with increasing salinity from 0 to 100 mM. Salt-stress induced the activities of catalase and peroxidase in all the cultivars but the magnitude of increase was more pronounced in the sensitive cultivars than in the tolerant cultivars. Contrarily, APX activity increased in the salt-sensitive cultivars but showed no significant change in the salt-tolerant cultivars. The amount of ascorbic acid content, reduced glutathione (GSH), reduced/oxidized glutathione (GSSG) ratio was higher in leaves of the tolerant cultivars than that of the sensitive cultivars under saline conditions. It is inferred that leaves of salt-tolerant cultivars tend to attain greater capacity to perform reactions

of antioxidative pathway under saline conditions to combat salinity-induced oxidative stress.

Keywords Antioxidant · Ascorbate · Glutathione · Rice · ROS scavenging enzymes · Salinity

Abbreviations

APX Ascorbate peroxidase
CAT Catalase
GR Glutathione reductase
POX Peroxidase
ROS Reactive oxygen species
SOD Superoxide dismutase

Introduction

Salinity is one of the major environmental factors that limits the productivity and quality of economically important crops throughout the world (Sharifi et al. 2007). The most common biological and physiological effects in plants experiencing salt stress are reduced water potential, ion imbalance and toxicity, diminished CO₂ assimilation and enhanced generation of reactive oxygen species (ROS) including superoxide radical (O₂⁻), hydrogen peroxide (H₂O₂), hydroxyl radical (OH⁻) and singlet oxygen (¹O₂) leading to oxidative stress. Excess of ROS triggers phytotoxic reactions such as lipid peroxidation, inactivating enzymes, protein degradation and denaturing DNA molecules (Jiang and Zhang 2001; Bor et al. 2003). To mitigate and repair the damage initiated by ROS, plant cells detoxify ROS by upregulating antioxidative system constituted of enzymatic and non-enzymatic components (Becana et al. 2000). Superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) are the enzymatic antioxidants while the non-

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enzymatic antioxidants include water soluble components like ascorbic acid, glutathione, flavonoids and the lipid soluble components such as carotenoids and α -tocopherol. A correlation between antioxidant capacity and NaCl stress has been demonstrated in several plant species (Broetto et al. 2002; Bor et al. 2003; Agarwal and Pandey 2004; Di Baccio et al. 2004; Amor et al. 2005). However, cooperative analyses of salinity-dependent antioxidant modulation between sensitive and tolerant cultivars of same plant species are still quite rare. Also, the precise mechanism that imparts salt-tolerance to plants still remains to be elucidated. This study was, therefore, undertaken to understand the salinity induced changes in antioxidant enzymes and metabolites in salt-tolerant and salt-sensitive cultivars of rice.

Materials and methods

Plant material

Rice seeds of salt-sensitive (MI-48, IR-28) and salt-tolerant (CSR-1, Pokkali) cultivars obtained from Central Soil Salinity Research Institute (CSSRI), Karnal, were grown hydroponically in specifically designed plastic trays having equidistant holes. Two seeds were placed in each hole and these were then put in other rectangular plastic trays containing 2.0 L of Yoshida nutrient solution (Yoshida et al. 1976). The nutrient solution was renewed once a week and water lost by evaporation was compensated by adding deionized water to each tray.

Salinity treatment

The chloride (Cl^-) dominated salinity was prepared by using a mixture of different salts such as NaCl, MgCl_2 , MgSO_4 and CaCl_2 where Na:Ca was in the ratio of 1:1 and Ca and Mg in the ratio of 1:3 and Cl and SO_4 ratio was 7:3 on a meq basis. At 25 days of sowing, the desired salinity (0, 50 and 100 mM) was created by supplementing the solution in the trays with salt solution and the sampling was done at 35 days seedling stage.

Enzyme extraction and assays

Extraction conditions were standardized w.r.t. type, molarity and pH of buffer, concentration(s) of stabilizing agents and other constituents of extraction medium to achieve maximum extraction of enzyme. All the steps of extraction were carried out at 0–4°C. Extraction medium for SOD, CAT, APX and GR consisted of 0.1 M phosphate buffer (pH 7.5) containing 5% (w/v) polyvinylpyrrolidone (PVPP), 1 mM EDTA, and 10 mM β -mercaptoethanol. For POX, however, the extraction buffer consisted of 0.01 M phosphate buffer (pH 7.5)

containing 3% (w/v) PVPP. The homogenate was prepared by grinding 1 g (fresh weight) of tissue in 5 ml of ice cold extraction medium in pre-cooled mortar and pestle. The homogenate thus prepared was centrifuged at 10,000 \times g for 15 min at 4°C. The supernatant was carefully decanted and used as the crude enzyme preparation. All the estimations were carried out in three replicates with two extractions for each replicate. The value reported for each parameter are, therefore, the means of six replicates.

SOD activity was determined by quantifying the ability of the enzyme extracts to inhibit light induced conversion of nitroblue tetrazolium (NBT) to formazan (Nishikimi et al. 1972). One enzyme unit was defined as the amount of enzyme which could cause 50% inhibition of the photochemical reaction. CAT and POX activities were assayed at 37°C as described by Sinha (1972) and Shannon et al. (1966), respectively. Method of Nakano and Asada (1981) was employed to assay APX. GR activity was determined at 30°C by adding 100 μ l of enzyme extract to 1 ml of 0.2 M phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.75 ml distilled water, 0.1 ml of 20 mM oxidized glutathione (GSSG) and 0.1 ml of 2 mM NADPH. Oxidation of NADPH by GR was monitored at 340 nm and the rate (nmol min^{-1}) was calculated using the extinction coefficient of $6.12 \text{ mM}^{-1} \text{ cm}^{-1}$ (Halliwell and Foyer 1978).

Extraction and estimation of ROS and metabolites

For the extraction of superoxide (O_2^-) radical, ascorbate and glutathione (reduced and oxidized), 1 g of the tissue from control and stressed plants were ground in 5 ml of chilled 0.8 N HClO_4 and centrifuged at 10,000 rpm for 25 min. The clear supernatant was decanted carefully and was used for estimation of superoxide radical. O_2^- radical was measured by monitoring the nitrite formation from hydroxylamine following the method of Elstner and Heupel (1976). Amount of NO_2^- formed which corresponded to O_2^- production was calculated from standard curve of NO_2^- (0–100 nmol).

H_2O_2 was extracted by homogenizing 4 g tissue in 5 ml of ice cold 0.01 M phosphate buffer (pH 7.0) and centrifuging the homogenate at 8,000 \times g for 10 min (Sinha 1972). Fifty μ l of the sample was added to 1.95 ml of 0.01 M phosphate buffer (pH 7.0). To the mixture, was added 2 ml of 5% potassium dichromate and glacial acetic acid (1:3, v/v). The optical density was read at 570 nm against the reagent blank without sample extract. The quantity of H_2O_2 was determined by comparing with the standard (10 to 160 nmol). Ascorbic acid was estimated according to the method of Roe (1964) which is based on the reduction of 2, 6-dichlorophenol indo-phenol by ascorbic acid. Method of Smith (1985) was employed for determining the level of oxidized, reduced and total glutathione.

Method of Griffith (1980) was employed for determining the level of oxidized, reduced and total glutathione.

Statistical analysis

The statistical analysis was done using analysis of variance. The complete randomized design (CRD) where each observation was replicated thrice and each replicate was estimated in duplicate was used. The critical difference (CD) among the variance was calculated at $P \leq 0.05$ (Panse and Sukhatme 1961).

Results and discussion

ROS production

Oxidative stress can be best assessed by the extent of ROS viz. superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) generation under saline conditions. Production of O_2^- was more in leaves of non-stressed plants of sensitive cultivars (34.76–36.13 $nmol\ g^{-1}\ f. wt.$) than that in resistant cultivars which had a range of 18.36–18.94 $nmol\ g^{-1}\ f. wt.$ Though the amount of O_2^- increased with the elevation in salinity stress in all the cultivars, but the level of increment was much higher and significant in the sensitive varieties and non-significant in tolerant cultivars (Fig. 1a). Hydrogen peroxide (H_2O_2) followed a similar pattern that was exhibited by O_2^- showing enhancement in H_2O_2 in all the cultivars at both the levels of salt-stress. However, the level of increase was much greater in the sensitive cultivars as compared to that in the resistant ones (Fig. 1b). The basal values of O_2^- and H_2O_2 in the salt-sensitive varieties were also about two fold the amount of these ROS in salt-tolerant cultivars (Fig. 1a). Superoxide ions and H_2O_2 has been reported to increase in wheat (Li et al. 2004) and maize (Jiang and Zhang 2001) under osmotic stress, in *Amaranthus* seedlings (Bhattacharjee and Mukherjee 2006) under heat and salt stress, in pea (Hernandez et al. 2004) under light stress and chickpea (Kukreja et al. 2006) under salt stress. Both O_2^- and H_2O_2 act as potential signaling molecules (Jiang and Zhang 2001) and play an important role in salt-stress tolerance in plants (Singha and Choudhuri 1990). Consistent higher values of O_2^- and H_2O_2 with significant higher increase under saline conditions in salt sensitive varieties may play an important role in inducing oxidative stress and membrane permeability by attacking membrane lipids in these varieties (Willekens et al. 1995).

To determine the role of ROS scavenging systems in combating the oxidative stress, enzymes and metabolites of antioxidant system were characterized in leaves of salt-sensitive and salt-tolerant cultivars. Fig. 2a reveals that SOD activity increased progressively with increase in salinity in salt-tolerant cultivars but receded in the sensitive varieties. The

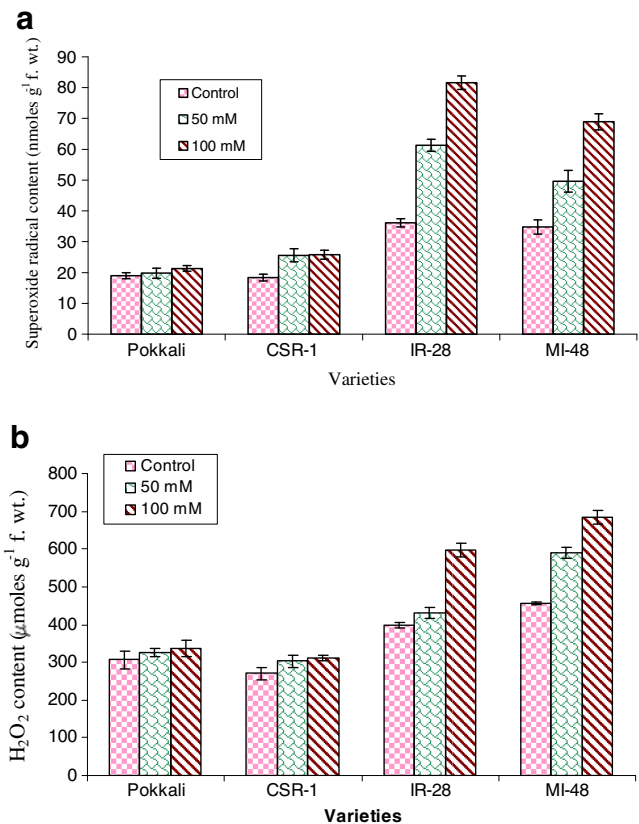


Fig. 1 a Effect of salinity on superoxide radical content in leaves of *Oryza sativa*. The bar (I) denotes \pm SE. [CD ($P \leq 0.05$) 3.12 (varieties), 2.20 (treatments), 5.39 (interactions)] b Effect of salinity on hydrogen peroxide in leaves of *Oryza sativa*. The bar (I) denotes \pm SE. [CD ($P \leq 0.05$) 31.66 (varieties), 22.39 (treatments), 54.83 (interactions)]

basal level of SOD activity was also significantly higher in salt-tolerant cultivars than in salt-sensitive cultivars. It was 459.6 and 498.1 units respectively in CSR-1 and Pokkali but was only 217.0 and 232.6 units in MI-48 and IR-28 respectively. These observations are in agreement with those reported earlier in *Solanum tuberosum* (Benavides et al. 2000), *Brassica juncea* (Kumar 2002) and *Najas graminia* (Rout and Shaw 2001) where tolerant-cultivars had higher basal level of the enzyme activity as compared to that in the sensitive ones. Similar to the present findings, SOD activity increased in salt-tolerant cultivars but depressed in salt-sensitive cultivar of wheat (Mandhanja et al. 2006), cotton (Meloni et al. 2002) and *Catharanthus roseus* (Jaleel 2009). Contrarily, salinity has been reported to stimulate SOD activity in both salt-tolerant and salt-sensitive cultivars of *B. juncea* with high basal level in salt-tolerant cultivar (Kumar et al. 2006). Increase in SOD activity upon salinization in leaves of all the tolerant cultivars could accelerate dismutation of superoxide ions generated upon salt-treatment, which may allow this variety to survive under oxidative stress (Desingh and Kanagaraj 2007). In salt-sensitive varieties, depreciation in SOD activity would limit its metabolic capacity to withstand

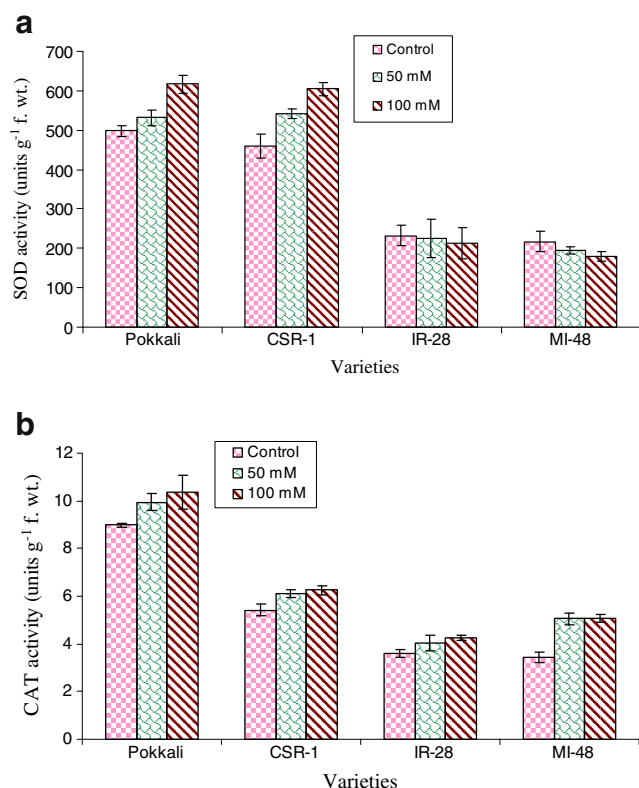


Fig. 2 **a** Effect of salinity on superoxide dismutase (SOD) activity in leaves of *Oryza sativa*. The bar (I) denotes \pm SE. [CD ($P \leq 0.05$) 20.41 (varieties), 14.83 (treatments), 35.36 (interactions)] **b** Effect of salinity on catalase (CAT) activity in leaves of *Oryza sativa*. The bar (I) denotes \pm SE. [CD ($P \leq 0.05$) 0.78 (varieties), 0.55 (treatments), 1.35 (interactions)]

oxidative stress. Thus increase in the activity of SOD on exposure to salinity with high basal activity in Pokkali and CSR-1 implies a possible involvement of enzyme in salt (Rout and Shaw 2001).

Like SOD, CAT activity was also higher in the leaves of non-stressed plants of tolerant cultivars (9.0 units in Pokkali and 5.4 units in CSR-1) as compared to that in salt-sensitive MI-48 (3.43 units) and IR-28 (3.6 units). It appreciated in all the cultivars at both the levels of stress but increase at 100 mM salinity was non-significant with respect to that at 50 mM salt stress (Fig. 2b). Catalase is one of hydrogen peroxide detoxifying enzymes that converts hydrogen peroxide into water and oxygen. The results obtained in this study are in accordance with those obtained by Pal et al. (2004) and Mutlu et al. (2009) who reported increase in CAT activity in both salt-tolerant and salt-sensitive cultivars of rice and wheat respectively. Azooz et al. (2009) observed that CAT activity increased gradually with increase in salt stress in the tolerant varieties but reduced significantly in the salt-sensitive cultivar of maize. Rout and Shaw (2001) and Dolatabadian et al. (2008) also observed increased CAT activity in aquatic macrophyte and *B. napus* on exposure to 200 mM NaCl stress. The induction of CAT activity was reported to be due to accumulation of H_2O_2 under

saline conditions (Gueta-Dahan et al. 1997) which seemingly is consistent with its role in scavenging enhanced H_2O_2 level (Gupta and Gupta 2005). Concomitant with results of the present study, the salt-tolerant genotypes of chickpea showed significantly higher CAT activity in comparison to susceptible genotypes at both pre and post flowering stages (Singh et al. 2005) which helps these genotypes to detoxify H_2O_2 generated under saline conditions.

Like CAT, POX activity also increased in all the cultivars subjected to 50 mM salt stress. However, further increment of salt stress to 100 mM, caused elevation in POX activity in tolerant cultivars and decline in the salt-sensitive cultivars (Fig. 3a). The results obtained in the present investigations are in accordance with those observed in *B. napus* (Dolatabadian et al. 2008), olive trees (Sofa et al. 2005), *Vigna* (Arulbalachandran et al. 2009) and rice (Kumar et al. 2009). Peroxidase activity has been shown to increase in both resistant and sensitive cultivars of wheat (Mandhanja et al. 2006), maize and *B. juncea* (Kumar et al. 2006) upon salinization with level of increment in the tolerant cultivars. The increase in peroxidase activity under saline conditions has been ascribed to either increased expression of peroxidase encoding genes or increased activation of the already existing enzyme molecules.

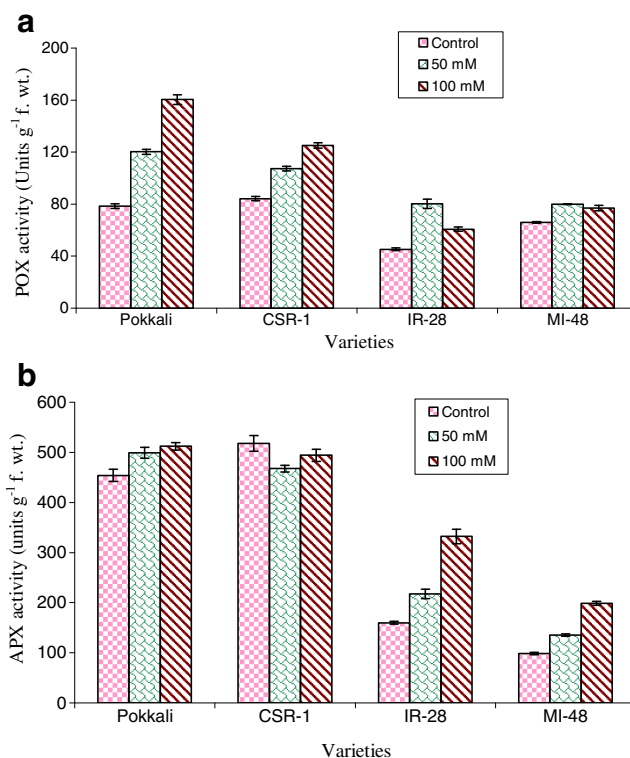


Fig. 3 **a** Effect of salinity on peroxidase (POX) activity in leaves of *Oryza sativa*. The bar (I) denotes \pm SE. [CD ($P \leq 0.05$) 1.94 (varieties), 1.37 (treatments), 3.36 (interactions)] **b** Effect of salinity on ascorbate peroxidase (APX) activity in leaves of *Oryza sativa*. The bar (I) denotes \pm SE. [CD ($P \leq 0.05$) 7.16 (varieties), 5.06 (treatments), 12.41 (interactions)]

Activity profile of APX (Fig. 3b) reveals that resistant cultivars had much higher APX level (453.9 and 518.2 units in Pokkali and CSR-1, respectively) as compared to sensitive genotypes (98.3, 160 units in MI-48 and IR-28, respectively). There was no appreciable change in APX activity in the tolerant varieties but increase was observed in the sensitive cultivars at both the levels of salinity stress. Imposition of salt stress up to 100 mM, though enhanced APX activity in MI-48 and IR-28, it was much lower than in non-stressed leaves of tolerant cultivars. These results are supported by Benavides et al. (2000) who reported an elevated basal level of APX in salt-tolerant potato clones than in salt-sensitive clone. Similarly, Meneguzzo et al. (1999) observed a much greater increase in APX activity in salt-sensitive cultivar than in salt-tolerant cultivar of wheat exposed to NaCl stress. Rout and Shaw (2001) reported an enhanced APX activity in salt-sensitive variety of aquatic macrophytes and reduced activity in tolerant cultivar in response to NaCl treatment. On the contrary, Neto et al. (2005) reported a higher increase in activity in leaves of salt-tolerant than salt-sensitive genotype of maize. With increase in salt-stress, APX activity also increased in wheat (Khan 2004; Heidari and Mesri 2008), cotton (Desingh and Kanagaraj 2007) and *Cakile maritima* (Amor et al. 2007) suggesting that high basal level of

APX and/or salt-induced increase in APX activity could impart tolerance by detoxifying H₂O₂ generated upon exposure of plants to saline conditions.

Glutathione reductase (GR) activity increased linearly in salt-tolerant cultivars with severity of salinization (Fig. 4a). On the other hand, the activity declined (45–50%) in salt-sensitive IR-28 but remained unchanged in MI-48 at both levels of salt stress. The results are in agreement with those obtained in *Calamus tenuis* (Khan and Patra 2007) and *Phaseolus vulgaris* (Nagesh Babu and Devaraj 2008) in which salt tolerance was correlated with elevated GR activity. Similarly, higher GR activity during salt-stress in tolerant cultivars and diminished activity in the sensitive cultivars was reported in pea (Hernandez et al. 2000) and wheat (Mandhania et al. 2006). However, Mittova et al. (2002) reported no change in GR activity in chloroplasts isolated from *Lycopersicon pennelli* plants subjected to salt stress. Thus, the results of the present study corroborate the previous reports (Benavides et al. 2000; Hernandez et al. 2000; Hossain et al. 2004; Verma and Mishra 2005) showing significantly higher GR activity in salt-tolerant cultivars with no change or slight decrease in salt-sensitive cultivars under salt-stress.

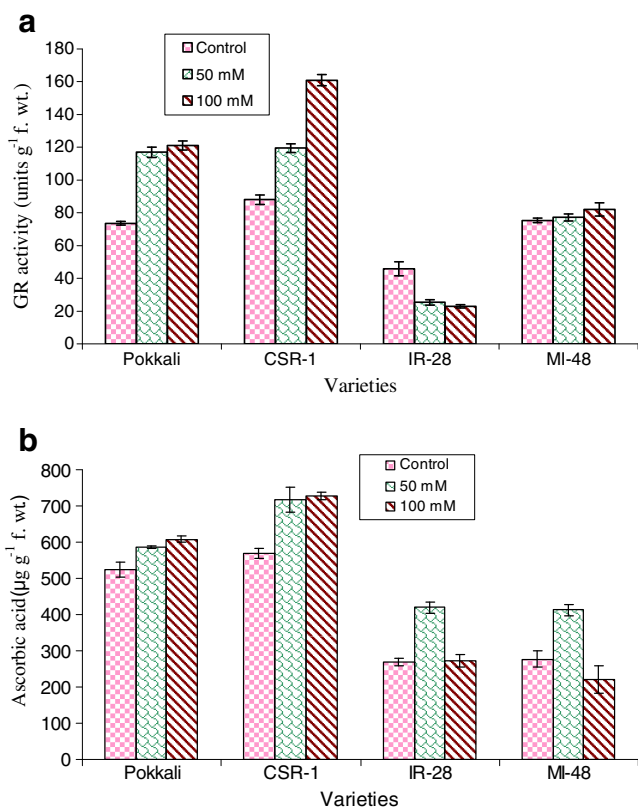


Fig. 4 a Effect of salinity on glutathione reductase (GR) activity in leaves of *Oryza sativa*. The bar (I) denotes±SE. [CD ($P\leq 0.05$) 2.26 (varieties), 3.60 (treatments), 3.92 (interactions)] b Effect of salinity on ascorbic acid content in leaves of *Oryza sativa*. The bar (I) denotes±SE. [CD ($P\leq 0.05$) 29.37 (varieties), 20.77 (treatments), 50.87 (interactions)]

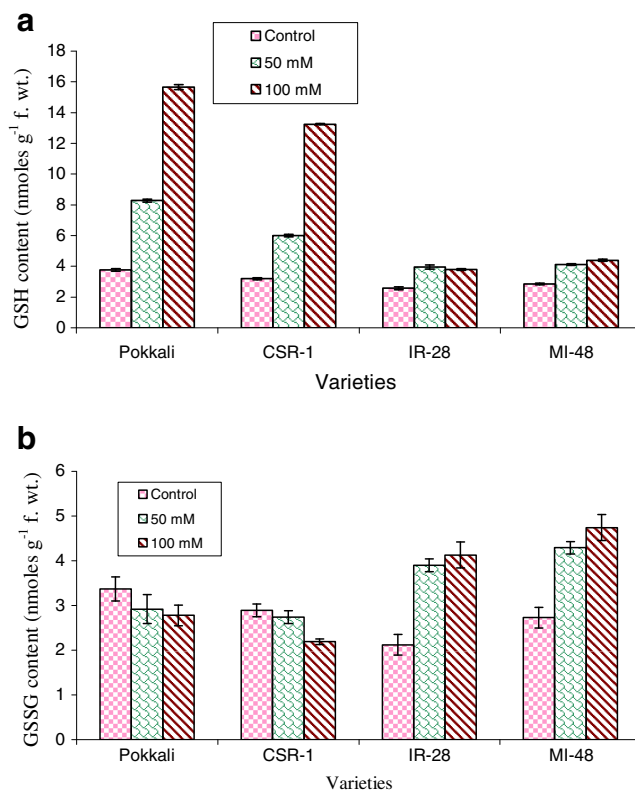


Fig. 5 a Effect of salinity on glutathione reduced (GSH) content in leaves of *Oryza sativa*. The bar (I) denotes±SE. [CD ($P\leq 0.05$) 0.15 (varieties), 0.10 (treatments), 0.25 (interactions)] b Effect of salinity on glutathione oxidized (GSSG) content in leaves of *Oryza sativa*. The bar (I) denotes±SE. [CD ($P\leq 0.05$) 0.46 (varieties), 0.32 (treatments), 0.79 (interactions)]

Ascorbic acid and reduced glutathione are important ROS scavenging metabolites. Results presented in Fig. 4b reveal that the leaves of salt-tolerant varieties of untreated plants had significantly higher ascorbic acid content (524.7–569.0 $\mu\text{g g}^{-1}$ f. wt.) as compared to that of salt-sensitive cultivars which possessed 268.7–276.6 $\mu\text{g g}^{-1}$ f. wt. ascorbic acid. At 50 mM salt-stress, ascorbic acid content increased in all the cultivars but 100 mM salt, it declined in the sensitive varieties and remained unchanged in tolerant varieties. Ascorbate plays an important role in affording protection against reactive oxygen species, as it acts as electron donor for ascorbate peroxidase. In accordance with the above results, Chen et al. (2007) observed maximum ascorbate content in the salt-tolerant cultivar as compared to the salt-sensitive cultivars of *Phragmites communis*. Enhancement in ascorbate under saline conditions in salt-tolerant cultivars was also reported in cotton (Gossett et al. 1994), potato (Benavides et al. 2000) and *Phaseolus vulgaris* (Nagesh Babu and Devaraj 2008; Telesinski et al. 2008). A positive correlation between salt-tolerance and ascorbate content has been demonstrated in leaves of *Cakile maritima* (Amor et al. 2006) and *Momordica charantia* (Agarwal and Shaheen 2007).

Data on reduced (GSH, Fig. 5a) and oxidized (GSSG, Fig. 5b) glutathione content reveal a linear increase in GSH with increasing salt concentration in leaves of all the varieties but the level of enhancement was much higher in salt-tolerant cultivars than salt-sensitive cultivars at both the stress levels (Fig. 5a). With the increase in salinity level, the oxidized glutathione (GSSG) content declined linearly in leaves of the salt-tolerant cultivars but increased in those of salt-sensitive cultivars (Fig. 5b).

Glutathione, a low molecular weight antioxidant, is a powerful regulator of major cell functions (Renenberg and Lamoureux 1990). GSH can react directly with free radicals; hence, preventing inactivation of enzymes due to oxidation of essential thiol groups (Wang et al. 1991).

The results presented above are consistent with those of Meneguzzo et al. (1999), Benavides et al. (2000), Amor et al. (2006, 2007) and Nagesh Babu and Devaraj (2008) who also observed that that GSH content significantly elevated in the tolerant cultivars as compared to the sensitive cultivars of wheat, potato, Jerba plants, *Cakile maritima* and French bean respectively, under saline conditions. On the contrary, Jaleel et al. (2008) reported a significant decrease in GSH content in salt-stressed *Withania somnifera* plants.

The results presented show that salt-stress induced oxidative stress, as indicated by increased production of $\cdot\text{O}_2^-$ and H_2O_2 in all the varieties. Stress imposition elicited activities of superoxide dismutase (SOD) and glutathione reductase (GR) in leaves salt-tolerant cultivars, whereas in the salt-sensitive cultivars the activities of both enzymes declined. However, activities of CAT and POX increased in all the cultivars while APX activity increased only in all the sensitive cultivars and remained

unchanged in the tolerant cultivars. However, the basal levels of CAT and APX were much higher in the tolerant cultivars. The ratio of glutathione reduced (GSH)/glutathione oxidized (GSSG) also increased in tolerant cultivars under saline conditions. From the present study, salt-tolerance seems to be conferred by an increased antioxidative capacity to detoxify ROS. The elevated enzyme activities and increased production of metabolites seems to confer tolerance to the plants. As the SOD activity decreased in the salt-sensitive cultivars, it could thus limit the ability of seedling to scavenge $\cdot\text{O}_2^-$ resulting in accumulation of ROS which ultimately led to membrane damage. This increased production of free radicals was more in the sensitive cultivars than tolerant cultivars and it may have led to loss of membrane integrity. Although the CAT and APX activity increased to a greater extent in the sensitive cultivars than tolerant cultivars but the basal levels were manifold higher in the tolerant cultivars, which may have resulted in faster depletion of H_2O_2 in tolerant varieties.

Thus, the increase in the activities of enzymes such as SOD, CAT, GR and higher basal level of APX accompanied by high GSH/GSSG ratio, may account for greater capability of Pokkali and CSR-1 to perform better under saline conditions than the salt-sensitive cultivars.

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