Antioxidant Response of Rice (*Oryza sativa* L.) Varieties to Salt Stress at Different Growth Stages

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ABSTRACT. *The mechanism(s) imparting salt tolerance in plants remains unresolved. The aim of this study was to identify and determine whether a highly salt tolerant variety (Pokkali) contains higher constitutive or inducible levels of antioxidants than moderately tolerant variety (At 353) or more salt sensitive (IR 28) variety at three growth stages (seedling, tillering and flowering) under induced salinity levels of 4, 6 and 8 dS/m. Chlorophyll content and rate of photosynthesis were also measured.*

The highly salt tolerant variety, Pokkali showed increased Superoxide Dismutase (SOD) activity and Catalase (CAT), and a decrease in Peroxidase (POD) activity and, virtually unchanged lipid peroxidation with the increase of salinity levels. Moderately tolerant variety At 353 had a slight increase in SOD, CAT and high content of POD and lipid peroxidation. At 353 showed symptoms of oxidative damage at 8 dS/m similar to that of IR 28. Increase EC in IR 28 resulted in a decrease SOD and CAT activities and increased the content of POD to a high level along with highly significant increase of lipid peroxidation. Induced salinity significantly decreased chlorophyll content and rate of photosynthesis in all varieties. These results indicated that SOD was the main antioxidant enzyme in Pokkali and POD in At 353 and IR 28. CAT plays an intermediate role between SOD and POD in all three varieties.

INTRODUCTION

In many areas of the rice (*Oryza sativa* L.) growing regions of the world secondary salinization is becoming a major obstacle and an increasingly serious production constraint. According to Thiruchelvam and Pathmarajah (2000), approximately 13% of the irrigated areas in Sri Lanka are affected by salinity. Excessive irrigation, poor quality of water supply and inadequate drainage are the principal causes of soil salinity (Thiruchelvam and Pathmarajah, 2000).

One of the biochemical changes possibly occurring when plants are subjected to salt stress is the production of reactive oxygen species (ROS). The chloroplasts and mitochondria of plant cells are important intracellular generators of ROS. Electrons that leak from electron transport chains of these organelles can react with O_2 during normal aerobic metabolism to produce ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2),

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and the hydroxyl radical (OH°). Plants posses a number of antioxidants that protect against the potentially cytotoxic species of ROS in varying degrees. Superoxide dismutase (SOD; EC 1.15.1.1) is a major scavenger of O_2^-, and its enzymatic action results in the formation of H_2O_2. Catalase (CAT; EC 1.11.1.6) and a variety of Peroxidase (POD; EC 1.11.1.7) catalyze the breakdown of H_2O_2 (Peiris *et al*., 1991). Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to damages such as high salinity, temperature extremes, drought *etc*. (Spychalla and Desborough, 1990).

This study was designed to determine, aside from the disturbance to photosynthesis, the effect of salt stress on antioxidant enzyme activities and lipid membrane peroxidation of rice varieties exhibiting differences in salinity tolerance. Comparison of these responses could be useful in identifying differences related to the relative ability of each variety to cope with salinity. Thus results from this study would provide information on the possible involvement of reactive oxygen species in the mechanism of damage by salt stress in rice plant, and also could allow deeper insights into the molecular mechanisms of tolerance to salt-induced oxidative stress.

MATERIALS AND METHODS

Experiments were conducted in a green house at the Department of Agricultural Biology, University of Peradeniya. Seeds of the rice varieties Pokkali (highly salt tolerant), At 353 (moderately salt tolerant) and IR 28 (salt sensitive) were obtained from the Rice Research Institute, Bathalagoda. Plants were grown under green house conditions in hydroponics using a nutrient medium (Yoshida *et al*., 1976, Appendix 1). Each variety had three replicates and seedling density was fifteen per 1 l plastic pot at the beginning and later at the flowering stage it was thinned out to two plants. Pots were inserted in to the tubes of the hydroponic system. The spacing between pots in the tube was 30 cm. The temperature varied from 28-31^{0}C during the day and 24 to 28^{0}C in the night.

Salt stress was imposed on 14-day old plants: A chloride dominant salt solution was added to the nutrient medium and a range of salinity levels was induced similar to that found under natural conditions (4, 6 and 8 dS/m) by adjusting the concentration of NaCl, $CaCl_2$, KCl and $MgSO_4$ (Appendix 2). Non - salinized nutrient medium (1.0 dS/m) was used as the control. Electrical Conductivity (EC) and pH of the growth medium (5.0-5.5) were checked and adjusted every two days. Solutions were renewed weekly till the 4th week after planting and then renewed twice a week until flowering.

Plants were collected for determining antioxidant enzymes activity at different growth stages *viz.,* seedling (19-25 days), tillering (35-42 days) and flowering (70-84 days) after salt stress was imposed. Samples were prepared for SOD, CAT, POD and lipid peroxidation determination by homogenizing 3 g of leaf material in 15 ml of ice-cold phosphate buffer (0.05M, pH 7.0). The extracts were centrifuged at 4^{0}C for 10 min. at 20,000 X g and the resulting supernatants were used as the crude extracts.

All spectrophotometric analyses were conducted in UV-VIS recording spectrophotometer (UV-1201, Shimadzu Corp., Kyoto, Japan). The total SOD was assayed by the combined method of Stewart and Bewley (1980) and Spychalla and Desborough (1990). One unit of SOD activity was defined as the amount of enzyme, which caused 50%

inhibition of the initial rate of reaction. The percentage inhibition was calculated and expressed as number of units of enzyme mg protein/ml/min. CAT activity was determined by monitoring the disappearance of H_2O_2 by measuring the decrease in absorbance during 1 min at 240 nm (Spychalla and Desborough, 1990). One unit of enzyme was defined as the amount necessary to decompose 1 μmol of substrate min^{-1} at 25^0C. POD activity was measured at 675 nm by monitoring the H_2O_2–dependent oxidation of reduced 2,6-dichlorophenol indophenol for 5 min. at 25^0C (Nickel and Cunningham, 1979). One unit of enzyme was defined as the amount necessary to decompose 1 μmol of substrate min^{-1} at 25^0C.

The extent of damage to the membrane (lipid peroxidation) was determined by measuring the amount of malondialdehyde (MDA) formation using the thiobarbituric acid method described by Stewart and Bewley (1980).

Rate of photosynthesis (μmol CO_2/m/s) was measured by a portable photosynthesis system (Li- 6400, Li- Co- R, Inc.) under full sunlight (9:30 am. to 11:30 pm.) at bi- weekly intervals synchronizing with the three growth stages (seedling, tillering and flowering). Leaf total chlorophyll content (mg/g fresh weight) in the 80% acetone extract was measured using the UV – VIS spectrophotometer (UV-1201, Shimadzu Corp., Kyoto, Japan).

Data were analyzed by the general linear model procedure using the statistical analysis system (SAS) version 6 - computer package. A model was chosen for factorial analysis indicating 3 way interaction and means were compared by the least square means (LSMEANS) test at P = 0.05.

RESULTS AND DISCUSSION

Superoxide dismutase activity

The increase in salinity had an effect on SOD activities of the three rice (*Oryza sativa* L.) varieties after exposure to salt stress at all growth stages (Table 1). During the seedling stage, Pokkali had significantly higher SOD activity, which was 64 and 19% higher than that of IR 28 (at 6 dS/m) and At 353 (at 8 dS/m) respectively (Table 1). Since SOD controls the concentration of oxygen radicals and its derivatives, it is defined as an essential antioxidant enzyme of plants, which protects chloroplasts from ROS and oxidative damage. The concentration of active oxygen was likely to be increased in plants suffering from salt stress. If the variety failed to adapt, the active mechanisms might soon be overburdened and would result in photo oxidative damage. This would be possible in IR 28 at 6 dS/m. SOD activity at seedling and tillering stages did not vary significantly among varieties (Table 1).

Generally, SOD activity increased during tillering and flowering stages in Pokkali and At 353 except in IR 28 at tillering stage with increasing salt levels. However, Pokkali and At 353 had higher SOD activity during flowering stage compared to tillering stage at all salt levels (Table 1). Manganese SOD has been demonstrated to increase dramatically in plant cells under stress as a specific defense against oxidative stress generated in mitochondria (Racchi *et al.,* 2001). The enzyme activity was a result of both synthesis and degradation. Increase in net SOD activity under salinity might be ascribed to increased synthesis of SOD and or decreased degradation. The highest SOD activity, 71%, was

observed in Pokkali during the flowering stage at 8 dS/m. It seems probable that tolerant phenotypes may have higher potential of the SOD enzyme than a sensitive one. Transgenic plants over expressing SOD have also shown increased resistance to oxidative stress and SOD gene expression seems to be modulated by the rate of oxy radical formation (McKersie *et al.,* 1999). In IR 28 and At 353, the SOD activity was reduced by 27% (at 6 dS/m) and 21% (at 8 dS/m) respectively at tillering stage (Table 1). This could diminish the ability to scavenge oxygen radicals favoring an accumulation of oxygen radical species, which could cause membrane damage to At 353 and IR 28. At 353 showed symptoms of oxidative damage at 8 dS/m similar to IR 28 and all plants of At 353 were dead in that condition before the next sampling. Lee *et al*. (2001) have also observed that NaCl increased SOD activity in rice seedlings under salt stress.

Table 1. The effect of salinity treatments on superoxide dismutase (SOD) activity in rice (*Oryza sativa* L.) leaves at different growth stages.

Growth stage	EC (dS/m)	Superoxide dismutase activity (Units x 10^3/g FW)		
		Pokkali	**At 353**	**IR 28**
Seedling	Control	5.12[a]	4.46[a]	3.21[a]
	4	5.70[a]	4.52[a]	3.90[a]
	6	6.01[b]	4.60[a]	3.34[a]
	8	6.49[b]	4.11[a]	*
	CV (%)	12.36	9.08	14.37
Tillering	Control	5.34[a]	4.97[a]	3.67[a]
	4	6.32[a]	4.99[a]	3.70[a]
	6	6.41[a]	5.30[b]	3.51[a]
	8	7.10[b]	4.41[a]	*
	CV (%)	10.32	11.24	12.52
Flowering	Control	6.02[a]	4.32[a]	4.13[b]
	4	7.92[b]	6.72[c]	5.69[c]
	6	8.10[c]	5.48[b]	3.01[a]
	8	10.32[d]	*	*
	CV (%)	11.25	13.21	12.27

Note: Values are means of nine observations. Means with the same letter in a column are not significantly different at $P < 0.05$ for the same parameters. * missing data.

Catalase activity

The elevated SOD activity without an accompanying increase in the ability to scavenge H_2O_2 can result in enhanced toxicity by the even more destructive hydroxyl radical generated from H_2O_2. The two main scavengers of toxic H_2O_2 are CAT and POD, which eliminate H_2O_2 by converting it into H_2O by a subsequent reaction. The constitutive level of CAT was significantly higher in Pokkali (62%) compared to their counterparts (IR 28 and At

353) at seedling stage (Fig. 1a). During tillering and flowering stages, CAT activity was increased significantly in all varieties (Figs 1b and c). Overall, Pokkali maintained higher levels of CAT activity compared to the other two varieties at all salt levels and growth stages (Figs 1a, b and c). The decline in CAT activity in At 353 and IR 28 would favor the accumulation of H_2O_2. Also, this decrease in CAT activity could be due to the inactivation by the accumulated H_2O_2 induced by salt stress and could be explained partly by the inactivation of the enzyme. Therefore, CAT plays a significant role in scavenging H_2O_2 and maintaining salt tolerance.

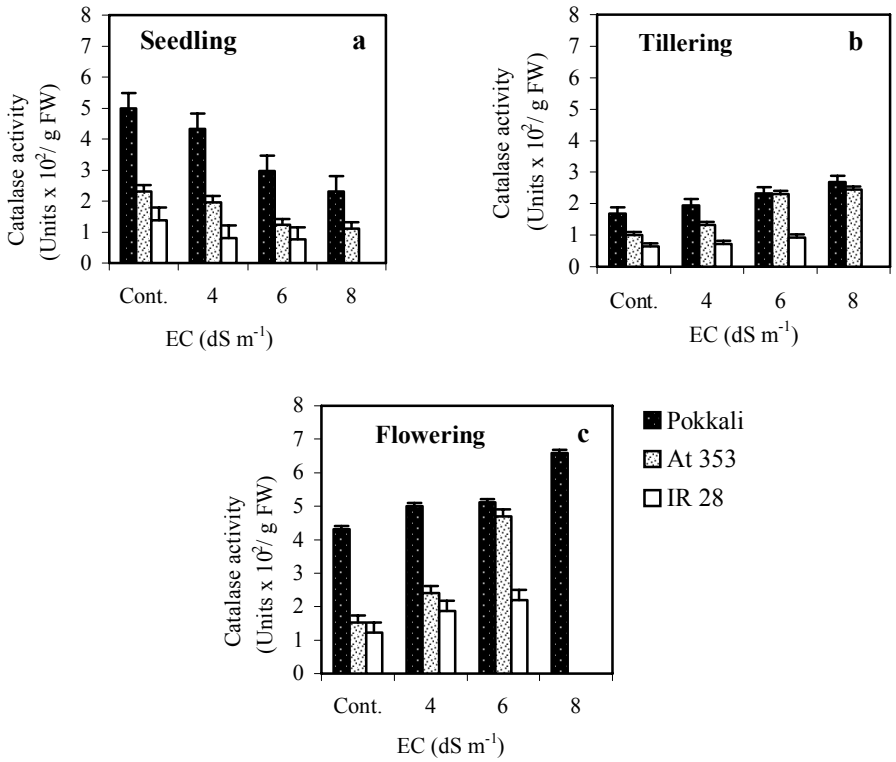

Fig. 1. Catalase activity in rice (*Oryza sativa* L.) leaves under different salt treatments at different growth stages.

Peroxidase activity

The second enzyme responsible for deactivation of H_2O_2 is peroxidase. POD activity increased significantly in IR 28 and At 353 grown at 4 and 6 dS/m during the seedling stage compared to Pokkali (Fig. 2a). It ranged from174% in At 353 to 116% in IR 28 compared to Pokkali at 6 dS/m (Figs 2a, b and c).

In contrast, POD activity in Pokkali decreased not significantly (P>0.05) at all salt levels and all growth stages (Figs 2a, b and c). Peroxidase (POD) catalyses hydrogen peroxide dependence of substrates (RH2) according to the general Equation 1,

$$RH_2 + H_2O_2 \text{ ----> } 2H_2O + R$$

Elevated H_2O_2 concentrations could release POD from membrane structures with which it is normally associated. POD activity increased in the sensitive variety compared to the highly tolerant variety with increasing salt levels. During the flowering stage, POD activity of IR 28 was increased by 432% compared to Pokkali at 6 dS/m (Fig. 2c). It is not clear whether the observed increase in POD activity in the sensitive variety under salt stress was due to increased activity of POD encoding genes or an increased activation of already existing pre enzymes. The higher increase in POD activity reflects severe salt stress and this could be an "early warning" marker of an incipient injury stage (Peiris *et al*., 1991).

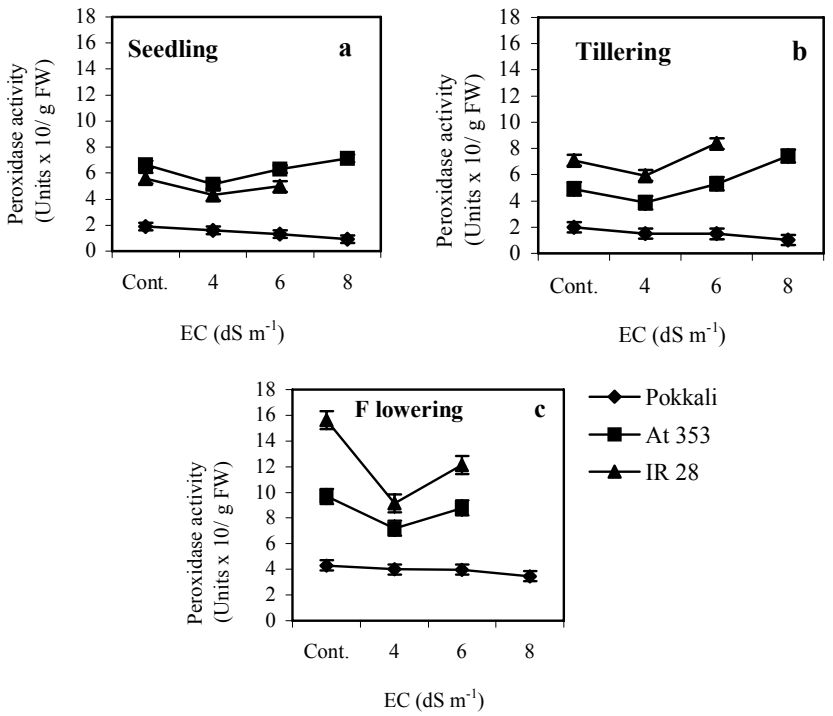

Fig. 2. Peroxidase activity in rice (*Oryza sativa* L.) leaves under different salt treatments at different growth stages.

The salt induced higher constitutive level of POD activity in IR 28 and At 353 compared to the highly tolerant variety indicates that the relatively sensitive and moderately tolerant varieties had a capacity for the decomposition of H_2O_2 generated by SOD.

The role of POD is complicated by their involvement in diverse physiological functions (Lee and Lin, 1995). However, increased POD activity might be a useful

adaptation under conditions requiring prevention of peroxidation of membrane lipids to a certain extent.

Lipid peroxidation of cell membrane

The extent of damage to the membrane was monitored by measuring the amount of thiobarbuteric-acid-reactive material (MDA) produced when polyunsaturated fatty acids in the membrane undergo peroxidation, by generating changes in unsaturated fatty acids that affect membrane structure and properties. With increasing salt level, the MDA content also was increased in the sensitive and moderately tolerant varieties (Table 2).

Table 2. The effect of salinity treatments on lipid peroxidation in rice (*Oryza sativa* L.) leaves at different growth stages.

Growth stage	EC (dS/m)	Lipid peroxidation (μm MDA x 10^3 g^{-1} fresh weight)		
		Pokkali	**At 353**	**IR 28**
Seedling	Control	8.45a	21.61a	31.61a
	4	9.26a	27.34a	39.73b
	6	9.47a	39.10b	46.80c
	8	11.31a	44.15c	*
	CV%	8.64	13.58	9.87
Tillering	Control	6.43a	16.10a	20.23a
	4	6.90a	21.32b	24.10a
	6	10.12b	29.25b	32.36b
	8	13.33b	33.74c	*
	CV%	12.45	11.21	10.32
Flowering	Control	5.11a	24.74a	34.12a
	4	8.69a	29.30a	51.30b
	6	8.99a	45.67b	62.16c
	8	11.43a	*	*
	CV%	9.27	9.61	10.29

Note: Values are means of nine observations. Means with the same letter in a column are not significantly different at $P < 0.05$ for the same parameters. * missing data.

On the other hand, Pokkali did not exhibit this increase significantly with increasing salt level (Table 2). The highest MDA content was observed in At 353 and in IR 28 during the flowering stage at 6 dS/m. This significant increase ranged from 82% in At 353 to 84% in IR 28 compared to control at flowering stage (Table 2). Salt sensitive variety may have also brought about an increase in membrane permeability or loss of membrane integrity. High concentrations of the various antioxidants, either constitutive or induced, in the highly tolerant variety could well explain the lesser amount of lipid peroxidation.

Since IR 28 exhibited an initial increase in lipid peroxidation above the levels observed in Pokkali, it might be argued that the sensitive variety has to devote more of its resources towards membrane repair than the tolerant varieties.

Effect of salinity on chlorophyll content and rate of photosynthesis (PR)

The total chlorophyll decreased significantly with increasing salinity. However, at 4 dS/m, it increased in values compared to the control in all varieties. Highly tolerant variety, Pokkali maintained high leaf chlorophyll content even after exposure to high salt treatment (Fig. 3).

The observed higher PR in Pokkali is well related with the maintenance of high total chlorophyll content even after imposed high salinity. Its percentage reduction was only 20% compared to 39% (At 353) and 64% (IR 28) at 8 dS/m and 6 dS/m respectively (Fig. 3). This indicates the lesser breaking down of chlorophyll molecules in Pokkali under high salinity. This also may be due to the inherent ability of the variety for salt tolerance.

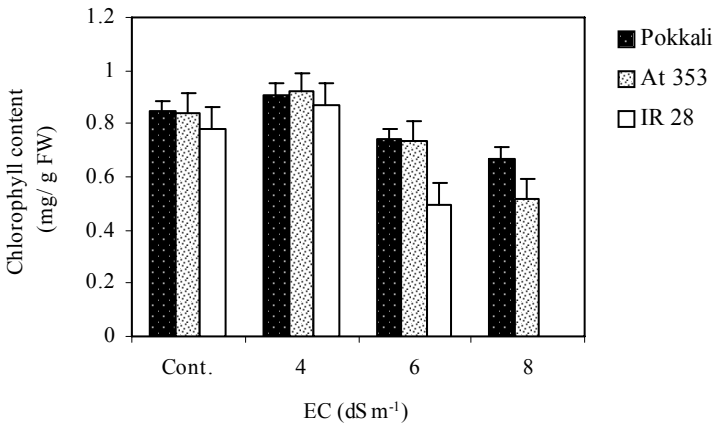

Fig. 3. Effect of salinity on chlorophyll content of rice (*Oryza sativa* L.) varieties in response to different salt treatment.

The decrease in total chlorophyll with increasing salt stress had severe impact on the rate of photosynthesis in all varieties at all sampling dates (Fig. 4). At the end of the salinization period, the salt level 4 dS/m significantly ($P > 0.05$) reduced the PR of Pokkali by 14%, At 353 by 18% and IR 28 by 24% compared to the control. However, it is considered that high salinity causes breakdown of chlorophyll molecules, because of the accumulation of toxic ions such as Na^+ and Cl^- (Brugnoli and Lauteri, 1991). Hence, the rate of photosynthesis was decreased significantly in moderately tolerant and sensitive varieties compared to the highly tolerant variety.

In chloroplasts of higher plants, photoreduction of molecular oxygen is an unavoidable process, which induces superoxide anion radical production. Also, the change in chlorophyll content suggests that a combination of antioxidant compounds and/ or

enzymes resulted in a greater protection of the photosynthesis system of the plant against toxic oxygen compounds as the plant developed. The disruption of the membrane system, as well as the direct effects on photosynthetic components and the integrity of thylakoid membranes might explain the salt induced declines in rate of photosynthesis (Gossett *et al.,* 1996). Although, CAT is apparently absent in the chloroplast, the POD decomposes H_2O_2 by oxidation of co substrate such as phenolic compounds (Lee and Lin, 1995).

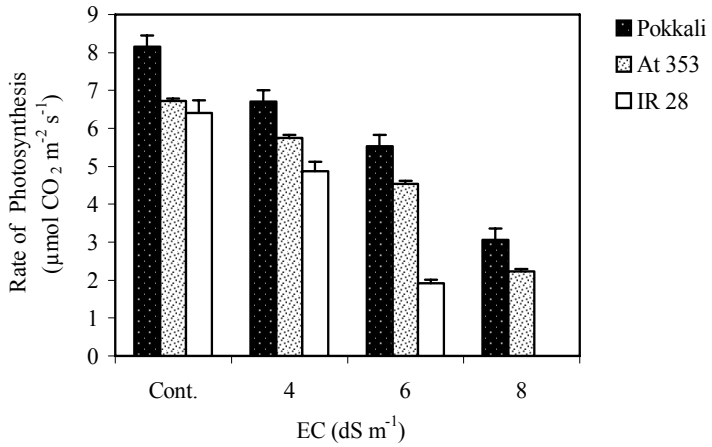

Fig. 4. Effect of salinity on rate of photosynthesis of rice (*Oryza sativa* L.) varieties in response to different salt treatment.

The reduction of chlorophyll content may have been responsible for the reduction in rate of photosynthesis in all varieties at all sampling dates. Also, the allocation of photosynthetic products during the early plant development could have an important implication on the overall plant growth under salinity. Since, there was reduction in chlorophyll content and PR with increasing salt levels, it is possible that salinity would cause significant reduction in yield.

CONCLUSIONS

Many environmental stresses cause damage to plants directly or indirectly through formation of reactive oxygen species. Among the three rice (*Oryza sativa* L.) varieties, Pokkali showed better enzymatic defense mechanisms against harmful H_2O_2 and other free radicals by way of elevated levels of enzymatic activity (antioxidants) of SOD and CAT than At 353 and IR 28. The increased tolerance was correlated with increased activities of some antioxidant systems. Moderately tolerant variety (At 353) showed a slight stimulation in SOD and CAT activities similar to that of Pokkali at 4 dS/m. However, At 353 and IR 28 had higher POD activity than the highly tolerant variety.

At 353 and IR 28 had higher content of MDA than Pokkali, and this was significantly increased with increasing salt levels. Induced salinity had significant effect on chlorophyll content and rate of photosynthesis in all varieties at all growth stages.

Therefore, it seems that SOD was the main antioxidant enzyme in highly tolerant varieties while POD played this role in moderately tolerant and sensitive varieties. CAT plays an intermediate role between SOD and POD in all three varieties. Such enhanced activity of antioxidant systems plays a role in the ability of plants to withstand high salt stress.

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APPENDICES

Appendix 1. Composition of the nutrient medium.

Compound	mmol/l
$NH_4 NO_3$	2.2
NaH_2PO_4	1.0
K_2SO_4	1.5
$CaCl_2$	2.0
$MgSO_4$	2.5
$MnCl_2.7H_2O$	0.006
$(NH_4)_6 Mo_7O_{24} 4H_2O$	0.0001
H_3BO_4	0.03
$ZnSO_4 7H_2O$	0.002
$CuSO_4 5H_2O$	0.0005
$FeCl_3 6H_2O$	0.115

Appendix 2. Composition (g dm^{-3}) of the salt mixture.

Compound	EC (dS/m)		
	4	6	8
NaCl	1.37	2.20	2.98
$CaCl_2$	0.07	0.13	0.18
KCl	0.05	0.09	0.12
$MgSO_4$	0.75	1.19	1.62