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Comparative biochemical analysis of wild introgression lines in response to short-term exposure to salinity

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The two high-yielding rice BC_2F_{6-7} (backcross populations of *O. sativa* (IR58025A/KMR3) x *O. rufipogon)* introgression lines (ILs) were used to analyze biochemical changes in response to salinity stress for 24 h on 10th day. Plants were grown in normal Hoagland's media for 10 days, treated with 150 mM NaCl for 24 h and were analyzed for chlorophyll, proline, antioxidant enzymes and sugar content. The results revealed ~two folds reduced concentrations of chlorophyll and ~three folds increased level of proline in K198 (salt-sensitive) under salt stress conditions. The salt-tolerant K478 showed chlorophyll reduction by ~one fold and proline increase by ~two folds in salt treated samples. The antioxidant enzyme activity in these contrasting introgression lines under short-term exposure to salt stress exhibited significant difference. An increased activity of superoxide dismutase and ascorbate peroxidase was recorded under salt stress conditions whereas, a decreased trend of catalase activity was observed in both the ILs. Peroxidase activity on the other hand, showed an increased trend in K478 (salt-tolerant) and decreased in K198 (salt-sensitive) under 150 mM 24 h NaCl treatment. A highly significant result was noticed in the content of total and reducing sugars and starch. The reduction over control in total sugar content was less in K478 and high reduction was recorded in K198. This further indicated possible salt tolerance of K478 in comparison to that of K198.

Key words : Introgression lines, Salinity, Total sugars, Reducing sugars, Starch, Antioxidant enzymes

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INTRODUCTION

Rice is an important cereal crops with economic agricultural important staple food especially in developing countries like India (IRRI, 2011). In fact, rice is the staple food of nearly half of the world population. It ranks third after wheat and maize in terms of worldwide production. Among the rice growing countries in the world, India has the largest area under rice crop about 45 million ha and ranks second in production next to China. Rice is the most important crop of India and it occupies 23.3 per cent of gross cropped area of the country. The average rice yield in India is only 2.09 t/ha, as compared to 6.58 t/ha in Japan and world average of 3.91 t/ha. The major inhibitors of increasing rice production are biotic and abiotic stresses and also soil health. In order to meet the ever growing population and food demand, rice production needs to be increased. Statistically, around 8.6 million ha cultivated land in India has been affected by salt, which contributes to the major part of the soil problems (Sahi *et al.*, 2006) by causing considerable reductions in yield. Rice is one of the important crops cultivated in these salinity affected soils. Salinity affects various stages of the rice plants like seed germination, seedling, vegetative and reproductive stages eventually causing economic and quality loss of rice yield (Sairam and Tyagi, 2004). Different mechanisms have been developed by the plants like rice to adopt or counteract the adverse effects of salt stress like high accumulation of osmolytes, polyamines and antioxidant defensiveness.

The salt stress responses may depend on various factors like salt concentration, salt forms and mainly on the elite genotypes. In this regard, many rice cultivars and elite lines characterized by tolerance to abiotic stresses have been developed through molecular breeding and transgenic approaches. For instance, the *codA* gene for choline oxidase from *Arthrobacter globiformis*, was transformed and the transgenic plants exhibited the ability to synthesize betaine

and confer enhanced tolerance to salt (Sakamoto and Murata, 1998). Many such genes identified for abiotic stress tolerance were tested in transgenic technologies and their role associated with biosynthesis of osmolytes, antioxidants, carbohydrates and ion homeostasis were reported (Kavi Kishor *et al*., 2005; Sangam *et al*., 2005).

In this direction, some of the biochemical constituents like chlorophyll, proline, soluble carbohydrates and antioxidant enzymes were affected by increasing concentrations of salt stress (Ali *et al*., 2004). Hakim *et al*. (2014) also reported that the chlorophyll content, proline, sugar content, soluble protein and free amino acid of many genotypes were significantly influenced by different salinity levels. Report by Kura-Hotta *et al*. (1987) on rice seedlings showed that the chlorophyll content decreases during senescence. Potato leaves showed a significant decline in chlorophyll content with increasing water deficit (Nadler and Bruvia, 1998). A decreased biosynthesis of chlorophyll and inefficiency in photosynthesis was also reported (Munns, 2002; Lichtenthaler *et al*., 2005). Like-wise, many reported on the role of proline in osmotic adjustment and detoxification of ions in plants under salt stress conditions (Kavi Kishor *et al*., 2005; Ashraf and Foolad, 2007). Interestingly, it has been reported in few studies that 50 per cent of the total osmotic potential is contributed by sugars under saline conditions (Cram, 1976).

Like-wise, an evidence of alleviation of oxidative damage, an increased salinity tolerance and other abiotic stresses are repeatedly correlated with anti-oxidative system (Acar *et al*., 2001; Sato *et al*., 2001; Bor *et al*., 2003). The antioxidant enzyme activity gets expedited under saline conditions and its influence on rice cultivars are well studied and reported (Dionisio-Sese and Tobita, 1998). High salt concentration alters the production of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD) and these parameters would serve as better indicators to understand the mechanism of salinity tolerance.

However, the crop improvement for salt tolerance is still remained elusive due to the fact that salt stress affect the plants biochemically (Murphy and Durako, 2003; Cuartero *et al.*, 2006) more intensely at cellular level. Hence, screening the elite wild-type derived rice seedlings for salt-stress tolerance through various biochemical parameters would help to facilitate the crop improvement. Biotic and abiotic stress tolerance has been recorded in *O. rufipogon* and *O. nivara* which are the wild genotypes and serve as important genetic resources (Brar and Khush 2002; Fu and Xue, 2010). The present study using wild introgression lines, various biochemical parameters like chlorophyll, proline, total sugars, reducing sugars, starch and antioxidant enzyme activity were investigated. The high-yielding KMR3 rice introgression lines (K478 - salt tolerant and K198 – salt sensitive) has revealed significant differences under short-term exposure to NaCl.

RESEARCH METHODOLOGY

Plant material and salinity treatments :

An advanced high-yielding backcross populations of *O. sativa* (IR58025A/KMR3) with *O. rufipogon* (BC_2F_{6-7}) *viz.*, K478 (salt-tolerant) and K198 (salt-sensitive) were used. These introgression lines (ILs) were analyzed for salt tolerance at early seedling stage under control and short-term exposure (24 h) to 150 mM NaCl treatments. The seeds were sown and grown in normal Hoagland's media for 10 days continuously and exposed to 150 mM NaCl hydroponic solutions for 24 h, by maintaining a separate control and salt treated batches. The biochemical parameters like chlorophyll, proline, total sugars, reducing sugars, starch and antioxidant enzyme activity were measured in these samples.

Chlorophyll estimation :

Chlorophyll content was analyzed by using fresh leaf samples of K478 and K198 control and short-term NaCl (24 h) treatments. The pigment was extracted using 80 per cent acetone (Palta, 1990). Fresh samples of leaves were analyzed for pigment contents. The samples (1.0 g) were ground to fine powder in liquid nitrogen in pestle and mortar. Around 3 ml of chilled 80 per cent acetone was added and incubated overnight. This extract was centrifuged at 3000 g for 10 min after incubation and the supernatant was measured at an optical density (OD) of 645 and 663 nm.

Proline estimation :

The control and 150 mM NaCl treated samples of K478 and K198 were collected on 10^{th} day after short-term exposure for 24 h to estimate the proline content. The samples were weighed (1.0 g) and homogenized in 1 ml of 3 per cent aqueous sulfo-salicylic acid and centrifuged at 7000 g for 12 min to remove the debris. The supernatant was mixed with 1ml of acid ninhydrin and 1 ml of glacial acetic acid. The mixture was then heated to 100°C in a water bath for 1 h. The reaction was stopped by removing the tubes from hot water bath and placing them on ice bath immediately. An equal volume of toluene was added to the mixture and vortex for 15 to 20 s. The chromophore formed was aspirated from the aqueous phase and the absorbance was measured at 520 nm by using the spectrophotometer (Bates *et al*., 1973).

Total, reducing sugars and starch estimation :

The seedlings of control and short-term (24 h) 150 mM NaCl treatment were oven dried at 70°C for 24 h, homogenized in 80 per cent ethanol and incubated in water bath at 80°C for 30 min. The homogenate was centrifuged at 3000 g for 5 min and washed twice with H_2O at room temperature. Each sample was re-suspended with 3 ml H_2O and boiled for 2 h. Total

sugar content was estimated calorimetrically using phenol sulphuric-acid method described by DuBois *et al*. (1956) and reducing sugars, starch by Nelson-Somogyi method as described by Oser (1979).

Antioxidant enzyme activity :

Enzymes extraction :

Enzyme extracts for superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POX) were prepared by freezing the freshly weighed leaf samples (1 g FW) of the K478 and K198 control and short-term (24 h) 150 mM NaCl treated seedlings in liquid nitrogen to prevent proteolytic activity and grounded with 10 ml extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA in case of SOD, CAT and POX and 0.5 mM EDTA and 1 mM ascorbic acid in case of APX. The mixture was passed through four layers of cheesecloth, filtered and centrifuged for 20 min at 15000 Xg and the supernatant was used as enzyme extract.

Superoxide dismutase (SOD) :

SOD activity was estimated by recording the decrease in optical density of formazone made by superoxide radical and nitro-blue tetrazolium dye by the enzyme (Dhindsa *et al.*, 1981). Three ml of the reaction mixture contained 13.33 mM methionine (0.2 ml of 200 mM), 75 µM nitroblue tetrazolium chloride (NBT) (0.1 ml of 2.25 mM) 0.1 mM EDTA (0.1 ml of 3 mM), 50 mM phosphate buffer (pH 7.8) (1.5 ml of 100 mM), 50 mM sodium carbonate (0.1 ml of 1.5 M), 0.05 to 0.1 ml enzyme and 0.9 to 0.95 ml of water (to make a final volume of 3.0 ml). The reaction was initiated by the addition of 2 µM riboflavin (0.1 ml) and placing the tubes under 15 W fluorescent lamps for 15 min. A complete blank reaction mixture, which gave the maximal color served as control. Transferring the tubes to dark stopped the reaction. A non-irradiated reaction mixture served as a blank (Yu and Rengel, 1999). The complete reaction mixture with KCN 3 mM (0.1 ml of 90 mM solution) was used to inhibit Cu/Zn-SOD. The reaction mixture with 3 mM KCN (0.1 ml of 90 mM solution) and 5 mM H_2O_2 (0.1 ml of 150 mM solution) inhibited both Cu/Zn-SOD and Fe-SOD activities. The absorbance was recorded at 560 nm and one unit of enzyme activity was expressed per min per g fresh weight.

Ascorbate peroxidase (APX) :

Ascorbate peroxidase was assayed by recording the decrease in optical density due to ascorbic acid at 290 nm (Nakano and Asada, 1981). The 3 ml reaction mixture contained a mixture of 50 mM potassium phosphate buffer (pH 7.0) (1.5 ml of 100 mM), 0.5 mM ascorbic acid (0.5 ml of 3.0 mM), 0.1 mM EDTA (0.1 ml of 3.0 mM), 0.1 mM H_2O_2 (0.1 ml of 3.0 mM), 0.1 ml enzyme and water 0.7 ml (to make a final volume of 3.0 ml). The reaction was initiated with the addition of 0.2 ml of hydrogen peroxide. The absorbance was measured at 290 nm in an UV-visible spectrophotometer. The initial and final contents of ascorbic acid were calculated by comparing with a standard curve drawn with the known concentrations of ascorbic acid. Enzyme activity was calculated as concentration of ascorbic acid oxidized (initial reading – final reading = quantity of ascorbic acid oxidized)/min/g fresh weight.

Catalase (CAT) :

The 3.0 ml reaction mixture consisted of potassium phosphate buffer 50 mM (1.5 ml of 100 mM buffer, pH 7.0), hydrogen peroxide 12.5 mM (0.5 ml of 75 mM H_2O_2), enzyme 50 µl and water to make up the volume to 3.0 ml. Addition of H_2O_2 initiated the reaction and decreased the absorbance at 240nm, which was recorded for 1min. The initial and final contents of hydrogen peroxide were calculated by comparing with a standard curve drawn with the known concentrations of hydrogen peroxide (Aebi, 1984). Enzyme activity was calculated as reduction in concentration of hydrogen peroxide (initial reading – final reading = quantity of hydrogen peroxide reduced)/min/g fresh weight.

Peroxidase (POX) :

Reaction mixture included phosphate buffer (50 mM, pH 6.1) 1.0 ml of 100 mM, guaiacol (16 mM) 0.5 ml of 96 mM H_2O_2 (2 mM) 0.5 ml of 12 mM, enzyme 0.1 ml and water 0.4 ml to make final volume of 3.0 ml. Absorbance was recorded at 470 nm and enzyme activity was calculated as per extinction co-efficient of its oxidation product, tetra-guaiacol 26.6/mM/cm. Enzyme activity was expressed as µmol tetra-guaiacol formed per min per g fresh weight (Castillo *et al*., 2004).

Statistical analysis :

One way analysis of variance (ANOVA) was performed on all biochemical parameters of chlorophyll, proline, total-reducing sugars, starch and antioxidant enzyme activity using SAS 9.3 software and the statistical significance of the parameter means were determined by performing the Fisher's LSD test.

RESEARCH FINDINGS AND ANALYSIS

Salt stress, one of the most serious problems in agriculture, has the disruptive effect on crop production. To overcome this constraint, improvement of crop for salt-tolerance is important in addition to reclamation of such soils through various methods. Many efforts has been attempted in past decades and still continued to exert all possible ways in crop improvement. In this direction, our study has demonstrated a comparative biochemical analysis between salt-tolerant and salt-sensitive introgression lines (ILs) to determine the biochemical changes in response to salinity stress.

In this study, total chlorophyll was noticeably decreased under short-term 150 mM NaCl treatment. The higher chlorophyll content was recorded in K478 introgression line and reduction over control was very less under short-term exposure to salt stress. On the contrary, K198 showed significantly lower chlorophyll content (Fig.1) under short-term NaCl treatment. The chlorophyll content reduction is due to loss of photosynthetic capacity and the inhibitory effect of accumulated ions during salt stress conditions. In rice, the chlorophyll pigments were more sensitive to salt stress in salt susceptible genotypes (Turan *et al*., 2009) not directly due to accumulation of ions but because of the hyper-sensitivity of chlorophyll-b to salinity than that of the chlorophyll-a (Mitsuya *et al*., 2004; Doganlar *et al*., 2010). Present results are in agreement with such reports where chlorophyll pigments were affected significantly under salinity stress conditions. Hence, the total chlorophyll content decreased with increased concentrations of salt in both the introgression lines with less significant reduction in K478. Like-wise, the chlorophyll a/b ratio was significant at P<0.001 for the short-term (24 h) 150 mM NaCl exposure on 10th day, but no significant trend of variations was observed. However, the results finally indicated that there was a significant influence of short-term salt stress on chlorophyll content in salt-sensitive IL K198.

Proline content increased with increasing salt concentrations in both the introgression lines. Similar results were reported even in the stem and leaves of tomato plants at 160 mM NaCl (Amini and Ali, 2005). The accumulation of proline has been reported to occur for up to three days after treatment with 200 mM NaCl in tobacco and up to 10 days under 100 mM NaCl in rice. In the present study, the short term exposure to NaCl stress exhibited ~two folds increase in the salt-tolerance of K478 whereas ~four folds in K198 (Fig.2). Proline accumulation is an important defense mechanism in higher plants under salt stress and protects chlorophyll, a photosynthetic pigment of the chloroplast. The free proline accumulation in rice genotypes has been reported to be significantly increased with increasing salinity levels (Wanichananan *et al*., 2003; Moradi and Ismail, 2007;

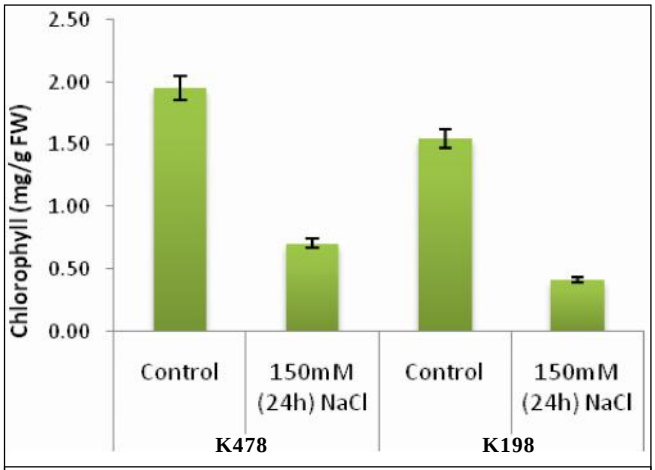

Fig. 1 : Chlorophyll content (mg/g FW) in seedlings of two rice introgression lines (ILs). *Error bars* represents the data series with 5 per cent value and each value represents mean of three replications

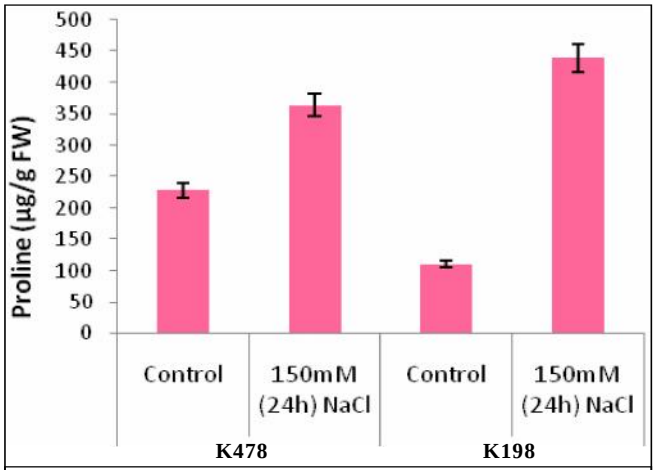

Fig. 2 : Proline content (µg/g FW) in seedlings of two rice introgression lines (ILs). *Error bars* represents the data series with 5 per cent value and each value represents mean of three replications

Table 1 : Total sugars, reducing sugars and starch content in salt-tolerant and salt-sensitive introgression lines (ILs) at seedling stage for short term exposure to 150 mM NaCl (24 h) treatment. Each value represents mean of three replications and the values in the same column below the means represent significant difference according to Fisher's LSD test

	Total sugars (mg/g DW)			Reducing sugars (mg/g DW)			Starch (mg/g DW)		
Genotypes	Control	Salt stress	Mean	Control	Salt stress	Mean	Control	Salt stress	Mean
K478	0.230	0.253	0.241	0.080	0.048	0.064	0.147	0.107	0.127
K198	0.146	0.157	0.152	0.054	0.024	0.039	0.107	0.034	0.070
Mean	0.188	0.205	0.197	0.067	0.036	0.052	0.127	0.071	0.099
LSD treatments (T)		0.003	P<0.001		0.001	P<0.001		0.003	P<0.001
LSD genotypes (G)		0.003	P<0.001		0.001	P<0.001		0.003	P<0.001
LSD (T×G)		0.007	P<0.001		0.001	P<0.001		0.007	P<0.001
CV (%)			1.246			1.962			2.076

Chutipaijit *et al*., 2009; Zahra *et al*., 2010; Hakim *et al*., 2014). Storey and Wyn Jones (1975) reported that the proline accumulation was 10-folds in shoots grown under 100 mM NaCl than in control.

Some studies in rice have reported that the sugar levels increased significantly under salinity stress conditions (Zahra *et al*., 2010). Like-wise, few cultivars of tomato showed significance difference in total and reducing sugars under salt stress conditions (Amini and Ali, 2005). Present study showed an increasing trend of total sugars under even short-term exposure of introgression lines (ILs) to 150 mM NaCl. The salt-tolerant K478 IL exhibited an increase of 10.11 per cent and K198 with 7.43 per cent of total sugars under short-term NaCl treatment (Table 1) with a co-efficient of variation of 1.246 per cent. Conversely, reducing sugars showed decreasing trend under salt-stress conditions, with 39.91 per cent reduction over control (ROC) in K478 and 55.11 per cent ROC in K198. Similar results were reported in tomato cultivars under salt stress conditions (Amini and Ali, 2005). A different trend of sugar content was observed in rice cultivars *i.e.*, an increased pattern of total sugars at 8 dS/m and decreased reducing sugars at 12 dS/m, respectively (Hakim *et al.*, 2014). Highly significant differences in reducing sugar content was observed for salinity treatments, genotypes and treatments x genotypes interaction ($P<0.001$) with co-efficient of variation of 1.962 per cent. The starch content was also measured in both the ILs after a short-term 150 mM NaCl exposure. The results inferred a decreased trend of starch content with increasing salinity stress. Though, the decrease was 26.77 per cent in K478 whereas, a drastic ROC of 68.48 per cent was recorded in K198 under short-term NaCl treatment (Table 1). Amirjani (2011) also reported a decreased content of starch in seedlings under NaCl stress conditions. Salinity treatments, genotypes and treatments over genotypes for starch content was found to be highly significant at ($P<0.001$) least significant differences (LSD).

The antioxidative response of plants in response to salinity stress has increased the activities of antioxidant enzyme activity (Lee *et al*., 2001; Rout and Shaw, 2001; Kim *et al*., 2005; Noreen and Ashraf, 2009; Wang *et al*., 2009; Tarchoune *et al*., 2010) while, Hernández *et al*. (2010) have reported decreased activities of antioxidant enzymes in *Brassica oleracea* upon short-term salt treatment. The current study of short-term salinity treatment on rice introgression lines has revealed significant difference in antioxidant enzyme activities. Superoxide dismutase (SOD) activity increased substantially at 24 h treatment of 150mM NaCl in both salt-tolerant and salt-sensitive ILs but the amount was lesser in K198 (salt-sensitive) in comparison to that of K478 (salt-tolerant) IL, suggesting possible involvement of SOD in salinity tolerance. The increase of SOD was 2.5 times more in K478 whereas, only 1.5 times higher in K198 under short-term salt treatment (Table 2).

The salt stress significantly increased ascorbate peroxidase (APX) activity over control treatment in both the ILs. The APX activity was higher in K478 with ~2.5 times over control and in K198 the increase was ~1.5 times (Table 2). The higher APX activity was reported in the leaves of salt-tolerant genotype of foxtail millet (Mandhania *et al*., 2006). Elimination of H_2O_2 molecules and its steady-state levels in tomato plants was found to be maintained by APX (Najami *et al.*, 2008).

Catalase (CAT) also has the ability to reduce H_2O_2 molecules to water but it possesses lower affinity for H_2O_2 than that of APX (Graham and Patterson, 1982). The CAT activity reduction over control was very less and not highly significant for short-term NaCl exposure, though some reports are there on enhancement of CAT activity in salt-tolerant and sensitive wheat cultivars under long exposure to salt stress conditions (Sairam *et al*., 2002).

Peroxidase (POX) activity showed contrasting trend under short-term salinity exposure in introgression lines. POX activity increased significantly upon short-term exposure to salt stress in IL K198 whereas an opposite trend of reduction was observed in K478. However, the two ILs did not show

Table 2 : Antioxidant enzyme activity in salt-tolerant (K478) and salt-sensitive (K198) introgression lines (ILs) at seedling stage for short term exposure to 150 mM NaCl (24 h) treatment. Each value represents mean of three replications and the values in the same column below the means represent significant difference according to Fisher's LSD test

Genotypes	Superoxide dismutase activity (units min^{-1} g^{-1} FW)			Ascorbate peroxidase activity (units min^{-1} g^{-1} FW)			Catalase activity (μmol H_2O_2 min^{-1} g^{-1} FW)			Peroxidase activity (? A_{470} min^{-1} g^{-1} FW)		
	Control	Salt stress	Mean	Control	Salt stress	Mean	Control	Salt stress	Mean	Control	Salt stress	Mean
K478	17.971	36.755	27.363	12.433	32.800	22.616	13.209	10.585	11.897	10.350	9.276	9.813
K198	17.645	28.008	22.826	10.986	24.000	17.493	11.949	9.538	10.744	8.120	10.210	9.165
Mean	17.808	32.382	25.095	11.710	28.400	20.055	12.579	10.061	11.320	9.235	9.743	9.489
LSD Treatments		2.191	P<0.001		1.838	P<0.001		0.152	P<0.001		0.014	P<0.001
LSD Genotypes		2.191	P<0.001		1.838	P<0.001		0.152	P<0.001		0.014	P<0.001
LSD Treatment × Genotypes		3.098	P<0.001		2.600	P<0.001		0.215	P<0.001		0.020	P<0.001
CV (%)			6.180			6.490			0.953			0.105

much variation in the activity (Table 2). Dionisio-Sese and Tobita (1998) reported a slight decrease in POX activity in the salt-tolerant rice cultivar upon increased salinity stress whereas, some other studies reported increased antioxidant enzymes in rice and cucumber in response to abiotic stress (Lee *et al*., 2001).

Conclusion :

Biochemical analysis of two wild introgression lines (ILs) of rice for short-term (24 h) 150 mM NaCl treatment has revealed noticeably significant differences. A higher concentration of chlorophyll pigment in salt-tolerant K478 and reduction over control was distinctly less under salinity stress conditions in comparison to that of salt-sensitive K198. The accumulation of osmolyte *i.e.*, proline was relevantly high in K198 under salt stress and indicated genotypic at most effort to exhibit defense mechanism. The results demonstrated that the antioxidant enzyme activity gets triggered upon short-term salinity exposure in both the introgression lines. An induction in the activity of superoxide dismutase and ascorbate peroxidase was significant. Catalase activity was reduced in both the lines. In addition, the content of total sugars was also stimulated just with short period exposure to salt stress especially in K478. The reducing sugars and starch content was noticeably decreased with 24 h salt stress on the introgression lines, though reduction was less in K478. The results further proved K478 as a salt-tolerant introgression line and would be useful in molecular breeding programs towards crop improvement.

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