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Article

Oxidative Stress Tolerance Mechanism in Rice under Salinity

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Abstract: The research was conducted to investigate comparative oxidative damage including probable protective roles of antioxidant and glyoxalase systems in rice (*Oryza sativa* L.) seedlings under salinity stress. Seedlings of two rice genotypes: Pokkali (tolerant) and BRRI dhan28 (sensitive) were subjected to 8 dSm^{-1} salinity stress for seven days in a hydroponic system. We observed significant variation between Pokkali and BRRI dhan28 in phenotypic, biochemical and molecular level under salinity stress. Carotenoid content, ion homeostasis, antioxidant enzymes, ascorbate and glutathione redox system and proline accumulation may help Pokkali to develop defense system during salinity stress. However, the activity antioxidant enzymes particularly superoxide dismutase (SOD), catalase (CAT) and non-chloroplastic peroxidase (POD) were observed significantly higher in Pokkali compared to salt-sensitive BRRI dhan28. Higher glyoxalase (Gly-I) and glyoxalase (Gly-II) activity might have also accompanied Pokkali genotype to reduce potential cytotoxic MG through non-toxic hydroxy acids conversion. However, the efficient antioxidants and glyoxalase system together increased adaptability in Pokkali during salinity stress.

Keywords: Reactive oxygen species (ROS); antioxidants; glyoxalase system; salinity; oxidative stress

Abbreviations

APX	Ascorbate peroxidase
ASA	Ascorbate
CAT	Catalase
DHAR	Dehydroascorbate reductase
Gly-I	Glyoxalase I
Gly-II	Glyoxalase II

GPX	Glutathione peroxidase
GR	Glutathionereductase
GSH	Glutathione
MDA	Malondialdehyde
MDHAR	Monodehydroascorbate dehydrogenase
MG	Methylglyoxal
NOX	NADPH; oxidases
POD	Peroxidase
ROS	Reactive oxygen species
SOD	Superoxide dismutase.

1 Introduction

Salt stress is one of the most substantial abiotic stresses limiting plant vigor, yield, and productivity [1]. More than 20% (45 million ha) of irrigated land worldwide is afflicted by salt stress, and quantity is rapidly increasing as a consequence of global warming and sea level rising [2]. Salt stress causes various alterations eventually lead to the inhibition of plant vigor and [3]. Rice is considered as one of the key cereal crops which feeds almost half of the world's total population and satisfies around 80% of their required calories. However, production of rice is harshly affected by salinity stress that cause a significant reduction in yield and productivity [4,5].

Salt stress causes osmotic stress, ionic toxicity, stomatal closure and production of reactive oxygen species (ROS) [6,3]. Accretion of ROS generation results in oxidative damage of membrane lipids, nucleic acids and proteins. ROS such as $O_2^{\bullet-}$ and H_2O_2 might be produced due to electron outflow from the photosynthetic and respiratory electron transport chains to oxygen. Salinity stress also produces ROS, such as singlet oxygen ($^1O_2$) and hydroxyl radicals ($OH^{\bullet}$) which can also cause oxidative damages to various cellular components [7]. Like ROS, methyl glyoxal (MG) is produced under salinity stress and is a potent cytotoxic compound that can act with and alter other molecules including DNA and proteins. Therefore, both ROS and MG are extremely poisonous and must be depolluted by cellular responses to persist under unfavorable growing condition [8]. To scavenge high ROS levels under salinity, an efficient system of non-enzymatic and enzymatic antioxidants is involved [9,10]. Those non-enzymatic and enzymatic antioxidants helped the plant to survive in stressful conditions. Among them, non-enzymatic antioxidants include phenolics, flavonoids, tocopherols, ascorbate (ASA), and glutathione (GSH) [11,12]. An enzymatic antioxidant include superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) as well as the enzymes of the ASA and GSH cycle: ASA peroxidase (APX), GSH reductase (GR), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR) [10].

The antioxidant enzymes such as SOD and APX play an essential part in ROS detoxification in cells. However, several rice cultivars were reported to have higher activity of ROS scavenging enzymes paralleled to the salt-sensitive rice cultivar under salinity stress. Although the defending roles of antioxidants have been widely studied in different plant species with no exception to rice, the core saline tolerant mechanism is clear yet. Salt tolerance is a multifaceted quantitative trait which is controlled by numerous genes [13], understanding the physiological mechanism of rice under salinity stress is more important to develop salt-tolerant cultivars. So, the present study was assumed to get a clear idea about antioxidant enzymes activities and other protein under saline condition in rice.

2 Materials and Methods

The experiment was conducted in the molecular breeding laboratory of Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur using two contrasting rice genotypes Pokkali (salt

tolerant) and BRRI dhan28 (salt susceptible). The seeds were collected from Bangladesh Rice Research Institute (BRRI), Joydebpur, Gazipur. Seeds were grown in a growth chamber, and five days old seedlings were transferred to greenhouse in hydroponic condition using Hoagland solution [14] as nutrient. After fifteen days of germination, the seedlings were exposed to 8 dS m^{-1} salinity stress by mixing salt with Hoagland nutrient solution. The experiment was conducted in a randomized complete block design with four biological replications. Each plastic pot contains fifteen rice plants. The seedling samples were collected for physiological and chemical analysis after seven days of applying salt stress treatment.

2.1 Determination of Chlorophyll Content

The contents of total chlorophyll (*a* + *b*), Chl *a*, Chl *b* and carotenoid were determined by homogenizing leaf samples (0.5 g), and the absorbance was taken at 663 nm and 645 nm and 470 nm, respectively by using UV-visible spectrophotometer (UV-1800, Shimadzu, Japan) following the method of Arnon [15].

2.2 Determination of Proline

Proline colorimetric determination was proceeded according to [16] based on proline's reaction with ninhydrin. 0.5 g fresh leaf tissue was homogenized in 10 mL of 3% sulfosalicylic acid in ice and centrifuged at 11,500 × g for 15 min. Two mL of the filtrate was mixed with 2 mL of acid ninhydrin and 2 mL of glacial acetic acid. After incubation at 100°C in water bath for 1 hr was cooled and 4 mL of toluene was added. The optical density of the chromophore-containing toluene was read spectrophotometrically at 520 nm using toluene as a blank. The amount of proline was determined by comparison with a standard curve.

2.3 Measurement of the $O_2^{\bullet -}$ Generation Rate

Superoxide radical was determined according to the method of [17] with slight modifications. Leaves (0.3 g) were homogenized in 3 ml of 65 mM (K-P) buffer (pH 7.8) on an ice bath and centrifuged at 4°C for 10 min at 5,000 × g. The supernatants (0.75 ml) were mixed with 0.675 ml of 65 mM K-P buffer (pH 7.8) and 0.07 ml of 10 mM hydroxylamine Chlorhydrate and incubated at 25°C. After 20 min, 0.375 ml of 17 mM sulfanilamide and 0.375 ml of 7 mM α-naphthylamine were added and incubated at 25°C for another 20 min before it was mixed with 2.25 ml of diethyl ether. The absorbance was measured at 530 nm, and the $O_2^{\bullet -}$concentration was calculated from a standard curve of $NaNO_2$.

2.4 Measurement of H_2O_2

Hydrogen peroxide was assayed according to the method described by [18]. H_2O_2 was extracted by homogenizing 0.5 g of leaf samples with 3 mL of 50 mM potassium-phosphate (K-P) buffer (pH 6.5) at 4°C and centrifuged at 11,500 × g for 15 min. Three milliliters of the supernatant was mixed with 1 mL of 0.1% $TiCl_4$ in 20% H_2SO_4 (v/v) and stored in room temperature for 10 min and centrifuged at 11,500 × g for 15 min. The optical absorption of the supernatant was measured spectrophotometrically at 410 nm to determine the H_2O_2 content and expressed as nmol g^{-1} fresh weight.

2.5 Measurement of Lipid Peroxidation

The level of lipid peroxidation was measured by estimating malondialdehyde (MDA) using thiobarbituric acid (TBA) as the reactive material following the method of [19].

2.6 Measurement of Methylglyoxal

About 0.3 g leaf tissue was extracted in 3 mL of 0.5 M perchloric acid and ice incubation for 15 min, then centrifuged at 4°C at 11,000 × g for 10 min. The supernatant was decolorized by adding charcoal (10 mg ml^{-1}), kept for 15 min at room temperature, and then centrifuged at 11,000 × g for 10 min at 20°C. Before using this

supernatant for MG assay, it was neutralized by incubating for 15 min with saturated potassium carbonate solution at room temperature and centrifuged again at 11,000 × g for 10 min. Neutralized supernatant was used for MG estimation following the method of [20] using N-acetyl-L-cysteine.

2.7 Extraction and Measurement of Ascorbate and Glutathione

Rice leaves (0.5 g fresh weight) were homogenized in 3 ml ice-cold acidic extraction buffer containing 5% meta-phosphoric acid and 1 mM EDTA using a mortar and pestle. The centrifugation of homogenates was performed at 4°C for 15 min at 11,500 × g, and the supernatant was preserved for ASA and GSH analysis. Ascorbate content was determined by the method of [21], and the glutathione pool was evaluated according to [18].

2.8 Assay of Enzymatic Activities

Leaf tissue (0.5 g) was homogenized was mixed in 1 mL 50 mM ice-cold K-P buffer (pH 7.0) containing 100 mM KCl, 1 mM ascorbate, 5 mM β-mercaptoethanol and 10% (w/v) glycerol. The centrifugation of homogenates was done at 11,500 × g for 10 min at 0–4°C and the supernatants were collected for determination of enzyme activity.

SOD (EC: 1.15.1.1): SOD activity was estimated according to [22] which was based on the xanthine-xanthine oxidase system. The reaction mixture contained KP buffer (50 mM), NBT (2.24 mM), xanthine oxidase (0.1 units), catalase (0.1 units), xanthine (2.36 mM), and enzyme extract. Catalase was added to avoid the H_2O_2-mediated possible inactivation of CuZn-SOD. SOD activity was expressed as units (amount of enzyme required to inhibit NBT reduction by 50%) min^{-1} mg^{-1} protein.

POD (EC: 1.11.1.7): POD activity was estimated according to [23]. The reaction mixture contained 25 mM K-P buffer (pH 7.0), 0.05% guaiacol, 10 mM H_2O_2 and the protein solution. The activity of POD was determined at 470 nm absorbance due to guaiacol oxidation for 1 min using an extinction coefficient of 26.6 $mM^{-1}cm^{-1}$.

CAT (EC: 1.11.1.6): CAT activity was measured according to the method of [24] by monitoring the decrease of absorbance at 240 nm for 1 min caused by the decomposition (or degradation) of H_2O_2. The reaction mixture contained 50 mM K-P buffer (pH 7.0), 15 mM H_2O_2, and enzyme solution in a final volume of 0.7 ml. The reaction was initiated with the addition of enzyme extract, and the activity was calculated using the extinction coefficient of 39.4 $M^{-1}cm^{-1}$.

APX (EC: 1.11.1.11): APX activity was assayed following the method of [25]. The reaction solution contained 50 mM K-P buffer (pH 7.0), 0.50 mM ASA, 0.10 mM H_2O_2, 0.1 mM EDTA, and enzyme extract in a final volume of 0.7 ml. The addition of H_2O_2 initiated the reaction$_2$, and the activity of APX was measured observing the absorbance at 290 nm for 1 min using an extinction coefficient of 2.8 $mM^{-1}cm^{-1}$.

GPX (EC: 1.11.1.9): GPX activity was measured as described by [26] using H_2O_2 as a substrate. The reaction mixture consisted of 100 mM sodium-phosphate buffer (pH 7.5), 1 mM EDTA, 1 mM NaN3, 0.12 mM NADPH, 2 mM GSH, 1 unit GR, 0.6 mM H_2O_2, and 5 µl of sample solution. The oxidation of NADPH was calculated by taking absorbance at 340 nm for 1 min, and the activity of GPX was calculated using an extinction coefficient of 6.62 $mM^{-1}cm^{-1}$.

GR (EC: 1.6.4.2): GR activity was measured following the method of [27]. The reaction mixture contained 0.1M K-P buffer (pH 7.8), 1 mM EDTA, 0.2 mM NADPH, 1 mM GSSG, and enzyme solution in a final volume of 1 ml. The decline in absorbance at 340 nm was recorded for 1 min and used to calculate the activity of GR using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$.

DHAR (EC: 1.8.5.1): DHAR activity was determined by the procedure of [25]. The reaction buffer contained 50 mM K-P buffer (pH 7.0), 0.1 mM dehydroascorbate (DHA) and 2.5 mM GSH. The reaction

was started with the addition of the sample solution to the reaction buffer. The change in absorbance at 265 nm estimates the activity using an extinction coefficient of 14 $mM^{-1}cm^{-1}$.

MDHAR (EC: 1.6.5.4): MDHAR activity was determined by the method of [27]. The reaction solution contained 50 mM Tris-HCl buffer (pH 7.5), 2.5 mM ASA, 0.2 mM NADPH, and 0.5 units of Ascorbate Oxidase (AO) and enzyme solution in a final volume of 0.7 ml. The addition of AO started the reaction. The activity of MDHAR was calculated from the change in ascorbate at 340 nm for 1 min using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$.

GST (EC: 2.5.1.18): GST activity was determined spectrophotometrically according to [28]. The reaction mixture 100 mM Tris-HCl buffer (pH 6.5), 1 mM 1-Chloro-2, 4-dinitrobenzene (CDNB), 1.5 mM GSH, and enzyme solution in a final volume of 0.7 ml. The addition of CDNB initiated the enzyme reaction, and the increase in absorbance was measured at 340 nm for 1 min. The activity was calculated through the extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

Gly-I (EC: 4.4.1.5): Gly-I assay was Carried out according to [29]. Briefly, the assay mixture contained 100 mM K-P buffer (pH 7.0), 15 mM magnesium sulfate, 1.7 mM GSH, and 3.5 mM MG in a final volume of 0.7 ml. The reaction was started by the addition of MG, and the increase in absorbance was recorded at 240 nm for 1 min. The activity was calculated using the extinction coefficient of 3.37 $mM^{-1}cm^{-1}$.

Gly-II (EC: 3.1.2.6): Gly-II activity was determined according to the method of [30] by monitoring the formation of GSH at 412 nm for 1 min. The reaction mixture contained 100 mM Tris–HCl buffer (pH 7.2), 0.2 mM 5,5'-Dithiobis 2-nitrobenzoic acid (DTNB), and 1 mM S-D-lactoylglutathione (SLG) in a final volume of 1 ml and the activity was calculated using the extinction coefficient of 13.6 $mM^{-1}cm^{-1}$.

Lipoxygenase (LOX, EC: 1.13.11.12): LOX activity was measured following [31]. The substrate solution was prepared by adding 35 µl linoleic acid to 5 ml distilled water with 50 µl Tween-20. LOX activity was determined spectrophotometrically by adding 10 µl of sample to 590 µl substrate solution. The increase in absorbance at 234 nm was measured for 1 min at 25°C. The activity was expressed as µM hydroperoxide formed $min^{-1}mg^{-1}$ protein using a molar extinction coefficient of 25,000 $M^{-1}cm^{-1}$.

2.9 Determination of Protein

For protein content, the Bradford reagent was prepared. Hundred milligrams of Comassie Brilliant Blue G-250 was dissolved in 50 ml 95% ethanol, and then 100 ml 85% (w/v) phosphoric acid was added and finally reagent was filtered. For protein assay, 5 µl supernatant was pipetted into test tube. Five milliliters of protein reagent was added to the test tube and the contents mixed either by inversion or vortexing. The absorbance at 595 nm was measured after 2 min [32] while Albumin from Bovine Serum (BSA) was used as standard.

2.10 Native PAGE and Activity Staining

Changes in proteins having isoenzymic activity of the ROS scavenging enzymes were studied using PAGE under non-reduced, non-denatured conditions at 4°C according to [33]. Native PAGE analysis was performed for various enzymes involved in the ascorbate-glutathione cycle on a gel (10% for SOD and 8% for others). 50 µg protein sample was run it with running buffer for 1.5 hours at 100 volt. For SOD staining 50 mM Na-P buffer containing 0.24 mM NBT with riboflavin was used for 20 minutes. Then emerged in 50 mM Na-P buffer containing 28 mM TMED appeared bands in light exposure. POD staining was done by immerging in 50 mM KP buffer (pH 7) containing 10 mM H_2O_2 and 10 mM Guaicol and shake until the band appeared. GPX staining was done by immerging gel in 1 mM GSH solution and final incubation was done by 0.008% cumine hydroperoxide for ten minutes. GR staining was done by using 100 mM Tris-HCl (pH 7.6) containing 4 mM GSSG, 1.5 mM NADPH and 2 mM NBT, after twenty minutes shaking band appeared. For NOX staining gel was immerged in 50 mM

Tris-HCl (pH 7.4) buffer containing 0.2 mM NBT; 0.1 mM $MgCl_2.6H_2O$ and 1 mM $CaCl_2$ for twenty minutes in dark. Then 0.2 mM NADPH was added in the solution and shake until the band appeared.

2.11 Histochemical Detections of $O_2^{\bullet-}$ and H_2O_2

Superoxide ($O_2^{\bullet-}$) and H_2O_2 were visualized in leaves according to the method of Rohman et al. [20] with modifications. Briefly, the second leaves were stained in 0.1% NBT or 0.1% 3,3-diaminobenzidine (DAB) solution for 8 h under dark and light, respectively. Incubated leaves were then decolorized by immersing in boiling ethanol which allowed visualization of bluish insoluble formazan (for $O_2^{\bullet-}$) or deep brown polymerization product (for H_2O_2). After cooling, glycerol was used to open the leaves and photographs were taken by placing the leaves between two transparent sheets.

2.12 Determination of Genotypic Difference

Genomic DNA from fresh leaves of 15-days-old seedlings was extracted. Five primers exhibiting good quality banding patterns and sufficient variability were selected for further analysis. Following electrophoresis, the sizes of amplification products were estimated by comparing the migration of each amplified fragment with that of known size fragments of molecular weight markers: 100 bp DNA ladder and using DNA FRAG, (version 3.03) [34]. Each distinct bands or fragments (RAPD markers) were thereby given an identification number according to their position on the gel and were scored visually based on their presence (1) or absence (0), separately for each individual and each primer.

2.13 Statistical Analysis

Data generated from this study were analyzed by STATISTIX 10 software where need. Data were analyzed following CRD design with five replications. Means were separated by Honest Significant Difference (HSD) test, and $P \leq 0.05$ was considered as the significance level. The graphs were prepared in MS Excel, 2010. Mean values ± standard error (SE) was presented in graphs from at least three independent experiments, each containing five replications.

3 Results

The rice plant growth was severely affected after 7 days of 8 dSm^{-1} salt stress. A noticeable phenotypic difference was observed in both genotypes salt susceptible BRRI dhan28 and tolerant Pokkali. Stress symptoms such as browning of lower leaves, burning of leaf tips, and also yellowing of leaves were observed more prominently in BRRI dhan28 than Pokkali (Fig. 1).

Both varieties exhibited a decreasing trend in chlorophyll pigments and an increase in carotenoid content, but pigments degradation rate was higher in BRRI dhan28 than Pokkali under salinity stress (Tab. 1). Noticeable increase in Na^+ uptake was observed in leaves, shoots, and root of the rice seedlings grown in saline condition as compared to the control of both genotypes (Figs. 2A, 2B, and 2C). Proline accumulation was significantly higher in salt tolerant Pokkali rice (Fig. 2D).

Excessive accumulation of ROS resulted in oxidative damage to the treated seedlings leading to increased lipid peroxidation and also due to cell membrane damage. Histochemical staining displayed with dark blue and brown spots, indicate over-production of $O_2^{\bullet-}$ and H_2O_2 respectively. The NaCl treated BRRI dhan28 seedlings exhibited more spots due to overproduction of $O_2^{\bullet-}$ and H_2O_2 compared to NaCl treated Pokkali. In BRRI dhan28 $O_2^{\bullet-}$ increased by 162.41% compared to Pokkali increased by 59.1% whereas, the level of H_2O_2 increased by 435.7% and 233.5% in BRRI dhan28 and Pokkali, respectively (Figs. 3A and 3B). The MDA content increased in salt-treated leaves by 113.6% in susceptible BRRI dhan28, and only 59.7% increase was found in salt tolerant Pokkali (Fig. 4C). Histochemical staining also showed higher lipid peroxidation indicated by concentrated pink-red color

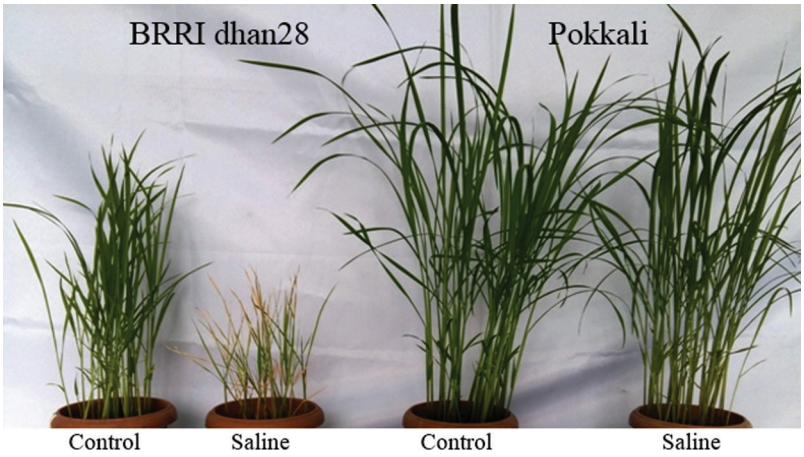

Figure 1: Phenotypic difference of seedlings after seven days salt stress

Table 1: Chlorophyll and carotenoid contents in leaf of rice seedlings under salt stress

Treatment	Chl *a*		Chl *b*		Total Chl		Carotenoid	
	BRRI dhan28	Pokkali	BRRI dhan28	Pokkali	BRRI dhan28	Pokkali	BRRI dhan28	Pokkali
Control	0.436 ± 0.006^{a}	0.325 ± 0.016^{b}	0.406 ± 0.009^{a}	0.203 ± 0.005^{b}	0.843 ± 0.014^{a}	0.529 ± 0.019^{b}	0.132 ± 0.001^{b}	0.186 ± 0.003^{a}
Saline	0.237 ± 0.009^{c}	0.277 ± 0.007^{bc}	0.195 ± 0.004^{b}	0.168 ± 0.002^{b}	0.432 ± 0.01^{c}	0.445 ± 0.008^{bc}	0.123 ± 0.001^{c}	0.181 ± 0.001^{a}

and higher loss of plasma membrane integrity specified by dark blue color in the roots of NaCl treated rice seedlings (Figs. 3C and 3D).

LOX activity was increased due to exposure in NaCl stress in both varieties, but both in stress and non-stress condition LOX value was lower in Pokkali compared to BRRI dhan28. Approximately 30% increase of LOX activity was found in salt stressed BRRI dhan28 leaf, and 29% was observed in salt stress Pokkali leaf whereas in root it's activity increased by 40.5% and 47.8% in BRRI dhan28 and Pokkali, respectively (Fig. 4D).

Under salinity stress, 50% decrease in ASA content was observed in BRRI dhan28 whereas in Pokkali the decreasing percentage was 21.95%. Dehydroascorbate (DHA) content increased in all stress treated seedlings (Fig. 5B). The ratio of ASA/DHA decreased with salt stress (Fig. 5C). GSH content significantly decreased but GSSG content increased in the salt-treated rice seedlings compared to the control (Figs. 5D and 5E). The ratio of GSH/GSSG decreased varies from 51.98% and 23.29% in BRRI dhan28 and Pokkali, respectively, compared to their control value (Fig. 5F).

In case of antioxidant enzymes activities, SOD showed higher activity in both varieties under salt stress. In leaves, SOD activity increased by 25% and 47%, compared to their control in BRRI dhan28 and Pokkali, respectively whereas, SOD activity in root increased by 28% and 39% in BRRI dhan28 and Pokkali, compared to control seedlings (Fig. 6A). A similar pattern of expression of SOD isozymes (SOD1, SOD2, and SOD3) was found in gel electrophoresis (Fig. 6B).

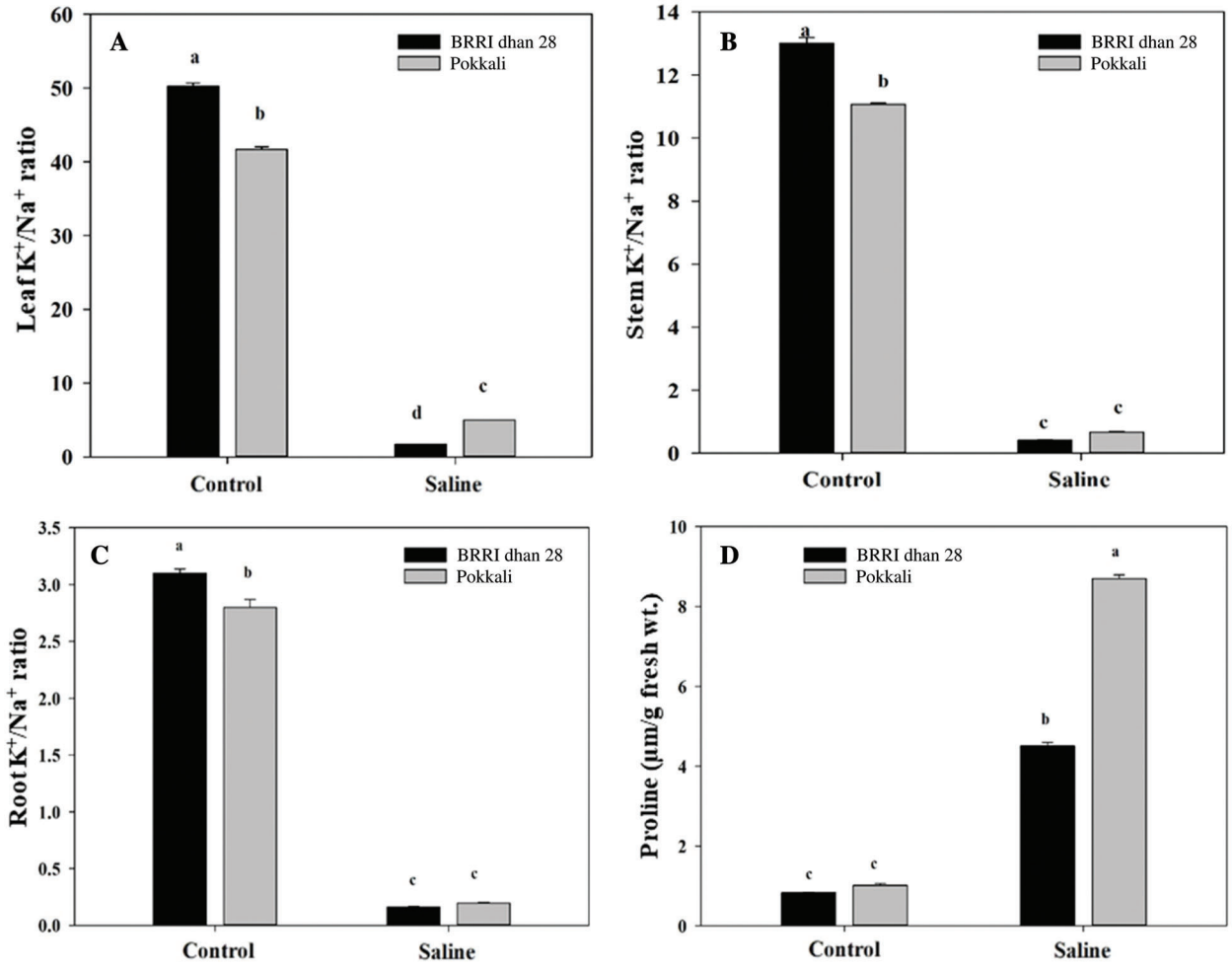

Figure 2: K^+ and Na^+ ratio in leaf (A), stem (B), root (C) and contents of proline in leaves (D) of rice seedlings under salt stress of rice seedlings

Under NaCl stress, POD activity increased by 68.37% and 248.9% in the leaves of BRRI dhan28, and Pokkali, respectively compared to control but in case of root 59% increase was found in both varieties (Fig. 6C). POD activity was approximately similar in both controls. However, in stress condition POD activity was found much higher in Pokkali leaf compared to BRRI dhan28. In root, POD activity was higher in BRRI dhan28 compared to Pokkali both in control and salt stress condition, although the percent of the increase was approximately similar (Fig. 6C). In-gel activity staining two isoforms of POD (POD1 and POD2) was found. The uppermost band showed higher intensity in Pokkali compared to BRRI dhan28 under salt stress condition, and the lower band was more prominent in salt stress condition compared to the control of both varieties (Fig. 6D).

Catalase activity increased by 19.45% in BRRI dhan28 leaf treated with NaCl stress and raised 106.5% in Pokkali leaf. In root, catalase activity increased by 39.1% and 28% in BRRI dhan28 and Pokkali, respectively (Fig. 7A). A strong alteration in CAT activity was found in the two cultivars throughout the experiment. Pokkali showed higher catalase activity in control as well as stressed seedlings as compared to BRRI dhan28. Gel analysis also showed a similar result in the band of the CAT isozymes (CAT1) (Fig. 7B).

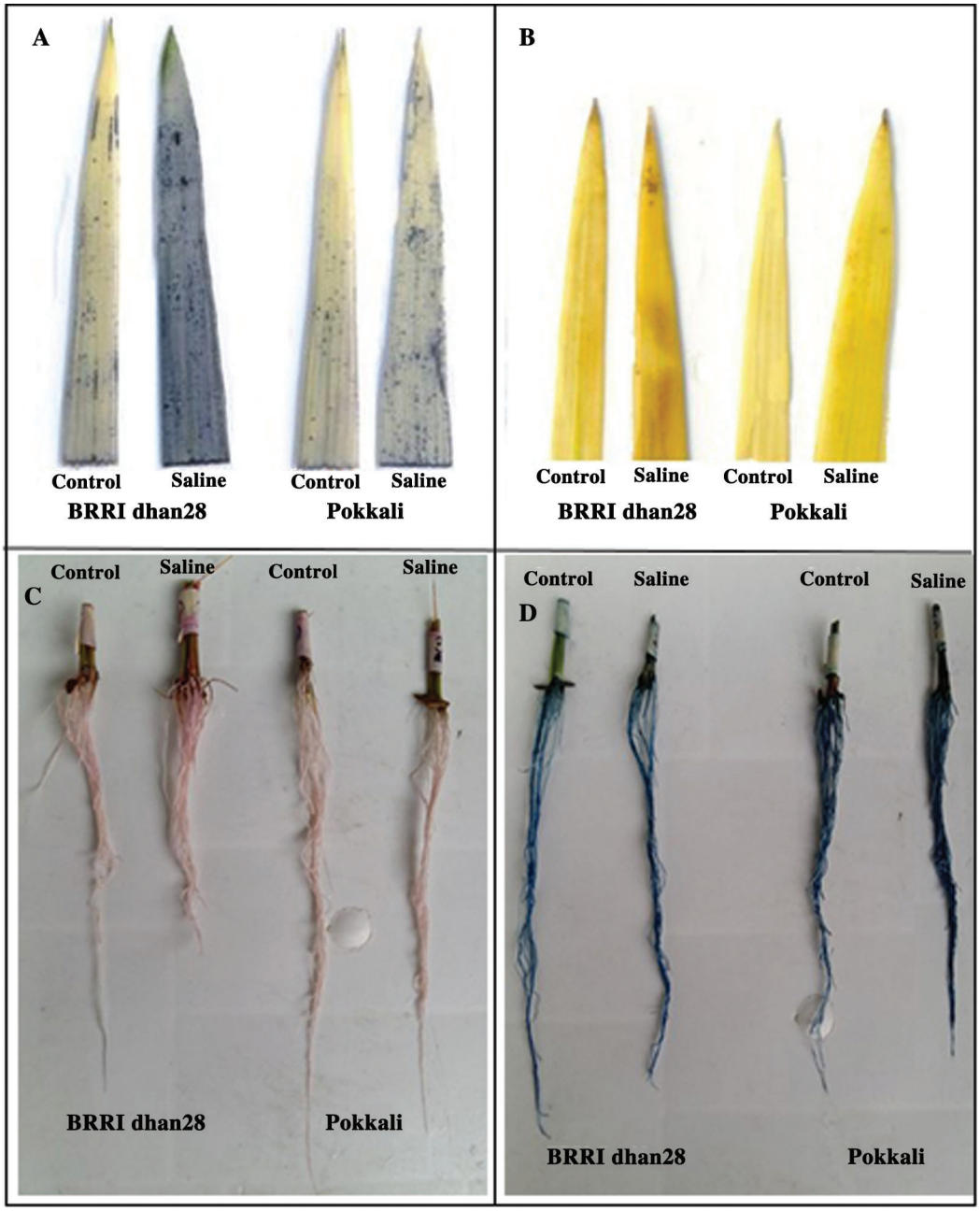

Figure 3: Histochemical detection of $O_2^{\bullet-}$ (blue dots) (A), H_2O_2 (brown spot) (B) in leaf, lipid peroxidation (C) and loss of plasma membrane integrity (D) in root of rice seedlings under salt stress

APX activity decreased sharply in leaf of both cultivars at NaCl stress. In BRRI dhan28 APX activity decreased by 6.61% and in Pokkali it was 46%. However, in root APX activity increased 39.9% and 18.4% in BRRI dhan28 and Pokkali, respectively (Fig. 7C). APX activity decreased sharply in both cultivars leaf at NaCl stress. However, in root APX activity increased 39.9% and 18.4% in BRRI dhan28 and Pokkali, respectively (Fig. 7C). Isozymes (APX1, APX2, APX3, and APX4) of APX gave similar band as the activity of APX in the gel (Fig. 7D). Compared with control, GPX activity decreased at NaCl stress by

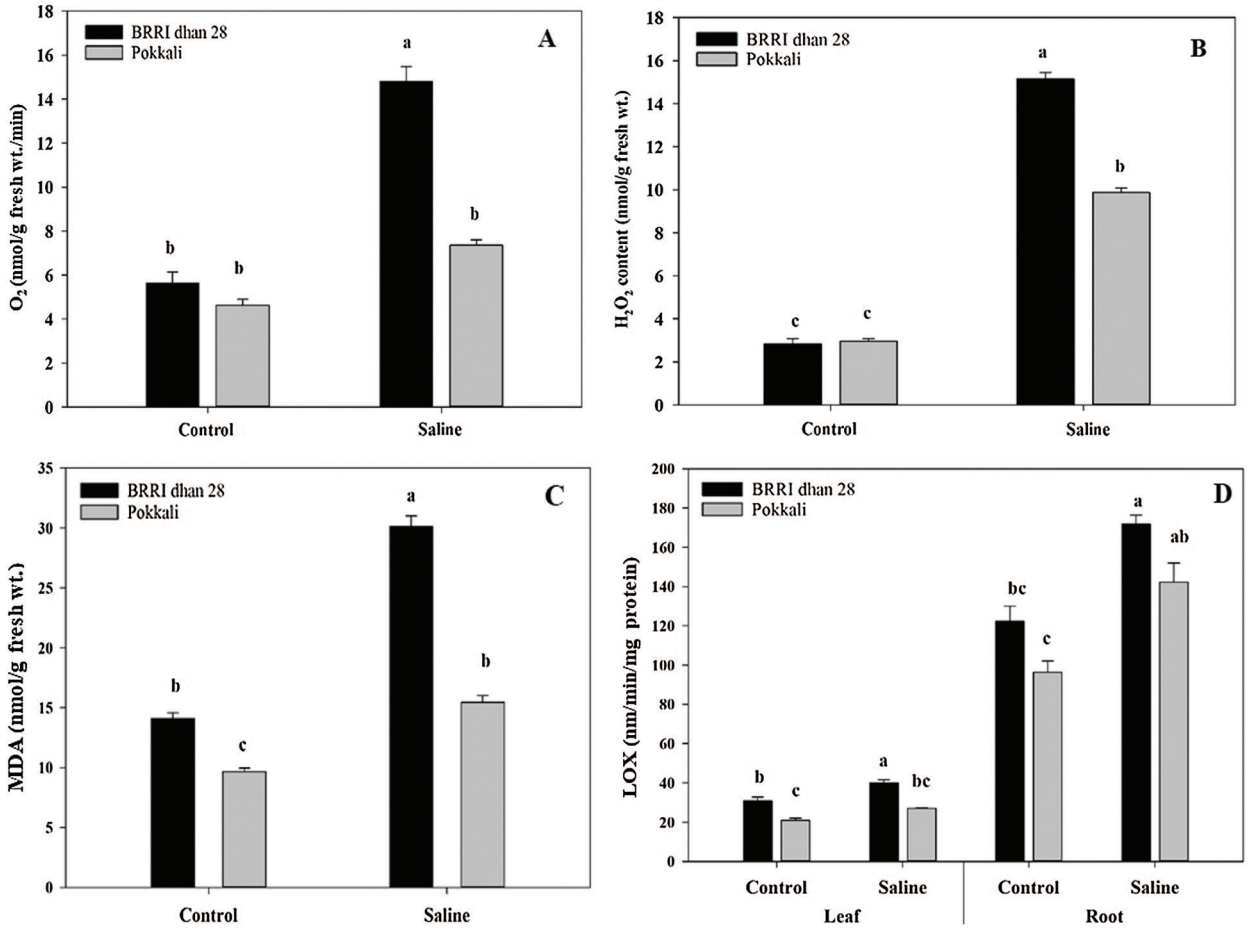

Figure 4: Generation rate of $O_2^{\bullet-}$ ion (A), contents of H_2O_2 (B), lipid peroxidation (MDA content) (C) and LOX activity (D) in leaves of rice seedlings at 7 days under salt stress with control

18% in BRRI dhan28 and 10.4% in Pokkali leaf (Fig. 8A). The similar banding pattern of GPX isozymes (GPX1) in gel electrophoresis also proved the similar activity (Fig. 8B). However, in case of root, GPX activity increased by 37.7% and 26.2% in BRRI dhan28 and Pokkali, respectively compared to their control (Fig. 8A).

Due to NaCl stress MDHAR activity increased in BRRI dhan28 and Pokkali leaf by 26.26% and 18.23%, respectively whereas, in the root, MDHAR activity increased by 12.52% and 22.59% in BRRI dhan28 and Pokkali compared with their control seedlings (Fig. 8C). DHAR activity also showed a similar increasing trend under salinity stress. In leaf, DHAR activity increased by 42.35% and 33.8% in BRRI dhan28 and Pokkali while in root it was raised by 75.16% and 90.30% in BRRI dhan28 and Pokkali, respectively (Fig. 8D).

GR activity decreased by 3.85% in BRRI dhan28 leaf. However, in salt tolerant Pokkali GR activity increased by 61.48%. In root, GR activity increased by 13.75% and 14.92% in BRRI dhan28 and Pokkali respectively (Fig. 9A). Gel electrophoresis of GR showed similar activity isozymes (GR1 and GR2). The lower band was more or less similar. However, the upper band was different in two varieties. The isoform of tolerant Pokkali in NaCl stress was more prominent, compared to susceptible BRRI dhan28 (Fig. 9B).

GST activity increased in both cultivars when exposed to NaCl stress. In BRRI dhan28, leaf GST activity increased by 9.43% and in tolerant Pokkali rice seedling GST activity increased by 39.17% compared to

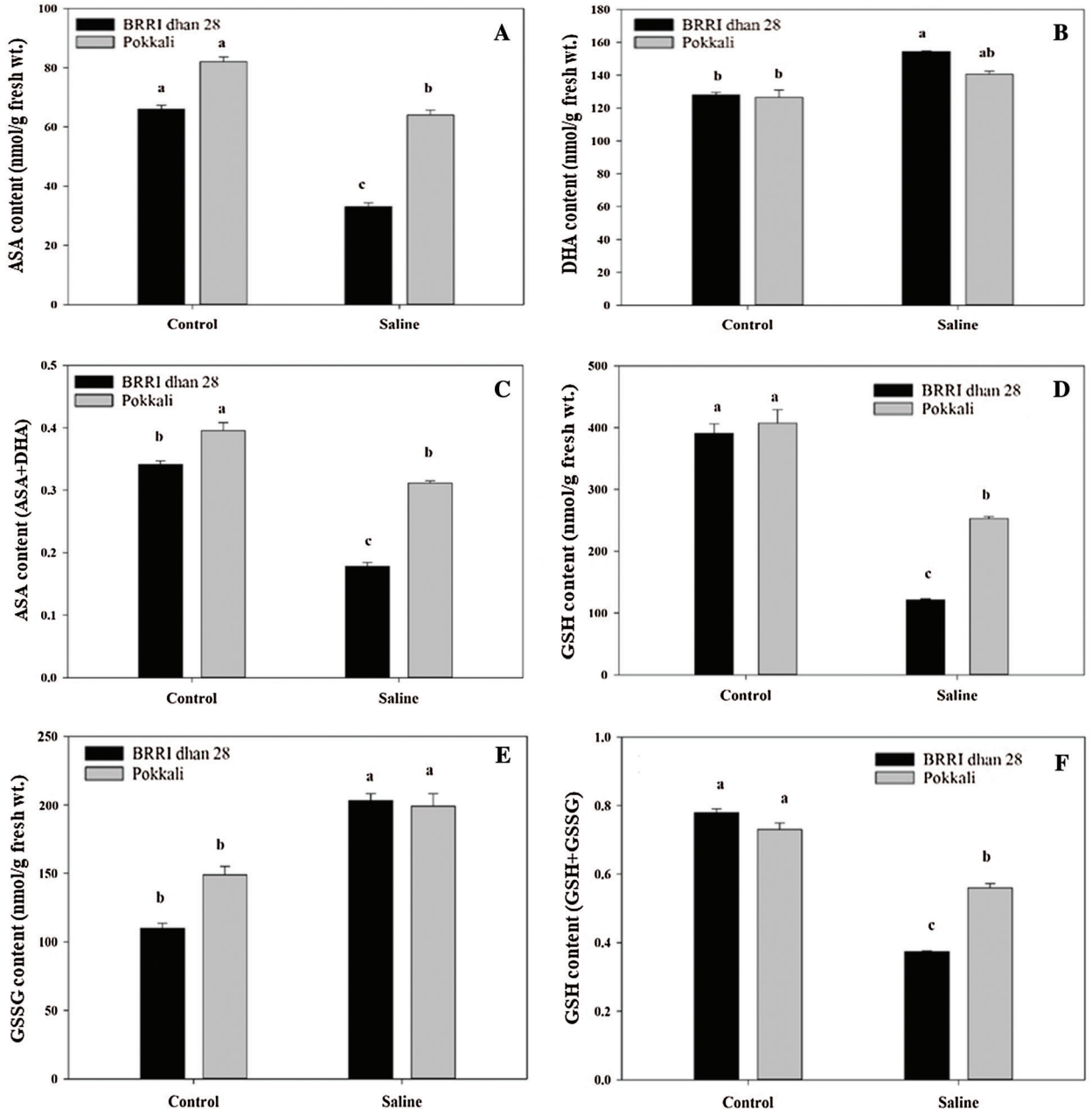

Figure 5: ASA content (A), DHA content (B), AsA/DHA ratio (C), GSH content (D), GSSG content (E) and GSH/GSSG ratio (F) in leaf of rice seedlings under salt stress

non-stressed seedlings. In root 7.81% and 3.57% increase was observed in BRRI dhan28 and Pokkali respectively (Fig. 9C). NOX is the most potent source of endogenous $O_2^{\bullet-}$ production. In gel electrophoresis, two isoforms (NOX1 and NOX2) were found (Fig. 9D). The bands of Pokkali showed higher intensity compared to BRRI dhan28. Moreover, in NaCl stress bands were more prominent, especially in Pokkali (Fig. 9D). In BRRI dhan28, 151.6% increase was found under salt stress condition; on the other hand, only 41.8% increase was found in Pokkali (Fig. 10A). Glyoxalase I and Glyoxalase II activity increased in the both NaCl stress-treated rice genotype. In leaf, Gly-I activity increased by

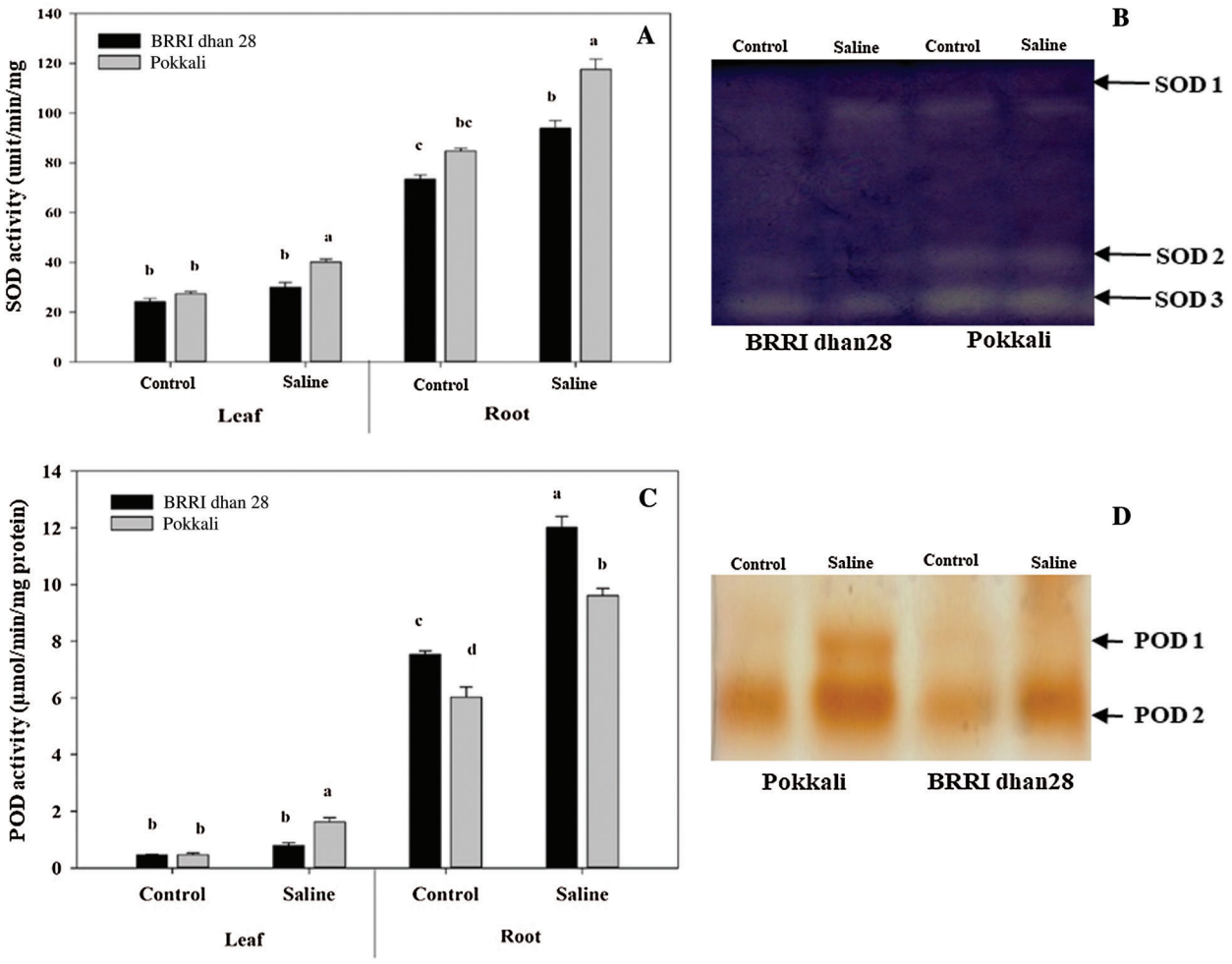

Figure 6: Activities of SOD (A), expression of SOD isozymes in gel electrophoresis (B), Activities of POD (C) and expression of POD isozymes in gel electrophoresis (D) in leaves of rice seedlings

13.69% and 55.02% in BRRI dhan28 and Pokkali, respectively and in the root, Gly-I activity increased by 12.72% and 40.89% in BRRI dhan28 and Pokkali (Fig. 10B). The Gly-II activity also showed a similar increasing pattern when they are exposed to NaCl stress. In leaf 31.1% and 27.4% increase was found in BRRI dhan28 and Pokkali, respectively but in the root increment percentage was observed 75.18% and 71.17% in BRRI dhan28 and Pokkali, respectively (Fig. 10C).

3.1 Genotypic Differences

In this study, RAPD marker was used to confirm the genetic variation among two contrasting genotypes. Five primers exhibiting good quality banding patterns and sufficient variability were selected for the study. Selected five primers (OPA-02, OPC-01, OPC-05, OPC-12, OPS-12) were found polymorphic. Different banding pattern was observed by different primers. The Noticeable difference was observed in two genotypes for RAPD band positions across five primers (Fig. 11).

The OPA-02 primer produced a total of seven bands and 28.57% polymorphism was found. One unique band was observed at 254 bp only in salt-treated Pokkali genotype. OPA-01 primer produced a total of five bands among them three was polymorphic, and 60% polymorphism was found. At 891 bp one unique band

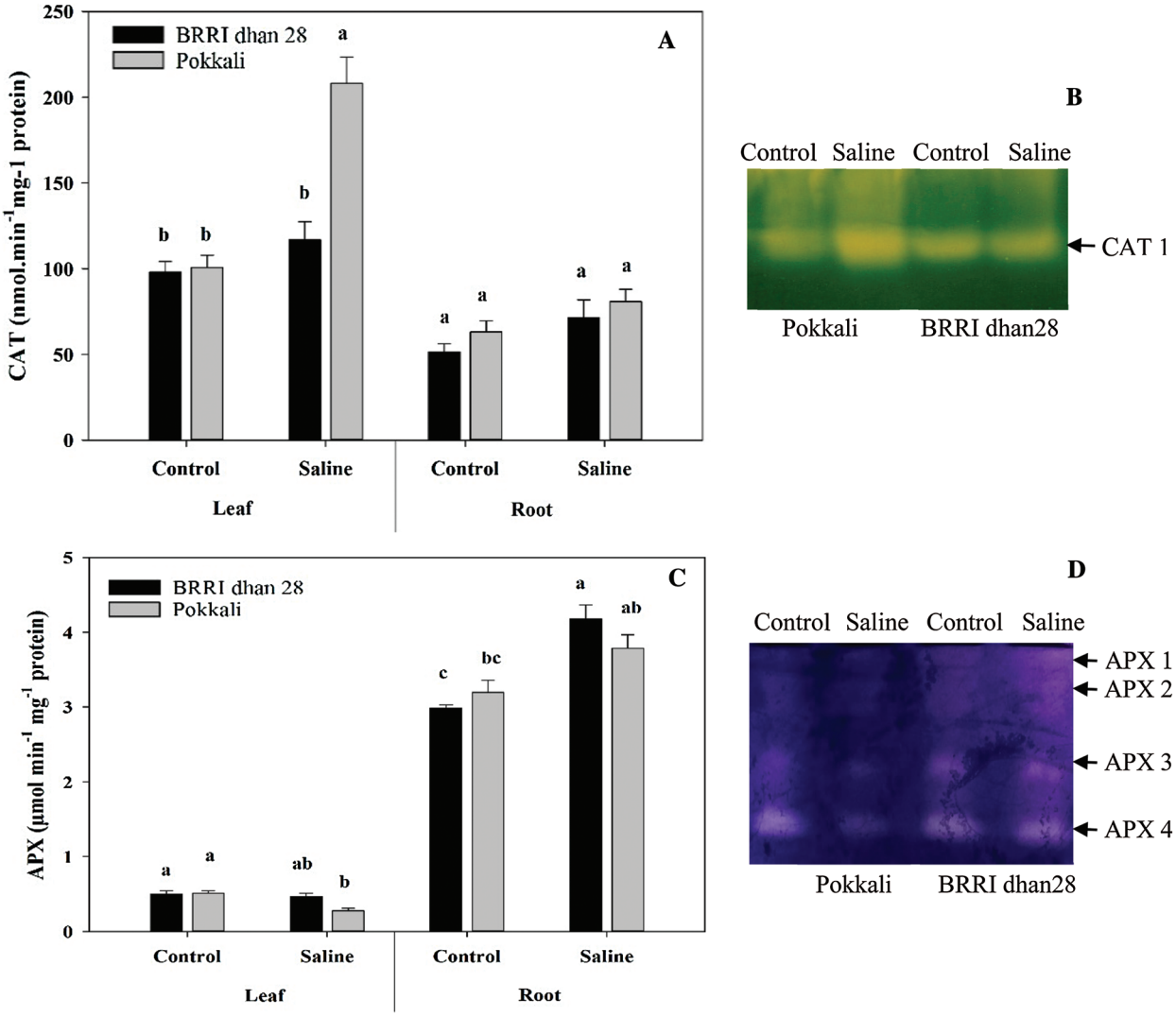

Figure 7: Activity of CAT (A), expression of CAT in gel electrophoresis (B), Activity of APX (C) and expression of APX in gel electrophoresis (D) in leaves of rice seedlings

was observed in salt-treated Pokkali. Primer OPC-05 produced the lowest number (04) of band, and among them only one band was polymorphic. Only 25% of polymorphism was observed. One unique band was found at 368 bp in salt-treated Pokkali. OPC-12 primer produced, the highest number of band (09) among them eight was polymorphic band and 88.88% polymorphism observed. In salt treated Pokkali, one unique band was observed at 400 bp. OPS-12 primer produced total of five bands with 60% polymorphism like OPA-01. A unique band was observed at 541 bp in salt-treated Pokkali only. By using those five primers five unique bands were observed, that band was present only at salt treated Pokkali rice and absent in BRRI dhan28 and even was absent in Pokkali control. Genotypic differences were confirmed by observing different banding pattern among BRRI dhan28 and Pokkali (Tab. 2).

4 Discussion

Rice is generally reflected as a salt-sensitive crop. Inhibitions of the shoot and root growth because of salt stress have been reported even in crops considered as tolerant [35,36]. Under salt stress conditions,

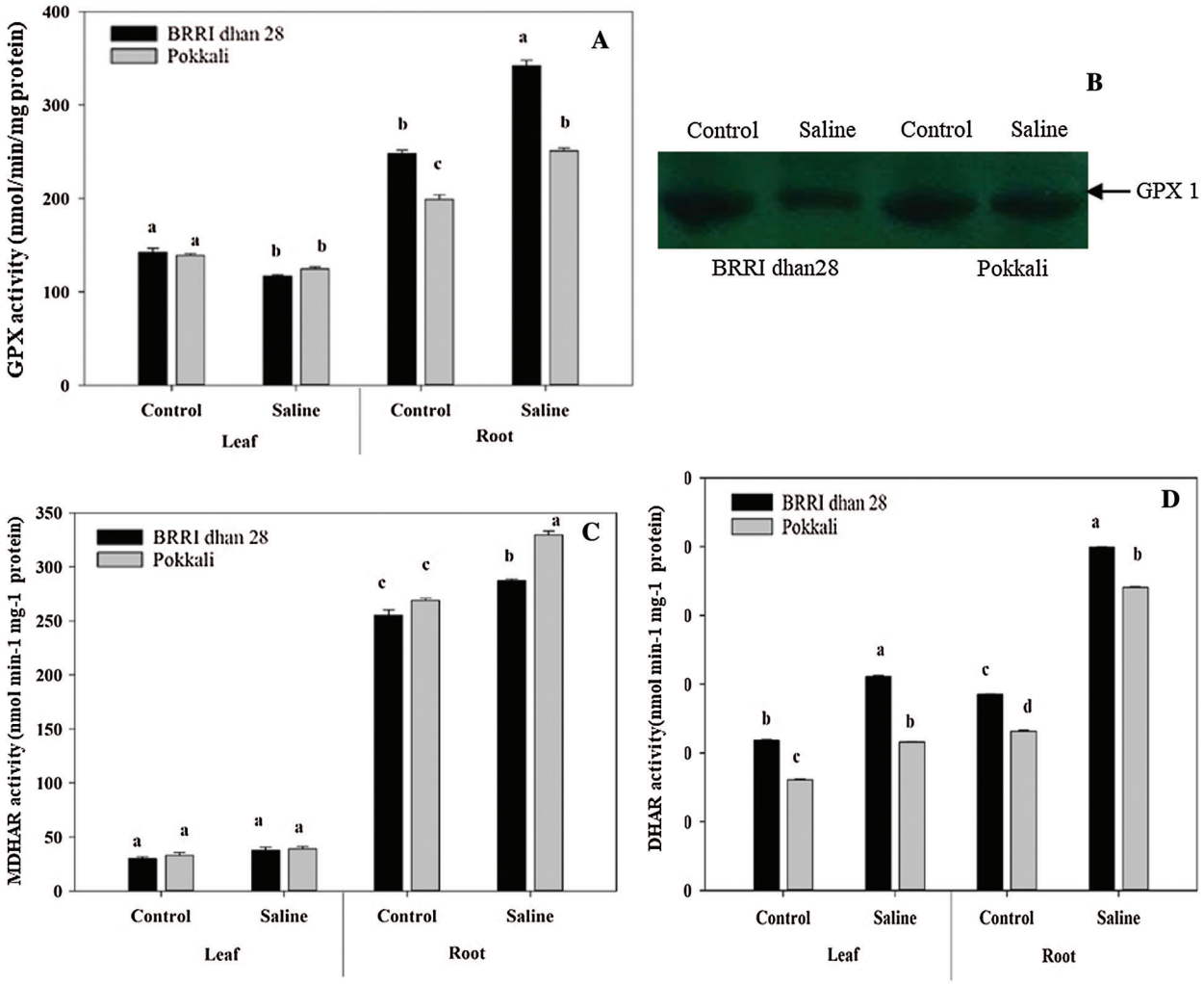

Figure 8: Activity (A) and expression of GPX in gel electrophoresis (B), activity of MDHAR (C) and DHAR (D)

higher accumulation of Na^+ disrupts ion homeostasis which leads to osmotic stress and inhibits growth [37]. Pokkali conserved the lowest Na^+/K^+ ratio under salt stress. The ability of plants to preserve K^+ ions to maintain Na^+/K^+ homeostasis has always remain a concerned feature of salt-tolerant [38]. In this study, NaCl stress increased Na^+ content and decreased K^+ content in the leaves, stems and roots of rice seedlings which might be due to entry of higher amount of Na^+ into plant by non-selective cation channel (NSCC) that caused K^+ efflux or leakage through NSCC and guard cell outward rectifying potassium channels (GORK) channel. Higher Na+ accumulation also results in a higher lower K^+/Na^+ ratio, which disrupts ion homeostasis by decreasing Mg, Mn and Zn contents. The Na^+ influx and K^+ leakage might be also for higher ROS production that can also activate NSCC [57]. Although higher K^+/Na^+ ratio in Pokkali might be due to vascular trafficking of the ion.

Proline has been suggested to act as a functional osmoprotectant with antioxidant prospective, which plays a dynamic role in abiotic-stress tolerance to scavenges free radicals and suppresses ROS accumulation in plants [36,39]. Under salt stress higher Proline accumulation was observed, which indicated a stress-induced water imbalance besides osmotic stress. Many studies also declared the

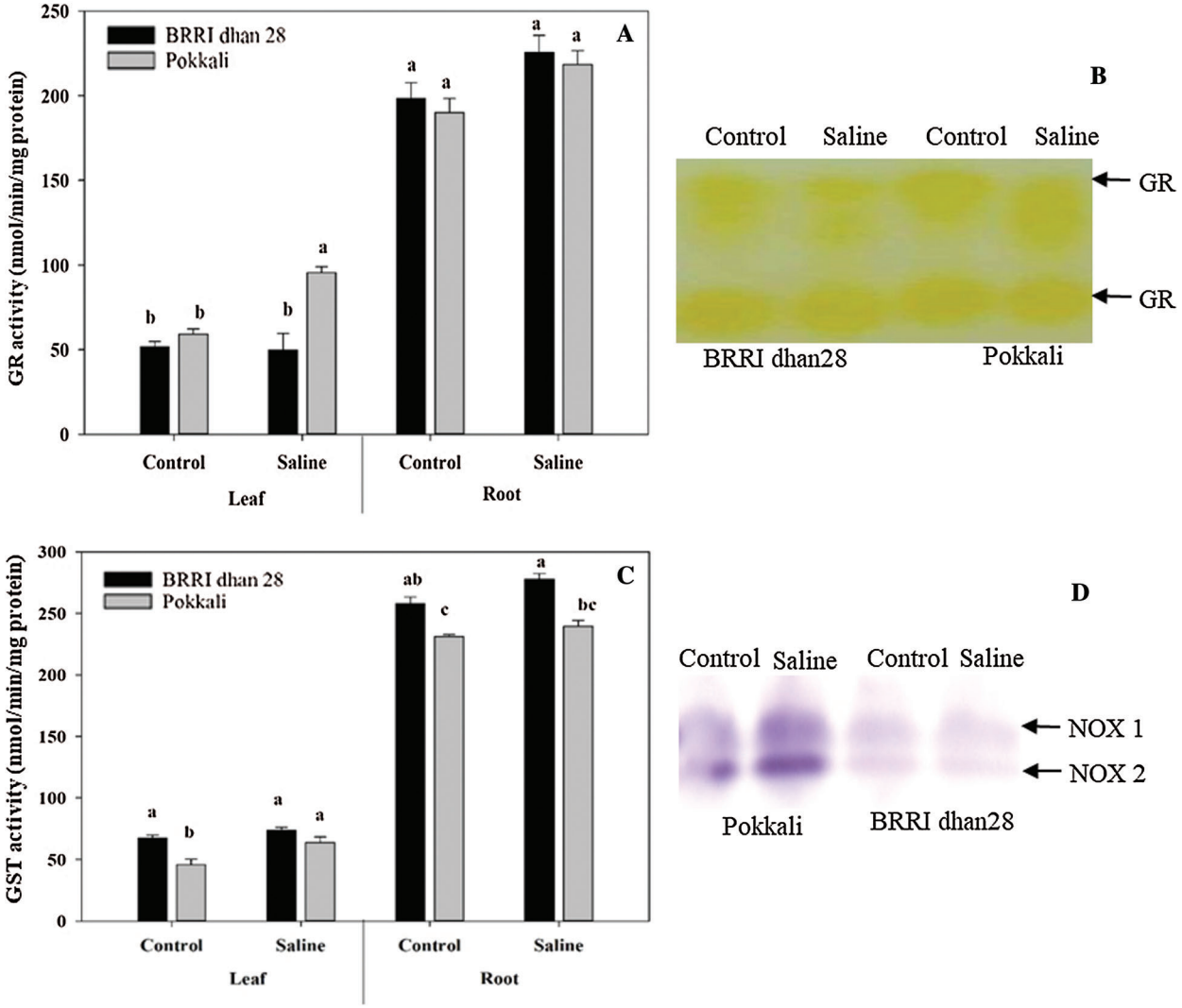

Figure 9: Activities (A) and expression of GR in gel electrophoresis (B); Activities of GST (C) and expression of NOX in gel electrophoresis (D) in leaves of rice seedlings

correlation of salt tolerance with an improved aptitude of the antioxidant scheme and proline accumulation [36,40,13,41]. Redused chlorophyll content under salt stress, is an extensive phenomenon [42]. Stress undermines cell membrane permeability and causes the leakage of numerous chl molecules and reduces chlorophyll contents may be due to higher accumulation of ROS [43]. The concentration of the pigment fractions in the leaves of all the rice varieties clearly demonstrated that the biosynthesis of photosynthetic pigments was affected by NaCl stress in both genotypes. However, Pokkali maintained higher chlorophyll of Pokkali ensure the tolerance in salt stress. Therefore, Chl might also be inhibited due to lower Mg uptake. The higher carotenoid in Pokkali under salinity can play role in reducing ROS like $^1O_2$ as well as beta-oxidation [20].

ROS are mainly produced at low levels in chloroplasts, mitochondria, and peroxisomes and can be scavenged by various defense mechanisms. During abiotic stress, the production rate of the ROS is increased. The tolerance of plant during abiotic stress mainly depends on the balance between the

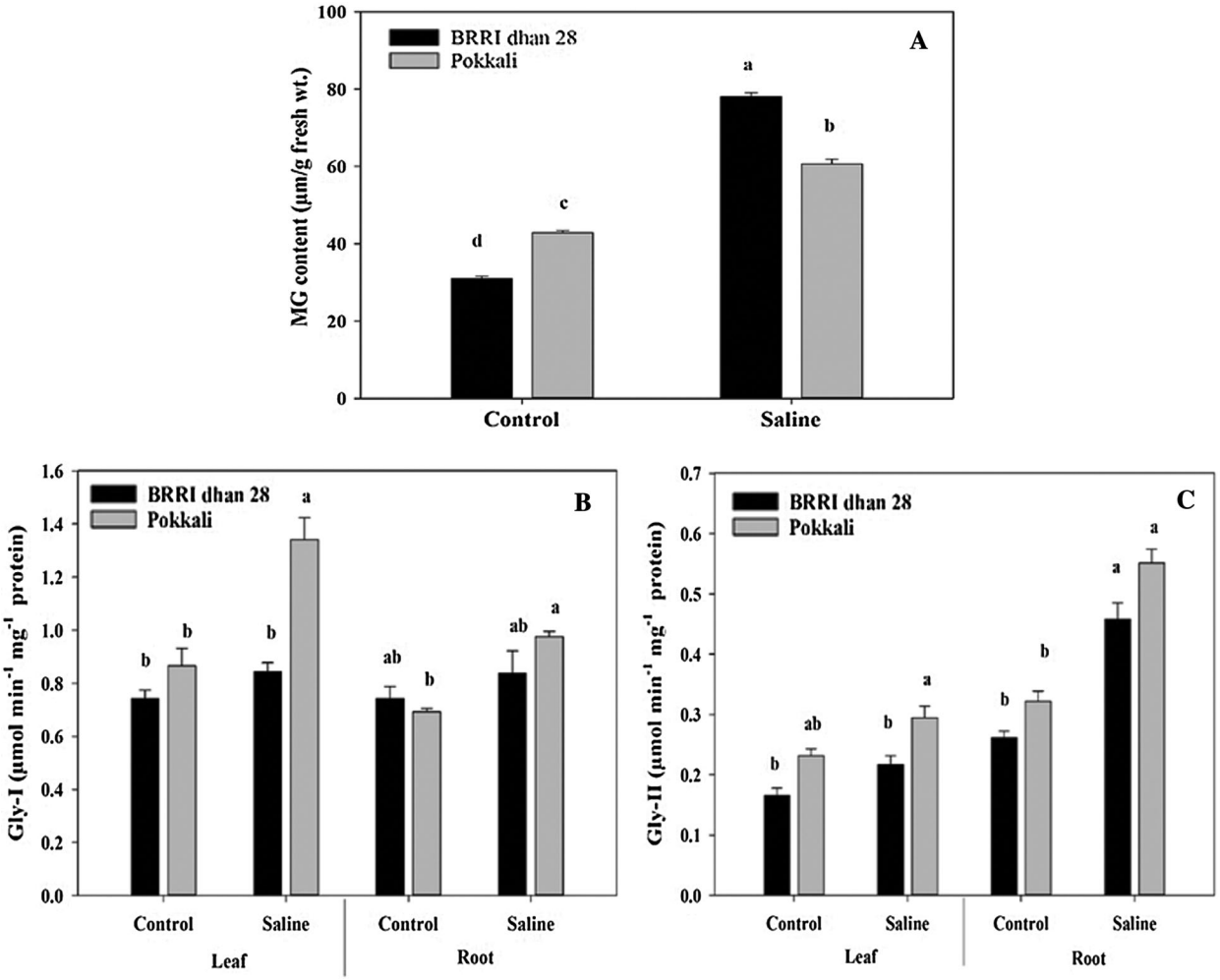

Figure 10: Contents of MG in leaves (A), activities of Gly-I (B) and Gly-II (C) in presence or absence of salt stress

production and the scavenging of ROS [10]. Superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), lipid peroxidation, secondary metabolites of ROS, LOX activity were found higher in case of BRRI dhan28 which may cause damage to plasmalemma and organelle membranes, enzyme inhibition and eventually lead to cell death [10]. Histochemical detection of $O_2^{\bullet-}$ and H_2O_2 in rice leaves also strongly support the findings.

A higher ratio of GSH/GSSG and ASA/DHA ratio found in Pokkali may play a vital role in maintaining cellular redox potential for salt stress tolerance by scavenging overproduced ROS [44,45,46]. MDHAR and DHAR activity was higher in BRRI dhan28, but the increasing rate of GR activity was much lower in BRRI dhan28, which may hamper ASA regeneration as AsA content was significantly lower in BRRI dan28 compared to Pokkali. In ASA-HSH cycle, ASA oxidized to MDHAR to scavange of H_2O_2 by APX. The produced MDHAR disappropriate to either ASA or dehydroascorbate (DHA) (Apel and hirt, 2004). On the other hand, MDHAR and DHAR regenerate ASA where MDHAR use NADPH as reductant, and DHAR used GSH. In this study, the higher activities of MDHAR and DHAR activities in Pokkali than BBRI dhan28 could maintain better ASA. Higher GR activity might be helpful to regenerate ASA in tolerant Pokkali. As GR activity is highly increased in Pokkali in compare to BRRI dhan28, higher GSH

Table 2: Polymorphism of the RAPD-PCR primers between salinity susceptible and tolerance varieties

Primer code	Sequence (5'-3')	Size range of the scorable Bands (bp)	Total bands	No. of monomorphic bands	No. of polymorphic bands	Unique bands (bp)	% Polymorphism
OPA-02	TGCCGAGCTG	254-2998	7	5	2	1 (254)	28.57
OPC-01	TTCGAGCCAG	364-1885	5	2	3	1 (891)	60.00
OPC-05	GATGACCGCC	368-3538	4	3	1	1 (368)	25.00
OPC-12	TGTCATCCCC	229-3402	9	1	8	1 (400)	88.88
OPS-12	CTGGGTGAGT	421-2000	5	2	3	1 (541)	60.00
Total			**30**	**13**	**17**	**5**	**262.45**
Mean			**6**	**2.6**	**3.4**	**1**	**52.49**

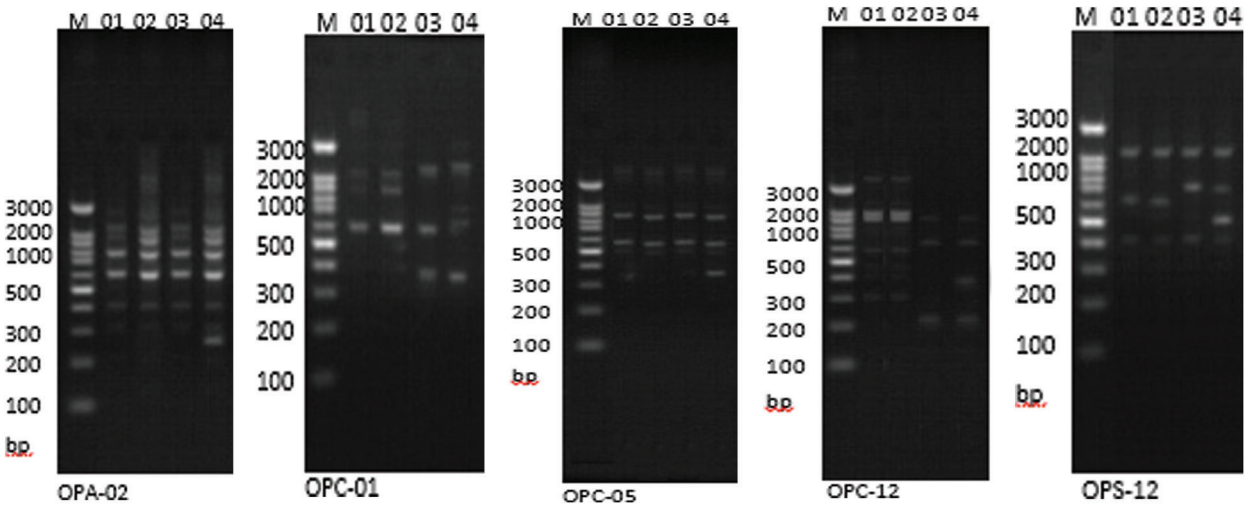

Figure 11: Electrophoretic banding pattern amplified by RAPD primer Lane 01-02: BRRI dhan-28, Lane 01: Control, Lane 02: Treatment, Lane 03-04: Pokkali, Lane 03: Control, Lane 04: Treatment: M: Molecular wt. marker 1 kb DNA Ladder

was observed in Pokkali. GR plays a vital role in ASA regeneration, and previous studies showed a similar result in rice showing high tolerance with higher GR, SOD, APX [47]. Higher SOD, POD, CAT activity in Pokkali genotype may be involved in ROS detoxification under salt stress. GST and GPX also work together and involve in the removal of ROS in a stressed condition [48,49] which was found higher in Pokkali rice than BRRI dhan28. In this study, all antioxidant enzymes were found to increase in the root of both varieties when exposed under salt stress. However, most of the cases there was no significant differences among the roots of two salt treated contrast genotypes. SOD and MDHAR activity was significantly higher in salt-treated Pokkali root.

The glyoxalase system consists of two enzymes (Gly-I and Gly-II) acts to alter the cytotoxic MG to non-toxic hydroxy acids such as lactate [49]. In stress conditions, cell enzymes of glycolysis and TCA cycle showed increased activity because of rapid activation of cell [50,51,52] which may in turn generate MG [53,20]. Higher expanse of methylglyoxal production under several stress condition was also reported by

[54,55,39]. In our study, higher MG content was found, and both Gly-I and Gly-II activity increased under salinity. MG content was higher in BRRI dhan28 compared to Pokkali, and higher Gly-I and Gly-II activity were observed in salt tolerant Pokkali that is inconsistent with the findings of previous studies [56,57].

5 Conclusion

Salt stress disrupts ion homeostasis, the antioxidant defense, and glyoxalase systems by increasing Na^+ uptake, ROS production, and MG formation, respectively. Along with physiological parameters, RAPD primers also confirmed the genotypic difference. Five unique bands were observed in salt-treated Pokkali and also those may promote the appearance of specific proteins or active any specific gene in salt-treated Pokkali. By scavenging free radicals and suppressing ROS, higher accumulation of Proline might be helpful for Pokkali to survive in salt stress. ROS-scavenging enzymes and antioxidant molecules in plants avert the damage from $O_2^{\bullet-}$ and H_2O_2. However, downstream production of $O_2^{\bullet-}$ may be due to lower NOX enzyme in Pokkali, as NOX is the most potent source of endogenous $O_2^{\bullet-}$ production. From our present study, it is suggested that enzymatic antioxidants SOD, CAT, and non-chloroplastic POD plays an essential role for salt tolerance in Pokkali compared to salt-sensitive BRRI dhan28. MG content was higher in BRRI dhan28 compared to Pokkali, and higher Gly-I and Gly-II activity were witnessed in salt tolerant Pokkali which helped to alter the cytotoxic MG to non-toxic hydroxy acids. Study on physiological mechanism may help us to find distinguishable differences between tolerant and susceptible variety which can furthermore help us to develop new salt-tolerant variety through advanced biotechnological approaches. In this study, significantly higher CAT and SOD was found as key survival mechanism in Pokkali. Further study should be undertaken transferring these enzymes through biotechnological approach. However, more study should be needed to identify either mitochondrial SOD contributed most or SOD localized in cytosol.

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