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Research Paper

Screening of salt tolerant landraces of rice grown in Sundarban, Bangladesh based on oxidative stress and redox metabolism during germination

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Abstract

In this work, an effort has been made to screen salinity tolerant landraces of rice (*Oryza sativa* L.) commonly grown in Sundarban, Bangladesh based on their redox regulatory attributes during early germination, grown under NaCl salinity stress. Five landraces of rice (Kajolshail, Jotaibalam, Charobalam, Kutepatnai and Nonakochi) of Sundarban, Bangladesh have been selected and imposed post-imbibitional salinity stress (200mM NaCl). Germination and early growth attributes were assessed in terms of germination rate, relative germination performance, t_{50} value of germination, relative growth index and vigor index. When salinity stress responsiveness in terms of germination and early growth performance of the experimental landraces was assessed and compared, the salinity resistance was noticed in the order Kutepatnai>Nonakochi>Kajolshail>Jotaibalam>Charobalam. Changes in pro-oxidant level and the sensitive redox parameters - Hydroperoxide, Conjugated diene, Free carbonyl content, TBARS content and Lipoxygenase activity were assessed to compare the oxidative stress responsiveness of all the experimental landraces of rice grown under salinity stress. When compared, Hydroperoxide, Free carbonyl content and TBARS content were found to be accumulated in the order Charobalam>Jotaibalam>Kajolshail>Nonakochi>Kutepatnai. Germination attributes and parameters of oxidative membrane damage showed a strong correlation, hinting the significance of redox metabolism in regulating germination, which may be exploited for screening the salt tolerant rice germplasm.

Keywords: Salinity stress, Landraces, Redox status, Oxidative damage.

INTRODUCTION

Rice is the staple food for one third of the world's population and occupies almost one-fifth of the total land area covered under cereals. Approximately 11% of the world's arable land is planted annually to rice, and it ranks next to wheat (Chakravarthi and Naravaneni, 2006). Coastal agriculture is highly vulnerable to salinity primarily due to climate change. Salinity is the second most widespread soil problem in rice growing countries after drought and is considered as a serious constraint to increasing rice production worldwide (Munns, 2005). In Bangladesh, the total saline area is one third of the 9 million hectares of total national cultivated area (Anon., 2006). IPCC estimated that a 0.5 degree Celsius increase in mean temperature and a 10cm rise in sea level could lead to inundation of 15 percent (approximately 750km^2) of the Sundarban forest, the largest mangrove ecosystem in Asia (Intergovernmental Panel on Climate Change - IPCC, 2001). Since the whole area of Sundarban is tidal, the saline water cannot be used for irrigation purposes, which have a significant negative impact on agriculture as well as rice cultivation.

Salinity is one of the most important abiotic stresses can directly affect on plant growth and development (Arshad et al., 2012; Lauchli and Grattan, 2007; Muhling and Lauchli, 2001; Galvani, 2007). Salinity affects crop by

imposing ionic and osmotic stress, causing ion toxicity and metabolic dysfunctions (Hossain et al., 2015; Chen et al., 2007). Salinity stress apart from imposing ionic and dehydration induced metabolic dysfunction and injury, inevitably cusses loss of redox-homeostasis due to the excessive generation of ROS, which eventually serious oxidative damage to cellular macromolecule (Hossain and dietz, 2016; Pandolfi et al., 2012). Rice is relatively tolerant to salinity at the germination stage but its panicle initiation and pollination stage are two most salinity-sensitive growth stages, which directly related to crop yield. Screening of rice genotypes at the seedling stage is comparatively easier than reproductive stage and also rapid. It is very difficult at the reproductive stage. The conventional method of plant selection for salt tolerance is not easy because of the large effects of the environment and low narrow sense heritability of salt tolerance (Gregorio et al., 1997). The redox parameter is very sensitive parameters which reflect the physiological status and the sensitivity of the germplasms towards salinity stress. So the differential redox parameters and oxidative deterioration can be useful biomarkers for determining salinity resistance and screening salinity tolerant plants.

As reclamation of saline soils is laborious and almost impossible, development or selection of salt tolerant crop species is one of the possible means for extension of the crop area. For further improvement in total rice production, rice breeders must develop high yielding varieties with tolerance against abiotic stress for unfavorable ecosystems. There are huge traditional cultivars and landraces of rice which have been neglected for a long time because of high yielding varieties but they have the great capacity to combat stress. These rice are more tolerant than many elite cultivars to various abiotic stresses. These resistant genotypes are considered to be good sources of tolerance traits. Appropriate salt tolerant high yielding varieties that can fit into the rice-growing ecosystem in the coastal areas of Bangladesh will boost up the country's rice production.

At present, our ability to improve salinity stress tolerance of indica rice cultivars is limited by our poor understanding of extremely complex nature of stress acclimation mechanism, particularly the redox regulatory network. Research activities on the performance of rice against salinity stress may be helpful in breeding of salt tolerant advanced lines and to find out the suitable plant type with improved characters by investigation of the physiological and biochemical mechanisms related to salt tolerance. The objective of the present study is therefore to screen some landraces of rice of Sundarban area based on some redox biomarkers.

MATERIALS AND METHODS

Plant growth and treatment of NaCl to induce post-imbibitional salinity stress

Seeds of five Land races of rice (*Oryza sativa* L., landraces Nonakochi, Kajolshail, Kutepatnai, Jotaibalam and Charobalam), selected as experimental material, have been collected from BARSIC, Munsiganj, Satkhira, Bangladesh. Seeds of the rice were washed with distilled water and were treated with 0.2% $HgCl_2$ for 5min and then washed thrice with sterile distilled water. The surface sterilized seeds were imbibed in distilled water for 48 h in darkness at $25^{\circ}\pm2^{\circ}$C and thereafter, were sown on moist filter paper in Petri plates and were placed in standardized conditions of thermostat-controlled seed germinator cum stability chamber (Remi 82 BL, India) maintained at $25^{\circ}\pm2^{\circ}$C. For imposing post imbibitional salinity stress, water imbibed seed lots were treated with 200mM NaCl for 7days, with intermittent change of treating solutions in petriplates (24h interval). For untreated control set, water imbibed seeds were sown directly in petriplates. All the seed lots were allowed to grow at $25^{\circ}\pm2^{\circ}$C with 14h photo period (light intensity 270µmol $m^{-2}s^{-1}$) and 78 ± 2% relative humidity. For all biochemical analysis, 168h old seedlings raised from aforesaid conditions were used.

Determination of reactive oxygen species (ROS)

Estimation of H_2O_2 generation

Hydrogen peroxide was estimated by the procedure of MacNevin and Uron (1953) using titanic sulfate. For this, 1g of tissue was extracted with 5mL of cold acetone and filtered through Whatman No.1 filter paper and volume made up to 10mL with distilled water. Now 1mL of 5% titanic sulfate (in 20% H_2SO_4) was added to this, which was followed by addition of 2mL of concentrated NH_4OH and finally centrifuged at 6000rpm for 10min. Pellet obtained was washed with 5mL of acetone (thrice) and then centrifuged at 5000rpm for 10min. Then, the pellet was dissolved in 3mL of 2(N) H_2SO_4 and absorbance was taken at 420nm against a blank.

Determination of indices of oxidative membrane damage (Hydroperoxide, Conjugated Diene, Lipoxygenase activity, Thiobarbituric acid reactive substances and Free carbonyl content)

Hydroperoxide

To estimate Hydroperoxide, 500mg of tissue was homogenized in 150mM Tris HCl (P^H-6.8), centrifuged at 5000 rpm and assayed as described by Simon (1904). The reaction mixture contained 250mM ammonium ferrous sulfate, 100mM xylenol orange, 0.25mM H_2SO_4, 4mM BHT prepared in 90% (v/v) methanol and an aliquot of the sample. After 30min of incubation at room temperature, the reaction was added with 100mM triphenyl phosphine to specifically reduced hydroperoxides to distinguish from H_2O_2 (Nourooz-Zadeh et al., 1994). Absorbance was taken at 560nm.

Conjugated Diene

For estimating conjugated dienes, the process of Buege and Aust (1978) was followed. 500mg of tissue extracted with chloroform: methanol mix (2:1) followed by vigorous vortexing and centrifugation at 2000rpm for 10min. The upper layer obtained was discarded along with the proteins, while the lower chloroform layer was dried under a stream of nitrogen at 45°C. The residue obtained was dissolved in cyclohexane and absorbance was taken at 230nm against cyclohexane (standard 1 O.D. = 37.5nmoles).

Lipoxygenase activity

Lipoxygenase activity was determined according to Peterman and Siedow (1985). 200mg of tissue was homogenized with 5mL of 50mM Na-phosphate buffer (p^H-6.5) and centrifuged at 5000rpm for 5min. The Supernatant was taken and re-centrifuged at 17000rpm for 10min in cold. The reaction mixture contained 1mL of 1.65mM Na-phosphate buffer (P^H-6.5) and 1mL of 1.3mM linoleic acid. After 1hr of incubation at 25°C absorbance was read at 234nm.

Thiobarbituric acid reactive substances

To estimate membrane lipid peroxidation, test for thiobarbituric acid reactive substances (TBARS) was performed using the procedure of Heath and Packer (1968). 200mg of sample was homogenized in 5mL 0.1% trichloroacetic acid (TCA) and then centrifuged at 10,000 rpm for 15min and finally, the supernatant was taken. To 1mL of supernatant, 3mL of 5% TCA containing 1% thiobarbituric acid (TBA) was added and heated in a hot water bath for 30 min and cooled quickly in cold water bath. It was finally centrifuged at 10,000rpm for 10min. The absorbance of the supernatant was measured at 530nm. The concentration of TBARS was measured from its extinction coefficient of 155µM cm^{-1}. The non-specific turbidity was corrected by subtracting A_{600} from A_{530} value. The formula employed as:

Conc. of unknown (CU)

$$= \frac{\text{The absorbance of the unknown at 530 nm mols/l}}{\text{The diameter of cuvette} \times 155}$$

The TBARS content is finally expressed in n mol g^{-1} dry mass of tissue.

Free carbonyl content

Oxidative damage to proteins was estimated as the content of carbonyl groups following the procedure of Jiang and Zhang (2001). 500mg of tissues (root and shoot) were homogenized with 3mL of 50mM potassium phosphate buffer (pH 7.0) containing 1mM EDTA (ethylene diamine tetra acetic acid), 1mM PMSF (phenyl methyl sulfonyl fluoride), 10mM DTT (dithithreitol) and 5µg mL^{-1} leupeptin (protease inhibitors). The homogenate was centrifuged at 15000×g for 25min and the supernatant was made free from contaminating nucleic acids by treatment with streptomycin sulfate. An equal volume of 10mM DNPH in 2M HCl was added to supernatant containing the oxidized protein. These were allowed to stand in the dark at room temperature for 1h, with vortex every 10min. Samples were precipitated with trichloroacetic acid (TCA; 20% final concentration) and centrifuged in a table-top micro centrifuge for 5min. The supernatants were discarded and the protein pellets were washed twice more with TCA, and then washed three times with 1mL portions of ethanol/ethylacetate (1:1) to remove any free DNPH. The protein samples were re-suspended in 1mL of 6M guanidine hydrochloride (dissolved in 20mM phosphate buffer, pH 2.3) at 37°C for 15min with vortex mixing. Carbonyl contents were determined from the absorbance at 370nm using a molar absorption coefficient of 22mM cm^{-1}.

Determination of germination and early growth performances

For studying early growth performances, Germination rate (GR), Co-efficient of velocity of germination(CVG), Germination energy (GE), Germination rate index (GRI), Mean germination time (MGT), relative growth index (RGI), t_{50} value, relative germination performance (RGP) and vigor index (VI) were calculated according to Rubio-Casal et al. (2003) and Bhattacharjee (2008).

Germination rate (GR) was calculated as:

GR = number of germinated seeds/days to first count +number of germinated seeds/days to second count +……..+ number of germinated seeds/ days to the final count.

t_{50}value = Time (in h) of 50% germination of seeds sown.

Relative germination performance (RGP) was measured as:
RGP = (Percentage of germination under treatment /Percentage of germination under control) ×100

Relative growth index was calculated as:
RGI = (average dry mass of ten treated seedlings + average dry mass of ten control seedlings) ×100

Vigor index (VI) was measured as:
VI = (Mean shoot length + mean root length)/Percentage of final germination.

Statistical analysis

Results are mean of three replicates ± standard error. For statistical analysis of the data for significance, the paired two sample t test was done with the help of Microsoft Excel 2013, which shows the significant variations between untreated control and different post-imbibitional salinity stress-raised seedlings.

RESULTS

Differential oxidative damage to newly assembled membrane lipid and protein in rice landraces under extremes of salinity

In order to investigate whether there is any genotypic difference of oxidative damage to newly assembled membrane system in response to salinity stress during early germination, assessment of free carbonyl content (RC=O), accumulation of TBARS, H_2O_2 content, estimation of hydroperoxide and conjugated diene and determination of lipoxygenase activity were done for the five landraces of rice namely Nonakochi, Kajolshail, Kutepatnai, Jotaibalam and Charobalam. Salinity stress caused significant enhancement in the accumulation of H_2O_2 (**Fig.1A**) and Hydroperoxide (**Fig.1B**) as well as TBARS and RC=O (indices of membrane protein oxidation and lipid peroxidation (**Fig.1C and 1F**) for the germinating tissues (168h old seedlings) of all the rice land races. However, when compared, the extent of oxidative membrane damage, measured in terms of accumulation of TBARS, RC=O, H_2O_2 and Hydroperoxide was found to be significantly higher (compared to control) for post imbibitional salinity stress-raised seedlings of landraces Charobalam and Jotaibalam as compared to other landraces (**Fig.1**). Estimation of hydroperoxide and conjugated diene and lipoxygenase activity an important parameter of determination of redox status of the cell. In case of the lipoxygenase activity, the highest lipoxygenase activity was found in Charobalam as compared to control (**Fig.1E**). Conjugated diene content was found to be significantly higher in salinity stress raised germinating tissue of Jotaibalam and Charobalam as compared to untreated control (**Fig. 1D**).

Effect of post imbibitional salinity stress on germination and early growth performances of rice landraces

When post-imbibitional salt stress (PISS) was imposed to the seeds of five rice Landraces, the land race Charobalam exhibited delayed germination and early growth performances as compared with untreated control (measured in terms of germination rate, t_{50} value, relative germination performance, relative growth index and vigor index (**Table 1**). However, the effect of post-imbibitional salt stress on germination early growth performances of rice landraces Kutepatnai, Nonakochi and Kajolshail was marginal (**Table 1**). The result in general shows inhibition of germination and early growth performances under the exposure of salt stress of all the rice landraces. The relative growth index (RGI) for post-imbibitional salt stress-raised seedlings of rice landraces Charobalam and Jotaibalam have been reduced to 20.84 and 28.77%, respectively (**Table 1**), Similarly the relative germination performance (RGP) of post-imbibitional salt stress-raised rice seeds of landraces Charobalam and Jotaibalam have been reduced to 64.28 and 77.77% respectively (**Table 1**), hinting more early growth impairment suffered by the landraces Charobalam and Jotaibalam than other three landraces under the same magnitude of corresponding salt stress. Assessment of other parameters of germination and early growth impairment, like germination rate, t_{50} value, vigor index etc. also corroborate the finding that germination continued even at salinity stress but early growth was inhibited and delayed more for the landraces Charobalam and Jotaibalm as compared to other landraces (**Table1**).

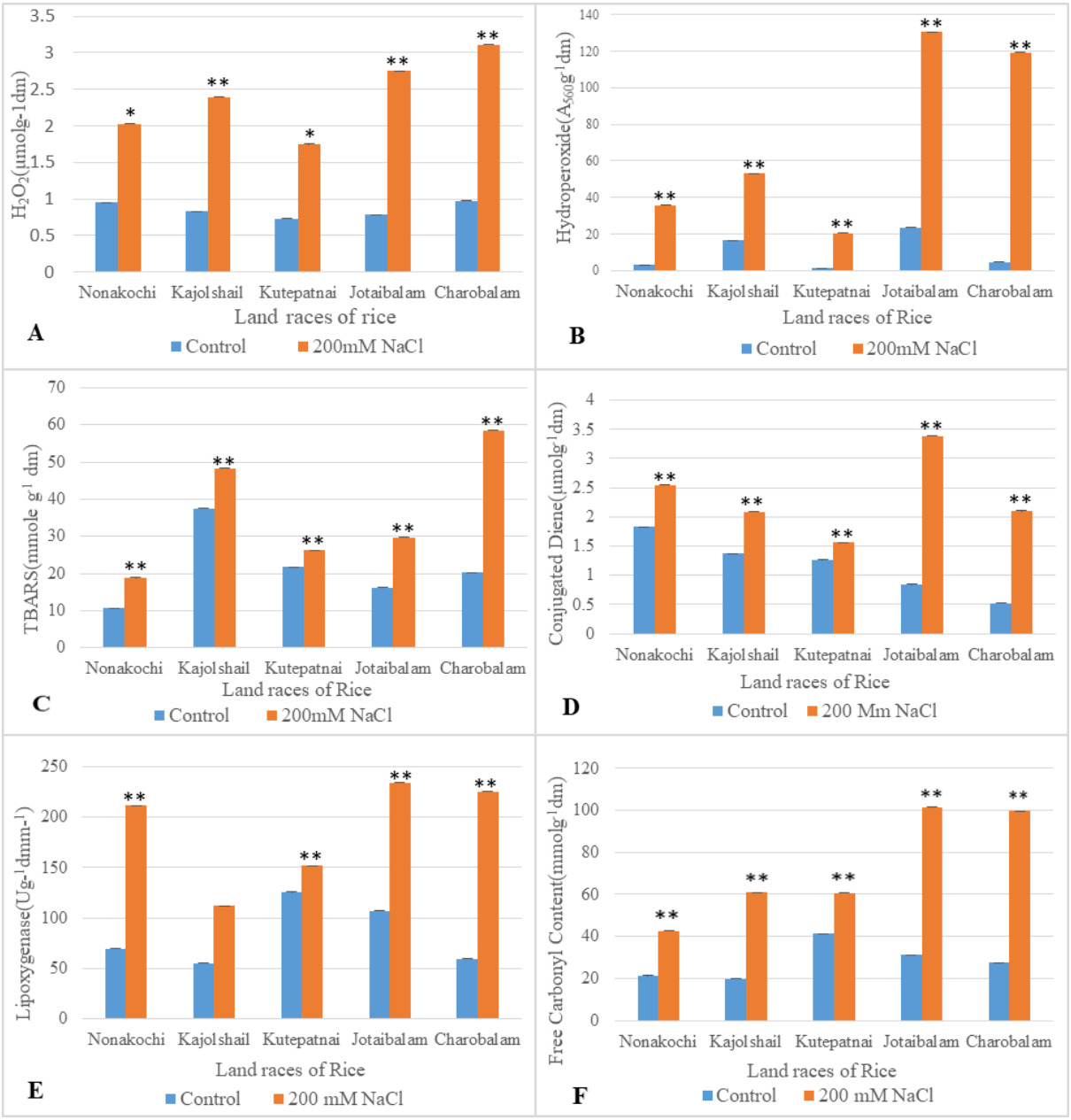

Fig. 1: Estimation of redox biomarkers assessed in terms of parameters of oxidative membrane damage to the seedling of five land races of rice of Sundarban, Bangladesh raised from post-imbibitional salinity stress (PISS-200mM/0.18Scm^{-1} NaCl). Results are mean of three replicate ± standard error * and **represent significant differences at 0.05 and 0.01 level.

Table 1: Impact of post-imbibitional salinity stress (PISS-200mM/0.18 Scm^{-1}NaCl) on Germination and early growth performance of five landraces of Rice of Sundarban, Bangladesh. Results are mean of three replicate ± standard error. * and ** represent significant differences at 0.05 and 0.01 level.

Landraces of Rice	Treatment	Germination and Early Growth Performance				
		GR	t_{50} (hrs)	RGP (%)	RGI (%)	VI
Nonakochi	Untreated Control	1.6 ± 0.007	28.5± 0.272	96.67±0.223	100±0	0.078±0.002
	200mM NaCl	1.24± 0.031*	39.17±0.207**	95.00±0.120	45.16±0.081**	0.027±0.001*
Kajolshail	Untreated Control	1.35± 0.018	23.67 ±0.207	100±0	100±0	0.092±0.001*
	200mM NaCl	0.95± 0.063	43.5±0.360**	90 ±0.152**	34.11±0.177**	0.018±0.001
Kutepatnai	Untreated Control	1.66± 0.021	20.5±0.136	100 ± 0	100±0	0.073±0.003
	200mM NaCl	1.43± 0.042*	24.67 ±0.208*	98.69 ±0.316	71.24±0.070**	0.025±0.001
Jotaibalam	Untreated Control	1.59± 0.011	29.33 ±0.342	99.25 ±0.350	100±0	0.082±0.003
	200mM NaCl	0.88± 0.025**	51.83 ±0.477**	77.77 ±0.171**	28.77±0.106	0.033±0.0017
Charobalam	Untreated Control	1.82± 0.015	20.83 ±0.157	93.33 ±0.152	100±0	0.146±0.004
	200mM NaCl	0.68±0.012**	60.83 ±0.079**	64.28 ±0.231**	20.84±0.139	0.018±0.000*

DISCUSSION

Excess production of ROS, a deleterious process is toxic to plants and causes oxidative damage to cellular constituents, leading to cell death (Noctor and Foyer, 1998; Hasegawa et al., 2000; Banu et al., 2009, 2010). Adverse environmental factors like salinity stress result in increased levels of ROS that are detrimental to the plant (Asada, 1999; Borsani et al., 2001). H_2O_2 (a ROS species) at low concentrations acts as a signalling molecule, inducing tolerance to several biotic and abiotic stresses, but at high concentrations triggers apoptosis-like and autophagic cell death (Love et al., 2008; Quan et al., 2008). In the present study, the production of H_2O_2 was observed to be higher (compared to control) in the landraces Charobalam. The findings in our study are in good agreement with Shahid et al. (2012) who found that salt stress enhanced H_2O_2 accumulation in the salt sensitive genotypes of pea.

Lipid peroxidation is the process where ROS remove electrons from the lipids in the cell membranes thereby damaging the cells (Catala, 2006). Thiobarbituric acid reactive substances (TBARS) assay is the most widely used for determining lipid peroxidation (Yagi, 1998). During the process of lipid peroxidation, the Malondialdehyde (MDA) is formed by the decomposition of polyunsaturated fatty acids which reacts with thiobarbituric acid. Oxidation of unsaturated fatty acids at a low level is a normal reaction in living cells. Enzymatic oxidation is catalyzed by lipoxygenases (LPOX). It has been reported that stimulation of LPOX activity under stress conditions reflects higher lipolytic activity in membranes and oxidation of membrane-bound fatty acids by causing propagation of lipid peroxidation (Molassiotis et al., 2006). Hydroperoxides are some of the first semi-stable products of the interaction between free radicals (and other reactive oxygen species) with biological systems so that they are potential indicators of the formation and effects of these reactive molecules. In our study, the highest lipoxygenase activity was found in Charobalam and significantly higher hydroperoxide was found (compared to control) in landraces Charobalam and Jotaibalam.

The Conjugated dienes (CD) provide a marker of the early stages of lipid peroxidation (Halliwell and Gutteridge, 1985) and measurement of conjugated dienes is a useful index of the early stages of peroxidation in studies with pure lipids and isolated lipoproteins (Dekkers et al., 1996). Protein carbonylation is significantly more sensitive to oxidative stress than lipid

peroxidation as the later gets catabolized rapidly than oxidized protein (Palma et al., 2002). The extent of oxidative damage assessed in terms of accumulation of free carbonyl content and thiobarbituric acid reactive substances (RC=O and TBARS) and estimation of hydroperoxide, conjugated diene and lipoxygenase activity, caused by salinity stress, generally correlated with germination and early growth performances and internal osmotic status of germinating seedlings (assessed in terms of vigor index, relative growth index, germination rate, t50 value and relative germination performance) of all five landraces of rice. Although oxidative membrane injury and early growth performances are not completely linked, but for all the landraces of rice, the early growth performances declined with higher accumulation of RC=O and TBARS. This finding strongly suggests that early growth performances under salinity stress require the ability to tolerate and mitigate oxidative damages to newly assembled membrane system of all five germinating experimental rice cultivars (Gong et al., 1988; Larkindale and Huang 2004; He et al., 2009; Bhattacharjee, 2008, Chakraborty and Bhattacharjee, 2015; Bhattacharjee and Dey, 2018). It is also quite evident that landrace Kutepatnai and Nonakochi were more tolerant in relation to oxidative injury and maintenance of internal water status of juvenile germinating tissues, which might be further corroborated by the data of redox status of the tissue under salinity stress.

CONCLUSION

Five experimental land races of Rice (Nonakochi, Kutepatnai, Kajolshail, Jotaibalam and Charobalam) of Sundarban, Bangladesh exhibited differential oxidative damage to the membrane lipid and protein assessed in terms of accumulation of Hydroperoside, Thiobarbituric acid reactive substances (TBARS), Conjugated Diene, RC=O and activities of LPOX. When compaired, there seems to be a close connection between oxidative membrane lipid & protein damage and germination & early growth performance. Land races exhibited more oxidative membrane lipid and protein damage showed significant germination and early growth impairment. It proves that, these parameters are very sensitive parameter which reflects the physiological status and the sensitivity of the Rice germplasms towards the salinity. In this study, the redox biomarkers based screening showing the land races of Rice of Sundarban, Bangladesh for salinity tolerant as Kutepatnai > Nonakochi > Kajolshail > Jotaibalam > Charobalam. So, the redox metabolic parameters offer a sensitive tool as a biomarker for the assessment and screening salinity tolerant germplasms of rice of Sundarban, Bangladesh.

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