Heat and chilling induced disruption of redox homeostasis and its regulation by hydrogen peroxide in germinating rice seeds (*Oryza sativa* L., Cultivar Ratna)

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Abstract Extremes of temperature (both heat and chilling) during early inhibitional phase of germination caused disruption of redox-homeostasis by increasing accumulation of reactive oxygen species (superoxide and hydrogen peroxide) and significant reduction of antioxidative defense (assessed in terms of total thiol content and activities of superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase) in germinating tissues of rice (*Oryza sativa* L., cultivar Ratna). Imbibitional heat and chilling stress also induced oxidative damage to newly assembled membrane system by aggravating membrane lipid peroxidation and protein oxidation [measured in terms of thiobarbituric acid reactive substances (TBARS), free carbonyl content (C = O groups) and membrane protein thiol level (MPTL)]. Treatment with standardized low titer hydrogen peroxide during early imbibitional phase of germination caused significant reversal in oxidative damages to the newly assembled membrane system imposed by heat and chilling stress [evident from the data of TBARS, C = O, MPTL, ROS accumulation, membrane permeability status, membrane injury index and oxidative stress index] in seedlings of experimental rice cultivar. Imbibitional H₂O₂ pretreatment also caused up-regulation of antioxidative defense (activities of superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase and total thiol content) in the heat and chilling stress-raised rice seedlings. When the parameters of early growth performances were assessed (in

terms of relative growth index, biomass accumulation, relative germination performance, mean daily germination, T₅₀ value), it clearly exhibited significant improvement of early growth performances of the experimental rice cultivar. The result proposes that an ‘inductive pulse’ of H₂O₂ is required to switch on some stress acclimatory metabolism through which plant restores redox homeostasis and prevents or repairs oxidative damages to newly assembled membrane system caused by unfavorable environmental cues during early germination to the rice cultivar Ratna. The importance of mitigating oxidative damages to membrane lipid and protein necessary for post-germinative growth under extremes of temperature is also suggested.

Keywords Temperature stress · Oxidative membrane damage · Hydrogen peroxide · Redox regulation · Antioxidative defense · Protein oxidation · Lipid peroxidation · Rice

Introduction

Abiotic stresses, such as extremes of temperature, drought, excess photochemical energy, salinity etc. disrupt cellular redox homeostasis; greatly affect plant metabolism, and productivity (Basu et al. 2010; Saidi et al. 2011; Long and Ort 2010; Essemine et al. 2010; Boyer 1972; Arora et al. 2002). An extreme of temperature is one of the most important environmental constraints for crop productivity. Indica rice varieties exhibit temperature susceptibility during early germination and flowering stage (Pareek et al. 1999; Hurry and Hunar 1992). When sown under extremes of temperature that prevails during sowing, seedlings exhibit stunted growth and low photosynthetic ability (Hurry and Hunar 1992).

To survive, plants have developed a complex signaling network involving different endogenous growth regulators that sense and protect them from environmental stresses.

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One of the common responses to different both abiotic and biotic environmental stresses, is the accelerated generation of reactive oxygen species (ROS) (Bhattacharjee 2005, 2010; Suzuki and Mittler 2006). Exposure of plants to both abiotic and biotic stresses cause activation of plasma membrane bound NADPH-dependent superoxide synthase (Sagi and Fluhr 2006), producing superoxide and subsequently H_2O_2 by superoxide dismutase.

Experimental evidences suggest that ROS, especially H_2O_2 is an active signaling molecule and its accumulation (oxidative stress) through redox- sensing leads to variety of cellular responses (Mylona and Palidoros 2010; Kovalchuk 2010; Mahalingam and Fedroff 2003). In fact, most of the recent works suggest that plant responses to ROS are dose-dependent (Kovalchuk 2010; Mahalingam and Fedroff 2003). High concentration of H_2O_2 results in cellular damage or even hypersensitive cell death, whereas low concentration of ROS functions as developmental signal, controlling growth, morphogenesis and plant responses to environmental stress (Bhattacharjee 2005; Kovalchuk 2010). Additionally, preexposure to abiotic and biotic stresses that induce an 'oxidative burst' can trigger a protective function or immunize plants against environmental stresses and thus can play a role in acclimatory stress tolerance (Foyer 1997; Bhattacharjee 2005; Kovalchuk 2010).

Further evidences suggest that treatment of leaves with H_2O_2 and then exposure to excess light (EL) caused significantly greater induction of ascorbate peroxidase (APX2) than control and excess light alone (Santos et al. 1996). Recent works also suggest that hydrogen peroxide seems to serve as a common stress signal under different environmental cues in plants. The stress signal in downstream in most of the cases works via Ca^{2+} , ABA and H_2O_2 etc., ultimately affecting common set of transcription factors associated with antioxidative defense enzymes (Mahalingam and Fedroff 2003; Bhattacharjee 2005; Agarwal et al. 2005; Mylona and Palidoros 2010). Keeping this in view, the present experiment aims at understanding the mechanistic aspect of H_2O_2 mediated acclimatization, especially the role of the molecule on oxidative stress metabolism and oxidative membrane damages under extremes of temperature in rice during early germination. In view of the above information, attempts were also made to develop a simple agro-technique as a short term strategy to overcome the problem of oxidative stress imposed by unfavorable environmental cues in rice during germination and early growth.

Materials and methods

Seeds of a cultivar of rice (*Oryza sativa* L.), Ratna, selected as experimental material, were collected from Chinsurah Rice Research Center (Government of West Bengal),

Chinsurah, West Bengal, India. Experimental Seeds were washed with distilled water, immersed in a solution of 0.2 % HgCl_2 for 5 min and then washed thrice with sterile distilled water. The surface sterilized seeds were imbibed in distilled water for 48 h in darkness at $25^\circ \pm 2^\circ \text{C}$ and thereafter were sown on moist filter paper in Petri plates (30 seeds / plate) with thin cotton pad soaked with H_2O_2 solution (100 μM) and kept in darkness at $25^\circ \pm 2^\circ \text{C}$ for 24 h. Two changes with freshly prepared H_2O_2 solutions were made in between to reduce the chances of degradation of H_2O_2 . After that, H_2O_2 pretreated seeds were again transferred to petriplates (30 seeds / plate) on filter paper soaked with H_2O and were placed in two different thermostat-controlled seed germination chamber (Bicon, India) exposed at 40°C and 8°C temperatures for duration of 16 h each to impose high and low temperature stress respectively. For direct stress treatment (without H_2O_2 pretreatment), water imbibed seeds were sown in petriplates (30 seeds/plate) and exposed to 40°C and 8°C temperature for duration of 16 h to impose supra- and sub- optimal temperature stress respectively. In another set, petriplates (30 seeds / plate) were exposed at $25^\circ \pm 2^\circ \text{C}$ after treating with 100 μM H_2O_2 solution (with two changes at an interval of 8 h) at 25°C in darkness for 24 h. For untreated control set, water imbibed seeds were sown directly in petriplates (30 seeds / plate) and exposed at $25^\circ \pm 2^\circ \text{C}$ without H_2O_2 pretreatment. Thereafter, all the seed lots were allowed to grow at $25^\circ \pm 2^\circ \text{C}$ with 12 h photo period ($270 \text{ E m}^{-2} \text{ s}^{-1}$) and $78 \pm 2\%$ RH.

For studying survival and early growth performances, relative germination performance (RGP), relative growth index (RGI), biomass accumulation, vigor index, mean daily germination (MDG), t_{50} value of germination and germination rate (GR) were calculated according to Rubio-Casal et al. (2003) and Bhattacharjee (2008).

To estimate membrane lipid peroxidation, test for thiobarbituric acid reactive substances (TBARS) was performed using the procedure of Heath and Packer (1968). 200 mg of sample was homogenized in 5 ml 0.1 % trichloroacetic acid (TCA), centrifuged at 10,000 rpm for 15 min and finally supernatant was taken. To 1 ml of supernatant 3 ml 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 10,000 rpm for 10 min. The absorbance of the supernatant was measured at 530 nm. The concentration of TBARS was measured from its extinction coefficient of $155 \mu\text{M cm}^{-1}$. The non-specific turbidity (if any) was corrected by subtracting A_{600} from A_{530} value.

The TBARS content is finally expressed in n mol / g dry mass of tissue.

For the determination of membrane protein thiol level (MPTL), the membrane was prepared according to Singh (1997) with some necessary modifications. 1 g of plant tissue was homogenized in 10 ml ice cold buffer (0.05 M Tris-HCl, pH 7.0). The homogenate was centrifuged at

10,000 g at 4 °C for 30 min and the pellet was discarded. The membranes were then sedimented at 1,00,000 g at 4 °C for 3 h and the pellet containing the membrane fractions was suspended in ice cold buffer (0.05 M Tris–HCl, pH 7.0). The membrane fractions were stored under ice. The membrane associated protein bound thiol group was assayed after protein precipitation with TCA (10 % mass/volume) and quantified with DTNB [5,5'-dithio-bis(2-nitrobenzoic acid)] following the procedures of Ellman (1959) and Dekok and Kuiper (1986).

Oxidative damage to proteins was estimated as the content of carbonyl groups (Jiang and Zhang 2001). 500 mg of tissues (root and shoot) were homogenized with 3 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM PMSF, 10 mM DTT and 5 µgml⁻¹ leupeptin, 5 µgml⁻¹ aprotinin and 5 µgml⁻¹ antipain (protease inhibitors). The homogenate was centrifuged at 15,000×g for 25 min and the supernatant was made free from contaminating nucleic acids by treatment with streptomycin sulfate. An equal volume of 10 mM DNPH (2,4-dinitrophenylhydrazine) in 2 M HCl was added to supernatant containing the oxidized protein. These were allowed to stand in the dark at room temperature for 1 h, with vortexing every 10 min. Samples were precipitated with trichloroacetic acid (TCA; 20 % final concentration) and centrifuged in a tabletop micro centrifuge for 5 min. The supernatants were discarded and the protein pellets were washed twice more with TCA, and then washed three times with 1 ml portions of ethanol/ethylacetate (1:1) to remove any free DNPH. The protein samples were re-suspended in 1 ml of 6 M guanidine hydrochloride (dissolved in 20 mM phosphate buffer, pH 2.3) at 37 °C for 15 min with vortex mixing. Carbonyl contents were determined from the absorbance at 370 nm using a molar absorption coefficient of 22 mMcm⁻¹.

Membrane injury index was measured according to the process of Bhattacharjee and Mukherjee (2003). Root and shoot tissues of the seedlings (200 mg of each) from each treatment were placed in vials containing 15 ml of deionised water and incubated at 25 °C for 24 h. Electrical conductivity (EC) of the bathing medium was measured at 25 °C with conductivity meter. The tissue with leachate was then autoclaved (at 6.6×10⁶ P) and brought to 25 °C and EC was measured again. MII was calculated using the formula of Sullivan (1972). $MII = [1 - (T_1/T_2) / 1 - (C_1/C_2)] \times 100$, where C_1 and C_2 are the ECs of untreated control sample before and after autoclaving and T_1 and T_2 are the ECs of treated sample before and after autoclaving. Oxidative stress index was calculated according to Bhattacharjee and Mukherjee (2006) as $OSI = \text{membrane lipid peroxidation of treated sample} / \text{membrane lipid peroxidation of untreated control} \times 100$. For the assessment of membrane permeability status, EC of tissue leachate were assessed following the same process as described for MII. The α -NH₂ and soluble carbohydrate content of the tissue leachate were

estimated by ninhydrin (Moore and Stein 1948) and anthrone reagent (Mc Cready et al. 1950).

Hydrogen peroxide was extracted and estimated following the procedure of MacNevin and Uron (1953) using titanous sulfate. For this, 1 g of tissue was extracted with 5 ml of cold acetone and then filtered through Whatman No. 1 filter paper and volume made up to 10 ml with distilled water. To this, 1 ml of 5 % titanous sulphate (in 20 % H₂SO₄) was added. After that 2 ml of concentrated NH₄OH was added and finally centrifuged at 6,000 rpm for 10 min pellet obtained was washed with 5 ml of acetone (thrice) and then centrifuged at 5,000 rpm for 10 min pellet was then dissolved in 3 ml of 2(N) H₂SO₄ and read at 420 nm against a blank.

For the determination of superoxide, the method of Chaitanya and Naithani (1994) was followed with some necessary modifications. 500 mg of tissues was homogenized in cold with 5 ml of 0.2 M sodium phosphate buffer, pH 7.2, with addition of diethyldithiocarbamate (10⁻³M) to inhibit SOD activity. The homogenates were immediately centrifuged at 2,000 g at 4 °C for 1 min. In the supernatant, superoxide anion was measured by its capacity to reduce nitrobluetetrazolium (2.5×10⁻⁴M). The absorbance of the end product was measured at 540 nm. Formation of superoxide was expressed as $\Delta A_{540} \text{ g}^{-1} \text{ (d.m.) min}^{-1}$.

For the extraction and estimation of catalase (CAT) and superoxide dismutase (SOD) the methods of Snell and Snell (1971) and Giannopolitis and Ries (1977) were followed. Ascorbate peroxidase (APOX) activity was determined according to Nakano and Asada (1981) using homogenates previously supplemented with 0.5 mM ascorbic acid and 0.1 mM EDTA. Parallel experiments in presence of p-chloromercuribenzoate (50 µM) were performed to rule out any interference from guaiacol peroxidases. Glutathione reductase (GR) activity was measured according to Schaedle and Bassham (1977). The reaction mixture contained 50 mM Tris–HCl (pH 7.6), 0.15 mM NADPH, 0.5 mM oxidized glutathione (GSSG), 3 mM MgCl₂ and 100 µl homogenate (7 mg protein ml⁻¹). NADPH oxidation was followed at 340 nm.

For the estimation of total thiol content the process of Tietze (1969) was followed. Total–SH content was assayed in acid soluble extract (500 mg of tissue in 3 % w/v TCA solution) followed by a brief centrifugation. The supernatant was then diluted 10-fold in 100 mM phosphate buffer (pH 7.5). Thiol contents were determined measuring absorbance at 412 nm in presence of 0.5 mM 5, 5'-dithiobis 2-nitrobenzoic acid (DTNB), 0.5 U ml⁻¹ glutathione reductase and 0.2 mM NADPH. The enzyme activity in all cases was expressed as enzyme unit min⁻¹ g⁻¹ d.m. according to Fick and Qualset (1975).

Statistical analysis

Each treatment consisted of three replicates and each experiment was carried out twice at different times. For statistical

analysis of the data for significance, the two sample *t*-test was employed with the help of Minitab 15 package, showing the significant variations between untreated control and temperature stress-raised seedlings. Test for significant variations between H₂O₂ pretreated-temperature stress raised seedlings and untreated temperature stress-raised seedlings was also employed.

Results

The obtained data indicates that both imbibitional chilling (8 °C for 16 h) and heat (40 °C for 16 h) stress imposed to the seeds of a rice cultivar Ratna, caused less survival (measured in terms of relative germination performance, germination rate, mean daily germination, *t*₅₀ value, final germination percentage, Table 1) and delayed early growth performances (measured in terms of vigour index, relative growth index, biomass accumulation, seedling length, Table 2). The result, in general, shows inhibition of germination and early growth performances under the exposure of extremes of temperature (both imbibitional chilling and high temperature) of the experimental rice cultivar (Ratna). The relative germination performance (RGP) of imbibitional chilling and heat stress-raised rice seeds of cultivar Ratna have been reduced to 90.2 and 91.4 % respectively (Table 1). Similarly the relative growth index (RGI) for imbibitionally chilling and heat stress-raised seedlings of rice cultivar Ratna have been reduced to 81.2, 82.4 % respectively (Table 1), hinting significantly more early growth impairment compared to germination by the cultivar Ratna under chilling and heat stress. Assessment of other parameters of germination and early growth impairment, like germination rate, mean daily germination, *t*₅₀ value, vigor index etc. also corroborate the

finding that germination continued even at supra and sub-optimum temperature but early growth was inhibited and delayed.

In order to establish whether the early growth inhibition caused by elevated and low temperature during the early imbibitional phase of germination was due to disruption of redox homeostasis and associated oxidative damages of the germinating tissue, the accumulation of thiobarbituric acid reactive substances (TBARS), membrane protein thiol level (MPTL), free carbonyl content (C=O), H₂O₂ and O₂⁻ content were assessed. The result clearly shows that both imbibitional heat and chilling stress caused significant enhancement in the accumulation of TBARS, free carbonyl content and ROS (Table 3) along with concomitant reduction in membrane protein thiol level (Table 3) for the rice cultivar Ratna. The same trends of result have been noticed when membrane injury index, oxidative stress index, membrane permeability status (measured in terms of electrical conductivity, α-NH₂ and soluble carbohydrate content of tissue leachate) was measured (Table 4) for both the heat and chilling stress-raised seedlings of rice.

To assess whether a pulse of low titer reactive oxygen species H₂O₂ plays any acclimatory role in restoring redox homeostasis and improving survival and early growth performances of the experimental rice cultivar, standardized low concentration H₂O₂ pretreatment (100 μM) have been made during early imbibitional phase of germination prior to the exposure of unfavorable temperature. H₂O₂ pretreatment clearly exhibited significant reversal in oxidative membrane damages which can be vouched from the reduction in accumulation of TBARS, C=O, restoration of MPTL (Table 3) and improvement of membrane permeability status (Table 4) as well as improvement in early growth performances for the cultivar Ratna (Tables 1 and 2). Surprisingly, the result

Table 1 Effect of imbibitional H₂O₂ pre-treatment (100 μM) on chilling (8 °C for 16 h) and heat (40 °C for 16 h) stress induced changes in germination rate, *T*₅₀ value, mean daily germination (%) and final

germination percentage and relative germination performance (%) of rice (*Oryza sativa* L, cultivar Ratna)

Rice Cultivar	Treatment	Germination rate	<i>T</i> ₅₀ (h)	Mean Daily germination (%)	Final germination percentage (%)	Relative Germination Performance (%)
Ratna	Untreated control	19.23±0.15	8.66±0.18	17.1±0.14	88.6±0.63	100
	Control + H ₂ O ₂	18.54±0.12	20.01±0.14**	16.98±0.17	86±0.54*	97*±0.65
	IHS	16.47±0.16**	20.49±0.24**	16.25±0.16	81±0.71**	91.4**±0.72
	ICS	14.86±0.11**	23±0.22**	15.32±0.13	80±0.57**	90.2**±0.88
	H ₂ O ₂ + IHS	16.91±0.18	21.39±0.21**	16.28±0.15	85.74±0.69**	96.7*±0.80
	H ₂ O ₂ + ICS	15.26±0.17*	21.16±0.19**	15.92±0.12	85.17±0.62**	96.1*±0.94

Results are mean of three replicate±Standard Error

IHS imbibitional heat stress

ICS Imbibitional chilling stress

*Significant from control at 0.05 level (*t*-test)

**Significant from control at 0.01 level (*t*-test)

Table 2 Effect of imbibitional H₂O₂ pretreatment (100 μ M) on chilling (8 °C for 16 h) and heat (40 °C for 16 h.) stress induced changes in early growth performance (measured in terms of relative growth index,vigor index, biomass accumulation and seedling length, 168 h old) of rice (*Oryza sativa* L., cultivar Ratna)

Rice Cultivar	Treatment	Biomass accumulation(mg)	Vigor index	Relative growth index (%)	Seedling length(mm) [7th day]	
					Root	Shoot
Ratna	Untreated control	150.80 $\pm$ 0.51*	609.87 $\pm$ 0.49	100	4.59 $\pm$ 0.043	2.42 $\pm$ 0.051
	Control + H ₂ O ₂	145.8 $\pm$ 0.56**	582.22 $\pm$ 0.45**	96.6 $\pm$ 0.21**	4.39 $\pm$ 0.072*	2.38 $\pm$ 0.029
	I H S	124.4 $\pm$ 0.58**	498.1 $\pm$ 0.37**	82.4 $\pm$ 0.17**	3.87 $\pm$ 0.054*	1.99 $\pm$ 0.039**
	I C S	122.6 $\pm$ 0.49**	441.84 $\pm$ 0.32**	81.2 $\pm$ 0.14**	3.59 $\pm$ 0.048*	1.67 $\pm$ 0.031**
	H ₂ O ₂ + IHS	140.2 $\pm$ 0.43**	510.84 $\pm$ 0.29**	92.9 $\pm$ 0.20**	3.89 $\pm$ 0.051*	2.05 $\pm$ 0.046*
	H ₂ O ₂ + ICS	143.8 $\pm$ 0.52**	459.85 $\pm$ 0.39*	95.3 $\pm$ 0.32**	3.69 $\pm$ 0.067**	1.72 $\pm$ 0.041*

Results are mean of three replicates $\pm$ Standard Error

IHS imbibitional Heat Stress

ICS imbibitional Chilling Stress

*Significant from control at 0.05 level (*t*-test)**Significant from control at 0.01 level (*t*-test)

also exhibited that H₂O₂ pretreatment (100 μ M) prior to imbibitional heat and chilling stress reduced its own accumulation as well as the accumulation of superoxide in root and shoot tissue of cultivar Ratna when compared with untreated heat and chilling stress raised seedlings (Table 3). The data of membrane injury index and oxidative stress index

(Table 4) further strengthen the fact that H₂O₂ pretreatment prior to imbibitional heat and chilling stress caused significant reduction of oxidative membrane deterioration for the rice cultivar Ratna.

Extremes of temperature during early imbibitional phase of germination significantly reduce the efficiency of

Table 3 Effect of imbibitional H₂O₂ pre-treatment (100 μ M) on chilling (8 °C for 16 h) and heat (40 °C for 16 h) stress induced changes in membrane injury index (MII), oxidative stress index (OSI) andmembrane permeability status of *Oryza sativa* L. (Cultivar Ratna) seedlings (168 h old)

Rice Cultivar	Treatment	MII (%)	OSI (%)	Membrane permeability status		
				EC (mS cm ⁻¹ g ⁻¹ dm) of tissue leachate	Soluble carbohydrate content of tissue leachate (mg g ⁻¹ dm)	α -NH ₂ content of tissue leachate (mg g ⁻¹ dm)
Ratna	Unt. con Root	0	0	1.701 $\pm$ 0.0028	11.982 $\pm$ 0.0048	1.52 $\pm$ 0.0061
	IHS Root	22.4 $\pm$ 0.03*	186 $\pm$ 0.40**	1.920 $\pm$ 0.0037	13.019 $\pm$ 0.0047	1.86 $\pm$ 0.0054
	ICS Root	32.1 $\pm$ 0.04**	181 $\pm$ 0.52**	2.127 $\pm$ 0.0046*	13.987 $\pm$ 0.0057*	1.92 $\pm$ 0.0047
	H ₂ O ₂ + Con Root	6.5 $\pm$ 0.003	23 $\pm$.05	1.821 $\pm$ 0.0054	12.113 $\pm$ 0.0039	1.64 $\pm$ 0.0053
	H ₂ O ₂ + IHS Root	17 $\pm$ 0.02*	132 $\pm$ 0.46**	1.890 $\pm$ 0.0029*	12.741 $\pm$ 0.043	1.75 $\pm$ 0.0072
	H ₂ O ₂ + ICS Root	26.2 $\pm$	141 $\pm$ 0.32**	1.989 $\pm$ 0.0038**	12.998 $\pm$ 0.0048	1.85 $\pm$ 0.0045
	Unt. con Shoot	0	0	1.429 $\pm$ 0.0052	1.412 $\pm$ 0.0043	2.54 $\pm$ 0.0054
	IHS Shoot	36 $\pm$ 0.04*	159 $\pm$ 0.60**	1.625 $\pm$ 0.0071*	2.899 $\pm$ 0.0057**	2.98 $\pm$ 0.0065*
	ICS Shoot	44 $\pm$ 0.06**	151 $\pm$ 0.52**	1.834 $\pm$ 0.0051**	3.976 $\pm$ 0.0061**	3.10 $\pm$ 0.0075**
	H ₂ O ₂ + Con Shoot	7 $\pm$ 0.002	20.5 $\pm$.04*	1.502 $\pm$ 0.0055	1.689 $\pm$ 0.0052	2.73 $\pm$ 0.0058
	H ₂ O ₂ + IHS Shoot	34 $\pm$ 0.03	138 $\pm$.41**	1.579 $\pm$ 0.0047	2.145 $\pm$ 0.0047**	2.81 $\pm$ 0.0054
	H ₂ O ₂ + ICS Shoot	37 $\pm$ 0.04**	131 $\pm$.22**	1.712 $\pm$ 0.0038**	3.024 $\pm$ 0.0059**	2.89 $\pm$ 0.0058*

Results are mean of three replicates $\pm$ Standard Error

IHS imbibitional heat stress

ICS imbibitional chilling stress

*Significant from control at 0.05 level (*t*-test)**Significant from control at 0.01 level (*t*-test)

Table 4 Effect of imbibitional H₂O₂ pre-treatment (100 µM) on chilling (8 °C for 16 h) and heat (40 °C for 16 h) stress induced changes in the oxidative damage [measured in terms of accumulation of thiobarbituric acid reactive substances (TBARS), membrane protein thiollevel (MPTL), free carbonyl content (C=O), reactive oxygen species accumulation (O₂⁻, H₂O₂)] of *Oryza sativa* L (Cultivar Ratna) seedlings (168 h old)

Rice Cultivar	Treatment	MPTL (n mol g ⁻¹ d.m.)	C=O content mmol g ⁻¹ (d.m.)	TBARS content (n mol g ⁻¹ d.m.)	O ₂ ⁻ content (ΔA ₅₄₀ g ⁻¹ d.m.)	H ₂ O ₂ content (µmol g ⁻¹ dm)
Ratna	Unt. con Root	112±0.29	172±0.52	153±0.40	6.5±0.01	0.879±0.003
	IHS Root	68±0.53**	248**±0.88	284±0.48**	16.8±0.04**	2.043±0.005**
	ICS Root	71±0.44**	226**±0.92	275±0.53**	13.0±0.03**	1.930±0.002**
	H ₂ O ₂ + Con Root	108±0.44	161±0.54	199±0.47**	6.8±0.01*	1.019±0.006*
	H ₂ O ₂ + IHS Root	84±0.40**	202*±0.80	202±0.48**	11.2±0.03**	1.845±0.005**
	H ₂ O ₂ + ICS Root	88±0.60**	192*±0.90	214±0.52**	10.3±**	1.645±0.007*
	Unt. con Shoot	162±0.81	204±0.55	197±0.44	8.8±0.02*	2.13±0.009
	IHS Shoot	89±0.42**	284**±0.90	301±0.56**	21.8±0.04*	3.071±0.011**
	ICS Shoot	98±0.41**	242**±0.50	299±0.51**	20.1±0.03**	2.685±0.007*
	H ₂ O ₂ + Con Shoot	164±0.88	193±0.68	201±0.32*	8.5±0.01**	1.396±0.0012**
	H ₂ O ₂ + IHS Shoot	112±0.72**	242*±0.42	289±0.38**	15.5±0.06*	2.584±0.006**
	H ₂ O ₂ + ICS Shoot	117±0.66**	218±0.84	244±0.41**	16.2±0.02	2.276±0.008

Results are mean of three replicates ± Standard Error

IHS imbibitional heat stress

ICS imbibitional chilling stress

*Significant from control at 0.05 level (*t*-test)**Significant from control at 0.01 level (*t*-test)

antioxidative defense, assessed in terms of activities of superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase and total thiol content (Table 5) of the seedlings of the cultivar Ratna. When the impact of imbibitional H₂O₂ pretreatment on the antioxidative defense of imbibitionally chilling and heat stressed experimental rice seedlings were assessed for seedlings of cultivar Ratna, based on the same parameters, it clearly exhibited significant increment in the activity of the antioxidative enzymes particularly ascorbate peroxidase and glutathione reductase as well as the level of total thiol content (Table 5).

Discussion

The obtained data exhibited induction of heat and chilling-induced disruption of redox homeostasis and oxidative damage to the newly assembled membrane lipid and protein (evident from the data of MPTL, C=O and TBARS) in the germinating experimental rice cultivar, which continued even after seven days of treatment. Protein carbonylation and the loss of membrane protein thiol level under extremes of temperature, the signs of irreversible oxidation processes that leads to loss of protein function is more sensitive indicator of oxidative stress than lipid peroxidation, because the later may be catabolized more rapidly than oxidized proteins (Palma et al. 2002). The susceptibility of newly

assembled membrane system towards oxidative damages (by aggravating membrane lipid peroxidation, protein carbonylation and reducing membrane protein thiol content) strongly suggests the importance of intact membrane system in post-germinative growth (Bhattacharjee and Mukherjee 2003/4). This concurs with the result described by Larkindale and Huang (2004), Gong et al. (1998), Bhattacharjee (2008). The loss of redox homeostasis and the oxidative damage suffered by germinating rice seeds under extremes of temperature (measured in terms of MPTL, C=O and TBARS) generally correlated with reduced early growth performances. Although parameters of oxidative damage and early growth performances are not completely linked, in all experiments, the survival and early growth performances declined with higher contents of TBARS, RC=O and lower MPTL. This strongly suggests that survival and subsequent growth during early germination after heat and chilling stress requires ability to tolerate and mitigate oxidative damages to membrane lipid and protein (Larkindale and Huang 2004; Gong et al. 1998; Bhattacharjee and Mukherjee 2006; Bhattacharjee 2008).

All the results pertaining to oxidative membrane injury, ROS accumulation and antioxidative defense clearly indicate that low titer of H₂O₂ pretreatment (100 µM) prior to imbibitional heat and chilling stress causes significant up-regulation of antioxidative defense and management of oxidative stress. Since oxidative membrane lipid peroxidation

Table 5 Effect of imbibitional H₂O₂ pre-treatment (100 μ M) on chilling (8 °C for 16 h) and heat (40 °C for 16 h) stress induced changes in antioxidative defense (measured in terms of activities of superoxidedismutase, catalase, ascorbate peroxidase, glutathione reductase and total thiol content) of *Oryza sativa* L. (Cultivar Ratna) seedlings (168 h old)

Rice Cultivar	Treatment	Antioxidative Defense				
		Superoxide dismutase (U g ⁻¹ d.m. min ⁻¹)	Ascorbate Peroxidase (U g ⁻¹ d.m. min ⁻¹)	Catalase (U g ⁻¹ d.m. min ⁻¹)	Glutathione reductase (U g ⁻¹ d.m. min ⁻¹)	Total Thiol Content (μ mol g ⁻¹ d.m)
Ratna	Unt. Con. Root	3.05±0.01	8.811±0.045	2.001±0.0057	0.340±0.002	1.98±0.04
	H ₂ O ₂ + Con. Root	3.15±0.02*	7.002±0.04	1.980±0.0031	0.318±0.003	2.10±0.03
	IHS Root	1.52±0.01**	4.243±0.05**	1.231±0.0043**	0.174±0.001**	0.98±0.01**
	ICS Root	1.84±0.04**	5.056±0.05**	1.879±0.0046	0.244±0.003**	1.10±0.02**
	H ₂ O ₂ + IHS Root	2.10±0.03**	6.531±0.05**	1.802±0.0038*	0.248±0.004**	1.55±0.01**
	H ₂ O ₂ + ICS Root	2.34±0.01**	5.904±0.06**	1.930±0.0041*	0.294±0.001	1.40±0.07**
	Unt. Con. Shoot	2.14±0.01	12.96±0.07	5.125±0.0068	0.300±0.001	1.84±0.04
	H ₂ O ₂ + Con Shoot	2.01±0.02	12.998±0.06	4.817 ±0.0063*	0.305±0.004	1.80±0.06
	IHS Shoot	0.94±0.04**	9.012±0.07**	4.401±0.0056**	0.146±0.003**	1.00±0.02**
	ICS Shoot	1.51±0.02**	10.36±0.06**	4.203±0.0045**	0.160±0.006*	1.05±0.02**
	H ₂ O ₂ + IHS Shoot	1.94±0.01	11.427±0.05	4.723±0.0041*	0.245±0.004	1.48±0.01*
	H ₂ O ₂ + ICS Shoot	1.98±0.01	12.998±0.06	4.817±0.0043*	0.210±0.002*	1.35±0.02**

Results are mean of three replicates $\pm$ Standard Error

IHS imbibitional heat stress

ICS imbibitional chilling stress

*Significant from control at 0.05 level (*t*-test)**Significant from control at 0.01 level (*t*-test)

is detrimental and one of the significant causes abiotic stress induced damages, research into this process was quite intense during last several years (Bhattacharjee 2005, 2010; Foyer et al. 1997; Suzuki and Mittler 2006). Excess accumulation of ROS (like H₂O₂ and O₂⁻) or imposed secondary oxidative stress during early germination might cause germination and early growth impairment (Gong et al. 1997, 1998; Bhattacharjee 2008). In several studies lower efficiency of antioxidative defense along with aggravation of oxidative damages to newly assembled membrane system has been held responsible for poor germination and early growth performances (Wahid et al. 2007, 2008; Devi et al. 2008; Bhattacharjee 2008). In contrary to this, several workers highlighted the impact of the oxidative stress in response to fluctuation of environmental condition on acclimatory responses to plant (Devi et al. 2008; He et al. 2009; Foyer et al. 1997; Krause 1988; Prasad et al. 1994; Russell et al. 1995; Wahid et al. 2007). When a leaf experiences a set of condition such as excess light or temperature fluctuation, it immediately responded by intracellular signaling and by up-regulating the activities of antioxidant defense genes elicited by redox changes followed by increase in H₂O₂ level (Karpinski et al. 1999). Prolong exposure in such a condition led to death of such a leaf. However, leaves suffering from environmental stress also produce systemic signal, a component of which may be H₂O₂, which set up an acclimatory response in

unstressed region of plant. The signaling mediated by H₂O₂ led to an increase capacity to tolerate further episode of environmental stress by activation of antioxidative defense that is systemic acquired acclimation. Treatment of a leaf with H₂O₂ and then its exposure to excess light caused significantly greater induction of APX-2 gene coding the enzyme ascorbate peroxidase (Krause 1988; Foyer et al. 1997). Protective effects of H₂O₂ pretreatment have been described for maize seedling under chilling stress (Prasad et al. 1994) and have been explained on the basis of triggering various stress defense mechanisms. Treatment of winter wheat with low concentration of H₂O₂ and inhibition of catalase did induce synthesis of polypeptide similarly to those synthesized during chilling stress (Krause 1988). Prasad et al. (1994) also reported that maize seedling became more chilling tolerant following H₂O₂ pretreatment. The present work clearly demonstrates the fact that imbibitional H₂O₂ pretreatment in general causes up regulation of antioxidative defenses and mitigation of oxidative damages to the newly assembled membrane system of the heat and chilling stress-raised rice seedlings. The alleviation of oxidative stress by H₂O₂ pretreatment is reflected by significant improvement in early growth performance of rice cultivar Ratna. Although no direct evidence of beneficial effect of H₂O₂ on membrane architecture is available in literature, the present work strongly favors the view that ROS H₂O₂ at low concentration prevents oxidative damage to membrane lipid

and protein probably by up-regulating antioxidative defense and by restoring redox balance.

Hydrogen peroxide is a membrane permeable molecule with moderately more half-life compared to other ROS and has been demonstrated to function as diffusible intercellular signal (Levine et al. 1994; Bestwick et al. 1997; Wahid et al. 2007; Kovalchuk 2010). Transmembrane movement of H_2O_2 probably involves controlled passage through aquaporin. At lower levels of H_2O_2 treatment, transport through aquaporins may be important in eliciting intracellular response (Allan and Fluhr 1997). Wahid et al. (2007, 2008) suggested that passive absorption of H_2O_2 by germinating seeds up-regulates antioxidative defense, which even persists in seedlings to offset oxidative damage, which led to improved physiological attributes. The increase in both imbibitional heat and chilling stress tolerance in H_2O_2 -pretreated seedlings of rice reinforced the idea that the oxidant H_2O_2 might be required in some signaling pathway leading to better acclimatory performance and improved survival and early growth performances (He et al. 2009; Yu et al. 2003; Wahid et al. 2007). This suggests that H_2O_2 is required to switch on some stress acclimatory metabolism by which plant prevent or repair oxidative damages caused by unfavorable environmental cues (Prasad et al. 1994; Devi et al. 2008; Foyer et al. 1997). A transient increase in H_2O_2 (by pretreatment) might cause signal activation of protective mechanism by up-regulating antioxidative defense and reducing membrane lipid and protein damages (by reducing lipid peroxidation, protein oxidation and restoring membrane protein thiol level) for acclimation to heat and chilling stress of the rice cultivar. Several workers (Kovalchuk 2010; Karpinski et al. 1999; Neill et al. 1999; Wahid et al. 2007) proposed that H_2O_2 generation should be considered as central trigger for defense mechanism following exposure to abiotic and biotic stress. The subject of the present work strongly favor the concept that elevated level of ROS which are generally used in breaking seed dormancy (Fontaina et al. 1994) might also be used in ameliorating oxidative stress imposed by extremes of temperature in experimental rice cultivar. The present work, therefore, proposes that an 'inductive pulse' of H_2O_2 is necessary to switch on some stress acclimatory metabolism by restoring redox homeostasis, through which plant prevent or repair oxidative damages to membrane lipid and protein during the recovery phase of post-germination event in rice.

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