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Contrasting Physiological Response to Oxidative Stress in Rice Genotypes Differing in Tolerance to Salt and Flooding Stresses

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All abiotic stresses lead to evolution of oxidative stress. Relatively, research addressing the physiological basis of oxidative stress tolerance are scanty. Hence, the study was designed to induce oxidative stress, by *in vivo* treatment with hydrogen peroxide (H_2O_2) in rice genotypes showing contrasting behavior to flooding and salt stress. Four genotypes namely, FR 13A (tolerant to flooding stress); Co 43 (susceptible to flooding stress); FL 478 (tolerant to salinity stress) and IR29 (susceptible to salinity stress) were taken to study important physiological traits like chlorophyll contents, cell Membrane Stability (CMS), lipid peroxidation, soluble sugars, Soluble protein content and Nitrate reductase (NR) activity under oxidative stress. The flooding and saline tolerant genotypes showed a lesser per cent reduction in the chlorophyll contents and were able to maintain higher sugar contents on exposure to H_2O_2 treatments compared to the susceptible ones. Degradation of protein contents and reduction in CMS with increased lipid peroxidation was observed in all the varieties and the protein contents decreased with increase in concentration of H_2O_2 treatment. H_2O_2 treatments also showed a linear decrease in NRase activity in all the genotypes taken for study.

Key words: Chlorophyll, Cell Membrane Stability, Lipid peroxidation, Soluble protein content, Soluble sugars, Nitrate reductase activity.

Rice is a salt and flooding sensitive crop, yet it is the only cereal that can grow fairly well in salt and flood affected soils because of its ability to grow in standing water that can help leach salts from top soils to a level lower enough for subsequent crops (Bhumbla and Abrol 1978), as well it thrives during rainy season in coastal saline areas, where other crops cannot survive recurrent flooding. High salinity and submergence stress induce oxidative stress by accumulation of H_2O_2 (Hernandez *et al*., 2000; Gosset *et al*., 1996; Góemez *et al*., 1999; Savoure *et al*., 1999). Oxidative stress is known to be generated by a number of environmental factors including light, temperature, water, mineral deficiency, toxic metals and air pollutants such as O_3, SO_2 and NO_x (Hendry 1994).

Stress often leads to the production of Reactive Oxygen Species (ROS) such as O_2^- and H_2O_2 in plant tissues (Desikan *et al*., 2004). H_2O_2 is produced and accumulated, leading to oxidative stress in plants. Hydrogen peroxide (H_2O_2) is a versatile molecule that is involved in several cell processes under normal and stress conditions (Quan *et al*., 2008). H_2O_2 are highly reactive to membrane lipids, protein and DNA; they are believed to be the major contributing factors to stress injuries and cause rapid cellular damage (Hariyadi and Parkin, 1993; O'Kane *et al*., 1996; Prasad 1996).

Plants after reacting with oxygen can exhibit a broad range of physiological responses including changes in gene expression (Elstner and Osswald 1994). H_2O_2 being a strong oxidant can initiate localized oxidative damage in leaf cells leading to disruption of metabolic function and degradation of pigment composition and protein contents leading to loss of cellular integrity resulting in senescence promotion (Sairam and Srivastava, 2000).

Oxidative stress greatly impacts chlorophyll biosynthesis, which is involved in various potent photosensitizing tetrapyrrole intermediate molecules (Aarti *et al*., 2006) and is also required to maintain stability of the light-harvesting complex proteins (Kuttkat *et al*., 1997). Apart from the chlorophyll damage, synthesis of organic solutes, the soluble sugars are also greatly affected by oxidative stresses. Sugars act as osmolytes and participate in retaining water, which allows the plant to maintain its physiological functions (Hasegawa *et al*., 2000). The presence of various ROS generated as a result of oxidative stress can cause severe oxidative damage to biomolecules like proteins and nucleic acids (Alia *et al*., 1995; Gille and Singler 1995). Hence, studies on NRase activity has been used as bioindicators in determining oxidative stress damage (Panda and Choudhury, 2005). Generation of ROS or oxidative stress also leads to lipid peroxidation (Chen *et al*., 2000). With physiological parameters such as Cell Membrane Stability (CMS), it is now possible to measure the membrane integrity under various stresses.

All the abiotic stresses lead to development of oxidative stresses. Though the physiological mechanisms underlying the salt and flood tolerance

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in rice genotypes have been extensively studied, yet, the physiological basis of oxidative stress response is not very clear. Hence, key physiological traits such as pigment composition, sugar and protein contents, Nitrate reductase (NRase) activities, membrane stability, which take lead in identifying rice genotypes for multiple stress tolerance were studied on the induction of oxidative stress in flooding, salt tolerant and susceptible rice genotypes in order to understand the physiological mechanisms underlying oxidative stress tolerance.

Materials and Methods

Plant material, growth and treatment conditions

Rice genotypes (*Oryza sativa L.*) cvs. FL 478 (salt tolerant); IR29 (salt susceptible); FR 13A (flooding tolerant) and Co 43 (flooding susceptible) were planted in earthen pots (medium size) filled with 10 kg mixture of tank silt and farm yard manure in 5:1 ratio. Each pot was fertilized with N,P,K corresponding to 150,50,50 kg/ha, respectively. Three seedlings were maintained in each pot. A total of sixty pots were maintained with three pots for each treatment in a variety. Plants were watered regularly. Samples for various assays/estimations were taken on 30-35 days after sowing. Assays were performed in the first fully expanded leaves. Samples collected in ice bucket were washed with tap water and then with double distilled water. Leaf strips of uniform size were submerged in about 150 cm^3 of various concentrations of H_2O_2 (0.05, 0.1, 0.15 and 0.2 mM) in 0.1M potassium phosphate buffer, pH 7.5 contained in 250 cm^3 beakers and incubated for 6 h in dark at 25^o C. Samples incubated in phosphate buffer served as control. After incubation the samples were twice washed with double distilled water and soaked dry and processed for various observations.

Determination of chlorophyll contents and soluble sugars

Measurements on Chlorophyll 'a', 'b' and total were made by following the protocol of Yoshida *et al*. (1976). About 0.1g of leaf samples was used for estimation of chlorophyll contents and expressed in mg/g.

Chlorophyll 'a' = (12.7* OD at 663nm) – (2.67 * OD at 645nm) * volume/ (1000 * weight)

Chlorophyll 'b' = (22.9* OD at 645nm) – (4.68 * OD at 663nm) * volume/ (1000 * weight)

Total chlorophyll = chlorophyll a + chlorophyll b

Soluble sugars (glucose, fructose, and saccharose) were determined in samples of leaf tissue macerated with 80% ethanol. The alcoholic extracts were transferred to 15 mL tubes and warmed in an oven at 30º C for 30 min, then centrifuged at 1957 × g for 10 min. The pellet was extracted second time using the same procedure. The two alcoholic extracts were then combined and treated with an anthrone solution. Soluble sugars were then quantified using a molecular absorption spectrophotometer at 254 nm (Hewitt, 1958).

Protein determination and NRase assays

The soluble protein content of the leaves was determined by measuring the colour developed by the reduction of Folin-Ciocalteau reagent by the amino acids like tyrosine and tryptophan of protein, following the method of Lowry *et al*. (1951) and expressed in mg g^{-1} FW. Nitrate reductase was determined as per the method of Hageman and Hucklesby (1971). The analysis was carried out using the physiologically matured leaf (third leaf from top) and the activity was expressed as µ moles of NO_2 g^{-1} hr^{-1} FW.

Lipid peroxidation and Cell membrane Stability

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content in 1 g leaf FW according to Koca *et al*. (2007). Malondialdehyde is a product of lipid peroxidation by thiobarbituric acid reaction. The concentration of MDA was calculated from the absorbance at 532 nm (correction was done by subtracting the absorbance at 600 nm for unspecific turbidity) by using extinction coefficient of 155 mM^{-1} cm^{-1}. Measurements of CMS were made by following the protocol of Blum and Ebercon (1981). CMS was calculated using the equations of Blum and Ebercon (1981) as given below.

CMS (%) = [(1-(T1/T2)) / (1-(C1/C2))]* 100,

where, T and C refer to the stress and control samples, respectively; the subscripts 1 and 2 refer to the initial and final conductance readings, respectively.

Statistical analysis

The experiment was conducted with a completely randomized design with four (oxidative stress levels: 0.05, 0.1, 0.15,0.2 mM H_2O_2) × four (genotypes), with five replicates. The data were analyzed statistically with SPSS-17 statistical software (SPSS, Chicago, Illinois, USA). Means were statistically compared by Duncan's multiple range test (DMRT) at $P < 0.05$ level.

Results and Discussion

Hydrogen peroxide is a potent cytotoxic compound produced during salinity, drought, high and low temperature stresses (Sairam and Srivastava, 2000). In order to understand the physiological mechanisms underlying oxidative stress tolerance in rice, four rice genotypes differing in their tolerance behavior to salinity (FL 478 and IR 29) and submergence (FR 13A and Co 43) were subjected to oxidative stress by exposing them to various H_2O_2 concentrations to study the changing patterns of chlorophyll, soluble sugars, protein contents, membrane stability and activities of nitrate reductase.

Changes in chlorophyll contents

The study clearly indicated that the genotypes varied significantly in chlorophyll degradation pattern. Hydrogen peroxide treatment resulted in decrease in chlorophyll contents (Chlorophyll a, b and total chlorophyll) and the decrease increased with increase in concentration of hydrogen peroxide (Fig.1). Though

the susceptible genotypes, Co 43 (2.66 mg/g) and IR 29 recorded higher chlorophyll contents under control conditions, there was a sharp decline in the chlorophyll contents with H_2O_2 treatments. But, the saline tolerant genotype FL 478 and flooding tolerant FR 13A showed a lesser per cent reduction in the chlorophyll contents on exposure to H_2O_2 treatments, when compared to the other two rice varieties. FR 13A was able to maintain higher chlorophyll contents (2.04mg/g) even when the leaves were exposed to 0.20mM H_2O_2. The chlorophyll a and chlorophyll b contents also followed a similar trend. Rice genotypes tolerant/ susceptible to salinity and flooding stress show differential H_2O_2 accumulation and physiological response (Lee *et al*., 2001; Khan and Panda 2008; Stanisavljevic *et al*., 2011; Blokhina *et al*. 2001). The findings of the present study are in line with the findings of Upadhyaya *et al*. (2007); Lin and Kao (1998) and Patra and Panda (1998), where the authors concluded that the H_2O_2 induced an increase in chlorophyll damage. Similarly, Aarti *et al*. (2006) have reported that 100 mM methyl viologen induced oxidative stress and impeded with key steps in chlorophyll biosynthesis.

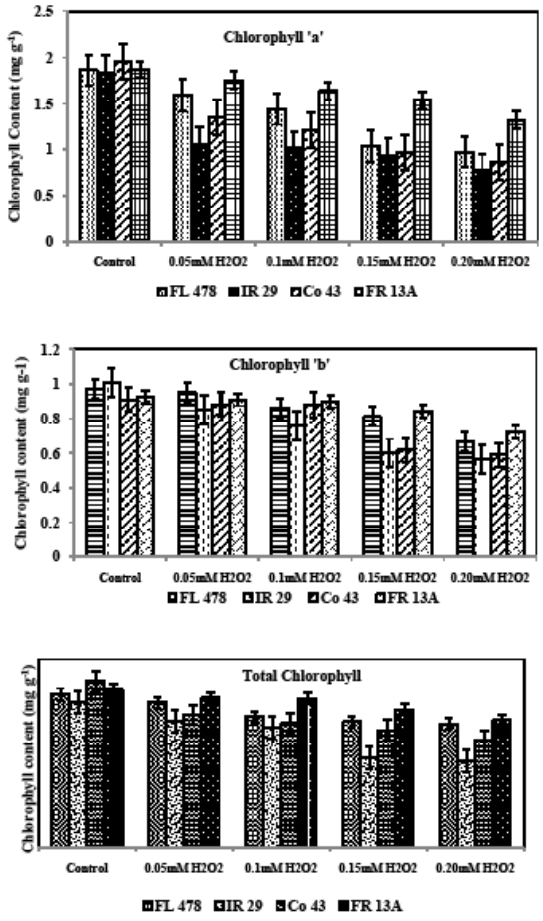

Fig. 1. Response of rice genotypes to different concentrations of hydrogen peroxide treatment on the chlorophyll contents (chlorophyll a, chlorophyll b and total chlorophyll in mg/g)

Accumulation patterns of soluble sugar

The synthesis of osmotically active compounds such as soluble sugars is a strategy with high energy cost for plants, but it is necessary when the level of stress increases; they may alter cellular metabolism (Serraj and Sinclair, 2002). A wide range of other molecules such as saccharose, pinnitol, and mannitol may accumulate in leaf tissues to a lesser degree (Hasegawa *et al*., 2000). In this study, the tolerant genotypes (FL 478 and FR 13A) showed capacity for osmotic adjustment by synthesizing soluble sugars from 0.15mM to 0.2. mM H_2O_2 stress. FL 478 the soluble sugar content increased from 62.02 mg g^{-1} DW in control to 84.82 mg g^{-1} DW, when the plants were experiencing an oxidative stress of 0.20 mM H_2O_2. Similarly, in flood tolerant genotype, FR 13A also showed an increase in sugar content by 43.48% over control. The sugar content values increased from 60.05 mg g^{-1} DW to 86.16 mg g^{-1} DW. Drought stress was found to increase the sugar contents in rice anthers (Fu Guan-fu *et al*., 2011). The increase in sugar content also acts as an osmolyte and contributes to the maintenance of plasma membrane integrity (Pareddy and Greyson, 1989; Tremblay and Tremblay, 1991). Soluble sugars can be involved in, or related to, ROS-producing metabolic pathways. In reverse, soluble sugars can also feed NADPH-producing metabolic path- ways, such as the oxidative pentose-phosphate (OPP) pathway, which can contribute to ROS scavenging (Ivan Coue *et al*., 2013).

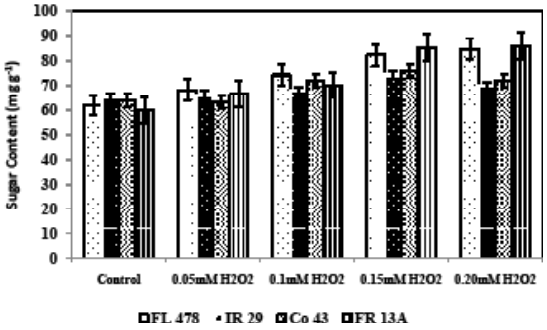

Fig. 2. Effects of hydrogen peroxide treatment on accumulation of soluble sugars in rice genotypes

Soluble protein profiles

With respect to the soluble protein contents, hydrogen peroxide treatment showed pronounced and adverse effects on soluble protein contents irrespective of the varieties taken for the study. Hydrogen peroxide treatment resulted in decrease in protein contents and the decrease, increased with increase in concentration of hydrogen peroxide treatment (Table 1). In genotypes FL 478 and FR 13A, the protein content gradually decreased with H_2O_2 treatments (FL 478: 20.15 to 16.12 mg/g; FR 13A: 20.15 to 16.53 mg/g), while in varieties IR 29 and Co 43, the protein contents gradually decreased upto 0. 1mM H_2O_2 and thereafter there was a steep decline in the protein contents at 0.15mM and 0.2mM H_2O_2 treatments. It is known that water, salt, metal toxicity and other stress factors induce endogenous H_2O_2 accumulation

(Upadhyay *et al*., 2007). H_2O_2 treatment of primary rice leaves induced an increase in chlorophyll, carotenoid and protein degradation in senescing leaves as observed also for other abiotic stresses (Sairam *et al*., 1997; Panda 2002). The protein degradation in senescing leaves may be due to a cytotoxic effect of H_2O_2 (Mukherhee and Choudhuri, 1983; Menconi *et al*., 1995; Khan and Panda, 2002).

Table 1. Response of rice genotypes to different concentrations of hydrogen peroxide treatment on soluble protein contents (mg/g).

Treatments	Rice genotypes			
	FL 478	IR 29	Co 43	FR 13A
Control	20.15±0.40	23.62±0.46	21.22±0.42	20.15±0.40
0.05mM H_2O_2	19.82±0.39	19.51±0.38	19.11±0.38	18.23±0.36
0.1mM H_2O_2	19.54±0.38	18.75±0.37	19.05±0.37	18.15±0.36
0.15mM H_2O_2	18.75±0.37	15.23±0.30	16.44±0.32	17.25±0.34
0.20mM H_2O_2	16.12±0.32	15.00±0.29	16.01±0.31	16.53±0.32
SEd.		0.51		
CD (P=0.05)		1.04*		

Values correspond to the average of five replicates ± standard error.

Activities of Nitrate reductase enzymes

Nitrate reductase activity (NRase) in untreated leaves was initially higher in all the rice genotypes taken for study and was the highest (0.789 µg of NO_2/g/hr) in Co 43. H_2O_2 treatments caused a linear decrease in NRase activity and the magnitude of reduction was very less in Co 43 compared to other genotypes. Among the treatments, 0.2mM H_2O_2 caused severe degradation of nitrate reductase activity in all the genotypes. IR 29 recorded a very low activity of 0.311 µg of NO_2/g/hr at the above treatment (Fig. 3). Inhibition of nitrate reductase activity has been reported under induction of oxidative stress caused due to Zn and Pb activity (Luna *et al*., 2000).

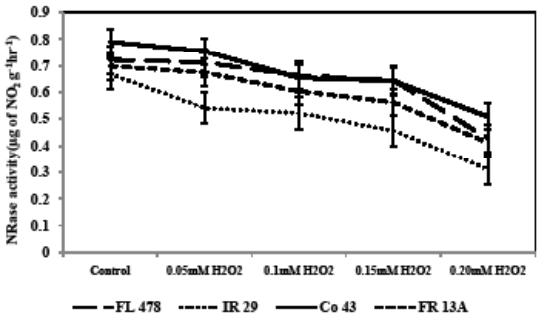

Fig. 3. Changes in Nitrate Reductase activity (µg of NO_2/g/hr) upon Hydrogen peroxide treatment

Membrane stability index

Evidence suggests that membranes are the primary sites of stress injury to cells and organelles, because ROS can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in plasmalema or intracellular organelles (Esfandiari *et al*., 2007). Cell membrane stability has long been taken as an indicator of stress tolerance (Ashraf and Ali, 2008). Cell membrane stability (CMS) decreased with increasing concentration of H_2O_2 treatments in all the four varieties taken. FR 13A showed lower CMS than all the other varieties at all levels of stress treatments. FL 478 had very good CMS initially (88.0%) under control and the per cent decrease in CMS was very less compared to other varieties upon treatment. The genotypes maintained a high CMS (77.0%) even at 0.20 mM H_2O_2 treatment (Table 2). Statistically significant changes were observed in the membrane stability within the genotypes and treatments.

Table 2. Response of rice genotypes to different concentrations of hydrogen peroxide treatment.

Treatments	Rice genotypes / CMS (%)			
	FL 478	IR 29	Co 43	FR 13A
Control	88±1.73	84±1.65	80±1.57	80±1.57
0.05mM H_2O_2	84±1.65	82±1.61	78±1.53	76±1.49
0.1mM H_2O_2	82±1.61	77±1.51	77±1.51	72±1.41
0.15mM H_2O_2	80±1.57	72±1.41	75±1.47	70±1.37
0.20mM H_2O_2	77±1.51	69±1.35	73±1.43	65±1.28
SEd.		2.15		
CD (P=0.05)		4.37*		

Values correspond to the average of five replicates ± standard error.

Lipid peroxidation and MDA contents

Lipid peroxidation in leaves of all the rice genotypes was measured using MDA content analysis. Oxidative stress increases lipid peroxidation (Nisha Kumari *et al*., 2013). MDA contents increased with increase in oxidative stress levels in all the genotypes (Fig. 4). Lipid peroxidation was observed with increasing H_2O_2 concentrations (Upadhyay *et al*., 2007). Higher MDA content in all stress levels was observed in the sensitive varieties (IR 29 and CO 43), thus indicating an increase in lipid peroxidation. These findings are in line with the findings of Olmos *et al*. (1994) and Nisha Kumari *et al*., (2013). MDA contents reached 12.04 µmmol g^{-1} fw and 12.24 µmmol g^{-1} fw under 0.20mM H_2O_2 in IR 29 and Co 43 respectively. The tolerant genotypes, FL 478 and FR 13A were found to record low MDA contents of (6.55 and 6.47 under 0.2 mM H_2O_2 stress respectively) exhibiting decreased lipid peroxidation at all levels of oxidative stresses.

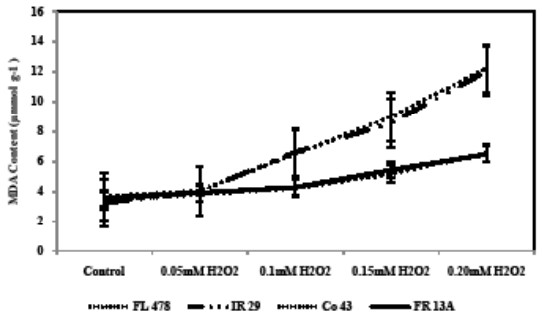

Fig. 4. Effects of hydrogen peroxide treatment on the MDA content (degree of lipid peroxidation)

The study clearly stated a strong negative correlation of lipid peroxidation and tolerance behaviour of the plants, as higher lipid peroxidation was found to be associated with lesser chlorophyll contents (Fig. 5). Hence, this trait is recently been used as an effective selection criterion for stress tolerance in plant species such as *Brassica napus* (Ashraf and Ali, 2008) and wheat (Sairam *et al*., 2002; Farooq and Azam, 2006). Studies clearly concluded that the oxidative damage of lipids, proteins, nucleic acids and alteration of normal cellular metabolism are important impacts of ROS (Munns, 2002; Tammam *et al*., 2008).

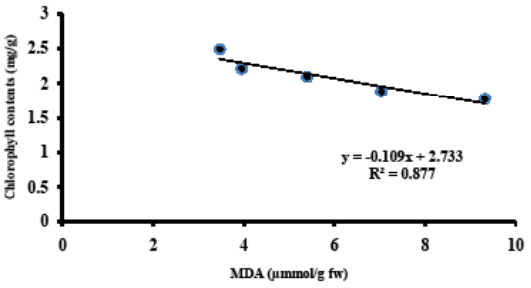

Fig. 5. Co-relation between the MDA and chlorophyll contents in rice genotypes exposed to hydrogen peroxide treatment

Conclusion

Induction of oxidative stress by *in vivo* treatment with hydrogen peroxide in rice genotypes varying in their tolerance behaviour to different abiotic stresses (submergence and salt stress) gave a clear understanding of the signaling role of H_2O_2. Slow degradation of chlorophyll contents and protein pool increased sugar accumulation, lesser lipid peroxidation and lesser reduction in cell membrane stability and stability of nitrate reductase activity in the tolerant genotypes (FR 13A and FL 478), which could explain the physiological basis of stress tolerance.

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