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Enhanced oxidative stress tolerance in rice plants is associated with membrane stability, pigment composition and scavenging of reactive oxygen species

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ABSTRACT

Oxidative stress was induced by in vivo treatment with hydrogen peroxide in rice genotypes showing contrasting behavior to flooding and salt stress. Four rice genotypes 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 used to study important physiological traits like chlorophyll contents, Cell Membrane Stability, Nitrate reductase activity and antioxidant capacity under oxidative stress. H_2O_2 treatments caused degradation in chlorophyll contents, decreased membrane stability and reduced the activities of Nitrate reductase in all the genotypes. A gradual increase in the activities of catalase and peroxidase were recorded under H_2O_2 treatments. Significant upregulation of antioxidant enzyme systems and slow degradation of chlorophyll contents, with less reduction in cell membrane stability and Nitrate reductase activity in the tolerant genotypes (FR 13A and FL 478) play important roles in stress protection.

Key words: *Chlorophyll contents, cell membrane stability, catalase, nitrate reductase activity, peroxidase*

INTRODUCTION

Stress often leads to the production of Reactive Oxygen Species (ROS) such as superoxide O_2 and hydrogen peroxide (H_2O_2) in plant tissues (Desikan et al. 2004). H_2O_2 is produced and accumulates, leading to oxidative stress in plants. Plants have evolved complex regulatory mechanisms in adapting to various environmental stresses. Recently, H_2O_2, in addition to being a toxicant, has been regarded as a signaling molecule (Hung et al., 2005). Therefore, the control of H_2O_2 concentration is critical for cell homeostasis.

High salinity and submergence stress induces oxidative stress by accumulation of H_2O_2 (Gosset et al., 1996; Goemez et al., 1999; Savoure et al., 1999; Hernandez et al., 2000). 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 to cause rapid cellular damage (Hariyadi and Parkin, 1993; O'Kane et al., 1996; Prasad, 1996; Azevedo Neto et al., 2008). 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.

Biochemical strategies used to enhance oxidative stress tolerance in plants include synthesis of osmotic regulators and induction of oxidative enzymes and certain hormones (Nakamura et al., 2002). Under physiological steady-state conditions, there is a balance between the production and scavenging of ROS (Skopelitis et al., 2006). Plants have evolved complex defense mechanisms to avoid an imbalance between generation and scavenging of ROS (Gill and Tuteja, 2010). Enzymes, including superoxide dismutase (SOD),

catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR) (Zhang et al., 1995; Lee and Lee, 2000), and non - enzymatic antioxidants such as tocopherols, ascorbic acid (AsA), and glutathione (GSH) (Wingsle and Hallgren, 1993; Kocsy et al., 1996; Noctor et al., 1998) work in concert to detoxify ROS. ROS may be scavenged by both enzymatic and non-enzymatic pathways; nonetheless, the failure to control ROS may lead to oxidative stress (Wang et al., 2013).

Rice genotypes tolerant/susceptible to salinity and flooding stress show differential H_2O_2 accumulation, physiological response and antioxidant activity (Blokhina et al., 2001; Lee et al., 2001; Khan and Panda, 2008; Stanisavljevic et al., 2011). However, how a plant perceives environmental changes and how it subsequently triggers signals to activate the physiological response are yet to be explored. Besides, the physiological mechanisms underlying the oxidative adaptive responses are not very clear. In order to improve our understanding of the physiological basis of the adaptive response, this article studied the induction of oxidative stress in salt tolerant (FL 478); salt susceptible (IR29) ; flooding tolerant (FR 13A) and flooding susceptible (CO 43) rice genotypes to understand the mechanisms underlying abiotic stress tolerance.

MATERIALS AND METHODS

Plant Growth Conditions and Treatments

Rice genotypes (*Oryza sativa* L.) cvs. FL 478 (used as salt tolerant check); IR29 (used as salt susceptible check); FR 13A (used as flooding tolerant check) and CO 43 (used as flooding susceptible check) obtained from Paddy Breeding Station, Tamil Nadu Agricultural University, 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, 0.05, 0.1, 0.15 and 0.2 mM) in 0.1M potassium phosphate buffer, pH 7.5 contained in 250 cm3 beakers and incubated for 6 h in dark at 25°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 (Sairam and Srivastava, 2000).

Pigment composition and Membrane Stability

Measurements on Chlorophyll 'a', 'b' and total were made by following the protocol of Yoshida et al. (1971). About 0.1g of leaf samples was used for estimation of chlorophyll contents. Measurements of Cell Membrane Stability (CMS) were made by following the protocol of Blum and Ebercon (1981).

Evaluation of Nitrate reductase and antioxidant enzymes

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 (Physiologically active leaf) from top) and the activity was expressed as μ moles of NO_2 $g^{-1}hr^{-1}$ FW. Catalase activity was determined following the method of Luck (1974). One gram of the sample was macerated and extracted in 0.067 M phosphate buffer (pH 7.0). A known volume of the extract was added to the experimental cuvette containing three ml H_2O_2 - PO_4 buffer. The time taken for per cent change in absorbance (Δt) at 240 nm was recorded for calculating the enzyme activity and expressed as enzyme units g-1 tissue. All the operations were carried out at 0 - 5° C. Peroxidase activity Peroxidase activity (Δ420 g^{-1} fresh weight min^{-1}) was determined according to Peru (1962) and Angelini et al. (1990). One gram of leaf was extracted in 0.1 M phosphate buffer (pH 7.0). A known volume of the extract was added to a cuvette containing 3 ml phosphate buffer and 3 ml pyrogallol was added and the increase in absorbance at 420 nm was recorded every 30s for 2 min. The change in absorbance in minutes was used to calculate the enzym*e* activity.

Statistical analysis

The experiment was designed as a completely randomized design and each treatment had three

replicates. Therefore, data from the experiments for each variable were subjected to analysis of variance (ANOVA) and then means were separated using Least Significant Differences (LSD) test. The genotypes, treatments and their interaction effects were significant at $P < 0.05$.

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 salinity and flooding stress tolerance in rice, genotypes exhibiting contrasting tolerance behavior to these stresses were subjected to oxidative stress by exposing them to various H_2O_2 concentrations to study the altered patterns of chlorophyll, membrane stability, activities of Nitrate reductase and antioxidant enzymes.

The results revealed that the hydrogen peroxide treatment showed significant reduction in the chlorophyll contents in all the genotypes taken for the study. Hydrogen peroxide treatment resulted in decrease in chlorophyll contents (Chlorophyll a, b and total chlorophyll) and the decrease was more prominently enhanced with increase in concentration of hydrogen peroxide (Table 1). Though the variety, CO 43 recorded higher chlorophyll contents (2.66 mg/g) under control conditions there was a sharp decline in the chlorophyll contents with H_2O_2 treatments. Similarly, IR 29 also showed a steep decline in chlorophyll contents (1.38 mg/g) with H_2O_2 treatments. But, the saline tolerant genotype FL 478 and flooding tolerant FR 13A showed a lesser percent 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. 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 et al., 2002). The findings of the present study are in line with the findings of Lin and Kao, (1998) and Patra and Panda, (1998) where the authors concluded that the H_2O_2 induced an increase in chlorophyll damage.

CMS is an index of stress tolerance. Oxidative stress increases lipid peroxidation (Nisha Kumari et al., 2013). CMS decreased with increasing concentration of H_2O_2 treatments in all the four varieties. Lipid

Table 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). Data significant at ($P< 0.05$).

Varieties	Treatments		Chlorophyll a	Chlorophyll b	Total Chlorophyll
G_1	T_1	Control	1.863±0.04[b]	1.863±0.04[a]	2.45±0.05[c]
FL 478[a]	T_2	0.05mM H_2O_2	1.592±0.03 [b]	1.592±0.03[d e]	2.32±0.05[d]
	T_3	0.1mM H_2O_2	1.441±0.03[a]	1.441±0.03[f g]	2.09±0.04[f]
	T_4	0.15mM H_2O_2	1.037±0.02 [b]	1.037±0.02[f]	2.01±0.04[f]
	T_5	0.20mM H_2O_2	0.975±0.02[d]	0.975±0.02[b]	1.98±0.04[g]
G_2	T_1	Control	1.834±0.04[i]	1.010±0.02[h]	2.33±0.05[d]
IR 29[c]	T_2	0.05mM H_2O_2	1.055±0.02[g]	0.851±0.02[g h]	2.02±0.04[f]
	T_3	0.1mM H_2O_2	1.012±0.02[c]	0.762±0.01[f g]	1.91±0.04[h]
	T_4	0.15mM H_2O_2	0.933±0.02[f]	0.599±0.01[c]	1.44±0.03[j]
	T_5	0.20mM H_2O_2	0.772±0.02 [I J]	0.564±0.01[i]	1.38±0.03[k]
G_3	T_1	Control	1.952±0.04[h]	0.910±0.02[g h]	2.66±0.05[a]
Co 43[e]	T_2	0.05mM H_2O_2	1.355±0.03[d]	0.883±0.02[f g]	2.12±0.04[e]
	T_3	0.1mM H_2O_2	1.213±0.02 [i]	0.880±0.02[d]	1.99±0.04[g]
	T_4	0.15mM H_2O_2	0.969±0.02[k]	0.619±0.01[j k]	1.88±0.04[h]
	T_5	0.20mM H_2O_2	0.872±0.02[j k]	0.590±0.01[j]	1.71±0.03[i]
G_4	T_1	Control	1.869±0.04[e]	0.925±0.02 [h]	2.52±0.05[b]
FR 13A[b]	T_2	0.05mM H_2O_2	1.752±0.03 [j k]	0.912±0.02[e]	2.40±0.05[d]
	T_3	0.1mM H_2O_2	1.641±0.03[m]	0.899±0.02[k]	2.39±0.04[d]
	T_4	0.15mM H_2O_2	1.537±0.03[l]	0.846±0.02[j k]	2.21±0.04[e]
	T_5	0.20mM H_2O_2	1.322±0.03[g]	0.725±0.01[i]	2.04±0.04[f]

* Values indicated are mean (n=3) ± SE.m. ANOVA was performed and mean comparison by LSD is indicated at $P < 0.05$.

Table 2. Cell Membrane Stability (CMS %) exhibited by rice genotypes in response to different concentrations of Hydrogen Peroxide treatments. * Values indicated are mean (n=3) ± SE.m. ANOVA was performed and mean comparison by LSD is indicated at $P < 0.05$.

Treatments		Rice genotypes			
		FL 478[a]	IR 29[c]	Co 43[c]	FR 13A[b]
Control	T_1[a]	88±1.73[a]	84±1.65[b c]	80±1.57[d e]	80±1.57[h i]
0.05mM H_2O_2	T_2[a b]	84±1.65[b]	82±1.61[d e]	78±1.53[g h i]	76±1.49[d e]
0.1mM H_2O_2	T_3[b c]	82±1.61[c d]	77±1.51[e f]	77±1.51[c d]	72±1.41[i]
0.15mM H_2O_2	$T_{4b c}$	80±1.57[c d]	72±1.41[b c]	75±1.47[g h i]	70±1.37 [f h]
0.20mM H_2O_2	T_5[c]	77±1.51[b]	69±1.35[d e]	73±1.43 [e f g]	65±1.28[j]

peroxidation was observed with increasing H_2O_2 concentrations (Upadhyay et al., 2007). 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 percent decrease in CMS was very less compared to other varieties upon treatment. The genotypes maintained a high CMS (77.0%) even at 0.20mM H_2O_2 treatment (Table 2). Statistically significant changes were observed in the membrane stability within the genotypes and treatments. The extent of damage to lipid membrane was very high in salt sensitive species of Pisum sativum and America cotton (Nisha Kumari et al., 2013).

Nitrate reductase activity (NRase) in untreated leaves was initially higher in all the rice genotypes and was highest (0.789 µg of NO2/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 H2O2 treatments, 0.2mM H_2O_2 treatments caused severe degradation of NRase 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. 1). Inhibition of Nitrate reductase activity has been reported under induction of oxidative stress caused due to Zn and Pb activity (Luna et al., 2000). Not much research on Nitrate reductase activity and oxidative stress has been reported.

POX, CAT, SOD are the three major antioxidant enzymes responsible for scavenging the reactive oxygen species generated via different

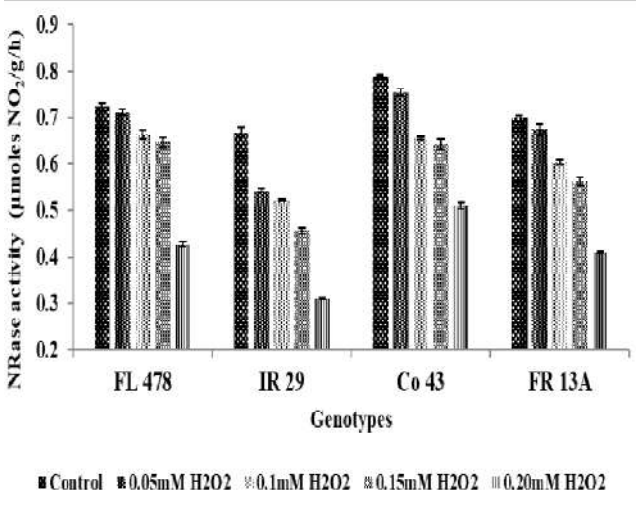

Fig. 1. Effect of Hydrogen peroxide treatment on the Nitrate Reductase activity (µg of NO_2/g/h) in rice genotypes. The bar graph of each treatment is from the mean values of data recorded for each of the genotype. The mean values been clculated the n =3 (replicates). The error bars indicated for each treatment are the SE.m. values.

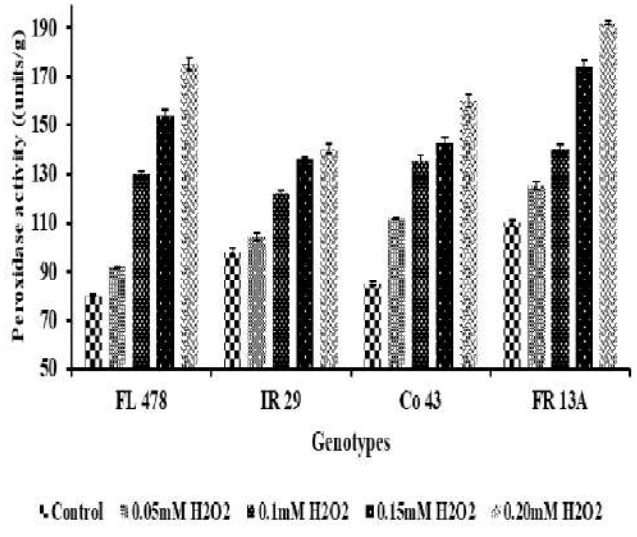

Fig. 2. Effect of Hydrogen Peroxide treatment on the catalase activity (µmolesH_2O_2/g/s) in rice genotypes. The bar graph of each treatment is from the mean values of data recorded for each of the genotype. The mean values been calculated the n =3 (replicates). The error bars indicated for each treatment are the SE.m. values.

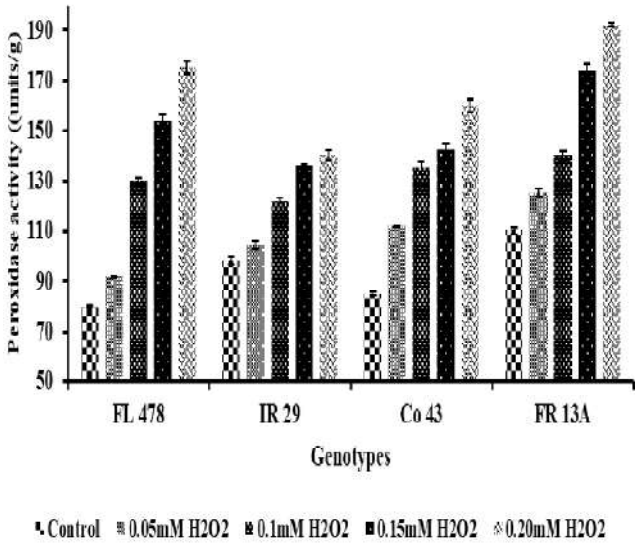

Fig. 3. Effect of Hydrogen Peroxide treatment on the peroxidase activity (units/g) in rice genotypes. The bar graph of each treatment is from the mean values of data recorded for each of the genotype. The mean values been calculated the n =3 (replicates). The error bars indicated for each treatment are the SE.m. values.

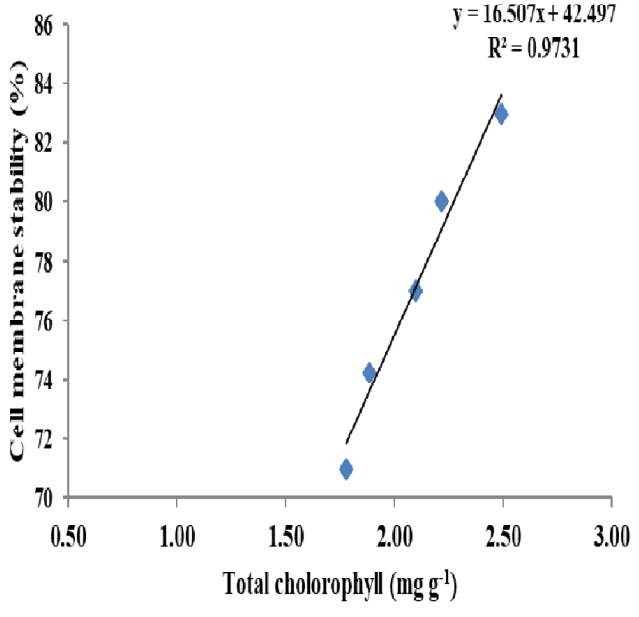

Fig. 4. Effect of H_2O_2 treatment on CMS (%) and Total chlorophyll content (mg g^{-1}). The figure shows a highly negative correlation (r^2= 0.97) indicating that when cell membrane stability decreases then the total chlorophyll content decreases. The correlation graphs were plotted by taking the treatment means of the genotypes (n=3).

the basis to study the activities of two major antioxidant enzymes POX and CAT. Activities of catalase and peroxidase showed increasing trends with increasing H_2O_2 treatments in all the varieties. The increased CAT and POX activities point to a signaling role of H_2O_2 in the induction of H_2O_2 synthesis detoxifying enzymes in rice leaves, as reported for other abiotic stresses (Guo et al., 1997; Sairam and Srisvastava 2000; Lee et al., 2001; Mittova et al., 2002). FR 13A manifested higher activity of catalase and peroxidase than the other genotypes at all concentrations of H_2O_2 treatments. In this genotype the catalase activity ranged from 0.455 µmoles H_2O_2/g/s under control to 1.526 µmoles H_2O_2/g/s at 0.2mM H_2O_2 treatments (Fig. 2). Same pattern was also observed with peroxidase activity where FR 13A recorded 110 (Δ420 g^{-1} fresh weight min^{-1}) under control and increased upto 192 (Δ420 g^{-1} fresh weight min^{-1}) at 0.2mM H_2O_2 treatments (Fig. 3). Similar findings have been reported by Smith et al. (1990) with exposures to O_3 and SO_2, heat shock or drought stresses.

CONCLUSION

From the study it is clear that induction of oxidative stress, by in vivo treatment with hydrogen peroxide in rice genotypes varying in their tolerance behavior to different abiotic stresses (Submergence and Salt stress) proved the signaling role of H_2O_2. It was found that as the H_2O_2 concentrations increased there was an increase in the peroxidase activity and CMS which was accompanied by a increase in total chlorophyll contents (Fig. 4). Similar reports of H_2O_2 accumulation in wheat has lead to enhanced antioxidative responses as evident in the differential or varied levels of antioxidative enzyme activities, accumulation of different antioxidants like photosynthetic pigments and improved CMS (Chakraborty and Pradhan, 2012). Similarly, a positive correlation between NRase activity and pigment compositions, both under control and stress situations was observed. To conclude, the lesser percent reduction in the chlorophyll contents and maintenance of higher NRase activity on exposure to H_2O_2, coupled with significant up regulation of antioxidant enzyme systems and lesser reduction in cell membrane stability in the tolerant genotypes (FR 13A and FL 478) could explain the physiological basis of tolerance and play important roles in stress protection.

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