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ORIGINAL ARTICLE

Induction of Oxidative Stress by Hydrogen Peroxide Treatment in Rice Genotypes to Study the Osmolyte Accumulation Pattern and Antioxidant Capacity

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The aim of the study was to compare the rice genotypes for oxidative stress tolerance. Induction of oxidative stress, by *in vivo* treatment with hydrogen peroxide (H_2O_2) in rice genotypes to study the osmolyte accumulation pattern and antioxidant capacity was investigated. Leaf strips of uniform size from rice genotypes FL 478, IR 29,Co 43 and FR13A were subjected to various concentrations of H_2O_2 (0, 0.05, 0.1, 0.15 and 0.2 mM). All the four rice genotypes exhibited varied responses to proline accumulation. FL 478 and Co 43 exhibited an increase in the accumulation of proline contents initially with low concentrations of H_2O_2, and thereafter showed a sharp decline in proline contents with higher concentrations. Degradation of protein contents in rice leaves was observed in all the varieties and the protein contents decreased with increase in concentration of hydrogen peroxide treatment. 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 protein contents in the tolerant genotypes (FR 13A and FL 478) play important roles in stress protection.

Key words: Catalase, Peroxidase, Proline, Oxidative stress, Rice, Soluble protein

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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 accumulates, leading to oxidative stress in plants. High salinity and submergence stress induces oxidative stress by accumulation of H_2O_2 (Hernandez *et al*., 2000; Gosset *et al.,* 1996; Gomez *et al*., 1999; Savoure *et al*., 1999). 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).

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 signalling molecule (Hung *et al.,* 2005). Therefore, the control of H_2O_2 concentration is critical for cell homeostasis. Plants require biochemical and molecular strategies to survive the problem of salinity. 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). 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 nonenzymatic 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.

The other way by which plants adjust to any environmental stresses is by increasing their tissue osmotic potential. Thus the cells tend to accumulate osmoprotective compounds which increases the osmotic potential of the cell. Proline is the major compound that protects cells by stabilizing proteins and cellular membranes (Kumar *et al*., 2003;Martinez *et al*., 2003).

Rice genotypes tolerant/ susceptible to salinity and flooding stress show differential H_2O_2 accumulation, physiological response and antioxidant activity (Lee *et al*., 2001; Khan and Panda., 2008; Stanisavljevic *et al.,* 2011; Blokhina *et al.,* 2001). H_2O_2 induced an increase in membrane permeability, chlorophyll damage, lipid peroxidation (Lin and Kao, 1998; Patra and Panda, 1998). However, how a plant perceives environmental changes and how it subsequently triggers signals to activate the physiological response are yet to be explored. Hence, the present study was aimed to study the effect of various H_2O_2 concentrations on oxidative stress, osmolyte accumulation and antioxidant activity in a salt tolerant (FL 478); salt susceptible (IR29); flooding tolerant (FR 13A) and flooding susceptible (Co 43) rice genotypes.

MATERIALS AND METHODS

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, 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° 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.

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. Proline was estimated by selective extraction with three per cent aqueous sulphosalicylic acid after removing the interfering proteins. The chromophore developed, while reacting with acid ninhydrin, was estimated spectrophotometrically adopting the procedure of Bates *et al*. (1973) and expressed in mg g^{-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 was determined by adopting the method of Malik and Singh (1980). One gram of leaf was macerated and extracted in 0.1 M phosphate buffer (pH 7.0). A known volume of the extract was added to an experimental cuvette containing three ml phosphate buffer and 0.05 ml guaiacol reagent and then 0.03 ml of H_2O_2 solution was added rapidly and the increase in absorbance at 436 nm was recorded. This Δt in minutes was used to calculate the enzyme activity. The enzyme activity was expressed as enzyme units per litre. All the operations were carried out at 0 – 5°C.

RESULTS

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 osmolyte accumulation pattern, protein degradation and antioxidant activity. The results revealed that the genotypes FL 478 and Co 43 exhibited an increase in the accumulation of proline contents with 0.5 mM H_2O_2 and 1 mM H_2O_2, and thereafter showed a sharp decline in proline contents when exposed to 0.15mM H_2O_2 and 0.20mM H_2O_2. The genotypes IR 29 and FR 13A showed a decline in proline contents with exposure to increasing concentrations of H_2O_2 (Table 1). Among the genotypes taken for the study, FR 13A recorded a higher content of proline (1000 µg/g) under control conditions and was also able to maintain a higher proline content (840 µg/g) compared to other genotypes even after exposure of 0.20mM H_2O_2. Statistically significant changes were observed in the

accumulation of proline contents within the genotypes and treatments.

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 (Fig.1). In genotypes FL 478 and FR 13A the protein content gradual 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.

The antioxidant activity was studied by observing the ROS scavenging enzymes namely catalase and peroxidase. Activities of catalase and peroxidase showed increasing trends with increasing H_2O_2 treatments in all the varieties. 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 (Table 2). Similar pattern was also observed with peroxidase activity where FR 13A recorded 110 enzyme units/g under control and increased upto 192 enzyme units/g at 0.2mM H_2O_2 treatments (Table 3).

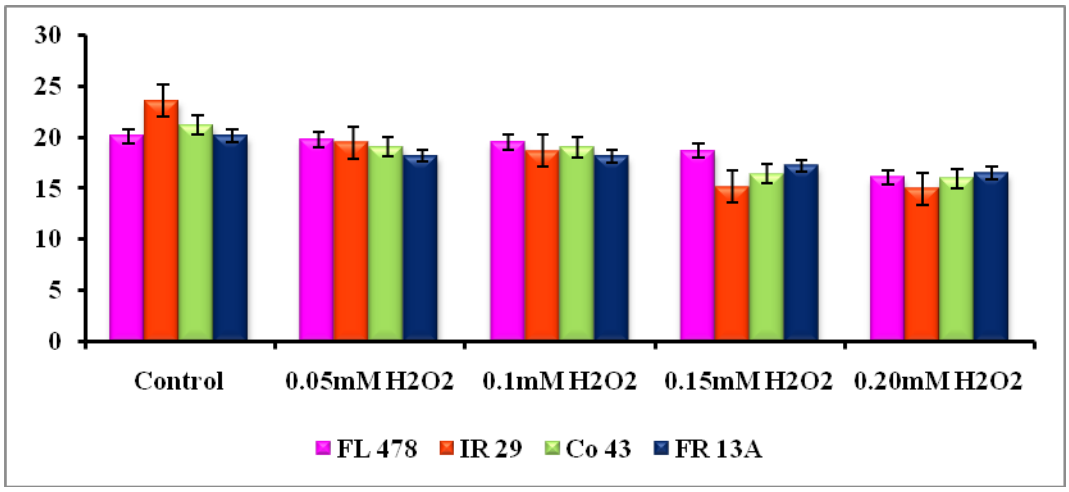

Figure 1. Effect of Hydrogen peroxide treatment on the soluble protein contents (mg/g) in rice genotypes

Table 1 Effect of Hydrogen peroxide treatment on the proline contents (µg/g) in rice genotypes

Treatments	Rice genotypes			
	FL 478	**IR 29**	**Co 43**	**FR 13A**
Control	955±18.75	892±17.51	908±17.82	1000±19.63
0.05mM H_2O_2	986±19.36	875±17.18	926±18.18	978±19.20
0.1mM H_2O_2	942±18.49	855±16.78	922±18.10	972±19.08
0.15mM H_2O_2	915±17.96	842±16.53	911±17.88	940±18.45
0.20mM H_2O_2	802±15.74	692±13.58	799±15.68	840±16.49
SEd.	25.75			
CD (P=0.05)	52.036*			

Table 2 Effect of Hydrogen peroxide treatment on the Catalase activity (µmoles H_2O_2/g/s) in rice genotypes

Treatments	Rice genotypes			
	FL 478	**IR 29**	**Co 43**	**FR 13A**
Control	0.422±0.01	0.399±0.01	0.402±0.01	0.455±0.01
0.05mM H_2O_2	0.578±0.01	0.485±0.01	0.499±0.01	0.502±0.01
0.1mM H_2O_2	0.644±0.01	0.594±0.01	0.591±0.01	0.679±0.01
0.15mM H_2O_2	0.754±0.01	0.667±0.01	0.753±0.01	0.825±0.02
0.20mM H_2O_2	1.322±0.03	0.699±0.01	0.984±0.02	1.526±0.03
SEd.	0.021			
CD (P=0.01)	0.056**			

Table 3 Effect of Hydrogen peroxide treatment on the Peroxidase activity (units/g) in rice genotypes

Treatments	Rice genotypes			
	FL 478	**IR 29**	**Co 43**	**FR 13A**
Control	80±1.57	98±1.92	85±1.67	110±2.16
0.05mM H_2O_2	92±1.81	104±2.04	112±2.20	125±2.45
0.1mM H_2O_2	130±2.55	122±2.39	135±2.65	140±2.75
0.15mM H_2O_2	154±3.02	136±2.67	142±2.79	174±3.42
0.20mM H_2O_2	175±3.44	140±2.75	160±3.14	192±3.77
SEd.	202.88			
CD (P=0.01)	410.04 ^{ns}			

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 behaviour to these stresses were taken for the study. The study clearly indicated that the genotypes varied significantly in the proline accumulation pattern and there was no uniform trends observed with the proline accumulation. The slight increase in proline, an osmoprotectant in H_2O_2 (0.5 and 1.0 mM) in FL 478 and Co 43 may be attributed to the free radical scavenging function of proline as reported (Smirnoff, 1993; Sairam and Srivastava, 2000; Matysik, 2002). Enhanced accumulation of proline in FL 478 might be the basis for its salinity tolerance. These findings are in line with Yazici *et al.,* (2007) who has reported improved salt tolerance of *Portulaca oleracea* L.with proline accumulation. Proline accumulation and stress tolerance correlation have been reported in different studies, and it has been observed that proline concentrations are higher in stress-tolerant plants than in stress-sensitive plants (Misra and Gupta, 2005). Although a positive correlation between abiotic stress tolerance and free proline accumulation has been reported (Martinez *et al.,* 2003), a negative correlation between proline accumulation and submergence tolerant line (FR 13A) was observed in the present study. FR 13A showed a decline in proline contents with exposure to increasing concentrations of H_2O_2.

This is in line with the findings of Lutts et al.who showed that stress sensitive rice cultivars accumulated more proline than the tolerant ones.

It is known that water, salt, metal toxicity and other stress factors induced 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 *et al.,* 2002). In this study also it was found that in all 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. In the stress tolerant lines (FR 13A and FL 478) the protein content gradually decreased, while in the other varieties (Co 43 and IR 29) there was a steep degradation of protein contents at 0.15mM and 0.2mM H_2O_2 treatments. 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).

POX, CAT, SOD are the three major antioxidant enzymes responsible for scavenging, the reactive oxygen species generated via different mechanisms in plant cells (Winston, 1990). This formed 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 signalling 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). The study clearly stated that under conditions of oxidative stress, such as exposure to H_2O_2 the antioxidant levels increase in plant tissues and was much higher in the tolerant genotypes. Similar findings has been reported by Smith *et al.,* 1990 with exposures to 0_3, SO_2, heat shock or drought stresses.

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 behaviour to different abiotic stresses (Submergence and salt stress) gave a clear understanding of the signalling role of H_2O_2. There was no uniform pattern in the accumulation of the osmolytes but significant upregulation of antioxidant enzyme systems and slow degradation of protein contents 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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