HYDROGEN PEROXIDE INDUCES OXIDATIVE STRESS IN DETACHED LEAVES OF *ORYZA SATIVA* L.

H. Upadhyaya[1]*, M. H. Khan[1], S. K. Panda[1,2]

[1]Plant Biochemistry Laboratory, School of Life Sciences, Assam (Central) University, Silchar–788011, Assam, India
[2]Research Institute for Bioresouces, Okayama University, Kurashiki, 710-0046 Japan

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Summary. Induction of oxidative stress and possible involvement of antioxidants in rice (*Oryza sativa* L) leaves after *in vivo* treatment with hydrogen peroxide (H_2O_2) was investigated. Degradation of chlorophyll, carotenoid and protein contents in rice leaves was observed which showed a recovery under free radical scavenger treatments. An increase in both total peroxide accumulation and lipid peroxidation was observed with increasing H_2O_2 concentrations whereas lipid peroxidation decreased under free radical scavenger treatments. A gradual decrease in SOD and GPx activity as well as an increase in CAT and GR activities were recorded under H_2O_2 treatments. In addition, proline accumulation accompanied by a decrease in ascorbate and glutahione content was observed in senescing rice leaves under H_2O_2 treatments.

Key words: catalase, chlorophyll, glutathione reductase, guaiacol peroxidase, lipid peroxidation, mannitol, reactive oxygen species, senescence, sodium benzoate, superoxide dismutase, *Oryza sativa*.

Abbreviations: CAT – catalase; GR - glutathione reductase; GPx - guaiacol peroxidase; H_2O_2 - hydrogen peroxide; ROS - reactive oxygen species; SOD - superoxide dismutase.

*Corresponding author, e-mail: hkupbl_au@rediffmail.com

INTRODUCTION

Plants functioning in an aerobic environment are often subjected to continuous threat from molecular oxygen which is due to toxic reactive oxygen species (ROS) like superoxide radical ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), alkoxyl radical ($RO^{\bullet}$) and singlet oxygen ($^{\bullet}O_2$) (Khan and Panda, 2002; Panda, 2002). These reactive oxygen species have the capacity to degrade almost all cell components including membrane lipids, proteins and DNA (Hendry, 1993, Casano et. al., 1994). Toxic hydrogen peroxide is a product of peroxisomal and chloroplast oxidative reactions and can act both as an oxidant and reductant. It is the most stable form of the ROS and is capable of rapid diffusion across cell membrane (del Rio et. al., 1992). Environmental stresses are known to induce H_2O_2 and other toxic oxygen species production in cellular compartments and result in acceleration of leaf senescence through lipid peroxidation and other oxidative damage. H_2O_2 being a strong oxidant can initiate localized oxidative damage in leaf cells leading to disruption of metabolic function and loss of cellular integrity resulting in senescence promotion. It also changes the redox status of surrounding cells where it initiates an antioxidative response by acting as a signal of oxidative stress (Begam and Choudhury, 1992; Prasad et al., 1994; Alscher et al., 1997; Lin and Kao, 1998; Sairam and Srivastava, 2000). Many substances are known to play an important role in protecting plants from free radicals. These are mannitol, sodium formate, sodium benzoate, etc. which are synthesized in various plant parts, and particularly in the chloroplast (Shen et al, 1997). To test the hypothesis that H_2O_2 accelerates senescence by inducing oxidative reactions, the present investigation has been undertaken with the aim to study the senescence-promoting effect of H_2O_2 and the role of different free radical scavengers in excised senescing rice leaves.

MATERIALS AND METHODS

Plant material

Rice (*Oryza sativa* L. cv. Longai) seeds were collected and surface

sterilized with 0.01 % $HgCl_2$ for 5 min and washed with double dH_2O. Seeds were then germinated in sterilized Petri dishes in sterilized cotton soaked with dH_2O at 29 ^{0}C. Germinated seeds were transferred to plastic glasses containing Yoshida solution and grown in a growth chamber under cool, fluorescent white (*Philips*, 20W) light of 52 µmol m^{-2} s^{-1} (PAR) intensity at a 12-h photoperiod. On the10th day primary leaves were excised and submerged in 20 ml of various concentrations of H_2O_2 (0, 0.01, 0.05, 0.1, 0.2, 0.3 and 1 mM) and 0.2 mM H_2O_2 + mannitol (10 mM), 0.2 mM H_2O_2 + sodium benzoate (10 mM) solution and incubated at 25 ^{0}C in the dark for 6 h. After that primary leaves were washed thrice with double dH_2O and used for biochemical analysis.

Extraction and estimation of metabolites

Senescing primary rice leaves were directly homogenized in cold 80% acetone and chlorophyll and carotenoid pigments were extracted and estimated according to the method of Arnon (1949). For proline estimation the primary leaves were homogenized with 3% aqueous sulfosalicylic acid and centrifuged at 3,000 *g* for 10 min. Proline from the supernatant was estimated using the method of Bates et al. (1973). Leaves (0.2 g) were homogenized with 5% trichloroacetic acid (TCA) and the homogenate was used for the extraction and estimation of total peroxide content (Sagisaka, 1976). Protein was estimated by the method of Bradford (1976).

Lipid peroxidation was measured as the amount of thiobarbituric acid reactive substance (TBARS) determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). The leaf tissues were homogenized in 0.1% (w/v) tricholoacetic acid (TCA). The homogenate was centrifuged at 10,000 *g* for 20 min. To l ml of the resulting supernatant, l ml of TCA (20%) containing (0.5 w/v) of TBA and 10 µl of butylated hydroxy tolune (BHT), (4% in ethanol) were added. The mixture was heated at 95 ^{0}C for 30 min and then cooled in ice followed by centrifugation at 10,000 *g* for 15 min. The absorbance was measured at 532 nm and corrected for 600 nm. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

The extraction and estimation of glutathione was done by the method

of Griffith (1980). The primary leaf tissue was homogenized in 5% (w/v) sulfosalicylic acid and centrifuged at 10,000 *g* for 10 min. The supernatant (l ml) was neutralized with 0.5 ml of 0.5 M potassium phosphate buffer (pH 7.5). Total glutathione was measured by adding l ml neutralized supernatant to a standard solution mixture consisting of 0.5 ml of 0.1 M sodium phosphate buffer (pH 7.5) containing EDTA, 0.2 ml of 6 mM 5,5[1] - dithiobis (2- nitrobenzoic acid), 0.1 ml of 2 mM NADPH and l ml of 1 U yeast GR Type III (Sigma Chemical, USA). The changes in the absorbance at 412 nm were followed at 25 ± 2 ^{0}C until the absorbance reached 5 U. The extraction and estimation of ascorbate was done by the method of Oser (1979). The reaction mixture consisted of 2 ml 2% sodium molybdate, 2 ml 0.15 N H_2SO_4 , l ml 1.5 mM Na_2HPO_4 and l ml of tissue extract. The mixture was incubated at 60 ^{0}C in a water bath for 40 min. After centrifugation at 3,000 *g* for 10 min the absorbance was read at 660 nm.

Extraction and estimation of enzyme activities

The leaf tissues were homogenized with 0.1 M phosphate buffer pH 6.8 in a prechilled mortar. The homogenate was centrifuged at 4 ^{0}C for 15 min at 17,000 *g*. The supernatant was used for the assay of catalase (CAT), guaiacol peroxidase (GPx) and superoxide dismutase (SOD) activities. The CAT and GPx activities were assayed by the method of Chance and Maehly (1955). The mixture (5.0 ml) comprised of 3.0 ml phosphate buffer (0.1 M) (pH 6.8), l ml (30 mM) H_2O_2, and l ml enzyme extract. The reaction was stopped by adding 10 ml of 2% H_2SO_4 after l min incubation at 20 ^{0}C. The acidified reaction mixture was titrated against 0.01 N $KMnO_4$ to determine the quantity of H_2O_2 utilized by the enzyme. CAT activity was expressed as µ mole H_2O_2 destroyed min^{-1} g^{-1} FW. For guaiacol peroxidase acivity assay the reaction mixture (3.0 ml) contained 0.1 M phosphate buffer (pH 6.80), guaiacol (30 mM), H_2O_2 (30 mM) and 0.3 ml enzyme extract. The absorbance was read at 420 nm using an UV visible spectrophotometer (Systronics, India). The enzyme activity was expressed as µmole H_2O_2 destroyed min^{-1} g^{-1} FW. The activity of SOD was measured using the method of Giannopolitis and Reis (1977). The assay mixture (3 ml) for SOD contained 79.2 mM Tris-HCI buffer (pH 8.9), containing 0.12 mM

EDTA and 10.8 mM tetra ethylene diamine, bovine serum albumin (3.3 x 10^{-3} %), 6 mM nitroblue tetrazolium (NBT), 600 µM riboflavin in 5 mM KOH and 0.2 ml enzyme extract. The reaction mixture was illuminated by two tube lights (Philips 20W). By switching the light on and off, the reaction mixture was illuminated and the illumination terminated. The increase in absorbance due to formazan formation was read at 560 nm. The increase in absorbance in the absence of enzyme was taken as 100% and 50% initial was taken as equivalent to 1 unit of SOD activity.

Glutathione reductase (GR) was assayed by the method of Smith et al. (1988). The reaction mixture contained 1 ml of 0.2 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.5 ml of 3 mM DTNB (5, 5 – dithiobis – 2 nitrobenzoicacid) in 0.01 M potassium phosphate buffer (pH 7.5), 0.1 ml of 2 mM NADPH, 0.1 ml enzyme extract and dH_2O to make up a final volume of 2.9 ml. Reaction was initiated by adding 0.1 ml of 2 mM GSSG (oxidised glutathione). The increase in absorbance at 412 nm was recorded at 25 ^{0}C over a period of 5 min spectrophotometrically. The activity is expressed as absorbance change (ΔA_{412}) g $^{-1}$ FW sec^{-1}.

Each experiment was repeated thrice with three replicates each and the data presented are means ±SE.

RESULTS

The effect of H_2O_2 and its subsequent interaction with free radical scavengers like mannitol and sodium benzoate on the changes in chlorophyll, carotenoid and protein content in primary leaves of rice is shown in Figure 1. A decrease in cholrophyll content was observed with increasing H_2O_2 concentrations (Fig. 1A). However, the addition of mannitol and sodium benzoate led to an increase in cholrophyll content (Fig. 1B). Similar results were observed for carotenoid content under H_2O_2 and mannitol treatment. Protein content decreased with increasing H_2O_2 concentration whereas mannitol and sodium benzoate caused an increase in protein content.

An increase in total peroxide levels was observed with increasing exogenous concentrations of H_2O_2. A slight decrease in peroxide levels was observed at mannitol and sodium benzoate treatment as compared to 0.2 mM H_2O_2 treatment. Lipid peroxidation measured in terms of

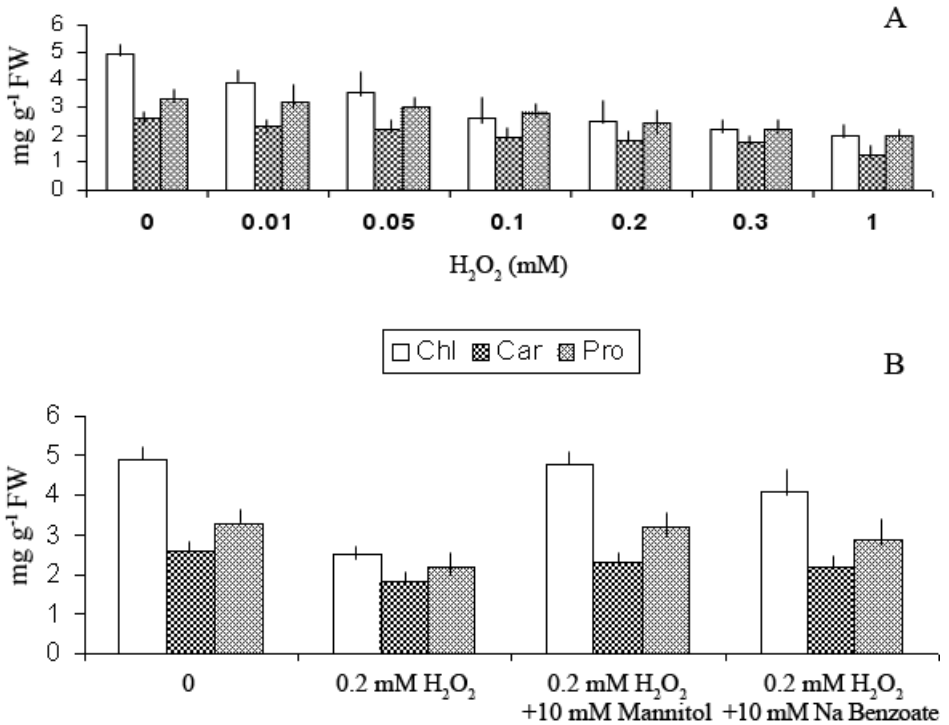

Fig. 1. Changes in chlorophyll, carotenoid and protein contents (A) after treatment with H_2O_2 and (B) its interaction with the free radical scavengers mannitol (10 mM) and sodium benzoate (10 mM) in primary detached rice leaves. Data represented are means of three separate experiments ± SE.

thiobarbituric acid reactive substance (TBARS) also increased with increasing H_2O_2 concentration whereas the free radical scavenger treatment led to a minimum decrease in TBARS level (Fig. 2A, B).

A gradual decrease in SOD activity under H_2O_2 treatment and a recovery in free radical scavengers treatment were observed (Fig. 3). However, GPx activity showed no effective changes due to H_2O_2 treatment as well as the scavengers tested. Both CAT and GR showed increased activities under different H_2O_2 concentrations, however, they decreased under free radical scavengers treatment (Fig. 3A, B).

Increased proline accumulation was observed with increasing H_2O_2 concentration and free radical scavengers were found to have no significant

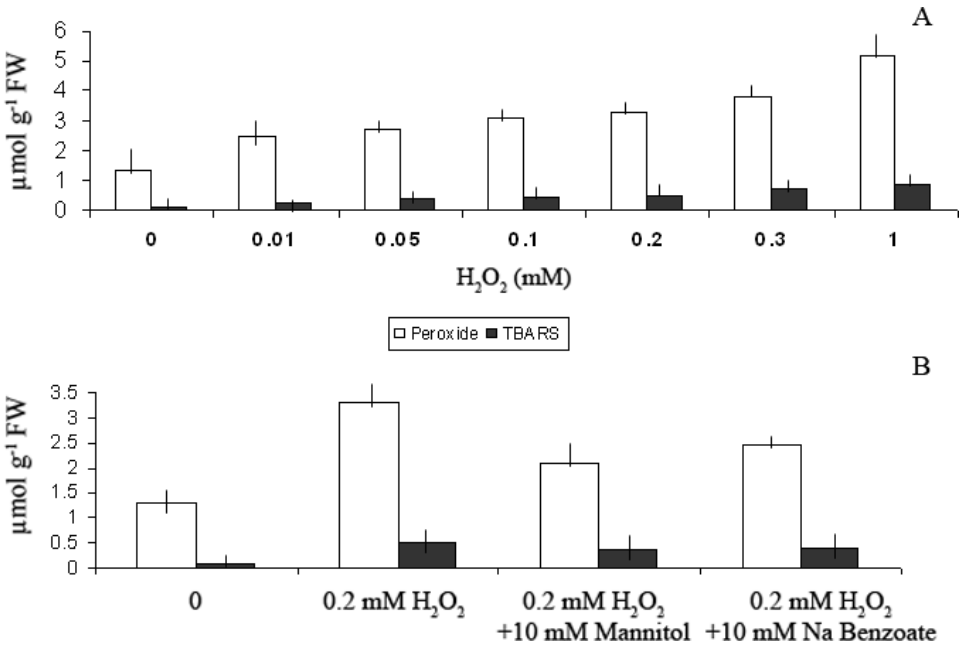

Fig. 2. Changes in the thiobarbituric acid reactive substance (TBARS) and total peroxide content (A) after treatment with H_2O_2 and (B) its interaction with the free radical scavengers mannitol (10 mM) and sodium benzoate (10 mM) in primary detached rice leaves. Data represented are means of three separate experiments ± SE.

effect on its accumulation. A decrease in ascorbate and glutathione content was observed for primary rice leaves under H_2O_2 treatment and its subsequent interaction with mannitol and sodium benzoate (Fig. 4A, B).

DISCUSSION

H_2O_2 treatment of primary rice leaves induced an increase in chlorophyll, carotenoid and protein degradation, however, these parameters were found to decrease due to the addition of the free radical scavengers mannitol (10 mM) and sodium benzoate (10 mM) in senescing leaves as observed also for other abiotic stresses (Sairam et al., 1997; Panda et al., 2002). It is known that water, salt, metal toxicity and other stress factors induced endogenous H_2O_2 accumulation. The pigment and protein degradation in

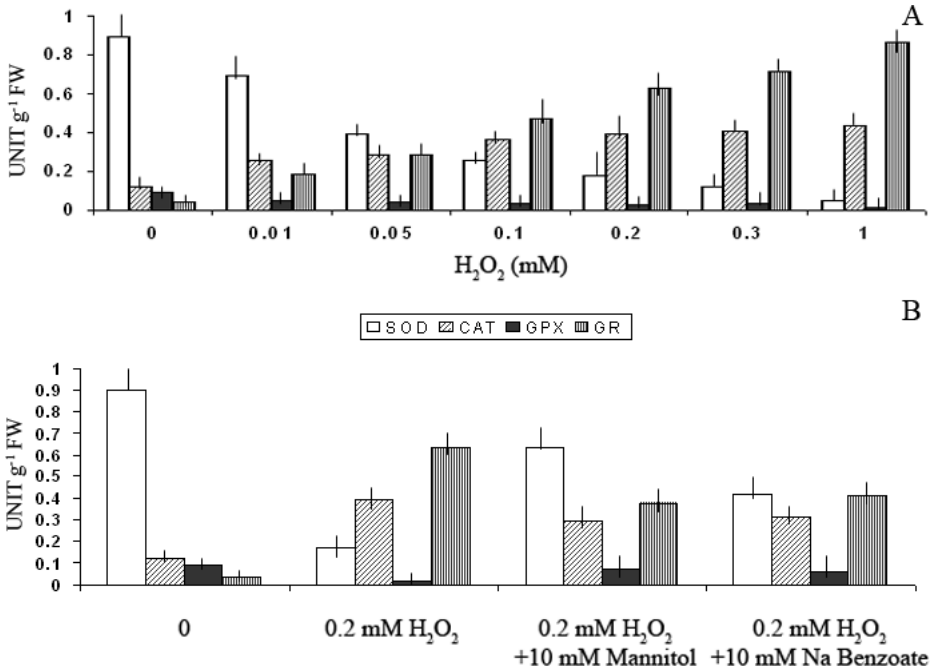

Fig 3. Changes in SOD, CAT, GPx and GR activities (A) after treatment with H_2O_2 and (B) its interaction with the free radical scavengers mannitol (10 mM) and sodium benzoate (10 mM) in primary detached rice leaves. Data represented are means of three separate experiments ±SE.

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).

H_2O_2 treatment increased lipid peroxidation, endogenous accumulation of H_2O_2 and decreased the membrane stability as observed earlier (Sarkar and Choudhury 1981; Lin and Kao, 1998). Decreased lipid peroxidation and H_2O_2 accumulation in the presence of the free radical scavengers mannitol and sodium benzoate suggested the involvement of H_2O_2 in producing $^{\bullet}OH$ radical via a Fenton type reaction and ultimately accelerating leaf senescence (Lin and Kao, 1998; Sairam and Srivastava, 2000; Panda and Patra, 2000).

The slight increase in proline, an osmoprotectant in H_2O_2 (0.2 mM)-stressed senescing rice leaves may be attributed to the free radical scavenging function of proline as reported elsewhere (Smirnoff, 1993; Matysik, 2002). However,

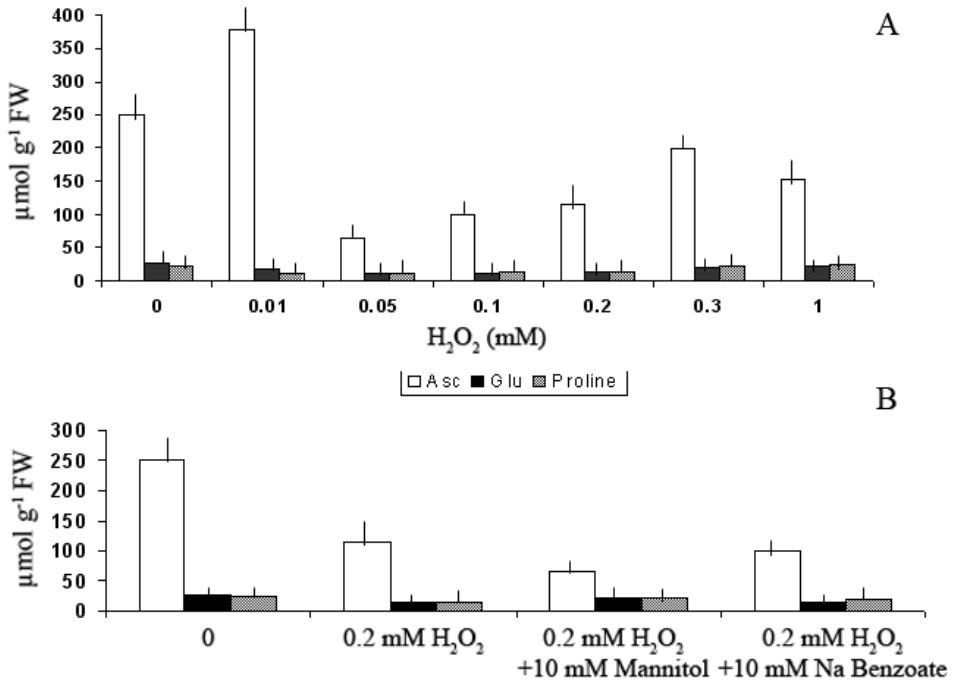

Fig. 4. Changes in ascorbate, glutathione and proline content (A) after treatment with H_2O_2 and (B) its interaction with the free radical scavengers mannitol (10 mM) and sodium benzoate (10 mM) in primary detached rice leaves. Data represented are means of three separate experiments ±SE.

the interaction with scavengers had no significant effect on osmoprotection. The decrease of ascorbate and glutathione content in H_2O_2-stressed leaves showed a poor non – enzymatic protection from oxidative stress (Gallego et al., 1996). The decreased SOD activity in senescing rice leaves and its recovery under free radical scavengers treatment might be due to the generation of oxidative stress by H_2O_2 and the possible inactivation of SOD (Casano et al., 1997; Panda and Patra, 2000). The increased CAT and GR activities point to a signaling role of H_2O_2 in the induction of H_2O_2 synthesis detoxifying enzymes in senescing 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 decrease in CAT activities due to the tested free radical scavengers could be attributed to decreased H_2O_2 levels being not

sufficient to activate the antioxidative property of the enzyme. At the same time the decrease in GR activities suggested also that Halliwel-Asada pathway was not active in the detoxification of H_2O_2 mediated by the tested scavengers. Thus, it can be assumed that the slight decrease in H_2O_2 content minimizing the effect of 0.2 mM H_2O_2 treatment by an interactive effect of the scavengers tested might be due to activation of Fenton type reaction producing $OH^{\cdot}$ in which the applied free radical scavengers might have some role to scavenge the free radicals produced in the process (Shen et al., 1997).

In conclusion, the induction of CAT and GR may not be sufficient to quantify sensecence in rice leaves where H_2O_2 was found to promote senescence based on chlorophyll and protein degradation, accumulation of H_2O_2, a decrease in membrane stability, increased lipid peroxidation and decreasd SOD and GPx activities which were partially regulated in the presence of free radical scavengers. As sodium benzoate and mannitol did not affect significantly the content of TBARS, H_2O_2 and the activities of CAT, the harmful effect could be attributed to superoxide radical rather than H_2O_2. The protective effect of the tested scavengers was more likely connected with increased SOD activity.

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