Induction of oxidative stress and antioxidant activity by hydrogen peroxide treatment in tolerant and susceptible wheat genotypes

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Abstract

We induced an oxidative stress by means of exogenous hydrogen peroxide in two wheat genotypes, C 306 (tolerant to water stress) and Hira (susceptible to water stress), and investigated oxidative injury and changes in antioxidant enzymes activity. H_2O_2 treatment caused chlorophyll degradation, lipid peroxidation, decreased membrane stability and activity of nitrate reductase. Hydrogen peroxide increased the activity of antioxidant enzymes, glutathione reductase and catalase. These effects increased with increasing H_2O_2 concentrations. However, no change was observed in the activity of superoxide dismutase and proline accumulation.

Additional key words: active oxygen species, catalase, chloroplast, glutathione reductase, lipid peroxidation, nitrate reductase, proline, superoxide dismutase, *Triticum aestivum*.

Introduction

Hydrogen peroxide accumulation, along with superoxide radical and hydroxyl radical as a result of drought, high/or low temperature or salinity stress are the main causes of oxidative stress. Superoxide radical and its reduction product, hydrogen peroxide can directly attack membrane lipids and inactivate HS-containing enzymes (Armstrong and Buchanan 1978, Navari-Izzo *et al.* 1994). Beside this, these reactive oxygen species (ROS) cause damage to DNA, proteins, lipids, chlorophyll and almost any other organic constituent of a living cell (Fridovich 1986, Liebler *et al.* 1986, Halliwell 1987, Davies 1987, Imlay and Linn 1988, Wise and Naylor 1987). H_2O_2 produced during environmental stresses is reported to act both as an agent of oxidative stress and a regulator of various antioxidant enzymes involved in the amelioration of oxidative stress (Prasad *et al.* 1994). H_2O_2 induced an increase in membrane permeability, chlorophyll damage, lipid peroxidation (Lin and Kao 1998, Patra and Panda 1998), increase in activity of glutathione reductase, ascorbate peroxidase and superoxide dismutase (Pastori and Trippi 1992, Guo *et al.* 1997) and synthesis and activity of catalase and peroxidase (Prasad *et al.* 1994) have been reported in many plants. Since wheat genotypes tolerant/susceptible to water and high temperature stresses show differential H_2O_2 accumulation, lipid peroxidation as well as activity of various antioxidant enzymes (Sairam *et al.* 1997, 1997/98, 1998, Sairam and Saxena 2000), an experiment was conducted with two wheat genotypes, differentially tolerant to water deficit, to elucidate role of H_2O_2 in response to drought stress. We studied effect of various H_2O_2 concentrations on oxidative stress and antioxidant activity in drought tolerant and susceptible wheat.

Materials and methods

Wheat (*Triticum aestivum* L.) cvs. C 306 (tolerant to water stress) and Hira (recommended for irrigated condition, susceptible to water stress) were planted in earthen pots (30 × 30 cm), filled with 10 kg mixture of

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Abbreviations: Cat - catalase; Chl - chlorophyll; GR - glutathione reductase; MDA - malondialdehyde; NR - nitrate reductase; SOD - superoxide dismutase; TBA - thiobarbituric acid; TCA - trichloroacetic acid.

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sandy loam soil and farm yard manure in 6:1 ratio. Each pot was fertilized with N, P and K corresponding to 120, 60 and 60 kg ha^{-1}, respectively. Four seedlings were maintained in each pot. Plants were watered when required to keep them fully turgid. Samples for various assays/estimations were taken 30 - 35 d after sowing from first fully expanded leaves at 08:00. Samples collected in ice bucket were washed with tap water and then with double distilled water. Leaf stripes of uniform size (8 - 10 mm) and mass (2 g) 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 0.1 M 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 a control. After incubation the samples were twice washed with double distilled water and soaked dry, and processed for various observations.

Chlorophyll content was estimated by the non-maceration method of Hiscox and Israelstam (1979). Leaf strips (0.1 g) were incubated in 10 cm^3 of DMSO at 65 °C for 4 h and absorbance was recorded at 645 and 663 nm. Chloropyll content was calculated according to the formula of Arnon (1949).

Membrane stability was estimated according to Sairam *et al.* (1997). Leaf material (0.1 g) was taken in 10 cm^3 of double distilled water in two sets. One set was subjected to 40 °C temperature for 30 min and conductivity of medium was recorded using a conductivity bridge (C1). Second set was kept in a boiling water bath (100 °C) for 10 min and its conductivity was also recorded (C2). Membrane stability index (MSI) was calculated as below:

$$MSI = [1 - (C1/C2)] \times 100$$

The lipid peroxidation was measured in terms of malondialdehyde (MDA) content following the method of Heath and Packer (1968). A leaf sample (0.5 g) was homogenized in 10 cm^3 of 0.1 % trichloroacetic acid (TCA). The homogenate was centrifuged at 15 000 *g* for 10 min. To 2.0 cm^3 aliquot of the supernatant 4.0 cm^3 of 0.5 % thiobarbituric acid (TBA) in 20 % TCA were added. The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10 000 *g* for 10 min, the absorption of the supernatant was recorded at 532 nm. The MDA content was expressed as nmol MDA per g dry mass.

Free proline concentration in the leaves was determined following the method of Bates *et al.* (1973). Leaf sample (0.5 g) was homogenized with 5 cm^3 of sulfosalicylic acid (3 %) using mortar and pestle and filter through *Whatman No. 1* filter paper. The volume of filtrate was made up to 10 cm^3 with sulfosalicylic acid. Two cm^3 of filtrate was incubated with 2 cm^3 of glacial acetic acid and 2 cm^3 ninhydrin reagent and boiled in a water bath at 100 °C for 30 min. After cooling the reaction mixture, 6 cm^3 of toluene was added, the chromophore containing toluene was separated and absorbance read at 520 nm spectrophotometrically (UV-visible spectrophotometer *Beckman M-36*).

Nitrate reductase activity was assayed *in vivo* (Klepper *et al.* 1971). Leaf samples cut in to 8 - 10 mm segments (0.2 g) were incubated with 2.5 cm^3 each of potassium phosphate buffer (0.1 M, pH 7.5) and potassium nitrate (0.1 M) and vacuum infiltrated using vacuum pump for 1 - 2 min, then the tubes were incubated at 30 °C in dark for 30 min. Keeping the tubes in boiling water for 2 min terminated the reaction. The tubes were then cooled and 0.1 cm^3 aliquot was taken and mixed with 1 cm^3 each of sulfanilamide (1 % in 1 M HCl) and N-(1,naphthyl-ethylene) diamine dihydro-chloride (0.02 % in water) for nitrite estimation spectrophotometrically at 540 nm.

Enzyme extract for superoxide dismutase (SOD), glutathione reductase (GR) and catalase (Cat) was prepared by grinding 0.5 g leaf material with 10 cm^3 of chilled buffer. The extraction medium consisted of 0.1 M potassium phosphate buffer (pH 7.5) containing 0.5 mM EDTA. The brie was filtered through cheesecloth and the filtrate was centrifuged in a *Beckman model J2 - 21* (Geneva, Switzerland) refrigerated centrifuge at 4 °C for 15 min at 20 000 *g*. Superoxide dismutase activity was estimated according to the method of Dhindsa *et al.* (1981). The 3.0 cm^3 reaction mixture contained 13 mM methionine, 25 μM NBT, 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM sodium bicarbonate and 0.1 cm^3 enzyme. Reaction was started by adding 2 μM riboflavin and placing the tubes below two 15 W fluorescent lamps (irradiance of 85 μmol m^{-2} s^{-1}) for 15 min. Reaction was stopped by switching off the light. A non-irradiated complete reaction mixture did not develop colour and served as a blank. Absorbance was recorded at 560 nm and one unit of enzyme activity was taken as that quantity of enzyme, that reduced the absorbance reading to 50 % in comparison with the tubes lacking enzyme. Catalase was assayed by measuring the disappearance of H_2O_2 as per the method of Teranishi *et al.* (1974). The 3.0 cm^3 reaction mixture contained 50 mM phosphate buffer, pH 7.0, 2 mM H_2O_2 and 0.1 cm^3 diluted (10 times) enzyme. The reaction was stopped after 5 min by the addition of 2 cm^3 of titanium reagent, which also forms coloured complex with residual H_2O_2. Aliquot was centrifuged at 10 000 *g* for 10 min and absorbance of the supernatant was recorded at 410 nm on a *Beckman M-36* spectrophotometer. Glutathione reductase was assayed by the method of Smith *et al.* (1988). The reaction mixture contained 1 cm^3 of 0.2 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.5 cm^3 of 3 mM DTNB (5,5-dithiobis-2-

nitrobenzoic acid) in 0.01 M potassium phosphate buffer (pH 7.5), 0.1 cm^3 of 2 mM NADPH, 0.1 cm^3 enzyme extract and distilled water to make up a final volume of 2.9 cm^3. Reaction was initiated by adding 0.1 cm^3 of 2 mM GSSG (oxidized glutathione or glutathione disulphide). The increase in absorbance at 412 nm was recorded at 25 °C over a period of 5 min spectrophotometrically. The activity is expressed as absorbance change (ΔA_{412}) per g fresh mass per s. All observations are means of six replicates and data were analyzed by analysis of variance.

Results

Hydrogen peroxide treatment resulted in decrease in total chlorophyll content and the damaging effect increased with increasing H_2O_2 concentration (Fig. 1*A*). Though cv. Hira had higher chlorophyll (Chl) content under control condition, hydrogen peroxide treatments, 0.1mM and above resulted in more steep decline in Chl content then in C 306. Chlorophyll *a/b* ratio did not show consistent changes under different H_2O_2 treatments, but it mostly decreased with increasing H_2O_2 concentrations (Fig. 1*B*). Though treatment effects were significant compared to control, genotypic variation was non-significant at all H_2O_2 concentrations.

Lipid peroxidation estimated as malondialdehyde (MDA) content was minimal in untreated leaves, and increased with increasing H_2O_2 concentration in both the genotypes (Fig. 1*C*). C 306 showed consistently lower

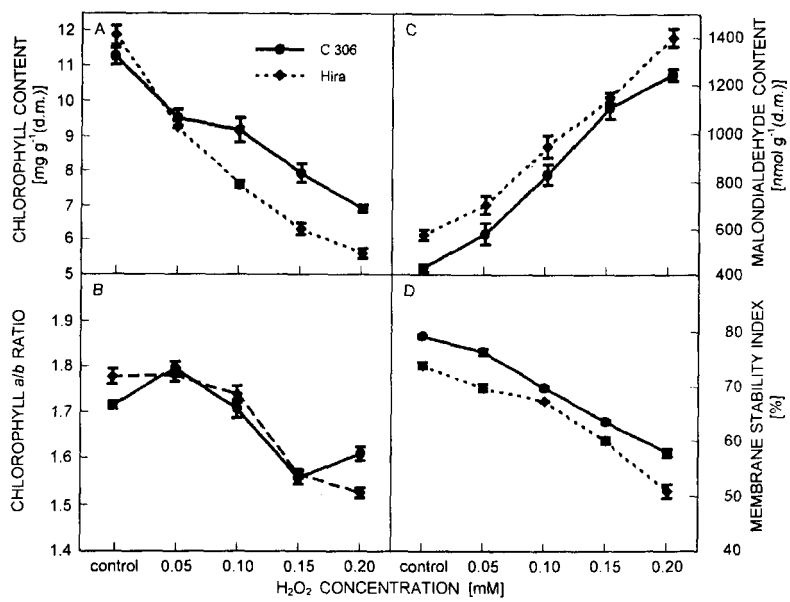

Fig. 1. Effect of H_2O_2 treatments on chlorophyll content (*A*), chlorophyll *a/b* ratio (*B*), lipid peroxidation (*C*),and membrane stability index (*D*) in drought stress tolerant (C 306) and susceptible (Hira) wheat cultivars. Vertical bars indicate ± SE of mean.

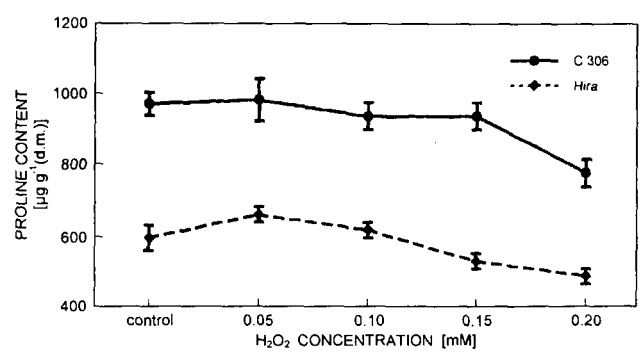

Fig. 2. Effect of H_2O_2 treatments on proline content in drought stress tolerant (C 306) and susceptible (Hira) wheat cultivars. Vertical bars indicate ± SE of mean.

MDA content at all H_2O_2 treatments as compared to Hira. Membrane stability index (MSI) decreased with increasing H_2O_2 concentrations in both the genotypes (Fig. 1*D*). Hira showed lower membrane stability than C 306 at all the levels of H_2O_2 concentrations.

Proline content was significantly higher in C 306 in control and H_2O_2 treated leaves than in Hira (Fig. 2). Proline content in C 306 did not show much variation up to concentration 0.15 mM H_2O_2, but recorded a steep decline at 0.2 mM H_2O_2 concentration. In case of Hira, the proline content slightly increased at 0.05 mM H_2O_2 concentration, but at higher concentrations it declined as compared to untreated control leaves.

Nitrate reductase (NR) activity in untreated leaves was initially higher in Hira than C 306 (Fig. 3*A*). H_2O_2 treatments caused linear decrease in NR activity in both genotypes, though the magnitude of decline was higher in Hira at all H_2O_2 concentrations.

Superoxide dismutase (SOD) activity was higher in C 306 than Hira and decreased gradually up to 0.15 mM and steeply at 0.2 mM H_2O_2 in C 306, while in case of Hira the gradual decline was only up to 0.1 mM and more steep decline was observed at 0.15 and 0.2 mM H_2O_2 concentrations (Fig. 3*B*).

Activities of glutathione reductase (GR) and catalase (Cat) showed increasing trends with increasing H_2O_2 concentrations (Figs. 3*C,D*) in both C 306 and Hira. C 306 manifested higher activity of glutathione reductase and catalase than Hira at all concentrations of H_2O_2.

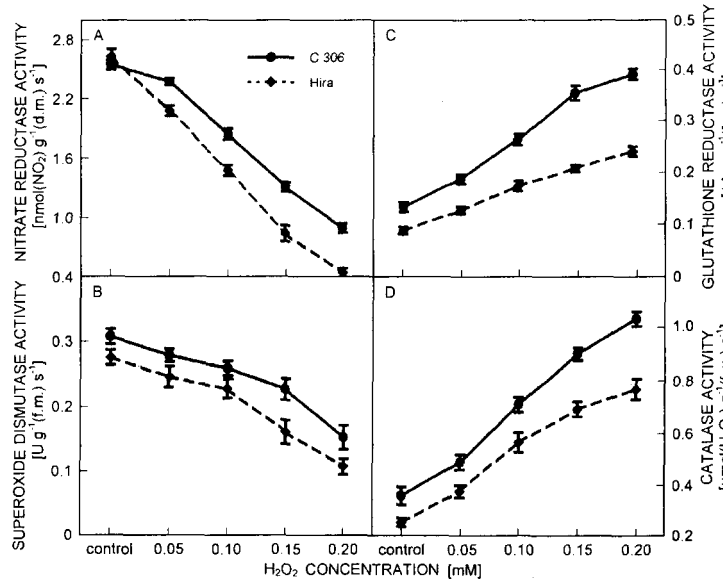

Fig. 3. Effect of H_2O_2 treatments on the activity of nitrate reductase (*A*), superoxide dismutase (*B*), glutathione reductase (*C*), and catalase (*D*) in drought stress tolerant (C 306) and susceptible (Hira) wheat cultivars. Vertical bars indicate ± SE of mean.

Discussion

Hydrogen peroxide is a potent cytotoxic compound produced during drought, high/low temperature and salinity stresses. With the increase in the magnitude of stress, the content of H_2O_2 in tissue increased along with a concurrent increase in various antioxidant enzymes (Mukherjee and Choudhuri 1983, Prasad *et al.* 1994, Menconi *et al.* 1995, Sairam *et al.* 1998, Sairam and Saxena 2000). Treating the leaf tissue with H_2O_2 *in vivo* induced chlorophyll degradation similar to what is usually observed under water and salinity stresses (Kalir 1981, Pastori and Trippi 1992, Moran *et al.* 1994, Sairam *et al.* 1997, 1997/98), suggesting that H_2O_2 or other active oxygen radicals might be responsible for chlorophyll damage under environmental stresses. The decrease in Chl *a/b* ratio suggests that functional Chl *a* is more sensitive to H_2O_2 than Chl *b*. The decrease in Chl *a/b* ratio following H_2O_2 treatment could also mean greater sensitivity of proteins in reaction centre (PS 2) with a consequence of Chl *a* degradation. Increasing concentrations of H_2O_2 induced lipid peroxidation and consequently decreased membrane stability in both the wheat genotypes. Pastori and Trippi (1992) and Lin and Kao (1998) have also reported increased lipid peroxidation, membrane permeability and chlorophyll damage in H_2O_2 treated tissue as compared to control. The deleterious effect of H_2O_2 was more expressed in Hira than in drought tolerant C 306. It was in agreement with our earlier studies where we observed that drought

and high temperature stress induced H_2O_2 accumulation was associated with lipid peroxidation and membrane injury (lower membrane stability) (Sairam *et al.* 1997, 1997/98, 1998, Sairam and Saxena 2000).

The magnitude of proline accumulation under water stress has been reported to be associated with the ability of the species/cultivars to withstand stress (Singh *et al.* 1972, Waldren and Teare 1974, Singh and Singh 1983, Quarrie 1980, Sairam and Dube 1984a). Though tolerant cv. C 306 showed more proline accumulation than susceptible cv. Hira, however, there was no change in proline content by H_2O_2 untill concentration 0.15 mM and a slight decrease at concentration of 0.2 mM H_2O_2 in both the genotypes. The results suggest that water stress induced oxidative stress (H_2O_2 and other active oxygen radicals) have no relationship with proline accumulation under similar conditions, however a higher H_2O_2 concentration might have some inhibitory effect on enzymes associated with proline synthesis from glutamate leading to lowering of proline content.

Nitrate reductase activity was affected by H_2O_2 treatment in a way similar to observed for water or temperature stress (Morilla *et al.* 1973, Sairam and Dube 1984b, Sairam *et al.* 1990, Sairam 1994). Environmental stress induced reactive oxygen intermediates are reported to inactivate the enzymes with active HS-groups and thus decreases their activity (Armstrong and Buchanan 1978, Navari-Izzo *et al.* 1994). The inhibitory effect of treatment was less pronounced in C 306 than in Hira.

Unlike drought and temperature stress induced increase in superoxide dismutase activity (Upadhyaya *et al.* 1990, Dhindsa 1991, Olmos *et al.* 1994, Menconi *et al.* 1995, Sairam *et al.* 1998), H_2O_2 treatment resulted in a gradual decline in SOD activity. This suggests that H_2O_2 alone does not has a promotive effect on SOD. This is in contradiction to the findings of Pastori and Trippi (1992) who reported H_2O_2 induced increase in SOD activity. A decrease in SOD activity observed after H_2O_2 treatment might be due to inhibition of CuZn-SOD and Fe-SOD by peroxide. However, H_2O_2 treatment has a significantly pronounced effect on glutathione reductase and catalase activity, which increased with increasing H_2O_2 concentration. It shows that H_2O_2 regulates the activity or induce the synthesis of these H_2O_2 scavenging enzymes. Similar results were reported by Pastori and Trippi (1992), Prasad *et al.* (1994), Guo *et al.* (1997) and Patra and Panda (1998).

The results clearly shows that H_2O_2 has two contradictory functions in cells, one as an oxidant, responsible for lipid peroxidation, membrane injury, pigment bleaching, protein/enzyme inactivation, and the other of an inducer of antioxidant enzymes (GR and Cat), which are responsible for its own scavenging. However, the induced antioxidant activity was not sufficient to scavenge the oxidative damage, as is clear from increased MDA content and chlorophyll damage, and decreased NR activity and lower membrane stability under H_2O_2 treatment as compared to control leaves.

Though both genotypes were subjected to same H_2O_2 concentration under similar conditions, drought tolerant C 306 performed better response than drought susceptible Hira, both in terms of lower oxidative damage as well as increased antioxidant activity. This suggests that H_2O_2 treatment of leaf tissue at an early stage (30 - 40-d-old plants) could be used as an easy and accurate test of stress tolerance of crop plants.

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