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Role of antioxidant systems in wheat genotypes tolerance to water stress

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Abstract

The role of plant antioxidant systems in stress tolerance was studied in leaves of three contrasting wheat genotypes. Drought imposed at two different stages after anthesis resulted in an increase in H_2O_2 accumulation and lipid peroxidation and decrease in ascorbic acid content. Antioxidant enzymes like superoxide dismutase, ascorbate peroxidase and catalase significantly increased under water stress. Drought tolerant genotype C 306 which had highest ascorbate peroxidase and catalase activity and ascorbic acid content also showed lowest H_2O_2 accumulation and lipid peroxidation (malondialdehyde content) under water stress in comparison to susceptible genotype HD 2329 which showed lowest antioxidant enzyme activity and ascorbic acid content and highest H_2O_2 content and lipid peroxidation. HD 2285 which is tolerant to high temperature during grain filling period showed intermediate behaviour. Superoxide dismutase activity, however, did not show significant differences among the genotypes under irrigated as well as water stress condition. It seems that H_2O_2 scavenging systems as represented by ascorbate peroxidase and catalase are more important in imparting tolerance against drought induced oxidative stress than superoxide dismutase alone.

Additional key words: active oxygen species, ascorbate peroxidase, ascorbic acid, catalase, hydrogen peroxide, oxidative injury, oxidative stress, superoxide dismutase, *Triticum aestivum*.

Introduction

Closure of stomata as a result of desiccation and consequent decrease in CO_2 concentration in leaf mesophyll tissue result in an accumulation of NADPH. Under such conditions where NADP is limiting, oxygen acts as an alternate acceptor of electrons resulting in the formation of superoxide radical ($O_2^{\cdot-}$) (Egneus *et al.* 1975, Cadenas 1989). Superoxide radical and its reduction product H_2O_2 are potentially

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Abbreviations: APO - ascorbate peroxidase; Asc - ascorbic acid; Cat - catalase; DAA - days after anthesis; LPO - lipid peroxidation; MDA - malondialdehyde; $O_2^{\cdot-}$ - superoxide radical; SOD - superoxide dismutase.

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toxic compounds and can also combine by Haber-Weiss reaction to form highly toxic hydroxyl radical (OH^-) (Elstner 1987). Active oxygen species, such as superoxide radical, hydrogen peroxide and hydroxyl radical can cause lipid peroxidation and consequently membrane injury, protein degradation, enzyme inactivation, pigment bleaching and disruption of DNA strands (Fridovich 1986, Liebler *et al.* 1986, Davies 1987, Imlay and Linn 1988). The detoxification of superoxide radical and hydrogen peroxide consequently is of prime importance in any defence mechanism.

Plants protect cell and subcellular systems from the cytotoxic effects of these active oxygen radicals using enzymes such as superoxide dismutase, ascorbate peroxidase, glutathione reductase, catalase and metabolites like glutathione, ascorbic acid, α-tocopherol and carotenoids (Liebler *et al.* 1986, Elstner 1987, Larson 1988). Modulation in the activities of these enzymes may be important in plant resistance to environmental stresses (Allen 1995). The objective of the present investigation was to study the effect of water stress on some of the plant antioxidant systems in order to analyze the relative significance of these antioxidant systems in imparting wheat genotypes tolerance to water stress.

Materials and methods

Wheat (*Triticum aestivum* L.) cvs. C 306 (water stress tolerant), HD 2285 (heat tolerant during grain filling period) and HD 2329 (water stress susceptible) were planted under normal (26 Nov. 1996) and late sown (30 Dec. 1996) conditions. Sowing was done in earthen pots (30 × 30 cm), filled with 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 as and when required and pots were saturated with water before starting the moisture stress treatment which was imposed by withholding water supply for 8 d and thereafter the plants were irrigated. The first moisture stress treatment was started when approximately 50 % main shoot ears showed anthesis and second 15 d after anthesis (DAA). Samples of soil from control and treated plants were taken at the end of the treatments (8 and 23 DAA). The mean soil moisture content in control pots was 29.57 % and in stressed pots it varied from 11.95 to 12.85 % (of oven-dried soil).

Leaf relative water content (RWC) was estimated according to the method of Weatherley (1950). Hydrogen peroxide was estimated with titanium reagent (Teranishi *et al.* 1974). One gram of titanium dioxide and 10 g of potassium sulphate were mixed and digested with 150 cm^3 of concentrated sulphuric acid for 2 h on a hot plate. The digested mixture was cooled and diluted to 1.5 dm^3 with distilled water and used as titanium reagent. Sample preparation and H_2O_2 estimation was done as described by Mukherjee and Choudhuri (1983). Plant material (0.5 g) was homogenised in 10 cm^3 of cold acetone. The homogenate was filtered through *Whatman No. 10* filter paper. To whole of the extract 4 cm^3 of titanium reagent were added followed by 5 cm^3 of concentrated ammonium solution to precipitate the peroxide-titanium complex. After centrifugation for 5 min at 10 000 *g*, the supernatant was discarded and precipitate was dissolved in 10 cm^3 of 1 M H_2SO_4. It

was re-centrifuged to remove the undissolved material and absorbance was recorded at 415 nm against blank. Concentration of H_2O_2 was determined using a standard curve plotted with known concentration of H_2O_2. The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content, a product of lipid peroxidation following the method of Heath and Packer (1968). A leaf sample (0.5 g) was homogenised in 10 cm^3 of 0.1 % trichloroacetic acid (TCA). The homogenate was centrifuged at 15 000 *g* for 5 min. To 1.0 cm^3 aliquot of the supernatant 4.0 cm^3 of 0.5 % thiobarbituric acid (TBA) in 20 % TCA was added. The mixture was heated at 95 °C for 30 min and then quickly cooled in a ice bath. After centrifugation at 10 000 *g* for 10 min, the absorbance of the supernatant was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The MDA content was calculated using its absorption coefficient of 155 $mmol^{-1}$ cm^{-1} and expressed as nmol MDA g^{-1}(d.m.). Ascorbic acid was estimated as described by Mukherjee and Choudhuri (1983). Leaf material (250 mg) was extracted with 10 cm^3 of 6 % trichloroacetic acid. Four cm^3 of the extract were mixed with 2 cm^3 of 2 % dinitrophenylhydrazine (in acidic medium) followed by the addition of 1 drop of 10 % thiourea (in 70 % ethanol). The mixture was boiled for 15 min in a water bath and after cooling at room temperature 5 cm^3 of 80 % (v/v) H_2SO_4 were added to the mixture at 0 °C. The absorbance was recorded at 530 nm. The concentration of ascorbic acid was calculated from a standard curve plotted with known concentration of ascorbic acid.

Enzyme extract for superoxide dismutase (SOD), ascorbate peroxidase (APO) and catalase (Cat) was prepared by grinding 0.5 g leaf material with 10 cm^3 of chilled buffer in a prechilled mortar and pestle. For SOD and Cat the extraction medium was 0.1 M potassium phosphate buffer (pH 7.5) containing 0.5 mM EDTA and for APO the extraction was done with 0.1 M phosphate buffer pH 7.0 containing 1 mM ascorbic acid. The brei was filtered through cheese cloth and the filtrate was centrifuged in a *Beckman* (Geneva, Switzerland) model *J2-21* refrigerated centrifuge for 15 min at 20 000 *g*. The supernatant is referred to as enzyme extract. All operations were carried out at 4 °C. 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 mM nitroblue tetrazolium chloride (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 for 15 min. Reaction was stopped by switching off the light and covering the tubes with black cloth. Tubes without enzyme developed maximal colour. 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 which reduced the absorbance reading to 50 % in comparison with the tubes lacking enzyme. The ascorbate peroxidase activity (APO) was assayed according to Nakano and Asada (1981) by recording the decrease in ascorbate content at 290 nm, as ascorbate was oxidized. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM H_2O_2 and 0.1 cm^3 of diluted enzyme in a total volume of 3.0 cm^3. The reaction was started with the addition of H_2O_2 and absorbance was recorded at 290 nm spectrophotometrically for 5 min. Catalase was assayed by

measuring the disappearance of H_2O_2 according to Teranishi *et al.* (1974). The 3.0 cm^3 reaction mixture contained 50 mM phosphate buffer, pH 7.0, 20 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 in a *Beckman* (Geneva, Switzerland) *model-36* UV-visible spectrophotometer.

Only the effects of water stress and genotypes are reported and all observations are mean of six replicates and two dates of sowing. Data was analyzed by analysis of variance.

Results

Relative water content (RWC) in leaves of wheat cultivars under irrigated and stress conditions (Fig. 1) showed a decreasing trend with age in all the genotypes. There was significant reduction in RWC under moisture stress in all the cultivars. Genotypes C 306 maintained significantly higher RWC both under drought and control conditions, HD 2285 exhibited medium behaviour, while HD 2329 showed lowest value under water stress. Hydrogen peroxide accumulation increased under water stress at both developmental stages (Fig. 2*A*). Lowest H_2O_2 content were observed in C 306 and highest in HD 2329 both under control and water stress conditions at these two stages. H_2O_2 content under water stress slightly declined at the second stage.

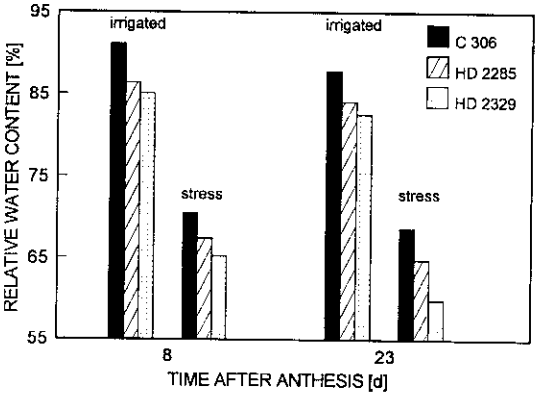

Fig. 1. Effect of water stress on relative water content in wheat genotypes. LSD (P = 0.05) 0.55, 0.34; 0.45, 0.28; 0.78, 0.49, at the two stages for cultivars, stress and interactions, respectively.

Lipid peroxidation (LPO) estimated as MDA content, showed marked increase under water stress at both stages (Fig. 2*B*). Maximum and minimum LPO was observed in HD 2329 and C 306, respectively, both under irrigated and water stress conditions at the two stages. HD 2285 showed intermediate response. Ascorbic acid

Asc acid content was observed in C 306, closely followed by HD 2285 at most of the samplings. HD 2329 showed significantly lower Asc acid content at the two stages under irrigated and water stress conditions.

Superoxide dismutase activity increased significantly under water stress at both stages (Fig. 2*D*). However, the genotypic response was inconsistent and did not follow any pattern *vis-a-vis* tolerant and susceptible genotypes. The genotypic response was non-significant at the second stage. Ascorbate peroxidase activity (APO) showed significant increase under water stress as well as with age (Fig. 2*E*). C 306 exhibited highest APO activity under stress and control conditions, followed by HD 2285 and HD 2329 in decreasing order. The percentage increase in activity under stress was also highest in C 306. Catalase activity also showed significant increase under water stress at these two stages of sampling (Fig. 2*F*). Under irrigated condition at both stages HD 2285 showed highest Cat activity followed by C 306,

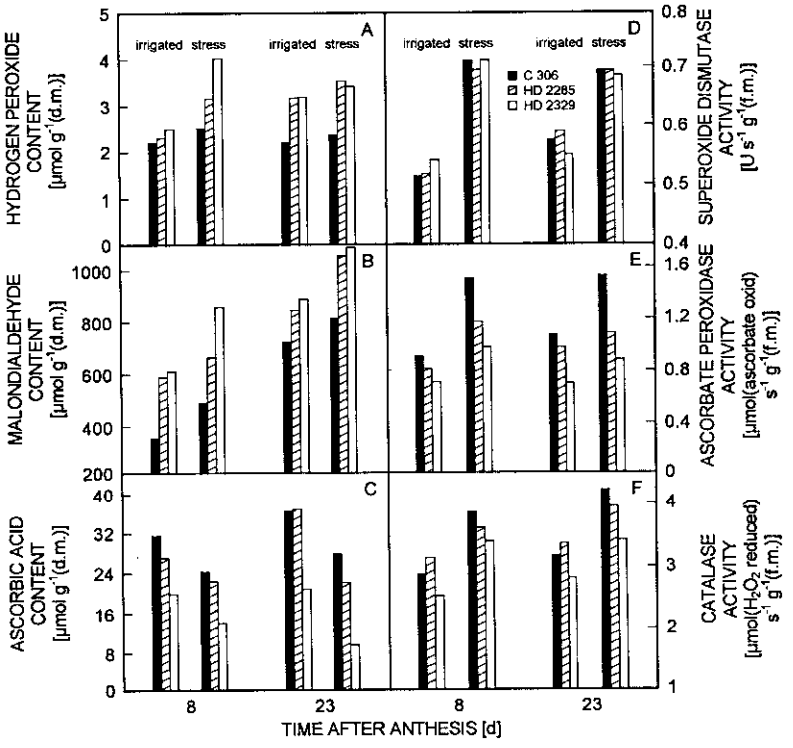

Fig. 2. Effect of water stress on hydrogen peroxide content (*A*), lipid peroxidation (malondialdehyde content) (*B*), ascorbic acid content (*C*), and superoxide dismutase (*D*), ascorbate peroxidase (*E*), and catalase (*F*) activities in wheat genotypes. LSD ($P = 0.05$): H_2O_2 - 0.085, 0.083; 0.069, 0.067; 0.120, 0.118; MDA - 25.64, 24.36; 20.94, 19.91; 36.28, 34.48; Asc - 0.542, 0.589; 0.441, 0.480; 0.765, 0.832, SOD - 0.013, NS; 0.011, 0.015; 0.018, 0.026; APO - 0.038, 0.036; 0.031, 0.029; 0.053, 0.051; Cat - 0.047, 0.055; 0.039, 0.044; 0.067, 0.077, at the two stages for cultivars, stress and interactions, respectively.

while HD 2329 recorded the lowest activity. However, under water stress C 306 showed consistently highest Cat activity followed by HD 2285 and HD 2329.

Discussion

Genotypes respond differentially to oxidative injury as a result of variations in their antioxidant systems (Pastori and Trippi 1992, Turcsanyi *et al.* 1994, Kraus *et al.* 1995). In this study C 306 which is recommended for rainfed cultivation showed comparatively higher RWC and the lowest hydrogen peroxide content and lipid peroxidation under water stress in comparison to susceptible genotype HD 2329 while HD 2285 which is tolerant to high temperature during grain filling showed intermediate response. Hydrogen peroxide is a toxic compound produced as a result of scavenging of superoxide radical, and its higher concentration is injurious to cell/plant resulting in lipid peroxidation and membrane injury (Pastori and Trippi 1992, Baisak *et al.* 1994, Menconi 1995).

Ascorbic acid content is associated with hydrogen peroxide scavenging *via* APO. A higher Asc content along with higher APO activity in C 306 showed its superior tolerance mechanism in terms of H_2O_2 scavenging over other genotypes. Importance of ascorbic acid and ascorbate peroxidase in amelioration of oxidative stress has also been reported by other workers (Gillham and Dodge 1987, Pastori and Trippi 1992, Baisak *et al.* 1994, Menconi *et al.* 1995). Though under irrigated condition Cat activity was highest in HD 2285 and it increased further at second stage, signifying its relative tolerance to high temperature during grain filling period, however, under water stress condition the highest activity was observed in C 306 at both stages. Catalase is also associated with scavenging of H_2O_2 and an increase in its activity is related with increase in stress tolerance (Upadhyaya *et al.* 1990, Olmos *et al.* 1994, Kraus *et al.* 1995).

A perusal of the results show that drought tolerant genotype C 306 which had the lowest H_2O_2 content, a potent oxidant, and the lowest lipid peroxidation also showed the highest Asc acid content and activities of APO and Cat while the drought susceptible HD 2329 showed highest H_2O_2 content, LPO (MDA content) and lowest activities of APO and Cat and Asc content. In our earlier studies we also observed a remarkably higher glutathione reductase, peroxidase and ascorbate peroxidase activity (all associated with H_2O_2 scavenging) in tolerant wheat genotypes compared to susceptible ones (Sairam *et al.* 1997, Sairam *et al.* 1997/98). Since wheat genotypes did not show significant differences in SOD activity nor any pattern was decernible *vis-a-vis* tolerant and susceptible genotypes, can it be said that H_2O_2 scavenging is more important in imparting tolerance against oxidative stress than scavenging of superoxide radical? Though such a statement seems premature, but observations to this effect have also been reported by other workers. Saruyama and Tanida (1995) did not observe any variation in SOD activity in chilling resistant and tolerant rice genotypes and concluded that chilling resistance was closely linked to Cat and APO activities. Similarly Oswald *et al.* (1992) suggested that H_2O_2 scavenging systems like catalase and glutathione reductase were more important in imparting tolerance against oxidative stress than SOD activity alone. A regulatory

role of catalase in H_2O_2 scavenging and chilling resistance has also been reported by Prasad *et al.* (1994). Though significance of SOD in scavenging of superoxide radical can not be belittled, however, it seems plausible that SOD activity may not be limiting in susceptible genotypes under control or stress conditions. However, the H_2O_2 scavenging systems as represented by APO and Cat are clearly limiting or less efficient in susceptible genotypes leading to an accumulation of H_2O_2 and consequently lipid peroxidation and a wide range of other deleterious effects.

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