

Increased antioxidant activity under elevated temperatures: a mechanism of heat stress tolerance in wheat genotypes

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Abstract

An experiment was conducted with three wheat (*Triticum aestivum* L.) genotypes C 306, HD 2285 and HD 2329 (differently susceptible to water and temperature stress) to study the extent of oxidative injury and activities of antioxidant enzymes in relation to heat stress induced by manipulating dates of sowing. Increase in temperature by late sowing significantly decreased leaf relative water content (RWC), ascorbic acid content, and increased H₂O₂ content and lipid peroxidation in all the genotypes at 8 and 23 d after anthesis. Temperature tolerant genotypes C 306, closely followed by HD 2285 were superior to HD 2329 in maintaining high RWC, ascorbic acid content, and lower H₂O₂ content and lipid peroxidation (malondialdehyde content) under high temperature (late sowing) at the two stages. Activities of superoxide dismutase and catalase were highest in HD 2285 followed by C 306 and minimum in HD 2329 while ascorbate peroxidase activity was highest in C 306.

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

Introduction

Crop plants experience various stresses during their life cycle, such as high or low temperature, drought and salinity, which result in the formation of various active oxygen species such as superoxide radical (O₂⁻), hydroxyl radical (OH⁻), and hydrogen peroxide (H₂O₂), which together constitute the oxidative stress (Egneus *et al.* 1975, Elstner 1987, Cadenas 1989). High temperature induced oxidative stress in various higher and lower plants have been reported by many workers (Upadhyaya *et al.* 1990, Jagtap and Bhargava 1995, Davidson *et al.* 1996). Active oxygen species, 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 antioxidant 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). Tolerance to high temperature stress in crop plants have been reported to be associated with an increase in antioxidant enzymes activity (Rui *et al.* 1990, Gupta *et al.* 1993, Badiani *et al.* 1994, Zhau *et al.* 1995). Modulation in the activities of these antioxidant enzymes and metabolites may be important in plant's resistance to environmental stresses. The objective of the present investigation was to study the effect of high temperature stress (late sowing) on wheat antioxidant systems and to analyze the significance of these systems in imparting temperature stress tolerance to wheat genotypes.

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Abbreviations: APO - ascorbate peroxidase; Asc - ascorbic acid; Cat - catalase; DAA - days after anthesis; LPO - lipid peroxidation; MDA - malondialdehyde; RWC - relative water content.

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Materials and methods

Wheat (*Triticum aestivum* L.) cvs. C 306 (tolerant to water and temperature stress), HD 2285 (recommended for late sowing, tolerant to temperature stress during post anthesis phase), and HD 2329 (recommended for irrigated conditions, susceptible to water and temperature stress) (Tandon 1993) were sown on 26 November 1996 and 30 December 1996 (normal and late sown conditions, respectively) into earthen pots (30 × 30 cm), filled with 10 kg mixture of 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 to keep them fully turgid. Flag leaf was used for various enzymatic and chemical estimations and samples for various assays/estimations were taken between 09:30 to 10:30 on 8 and 23 DAA. Anthesis was considered to have occurred when approximately 50 % of main shoot ears showed anther dehiscence.

Leaf relative water content (RWC) was estimated according to Wetherley (1950). Hydrogen peroxide was estimated with titanium reagent (Teranishi *et al.* 1974). One gramme of titanium dioxide and 10 g of potassium sulphate were mixed and digested with 150 cm³ of concentrated sulphuric acid for 2 h on a hot plate. The digested mixture was cooled and diluted to 1.5 dm³ with distilled water and used as titanium reagent. Sample preparation and H₂O₂ estimation was done as described by Mukherjee and Choudhuri (1983). Leaf material (0.5 g) was homogenised in 10 cm³ of cold acetone. The homogenate was filtered through Whatman No. 10 filter paper and to filtrate 4 cm³ of titanium reagent was added followed by 5 cm³ of concentrated ammonium solution to precipitate the peroxide-titanium complex. The reaction mixture was centrifuged in a Beckman (Geneva, Switzerland) model J2-21 refrigerated centrifuge for 5 min at 10 000 g, the supernatant was discarded and precipitate was dissolved in 10 cm³ of 2 M H₂SO₄. It was re-centrifuged to remove the undissolved material and absorbance was recorded at 415 nm against blank. Concentration of H₂O₂ was determined using a standard curve plotted with known concentration of H₂O₂.

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 cm³ of 0.1 % trichloroacetic acid (TCA). The homogenate was centrifuged at 15 000 g for 10 min. To 2.0 cm³ aliquot of the supernatant 4.0 cm³ 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 an 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 tissue (250 mg) was extracted with 10 cm³ of 6 % trichloroacetic acid. Four cm³ of the extract was mixed with 2 cm³ 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 to room temperature, 5 cm³ of 80 % (v/v) H₂SO₄ was added to the mixture at 0 °C (in an ice bath). 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 cm³ 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 J2-21 refrigerated centrifuge for 15 min at 20 000 g. The supernatant was 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 cm³ 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 cm³ 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 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 the method of 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 H₂O₂ and 0.1 cm³ of diluted enzyme in a total volume of 3.0 cm³. The reaction was started with the addition of H₂O₂ and absorbance was recorded at 290 nm spectrophotometrically for 5 min.

Catalase was assayed by measuring the disappearance of H₂O₂ according to Teranishi *et al.* (1974). The 3.0 cm³ reaction mixture contained 50 mM phosphate buffer, pH 7.0, 2 mM H₂O₂ and 0.1 cm³ diluted (10 times) enzyme.

The reaction was stopped after 5 min by the addition of 2 cm³ of titanium reagent, which also forms coloured complex with residual H₂O₂. Aliquot was centrifuged at 10 000 g for 10 min and absorbance of the supernatant was

recorded at 410 nm in a *Beckman* model 36 UV-visible spectrophotometer (Geneva, Switzerland).

All observations are means of six replicates and data were analyzed by analysis of variance.

Results

Mean maximum and minimum temperatures increased about 4.9 °C at first stage (8 DAA) and 3.3 to 4.7 °C at second stage of sampling (23 DAA) under late sowing in comparison to normal sowing (Fig. 1, Table 1). Relative water content (RWC) in leaves of wheat cultivars under normal and late sowings (Table 2) decreased with age in all the genotypes. There was significant reduction in RWC under late sowing in all the cultivars. C 306 maintained significantly higher RWC both under normal and late sown

conditions, HD 2285 exhibited medium behavior, while HD 2329 showed lowest value under late sowing. Hydrogen peroxide accumulation increased under late sowing as well as with age except in C 306, which registered a decline under late sowing at first stage (Fig. 2A). The lowest H₂O₂ content were observed in C 306 and the highest in HD 2329 at the two stages. Maximum increase in H₂O₂ content under late sowing was observed in susceptible genotype HD 2329.

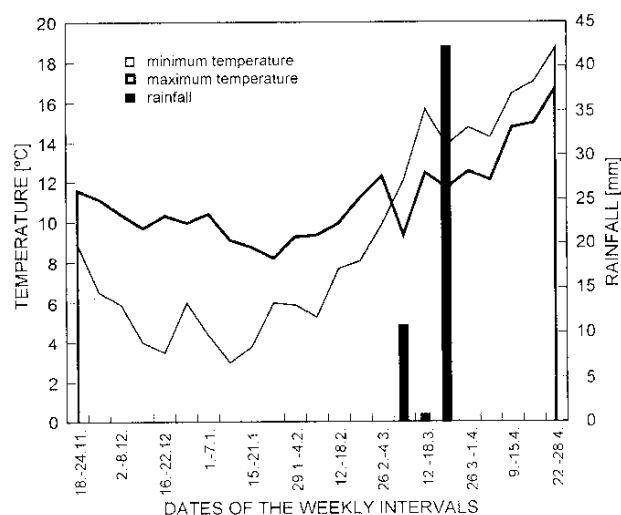


Fig. 1. Changes in mean maximum and minimum temperatures and rainfall during the crop growth period.

Table 1. Dates of anthesis and two sampling stages, and corresponding mean maximum and minimum temperatures.

		Normal sowing			Late sowing		
		C 306	HD 2285	HD 2329	C 306	HD 2285	HD 2329
Anthesis	date	03/3	25/2	25/2	18/3	17/3	20/3
	max.	27.7	25.3	25.3	28.1	28.1	26.4
	min.	9.9	8.1	8.1	15.7	15.7	13.9
	mean	18.8	16.7	16.7	21.9	21.9	20.2
1 st sampling	date	11/3	05/3	05/3	26/3	25/3	28/3
	max.	21.3	21.3	21.3	28.3	28.3	28.3
	min.	12.1	12.1	12.1	14.8	14.8	14.8
	mean	16.7	16.7	16.7	21.6	21.6	21.6
2 nd sampling	date	26/3	20/3	20/3	10/4	09/4	12/4
	max.	28.3	26.4	26.4	33.3	33.3	33.3
	min.	14.8	13.9	13.9	16.5	16.5	16.5
	mean	21.6	20.2	20.2	24.9	24.9	24.9

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Table 2. Effect of temperature stress (comparison of normal and late sowing) on relative water content [%] in wheat genotypes at 8 and 23 DAA. Means \pm SE, $n = 6$.

	8 DAA		23 DAA	
	normal sowing	late sowing	normal sowing	late sowing
C 306	91.11 \pm 1.040	86.54 \pm 0.678	87.85 \pm 0.042	83.13 \pm 0.531
HD 2285	86.38 \pm 0.102	83.78 \pm 0.531	84.14 \pm 0.270	79.54 \pm 0.506
HD 2329	85.03 \pm 0.470	83.52 \pm 1.017	82.62 \pm 0.100	77.17 \pm 0.457
LSD _{0.05} date	0.924		0.482	
LSD _{0.05} genotype	1.132		0.590	
LSD _{0.05} interactions	1.601		0.834	

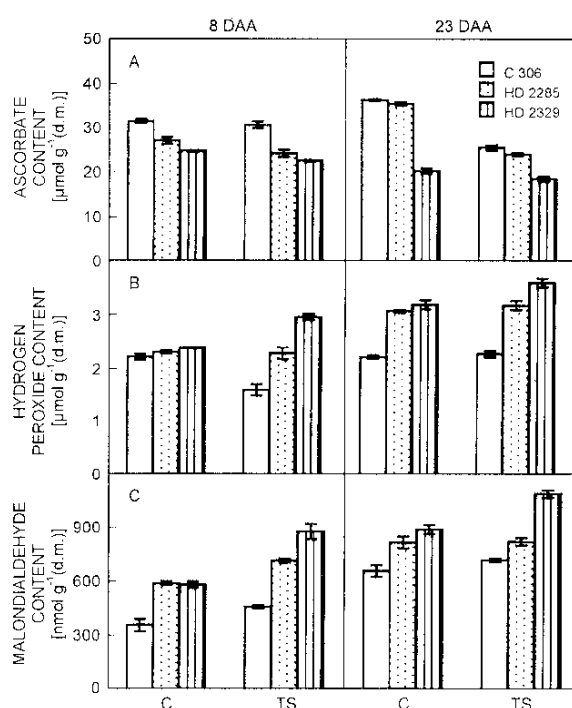


Fig. 2. Effect of temperature stress induced by late sowing on hydrogen peroxide content (A), lipid peroxidation (malondialdehyde content) (B), and ascorbic acid content (C) in different wheat genotypes at 8 and 23 DAA. Vertical bars show SE, differences between normal and late sowing were significant at $P = 0.05$.

Lipid peroxidation (LPO) as MDA content increased under late sowing at both the stages in all the cultivars (Fig. 2B). Maximum and minimum lipid peroxidation was observed in HD 2329 and C 306, respectively, under late sown conditions at the two stages. HD 2285 showed intermediate response. Ascorbic acid (Asc) content decreased under late sowing (Fig. 2C). However, the highest Asc content was observed in C 306, followed by HD 2285 at the two stages under both the sowings. HD 2329 showed the lowest Asc content at the two stages under late sown condition.

Superoxide dismutase activity increased significantly under late sown conditions at both the stages (Fig. 3A). Though, the genotypic response was non-significant at the

first stage, significant differences were observed at the second stage. HD 2285 showed maximum activity under late sown condition closely followed by C 306, while HD 2329 exhibited minimum activity. Ascorbate peroxidase activity (APO) increased under late sowing as well as with age in HD 2285 and HD 2329 (Fig. 3B). APO activity of C 306 was higher than that of HD 2285 and HD 2329, but it decreased under late sowing at 8 DAA. Catalase activity slightly increased with age and under late sowing (Fig. 3C). HD 2285 showed highest Cat activity at both the stages followed by C 306, while HD 2329 recorded the lowest Cat activity. Maximum increase in catalase activity under elevated temperature was also observed in HD 2285.

Discussion

Genotypes respond differentially to various stresses 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 tolerant to water and temperature stress, showed higher RWC and lowest hydrogen peroxide content and lipid peroxidation under elevated temperature 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 a higher concentration is injurious to cell/plant resulting in lipid peroxidation and membrane injury (Pastori and Trippi 1992, Baisak *et al.* 1994, Menconi *et al.* 1995). Dhindsa *et al.* (1981) and Sairam *et al.* (1997/98) have also reported that increase in lipid peroxidation is associated with cell membrane injury.

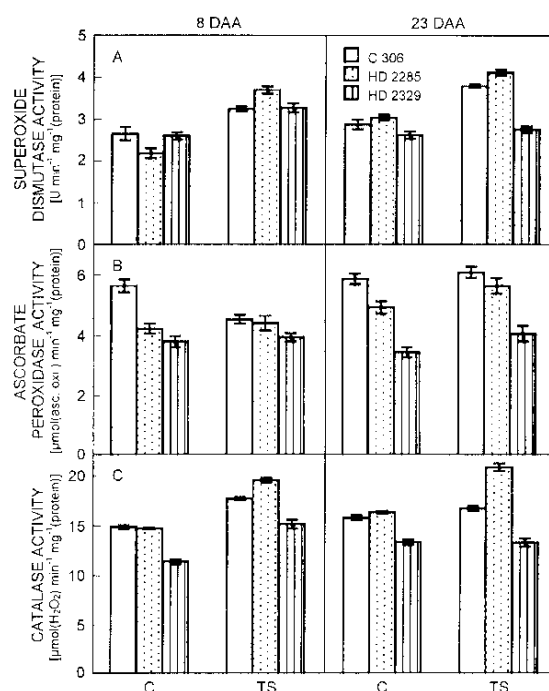


Fig. 3. Effect of temperature stress induced by late sowing on the activities of superoxide dismutase (A), ascorbate peroxidase (B), and catalase (C) in different wheat genotypes at 8 and 23 DAA. Vertical bars show SE, differences between normal and late sowing were significant at $P = 0.05$.

Ascorbic acid content is associated with hydrogen peroxide scavenging via APO. A higher Asc content along with higher APO activity in C 306 demonstrates its superior tolerance mechanism in terms of H_2O_2 scavenging over other genotypes. Other workers (Gillham and Dodge 1987, Pastori and Trippi 1992, Baisak *et al.* 1994, Menconi *et al.* 1995) have also reported importance of Asc and APO in amelioration of oxidative stress. Highest SOD and Cat activity under elevated temperature in HD 2285 closely followed by C 306, both temperature tolerant genotypes, showed the significance of these two enzyme systems in imparting tolerance to these genotypes. Significance of SOD in temperature stress tolerance has also been reported by many workers (Upadhyaya *et al.* 1991, Gupta *et al.* 1993, Jagtap and Bhargava 1995, Davidson *et al.* 1996). Catalase is also associated with the 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). Prasad *et al.* (1994) and Anderson *et al.* (1995) have suggested that an increase in Cat activity in acclimatized maize seedlings under chilling temperature as compared to control represent a potential defence mechanism against oxidative damage.

A perusal of the results show that temperature and water stress tolerant genotype C 306 which had lowest H_2O_2 content, a potent oxidant, and lowest lipid peroxidation also showed highest Asc acid content and ascorbate peroxidase activity. While temperature tolerant genotype HD 2285 showed highest increase in SOD and Cat activity under elevated temperature accompanied by intermediate levels of Asc, H_2O_2 and lipid peroxidation. Temperature sensitive genotype HD 2329 on the other hand showed highest H_2O_2 content, LPO (MDA content) and lowest activities of SOD, APO and Cat and Asc content. In our earlier studies we

have also observed a remarkably higher glutathione reductase, peroxidase and ascorbate peroxidase activity (all associated with H₂O₂ scavenging) in tolerant wheat genotypes compared to susceptible ones (Sairam *et al.* 1997, Sairam *et al.* 1997/98).

It is further clear from the results that not all the genotypes tolerant to a particular stress show similar increase in the activities of various antioxidant enzymes under temperature stress. While HD 2285 showed highest SOD and Cat activity, suggesting that a major quantity of H₂O₂ was being scavenged by catalase route, in case of

C 306, APO activity was highest, indicating a major role of this enzyme in the scavenging of H₂O₂. Differential significance of SOD, Cat and APO in chilling resistance have also been reported by Anderson *et al.* (1995), Saruyama and Tanida (1995) and Prasad *et al.* (1994). It may thus be surmised that the temperature tolerance of wheat genotypes is associated with the level of antioxidant enzymes, though all the enzymes involved in amelioration of oxidative stress may not show uniform increase in activity in a given tolerant genotype.

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