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ROS production, H_2O_2 detection and biochemical characterization of water stressed wheat (*Triticum aestivum* L.) varieties

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Abstract
One month old plant of four varieties (MW, KD, GY and GN) of wheat (*Triticum aestivum* L.) was taken and subjected to water stress for 3, 6 and 9 days. RWC was found to be higher in case of GY and KD when compared to MW and GY. There was an initial enhancement in the activities of all five tested antioxidative enzymes- peroxidase, ascorbate peroxidase, catalase, glutathione reductase and superoxide dismutase in K and GN varieties, while in MW and GY, the activity of catalase and superoxide dismutase showed a decline at all periods of water stress. Peroxidase and glutathione reductase activities increased even on 9th day of stress in K and GN, but all other activities showed a decline after 3 days of stress. The accumulation of H_2O_2 showed an increase with increasing days of water stress but in K and GN there was a decline during prolonged water stress. Lipid peroxidation increased significantly which was higher in case of MW and GY. With increase in the duration of water stress proline, phenol and ascorbate content increased. Higher values of MSI and total antioxidant were observed in the cultivar KD and GY with increase in the severity of water stress than in MW and GY. After an initial enhancement the content of carotenoid increased followed by a decline. Total chlorophylls showed a general decline during water stress, but the ratio of chla/b showed an initial increase in the 3rd day of water stress which declined during the latter stages of water stress. Results of the present study indicate that two of the varieties- MW and GY are susceptible to water stress, while the other two-K and GN is tolerant.

Keywords: antioxidative enzymes, Lipid peroxidation, phenol, ascorbate, MSI, carotenoid

Since plants have limited mechanisms of drought stress avoidance, they require flexible means of adaptation to changing drought conditions (Zhang et al 2004). Tolerance to this abiotic stress is a complex phenomenon, comprising a number of physio-biochemical processes at both cellular and whole organism levels activated at different stages of plant development. Both enzymatic and non-enzymatic antioxidants provide protection against oxidative damage (Munne-Bosch and Alegre, 2000). Various tolerance mechanisms have been suggested on the basis of the biochemical and physiological changes related to drought (Quartacci *et al.* 1994, Quartacci *et al.* 1995, Sgheeri *et al.* 2000).

Reactive oxygen species (ROS) are known as toxic metabolic products in plants and other aerobic organisms. An elaborate and highly redundant plant ROS network, composed of antioxidant enzymes, antioxidants and ROS-producing enzymes, is responsible for maintaining ROS levels under tight control (Gechev et al 2006). ROS, resulting from excitation or incomplete reduction of molecular oxygen, are unwelcome harmful by-products of normal cellular metabolism in aerobic organisms (Halliwell, 2006) Plants, facing an even greater burden of excess ROS, initially developed various protective mechanisms, such as small antioxidant molecules and antioxidant enzymes, to keep ROS levels under control (Van, 2001).

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It is generally believed that maintaining a high reduced to oxidized ratio of ascorbic acid and glutathione is essential for the proper scavenging of ROS in cells. The ratio is mainly maintained by glutathione reductase (GR, EC 1.6.4.2) (Noctor and Foyer 1998; Asada 1999).

Moreover, it has been noted that the balance between ROS-scavenging enzymes is crucial for determining the steady-state level of superoxide radicals and hydrogen peroxide (Bowler *et al.* 1991). All cellular compartments are well-equipped with antioxidant enzymes and antioxidants. Therefore, ROS are normally scavenged immediately at the sites of their production by the locally present antioxidants. However, when this local antioxidant capacity cannot cope with ROS production (for example, during stress or temporarily reduced antioxidant levels due to developmental signals), H_2O_2 can leak into the cytosol and diffuse to other compartments. Plants can also deal with excess H_2O_2 by transporting it into vacuoles

for detoxification (Bienert, 2006 and Gould , 2002). Decline in the efficacy of the H_2O_2 decomposing system is probably responsible for the oxidative damage occurring in water stressed leaf (Baisak R et al,1994) This deficit has an evident effect on plant growth that depends on both severity and duration of the stress (Araus *et al.* 2002; Bartels & Souer 2004).

Considering the importance of water stress in crop production, the present work was undertaken to evaluate for wheat varieties on their tolerance and susceptibility

to the above stress in terms of defense responses.

Materials and Methods

Plant Material

Seeds of four varieties of wheat (*Triticum aestivum* L.) – Kedar (KD), MW, GY and GN were selected were initially surface sterilized with 0.1% (w/v) $HgCl_2$ for 3-4 minutes, washed with sterile distilled water and were then transferred to petriplates under aseptic conditions. These seeds were allowed to germinate in the petriplates for one week and then the seedlings were transferred to earthen pots. Plants were maintained in growth chamber at a temperature of 20-25°C, Relative Humidity (RH) 65 -70%, 16 h photoperiod and irradiance of 400 µmol $m^{-2}s^{-1}$. One month old plants (taken as control or zero day treated) were subjected to water stress by withholding water completely for specific period and the sampling was done after 3, 6 and 9 days and after each period of water stress. Morphological changes were noted and relative water content (RWC) of leaves was determined as described by Farooqui *et al.* (2000) calculated by the following formula:-

Various other biochemical assays were then performed as given below.

$$RWC(\%) = \frac{\text{fresh wt.} - \text{dry wt.}}{\text{fully turgid wt.} - \text{dry wt.}} X100$$

Antioxidant enzyme extraction and assays

Extraction for enzymatic and isozymatic analysis

Leaves of wheat seedlings were homogenized in 5mL of ice-cold 50 mM sodium phosphate buffer, pH 7.2, containing 1% (w/v) polyvinylpolypyrrolidone using liquid nitrogen in a chilled mortar and pestle. The homogenate was then centrifuged at 6708 g for 20 min at 4°C and the supernatant was directly used as crude extract for enzyme assays. Protein contents in extracts were quantified following the method of Lowry *et al.* (1951), using BSA as standard.

Assay

Peroxidase (POX: EC. 1.11.17) activity was assayed in 4802 UV VIS spectrophotometer (Cole Parmer, USA) at 460 nm by monitoring the oxidation of o-dianisidine in presence of H_2O_2 (Chakraborty *et al.* 1993). Specific activity was expressed as mmol O-dianisidine mg $protein^{-1}$ min^{-1}.

Ascorbate peroxidase (APOX: EC.1.11.1.11) activity was assayed as decrease in absorbance by monitoring the oxidation of ascorbate at 290 nm according to the method of Asada and Takahashi (1987) with some modification. Enzyme activity was expressed as mmol ascorbate mg $protein^{-1}$ min^{-1}.

Catalase (CAT: EC.1.11.1.6) activity was assayed as described by Beers and Sizer (1952) by estimating the breakdown of H_2O_2 which was measured at 240 nm. The enzyme activity was expressed as as µmol H_2O_2 mg $protein^{-1}$ min^{-1}.

Glutathione reductase (GR: EC 1.6.4.2) activity was determined by the oxidation of NADPH at 340 nm as described by Lee and Lee (2000). Enzyme activity was expressed as µM NADPH oxidized mg $protein^{-1}$ min^{-1}.

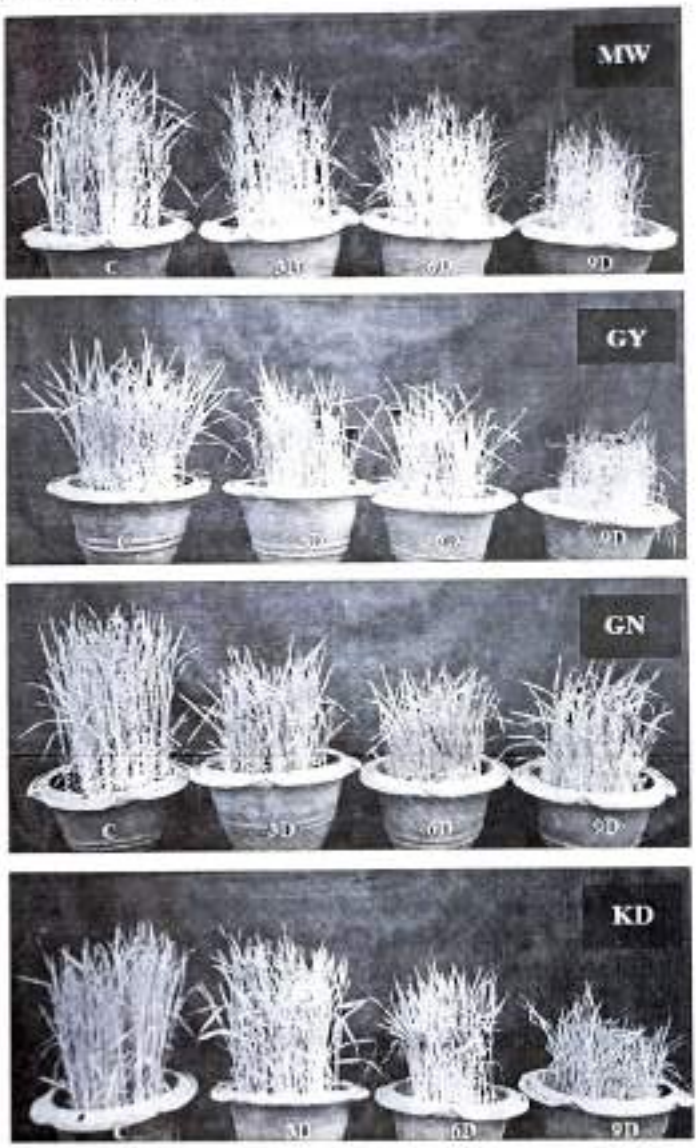

Fig 1. Different varieties of wheat seedlings subjected to water stress for 3, 6 and 9 days

Superoxide dismutase (SOD: EC 1.15.1.1) was assayed by monitoring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Dhindsa *et al.* (1981) with some modification. The absorbance of the samples was measured at 560 nm and 1 unit of activity was defined as the amount of enzyme required to inhibit 50% of the NBT reduction rate in the controls containing no enzymes.

Isozymatic analysis of Peroxidase and Catalase

POX isoenzymes were separated on 7.5% non-denaturating acrylamide gel run at 10 mA for 4 h at 4°C following the method of Davis (1964). Isoforms of POX were visualized as bright blue bands by staining the gel in 50 ml solution containing 1.04% of Benzidine, 9% of acetic acid and 3% H_2O_2 as described by Reddy and Gasber (1971). After development of the bands the reaction was terminated by arresting the reaction by immersing the gel into a large volume of 7% acetic acid for 10minutes.

Quantification of H_2O_2 and in-situ detection of H_2O_2

Table 1. Effect of water stress on APOX, POX, GR, CAT & SOD in different wheat varieties

Varieties	Days of treatment	CAT	GR	SOD	APOX	PER
MW	0d	1.67 ±0.07^a	0.14±0.02^a	0.086±0.008^a	0.116±0.007	0.020±0.006
	3d	1.43 ±0.02^b	0.40±0.03^b	0.073±0.006^a	0.730±0.010	0.029±0.013
	6d	1.19 ±0.01^b	0.33±0.01^b	0.055±0.005^b	0.263±0.006	0.038±0.015
	9d	1.09 ±0.02^b	0.23±0.01^b	0.041±0.004^b	0.168±0.004	0.026±0.009
GY	0d	1.77 ±0.02^a	0.18±0.03^a	0.059±0.006^a	0.089±0.005	0.023±0.005
	3d	1.46 ±0.04^b	0.22±0.01^a	0.055±0.005^a	1.017±0.003	0.048±0.007
	6d	1.41 ±0.02^b	0.15±0.01^a	0.036±0.003^b	0.385±0.004	0.051±0.006
	9d	1.27 ±0.01^b	0.11±0.01^a	0.027±0.002^b	0.244±0.006	0.044±0.008
KD	0d	1.77 ±0.03^a	0.13±0.01^a	0.043±0.003^a	0.189±0.011	0.045±0.007
	3d	2.76 ±0.06^b	0.28±0.02^b	0.068±0.002^b	0.633±0.005	0.062±0.006
	6d	1.25 ±0.03^b	0.76±0.04^b	0.020±0.001^b	0.067±0.009	0.095±0.007
	9d	1.13 ±0.02^b	1.53±0.08^b	0.015±0.002^b	0.044±0.004	0.193±0.007
GN	0d	1.18 ±0.01^a	0.17±0.01^a	0.036±0.003^a	0.065±0.004	0.035±0.010
	3d	2.19 ±0.03^b	0.32±0.02^b	0.061±0.003^b	0.166±0.003	0.042±0.011
	6d	1.73 ±0.02^b	0.51±0.03^b	0.022±0.002^b	0.107±0.004	0.072±0.009
	9d	1.50 ±0.04^b	0.97±0.04^b	0.013±0.001^b	0.068±0.008	0.126±0.004

Enzyme activities is expressed as APOX= m moles ascorbate mg protein^{-1} min^{-1}, POX= mmol o-dianisidine mg protein^{-1}min^{-1}, GR= µmoles NADPH oxidized mg protein^{-1} min^{-1}, SOD= EU mg protein^{-1} and CAT= μmole H_2O_2 mg protein^{-1} min^{-1}; ± = S.E.; Different superscripts indicate values differ significantly at 1% in t test

The quantification of H_2O_2 levels in the leaves were done according to the method given by Jena and Choudhuri (1981). The intensity of yellow color was measured at 410 nm in the spectrophotometer and the H_2O_2 levels were calculated using extinction coefficient 0.28 µmol^{-1} cm^{-1}. *In-situ* detection of H_2O_2 was done following the method of Thordal-Christensen et al (1997) with minor modifications using diaminobenzidine (DAB). H_2O_2 was visualized as reddish-brown colour at the site of DAB polymerization. DAB polymerizes instantly and locally at sites of peroxidase activity into a reddish-brown polymer.

Determination of lipid peroxidation

MDA content, a measure of lipid peroxidation was determined by the thiobarbituric acid (TBA) reaction. The absorbance was measured at 532 and 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968).

Extraction and estimation of non-enzymatic antioxidants and DPPH based free radical scavenging activity

The extraction and estimation of carotenoids were following the method described by Lichtenthaler (1987). Extraction was done in methanol and the extract was filtered and the absorbance of the filtrate was measured at 480, 663 and 645 nm in a VIS spectrophotometer. The carotenoid content was calculated using a standard formula. Ascorbic acid was extracted and estimated following the method described by Mukherjee and Chaudhuri (1983).

Total antioxidant activity or DPPH based free radical scavenging activity was measured by following the method of (Blois 1958) and expressed as percent (%) inhibition of DPPH absorbance which was measured at 515 nm. The inhibition percentage of the absorbance of DPPH solution was calculated using the following equation:

Inhibition % = (Absorbance at T_0 – Absorbance at T_{30}) / A T_0 X 100

Where, A T_0 was the absorbance of DPPH at time zero, and A T_{30} was the absorbance of DPPH after 30 min of incubation. Total antioxidant activity was thus measured as free radical scavenging ability in terms of inhibition of absorbance by DPPH.

Membrane stability index (MSI)

The leaf membrane stability index (MSI) was determined according to the method of Premchandra *et al.* (1990) as modified by Sairam (1994). The MSI was calculated as given by the following equation:

Membrane stability index (MSI) = [1 – (C1/C2)] x 100

Results and Discussion

Morphological symptoms of plants and RWC during stress

Severe wilting symptoms were observed in the four tested varieties only during the 9th day of stress and the plants showed little or no wilting symptoms morphologically during the initial stages of stress (Fig 1). The relative water content in the leaf of the plants declined significantly with induction and duration of water stress. It was noted that the decline in RWC (Fig 2) after 9 days in relation to control (0 day treatment) was lesser in KD and GN (35.7 and 36% respectively) compared to MW and GY (53 and 53.4% respectively). It has also been reported in previous studies that drought resistant cultivars maintain higher RWC during water stress, while in susceptible cultivars RWC shows greater decline (Farooqui *et al.* 2000, Chakraborty *et al.* 2002,

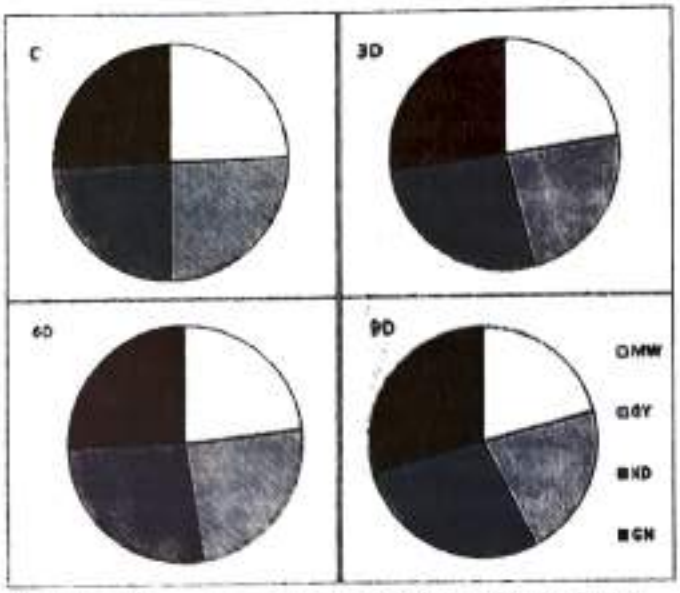

Fig 2. Relative water content of four varieties of wheat subjected to water stress. Results are expressed as the mean of three replicates (10 plants each). C-0 day; 3, 6 and 9 D. days after withholding water.

Iqbal and Bano 2009).

Effect of water stress on antioxidative enzymes and isozymatic patters in wheat varieties

After 3 days of water stress the activities of APOX, POX and GR (Table 1) showed an initial enhancement in all four varieties. With increase in the duration of stress, the activities of POX, APOX and GR showed a decrease in the case of MW and GY whereas POX and GR increased with prolonged water stress in KD and GN. However, the activity of APOX decline after 3 days in these two varieties. It was observed that at all periods of water stress activities of CAT and SOD (Table 1) declined in MW and GY, whereas it showed an initial increase followed by a decline in case of KD and GN. The data of the present study thus reveal that in KD and GN, which are more tolerant than the other two, activities of all antioxidant enzymes increased initially and activities of POX and GR continued to increase, indicating their involvement in tolerance, whereas APOX, CAT and SOD did not contribute directly to tolerance. Previous workers have also reported differential responses of genotypes to water stress with respect to antioxidant enzymes (Dhanda *et al.* 2004, Nair *et al.* 2008). It is evident from results of the present study that POD has a greater role in tolerance than CAT during prolonged water stress. Chakraborty *et al.* (2002) also reported that peroxidase activities increased initially in all tea cultivars following water stress, but in tolerant cultivars it increased even with prolonged periods. Iqbal and Bano (2009) obtained greater increase in activities of POD and CAT in wheat accessions which were tolerant to water stress than those which were less tolerant. Enhancement of GR activity in tolerant varieties indicated that tolerant plants exhibit a more active ascorbate- glutathione cycle than the less tolerant cultivars (Chai *et al.* 2005). This cycle has been implicated in mitigating the effects of reactive oxygen species (Molina *et al.* 2002; Mandhania *et al.* 2006). Under drought stress, enhanced SOD activity was found in pea, tobacco and bean (Moran *et al.* 1994, Van Rensburg *et al.* 1994, Zlatev *et al.* 2006), decreased SOD activity in sunflower seedlings and banana (Quartacci and Navaro 1992, Chai *et al.* 2005) and unaffected SOD activity in maize (Luna *et al.* 1985). In wheat, SOD activity increased or remained unchanged in the early phase of drought but decreased with prolonged water stress (Zhang and Kirkham 1995), as was also obtained in the present study.

Isozyme analysis of POX and CAT (Fig 3) revealed that water stress induced the over expression of isozymes in both tolerant and less tolerant varieties. Hence assay of activity and isozyme analysis could not be directly correlated.

In-situ detection of *accumulation of H_2O_2 and MDA content in the leaf after water stress*

In situ detection of H_2O_2 in leaf tissues and microscopic observations revealed darker staining in tissues subjected to prolonged drought stress, especially in the less-tolerant varieties (Fig 4). A decline in CAT activity following water stress was correlated with increased accumulation of H_2O_2 as well as increased lipid peroxidation in all varieties. However, both H_2O_2 accumulation and lipid peroxidation (Fig. 5) were significantly higher in susceptible varieties in comparison to tolerant ones. The present results are in conformity with those of several previous workers (Chai *et al.* 2005, Zlatev *et al.* 2006). Increased concentrations of H_2O_2, a strong oxidant causes localized oxidative damage, disruption of metabolic functions and lipid peroxidation (Foyer *et al.* 1997, Velikova *et al.* 2000, Zlatev *et al.* 2006). However, besides being an ROS, H_2O_2 is also a signal molecule which is involved in signal transduction mechanisms for several processes in plants such as stomatal closure, root growth and responses to pathogen challenge (Neill *et al.* 2002, Laloi

Table 2.Effect of water stress on cell membrane stability expressed as percent (%) relative injury and free radical scavenging activity (total antioxidant activity) expressed as percent (%) inhibition of DPPH absorbance

Var.	Days of treatment	Relative Injury (%)	Total atioxi-dant activity
MW	0d	64.86	8.43
	3D	58.18	4.49
	6D	51.61	10.53
	9D	20.27	3.33
KD	0d	91.12	8.81
	3D	88.70	21.43
	6D	89.21	29.71
	9D	37.75	56.37
GN	0d	87.42	4.83
	3D	91.51	23.61
	6D	64.09	26.83
	9D	49.78	36.02
GY	0d	95.12	4.56
	3D	80.37	5.47
	6D	64.68	9.54
	9D	49.78	4.11

For cell membrane stability K=0.946, cell constant=1, solution condition=84μS,coefficient-1 at 25°C

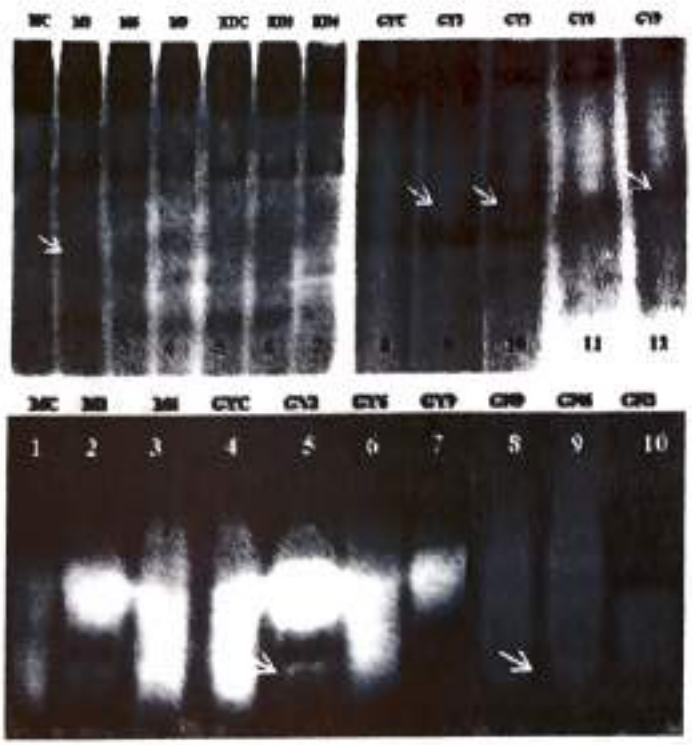

Fig 3. Peroxidase isozyme analysis (upper) of MW, KD and GY during water stress; ln1, 5, 8: Control (0 day), ln 2, 6, 9, 10: 3rd of day, ln3, 7, 11: 6th day, ln4, 12: 9th day and catalase isozyme analysis (lower) of MW, GY and GN during water stress; ln1, 4: Control (0 day), ln2, 5, 10: 3rd day, ln3, 6, 9: 6th day, ln 7, 8: 9th day

et al. 2004, Desikan *et al.* 2005). Thus levels of H_2O_2 are efficiently controlled to maintain balance between production and breakdown. In the present study, though H_2O_2 accumulation increased during water stress, in tolerant varieties, after a period of prolonged drought there was a decrease in H_2O_2 levels indicating greater antioxidant activity.

The peroxidation of lipids in the cell membrane is one of the most damaging cellular responses observed in response to water stress (Thankamani *et al.* 2003) and the amount of lipid peroxidation is considered as one of the determinants which indicate the extremity of stress experienced by a plant. It was observed that during water stress, MDA content which is a measure of lipid peroxidation, increased in all varieties. After 9 days of stress the MDA content in susceptible varieties was more than 3 times that of tolerant varieties.

Change in the content of non-enzymatic antioxidants, total antioxidants and relative injury of the leaf during water stress

The accumulation of ascorbic acid and carotenoid (Fig 6) in plants showed a significant increase in all four varieties. However, it was noted that the accumulation of ascorbate was enhanced all four varieties even after 9 days of water stress; on the other hand, in MW and GY carotenoids declined after 3 days and after 6 days in K and GN. The data was in accordance with the accumulation of total antioxidants in the plants under stress. The value total antioxidant was found to be higher in case of tolerant varieties KD and GN (Table 2) and in the more susceptible varieties, i.e., MW and GY it decreased during prolonged stress. The cell membrane

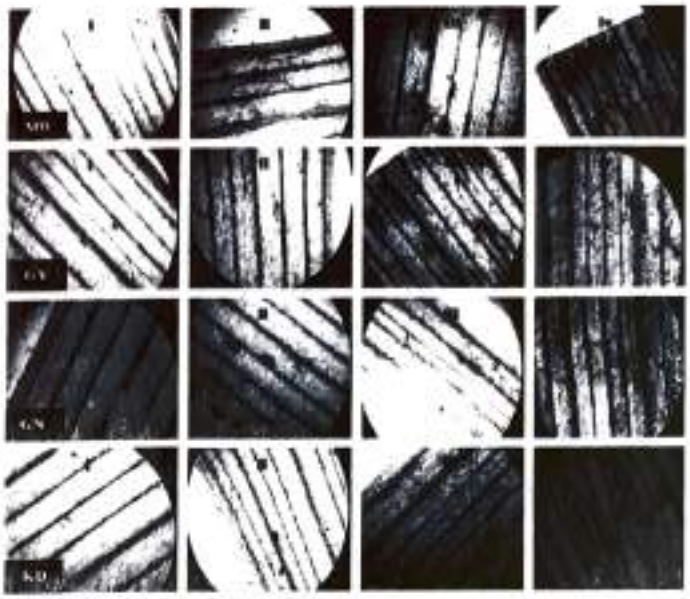

Fig 4. *In situ* detection of H_2O_2 in mid-portions of leaves of four wheat varieties (1st, 2nd, 3rd & 4th row = variety of MW, GY, GN & KD respectively) of wheat following drought, i-0d, ii-3d, iii-6d & iv-9d

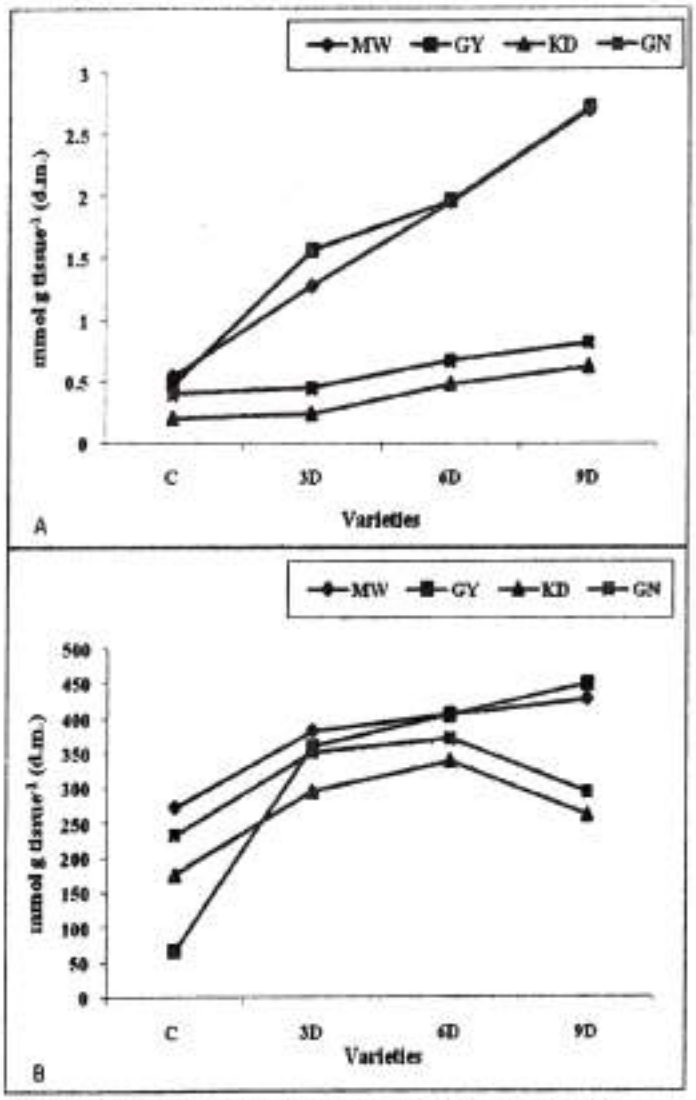

Fig 5. Effect of water stress on the accumulation of MDA (A) and H_2O_2 (B) in four varieties of wheat C= 0 day treatment

stability expressed as percent relative injury was lower in case of varieties which were susceptible and the tolerant varieties showed higher percentage (Table 2). In an earler study, Nair *et al.* (2008) reported that ascorbic acid contents in cowpea decreased with severity of water

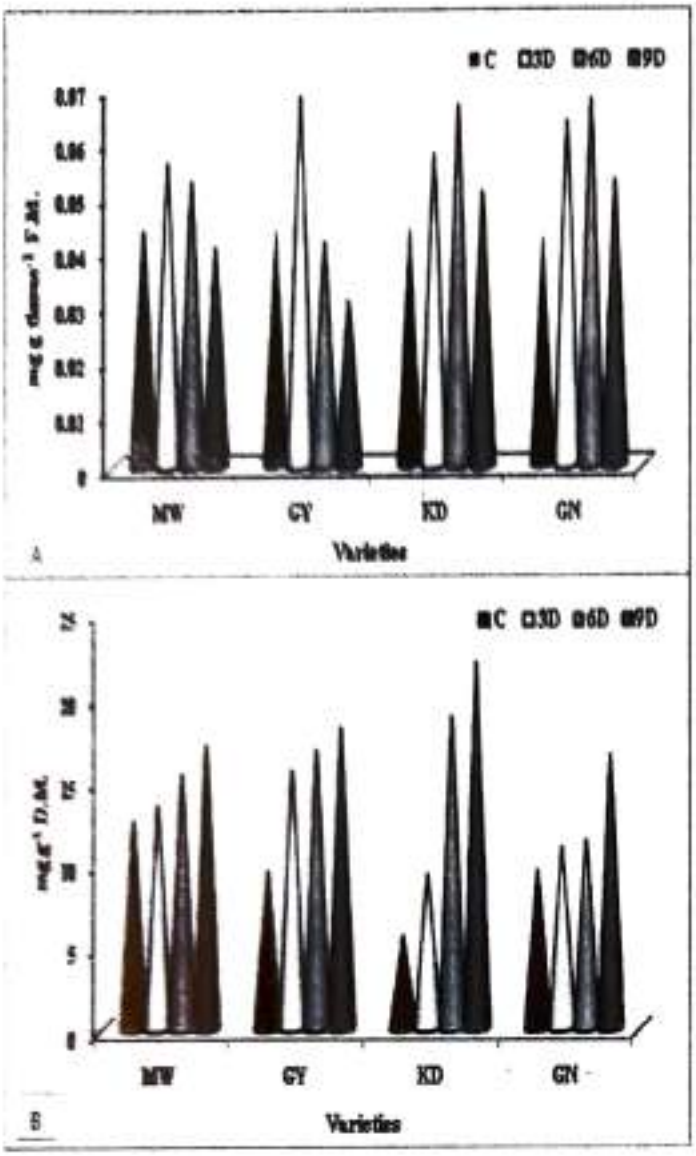

Fig 6. Carotenoid (A) and ascorbate (B) content in the leaves of four wheat varieties subjected to water stress. Means ± S.E., n=10, C= 0 day treatment

stress but tolerant cultivars had higher ascorbic acid contents during severe stress in comparison to susceptible cultivars. Jaleel (2009) reported enhanced accumulation of ascorbic acid during water stress in *Withania somnifera*. L-ascorbic acid is a strong antioxidant but also performs several other functions in the plant (Noctor and Foyer, 1998). In the present study, increase in ascorbate, along with glutathione reductase, indicates the involvement of ascorbate-glutathione cycle as a predominant mechanism of oxidative stress detoxification.

Results of the present study clearly indicate that while water stress induced oxidative damage in wheat varieties as evidenced by decrease in RWC, increased lipid peroxidation, accumulation of H_2O_2, antioxidative mechanisms including enhanced activities of antioxidative enzymes, accumulation of other antioxidants. Antioxidative mechanisms were much more pronounced in two of the varieties- Kedar (KD) and Gandhari (GN), and hence, these were protected from oxidative damage to a great extent. Taking into consideration all the available data, it is concluded that while Kedar and Gandhari could be considered as tolerant, Mohan Wonder and Gayetri were susceptible to water stress.

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