



Full Length Article

Evaluation of Oxidative Stress Tolerance in Two Wheat (*Triticum aestivum*) Cultivars in Response to Drought

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ABSTRACT

Acclimation of plants to mild or sub-lethal stress condition leads to development of resistance to severe or lethal stress condition. Present study was conducted to evaluate the role of oxidative stress management in two wheat (*Triticum aestivum* L.) cultivars; Veery (drought resistant) and Sids (drought susceptible). The activities of superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT), as well as the levels of ascorbate pool (ASA) and hydrogen peroxide (H₂O₂) were studied after exposure to drought stress phases interrupted by rewatering period (drought acclimation). Drought stress induced oxidative stress for plants which exhibited high H₂O₂ and oxidized ascorbate levels in relation with the prolonged drought period. Also, weak antioxidant enzymes response leading to enhanced membrane damage during severe drought stress, indicated by the accumulation of malondialdehyde (MDA). The drought acclimated leaves exhibited systematic increase in the activity of H₂O₂ scavenging enzymes particularly APX and CAT and maintenance of ascorbate redox pool by efficient function of APX enzyme. As a result, lower membrane injury indicated by lower MDA content was observed in drought acclimated plants. Results showed the ability of wheat plants to acclimate and induce the antioxidant defense system under drought stress.

Key Words: Antioxidant enzymes; Ecophysiological studies; Drought; Ascorbic acid; Wheat; Oxidative stress

INTRODUCTION

With progressive global climate change and increasing shortage of water resources and worsening eco-environment, wheat production is influenced greatly (Vasil, 2003). Anti-oxidative performance in higher plants has been thought to be one of the main physiological mechanisms and be central in responses to soil water deficits (Jiang & Zhang, 2004). The imposition of biotic and abiotic stress conditions can give rise to excess concentrations of active oxygen species (AOS), resulting in oxidative damage at cellular level. AOS are by-products of aerobic metabolism and their production is enhanced during drought conditions through the disruption of electron transport system (Asada, 1999; Van Breusegem *et al.*, 2001). Therefore, a consequence of drought is the limitation of photosynthesis and usually accompanied with the formation of (AOS) in chloroplasts such as the superoxide radical (O₂⁻), hydrogen peroxide (H₂O₂) and the hydroxyl radical (OH⁻) (Foyer *et al.*, 1994; Asada, 1997).

Survival under this stressful condition depends on the plant's ability to perceive the stimulus, generate and transmit the signals and initiate various physiological and chemical changes (Shao *et al.*, 2005). The antioxidant defenses appear to provide crucial protection against oxidative damage in cellular membranes and organelles in plants grown under un-favorable conditions (Kocsy *et al.*, 1996). Thus, plants

are equipped with complex and a highly efficient antioxidative defense system, which can respond and adapt to drought stress, composed of protective non-enzymatic and enzymatic protection mechanisms function to interrupt the cascades of un-controlled oxidation in some organelles (Noctor & Foyer, 1998) and serve to maintain the antioxidants in their reduced functional state, that efficiently scavenge AOS and prevent damaging effects of free radicals (Schwanz *et al.*, 1996; Shalata & Tal, 1998).

Acclimation of plants to drought is considered to promote antioxidants defense systems to face the increased levels of activated oxygen species (AOS), which in turn, cause membrane damage by lipid peroxidation and indicated by malondialdehyde (MDA) content, which is one main parameter for evaluating membrane oxidation extent and are toxic for the cells (Chaves *et al.*, 2003; Shao *et al.*, 2005).

Antioxidative enzymes (e.g., superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and ascorbic peroxidases (APX, EC 1.11.1.11) have been related with water deficiency and are considered the main components of anti-oxidative machinery for drought resistance in higher plants (Gogorcena *et al.*, 1995; Bergmann *et al.*, 1999). Detoxification of AOS involves dismutation of superoxide radicals by SOD to hydrogen peroxide, a partially reduced AOS (Bowler *et al.*, 1992) and (Mascher *et al.*, 2002).

Hydrogen peroxide is commonly taken as an indicator of oxidative stress, because it is induced by AOS and also influencing the level of lipid peroxidation (Mittler, 2002). The enzymes catalase (CAT) and ascorbic peroxidases (APX) detoxify the cellular hydrogen peroxide (Bowler *et al.*, 1992) and (Fridovich, 1983). Furthermore, antioxidant isoenzymes particularly CAT and APX isoenzymes play an important role in eliminating H_2O_2 and are distributed in distinct cell compartments (Ishikawa *et al.*, 1998; Caldwell *et al.*, 1998). The antioxidant non-enzymatic system includes ascorbate and glutathione, two constituents of the antioxidative ascorbate–glutathione cycle which detoxify H_2O_2 in the chloroplasts (Asada, 1999) and are located both within the cell in the apoplast (Horemans *et al.*, 2000). Ascorbic acid (ASA) is an essential component of the cellular antioxidative defense system, which keeps active oxygen species (AOS) under control and functions as a reductant for many free radicals, thereby minimizing the damage caused by oxidative stress (Noctor & Foyer, 1998). Moreover, ASA involved in other functions such as plant growth, gene regulation, modulation of some enzymes and redox regulation of membrane-bound antioxidant compounds (Horemans *et al.*, 2000). Erdei *et al.* (2002) analyzed the physiological responses of Hungarian wheat cultivars to soil water deficits. They found that drought resistance in different cultivars was significantly different ($p \leq 0.05$). The best option for crop production, yield improvement and yield stability under soil moisture deficient conditions is to develop drought tolerant crop varieties. A physiological approach would be the most attractive way to develop new varieties rapidly (Klein *et al.*, 2001). Only few studies highlighted the importance of antioxidant enzymes during drought stress. In this investigation two wheat cultivars differing in their sensitivity to drought were examined after two drought periods interrupted by rehydration period to determine the changes in some antioxidant enzymes such as SOD, CAT and APX and their roles in scavenging AOS by monitoring their activities. Furthermore, the level of some compounds (ascorbic acid pool, MDA & H_2O_2) have been considered and discussed in an effort to identify the mechanism (s) by which drought stress decreases plant growth.

MATERIALS AND METHODS

Two wheat (*Triticum aestivum* L.) cultivars, namely Veery (drought tolerant) and Sids-1 (drought sensitive) were used in this investigation. Seeds were grown in plastic pots (15 cm diameter x 20 cm height; 40 pots per cultivar) in the growth chamber of Teacher's college, King Saud University, Saudi Arabia. Seeds were soaked in continuously aerated distilled water for 24 h in darkness. At the end of soaking period, twelve seeds were sown in each pot containing 1700 g sterilized sandy soil (70%) and vermiculite (30%) under 16/8 h day/night cycle. Light intensity was $420 \mu\text{mol m}^{-2}\text{s}^{-1}$ at the canopy of plant supplied by a mixture of fluorescent and incandescent lamps and at controlled temperature of 28/26°C

and 55/60 relative humidity and the pots were irrigated by distilled water day after day. After 15 days from sowing, the pots were irrigated with half strength of Hoagland solution only up to twenty eight days then the pots for each cultivar were grouped into two sets. In the first set, control (non-stressed) plants were grown under these conditions throughout the whole experimental period. In the second set, plants were drought pretreated (drought acclimated) by cessation of watering for 8 days. After this drought period, the same plants were re-watered for 48 h and then subjected to second drought period extended for 12 days.

To eliminate the indirect effect of drought on plant development, all measurements were carried out with the leaves at the same developmental stage (developmental control). Sampling was done around midday between 11:00 and 12:00h from control and stressed/rewatered plants for quantifying the H_2O_2 level and antioxidant defense components. Quantification of H_2O_2 and MDA were performed immediately after sampling of the tissue. For the antioxidant metabolites and enzyme assays, the leaves were cut into small pieces, weighed 0.2 g in replicates, frozen in liquid nitrogen and stored at -20°C . Three replicates were maintained for all the measurements.

Oxidative Stress Indexes

Lipid peroxidation. Lipid peroxidation was estimated *in vitro* after the formation of malondialdehyde (MDA), a by-product of lipid peroxidation that reacts with thiobarbituric acid (TBA). Concentration of MDA was estimated according to the method of Dhinds and Matowe (1981). The resulting chromophore absorbs at 535 nm and the concentration was calculated directly using the extinction coefficient of $1.55 \times 10^5 \text{ mM cm}^{-1}$. Ground frozen tissue (0.2 g) was transferred to a screw-capped 1.5 mL eppendorf tube and homogenized following addition of 1 mL of TCA–TBA–HCl reagent [15% (w/v) trichloroacetic acid (TCA), 0.37% (w/v) 2-thiobarbituric acid (TBA), 0.25 M HCl & 0.01% butylated hydroxytoluene]. After homogenization, samples were incubated at 90°C for 30 min in a hot block. Then chilled in ice and centrifuged at $12\,000 \text{ g}$ for 10 min. Absorbance was measured at 535 nm and 600 nm.

Determination of H_2O_2 . Hydrogen peroxide level was measured colorimetrically as described by Mukherjee and Choudhuri (1983). H_2O_2 was extracted by homogenizing 0.5 g of leaf tissue with 3 mL of phosphate buffer (50 mm, pH 6.5). The homogenate was centrifuged at $6,000 \text{ g}$ for 25 min. To determine H_2O_2 levels, 3 mL of extracted solution was mixed with 1 mL of 0.1% titanium sulfate in 20% H_2SO_4 (v/v). The mixture was then centrifuged at $6,000 \text{ g}$ for 15 min. The intensity of the yellow color of the supernatant was measured at 410 nm (Extinction coefficient = $0.28 \mu\text{mol cm}^{-1}$).

Antioxidant Enzymes

Extraction of enzymes. The overall procedure was carried out at 0°C to 4°C . Samples (0.5 g) of leaf tissue, were ground and homogenized in 20 mL ice-cold extraction buffer (100 mm $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ (pH 7.8), 300 mg polyvinyl

pyrrolidone (PVP), 1% (v/v) Triton X-100 and 5 mm ascorbate (Schwanz *et al.*, 1996). The homogenate was centrifuged at $25,000 \times g$ (20 min, 4°C). The supernatant was used for the determination of SOD, CAT and APX.

Enzymes Assay

SOD. (EC 1.15.1.1) activity was measured according to the method of Stewart and Bewley (1980). One unit of SOD activity was the amount of enzyme activity that caused 50% inhibition of the initial rate of the reaction in the absence of enzyme.

CAT. (EC 1.11.1.6) activity was assayed by monitoring the decomposition of H_2O_2 spectrophotometrically at 240 nm (Aebi, 1983). One unit of enzyme activity is equal to 1 μmol of H_2O_2 decomposed per min.

APX. (EC 1.11.1.11) activity was assayed according to Asada (1992). The reaction was initiated by the addition of H_2O_2 . The H_2O_2 dependent oxidation of ascorbate was followed by monitoring the decrease in absorbance at 290 nm due to the oxidation of AsA in the first 30 s from the start of the reaction (extinction coefficient = $2.8 \text{ mm}^{-1} \text{ cm}^{-1}$). One unit of APX was the amount of enzyme that oxidized 1 μmol of ascorbate per min at room temperature.

Ascorbate level. A 0.5 g aliquot of leaves was homogenized in 1.0 mL of ice-cold $HClO_4$ (2.5 N). The homogenate was filtered through three layers of cheesecloth (Miracloth) and then centrifuged at $15,000 \times g$ for 5 min. The supernatant was neutralized with 5 M K_2CO_3 to pH 4.5 for ascorbate determination.

Ascorbic acid was measured spectrophotometrically by reading absorbance at 265 nm due to ascorbate oxidation by ascorbate oxidase, according to Foyer *et al.* (1983). For measurements of total ascorbate (ASA+DHA), 300 μL of neutralized extract were added to 1.2 mL of a reaction mixture containing 20 mM dithiothreitol (DTT) in 50 mM HEPES-KOH, pH 7.0. After incubation for 10 min at 25°C in a water bath, 100 μL of 0.5 M *N*-ethylmaleimide were added to remove DTT. The reaction was started with the addition of five units of ascorbate oxidase. For reduced ascorbate (ASA) determination, 300 μL of neutralized extract were added to 1.2 mL of a reaction mixture containing 150 mM K_3PO_4 , pH 7.4, 5 mM EDTA and five units of ascorbate oxidase. Total ascorbate (AsA) and dehydroascorbate (DHA) contents were analyzed in absorbance at 520 nm. The concentration of dehydroascorbate (DHA) was calculated as the difference between total ascorbate and AsA.

Statistical analysis. The experiment was conducted in completely randomized design. Results are the mean of four measurements per treatment. The significance of difference between means values was determined by one-way analysis of variance. Duncan's multiple range test was used to compare the means of treatments at $P \leq 0.05$.

RESULTS

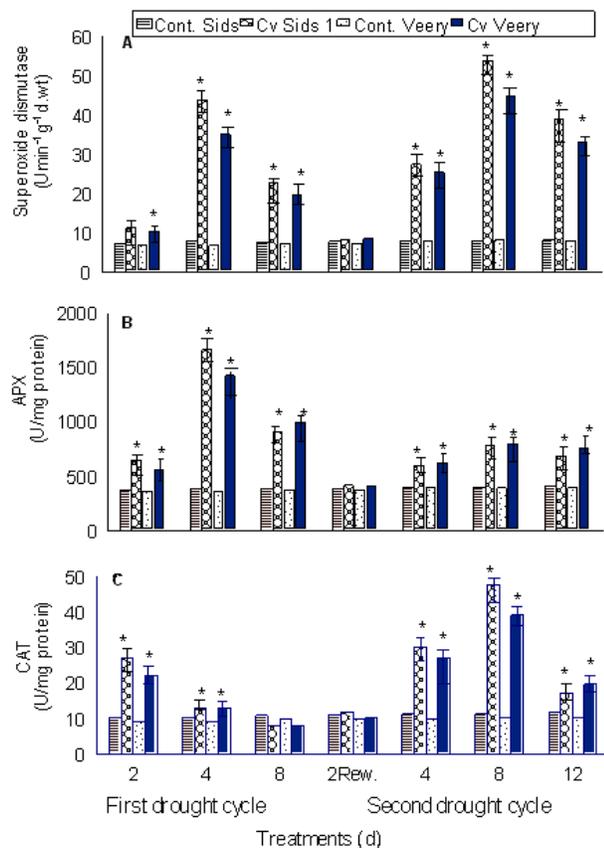
The occurrence of oxidative stress induced by drought stress was monitored by determining oxidative stress

indexes such as H_2O_2 , ascorbate status and by analyzing membrane damage through measurement of MDA levels. During the first drought phase H_2O_2 and MDA were increased progressively in both cultivars relative to control, however slight increase was observed in sensitive cultivar (*cv* Sids-1) more than tolerant one (*cv* Veery). After completion of the first drought phase and in comparison to control, H_2O_2 and MDA contents of *cv* Sids-1 were increased significantly and reached 4.4 and 2.4 times, respectively. The corresponding values for *cv* Veery were 3.8 and 2.3 times, respectively. On the contrary, after 2d rewatering, both cultivars showed high significant reduction in H_2O_2 and MDA contents, which they decreased by approximately 65% and 50%, respectively and compared with those estimated after the completion of first drought phase (Fig. 1). After rewatering and in the fourth day of the second drought phase, slight increase in H_2O_2 and MDA levels occurred in both cultivars relative to control, therefore, H_2O_2 and MDA contents obtained in *cv* Sids-1 wheat leaves were 1.6 and 1.4 times, respectively more than the control. The corresponding values for *cv* Veery were 1.3 and 1.1, respectively compared with control. However, the maximum increase in both H_2O_2 and MDA contents appeared after the completion of the second drought phase treatment, but still significantly lower than the highly significant increase occurred after the completion of the first drought phase. For example, after completion of the second drought phase for 12 d, the contents of H_2O_2 and MDA in *cv* Sids-1 were decreased 19% and 18% less than their contents estimated after 8 d drought stress in non-acclimated plants. The corresponding values for *cv* Veery were 31% and 27%, respectively (Fig. 1).

In well watered seedlings the DHA content was almost a 16% of the reduced form. Whereas after the completion of the first drought phase for *cv* Sids-1 and *cv* Veery, the DHA content increased significantly in sensitive cultivar (*cv* sids) compared with the increase in tolerant cultivar (*cv* Veery) and reached 95% and 159% of the reduced form, respectively. The ASA/DHA ratios decreased significantly and were positively related to first drought phase. After 8 d drought the ratio compared to control were decreased by 87% and 81% in *cv* sids and *cv* veery, respectively. However, after two days rewatering, the ratios of ASA/DHA increased significantly in both cultivars and reached to control. Whereas, during the second drought phase, ASA/DHA ratio increased only after 4 d drought compared to control. Subsequently, both cultivars, showed significant decrease after 8 and 12 d, but still lower than the ratios in non acclimated plants (Fig. 2).

The effects of water stress on the activities of several important antioxidant enzymes such as SOD, CAT and APX in wheat leaves were investigated and the results are shown in Fig. 3. Changes of SOD activity had a similar tendency in both cultivars. SOD activity in the two cultivars increased 1-2 d of the first drought phase, but statistically significant increase activity (3.8 fold) was found only after 4

Fig. 1. Hydrogen peroxide concentration (A) and malondialdehyde concentration (B) of well watered and drought stressed wheat cultivars CV sids (drought sensitive) and CV veery (drought Tolerant) subjected to two drought phases interrupted by 2d rewating. Cont. Sids-1; Control of sensitive cultivar, Cv Sids-1; drought stressed sensitive cultivar, Cont. Veery; Control of tolerant cultivar, Cv Veery; drought stressed tolerant cultivar, 2 Rew; 2 days rewating. Each value represents the mean \pm SE of five replicates. Significant differences ($P < 0.05$) between treatments according to LSD test are shown by an asterisk



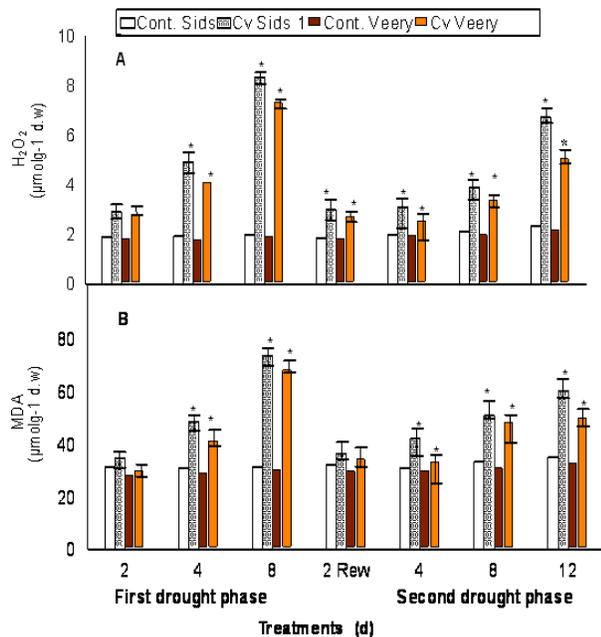
d as compared to control. After completion of the first drought phase, SOD activity was significantly decreased, therefore, for *cv* Sids-1 and *cv* Veery were dropped to 51% and 44% ($P < 0.05$), respectively but still higher than the corresponding control. The first drought stressed leaves exhibited an increased APx activity in both cultivars, the maximum increase in APX activity ($P < 0.05$) appeared after 4 d of the first drought imposition (430%) and the lowest after 2d (170%) compared to control. On the other hand, activity of CAT, in the first drought phase, tended to increase only after 2 d, reached 265% followed by progressive decrease with 4 and 8 d drought, but a statistically significant ($P < 0.05$) decrease as compared to control leaves was observed only after 8 d (27% & 19) for sensitive and tolerant cultivars, respectively (Fig. 3C).

Generally, after rewating, the activities of SOD, APX and CAT were regulated after the 2 d rewating phase and their values were lower than those found in the first drought-stressed plants (Fig. 3). During rewating period the activities of SOD, APX and CAT were significantly decreased in both cultivars, whereas the decrements of activities were more marked in *cv* sids than in *cv* veery, reaching values similar to those found in the corresponding control (Fig. 3). However, after rehydration, exposure of plants to the second drought phase showed a tendency of increase in the activities of these antioxidant enzymes even after 8 d drought in both cultivars. Thereafter, subsequent decrement occurred after 12 d drought stress and more significant in the sensitive cultivar. The decrement after the completion of the second 12 d drought phase were nearly similar to the values obtained after the achievement of the first drought phase in non-acclimated plants. No significant changes in the activities of these enzymes in control were observed during experimental period.

DISCUSSION

The response of resistant and susceptible wheat genotypes to water deficit-induced oxidative stress and antioxidant management, at a particular growth stage has been reported in many literatures (Loggini *et al.*, 1999; Sgherri *et al.*, 2000; Lascano *et al.*, 2001). However their response has not been evaluated to multiple cycles of drought stress with intermittent recovery by rehydration. Drought tolerance of crop plants is a genetically determined character but interaction with environment determines the expression of the plant traits. Lascano *et al.* (2001) observed no clear correlation between water-stress tolerance and antioxidant system behavior between drought-tolerant and susceptible wheat cultivars under field conditions. The present study showed clear difference in the participation of antioxidant defense system in the drought tolerance of wheat (*T. aestivum* L.) cultivars when subjected to drought stress with and without acclimation treatment. The exposure of water deficit of severe nature led to differential H_2O_2 accumulation, membrane damage (MDA) and AOS scavenging response in the acclimated and non-acclimated for both cultivars. After completion of first drought cycle, non-acclimated plants of both cultivars showed enhanced SOD activity without concomitant increase in H_2O_2 scavenging CAT and APX leading to increase in H_2O_2 levels in these plants (Fig. 1 & Fig. 3). In contrast, after two days rewating, acclimated plants showed less increase in H_2O_2 level due to lower induction in SOD and higher APX activity. Differential $O_2^{\bullet-}$ radical generation under drought stress in susceptible and tolerant wheat cultivars has been linked to lipid composition of membrane (Sgherri *et al.*, 1996). Our results suggested that maintenance of favorable water relations in the drought-acclimated plants might have contributed towards the regulation of ROS generation (Selote *et al.*, 2004). There is need to understand the biochemical

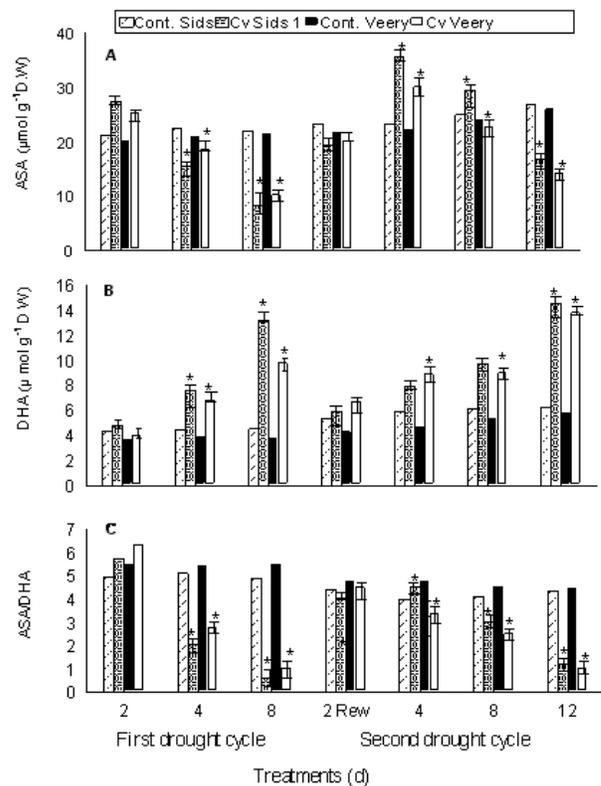
Fig. 2. Changes in ascorbate status, DHA and ASA/DHA ratio in wheat cultivars Cv sids and Cv veery exposed to two drought cycles interrupted by 2d rewatering. Each value represents the mean \pm SE of five replicates. Cont. Sids-1; Control of sensitive cultivar, Cv Sids-1; drought stressed sensitive cultivar, Cont. Veery; Control of tolerant cultivar, Cv Veery; drought stressed tolerant cultivar, 2 Rew; 2 days rewatering. Significant differences ($P < 0.05$) between treatments according to LSD test are shown by an asterisk



and molecular mechanisms underlying the regulation of ROS generation under acclimated conditions. Non-acclimated wheat leaves, for both cultivars, exhibited significantly higher membrane damage due to severe water stress as compared to drought-acclimated plants (Fig. 2). This is due to systematic increase in H₂O₂ levels due to lack of increase in H₂O₂ scavenging APX and CAT. In contrast, drought-acclimated plants showed increase in overall enzyme activities (Fig. 3). We have observed less membrane lipid peroxidation in drought-acclimated as compared to non-acclimated plants in both cultivars. These results are in agreement with data presented by Selote *et al.* (2004).

An increase in ASA and an induction of ascorbate-glutathione cycle enzymes during water stress minimized the oxidative damage, but decrease in AsA content and AsA-GSH cycle enzymes intensified oxidative processes during severe water stress conditions (Sgherri & Navari-Izzo, 1995). Lesser oxidative damage in the tolerant wheat cultivar during osmotic stress has been attributed to higher AsA and induction of AsA-GSH cycle enzymes (Lascano *et al.*, 2001). In the present study, susceptibility of veery leaves during severe water stress was evident from the failure in H₂O₂ management (Fig. 1) and by drastic oxidation of ascorbate-glutathione pool and significant

Fig. 3. Antioxidant enzymes activities SOD (A), APX (B) and CAT (C) of well watered and drought wheat cultivars cv Sids-1 and cv Veery subjected to two drought cycles interrupted by 2d rewatering. Cont. Sids-1; Control of sensitive cultivar, Cv Sids-1; drought stressed sensitive cultivar, Cont. Veery; Control of tolerant cultivar, Cv Veery; drought stressed tolerant cultivar, 2 Rew; 2 days rewatering. Each value represents the mean \pm SE of five replicates. Significant differences ($P < 0.05$) between treatments according to LSD test are shown by an asterisk



reduction in AsA/DHA ratio (Fig. 2). The high H₂O₂ level and/or oxidation of ascorbate pool might have inhibitory effect on antioxidant enzymes particularly APX and CAT in the susceptible sids leaves and in non-acclimated veery leaves (Shigeoka *et al.*, 2002). Drought-tolerant cv. Veery exhibited an excellent recovery capacity than susceptible cv. sids in terms of membrane stability (Fig. 2) due to down-regulation of SOD and up-regulation of H₂O₂ scavenging enzymes (Fig. 1 & Fig. 3) and regeneration of ascorbate pool (Fig. 2). To sum it up, the present data have shown that antioxidant enzymatic response to drought stress activated markedly in *T. aestivum* leaves after rehydration period especially the sensitive cultivar.

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