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ARTICLE

Antioxidant enzyme changes in response to osmotic stress in wheat (*Triticum aestivum* L.) seedling

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ABSTRACT In order to evaluate the effects of osmotic stress on behavioral responses of antioxidant enzymes including superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), glutathione reductase (GR) and ascorbate peroxidase (APX), a factorial experiment was carried out under laboratory conditions with three groups of wheat genotypes (tolerant, intermediate and susceptible) and three osmotic stress levels induced by PEG (control, mild and severe). Electrophoretic analyses were performed for three antioxidant enzymes SOD, POX and CAT in shoots of wheat seedlings using 7.5% slab polyacrylamide gels. The activities of GR and APX were determined spectrophotometrically. For SOD, POX and CAT, two, seven and one isozymes were observed, respectively. Statistical analysis showed that osmotic stress has a significant effect on enzymatic activities in wheat seedlings. POX, CAT, GR and APX activities were increased significantly in the severe stress compared with control condition about 31, 61, 129 and 149 percent, respectively. Whereas, SOD activity increased significantly by 41% in the mild stress compared with control treatment. The highest enzymatic activity was belonged to tolerant group under severe stress conditions for almost all of isozymes and enzymes. Among the antioxidant enzymes, APX activity was increased most drastically in severe stress condition. The extent of damage to the wheat seedlings seems to depend on genotype and severity of osmotic stress.
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KEY WORDS

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The stress factors especially drought, negatively affect plant growth and development and causes a sharp decrease of plants productivity. The limitation in the availability of water induces osmotic stress (Molinari et al. 2007). In certain tolerant crop plants morphological and metabolic changes occur in response to osmotic stress, which contribute towards adaptation to such unavoidable environmental constraints (Sinha et al. 1986). Among crop plants, wheat (*Triticum aestivum* L.), is an attractive study system because of the natural genetic variation in traits related to water deficit tolerance (Loggini et al. 1999). Water deficit stress induces oxidative reactions by producing reactive oxygen species (ROS), which attack the most sensitive biological macromolecules and membranes to impair their function (Foyer et al. 1994; Noctor and Foyer 1998; Mitller 2002). Strategies to minimize oxidative damage are a universal feature of plant defense responses. The plant response to water deficit stress would depend on the species inherent "strategy" as well as on the duration and severity of the stress period.

Mechanisms of ROS detoxification in several plants by enzymatic and non-enzymatic antioxidants are well documented (Dhindsa et al. 1981; Asada and Takahashi 1987; Foyer and Noctor 2000). The measure of specific antioxidant enzyme activities and/or expression analysis during water deficit stress treatments has been generally accepted as an approach to assess the involvement of the scavenging system during water stress (Cruz de Carvalho 2008). However, contradictory results (increment, reduction or remaining unchanged) have been obtained through the much more analysis of plants antioxidant in different conditions. In sunflower seedlings and in grass plants (*Aegilops squarrosa*) a decrease in SOD activity was detected under water deficit stress (Badiani et al. 1990). The reverse was those also found in wheat (Badiani et al. 1990) and rice (Sharma and Dubey 2005) where water stress increased SOD activity. Simova-Stoilova et al. (2010) reported increased CAT activity in wheat under drought stress being higher especially in sensitive varieties. In another study, Sharma and Dubey (2005) reported a decrease in CAT activity in rice seedlings following drought stress. It was shown that APX and/or GR activities were enhanced during water stress in wheat seedlings (Keles and Oncel 2002) and alfalfa (Rubio et al. 2002). A time course measure of APX and GR activities under a mild water stress imposed by a PEG treatment (-0.7 MPa) on maize detached leaves also showed a significant increase in both above mentioned enzyme activities (Jiang and Zhang 2002). In a field study, it was observed that when plants subjected to mild drought stress in the seedling stage,

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Table 1. List and characteristics of studied wheat genotypes belonging to three groups.

No.	Families	Group	Reference
1	Unknown-1	Tolerant	Mohammadi et al. 2010
2	1-27-6149/Sabalan// 84.40023	Tolerant	Mohammadi et al. 2010
3	Ghafghaz//F9.10/Maya"s" IRW92-1-D-474-OMA-OMA-OMA-OMA-IMA-OMA	Tolerant	Mohammadi et al. 2010
4	DARIC95-010-OMA-OMA-OMA-OMA-6MA-OMA	Tolerant	Roostaei 2008; Valizadeh et al. 2012
5	Azarbaijan/Gobostan	Tolerant	Mollasadeghi et al. 2011
6	Azarbaijan/Roozi-84	Tolerant	Mollasadeghi et al. 2011
7	Tous	Tolerant	Mollasadeghi et al. 2011
8	Azar-2	Tolerant	Mohammadi et al. 2010
9	Sardari	Tolerant	Mohammadi et al. 2010
10	DARIC95-010-OMA-OMA-OMA-OMA-8MA-OMA	Intermediate	Roostaei 2008; Valizadeh et al. 2012
11	Manning/Sdv1//Dogu88	Intermediate	Mohammadi et al. 2010
12	RECITL/TIA.2//TRK13	Intermediate	Mohammadi et al. 2010
13	Vrz/3/Orf1.148/Td1/Blo/4/Sabalan	Intermediate	Roostaei 2008; Valizadeh et al. 2012
14	HK16/7/KVZ/T171/3/MAYA//BB/INIA/4/KAR/JCWH99034-OAP- OAP-OAp-OMAR-6MAR	Susceptible	Roostaei 2008; Valizadeh et al. 2012
15	FKG13/4/NWT/3/TAST/SPRW// TCI98-0139-OAP-OAP-OMAR-5MAR	Susceptible	Roostaei 2008; Valizadeh et al. 2012
16	JANZ QT3685-OAUS	Susceptible	Roostaei 2008; Valizadeh et al. 2012
17	RINA-11	Susceptible	Roostaei 2008; Valizadeh et al. 2012
18	Azarbaijan/Saratoveskaya-29	Susceptible	Mollasadeghi et al. 2011
19	Cimmyt/Saysonz	Susceptible	Mollasadeghi et al. 2011

the drought-tolerant wheat cultivar acclimatized better than the drought- susceptible cultivar by maintaining favorable water relations and lower membrane injury due to low H_2O_2 accumulation and antioxidant defense in the leaves under severe water-deficit conditions (Khanna-Chopra and Selote, 2007).

Polyethylene glycol (PEG) compounds have been used to simulate water stress effect in plants (Murillo-Amadaor et al. 2002). PEG of higher molecular weight is considered to cause blockage of the pathway of water movement, reducing water absorption and causing desiccation of the plant (Lawlor 1970). It is envisaged from the above findings that PEG solution can be frequently used in the laboratory for screening drought tolerant genotypes at early stage.

Therefore, the present study aimed to determine the effect of PEG-induced osmotic stress on enzymatic antioxidant systems in tolerant, intermediate and susceptible wheat genotype's seedlings and to evaluate the activity changes in antioxidant enzymes in the three groups of wheat genotypes.

Material and Methods

Plant material and experimental conditions

The experiment was conducted in factorial form, using a completely randomized design with three replications. Three groups of wheat genotypes (9 tolerant, 4 intermediate and 6 susceptible), (Roostaei 2008; Mohammadi et al. 2010; Mollasadeghi et al. 2011) (Table 1) were evaluated under laboratory conditions. Seeds of wheat genotypes were surface sterilized with 0.01% $HgCl_2$ solution for three minutes, followed by washing several times by distilled water. Ten seeds of each genotype then were placed on the moist Whatman germination papers in Petri dishes and were germinated using distilled water for 3 days under control conditions (light/dark regime of 16/8 h at 25/20 ºC, relative humidity of 60-70%, Light intensity during the daytime was 350 μmol m^{-2} s^{-1}). After 3 days, osmotic stress was imposed by application of PEG-6000 (polyethylene glycol) for 5 days. Using the Michel-Kaufmann equation, 139 and 203 g of PEG-6000 was dissolved in 200 ml of distilled water and total volume was raised up to one liter to produce solutions of mild (–0.4MPa) and severe (–0.8MPa) osmotic potential, respectively (Michel and Kaufmann 1973). The activities of five antioxidant enzymes were evaluated on shoots of 8-day-old seedlings in Faculty of Agriculture, University of Tabriz during 2012.

Native polyacrylamide gel electrophoresis

The activities of SOD, POX and CAT were determined in native PAGE (Polyacrylamide gel electrophoresis). The crude extract of fresh and healthy shoots were prepared with separate mortars and pestles in a Tris-HCl extraction buffer pH 7.5 (Tris 50 mM, sucrose 5%, ascorbic acid 50 mM, sodium metabisulfite 20 mM, PEG 2% and 2-mercaptoethanol 0.1%) before use with a ratio of 0.1 gr μl^{-1} (W:V) and centrifuged (Model EBA 12R) at 4 ºC and 10 000 rpm for 10 minutes (Valizadeh et al. 2011). Enzyme extracts were immediately absorbed onto 3×5 mm wicks cut from Whatman 3 mm filter paper and loaded onto 7.5% horizontal slab polyacrylamide gels (0.6×15×12 cm), prepared by Poulik buffer (Soltis and

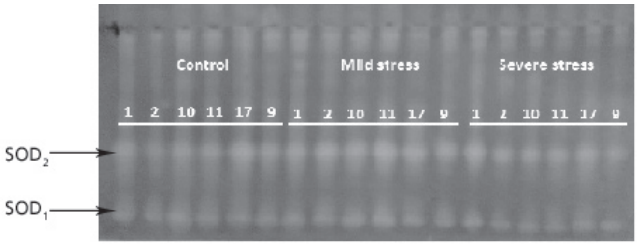

Figure 1. Example of isozyme pattern and relative activity of SOD in the shoots of wheat seedlings for three stress conditions (No.1, 2, 9: tolerant- 10, 11: intermediate and 17: susceptible genotypes).

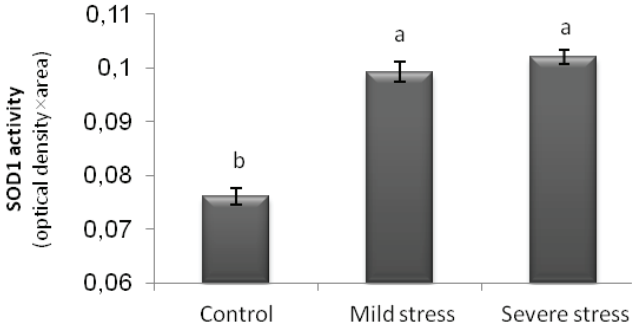

Figure 2. Mean comparison of SOD_1 in three stress conditions.

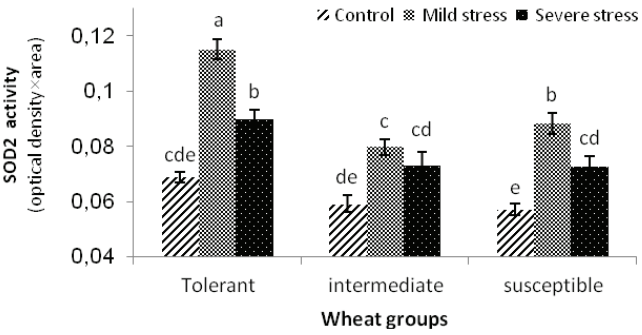

Figure 3. Treatment combination means of wheat groups and PEG-mediated osmotic stress for SOD_2.

Soltis 1990) using TBE (Tris-Borate-EDTA) electrode buffer (pH 8.8). Electrophoresis was carried out at 4 °C for 3 hours (constant current of 30 mA, and voltage of about 180 V). For each genotype, analysis was repeated three times, each time from bulked material of at least five seedlings. After electrophoresis, two slices of slab gel were prepared. The staining protocol for SOD and CAT was performed according to Soltis and Soltis (1990) and POX according to Olson and Varner (1993). The gels were fixed and scanned immediately after staining. An image analysis program (MCID Analysis Evaluation 0.7) was used to measure D×A (optical density × area) parameter for each isozyme band to evaluate the enzymatic activity.

Spectrophotometer analysis

The activities of GR and APX were determined spectrophotometrically (Model RAY LEIGH UV-2601). GR activity was determined by measuring the reduction kinetics of oxidized glutathione (O'Kane et al. 1996). APX activity was determined following the oxidation of ascorbate to dehydroascorbate, as described by Nakano and Asada (1981).

Protein determination

The protein contents of the enzyme extracts were determined by Bradford (1979) method using bovine serum albumin (BSA) as a standard.

Statistical analysis

Data were analyzed using the general linear model procedure in SAS program (SAS Institute, Cary, USA). The assumptions of variance analysis were tested by ensuring that the residuals were random and homogenous, with a normal distribution. Enzymatic activity means were compared by LSD and SNK using the SAS program.

Results and Discussion

Assessment of electropherograms for SOD, CAT and POX in 19 wheat genotypes displayed two, one and seven isozymes, respectively. Analysis of variances for 10 above mentioned isozymes activities and two antioxidant enzymes, including GR and APX, studied spectrophotometrically, showed that the osmotic stress has a significant effect on enzymatic activities in wheat seedlings. But, the differences between three groups of wheat (susceptible, tolerant and intermediate) were significant only for one SOD (SOD_2) and two POXs (POX_1 and POX_7). Stress × wheat groups interactions were significant for all enzymes except one SOD (SOD_2) and one POX (POX_2) isozymes (variance analysis not shown).

Superoxide dismutase

The specific SOD activity was increased in the shoots of wheat seedlings under stress conditions. Increase in enzyme activity coincided with a variable increment in the individual isoform expression. Two isozymes (SOD 1 and 2) were detected in the shoot with SOD_2 being the major one (Fig. 1). Mean comparison of SOD_1 in three levels of stress conditions is presented in Figure 2. SOD_1 activity was significantly increased in both mild and severe stress conditions, having non-significant stress wheat group interaction. The SOD_2 expression showed a concomitant increase with the total SOD activity. The stress × wheat group interaction was significant for SOD_2 (Fig. 3). A significant difference between severe and mild stress was observed in tolerant and susceptible groups

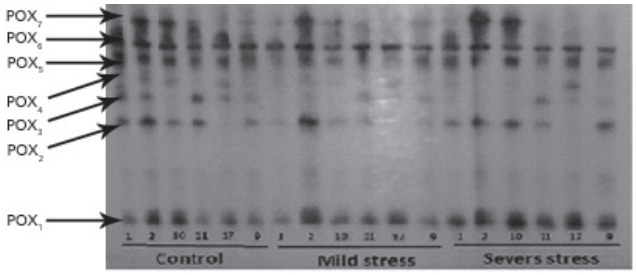

Figure 4. Example of isozyme pattern and relative activity of POX in the shoots of wheat seedlings for three stress conditions (No.1, 2, 9: tolerant- 10, 11: intermediate and 17: susceptible genotypes).

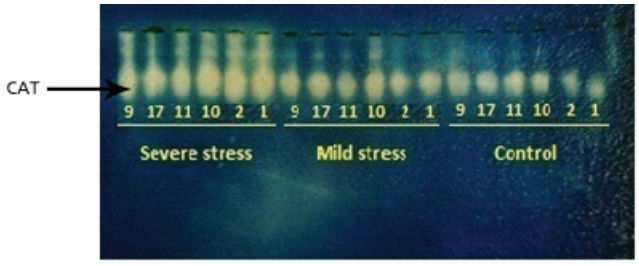

Figure 6. Example of enzyme pattern and relative activity of CAT in the shoots of wheat seedlings for three drought conditions (No.1, 2, 9: tolerant- 10, 11: intermediate and 17: susceptible genotypes).

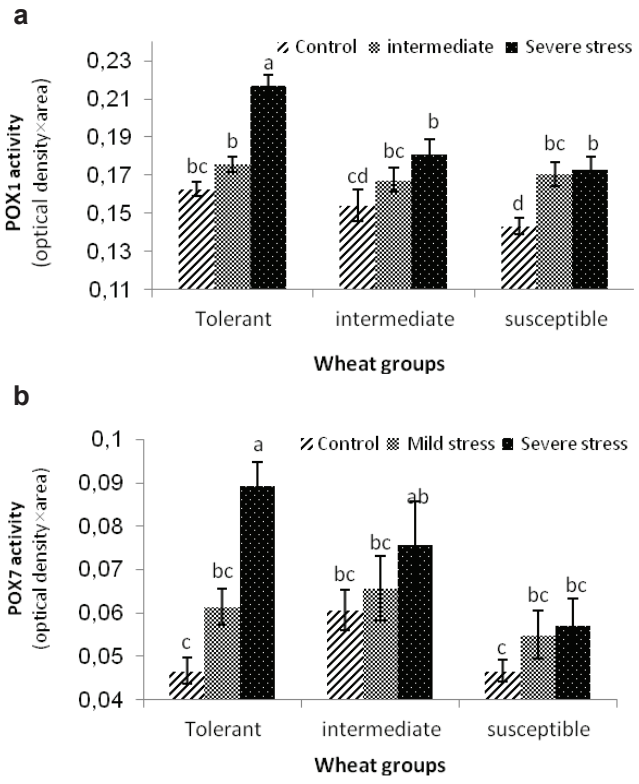

Figure 5. Treatment combination means of wheat groups and PEG-mediated osmotic stress for POX_1 (a) and POX_7 (b).

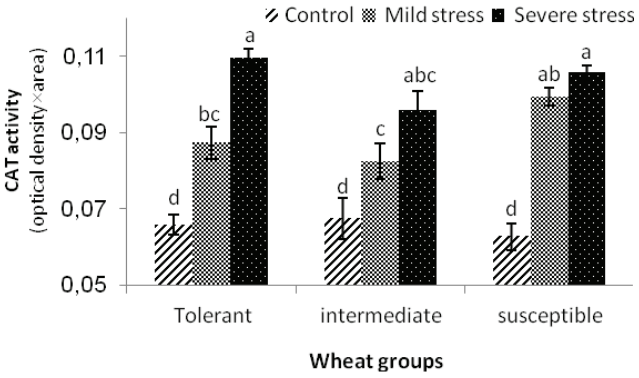

Figure 7. Treatment combination means of wheat groups and PEG-mediated osmotic stress for CAT activity.

for SOD_2, whereas in intermediate group there was no significant difference and the maximum activity was obtained in tolerant group at mild (-0.4 MPa) stress.

Interestingly, mild water stress resulted in a maximum and significant up-regulation of SOD_2 in both tolerant and susceptible groups compared with intermediate group (Fig. 1 and 3). The ability of plants to overcome oxidative stress partly relies on the induction of SOD activity and subsequently on the up-regulation of other downstream antioxidant enzymes (Alscher et al. 2002). According to this fact that SOD processing is known to be substrate inducible) Tsang et al. 1991), an increment in the SOD activity may be attributed to the increased production of the superoxide ($O_2^{\bullet-}$) as substrate that lead to induced expression of genes encoding SOD. In sunflower seedlings a decrease in SOD activity was detected under water stress (Badiani et al. 1990). The reverse was true in wheat (Badiani et al. 1990; Bakalova et al. 2004) and rice (Sharma and Dubey 2005) where water stress increased SOD activity. Our results are consistent with Badiani et al. (1990) and Bakalova et al. (2004). Higher SOD activity in tolerant group compared with susceptible and intermediate groups can also be explained by less efficiency susceptible group in scavenging of $O_2^{\bullet-}$ under severe stress conditions.

Peroxidase

Seven isozymes were detected (Fig. 4) in the shoots of wheat seedlings with POX_1 and POX_7 being the major ones, having significantly different activities between wheat groups. Differences of genotypes within group and stress × wheat group interaction were significant for all POX isozymes except for POX_2 (data not shown). The highest enzymatic activity increment was belonged to tolerant group of wheat under severe

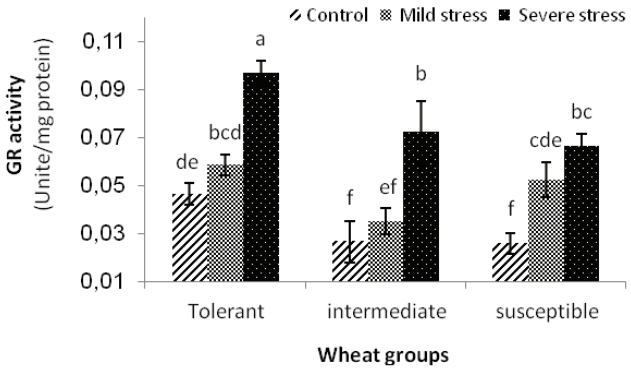

Figure 8. Treatment combination means of wheat groups and PEG-mediated osmotic stress for GR activity.

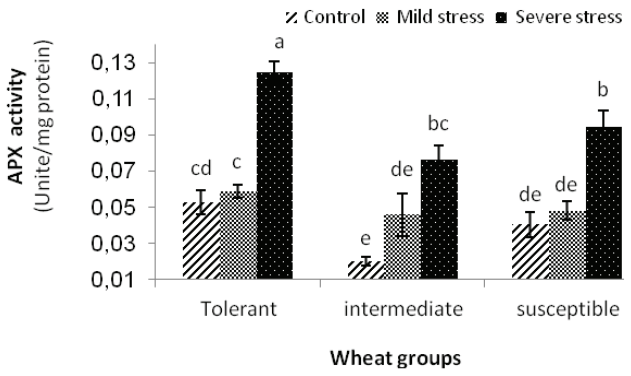

Figure 9. Treatment combination means of wheat groups and PEG-mediated osmotic stress for APX activity.

stress conditions (-0.8 MPa) for most of POX isozymes, especially for POX_1 and POX_7 (Fig. 5a, b).

POX, APX and CAT are three important H_2O_2 scavenging antioxidant enzymes functioning in different sub cellular compartments (Noctor and Foyer 1998). Increase in POX activity in leaves of drought tolerant and susceptible wheat cultivars has also been reported earlier under water deficit stress. Sairam and Saxena (2000) reported that tolerant genotypes, with highest peroxidase activity, had the lowest lipid peroxidation and highest membrane stability under water stress at different stages after anthesis, while the susceptible genotypes exhibited the lowest antioxidant enzyme activity, membrane stability and the highest lipid peroxidation.

Acar et al. (2001) reported an increase in POX activity in tolerant barely variety but non changed activity in susceptible one. Shao et al. (2005) observed variation in peroxidase activity in wheat genotypes under soil water deficits at maturation stage and suggested that water stress tolerance was closely associated with POX activities. An increase in POX activity in drought tolerant as well as susceptible maize genotypes at seedling stage under 72 h drought stress has been reported by Chugh et al. (2011). In a review applied at seedling stage of wheat cultivars, antioxidant enzymes activities were increased with the decrease of osmotic potential in both tolerant and sensitive cultivars. The tolerant cultivar exhibited a higher antioxidant activity compared to the sensitive one (Valifard et al. 2012), supporting our results in the present study.

Catalase

A single band of CAT was detected in the shoots of wheat seedlings upon native PAGE separation. In severe stress condition, CAT showed higher activity as compared with mild stress and control condition (Fig. 6). Stress × wheat group and stress × genotype within group interaction for CAT were also significant (data not shown). A significant difference was observed between severe and mild stress in tolerant wheat group alone (Fig. 7).

Reports on catalase activity under stress condition are heterogeneous. CAT activity has been shown to increase in maize (Kolarovic et al. 2009), wheat (Luna et al. 2004) and also to remain unchanged or even decrease under water stress in sunflower (Zhang and Kirkham 1992). Luna et al. (2004) reported leaf H_2O_2 content increased even though total CAT activity doubled in wheat seedling under severe stress conditions. Our results are consistent with works reporting the increased CAT activity in response to osmotic stress in wheat seedling.

Glutathione reductase

Effect of water deficit stress was significant on GR activity, measured spectrophotometrically. Stress × wheat group interactions and genotype within group were significant. Figure 8 indicates the mean GR activity values, for treatment combinations. The highest and significant GR activity was obtained for tolerant wheats in severe stress conditions.

Several authors have reported increased activity of GR in rice seedlings (Sharma and Dubey 2005) and alfalfa (Rubio et al. 2002) under environmental stresses. Lascano et al. (2001) reported an increase in glutathione reductase (GR) activities in the tolerant wheat cultivars and a higher decline in reduced glutathione (GSH), ascorbate content and less oxidative damage than in the susceptible cultivar.

Ascorbate peroxidase

APX activity showed significant difference for osmotic stress, genotype within group conditions, stress × wheat group and stress × genotype within group interactions. In spite of genotype differences within groups (data are not shown), the interaction effect between stress and groups of wheat

revealed that (Fig. 9), the tolerant group of wheats under severe stress displays a substantially increased activity for APX as for GR.

Khanna-Chopra and Selote (2007) reported that drought tolerant wheat cultivars had the highest APX activity during severe water deficit stress in post-anthesis period as our finding for wheat seedling. Al-Ghamdi (2009) reported that drought acclimated (by cessation of watering for 8 days) wheat seedling exhibited systematic increase in the activity of H_2O_2 scavenging enzymes, particularly APX and CAT and maintenance of ascorbate redox pool by efficient function of APX enzyme.

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