Anti-oxidative response of different wheat genotypes to drought during anthesis

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Abstract

The aim of this work was to study drought effects on lipid peroxidation, antioxidant compounds, and anti-oxidative enzymes activities in two tolerant (Daric and 92 Zhong), two moderately tolerant (Sabalan and DH-2049-3) and two sensitive (Shark and Tevees) wheat genotypes. Malondialdehyde content and electrolyte leakage increased significantly ($P<0.05$) in flag leaves under drought. The increase was higher in sensitive genotypes compared to the others. Protective enzymes activities (ascorbate peroxidase, peroxidase, superoxide dismutase, catalase and glutathione reductase) and non- enzymatic antioxidants contents (ascorbate and glutathione) increased under drought stress and this increase was higher in tolerant genotypes. There were significant positive correlations ($P<0.05$) between anti-oxidative enzyme activities and non- enzymatic antioxidants. It seems that peroxidase and superoxide dismutase - that showed higher increase under stress- had an important role in drought tolerance.

Key words: anti-oxidative enzymes; abiotic stress; antioxidants; free radicals; tolerance

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Introduction

Environmental stresses especially drought have negative effects on plant growth, development, and productivity. The limitation in water availability induces osmotic stress (Naderi *et al*., 2014). Wheat (*Triticum aestivum* L.) is an attractive plant because of natural genetic variation related to drought tolerance. Wheat is a main food for more than 35% of the world people and it is the first grain crop in Iran (Mohammadi *et al*., 2006). Wheat often experiences drought stress conditions during crop cycle. Therefore, improvement of wheat produce for drought tolerance is one of the major aims in plant breeding programs for arid and semi-arid regions (Ahmadizadeh *et al*., 2011).

Acclimatization of plants to drought induces antioxidants defense systems to confront the high levels of reactive oxygen species (ROS) that causes membrane damage by lipid peroxidation. Malondialdehyde (MDA) content is an indicator of lipid peroxidation and could reflect the degree of damage at stress conditions (Weng *et al*., 2015).

These adverse conditions increase the rate of reactive oxygen species (ROS) production. Plants generate very effective enzymatic (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; peroxidase, POX and glutathione reductase, GR) and non-enzymatic (ascorbic acid and glutathione) antioxidant defense systems which protect cells against

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oxidative damage by scavenging ROS. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide to oxygen and hydrogen peroxide. H_2O_2 can be eliminated by CAT, APX, and POX (Hasheminasab *et al*., 2012).

Drought stress increased SOD activity in wheat (Naderi *et al*., 2014) and rice (Sharma and Dubey, 2005). Grzesiak *et al*. (2013) reported that increase of GR activity in wheat plants under drought stress was higher in sensitive varieties. It was shown that APX and CAT activities were increased during water stress in wheat seedlings (Hasheminasab *et al*., 2012).

In this study six wheat genotypes were evaluated from the view point of antioxidant system response to drought stress. Drought-induced alternations in six wheat genotypes, lipid peroxidation level, antioxidant compounds and enzymes activities under drought stress after pollination were studied. Considering the importance of reproductive stage for wheat, the pollination period was chosen for applying drought stress and our results were obtained in reproductive period. Previous studies on wheat have been conducted usually in vegetative phase. Also our wheat plants were lines and we studied them for the first time in reproductive phase.

Materials and Methods

Growth conditions

Wheat (*Triticum aestivum* L.) genotypes SHARK-4-0YC-0YC-0YC-5YC-0YC and TEVEE'S'//CROW/VEE'S (sensitive to drought stress), Sabalan and DH-2049-3 (moderately tolerant to drought stress), DARIC 98-95 and 92 ZHONG257 (tolerant to drought stress) were grown in the field of Agricultural Dryland Research Station, Maragheh, Iran. Experiment was done for two years. The seeds were sown in 5 rows with 20 cm row spacing and interplant space of 10 cm adjusting seeding rate of 200 seeds m^{-2}. Soil texture was clay loam with electrical conductivity (EC) 1.4 $ds.m^{-1}$, pH 7.5 and sodium adsorption ratio (SAR) of 1.32. Fertilizer was applied at the rate of 110: 65: 60 kg ha^{-1} N: P: K as a split dose, first at 20 days after sowing (DAS) at the rate of 60: 65: 60 kg ha^{-1} N: P: K and second at 85 DAS at the rate of 50:0:0 kg ha^{-1} N: P: K.

Plants were watered when required to keep them fully turgid. The experiment was designed as a Randomized Complete Block with three replicates. Drought treatments were applied by maintaining soil moisture at 50% of field capacity by weighting. The water stress was applied at 7, 17, and 27 days after pollination for a uniform period of 18 days at each treatment. Samples for various assays were taken from flag leaves between 10:30 and 11:30 h at the end of each treatment period. Anthesis in each variety was considered to have occurred when approximately 50 % of main shoot ears showed anther dehiscence (Sairam and Srivastava, 2001). However, the duration of anthesis was shortened by 4–5 days due to drought stress in all six genotypes. The average annual temperature was 14.5 °C and the mean absolute temperature was 37 °C in July and -25.5 °C in January. Total rainfall received by wheat plants from sowing to physiological maturity was 2.2 cm. Rainfall was meager between anthesis and post-anthesis periods.

Measurement of lipid peroxidation and electrolytes leakage

The level of membrane damage was determined by measuring malondialdehyde (MDA) by De Vos *et al.* (1991) method. Briefly, leaf samples were homogenized in 10% (w/v) TCA. The homogenate was centrifuged at 10,000 *g* for 15 min. 20 % TCA containing 0.5 % thiobarbituric acid (TBA) was added to the supernatant and the mixture was heated at 95 °C in a water bath for 25 min, followed by cooling before centrifugation. MDA content was determined from the absorbance at 532 nm, followed by a correction for the non-specific absorbance at 600 nm.

Membrane integrity was determined by Nunes and Smith (2003) method. Electrolyte leakage of leaf segments floating on distilled water for 24 h at 4 °C measured using a conductivity meter (Model Kent EIL 5007), and expressed in percentage of the total leaf electrolyte content obtained after boiling the segments.

Antioxidant enzyme assays

Ascorbate peroxidase (APX) was assayed by Nakano and Asada (1981) method. Reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 0.5 mM ascorbic acid, 0.1 mM EDTA, 1.5 mM H_2O_2 and 0.1 ml enzyme extract. The reaction was started with the addition of H_2O_2. Absorbance was measured at 290 nm for 3 min.

Peroxidase (POX) activity was determined following the method of Ghanati *et al.* (2002). The POX activity was assayed by the oxidation of guaiacol in the presence of H_2O_2; the increase in absorbance was recorded at 470 nm. The reaction mixture contained 100 µl crude enzyme extract, 5 mM H_2O_2, 28 mM guaiacol and 60 mM potassium phosphate buffer (pH 6.1).

The GR activity was determined as described by Barata *et al.* (2000) in a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM 5,5'-dithiobis (2-nitrobenzoic acid), 1 mM oxidized glutathione and 0.1 mM NADPH. The reaction was initiated by the addition of 50 µl of plant extract. The rate of reduction of oxidized glutathione was followed by monitoring the increase in absorbance at 412 nm over 2 min.

Superoxide dismutase (SOD) activity was assayed according to the method of Giannopolitis and Ries (1977). The reaction mixture contained 1 µM riboflavin, 12 mM L–methionine, 0.1 mM EDTA (pH 7.8), 50 mM Na_2CO_3 (pH 10.2), and 75 µM nitroblue tetrazolium (NBT) in 25 mM sodium phosphate buffer (pH 6.8), with 200 µl crude enzyme extract added to a final volume of 3 ml. SOD activity was assayed by measuring the ability of the enzyme extract to inhibit the photo reduction of NBT. Glass test tubes containing the mixture were illuminated with a fluorescent lamp (120 W); identical tubes that were not illuminated served as blanks. After illumination for 15 min, the absorbance was measured at 560 nm. One unit of SOD was defined as the amount of enzyme activity that was able to inhibit the photoreduction of NBT to blue formazan by 50 %.

Catalase (CAT) activity was assayed by the method of Cakmak and Horst (1991). Tissues (0.5 g of frozen leaves) were homogenized using ice–cold extraction buffer (25 mM sodium phosphate, pH 7.8). The homogenate was centrifuged at 18,000 *g* for 30 min at 4 °C. The reaction mixture contained enzyme extract, 10 mM H_2O_2, and 25 mM sodium phosphate buffer. The decrease in the absorbance at 240 nm was recorded for 1 min by spectrophotometry.

H_2O_2 assay

Hydrogen peroxide was assayed with titanium reagent (Teranishi *et al*., 1974). Sample preparations were determined as described by Mukherjee and Choudhuri (1983).

Non enzymatic antioxidants determination

Ascorbic acid was assayed according to the method of Luwe *et al*. (1993). The samples were extracted with 5% metaphosphoric acid. The homogenate was centrifuged at 10,000 g at 2 °C for 10 min. Ascorbic acid (AA) was determined in a reaction mixture containing supernatant and 0.1 M phosphate buffer (pH 6.8) following the decrease of absorbance at 265 nm after addition of 1 U of purified ascorbate oxidase (EC 1.10.3.3).

The glutathione (GSH) content was measured as described by Griffith and Meister (1979). Fresh leaves were homogenized in 2 % metaphosphoric acid and centrifuged at 17,000 *g* for 10 min. The addition of 10% sodium citrate neutralized the supernatant. Assay mixture was prepared by adding extract, distilled water, 6 mM 5,5–dithio–bis–(2–nitrobenzoic acid) (DTNB), and 0.3 mM NADPH. The mixture was stabilized at 25° C for 3–4 min. Then, 10 µl of glutathione reductase was added, and the absorbance was measured at 412 nm.

Statistical analysis

Statistical analyses were done using the SPSS (Version 19.0). The mean values of three replicates and standard error were calculated. Duncan's multiple range tests ($P<0.05$) and GLM (General Linear Model) was performed to determine the significance of the results. Correlations between different factors were calculated.

Table 1
Analysis of variance (mean square) of antioxidants for the studied genotypes

	MDA	EL	APX	POX	GR	SOD	CAT	H_2O_2	AA	
Year	0.048	82.73	0.06*	0.018	67.14*	0.168	77.95*	4.02*	21.16	13.55
Treatment	35.43*	21807.96*	0.79*	53.92*	1981.03*	189.19*	2300.41*	102.14*	9137.54*	3898.4*
Year*Treatment	0.002	3.56	0.001	0.002	2.09	0.003	47.01*	0.106	0.719	1.61

* indicates significant level at P<0.05 according to Duncan's test.

Table 2
Pearson correlations coefficients between studied traits in wheat genotypes under drought stress

	MDA	EL	APX	POX	GR	SOD	CAT	H_2O_2	AA	GSH
MDA	1									
EL	0.470**	1								
APX	-0.353**	0.204**	1							
POX	-0.105	0.405**	0.907**	1						
GR	-0.378**	0.255**	0.962**	0.898**	1					
SOD	-0.256**	0.246**	0.912**	0.885**	0.880**	1				
CAT	-0.112	0.392**	0.846**	0.889**	0.828**	0.838**	1			
H_2O_2	0.860**	0.598**	-0.225**	0.083	-0.205**	-0.126*	0.073	1		
AA	-0.099	0.459**	0.875**	0.964**	0.888**	0.834**	0.885**	0.112	1	
GSH	-0.325**	0.261**	0.963**	0.925**	0.977**	0.901**	0.869**	-0.135*	0.907**	1

* and **: Significant at the 0.05 and 0.01 probability levels, respectively; ns: non-significant according to Duncan's test

Results

MDA content and electrolyte leakage

MDA content and electrolyte leakage increased under drought conditions compared to control (Fig. I); this increase was higher in sensitive genotypes (Shark and Tevees) compared to moderately sensitive (Sabalan and DH-2049-3) and also in moderately sensitive ones compared to tolerant genotypes (Daric and 92 Zhong). Electrolyte leakage was higher 17 days after pollination than others. The difference in MDA content and electrolyte leakage among treatments (steps after drought stress) was significant (P<0.05), but the effects of year and treatment × year was not significant (Table 1).

Enzymatic antioxidants activity

Our results showed that antioxidative enzymes activity (ascorbate peroxidase, peroxidase, glutathione reductase, and superoxide dismutase) increased in flag leaves of all genotypes under drought stress (Figs. II and III), so that the tolerant genotypes (Daric and 92 Zhong) had a higher activity compared to the moderately sensitive genotypes (sabalan and DH-

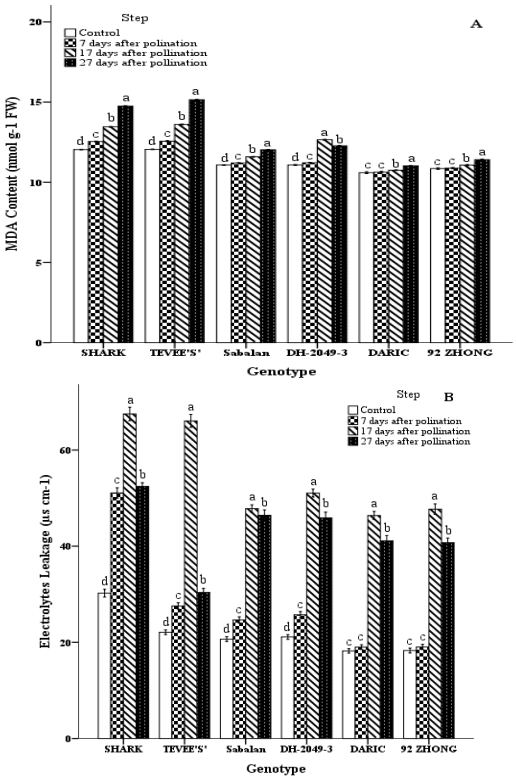

Fig. I. MDA content (A) and electrolytes leakage (B) in flag leaves of six wheat genotypes [SHARK-4-0YC-0YC-0YC-5YC-0YC, TEVEE'S'//CROW/VEE'S, Sabalan, DH-2049-3, DARIC 98-95 and 92 ZHONG257] grown under drought stress compared to control; Different letters above the columns indicate significant difference between the treatments according to Duncan's test.

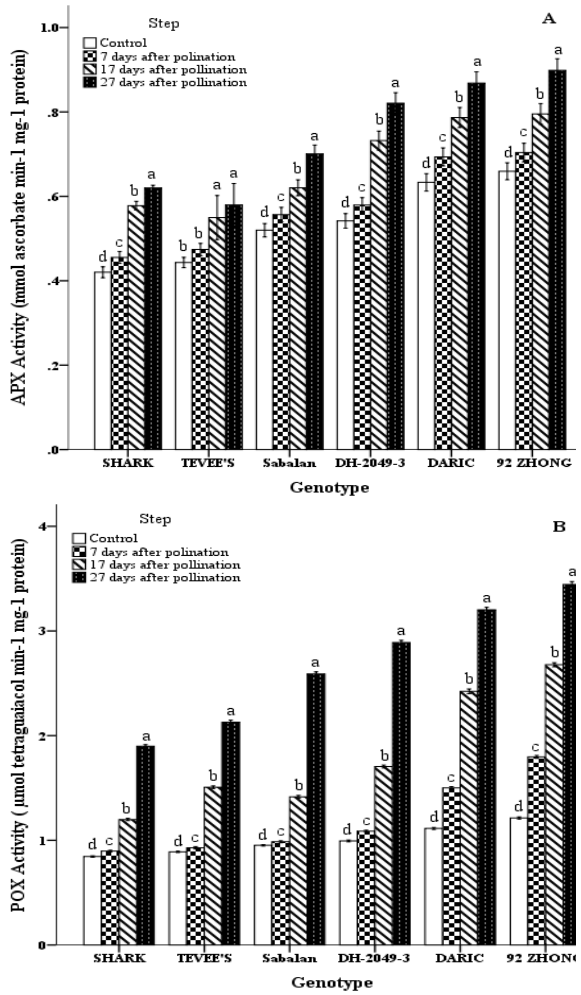

Fig. II. Ascorbate peroxidase (A) and peroxidase (B) activity in flag leaves of six wheat genotypes [SHARK-4-0YC-0YC-0YC-5YC-0YC, TEVEE'S'//CROW/VEE'S, Sabalan, DH-2049-3, DARIC 98-95 and 92 ZHONG257] grown under drought stress compared to control; Different letters above the columns indicate significant difference between the treatments according to Duncan's test.

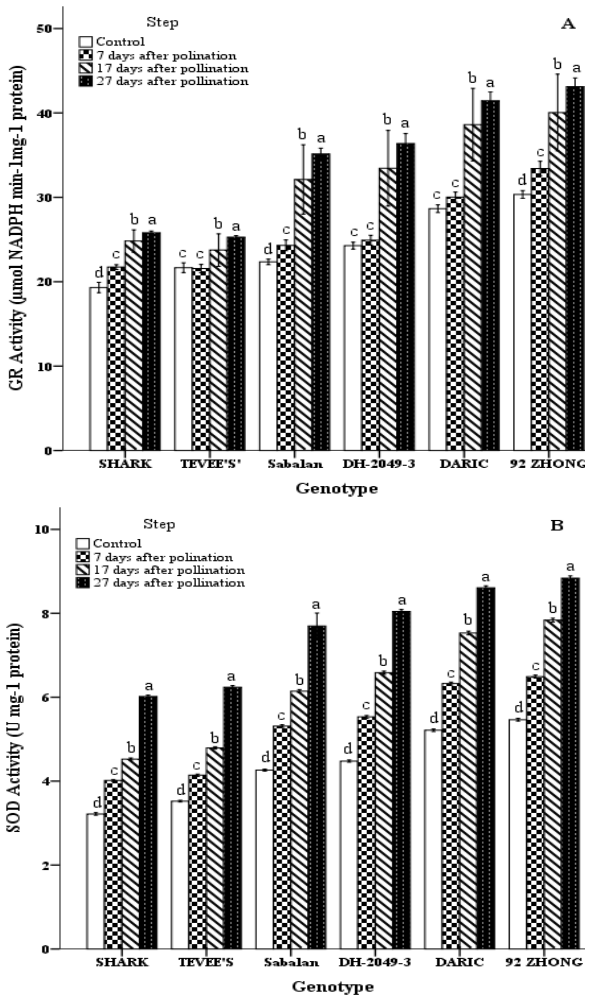

Fig. III. Glutathione reductase (A) and superoxide dismutase (B) activity in flag leaves of six wheat genotypes [SHARK-4-0YC-0YC-0YC-5YC-0YC, TEVEE'S'//CROW/VEE'S, Sabalan, DH-2049-3, DARIC 98-95 and 92 ZHONG257] grown under drought stress compared to control; Different letters above the columns indicate significant difference between the treatments according to Duncan's test.

2049-3), and also moderately sensitive genotypes showed a higher enzymatic activity compared to sensitive genotypes (Shark and Tevees).

All differences in ascorbate peroxidase (APX) activity among genotypes were significant ($P<0.05$), except the difference between Tevees and Shark. Also the difference between steps and year was significant (Table 1).

Regarding peroxidase (POX), glutathione reductase (GR), and superoxide dismutase (SOD) activities the difference among steps after drought stress was significant ($P<0.05$), but the effects of treatment × year was not significant.

Catalase (CAT) activity enhanced in flag leaves of all genotypes under drought stress compared to control and this increase was lower than other antioxidant enzymes (Fig. IV A). The tolerant genotypes showed a higher activity than others. About CAT the effect of treatment, year and treatment × year was significant (Table 1).

H_2O_2 content increased in the genotypes under drought stress and the increase in sensitive genotypes (Shark and Tevees) was higher than others (Fig. IV. B). The tolerant genotypes showed a lower H_2O_2 content compared to others. The difference in H_2O_2 content between steps and year was significant ($P<0.05$).

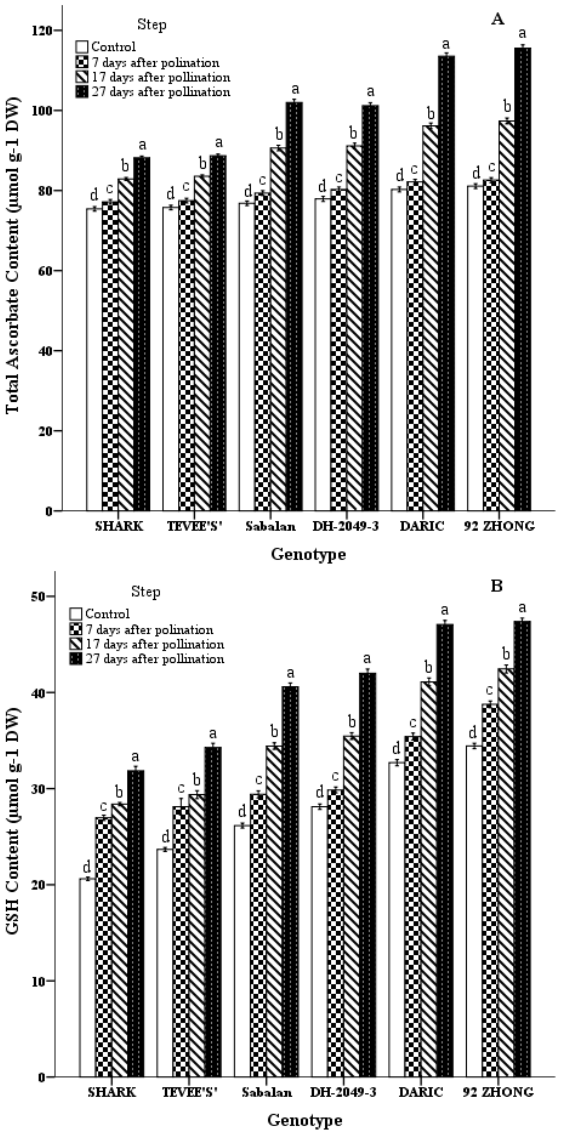

Fig. IV. Catalase activity (A) and H_2O_2 content (B) in flag leaves of six wheat genotypes [SHARK-4-0YC-0YC-0YC-5YC-0YC, TEVEE'S'//CROW/VEE'S, Sabalan, DH-2049-3, DARIC 98-95 and 92 ZHONG257] grown under drought stress compared to control; Different letters above the columns indicate significant difference between the treatments according to Duncan's test.

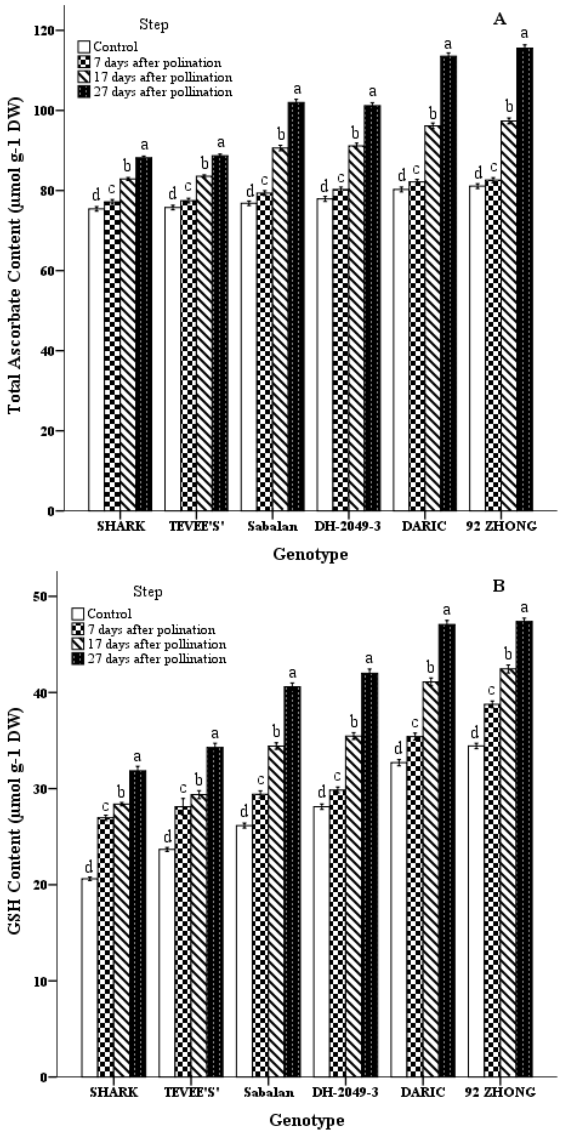

Fig. V. Ascorbate (A) and glutathione (B) content in flag leaves of six wheat genotypes [SHARK-4-0YC-0YC-0YC-5YC-0YC, TEVEE'S'//CROW/VEE'S, Sabalan, DH-2049-3, DARIC 98-95 and 92 ZHONG257] grown under drought stress compared to control; Different letters above the columns indicate significant difference between the treatments according to Duncan's test.

Non enzymatic antioxidants contents

Non enzymatic antioxidants contents (ascorbate and glutathione) increased under drought stress in our genotypes (Fig. V), where the increase in tolerant genotypes (Daric and 92 Zhong) was higher than moderately sensitive genotypes (Sabalan and Dh-2049-3) and also it was higher in moderately sensitive genotypes compared to the sensitive genotypes (Shark and Tevees). The difference in ascorbate and glutathione among all genotypes and steps after drought stress was significant ($P<0.05$).

Discussion

Higher plants have evolved a wide range of defense systems to survive under biotic or environmental conditions. If environmental stresses are severe and do not allow short-term metabolic control, stress induces damage, but

there is an obvious stress threshold (Shao et al., 2005). In this situation changes in gene expression are induced. If genomic responses are insufficient or not appropriate, new genes cannot induce stress tolerance, primary metabolism is impaired, oxidative stress happens, and cell death responses are triggered (Chen and Gallie, 2004). One of the first responses of plants to abiotic stresses is the accumulation of reactive oxygen species such as superoxide, hydroxyl radicals, hydrogen peroxide and singlet oxygen. This is observed in wheat exposed to environmental stresses.

Common signal transduction pathways are activated that can lead to stress acclimation or cell death depending on the degree of oxidative stress. Wheat responses to stresses involve acclimation and repair damage which is the basic common feature of organisms. POD, SOD, CAT and MDA are important indicators for evaluating the status of wheat tolerance, where high activities of the enzymes and low MDA content show higher anti oxidative ability (Chen and Gallie, 2004). Weng *et al.* (2015) reported MDA content increased in wheat genotypes under drought stress that was higher in sensitive genotypes. This is consistent by the results of the present study on tolerant genotypes (Daric and 92 Zhong) that showed a lower MDA content also had a higher anti oxidative enzymes activity compared to other genotypes. There was a significant negative correlation ($P<0.05$) between MDA content and anti oxidative enzymes activity in wheat genotypes.

The ability of plants to overcome oxidative stress partly depends on SOD activity and also up-regulation of other antioxidant enzymes. Bakalova *et al*. (2004) and Salekjalali *et al*. (2012) reported that drought stress increased SOD activity in wheat and barley, respectively, so that SOD activities was higher in severe stress compared to moderate stress conditions. This was also verified by the results of the present study. Higher SOD activity in tolerant plants (Daric and 92 Zhong) compared to moderately tolerant (Sabalan and DH-2049-3) and sensitive plants (Shark and Tevees) can also be explained by their higher efficiency in scavenging of free radicals under drought conditions. Tolerant genotypes showed lower MDA content and electrolyte leakage. The indexes at reproductive stage and drought effects at this stage were severe compared to the vegetative stage.

Sairam and Saxena (2000) reported that tolerant genotypes, with higher peroxidase activity, showed lower lipid peroxidation and higher membrane stability under water stress at different stages after anthesis, while the susceptible genotypes exhibited lower antioxidant enzyme activity and membrane stability and higher lipid peroxidation. Shao *et al.* (2005) observed changes in peroxidase activity in wheat genotypes under soil water deficits at maturation stage and suggested that drought tolerance was closely associated with POX activities. Hasheminasab *et al*. (2012) reported that peroxidase activity increased significantly under water stress and that the highest activity was observed in drought tolerant genotypes. This is also consistent with results of the present study. Our tolerant genotypes (Daric and 92 Zhong) that showed the lowest lipid peroxidation, also had a highest antioxidant enzymes activity while the lowest activities belonged to sensitive genotypes. Acar *et al.* (2001) reported an increase in POX activity in tolerant barely variety but no changes in activities were observed in susceptible genotypes. Both tolerant and sensitive genotypes n this study showed increase in POX activity and this was not consistent with Acar *et al*. (2001) study.

Luna *et al*. (2004) reported CAT activity increased in wheat, also leaf H_2O_2 content increased even though total CAT activity doubled in wheat seedlings under severe stress conditions. Also Chakraborty and Pradhan (2012) reported higher accumulation of H_2O_2 content in sensitive wheat varieties under drought stress. This is consistent with the findings of the present study. H_2O_2 content increased in sensitive genotypes (Shark and Tevees) more than others, but CAT activity increased more in tolerant ones. Changes in CAT activities were low in our genotypes and it was not consistent with Luna *et al.* (2004). It seems that CAT had not an important role for the genotypes in this study.

Sharma and Dubey (2005) and Naderi *et al.* (2014) reported that GR activity of rice and wheat genotypes increased under environmental stresses, respectively. Lascano *et al.* (2001) reported a higher increase in glutathione reductase (GR) activity and a higher decrease in

reduced glutathione (GSH) and ascorbate content in tolerant wheat cultivars compared to sensitive ones. Whereas in our study GR activity, GSH, and ascorbate content increased under drought conditions after pollination, where the increase was higher in tolerant genotypes (Daric and 92 Zhong) compared to moderately tolerant (Sabalan and DH-2049-3) and sensitive genotypes (Shark and Tevees). Previous studies reported increase in GR activity under drought in vegetative phase while the findings of the present study are related to reproductive phase. There were a significant positive correlation ($P<0.05$) between anti oxidative enzymes activity and antioxidant contents in wheat genotypes in this study. Table 2 shows Pearson correlations coefficients between studied traits in wheat genotypes under drought stress.

Khanna-Chopra and Selote (2007) reported that drought tolerant wheat cultivars had the highest APX activity during severe drought stress in post-anthesis period which is consistent with our findings for wheat genotypes. Al-Ghamdi (2009) reported that drought acclimated wheat seedlings exhibited increase in activity of H_2O_2 scavenging enzymes, particularly APX and CAT. Our results verified all of these reports about wheat genotypes, but our genotypes did not show higher variation in CAT activity.

In conclusion the results showed that our genotypes responses to drought stress was significantly different ($P<0.05$) as a result of changes in their antioxidant defense systems. Our tolerant wheat genotypes that showed efficient antioxidant system (enzymatic and non-enzymatic) had a lower lipid peroxidation, electrolytes leakage, and H_2O_2 content under drought stress after pollination. Our results reveal the appropriate relationship between enzymatic and non- enzymatic antioxidants under drought stress in wheat. It seems that POX and SOD enzymes -that showed higher increase under stress conditions- had a strong role in drought tolerance. The difference between drought steps for all studied factors was significant ($P<0.05$).

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