The Journal of Animal & Plant Sciences, 26(6): 2016, Page: 1740-1749
ISSN: 1018-7081

CHANGES IN BIOCHEMICAL CHARACTERISTICS OF THIRTY-SIX IRANIAN WHEAT LANDRACES IN RESPONSE TO DROUGHT STRESS AND THEIR CLASSIFICATION USING MULTIVARIATE ANALYSIS

S. Dorostkar[1], A. Dadkhodaie[1,2*] and B. Heidari[1,2]

[1]Department of Crop Production and Plant Breeding, School of Agriculture, Shiraz University, Shiraz, Iran
[2]Drought Research Center, Shiraz University, Shiraz, Iran
[*]Corresponding author e-mail: dadkhodaie@shirazu.ac.ir

ABSTRACT

Drought stress is one of the most important factors limiting crop production in dry regions. In order to study the effects of drought stress on antioxidant enzymes and physiological characteristics, thirty six bread wheat genotypes were evaluated at the research station, School of Agriculture, Shiraz University, Iran during 2010-2011 and 2011-2012 growing seasons. The experiment was conducted as a split-plot design with irrigation treatments (well-irrigation and non-irrigation) as main plots in three replications and genotypes as sub-plots. Water stress was applied when 50% of ears emerged. Two weeks after applying stress, the youngest leaves were collected to extract enzymes. Results showed that the activity of all enzymes increased under stress, with APX and proline showing the highest increase. Therefore, these factors were considered suitable to screen drought tolerant genotypes. The genotypes KC4856, KC4848, KC4512, KC3893, KC3892 and KC4929 had high anti-oxidant activity and high yields but showed a low decrease in MSI and hence were classified as tolerant. These results suggest that oxidative stress tolerance is closely associated with increase in antioxidant enzymes and proline and decrease of MSI.

Keywords: anti-oxidant enzyme, crop production, hexaploid wheat, membrane stability index, proline.

Abbreviations: APX= ascorbate peroxidase, MSI= membrane stability index, ROS= reactive oxygen species, O_2^-= superoxide radicals, H_2O_2= hydrogen peroxide, OH^-= hydroxyl radical, SOD= super oxide dismutase, CAT= catalase, POD= peroxidase, RWC= relative water content, PVP= polyvinylpyrrolidone, NBT= nitroblue tetrazolium, EC= electrical conductivity, PRO=proline**,** PCA= principal component analysis

INTRODUCTION

Drought stress is known to be the major limiting factor of crop production worldwide. This multi-dimensional stress affects plants at different organizational levels (Blum, 1996) and its effects are expected to increase by the global climate change in future (IPCC, 2013). The response of plants to drought stress is complex because it not only reflects stress effects but also the corresponding responses at all organizational levels. Siddique *et al.* (1999) reported drought as the most important factor affecting plants' growth and yield. Allen (1995) pointed to the reduction of plant growth and productivity in response to water shortage and oxidative damage.

Likewise, studies have shown that photosynthesis decrease under stress which could be explained by stomata closure and high concentrations of CO_2 (Sairam and Saxena, 2000). Plant stomata become closed when water is short, after which CO_2 concentration reduces in mesophyllic tissue. Therefore, dark reactions of photosynthesis are confused and products of light reactions (ATP and NADPH) are not consumed. In such situations, oxidation of NADPH molecules and consequently $NADP^+$ consumption for receiving electrons is reduced. As a result, the oxygen molecules act as alternative receptors of electron in the electron transport chain that leads to the formation of reactive oxygen species (ROS) such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Arora *et al.* 2002). Reactive oxygen species may cause damages such as lipid oxidation (change in membrane structure), protein structure change and oxidization of sulfhydryl groups (-SH), inactivation of enzymes, loss of chlorophyll and other pigments. ROS also continually attack organic molecules such as DNA and consequently lead to the impairment of DNA strands (Mittler, 2002).

Wheat has developed an efficient defense system (enzymatic and non-enzymatic) against oxidative stress that can neutralize or destroy free radicals. This system consists of enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) (Idso *et al.* 1981). Non-enzymatic compounds involve proline, ascorbate, tocopherol and carotenoids (Sheoran *et al.* 2015). These defensive molecules are interconnected and disable radicals as fast as they are formed and consequently reduce cell injury and death (Sairam and Srivastava, 2002).

Several reports have shown strong relationships between tolerance to adverse conditions and increase in concentrations of antioxidant enzymes in plants (Sairam and Saxena, 2000; Sairam and Srivastava, 2001 and 2002). The activity of SOD, CAT, POD and APX increased in tolerant wheat genotypes in response to stress (Lascano *et al.* 2005; Sheoran *et al.* 2015). Johnson *et al*. (1984) also reported that wheat genotypes with high enzymatic activity showed higher relative water content (RWC), concentrations of proline and soluble sugars and less grain yield loss under stress condition. They also expressed that these genotypes had high value of membrane stability index (MSI) under both stress and non-stress conditions. Similar results showed that tolerant genotypes have high anti-oxidant enzymes (POD, CAT, APX and SOD) activity, low concentration of free radicals, high MSI and consequently lower cell death (Renu and Devarshi, 2007). Increase in the activities of these enzymes enables tolerant genotypes to endure oxidative stress while too little increase is observed in sensitive ones. Therefore, selecting and breeding wheat genotypes with high antioxidants could be useful in improving agronomic traits when associated with enhanced quality and stability of wheat products (Martine *et al.* 1993). Despite the importance of biochemical and physiological indices in breeding for drought resistance, researches have emphasized on their role in commercial cultivars while wheat landraces have received little attention. Therefore, this research was conducted to evaluate the response of Iranian wheat landraces to drought stress using anti-oxidant and non-anti-oxidant enzymes.

MATERIALS AND METHODS

To study enzymes' activity, 36 wheat genotypes were evaluated at the research station, School of Agriculture, Shiraz University (1810 m above sea level with 52˚ 32´ E longitude and 29˚ 36´ N latitude), Iran during 2010-2011 and 2011-2012 growing seasons. The genotypes included thirty four landraces preceded with KC (4567, 4630, 4538, 4606, 4608, 4580, 4862, 4856, 4920, 4848, 4575, 4577, 4508, 4565, 2195, 4512, 4623, 4642, 4847, 4860, 4595, 4564, 4687, 4633, 4545, 4557, 2177, 4562, 4535, 4619, 3893, 3892, 4929 and 4646) and the commercial cultivars i.e., Shiraz and Cross Boolani, as controls. An experiment was performed in the field as split-plot design where irrigation (well-irrigation, non-irrigation after anthesis) was used in main plots in a randomized complete block design with three replications and genotypes were allocated to sub-plots.

The genotypes were planted on 13th and 2nd of November in 2010-2011 and 2011-2012, respectively, in four rows of three meter length each at a rate of 250 seeds per square meter (the soil was fine mixed, Mesic Calcixerpets, Xerochrepts). Nitrate fertilizer (@ 300 kg ha^{-1}) was split into two parts and applied at the planting and ear-emerging stages. All plots were irrigated at 100% field capacity until 50% ear emergence when stress was applied. Weather information of the experimental site is given in Table 1.

Fifty cm on either side of each row was considered as border and two weeks after applying stress, flag leave samples were collected which were immediately placed in liquid nitrogen and stored in refrigerator for further analyses.

Table 1. Some weather parameters for the experimental site during 2010-2011 and 2011-2012 growing seasons.

	Temperature (ºC)				Relative humidity (%)		Precipitation (mm)	
	2010-11		2011-12					
Month	**Min**	**Max**	**Min**	**Max**	**2010-11**	**2011-12**	**2010-11**	**2011-12**
November	- 6.94	18.20	- 3.50	12.47	30.85	52.67	0.00	79.5
December	- 5.79	12.30	- 4.11	12.88	42.93	58.28	48.5	61.0
January	- 1.30	10.26	- 2.52	10.48	48.98	55.45	107.5	127.0
February	0.89	16.27	- 1.72	13.43	49.47	42.82	76.8	27.0
March	3.32	20.31	3.17	19.06	50.02	43.35	30.5	45.0
April	7.83	27.50	7.51	26.82	48.27	35.05	0.00	0.00
May	12.39	34.10	11.58	31.43	24.47	26.15	0.00	0.00
June	15.30	35.77	14.08	35.23	20.92	22.19	0.00	0.00
			Total				**262.8**	**339.5**

Methods of extracting and measuring biochemical traits: To extract enzymes, 0.5 g of frozen tissue was homogenized using 2 ml buffer (pH=7.8) which consisted of 0.607 g Tris, 0.05 g PVP (polyvinylpyrrolidone) and 50 ml water. Then the homogenate was transferred to a new tube and centrifuged at 13000 rpm for 15 min at 4˚C. Finally, transparent phase was kept as enzyme extract (Sairam and Srivastava, 2001; Sairam and Saxena, 2000). The concentration of ascorbate peroxidase was measured using the method of Nakano and Asada (1981). Peroxidase activity was measured based on guaiacol oxidation as described by Chance and Maehly (1995). The method of CAT enzyme measurement was similar to that of POD with the exception that it is based on the

demolition speed of H_2O_2 (Chance and Maehly, 1995). Superoxide dismutase (SOD) activity was measured based on its ability to stop light reviving of NBT in the presence of riboflavin and light using Beauchamp and Fridovich (1971) method.

To measure proline concentration, the following formula (Bates *et al.* 1973) was used.

Proline (µM g^{-1} fresh wt) = M×T×W/115.5

Where M is the value for each sample shown by the spectrophotometer, T is toluene volume (ml) and W is tissue weight (g).

MSI was calculated as $[1- (\frac{C_1}{C_2})] \times 100$ based on the method of Sairam (2000), where C_1 and C_2 represent EC at 40 and 100 ˚C, respectively.

Statistical analysis for each year (under combined conditions of both stress and non-stress) and combined years (both of 2010-2011 and 2011-2012) were performed using EXCEL and SAS 9.2 (SAS, 2004) softwares. Genotypes were clustered and least significant difference (LSD) at 5% probability level was used to compare means.

RESULTS AND DISCUSSION

Analysis of variance indicated that both irrigation treatments and genotypes were significantly different in both years for APX, POD, SOD and CAT and proline but the interaction of these factors was only significant for CAT and POD in one year and proline for both years (Table 2). The effects of irrigation, genotypes and their interaction was significant for MSI in one year.

Drought stress increased the activity of APX (Figure 1-1, further details for each year could be found in supplementary data). The highest and lowest APX activity belonged to genotypes number 17 (834.0 U g^{-1} FW[1]) and 3 (377.4 U g^{-1} FW), for 2010-2011 under combined conditions of stress and non-stress, respectively (Table 3). Genotypes number 33, 8, 34 and 35 with 697.2, 623.4, 562.9 and 546 U g^{-1} FW also produced high APX in this year. However, they did not differ significantly from genotype number 17 that showed the highest activity. For 2011-2012, the highest and lowest APX activity belonged to genotypes number 27 (576.2 U g^{-1} FW) and 23 (342.7 U g^{-1} FW) under combined conditions, respectively. The high-yielding tolerant genotypes (based on drought tolerance indices and yield loss; data not shown), 33, 17, 35, 34 and 8 with 561.9, 535.7, 530.4, 517.9 and 430.3 U g^{-1} FW did not significantly differ from genotype number 27 (Table 3). APX activity in 2011-2012 was lower than that of 2010-2011 which is due to differential response of genotypes to year effect or their different potential or stability. Thus, genotypes with high enzyme activities in different environments could be used in breeding programmes.

Mean of two-year data revealed that the tolerant genotypes 17 (684.8 U g^{-1} FW) and 33 (629.5 U g^{-1} FW) had higher APX concentrations (Table 3). These genotypes withstood detrimental effects of drought and produced acceptable yields (5328 and 4824 kg ha^{-1}, respectively) most likely due to APX increase. This enzyme removes free oxygen radicals (Hus and Kao, 2007) and protects cells against their destructive effects. On the other hand, this enzyme can create a balance between formation and removal of reactive oxygen radicals and keep H_2O_2 in a balanced level (Munns and Tester, 2008).

As genotypes with higher APX have higher yield stability and sustain less injury under stress condition, this enzyme can be beneficial in screening tolerant genotypes under drought and oxidative stresses. Amjad *et al.* (2011) highlighted the role of APX in scavenging ROS and cell protection from injury under stress conditions. Similarly, Renu and Devarshi (2007) reported higher APX activity in tolerant wheat cultivars under drought stress.

For 2010-2011, the highest and lowest POD belonged to genotypes number 23 (476.6 U g^{-1} FW) and 13 (129.0 U g^{-1} FW) in combined conditions (Table 3). In this year, genotypes 25, 33, 27, 21, 15 and 17 with 441.4, 401.2, 397.1, 368.9, 358.0 and 348.6 U g^{-1} FW ranked second to fifth, respectively. However these genotypes did not significantly differ from genotype 23 that had the highest POD. For 2011-2012, genotypes 16 and 24 with 507.3 and 223.3 U g^{-1} FW had the highest and lowest POD activity, respectively in combined conditions of stress and non-stress. This enzyme increased in all genotypes under drought (Figure 1-2) because under such conditions, plants use their full capacity to combat or escape drought and survive its harsh conditions (Shao *et al.* 2005). Kim *et al.* (2005) indicated that increase in this enzyme could protect plants from stress effects, thus, maintaining their high performance. POD reduces oxidative damage through decomposing H_2O_2; consequently leading to lower decrease in MSI. These results are in agreement with the findings of McDonald (2004) who observed higher POD concentration in tolerant genotypes and reported its role in defense against oxidative stress. Similar results were also reported by Bailly (2004) in crops under stress.

For 2010-2011, the highest and lowest CAT belonged to genotypes number 25 (91.1 U g^{-1} FW) and 26 (34.1 U g^{-1} FW) in combined conditions. Genotypes number 23, 10, 33, 16, 21, 28, 27, 17 and 11 with 89.2, 84.8, 80.7, 80.5, 80.0, 75.8, 74.1, 71.8 and 71.8 U g^{-1} FW ranked second to ninth, respectively. However their difference was not significant from genotype 25 that had the highest CAT concentration (Table 3). For 2011-2012,

[1]- Unit/gram Fresh Weight

genotypes number 7 and 17 with 95.3 and 54.40 U g $^{-1}$ FW had the highest and lowest CAT activity, respectively (Table 3). CAT concentration in genotypes differed in both years which refers to either the significant year effect and/or its sensitivity to light or temperature under stress condition which causes fluctuation in different genotypes.

Results for 2010-2011 showed the highest and lowest SOD belonged to genotypes number 33 (145.2 U g^{-1} FW) and 5 (16 U g^{-1} FW), respectively and for 2011-2012, genotypes number 20 and 11 with 120.9 and 53.61 U g $^{-1}$ FW showed the highest and lowest SOD in combined conditions of stress and non-stress, respectively (Table 3). The year effect was significant which means different genotypes performed differently every year. SOD accumulation in the tolerant genotype e.g. 33 (145.2 and 82.3 U g^{-1} FW in 2010-2011 and 2011-2012, respectively, under combined conditions of stress and non-stress) was higher compared to sensitive ones (e.g. 25 with 51.5 and 73.7 U g^{-1} FW in 2010-2011 and 2011-2012, respectively, under the same conditions) which refers to SOD efficiency in reducing destructive free oxygen radicals and oxidizing lipids (Alscher *et al.* 2002). This enzyme converts superoxide radicals (O_2^-) to hydrogen peroxide in chloroplasts, mitochondria, cytoplasm and peroxisome, and has been regarded as one of the best defense systems (Mittler, 2002).

Means over years showed that genotypes 25 and 10 with 83.5 and 80.4 U g $^{-1}$ FW had the highest CAT activities (Table 3). However, these genotypes were sensitive to drought and had yield losses of 42.5 and 48.1%, respectively (Suppl. 1). Therefore, they are not suitable for stress condition. The lowest CAT activity belonged to genotypes 35 and 24 with 52.5 and 50.1 U g $^{-1}$ FW. Genotype 24 had high grain yield loss along with lower enzyme activity under stress which makes it only suitable for non-stress conditions. Genotype 35 had a high yield of 7,493 and 4,893 kg ha^{-1} under combined conditions of stress and non-stress in 2010-2011 and 2011-2012, respectively, while the difference between genotypes 35 and 24 in terms of CAT activity was not significant (Table 3). Based on these results, CAT enzyme is not suitable in identifying tolerant genotypes because no special pattern was detected among tolerant and sensitive genotypes.

Results showed that the highest and lowest SOD belonged to genotypes number 33 (113.8 U g^{-1} FW) and 5 (50.8 U g^{-1} FW), respectively. Genotype 34 did not show a significant difference from genotype 33 (Table 3) which has been classified as tolerant in previous findings (data not shown). These two genotypes could be used in breeding programmes because lower SOD activity correlates with higher amounts of superoxide radicals. These radicals synthesize hydrogen peroxide and create hydroxyl which is an extremely dangerous radical that destroys bio-molecules (Asada, 2000). Similar results have been also reported by Hidalgo *et al.* (2006) in wheat who concluded that tolerant genotypes had higher SOD amounts associated with less free radicals compared to sensitive ones under stress condition. Also, Stepien and Klobus (2005) reported that antioxidant defense plays an important role in both C_3 and C_4 plants tolerance to environmental stresses.

Analysis of variance for proline showed irrigation regime, genotype and their interaction were significantly different in 2010-2011 and 2011-2012 (Table 2). The highest and lowest amount of proline belonged to genotypes number 33 (156.8 µmol g^{-1} FW) and 13 (33.3 µmol g^{-1} FW) under combined conditions of well irrigation and non-irrigation regimes, respectively, in 2010-2011 (Table 3). Genotypes number 12, 35, 34, 5 and 7 with 131.5, 122.5, 120, 118.8 and 114.8 µmol g^{-1} FW also showed high proline content which were significantly different from genotype 33.

Table 2. Analysis of variance for enzymes, proline amino acid and MSI of 36 hexaploid wheat genotypes under two irrigation regimes as main plot with three replications in combined years.

S.O.V	df	APX	POD	CAT	SOD	Proline	MSI
Y	**1**	**886179.9****	**953390.6****	**18617.6****	**30202.4****	**119933.5****	**31306.3****
Rep (Year)	4	495772.7	250905*	1341.5	5551.7	2093.8	654.3
I	1	2195004.2**	16274550**	95480.6**	106388.7**	72182.7**	10005.6**
Y×I	1	876694.2*	143737.5*	34642.8**	526.7	22933.5**	285.4**
Error	4	129509.6	292081.9	2128.8	613.5	882.9	1703.0
G	35	44403.2	34859.6*	879.3*	2814.3**	2787.8**	160.9**
Y×G	35	42415.0*	39019.4*	994.3**	3461.9*	2548.0**	145.7**
I×G	35	33806.3**	49260.7**	568.5	1221.4	1311.1**	92.1
Y×I×G	35	19980.2**	61224.1**	783.3	1551.7	1070.8**	83.5
Erorr	280	36895.9	24777.7	560.9	1663.8	237.2	74.3

B: block, I: irrigation, G: genotype, Y: year, Rep (Replication), MS: mean square

df; degrees of freedom, APX; ascorbate peroxidase, POD; peroxidase, CAT; catalase, SOD; super oxide dismutase, MSI; membrane stability index. ** and * means significant differences at *1* and *5*% levels of probability, respectively.

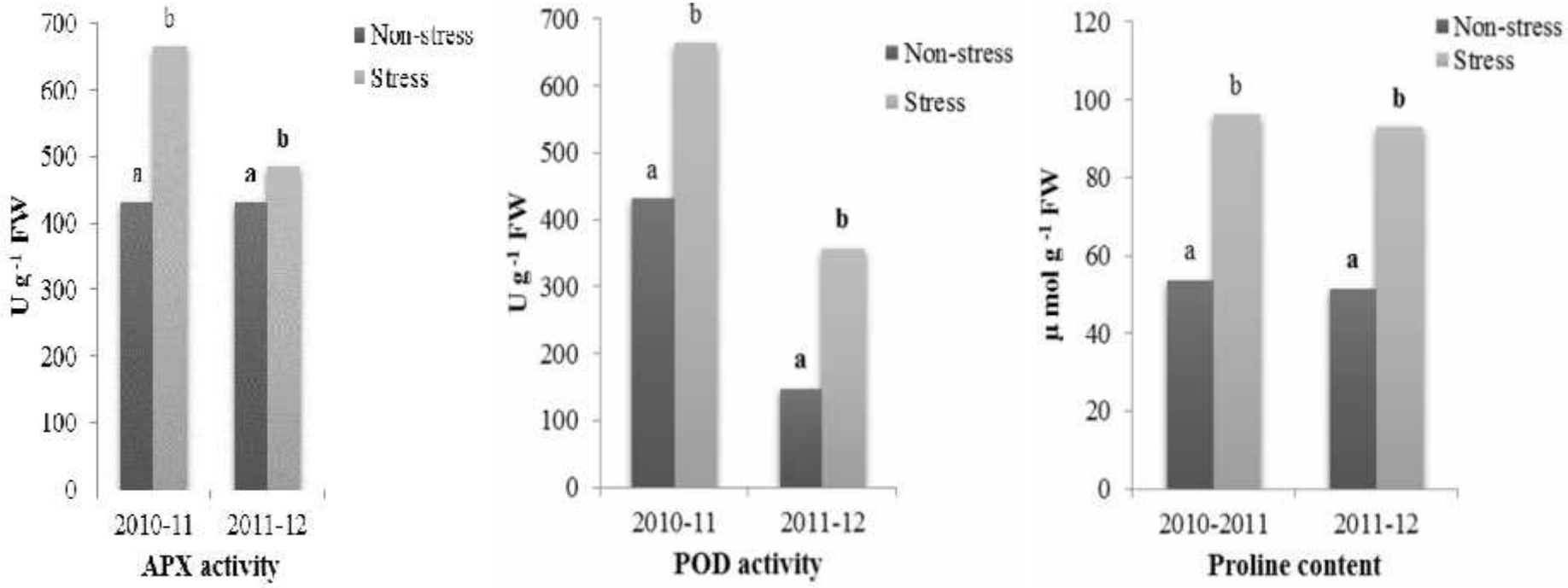


Figure 1. Activities of APX (1-1), POX (1-2) and proline (1-3) in 36 wheat genotypes under both stress (S) and non-stress (NS) conditions in 2010-2011 and 2011-2012 growing seasons. APX; ascorbate peroxidase, POD; peroxidase. Different letters indicate significant differences.

Table 3. Average of APX, POD, CAT and SOD (U g $^{-1}$ FW) and proline (µ mol g $^{-1}$ FW) and MSI (%) of 36 common wheat genotypes in 2010-2011 and 2011-2012 growing seasons and two-year average.

Genotype	APX			POD			CAT			SOD			Proline			MSI		
	2010-11	2011-12	Mean	2010-11	2011-12	Mean	2010-11	2011-12	Mean	2010-11	2011-12	Mean	2010-11	2011-12	Mean	2010-11	2011-12	Mean
1(KC 4567)	475.9	430.0	452.9	277.4	281.6	279.5	64.5	55.7	60.1	27.1	81.6	54.4	78.4	75.7	77.0	36.4	15.1	25.7
2(KC 4630)	503.4	462.9	483.1	170.9	376.9	273.9	44.9	71.6	58.2	34.0	94.9	64.5	68.3	65.9	67.1	27.8	21.9	24.8
3(KC 4538)	377.4	405.8	391.6	227.3	414.6	320.9	47.4	75.3	61.4	97.5	93.2	95.4	82.2	79.4	80.8	31.0	19.1	25.1
4(KC 4606)	476.2	398.0	437.1	308.5	367.1	337.8	63.1	75.4	69.3	64.9	56.0	60.5	33.7	32.1	32.9	39.1	17.9	28.5
5(KC 4608)	572.2	489.1	530.6	294.5	399.6	347.1	59.7	65.3	62.5	16.0	85.6	50.8	118.8	115.1	116.9	36.1	33.2	34.6
6(KC 4580)	576.7	421.9	499.3	143.4	293.6	218.5	46.5	74.0	60.2	51.1	66.6	58.9	66.2	65.7	64.9	35.2	18.4	26.8
7(KC 4862)	399.2	545.2	472.2	247.6	490.8	369.2	56.9	95.3	76.1	65.9	104.7	85.3	114.8	111.2	112.9	33.6	19.4	26.5
8(KC 4856)	623.4	430.3	526.8	130.0	389.8	259.9	61.0	81.5	71.2	56.8	117.0	86.9	107.5	104.1	105.7	38.6	13.7	26.1

9(CrossBoolani)	387.2	439.7	413.4	252.9	402.4	327.7	57.3	82.8	70.0	48.1	104.2	76.2	74.9	74.5	74.7	33.9	15.6	24.8
10(KC 4920)	493.6	418.7	456.1	181.3	362.0	271.7	84.8	76.0	80.4	30.6	89.2	59.9	77.2	72.3	74.5	43.6	17.4	30.5
11(KC 4848)	599.7	407.1	503.4	286.1	438.3	362.2	71.8	88.0	79.9	65.0	53.6	59.3	101.3	98.0	99.6	42.9	19.5	31.2
12(KC 4575)	640.7	460.2	550.4	261.2	342.9	302.1	54.9	69.2	62.1	63.0	74.3	68.7	131.5	127.4	129.4	37.0	18.6	27.8
13(KC 4577)	628.8	380.5	504.6	129.0	380.9	255.0	47.2	68.8	58.0	93.8	74.6	84.2	33.3	31.8	32.5	31.8	21.0	26.4
14(KC 4508)	467.7	421.3	444.5	211.7	419.1	315.4	46.1	76.2	61.1	59.1	86.5	72.8	48.2	46.3	47.2	29.8	15.2	22.5
15(KC 4565)	504.0	391.0	447.5	358.0	487.1	422.6	69.7	70.4	70.0	108.1	74.5	91.3	65.0	62.6	63.8	40.6	18.7	29.6
16(KC 2195)	666.5	440.1	553.3	307.9	507.3	407.6	80.5	70.9	75.7	34.1	96.5	65.3	60.9	58.7	59.8	39.2	6.5	22.9
17(KC 4512)	834.0	535.7	684.8	348.6	238.7	293.6	71.8	54.4	63.2	57.0	58.8	57.9	62.7	60.4	61.5	35.5	13.2	24.3
18(KC 4623)	588.9	383.2	486.0	263.8	399.0	331.4	52.1	74.4	63.3	63.6	101.5	82.6	68.1	63.8	65.4	38.9	16.4	27.7
19(KC 4642)	607.4	373.7	490.5	182.6	460.4	321.5	43.1	75.3	59.2	70.1	58.1	64.1	85.1	82.2	83.6	39.9	18.0	28.9
20(KC 4847)	625.4	514.9	570.1	298.8	332.5	315.7	53.7	62.1	57.9	82.3	120.9	101.6	92.1	89.0	90.5	20.5	11.5	16.0
21(KC 4860)	619.6	392.4	506.0	368.9	313.7	341.3	80.0	63.2	71.6	140.2	60.8	100.5	46.7	44.8	45.5	32.8	15.7	24.2
22(KC 4595)	599.5	377.3	488.4	262.6	272.2	267.4	55.1	71.6	63.3	72.0	82.4	77.2	54.4	52.3	53.3	39.4	17.7	28.5
23(KC 4564)	450.4	342.7	396.5	476.7	291.5	384.1	89.2	65.1	77.1	78.2	88.5	83.4	58.0	55.8	56.8	32.0	22.4	27.2
24(KC 4687)	525.7	403.9	464.8	177.9	223.3	200.6	38.3	61.9	50.1	70.1	109.2	89.7	45.4	43.5	44.4	32.2	11.0	21.6
25(KC 4633)	466.8	474.4	470.6	441.5	296.5	369.0	91.1	75.8	83.5	51.5	73.7	62.6	65.8	63.5	64.6	37.9	15.7	26.8
26(KC 4545)	514.3	417.1	465.7	184.3	298.0	241.1	34.1	80.7	57.4	84.2	92.5	88.4	52.2	50.2	51.1	39.3	18.4	28.8
27(KC 4557)	527.6	576.2	551.9	397.2	299.9	348.6	74.1	66.2	70.1	50.4	80.7	65.6	44.4	42.5	43.8	29.9	25.2	27.6
28(KC 2177)	586.4	481.4	533.9	313.9	334.7	324.3	75.8	78.5	77.1	62.3	79.7	71.0	37.6	36.0	36.78	36.8	13.1	24.9
29(Shiraz)	701.7	471.3	586.5	247.9	320.1	284.0	47.4	67.5	57.4	92.1	77.1	84.6	45.5	43.6	44.5	30.9	16.1	23.5
30(KC 4562)	445.4	569.1	507.2	247.8	313.8	280.8	58.1	65.6	61.8	63.9	72.1	68.0	55.9	53.7	54.8	36.0	17.3	26.6
31(KC 4535)	470.6	536.0	503.3	177.0	341.0	259.0	39.3	81.2	60.2	57.8	73.4	65.6	60.1	57.8	58.9	35.7	31.5	33.6
32(KC 4619)	378.7	563.4	471.0	264.6	320.0	292.3	57.6	77.8	67.7	68.4	81.7	75.1	67.0	65.8	65.8	32.1	12.9	22.5
33(KC 3893)	697.1	561.9	629.5	401.2	449.9	425.6	80.7	75.6	78.2	145.2	82.3	113.8	156.8	152.1	154.4	41.5	24.5	33.0
34(KC 3892)	562.9	517.9	540.4	280.1	396.3	338.2	47.1	71.6	59.4	81.3	85.7	83.5	120.0	118.7	119.3	36.1	27.4	31.7
35(KC 4929)	546.0	530.5	538.2	214.6	257.0	250.8	37.9	67.1	52.5	30.9	78.2	54.6	122.5	116.3	119.4	36.8	19.5	28.1
36(KC 4646)	593.8	512.4	553.1	228.9	406.4	317.7	45.6	69.4	57.5	47.4	72.8	60.1	94.3	91.2	92.7	33.9	21.8	27.9
Average	548.2	457.7	502.9	265.7	359.7	312.7	59.1	75.2	65.6	66.2	82.8	74.5	74.9	72.3	73.5	35.41	18.3	26.9
LSD (5%)	275.27	142.72	196.8	160.9	196.6	128.61	31.85	21.14	19.13	63.02	19.07	32.78	21.51	20.97	12.37	13.17	4.47	7.46

APX: ascorbate peroxidase, POD: peroxidase, CAT: catalase, SOD: super oxide dismutase, MSI: membrane stability index, LSD; least significant differences.

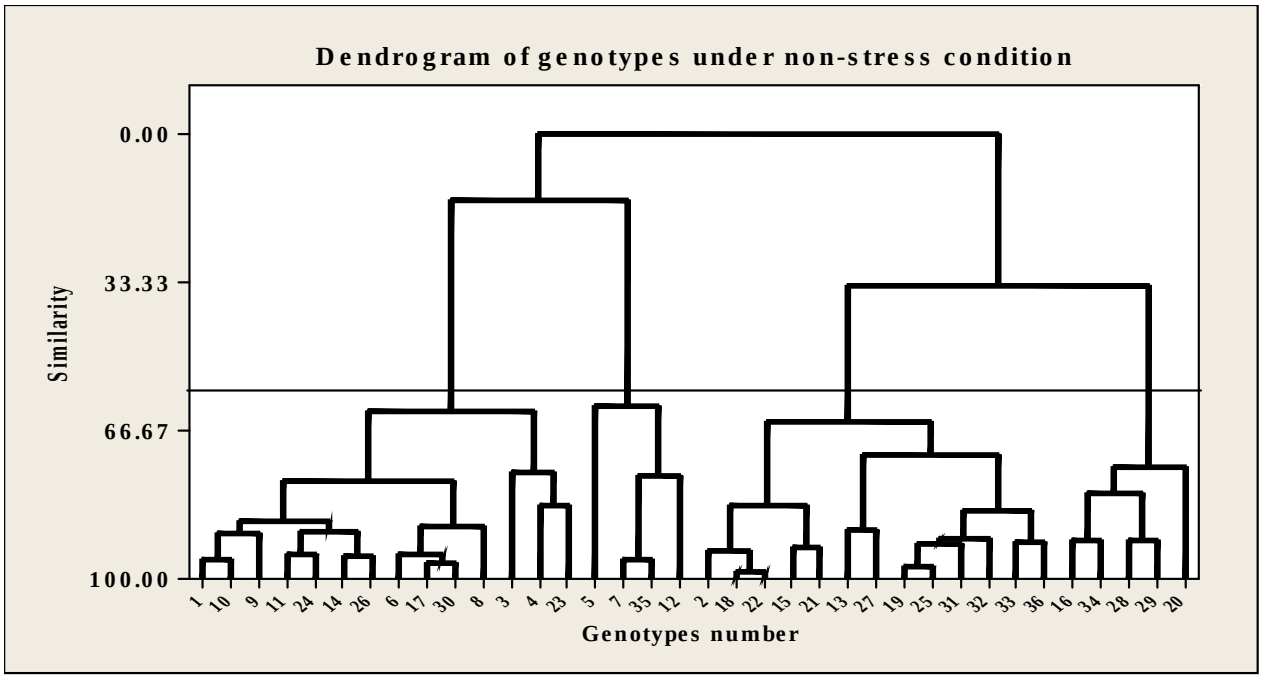
Similarly, the highest and lowest amounts of proline for 2011-2012 belonged to genotypes number 33 (152.1 µmol g^{-1} FW) and 13 (31.8 µmol g^{-1} FW) under combined conditions, respectively. The same genotypes; i.e., 12, 34, 35, 5 and 7 with 127.4, 118.7, 116.3, 115.1 and 111.2 µmol g^{-1} FW were significantly different from genotype number 33 (which had the highest proline concentration) and ranked second to sixth, respectively. The control genotypes, 9 and 29 showed the lowest amount of proline and were not significantly different from genotype 13. These results showed all genotypes except tolerant ones had similar concentration of proline in two years (Figure 1-3). Drought stress reduces cell's water and in turn, plants react by increasing proline concentration to reduce its effects (Bhaskaran *et al*. 1985). Therefore, proline increase causes osmotic adjustments in plants (Hanson *et al.* 1997) which leads to more water absorption and consequently results in yield improvement in tolerant genotypes.

These results are similar to those reported by Pierivatolum *et al.* (2010) where they found tolerant wheat genotypes produced higher amounts of proline that can reiterate this amino acid's role for screening genotypes under drought stress. Some studies have used proline as a control for biochemical characteristics because of its biosynthesis via an integrative mechanism and its role in osmotic adjustment. Therefore, proline increase can be considered an effective and feasible approach to improve plant's tolerance to multiple abiotic stresses without causing phenotypic defects (Bhaskaran *et al.* 1985; Sarvajeet and Narendra, 2010).

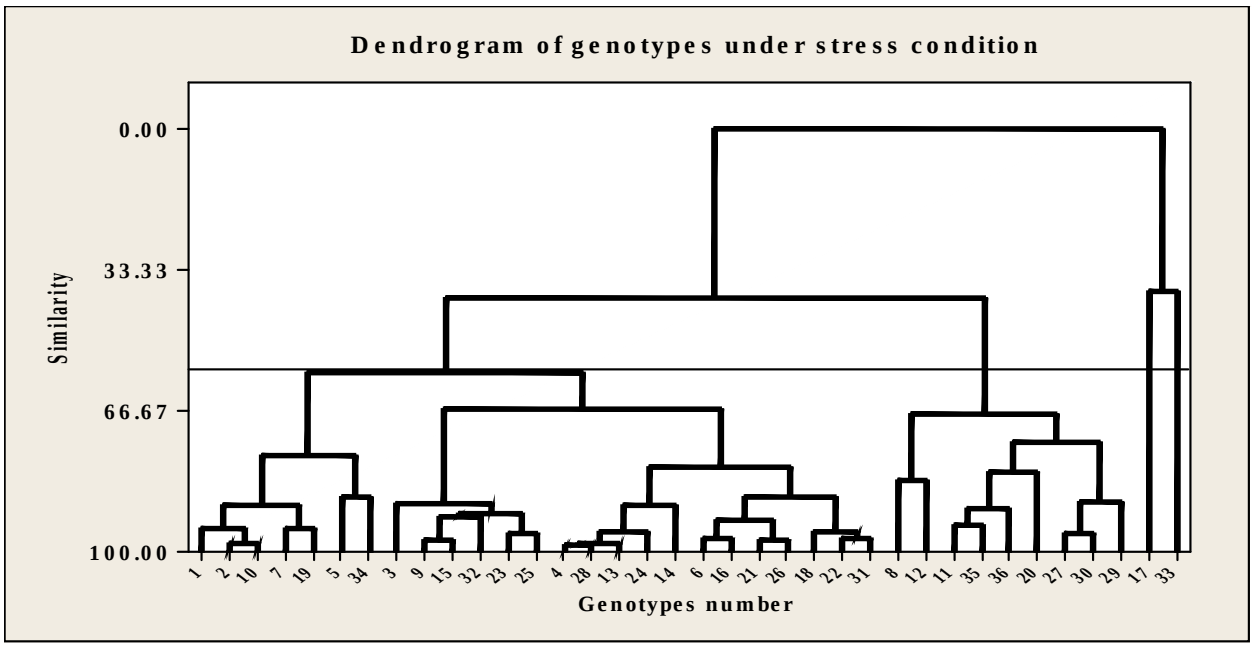
Results showed that genotypes were significantly different for MSI in 2011-2012 (Table 2). The highest and lowest MSI belonged to genotypes number 5 and 16 with 33.2% and 6.5%, respectively (Table 3). Means over years showed genotypes 5 (34.6%) and 31 (33.6%) had the highest MSI and genotypes 20 (16.0%) and 24 (21.6%) had the lowest MSI, respectively (Table 3). Genotypes number 33, 34 and 11 with 33.0, 31.7 and 31.2 % showed no significant difference from genotype number 5 that had highest MSI. The tolerant genotypes 11 (KC4848), 33 (KC3893) and 34 (KC3892) had a slight decrease in MSI, higher anti-oxidant enzyme and proline activities under drought. The activity of enzymes decreases the destructive effects of O_2^- radicals and lipid oxidation using H_2O_2 decomposition and leads to lower MSI reduction and consequently yield stability. Therefore, genotypes 33 and 34 are suitable for arid and semi-arid regions. Genotypes with higher anti-oxidant enzymes reduce free radical effects and adjust osmotic pressure through increasing proline and soluble sugars. Consequently, this leads to less MSI reduction and yield improvement which are defense systems against stress. These results are similar to those reported by Amjad *et al.* (2011) in wheat who stated free oxygen radicals, cell death and MSI are appropriate indices to screen sensitive and tolerant wheat genotypes. Similar results were also obtained by Bajii *et al.* (2001) and Renu and Devarshi, (2007) who showed proline and soluble sugars increased under stress condition and had a positive correlation with antioxidant enzymes activities.

Genotypes were classified into four groups under both non-stress and stress condition based on average linkage and Pearson distance using enzymes activity, proline and MSI index under stress and non-stress conditions (Figures 2-1 and 2-2). Under stress condition, the first two groups consisted of genotypes 33 and 17 which were highly tolerant and tolerant, respectively. The third group included nine genotypes that showed high enzymatic activity, MSI and proline and therefore, were named semi-tolerant. Of them, genotypes 8, 11, and 35 produced high yield. The fourth group which composed of the remaining 23 genotypes was named stress sensitive group because they showed low enzymatic activity, proline and MSI (Figure 2-2). Under non-stress conditions, the four groups composed of 14, 4, 13 and 5 genotypes. Similar to stress conditions, the genotypes 33 and 17 were placed in different groups. However, their corresponding groups composed of 13 and 14 genotypes, respectively.

Principal component analysis (PCA) was performed using correlation matrix to compare different genotypes by different anti-oxidant enzymes, proline and MSI index. The PC1 and PC2 explained 32.1% and 23.0 % of variations, respectively (Table 4) and were named as the first and second remover of H_2O_2 component (Figure 3). These indices can separate tolerant and sensitive genotypes. The genotypes 8, 10, 11, 12, 17, 33, 34 and 35 with 6290, 5987, 5255, 5254, 5328, 48246, 5121 and 5566 kg ha^{-1}, respectively, had high yield in combined analysis of 2010-2011 and 2011-2012. These genotypes also had high anti-oxidant enzymes and proline (Table 3) and less grain yield loss under stress and therefore are appropriate for both stress and non-stress conditions. Genotypes 3, 4, 13, 14, 20, 24, 26 and 32 with both low PC1 and PC2 are suitable neither in stress nor non-stress conditions because they are sensitive to end-season drought. Therefore, their cultivation is not recommended. Genotypes number 1, 2, 6, 19, 22, 29, 30, 31 and 36 with low PC1 and high PC2 and genotypes number 5, 7, 9, 15, 16, 18, 21, 23, 25, 27 and 28 with low PC1 and high PC2 were semi-sensitive to drought condition (Figure 3) and can be used in breeding programms or crossing nurseries to improve their undesirable traits.



2-1



2-2

Figure 2. Tree dendrogram of 36 wheat genotypes using different enzymatic and non-enzymatic characteristics (1) non-stress and (2) stress conditions in 2010-2011 and 2011-2012 growing seasons.

Table 4. Principal component loading for anti-oxidant enzymes (APX, POD, CAT, SOD), proline content and MSI in 2010-2011 and 2011-2012 growing seasons.

Component	Proportion of total variation (%)	Cumulative percent	APX	POD	CAT	SOD	proline	MSI
PC1	32.1	32.1	0.11	0.60	0.53	0.08	0.41	0.38
PC2	23.0	55.1	0.47	-0.28	-0.36	-0.41	0.46	0.41

APX: ascorbate peroxidase, POD: peroxidase, CAT: catalase, SOD: super oxide dismutase, MSI: membrane stability index.

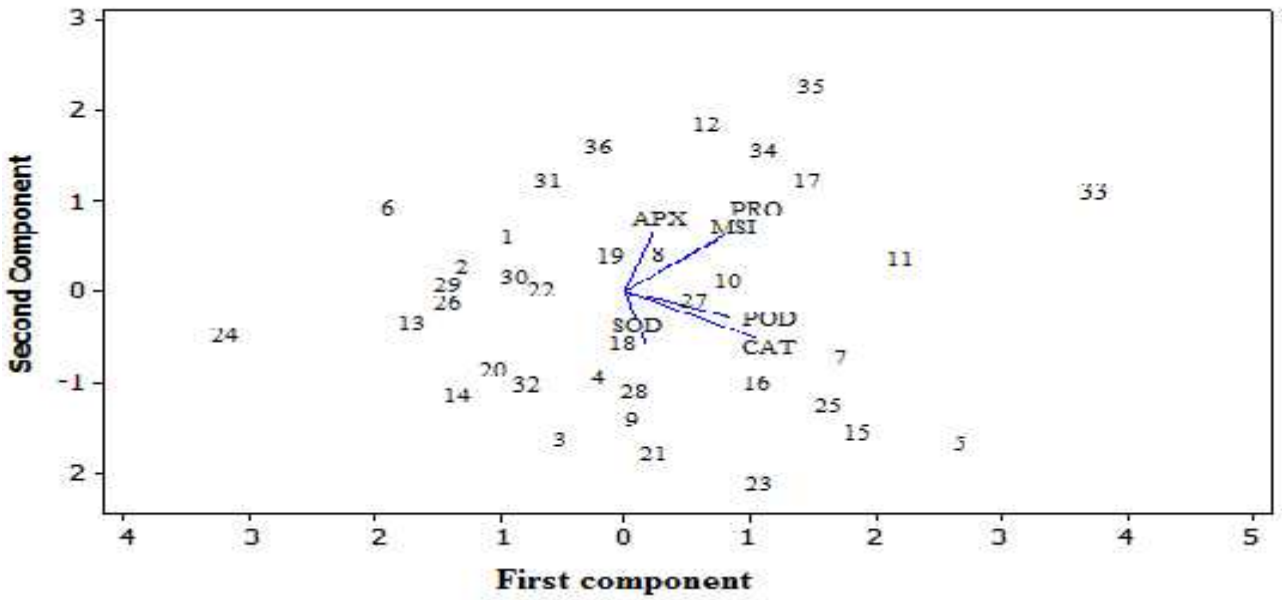


Figure 3. Biplot for anti-oxidant enzymes, proline and MSI index. APX: ascorbate peroxidase, POD: peroxidase, SOD: superoxide dismutase, CAT: catalase, PRO: proline and MSI: membrane stability index.

Conclusion: Drought stress with associated oxidative stress leads to enhanced accumulation of ROS, $O_2^-$and H_2O_2 in chloroplasts, mitochondria and peroxisomes. Therefore, plants induce anti-oxidant activity to overcome stresses (Alscher *et al.* 2002). Antioxidants prevent production of free radicals which occur by decomposition of peroxides and consequently, lead to drought tolerance (Rice-Evans and Burdon, 1994). Results of present study showed the activities of CAT, POD, APX and SOD and proline content increased significantly ($P < 0.01$) while MSI decreased significantly. Based on the results, the high yielding genotypes KC4848, KC3893 and KC3892 were categorized as tolerant. These genotypes are of great value for potential use in breeding programs. Also, it would be of interest to screen them using anti-oxidants such as malondialdehyde, glutathione, glutathione reductase, glutathione peroxidase and non-anti-oxidant enzymes such as carotenoid because of their important role in protection against stress. Additionally, genes responsible for these mechanisms can be identified in future studies which are likely to help breeders to increase plant resistance to stress.

REFERENCES

Allen, R.D. (1995). Dissection of oxidative stress tolerance using transgenic plants. J. Plant Physiol. 153: 1049-1054.

Alscher, R.G., N. Erturk, and L.S. Heath (2002). Role of superoxide dismutases (SOD) in controlling oxidative stress in plants. J. Exp. Bot. 153: 1331-1334.

Amjad, H., B. Noreen, A. Javed, and I. Nayyer (2011). Differential changes in antioxidants, proteases, and lipid peroxidation in flag leaves of wheat genotypes under different levels of water deficit conditions. J. Plant Physiol. Biochem. 49: 178-185.

Arora, A., R.K. Sairam, and G.C. Sriuastava (2002). Oxidative stress and anti-oxidative system in plants. Curr. Sci. 82: 1227-1238.

Asada, K. (2000). The water-water cycle as alternative photon and electron sinks. Philos. Trans. Royal Soc. Lond. B Biol. Sci. 355: 1419-1431.

Bailly, C. (2004). Active oxygen species and antioxidants in seed biology. Seed Sci. Res. 14: 93-107.

Bajii, M., S. Lutts, and J. Kinet (2001). Water deficit effect on solute contribution to osmotic adjustment as a function of leaf ageing in three wheat cultivars performing differently in arid conditions. Plant Sci. 160: 669-681.

Bates, L.S., R.W. Waldern, and L.D. Treare (1973). Rapid determination of free proline for stress studies. Plant Soil 23: 205-207.

Beauchamp, C., and I. Fridovich (1971). Superoxide dismutases: Improved assays and an assay predictable to acrylamide gels. Anal. Biochem. 44: 276-287.

Bhaskaran, S., R.H. Smith, and R.J. Newton (1985). Physiological change in cultured sorghum cell in response to induced water stress. J. Plant Physiol. 79: 266-269.

Blum, A. (1996). Crop responses to drought and the interpretation of adaptation. J. Plant Growth Regul. 20: 135-148.

Chance, B., and A.C. Maehly (1995). Assay of catalase and peroxidase. J. Plant Physiol. 2: 764-791.

Hanson, A.D., C.E. Nelsen, and E.H. Eversonl (1997). Evaluation of free proline accumulation as an index of drought resistance using two contrasting barley cultivars. Crop Sci. 17: 720-726.

Hidalgo, A., A. Brandolini, C. Pompei, and R. Piscozzi (2006). Carotenoids and tocols of einkorn wheat (*Triticum monococcum* L.). J. Cereal Sci. 44:182-193.

Hus, Y.T., and C.H. Kao (2007). Heat shock-mediated H_2O_2 accumulation and protection against Cd toxicity in rice seedlings. Plant Soil 300: 137-147.

Idso, S.B., R.D. Jackson, J.P.J. Pinter, R.J. Reginato, and J.L. Hatfield (1981). Normalizing the stress degree day for environmental variability. J. Agric. Res. 24: 45-55.

IPCC (Intergovermental Panel on Climate Change). (2013). Climate Change 2013. Available at http://www.ipcc.ch. [10 May 2013].

Johnson, R.C., H.T. Nguyen, and L.I. Croy (1984). Osmotic adjustment and solute accumulation in two wheat genotypes differing in drought resistance. Crop Sci. 24: 951-962.

Kim, S.Y., J.H. Lim, M.R. Park, Y. Kim, Y.S. Won, K.G. Choi, and S.J. Yun (2005). Enhanced antioxidant enzymes are associated with reduced hydrogen peroxide in barely roots under saline stress. J. Biochem. Mol. Biol. 38: 218-224.

Lascano, H.R., G.E. Antonicelli, C.M. Luna, M.N. Melchiorre, L.D. Gomez, R.W. Racca, V.S. Trippi, and L.M. Casano (2005). Antioxidant system response of different wheat cultivars under drought: Field and in vitro studies. J. Plant Physiol. 28: 1095-1102.

Martine, M., F. Micell, J.A. Morgan, M. Scalet, and G. Zerbi (1993). Synthesis of osmotically active substances in winter wheat leaves as related to drought resistance of different genotypes. J. Agron. Crop Sci. 17: 176-184.

McDonald, M.B. (2004). Seed deterioration: Physiology, repair, and assessment. Seed Sci. Technol. 27:177-237.

Mittler, R.M. (2002). Oxidative stress, antioxidant and stress tolerance. Trends Plant Sci. 7: 405-410.

Munns, R., and M. Tester (2008). Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. 59: 651-681.

Nakano, Y., and K. Asada (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22: 867-880.

Pierivatolum, J., N. Qasimov, and H. Maralian (2010). Effect of soil water stress on yield and proline content of four wheat lines. J. Biotechnol. 9: 036-040.

Renu, K.C., and S. Devarshi 2007. Acclimation to drought stress generates oxidative stress tolerance in drought-resistance than susceptible wheat cultivars under field conditions. J. Exp. Bot. 60: 276-283.

Rice-Evans, C.A., and K.M. Burdon (eds), (1994). Free Radical Damage and its Control. Elsevier, Amsterdam, pp. 389-390

Sairam, R.K. (2000). Induction of oxidative stress and antioxidant activity by hydrogen peroxide treatment in tolerant and susceptible wheat genotypes. Biol. Plant 3: 81-86.

Sairam, R.K., and D.C. Saxena (2000). Oxidative stress and antioxidant in wheat genotypes: Possible mechanism of water stress tolerance. J. Agron. Crop Sci. 184: 55-61.

Sairam, R.K., and G.C. Srivastava (2001). Water stress tolerance of wheat *Triticum aestivum* L.: Variation in hydrogen peroxide accumulation and antioxidant activity in tolerant and susceptible genotypes. J. Agron. Crop Sci. 186: 63-70.

Sairam, R.K., and G.C. Srivastava (2002). Changes in antioxidant activity in sub-cellular fractions of tolerant and susceptible wheat genotypes in response to long term salt stress. Plant Sci. 162: 897-904.

Sarvajeet, S.G., and T. Narendra (2010). Reactive oxygen species and antioxidant machinery in a biotic stress tolerance in crop plants. Plant Physiol. Biochem. 3: 1-22.

Shao, H.B., Z.S. Liang, M.A. Shao, and B.C. Wang (2005). Changes of some anti-oxidative enzymes under soil water deficits among 10 wheat genotypes at maturation stage. Colloids Surf. B Biointerfaces 45: 7-13.

Sheoran, S., V. Thakur, S. Narwal, R. Turan, H.M. Mamrutha, V. Singh, and I. Sharma (2015). Differential activity and expression profile of antioxidant enzymes and physiological changes in wheat (*Triticum aestivum* L.) under drought. Appl. Biochem. Biotechnol. 177: 1282-1298.

Siddique, M.R.B., A. Hamid, and M.S. Islam (1999). Drought stress effects on photosynthetic rate and leaf gas exchange of wheat. Bot. Bull. Acad. Sin. 40: 141-145.

Stepien, P., and G. Klobus 2005. Antioxidant defense in the leaves of C_3 and C_4 plants under salinity stress. Physiol. Plant 125: 31-40.