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Full Length Research Paper

Water stress induced changes in antioxidant enzymes, membrane stability and seed protein profile of different wheat accessions

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Water stress induced changes in antioxidant enzymes membrane stablity index and seed protein profiling of four different wheat (*Triticum aestivum* L.) accessions (011251, 011417, 011320 and 011393) were determined in a pot study under natural condition during the wheat-growing season 2005 and 2006. Sampling was done 3, 6 and 9 days after induction of water stress. Recovery was studied at 48 and 72 h of re-watering. Marked increase in leaf antioxidant enzymes associated with a decrease in membrane stability index occurred under water stress. Accession 320 showed the least increase in catalase and peroxidase activity but maximum decrease in membrane stability index. The inhibitory effects of water stress on plants were ameliorated by exogenous application of ABA and this ameliorating effect was found to be more significant at booting stage as compared to grainfilling particularly in the accession 320. The accessions 417 and 320 (which were most dissimilar on the basis of physiology under water stress, one most tolerant and the other most sensitive to water stress) showed least polymorphism among the four accessions on the basis of RAPD (Random Amplified Polymorphic DNA) analysis. Seed protein composition was found to be mainly controlled by genetic factors rather than water stress.

Key Words: Grainfilling, booting, protein, antioxidants, wheat.

INTRODUCTION

The global water crisis seriously influences crop productivity particularly in most of the Asian countries where irrigated agriculture accounts for 90% of total diverted fresh water (Huaqi et al., 2002). The intensity of the response to water stress depends on the stress severity and its duration, as well as the plant developmental stage. Wheat crop needs water for the entire period of growth, but some stages are more vulnerable to water shortage and moisture stress during this period may result in significant yield losses, noteworthy in this regard are the phases of crown root initiation, booting and early grain fill period (Anonymous, 2007). But it is considered that water stress is usually less detrimental to grain yield when occurring early in the crop cycle (Blum, 1996).

The productivity of crop plants could be increased through studying the nature of adaptation of cereal crops to water stress and by identifying the possible physiological markers for evolving the best-adapted and high yielding wheat varieties for areas affected by water stress (Mujtaba and Alam, 2002).

During water stress, there is considerable potential for increased accumulation of superoxide and hydrogen peroxide resulting from the increased rate of O_2 photoreduction in chloroplasts (Robinson and Bunce, 2000). In fact it has been reported that much of the injury to plants caused by exposure to various stresses is associated with oxidative damage at the cellular level (Allen, 1995). Mechanisms of ROS detoxification exist in all plants (Mundree et al., 2002). Changes of antioxidants reflect the impact of environmental stresses on plant metabolism (Herbinger et al., 2002). The level of response depends on the species, the development and the metabolic status of the plant, as well as the duration and intensity of the stress.

The RAPD technique (Williams et al., 1990) has been widely applied in studies of genetic diversity in wheat

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(Mukhtar et al., 2002) genotypes. Although grain protein composition is also applied in studying the genetic diversity as it depends primarily on genotype (Jha and Ohri, 1996) but it is significantly affected by environment factors and their interactions (Zhu and Khan, 2001). SDS-PAGE is a widely used method for the detection of proteins due to its simplicity and effectiveness (Javaid et al., 2004). Despite the fact that the response of protein composition to environmental factors in mature wheat grain results from changes in protein deposition during plant development, very few studies has examined the effects of water stress and the role of ABA on protein profiling of grains. Therefore this study was planned to examine effect of water stress and ABA seed soaking on protein profile; moreover the interaction of genotype and water stress was also determined. For determining the genetic variability among the four wheat accessions belonging to different geographical areas of the country, Random Amplification of Polymorphic DNA (RAPD) analysis was performed. The activity of antioxidant enzymes, which can be correlated with the plant's resistance to water stress, was also evaluated.

MATERIALS AND METHODS

The experiment was conducted in the wire house of Quaid-i-Azam University, Islamabad (latitude, 33°44′ N, longitude73° 08′ E and altitude 2021 ft) during the wheat-growing seasons of 2005 and 2006. Seeds of four selected local accessions (011251, 011417, 011320 and 011393) of wheat (*Triticum aestivum* L.), all of which are primitive cultivars/land races belonging to different areas having rainfall less than 250 m, were obtained from Plant Genetic Resource Institute (PGRI), National Agriculture Research Centre (NARC), Islamabad. These landraces were selected assuming that due to their arid origin they must have more chances to be adaptive to water stress conditions. The seeds were sown in earthen pots (20 x 30 cm^2) containing soil, sand and farmyard manure in a ratio of 3:1:1. Recommended doses of nitrogen phosphorus and potassium fertilizers were applied. Pots were arranged in Randomized Complete Block Design (RCBD) and were protected from rain. A week after germination the plants were thinned to five per pot. The plants were watered as required. Prior to sowing, surface sterilization of seeds was done with 10% (v/v) chlorox followed successive washings with distilled water. Prior to sowing, seeds were soaked for 8 h in aqueous solution of ABA (10^{-6} M) and for control seeds were soaked in sterilized water for equal period of time. Water stress was imposed by withholding water supply for a period of 9 days and thereafter the plants were re-watered. The first water stress treatment was started at 50% booting (85 - 95 DAS) and the second at 50% grainfilling (125 - 140 DAS).

Leaf relative water content

The leaves from plants in the pots were harvested. Then fresh weight of these harvested leaves was recorded. The leaves were then immersed in distilled water in beakers and left for 24 h. Thereafter, fully turgid leaves were again weighed. Then leaves were dried in oven for 72 h at 7°C, until constant weight of leaves was obtained.

Relative water content (RWC) of flag leaf was estimated according to Weatherley (1950) and calculated as follows:

RWC = [(fresh mass - dry mass)/(saturated mass - dry mass)] × 100.

Assays of antioxidant enzymes

Extraction

For extracting antioxidant enzymes, leaves (0.5 g) were ground in 5 ml. of 50 mM potassium phosphate buffer (pH 7) in pastel and mortar placed in ice bath. The homogenate was centrifuged at 13000 g for 20 min at 4°C. The supernatant was used for assay of the activities of enzymes.

Peroxidase activity

The POD activity was measured by the method of Vetter et al. (1958) as modified by Gorin and Heidema (1976). The assay mixture contained 100 µl enzyme extract, 1.35 mL 100 mM MES buffer (pH 5.5), 0.05% H_2O_2 and 0.1% ρ-phenylenediamine. Changes in absorbance were recorded at 485 nm for 3 min with the spectrophotometer. The one unit of POD was defined as change in OD 485 nm /min. The activity of POD was presented as units/g fwt of leaves.

Catalase activity

Catalase activity was assayed by measuring the disappearance of H_2O_2 (Teranishi et al., 1974). The reaction mixture (3 mL.) contained 50 mM phosphate buffer (pH 7.5) 20 mM H_2O_2 and 0.1 mL enzyme extract. The reaction was stopped after 5 min by the addition of 2 mL titanium reagent (20% (v/v) titanium tetrachloride in Conc HCl), which also formed coloured complex with the residual H_2O_2. Aliquot was centrifuged at 10000 rpm for 10 min and the absorbance of the supernatant was recorded at 410 nm.

Membrane stability index (MSI)

Leaf membrane stability index (MSI) was determined according to the method of Premchandra et al. (1990) as modified by Sairam (1994). Leaf discs (100 mg) were thoroughly washed in running tap water followed by washing with double distilled water thereafter the discs were heated in 10 ml of double distilled water at 40°C for 30 min. Then electrical conductivity (C1) was recorded by EC (Electrical Conductivity) meter. Subsequently the same samples were placed in a boiling water bath (100 °C) for 10 min and their electrical conductivity was also recorded (C2). The MSI was calculated as:

Membrane stability index (MSI) = [1 - (C1/ C2)] × 100.

The data were subjected to factorial ANOVA and the mean values were compared with Duncan's Multiple Range Test (DMRT) using MSTAT-C version 1.4.2.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Seeds were ground with the help of mortar and pestle. To 10 mg seed flour 400 µl of protein extraction buffer (0.05 M Tris-HCl pH 8.0, 0.2% (w/v) SDS, 5 M Urea, 1% (v/v) ß- mecaptoethanol) was added. The contents were vortexed and stored at -20°C till further analysis. One dimentional SDS-PAGE was carried out according to the method of Laemmli (1970) in a linear Polyacrylamide resolving gel (12.25%) and with stacking gel (4.5 %). After centrifugation of sample at 13, 000 rpm for 10 min. 15 µl of the sample was loaded

Table1. Sequence of 15 random primers used for RAPD analysis.

No.	Primer code	Primer Sequence
1	OPA-05	5′-AGGGGTCTTG-3′
2	OPA-10	5′-GTGATCGCAG-3′
3	OPA-11	5′-CAATAGCCGT-3′
4	OPB-01	5′-GTTTCGCTCC-3′
5	OPB-11	5′-GTAGACCCGT-3′
6	OPC-07	5′-GTCCCGACGA-3′
7	OPC-10	5′-TGTCTGGGTG-3′
8	OPD-08	5′-GTGTGCCCCA-3′
9	OPF-06	5′-GGGAATTCGG-3′
10	OPF-15	5′-CCAGTACTCC-3′
11	OPJ-01	5′-CCCGGCATAA-3
12	OPJ-08	5′-CATACCGTGG-3′
13	OPK-12	5′-GTGCAACGTG-3′
14	OPK-15	5′-CTCCTGCCAA-3′
15	OPK-17	5′-CCCAGCTGTG-3′

into the well. Protein molecular weight marker was also loaded in the similar manner and electroporesed at 100 V until the dye front reached the bottom of the gel. Gels were removed from the plates and were shaken in staining solution (440 ml methanol, 60 ml acetic acid, 500 ml distilled water, 2.25 g Coomassie Brilliant Blue) for 2 h, then transferred to a destaining solution (200 ml methanol 50 ml acetic acid and 750 ml distilled water) and left for shaking for several hours until the protein bands appeared. The gels were observed under gel documentation system (S.N. 76 S/ 04069, Bio-RAD, Italy) and photographed.

Random amplified polymorphic DNA (RAPD) analysis

Total genomic DNA was extracted from dried seeds of each accession according to the method described by Kang et al. (1998) with minor modification. Seeds (3 - 5) were placed in a micro centrifuge tube (1.5 ml.). 400 µl of extraction buffer (200 mM Tris-HCl (pH 8.0), 25 mM EDTA, 200 mM NaCl, 0.5% (w/v) SDS) containing Proteinase K (50 µg) was added to it. Tubes were incubated at 37°C for 1 h and seeds were ground in the extraction buffer with a glass rod. 400 µl of 2% CTAB solution (100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2 % (w/v) CTAB, 1% (w/v) PVP “polyvinylpyrrolidone 40,000) was added. Extraction was done in chloroform and isoamyl alcohol in the ratio of 24:1 with 5% (v/v) phenol. Samples were centrifuged at 12,000 rpm for 10 min at 4°C. Two third volume of isopropanol was added to the supernatant and tubes were incubated at room temperature for 10 min to precipitate DNA. Tubes were centrifuged at 12,000 rpm for 5 min and supernatant was removed. The DNA pellets were washed with 70% Ethanol (500 µl) and centrifuged at 12,000 rpm for 5 min. at room temperature. The DNA pellet so obtained was air-dried for 5 - 10 minutes and re-suspended in 100 µl of TE buffer, RNA was removed by adding 1 µl of RNase (10 mg/ml).

After isolation of DNA from dried seed samples, DNA concentration and purity of each genotype was determined spectrophotometrically at a wavelength of 260 and 280 nm using NanoDrop ND-1000 Spectrophotometer. The ratio between absorbance at 260 and 280 nm (260/280) was used to estimate DNA purity. DNA of each accession was diluted to a working concentration of 15 ng/µl for PCR/RAPD analysis.

A modified RAPD method based on Williams et al. (1990) was used with a thermal cycler (model 9700, Applied Biosystems, USA). After standardization of PCR, 20 µl reaction mixture containing 1x PCR buffer [10 mM Tris HCl (pH 8.3), 50 mM KCl], 1.5 mM $MgCl_2$, 200 µM each deoxynucleotide triphosphate (dNTP), 0.4 µM of 10-mer primer (Table 1) (Operon Technologies Inc., Alameda, CA), 1 unit AmpliTaq Gold DNA polymerase and approximately 15 ng of template DNA was found optimum for the amplification of wheat genomic DNA. Taq DNA polymerase and reaction buffer were purchased from Applied Biosystems, Japan. DNA amplification was performed in DNA thermal cycler. The thermal cycler was programmed to 1 cycle of 5 min at 94°C for initial strand separation. This was followed by 45 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 36°C and primer extension for 2 min at 72°C. Finally, 1 cycle of 7 min at 72°C was used for final extension, followed by soaking at 4°C.

After amplification, 3 µl of gel loading dye buffer (0.02% bromophenol blue, 0.02% (w/v) xylene cyanol FF, 50% (v/v) glycerol and 1% (w/v) SDS) were added directly to the reaction tubes and spun for a few seconds in a micro centrifuge after mixing with the entire reaction mixtures. Aliquots of amplification products (15 µl) plus loading dye were then loaded in 1.5% agarose gels for electrophoresis in 1 x TBE (10 mM Tris-Borate, 1 mM EDTA) buffer and run at 100 V for 40 min to separate the amplified products. 1 Kb DNA was used as a molecular weight marker. After electrophoresis, the gels were photographed under UV light using black and white film # 667 (Polaroid, Cambridge, Mass., USA).

Data analysis

Photographs from ethidium bromide stained agarose gels were used to score the data for RAPD analysis. Each DNA fragment amplified by a given primer was treated as a unit character and the RAPD fragments were scored as present (1) or absent (0) for each of the primer-accession combinations. Since DNA samples consisted of a bulk sample of DNA extracted from 5 - 10 seeds, a low intensity for any particular fragment may be explained by the lesser representation of that specific sequence in the bulk sample of DNA. Therefore the intensity of the bands was not taken into account and the fragments with the identical mobility were considered to be the identical fragments. Only major bands were scored.

The presence and absence of the bands was scored in a binary data matrix. Pair-wise comparisons of the accessions based on the presence or absence of unique and shared amplification products were used to generate similarity coefficients. The resulting similarity coefficients were used to evaluate the relationships among accessions with a cluster analysis using an un-weighted pair-group method with arithmetic averages (UPGMA) and then plotted in the form of a dendrogram. All computations were carried out using the computer program NTSYS, version 2.1 (Applied Biostatistics Inc., USA).

RESULTS

Relative water content

The analysis of data (Table 2) showed that with the increase in the duration of water stress period there was a progressive decrease in the relative water content of flag leaves.

At booting stage maximum reduction (16%) in RWC was found in the leaves of accession 251 (Table 2) after 3 d (SMC = 12.2 - 13.1%) induction of water stress

Table 2. Effect of water stress and abscisic acid (ABA) on Relative Water Content (%) of leaves at booting and grainfilling stages of four wheat accessions.

	Accession 251					Accession 417					Accession 320					Accession 393				
Treatments	**3d**	**6d**	**9d**	**48h (rw)**	**72h (rw)**	**3d**	**6d**	**9d**	**48h (rw)**	**72h (rw)**	**3d**	**6d**	**9d**	**48h (rw)**	**72h (rw)**	**3d**	**6d**	**9d**	**48h (rw)**	**72h (rw)**
T_{0a}	74.9 a	70.9 bcd	74.6 a	76.5 a	75.9 a	73.3 hij	77.1 def	79.01 abcd	74.5 ghi	77.2 cdef	62.1 fgh	60.1 gh	67.7 bc	69.3 ab	62.3 Efg	73.2 ab	72.1 bcd	69.9 def	75.3 a	70.1 cdef
T_1	63 jk	51.3 q	40.2 t	58.3 bcde	69.5 bc	67 mn	70.1 kl	55.2 s	69.2 lm	72.1 ijk	53.1 kl	42.1 rs	27.3 v	40.9 s	42.3 Rs	60.1 no	49.2 r	39.3 tu	54.9 p	65.9 hijk
T_2	71.1 bc	71.5 b	71.9 b	68.9 bcde	67.6 efg	78.5 bcde	77.4 cdef	76.3 efg	75.6 fgh	78.6 bcde	66.1 cd	62.9 ef	70.1 a	69.3 ab	68.9 Ab	72.1 bcd	73.9 ab	71.9 bcd	75.2 a	72.3 bc
T_3	66 fgh	56.7 nop	44.7 s	69.3 cdef	70.1 bcde	79.6 abc	69.7 kl	60.7 qr	80.2 ab	81.1 A	55.9 j	49.3 mno	38.5 t	50.9 lm	55.4 Jk	62.3 mn	53.4 pq	48.2 r	58.3 o	70.2 cde
T_{0b}	65.9 ghi	63.3 ijk	62.1 kl	68.3 defg	64.9 hij	69.7 kl	70.1 kl	72.1 ijk	70.3 kl	73.1 Ij	60.2 gh	57.3 ij	59.7 h	58.9 hi	57.2 Ij	67.3 ghij	68.4 efg	65.2 jkl	64.3 lm	64.1 klm
T4	59.3 mn	47.2 rs	37.8 t	55.4 p	59.3 mn	64.2 op	56.3 s	45.2 u	62.1 pq	65.1 No	49.2 mno	40.1 st	29.3 v	35.3 u	46.3 Pq	58.3 o	45.5 s	38.1 u	52.1 q	55.3 p
T_5	68.7 cde	71.2 bc	69.3 bcde	68.4 defg	68.8 cde	70.2 kl	71.3 jkl	71.2 jkl	73.2 ij	71.1 Jkl	62.3 efg	64.5 de	63.1 ef	61.1 fgh	59.9 H	68.2 efgh	69.3 efg	67.5 ghij	65.6 Ijkl	67.8 fghi
T_6	62.1 kl	49.1 qr	40.1 t	56.4 op	60.3 lm	64.3 op	59.3 r	48.4 t	64.2 op	64.9 No	50.1 mn	44.2 qr	33.1 u	47.3 op	48.1 nop	58.1 o	45.2 s	41.3 t	55.3 p	60.2 no
LSD values	2.672 at alpha = 0.05					2.487 at alpha = 0.05					2.370 at alpha = 0.05					2.381 at alpha = 0.05				

T0a = control at booting, T1 = water stress at booting, T2 = ABA seed soaking (booting), T3 = water stress at booting + ABA seed soaking, T0b = control at grainfilling, T4 = water stress at grainfilling, T5 = ABA seed soaking (grainfilling), T6 = water stress at grainfilling +ABA seed soaking, d = days after induction of water stress, rw = rewatering.
All such means, which share common letters, do not differ significantly.

Below 50% , 50 - 70% , Above 70%

followed by accession 320 (Table 2) in which case a reduction of about 14% was noted as compared with the control. But in the longer duration of water stress (after 6 (SMC = 8.2 - 9.5%) and 9 d (SMC = 6.9 - 7.3%) of water stress) maximum reduction (30 - 60%) was found in accession 320 (Table 2). Significantly ($P < 0.05$) less decrease in RWC was observed in accession 417 (Table 2) as compared to all other accessions which showed only 8-9% reduction after 6 d of water stress as compared to control plants and 30% decrease after 9 d of water stress period. The recovery after re-watering was significantly low in accession 320 (Table 2) as compared to other three accessions. While maximum and rapid recovery was found in accession 417 in which case after 48 h of re-watering the value of RWC remained only 7% below control level (Table 2) while in other accessions recovery was slow. The RWC remained significantly higher during stress period in the leaves of plants pre-treated with ABA (10^{-6} M) than the untreated control. Accession 320 (Table 2) appeared to be the most responsive to ABA followed by accession 393, as these were able to maintain significantly ($P < 0.05$) higher RWC under water stress when treated with ABA. While accession 417 was the least responsive to ABA among all the accessions.

At grainfilling stage changes in RWC of flag leaves were similar to that of booting stage but the RWC of leaves was significantly ($P < 0.05$) lower at grainfilling stage under all the treatments in all the accessions. Moreover the magnitude of recovery observed after re-watering the stressed plants was also significantly less at grainfilling stage as compared to recovery observed at booting stage. The effect of ABA was less prominent at grainfilling stage in all the accessions.

Table 3. Effect of water stress and abscisic acid (ABA) on peroxidase (POD) activity (Units/ g fwt.) of leaves at booting and grainfilling stages of four wheat accessions.

	Accession 251					Accession 417					Accession 320					Accession 393				
Treatments	**3d**	**6d**	**9d**	**48h (rw)**	**72h (rw)**	**3d**	**6d**	**9d**	**48h (rw)**	**72h (rw)**	**3d**	**6d**	**9d**	**48h (rw)**	**72h (rw)**	**3d**	**6d**	**9d**	**48h (rw)**	**72h (rw)**
T_{0a}	30.5	29.9 nop	30.1 mnop	28.7 p	30.4 mnop	26.2 lmno	25.4 lmno	28.3 klm	24.3 mnop	27.4 lmn	32.3 nop	30.2 pqr	28.9 qr	33.4 lmno	27.7 r	34.1 gh	29.3 lm	28.4 m	31.5 hijkl	30.4 jklm
T_1	41.9 j	53.2 f	65.3 b	59.2 cd	44.1 ij	34.1 ijk	47.4 e	73.4 bc	39.1 fghi	33.7 jkl	37.3 ijk	42.1 ef	50.3 c	44.3 de	38.4 hij	42.1 ef	55.2 d	64.9 b	56.3 cd	40.6 f
T_2	32.3 klmn	28.9 op	31.7 lmnop	30.7 mnop	33.1 klmn	24.9 mnop	27.3 klmn	25.9 lmno	26.4 lmno	25.8 lmno	29.9 pqr	33.4 lmno	32.5 mnop	31.6 opq	28.8 qr	34.8 g	32.9 ghijk	30.7 ljklm	32.1 ghijkl	29.3 lm
T_3	49.3 g	59.2 cd	70.2 a	56.4 de	45.4 hi	36.2 ijk	47.9 de	75.5 bc	40.9 fgh	32.3 jkl	45.9 d	50.7 c	57.2 a	43.7 de	39.2 ghi	44.3 e	58.7 c	67.4 ab	57.1 cd	43.2 ef
T_{0b}	33.2 klmn	32.1 klmno	31.9 klmnop	34.1 kl	33.3 klm	25.9 lmno	24.3 mnop	26.1 lmno	25.3 mno	24.8 mnop	35.2 klmn	36.1 jkl	34.9 klmn	35.6 jkl	34.3 lmno	33.3 ghij	29.9 klm	34.1 gh	32.3 ghijkl	33.7 ghi
T4	43.1 ij	54.2 ef	66.3 b	60.1 c	47.3 gh	40.4 gh	50.2 d	77.3 a	41.2 fgh	35.5 ijkl	40.2 fgh	44.4 de	53.9 b	49.2 c	42.1 ef	44.2 e	57.1 cd	66.8 ab	55.6 d	42.4 ef
T_5	31.9 klmnop	33.1 klmn	34.3 kl	35.2 k	34.9 kl	27.1 klmn	26.3 lmno	25.8 lmno	27.2 lmn	24.9 mnop	34.2 lmno	33.9 lmno	35.4 klm	36.1 jkl	35.2 klmn	34.2 gh	33.3 ghij	31.9 ghijkl	34.1 gh	32.8 ghijk
T_6	44.3 ij	56.2 de	66.9 b	62.1 c	47.9 gh	41.1 fgh	51.3 d	78.2 a	42.3 fg	35.4 ij	42.1 ef	45.3 d	53.6 b	50.1 c	41.7 efg	45.1 e	56.9 cd	68.2 a	57.4 cd	43.7 e
LSD values	2.814 at alpha= 0.050					2.097 at alpha= 0.050					2.550 at alpha= 0.050					2.682 at alpha= 0.050				

T0a= control at booting, T1= water stress at booting, T2= ABA seed soaking (booting), T3 =water stress at booting + ABA seed soaking, T0b= control at grainfilling, T4 =water stress at grainfilling, T5= ABA seed soaking (grainfilling), T6 =water stress at grainfilling +ABA seed soaking, d= days after induction of water stress, rw= rewatering. All such means which share common letters do not differ significantly.

Above 60 = , 40 – 60 = , below 40 =

Peroxidase (POD) activity

The basal level of POD was found to be different among the accessions being minimum in accession 417 (Table 3). On imposition of water stress at booting stage, a linear increase was observed in POD activity in all the accessions. During short term of water stress (3 d of water stress) maximum increase (37%) in POD activity was recorded in accession 251 (Table 3) and after 6 d of water stress treatment maximum increase (88%) was observed in accession 393 (Table 3) while during long term water stress (after 9 d of water stress) accession 417 (Table 3) exhibited maximum increase (159%). The POD activity was minimum in accession 320 under water stress (Table 3). The decrease in POD activity on re-watering was greater and earlier in accession 417 (Table 3). ABA did not show significant effect on POD activity under unstressed condition. But under water stress condition, ABA treatment significantly increased the POD activity in the leaves of accession 251 (Table 3) and accession 320 (Table 3) at booting stage whereas in other two accessions there occur no significant effect of ABA under water stress.

At grainfilling stage under unstressed condition no significant ($P < 0.05$) difference was noted in POD activity as compared to that of booting stage. Under water stress condition in accession 251 (Table 3) percentage increase in POD activity was significantly ($P < 0.05$) lower as compared to that of booting stage but accession 417 (Table 3) showed greater POD activity at grainfilling under water stress. Whereas, in accession 320 (Table 3) higher POD activity was recorded under un-stresses condition at grainfilling compared to that of booting but under stress treatment percentage increase was less.

Table 4. Effect of water stress and abscisic acid (ABA) on catalase activity (µmol H_2O_2 reduced /g fwt.) of leaves at booting and grainfilling stages of four wheat accessions.

	Accession 251					Accession 417					Accession 320					Accession 393				
Treatments	**3d**	**6d**	**9d**	**48h (rw)**	**72h (rw)**	**3d**	**6d**	**9d**	**48h (rw)**	**72h (rw)**	**3d**	**6d**	**9d**	**48h (rw)**	**72h (rw)**	**3d**	**6d**	**9d**	**48h (rw)**	**72h (rw)**
T_{0a}	2.35 vw	2.4 uv	2.34 w	2.39 uvw	2.42 Tu	2.35 o	2.41 lmn	2.37 no	2.42 klmn	2.38 mno	2.39 o	2.41 no	2.37 o	2.42 mno	2.39 o	2.29 no	2.31 lmn	2.32 lmn	2.25 o	2.3 mno
T_1	3.1 l	3.7 i	4.45 cd	4.42 d	4.01 f	3.03 i	3.69 g	4.47 a	4.08 c	3.95 e	2.52 jk	2.93 g	3.22 c	3.2 c	3.17 c	2.95 j	3.62 h	4.34 abc	4.3 bc	4.09 f
T_2	2.51 pqrs	2.47 st	2.55 nop	2.49 qrs	2.52 opqrs	2.47 jkl	2.45 jkl	2.49 j	2.48 jk	2.45 jkl	2.45 lmn	2.47 jklm	2.46 klmn	2.49 jkl	2.47 jklm	2.34 k	2.34 klmn	2.35 klmn	2.36 kl	2.35 klmn
T_3	3.17 k	3.91 h	4.52 b	4.45 cd	4.13 e	3.09 h	3.71 fg	4.5 a	4.1 b	3.93 d	2.71 h	2.99 ef	3.35 a	3.29 b	3.2 c	3.01 i	3.65 gh	4.38 a	4.32 bc	4.13 ef
T_{0b}	2.53 nopqr	2.49 qrs	2.55 nop	2.57 mno	2.48 rs	2.45 jkl	2.41 lmn	2.44 jklm	2.43 jklm	2.44 lmn	2.51 jkl	2.49 klmn	2.5 jkl	2.48 jkl	2.53 j	2.33 klmn	2.34 klmn	2.3 mno	2.34 klmn	2.35 klmn
T4	3.24 j	3.95 gh	4.63 a	4.5 bc	4.45 cd	3.14 h	3.75 f	4.48 a	4.17 b	4.02 d	2.62 i	2.75 h	3.04 de	2.98 fg	2.93 g	3.02 i	3.69 g	4.35 ab	4.32 bc	4.16 de
T_5	2.54 nopq	2.48 rs	2.58 mn	2.61 m	2.57 mno	2.47 jkl	2.45 jklm	2.44 jklm	2.41 lmn	2.43 jklm	2.49 jkl	2.52 jk	2.48 jkl	2.53 j	2.49 jkl	2.34 klmn	2.36 Klm	2.32 lmn	2.3 mno	2.34 klmn
T_6	3.22 jk	3.97 fg	4.59 a	4.51 b	4.43 d	3.14 h	3.72 fg	4.49 a	4.2 b	4.01 d	2.64 i	2.74 h	3.08 d	3 ef	2.97 fg	2.99 ij	3.7 g	4.34 abc	4.29 c	4.19 d
LSD values	0.0514 at alpha = 0.050					0.01817at alpha = 0.050					0.05140at alpha = 0.050					0.05140 at alpha= 0.050				

T0a = control at booting, T1 = water stress at booting, T2 = ABA seed soaking (booting), T3 = water stress at booting + ABA seed soaking, T0b = control at grainfilling, T4 = water stress at grainfilling, T5 = ABA seed soaking (grainfilling), T6 = water stress at grainfilling +ABA seed soaking, d = days after induction of water stress, rw = rewatering
All such means which share common letters do not differ significantly.

Above 4 = , 3 - 4 = , below 3 =

Catalase (CAT) activity

The basal level of catalase was lower in accession 393 as compared to other accessions. Activity of catalase (CAT) was significantly ($P < 0.05$) increased with the increase in the duration of water stress at both booting and grainfilling stages. At booting no significant ($P < 0.05$) difference was observed among the accessions in terms of percentage increase (29 - 90%) during water stress except for accession 320 (Table 4) in which case after 9 d of water stress minimum increase (36%) was observed as compared to other accessions. ABA seed soaking treatment had significantly ($P < 0.05$) stimulated the CAT activity in accession 251 (Table 4) and accession 320 (Table 4); the magnitude of stimulation was higher in accession 320 (Table 4). While in other two accessions no significant ($P < 0.05$) effect of ABA seed soaking was observed. On re-watering, the CAT activity was decreased in all the accessions showing maximum decrease in accession 417 (Table 4). This decrease was rapid within first 48 h of re-watering which slowed down thereafter.

At grainfilling stage, similar changes in CAT activity were recorded as that of booting. But the CAT activity was higher at grainfilling than that of booting. ABA seed soaking treatment had no significant ($P < 0.05$) effect on CAT activity at grainfilling stage.

Membrane stability index (MSI)

Water stress had significant ($P < 0.05$) effect on membrane stability index (MSI) (Table 5). Linear decrease occurred in membrane stability index with the increase in the duration of stress. Significantly ($P < 0.05$) higher MSI values were

Table 5. Effect of water stress and abscisic acid (ABA) on membrane stability index (MSI) (%) of leaves at booting and grainfilling stages of four wheat accessions.

	Accession 251					Accession 417					Accession 320					Accession 393				
Treatments	**3d**	**6d**	**9d**	**48h (rw)**	**72h (rw)**	**3d**	**6d**	**9d**	**48h (rw)**	**72h (rw)**	**3d**	**6d**	**9d**	**48h (rw)**	**72h (rw)**	**3d**	**6d**	**9d**	**48h (rw)**	**72h (rw)**
T_{0a}	78.2 bc	80.1 a	79.3 ab	78.5 bc	79.4 ab	85.4 ab	84.9 abc	85.2 ab	84.8 abc	85.7 a	75.4 def	74.9 efg	75.6 cde	75.2 def	74.1 fgh	86.3 ab	85.9 ab	84.8 bcd	85.2 bc	85.5 ab
T_1	72.4 g	63.5 j	57.4 p	59.2 mno	62.1 jk	76.7 f	69.9 i	60.2 m	66.8 j	70.2 i	69.1 k	57.2 n	49.2 tu	51.4 rs	54.3 op	77.2 g	70.3 j	61.7 o	67.9 k	71.7 i
T_2	79.5 ab	80.5 a	80.4 a	78.5 bc	79.2 ab	84.7 abc	85.3 ab	85.9 a	84.8 abc	85.6 a	76.9 abc	77.1 ab	76.5 abcd	77.8 a	76.2 bcde	85.9 ab	86.8 a	85.2 bc	85.7 ab	86.3 ab
T_3	75.2 ef	70.5 h	63.2 j	67.5 i	70.1 h	76.8 f	71.2 hi	62.3 l	67.1 j	72.2 h	72.3 j	59.3 m	53.2 pq	55.1 o	57.3 n	79.2 f	72.3 i	64.2 mn	69.7 j	74.2 h
T_{0b}	75.5 def	76.2 def	77.3 cd	75.5 ef	74.9 f	83.5 cd	82.9 de	83.4 cd	81.8 e	83.1 de	73.2 hij	72.9 hij	73.1 hij	72.8 hij	72.5 ij	83.9 cde	82.7 e	82.4 e	83.7 de	82.8 e
T4	68.3 i	59.9 mn	53.5 q	57.9 op	60.3 lm	73.9 g	66.8 j	57.2 n	63.1 kl	65.9 j	65.2 l	55.1 o	47.8 v	49.1 tu	52.2 qr	72.5 i	65.5 lm	57.8 p	63.2 n	68.3 k
T_5	76.5 de	75.4 ef	76.2 def	76.5 de	74.9 f	82.9 de	83.4 cd	82.7 de	83.5 cd	83.9 bcd	73.8 ghi	73.2 hij	72.9 hij	72.5 ij	73.6 ghij	82.6 e	83.5 de	82.4 e	83.6 de	82.9 e
T_6	68.2 i	58.4 op	54.1 q	58.7 nop	61.4 kl	74.1 g	67.2 j	58.1 n	63.7 k	66.3 j	65.5 l	56.9 n	48.2 uv	50.2 st	53.9 op	73.1 hi	66.2 l	57.5 p	62.9 no	69.1 jk
LSD values	1.363 at alpha= 0.050					1.322 at alpha= 0.050					1.203 at alpha= 0.050					1.305 at alpha= 0.050				

T0a = control at booting, T1 = water stress at booting, T2 = ABA seed soaking (booting), T3 = water stress at booting + ABA seed soaking, T0b = control at grainfilling, T4 = water stress at grainfilling, T5 = ABA seed soaking (grainfilling), T6 = water stress at grainfilling +ABA seed soaking, d = days after induction of water stress, rw = rewatering.
All such means which share common letters do not differ significantly.

Below 55% = , 55 - 70% = , above 70% =

observed in accession 417 (Table 5) and accession 393 (Table 5) under water stress as com-pared to other accessions. At booting stage, after 3 d of water stress minimum decrease (7%) in membrane stability index (MSI) values was noticed in accession 251 (Table 5) and maximum (10%) in accession 393 (Table 5). While after 6 d and 9 d of water stresses, maximum decreases were recorded in accession 320 (Table 5). Re-watering the water stressed plants improved the membrane stability index (MSI) to some extent, the maximum increase in MSI over that of stressed condition was found in accession 393 (Table 5) but accession 320 showed least increase (Table 5). Under control condition ABA seed soaking treatment did not affect the mem-brane stability index (MSI) values significantly ($P < 0.05$) except for accession 320 in which case upto 3% higher MSI values were observed due to ABA treatment. Under water stress condition ABA seed soaking helped to maintain significantly higher MSI at booting stage, accession 417 (Table 5) being the exception for which significant ($P < 0.05$) effect of ABA was noticed only after 9 d of water stress.

Membrane stability index (MSI) values were significantly ($P < 0.05$) lower at grainfilling as compared to booting. But the pattern of changes was similar as that of booting stage.

Protein profiling of grains by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The variations in banding pattern (Figure 1) were more pronounced in genotypes but the effects of treatments were less. The SDS-PAGE results revealed no effects of treatments on the protein

Table 6. Distribution pattern of protein bands in four wheat accessions under different treatments.

Treatments	1	2	3	4	5	6	7	8	9	10	11	12	13
Approximate MW in KDa.	**130**	**110**	**100**	**95**	**80**	**70**	**65**	**60**	**55**	**50**	**47**	**43**	**35**
V1T0	-	+	+	-	+	-	+	+	-	+	+	-	+
V1T1	-	+	+	-	+	-	+	+	-	+	+	-	+
V1T2	-	+	+	-	+	-	+	+	-	+	+	-	+
V1T3	-	+	+	-	+	-	+	+	-	+	+	-	+
V1T4	-	+	+	-	+	-	+	+	-	+	+	-	+
V1T5	-	+	+	-	+	-	+	+	-	+	+	-	+
V2T0	-	+	-	+	+	-	+	+	+	+	+	+	+
V2T1	-	+	-	+	+	-	+	+	+	+	+	+	+
V2T2	-	+	+	-	+	-	+	+	+	+	+	+	+
V2T3	-	+	-	+	+	-	+	+	+	+	+	+	+
V2T4	-	+	+	-	+	-	+	+	+	+	+	+	+
V2T5	-	+	+	-	+	-	+	+	+	+	+	+	+
V3T0	+	+	-	+	+	+	+	+	+	+	+	+	+
V3T1	+	+	+	+	+	+	+	+	+	+	+	+	+
V3T2	+	+	+	+	+	+	+	+	+	+	+	+	+
V3T3	+	+	-	+	+	+	+	+	+	+	+	+	+
V3T4	+	+	+	+	+	+	+	+	+	+	+	+	+
V3T5	+	+	-	+	+	+	+	+	+	+	+	+	+
V4T0	+	+	-	+	-	+	+	+	+	+	+	+	+
V4T1	+	+	-	+	+	+	+	+	+	+	+	+	+
V4T2	+	+	-	+	+	+	+	+	+	+	+	+	+
V4T3	+	+	-	+	+	+	+	+	+	+	+	+	+
V4T4	+	+	-	+	+	+	+	+	+	+	+	+	+
V4T5	+	+	-	+	+	+	+	+	+	+	+	+	+

(+) = Presence; (-) = absence.
V1 = 251, V2 = 417, V3 = 320, V4 = 393.
T0 = control T1 = water stress at booting, T2 = water stress at grainfilling T3 = ABA seed soaking T4 = water stress at booting + ABA seed soaking, T5 = water stress at grainfilling +ABA seed soaking.

banding patterns in accessions 251 and 393.

In accession 417 band number 3 (MW ≅ 100 KDa) was present in the grains obtained from plants exposed to water stress at grainfilling stage and also in grains from the plants treated with ABA and exposed to water stress at booting and grainfilling stages. While band No.4 (MW ≅ 95 KDa) was absent (Table 6) in these treatments. This band (4) along with band 1 (MW ≅ 130), band 9 (MW ≅ 50 KDa) and band 12 (MW ≅ 40 KDa) were also absent from the grains of accession 251 (Table 6). In case of accession 320 band 3 was absent (Table 6) from grains of control plant also from plants exposed to water stress at grainfilling (Table 6) Accession 393 also lack this band along with band no 5 (MW ≅ 80 KDa).

Random amplification of polymorphic DNA (RAPD)

Genetic diversity among 4 wheat accessions was investigated at DNA level using randomly amplified polymorphic DNA (RAPD) technique. A band was considered as polymorphic if the band differentiates at least any 2 of the 4 accessions. The 4 wheat accessions evaluated in this study were evaluated genetically using 15 RAPD primers which generated a total of 75 RAPD bands. Of these bands, 37 were found to be polymorphic across the 4 wheat accessions. The number of amplification products per primer varied from 3 to 6, with a mean of 5 (Table 7) These primers produced fragments, which fall in the range of 250 to 2000 bp in size (Figure 2) The genetic similarities based on RAPD patterns have been presented in the form of similarity coefficient in Table 8 and Figure 3. The calculated coefficient of similarity between all accessed genotypes varied between 0.627 and 0.76. Maximum coefficient of similarity (Table 8) was recorded between the accession 320 and 417 and minimum similarity co-efficient (Table 8) was recorded between accessions 251 and 393. The lower values of similarity index indicated the genetic diversity among the accessions.

DISCUSSION

Oxidative injury at the cellular level as a result of water or

Table 7. Detected polymorphism of 15 selected primers for RAPD analysis.

No.	Primer code	No. of amplified bands	No. of polymorphic bands	Degree of Polymorphism (%)
1	OPA-05	6	6	83.3
2	OPA-10	4	2	50
3	OPA-11	5	2	40
4	OPB-01	5	5	100
5	OPB-11	3	1	33.3
6	OPC-07	4	2	50
7	OPC-10	5	0	0
8	OPD-08	6	5	83.3
9	OPF-06	6	5	83.3
10	OPF-15	4	3	75
11	OPJ-01	5	1	20
12	OPJ-08	6	2	33.3
13	OPK-12	5	1	20
14	OPK-15	6	3	50
15	OPK-17	5	2	40
	Total	75	40	53.3

Table 8. Coefficient of similarity among the four wheat accessions.

Accessions	251	417	320	393
251	1			
417	0.746667	1		
320	0.693333	0.76	1	
393	0.626667	0.693333	0.746667	1

other stresses (temperature, pollution etc.) is a major cause of crop damage (Allen, 1995). Under such stressed conditions plant membranes are subjected to changes often associated with the increase in permeability and loss of integrity (Blokhina et al., 2003). Changes of antioxidants reflect the impact of environmental stresses on plant metabolism (Herbinger et al., 2002). Present investigation revealed that activities of peroxidase (POD) (Table 3) and catalase (CAT) (Table 4) were significantly ($P < 0.05$) increased but MSI (Table 5) decreased with the increase in the duration of water stress at both booting and grainfilling stages in all the accessions. Accessions respond differently to water stress in terms of POD activity. Noteworthy, at booting stage, accession 251 responded to short term exposure to water stress showing maximum increase in POD activity (Table 3a) while after long term exposure accession 417 (Table 3b) exhibited maximum increase though this accession was able to maintain higher relative water content of leaves thus indicating the sensitivity of its antioxidant system to smaller changes in leaf water status. Least increase in activities of both antioxidant enzymes was found in accession 320. These differences in response to water stress suggest the different inherent capability of accessions to combat oxidative damage by increasing the activities of antioxidant enzymes. Rewatering caused maximum decrease in POD and CAT activity in accession 417 though the value was significantly higher than control possibly to counteract the remaining ROS (Reactive Oxygen Species) produced during stress. ABA seed soaking treatment showed significant stimulating effects in accession 251 (Table 3a) and accession 320 (Table 3c) at booting stage. ABA is reported to play an important role in water stress induced accumulation of antioxidants (Jiang and Zhang, 2002). Significantly ($P < 0.05$) higher MSI values were observed in accession 417 (Table 5) and accession 393 (Table 5) under all the treatments. Re-watering was least effective in recovery process of accession 320 (Table 5) possible cause appears to be less production of antioxidant enzymes which resulted in higher accumulation of ROS, which is the cause of water stress, induced damage to membranes subsequently decreasing the MSI as reported by Menconi et al. (1995). Kraus et al. (1995) reported that genotypes respond differentially to stresses as a result of variations in their antioxidant systems. Different wheat accessions had discrete water stress threshold and therefore they had different physiological adaptive

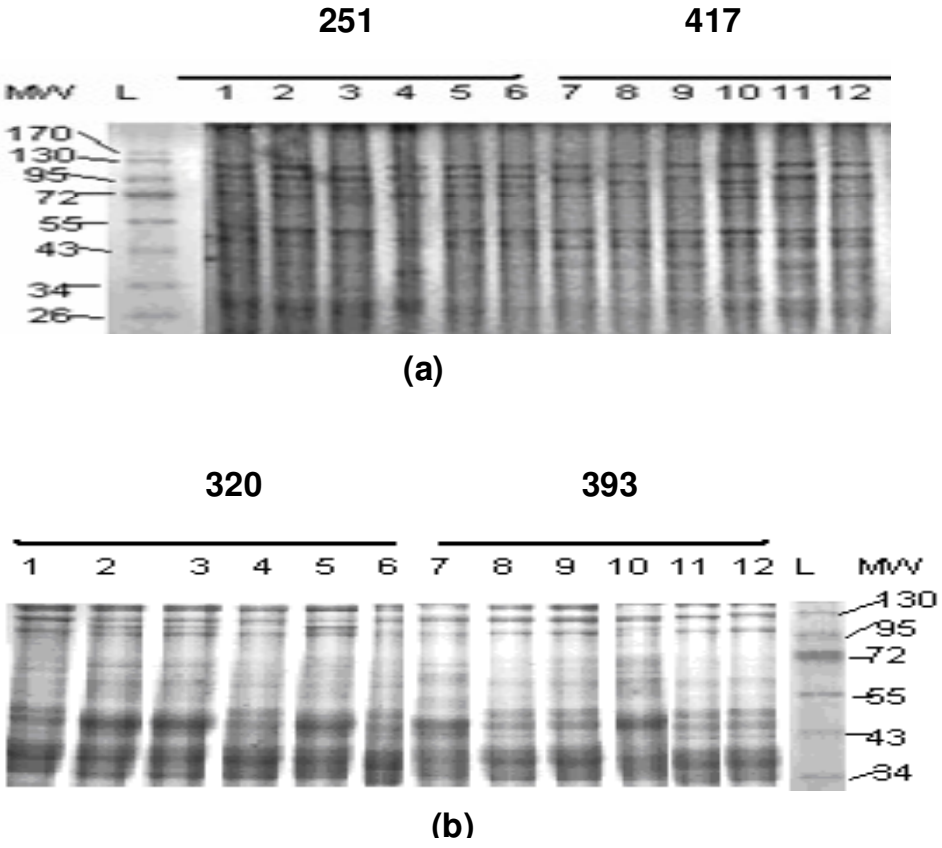

Figure 1. Effect of water stress and ABA seed soaking on protein profile of wheat grains. Lane No. 1/7 = control, 2/8 = water stress at booting, 3/9 = water stress at grainfilling, 4/10 = ABA seed soaking, 5/11 = water stress at booting + ABA seed soaking, 6/12 = water stress at grainfilling +ABA seed soaking.

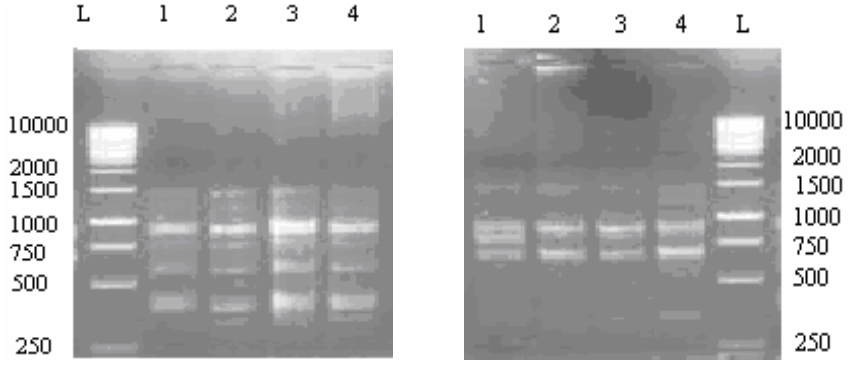

Figure 2. RAPD profile of four wheat accessions generated by random primers (OPK 15 and OPJ 080.
(L = DNA Ladder (bp), 1 = 251, 2 = 417, 3 = 320, 4 = 393).

mechanism to regulate their redox status (Shao et al., 2005). The POD, CAT and MSI are common and important indices for evaluating the redox status of plants. Dhanda et al. (2004) reported that higher anti-oxidative ability and higher MSI reflects higher water stress resistance as observed in accession 417. The increased activities of antioxidant enzymes act as a damage control system and thus provide protection from oxidative stress, which otherwise could cause lipid peroxidation resulting in damage to the cell membrane and organelles, protein and DNA structure and inhibit photosynthesis and other enzyme activities (Perhaps the higher activities of CAT

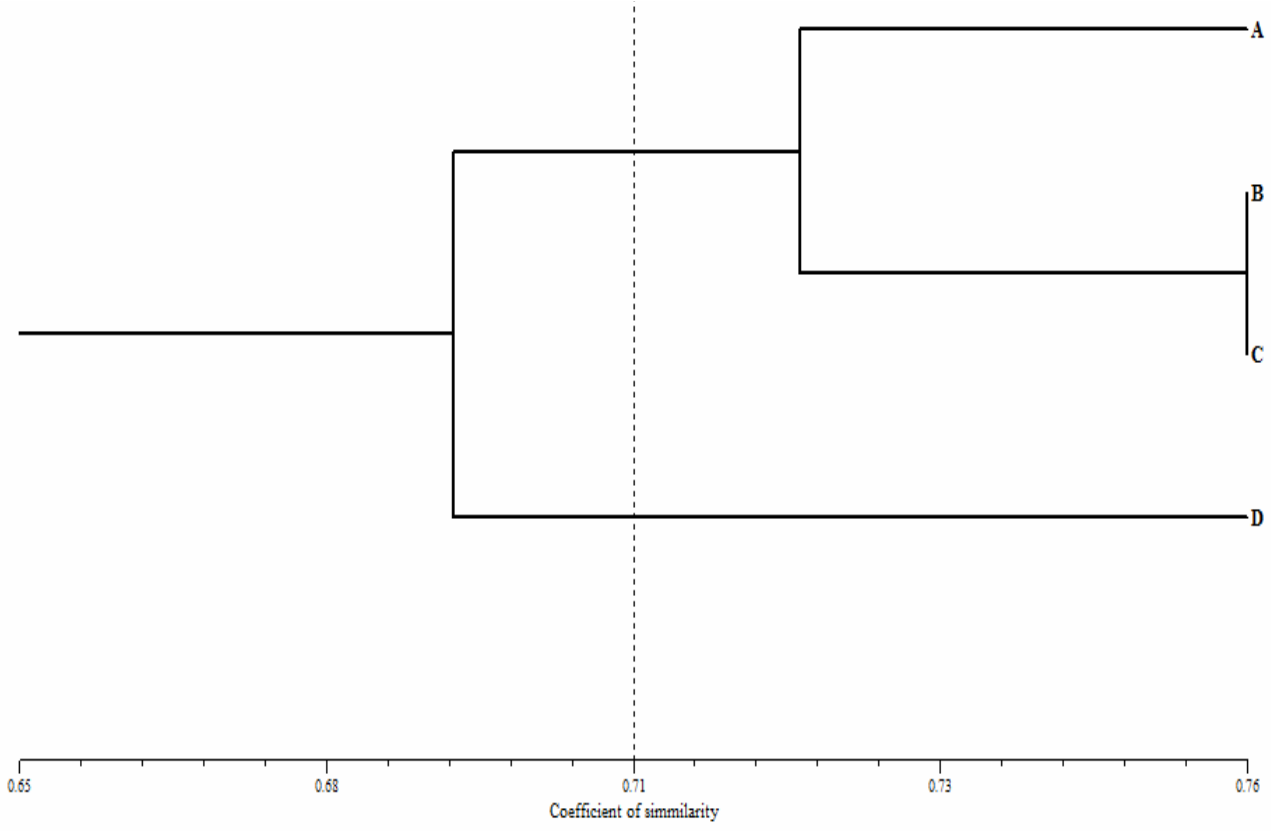

Figure 3. UPGMA Dendrogram illustrating the genetic relationships of the 4 wheat accessions based on their coefficient of similarities. (A = 251, B = 417, C = 320 and D = 393).

and POD have removed the O_2^- radicals and its product H_2O_2 induced by water stress (Gupta and Gupta, 2005). Lower MSI at grainfilling might be due to the enhanced production of damaging ROS molecules. Possibly less effective antioxidant system in accession 320 as indicated by least increase in antioxidant enzymes POD and catalase hence the higher accumulation of ROS causing damage to membranes subsequently decreasing MSI. The mechanism of ABA induced increase in MSI appears to be mediated via enhanced activities of antioxidants.

Though variations in proteins bands which correspond to both low (10 - 70 KDa) and high molecular weight (80 - 130 KDa) glutenin subunits (Bietz and Wall, 1972) was observed in the present study but according to the results of the SDS-PAGE, the overall pattern of seed storage-proteins shows low degree of heterogeneity. Moreover the changes which were observed were mainly due to the differences in the genome rather than the treatment indicating that seed protein composition is mainly controlled by genetic factors rather than environment. Due to this reason seed storage proteins have been used mostly as genetic markers for analyzing the genetic diversity within and between species (Ahmad et al., 2008). Under control condition the magnitude of differences in the protein banding pattern between accession 417 and 320 were low, but under water stress the differences were high. These two accessions also showed maximum coefficient of similarity on the basis of RAPD analysis indicating that though there occurred some genetic similarity between these two accessions but the differences present in their genome are very important in controlling their physiology as well as seed storage proteins composition during water stress. The differences in grain protein composition due to water stress and other environmental factors have been reported earlier (Beltrano et al., 2006). It is also evident from the present investigation that the changes in grain protein compositions due to water stress and ABA application are also genotype specific.

Differences between results of physiological and molecular analysis could have resulted from interaction of environment and genotype in modifying a physiological trait under water stress condition (Lepse et al., 2005). Polymorphism among the drought tolerant as well as sensitive genotypes on the basis of RAPD markers have also been detected previously (Ahire et al., 2005). Genetic differences among these wheat accessions (landraces) which belong to different geographical areas of the country could be referred to ecological and genetic

isolation (Sawalha et al., 2008).

Conclusion

The inhibitory effects of water stress on plants at booting and grainfilling stages of wheat can be alleviated by exogenous application of ABA used as seed soaking particularly in relatively sensitive wheat genotypes. Effect of ABA appears stage specific and booting stage was found to be more responsive the possible reason may be that ABA applied at seed soaking might have metabolized and influenced changing ratio of inhibitors and promoters at grainfilling. Random amplification of Polymorphic DNA (RAPD) and protein profiling can be implicated along with physiological parameters to have a detailed insight into the mechanism of tolerance to water stress. ABA seems to have important role in protecting the plants from oxidative damage under water stress through induction of antioxidant enzymes thereby affecting MSI.

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