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ABSCISIC ACID AND SALICYLIC ACID SEED TREATMENT AS POTENT INDUCER OF DROUGHT TOLERANCE IN WHEAT (*TRITICUM AESTIVUM* L.)

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Abstract

Two experiments were conducted to study the abscisic (ABA) acid and salicylic acid (SA) seed soaking induced regulatory role on protein content, soluble sugars, membrane stability index (MSI) and protein profiling through SDS-PAGE in two wheat varieties viz.,Wafaq-2001 and Punjab-96. In the pot study significant interaction between treatments and cultivars for soluble protein content was observed where drought stress and Drought + ABA caused increase in soluble proteins in cv. Wafaq-2001. There occurred decrease in MSI (%) in Punjab-96 whereas the effects of ABA and SA were at par to increase the MSI (%). The soluble sugar contents significantly increased under drought stress. Drought + ABA and Drought + SA caused 29% and 37% increase in soluble sugars of Wafaq-2001 respectively. Protein profiling through SDS-PAGE revealed the presence of polypeptides of 18, 25 and 38 kDa under drought and control in both the cultivars. ABA seed treatment induced a new protein of 18 kDa in cv. Punjab-96. Similarly; SA treatment induced a new protein of 45 kDa in the same cultivar under drought stress.

The beneficial effects of ABA and SA pre-soaking were also reflected in the form of increased grain yield. Results of the current research investigation provide new insights that can lead to a better understanding of the molecular basis of drought-sensitivity in wheat and its possible mitigation by ABA and SA seed treatments. Therefore, the current findings could be used by plant breeders to develop drought resistant as well as ABA and SA responsive wheat cultivars, in order to improve the wheat yield in the dry areas.

Introduction

Plants are exposed to various environmental stresses during the course of their life cycle. Among these are drought, temperature, salinity and cold stress etc. To survive under these abiotic stresses plants have developed various adaptive strategies that manifest in them morphological, physiological, developmental and molecular changes (Bohnert *et al*., 1995; Bray, 1997). Till date, the intracellular pathways leading to these adaptive strategies have not been fully understood (Mikolajczyk *et al.*, 2000). Drought tolerance is a multifaceted phenomenon in which several characteristics influence plant success during vegetative period (Ingram & Bartels, 1996; Khan & Khan, 2010). It is accomplished by modulation of gene expression and accumulation of specific protective proteins and metabolites (Reddy *et al*., 2004; Zang & Komatsu, 2007). Water stress tolerance has been reported in almost all plants but its extent varies from species to species (Chaitanya *et al*., 2003). Under water stress plants induce the accumulation of compatible solutes such as soluble sugars, proline and proteins. This phenomenon is called osmotic adjustment. Osmotic adjustment is accepted as an effective way of drought resistance in many crops (Kramer & Boyer, 1995; Zhang *et al*., 2005). Accumulation of sugars in response to drought stress is also well documented (Alhakimi, 2006). A multifaceted essential role of soluble sugars in plant metabolism is well known as products of hydrolytic processes, energy production but also in a sugar sensing and signaling systems. Proline accumulation under drought stress help in osmotic adjustment and other possible functions of proline include the protection of plasma membrane integrity, the prevention of protein denaturation, being a sink of energy or reducing power, a source for carbon and nitrogen and acting as a hydroxyl radical scavenger (Hare *et al*., 1998; Izanloo *et al*., 2008). Plants produce proteins in response to abiotic stresses and many of these proteins are induced by phytohormones such as ABA and SA (Jin *et al*., 2001). Dehydrins have been found to be induced by ABA in some species (Cellier *et al*., 1998; Giordani *et al*., 1999). Similarly protein synthesis occurs under water stress, heat and osmotic shock etc. There is synthesis of some proteins and decrease in the others, with or without initiation of unique stress proteins. Late embyogenesis abundant (LEA) proteins have essential role in the protection of plants under water or salt stress (Chandra & Tayagi, 2004). Dehydrin proteins along with other LEA proteins have been proposed to play important role in membrane protein stability and osmotic adjustment (Carpenter & Crowe, 1988; Dure *et al*., 1989). Dehydrins may have a cryoprotective role in macromolecules stabilization by binding water molecules to their hydrophilic surfaces, which reverses or prevents further denaturation of cellular proteins (Close, 1996). These proteins also seem to respond similarly to the application of ABA (Chaves *et al*., 2003; Kullertz *et al*., 1999). The level of endogenous ABA increases in tissues under osmotic stress, there is activation of genes that can be induced in unstressed tissues by application of exogenous ABA (Esther *et al*., 2000). Drought triggers the production of the abscisic acid (ABA), which in turn causes stomatal closure and induces expression of stress-related genes. Several drought-inducible genes are induced by exogenous ABA treatment, while others are not affected. There is supporting data demonstrating the presence of both ABA-independent and ABA-dependent regulatory systems governing drought-inducible gene expression (Yamaguchi-Shinozaki & Shinozaki, 2005).

Salicylic acid (SA) is a potent signaling molecule in plants and is involved in eliciting specific responses to biotic and abiotic stresses (Krantev *et al*., 2006). Similarly salicylic acid now being included in the category of phytohormones has been reported to regulate various physiological processes in plants such as stomatal closure, nutrient uptake, chlorophyll and protein synthesis, inhibition of ethylene biosynthesis, transpiration and photosynthesis (Raskin, 1992; Khan *et al*., 2003). Further the exogenous application of SA in the maintenance of osmoregulation in plants under water stress have been well documented (Shakirova *et al*., 2000; El-Tayeb, 2005; Sivakumar *et al*., 2001). Further the research studies conducted so far indicate that SA is a natural and hormone like signal molecule involved in the activation of plant defenses (Klessing & Malamy, 1994). However, the molecular mechanism involved in SA signaling is not yet fully understood.

The current research study was undertaken to ascertain whether total soluble proteins total soluble sugars, MSI, dehydrin expression under drought as well as exogenously applied ABA and SA had the potential for development into a screening technique to evolve drought tolerant wheat cultivars.

Materials and Methods

Plant materials and growth conditions: The present research study was conducted at Plant Physiology Program, Crop Sciences Institute, National Agricultural Research center (latitude 33 38˚ N, longitude 73˚ 00 E), Islamabad, during 2006-07. The first experiment was conducted in hydroponic culture to perform SDS-PAGE. Caryopses of two wheat cultivated varieties viz., Wafaq-2001 and Punjab-96 were obtained from wheat program National Agricultural Research Center, Islamabad. The caryopses were surface sterilized in 5% solution of Clorox (Sodium hypochlorite) and soaked in 10^{-5} M aqueous solutions of ABA and Salicylic acid in 500 ml flasks for 24 hours. Caryopses were continuously aerated by means of aquarium pump. The seeds were sown in plastic trays on two layers of filter paper at 25˚C in an incubator. After germination, five days old seedlings were transferred to plastic pots (30 × 25 cm^2) filled with sand and irrigated with half strength of Hoagland nutrient solution. After fifteen days seedlings were washed with distilled water and transferred to hydroponics culture of aerated plastic boxes measuring 15 × 25 cm^2 and 12 cm depth, containing 20% solution of polyethylene glycol 6000 to achieve osmotic potential of -1.3 MPa as water stress treatment for 24 hours. The plastic boxes containing Hoagland solution served as control. The seedlings from control, drought, Drought+ABA and Drought+SA treatments were sampled for protein pattern analysis using SDS-PAGE.

The 2nd experiment comprised a pot study conducted under natural conditions with average day/night temperature 30 ± (8˚C) and 13 ± (5˚C) respectively and photoperiod ranging from 10-13.5h. The following schedule of treatments was followed: Control, Drought, Drought + ABA and Drought +SA. For ABA and SA treatments, caryopses were soaked in aqueous solutions of 10^{-5} M ABA and Salicylic acid (Sigma Aldrich, USA) for 24 hours. The seeds were sown in earthen pots measuring 24 × 30 cm^2 filled with soil and sand mixture (3:1). Eight caryopses were sown in four holes per pot. Every 3rd day pots were rotated to minimize the positional effects. The irrigation was provided when required to the treatment and control pots throughout the study period. The plants were protected from rain by placing in glass house. The drought stress was imposed by withholding water supply for 7-10 days at anthesis (80 DAS) stage till the symptoms of temporary wilting/leaf rolling appeared. The 2nd leaf starting from the top from different treatments was sampled and replicated thrice for measurement of soluble proteins, total soluble sugars and membrane stability index (%).

Soluble protein content: Leaf soluble proteins were determined by the dye binding method of Bradford (1976). 0.5 g leaf tissue was homogenized in 0.15 M in 5 ml NaCl solution. The homogenate was centrifuged at 1000 rpm for 15 minutes. 10 µl of supernatant and 50 µl distilled water were added to 3 ml 5 fold diluted Bradford reagent (Bio-Rad protein assay dye reagent). 0.1 g BSA was dissolved in 100 ml distilled water. From this 1 ml was taken and diluted to 10 ml. From the stock solution a calibration curve consisting of a series of 10, 20, 30, 40 and 50 µg ml^{-1} BSA was run to plot a standard curve. The absorbance of the standards and samples was measured at 595 nm on spectrophotometer (Unico UV-2100, Japan). Protein concentration of the sample was calculated using the calibration curve of Bovine serum albumin and expressed on fresh weight basis.

Membrane stability index (%): The membrane stability index was determined according to Sairam *et al*., (1994). Leaf samples (0.1 g each) were cut into discs of uniform size and placed in 10 ml of double- distilled water in two sets. One set was kept at 40˚C for 30 min., and its conductivity was (C1) recorded using conductivity meter. The second set was kept in a boiling water bath (100˚C) for 15 min., and its conductivity also recorded (C2). The membrane stability index (MSI) was calculated as:

$$MSI = [1-(C1/C2)] \times 100$$

Protein extraction and electrophoresis by SDS-PAGE:

Fifteen days wheat shoots were grinded to fine powder using liquid nitrogen. 400 ul of protein extraction buffer (0.05 M Tris-HCl, 2.5% SDS, 10% Glycerol, 5M urea, 5% β-mercaptaethanol and 0.2% bromophenol blue) was added to 0.1 g of leaf powder and vortexed thoroughly for homogenization. The homogenate was centrifuged at 13000 rpm for 10 min at 4˚C in refrigerated centrifuge. Protein samples for SDS-PAGE were boiled for 10 min, cooled on ice for 5 min., and centrifuged at 10,000 × g for 10 min. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was used for determining the molecular weight of proteins according to Laemmli (1970). PAGE of proteins was performed by standard cooled dual vertical slab unit SE-600 (Hoefer Scientific Instruments). Aliquots (20-50 µg protein per lane) were loaded on 1.5 mm thick 15%

denaturing gels and run at 4˚C. A known molecular weight standard protein marker, Fermentas Germany of 10-200 kDa, # SM-0661 was also run with the samples to determine the mol. weight of corresponding protein. Electrophoresis was performed under constant current of 15 mA per gel until the bromophenol blue marker reached the bottom of the gel. The proteins were stained with 0.2% Coomassie Brilliant Blue R-250 for 1 h followed by several times destaining with 15% methanol, 7.5% acetic acid. The appearance or disappearance of proteins was identified visually of control, drought, Drought + ABA and Drought + SA treated plants. The relative movement of the protein bands against the position of known molecular markers of the coomassie stained gel was carefully measured with a ruler.

Statistical analysis: The experiment was laid out in a completely randomized design with factorial arrangement possessing three replicates. Statistical analysis of the results was performed using Minitab ver.13.2 and treatment means were compared by Duncan's multiple range test (DMRT) at $p<0.01$ and 0.05 (Steel & Torrie, 1980).

Results

The effect of Abscisic acid and Salicylic acid seed treatment was investigated on the change of protein pattern in 15 days old wheat seedlings grown under control and water stressed conditions of two wheat varieties ; cvv. Wafaq-2001 and Punjab-96 (Fig. 1.).

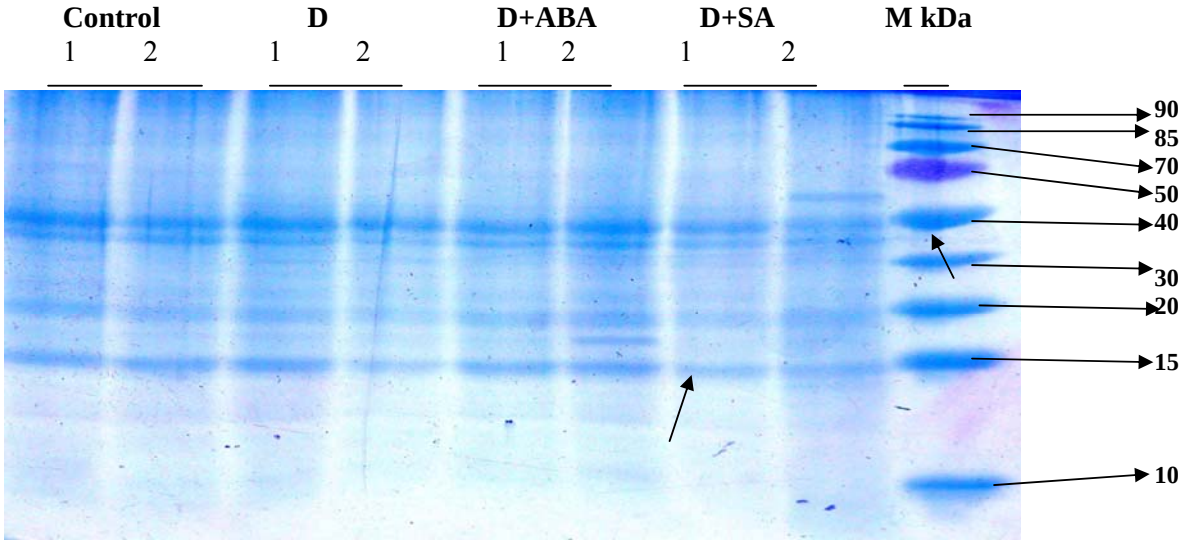

Fig. 1. Leaf protein profile of 15 days old seedlings of two wheat varieties (1=Wafaq-2001 and 2= Punjab-96) under control, drought, ABA and SA seed treatment under drought stress. The arrows represent two new polypeptides bands induced by ABA and SA seed treatment under drought. Molecular marker used in this gel was SM-0661, 10-200 kDa mol. wt. from FERMENTAS, Germany.

The SDS-PAGE analysis of soluble proteins from leaf revealed the presence of several polypeptides of 18, 25 and 38 kDa under drought stress in both wheat cultivars. The protein profile of drought susceptible cv. Punjab-96 showed decrease in the intensity of 50 kDa band under drought stress. However, ABA seed pre-soaking seemed to ameliorate the adverse effects of drought stress in Punjab-96 on the same polypeptide band (15KDa). In contrast, SA seed pre-soaking under drought stress was unable to avoid the deleterious effects of drought stress in cv.Punjab-96 through expression of above mentioned protein band (Fig. 1; Table 2). The protein pattern analysis further showed that a new protein of 18 kDa occurred in the Drought + ABA treated wheat seedlings of cv. Punjab-96. Similarly SA treated seedlings revealed the presence of a new protein of molecular weight of 45 kDa in cv. Punjab-96.

The interaction among treatments and cultivars was non-significant (Fig. 2; Table 1) for membrane stability index ($p>0.05$). However, the magnitude of decrease was greater in cv. Punjab-96. Both ABA and SA seed treatments were ineffective to increase the MSI (%) in both the wheat cultivars.

The results demonstrated that interaction between treatments and cultivars for total soluble sugars differed significantly (Fig. 3, Table 1; $p<0.001$). The soluble sugars content of cv. Punjab-96 significantly increased in response to drought stress. In cv. Wafaq-2001, both ABA and SA significantly increased (29% and 37% respectively) the accumulation of soluble sugars under drought stress. In contrast, cv. Punjab-96 exhibited no such increase under drought stress being supplemented either with ABA or SA as seed pre-soaking.

There occurred significant (Table 1; $p<0.001$) differences in the interaction between treatments and cultivars for soluble protein contents (Fig. 4). The drought stress caused increase in total soluble proteins in cv. Wafaq-2001; whereas; cv. Punjab-96 was unable to accumulate soluble proteins in response to drought stress (Fig. 4). Further, ABA was ineffective to increase the soluble proteins under drought stress. However, SA treatment increased (19%) the soluble proteins in the leaves of cv. Wafaq-2001 under drought stress.

Table 1. F values from analysis of variance of data for Membrane stability index, Soluble sugars, Soluble proteins and Grain yield of two wheat cultivars with ABA and SA seed treatment under drought stress conditions (n=3).

SOV	d.f	MSI (%)	Soluble sugars	Soluble proteins	Grain yield
H	3	139.19^{***}	164.34^{***}	83.13^{***}	51.26^{***}
V	1	249.07^{***}	17.89^{***}	143.90^{***}	215.88^{***}
GS	2	3.97^{*}	204.35^{***}	6.12^{*}	216.42^{***}
H*V	6	1.34^{ns}	4.44^{***}	6.88^{***}	7.97^{***}
H*GS	3	9.41^{***}	18.70^{***}	2.50^{*}	2.52^{*}
V*GS	2	0.62^{ns}	1.29^{ns}	0.64^{ns}	2.61^{ns}
H*V*GS	6	0.74^{ns}	0.25^{ns}	1.46^{ns}	4.57^{***}
Error	48				
Total	71				

H= Hormones, V= Varieties, GS= Growth stages, MSI= Membrane stability index
*, **, *** = Significant at 0.05, 0.01 and 0.001 levels, respectively, ns= Non-significant

Table 2. Distribution pattern of protein bands in two wheat cultivars under drought, ABA and SA seed treatments. (+) and (-) signs indicate the presence and absence of protein bands.

Band Mol. Wt.	Control		Drought		D+ABA		D+SA	
	Wafaq-2001	Punjab-96	Wafaq-2001	Punjab-96	Wafaq-2001	Punjab-96	Wafaq-2001	Punjab-96
45 kDa	-	-	-	-	-	-	-	+
40 kDa	+	+	+	+	+	+	+	+
35kDa	+	+	+	+	+	+	+	+
18 kDa	-	-	-	-	-	+	-	-

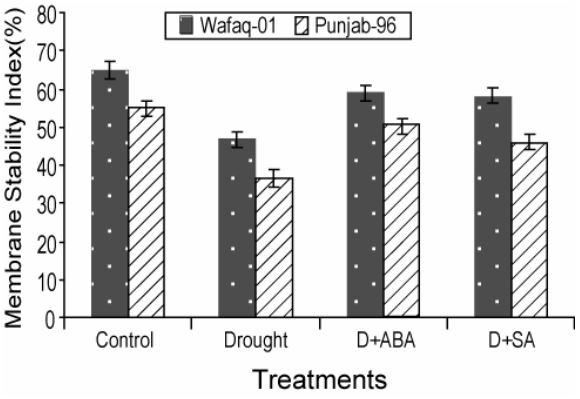

Fig. 2. Effect of Drought, ABA and SA seed treatment on Membrane stability in dex (%) of wheat leaves. Results are the mean ± SE of means (n=3).

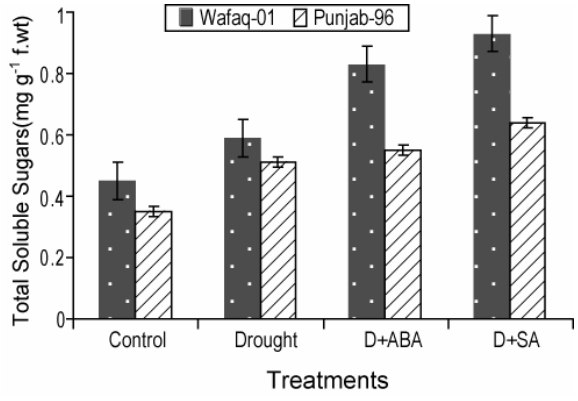

Fig. 3. Effect of Drought, ABA and SA seed treatment on Soluble sugar content (mg g^{-1} f.wt) of wheat leaves. Results are the mean ± SE of means (n=3).

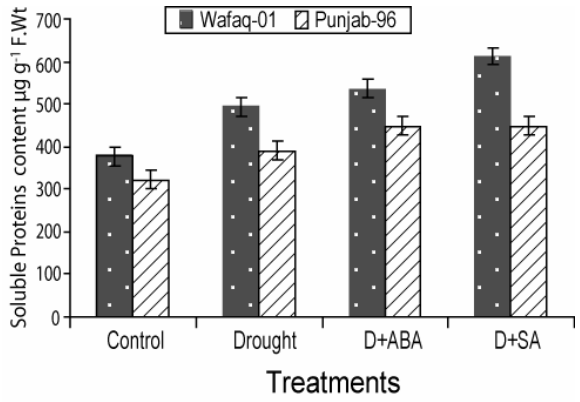

Fig.4. Effect of Drought, ABA and SA seed treatment on Soluble protein contents (μg^{-1} f.wt) of wheat leaves. Results are the mean±SE of means (n=3).

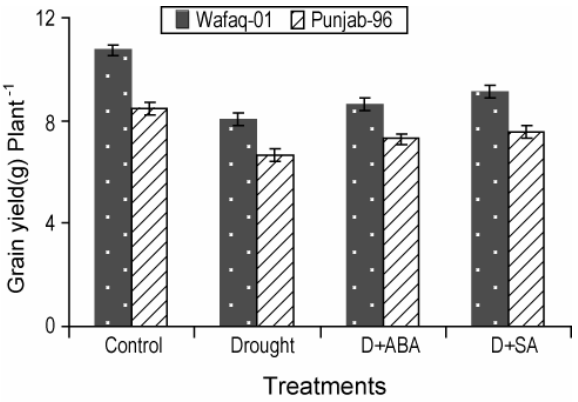

Fig. 5. Effect of Drought, ABA and SA seed treatment on Grain Yield $Plant^{-1}$ of wheat. Results are the mean ± SE of means (n=3).

Significant interaction for grain yield was observed between treatments and cultivars at $p<0.01$ (Fig. 5; Table 1). Drought stress significantly decreased the grain yield in both the wheat cultivars. The ABA seed pre-soaking significantly ameliorated the adverse effects of drought stress on grain yield. Nevertheless, the magnitude of increase was significantly higher in cv. Punjab-96 than cv. Wafaq-2001. The SA also increased the grain yield under drought stress in both the wheat cultivars. However, the magnitude of increase was significantly greater in cv. Punjab-96. It was found that SA was more effective to increase the grain yield in both wheat cultivars under drought stress than ABA.

Discussion

The results of the present study are in consonant with those of Din & Flower (2002) who reported 15, 18 and 23 kDa proteins in ABA seed treated wheat seedlings under non-stress conditions. Similarly, Reddy *et al*., (1993) found 15 and 23 kDa proteins under ABA seed treatment in rice callus under water stress conditions. The plants produce some proteins in response to biotic and abiotic stresses, that some of these proteins deduct by phytohormones such as salicylic acid (Hussein *et al.,* 2007).

Several studies supported a major role of SA in mediating the plants responses to abiotic stresses; however, the molecular events in SA signaling are not yet completely understood. During current research investigations, SA seed treatment induced a new protein of 45 kDa in cv. Punjab-96. Salicylic acid induced protein kinase of 40 and 48 kDa molecular weight has been reported in tobacco cells activated by various stress stimuli (Hoyos & Zhang, 2000). The findings of the present study and similarities between the same proteins induced by drought in some other crop species are consistent with the observations of other workers (Mundy & Chua, 1998; Skriver & Mundy, 1990; Ben-Hayyim *et al.,* 1993; Reddy *et al.,* 1993) and suggest that these new proteins induced by ABA and SA seed treatments under drought seems to be responsible for wheat plants adaptation to drought stress.

Salicylic acid seed treatment caused highest accumulation of leaf proteins in cv. Wafaq-2001), whereas in cv. Punjab-96 the effects of ABA and SA were at par (Fig. 1). The higher soluble proteins accumulation by Wafaq-2001 under drought stress indicates the better adaptability of this cultivar under drought stress. Similar results were found in some earlier work by Labhilili *et al*., (1995) in which drought tolerant cultivars of wheat possessed higher soluble proteins. The SA was highly effective to increase the soluble protein content of wheat leaves. The results of present study are in agreement with those of Singh & Usha (2003) who reported that exogenously applied SA enhanced soluble protein contents in wheat seedlings under water stress conditions.

In the present research investigation relatively more reduction in membrane stability index (MSI) was found in drought susceptible cv. Punjab-96 under drought stress. The results of the current study confirmed the findings of Sairam & Srivastava (2001) who observed that drought tolerant wheat genotypes showed higher MSI than susceptible ones. Membrane stability index is an indicator of drought tolerance has been also reported by Premchandra *et al*., (1990).

Soluble sugars and proline are responsible for osmoregulation phenomenon in the expanded leaves of many species (Morgan, 1994). Soluble sugar may function as a typical osmoprotectant, stabilizing cellular membranes and maintaining turgor pressure. In the present research study, there occurred accumulation of soluble sugars under drought stress in both the wheat varieties. Both ABA and SA increased the soluble sugar accumulation under drought stress in Wafaq-2001. Mohsina *et al*., (2008) observed that reducing sugars increased more in salinity tolerant wheat variety S-24 than Inqalab-91 under imposed salinity stress by salicylic acid seed treatment. It is likely that both ABA and SA seed soaking triggered the drought tolerance mechanism in Wafaq-2001 through enhanced sugar accumulation.

The application of ABA has been found to increase yield of wheat in the field under water stress conditions ordinarily at anthesis and post-anthesis stages through the mobilization of photosynthates to the grains (Travaglia *et al*., 2007). The ABA applied beneficial effects were prominent in the presence of water stress only.

Under water stress, the decrease in seed set and grain growth in wheat has been reported by several workers (Morgan, 1980; Saini & Aspinall, 1982; Ahmadi & Baker, 1999). There are also a number of research reports in which ABA applications were able to promote dry matter accumulation in the reproductive organs and its levels were linked with the growth rate of seeds or fruits (Schussler *et al*., 1991; Wang *et al*., 1987; Ross & MacWha, 1990; Kato *et al*., 1993; Wang *et al*., 1998; Yang *et al*., 2001). The enhanced movements of photosynthetic assimilates by ABA towards developing seeds have been reported (Ackerson, 1985; Brenner & Cheikh, 1995; Yang *et al*., 2004). The beneficial effects of SA on seed yield under drought stress has been reported by several workers like Maibangsa *et al.,* (2001), Shehata *et al*., (2001), Abdel-Wahed *et al*., (2006) and Iqbal & Ashraf (2006). The possible mechanism of SA to exert positive effects on grain yield might be due to its beneficial effects on membrane stability, oxidative stress minimization and osmoprotectants (protein, proline, sugars) accumulation.

Conclusions

ABA and SA seed priming elicited the drought tolerance mechanism in wheat through osmotic adjustment (soluble protein and soluble sugar accumulation). Further, both the phytohormones treatments provoked stability to membranous system and photosynthetic machinery in both the wheat varieties but greater being in the tolerant Wafaq-2001. The current research findings suggested developing ABA and SA responsive wheat cultivars to enhance wheat yield in the water stress conditions.

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