Journal of Plant Process and Function, Vol. 5, No. 18, Year 2017

Effects of water deficit on the physiological response, total protein, and gene expression of *Rab17* in wheat (*Triticum aestivum)*

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(Received: 21/11/2015-Accepted: 20/04/2016)

Abstract:

Drought is the cause of adverse environmental impacts on plant growth and crop yield. In this study, the effects of water deficit on plant molecular and physiological responses were investigated using two cultivars (namely, Sardari and Zarin) of bread wheat selected based on the results of a three-year research. For the purposes of this study, they were grown in plastic pots containing field soil and maintained in growth chambers (30/20 °C, 14/10 h day/night, 60% R.H). The cultivars had been The experiment was conducted in a completely randomized design with three replications. Plant response to water stress was evaluated at the physiological level by determining the relative water content (RWC) as well as chlorophyll, free proline, and total protein contents following the drought treatment with subsequent re-irrigation. All the physiological parameters were found to be affected by drought stress. The Zarin cultivar exhibited a significant decrease in its RWC. Chlorophyll a and total chlorophyll in both the cultivars showed significant decreases but chlorophyll b did not exhibit any significant variation in either cultivar. The free proline content increased significantly in both cultivars such that they were both restored their normal proline contents life when irrigation was resumed after a short-term experimental drought stress. SDS-PAGE electrophoresis of leaf proteins in both control and experimental samples revealed regulating adjustments in protein contents. Modification in the expression level of the dehydrin (DHN) gene (that is, *Rab17*) was also analyzed by reverse transcription–polymerase chain reaction (RT-PCR). This gene was expressed slightly in the well watered plants of the two cultivars, but the gene expression in the Sardari cultivar increased significantly after the long-term experimental drought.

Key words: Drought Stress, Gene expression *Rab17*, Semi-Quantitative RT-PCR.

Introduction:

Water deficit is considered to be among the most severe environmental stresses that poses a major constraint on plant productivity; losses in crop yield due to water stress probably exceed the losses from all other causes combined (Kramer, 1980). This deficit has an evident effect on plant growth that depends not only on the severity and duration of the stress event but also on the developmental stage and morphological/anatomical parameters of the plants (Rizhsky *et al.,* 2002; Bartels *et al.,* 2004). The expression of many genes is induced by drought, and their gene products function directly in stress tolerance, regulation of gene expression, and signaling transduction in stress responses (Zhou *et al.,* 2010). Soon after the perception and recognition of external changes, different signaling pathways are activated in order to convert a physical stress into a biochemical response, each of which then promote the expression of a set of stress-responsive genes. The full activation of all signal cascades induced by a given stress event promotes acclimation and leads to stress tolerance. Transcriptomics, proteomics, and gene expression studies have identified the activation and regulation of several stresses related transcripts and proteins, which are generally classified into two major groups. One group is involved in signaling cascades and in transcriptional control, whereas members of the other group (including osmoprotectants, antioxidants, and reactive oxygen species (ROS) scavengers) are involved in membrane protection (Shinozaki and Yamaguchi-Shinozaki, 2007).

Many genes respond to drought, salt, or cold stresses at the transcriptional level and the products of these genes function in the stress response and tolerance. Either these genes are involved in signaling and regulatory pathways, or encode enzymes leading to the synthesis of functional and structural protectants (Cuming *et al.,* 2007; DeMarcelo *et al.,* 2007; FuD *et al.,* 2007; Hussain *et al.,* 2011). Some are controlled by abscisic acid (ABA) while others are not, representing the involvement of both ABA-dependent and ABA-independent regulatory systems for stress responsive gene expression. One response by plants under stress is accumulation of novel protective proteins, including

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[DOR: 20.1001.1.23222727.1395.5.18.1.0]

heat shock proteins (HSPs) and late embryogenesis abundant (LEA) proteins. Similarly, aquaporins are involved in the regulation of water transport, particularly under abiotic stresses. A large set of highly hydrophilic proteins, called LEA proteins, accumulate naturally in some desiccation tolerant plant structures, such as the seed; these proteins are also induced in vegetative plant tissues under water limited conditions (Jyothsnakumari *et al.*, 2009; Liu *et al.*, 2010). LEA type proteins are encoded by RD (responsive to dehydration), ERD (early responsive to dehydration), KIN (cold inducible), COR (cold regulated), and RAB (responsive to abscisic acid) coding genes in different plant species (Roychoudhury *et al.*, 2007). It has been indicated that the LEA type proteins act as water binding molecules in seeds where they protect other proteins from the harmful effects of desiccation, including ion sequestration and macromolecule and membrane stabilization against freeze-induced injury (Liu *et al.*, 2010; Jyothsnakumari *et al.*, 2009; Roychoudhury *et al.*, 2007; Tunnacliffe and Wise, 2007). Besides the protective chaperone-like functions of the LEA proteins, their action against cellular damage has been proposed, indicating the role of LEA proteins in anti-aggregation of enzymes under dehydration or freezing stress (Shao *et al.*, 2007). The maize (*Zea mays* L) responsive *Rab17* gene is induced during late embryogenesis when ABA levels are high, and it is also ABA that is water stress inducible in the embryo and vegetative tissues (Roychoudhury *et al.*, 2007). This gene belongs to Group 2 LEA proteins. Several gene products homologous to *Rab17*- encoded proteins (also known as dehydrins and late embryogenesis abundant LEA proteins) have been identified in different plant systems. Based on the highly conserved protein structure and regulation of the rab genes, theoretical models have been developed implicating the protective roles of these proteins (Reviewed by Dure 1993). In the present work, two cultivars of bread wheat (Sardari and Zarin) with different levels of tolerance to drought were used to study the effects of drought stress on physiological responses (Relative Water (RWC), proline, chlorophyll, and total protein contents) and molecular responses (modification in the expression level of dehydrin (DHN) gene (*Rab17*).

Materials and Methods:

Plant material and treatment: Two cultivars of bread wheat (namely, Sardari as the drought-resistant cultivar and Zarin a the sensitive one) were selected based on the results of a three-year research described in Heidari *et al.* (2003). The plants were cultivated in a completely randomized design with three replications in plastic pots containing field soil and kept in growth chambers (30/20 °C, 14 h day/10 night, and 60% R.H). At the two-leaf seedling stage, one group of the plants from both genotypes was irrigated daily and maintained under optimum irrigation conditions (used as control) while another was subjected to water deficient conditions (suspended irrigation for a period of 7 days re-watered for 3 days, and suspended irrigation for a second period of 7 days). Drought stress was imposed by withholding water supply. The samples were collected from both control and stressed plants on days one, seven, and ten of the water withholding period. The 7-day drought-treated plants were re-watered for 3 days every day.

RWC measurements: Leaf relative water content (RWC) was estimated according to Ekanayake *et al* (1993). The third or fourth leaves were cut to 1 cm segments to determine fresh weight (W_F) before they were soaked in water at 4 °C in the dark for 24 h when the turgid weights (W_T) were recorded. Finally, the segments were dried in an oven at 80 °C for 24 h and their dry weights (W_D) were measured. The percentage of relative water content (RWC) was calculated using the following formula:

$$RWC= W_F-W_D/W_T-W_D \times 100$$

Proline measurements: Free proline in 100 mg of leaf samples was measured according to Bates et al (1973). The plant material (100 mg) was ground in 10 ml of 3% sulfosalicylic acid and the homogenate was filtered. To 2 ml fractional were added 2 ml of acetic acid glacial, 2 ml of acid phosphoric 6 M, and 2ml of acidic ninhydrin reagent. The mixture was then stirred and incubated in a boiling water bath for 1 h before 4 ml of toluene was added to the mixture. After 30 s of shaking, two phases were separated and the absorbance of the upper phase was read at 520 nm. The proline concentration was determined based on comparison with 0–160 µM proline standard curve and expressed on a ($\mu M\ g^{-1}$) FW basis.

Chlorophyll content measurements*:* Chlorophyll content was determined in 80% acetone extract. After centrifugation (13000g, 15 min), the absorbance was read spectrophotometrically at 645 and 663 nm. Total chlorophyll as well as chlorophyll a and b concentrations were calculated according to Arnon (1949).

Total Protein extraction*:* In a mortar and pestle, 100 mg of frozen leaf tissues was powdered and homogenized in 200 µl of the extraction buffer consisting of 50 mM Tris - HCl pH 7.5, 1 M EDTA and 2-mercaptoethanol 0.04% (v/v) and finally vortexed for 40s. After incubation on ice for 30 min, the homogenate was centrifuged at 13000× g at 4 °C for 15 min. The supernatant was transferred into fresh tubes and stored at –20 °C. The protein content was determined according to the method of Bradford (1976) using bovine serum albumin as the standard.

Sodium Dodecyl Sulfate Polydactyl Amide Gel Electrophoresis (SDS- PAGE): The discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the qualitative analyses of protein (Askar *et al.*, 2011).

Optimization of semi quantitative PCR conditions*:* RNA was extracted from leaf samples using

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the RNXplus Kit (Cinnagen, Iran) and transcribed to cDNA using the Rivert Aid™ first strand cDNA synthesis kit (Roch) according to the manufactuer's instructions. The primers were then designed by OLIGO 5 software according to the BLAST results of different *Rab17* and *ß-Tubuline* genes or ESTs recorded in Genebank. The Rab17 forward primer was 5´-AGACGGGGCAGACCATTCA-3 and its reverse was 5´- TAGCGAAACAGAAGGAGGG-3´. The tubuline gene forward primer was designed as: 5´-GCTTTCAACAACTTCTTCAG -3´ and the reverse was 5´- GGGGCGTAGGAGGAAAGCA- 3´. Semi-quantitative RT-PCR was set up using *ß-Tubuline*, as a housekeeping gene. Final RT-PCR was performed at 94 °C for 5 minutes followed by 94 °C for 1 minute, 58 °C for 1 minute, and 72 °C for 1 minute over 35 cycles with an ultimate extension temperature of 72°C for 10 minutes. The polymerization chain reaction contained 1X PCR buffer, 1.5mM $MgCl_2$, 0.2 mM dNTP, 10 pmol from each of the reverse and forward primers, and 2.5 U *Taq* DNA Polymerase in a 20µl reaction. This procedure was performed for the different experimental treatments in two biological replicates and two RT-PCRs for each sample (Watpade *et al*., 2012).

Statistical Analysis: SPSS software (version, 14) was used to analyze the data. Analysis of variance was performed on the target traits. Duncan's multiple range tests were used to compare means and the Excel software was used to construct diagrams. Test of normalization was carried out using the Kolmogorov smirrov test. RT-PCR gels were recorded and band intensities were assessed using the Total Lab V.L 10 software.

Result:

Effects of water deficit on RWC: Results revealed a slight decrease in relative water content on the first day of the experimental treatments, which rose to significant levels after water was withheld for 10 days. As shown in Fig. 1, the average values of 84.14% and 74.4% represent the reference points for the RWCs of the well-watered Sardari and Zarin cultivars, respectively. These values later reduced to 79.33% and 68.52%, respectively, after the 7-day drought period to subsequently return to normal after watering was resumed. In the cultivars subjected to the 10-day drought treatment, these values further reduced to 71.78% and 55.13% in the Sardari and Zarin cultivars, respectively.

Effects of water deficit on chlorophyll content*:* Drought stress had a significant (p<0.01) effect on chlorophyll a and total chlorophyll contents but no such effect on chlorophyll b rate in either of the cultivars (Fig. 2). Chlorophyll a, b, and total chlorophyll contents decreased slightly in Zarin during the 7-day drought but they increased in Sardari when subjected to the 10-day drought stress. Both cultivars exhibited slightly reduced chlorophyll (a, b and total) content in the 10-day treatment (Fig. 2).

Effects of water deficit on proline accumulation: The free proline content of the control Zarin and Sardari cultivars was found to lie within a range of 3.2–15.76 and 3.03–11.5 µmol −1 leaf fresh weight, respectively (Fig. 3). Withholding water for 7 days increased the proline content in both cultivars but returned to the level of control plants after an irrigation rewater of 3 days. Interestingly, when the drought condition was extended to 10 days, the free proline contents in Zarin and Sardari cultivars**,** respectively**,** increased to 5 and 4 times that of the control. This is in agreement with previous reports that water stress induces higher proline contents in wheat plants (Vendruscolo *et al.*, 2007). Mastrangelo (2009) also reported that the greatest amounts of free proline accumulate in the leaf tissues of crops under stress, (Cuming *et al*., 2007; De Marcelo *et al*., 2007; FuD *et al*., 2007; Hussain *et al*., 2011; Shamsi, 2010; Shao *et al.*, 2007).

Effects of water deficit ontotal protein content*:*One-dimensional electrophoresis was used to analyze and compare the leaf protein patterns of the one-day stress treated Sardari and Zarin plants. Results showed that the synthesis of two polypeptides of 9 and 10 KDa increased but that of eleven polypeptides of 2, 3, 15, 40, 42, 44, 45, 66, 85, 100, and 105 KDa decreased in Zarin cultivar. In the 7-day treated plants, the 2, 15, and 20 KDa polypeptides increased in both cultivars as compared to their quantities in the control plants. Moreover, it was found that the eight polypeptides of 8, 26, 43, 45, 47, 53, and 61 KDa in Zarin and the 12 and 75 KDa ones in Sardari increased relative to their quantities in the control plants. This is while decreases were observed in the quantities of the 15, 40, and 63 KDa polypeptides in Zarin and in that of the 12 KDa polypeptide in Sardari relative to the quantities of the same polypeptides in the control. In the rewatered plants, the amounts of four polypeptides with 37.5, 85, 90, 120, and 130 KDa reduced in both cultivars as compared to the control. In addition, the 200, 190, 55, and 30 KDa polypeptides decreased in Sardari as did the 145, 115, 100, and 40 KDa polypeptides in Zarin. On the other hand, seven polypeptides with molecular weights of 5, 21, 43, 65, 67.5, 100, and 170 KDa in Sardari and 10 polypeptides with 2, 10, 12, 14, 15, 25, 37, 40, 80, and 190 KDa in Zarin increased relative to their reference quantities in the control plants. In the 10-day stress treated Zarin and Sardari plants, five polypeptides with 50, 55, 110, 117, and 200 KDa and four polypeptides with 37, 46, 48.5 and 54 KDa exhibited incerements in their quantities as compared to the control plants. Finally, thirteen polypeptides with molecular weights of 20, 32, 39, 42, 46, 55, 69, 77, 85, 105, 129, 130, and 180 KDa and four polypeptides with molecular weights of 12, 34, 80 and 160 KDa reduced in Zarin and Sardari, respectively, while a 105 KDa polypeptide rose in quantity in Sardari but not in Zarin.

Effects of water deficit on expression pattern of the Rab17 gene: A semi-quantitative RT-PCR method was developed to reveal the changes in gene expressions

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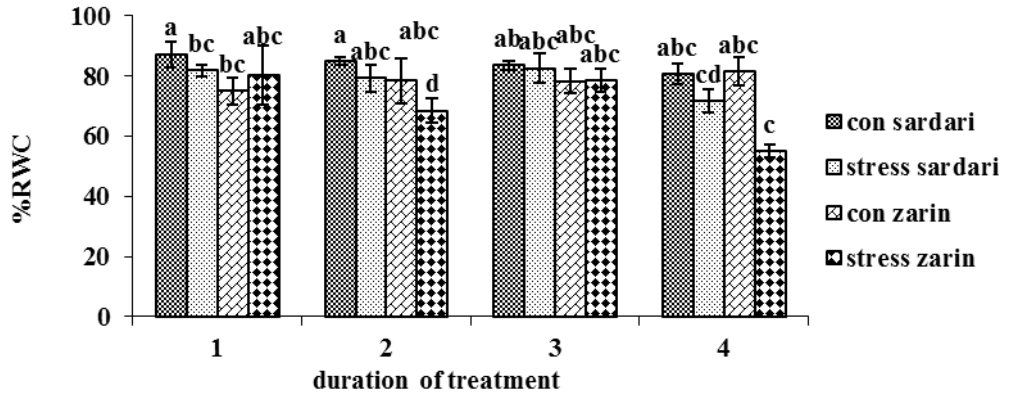


Figure 1. Effects of water withholding and rewartering on RWC in the two experimental cultivars (Sardari and Zarin) of bread wheat (Triticum aestivum). Samples were harvested on days one, seven, and ten after watering (control) or the water withholding (stress) period. First day=control or non-Stress, seventh day=suspended irrigation for a period of 7 days, RE= re-watering, tenth day= suspended irrigation for a period of 10 days.

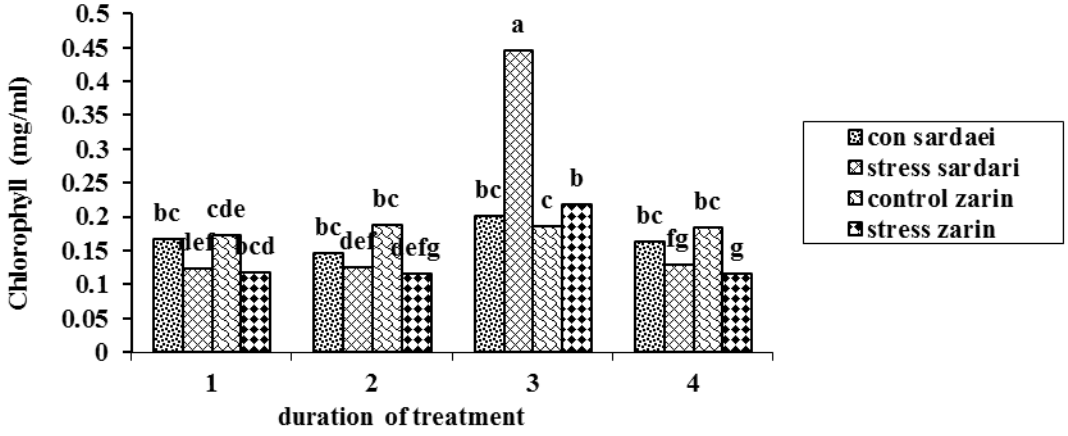


Figure 2. Effects of water withholding and rewatering on chlorophyll content in the two (Sardari and Zarin) cultivars of bread wheat (*Triticum aestivum*). Samples were harvested on days one, seven, and ten after watering (control) or the water withholding (stress) period. First day=control or non-Stress, seventh day=suspended irrigation for a period of 7 days, RE= re-watering, tenth day= suspended irrigation for a period of 10 days.

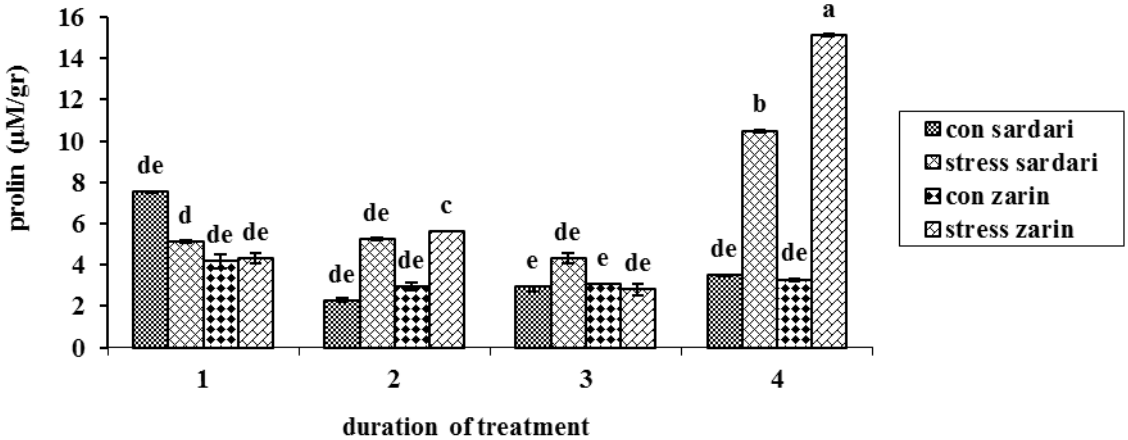


Figure 3. Changes in proline contents of the two control Sardari and drought treated Zarin cultivars of bread wheat. First day=control or non-Stress, seventh day=suspended irrigation for period of 7 days, RE= re-watering, tenth day= suspended irrigation for period of 10 days.

at the transcriptional level. The data were initially normalized against the *ß-tubuline* gene and, as a housekeeping gene; a fragment of 500 bp was resolved in both treated and non-treated plants. Two replicates of

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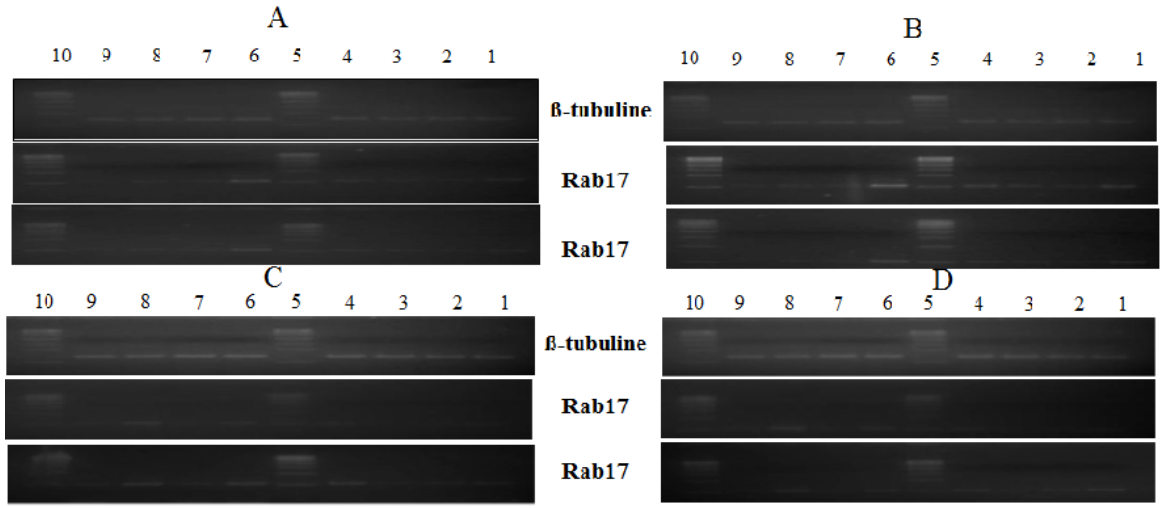


Figure 4. a) Patterns of gene expression of Rab17 Sardari in the first replicate of the PCR reaction 1: 1) 0-day stress treated plants, 2) 7-day stress treated plants, 3) re-watered plants, 4) 10-day stress treated plants, 6,7,8,9) control plants from each treatment of 5, and 10) 1 Kb DNA ladder; b) Patterns of gene expression of Rab17 Sardari in the first replicate of the PCR reaction 2: 1) 0-day stress treated plants, 2) 7-day stress treated plants, 3) re-watered plants, 4) 10-day stress treated plants, 6,7,8,9) control plants from each treatment, 5, and 10) 1 Kb DNA ladder; c) Patterns of gene expression of Rab17 Zarin in the first replicate of the PCR reaction 1: 1) 0-day stress treated plants, 2) 7-day stress treated plants, 3) rewatered plants; c) Patterns of gene expression of Rab17 Zarin in the first replicate of PCR reaction 2: 1) 0-day stress treated plants, 2) 7-day stress treated plants, 3) rewatered plants, 4) 10-day stress treated plants, 6, 7, 8, 9) control plants from each treatment, 5, and 10) 1 Kb DNA ladder. Watered plants, 4) 10-day stress treated plants, 6, 7, 8, 9) control plants from each treatment, 5, and 10) 1 Kb DNA ladder.

the total RNA extracted from the treated and non-treated plants and two replicates of the PCR reaction were compared. Compared to the control plants, the gene expression of *Rab17* decreased in the 7-day stress treated plants in both cultivars (Figs. 4a, b, c, and d)as. Although this reduction was not significant, it became meaningful after rewatering. In the 10-day stress treated plants, *Rab17* expression was eminent and showed increments of 1.5 and 2 folds in Zarin and Sardari, respectively. This enhancement was significant in Sardari.

Discussion:

The effects of drought stress were investigated on the physiological responses, total protein content, protein patterns, and gene expression of Rab17 in the two bread wheat cultivars of Sardari and Zarin. When the plants were subjected to stress, their cellular metabolism was readjusted to the new conditions in response to the development of the specific tissues and cell types. One such response was the proline accumulation in leaves. Results showed accumulation of proline in plants subjected to drought conditions (Fig. 3). This finding is in agreement with previous stuides correspond that found similar declining trends in leaf proline content and RWC approximately 10 days after their drought treatments (Jyothsnakumari *et al.*, 2009; Roychoudhury *et al*., 2007; Tunnacliffe and Wise, 2007; Liu *et al.*, 2010; Wang *et al*., 2008). However, the decline has been reported to be reversible so that the proline content was restored quickly after re-watering. In fact, drought stress decreased RWC in all the cultivars investigated. Similar results have been reported on beans (*Phaseolus vulgar*) (Vendruscolo *et al*., 2007). On the other hand, the differences observed in the RWCs of the different cultivars under drought stress may be explained by either differences in plants' ability to uptake more water from the soil, or differences in the ability of their stomata to reduce water loss. Shinozaki *et al.* (2007) maintained that RWC in wheat usually, and only usually, decreases with increasing drought stress. In drought stress conditions, the drought-resistant cultivars gain a higher RWC. These results are confirmed by those reported by Sahmsi (2010) and those of the present study.

Protein synthesis is severely affected by drought stress. The drought-stressed plants of both tolerant and sensitive cultivars investigated in the present study exhibited changes in their overall patterns of protein synthesis (Fig. 5). Among the proteins in the mature embryo and vegetative tissues under drought stress and ABA, LEA proteins were found to be the main groups altering their gene expression. These proteins are essential for repairing damaged proteins and they serve as chaperons (Allagulova *et al.*, 2003). It seems that the initial increase in total soluble proteins during the drought stress was due to the expression of new stress proteins, but that the subsequent decrease occurred due to the severe declines in photosynthesis and chlorophyll content (Fig. 2). In the 7-day stress treated plants, dehydrin increased in both cultivars as did the polypeptides with molecular weights of 20, 15, and 2 KDa. Compared to the quantities of the corresponding polypeptides in the control plants, eight with molecular

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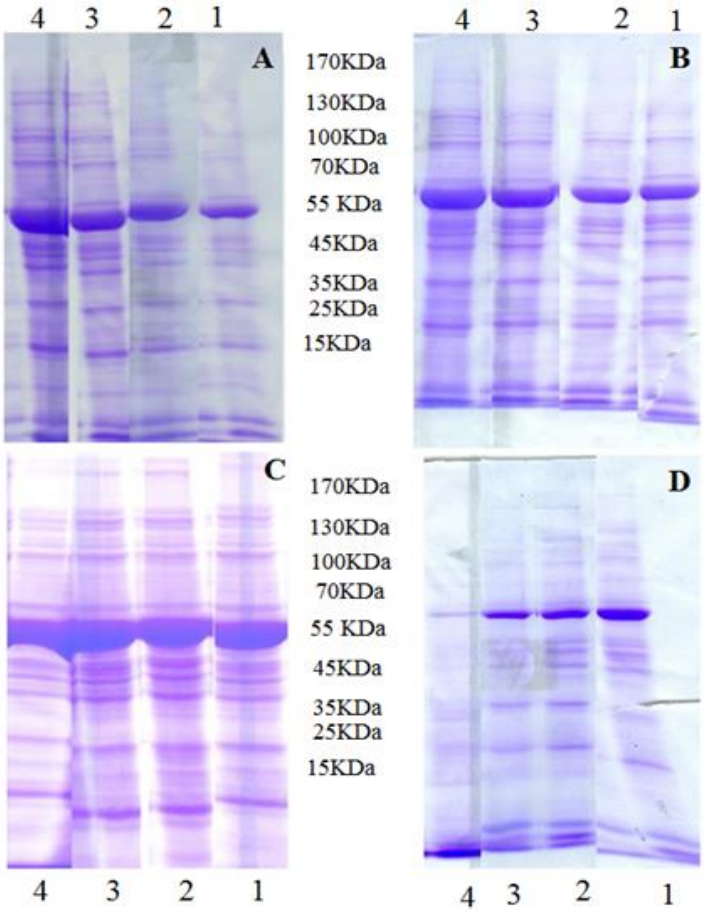


Figure 5. a) Patterns of protein leaf from 0-day stress treated plants of the Sardari and Zarin two cultivars separated by one-dimensional SDS-PAGE: 1) Sardari stress treated plants, 2) Zarin control plants, 3) Zarin stress treated plants, and 4) Sardari control plants; b) Patterns of protein leaf from 7-day stress treated plants of the two cultivars: 1) Zarin stress treated plants, 2) Sardari control plants, 3) Sardari stress treated plants, and 4) Zarin control plants; c) Patterns of protein leaf from the re-watered plants of the two cultivars: 1) Sardari stress treated plants, 2) Zarin control plants, 3) Zarin stress treated plants, and 4) Sardari control plants; and d) Patterns of protein leaf from the 10-day stress treated plants of the two cultivars: 1) Zarin control plants, 2) Zarin stress treated plants, 3) Sardari stress treated plants, and 4) Sardari control plants.

weights ranging from 8 to 61 KDa increased in the Zarin cultivar and the two with 12 and 75 KDa were observed to increase in the Sardari cultivar. This is while the polypeptides with molecular weights of 15, 63, and 40 KDa and a 12 KDa polypeptide reduced in Zarin and Sardari, respectively. Meanwhile, in the 10-day stress treatment, five polypeptides with molecular weights ranging from 50 to 200 KDa and four with molecular weights from 37 to 54 KDa increased in Zarin and in Sardari, respectively. However, thirteen polypeptides with molecular weights ranging from 20 to 130 KDa and four with molecular weights ranging from 12 to 180 KDa, respectively, decreased in increased in Zarin and Sardari. The variations observed in total soluble proteins of wheat under the drought conditions in this study are consistent with those reported by Riccardi *et al*. (1998) and Ti-da *et al* (2006) on maize and Bensen *et al*. (1988) on soybean (*Glycine max*). These authors reported that drought stress led to increases in certain proteins but decreases in others.

In summary, it was found that the differentces in the responses of the two cultivars with different levels of drought tolerance can be explained by the expression severity of the single gene, Rab17, rather than by the expression of new genes involved in the tolerance or susceptibility of the cultivars. Hence, it might be hypothesized that as plant tolerance to abiotic stresses is independent of particular gene/genes expression, it must be indirect effects of genes on such chaperon molecules as proline that confer tolerance unto plants. Furthermore, the patterns of proteins examined in this study revealed significant alternations that lead to the presence or absence of certain polypeptides in the tolerant and susceptible wheat cultivars under stress conditions. This finding bears witness to the important roles played by the protein patterns in the wheat tolerance mechanisms, which is suggested to be further investigated in future research.

Acknowledgements:

This study was supported partly by the National Institute of Genetic Engineering and Biotechnology

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(NIGEB) under Grant No. 271 and partly by Mohaghegh Ardebili University.

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