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High Temperature Responses of *Aegilops biuncialis* Species and *Triticum durum* Cultivar

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Abstract: Thermal tolerances and heat shock protein (s) [HSP(s)] of wild wheat species (*Aegilops biuncialis* Vis.) and *Triticum durum* Desf. cv. Harran-95 were investigated in etiolated seedlings. The thermal tolerances of the genotypes were determined by growth experiment and cell viability test. The HSPs were separated by two-dimensional (2-D) gel electrophoresis technique. In all the experiments, the two-day old etiolated seedlings were exposed to control (23°C, 24 h), acclimation (37°C, 24 h) and heat stress (37°C, 24 h→50°C, 1 h) temperature treatments. In growth experiment, there was no significant difference between the values of acquired thermal tolerance (%) of *Ae. biuncialis* and cv. Harran-95 in all treatments. In cell viability test, the values of acquired thermal tolerance (%) for *Ae. biuncialis* and cv. Harran-95 were detected as 54.5 and 28.4%, respectively. The molecular weights of HSPs synthesized in coleoptile tissues ranged from 19.7 kDa to 67.5 kDa and pI values ranged from 6.3 to 7.3. Most of the HSPs synthesized at acclimation treatment disappeared at heat stress treatment in *Ae. biuncialis*. Four newly synthesized HSPs with molecular weights of 19.7, 19.7, 25.9 and 26.6 kDa (pI values of 6.6, 6.5, 6.6 and 6.4, respectively) were common between *Ae. biuncialis* and cv. Harran-95 at both acclimation and heat stress treatments compared to control.

Key words: *Aegilops biuncialis*, *Triticum durum* cultivar, growth, cell viability, thermal tolerance, heat shock proteins

INTRODUCTION

High temperature stress is an important environmental factor, significantly affecting crop productivity in many regions. The rate of temperature change and the duration and degree of the elevated temperatures affect the intensity of heat stress (Rane and Nagarajan, 2004). Plant response to heat stress depends on thermal adaptation, the duration of exposure and the stage of growth (Gusta and Chen, 1987). Many organisms, including plants, can survive at lethal high temperature treatments (heat stress) if they are first subjected to non-lethal high temperatures (acclimation) as a pre-heat treatment. This phenomenon is called acquired thermal tolerance which is a complex physiological phenomenon (Burke *et al*., 2000). Although several methods have been developed for measuring the heat tolerance of crop plants (Chen *et al*., 1982), the cell viability test [triphenyltetrazolium chloride (TTC) reduction test] has been used successfully to evaluate stress response and acquired thermal tolerance for variety of organism (Chen *et al*., 1982; Ibrahim and Quick, 2001; Mullarkey and Jones, 2000; Porter *et al*., 1994). The ability of viable cells to reduce various tetrazolium salts has been reported for a variety of organisms, reduction apparently occurring in the mitochondria by tetrazolium salt accepting electrons from the electron transport chain (Porter *et al*., 1994).

The exposure of wheat coleoptiles to a temporary sub-lethal high temperature stress (acclimation) results in the synthesis of a group of proteins known as the heat shock proteins, HSPs (Blumenthal *et al*., 1990). HSPs have been found in every eukaryotic and prokaryotic organism analyzed. HSPs are usually divided into five unique classes: HSP 100, HSP 90, HSP 70, HSP 60 and low molecular weight (LMW) HSPs (17-30 kDa) (Waters *et al*., 1996). The appearance of HSPs depends on transient transcriptional activation of the heat-shock genes (Bienz, 1985). The causal involvement of several HSPs in acquired thermal tolerance of plants has been demonstrated (Burke *et al*., 2000). It is thought that HSPs function as molecular chaperones by binding to partially folded or denatured proteins, thereby preventing irreversible aggregation and promoting correct folding (Lee *et al*., 1997).

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In this research, high temperature responses of wild wheat species (*Aegilops biuncialis* Vis.) and cultivated durum wheat cultivar (*Triticum durum* Desf. cv. Harran-95) were assessed in etiolated seedlings exposed to 23, 37 and 37→50°C temperature treatments using recovery growth experiment, cell viability test and 2-D gel electrophoresis technique.

MATERIALS AND METHODS

Plant material and growth conditions: In this study, the seeds of *Aegilops* L. species (*Ae. biuncialis* Vis.) and *Triticum durum* Desf. cv. Harran-95 were used. Seeds of *Ae. biuncialis* was collected from an uncultivated area in Beytepe-Ankara/Turkey, while the seeds of cv. Harran-95 were obtained from Southeast Anatolia Agricultural Research Institute. The imbibed seeds were germinated on moist filter paper (with distilled water) in germination cups in a controlled growth chamber in the dark at 23°C, 50-60% humidity for 48 h.

Temperature treatments: Two-day-old etiolated seedlings (the lengths of coleoptiles and seminal roots were approximately same lengths) were exposed to control (C; 23°C, 24 h), acclimation (T_1; 37°C, 24 h), acclimation following by heat stress (T_2; 37°C, 24 h→50°C, 1 h) and control following by heat stress (T_3; 23°C, 24 h→50°C, 1 h) treatments. After the temperature treatments, the etiolated seedlings were transferred to 23°C and then sampled at the end of 24, 48 and 72 h recovery growth periods. The seedlings were watered with half-strength Hewitt's solution once each day. The experiments were carried out with three replicates, each replicate consisted of four seedlings in recovery growth and viability test.

Growth parameters: In the recovery growth experiments, the shoot length (the part from the crown to the tip of the first true leaf) and the seminal root (the part from the crown to the tip of the longest seminal root axis) were measured as cm. seedling^{-1} after each recovery growth period. The seedlings were not survived at 23→50°C (T_3). After the recovery growth period, acquired thermal tolerance (ATT%) to high temperature was expressed in terms of the length of shoot and seminal root at T_1 and T_2 treatments, relative to the values of the control seedlings.

Cell viability test [2, 3, 5-triphenyltetrazolium chloride (TTC) reduction test]: The segments of coleoptiles (1 cm long cut just over the crown) from 3-day-old etiolated seedlings were used after C (23°C, 24 h) and T_1 (37°C, 24 h) temperature treatments. Some of the segments of coleoptiles from C and T_1 treatments were incubated at 50°C for 1 h [$C_{(50°C)}$ and $T_{1(50°C)}$], while the others were incubated in boiling water for 1 min [$C_{(boiled)}$ and $T_{1(boiled)}$]. Immediately following the temperature treatments, the segments of coleoptiles transferred to TTC solution. TTC experiment was performed using the protocol of Porter *et al.* (1994) with minor modifications. At the end of each treatment, the level of formazan which was produced as the result of TTC reduction in all genotypes was determined by measuring the optical density at 475 nm with a double beam spectrophotometer (Jenway 6105 U.V./Vis.). Acquired thermal tolerance (ATT%) is the measure of cell viability in the non-acclimated and acclimated coleoptile segments expressed by following formulas (Porter *et al.*, 1994):

ATT% for non-acclimated coleoptile segments = $[C_{(50°C)} - C_{(boiled)}] \times 100/C - C_{(boiled)}$

ATT% for acclimated coleoptile segments = $[T_{1(50°C)} - T_{1(boiled)}] \times 100/T_1 - T_{1(boiled)}$

Protein extraction and two-dimensional gel electrophoresis: Two day-old etiolated seedlings were exposed to control (C; 23°C, 24 h), acclimation (T_1; 37°C, 24 h) and acclimation following by heat stress (T_2; 37°C, 24 h→50°C, 1 h) treatments. In brief, the coleoptile tissues from *Ae. biuncialis* and Harran-95 seedlings were frozen and ground in liquid nitrogen and total soluble proteins were extracted by addition of 5 mL of 10% trichloroacetic acid in acetone with 0.07% β-mercaptoethanol (Damerval *et al.*, 1986). Protein concentrations of samples were determined according to Bradford (1976) and Ramagli and Rodriguez (1985). The characterization of protein samples was performed by 2-D gel electrophoresis. In the first dimension, isoelectric focusing (IEF) was performed (O'Farrell, 1975). IEF gel solution was prepared with 10 g urea, 3 mL of acrylamide-bis acrylamide, 0.2 mL ampholines (pH: 5-8), 0.8 mL ampholines (pH: 3-10), 0.1 mL Nonidet P-40 (detergent) and 0.3 g 3-[(3-Cholamidopropil) dimethyl-ammonio]-1-propane sulfonate (Sigma, St. Louis, MO) and 7.4 mL distilled water (Hochstrasser *et al.*, 1988). Sample preparation and application for IEF was carried out according to the method of Naqvi *et al.* (1994). A blank gel without protein sample was prepared in the same way for determination of the pH gradient. The cathodic solution was 20 mM sodium hydroxide while the anodic solution was 100 mM phosphoric acid. Separation was achieved under constant voltage of 400 V for 3 h followed by 800 V for 17.15 h. After electrophoresis in the first dimension was completed, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% acrylamide gels was performed (Laemmli, 1970). In the second dimension, on top of the gel, 0.5% agarose with 0.01% bromphenol blue was layered. Commercial protein molecular weight markers (10-70 kDa; Sigma) were prepared by Sigma commercial catalogue and used. Separation was performed using a 10 mA constant current for the first 30 min and then 25 mA for the rest of the run. The gels were stained with silver dye (Blum *et al.*, 1987)

and dried (Krishnan and Nguyen, 1990). Isoelectric points (pI was obtained with measuring pH value of the blank gel without protein sample) and molecular weights (kDa) of heat shock proteins were determined by reference to standards.

Experimental design and statistical analysis: The experiments were performed in a randomized design. Differences between the temperature treatments as well as between the genotypes for all recovery growth periods in growth experiments and differences between the genotypes for cell viability assay data were tested using SPSS statistical program. Statistical variance analysis of the independent data with twelve replicates (n = 12) was performed using analysis of variance (ANOVA) and compared with Least Significant Difference (LSD) at the 5% level.

RESULTS

Growth parameters and cellular thermal tolerance: The shoot and seminal root lengths of all genotypes were decreased significantly at T_1 and T_2 temperature treatments compared to control. However, the seedlings survived at acclimation following by heat stress treatment (50°C, 1 h) (data not shown). For the shoot and seminal root lengths (%), ATT_2 values of *Ae. biuncialis* and cv. Harran-95 were significantly lower than ATT_1 at the end of 48 and 72 h recovery growth periods (Fig. 1a and b). ATT values for the shoot lengths of *Ae. biuncialis* fallen from 82.5% (ATT_1) to 53.2% (ATT_2) and these values for cv. Harran-95 were found as 83.1 and 53.9% at the end of 72 h recovery growth period. In addition, ATT values for the seminal root lengths of *Ae. biuncialis* fallen from 86.0% (ATT_1) to 59.1% (ATT_2) and these values for cv. Harran-95 were found as 78.0 and 45.2%. There was no significant difference between *Ae. biuncialis* and cv. Harran-95 with respect to ATT values of the shoot and seminal root lengths (Fig. 1a and b).

The difference in the level of TTC reduction by non-acclimated seedlings was significant in non-stressed tissue (23°C) ($p \leq 0.05$). The non-acclimated coleoptile tissues were not able to survive the 50°C (1 h) treatment. The values of acquired thermal tolerance (%) were very low and negative. This result clearly showed that non-acclimated tissue was not capable of ATT (Table 1).

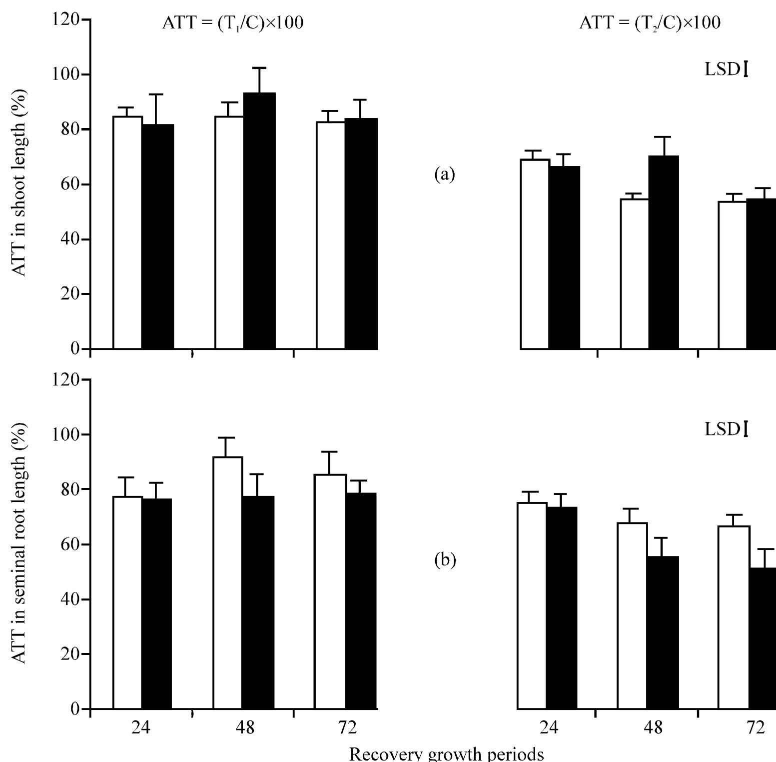

Fig. 1: Acquired thermal tolerance values (%) for the lengths of shoot and seminal root of *Ae. biuncialis* (□) and cv. Harran-95 (■). The means are calculated from values which are measured at the end of 24, 48 and 72 h recovery growth periods after exposed to C (23°C, 24 h), T_1 (37°C, 24 h) and T_2 (37°C, 24 h→50°C, 1 h) temperature treatments. Each mean is obtained from independent data with twelve replicates. Least Significant Difference (LSD) test was used to determine significant differences of means at a 5% level. Bar on each column represents±SE of mean

Table 1: Cellular thermal tolerance values of non-acclimated and acclimated *Aegilops biuncialis* Vis. and *Triticum durum* Desf. cv. Harran-95 measured by TTC reduction of coleoptile tissue

	Non-acclimated coleoptile segments				Acclimated coleoptile segments			
	23°C [C]	50°C [$C_{50°C}$]	Boiled [C_{Boiled}]	ATT	37°C [T_1]	50°C [$T_1(_{50°C})$]	Boiled [$T_{1(Boiled)}$]	ATT
Cultivars		OD 475		%		OD 475		%
Ae. biuncialis	1.061A*	0.004A	0.011A	-0.7A	0.914A	0.504A	0.012A	54.5A
cv. Harran-95	1.359B	0.019B	0.016B	0.2A	2.541B	0.727B	0.008B	28.4B

*Means followed by the different letter(s) within each column are significantly ($p \leq 0.05$) different according to LSD

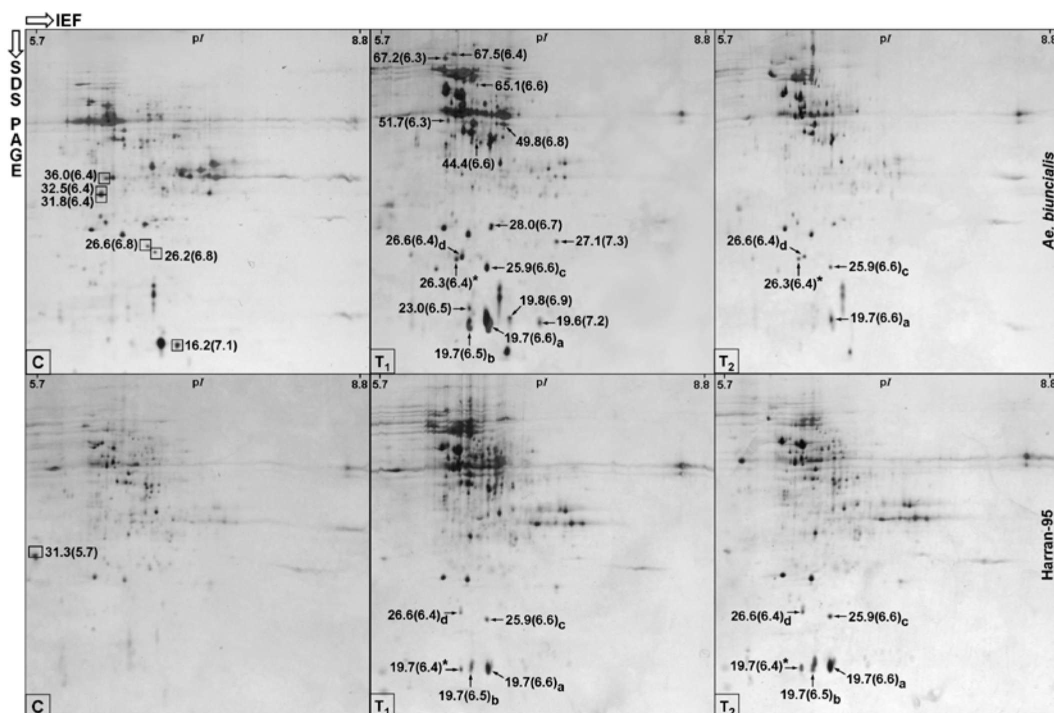

Fig. 2: 2-D gel electrophoresis protein profiles of total coleoptile tissues of *Ae. biuncialis* and cv. Harran-95 at 23°C (C), 37°C (T_1) and 37→50°C (T_2) temperature treatments. The normal cellular proteins disappeared at T_1 and T_2 temperature treatments are shown into squares on C gels. The HSPs are indicated by arrows. The same letters (a-d) indicate the common HSPs synthesized in two genotypes. The decimal numbers [molecular weight (kDa) and isoelectric point (pI), respectively] without asterisk indicate the HSPs synthesized at a temperature treatment within each genotype, while the others with asterisk indicate the HSPs synthesized at T_1 and T_2 temperature treatments within each genotype

In coleoptile tissues of acclimated (37°C, 24 h) seedlings (Table 1), the capacity of the tissue to ATT, the level of diversity of the response was significantly different ($p \leq 0.05$). The coleoptile tissues of acclimated seedlings were able to survive the 50°C (1 h) treatment. The values of ATT were 54.5% for *Ae. biuncialis* and 28.4% for cv. Harran-95.

Heat shock proteins in coleoptile tissues of etiolated seedlings: Total protein profiles of coleoptile tissues of *Ae. biuncialis* and cv. Harran-95 were investigated by two-dimensional gel electrophoresis followed by silver staining. Similar data were observed in three electrophoresis repeats. The electrophoretic profiles of these genotypes were showed in Fig. 2. In coleoptile protein profile of *Ae. biuncialis*, as a result of both high temperature treatment (37 and 37→50°C), six normal cellular proteins with molecular weights ranged from 16.2 to 36.0 kDa (pI values ranged from 6.4 to 7.1) were disappeared, relative to control (C gel in Fig. 2). In addition, one normal cellular protein (31.3 kDa; pI 5.7) was disappeared in cv. Harran-95. The molecular weights of

HSPs synthesized in coleoptile tissues ranged from 19.7 to 67.5 kDa and pI values ranged from 6.3 to 7.3. Most of the HSPs synthesized at acclimation treatment disappeared at heat stress treatment in *Ae. biuncialis* (T_1 and T_2 gel in Fig. 2). Four newly synthesized HSPs with molecular weights of 19.7, 19.7, 25.9 and 26.6 kDa (pI values of 6.6, 6.5, 6.6 and 6.4, respectively) were common between *Ae. biuncialis* and cv. Harran-95 at both acclimation and heat stress treatments compared to control (T_1 and T_2 gels in Fig. 2). One newly protein (26.3 kDa; pI 6.4) synthesized in only *Ae. biuncialis* was detected at both high temperature treatments and also one newly HSP (19.7 kDa; pI 6.4) synthesized in only cv. Harran-95 was detected (shown with asterisk on T_1 and T_2 gels in Fig. 2).

DISCUSSION

The recovery growth experiment results showed that none of the seedlings survived at control (23°C for 24 h) following by heat stress treatment (50°C, 1 h). In contrary to this, the seedlings survived after acclimation (37°C for 24 h) following by heat stress treatment, thus the seedlings were protected by acclimation temperature treatment (data not shown). ATT_1 (%) values of the shoot lengths were over 80%, but ATT_2 (%) values fell below 70%. Similar decrements were also obtained for the seminal roots. These results show that the acclimation temperature at 37°C for 24 h used in this study was sufficient to give thermo-protection to seedlings. The acclimated (37°C, 24 h→50°C, 1 h) seedlings gained over 50% thermal tolerance, relative to non-acclimated (23°C, 24 h→50°C, 1 h) seedlings. Similar results also reported in soybean *Glycine max* var. Wayne seedlings (Lin *et al*., 1984), *Triticum aestivum* L. cultivars (Blumenthal *et al*., 1990), sunflower seedlings (Kumar *et al*., 1999), *Arabidopsis thaliana* hypocotyl (Hong and Vierling, 2000) and *Pisum sativum* L. varieties (Ganeshkumar *et al*., 2002).

TTC reduction occurs in mitochondria via electron transport chain. Two aspects of cell damage (i.e., membrane stability and enzyme activity) following heat stress are being assayed at once with TTC test (Porter *et al*., 1994). TTC reduction test was used effectively in the selection of thermo-tolerant mutants of wheat cv. Guardian (Mullarkey and Jones, 2000) and in genetic variability and heritability of acquired thermal tolerance of fourteen diverse winter and spring wheat lines (Ibrahim and Quick, 2001). The inability to detect genotypic differences in levels of acquired thermal tolerance on non-acclimated seedlings, subjected to a normally lethal temperature treatment (50°C for 1 h), is consistent with reports on winter wheat cultivars and two genotypes each of bean, potato, soybean, tomato (Chen *et al*., 1982; Porter *et al*., 1994). In this study, TTC cell viability test was well suited to measure genotypic differences in acquired thermal tolerance in *Ae. biuncialis* and cv. Harran-95. Acquired thermal tolerance in acclimated seedlings was significantly higher than non-acclimated seedlings. Although acclimation at 37°C for 24 h was sufficient to achieve thermal tolerance, injury in the acclimated coleoptile tissue of cv. Harran-95 was over 70% following high temperature treatments. There was significant genotypic difference in acclimated seedlings. Porter *et al*. (1994) suggested that the identification of genotypes with differential capacities to acquire thermal tolerance provides the germplasm base needed for further characterization of the physiological and biochemical bases for acquired thermal tolerance and identifies genotypes to be included in a program for improving plant performance under semi-arid environments.

Heat shock proteins have been studied extensively in different tissues of soybean, rice, pea, sunflower, tobacco, wheat (Blumenthal *et al*., 1990; Ganeshkumar *et al*., 2002; Kumar *et al*., 1999; Lin *et al*., 1984; Mariamma *et al*., 1997; Park and Hong, 2002). These researchers reported that the plants exposed to different temperature treatments synthesized different classes of heat shock proteins with high- (HMW), intermediate- (IMW) and low- (LMW) molecular weights. The synthesis of HSPs has been related to the acquisition of thermal tolerance. Additionally, LMW HSPs are unique to plants and associated with organelles. In our study, most of the HSPs were LMW HSPs (19.7-28.0 kDa) with acidic character (< pI 7). There was variation in the LMW HSP response between *Ae. biuncialis* and cv. Harran-95. The variability in heat shock proteins was observed among Chinese Spring, Moisson and Selkirk wheat varieties (Zivy, 1987) and among accessions (M3, M5 and M9) of *Triticum monococcum* L. (Vierling and Nguyen, 1990). Genetic variability for thermal tolerance and expression of individual HSPs were noted in cultivated cereals (Burke, 2001; Maestri *et al*., 2002). Some researchers suggested that this variation in HSPs was correlated with the intrinsic thermal tolerances of the genotypes (Park *et al*., 1997) or environmental conditions within the species ranges (Downs *et al*., 1998). In our study, the number of LMW HSPs (15 protein spots) was more than IMW- (3 protein spots) and HMW- (3 protein spots) HSPs. In addition, especially 19.7 kDa (pI 6.6) HSP was accumulated as a big spot in *Ae. biuncialis* and cv. Harran-95. Vierling and Nguyen (1990) suggested that the abundance and heterogeneity of LMW HSPs may have unique physiological functions in plant somatic tissues.

On the other hand, the electrophoretic profiles showed that synthesis of some cellular proteins observed in the control gels disappeared at both acclimation and heat stress treatments. Blumenthal *et al.* (1990) reported that the appearance of HSPs was associated with a concomitant reduction in normal protein synthesis and has been correlated with the acquisition of thermal tolerance (assessed as growth of coleoptiles). Most of the HSPs synthesized at acclimation treatment disappeared at heat stress treatment in *Ae. biuncialis*. Necchi *et al.* (1987) reported that gradually synthesis of new HSPs (103-70, 59-32 and 16-17 kDa) was described in the coleoptiles of some monocotyledons at 40°C for 1, 2 and 4 h.

In conclusion, the measurement of seedling growth may be used to screen a large number of genotypes with respect to acquired thermal tolerance. On the other hand, further work in cell viability test is needed in cultivated and closely related wheat to characterize the plant's capacity to acquire thermal tolerance at the critical flowering and grain-filling stages and to establish the relationship between acquired thermal tolerance at the seedling and further critical stages. Being sensitive and reproducible, 2-D gel electrophoresis followed by silver staining is a convenient procedure for detection of changes in protein profiles of tissues (Naqvi *et al.*, 1994). HSPs in coleoptile tissues of *Ae. biuncialis* and cv. Harran-95 were well separated using 2-D gel electrophoresis technique.

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