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PHYSIOLOGICAL AND HEAT SHOCK PROTEIN RESPONSES IN BELL PEPPER PLANTS UNDER HEAT STRESS

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Abstract
The effects of heat stress on the relative water content (RWC), turgidity loss, total soluble protein (TSP), SDS-PAGE protein profiles, and heat shock protein of bell pepper (Capsicum annuum L. cv. "Kandil") leaves were studied. During the seedling stage, leaf samples were obtained from the plants and subjected to heat stress treatments in a water bath at 35, 40, 45, 50, 55, and 60 °C in 30-minute increments. Heat stress tolerance (LT50) was then computed. The results revealed that the LT50 value for the bell pepper cultivar was 41.2 °C. As temperature rose, leaf RWC decreased and turgor loss increased. The TSP content was higher in the 35 and 40 °C treatments compared to the control group, a significant decrease was observed at 60 °C. SDS-PAGE analysis revealed various polymorphic protein bands ranging from 7 to 54 kDa. The HSP60 antibody identified a band around 54 kDa under all temperature treatments. Moreover, the intensity of 54 kDa protein in plants at 35, 40, and 55 °C was higher than in the control group. Furthermore, the strength of this band fell considerably at 60 °C. These results suggest that the 54 kDa protein may play a role in enhancing HST in bell pepper plants.

Keywords: Capsicum annum L., LT50, high temperature stress, protein expression, relative water content.

1. INTRODUCTION

Temperature is one of the most important environmental factors for the survival of plants. Capsicum crops thrive between 20-25 °C, while temperatures exceeding 32 °C cause heat stress, which reduces growth (Saha et al., 2010). Heat stress reduces photosynthesis, stunts plant development, and causes aborted fruits, all of which have an impact on production (Angon et al., 2024). Heatwaves are anticipated to rise by 50% by 2050, considerably increasing the risk for producers (Hassan et al., 2024). Heat waves that strike during important growth periods can cost growers between £5,000 and £10,000 per hectare (European Commission, 2021).

Heat stress increases transpiration, which causes dehydration and a water deficit in plants (Zhao et al., 2020). High temperatures damage root function, limiting water absorption and aggravating stress levels (González-García et al., 2023). Water deficiency causes the creation of abscisic acid (ABA), which signals stomatal closure to conserve water but inhibits photosynthesis (Buckley, 2019). Heat stress compromises membrane integrity, resulting in ion leakage and impaired cell activity. Leakage of electrolytes from cells is a common indicator of membrane damage severity during heat stress (Ilik et al, 2018). Heat-induced oxidative stress accelerates lipid peroxidation, which further destabilizes membrane lipids and structures (Sachdev et al., 2021).

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Plants have evolved extensive biochemical defensive mechanisms to deal with heat stress, which disrupts cellular structure and function. These defenses include heat shock proteins (HSPs), antioxidants, carbohydrates, lipids, secondary metabolites, and phytohormones (Hasanuzzaman et al., 2013). Heat stress typically causes a decrease in normal protein synthesis, which is followed by an increase in the transcription and translation of novel proteins known as HSPs. HSPs and other stress proteins have been shown to protect cells against the detrimental effects of stress (Ul Haq et al., 2019). HSPs function as molecular chaperones, inhibiting protein denaturation under heat stress conditions (Ahuja et al., 2010). Their production is increased in response to high temperatures to protect cellular proteins. HSPs are categorized into sub-families based on their molecular weight, including HSP100, HSP90, HSP70, HSP60, and small HSPs (Shamovsky and Nudler, 2008; Guo et al., 2015). The HSP60/70/90 families are the most extensively investigated HSPs (Hu et al., 2020). Among them, it is known that HSP60 plays a function in protein folding and assembly. Protein folding requires collaboration between HSP70, HSP60, and HSP10 (Langer et al., 1992). HSPs stabilize proteins, keeping them functioning even when exposed to high temperatures (Park and Seo, 2015). They help to refold or repair proteins damaged by thermal stress, restoring function. HSPs direct permanently damaged proteins toward destruction via the ubiquitin-proteasome pathway (Hu et al., 2020). Overexpression of HSP genes in transgenic plants promotes their survival and development under heat stress (Wang et al., 2020).

With global warming, high temperature is predicted to be one of the limiting factors for cultivation of pepper and other plants in the future. Because of their high heat need, peppers are produced in tropical regions or in subtropical and temperate zones throughout the summer months. It is also cultivated under greenhouses. As a result, they are susceptible to extreme temperatures throughout their lives. Heat stress is a major danger to bell pepper growth. Heat resilience is becoming more significant. Exploring physiological reactions as well as HSP pathways will help us understand how to create varieties that can resist rising temperatures and retain productivity in the face of climate change. This study looked into the impact of high temperatures on pepper plants in the context of global warming and harsh climate changes.

2. MATERIALS AND METHODS

This study looked at heat stress-induced physiological changes and heat shock proteins (HSPs) in bell pepper plants (*Capsicum annuum* L. cvs. 'Kandil'). Samples were gathered from seedling plants grown in a field under favorable conditions in Eskisehir, Türkiye (longitude: 39° 45' 38" N, latitude: 30° 28' 47" E). The average temperature was recorded as 22-24 °C. The leaves were treated to heat stress treatments in a water bath at 35, 40, 45, 50, 55, and 60 °C, with half-hour increments (Arora et al., 1998). Half of the samples obtained at each treatment temperature were used for membrane thermostability, leaf RWC and loss of turgidity analyses while the other half were immediately frozen in liquid nitrogen and stored at -80 °C for protein analysis. The approach of Arora et al. (1998) was followed, with few modifications, to determine membrane thermostability. The heat stress tolerance (HST; LT_{50}) was calculated as the temperature that caused half of the maximum percent injury (50%) based on electrolyte leakage (EL). Leaf discs with a diameter of 1.5 cm were removed from leaves, washed with deionized water, and inserted in tubes containing 15 mL of distilled water for EL measurements. After shaking the samples at 100 rpm for 4 hours at room temperature, the amount of EL (EC1) was measured using an EC meter (Mettler Toledo, SevenEasy S30, Colombus, Ohio, USA). The tubes were autoclaved at 121°C for 15 minutes. Total EL (EC2) was measured and estimated using the equation.

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EL (%) = (EC1/EC2) x 100.
The Barr and Weatherley (1962) method was used to determine leaf RWC (%) and turgidity loss. Briefly, 1.5 cm leaf discs were collected, and the fresh weight (FW) was recorded. The leaf discs were then floated in distilled water on a petri plate for 4 hours at room temperature before being removed, blotted, and measured for turgid weight (TW). The leaf discs were oven-dried for 48 hours at 70 °C to determine their dry weight (DW). The leaf RWC and loss of turgidity were computed as follows:
RWC (%) = [(FW-TW)/(TW-DW)]*100.
Loss of turgidity (%),= [(TW-FW)/TW]*100.
Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with anti-HSP60 monoclonal antibody were used to investigate protein patterns in heat stress. Total soluble proteins were extracted using the Shen et al. (2003) technique, with a few changes. In summary, 0.25 g of each sample was homogenized in 1 ml of homogenate buffer comprising 25 mM Tris-base (pH 7.8), 275 mM sucrose, 2 mM EDTA, 10 mM Dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 1% polyvinylpolypyrolidone (PVPP). The homogenate was transferred to an eppendorf tube and centrifuged at 10,000 rpm for 10 minutes at 4 °C. The protein content was determined using the Bradford technique (Bradford, 1976). SDS-PAGE was carried out on a PROTEAN tetra vertical electrophoresis unit with 0.04 stacking gel and 0.125 separation gel. Each sample received an identical amount of total protein, and the gels were stained with Coomassie Brillant Blue. Western blotting was used to detect HSPs, as described by Arora and Wisniewski (1994). HSP60 [Monoclonal Anti-Heat Shock Protein 60 antibody developed in mice by Sigma] was used. The alkaline phosphatase assay was performed using the ProtoBlot Western Blot AP Kit (Promega) to detect immunoreactive bands. The band pictures on the membranes were acquired with a scanner and transferred to digital media. Densitometric studies of the bands were carried out with the Public Domain NIH Image program (available at http://rsb.info.NIH.gov/nih-image/), also known as ImageJ software.
The experiment was carried out in three replications using a completely randomized design. The data were analyzed with SPSS Statistics for Windows 22. The Duncan test was used to detect significant differences between treatments at a significance level of 0.05.

3. RESULTS AND DISCUSSIONS

The calculated leaf cell membrane injury percentages and stress tolerance point (LT_{50}) values of the bell pepper plants are shown in Figure 1. Membrane damage increased proportionally with temperature, and the increase was noticeable at 45 °C. While the injury rate was 46.32% at 40 °C, it increased to 67.58% at 45 °C, and reached 76.00% at 60 °C. Statistical analyses revealed that temperature had significant effects on membrane injury (Table 1). Besides, the stress tolerance level (LT_{50}) of cv. “Kandil” bell pepper was determined as 41.12 ºC. (Figure 1). Heat stress led to higher injury rates in strawberry (Kesici et al., 2013), green bean (Tokyol and Turhan, 2019), tomato (Turhan et al., 2022), and watermelon (Aydogan and Turhan, 2024) plants. Cell membrane thermostability is a useful measure for detecting heat tolerance in plants (Usman et al., 2015). Heat stress causes severe changes in the stability of the cell membrane, influencing the sensors that are present in it (Suzuki and Mittler, 2006). According to Craufurd et al. (2003), cell membrane thermostability greater than 60% is heat-tolerant, 30% to 60% moderate, and less than 30% sensitive to heat stress with minor variations. Gajanayake et al. (2011) for ornamental peppers and Usman et al. (2015) for chili peppers identified thermotolerant genotypes as those with cell

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membrane thermostability more than 59.50% and 60%, respectively. As a result, the Kandil bell pepper cultivar employed in this study was classified as moderately heat resistant. Turhan et al. (2015) found that under HS, the HST (LT_{50}) was 36.9 ºC, 37.0 ºC, and 42.3 ºC for pepper plants cvs. Çarli, Çoban, and Demre, respectively.

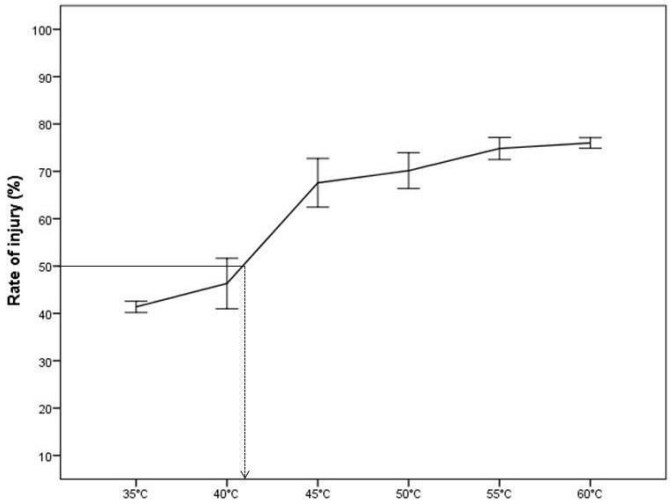

Figure 1. High temperature tolerance value of bell pepper plants depending on high temperature applications. Vertical bars show the ± SD of the repetitions.

Table 1. The rate of injury, Leaf RWC, Loss of turgidity and TSP content of bell pepper plants at different temperatures.

Temperature	Rate of injury	Leaf RWC	Loss of turgidity	TSP
Control	-	85.50^{a}	10.31^{b}	12.07^{bc}
35 °C	41.39^{b}	81.41^{ab}	18.06^{a}	13.03^{ab}
40 °C	46.32^{b}	76.13^{bc}	20.64^{a}	13.55^{a}
45 °C	67.58^{a}	74.91^{bc}	21.40^{a}	12.16^{bc}
50 °C	70.15^{a}	74.29^{bc}	22.37^{a}	11.85^{c}
55 °C	74.83^{a}	72.00^{c}	24.09^{a}	11.52^{c}
60 °C	76.00^{a}	71.31^{c}	24.49^{a}	6.98^{d}
F value	12.264	4.702	5.821	46.461

Data are the means of three replicates. Mean values labeled with different letters in columns were significantly different at $p<0.05$

Figure 2 shows the changes in RWC and turgidity loss in bell pepper plants under heat stress. The RWC began to decline from 35 °C onward and continued to decrease in parallel with rising temperatures. Overall, RWC values dropped from 85.50% under control conditions to 81.41% at 35 °C, 76.13% at 40 °C, 74.91% at 45 °C, 74.29 % at 50 °C, ~ 72,00% at 55 and 60 °C (Figure 2A). The effect of heat stress treatment on RWC was statistically significant (Table 1). Additionally, turgidity loss values increased progressively with heat stress treatments in bell pepper plants. The turgidity loss value, which was 10.31% in the control group plants, reached 24.49% at 60 °C. The effect of temperature treatments on turgor loss was found to be statistically significant and all

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treatments except control were in the same statistical group (Table 1) (Figure 2B). Relative water content could be used as a measure of plant water status, indicating the balance of water absorbed by the plant and consumed by transpiration (Arjenaki et al., 2012). Leaf RWC decreased in response to high temperatures in bean (Tokyol and Turhan, 2019), several cucurbits (Ergin et al., 2021), and tomato (Turhan et al., 2022). Heat stress reduces leaf RWC and turgidity as a result of increased transpiration (Cansev, 2012). In the present study, loss of turgidity increased with temperature treatments. This was consistent with the findings of previous investigations on tomatoes (Turhan et al., 2014), beans (Tokyol and Turhan, 2019), and cucurbits (Ergin et al.,2021).

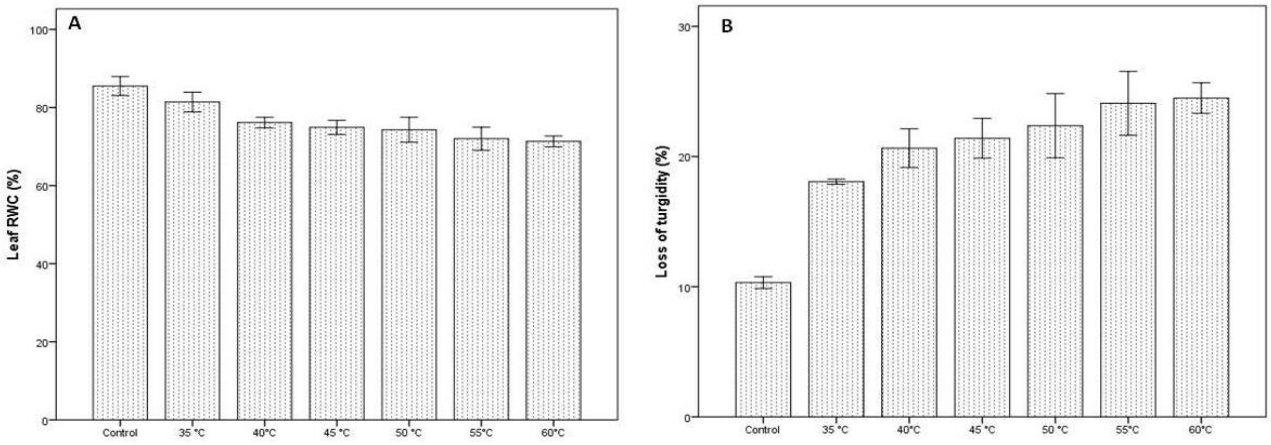

Figure 2. Leaf RWC (Panel A) and loss of turgidity (Panel B) of bell pepper plants. The vertical bars show the repetitions' standard deviation (SD).

Figure 3 shows how temperature applications affect the TSP content of cv. Kandil. At 35 and 40 °C, TSP concentration in leaf tissues rose considerably compared to the control treatment. TSP concentration in leaf tissues decreases at temperatures above 45 °C. TSP concentration reduced substantially at 60 °C. The highest TSP concentration was recorded at 40 °C (13.55 mg protein/gFW), and the lowest at 60 °C (6.98 mg protein/gFW). The statistical analysis revealed that temperature had a significant effect on TSP content (Table 1). Aydogan and Turhan (2022) also showed that high temperatures reduces TSP in reddish beans. The TSP concentration of watermelon and zucchini remained unchanged when the temperature rose, whereas it was reduced in cucumbers; melon cultivars showed a considerable decrease in TSP at 60°C, but no discernible change until 55°C (Ergin et al., 2021).The results of the current study indicate that while bell pepper plants adjust their protein metabolism under heat stress, the plant maintains a stronger TSP response especially at 40 °C. The observed increase in total protein content under high-temperature conditions up to 40 °C is likely due to the enhanced synthesis of stress-related proteins, particularly heat shock proteins (HSPs). This upregulation reflects the activation of the plant's defense mechanisms in response to thermal stress, depending on the severity and duration of the exposure (Hasanuzzaman et al., 2013).

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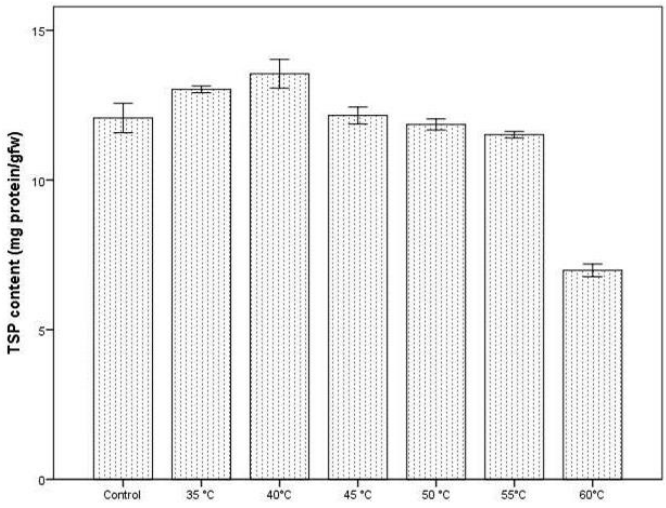

Figure 3. TSP content of bell pepper plants depends on high-temperature applications. Vertical bars show the ± SD of the repetitions.

Figure 4A presents the protein profile from the SDS-PAGE analysis of bell pepper leaf tissues. A 40 kDa protein band was clearly visible in all treatments and is presumed to be a structural protein. Proteins of 7 and 54 kDa were prominent at higher temperatures except 60 °C compared to control. The overall intensity of protein bands declined with rising temperature, with a more pronounced reduction observed at 60 °C.

Figure 4B presents the HSP profile identified through immunoblot analysis following high temperature treatments. HSP60 levels nearly doubled at 35 °C compared to the control. At 40 °C, HSP60 was higher than control. However, at 45 and 50 °C, the level declined to near-control values, and at 55 °C HSP levels peaked and increased almost 3 times compared to the control. At 60 °C, HSP 60 decreased dramatically. Shortly, the cv. "Kandil" showed a steady increase in HSP60 levels with rising temperature. Although the increase at 45 and 50 °C was lower than that at 40 °C, it remained close to control values, indicating that HSP60 may play a key role in sustaining heat tolerance in the cv. "Kandil". Under elevated temperatures, the expression of HSPs is significantly upregulated in many plant species as part of the protective mechanism against protein misfolding and aggregation (Hasanuzzaman et al., 2013). The majority of HSP60 family proteins are induced by heat and play essential roles in preventing protein aggregation as well as facilitating protein folding and refolding within mitochondria under heat stress conditions (Kang et al., 2022). Several studies have investigated the expression of heat shock protein (HSP) genes in *Capsicum annuum* under temperature stress, revealing their critical role in heat stress tolerance. Multiple members of the HSP family, such as CaHSP70, CaHSP60, CaHSP20, and CaHSP16.4, have demonstrated upregulation in response to thermal stress (Guo et al., 2015; Usman et al., 2015; Feng et al., 2019; Haq et al., 2019). Among these, the HSP70 gene, which confers thermotolerance, showed a markedly higher expression in thermotolerant pepper lines at 42 °C than in thermosensitive ones (Usman et al., 2015). Furthermore, 15 CaHSP60 genes were found to be elevated in both heat and cold stress situations by Haq et al. (2019). These results imply that the variation in heat stress tolerance shown across several pepper genotypes may be attributed to differential regulation of HSP genes, namely within the HSP60 family.

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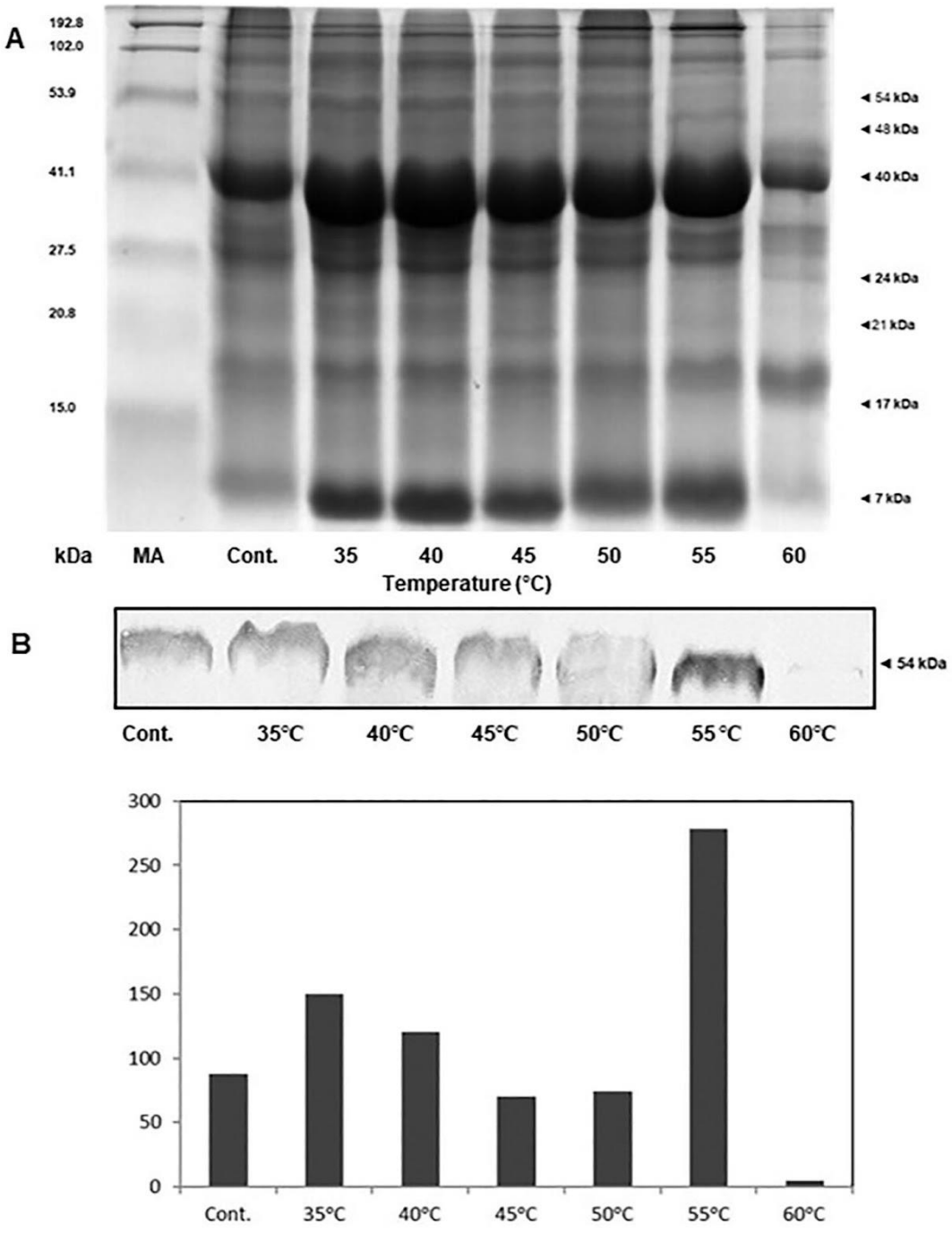

Figure 4. Total protein profiles (Panel A) and status of HSP60 (Panel B) determined due to high-temperature treatments in bell pepper leaf tissues.

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4. CONCLUSIONS

HSPs support cell survival by preventing the oxidative stress-induced aggregation of misfolded proteins. The increased synthesis of stress-related proteins, especially heat shock proteins (HSPs), is probably the cause of the observed rise in total protein content under high temperature settings. By reducing proteolytic degradation and refolding denatured proteins, HSPs stabilize the proteome in adverse environments. By promoting lipid peroxidation and protein denaturation, heat stress also damages membrane structure, increasing membrane permeability and causing cellular homeostasis to be lost. The current study found that high temperatures caused damage to the membranes, indicating that cell membranes are extremely vulnerable to oxidative stress brought on by heat. By safeguarding membrane-associated proteins and maintaining the integrity of membrane constituents, the increased amounts of HSP60 may aid in membrane stabilization. Furthermore, a considerable decrease in relative water content (RWC) was the outcome of high temperature stress, suggesting decreased water intake and increased water loss. In this case, by maintaining protein structure and promoting stress recovery, the elevated production of HSP60 may aid in maintaining cellular activities in spite of dehydration. In this study, heat stress treatments resulted in an increase in leaf membrane injury in cv. “Kandil”. The bell pepper cv. “Kandil”, with an LT50 value of 41.12 °C, was shown to have moderate heat tolerance. Leaf RWC and TSP content data also supported this result. Heat stress treatment induced HSP60 synthesis in pepper plants. Indeed 54 kDa protein may be responsible for heat stress tolerance in cv. “Kandil”. In addition, by increased expression of the 54 kDa HSP, the structural stability of cellular membranes of pepper leaf tissues of the cultivar “Kandil” was enhanced under heat stress. 54 kDa HSP60 can be used as a marker. It will assist in determining the genes responsible for high-temperature stress in pepper. Conclusively, the observed elevations of HSP60 and total protein levels, in addition to increased membrane damage and decreased RWC, underscore the intricate yet synchronized reaction of pepper plants to high temperature stress. HSPs, especially HSP60, seem to be essential for improving plant thermotolerance because they preserve physiological and molecular stability in the face of heat stress.

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