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**Heat stress responses in common bean genotypes: Membrane damage, proline accumulation, and heat shock protein expression**

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Running Title: PHYSIOLOGICAL MARKERS OF HEAT STRESS IN COMMON BEAN

**Abstract** – This study investigated the physiological and molecular responses of two common bean (*Phaseolus vulgaris* L.) genotypes, “Balkız” and “Local Genotype”, which differ in their relative heat tolerance, with the “Local Genotype” being more tolerant than “Balkız”, to high-temperature stress during the seedling stage, aiming to identify genotype-specific physiological and biochemical responses and to determine reliable indicators of thermotolerance. Leaf relative water content (RWC), turgor loss, membrane damage, proline levels, total soluble protein (TSP), and the expression of heat shock proteins HSP23 and HSP60 were examined. The “Local Genotype” exhibited greater thermotolerance, with lower membrane damage while the temperature at which 50% injury occurs ($LT_{50}$) was higher, despite the accumulation of less proline than “Balkız”. Genotype-specific variations detected in protein expression indicate distinct biochemical responses to heat stress. HSP23 showed a rapid, transient increase at moderate temperature (40 °C) in both genotypes, suggesting it has a role in early heat stress response. HSP60 levels were maintained near $LT_{50}$ temperatures, supporting sustained stress adaptation, but declined at 48 °C, reflecting extreme stress rather than genotype-specific differences. These findings emphasize $LT_{50}$ and HSP dynamics as key indicators of thermotolerance and provide insights for understanding heat stress responses in common bean seedlings.

**Keywords:** High temperature stress, HSP23, HSP60, $LT_{50}$, *Phaseolus vulgaris* L., soluble protein, thermotolerance, water balance

## Introduction

Abiotic stressors are major contributors to crop losses worldwide. Among these, high temperatures disrupt plant growth and development, limit productivity, and, in extreme situations, cause plant death (Raza et al. 2023). Rising global temperatures are expected to drastically affect agricultural productivity, particularly in moderate zones, where many major crop production areas are located. Heat stress, changing precipitation patterns, and more frequent extreme weather events are predicted to reduce global yields of major staple crops, including wheat by 6.0%, rice by 3.2%, maize by 7.4%, and soybean by 3.1%, posing a significant threat to global food security (Zhao et al. 2017). Although some high-latitude locations may benefit modestly, evidence suggests that climate change has slowed the growth of agricultural productivity worldwide, and the net effect in many regions is unfavourable (IPCC 2023).

Temperature elevation triggers a range of biochemical and physiological disruptions in plants. High temperatures promote protein denaturation and reduce membrane fluidity, leading to oxidative stress (Pant et al. 2025). The increase in the kinetic energy and mobility of biomolecules under heat stress weakens chemical bonds, resulting in membrane injury (Zhao et al. 2021). Leaf relative water content (RWC) is a widely used physiological indicator for

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assessing plant water balance and stress adaptation (Soltys-Kalina et al. 2016). This parameter provides valuable insight into the balance between water uptake and transpiration, which directly influences growth, gas exchange, and metabolic activity. Likewise, the maintenance of turgor has been shown to be critical for normal cell function and for supporting growth (Kumari et al. 2021). Previous studies have reported that high temperatures reduce leaf RWC and increase turgidity loss by enhancing transpiration (Kumar et al. 2011, Tokyol and Turhan 2019).

Proline is an essential molecule that acts as an osmoprotectant and molecular chaperone (Meena et al. 2019). It aids in osmotic adjustment and protects cellular structures under stress (Kavi Kishor et al. 2022). However, its integrative role in legumes under heat stress and its precise physiological functions remain unclear. Although increased proline accumulation is generally associated with improved thermotolerance in crops like common bean and moth bean (Chavez-Arias et al. 2018, Tiwari et al. 2018), this relationship is not universal; in *Vigna aconitifolia*, proline accumulation under heat stress was not associated with thermotolerance (Harsh et al. 2016). Heat stress also induces the accumulation of heat shock proteins (HSPs), which play a critical role in maintaining protein structure and cellular homeostasis under stressful conditions (Scharf et al. 2012). While their general function as molecular chaperones is well established, recent studies have revealed genotype-specific variations in HSP expression and their contributions to thermotolerance (Ergin et al. 2016, Haq et al. 2019). Comparative analyses across crop species indicate that genotypes with higher and sustained HSP levels generally exhibit enhanced membrane stability, improved water retention, and better growth recovery following heat exposure (Xu et al. 2011, Kumar et al. 2024).

Common bean (*Phaseolus vulgaris* L.) is an important legume crop valued for its protein, vitamin, and mineral content. It also contributes to soil fertility through biological nitrogen fixation (Blair 2013). However, high temperatures are a major limiting factor for common bean production and quality. Heat stress reduced yield by 26–37%, primarily due to impaired pollen viability, defective seed set, and poor seed filling (Vargas et al. 2021). Most studies on abiotic stress in common bean have addressed drought, salinity, and cold (Jha et al. 2024), whereas research on heat stress has largely focused on reproductive-stage responses, such as pollen viability (Soltani et al. 2019, da Silva et al. 2020, Vargas et al. 2021). Relatively few studies (Chavez-Arias et al. 2018, Tokyol and Turhan 2019) have examined the seedling stage, which is critical for successful plant establishment under stress conditions. Moreover, integrating physiological and molecular investigations provides a more comprehensive understanding of the mechanisms underlying plant thermotolerance (Tiwari et al. 2018). In this study, we examined proline accumulation and the expression patterns of HSP23 and HSP60, alongside physiological parameters such as RWC, turgor maintenance, and membrane integrity in two *Phaseolus vulgaris* genotypes which differ in their relative heat tolerance, to elucidate the genotype-specific molecular mechanisms underlying heat tolerance in common bean seedlings. Our findings provide new insights into early-stage thermotolerance mechanisms in common bean.

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## Material and methods

### Plant material and growth conditions

In this experiment, the genotypes “Balkız”and “Local Genotype” of common bean (*Phaseolus vulgaris* L.) were used. In our previous study, the “Local Genotype” was shown to be relatively more heat-tolerant than “Balkız” (Tokyol and Turhan 2019), which guided the selection of these genotypes for comparative analysis. Seeds of each genotype were obtained from our laboratory common bean germplasm collection and sown in pots filled with a 1:1:1 mixture of peat, perlite, and soil. The plants were kept for four weeks in a controlled greenhouse environment with 65% relative humidity (RH) and temperatures ranging from 15 to 30 °C

(night/day). After emergence, seedlings were irrigated using a pot tensiometer (Irrometer Co. Riverside, Calif, USA), ensuring uniform water availability across all pots. The greenhouse phase of the experiment was conducted from 15 October to 30 November 2019, followed by heat-stress treatments completed during the first two weeks of December 2019.

**Heat stress treatments**

When the seedlings reached the 5-6 leaf stage, they were transferred to a growth chamber (DAIHAN WGC-1000, South Korea) set at 65% humidity and a light intensity of 450 μmol $m^{-2}$ $s^{-1}$ (Tokyol and Turhan 2019). The temperature was gradually increased from 30 °C by 2 °C per hour until it reached 40 °C. Seedlings were maintained at 40 °C for 2 hours, after which samples were collected. The temperature was then gradually increased by 2 °C per hour to 44 °C, maintained for 2 hours, and sampled again. Finally, the temperature was gradually increased by 2 °C per hour to 48 °C, maintained for 2 hours, and sampled. For each genotype and temperature treatment, five pots (each containing one seedling) were used per replicate. The experiment was conducted with three independent biological replicates, each performed at a different time. Control seedlings were maintained under standard greenhouse conditions (30 °C, 65% RH) without heat stress.

**Sampling for analyses**

After each treatment, leaf samples were collected and divided into two groups. One group was used to measure membrane injury, leaf RWC, and turgor loss, while the second group was immediately frozen in liquid nitrogen and stored at –80 °C for proline and protein analyses. Fully expanded young leaves were collected for all measurements, with approximately 2.5-3 g of tissue per replication. For each genotype and temperature treatment, leaf samples were collected from five plants per replicate, with three biological replicates in total.

**Cell membrane injury (CMI)**

The method of Arora et al. (1998) was used with some modifications to assess CMI according to the percentage of total electrolyte leakage (EL). Leaf discs (1.5 cm in diameter) were collected from both control and high temperature-treated plants of each bean genotype. The discs were placed in test tubes containing 15 mL of double-distilled water ($ddH_2O$). The samples were incubated on an orbital shaker (Thermo SCIENTIFIC, 4334, USA) for 4 h. The electrical conductivity (EC) of the solution was measured using a conductivity meter (Mettler-Toledo, Seven Compact Conductivity S230, Switzerland) to determine initial ion leakage (EC1). The samples were then autoclaved (ALP, CLG-32L, Japan) at 121 °C for 15 min, and incubated again on the orbital shaker for 4 h before the final conductivity reading (EC2) was made. Electrolyte leakage (EL, %) was calculated using the following equation:

$$EL (\%) = (EC1/EC2) \times 100$$

The percentage of cell membrane injury (CMI, %) was determined according to Arora et al. (1998) using the formula:

$$CMI (\%) = [EL(t) - EL(c)] / [100 - EL(c)] \times 100$$

where EL(t) and EL(c) refer to the EL values of the treatment and control samples, respectively.

Additionally, $LT_{50}$ (semi lethal temperature), defined as the temperature at which 50% CMI occurs, was calculated by nonlinear regression analysis of CMI values derived from EL measurements.

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**Leaf relative water content (RWC) and turgor loss**

Leaf RWC (%) and turgor loss were determined using the method described by Barr and Weatherley (1962). Leaf discs (1.5 cm in diameter) were obtained from each sample. The fresh weight (FW) of the discs was recorded immediately. The discs were then soaked in distilled water for 4 h to reach full turgor, after which their turgid weight (TW) was measured. Finally, the discs were dried in an oven at 70 °C for 24 h, and the dry weight (DW) was recorded.

Leaf RWC and turgor loss were calculated using the following formulas:

$$\text{RWC (\%)} = [(\text{FW} - \text{DW}) / (\text{TW} - \text{DW})] \times 100$$
$$\text{Turgor loss (\%)} = [(\text{TW} - \text{FW}) / \text{TW}] \times 100$$

**Proline content**

Proline content was determined using the method described by Bates et al. (1973). For the analysis, 200 mg of plant material previously stored at -80 °C was homogenized in 1 mL of 3% sulfosalicylic acid and centrifuged at 5000 g for 15 min at 4 °C. To 200 µL of the resulting supernatant, 400 µL ninhydrin reagent, 400 µL glacial acetic acid, and 200 µL of 3% sulfosalicylic acid were added and mixed thoroughly. The mixture was incubated in a water bath at 100 °C for 1 h. The samples were then placed on ice, 2 mL of toluene was added, and the mixture was centrifuged for 20 s at 4000 g. Proline content was quantified spectrophotometrically at 520 nm (Perkin Elmer Lambda 25, USA) using a standard curve prepared with L-proline (0, 50, 100, 200, 400, 800 µM).

**Protein content and expression**

The extraction method for total soluble protein (TSP) followed the procedure described by Shen et al. (2003) with some modifications. The extraction solution was prepared by adjusting the pH to 7.8 using 2-morpholinoethanesulfonic acid monohydrate (MES) and contained 25 mM Tris-base, 275 mM sucrose, 2 mM EDTA, 10 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). For the extraction, 250 mg of plant material was homogenized with 1 mL of the extraction solution. The samples were then centrifuged at 10000 rpm for 10 min at 4 °C. TSP concentration was determined using the Bradford method (1976), with absorbance measured at 595 nm.

Protein profiles in leaf tissues were evaluated using a Mini PROTEAN Tetra electrophoresis device (Bio-Rad, Hercules, USA). An electrical current of 150 V was applied to the gel using a Bio-Rad PowerPac Basic power supply. For protein separation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels were prepared manually. A 4% stacking gel and a 12.5% separating gel were used. The gels were cast immediately before use. The samples were run on the gel for 1 h and 20 min at ambient temperature. Prior to electrophoresis, samples were mixed with 6× sample buffer containing 1 M Tris (pH 6.8), glycerol, SDS, DTT and bromophenol blue and denaturated at 100 °C for 3 min. The samples were then cooled on ice for 5 min, and 10 µg of protein was loaded for each sample. Coomassie Brilliant Blue G-250 dye was used to visualize the protein bands in the gel, which were then imaged using Vilber's Quantum ST4 Gel Imaging System (France). Molecular weights were determined using the SDS-PAGE molecular weight standard (Bio-Rad, Precision Plus Protein Unstained Standard).

Western blotting was performed to detect HSPs according to the method of Arora and Wisniewski (1994). Proteins were transferred from the gel onto nitrocellulose membranes (Nitrocellulose membranes BioRad) using a semi-dry transfer system (Bio-Rad Trans-Blot Turbo). The proteins were probed with a 1:1500 dilution of the antibody directed against HSP23

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[Monoclonal Anti-Heat Shock Protein 23 (rabbit, Sigma)] and HSP60 [Monoclonal Anti-Heat Shock Protein 60 (mouse, Sigma)]. The alkaline phosphatase assay was performed with a ProtoBlot Western Blot AP Kit (Promega) to detect immunoreactive bands. The band images that appeared on the membranes were captured using a scanner and transferred to digital media. The experiments were repeated three times, and densitometric analysis of the bands was performed in triplicate using the Public Domain NIH Image program (available at http://rsb.info.NIH.gov/nih-image/), also known as ImageJ Software. Band intensities were normalized relative to the control sample of the "Balkız" genotype, which was set to 100%, and all other treatments were expressed relative to this.

**Statistical analysis**

The experiment was conducted as a completely randomized design with three independent biological replicates, each consisting of five pots (one plant per pot). Percentage-based traits (CMI, RWC and turgor loss) were arcsine square root transformed to satisfy the assumptions of normality and homoscedasticity. Normality and homoscedasticity of residuals were checked using standardized residual plots and Shapiro-Wilk tests ($P > 0.05$). Data were analyzed using SPSS software (version 22, Chicago, IL, USA) using two-way ANOVA followed by Tukey HSD post hoc test. Data are presented as mean ± standard error (SE) of three biological replicates.

## Results

**Cell membrane injury (CMI) and high temperature tolerance ($LT_{50}$)**

The leaf CMI percentages and $LT_{50}$ values for both genotypes are presented in Fig. 1. The "Balkız" genotype exhibited a higher CMI percentage (42.09%) than the "Local Genotype" when averaged across all temperature treatments. CMI percentage increased proportionally with temperature, with the most pronounced rise observed at 48 °C. From 40 to 44 °C, CMI increased approximately 3.3-fold in the "Balkız" genotype and 3.8-fold in the "Local Genotype". A further increase from 44 to 48 °C resulted in an additional 2.9-fold rise in "Balkız" and a 7.9-fold rise in the "Local Genotype". The higher relative increase observed in the "Local Genotype" is attributable to its considerably lower initial CMI value at 40 °C. Nevertheless, absolute CMI values at 48 °C were comparable between the genotypes. $LT_{50}$ values further indicated that the "Local Genotype" (46.2 °C) exhibited relatively greater heat tolerance than "Balkız" (45.3 °C) (Fig. 1).

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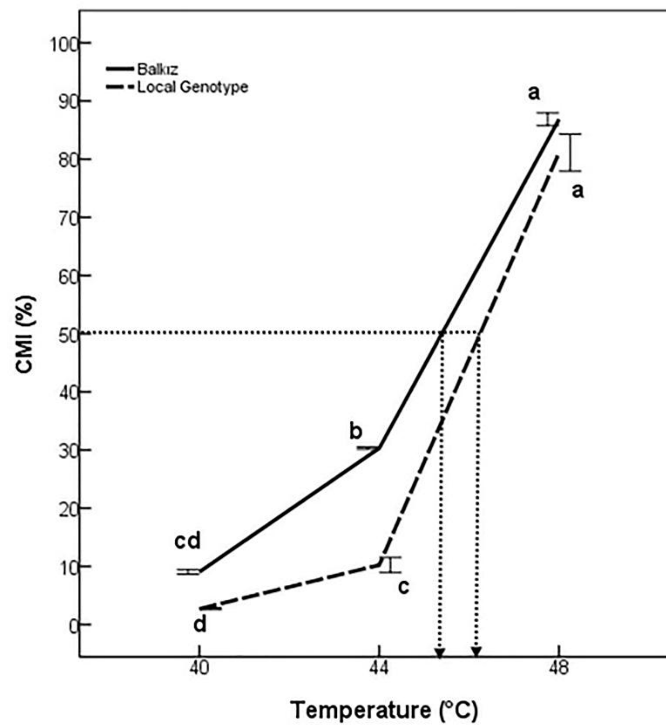


**Fig. 1.** Cell membrane injury (CMI, %) of "Balkız" and "Local Genotype", two genotypes differing in heat tolerance, measured at 40, 44, and 48 °C. For each temperature treatment, leaves were collected from all five seedlings within each pot representing one replicate, and the experiment was conducted with three independent biological replicates. The horizontal dotted line shows the 50% CMI threshold, and vertical arrows indicate the $LT_{50}$ (semi lethal temperature) of each genotype, calculated from electrolyte leakage (EL; %) using nonlinear regression. Different lowercase letters indicate significant differences among temperature treatments within each genotype ($P < 0.05$). Vertical bars represent ± standard errors (SE) of three replicates.

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A two-way ANOVA revealed that temperature, genotype, and their interaction had significant effects on membrane injury (Tab. 1).

**Tab. 1.** Results of analysis of variance (ANOVA) showing the effects of genotype, temperature, and their interactions on cell membrane injury, relative water content (RWC), turgor loss, proline content, total soluble protein (TSP) content and expression of heat stress proteins (HSP) in leaf tissues. Seedlings of two common bean genotypes differing in heat tolerance, "Balkız" and "Local Genotype", were exposed to different temperature treatments (30, 40, 44, and 48 °C). For each temperature, measurements were performed on leaves from three biological replicates. df1 and df2 denote degrees of freedom for the factor and error, respectively. F and P values were obtained from two-way ANOVA.

| Dependent variable | Independent variable | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Genotype | | | Treatment | | | Genotype x Treatment | | |
| | ($df_1$, $df_2$) | F | P | ($df_1$, $df_2$) | F | P | ($df_1$, $df_2$) | F | P |
| **Injury** | (1, 12) | 90.620 | <0.000 | (2, 12) | 1129.511 | <0.000 | (2, 12) | 10.265 | <0.003 |
| **RWC** | (1, 14) | 2.072 | =0,172 | (3, 14) | 82.219 | <0.000 | (3, 14) | 1.055 | =0.399 |
| **Turgor loss** | (1, 15) | 3.598 | =0,077 | (3, 15) | 40.124 | <0.000 | (3, 15) | 2.435 | =0.105 |
| **Proline** | (1, 16) | 21.493 | <0.000 | (3, 16) | 96.838 | <0.000 | (3, 16) | 12.340 | <0.000 |
| **TSP** | (1, 16) | 2.023 | =0.193 | (3, 16) | 218.516 | <0.000 | (3, 16) | 37.829 | <0.000 |
| **HSP23** | (1, 16) | 0.969 | =0.339 | (3, 16) | 186.243 | <0.000 | (3, 16) | 32.736 | <0.000 |
| **HSP60** | (1, 16) | 88.932 | <0.000 | (3, 16) | 199,696 | <0.000 | (3, 16) | 20.970 | <0.000 |

## Leaf RWC and turgor loss

In both genotypes, leaf RWC began to decline noticeably from 40 °C onward and continued to decrease in parallel with rising temperatures (Fig. 2A). At 48 °C, the RWC of the "Local Genotype" was still higher (40.32%) than that of the "Balkız" genotype (28.97%) (Fig. 2A). In the "Balkız" genotype, RWC decreased by approximately 38% from control to 40 °C, followed by additional decreases of 18% (40–44 °C) and 32% (44–48 °C). In the "Local Genotype", the corresponding decreases were approximately 38%, 13%, and 10%, respectively. Although, the "Local Genotype" maintained higher RWC than the "Balkız" genotype at 44 °C and 48 °C, the differences between genotypes were not statistically significant ($P < 0.05$). A two-way ANOVA revealed a significant effect of temperature on RWC ($P < 0.05$), while the genotype × temperature interaction was not significant (Tab. 1).

Additionally, turgor loss increased progressively with rising temperature in both genotypes (Fig. 2B). In the "Balkız" genotype, turgor loss increased by approximately 184% from 30 to 40 °C, followed by further increases of 20% (40–44 °C) and 42% (44–48 °C). In the "Local Genotype", the corresponding increases were approximately 193%, 14%, and 5%, respectively. A two-way ANOVA revealed a significant effect of temperature on turgor loss (30°C < 40°C < 44°C < 48°C; $P < 0.05$), whereas neither the genotype effect nor the genotype × temperature interaction were significant (Tab. 1).

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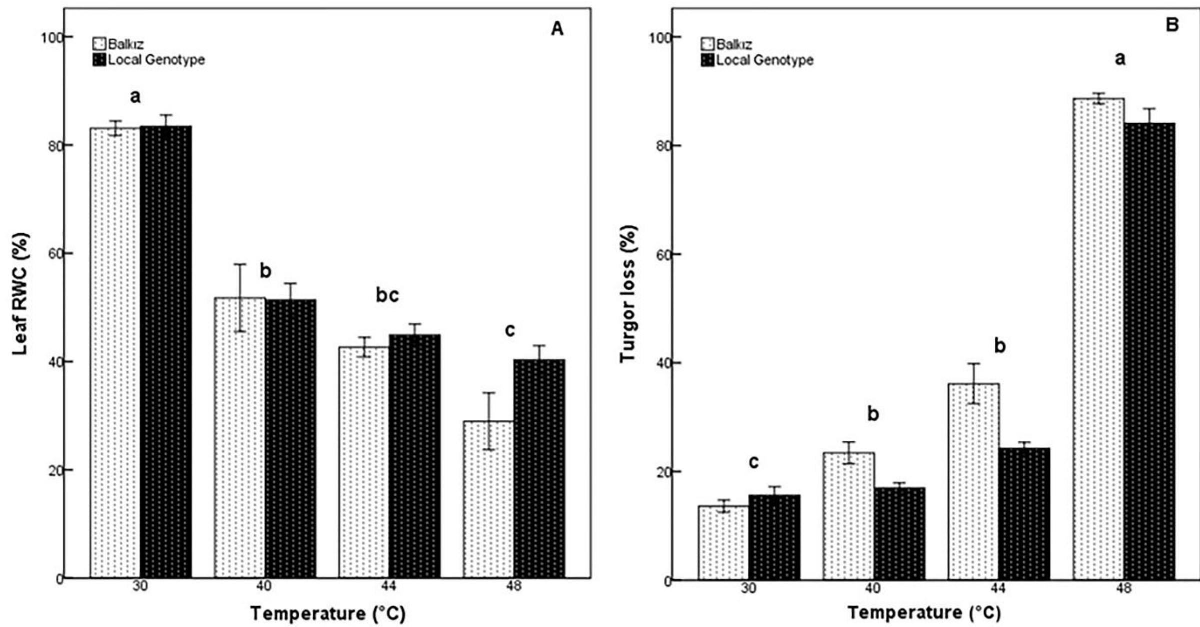


**Fig. 2.** Relative water content (RWC) (A) and turgor loss (B) of two common bean genotypes differing in heat tolerance, "Balkız" and "Local Genotype", under different temperature treatments. Control conditions were 30 °C and 65% relative humidity. Two-way ANOVA revealed a significant effect of temperature ($P < 0.05$), while genotype and genotype × temperature interaction were not significant. Different small letters above the bars indicate statistically significant differences among temperature treatments (Tukey's test, $P < 0.05$). Vertical bars represent ± standard errors (SE) of three replicates.

**Proline content**

Proline content increased progressively with rising temperature in both genotypes (Fig. 3A). In the "Balkız" genotype, proline increased by approximately 39% at 40 °C, 155% at 44 °C, and 308% at 48 °C relative to the control. In the "Local Genotype", increases were about 18%, 42%, and 127% at the same temperatures, respectively. Across all treatments, the "Balkız" genotype had a higher average proline content than the "Local Genotype". At elevated temperatures (44 and 48 °C), "Balkız" accumulated significantly more proline than the "Local Genotype" (Tukey, $P< 0.05$), and within each genotype, all temperature points differed significantly from each other. Two-way ANOVA showed significant effects of temperature, genotype, and their interaction ($P < 0.05$), indicating a strong, genotype-dependent, temperature response (Tab. 1).

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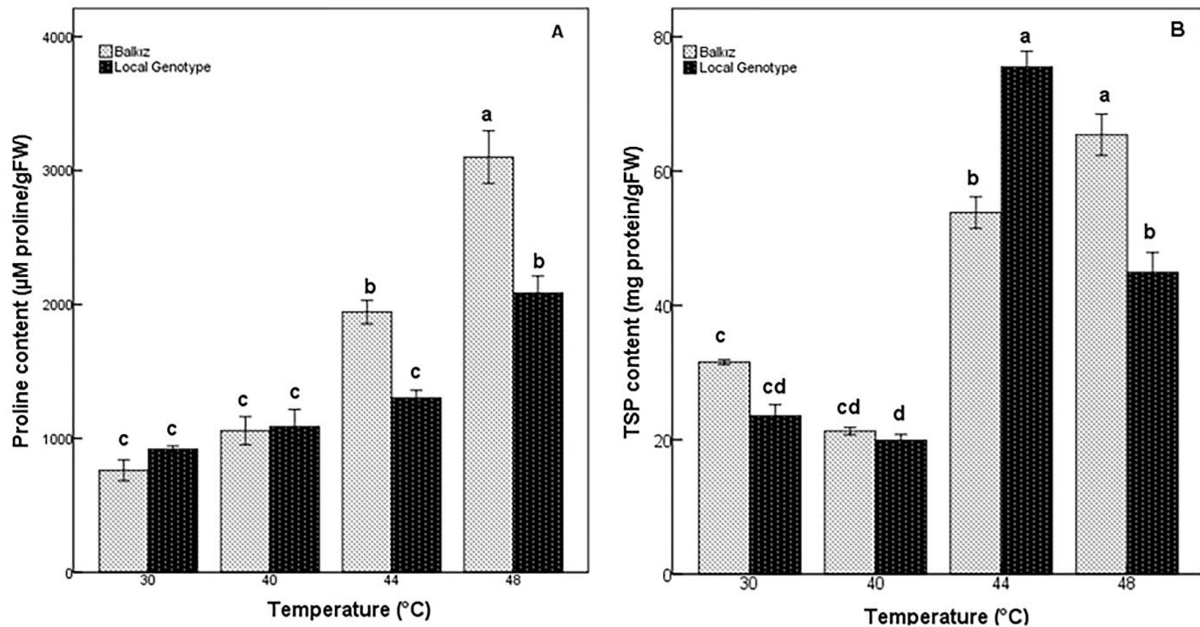


**Fig. 3.** The content of proline (A) and TSP (B) in common bean genotypes "Balkız" and "Local Genotype" differing in heat tolerance, under heat-stress treatments. Control conditions were 30 °C and 65% relative humidity. Two-way ANOVA revealed significant effects of temperature, genotype, and genotype × temperature interaction ($P < 0.05$). Different small letters above the bars indicate statistically significant differences among temperature treatments within each genotype (Tukey's test, $P < 0.05$). Vertical bars represent ± standard errors (SE) of three replicates.

**Protein content and expression pattern**

Total soluble protein (TSP) content changed with increasing temperature in both genotypes (Fig. 3B). Relative to control, TSP in "Balkız" changed by approximately -33% at 40 °C, +71% at 44 °C, and +107% at 48 °C, whereas in the "Local Genotype", the corresponding changes were about -16%, +220%, and +91%. Overall, both genotypes showed a temperature-dependent TSP pattern, with significantly higher values at 44 and 48 °C than in control (30 °C) and at 40 °C (Tukey, $P < 0.05$). Two-way ANOVA indicated a significant effect of temperature and a significant genotype × temperature interaction ($P < 0.05$), whereas the main effect of genotype was not significant.

In the SDS-PAGE analysis, protein expression patterns differed between the two genotypes (Fig. 4A). In the "Balkız" genotype, the strongest overall band intensities were observed at 40 °C, followed by a clear reduction at 44 °C and an even weaker expression at 48 °C. In contrast, the "Local Genotype" displayed its highest protein expression at both 40 and 44 °C, with a decrease at 48 °C. In the "Balkız" genotype, the 43, 64, and 120 kDa bands increased at 40 °C compared with the control (30 °C), decreased slightly at 44 °C, and were strongly reduced or absent at 48 °C. In the "Local Genotype", the 43 kDa band increased at 40 °C, decreased at 44 °C, and disappeared at 48 °C, while the 64 and 120 kDa bands, not detected at control, appeared at 40 and 44 °C but were absent at 48 °C. In the "Balkız" genotype, the 13 and 14 kDa protein bands increased at 40 and 44 °C, a pattern not observed in the "Local Genotype". Proteins of 22, 25, and 38 kDa were more prominent at higher temperatures in both genotypes compared to the control except for the 22 and 25 kDa bands at 48 °C. Notably, the 23 kDa band increased at 48 °C in the "Balkız" genotype. A 57 kDa protein band was clearly visible in both genotypes and is presumed to be a structural protein. The overall intensity of

protein bands declined with rising temperature, with the most pronounced reduction observed at 48°C in both genotypes.

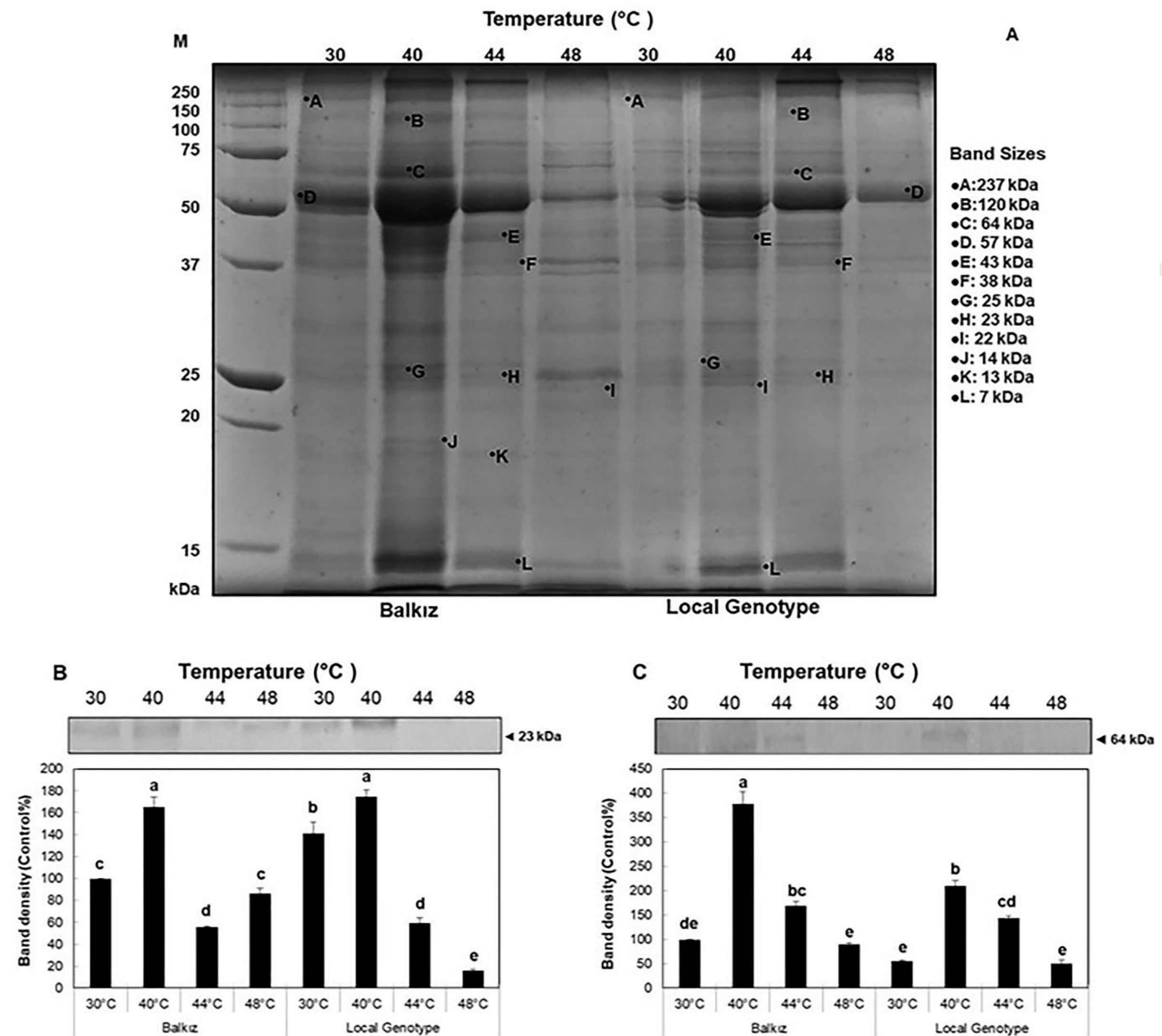


**Fig. 4.** Total protein profiles (A), along with the expression of heat shock proteins HSP23 (B) and HSP60 (C) in two common bean genotypes “Balkız” and “Local Genotype” differing in heat tolerance, under heat-stress treatments. Control conditions were 30 °C and 65% relative humidity. M: MW standard. For each sample, 10 µg of protein was loaded. Different letters indicate significant differences among temperature treatments within each genotype according to Tukey’s test ($P < 0.05$). Vertical bars represent ± standard errors (SE) of three replicates.

Fig. 4B and 4C presents the HSP profiles identified through immunoblot analysis following high temperature treatments. A 23 kDa band corresponding to HSP23 was detected in both genotypes (Fig. 4B). In the “Balkız” genotype, HSP23 expression increased significantly at 40 °C, dropped sharply below control value at 44 °C, and returned to control levels at 48 °C. HSP23 expression in the “Local Genotype”, which was higher than in the “Balkız” genotype at 30 °C (basal expression), increased markedly at 40 °C, fell below control levels at 44 °C, and reached its minimum at 48 °C. These patterns indicate clear genotype-specific differences in HSP23 response, with the “Balkız” genotype maintaining expression at 48 °C, unlike the “Local Genotype”. A 64 kDa protein corresponding to HSP60 was detected,

and its accumulation varied depending on genotype and temperature (Fig. 4C). In the "Balkız" genotype, HSP60 expression rose sharply at 40 °C, and although it declined at 44 °C, it remained above control levels, and returned to control levels at 48 °C. A similar pattern was observed in the "Local Genotype", i.e. HSP60 accumulation increased significantly at 40 °C, remained elevated above control at 44 °C, and returned to control levels at 48 °C. The findings reveal genotype-specific differences in HSP60 dynamics, with the "Balkız" genotype showing higher accumulation at 40 °C (response to moderate heat stress), while the "Local Genotype" maintains HSP60 more steadily at 44 °C.

## Discussion

Measuring ion leakage is one of the most effective methods for detecting cell membrane damage caused by high-temperature stress. In this study, CMI increased with rising temperature in both genotypes, and was consistently higher in the "Balkız" genotype, resulting in an $LT_{50}$ difference of 0.86 °C, which supports the superior thermotolerance of the "Local Genotype". Similar results have also been reported in other species, including strawberry (Ergin et al. 2016) and cucumber (Ali et al. 2019), as well as in common bean (Tokyol and Turhan 2019). Mechanistically, high temperature perturbs lipid–protein interactions and increases membrane fluidity, which, together with protein denaturation, compromises barrier function and promotes ion efflux. Elevated temperatures also enhance reactive oxygen species (ROS) production, driving lipid peroxidation, which further disrupts membrane architecture and permeability (Bita and Gerats 2013). Plants counter these effects by accumulating protective molecules and chaperones: for example, heat-tolerant accessions have been shown to increase membrane stabilizers including proline, HSPs, and saturated lipids to maintain membrane integrity under heat stress (Niu and Xiang 2018). Together, these processes explain how differences in membrane stabilization and cellular protection, rather than baseline water status alone, may underlie the genotypic variation in thermotolerance observed here.

Relative water content (RWC) reflects the balance between leaf water status and transpiration rate and is a key indicator for evaluating plant resistance to stress (Mullan and Pietragalla 2012). High-temperature exposure has been shown to negatively affect water retention in legumes. Kumar et al. (2011) reported a reduction in leaf RWC in mung bean, while Tokyol and Turhan (2019) observed both a decline in leaf RWC and increased turgor loss with rising temperature in common bean. Besides, it has been found that the increase in RWC and the decrease in turgor loss are associated with heat tolerance in moth bean (Tiwari et al. 2018). Lower leaf RWC and higher turgor loss observed in both genotypes in the present study suggest that high-temperature stress disrupts normal physiological processes. However, these values did not differ significantly between genotypes, indicating that these physiological traits are not the primary determinants of thermotolerance in the common bean genotypes examined. This finding underscores the importance of integrating molecular and biochemical markers to better characterize genotype-specific responses to heat stress.

Proline accumulation is a common response in plants to various abiotic stresses. While moderate proline levels help stabilize membranes, protect enzymes, and scavenge ROS (Kavi Kishor et al. 2022), excessive amounts can disrupt cellular homeostasis or increase oxidative damage via the proline/P5C cycle (Spormann et al. 2023). In the present study, the "Balkız" genotype accumulated more proline than the "Local Genotype" but experienced greater membrane injury, suggesting that proline accumulation alone does not confer thermotolerance. These results, which are consistent with observations under heat, drought, and salinity stress (Raza et al. 2023), indicate that proline's protective role is context-dependent and relies on coordination with other antioxidant and protective mechanisms. To enhance stress adaptation, breeding strategies should target genotypes that maintain optimal proline levels while ensuring efficient redox regulation.

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High-temperature stress induces complex changes in protein metabolism, including the degradation of existing proteins and the synthesis of stress-related proteins, which can vary depending on genotype-specific tolerance mechanisms and the severity of the stress (Kosová et al. 2011). In many species, high temperatures lead to a reduction in TSP levels, due to protein denaturation and proteolysis, as reported in strawberry (Ergin et al. 2016) and moth bean (Tiwari et al. 2018). However, Tiwari et al. (2018) also noted that heat-tolerant moth bean genotypes exhibited only a slight reduction in protein content compared with sensitive genotypes. In contrast, in the present study, although TSP levels showed a slight decrease at 40 °C in both genotypes, this change was not considered significant. However, at 44 °C and 48 °C, TSP content increased markedly and significantly in both genotypes, indicating that, beyond a moderate threshold, high-temperature exposure predominantly triggers the accumulation of temperature-responsive proteins rather than their degradation. Moreover, the results indicate that while both genotypes adjust their protein metabolism under heat stress, the "Balkız" genotype maintains a stronger TSP response at higher temperatures. Rani et al. (2016) reported the induction of two protein bands (25.8 and 30.7 kDa) in mustard subjected to 45 °C. Similarly, dehydrin proteins of 21, 23, and 27 kDa were detected in sugarcane leaves under high-temperature stress (Wahid and Close 2007). In line with these findings, the SDS-PAGE results in the present study showed both the disappearance of some protein bands and the appearance of new bands at higher temperatures, indicating active proteome reconfiguration. However, in the present study, SDS-PAGE profiles displayed the strongest banding at 40 °C and progressively weaker, less-defined bands at higher temperatures. This pattern, which appears inconsistent with the TSP results, can be explained by heat-induced changes in protein solubility and stability; severe heat promotes protein unfolding, aggregation and chaperone association, shifting a portion of the proteome into aggregated or chaperone-bound forms that are less resolved by SDS-PAGE, even as newly synthesized, highly soluble stress proteins such as small HSPs and dehydrins elevated TSP levels (Kosová et al. 2011, McLoughlin et al. 2019).

Heat shock proteins (HSPs) are evolutionarily conserved molecular chaperones that assist in the refolding of stress-damaged proteins (McLoughlin et al. 2019). HSP23, HSP36, and HSP66 have been linked to thermotolerance in turf grasses exposed to both gradual and sudden heat stress (Xu et al. 2011). According to Ergin et al. (2016), greater accumulation of a 23 kDa HSP was associated with improved heat tolerance in strawberry cultivars. Silencing of CaHSP60-6 in pepper reduced antioxidant enzyme activities under heat stress, indicating that HSP60 not only induces antioxidant enzyme synthesis but also preserves their functional structure as a molecular chaperone (Haq et al. 2019). In the present study, HSP23 accumulated transiently at 40 °C in both genotypes, but the expression was higher in the "Local Genotype", than in the "Balkız" genotype at 30 °C (basal expression). Although HSP23 expression in the "Local Genotype" declined at higher temperatures, the genotype exhibited better thermotolerance than "Balkız", likely due to its higher basal HSP23 expression at 30 °C and the combined effect of other protective mechanisms. This also indicates that HSP23 expression alone does not reflect heat tolerance. Accumulation of HSP60 also increased in both genotypes, showing a greater absolute band intensity in "Balkız" but a larger relative increase from control (30 °C) in the "Local Genotype". Relatively higher HSP60 levels persisted at higher temperatures in the "Local Genotype," suggesting genotype-specific regulatory mechanisms that enhance protein stability and organellar integrity. HSP60, a chaperonin located in mitochondria and plastids, is essential for maintaining protein homeostasis under stress. It facilitates protein import across the mitochondrial membrane and proper folding within organelles, thereby supporting chloroplast protein maturation and Rubisco assembly (Roy et al. 2019). HSP60 functions in an ATP-dependent manner and exhibits stress- and species-specific expression, with minimal responsiveness to low-temperature or osmotic stressors (Kumar et al.



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2024). These findings suggest that prolonged HSP60 accumulation contributes to thermotolerance by stabilizing organellar proteins and maintaining cellular homeostasis.

## Conclusions

High temperature is a major environmental factor negatively affecting plant growth and development. In this study, thermotolerance was primarily associated with $LT_{50}$ and HSP expression patterns. According to $LT_{50}$ values, the "Balkız" genotype showed lower heat tolerance than the "Local Genotype". HSP23 contributed to the early phase of heat stress response at moderate temperatures (around 40 °C), while HSP60 provided a sustained response at temperatures below or around $LT_{50}$, contributing to stress adaptation. Notably, extreme temperature (e.g. 48 °C) overwhelmed both genotypes, emphasizing the physiological limits of seedlings under heat stress. Together, $LT_{50}$ measurements and HSP dynamics emerge as robust indicators for screening thermotolerant genotypes, providing useful information for breeding heat-resilient common beans. However, to fully understand genotype-specific responses, future studies should also investigate additional physiological, biochemical, and molecular mechanisms that may contribute to thermotolerance, alongside regulatory mechanisms controlling HSP expression.

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**Author contribution statement**

Tugba Bicer: Methodology, Investigation, Data curation, Formal analysis, Validation, Writing- Original draft preparation. Ece Turhan: Conceptualization, Methodology, Writing- Reviewing and Editing, and Supervision.

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