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Research article

Effects of cold stress on protein metabolism of certain walnut cultivars

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Abstract

To investigate the effects of heat shock proteins (HSPs) on walnut (*Juglans regia* L.) plants under low-temperature stress, first of all, low-temperature tolerances of ten walnut cultivars (Chandler, Fernor, Franquette, Pedro, Bilecik, Kaman-I, Kaman-II, Kaman-III, Sebin, and Yalova I) were determined. One-year-old shoot samples were taken from the plants in two different periods, cold-acclimated (CA) and non-acclimated (NA), and were exposed to +5°C, -5°C, -15°C and -25°C for 12 h. Cold injury was determined by ion leakage analysis in the thawed bark tissues. According to the results of this analysis, two cultivars were determined as cold-sensitive (Chandler) and cold-tolerant (Fernor) in terms of low-temperature tolerance. To examine the effects of HSPs on cold tolerance in walnut plants, the protein profiles, the amount of total protein, and the HSPs of these two cultivars were determined. As a result of the immunoblot analysis, it was determined that 44 kDa HSP23 and 59 kDa HSP60 are responsible for low-temperature tolerance in walnut plants.

Keywords: *Cold acclimatization; heat shock proteins; HSP23; HSP60; Juglans regia; low temperature stress*

1. Introduction

Cold stress is one of the important factors affecting plant growth and the geographical distribution of plants in the world. Plants can acclimate to cold temperatures by being previously exposed to low, non-freezing temperatures. This exposure activates adaptation mechanisms that play a crucial role in increasing cold tolerance (Kerbler and Wigge, 2023). During the process of cold acclimation, plants undergo physiological changes such as increased sugar accumulation, altered lipid composition, activation of genes responsible for stress proteins, and reinforcement of their antioxidative mechanisms (Dou et al., 2024). Acclimation begins in the autumn with cessation of growth and development in plants, while freezing damage of plants is lower in this period, in the spring and the summer, freezing injury can be severe as plants are deacclimate (Riikonen et al., 2023). Survival throughout the freeze-thaw cycle depends on maintaining the structural and functional characteristics of the plasma membrane, which serves as a vital barrier between the cytoplasm and the extracellular environment and is thought to be the primary site of freezing damage (Takahashi et al., 2021). Although there are many methods for determining freeze damage, the most widely used is the electrolyte leakage conductivity method (Rezaei and Rohani, 2023).

Cell survival under stress conditions depends on the preservation of protein structures and functions (Hu et al., 2022). Heat shock proteins (HSPs), which are associated with heat shock, are widely found in plant and animal cells. But they are now known to be responsible for a wide variety of stresses such as cold, UV light, wound healing, tissue remodelling, or biotic stresses (Abdullah et al., 2022). In plants, HSPs are divided into five classes based on their molecular weight: HSP100, HSP90, HSP70, HSP60, and small HSPs (sHSPs). Most HSPs act as chaperones and are essential for the correct three-dimensional folding of newly produced proteins in the cell. They also play a critical role in repairing damaged proteins caused by stress

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(Almalki et al., 2021). For this reason, many chaperones are considered HSPs due to their nature to aggregate when denatured by heat stress (Yurina, 2023). The accumulation of HSPs under heat stress and the correlation between HSP accumulation and increased thermotolerance have been well described in previous studies (Ergin et al., 2016; McLoughlin et al., 2016; Yang et al., 2020). Cold stress affects enzymes, and cellular membranes, alters physiology and metabolism, and sometimes causes water starvation and dehydration (Zinta et al., 2022). Cold stress can also cause protein dysfunction and denaturation. In plant cells, the synthesis of HSPs plays a crucial role in protein folding, assembly, degradation, and translocation. These proteins are essential for preventing cellular damage, maintaining cellular homeostasis, and developing tolerance to cold stress (Batool et al., 2022). However, studies on their role in cold stress are quite scarce (Ré et al., 2017; Bourgine and Guihur, 2021).

Cold stress is an important risk in walnut cultivation, which has a high economic value in the world. Especially, late spring frosts and early autumn frosts pose a great risk for crop losses in woody plants (Drepper et al., 2022; Tadić et al., 2023). Cold acclimation caused an increase in cold tolerance of woody plants under winter conditions. However, in the non-acclimated (NA) period, when the plants are not accustomed to the cold, cold damage is more and this causes economic product losses to be high. Although there are studies on the cold tolerance of walnut cultivars in winter and autumn, studies on the responses of walnut cultivars in the NA period, which is a sensitive period for their culture, are limited (Poirier et al., 2004; Aletà et al., 2013; Charrier et al., 2018a,b). Furthermore, comparative research conducted during the cold-acclimated (CA) and NA periods is crucial to understanding the cold tolerance of cultivars. In this regard, selecting frost-tolerant cultivars and understanding the cold tolerance mechanism can greatly improve frost tolerance for walnuts. In the present study, frost tolerance was compared on 10 walnut cultivars (Chandler, Fernor, Franquette, Pedro, Bilecik, Kaman I, Kaman-II, Kaman-III, Sebin, and Yalova I) under CA and NA periods. According to the injury results, two cultivars were determined as cold-sensitive (Chandler) and cold-tolerant (Fernor) in terms of frost tolerance. Although there are many studies on the mechanism of frost tolerance in plants, the effectiveness of HSPs in cold tolerance has not been fully elucidated. In this study, the effects of HSPs on frost tolerance in walnut plants were examined by using frost-tolerant and frost-sensitive walnut cultivars.

2. Materials and methods

2.1. Plant material and low-temperature applications

One-year-old shoot samples were collected randomly from 4-5 trees in 8 years old plants in Kutahya- Türkiye (in Altintas region, latitude 39° 3′ 41″ N, longitude 30° 6′ 35″ E) in different periods CA (in January) and NA (in July). The average temperature in January was 0.3°C (-3.3°C to 4.6°C) and the average temperature in July was 20.7°C (13.0°C to 28.0°C).

The shoot samples were prepared for low-temperature tests in the way that Turhan and Ergin (2012) suggested. For this purpose, the shoots wrapped in moist paper and aluminium foil were placed into a manually controlled freezer. Also, a sample of plant tissue that was not exposed to low temperatures was used as a control.

Prepared samples were exposed to +5°C, -5°C, -15°C and -25°C for 12 hours, and the temperature was reduced gradually to 5°C/h. The samples were removed from the freezer at the end of the 12 hours and left at 4°C for a gentle overnight thawing. Ion leakage analysis was performed using the bark samples that were removed from the shoots. For further analysis, the remaining bark sample portions were stored at -80°C.

2.2. Determination of freezing injury

According to Turhan and Ergin (2012), electrolyte leakage analysis was used to detect freezing injury on shoots. The electrical conductivity of each sample was measured using a conductivity meter (Mettler Toledo, SevenEasy S30, Colombus Ohio, USA). The proportion of injury at each temperature was determined using the ion leakage data and the following formula: Injury% = [(L(t)% − L(c)%)/(100 − L(c)%)] × 100, where L(t) represents the treatment's ion leakage percentage and L(c) the control's ion leakage percentage (Arora et al. 1992).

2.3. Protein analysis

One gram of bark tissue was homogenized in borate buffer (pH 9.0; 1 mM PMSF, 50 mM sodium tetraborate, 50 mM ascorbic acid, and 1% β-mercaptoethanol) at 4°C to extract all of the soluble proteins (Arora et al., 1992). The homogenates were centrifuged for 1.5 hours at 4°C at 26000 × g. Using the Bradford method, the TSP content in preserved supernatants was determined (Arora and Wisniewski, 1994). Trichloroacetic acid solution (10%) was used to precipitate the proteins (Lim et al., 1999). Pellets were dried and rinsed three times with cold acetone before adding 100 µL of loading buffer. An equivalent amount of sample (30 µg) was placed into each well, and the proteins were separated using SDS-PAGE. The gels were stained with Coomassie dye (Arora et al., 1992). Antibodies of HSP23 [Anti-Heat Shock Protein 23 produced in rabbit affinity isolated antibody (Sigma)] and HSP60 [Monoclonal Anti-Heat Shock Protein 60 antibody produced in mouse (monoclonal LK2, ascites fluid (Sigma)] were diluted 1:1500 and used for immunoblot analyses. Blots on the membrane were detected by the alkaline phosphatase assay using the ProtoBlot Western Blot AP Kit (Promega). Public Domain NIH Image program was used to compare the intensities of the bands (NIH Image, 2024).

2.4. Statistical analysis

Each trial was repeated three times. The obtained data were determined by Duncan's test at $p \leq 0.05$. Statistical analyses were performed with SPSS for Windows software.

3. Results

3.1. Freezing injury

Effects of low-temperature applications on the cell membrane injury of walnut cultivars in cold-acclimated (CA, in January) and non-acclimated (NA, in July) periods were shown in Fig. 1. In general, it has been found that the injury in the NA period is more than in CA period. While the injury did not reach 50% in any cultivar in the CA period at any low-temperature application, it was about 50% at all temperature applications (except for Fernor at 5°C) in the NA period. Significant differences were detected between periods, cultivars, temperatures, and interactions (Table 1).

Table 1
According to the analysis of variance (ANOVA), the factors of period (P), cultivar (Cv.), and temperature (T), as well as their interactions, on injury and total soluble proteins (TSP). Numbers show F values at the 0.05 level.

Dependent variable	Independent variable						
	P	Cv.	T	P × Cv.	P × T	Cv.×T	P × Cv. × T
Injury	54025.68*	57.14*	886.39*	42.71*	50.61*	14.34*	14.46*
TSP	967.25*	109.28*	192.63*	11.43*	134.78*	30.92*	13.51*

* Significant at $p < 0.05$.

The lowest average freezing injury in the NA period was observed in Fernor at 5°C (43.17%) whereas the highest one was in Chandler, at -25°C (96.33%). Besides, the lowest average freezing injury in the CA period occurred in Fernor at 5°C (7.07%) whereas the highest one was in Bilecik at -25°C (33.69%). Based on the responses of the plants in the NA period, a sensitive cultivar (Chandler) and a tolerant cultivar (Fernor) to low temperatures were determined according to the injury results.

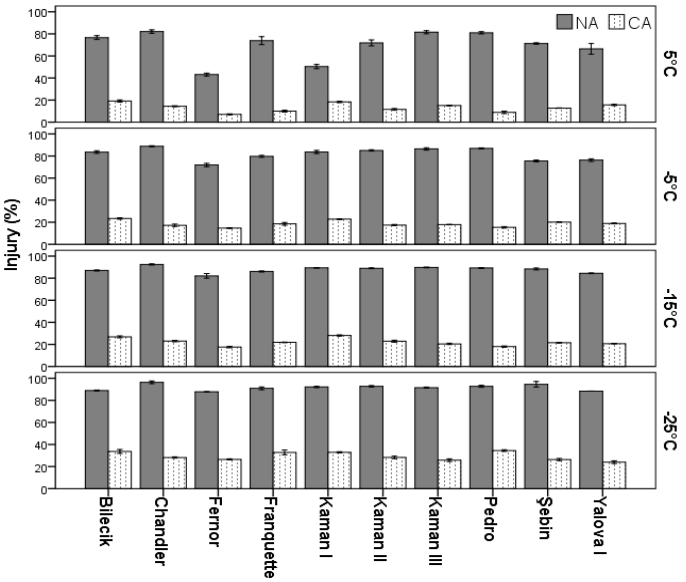

Fig. 1. Variations in injury rates among walnut cultivars exposed to cold temperatures during the NA and CA periods. The error bars indicate ± SE of three replications.

3.2. Protein analysis

Protein and immunoblotting analysis were performed in the Chandler and Fernor, which are cold sensitive and cold tolerant plants, respectively. Total soluble protein contents of varieties were higher in the CA period than in the NA period (Fig. 2). In the NA period, TSP content of Fernor was increased in response to the low temperature applications especially at -15°C (6.2 mg /gFW) and -25°C (6.0 mg /gFW) but Chandler's TSP content remained constant. In the CA period, TSP contents of varieties were increased at -25°C and it was determined as 8.33 mg /gFW in Chandler and 9.63 mg /gFW in Fernor. Significant differences were detected between periods, cultivars, temperatures and interactions (Table 1).

Total protein profiles of Chandler and Fernor cultivars due to low-temperature applications in NA and CA periods were shown in Fig. 3 and Fig. 4, respectively. In both cultivars, protein bands with sizes of 66 kDa, 59 kDa, and 44 kDa were determined; the densities of these bands changed in response to low-temperature treatments. It was determined that the intensity of the 66 kDa protein band increased especially at -15°C and -25°C with low temperatures in both cultivars in the NA period (Fig. 3), the increase in intensity was observed more clearly in Fernor. A similar situation was also detected for the 59 kDa protein band. Low-temperature treatments caused the 44 kDa protein band's intensity to rise in Fernor and decrease in Chandler.

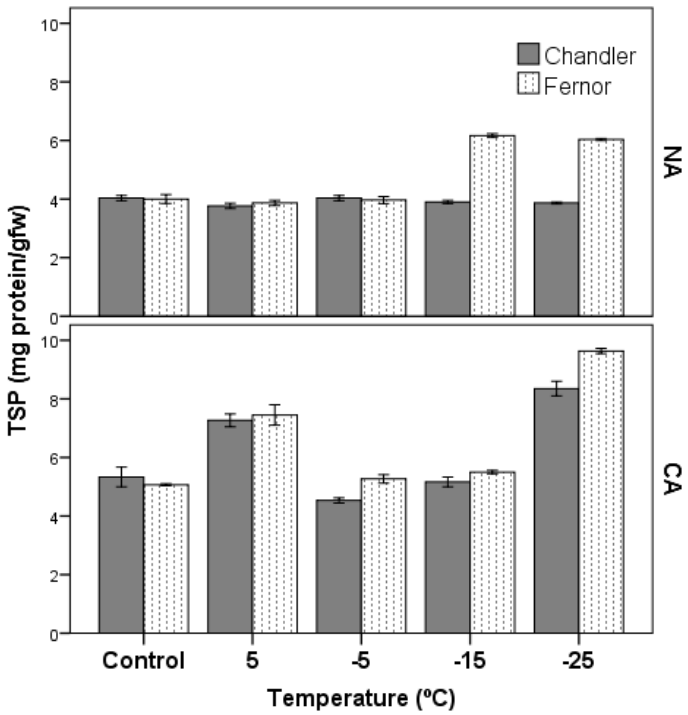

Fig. 2. Effect of low temperature applications on total soluble protein (TSP) contents in walnut cultivars at NA and CA periods. The error bars indicate ± SE of three replications.

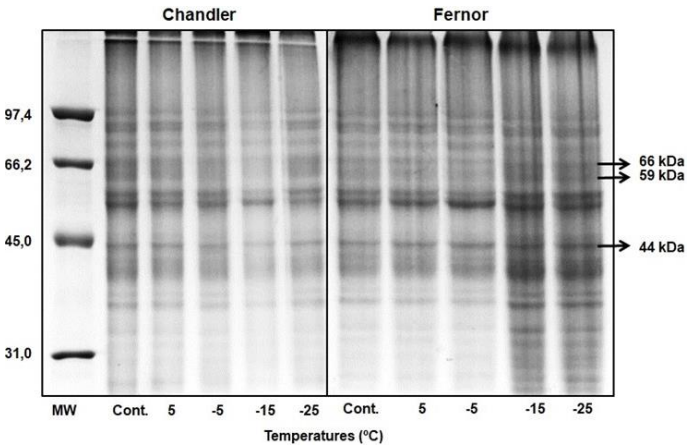

Fig. 3. SDS-PAGE protein profiles of walnut cultivars during the NA period based on low temperature applications. Each well was loaded with 30 µg of protein. The molecular weight (Mw) of the marker used is displayed on the left in units of kDa. The arrows on the right represent the locations of proteins that varied during low temperature stress.

While the intensity of the 66 kDa protein band was indeterminate in Chandler, it was increased with low

temperatures in Fernor at CA period (Fig. 4). It was observed that the 59 kDa protein band increased at 5°C in Chandler but the other temperatures did not have a significant effect, whereas in Fernor it increased at -15°C and -25°C. While the intensity of the protein band, which was determined as 44 kDa, decreased in Chandler compared to the control due to low-temperature applications, it was determined that it increased at -15°C and -25°C in Fernor.

The immunoblotting results for HSP23 showed that there was a 44 kDa protein band, with varying band intensities based on temperatures and cultivars in the NA and CA periods (Fig. 5 and Fig. 6). Intensity of HSP23 was higher in the CA period than in NA period in both cultivars. Besides, it was determined that the intensity of HSP23 in Fernor was higher than in Chandler in both periods.

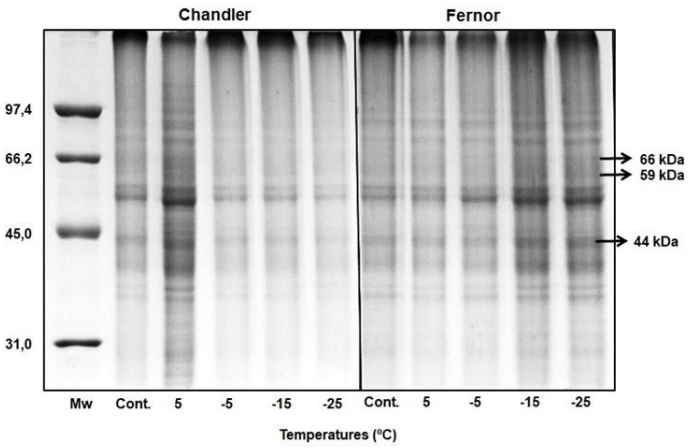

Fig. 4. SDS-PAGE protein profiles of walnut cultivars during the CA period based on low temperature applications. Each well was loaded with 30 µg of protein. The molecular weight (Mw) of the marker used is displayed on the left in units of kDa. The arrows on the right represent the locations of proteins that varied during low temperature stress.

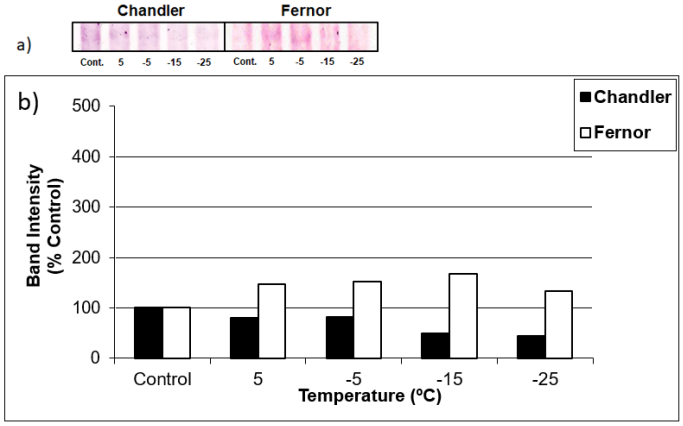

Fig. 5. Appearance of 44 kDa HSP23 protein (a) and band intensities (b) of cultivars due to low temperature applications in NA period.

During the NA period, the intensity of HSP23 in "Chandler" was decreased depending on the low-temperature application compared to the control (Fig. 5). However, synthesis of HSP23 was increased depending on the low-temperature applications in Fernor and it was reached the highest level at -15ºC (168%), and then it was decreased at -25ºC (133%).

In the CA period, HSP23 has reached the highest level at 5ºC (162%) in Chandler compared to the control, thereafter it decreased depending on the low temperature (Fig. 6). In Fernor, the synthesis of HSP23 was increased depending on the low-temperature application and it was reached the highest level at -25ºC (447%).

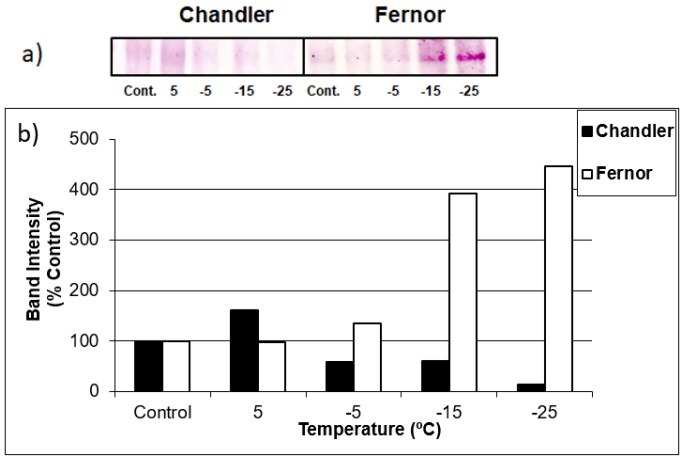

Fig. 6. Appearance of 44 kDa HSP23 protein (a) and band intensities (b) of cultivars due to low temperature applications in CA period.

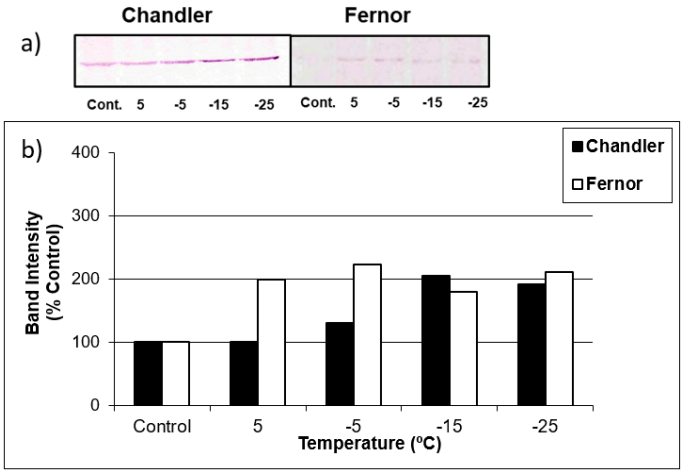

Fig. 7. Appearance of 59 kDa HSP60 protein (a) and band intensities (b) of cultivars due to low temperature applications in NA period.

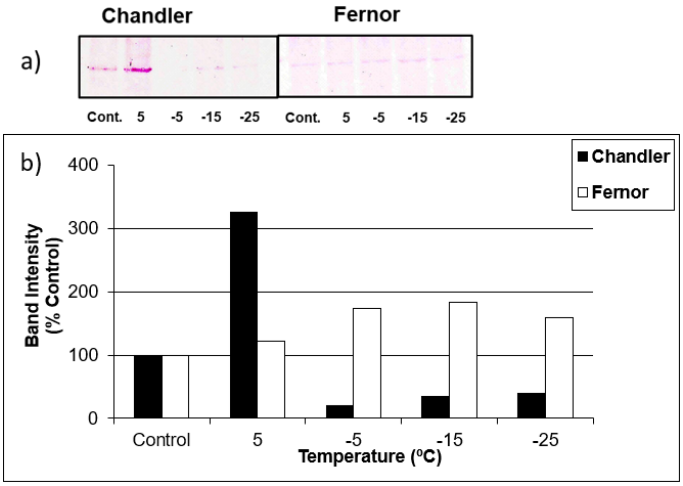

Fig. 8. Appearance of 59 kDa HSP60 protein (a) and band intensities (b) of cultivars due to low temperature applications in CA period.

The immunoblotting investigation results for HSP60 suggested that 59 kDa protein band with various band intensities depending on the cultivars and low temperatures with NA and CA periods (Fig. 7 and Fig. 8). As shown in Fig. 7, during the

NA period, the synthesis of the HSP60 was increased to -15ºC (205%) and then it was decreased at -25ºC (191%) in Chandler. The intensity of HSP60 in Fernor was increased until -5ºC (223%), then it was decreased at -15ºC (180%), after it was increased again -25ºC (211%). In the CA period, an increase was measured for HSP60 at 5ºC (325%) in Chandler, it was found at quite low levels at the other low-temperature applications as compared with the control (Fig. 8). In Fernor the HSP60 was increased until -15ºC (184%), then it was decreased at -25ºC (154%). The intensity of HSP60 was calculated according to the control of varieties. Generally, the synthesis of HSP60 was more intense in Fernor than in Chandler at all low temperatures in both periods.

4. Discussion

The plasma membrane has a highly dynamic structure to preserve the integrity and identity of closed structures (Casares et al., 2019). Compositional alterations in the plasma membrane, one of the primary sites of freezing injury, are required to achieve cold acclimatization (Miki et al., 2019). The reactions of walnut cultivars to cold stress may vary depending on the season and the region where they are grown. Actually, Aleta et al. (2013) found that, in their research in northeast Spain, the Fernor variety was more tolerant than Chandler in the fall, in contrast, Chandler was more tolerant in the winter. Poirier et al. (2004) according to their research conducted in south-central France, they stated that the Fernor variety was more tolerant than Chandler among seven varieties in the autumn, winter and spring seasons, which is similar to the present study. Since plants are not accustomed to cold during the NA period, it is important to know their reactions to cold stress in this period in order to explain their cold tolerance. Therefore, in this study, two cultivars were determined as cold-sensitive (Chandler) and cold-tolerant (Fernor) in terms of low-temperature tolerance in the NA period.

Cold acclimation boosted the cold tolerance of all walnut cultivars. Low temperatures that happen during the NA period, when plants are not used to the cold, severely harm plants and result in losses of valuable products. For this reason, similar to this study, freezing damage in the field is generally much greater in non-acclimated seasons than the damage that occurs during the winter (Turhan and Ergin, 2012). Especially, late spring frosts and early autumn frosts pose a great risk for crop losses (Heberling and Muzika, 2023). Therefore, among the ten cultivars used in this study, the Chandler with the most damage in the NA period was determined to be cold sensitive and the Fernor, which showed the least damage, was determined to be cold tolerant.

Through a complicated adaption process called “cold acclimation”, plants adapt to withstand freezing temperatures. During the cold acclimation, ultrastructural, compositional, and biochemical changes occur in plant cells (Vafadar et al., 2024). However, the significance of these changes in acquiring frost tolerance is still unclear. To understand frost tolerance, which is a result of cold acclimation, the molecular basis of cold acclimation in plants should be elucidated. In addition to these adaptations, at the onset of stress, normal protein production is reduced and the production of heat shock proteins (HSPs) is increased in plant cells (ul Haq et al., 2019). As previously reported, it was shown that while the synthesis of some other proteins decreased, the synthesis of stress-related proteins increased in the current study. In the present study, it was determined that 66 kDa, 59 kDa, and 44 kDa protein bands were effective in adaptation to cold in both cultivars. Nevertheless, the increment of these bands is more prominent in Fernor, due to the better cold adaptation of this cultivar.

HSPs prevent stress-induced misfolding and play a role in preventing denatured and aggregated proteins under stress conditions, they are also responsible for maintaining cell homeostasis under normal conditions, transporting newly produced proteins between the organelles of cells and folding (Shahbaz, 2024). HSPs are produced not only under heat stress but also under other stress situations (ul Haq et al., 2019). In Arabidopsis, pea, poplar, and rice, several HSPs were up-regulated in response to cold stress (Bae et al., 2003; Renaut et al., 2004; Cui et al., 2005; Taylor et al., 2005). Like other HSPs, sHSPs work as molecular chaperones and supply protection by binding to non-native forms of proteins and inhibiting the aggregation (Dou et al., 2024). Cold-induced accumulation of sHSPs has been reported in some plant species (Zhang et al., 2020). In this study, the synthesis of HSP23, which belongs to sHSP family, was in the NA period lower than in the CA period. The fact that, the synthesis of HSP23 was significantly increased at the CA period especially in Fernor, which was cold tolerant, suggests that HSP23 is quite effective in gaining cold tolerance in walnut plants.

Correlatively, the synthesis of the HSP60 was higher in Fernor than in Chandler in both periods. The stable increase in HSP60 due to low temperatures in Fernor during the CA period, can be explained by the effect of HSP60 on the cold tolerance of this cultivar. In the CA period, maximum HSP60 synthesis was measured at 5ºC in Chandler, it was found at quite low levels at the other low-temperature applications. In this case, it could be this cultivar is tolerant down to 5°C, but not tolerant to lower temperatures. As noted in previous studies (Nagaraju et al., 2021, Tian et al., 2021), it was determined that HSPs act as molecular chaperones that prevent cold-induced protein denaturation and promote the refolding of partially denatured proteins, and therefore, they are closely related to cold tolerance in the walnut plant. In general, the synthesis of HSPs is more intense in Fernor than in Chandler at all low temperatures of the NA and CA periods, due to the better cold tolerance of this cultivar than the other one.

Global warming and climate change brought about by the world’s rapidly growing industrialization and urbanization have severe consequences for plant growth, development, yield, and quality, and occasionally even threaten plant existence (Janni et al., 2024). In order to avoid nutritional problems in the future, it is necessary to investigate, identify, and confirm certain traits related to stress tolerance in plants, and develop transgenic plants by revealing and manipulating stress-related genes (Sharma et al., 2023). Related to this, HSPs have a broad function in stress tolerance. Further research is required to fully comprehend the overall function of HSPs in connection to plant cold adaptation. Various scientific studies on the roles of HSP families against various biotic and abiotic stresses in different cultivated plants will be helpful in terms of developing stress-tolerant varieties.

5. Conclusion

The Chandler and Fernor cultivars were identified to be sensitive and tolerant, respectively, among the ten walnut cultivars examined during the NA and CA periods. The differences between these two cultivars in terms of cold

tolerance were investigated in terms of HSPs. The results were emphasized that cold acclimatization is effective in gaining cold tolerance in walnut plants. It was determined for the first time that HSP23 and HSP60 were effective in cold tolerance in walnut plants. According to these findings, to understand HSP's functions in woody plants under cold stress, it is recommended that additional analyses should be performed.

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Conflict of interest: The authors declare that they have no conflict of interests.

Informed consent: The authors declare that this manuscript did not involve human or animal participants and informed consent was not collected.

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