International Journal of Horticultural Science and Technology
Journal homepage: https://ijhst.ut.ac.ir

Assessment of Chilling Stress Effects on Physiological and Biochemical Attributes and Gene Expression on 'Abbas Ali' Pistachio Cultivar

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ARTICLE INFO

Article history:

Received: 24 January 2025,
Received in revised form: 7 May 2025,
Accepted: 9 May 2025,

Article type:

Research paper

Keywords:

Abiotic stress,
Antioxidant enzymes,
Cold damage,
Proline,
Soluble carbohydrate,
Spring frosts

ABSTRACT

Pistachio is sensitive to late-spring frost, and cold injury may occur when temperatures decline rapidly. In this study, selected physiological traits and the expression of specific genes associated with frostbite-induced temperature hypersensitivity, namely *CLO*, *ICE1*, and *Dehydrin*, were evaluated. The results demonstrated that physiological parameters such as proline content, electrical conductivity (EC), and the activity of enzymes associated with cold stress tolerance, including catalase, superoxide dismutase (SOD), and ascorbate peroxidase (APX), increased in reproductive organs (flowers and fruits) under temperature treatments, as expected. The levels of soluble sugars, ion leakage, and proline varied between organs and treatments, with the highest concentrations observed in both flowers and fruits at –2 °C. In contrast, the highest chlorophyll content in flowers was recorded at 2 °C. Enzyme activities (catalase, SOD, and APX) peaked in flowers at 0 °C. Gene expression analysis revealed that the *CLO* gene showed the highest expression at –2 °C in the flower organ, while the lowest expression level across all 24 experimental groups was recorded for the *CLO* gene in the fruit organ at 4 °C. In conclusion, the response of pistachio to cold stress varies considerably depending on temperature and organ type. Overall, decreasing temperatures triggered accelerated and enhanced gene expression, whereas higher temperatures were associated with the down-regulation of these cold-responsive genes.

Introduction

The overall performance of fruit orchards is closely linked to soil characteristics and climatic conditions. Among these, unfavorable climatic factors—particularly winter frost damage (including chilling and freezing) and spring cold—are among the most critical determinants of species distribution and serve as key indicators for orchard site selection (Luedeling, 2012; Pechan et al., 2023). A decline in temperature during early spring, especially if it coincides with flower bud opening, can result in significant yield losses (Feng et al., 2021; Siebert et al., 2017). Cold stress is a major abiotic factor that can cause frostbite and alter physiological, biochemical, and molecular processes in plants, ultimately leading to reduced productivity (Feng et al., 2021; Ritonga and Chen, 2020). Agricultural statistics from recent decades suggest that climate fluctuations during the growing season have contributed to declines in both the quantity and quality of fruit production, particularly in tropical and subtropical fruit trees that are vulnerable to winter and spring frost damage (Mahlein et al., 2012; Upreti and Sharma, 2016).

Pistachio (*Pistacia vera*), one of the most economically significant horticultural crops globally and in Iran, is notably susceptible to frost injury, with its blossoms and young fruits damaged annually (Feng et al., 2021; Saxe et al., 2001). Native to west-

central Asia, pistachio is an important crop in tropical and subtropical regions and a valued edible product of the cashew family (*Anacardiaceae*), commonly cultivated in drylands with warm to temperate climates (Mandalari et al., 2021). Iran, with an annual production of 200,000 tons, ranks third globally—following the United States and Türkiye—in pistachio production, and is widely recognized for producing one of the world's most renowned and economically significant nuts (FAOSTAT—Crops, 2020; Rezaei et al., 2023).

As winter ends, pistachio trees rapidly lose their cold hardiness, becoming highly sensitive to low temperatures. Several studies have identified 4 °C as the threshold temperature for pistachio, below which metabolic activity declines, cellular structures are damaged, and plant growth is impaired. Although all growth processes, particularly reproductive functions, are sensitive to cold stress, sensitivity levels vary across plant tissues (Albertos et al., 2019). For instance, stems exhibit greater cold tolerance than roots, which are more susceptible to restricted growth, nutrient and water deficiency, and overall plant weakening under cold stress (Weiser, 1970b). Leaves also display limited adaptability to cold; during the initial signs of flowering, they are prone to frostbite (Pope et al., 2015). At this stage, sudden temperature drops near or below 0 °C reduce flower bud viability and ultimately diminish the expected quality of the harvest (Mannino et al., 2019).

Studies have shown that cold stress significantly influences various physiological and biochemical processes in plants, including the accumulation of soluble sugars and proline, which function as important osmoprotectants (Chang et al., 2021). Low temperatures affect photosynthetic activity by altering stomatal movement and reducing the synthesis of photosynthetic pigments such as chlorophyll a, chlorophyll b, and chlorophyll ab. Under cold stress, the metabolism of plant cells, particularly in leaves, is severely inhibited due to reduced carbon dioxide fixation, disrupted photosynthetic cycles, and impaired carbohydrate synthesis and distribution. These effects collectively lead to a decline in chlorophyll content (Azami et al., 2021).

Physiological indicators such as proline content, soluble carbohydrates, electrical capacitance, chlorophyll and carotenoid concentrations, and antioxidant enzyme activity are closely associated with cold tolerance in plants and can serve as useful indices for screening cold-resistant varieties (Li et al., 2012). Cold stress adaptation is governed by complex molecular and biochemical mechanisms, which involve the regulation of gene expression through activation and repression of specific genes (Guo et al., 2018; Ritonga & Chen, 2020). Comparing gene expression profiles under stress and non-stress conditions is a common approach to identify genes that mediate plant responses to environmental factors (Swindell, 2006).

Abiotic stress tolerance in plants is influenced by several genetic components, including transcription factors (TFs), microRNAs (miRNAs), epigenetic modifications, non-coding RNAs, stress-responsive genes, and genetic engineering or breeding approaches. These elements collectively contribute to plant adaptation. In the present study, the genes examined include *dehydrin* and *CLO* (stress-responsive genes) and *ICE1* (a transcription factor). Transcription factors regulate the expression of stress-responsive genes; notable examples include DREB (Dehydration-Responsive Element Binding) proteins and NAC (NAM, ATAF1/2, and CUC2) transcription factors.

Stress-responsive genes encode proteins such as heat-shock proteins (HSPs) and antioxidant enzymes. *CLO* (Caleosin) genes contribute to chilling tolerance by regulating oxidative stress through modulation of antioxidant enzyme activity, influencing the expression of other stress-responsive genes, and maintaining membrane stability (Zhenfeng et al., 2022). *ICE1* is a transcription factor that activates C-repeat binding factors (CBFs), which in turn regulate the expression of cold-responsive genes (Chinnusamy, 2010). By inducing CBF expression, *ICE1* enhances cold acclimation and freezing tolerance. Its activity is modulated by post-translational modifications, including ubiquitination and sumoylation, which affect its stability and function. This gene is highly conserved across plant species, and its overexpression in crops such as tomato and cucumber has been shown to increase chilling tolerance, demonstrating its importance in improving cold resilience (Badawi, 2008).

Dehydrin genes are key mediators of plant responses to chilling stress. They protect cellular structures against dehydration damage, stabilize proteins and membranes, and act as molecular chaperones to maintain cellular integrity during cold acclimation (Biologia Plantarum, 2007). Their expression is regulated by phytohormones such as abscisic acid (ABA), which are central to plant stress responses, thereby enhancing cold tolerance (Yang Liu & Qiping Song, 2017).

Accordingly, the present study aimed to evaluate physiological traits and stress-responsive genes that play key roles in frost-induced temperature hypersensitivity, as well as to examine their changes in different pistachio organs (flowers and fruits). Specifically, the study sought to address the following research questions: (1) Do gene expression patterns and physiological or biochemical responses in plants change under cold stress? (2) Are stress-responsive genes upregulated under cold conditions? and (3) If so, are the expression levels of these genes

different during the flowering and fruit formation stages?

Materials and Methods

The study was conducted in April 2022 in the pistachio orchards of Damghan City, Iran, located at 36.1679° N latitude and 54.34292° E longitude, at an elevation of 1,250 m in the southeastern Elburz Mountains. This region is classified as dry to semi-dry. The experimental orchard belongs to the Pistachio Research Center of Damghan and consists of 26-year-old trees of the Abbas Ali (*Abbasali*) pistachio cultivar.

The research was performed in two stages to assess the physiological and biochemical traits of pistachio flowers and fruits, alongside the expression of three cold-tolerance–related genes under chilling stress. Data collection occurred in two rounds.

In the first stage, which coincided with the onset of female flower opening in April, three random points within the orchard were selected. From each point, four branches were sampled from the periphery of the trees. The flowers were excised along with their branches, sprayed with distilled water, and immediately transferred to an incubator with the capacity to gradually adjust temperature. The ambient temperature at the time of harvest was approximately 21 °C, which was used as the reference treatment. Additional treatments included exposure to 4, 2, 0, and −2 °C. Flowers were held at each target temperature for 2 h once the desired temperature was reached.

In the second stage, approximately one week after flowering onset, fruits in the millet-shaped stage (post-pollination) were randomly collected. The fruits underwent the same incubation and temperature treatments as described for the flowers. Physiological and biochemical traits were subsequently measured for both flowers and fruits, and the data were analyzed.

Chlorophyll

Chlorophyll and carotenoid concentrations were determined using Arnon's (1949) method. Briefly, 0.25 g of pistachio leaf tissue was ground in 10 mL of 80% acetone to form a slurry. The mixture was centrifuged at 3,500 rpm for 10 min. Absorbance of the supernatant was measured at 645 nm and 663 nm using a Shimadzu Biospec 1601 UV-Vis spectrophotometer (190–1,100 nm, 230 V, 50/60 Hz, 160 W). Chlorophyll concentrations were then calculated using standard equations.

Proline

Specifically, 0.5 g of the leaf of pistachio was pounded with 5 mL of 95% ethanol and after centrifugation, 1 mL of the extract was diluted with 10 mL of distilled water, 5 mL of ninhydrin which is a mixture of 1.25 g of ninhydrin in 30 mL of glacial acetic acid, and 20 mL of 6 M phosphoric acid. The amount of proline was measured by a spectrophotometer at a wavelength of 515 nm. The final amount of protein was calculated in µmoles proline g^{-1} of fresh-weight material based on the standard curve (Bates et al., 1973).

Total soluble carbohydrate (TSC)

Total soluble carbohydrates were determined following the method of Irigoyen et al. (1992). A 0.5 g leaf sample was homogenized with 5 mL of 95% ethanol. Then, 0.1 mL of the alcoholic extract was mixed with 3 mL of freshly prepared anthrone reagent (150 mg anthrone in 100 mL of 72% sulfuric acid) and incubated in a hot water bath for 10 min. Absorbance was read at 625 nm using a spectrophotometer (Irigoyen et al., 1992).

Electrical conductivity

Electrolyte leakage was assessed according to Sairam and Srivastava (2002). A 0.1 g sample was washed with 10 mL of double-deionized water, and initial electrical conductivity was measured using an EC meter. The sample was then placed in a bain-marie at 100 °C, and final conductivity was measured to calculate electrolyte leakage (Sairam and Srivastava, 2002).

$$Electrical\ Capacitance\ percentage = \frac{Initial\ leakage}{Final\ leakage} \times 100$$

Ascorbate peroxidase enzyme assay (APX) (EC1.11.1.1)

APX was assayed by the method of (Bradford, 1976). In this method, the reaction mixture consists of 50 mM potassium phosphate buffer (pH = 7), 0.5 mM ascorbic acid, 0.15 mM H2O2, 0.1 mM EDTA, and 50 µL of enzyme extract. Following the oxidation of ascorbic acid at the start of the reaction, 2 min after the start of the reaction, the extent of enzyme reduction absorption was calculated at 290 nm using the method of Bradford.

Superoxide dismutase enzyme assay (SOD) (-EC 1.15.1.1)

The reaction mixture was composed of 50 mM phosphate buffer (pH = 7), 0.075 µM NBT, 0.1 mM Na-EDTA, 75 µM riboflavin, 13 mM methionine, and 50 µL enzyme extract. The photo reduction of NBT (formation of formazan) was measured at 560 nm (Giannopolitis and Ries, 1977).

Catalase enzyme assay (CAT) (EC 1.11.1.6)

According to Dhindsa et al. method, the catalase enzyme activity was measured by calculating the reduction in H_2O_2 absorption (decline in the amount

of H_2O_2) at 240 nm. The reaction mixture included 50 mM potassium phosphate buffer (pH = 7) and 15 mM hydrogen peroxide. The reaction was initiated by adding 100 mL of enzyme extract to the mentioned mixture. Absorption variations (the difference between the absorption at the initiation time of the reaction and 1 min after the start time of the reaction) were calculated (Dhindsa et al., 1981).

Gene expression studies

To investigate gene expressions during cold stress induction at different temperature treatments in pistachio flowers and fruits, total RNA was extracted from the control group and treated plants using the YTzol Pure RNA extraction kit (YektaTajhiz, Tehran, Iran) A portion of the plant sample, frozen at -80 °C, was transferred into a porcelain mortar with liquid nitrogen. After nitrogen evaporation, the sample was powdered, and 100 mL of the powdered sample was added to a 2 mL microtube with 1 mL of FARB BUFFER and left at room temperature for 5 min. The sample was then microfuged for 3 min at 4 °C. The clear upper phase was transferred to a columnar microtube, and 600 µL of WASH BUFFER (1) was added and microfuged for 1 min at 4 °C. The liquid collected was removed, and 800 µl of WASH BUFFER (2) was added to the column, followed by microfugation for 1 min at 4 °C. This step was repeated 2 to 3 times. Finally, 100 µL of ELUTION buffer was added to the column, left for 3 min, and microfuged. The column was removed, and the microtube was placed in a -80 °C freezer for further experiments. Spectrophotometer (Wilmington, USA) and horizontal electrophoresis on 1% agarose gel were used to assess RNA presence, purity, and quantity of samples.

Primer design for RNA and its target gene

Sequence coding regions of the studied genes of pistachio were selected as the target genes while the GAPDH gene as the reference gene was taken from the NCBI (National Center for Biotechnology Information, an agency of the U.S. National Library of Medicine Website) (https://www.ncbi.nlm.nih.gov/). The primers were designed using Primer3 software and finally validated with an oligo analyzer (IDT) and Blast software. Note that primers were designed in the form of exon-exon junctions to ensure the absence of possible DNA contamination and prevent its replication. The designed primers were synthesized by Cinna Gen Co Iranian Biotechnology Company. The primer's information is given in the table below (Table 1).

Table 1. Designed primers for the PCR reaction.

Gene	Sequence	Tm	PCR Product
Dehydrin -Forward	GGCGTAGAGAAGAAGCACCA	59.75	85
Dehydrin -Reverse	TCGCTAACCCCATTCACCTAT	58.60	
Ice1- Forward	TGATGTGGAAGACGTGAGCA	59.32	271
Ice1 - Reverse	TGAAGCCCTGTCCATTTTGC	59.03	
Clo - Forward	AGGTGGCAGATCCTGTAGTCT	59.99	361
Clo- Reverse	GCTCCCCACTGAAATCCTTGA	60.00	
Gapdh- Forward	CAGCAATGCTTCTTGCACCACA	62.48	107
Gapdh - Reverse	CGGTGTAGGAGTGAGTAGTT	56.02	

cDNA synthesis.

For cDNA synthesis, the same RNA concentration of all samples was selected and according to the protocol of the cDNA synthesis kit (Cat: YT 4500) (YektaTajhiz, Tehran, Iran), reverse transcription was done. The procedures were briefly as follows. First, 1 µL random hexamer, 4 µL RNA sample, and 8.5 µL Decpce water were mixed and put promptly on ice. Subsequently, the mixture of 4 µL of 5X RT buffer, 4 µL M-MLV, 2 µL dNTP, and 0.5 µL RNasin was added to the micro tube to inactivate RT reverse transcriptase enzyme; the solution first was put on thermal cycler at 37 °C for 1 h, and then was placed at 70 °C for 5 min.

Real-time PCR

After RNA extraction and cDNA synthesis, a real-time PCR reaction was used to determine the expression level of target genes. Real-time was repeated twice and the GAPDH gene was used for normalization and control of gene expression. Real-time PCR reactions were done using Green SYBR solution (Fermentas company-Canada) in a volume of 10 µL, including 5 µL of Master Mix, 2 µL of forward primer (0.3 µM), 2 µL of reverse primer (0.3 µM), and 1 µL cDNA. (Applied bio systems 4389986, Fermentas method).

Statistical analyses

Statistical analyses for physiological and biochemical empirical data were done using the SAS9.3 and the experiments were analyzed as a factorial experiment based on randomized complete block design. The estimated means were compared using Duncan's test with a $P < 0.05$ level. The effect of cold stress on the studied characteristics was analyzed by Duncan's Multiple Range tests (DMRT. Finally, The analysis of gene expression was carried out using specialized software the REST 2009 software (version 2.0.13) and graphpad prism version 8 using statistical tests one way anova and T Test.

Descriptive statistical methods were also used to perform analysis of variance, mean and standard deviation calculations. $P < 0.05$ were considered statistically significant.

Results

The effect of cold stress on physiological characteristics

According to the results of Table 2, effect of low temporature (cold stress) was significant on all the investigated traits except soluble carbohydrates at the 5 and 1% probability level. Also, The difference between organs (flower/fruit) was also significant in all parameters ($P < 0.01$), and just proline content at 5% probability level was different in organs under temperature treatment. However, their interaction effect had significant effect in all the investigated traits except soluble carbohydrate parameter ($P = 0.02$).

Table 2. Analysis of variance for physiological characteristics of Abbas Ali pistachio cultivar (flowers and fruits) under cold-stress.

S.O.V	df	Means of square						
		Soluble carbohydrate	Carotenoid	Proline	Electrical capacitance	Chlorophyll total	Chlorophyll *b*	Chlorophyll *a*
Block	3	0.3993^{**}	0.0032^{**}	0.0002^{ns}	14.202^{**}	0.03^{ns}	0.03^{ns}	**0.00^{ns}**
Temperature (A)	4	0.0377^{ns}	0.0062^{**}	0.0005^{*}	1073.1^{**}	0.07^{**}	0.05^{*}	**0.00^{**}**
Organ (B) (flower/fruit)	1	0.4541^{**}	0.0198^{**}	0.0007^{*}	44.598^{**}	1.58^{**}	0.86^{**}	**0.10^{**}**
A × B	4	0.0200^{ns}	0.0039^{**}	0.0005^{*}	110.66^{**}	0.19^{**}	0.12^{**}	**0.01****
Error	27	0.033572	0.000418	0.000133	1.963987	0.0167	0.0155	**0.0008**
(CV) %		16.11	4.08	10.96	4.63	7.18	10.93	**4.10**

^{ns}, * and ** non-significant and significant at the probability levels of 5% and 1%, respectively.

The results presented in Table 3 indicate that the highest electrical capacitance was observed in flowers at −2 °C. Exposure to low temperatures significantly increased electrical conductivity under all cold-stress conditions compared with the control (21 °C). Moreover, under the same temperature treatments, flowers consistently exhibited higher electrical capacitance than fruits.

Analysis of other physiological traits revealed that the maximum proline content (0.128 µmol g^{-1}) was recorded in fruits at 4 °C. In general, proline accumulation was higher in fruits than in flowers across all temperature treatments.

The interaction between organ type (flower vs. fruit) and temperature did not exert a significant combined effect on total soluble carbohydrate concentrations. In contrast, the interaction between organ and temperature significantly affected chlorophyll pigments. Both organ type and cold-stress conditions influenced chlorophyll content; however, no clear linear trend was observed across temperatures. At the same temperature, flowers generally contained higher levels of chlorophyll than fruits. Notably, the maximum concentrations of chlorophyll a (0.749 mg g^{-1}), chlorophyll b (1.335 mg g^{-1}), and total chlorophyll (2.0788 mg g^{-1}) were observed in flowers at 2 °C.

According to Table 4, the analysis of variance (ANOVA) for the effects of temperature, organ type (flower vs. fruit), and their interaction on the activities of ascorbate peroxidase (APX), superoxide dismutase (SOD), and catalase (CAT) showed distinct patterns. Both temperature and organ type exerted a highly significant effect on APX activity ($P < 0.01$), whereas neither factor had a significant effect on SOD or CAT activity when considered independently ($P > 0.05$).

Table 3. Comparison of average interaction effects of temperature × organ (flower and fruit) on the physiological characteristics in Abbas Ali pistachio cultivar.

Temperature× organ	electrical capacitance	Proline	Carotenoid	soluble sugar	Chlorophyll (mg g^{-1} FW)		
	(%)	(µmol g^{-1} FW)	(mg g^{-1} FW)	(mg g^{-1} FW)	Total	*b*	*a*
	Mean± Std.Error	Mean± Std.Error	Mean± Std.Error	Mean± Std.Error	Mean± Std.Error	Mean± Std.Error	Mean± Std.Error
Flower (-2 °C)	50.32±0.11^{a}	0.10±0.00^{bc}	0.53±0.01^{ab}	1.01±0.19^{b}	1.96±0.00^{a}	1.25±0.00^{ab}	0.71±0.00^{ab}
Fruit (-2 °C)	43.65±0.18^{b}	0.11±0.00^{b}	0.55±0.00^{a}	1.36±0.01^{a}	1.89±0.05^{ab}	1.21±0.03^{abc}	0.70±0.01^{b}
Flower (0 °C)	39.39±0.13^{c}	0.10±0.00^{bc}	0.54±0.01^{ab}	1.11±0.14^{a}	2.01±0.04^{a}	1.31±0.04^{a}	0.71±0.00^{ab}
Fruit (0 °C)	27.97±1.15^{e}	0.12±0.01^{ab}	0.48±0.00^{cd}	1.35±0.02^{a}	1.64±0.03^{cd}	1.03±0.02^{cd}	0.61±0.01^{cd}
Flower (2 °C)	27.50±0.12^{e}	0.10±0.007^{bc}	0.49±0.03^{cd}	0.96±0.22^{b}	2.08±0.07^{a}	1.33±0.05^{a}	0.75±0.02^{a}
Fruit (2 °C)	35.75±0.63^{d}	0.11±0.00^{b}	0.47±0.00^{d}	1.17±0.02^{a}	1.25±0.17^{e}	0.67±0.1^{e}	0.58±0.01^{de}
Flower (4 °C)	21.53±0.12^{f}	0.10±0.00^{bc}	0.53±0.01^{ab}	1.03±0.18^{b}	2.06±0.059^{a}	1.32±0.045^{a}	0.74±0.01^{ab}
Fruit (4 °C)	21.53±1.58^{f}	0.13±0.01^{a}	0.44±0.00^{e}	1.19±0.02^{a}	1.49±0.054^{d}	0.93±0.03^{d}	0.56±0.02^{e}
Flower (21 °C)	17.69±0.12^{g}	0.10±0.00^{bc}	0.51±0.02^{bc}	1.04±0.18^{b}	1.884±0.04^{ab}	1.25±0.00^{abc}	0.71±0.00^{b}
Fruit (21 °C)	16.98±1.89^{g}	0.08±0.01^{c}	0.44±0.00^{e}	1.13±0.02^{a}	1.733±0.05^{bc}	1.21±0.034^{bcd}	0.70±0.02^{c}

According to Duncan's multi-range test, the numbers with the same letters in each column do not have a significant difference.

The interaction between temperature and organ type significantly influenced APX activity ($P < 0.01$) and had a moderate but significant effect on SOD activity ($P < 0.05$). In contrast, the interaction between temperature and organ type did not significantly affect CAT activity ($P > 0.05$).

Table 4. Analysis of variance for biochemical characteristics of 'Abbas Ali' pistachio cultivar (flowers and fruits) under cold-stress.

S.O.V	df	Means of square		
		Ascorbate peroxidase (U mg^{-1} Protein)	Catalase (U mg^{-1} Protein)	Superoxide dismutase (U mg^{-1} Protein)
block	3	0.3850*	0.0013^{ns}	0.0060^{ns}
Temperature (A)	4	0.7872**	0.0006^{ns}	0.0021^{ns}
Organ (B) (flower/fruit)	1	0.8191**	0.0005^{ns}	0.0210^{ns}
A×B	4	1.3837**	0.0008^{ns}	0.0041*
Error	27	0.1055	0.0014	0.0039
(CV) %		6.19	5.22	8.91

^{ns}, * and ** non-significant and significant at the probability levels of 5% and 1%, respectively.

Comparison of mean values indicated that variations in temperature and organ type (flower vs. fruit) had no significant effect on CAT activity. Furthermore, as shown in Table 5, the activities of SOD and APX exhibited no significant association with temperature or organ type and did not display any consistent trend across treatments. Nevertheless, a general increase in SOD and APX activity was observed in flowers compared to fruits. The highest concentration of catalase (0.728 U mg^{-1}), superoxide dismutase (0.788 U mg^{-1}), and ascorbate peroxidase enzyme (-6.453 U mg^{-1}) was found in flowers at 0 °C. Also, the maximum levels of ascorbate peroxidase and catalase enzyme, which were observed in cold stress, were still lower than the control group.

Table 5. Comparison of average interaction effects of temperature × organ (flower and fruit) on the biochemical characteristics in Abbas Ali pistachio cultivar.

Temperature×organ	Catalase (U mg^{-1} Protein)	Ascorbate peroxidase (U mg^{-1} Protein)	Superoxide dismutase (U mg^{-1} Protein)
	Mean±Std.Error	Mean± Std.Error	Mean± Std.Error
Flower (-2 °C)	0.699±0.011^{a}	5.084±0.198^{bcd}	0.701±0.025^{b}
Fruit (-2 °C)	0.694±0.016^{a}	4.754±0.016^{d}	0.673±0.016^{cd}
Flower (0 °C)	0.728±0.027^{a}	5.584±0.487^{b}	0.788±0.075^{a}
Fruit (0 °C)	0.705±0.016^{a}	4.844±0.016^{d}	0.666±0.016^{d}
Flower (2 °C)	0.695±0.009^{a}	4.844±0.060^{d}	0.715±0.033^{b}
Fruit (2 °C)	0.711±0.016^{a}	5.454±0.016^{bc}	0.671±0.016^{cd}
Flower (4 °C)	0.699±0.012^{a}	4.933±0.111^{cd}	0.693±0.020^{bc}
Fruit (4 °C)	0.708±0.016^{a}	5.45375±0.01^{bc}	0.689±0.016^{c}
Flower (21 °C)	0.731±0.029^{a}	5.08375±0.19^{bcd}	0.718±0.034^{b}
Fruit (21 °C)	0.702±0.016^{a}	6.45375±0.01^{a}	0.687±0.016^{c}

According to Duncan's multi-range test, the numbers with the same letters in each column do not have a significant difference.

Screening of frost resistance gene expression

According to Figure 1A, the expression of the *CLO* gene in flowers was significantly upregulated at −2 °C compared with the control group (21 °C). Gene expression did not change significantly at 2 °C or 0 °C; however, when the temperature declined to −2 °C, *CLO* expression increased significantly relative to all other treatments, particularly the control. In contrast, Figure 1B shows that in fruits, *CLO* gene expression did not differ significantly across any of the temperature treatments or compared with the control.

As illustrated in Figure 1C, the expression of the *Dehydrin* gene in flowers did not differ significantly among the −2 °C to 2 °C treatments. Nonetheless, all cold-stress conditions (−2 °C to 2 °C) showed significantly higher *Dehydrin* expression compared with the control group. Moreover, gene expression at 4 °C differed significantly from that at 0 °C and −2 °C. Figure 1D shows that in fruits, *Dehydrin* expression at 4 °C was not significantly different from the control, but as the temperature declined to −2 °C, its expression increased significantly, reaching the highest level at −2 °C. Overall, *Dehydrin* expression under all cold-stress conditions was higher than in the control, although this increase was only statistically significant at −2 °C.

The expression of the *ICE1* gene is presented in Figures 1E and 1F. In flowers (Figure 1E), the highest expression level occurred at −2 °C, which was significantly different from the control. Across the 4 °C to −2 °C range, expression showed a slight decline from 0 °C to −2 °C but did not differ significantly among the cold-stress treatments. However, all cold-stress treatments exhibited significantly higher *ICE1* expression than the control. In fruits (Figure 1F), *ICE1* expression also increased under cold-stress conditions (−2 °C to 4 °C) relative to the control, but these differences were not statistically significant ($P > 0.05$).

The results demonstrated a marked upregulation of *CLO* gene expression under −2 °C, showing an approximately 18-fold increase compared with the control group (21 °C). In fruits, *CLO* expression at −2 °C was approximately 2-fold higher than in the control, whereas expression in other temperature treatments exhibited only a gradual increase with a gentle slope.

Similarly, *Dehydrin* gene expression was significantly elevated at −2 °C, reaching approximately 13-fold higher than the control in flowers. A significant difference was also observed between the −2 °C and 4 °C treatments. In fruits, *Dehydrin* expression at 4 °C did not differ significantly from the control; however, lowering the temperature to −2 °C led to a significant increase, with expression reaching its maximum at this temperature. Overall, *Dehydrin* expression at −2 °C was approximately 6-fold higher than the control in fruits.

The *ICE1* gene exhibited a similar trend. In flowers, expression at −2 °C was significantly higher than the control, showing an almost 12-fold increase. Other cold-stress treatments induced expression levels above the control but lower than those observed at −2 °C. In fruits, *ICE1* expression increased by approximately 3.5-fold at −2 °C compared with the control. These differences were statistically significant ($P < 0.005$). Each data point represents the mean ± SD for the respective gene.

Discussion

Although pistachio is generally considered a plant adapted to adverse environmental conditions, several studies have reported its sensitivity to abiotic stress, particularly spring frost, which is one of the most critical climatic risks affecting pistachio yield and quality (Askari et al., 2011). For pistachio trees, the risk of late-spring frost injury begins at temperatures of 4 °C and below, with critical damage thresholds reported at −2 °C and −4 °C. When temperatures drop approximately 2 °C below the critical threshold, green tissues may suffer irreversible injury, often manifested as tissue browning and permanent structural damage (Kayimov et al., 2021).

In the present study, several physiological traits associated with cold-stress resistance in pistachio flowers and fruits were evaluated, including ion leakage (electrical capacitance), chlorophyll and carotenoid content, proline accumulation, and soluble sugar levels. Measurements of ion leakage indicated that flowers exhibited a stronger response to cold stress than fruits, as reflected in higher electrical capacitance. Increased electrical capacitance was associated with reduced resistance to cold stress.

Physiologically, the plasma membrane is the primary cellular site affected by cold stress, which leads to changes in cell tonicity, loss of turgor pressure, and disruption of cytoplasmic streaming. Increased electrical capacitance reflects ion movement toward lower concentration gradients and the loss of ionic balance across the membrane. Under severe cold stress, widespread opening of ion channels leads to ion leakage into the cytoplasm, ultimately resulting in membrane dysfunction and tissue damage. This process is also linked to altered enzyme activity and the degradation of cell wall components under low temperatures (Barrero-Sicilia et al., 2017; Petruccelli et al., 2022).

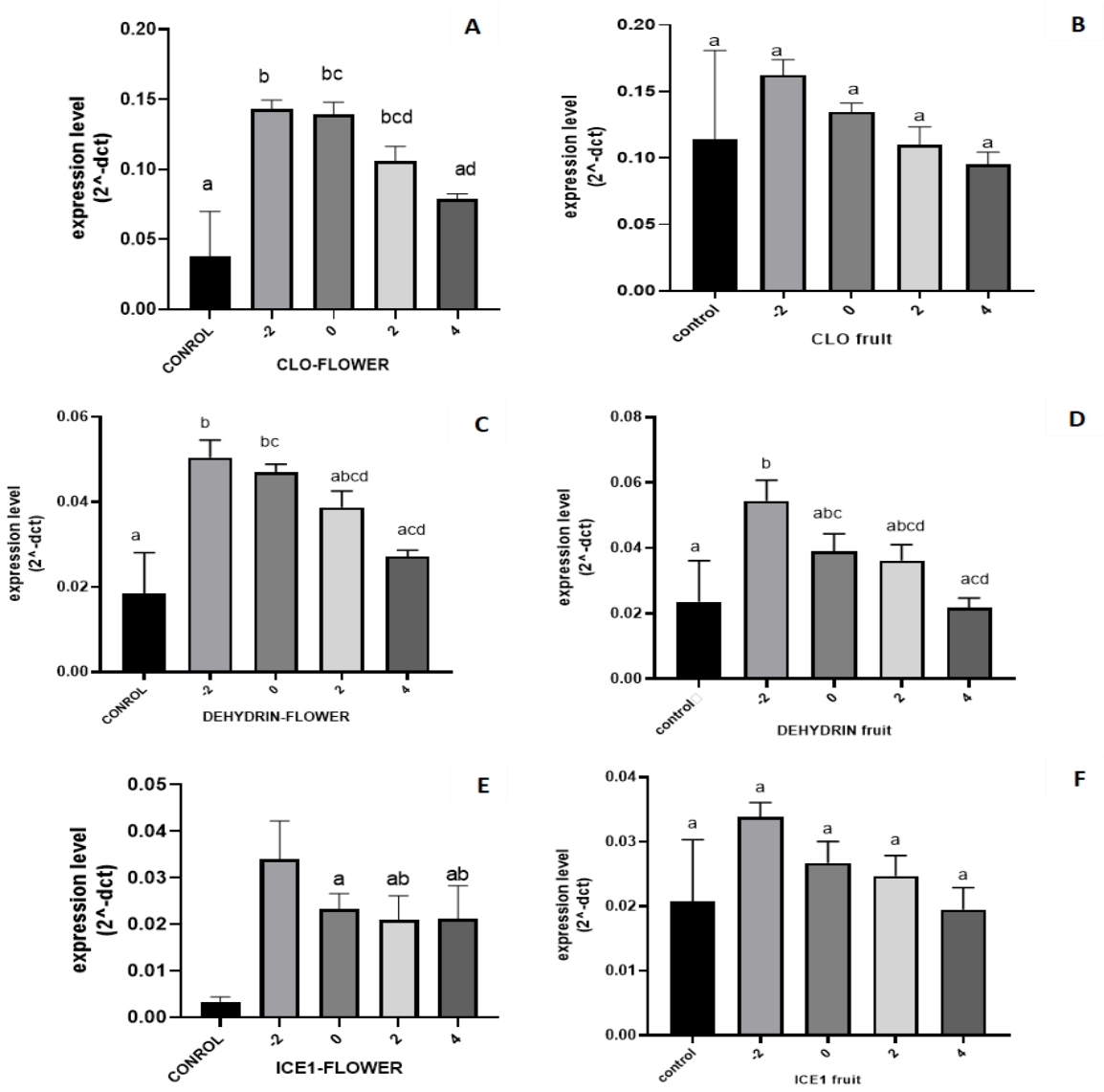

Fig. 1. (**A**) CLO gene expression in flower samples of 'Abbas Ali' pistachio cultivar in -2, 0, 2 and 4 °C as study groups compared to 21 °C as the control group; (**B**) CLO gene expression in fruit samples of 'Abbas Ali' pistachio cultivar in -2, 0, 2, and 4 °C as study groups compared to 21 °C as control group; (**C**) Dehydrin gene expression in flower samples of 'Abbasali' pistachio cultivar in -2, 0, 2, and 4 °C as study groups compared to 21 °C as the control group; (**D**) Dehydrin gene expression in fruit samples of 'Abbasali' pistachio cultivar in -2, 0, 2, and 4 °C as study groups compared to 21 °C as the control group; (**E**) ICE1 gene expression in flower samples of 'Abbasali' pistachio cultivar in -2, 0, 2, and 4 °C as study groups compared to 21 °C as the control group; (**F**) ICE1 gene expression in fruit samples of 'Abbasali' pistachio cultivar in -2, 0, 2, and 4 °C as study groups compared to 21 °C as the control group. The same letters above each column indicate insignificant difference from one another. a: significant when compared to control at $P < 0.05$; b: significant when compared to -2 °C group at $P < 0.05$; c: significant when compared to 0 °C group at $P < 0.05$.

Previous studies on cold stress in plants have reported a progressive increase in electrical capacitance as temperature decreases from 0 to −6 °C. Murray et al. (2006) demonstrated that electrical capacitance percentage is a reliable indicator for assessing cold and freezing stress in pistachio trees. Consistent with these findings, the present study showed that ion leakage, measured as electrical capacitance, was higher in flowers than in young fruits, suggesting that flowers are more susceptible

to cold-induced membrane damage during early spring.

According to Hayat et al. (2012), proline is one of the key amino acids that accumulates under various stress conditions. It functions as an excellent osmolyte, contributing to osmotic adjustment and stabilizing membranes by strengthening their structure, thereby reducing electrolyte leakage (Hayat et al., 2012; Spormann et al., 2023). In the present study, one of the notable findings was the increase in osmotic regulators, particularly proline, under decreasing temperatures (cold stress). Proline content was consistently higher in fruits than in flowers within the −2 to 4 °C range, reflecting its role as a stress-protective compound. As expected, the highest proline content in fruits was observed at 4 °C. Similar observations were reported by Li et al. (2019, 2021), who investigated the effects of temperature treatments on the physiological traits of *Paeonia lactiflora*. They found that the highest levels of soluble carbohydrates, proline, antioxidant capacity, and protease activity occurred at −4 °C under cold-stress conditions. In the present study, the correlation between proline and soluble sugars was positive, meaning that as soluble sugar content increased, proline content also increased. However, no significant increase in total soluble carbohydrates was observed in either fruits or flowers across the tested temperature treatments.

It is well established that plants accumulate soluble carbohydrates in cell membranes to enhance cold and freezing tolerance (Yuanyuan et al., 2009). In a study by Altaf et al. (2022), the effects of cold stress on physiological characteristics and antioxidant systems were evaluated in *Capsicum annuum L.* seedlings. Their results showed that the greatest accumulation of soluble carbohydrates and the highest activity of photosynthetic enzymes (Rubisco and fructose-1,6-bisphosphatase) occurred in the cold-sensitive cultivar, with sucrose being the primary accumulated soluble sugar. The discrepancy between these findings and the present study may be attributed to the timing of harvest and the gradual nature of cold acclimation, which allows soluble carbohydrates to accumulate progressively. In the current study, the lack of significant carbohydrate accumulation in fruits and flowers may reflect insufficient time for carbohydrate translocation to these organs under acute cold stress.

Our results also revealed that temperature and organ interaction significantly affected the levels of chlorophyll and carotenoid pigments; however, these changes did not follow a clear linear trend. At a constant temperature, flowers generally had higher chlorophyll content than fruits. This pattern is consistent with the findings of Zhou et al. (2021), who reported that cold stress significantly reduced chlorophyll content in three varieties of pitaya (*Hylocereus* spp.) and negatively affected chloroplast structure, ultimately reducing plant growth and yield. Similarly, El-Mahdy et al. (2018) evaluated two banana genotypes (*Williams* and *Grand Nain*) under chilling stress and found reductions in both chlorophyll a and b compared to the control. The *Williams* genotype maintained higher chlorophyll content and exhibited greater tolerance, suggesting that chlorophyll retention is associated with cold resistance. Previous research has also linked higher chlorophyll content to stress tolerance in other crops (Yadegari et al., 2007; Zhou et al., 2005). Interestingly, in herbaceous peony (*Paeonia lactiflora*), Tingting et al. (2020) observed that drought stress led to significant increases in chlorophyll, carotenoid, and flavonoid contents, which aligns with the results of the present study in demonstrating the role of pigment modulation in stress adaptation (Li et al., 2020).

Under cold stress, the activity of membrane-bound and membrane-associated enzymes is often disrupted (Dhaliwal & Angeles-Shim, 2022). Low temperatures can also affect plant metabolic pathways by damaging mitochondrial structures, reducing kinetic energy flow, and impairing enzyme function, which collectively lower the respiration rate (Ikkonen et al., 2020). To cope with such stress, plants activate enzymatic antioxidant defense systems, including catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD). High activities of these enzymes under cold stress typically indicate enhanced stress tolerance and cold resistance (Ritonga & Chen, 2020; Yadav, 2010).

The results of the present study revealed that temperature and organ type had a significant effect on APX activity, whereas neither factor significantly influenced SOD or CAT activity. Furthermore, the interaction between temperature and organ type did not show a consistent trend for SOD or CAT levels. Although variations were not always statistically significant, flowers generally exhibited higher SOD and APX activity than fruits. The maximum enzyme activities for CAT, SOD, and APX were observed in flowers at 0 °C, indicating that antioxidant responses were more pronounced in floral tissues than in young fruits under cold stress.

Given that genetic regulation is a key determinant of plant cold resistance, three cold-tolerance genes (*CLO*, *ICE1*, and *Dehydrin*) were further assessed. The *CLO* (Caleosin) gene family encodes multifunctional, calcium-binding proteins that are closely involved in stress signal transduction. Caleosins also function as peroxygenases and are encoded by small gene families in terrestrial plants, where they play critical roles in abiotic stress responses (Khalil, 2013; Kumar et al., 2015; Rahman et al., 2018; Fu et al., 2022). Analysis of *CLO* gene expression in the ‘Abbasali’ pistachio cultivar under four temperature treatments (−2, 0, 2, and 4 °C) and in both flowers and fruits revealed an initial increase

followed by a decline in expression across the cold-stress range. Compared with the control (21 °C), *CLO* expression was significantly induced at −2 °C (Figure 1A). The steepest increase in expression occurred in flowers at −2 °C, followed by a secondary peak at 0 °C. Across all treatments, *CLO* expression remained higher than in the control, suggesting that *CLO* responds dynamically to cold stress and that its expression pattern reflects its critical role in early stress signaling.

These results indicate that *CLO* genes may adopt distinct response patterns under cold-stress stimulation. Transcriptome analyses further support that *CLO* expression levels were consistently higher than those of the other two studied genes under cold conditions, particularly in floral tissues. The pronounced induction of *CLO* at −2 °C highlights this temperature as a critical threshold for triggering pistachio cold-stress responses and suggests that *CLO* plays a primary role in cold tolerance mechanisms.

Numerous studies have shown that abiotic stresses, including salt stress, disrupt plant metabolism and trigger excessive production of reactive oxygen species (ROS). Among antioxidant enzymes, superoxide dismutase (SOD) plays a critical role in detoxifying ROS and maintaining cellular homeostasis under stress conditions (Wang et al., 2018). Taken together with our results, this supports the conclusion that *CLO* genes have a positive regulatory role in cold tolerance.

Other stress-responsive genes, such as *Dehydrin* (DHN) and *ICE1*, have also been widely studied in the context of salt and cold stress. Salt tolerance and cold resistance often involve a combination of physiological and biochemical mechanisms, including the expression of protective proteins such as those in the Late Embryogenesis Abundant (LEA) gene family (Arif et al., 2020). LEA proteins, including dehydrins, are upregulated in response to abiotic stresses such as drought, cold, and osmotic stress. These stresses commonly induce cellular dehydration, forcing water efflux from the cytoplasm and triggering LEA protein accumulation (Close, 1996; Hughes & Graether, 2011; Marsh et al., 2006). Dehydrins, classified as LEA group II proteins, typically accumulate in seeds during maturation but are rarely found in vegetative tissues under non-stress conditions. When present, they are usually localized to actively dividing or elongating tissues, such as lateral and terminal buds, root tips, and petioles. However, under stress conditions associated with cellular water loss—such as drought, osmotic stress, salinity, and low temperature—dehydrins rapidly accumulate in vegetative tissues (Houde et al., 1992; Vítámvás et al., 2010). These intrinsically disordered proteins function as molecular shields, acting as osmoregulators during dehydration and protecting membranes and proteins from damage (Chen et al., 2022; Hughes et al., 2013; Xu et al., 2020).

Extensive research indicates that dehydrin gene expression increases markedly under abiotic stress conditions. Protein levels often rise at the onset of autumn and during early cold acclimation in winter, but gradually decline as the plant completes acclimation. Under stress-free conditions, dehydrin expression remains low, confirming its role as a stress-inducible protein (Sun et al., 2021; Vítámvás et al., 2019). High expression of *Dehydrin* genes is therefore considered a key indicator of plant stress tolerance.

Consistent with these findings, the present study demonstrated that cold stress induced *Dehydrin* gene expression in both flowers and fruits of pistachio, with higher expression in flowers. This is in agreement with Yuxiu et al. (2007), who observed enhanced *Dehydrin* expression under multiple abiotic stresses, and with Hanin et al. (2011), who linked dehydrin accumulation to improved plant survival under cold conditions. These results confirm that elevated *Dehydrin* expression contributes to cold tolerance and can serve as a molecular marker for assessing pistachio resistance to frost stress.

One of the most important gene families involved in plant cold-stress responses is the C-Repeat Binding Factor (CBF) family. CBF genes play a crucial role in regulating low-temperature responses (Tang et al., 2020). *ICE1* (Inducer of CBF Expression 1) is a constitutively expressed transcription factor that activates and regulates the transcription of CBF genes under cold-stress conditions. ICE1 binds to the promoters of CBF genes, triggering their expression, and thereby initiates downstream cold-response pathways. As a result, ICE1 is considered a central regulator of cold tolerance (Tang et al., 2020). A decline in *ICE1* expression leads to a significant reduction in plant chilling tolerance (Zhao et al., 2023).

In the present study, *ICE1* gene expression increased approximately 3.5-fold at −2 °C compared with the control (21 °C). All other cold-stress treatments also showed increased *ICE1* expression relative to the control, although the levels were lower than those observed at −2 °C. This pattern highlights *ICE1* as a key transcription factor activated by cold stress. Our findings are consistent with those of Dong et al. (2013), who demonstrated that transgenic tobacco overexpressing *VaICE1* exhibited enhanced SOD, POD, and CAT activities, as well as improved chlorophyll efficiency, cold tolerance, and survival rates. Similarly, Viswanathan et al. (2003) reported that *ICE1* interacts with multiple members of the CBF gene family, modulating their expression through various mechanisms, including the regulation of *CBF3* and downstream cold-responsive genes.

In a more recent study, Youssef et al. (2022) conducted gene set enrichment and protein-protein interaction analyses to identify conserved genes involved in crop cold tolerance. Their results revealed that pathways related to protein regulation, hormone metabolism, cell membrane integrity, and secondary metabolism were highly conserved, and *ICE1* was among the most highly interactive proteins within cold-tolerance networks. Collectively, the results of previous studies align with the findings of the present research, confirming that *ICE1* plays a pivotal role in orchestrating CBF-mediated cold-response pathways and enhancing pistachio tolerance to low temperatures.

Conclusion

In summary, this study demonstrated that pistachio exhibits variable physiological and molecular responses to cold stress, which differ depending on organ type and temperature conditions. Overall, key physiological parameters associated with cold tolerance, including proline accumulation, electrical capacitance, and the activities of antioxidant enzymes such as CAT, SOD, and APX, increased under cold-stress conditions, as anticipated. In contrast, soluble carbohydrate content and chlorophyll levels were generally lower than expected, indicating that pigment and sugar dynamics may be more sensitive to temperature fluctuations or require longer acclimation periods. At the molecular level, the expression of the evaluated cold-responsive genes (*CLO*, *Dehydrin*, and *ICE1*) increased in response to cold stress, confirming their involvement in pistachio's cold-tolerance mechanisms. However, the changes in both physiological and molecular parameters did not consistently follow a linear trend across temperature treatments, highlighting the complex and dynamic nature of pistachio's response to chilling conditions.

Acknowledgments

The authors would like to thank the Pistachio research center of the Damghan and Islamic Azad University of Damghan.

Author Contributions

MF; data curation, formal analysis, writing original draft, HA; Supervision, lab equipment's, editing paper, BK; lab equipment's, editing paper; GL; lab equipment's, editing paper, VZ; lab equipment's, editing paper, AT; lab equipment's, editing paper.

Funding

This research received no external funding.

Conflict of Interest

The authors indicate no conflict of interest in this work.

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