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ORIGINAL ARTICLE

Investigating the Effect of Cold Stress on the Physiological and Biochemical Traits of Leaves of Almond Cultivars

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K E Y W O R D S

Almond;
Antioxidant;
Cold stress;
Malondialdehyde;
Peroxide

A B S T R A C T

The late frost spring is one of the most important limiting factors for almond production. However, different cultivars of almonds have various reactions to this process, the selection and introduction of cultivars that tolerate late frost spring is the most effective way to avoid cold damage. The present study aimed to investigate the importance of several physiological and biochemical characteristics related to cold adaptation mechanisms and their influence on tolerance to late-spring frost in almond cultivars. The experiment was arranged in a completely randomized design (six cultivars of almonds and cold stress +4°C) with three replications, was carried out in the Biotechnology Laboratory at Mohaghegh Ardabili University in 2022. Cold stress (+4°C) was applied on current-year branches of early-flowering (Seffid (A1), Mamaei clone (A2)), mid-flowering (SH21, A1-16) and late-flowering (MS13, AD55) almond tree cultivars for three days. The investigated traits included ion leakage, proline, proteins and soluble sugars, photosynthetic pigments, hydrogen peroxide, malondialdehyde and some antioxidant enzymes. The results by the selection index of ideal genotype (SIIG) showed that late-flowering cultivars MS13 and mid-flowering A1-16 were the most tolerant; the mid-flowering cultivar SH21 was semi-tolerant, whereas late-flowering cultivars AD55 and early-flowering A1 and A2 were the most susceptible to cold stress.

Introduction

Almond (*Prunus dulcis* Mill.) belongs to the Prunoideae subfamily and the Rosaceae family (Zokaee-Khosroshah *et al*., 2014; Ghasemi *et al*., 2015; Estaji *et al.*, 2016). Almond trees usually require enough cold before spring to produce fruits afterwards. Almond flowers are susceptible to low temperatures and the occurrence of late-spring cold causes damage to crop load. The sporadic occurrence of this phenomenon has prompted a decrease in production, yield and economic efficiency in almond

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orchards and this is a threat to horticulture (Shahnavazi and Hosseini, 2011). The extent of damage caused by cold depends on the growth stage, cultivar and nutrition status of the plant (Rodrigo, 2000; Aslamarz *et al.*, 2010; Alonso *et al*., 2010; Ansari and Gharaghani, 2019; Imani *et al*., 2021). The most damaging of frost-risk occurred in around of flowering to final of growth stage in almond tree. But in general, in the stage of full bloom and growing fruits, they could tolerate up to -2.8°C and -1°C for half an hour without damage, respectively (Ghasemi *et al*., 2015; Imani *et al*., 2021).

Plants of temperate regions can increase their frost tolerance for some time by being exposed to cold and non-freezing temperatures (Aslamarz *et al.*, 2009). This process is known as cold acclimation (Levitt, 1980). During acclimation, plants sense environmental changes and develop a series of protective mechanisms, which includes various metabolic processes, gene expression reprogramming and physiological and biochemical changes (Hannah *et al*., 2005). The acclimation involves accumulated osmotic regulating substances such as amino acids, soluble carbohydrates, mineral ions and proteins at low temperatures. Proline is considered one of the active amino acids in osmotic regulation. It accumulates in various plant species in response to environmental stress and positively affects the stability of membranes and enzymes. It plays a role in adapting and adjusting osmotic backdrops in plants under stress conditions (Ashraf and Foolad, 2007; Aslani Aslamarz *et al.*, 2011). Soluble carbohydrates in plants usually serve as cellular protective compounds that can prevent or decelerate the formation of ice crystals in the cytoplasm (Karimi and Ershadi, 2015).

Previous research showed that the first place affected by frost damage is the cellular membrane. Frost changes the membrane structure from crystal-liquid to solid-gel and disrupts membrane functionality (Han and Bischof, 2004). Therefore, measuring the amount of ion leakage from tissues under cold stress is a reliable method for evaluating the resistance of plants (Aslamarz and Vahdati, 2010). Various types of abiotic stress, such as frost, may increase the accumulation of reactive oxygen species (ROS) such as superoxide anion radical (O^{2-}), hydroxyl radical (OH^{-}) and hydrogen peroxide (H_2O_2) in plant cells (Bacelar *et al*., 2007). A disproportionate accumulation of ROS leads to plant oxidative stress, whereby plants upregulate their antioxidant defence mechanisms to protect against the adverse effects of free radicals (Jan *et al*., 2018; Shangguan *et al*., 2018). Antioxidant enzymes such as SOD, APX, CAT and POX and the balance between these enzymatic activities in cells is crucial for maintaining a stable level of reactive oxygen species (Nasiriyan Jazi, 2020). Oxidative stress has reportedly increased lipid peroxidation and subsequent membrane leakage while decreasing membrane fluidity. Oxidative stress is a cause of damage to membrane-localized proteins, affecting ion channels, receptors, and enzyme functions (Das *et al*., 2014). In addition, cold stress affects protein stability and solubility, disrupting metabolic reactions for plant growth, including the Calvin cycle (Siddiqui *et al*., 2006).

Previous research has shown that physiological and biochemical parameters in different cultivars, including ion leakage, proline content, soluble sugars, proteins and enzymes, can vary in response to cold stress conditions (Aslamarz *et al.*, 2010; Afshari and Parvane, 2013; Mosavi *et al*., 2014; Sajadian *et al*., 2018; Ensafi Avval, 2019; Saadati *et al*., 2020; Vosnjak *et al*., 2022). The role of leaves is important not only in the production of photosynthetic materials, but also in the production of substances that cause resistance to cold (Afshari and Parvane, 2013). Therefore, this study aimed to determine cold tolerance in several almond cultivars and described the relationship between cold tolerance, biochemical traits, and enzyme activity in leaves of cultivars.

Materials and Methods

Plant materials and cold stress

The current research became arranged as a completely randomized design in three replications. The selected cultivars originated from the Karaj Horticultural Science Research Institute. They included early-flowering trees (Sefid (A1), Mamaei clone (A2)), mid-flowering (SH21, A1-16), and late-flowering (MS13, AD55) trees. At the early of May 2022 (at the stage of growth of young fruits), the current-year branches (30 cm long) were considered samples. Sampling involved taking two branch pieces from each tree (as a replication) and labeling them in a water bottle. The procedure was followed by transferring the samples to the Biotechnology Laboratory of Horticultural Sciences at Mohaghegh Ardabili University. Cold stress (+4°C) was applied to the samples for three days. Then, the leaf samples from each treatment were separated and packed in labeled aluminum foil and kept in a freezer at -80°C.

Determination of electrolyte leakage

To measure electrolyte leakage (EL), a method by Heidarpour *et al*. (2020) was used with a slight modification. A leaf was excised into pieces (one-centimeter diameter) from three mature fresh leaves of each sample. The samples were placed in a Falcon containing 10 ml of double-distilled water, which rotated at 110 rpm for 24 hours at room temperature on a shaker machine. Using a spectrophotometer (model SP-UV 200 Spectrum Instruments A. Perkin Elmer Company), the electrolytic conductivity was read at 280 nm (EC1). Then the samples were autoclaved at 121°C, with a pressure of 1.5 megapascals (MPa) for 20 min. After cooling, the electrolytic conductivity was read (EC2). The ion leakage (%) was measured according to equation 1:

$$EL\ \% = EC_1/EC_2 \times 100 \quad (1)$$

Determination of proline

To measure proline, 0.1 gram of frozen leaf sample was ground in 2 ml of 3% sulfuric acid in a mortar. After centrifuging of the solution for 10 min at 4000 rpm, 1 ml of supernatant extract was mixed with 1 ml of ninhydrin reagent and one ml of glacial acid and placed in a hot water bath at 90°C for an hour. Two ml of toluene was added to the cooled reaction solution before vigorous stirring for 20 seconds. Standards of pure proline (0, 50, 100, and 200 µM) were prepared and the absorption values of supernatants and standards were read via a spectrophotometer (model SP-UV 200) at a wavelength of 520 nm. Then, the amount of proline was calculated according to equation 2 in terms of µmole of proline per gram of fresh weight (Bates *et al*., 1973).

$$\mu mole\ per\ gram\ tissue = [(\mu g\ proline\ ml^{-1}) \times ml\ toluene)/115.13 \mu g\ \mu mole^{-1}] / [(g\ sample)/5] \quad (2)$$

Determination of soluble carbohydrate content

The soluble carbohydrate content was measured according to a method by Irigoyen *et al*. (1992). First, 0.16 grams of frozen leaf samples was ground with 1.6 mL of 95% ethanol in a porcelain mortar. The soluble part of the sample was separated using a sampler and poured into a falcon. The extraction process was repeated twice, each time using 1.6 ml of 75% ethanol. In the end, 100 ml of the alcoholic extract obtained from the centrifuge (3500 rpm for 10 min) were added to three ml of freshly prepared anthrone (150 mg of anthrone and 100 ml of sulfuric acid 72% (W/W)). The mixture was placed in a boiling water bath for 10 min to form a colored appearance. Then, the absorbance of each sample was read at 625 nm with a spectrophotometer (SP-UV 200). A standard curve (equation 3) was used for determining the amount of soluble carbohydrates in the samples. The result was expressed as mg per gram of leaf fresh weight.

$$Y= 0.0012x+ 0.1091 \quad R^2: 9927 \quad (3)$$

Soluble sugars (mg g^{-1}FW) = [X/1000 × V]/W (4)

X: Sugars of the extract

V: Sample volume of the extract

W: Wet sample weight

Determination of photosynthetic pigments

To measure photosynthetic pigments, 0.1 g of frozen leaf sample was ground in one ml of 80% ethanol at a low light intensity and cool temperature. After adding 4 ml of acetone to the samples, they were kept in a refrigerator at 4°C for 24 hours. Then, they were centrifuged at 3000 rpm for 10 min. The absorbance of supernatant solution was read at 663, 645, and 470 nm via a spectrophotometer (Model 6705 UV/Vis.). Using the following formulas (equation 5, 6, 7, 8), the chlorophyll content (a, b, total), and carotenoids were calculated as mg per gram of fresh weight (Arnon, 1967).

Chlorophyll a = [19.3(Absorption at 663nm) – 8.6(Absorption at 645nm)] ×V/ (1000×W) (5)

Chlorophyll b = [19.3(Absorption at 645nm) – 3.6(Absorption at 663nm)] × V/ (1000×W) (6)

Total chlorophyll = Chlorophyll a + Chlorophyll b (7)

Carotenoids = [100 (Absorption at 470 nm) – 3.27 (mg chlorophyll a) – 104 (mg chlorophyll b)]/227 (8)

In this regard: V: Sample volume of the extract

W: Wet sample weight

Determination of hydrogen peroxide (H_2O_2)

The amount of hydrogen peroxide (H_2O_2) was measured according to the reaction of hydrogen peroxide with potassium iodide. For this purpose, 0.1 g of frozen leaf tissue was treated with 1.5 ml of 0.5% TCA. The resultant extract was centrifuged for 15 min at 5000 rpm. Then, 500 mL of the supernatant extract was added to 500 mL of 100 mM sodium phosphate buffer (pH = 7), followed by adding 2 mL of 1 M potassium iodide. The resultant mixture was placed in the dark at room temperature for one hour. Then, the absorbance of each sample was read at 390 nm. Finally, the concentration of hydrogen peroxide was calculated using a standard curve and expressed in µmol per gram of fresh weight (Alexieva *et al*., 2001).

Determination of membrane lipid peroxidation Malondialdehyde (MDA)

To begin, 0.12 g of leaf was homogenized in 1.5 mL of 0.1% trichloroacetic acid (TCA).Then; the solution was centrifuged for 5 min at 10,000 rpm. The supernatant was separated, and 2 ml of thiobarbituric acid 0.5% was added to the solution. The samples were incubated for 30 min in a bain-marie apparatus (95°C) and immediately kept in ice water to stop the reaction. After centrifugation at 10,000 g for 10 min, the absorbance of the supernatant was read at 532 and 600 nm using a spectrophotometer (model 6705 UV/Vis. Spectrophotometer JENWAY) (Davey *et al*., 2005). Membrane lipid peroxidation was calculated via the extinction coefficient which equaled 155 mM^{-1} cm^{-1}, as nmol per gram of fresh weight, according to Equation 9.

$$MDA\ (nmol\ g^{-1}\ FW) = (A_{532} - A_{600}) \times V \times 1000/155 \times W \quad (9)$$

In this regard: V: Sample volume of the extract

W: Sample fresh weight in extraction

Extraction to measure total protein and enzymes

To extract and measure total soluble protein and enzymes, 2 ml of 100 mM sodium phosphate buffer (100 mM sodium phosphate with an acidity of 7.5, 2 mM EDTA and 0.7% PVP) were added to 0.1 g of powdered leaves in liquid nitrogen. After 30 Second vortexing, was centrifuged for 15 min at 14,000 rpm at 4°C. Supernatant extracts, after being transferred to 2 ml cold microtubes and centrifuged again (14000 rpm for 10 min at 4°C) were poured into microtubes (200 µL) using a sampler and and kept in a freezer at -80°C until the protein content and enzymes

concentration were measured (Zhang and Shao, 2015).

Determination of total protein

To measure total protein, 100 µL of supernatant extract and 2900 µL of Bradford reagent were poured into a plastic cuvette. The solution was vortexed manually. The absorbance of each sample was measured in the dark after 2 min at 595 nm by a spectrophotometer (SP-UV 200) (Bradford, 1976). Finally, a standard curve was drawn. Equation 10 was used for measuring the amount of soluble protein. The results were expressed as mg per gram of leaf fresh weight.

$$Y= 0.0008x + 0.0407 \quad R^2= 0.972 \qquad (10)$$

Determination of Guaiacol peroxidase (GPX) enzyme activity

The activity of guaiacol peroxidase (GPX) enzyme was measured by using reaction medium containing 50 mM phosphate buffer (pH=7), 10 mM guaiacol, and 20 mM H_2O_2. The increase in absorbance at 470 nm wavelength was recorded from the start of the reaction for 3 min. The final result was expressed as an extinction coefficient of 26.6 mM^{-1} cm^{-1} in µmol/min/mg of protein (ZHANG and SHAO, 2015).

Determination of ascorbate peroxidase (APX) enzyme activity

The activity of the APX enzyme by the Nakano and Asada (1981) with some modifications, two reaction solutions were used, comprising 250 µM ascorbic acid and 0.5 mM EDTA; 1.5 mM H_2O_2 and 50 mM sodium phosphate buffer (pH=7).The decrease in absorbance of the reaction solution was read at 290 nm for 2 min. The final result was expressed as an extinction coefficient of 2.8 mM^{-1} cm^{-1} in µmol/min/mg of protein. Equation 11 was used for calculating the above enzymes.

$$\text{Enzyme activity } (\mu M\ Min^{-1}.\ Mg^{-1}\ Pr) = [(\Delta Abs \times \text{Total assay volume}) / (\Delta t \times \varepsilon \times l \times \text{Enzyme sample volume})] / (W \times \text{total protein}) \qquad (11)$$

ΔAbs: Change in absorbance

Δt: Time of incubation (min)

ε: Extinction coefficient of substrates in units of mM^{-1} cm^{-1}

l: Cuvette diameter (1 cm)

W: Sample fresh weight in extraction

Calculation of ideal genotype selection index (SIIG)

To evaluate the cold tolerance of cultivars using all indices (plant properties) at the same time, the SIIG method was used. This index was calculated in several steps (Zali *et al*., 2015; Zali *et al*., 2016).

I - Creating the data matrix: based on the number of genotypes and the various factors analyzed, the data matrix is formed as follows:

$$D = \begin{bmatrix} x_{11} & x_{12} & \cdots & x_{1m} \\ x_{21} & x_{22} & & x_{2m} \\ \vdots & & \ddots & \vdots \\ x_{n1} & x_{n2} & \cdots & x_{nm} \end{bmatrix}$$

In this matrix, xij is the value of the i-th genotype (i = 1, 2, ... n) concerning the j-th attribute (j = 1, 2, ... m).

II- Transforming the data matrix into a normal matrix: the following relationship is operated to normalize the data (without joining the data):

$$r_{ij} = \frac{x_{ij}}{\sqrt{\sum_{i=1}^{n} x_{ij}^2}}$$

III- the Matrix R is defined as the following connection:

$$R = \begin{bmatrix} r_{11} & r_{12} & \cdots & r_{1m} \\ r_{21} & r_{22} & & r_{2m} \\ \vdots & & \ddots & \vdots \\ r_{n1} & r_{n2} & \cdots & r_{nm} \end{bmatrix}$$

IV- Discovering the ideal genotype and the non-ideal (weak) genotype for each trait: at this stage, according to the type of trait and the researcher's viewpoint on each feature, the best genotype (ideal) and the weakest (non-ideal) was selected.

V- Estimating the distance from the ideal genotype (di^+) and weak genotype (di^-): for each genotype, the distance from ideal genotypes (di^+) and weak (di^-) was calculated using relationships 4 and 5.

$$d_i^+ = \sqrt{\sum_{j=1}^{m}\left(r_{ij} - r_j^+\right)^2} \qquad i = 1, \dots, n$$

$$d_i^+ = \sqrt{\sum_{j=1}^{m}\left(r_{ij} - r_j^+\right)^2} \qquad i = 1, \dots, n$$

In the above relationships, rij is the normalized value of the ith genotype (i = 1, 2, ..., n) concerning the jth index (j = 1, 2, ...m). Meanwhile, d_i^+ and d_i^- are the normalized values of ideal and weak genotypes for each jth index (j = 1, 2, …, m). Also, di^+ is the distance from the ideal genotype and di^- is the distance from the weak genotype.

VI- Calculation of the ideal genotype selection index (SIIG): In the last step, the ideal genotype selection index for each genotype is calculated from the following relationship:

$$SIIG_i = \frac{d_i^-}{d_i^+ + d_i^-} \qquad i = 1, 2, \dots, n,$$

$$0 \le SIIG_i \le 1$$

Statistical analyses

Data analyses and the comparison of mean values were done by Duncan's Multiple Range Test ($P \le 0.05$) using SPSS 26 statistical software. Microsoft Excel was used for drawing the graphs, calculating the indices of cold tolerance, and finding the ideal genotype selection index.

Results

According to the results (Table 1), a statistically significant difference was observed among the cultivars regarding all traits, except the leaf protein content ($P \le 0.01$), which showed the various responses of the cultivars to cold stress.

Table 1. Variance analysis of the effect of cold stress treatment on some physiological and biochemical traits of different almond cultivars.

S.O.V	df	Mean squares					
		Electrolyte leakage (%)	Proline (µmol g^{-1} FW)	Soluble sugars (mg g FW^{-1})	Chlorophyll a (mg g^{-1}FW)	Chlorophyll b (mg g^{-1} FW)	Chlorophyll total (mg g^{-1} FW)
Cultivar	5	**S.O.V**	1.175**	126.30**	0.391**	0.023**	0.555**
Error	12	11.361	0.065	5.19	0.065	0.003	0.077
C.V. (%)		5.63	15.85	11.37	10.84	13.35	10.05
S.O.V	**df**	**Mean squares**					
		Carotenoids (mg g^{-1} FW)	H_2O_2 (µmol g^{-1} FW)	MDA (nmol g^{-1} FW)	Proteins (mg g^{-1} FW)	GPX (µmol min^{-1} mg^{-1} Pr)	APX (µmol min^{-1} mg^{-1} Pr)
Cultivar	5	0.125**	0.043**	36.981**	0.233ns	0.446**	0.097**
Error	12	0.007	0.008	6.763	0.081	0.000	0.006
C.V. (%)		20.06	28.75	8.56	15.44	0	20.11

The comparison of mean values (Fig. 1-A) demonstrated that the percentage of electrolyte leakage differed between the studied almond cultivars. Cultivar A1 (91.01%) had the maximum percentage, and cultivar MS13 (10.94%) had the minimum percentage of ion leakage. However, no statistically significant difference was observed between A1 and AD55 cultivars. Cultivars A2, SH21, and A1-16 were categorized in the second and fourth diagrams, respectively.

The comparison of mean values (Fig. 1-B) showed that all cultivars reacted to cold stress by generating different amounts of proline. Depending on the cultivar, the stress response was different. MS13 had the highest leaf proline content (2.54 µmol g^{-1} FW). No statistically significant difference was observed in the proline level among the A2, A1-16, and SH21 cultivars. The lowest amount of proline was in AD55 (0.905 µmolg^{-1} FW), which was not significantly different from A1 (0.962 µmol g^{-1} FW).

According to the comparison of mean values (Fig. 1-C), MS13 (31.5 mg g^{-1} FW) and A2 (13.76 mg g^{-1} FW) had the highest and lowest soluble sugar content, respectively. Cultivars SH21, A1-16, and AD55 were almost similar in their soluble sugar content and did not show statistically significant differences.

As shown (Fig. 1-D, E, F), leaf chlorophyll pigments responded differently to the low-temperature stress, as revealed by measuring the chlorophyll a and total chlorophyll content. Cultivar A1 had the highest chlorophyll content with a significant difference compared to the rest of the cultivars. Therefore, the maximum amounts of chlorophyll a and total chlorophyll were observed in cultivar A1 (3.03 mg g^{-1} FW and 3.56 mg g^{-1} FW, respectively) and their minimum values were observed in cultivar MS13 (1.94 mg g^{-1} FW and 2.25 mg g^{-1} FW, respectively). Regarding chlorophyll b, the highest concentration was observed in cultivar A1 (0.531 mg g^{-1} FW), which had no statistically significant difference with cultivars A1-16 (0.454 mg g^{-1} FW) and AD55 (0.437 mg g^{-1} FW). The lowest chlorophyll b concentration was observed in SH21 (0.304 mg g^{-1} FW), which did not show a statistically significant difference with MS13 (0.312 mg g^{-1}FW).

The comparison of mean values (Fig. 1-G) showed that the cold stress (+4 °C) significantly affected the carotenoid content in each cultivar. The highest carotenoid content was recorded in cultivar SH21 (0.735 mg g^{-1} FW). Meanwhile, A2 had the lowest carotenoid content (0.27 mg g^{-1} FW) but did not show statistically significant differences with AD55, A1-16, and A1 cultivars. Also, the carotenoid content in MS13 (0.613 mg g^{-1} FW) ranked second among the other cultivars.

The comparison of mean values (Fig. 1-H) showed that the highest amount of H_2O_2 (0.45 µmol g^{-1} FW) was recorded in cultivar SH21, whereas the lowest (0.144 µmol g^{-1} FW) occurred in cultivar A1. The amount of H_2O_2 in the SH21 cultivar was not significantly different from the AD55, MS13 and A1-16 cultivars.

Lipid peroxidation can be estimated quantitatively by measuring the malondialdehyde concentration. Accordingly, the highest concentration of malondialdehyde (34.68 nmol g^{-1} FW) was observed in the MS13 cultivar (Fig. 1-I). However, this concentration was not significantly different from that observed in A1 and AD55 cultivars. The lowest malondialdehyde concentration (25.65 nmol g^{-1} FW) was recorded in cultivar A1-16. These results are not consistent with previous reports by Aazami *et al*. (2021), Neisi *et al*. (2022), and Zhang *et al*. (2022) who reported that cold-tolerant cultivars show less lipid peroxidation when faced with cold stress.

The A1-16 cultivar had the highest GPX enzyme activity (0.987 µmol min^{-1} mg^{-1} Pr) compared to the other cultivars (Fig. 1-J). The lowest GPX activity (0.004 µmol min^{-1} mg^{-1} Pr) was observed in the A1 cultivar. The peroxidase enzyme (POX) is usually tasked with collecting reactive oxygen species to prevent damage on the plasma membrane (Hashempour *et al*., 2014) and ultimately increases tolerance to stress in plants.

The comparison of mean values (Fig. 1-K) in this experiment showed that the APX content was highest in cultivar MS13 (0.599 µmol min^{-1} mg^{-1} Pr), which was not significantly different from the APX content of A1-16 and SH21 cultivars. Also, the lowest concentration of this antioxidant (0.186 µmol min^{-1} mg^{-1} Pr) was observed in cultivar A2, but showed no significant difference with cultivars AD55 and A1.

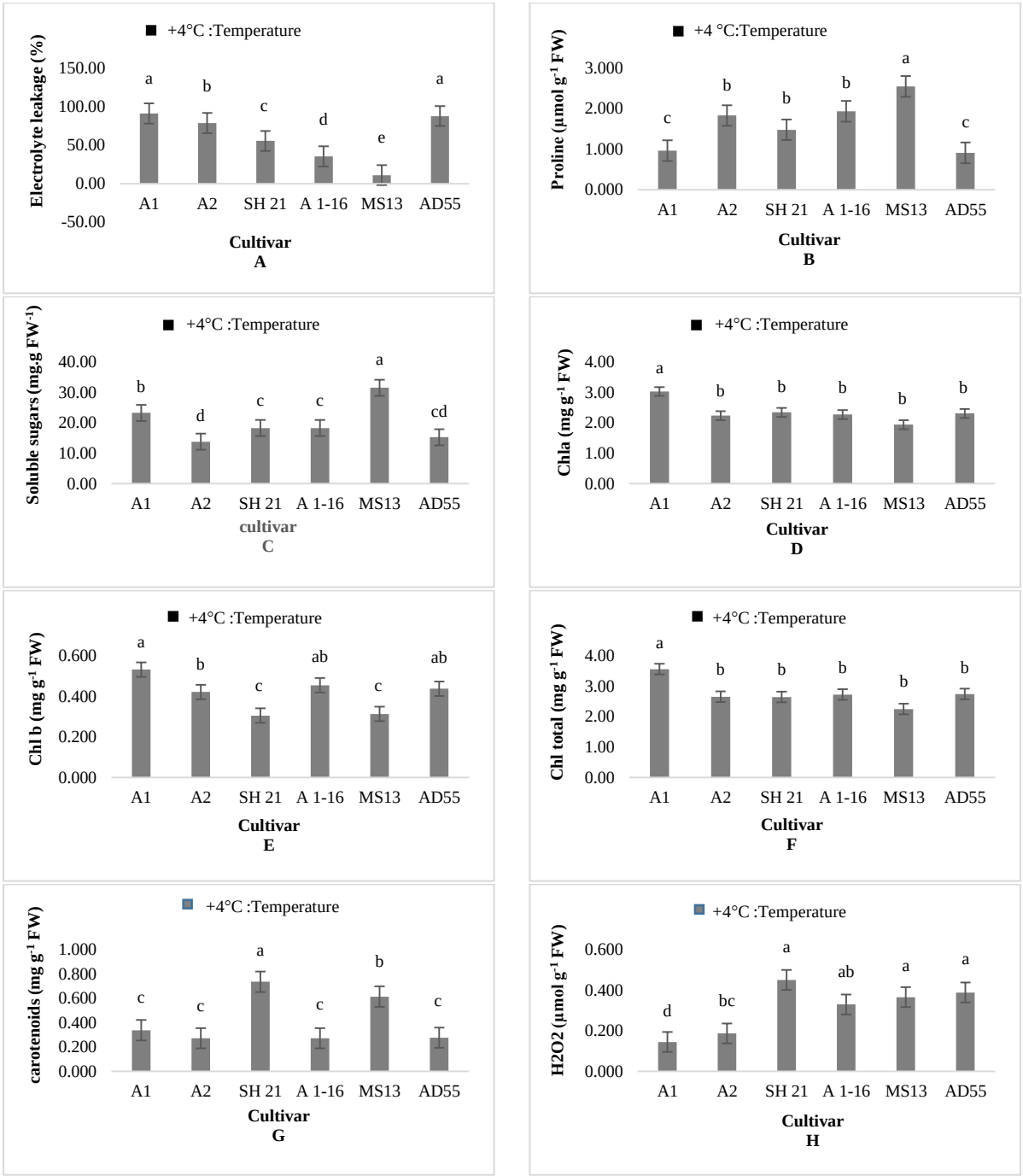

Fig. 1. Effect of cold stress +4 °C on electrolyte leakage (A), Proline (B), soluble sugars (C), chlorophyll a (D), b (E) and total (F), carotenoids (G), H_2O_2 (H), MDA (I), GPX (J) and APX (K) leaves of almond cultivars.
Different Alphabets indicate significant differences were observed by using Duncan's multi-range test at 5% probability level.

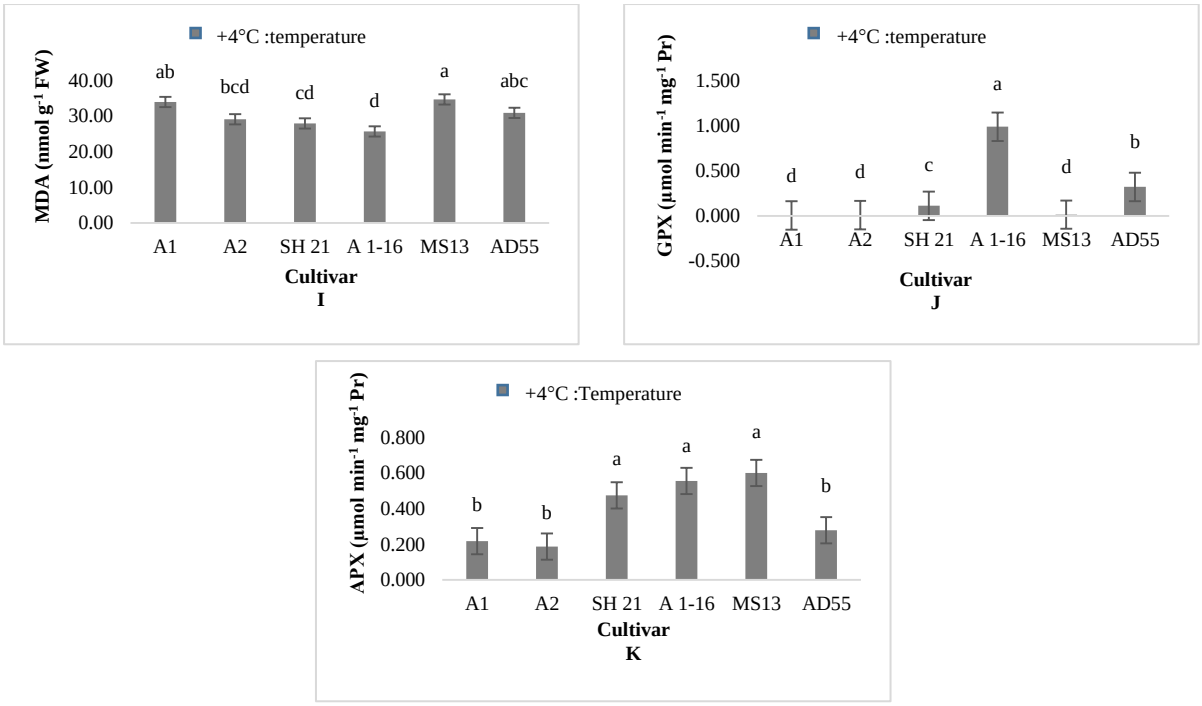

Fig. 1. Continued.

Discussion

Electrolyte leakage occurs in cells when the membrane is damaged due to stress (Afshari *et al*., 2014). Previous studies showed that the plasma membrane of cold-tolerant plants is less vulnerable to damage, compared to susceptible plants (Nazari *et al*., 2012). Accordingly, almond cultivars are among plants with electrolyte leakage values that increase in response to cold stress (Mousavi *et al*., 2014). Also, Imani *et al*. (2011) examined 60 cultivars of almonds, indicating that the severity of frost damage depends on the genotype of the plant, and more resistant genotypes show less ion leakage. Therefore, this trait can be used as an indicator to identify tolerant and susceptible cultivars. In the current research, MS13 showed the least amount of electrolyte leakage and probably had the smallest extent of damage and maximum tolerance to cold stress.

In cold-tolerant plants, proline was the main amino acid, which acted as a protective component against the cold. A high proline concentration in tissues serves as a mechanism for adaptation to cold stress (Aazami *et al*., 2021). The late-flowering cultivar AD55 had the lowest proline content, which shows that the proline content does not depend on the flowering time of the cultivars.

Total soluble sugars have a role in the osmotic regulation of cells during environmental stress, such as drought and low temperatures. Some plants tolerate low-temperature stress by accumulating large amounts of osmotic protective soluble substances, such as soluble sugars. According to previous research, osmotic protectors such as sugar and proline significantly increase the tolerance of plants to cold stress (Ben Ahmed *et al*., 2011; Zhang *et al*. 2012 ; Brown *et al*., 2019), which is consistent with the results of this research.

Accordingly, since chlorophyll and proline are both made of a common precursor (glutamate), an increase in proline content at times of stress may decrease the chlorophyll concentration (Khalid *et al*., 2010). The higher chlorophyll concentration in cultivar A1 was probably due to stress conditions of intracellular water loss, which caused the chlorophyll concentration to increase per unit area (Teulate *et al*., 1997). Also, it could be due to the characteristics of the cultivar that had more chlorophyll content than the

rest of the cultivars.

A high level of carotenoids may occur during cold stress and have a protective role (Amini, 2019). Cold resistance sometimes increases pigment concentration, such as carotenoids, which are oxidized at low temperatures. Their oxidization acts as a shield against the loss of chlorophyll a and b, thereby preventing frost damage (Nasiriyan Jazi, 2020).

The accumulation of H_2O_2 as a low-risk ROS, compared to highly toxic oxygen radicals such as superoxide, is tasked with signaling but can be eliminated in later stages by other components in the defense system, including antioxidant enzymes (APX, CAT, SOD, and GPX) (Amini,2019). Although excess ROS are toxic to plant cells, a certain level of their production is required for a successful response to stress. ROS has an important signaling role in plants and affects the expression of specific genes, thereby controlling many processes such as abiotic stress responses (Ahmad *et al*., 2018).

Mano *et al*. (2010) reported that several types of aldehydes are present in the leaves and roots of plants at micromolar levels under non-stress conditions. However, when exposed to abiotic stress, plants show an increased amount of these toxic substances. Accordingly, it can be assumed that the initial malondialdehyde concentration in the MS13 cultivar was high. But to make a more accurate assessment of cultivar reactions to lipid membrane damage, it is necessary to take more samples during the stress period and show precisely the changes in Cold concentration. Also, it may be possible that such results were specific to the anatomical, physiological and biochemical characteristics of the cultivars when studied against cold stress.

Zrig *et al*. (2015) reported that the increase in APX enzyme activity in the 'Mazzetto' almond cultivar had a protective role against abiotic stress, which is consistent with the current findings. The main ROS (reactive oxygen species) enzymes include SOD, APX, CAT and GPX. The balance between the activities of these enzymes in cells is crucial for maintaining a stable level of ROS (Nasiriyan Jazi, 2020). Under normal conditions, plant cells have a stable level of ROS. However, various stress conditions, including cold stress, lead to excessive production of ROS. Nonetheless, excessive levels of ROS are removed by increasing the activity of different antioxidant enzymes such as SOD, APX, CAT and GPX (Jan *et al*., 2018).

In the current research, the APX enzyme played a more significant role against cold stress compared to the GPX enzyme. Its activity was more timely and prominent than that of GPX. The cold-tolerant cultivar had higher APX activity, whereas the susceptible cultivars showed lower levels of both enzymes (GPX and APX), indicating severe damage to the tissues of these cultivars. According to Nourredine *et al*. (2015), GPX activity increased slowly in the first 5 days after applying the stress but then showed a significant increase in stress-tolerant samples in the subsequent 8 to 11 days. Also, Ensafi (2019) indicated that the weak activity of this enzyme in the shoot samples of grape cultivars can be attributed to its weak activity in the initial days of exposure to stress, compared to other antioxidant enzymes, thereby confirming our research. Amini (2019) studied cold stress on chickpea cultivars and reported that antioxidant enzymes are more active in tolerant cultivars, compared to susceptible cultivars. This stronger activity mitigated the severity of damage caused by H_2O_2 and EL when the plants were exposed to cold stress.

Cultivar MS13 was generally more adaptable to cold stress (+4°C), whereas the AD55, A1, and A2 cultivars were most susceptible.

To select stress-tolerant cultivars, paying attention to one index alone may not lead to favorable results, whereas relying on several indices can increase the chances of identifying ideal genotypes. Accordingly, the SIIG technique for selecting ideal genotypes has been used for the first time to integrate different indices of drought tolerance and increase the efficiency of selecting ideal genotypes. With the help

of the SIIG technique, all indices and traits become a single index, making the classification of genotypes much easier. In other words, negative and positive indicators can be used in a combined form in this technique. The best genotype is the closest genotype to the ideal genotype and the farthest from the non-ideal genotype (Zali *et al*., 2015; Zali *et al*., 2016). Therefore, an effective evaluation of cold tolerance in cultivars can be realized by using all available indices simultaneously. In this research, the SIIG index was calculated while considering 12 traits. The cultivars with the highest amounts of proline, soluble sugars, photosynthetic pigments, protein, and antioxidant enzymes, but the lowest amount of ion leakage, hydrogen peroxide, and malondialdehyde were considered ideal. The value of SIIG changed between zero and one. The closer it was to one, the more ideal the genotype was. According to this index, the late-flowering cultivar MS13 (0.899) and mid-flowering A1-16 (0.67) were the most tolerant. The mid-flowering cultivar SH21 (0.44) was semi-tolerant. Late-flowering cultivars AD55 (0.06) and early-flowering A1 (0.106) and A2 (0.164) were the most susceptible to frost (Table 2).

Table 2. The selection index of ideal genotype (SIIG) based on different indices of cold tolerance and distances from ideal and weak genotypes.

Cultivars	d^+	d^-	SIIG index	Cold tolerance
MS13	0.0472	0.4217	0.899	HT
A1-16	0.1427	0.2912	0.670	HT
SH21	0.2389	0.1878	0.440	MT
A2	0.3590	0.0706	0.164	LT
A1	0.4160	0.0491	0.106	LT
AD55	0.4035	0.0279	0.060	LT

Note: HT, high tolerance; MT, medium tolerance; LT, low tolerance.

Conclusions

Physiological and biochemical traits were measured in six almond cultivars. Since each cultivar had a different flowering time, each responded uniquely to low-temperature stress (+4°C). The cultivars had different physicochemical reactions to cold stress. The SIIG index revealed that the AD55 cultivar (late-flowering), A1 and A2 (early-flowering) were the most susceptible. The SH21 was semi-tolerant. MS13 and A1-16 were the most cold-tolerant. Thus, cold tolerance does not correlate with the date of flowering. Ultimately, the late-flowering cultivar AD55 was considered the most susceptible.

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Conflict of interests

The authors declare that they have no conflict of interest.

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