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ORIGINAL ARTICLE

Phenylalanine and Calcium Nitrate Alleviate Cold Tolerance of *Pistacia vera* L. Seedlings

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K E Y W O R D S

Carotenoid;
Chlorophyll;
Cold stress;
Pistacia vera L.;
Proline

A B S T R A C T

To study the effects of different concentrations of phenylalanine, and calcium nitrate under cold stress on *Pistacia vera* L. 'Abbas Ali' cultivar seedlings, a factorial experiment was performed with three factors of phenylalanine at three levels of calcium nitrate, and temperature based on a completely random design with three replications. The findings demonstrated that the effects of phenylalanine, calcium nitrate, temperature, as well as their interactions, were significant for all studied features. By simultaneous use of 2.5 mM of phenylalanine, and 5 ppm of calcium nitrate, electrolyte leakage reduced by 19% compared to the control sample at -3°C. Furthermore, when the temperature reduced from zero to -3°C, the concentration of proline increased from 2.3 to 3.1 μg g^{-1} fw compared to the control seedlings. By simultaneous use of the highest concentrations of phenylalanine, and calcium nitrate, phenolic compounds, and soluble sugar increased. However, the interactions of phenylalanine×calcium nitrate, cold×phenylalanine, and phenylalanine×calcium nitrate×cold were not significant on the content of proline. A significant positive correlation was between proline, and soluble sugar at the probability level of 1% (r=0.87**). A significant negative correlation was between electrolyte leakage and chlorophyll fluorescence at the probability level of 1% (r=- 0.68**).

Introduction

Pistacia vera L. belongs to the family Anacardiaceae. The fruit can be classified as a semi-dry seed that contains an edible (kernel), which is covered by testa and surrounded by green to reddish-yellow endocarp (Arjeh *et al*., 2020; Nazoori *et al*., 2022a, b) *P. vera* is one of the most important agricultural products in Iran (Shamshiri and Hasani, 2015; Alipour, 2018; Norozi *et al*., 2019; Sharifkhah et al., 2020).

Resistance of plant species to environmental stresses can determine their spread and survival. Annually, a lot of losing occurs in the agriculture and horticulture sectors in terms of plants being exposed to undesired environmental conditions (Mangrich, 2000). Frostbite is the physiological damage that occurs when plants and crops are exposed to low temperatures (often 1 to 10°C), particularly those of tropical and subtropical origin (Nobari *et al*., 2022; Ghasemi-Soloklui and Ershadi, 2023). Although *P. vera* is known as a plant that adapts to adverse environmental conditions, there are several reports about the sensitivity of *P. vera* to environmental stresses (Shamshiri, 2018; Alipour, 2018). The emergence of *P. vera* leaves is almost simultaneous

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with flowering, at which stage the plant is sensitive to spring cold. This product is frostbitten at the flowering stage, which usually occurs from late March to early May, and suffers a lot of damage from the spring cold. The phenomenon of frostbite occurs when the air temperature reaches the low threshold depending on plant species, which for *P. vera* is 4°C or less (Taiz & Zeiger, 2002). Cold stress is a severe abiotic stress that significantly limits the growth and yield of crops (Aazami *et al*., 2021; Feng *et al*., 2021). The exposure of plant tissue to low temperature causes the leakage of calcium ions into the cytoplasm, which is related to the change in enzyme activity in terms of the breakdown of cell walls (Habibie *et al*., 2019). When the membrane is broken, electrolyte leakage takes place (Aslamarz and Vahdati, 2010; Aslamarz *et al.*, 2010). According to the findings of the research done on plant tissue by Yokota *et al*., the treatment at -4°C caused the most electrolyte leakage, malondialdehyde, soluble sugar, proline, antioxidant capacity, and protease activity. Plants accumulate various soluble sugars that stabilize the cell membrane under cold stress (Aslamarz *et al*., 2011; Yokota *et al*., 2015). Furthermore, cold stress causes severe changes in the physiological, biochemical, metabolic, and molecular processes of the plant, and consequently, a significant reduction in crop efficiency (Hu *et al*., 2016; Nasibi *et al*., 2020; Repkina *et al*., 2021). Besides, the accumulation of osmolytes and sugars by improving the activities of catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD) reduces the content of ROS and malondialdehyde (MDA) under cold stress (Khandan-Mirkoh *et al*., 2017; MohammadRezakhani and Pakkish, 2017; Sun *et al*., 2019; Nasibi *et al*., 2020; Behzadi Rad *et al.*, 2021). Plants try to counteract the harmful effects of cold stress by increasing the synthesis of proline, glycine betaine, soluble protein, and soluble sugars, which vary by plant species and may be interchangeable (Erdal, 2012; Shao *et al*., 2008). Photosynthesis which is the main source of seed production is seriously affected by cold stress (Khan *et al*., 2017). Cold stress causes overstimulation of PSII, which increases energy loss by non-radiative reactions (Cvetkovic *et al*., 2017). Additionally, cold stress decreases stomatal conductance, Rubisco activity, electron transport, chlorophyll synthesis, and fluorescence, which significantly lowers yield and the amount of absorbed chemicals produced (Hussain *et al*., 2018). Therefore, it is necessary to propose methods to increase the cold tolerance of plants, and garden products, including the use of chemicals. Meanwhile, phenylalanine is an essential amino acid for producing the phenolic and aromatic compounds in the phenylpropanoid biosynthesis pathway. Phenylalanine is a nitrogen-containing molecule that is considered one of the essential amino acids in plants and as a precursor plays an important role in the activity of the phenylalanine ammonia-lyase enzyme in the production of phenolic compounds, such as anthocyanins and tannins (Garde-Cerdán *et al*., 2014). Results from earlier research indicated that phenylalanine consumption boosts antioxidant capacity and, ultimately, the quality of garden goods (Portu *et al*., 2015). Phenylalanine is the main precursor of enzyme, therefore increasing the levels of phenylalanine in the fruit increases the activity of this enzyme, and finally the production and accumulation of useful compounds such as anthocyanin and the quality of the fruit (Solecka & Kacperska, 2003). For physical properties, this substance is easily transferred via the plant and is used as an intermolecular message to create resistance in plants under stress (Gunes *et al*., 2007). By delaying the disintegration of the cell wall, preserving and stabilizing the membrane, and extending the membrane's ability to transmit cell signals, calcium also slows down the rate of aging, ripening, and lessens susceptibility to frost in a variety of fruits and vegetables (Chavoshi *et al*., 2022). This element plays a role in the structure of protein molecules, nucleic acids, growth regulators, enzymes, coenzymes, and cytochromes (Hassegawa *et al*., 2008). This research aimed to study the effect of

phenylalanine and calcium nitrate on the cold tolerance of *P. vera* Abbas Ali cultivar seedlings.

Materials and Methods

The effect of phenylalanine, calcium nitrate, and cold treatment on *P. vera* Abbas Ali cultivar, as well as their interactions, was investigated in a factorial experiment. The studied factors consisted of phenylalanine at three levels (0, 1 and 2.5 mM), calcium nitrate at three concentrations (0, 3 and 5 ppm) and temperature at three levels (0, -1, and -3 °C) based on a completely random design with three replications in 2022 in the laboratory of Damghan Islamic Azad University. The studied traits included total chlorophyll, carotenoid, total phenol, soluble sugars, soluble proteins, electrolyte leakage, proline, starch, and chlorophyll fluorescence. *P. vera* Abbas Ali cultivar trees were prepared at the research station in Damghan and planted in 30 cm long plastic pots and irrigated with distilled water. The treatments were applied when the seedlings reached 6-leaf stage؛Foliar application of phenylalanine, and calcium nitrate was twice at an interval of 3 days (water was used for the control treatment). Then, three days after the second foliar application, distilled water was applied to the bases placed in a cold incubator with the ability to gradually reduce the cold. The incubator was cooled down to 2°C with a freezing rate of 10°C per hour, then it continued with a freezing rate of 5°C per hour until the mentioned cold. The samples were kept in each temperature for 3 hours. Then, some leaves were separated from each seedling and transferred to the laboratory.

Chlorophyll and carotenoids

0.25 g of the base leaf of the pistachio cultivar Abbas Ali was ground in a porcelain mortar with 10 ml of 80% acetone until it became a uniform solution. Then, the solution reached a volume of 25 cc and the final volume of the extract extracted was centrifuged for ten minutes at 3500 rpm. Then, the light absorption of the solution was read using a spectrophotometer at wavelengths of 652, 645, 510, 480 and 663 nm and finally the concentration of chlorophyll and carotenoid was calculated (Arnon, 1967).

Proline

To extract proline, 0.5 g of the base leaf of the pistachio cultivar Abbas Ali was weighed and pounded with 5 ml of 95% ethanol in a porcelain mortar, and the extraction process was repeated twice, each time with 5 ml of 70% ethanol. For 10 minutes, the resulting solution was centrifuged at 3500 rpm. The supernatant was utilized for extraction after removal. To find the proline content, 1 ml of the aforementioned extract was diluted with 10 ml of distilled water, 5 ml of ninhydrin (1.25 g of ninhydrin in 30 ml of glacial acetic acid and 20 ml of 6 M phosphoric acid) was added, and the mixture was then heated to 95°C for 45 minutes. After removing the samples from hot water bath and cooling, 10 ml of benzene was added and mixed by a mechanical stirrer until proline entered the benzene phase. The samples were kept for 30 minutes, and then the absorption was measured by a UV/VIS spectrophotometer (PG Instruments, T80) at a wavelength of 515 nm (Bates, 1973; Paquin & Lechasseur, 1979).

Electrolyte leakage

To determine the stability of the cell membrane in the base leaf of the pistachio cultivar Abbas Ali, electrolyte leakage was measured. In this method, 0.1 g of sample was placed in 10 ml of double distilled water. After that, each sample's electrical conductivity (initial leakage) was measured using an electrical conductivity meter after being submerged in 40°C water for 30 minutes (EC meter). Then, the sample was placed in bain-marie at 100°C for 15 minutes, and the electrical conductivity (final leakage) was measured for the second time, and the electrolyte leakage percentage was calculated by the following equation (Sairan & Srivastava, 2002).

electrolyte leakage percentage = initial leakage / final leakage × 100

Soluble sugar

To measure soluble sugar, 0.5 g of the base leaf of the pistachio cultivar Abbas Ali was ground using 5 ml of 95% ethanol in a porcelain mortar and 0.1 ml of the alcoholic extract of anthrone was mixed with 3 ml of freshly prepared anthrone (150 mg of anthrone plus 100 ml of sulfuric acid (72%) and the solution was placed in a hot water bath for 10 minutes until the reaction was done and colored. The amount of dissolved sugars was then determined by measuring the solution's light absorption at a wavelength of 625 nm using a spectrophotometer (Irigoyen *et al*., 1992).

Total protein

To measure total soluble protein, 0.5 g of the sample was poured into a porcelain mortar. Next, 6.25 ml of extraction buffer (Tris-HCl and pH = 7.5) was added to the sample at several stages and the grinding process was continued for 30 minutes. To more effectively extract the protein and thoroughly dissolve the sample in the falcon tube buffer, the sample was placed into the 15 ml falcon. The samples were then centrifuged for 30 to 40 minutes at 2-4C and 1600 rpm. After centrifugation, 0.1 ml of the supernatant of the samples was taken and poured into a 15 ml falcon, and five ml of Bradford reagent was added to it and vortexed quickly. After 25 minutes, the light absorption of the resulting solution was read by a spectrophotometer at a wavelength of 595 nm (Bradlford, 1976).

Total phenol

To assess total phenol, 1.0 g of leaf sample was ground in 5 ml of 95% ethanol. Then, the resulting mixture was poured into test tubes and kept in the dark for 48 hours. Then, 1 ml of the supernatant was taken and 1 ml of 95% ethanol was added to it and the volume reached 5 ml with double distilled water. 0.5 ml of 50% Folin's reagent and 1 ml of 5% calcium carbonate were added to the above solution, leading to the black color of the samples. The tubes were kept in the dark for 1 hour, and then the light absorption was read by a spectrophotometer at a wavelength of 725 nm (Isfendiyaroglu & Zeker, 2002).

Starch

0.5 g of the plant sample was homogenized in 80% ethanol to isolate the starch. The mixture was centrifuged and the lower part was washed several times with 80% ethanol, and the residue was placed in a dry hot water bath. Then, 5 ml of distilled water and 6.5 ml of 52% perchloric is added and extracted at 0C. After centrifuging the mixture, the supernatant was removed with perchloric acid, centrifuged once more, and then diluted to 100 ml with purified water. 0.1-0.2 ml of supernatant reached 1 ml by deionized water and 4 ml of anthrone was added to each test tube and placed in a hot water bath at 100C for 8 minutes. Then, the tubes were quickly cooled, and color intensity was read by a spectrophotometer at a wavelength of 630 nm (Hedge & Hofreiter, 1962).

Chlorophyll fluorescence

After applying cold stress to pistachio cultivar Abbas Ali seedlings, chlorophyll fluorescence parameters were measured in developed leaves using Multi-Mode-Chlorophyll Fluorometer (OS5p) (USA). The capability of this device is used to estimate the damage caused to photosystem two in terms of cold stress (Gray *et al*., 1997; Rizza *et al*., 2001). By attaching specialized clips of the gadget to the leaf, 30 minutes of total darkness were produced for this purpose. The fluorometer was then attached to each of these clips, and measurements were taken by shining red light by the instrument.

Statistical analysis

After data collection, the data obtained from the experiment were analyzed by SAS 9.3. The mean comparison was done by Duncan's multi-range test at the probability level of 5%. Pearson's correlation

coefficient was used for the relationship among the variables.

Results

Chlorophyll fluorescence parameter F_v/F_m (PI)

The results showed that the effects of temperature, phenylalanine, calcium nitrate, and interactions were significant at the probability level of 1% on chlorophyll fluorescence (Table1).The mean comparison of the interaction of temperature×phenylalanine × calcium nitrate showed that the chlorophyll fluorescence index reduced at -3 °C, and F_v/F_m reduced compared to the control. The chlorophyll fluorescence index, however, was positively affected by the combined usage of phenylalanine and calcium nitrate and increased in comparison to the control seedlings. Furthermore, the results showed that the highest(PI) (7.1) was obtained from the interaction of temperature -1°C×1 mM of phenylalanine×3 ppm of calcium nitrate, and the lowest PI (1.6) was obtained from the interaction of temperature -3 °C×non-use of phenylalanine and calcium nitrate (Table 2). The results showed that with non-use of phenylalanine and calcium nitrate, PI reduced by reducing temperature. However, foliar application of calcium nitrate alone at -1 and -3°C increased PI compared to the control. In such a way that using 3 and 5 kg of calcium nitrate per 1000 liters of water, PI increased significantly ($p<0.01$). However, the highest increase in PI was obtained from 0°C with the simultaneous use of 2.5 mM of phenylalanine and 5 ppm of calcium nitrate (Table 3). A significant positive correlation was between PI, soluble proteins (r=0.51**) and starch (r=0.54**) at the probability level of 1 %, i.e. by increasing PI, the content of soluble proteins and starch increased. A significant negative correlation was between PI and electrolyte leakage (r = -0.68**) at the probability level of 1 %, i.e. by increasing PI, electrolyte leakage is reduced, which can lead to more seedling survival (Table 4).

Table 1. Analysis of variance of the effect of cold, amino acid phenylalanine and calcium nitrate on *Pistacia vera* L. Abbas Ali cultivar seedlings

Source	DF	Mean of Square								
		Chlorophyll fluorescence (Fv/Fm)	Total chlorophyll (mg g^{-1} fw)	Carotenoid (mg g^{-1} fw)	Total phenol µg g^{-1}) (fw	Soluble sugar (mg g^{-1} (fw	Soluble protein mg g^{-1}) (fw	Electrolyte leakage (%)	Proline µg g^{-1}) (fw	Starch (mg g^{-1} dw)
Cold	2	24.7**	6.0**	44.5**	1540.2**	4064.7**	0.003**	2571.2**	11.8**	13542.4**
Phenylalanine	2	41.2**	2.6**	3.1**	1380.2**	6301.3**	0.007**	1448.7**	19.6**	4351.0**
Calcium nitrate	2	7.1**	1.6**	0.5**	294.4**	3916.9**	0.003**	132.2**	18.6**	11603.8**
F*N	4	1.0**	0.1*	0.1**	58.4**	584.0**	0.002**	145.0**	1.0 ns	100.0**
cold*F	4	2.0**	3.4**	2.8**	127.4**	92.9**	0.001**	317.1**	0.4 ns	1323.2**
cold*N	4	2.0**	0.2**	0.3**	36.8**	106.9**	0.002**	184.0**	1.6*	240.0**
cold*F*N	8	0.9**	0.1**	0.1**	53.8**	233.4**	0.001**	194.1**	0.3 ns	671.8**
Error	54	0.041	0.040	0.003	9.000	8.890	0.000	9.01	0.490	2731.600
CV%		4.2	9.5	4.3	6.1	1.9	4.420	6.1	16.1	3.1

ns, * and ** insignificant and significant at the probability levels of 5% and 1%, respectively.

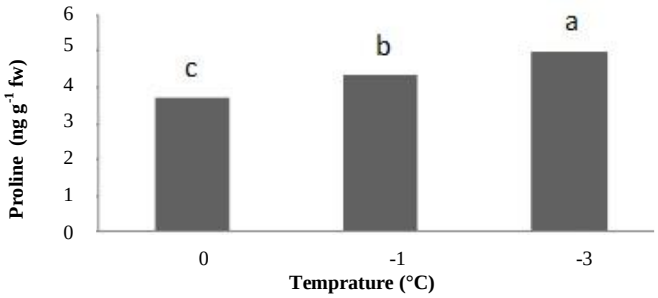

Fig. 1. Effect of temperature on proline in *Pistacia vera* L. Abbas Ali cultivar.

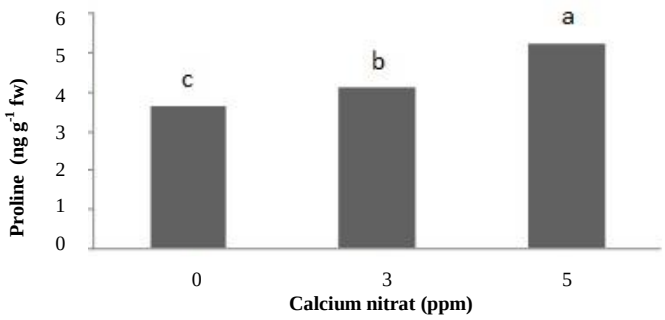

Fig. 2. Effect of calcium nitrate on proline in *Pistacia vera* L. Abbas Ali cultivar

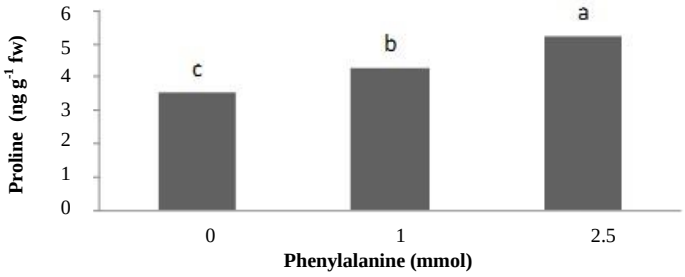

Fig. 3. Effect of phenylalanine on proline in *Pistacia vera* L. Abbas Ali cultivar.

In Figs. 1-3 according to Duncan's multiple range tests, numbers with different letters in each column are significantly different.

Table 2. Mean comparison of the interaction of cold×amino acid phenylalanine×calcium nitrate on the studied traits in *Pistacia vera* L. Abbas Ali cultivar seedlings

	Chlorophyll fluorescence (Fv/Fm)	Total chlorophyll (mg g^{-1} fw)	Carotenoid (mg g^{-1} fw)	Total phenol (µg g^{-1} fw)	Soluble mg) sugar (g^{-1} fw	Soluble protein mg g^{-1}) (fw	Electrolyte (%) leakage	Proline (µg g^{-1} fw)	Starch (mg (g^{-1} dw
Cold C^0-F(mM)									
0C^0-0	4.2 d	1.67 d	0.33 d	37.57 f	123.53 e	0.1 d	46.20 bc	3 .00 a	96.77 cd
0C^0-1	6.2 ab	1.77 cd	0.43 d	39.30 f	145.47 cd	0.13 b	38.77 de	3.73 a	112.47 bc
0C^0-2.5	6.6 a	2.00 b-d	0.47 d	49.37 cd	158.93 bc	0.15 a	37.07 e	4.37 a	137.77 a
-1 C^0-0	3.53 d	1.67 d	0.37 d	41.77 ef	138.00 d	0.09 d	51.53 b	3.43 a	85.2 de
-1 C^0-1	5.67 b	1.87 cd	0.5 d	50.13 cd	155.97 c	0.11 b-d	49.7 0 b	4.33 a	97.83 cd
-1 C^0-2.5	5.23 c	2.1 bc	0.5 d	52.20 c	169.37 ab	0.12 bc	41.03 c-e	5.20 a	120.9 ab
-3 C^0-0	2.3 e	1.9 cd	1.9 c	45.5 de	153.2 c	0.10 d	65.60 a	4.17 a	69.33 e
- 3C^0-1	3.77 d	2.3 b	2.3 b	59.70 b	171.47 ab	0.10 cd	69.40 a	4.77 a	77.13 de
-3 C^0-2.5	5.2 c	3.77 a	3.77 a	66.03 a	176.87 a	0.11 b-d	44.73 b-d	6.13 a	68.77 e
Cold C^0-n (g 1^{-1})									
0C^0-0	5.53 a	1.6 d	0.37 c	46.10 c-e	128.4 d	0.10 b	45.53 bc	3.07 d	97.07 c
0C^0-3	5.60 a	1.77 cd	0.40 c	41.87 de	146.53 c	0.12 b	41.13 cd	3.77 cd	104.2 c
0C^0-5	5.87 a	2.07 cd	0.47 c	38.27 e	153.00 bc	0.15 a	35.37 d	4.27 bc	145.7 a
-1C^0-0	3.77 c	1.67 d	0.43 c	49.13 b-d	142.37 cd	0.11 b	48.43 bc	3.87 cd	90.20 c
-1C^0-3	5.67 a	1.87 cd	0.43 c	49.6 b-d	154.47 bc	0.10 b	49.03 bc	4.03 b-d	93.40 c
-1C^0-5	5.00 ab	2.1 cd	0.5 c	45.37 de	166.5 ab	0.11 b	44.8 b-d	5.07 b	120.3 b
-3C^0-0	3.23 c	2.27 bc	2.27 b	62.23 a	157.43 bc	0.10 b	61.7 a	4.03 b-d	60.30 d
-3C^0-3	4.27 bc	2.93 a	2.93 a	54.93 ab	163.13 b	0.1 0 b	54.33 ab	4.57 bc	60.70 d
-3C^0-5	3.77 c	2.77 ab	2.77 a	54.07 bc	180.97 a	0.11 b	63.7 a	6.47 a	94.23 c
f_n									
0--0	3.20 d	1.73 cd	0.83 b	46.97 b-d	123.87 e	0.08 d	58.03 a	2.80 a	69.20 f
0--3	3.50 d	2.03 a-d	1.00 ab	42.00 de	147.60 d	0.10 c	52.87 ab	3.47 a	75.9 ef
0--5	3.33 d	2.23 a-c	1.03 ab	35.87 e	143.27 d	0.11 bc	52.43 ab	4.33 a	106.2 bc
1-0	4.50 c	1.53 d	0.73 b	51.80 a-c	149.23 d	0.10 c	51.5 ab	3.83 a	82.5 c-f
1-3	5.70 ab	2.10 a-d	1.20 a	50.83 a-c	154.23 cd	0.10 c	50.40 ab	4.10 a	81.7 d-f
1-5	5.43 a-c	2.00 b-d	1.07 ab	46.50 cd	169.43 b	0.14 a	55.97 a	4.90 a	123.23 ab
2.5--0	4.83 bc	2.27 a-c	1.50 a	58.70 a	155.10 cd	0.14 a	46.13 a-c	4.33 a	95.87 c-e
2.5--3	6.33 a	2.43 ab	1.57 a	53.57 a-c	162.30 bc	0.12 ab	41.23 bc	4.80 a	100.73 b-d
2.5--5	5.87 ab	2.70 a	1.63 a	55.33 ab	187.77 a	0.13 a	35.47 c	6.57 a	130.83 a

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

Table 3. Mean comparison of the interaction of temperature × amino acid phenylalanine × calcium nitrate on the studied traits in *Pistacia vera* L. Abbas Ali cultivar seedlings

Cold-F-N	Chlorophyll fluorescence (Fv/Fm)	Total chlorophyll (mg g^{-1} fw)	Carotenoid (mg g^{-1} fw)	Total phenol(µg g^{-1} fw)	Soluble sugar(mg g^{-1} fw)	Soluble protein(mg g^{-1} fw)	Electrolyte leakage(%)	Proline (µg g^{-1} fw)	Starch (mg g^{-1} dw)
0 C^0-0-0	4.9 d	1.5 i	0.3 k	42.3 g-i	99 r	0.09 h	48.1 f-g	2.3 a	76.1 ij
0 C^0-0-3	3.8 f	1.7 g-i	0.3 k	39.7 h-k	137.3 op	0.1 e-g	48.6 f-g	3.1 a	86.2 f-h
0 C^0-0-5	3.9 f	1.8 f-i	0.5 i	30.7 l	134.3 p	0.11 e-g	41.9 i- l	3.6 a	128 c
0 C^0-1-0	5.6 c	1.7 g-i	0.5 i	47 e-g	140 o	0.1 e-g	46.8 f-g	3.3 a	91.1 ef
0 C^0-1-3	6.2 b	1.8 f-i	0.5 i	36.5 kj	148 l-n	0.1 e-g	35.9 mn	3.7 a	92.3 e
0 C^0-1-5	6.8 a	2.1 c-f	0.5 i	34.4 kl	148.4 l-n	0.17 a	33.6 no	4.2 a	154 a
0 C^0-2.5-0	6.1 b	1.8 f-i	0.5 i	49 ef	146.2 mn	0.12 d	41.7 i-l	3.6 a	124 c
0 C^0-2.5-3	6.8 a	2.1 c-f	0.5 i	49.4 d-f	154.3 i-k	0.16 b	38.9 k-n	4.5 a	134.2 b
0 C^0-2.5-5	6.9 a	2.4 c	0.5 i	49.7 d-f	176.3 d	0.18 a	30.6 o	5 a	155.1 a
-1 C^0-0-0	3.1 g	1.5 i	0.3 k	46 gf	120 q	0.07 i	51.2 e-g	3 a	73 j
-1 C^0-0-3	3.7 f	1.6 hi	0.3 k	41.6 g-j	148.5 l-n	0.1 e-g	51.6 ef	3.3 a	75.1 j
-1 C^0-0-5	3.8 f	1.9 e-h	0.4 j	37.7 i-k	145.5 n	0.11 ef	51.8 ef	4 a	107.5 d
-1 C^0-1-0	4.3 e	1.5 i	0.4 j	49.3 ef	149.5 k-n	0.1 fg	48.8 g-f	4.2 a	85.4 gh
-1 C^0-1-3	7.1 a	1.7 g-i	0.4 j	55 cd	151.4 k-m	0.1 e-g	54.6 de	4 a	81 hi
-1 C^0-1-5	5.6 c	2.1 c-f	0.5 i	46.1 fg	167 ef	0.13 c	45.7 g-j	4.8 a	127.1 c
-1 C^0-2.5-0	3.9 f	1.f-i	0.4 j	52.1 de	157.6 h-j	0.16 b	45.3 h-j	4.4 a	112.2 d
-1 C^0-2.5-3	6.2 b	2 d-g	0.5 i	52.2 de	163.5 fg	0.11 e-g	40.9 j-m	4.8 a	124.1 c
-1 C^0-2.5-5	5.6 c	2.2 c-e	0.5 i	52.3 de	187 c	0.11 e-g	36.9 l-n	6.4 a	126.4 c
-3 C^0-0-0	1.6 i	1.6 hi	1.6 h	52.6 de	152.6 j-l	0.08 h	74.8 b	3.1 a	58.5 l
-3 C^0-0-3	3 g	3 b	3 d	44.7 f-h	157 h-j	0.1 fg	58.4 cd	4 a	66.4 k
-3 C^0-0-5	2.3 h	2.3 cd	2.3 e	39.2 i-k	150 k-n	0.11 ef	63.6 c	5.4 a	83.1 h
-3 C^0-1-0	3.6 f	3.6 a	3.6 c	59.1 bc	158.2 h-g	0.1 g	58.9 cd	4 a	71 jk
-3 C^0-1-3	3.8 f	3.8 a	3.8 b	61 b	163.3 fg	0.11 e-g	60.7 c	4.6 a	71.8 j
-3 C^0-1-5	3.9 f	3.9 a	3.9 a	59 bc	192.9 b	0.11 e	88.6 a	5.7 a	88.6 e-g
-3 C^0-2.5-0	4.5 e	1.6 hi	1.6 h	75 a	161.5 hg	0.13 c	51.4 ef	5 a	51.4 m
-3 C^0-2.5-3	6 b	2 d-g	2 g	59.1 bc	169.1 e	0.1 fg	43.9 h-k	5.1 a	43.9 n
-3 C^0-2.5-5	5.1 d	2.1 c-f	2.1 f	64 b	200 a	0.1 e-g	38.9 k-n	8.3 a	111 d

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

Table 4. Correlation coefficient of the effect of cold on the studied variables in *Pistacia vera* L. Abbas Ali cultivar

	Chlorophyll fluorescence	Total chlorophyll	Carotenoid	Total phenol	Soluble sugar	Soluble protein	Electrolyte leakage	Proline	Starch
Chlorophyll fluorescence	1.00								
Total chlorophyll	-0.16	1.00							
Carotenoid	-.410*	.858**	1.00						
Total phenol	0.04	0/31	.495**	1/00					
Soluble sugar	0.18	.493**	.451*	.562**	1.00				
Soluble protein	.510**	0.07	-0.19	-0.02	0.29	1.00			
Electrolyte leakage	-.682**	.462*	.650**	0/25	0/07	-.437*	1/00		
Proline	0.19	0.34	0.36	.498**	.874**	0.24	-0.09	1.00	
Starch	.548**	-0.05	-.444*	-0.37	0.19	.685**	-.594**	0.24	1.00

ns, * and ** insignificant and significant at the probability levels of 5% and 1%, respectively.

Total chlorophyll

The results showed that the effects of temperature, phenylalanine, calcium nitrate, and the interactions among the variables were significant on the total chlorophyll (Table 1). The mean comparison of interaction among temperature, phenylalanine, and calcium nitrate showed that the highest total chlorophyll (3.9 mg g^{-1} fw) was obtained from the interaction of temperature -3°C×1 mM of phenylalanine×5 ppm of calcium nitrate and the lowest total chlorophyll (1.5 mg g^{-1} fw) was obtained from the interaction of temperature -1°C×non-use of phenylalanine and calcium nitrate (Table 3). Total chlorophyll and carotenoid had a statistically significant positive association (r=0.85**) at the probability level of one percent, meaning that when total chlorophyll grew, carotenoid also increased (Table 4). The amount of chlorophyll in plants is one of the key elements in sustaining photosynthetic capability. Chlorophyll synthesis is one of the processes sensitive to cold changes and is used as a quantitative method to measure the sensitivity of different species to frostbite (Colom & Vazzana, 2001).

Carotenoid

The results showed that the effects of temperature, phenylalanine, calcium nitrate, and the interactions among the variables were significant at the probability level of 1% on carotenoids (Table1). The mean comparison of the interaction of temperature×phenylalanine × calcium nitrate showed that the highest carotenoid (3.9 mg g^{-1} fw) was obtained from the interaction of temperature -3°C×1 mM phenylalanine×5 ppm calcium nitrate. And the lowest carotenoid (0.3 mg g^{-1} fw) was obtained from the interactions of temperature -3°C×non-use of phenylalanine and calcium nitrate, and temperature -1°C ×non-use of phenylalanine×3 ppm calcium nitrate (Table 3). The results showed that using 2.5 mM phenylalanine compared to non-use increased carotenoids. On the other hand, with the simultaneous use of 2.5 mM of phenylalanine and 5 ppm of calcium nitrate, carotenoids at 0°C increased compared to the control. At -3°C, only a small amount of carotenoids reduced (Table 3). At the probability level of 1%, there was a substantial positive connection between carotenoid and electrolyte leakage (r = 0.65**), i.e., as carotenoid rose, electrolyte leakage increased (Table 4).

Total phenol

The results showed that the effects of temperature, phenylalanine, calcium nitrate, and interactions were significant at the probability level of 1% on total phenol (Table 1). The mean comparison of the interaction of temperature × phenylalanine × calcium nitrate showed that the highest total phenol (75 µg g^{-1} fw) was obtained from the interaction of temperature -3°C×2.5 mM phenylalanine non-use of calcium nitrate, and the lowest total phenol (30.7 µg g^{-1} fw) was obtained from the interaction of temperature 0°C×non-use of phenylalanine×5 ppm calcium nitrate (Table 4). The findings of the data's mean comparison also revealed that phenolic chemicals rose when temperature was lowered. The application of 2.5 mM phenylalanine alone also raised phenolic compounds, with the maximum total phenol concentration occurring at -3°C, which was substantially different from zero and -1 °C (Table 3). A positive significant correlation was between total phenol and soluble sugar at the probability level of 1% (r=0.56**), e. by increasing total phenol, soluble sugar increased (Table 4).

Soluble sugar

The results showed that the effects of temperature, phenylalanine, calcium nitrate, and their interactions were significant at the probability level of 1% on soluble sugar (Table 1). The mean comparison of the interaction of temperature × phenylalanine × calcium nitrate showed that the highest soluble sugar (200 mg

g^{-1} fw) was obtained from the interaction of temperature -3°C× 2.5 mM of phenylalanine×5 ppm of calcium nitrate, and the lowest soluble sugar (99 mg g^{-1} fw) was obtained from the interaction of temperature 0°C × no-use of phenylalanine and calcium nitrate. The content of soluble sugars rose from 99 to 200 mg g1 fw when the temperature dropped from zero to -3°C, as indicated in the mean comparison table (Table 3), and this rise was also seen when 3 ppm calcium was utilized in contrast to the control. Compared to the control, soluble sugars were increased when phenylalanine was used alone. Soluble sugars increased at 0°C when phenylalanine concentrations of 1 and 2.5 mM were used alone. However, the highest increase in soluble sugars at 0°C was obtained from the simultaneous use of 2.5 mM phenylalanine and 5 ppm calcium nitrate. A significant positive correlation was between soluble sugar, and proline at the probability level of 1% (r=0.87**), i.e. by increasing soluble sugars, proline increased too (Table 4).

Soluble protein

The results showed that the effects of temperature, phenylalanine, calcium nitrate, and their interactions were significant at the probability level of 1% on soluble proteins (Table 1). The mean comparison of the interaction of temperature × phenylalanine × calcium nitrate showed that the highest soluble protein (0.18 mg g^{-1} fw) was obtained from the interaction of temperature 0°C ×2.5 mM phenylalanine×5 ppm calcium nitrate and the lowest soluble protein (0.07 mg g^{-1} fw) was obtained from the interaction of temperature -1°C ×non-use of phenylalanine and calcium nitrate (Table 3). As shown, the lowest soluble protein was at -1°C but using 3 ppm calcium nitrate at 0°C increased soluble protein. using 2.5 mM phenylalanine alone at -1°C significantly increased soluble protein compared to the control at the same temperature. But using the most phenylalanine and calcium nitrate at 0°C simultaneously resulted in the highest soluble protein (Table 3). At the probability level of 1 percent, there was a substantial positive association between soluble protein and starch (r=0.68**), indicating that as soluble protein concentration grew, so did starch content (Table 4).

Electrolyte leakage

The results indicated that the effects of temperature, phenylalanine, calcium nitrate, and interactions were significant at the probability level of 1 % on electrolyte leakage (Table 1). the results indicated that the highest electrolyte leakage (88.6%) was obtained from the interaction of temperature -3°C×1 mM phenylalanine×5 ppm calcium nitrate, and the lowest electrolyte leakage (30.6%) was obtained at temperature 0°C ×2.5 mM phenylalanine×5 ppm calcium nitrate (Table 3). A significant negative correlation was between electrolyte leakage and starch at the probability level of 1% ($r = -0.59^{**}$), i.e. by increasing electrolyte leakage, starch reduced (Table 4). Electrolyte leakage increased in the treatments without phenylalanine and calcium nitrate when the temperature was lowered from 0 to -3 °C, as shown. On the other hand, electrolyte leakage from the leaves was decreased when phenylalanine was used alone. However, the highest reduction in electrolyte leakage occurred with the simultaneous use of 2.5 mM of phenylalanine and 5 ppm of calcium nitrate at 0°C.the results of the present study showed that phenylalanine and calcium nitrate treatments reduced electrolyte leakage from the membrane at the end of the cooling treatment.

Starch

The results indicated that the effects of temperature, phenylalanine, calcium nitrate, and their interactions were significant at the probability level of 1% on starch (Table 1). It was shown that the highest starch (154 and 155.1 mg g^{-1} dw) was obtained from the interactions of temperature 0°C×1 mM phenylalanine×5 ppm calcium nitrate, and temperature 0°C ×2.5 mM of phenylalanine×5 ppm of calcium nitrate; and the lowest starch (2.3 mg g^{-1} dw)

was obtained from the interaction of temperature 0°C ×non-use of phenylalanine and calcium nitrate (Table 3). As shown, leaf starch decreased when the temperature was lowered to -3 °C. However, starch increased when the temperature was lowered to 0 °C when 5 ppm calcium nitrate was added alone; there was no discernible change when 2.5 mM phenylalanine was added alone. However, leaf starch reached more than two times the level of the control when the greatest concentrations of phenylalanine and calcium nitrate were used at 0 °C. Leaf starch reached to its highest level with the simultaneous use of the highest mentioned treatments at -1 and -3°C. A significant positive correlation was between chlorophyll fluorescence, and starch content ($r=0.55^{**}$) and soluble protein ($r=0.68^{**}$) at the probability level of 1%, and a significant negative correlation between electrolyte leakage and starch content at the probability level of 1% ($r=-0.59^{**}$), i.e. by increasing electrolyte leakage, starch content reduced (Table 4).

Proline

The results of variance analysis (Table 1) of proline showed that cold, phenylalanine and calcium nitrate had a significant effect on this index. But, the interactions of phenylalanine × calcium nitrate, cold × phenylalanine, and phenylalanine × calcium nitrate × cold were not significant on proline. The results (Fig. 1) showed that proline increased by reducing the temperature (5.02 mg g^{-1} fw at -3°C). it was also indicated (Fig. 2) that proline increased by increasing calcium nitrate (5.27 mg g^{-1} fw using 5 ppm calcium nitrate). The results (Fig. 3) showed that proline increased by increasing amino acid of phenylalanine (5.23 mg g^{-1} fw using 2.5 mM of phenylalanine). The mean comparison table (Table 2) demonstrates that the most proline was produced at -3°C and 5 ppm calcium nitrate, while the lowest proline was obtained at 0°C and no calcium nitrate (3.07 mg g1 fw in the treatments with no calcium nitrate and phenylalanine). A significant positive correlation was between soluble sugar, and proline at the probability level of 1% (r=0.87**), i.e. by increasing soluble sugar, proline increased (Table 4).

Discussion

One of the most important reasons for the reduction of chlorophyll in the plants under environmental stresses is the degradation by reactive oxygen species (ROS) (Gharib, 2007; Gharaghani *et al.*, 2018). The main reason for the formation of ROS is the imbalance between light absorption and photosynthesis. Despite these conditions, the inability to utilise light when the temperature is lowered in the presence of light increases the danger of photooxidation. The study's findings indicated that the low temperature decreased total chlorophyll, which is likely because the cold and frostbite stressors interfered with the synthesis of chlorophyll and harmed the structure of the chloroplast (Gharib, 2007). Researchers have found that some amino acids, such as salicylic acid, by stimulating chlorophyll synthesis, increased photosynthetic pigments in treated plants (Gharib, 2007). F_v/F_m shows the efficiency of photosynthesis, under cold stress and its amount reduces in the plants sensitive to the cold. Chlorophyll fluorescence as an index for determining the efficiency of the photosystem can indicate the damage to the photosynthetic system and the ability of plants to tolerate various environmental stresses (Maxwell & Johnson, 2000). This value under normal conditions for a healthy plant leaf is reported to be 0.8 or 80%, which tends to zero under any stress condition. It is reported that stress-tolerant cultivars have F_v/F_m higher than sensitive cultivars (Liu & Huang., 2002). PI indicates the current physiological state of the plant, as well as the damage and/or adaptation of the photosynthetic system to deal with changing environmental conditions (Strasser *et al*., 2000). The results of the present study showed that cold stress reduced F_v/F_m and PI. A reduction in PI of π photosystem refers to a reduction in the speed of electron transfer in the chloroplast electron transport

chain, and the reduction in electron acceptors may cause a possible increase in the production of free radical species, which damage the components of the π photosystem. Majdi *et al*. (2007) reported that the reduction in ambient temperature caused a reduction in PI in sensitive and resistant cultivars of wheat, but this reduction in resistant cultivars was significantly less than in sensitive cultivars. Carotenoids are sometimes referred to as promoters of pigments that block light energy, both photosynthetic and non-photosynthetic. One of the most significant internal variables, photosynthetic pigments, is thought to sometimes be able to place restrictions on the photosynthesis center (Bertamini *et al*., 2007). Carotenoids include beta-carotene, xanthophyll, and lycopene. The antioxidant role of carotenoids is related to inhibiting free radicals production under stress. The study results showed that lowering the temperature caused a reduction in carotenoids, but using phenylalanine and calcium nitrate treatments under cold stress improved the content of carotenoids. It seems that the reduction in the plant carotenoid pigments under cold stress is in terms of the oxidation of this pigment by ROS, in which way chlorophyll protects against damage caused by singlet oxygen molecules (Tadjvar *et al*., 2011). It is well known that carotenoids can protect the photosynthetic system from damage caused by singlet oxygen molecules, by combining with oxygen radicals in a reversible way, and the formation of xanthophyll cycle prevents the degradation of chlorophyll. The study results are consistent with the results of Tadjvar *et al*. (2011) on tangerine.

Plants build up phenolic chemicals, which are connected to the plant's antioxidant ability, to adapt to the cold. ROS are poisonous and seriously harm plants (Imlay & Linn, 1998). Reducing temperature increases the accumulation of phenolic compounds in the plant which can act as a mechanism to adapt and overcome the oxidative stress caused by cold stress (Balasundram *et al*., 2007). To maintain the turgescence of cell, the plant produces substances that cause the water potential inside the cell to become more negative and maintain the turgescence of the cell, which are called osmolytes. Soluble sugars, which are part of these substances, have very high solubility and cause no toxicity to the cell at high concentrations. Additionally, by forming hydrogen bonds, carbohydrates make proteins and membranes more stable and stop proteins from degrading. Some hormones allow plants to accumulate carbohydrates, which along with the resulting osmotic gradient make the plant more resistant to water loss, raise the water content of the leaves, and speed up the development of plants under stress. Moreover, the accumulation of sugars, which are produced as a result of adaptation to stress, stabilizes and protects the membrane against freezing damage (Ashraf & Foolad, 2007). The increase in soluble sugars increases the adaptability of trees to the cold of autumn and winter. An increase in soluble sugars is related to an increase in resistance to cold stress, and sugars affect the plant against low temperature (Ranney *et al*., 1991). Stress tolerance is associated with changes in the osmotic substances inside the cell, and treatment with amino acids plays an osmotic role for the cell by stimulating the hydrolysis of insoluble sugars. Therefore, by increasing soluble sugars, cold tolerance should also increase. Besides, the treatment of some amino acids, such as salicylic acid reduces the occurrence of frostbite symptoms (Kafi & Mahdavi Damghani, 2002). Cold stress leads to the breaking of electron transport chain and production of active oxygen. Due to their strong affinity, oxygen radicals created as a consequence of stress cause the breakdown of membranes, nucleic acids, and cell proteins. The reduction in the production or increase in the breakdown of proteins or both under cold stress can be attributed to the increase or reduction in antioxidant activity (Peltzer *et al*., 2002). For the adaptation to cold, there are major changes in the amount and pattern of production of membrane proteins, which are due to the quick response of the plant to low cold. These changes include the

production of membrane repair proteins, proteins associated with osmotic stresses, and proteins with unknown functions (Uemura *et al*., 2006). Leaf electrolyte leakage is one of the important factors that show the plant's cold tolerance. According to the study's findings, electrolyte leakage was significantly increased by cold stress, and when phenylalanine and calcium nitrate were utilized instead of control bases, electrolyte leakage was significantly decreased. Under cold stress, one of the measures of plant resistance is the production of osmotic regulators, which are involved in creating resistance and osmotic regulation of the plant under cold conditions. The results of the present study showed that cold stress increased the osmotic regulators, such as proline, soluble sugars, and phenol compounds, and the simultaneous use of phenylalanine and calcium nitrate led to an increase in these parameters compared to the control. Protein and starch indices were evaluated, and cold stress caused a significant reduction. However, the aforesaid parameters were elevated when phenylalanine and calcium nitrate were used together as opposed to the control therapy. One of the active amino acids that rises in stressed plants and strengthens membranes under cold stress is proline. Free radicals increase under stress conditions which cause disturbances in electron transfer in plant tissues, and finally the degradation of membranes under stress (Hassibi *et al*., 2007). Proline plays an antioxidant role and consequently, it can protect proteins and membranes against oxidative damage (Zhang *et al*., 2000). Moradmand *et al*. (2015) reported that in bell pepper under cold stress, amino acid treatment increased proline in leaves compared to the control. It was reported in another study that amino acid treatment led to an increase in leaf proline compared to the control under cold stress (Ershadi & Taheri, 2013).

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Conflict of interests

The authors declare that there is no conflict of interest.

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