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Physiological Responses of Some Rootstocks and Interspecific Hybrids of Pistachio to Cold Stress under Greenhouse Conditions

Hossein Sajadian[1], Mansoore Shamili[*1], Hossein Hokmabadi[2], Ali Tajabadipour[3], Hojjat Hasheminasab[3]

[1] Department of Horticulture Science, University of Hormozgan, Hormozgan, Iran

[2] Department of Horticulture, Education and Extention Institute, AREEO, Tehran, Iran

[3] Pistachio Research Center, Horticultural Sciences Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Rafsanjan, Iran

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ABSTRACT

Cold is an environmental factor limiting the growth and yield of agricultural crops. To find cold-tolerant pistachio rootstocks, a factorial experiment was conducted at Pistachio Research Center (PRC), Rafsanjan, Iran during 2017-2018. The factors included six rootstocks (Badami Zarand, Ghazvini, Sarakhs, Integerrima × Badami Zarand, Integerrima × Ghazvini and Integerrima × Sarakhs) and four thermal treatments (4, 0, -2, and -4°C) with three iterations. Seedlings in 6-8 leaf stage were placed in thermal treatments for two hours. Ionic leakage, pH of leaked solution, chlorophyll fluorescence, photosynthesis efficiency index, chlorophyll (a, b, total) and carotenoid contents were the traits assessed in this study. Based on the results, a decrease in temperature made an increase in ionic leakage rate, conversely making decreases in pH of the leaked solution, photosynthetic pigments contents, Fv/Fm and PI index. In addition, the highest photosynthetic pigments, pH of leaked solution, Fv/Fm and PI index were observed in Ghazvini and Integerrima × Ghazvini rootstocks. Meanwhile, the highest ionic leakage was observed in Integerrima × Badami Zarand. In aggregate, Ghazvini and Integerrima × Ghazvini were identified as the most cold-tolerant rootstocks, while Integerrima × Badami Zarand was the most cold-sensitive rootstock.

Introduction

Cold stress is a key factor limiting the growth of plants, from germination to reproductive stage (Sakai & Larcher, 1987; Aslani Aslamarz *et al.*, 2010). Pistachio (*Pistacia vera* L.), a dioecious plant belonging to the family of Anacardiaceae, is believed to be one of the most important economic crops in Iran (15% of the Iran's orchards, Statistics, 2017) with 429535 ha cultivation area (FAO, 2017). Pistachio trees are susceptible to late-spring frosts. In 1997, late-spring frost damaged half of Kerman's (the main pistachio producing province in Iran) pistachio areas, which diminished Iran pistachios exports remarkably. Likewise, in 2004 and 2005, some pistachio areas in Kerman, e.g. Rafsanjan, were hurt from spring frost (Sohrabi *et al.*, 2009). Reducing temperature causes membrane disturbance and subsequent intracellular

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electrolytes leakage (Azzarello *et al*., 2009). In orange (Tignor *et al*., 1998), olive (Bartolozzi & Fontanazza, 1999) and pistachio (Hokmabadi *et al*., 2016, Afrousheh *et al*., 2018) electrolyte leakage has reported as a suitable index of frost tolerance.

Cold stress, through damage to photosystem II and other components of the electron transport chain, leads to severe deterioration or reduction of photosynthetic electron transfer. In this way, a great part of absorbed light is departed as heat and fluorescence (Roháček & Barták, 2008). Chlorophyll fluorescence is a suitable physiological indicator for determining induction changes in the photosynthetic system (Mehata *et al*., 2010, Bertin *et al*., 1996, Strauss *et al*., 2006).

The common pistachio rootstocks of Iran include Badami Zarand (in different regions of Kerman province), Badami (in Khorasan), Ghazvini (in Qazvin province), and Sarakhs (wild type of *Pistacia vera* in North East of Iran). Pistachio cultivated area in Khorasan province is higher in Qazvin province (Hokmabadi *et al*., 2016).

The *integerrima* species, a vigorous species, is originated in Mediterranean regions and usable for pistachio cultivars as rootstock (Hasheminasab & Afrousheh, 2018). Although in recent years, various environmental stresses have been major challenges for Iran pistachio industry and various studies have been conducted to ameliorate the effect of different abiotic stresses (Shamshiri and Hasani, 2015; Alipour, 2018), the existence of enormous genetic resources and the diversity of Iranian pistachio cultivars and genotypes have provided an exceptional opportunity to improve this plant (Hasheminasab & Afrousheh, 2018). Therefore, the main objective of the present study was to find cold-resistant interspecific hybrids and rootstocks based on physiological traits.

Materials and Methods

The present research was performed at Pistachio Research Center (PRC), Rafsanjan, Iran. Three pistachio rootstocks (including Badami Zarand, Ghazvini and Sarakhs) were selected as female parents and *Pistacia integerrima* was used as the male parent. All parents were 35 years old.

In mid-March 2017, female parents were labeled (three trees). Five branches of each tree (four branches for controlled pollination and one for open pollination) were chosen and tagged. Before full bloom, the clusters were treated with 70% alcohol, and then the branches were covered with two-layer pads to prevent unwanted pollination. Integerrima pollens as male parent were collected during late-March, 2017, when one-third of flower anthers opened and the red flowers turned to yellow. Then, the flowers were placed on a paper, under ambient temperature (25°C) and dry conditions. After 24 hours, the released pollens were transferred to clean filter paper, passed through a fine sieve, and placed in glass containers. The containers were kept in a freezer (-20°C) until being used for artificial pollination. The pollens grain viability was tested before control pollination (in mid-April 2017) to ensure about the germination efficiency. Control pollination was done from mid to late April 2017, due to the variety of female parent blooming period (when most of the female clusters were bloomed, with pink flowers and white-milky stigmas), three times (one-day interval) for each experimental unit. During late-April, when the stigma turned to brown and the small-sized fruits reached millet grain size, the cover of experimental branches were picked up, immediately. After growing the fruits over 140 - 150 days, the nuts were harvested during the mid-September 2017.

Over mid-March 2018, the seeds were sown in pots under greenhouse conditions in Pistachio Research Institute. The seeds of Badami Zarand, Integerrima × Badami Zarand, Ghazvini, Integerrima × Ghazvini, Sarakhs and Integerrima × Sarakhs were pre-soaked in distilled water (for 24 hours), and then disinfected with 5% sodium hypochlorite solution (for 10 minutes). Subsequent rinsing in distilled water (three times) was followed by a fungicide treatment (Captan® 0.2% for an hour). Finally, the seeds were sown in polyethylene growing pots, containing a mixture of ratio of 70:30 V/V coco peat and perlite. The seeds were germinated in April 2018. They were fertilized by Hoagland's complete nutrient solution (50ml/ pot, every three days). When the seedlings reached the 6 to 8 leaf stage, they were sprayed with distilled water and placed inside the incubator. Then, the seedlings were exposed to each thermal treatment (4, 0, -2, and -4°C) for two hours (Tajabadipour *et al*., 2018). Finally, the physiological parameters including ionic leakage, leaked solution pH, chlorophyll (a, b, total) content, carotenoids content, chlorophyll fluorescence (Fv/Fm) and photosynthetic efficiency index (PI) were measured as following procedures:

Measurement of ionic leakage and pH of leaf extract

The Sairam method (1994) was used to measure ionic leakage. Based on this method, 0.1g of leaf sample was put in 10ml of double distilled water and placed in bath water (40°C, 30min). The electrical conductivity (initial leakage) of each sample (EC_1) was measured using an EC meter (BC3020, Trans Instruments, UK) and initial pH (pH_1) was measured using a pH meter (BP3001, Trans Instruments, UK). Then, the samples were re-placed in water- bath (100°C, 15 min). The electrical conductivity (EC_2) and pH (pH_2) were measured, too. The ionic leakage was calculated by the following formula:

$$\text{Ionic leakage (\%)} = EC1/EC2\times100$$

Measurements of chlorophyll (a, b, total) and carotenoids

Measurements of chlorophyll a, b and total chlorophyll contents were performed by sampling from the fully developed leaves using Porra method (2002). To this end, 0.25 g of fresh leaves was extracted in 5 ml of acetone 80%. The samples were then centrifuged (3500 rpm, 10 min). In the next step, the spectrophotometer (T80 UV/VIS, PG Instruments, UK) was used to read the optical absorption at 470, 646.6 and 663.6 nm. Finally, the chlorophyll concentration was calculated using the following equations.

$$\text{Total chlorophyll } (\mu g / g \text{ FW}) = [(17.76 \times OD_{646.6}) + (7.34\times OD_{663.6})] \times [V/W]$$

$$(\mu g / g \text{ FW}) = [(12.25 \times OD_{663.6}) - (2.22 \times OD_{646.6})] \times [V/W] \text{ Chlorophyll a}$$

$$(\mu g / g \text{ FW}) = [(20.31 \times OD_{646.6}) - (4.91 \times OD_{663.6})] \times [V/W] \text{ Chlorophyll b}$$

To calculate carotenoids based on the Lichtenthaler and Wellburn method (1983), the following equation was used:

$$\mu g / g \text{ FW}) = (1000\ OD_{470} - 3.27[Chla] - 104\ [Chlb]) / 227 \text{ (Carotenoids}$$

OD: read absorption rate, V: Final extract volume, W: Wet sample weight

Chlorophyll fluorescence measurement (Fv/Fm) and photosynthesis efficiency index (PI)

The chlorophyll fluorescence of seedlings was measured by a chlorophyll fluorescence apparatus (Pocket PEA, Hansatech Instruments, UK). To this end, a seedling was selected from each pot and the middle leaf from top of the plant was placed in special clips for 20 min, Fv/Fm and the photosynthesis efficiency index (PI) were then recorded.

Statistical analyses

This factorial experiment was conducted in a randomized complete design. Data analysis was performed by means of SAS 9.1 software. Mean comparison was conducted with Duncan's multiple range test. Pearson correlation analysis was performed using SPSS software. Diagrams were drawn by Excel 2013 software.

Results

Ionic leakage

The results of variance analysis revealed that different thermal treatments had significant effect on leaf ionic leakage (Table 1). In this study, the ionic leakage sharply increased with decreasing temperature. The most and the least ionic leakages were observed under -4 °C and 4°C, respectively (Fig. 1a). There was a significant difference among rootstocks and the most value was observed in Integerrima × Badami Zarand hybrid. The least values belonged to Ghazvini and Integerrima × Ghazvini hybrid (Fig. 1b). According to the results, the interaction between the rootstock and temperature was not significant.

Table 1. The impact of thermal treatments on physiological characteristics of different pistachio rootstocks.

Source of variation	df	Mean Square								
		Ionic leakage	Initial pH	Final pH	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoid	Fv/Fm	PI
Rootstock	5	21.96604*	0.485776**	0.489997**	15608.82*	133172.4**	441494.6*	208.9353*	0.000833**	4.959905**
Cold	3	456.2194**	1.977383**	2.927409**	2344588**	3805894**	12088495**	6967.863**	0.002018**	100.7925**
Rootstock × Cold	15	10.66756^{ns}	0.070193^{ns}	0.074507^{ns}	7012.309^{ns}	10450.32^{ns}	247662.5^{ns}	82.04548^{ns}	0.000112^{ns}	0.255534^{ns}
Error	48	7.223638	0.054029	0.132519	6260.452	13913.12	146300.3	82.47661	0.00009390	0.6129
CV%		11.21576	3.783813	6.941660	4.029092	11.31743	12.85251	14.42501	1.203230	9.132479

ns, * and **: non-significant, significant at 5% and 1%, respectively

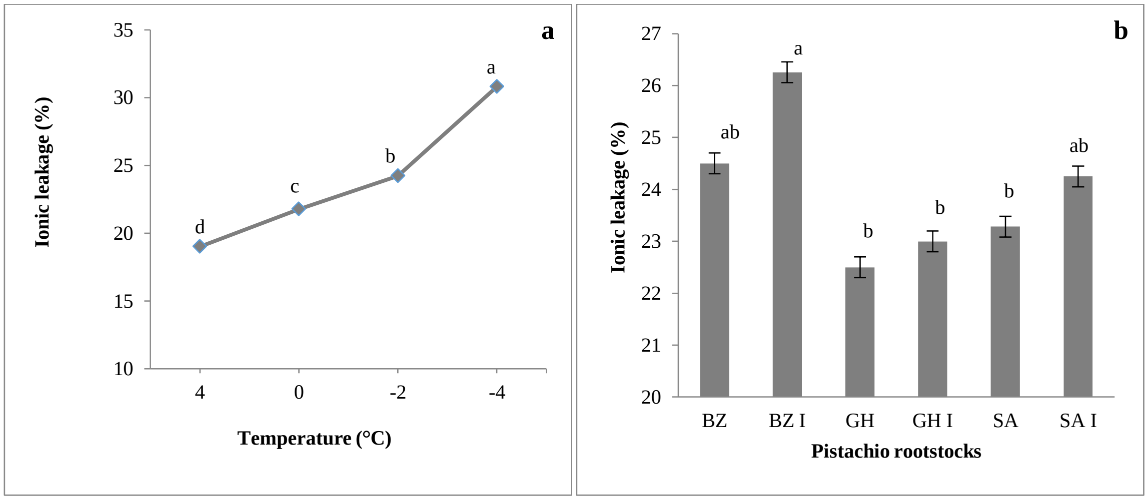

Fig. 1. The impact of thermal treatments (a) on ionic leakage of pistachio rootstocks (b): Badami Zarand (BZ), Integerrima ×Badami Zarand (BZ-I), Ghazvini (GH), Integerrima ×Ghazvini (GH-I), Sarakhs (SA), Integerrima × Sarakhs (SA-I). (Means followed by the same letter are not significantly different at 5% probability using Duncan's test)

pH of leaked solution

The analysis of variance showed a significant difference between the initial and final pH of leaked solution (1% level). Furthermore, there was a significant difference between the temperature treatments in terms of pH of the leaked solution at 1% level; however, there was not a significant interaction between the rootstock and the temperature (Table 1). The highest pH was observed under 4°C and the least value belonged to -4°C treatment (Fig. 2a). The results showed that the pH of the leaked solution significantly decreased with decreasing temperature. Moreover, according to results, the highest pH was related to the Ghazvini rootstock, while the least value was observed in Integerrima × Badami Zarand (Fig. 2b).

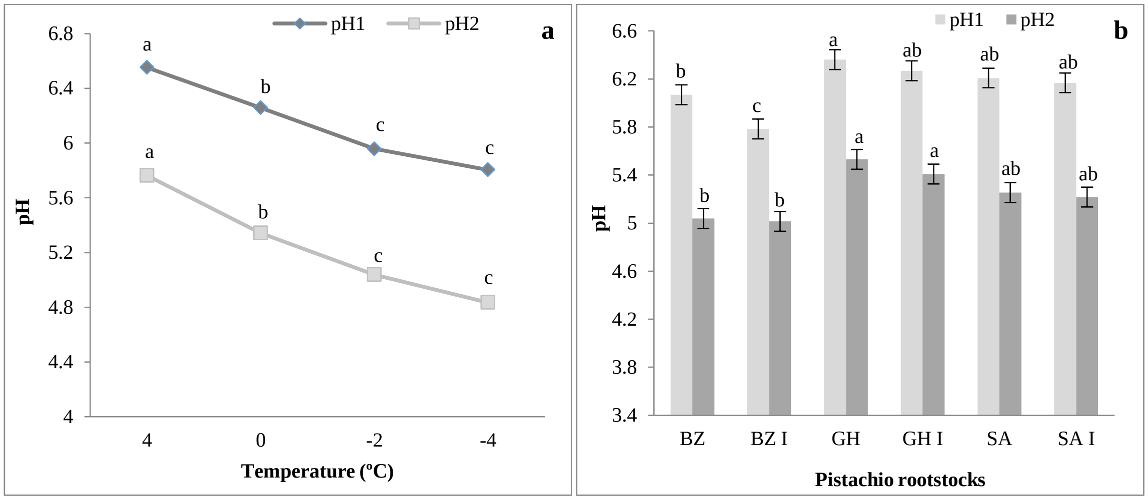

Fig. 2. The impact of thermal treatments (a) on initial and final pH of leaked solution of pistachio rootstocks (b): Badami Zarand (BZ), Integerrima×Badami Zarand (BZ-I), Ghazvini (GH), Integerrima×Ghazvini (GH-I), Sarakhs (SA), Integerrima × Sarakhs (SA-I). (Means followed by the same letter are not significantly different at 5% probability using Duncan's test)

Leaf chlorophyll a content

Leaf chlorophyll a content was significantly influenced by thermal treatments. The most leaf chlorophyll a content was observed under 4°C and the lowest value belonged to -4°C treatment (Fig. 3a). Based on mean comparison, the highest chlorophyll a content of the leaves belonged to Ghazvini and Integerrima × Ghazvini rootstocks trailing by Sarakhs, Integerrima × Sarakhs, Badami Zarand and Integerrima × Badami Zarand (Fig. 3b). According to the results, there was not a significant interaction between the rootstock and temperature.

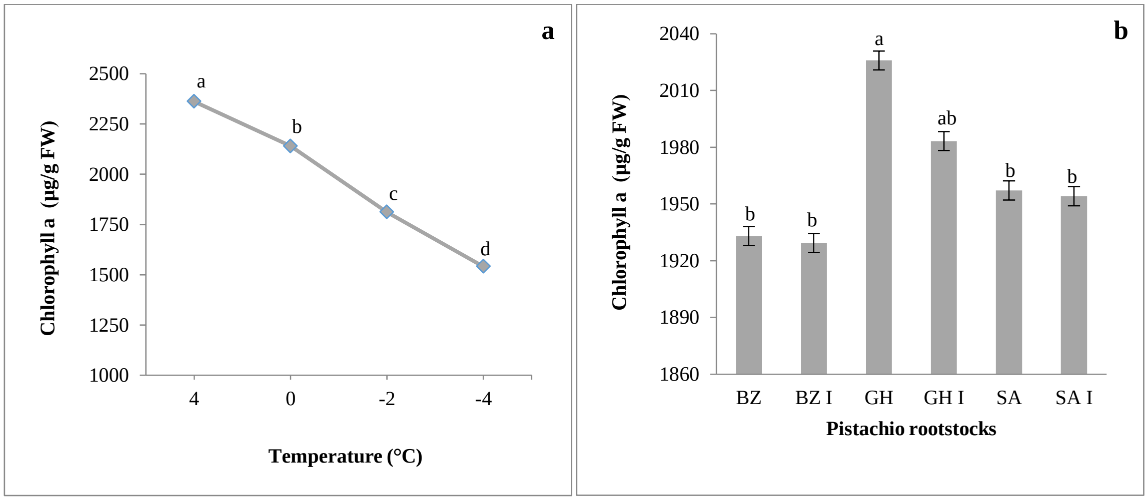

Fig. 3. The impact of thermal treatments (a) on chlorophyll a content of pistachio rootstocks (b): Badami Zarand (BZ), Integerrima×Badami Zarand (BZ-I), Ghazvini (GH), Integerrima×Ghazvini (GH-I), Sarakhs (SA), Integerrima × Sarakhs (SA-I). (Means followed by the same letter are not significantly different at 5% probability using Duncan's test)

Leaf chlorophyll b content

Analysis of variance revealed that chlorophyll b content of the leaf was affected by rootstock type and temperature treatments at 1% level; however, there was not a significant interaction between rootstock and temperature (Table 1). Ghazvini rootstock had the highest leaf chlorophyll b content, followed by the Integerrima × Ghazvini, Sarakhs, Integerrima × Sarakhs, Badami Zarand and Integerrima × Badami Zarand rootstocks (Fig. 4a). According to the results, the Integerrima × Badami Zarand, the most cold-sensitive rootstocks, had the lowest leaf chlorophyll b content. The highest chlorophyll b content was observed under 4 °C, which decreased by temperature reduction (Fig. 4b).

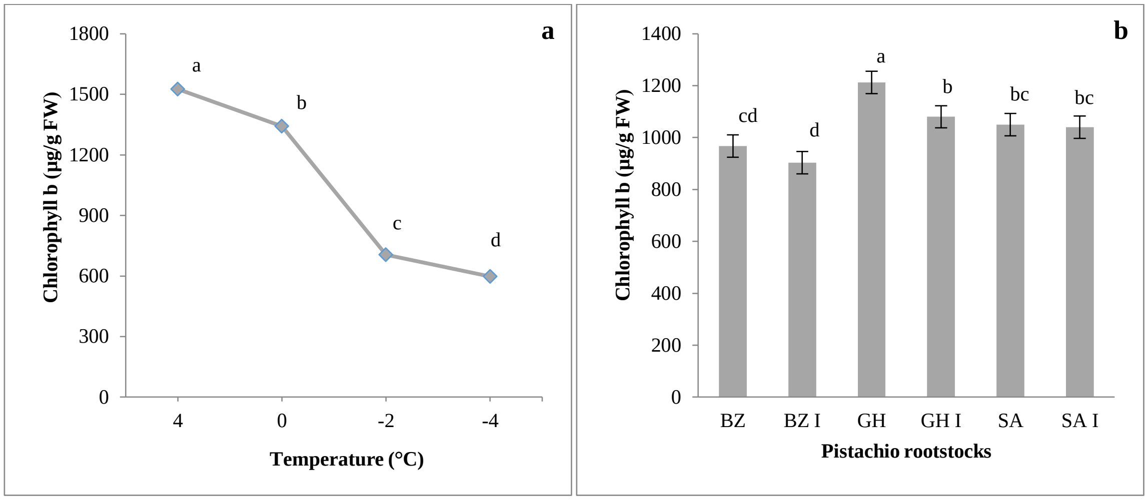

Fig. 4. The impact of thermal treatments (a) on chlorophyll b content of pistachio rootstocks (b): Badami Zarand (BZ), Integerrima×Badami Zarand (BZ-I), Ghazvini (GH), Integerrima×Ghazvini (GH-I), Sarakhs (SA), Integerrima × Sarakhs (SA-I). (Means followed by the same letter are not significantly different at 5% probability using Duncan's test)

Total chlorophyll content of leaf

Based on the results, there was a significant difference between thermal treatments for total chlorophyll content of leaf at 1% level (Table 1). The highest total chlorophyll content was at 4°C and the lowest was at -4°C (Fig. 5a). Based on the mean comparison, the highest total chlorophyll belonged to Ghazvini, Integerrima × Ghazvini, Sarakhs and Integerrima × Sarakhs rootstocks. The lowest level was found in the Integerrima × Badami Zarand (Fig. 5b). There was not a significant interaction between rootstock and temperature.

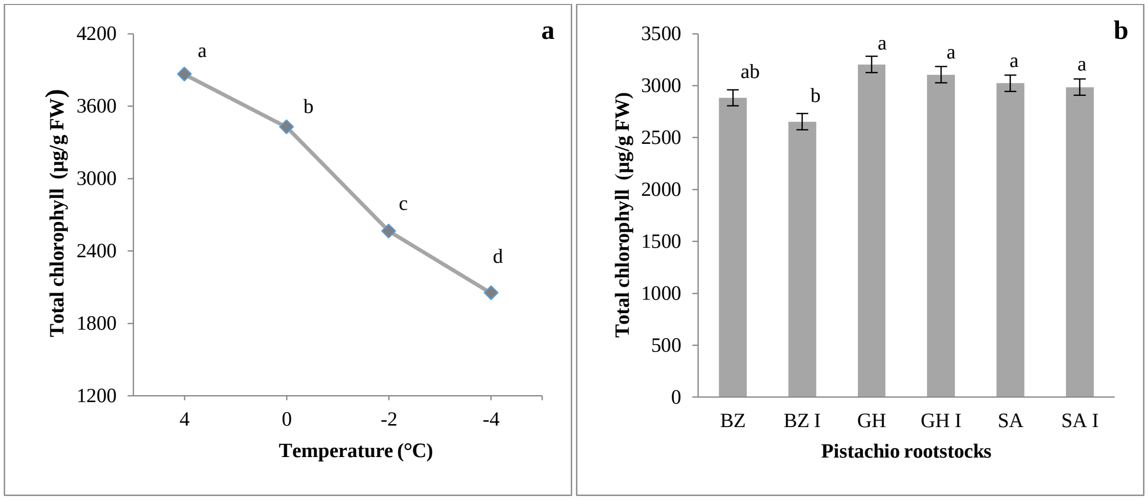

Fig. 5. The impact of thermal treatments (a) on total chlorophyll content of pistachio rootstocks (b): Badami Zarand (BZ), Integerrima×Badami Zarand (BZ-I), Ghazvini (GH), Integerrima×Ghazvini (GH-I), Sarakhs (SA), Integerrima × Sarakhs (SA-I). (Means followed by the same letter are not significantly different at 5% probability using Duncan's test)

Leaf carotenoids content

According to Table 1, the effect of thermal treatments on leaf carotenoids content was significant at 1% level. The highest carotenoids content was obtained under 4°C, significantly decreasing with temperature reduction (Fig. 6a). Based on the results, the highest levels of carotenoids were observed in Ghazvini and Integerrima × Ghazvini rootstocks (Fig. 6b). There was not significant interaction effects of rootstock and temperature.

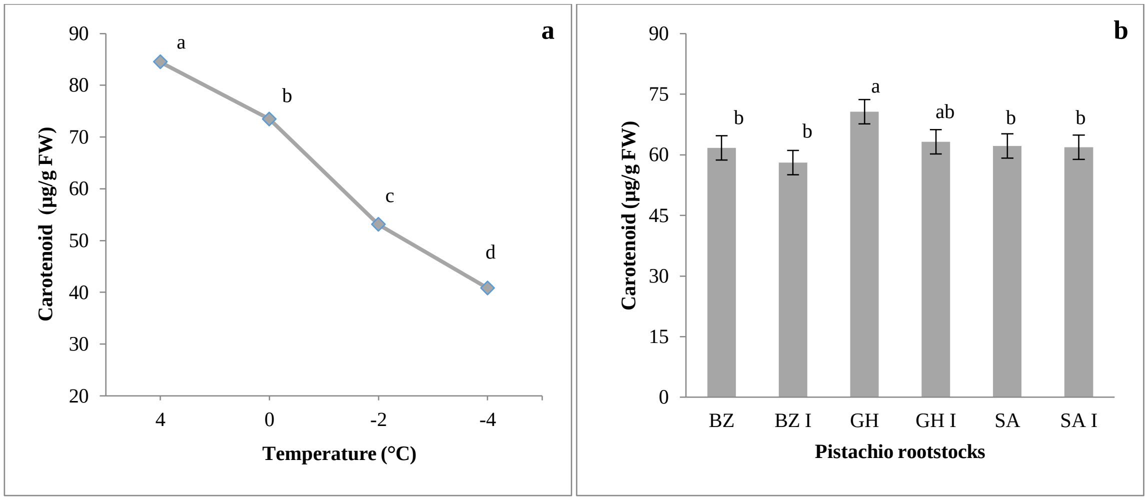

Fig. 6. The impact of thermal treatments (a) on carotenoids content of pistachio rootstocks (b): Badami Zarand (BZ), Integerrima×Badami Zarand (BZ-I), Ghazvini (GH), Integerrima × Ghazvini (GH-I), Sarakhs (SA), Integerrima × Sarakhs (SA-I). (Means followed by the same letter are not significantly different at 5% probability using Duncan's test)

Fluorescence chlorophyll (Fv/Fm)

There was a significant difference among different temperatures in terms of Fv/Fm (Table 1). The most Fv/Fm was observed under 4 and 0°C. Moreover, the least ratio was obtained from the plant exposed to -4°C (Fig. 7a). The highest Fv/Fm was associated with Ghazvini and then with Integerrima × Ghazvini, Sarakhs, Integerrima × Sarakhs, Badami Zarand and Integerrima × Badami Zarand rootstocks (Fig. 7b). The most cold-tolerant rootstock was Ghazvini. On the other hand, Integerrima × Badami Zarand was the most cold-sensitive one.

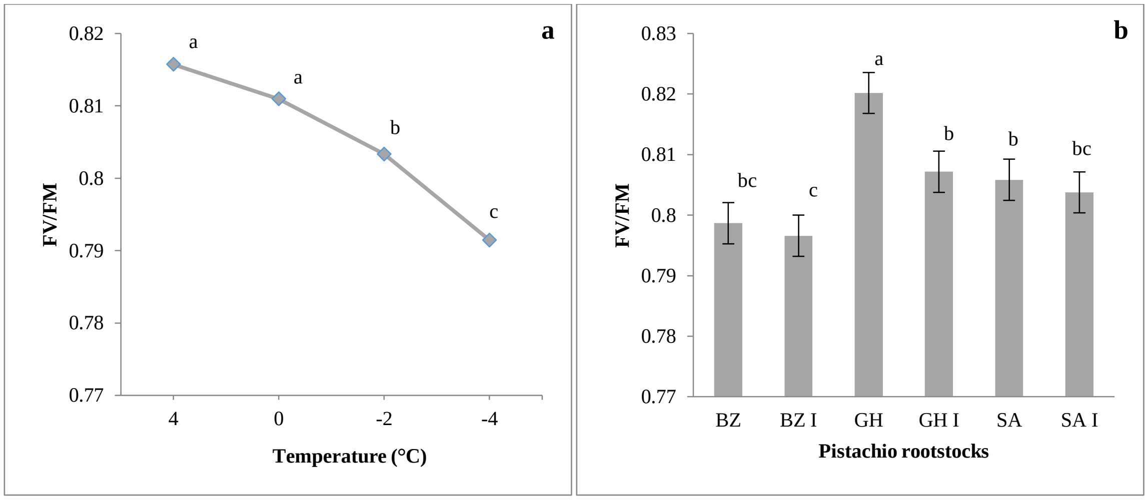

Fig. 7. The impact of thermal treatments (a) on Fv/Fm ratio of pistachio rootstocks (b): Badami Zarand (BZ), Integerrima×Badami Zarand (BZ-I), Ghazvini (GH), Integerrima×Ghazvini (GH-I), Sarakhs (SA), Integerrima × Sarakhs (SA-I). (Means followed by the same letter are not significantly different at 5% probability using Duncan's test)

Photosynthesis efficiency index (PI)

PI index was significant in different pistachio rootstocks and temperature treatments; nevertheless, the interaction effect of rootstock and temperature was not significant (Table 1). The most and the least PI indices were observed under 4 and -4°C, respectively (Fig. 8a). Ghazvini rootstock showed the highest, while Integerrima ×Badami Zarand indicated the lowest PI index (Fig. 8b).

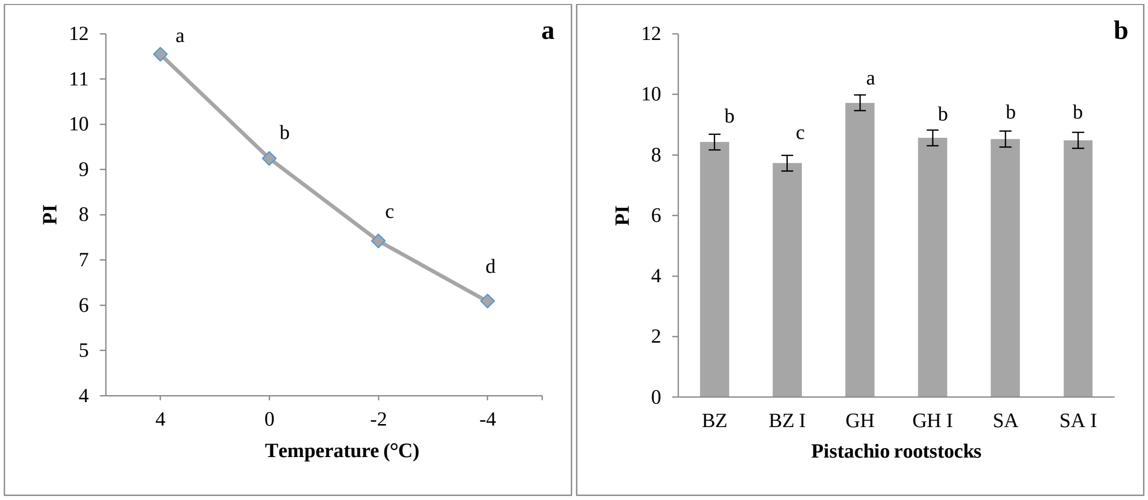

Fig. 8. The impact of thermal treatments (a) on PI index of pistachio rootstocks (b): Badami Zarand (BZ), Integerrima×Badami Zarand (BZ-I), Ghazvini (GH), Integerrima×Ghazvini (GH-I), Sarakhs (SA), Integerrima × Sarakhs (SA-I). (Means followed by the same letter are not significantly different at 5% probability using Duncan’s test)

Correlations

The results of the correlation showed a significant negative correlation between ionic leakage of cold-stressed leaves and the rest of traits (Table 2). In addition, there was a significant correlation between the initial pH and the final pH (0.846**). Chlorophyll a had the highest significant negative correlation with ionic leakage.

Table 2. Correlation analysis of some physiological Characteristics in cold-exposed pistachio rootstocks.

Characteristics	Ionic leakage	Initial pH	Final pH	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoid	Fv/Fm	PI
Ionic leakage	1								
Initial pH	-.836**	1							
Final pH	-.797**	.846**	1						
Chlorophyll a	-.910**	.825**	.854**	1					
Chlorophyll b	-.841**	.859**	.866**	.949**	1				
Total chlorophyll	-.875**	.848**	.900**	.937**	.929**	1			
carotenoid	-.893**	.851**	.874**	.958**	.954**	.957**	1		
Fv/Fm	-.816**	.772**	.765**	.750**	.780**	.731**	.761**	1	
PI	-.879**	.888**	.920**	.958**	.950**	.935**	.948**	.792**	1

* and **; significant at the 5% and 1%, respectively.

Discussion

Cold stresses disrupt cell membrane activity and cause intracellular electrolytes leakage (Azzarello *et al*., 2009; Palonen, 1999). In this study, ionic leakage as a sign of membrane damage at 4°C was significantly lower than -4°C. An increase in electrolyte leakage can be attributed to membrane degradation due to the cold stress. Temperature drop resulted in an increase electrolyte leakage in grape (Ershadi & Taheri, 2013), apricot (Afshari *et al*., 2014), olives (Barranco *et al*., 2005, Moshtaghi *et al*., 2009, Azzarello *et al*., 2009), and almond (Khorram *et al*., 2011). Moreover, low ionic leakage is related to cold-resistant rootstocks of grape (Lu *et al*., 2012) and almond (Imani *et al*., 2011). According to our results, Integerrima × Badami Zarand showed the most leakage; and Ghazvini and Integerrima × Ghazvini rootstocks experienced lower leakage. Hokmabadi *et al*., (2016) reported that the pH of the leaked solution could be a suitable tool to screen cold tolerant or sensitive pistachios. According to the obtained results, the pH of the solution strongly decreased with decreasing temperature.

Chlorophyll is the most important plant photosynthesis pigment playing a variety of physiological roles, such as absorbing light to be used in photosynthesis (Ahmadi *et al*., 2009). Low temperatures may disrupt chlorophyll synthesis and damage the chloroplast structure (Mirmohammadi Meybodi & Tarkesh Isfahani, 2004). The finding of the present study suggested that cold stress reduced chlorophyll a, b and total chlorophyll contents. Same results were reported in Mexican lime (Baghbanha *et al*., 2007), tangerine (Tajvar *et al*., 2011) and tomato (Jafari *et al*., 2006). According to the results, the most chlorophyll a, b and total chlorophyll contents were observed in Ghazvini and Integerrima × Ghazvini rootstocks; whereas, Integerrima × Badami Zarand had the least chlorophyll a, b and total chlorophyll contents. Keshavarz and Modaress sanavi (2014) reported less chlorophyll reduction in cold-resistant cultivars.

Carotenoids play a significant role in photosynthesis system protection (Niyogi, 1999). According to the results of this study, the reduction of temperature reduced the carotenoids. It seems that, under cold stress, the reduction of plant carotenoids is due to oxidation of this pigment by an active oxygen species (Berova *et al*., 2002). These results are consistent with the results of Jafari *et al*., (2006) and Tajvar *et al*., (2011) in tomato and tangerine, respectively.

The photosynthetic efficiency index reflects the current physiological state of the plant, as well as damage to photosynthetic system in stress-exposed plants (Strasser *et al*., 2000). Photosystem II is the first part of the plant photosynthetic system, reacting with environmental stresses (Baker, 1991, Terzaghi *et al*., 1989). The Fv/Fm, indicating photosynthetic yield, decreases in cold-exposed plants (Percival & Henderson, 2003, Liu & Huang, 2002). The results of the current study were consistent with Baghbanha *et al*., (2007) in Mexican lime, Hakam *et al*., (2000) in hybrid roses and Simkeshzadeh *et al*., (2010) in olive, who reported a decrease in Fv/Fm under low temperature conditions. Lahijanian *et al*., (2012) stated that cold-resistant cultivars have higher Fv/Fm and the ratio decreased with decreasing temperature. According to the results, the most and the least Fv/Fm and PI were observed in Ghazvini and Integerrima × Badami Zarand rootstocks, respectively.

Conclusions

In this research, cold stress made a reduction in chlorophyll (a, b, total) content and carotenoid contents, chlorophyll fluorescence and photosynthesis efficiency index of pistachio seedlings, while it significantly increased electrolyte leakage. The Integerrima× Ghazvini and Ghazvini rootstocks had the most chlorophyll (a, b, total) content, carotenoids, pH of leaked solution, Fv/Fm, and PI index and the least ionic leakage. Moreover, Integerrima × Ghazvini and Ghazvini rootstocks could be introduced as the most cold-resistant and Integerrima × Badami Zarand as the most cold-sensitive rootstocks.

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