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Article

Pomegranate freezing tolerance evaluation and its relationship with biochemical parameters

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Abstract: Low temperature is one of the most significant environmental factors that threaten the survival of subtropical and tropical plant species. By conducting a study, which was arranged in a completely randomized design with three replicates, the relative freezing tolerance (FT) of four Iranian pomegranate cultivars, including 'Alak Torsh', 'Tabestaneh Torsh', 'Poost Sefid', and 'Poost Syah', as well as its correlation with some biochemical indices, were investigated. From each cultivar, pieces of one-year-old shoot samples were treated with controlled freezing temperatures (−11, −14, and −17 ℃) to determine lethal temperatures (LT_{50}) based on survival percentage, electrolyte leakage, phenolic leakage, and tetrazolium staining test (TST) methods. Results showed that FT was higher in the second year with a lower minimum temperature and a higher concentration of cryoprotectants. The stronger correlation of electrolyte leakage with survival percentage ($r = 0.93$***) compared to the other three indices explained that this index could be the most reliable injury index in determining the pomegranate FT to investigate freezing effects. Of all four cultivars, 'Poost Syah' was the hardest by presenting a higher FT than ~ −14 ℃ in mid-winter. Accordingly, this pomegranate cultivar seems to be promising to grow in regions with a higher risk of freezing and to be involved in breeding programs to develop novel commercial cultivars.

Keywords: electrolyte leakage; lethal temperature; phenolic leakage; survival percentage; tetrazolium staining test

1. Introduction

Pomegranate (*Punica granatum* L.) is a subtropical fruit tree that grows in a wide range of climate conditions, from temperate to tropical regions, worldwide [1]. Iran is one of the largest pomegranate producers in the world, with a remarkable diversity of pomegranate genotypes and a high annual production of approximately 1 million tons. A limited number of native pomegranate cultivars in central Asia can survive at temperatures below −20 ℃, but most others cannot withstand temperatures below −15 ℃ for a long time [2]. Hence, severe freezes during the winter and temperature fluctuations impose irreparable economic losses on pomegranate orchards [3]. The adverse effects of this factor have been observed more in the margins of the desert, where the minimum temperatures may drop below −20 ℃ in winter. For example, in winter 2007, declining temperatures to −20 ℃ for three days severely damaged about 35,000 ha of pomegranate orchards in Iran [4].

Generally, plants from temperate and boreal zones can limit freezing-induced damage by developing an adaptive mechanism; named cold acclimation [5]. This complex and species-dependent mechanism can increase the freezing tolerance (FT) by intricate alteration in physiological and biochemical events taking place in cells. These modifications include changes in cell wall and membrane composition,

activation of antioxidant systems, cryoprotective compound production, and protein synthesis [6–8], all of which are regulated by transcriptional regulatory networks [9]. The accumulation of soluble carbohydrates, proline, and other low-molecular-weight osmolytes plays a pivotal role in developing the cold hardiness in plants through osmotic adjustment, scavenging reactive oxygen species (ROS), and starting up signaling transduction [10,11]. Low temperatures also induce secondary oxidative stress, which results in disturbing the redox homeostasis in cells by excessive accumulation of ROS. The main destructive effect of ROS is lipid peroxidation, which leads to damage to the unsaturated fatty acids and subsequently results in the formation of malondialdehyde (MDA) [12]. In order to scavenge ROS, plants possess a series of enzymatic and non-enzymatic antioxidant protective networks, which are normally responsible for keeping ROS levels in balance [13]. Phenolic compounds are one of the main elements of the non-enzymatic antioxidant system; their metabolism and accumulation are modified in response to biotic and abiotic stresses, for example, freezing stress [14]. Phenolic compounds are highly effective in free radical scavenging, electron-donating for stabilizing, delocalizing the unpaired electrons (chain-breaking function), chelating redox-active metal ions, and increasing the activity of oxidative enzymes [15,16]. A significant correlation was observed between specific metabolites and coping with adverse biotic and abiotic stresses [17,18]. Hence, they can be used as biomarkers to assess FT in plants. Reports validate the metabolite differences concerning adaptation and FT in different crops, including pomegranate [19,20].

One of the prerequisites for researching freezing stress in plants is exploring their FT status. Furthermore, the identification of more tolerant cultivars for breeding and cultivation programs passes through this process. For this purpose, the determination of minimum survival temperatures of plant genotypes or their tissues should be done under controlled freezing tests consistent with field conditions [21]. To enhance the reliability of the FT estimation, Sutinen et al. [22] suggested the use of two or more evaluation methods simultaneously and combined utilization of their results. Moreover, despite the ambiguous role of physiological parameters in relation to FT, the correlation of their changes seems to be an effective indicator in interpreting the results. Despite the spreading of commercial pomegranate cultivars to new areas, their low chill requirements (~200–600 h) [23], and high vulnerability to untimely warming and deacclimation in mid-winter [19], information about their physiological responses to freezing stress is limited. Accordingly, the present study aimed to (i) identify the relative FT of four Iranian pomegranate cultivars based on different freezing laboratory tests, (ii) compare different tests, and consequently detect the best fitting method, and (iii) analyze the correlation between some physiological indices and FTs.

2. Materials and methods

2.1. Plant materials

In this experiment, four ten-year-old pomegranate cultivars were used, which were supposed to differ compared with FT according to preliminary experiments during winter 2015 [24]. The cultivars namely; ‘Alak Torsh’, ‘Tabestaneh Torsh’, ‘Poost Sefid’, and ‘Poost Syah’ were growing at the Horticultural Research Station of

the University of Tehran, Karaj, Iran (35°48′ N, 50°57′ E, 1293 m a. s. l). In the experimental site, trees with a 3 × 4 m planting pattern were in similar conditions in terms of fertilization, irrigation, and other managing operations during growing seasons (March–October) in 2016 and 2017. Some meteorological data from two years of study related to the orchard site are presented in **Table 1**.

Table 1. Some meteorological data of the experimental site, Horticultural Research Station, University of Tehran, Iran.

Month	Temperature (°C)						Relative humidity (%)	
	Minimum		Maximum		Average			
	2016	2017	2016	2017	2016	2017	2016	2017
September	17.2	16.9	32.7	32.8	24.9	25.0	30.9	36.2
October	10.4	11.6	24.4	25.8	17.1	18.2	44.1	39.4
November	10.2	7.2	21.5	19.3	15.6	13.1	36.8	45.8
December	0.3	−1.7	10.9	8.7	5.6	3.2	50.4	50.2
January	2.3	0.0	12.3	9.2	6.7	4.4	48.5	59.8
February	−0.1	−1.6	8.6	6.1	4.2	1.9	64.4	68.9
March	5.3	2.1	16.5	13.4	11.2	7.6	51.3	51.8

The plant samples used in this experiment were one-year-old shoots without leaves, collected randomly from the middle parts of each pomegranate tree in mid-winter 2016 and 2017 (February). The collected shoots were transferred to the Biology Laboratory of the Department of Horticulture Science, University of Tehran. After the application of freezing treatments, a part of the samples was used for detecting FT status based on injury indices, including survival percentage, electrolyte leakage, phenolic leakage, and tetrazolium staining test (TST). The other part quickly froze in liquid nitrogen and was kept at −80 °C for measuring physiological parameters, including soluble carbohydrates, proline, total phenolic compounds, MDA, and DPPH scavenging capacity [25].

2.2. Freezing treatments

Shoot sections of each cultivar were put in a programmable freezing chamber after wrapping them in damp paper and sealing them in plastic bags. The freezing treatments, as the critical stressful temperatures, were determined for cultivars based on the previous experiments [19,24]. Accordingly, the freezing temperatures included −11, −14, and −17 °C, and the control (4 °C). To perform these treatments, stem segments were chilled from the starting temperature of 4 °C with the reduction rate of 2 °C h−1 until reaching the defined temperatures for each freezing treatment with a 6-hour stop in that temperature. After this period, the temperatures were returned to 4 °C at the same rate. Then, the samples were taken out of the freezer. For the control treatment, the shoot samples were kept at a constant 4 °C with similar time duration to other treatments [20].

2.3. Survival percentage

The survival percentage of pomegranate shoots was estimated as described by Balanian et al. [24]. For this purpose, twenty freezing-treated shoots from each cultivar

(with a length of 15 cm) were planted in pots filled with washed sand. They were transferred to the growth chamber with the average temperature of 23/18 ℃ (day/night), relative humidity of 75%–80%, photoperiod of 16/8 h (light/darkness), and light intensity of 200 μmols s^{-1} m^{-2}. After two-months maintaining under this condition and regular one-day interval irrigation, the survival percentage of samples were measured. The shoots with sprouted buds were considered alive, and those with non-sprouted buds were considered dead. The percentage of survival at each test temperature was defined as the number of live shoots/total shoots. This experiment consisted of three replications and each pot, which contained 20 shoots, was considered a replication.

2.4. Electrolyte leakage assay

A total number of three segments (each 1-cm long) were placed into 50-mL tubes containing 20 mL of deionized water as a replicate (three replications). Tubes were incubated on a shaker (250 rpm) for 24 h at room temperature, then the electrical conductivity (EC1) was measured. Subsequently, for determining the maximum electrolyte leakage, samples were autoclaved at 120 ℃ for 20 min, cooled at room temperature for 2 h, and again the EC (EC2) was measured [26]. Relative electrolyte leakage (REL) was calculated using the following equation:

$$REL = \left(\frac{EC1}{EC2}\right) \times 100 \quad (1)$$

2.5. Phenolic leakage assay

A modified method by Zieslin and Abolitz [27] was used to quantify the phenolic leakage of the stem samples. Some three stem segments with 1-cm long (~ 150 mg) from each treatment after rinsing with deionized water, were placed into 50-mL tubes containing 20 mL of distilled water and shook (250 rpm) for 24 h at room temperature. Then the presence of phenolic compounds leaking from the stems into the medium was determined spectrophotometrically by measuring the absorbance at 260 nm.

2.6. Tetrazolium staining test (TST)

The TST was based on the triphenyl tetrazolium chloride (TTC) reduction to the red formazan derivative by viable tissues. This assay was according to the modified method of Steponkus and Lanphear [28]. In brief, three segments with 1- cm long (~150 mg) were placed in 15-mL tube containing 5 mL of 1.0 % (w/v) TTC in 0.05 M Na2HPO4-KH2PO4 buffer (pH = 7.4). The tubes were subjected to vacuum to make sure uptake of the reagent uniformly by the tissues. After incubating at 25 ℃ for 15 h, the TTC solution was rinsed, and the tissues were briefly washed with distilled water. Then, the samples were extracted with 7 mL of 95% (v/v) ethanol in a boiling water bath for 10 min to recover the formazan from the tissues. The extracts were cooled and adjusted to a 20 mL volume with 95% ethanol. The absorbance of extract containing formazan was measured at 530 nm.

2.7. Quantitative evaluation of FT

FT was expressed as LT_{50} (the lethal temperature at which 50% of the tissues were dead or maximum electrolyte/phenolic leakage occurred) and calculated by fitting response curves with the following logistic sigmoid equation:

$$R = \frac{a}{1 + e^{b(x-c)}} + d \quad (2)$$

R was survival percentage, electrolyte leakage, phenolic leakage, or the amount of produced formazan, based on LT_{50} estimation method used; *x* is treatment temperature; *b* is slope of the function at the inflection point *c*, while *a* and *d* are the upper and lower asymptotes of the function, respectively [20].

2.8. Soluble carbohydrates

Soluble carbohydrates were quantified by the anthrone method, according to Yemm and Willis [29]. Soluble carbohydrate extraction was carried out and pooled three times by adding 1 mL of 80% ethanol to 0.1 g of ground stem tissues, then centrifuging for 15 min at 10,000 rpm, and separating supernatant. In the following, 1 mL of 0.2 % anthrone reagent (2 g anthrone in 1 L of 72% sulphuric acid) was added to 100 µL of the ethanolic extract. Then the reaction mixture was heated in a boiling water bath for 10 min and cooled on ice. The absorbance of samples was measured spectrophotometrically at 629 nm. Soluble carbohydrate concentration was calculated through a glucose calibration curve and reported as mg soluble carbohydrates g^{-1} fresh weight (FW).

2.9. Proline

According to Bates et al. [30], 0.1 g of ground stem samples were homogenized in 2 mL of 3% (w/v) aqueous sulfosalicylic acid and centrifuged at 10,000 rpm for 10 min. Afterward, 1 mL ninhydrin and 2 mL glacial acetic acid were added to 1 mL of supernatant. After boiling of mixture for 1 h, the reaction was stopped rapidly on ice. Then, 2 mL of toluene was added to the reaction mixture and vortexed for 10 s. The absorbance of the extract from the organic phase (upper phase) was monitored at 520 nm. Proline concentration was finally calculated through a calibration curve and expressed as µmole proline g^{-1} FW according to the following equation:

µmole proline g^{-1} FW= [(µg proline mL^{-1} × mL toluene)/115.5]/[(g sample)/5]

2.10. Total phenolic content

By applying the Folin–Ciocalteu method with some modifications, total phenolic content was measured in stem tissues based on Singleton and Rossi [31]. A volume of 300 µL of metanolic extract was added to 1.5 mL of 10 times diluted Folin–Ciocalteu reagent and 1.2 mL of 7.5% sodium carbonate. The mixture was allowed to stand for 90 min at room temperature in the dark before measuring the absorbance at 760 nm. Total phenolic content was expressed as mg gallic acid equivalent (GAE) g^{-1} FW against a calibration curve with gallic acid.

2.11. MDA content

The oxidative damage to lipids in the form of lipid peroxidation was determined by measuring the MDA content through a thiobarbituric acid (TBA) reaction, as reported by Health and Packer [32]. The amount of 0.1 g powdered stem was homogenized in 3 mL of 0.1% trichloroacetic acid (TCA) and then centrifuged at 12000 rpm for 10 min. Thereafter, 4 mL of 20% TCA containing 0.5% TBA was added to 1 mL of the supernatant aliquot. The mixture was heated at 95 ℃ for 60 min and rapidly cooled on ice. The supernatant absorbance was read at two wavelengths of 532 and 600 nm. After subtracting the non-specific absorbance at 600 nm, the MDA content was defined using an extinction coefficient of 155 mM cm^{-1} according to the following equation:

nmole MDA g^{-1} FW= (Abs532-Abs600 × 155) × (mL extract/g sample)

2.12. 1,1-diphenyl–2-picrylhydrazyl (DPPH)- scavenging capacity

The antioxidant activity of stem tissues was evaluated by the DPPH radical-scavenging method reported by Cheung et al. [33] with some modifications. Stem sections (1 g) were extracted with 10 mL of methanol. Then, 50 µL of the methanolic extract was added to 950 µL of 0.1 mM DPPH radical, vortexed, and incubated at room temperature in darkness for 30 min. Then, the absorbance was determined at 517 nm using a spectrophotometer. Methanol was used instead of the pomegranate stem sample as a control to determine the concentration of remaining DPPH. Antioxidant activity was expressed as the DPPH scavenging capacity using the following equation:

DPPH (%) = [(A517 control − A517 sample)/A517 control] × 100

2.13. Statistical analysis

The experiment was arranged based on a completely randomized design with three replicates (three trees per replication). Physiological indices and LT_{50} values were measured in February were analyzed by PROC GLM SAS 9.2 (SAS Institute, Inc., CARY, NC). Means were separated using Duncan's multiple range tests ($P \leq 0.05$). LT_{50} values were applied using the SigmaPlot software (Systa Software, Inc., California, USA). The data were presented as average, and figures were drawn by GraphPad Prism version 8 for Windows (GraphPad Software, La Golla California, USA).

3. Results and discussion

3.1. Freezing tolerance

According to the survival percentage-LT_{50} values, FT varied among cultivars in both years of the experiment. The highest FTs (lowest LT_{50} values) were observed in 'Poost Syah' by −14.32 and −14.66 ℃ for the first and second year, respectively. The results of the first year showed that 'Tabestaneh Torsh' (−10.42 ℃) was the most sensitive cultivar to freezing injury. However, in the second year, there was no significant difference between the FT of 'Alak Torsh' and 'Tabestaneh Torsh' (**Table 2**). A significant positive correlation was found between survival percentage, as the most similar index to field condition and other indices. The highest correlation was

observed between survival percentage and electrolyte leakage (r = 0.93***) (**Table 3**).

Table 2. LT_{50} values estimated by survival percentage (SP), electrolyte leakage (EL), phenolic leakage (PL), and tetrazolium staining test (TST) in four pomegranate cultivars in mid-winter.

Cultivar	LT_{50} values (°C)							
	SP		EL		PL		TST	
	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2
'Alak Torsh'	−11.40^{c} ±0.23	−13.21 ^{c} ±0.11	−11.98^{b} ±0.25	−13.13^{b} ±0.27	−11.00^{b} ±0.61	−12.34^{b} ±0.43	−11.57^{b} ±0.28	−12.11^{b} ±0.16
'Tabestaneh Torsh'	−10.42^{d} ±0.25	−13.56 ^{c} ±0.21	−12.18^{b} ±0.20	−13.68^{b} ±0.28	−11.14^{b} ±0.58	−12.21^{b} ±0.31	−10.50^{c} ±0.23	−11.98^{b} ±0.41
'Poost Sefid'	−13.73^{b} ±032	−13.95^{b} ±0.18	−13.66^{b} ±0.19	−13.68^{b} ±0.18	−11.15^{b} ±0.34	−11.98^{b} ±0.26	−11.31^{b} ±0.36	−12.45^{b} ±0.54
'Poost Syah'	−14.32^{a} ±0.31	−14.66^{a} ±0.43	−14.05^{a} ±0.43	−14.41^{a} ±0.24	−12.93^{a} ±0.32	−13.42^{a} ±0.41	−13.75^{a} ±0.82	−14.34^{a} ±0.37

Means in each column with the same letter are not significantly different at $P \leq 0.05$.

Table 3. Pearson correlation coefficients among LT_{50} values estimated by survival percentage (SP), electrolyte leakage (EL), phenolic leakage (PL), and tetrazolium staining test (TST) in four pomegranate cultivars in mid-winter.

Parameter	LT_{50} values (°C)			
	SP	EL	PL	TST
SP	1			
EL	0.93 ***	1		
PL	0.63 *	0.69 **	1	
TST	0.62 *	0.59 *	0.74 **	1

Significance is indicated by *** ($P \leq 0.001$), ** ($P \leq 0.01$), and * ($P \leq 0.05$).

As presented in **Table 2**, LT_{50} values of electrolyte leakage significantly divided cultivars into distinct tolerant and sensitive groups in both years. 'Poost Syah' with LT_{50} values at lower than −14.00 °C temperature had the highest FT, whereas 'Alak Torsh', 'Tabestaneh Torsh', and 'Poost Sefid' with LT_{50} values at higher than −13.70 °C temperature were the sensitive cultivars. Also, there were significant correlations between electrolyte leakage with survival percentage (r = 0.93***), phenolic leakage (r = 0.69**), and TST (r = 0.59*) (**Table 3**).

The FT of pomegranate cultivars was also investigated by the phenolic leakage in mid-winter. Although the relative tolerance of the cultivars was the same in both years, the FTs of the second year were higher than the first year, ~ 0.5–1.5 °C depending on the cultivar. The FT of 'Poost Sefid' and 'Poost Syah', based on phenolic leakage, were lower than values based on two former injury indices. Phenolic leakage, with less efficiency, could also sort out cultivars into two tolerant and sensitive groups in both years. 'Poost Syah' was the most cold-hardy cultivar, while 'Alak Torsh', 'Tabestaneh Torsh', and 'Poost Sefid', were categorized in the sensitive group with ~ 1.8 and ~ 1.3 °C less tolerance in the first and second year, respectively (**Table 2**). As shown in **Table 3**, phenolic leakage - LT_{50} values also significantly

correlated with three other injury indices - LT_{50} values, especially the survival percentage ($r = 0.63$*).

In this experiment, TST also significantly distinguished pomegranate cultivars in terms of FT. Based on the results of the first-year study, 'Poost Syah' and 'Tabestaneh Torsh' were the most tolerant (−13.75 °C) and the most sensitive (−10.50 °C) cultivars to freezing stress, respectively. However, 'Poost Sefid' (−11.01 °C) and 'Alak Torsh' (−11.57 °C), with moderate relative tolerance, were not significantly different (**Table 2**). In the second year, 'Poost Syah' was more tolerant up to ~ 2 °C than the other three cultivars. TST - LT_{50} values also had a significant correlation with the survival percentage - LT_{50} values, whereas the calculated coefficient was lower than that based on the electrolyte leakage index (**Table 3**).

3.2. Soluble carbohydrates

The effect of year on soluble carbohydrate concentration in four pomegranate cultivars was presented in **Figure 1a**. Accordingly, except for 'Poost Syah', the soluble carbohydrate concentration of three other cultivars was significantly lower in the first year than in the second year. With an increase of ~ 10 mg g^{-1} FW, 'Tabestaneh Torsh' showed the highest increase in the second year. However, the highest and the lowest soluble carbohydrate concentrations were detected in 'Poost Syah' and 'Tabestaneh Torsh', respectively, in both years of the experiment. Based on regression analysis of data, there was a linear relationship between soluble carbohydrates and LT_{50} values estimated based on all injury indices. Noticeably, soluble carbohydrate concentration negatively correlated with survival percentage ($r = -0.80$*), electrolyte leakage ($r = -0.75$*), phenolic leakage ($r = -0.91$**), and TST ($r = -0.97$***) LT_{50} values (**Figure 2a**).

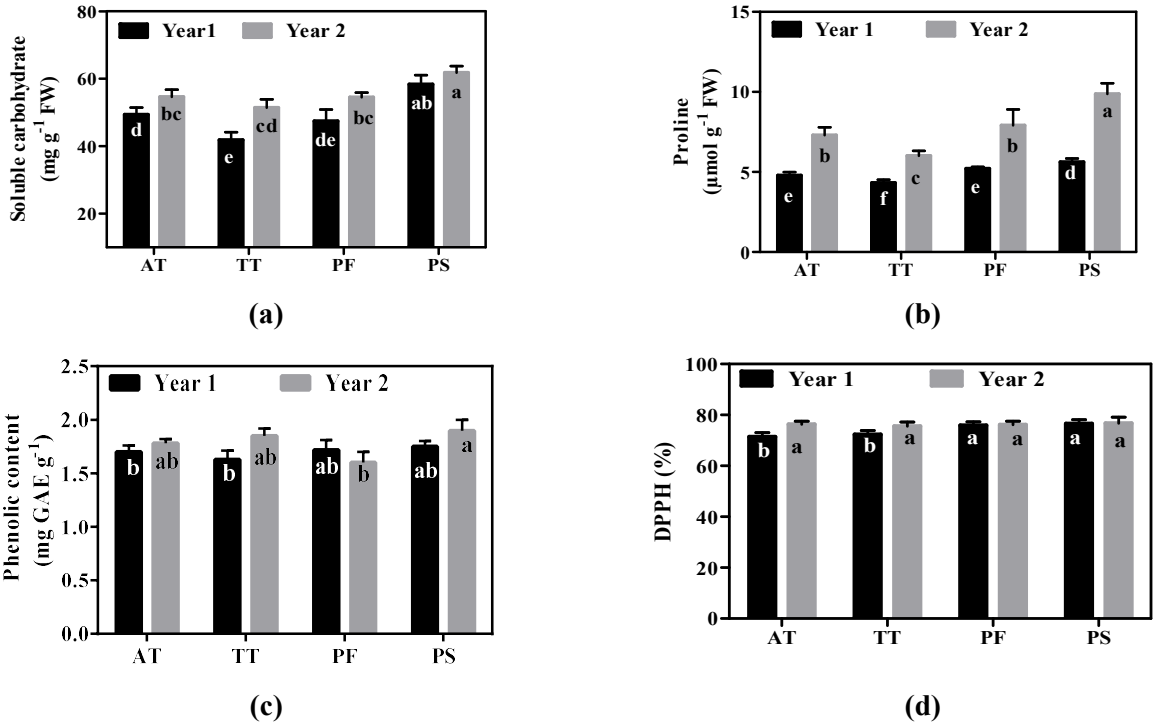
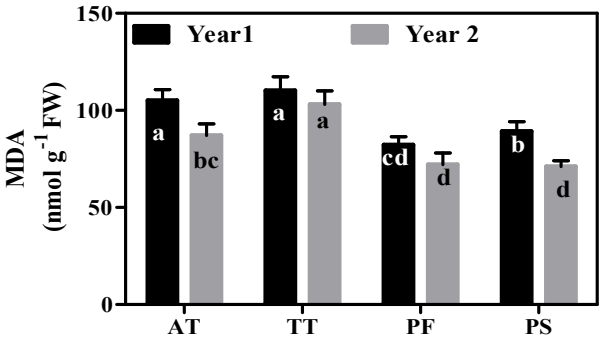
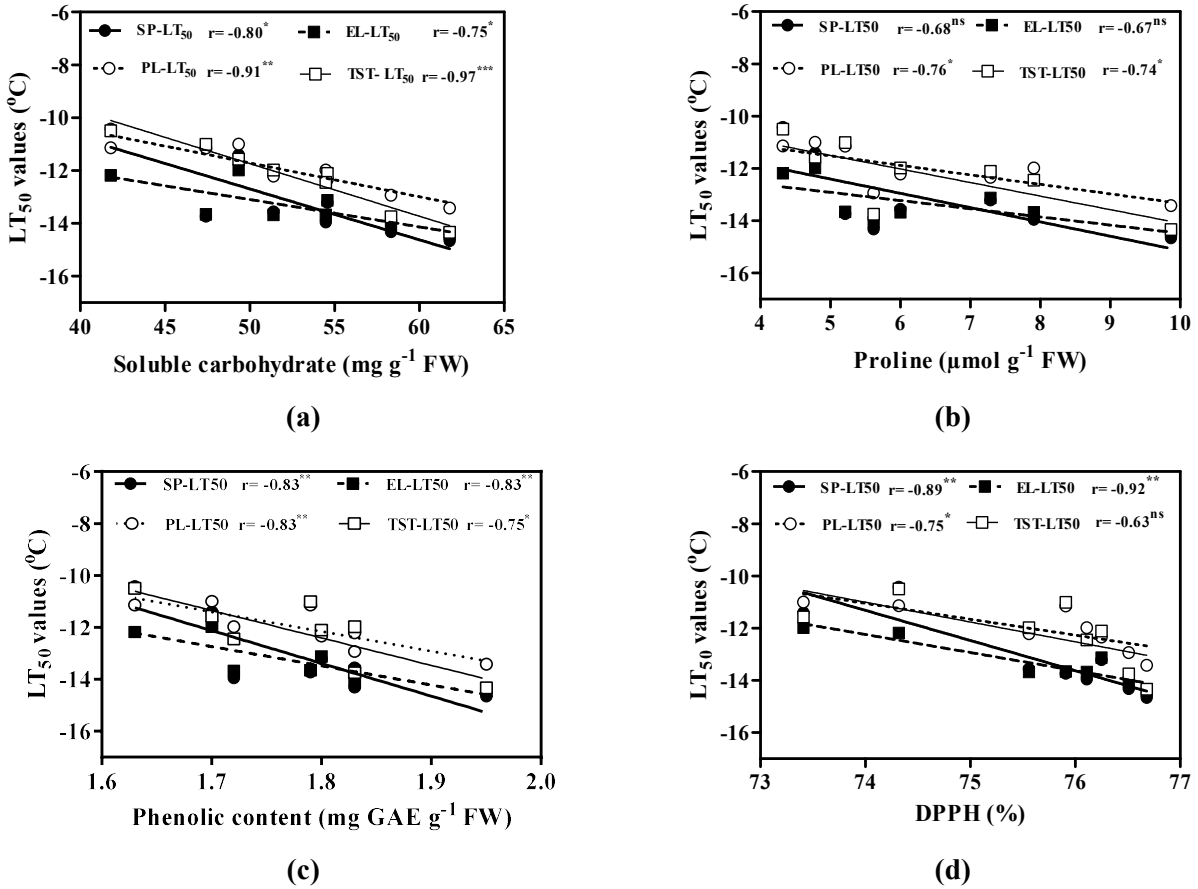


Figure 1. (*Continued*).



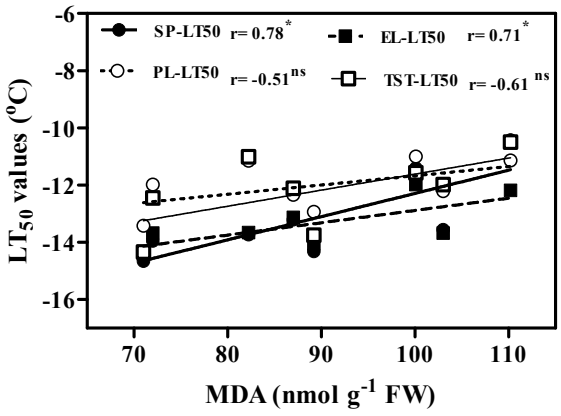
(e)

Figure 1. Physiological parameters of four pomegranate cultivars (AT = 'Alak Torsh', TT = 'Tabestaneh Torsh', PF = 'Poost Sefid', and PS = 'Poost Syah') in mid-winter. Similar letters on columns indicate non-significant differences among the cultivars at $P \leq 0.05$.



(a) **(b)** **(c)** **(d)**

Figure 2. (*Continued*).



(e)

Figure 2. Relationship between LT_{50} values estimated by survival percentage (SP), electrolyte leakage (EL), phenolic leakage (PL), and tetrazolium staining test (TST) and soluble carbohydrate **(a)**; proline **(b)**; phenolics content **(c)**; DPPH **(d)**; MDA **(e)** of four pomegranate cultivars. Significance is indicated by *** ($P \leq 0.001$), ** ($P \leq 0.01$), * ($P \leq 0.05$), or ns (non-significant).

3.3. Proline

As shown in **Figure 1b**, the proline concentration in the second year was ~1.3-fold higher than in the first year in all four cultivars. Higher proline content was observed by increasing the tolerance levels of all cultivars in both years. However, proline concentration had a narrow range of variation from 4.38 to 5.62 µmol g^{-1} FW among cultivars in the first year. In both years, 'Poost Syah', as the most tolerant cultivar, showed the highest proline concentration up to 5.62 and 9.78 µmol g^{-1} FW in the first and second year, respectively, while the least concentration was recorded in 'Tabestaneh Torsh' by 4.32 and 6.00 µmol g^{-1} FW in the first and second year, respectively, as the most sensitive cultivar. The regression analysis results showed a linear relationship between the proline concentration of the pomegranate cultivars and the degree of their FT. Also, significant negative correlations were observed between proline content with phenolic leakage ($r = -0.76$*) and TST ($r = -0.74$*) LT_{50} values (**Figure 2b**).

3.4. Phenolics content

According to the results, the phenolics content of studied cultivars, except for 'Poost Sefid, was higher in the second year compared to the first year. The cultivars did not differ significantly in terms of the phenolics content in the first year (**Figure 1c**). However, in the second year, the phenolics content significantly differed among cultivars so that 'Poost Sefid' (1.60 mg GAE g^{-1} FW), 'Alak Torsh' (1.78 mg GAE g^{-1} FW), 'Tabestaneh Torsh' (1.85 mg GAE g^{-1} FW), and 'Poost Syah' (1.90 mg GAE g^{-1} FW) showed the minimum to the maximum levels, respectively. A linear relationship significantly existed between total phenolics content and FT measurements (**Figure 2c**). Furthermore, phenolics content had a higher negative correlation ($r = -0.83$**) with survival percentage, electrolyte leakage, and phenolic leakage LT_{50} values as well as a lower correlation with TST - LT_{50} values ($r = -0.75$*) (**Figure 2c**).

3.5. DPPH scavenging capacity

DPPH scavenging capacity showed a low extent of increase in 'Alak Torsh' (~ 5%) and 'Tabestaneh Torsh' (~ 3%) in the second year, as compared to first year. Moreover, without any significant difference, two former cultivars showed the minimum antioxidant activity in the first year. The DPPH scavenging capacity of 'Poost Sefid' and 'Poost Syah' did not significantly differ compared to each other and did not change in both years (**Figure 1d**). There were also linear regressions between DPPH scavenging capacity and LT_{50} values measured by all four indices, with negative significant correlations between it and survival percentage (r = −0.89**), electrolyte leakage (r = −0.92**), and phenolic leakage (r = −0.75*) LT_{50} values (**Figure 2d**).

3.6. MDA content

Results presented in **Figure 1e** revealed that the MDA content was higher in the first year than in the second year in all four cultivars. In the first year, lower MDA content was observed with higher tolerance level. More tolerant cultivars, including 'Poost Sefid' (82.24 nmol g^{-1} FW) and 'Poost Syah' (89.31 nmol g^{-1} FW), showed the minimum MDA levels. 'Alak Torsh' and 'Tabestaneh Torsh', with the highest content of MDA, were not significantly different. Similarly, in the second year, 'Poost Syah' showed ~ 1.5-fold lower MDA content than 'Tabestaneh Torsh' (**Figure 1e**). According to **Figure 2e**, a linear regression existed between the MDA content and LT_{50} values estimated by survival percentage, electrolyte leakage, phenolic leakage, and TST measurement. MDA content also had significant positive correlations with survival percentage-LT_{50} values (r = 0.78*) and electrolyte leakage- LT_{50} values (r = 0.71*) (**Figure 2e**).

4. Discussion

In both years of the experiment, significant correlations among results of survival percentage, electrolyte leakage, phenolic leakage, and TST (**Table 2**) illustrated that these different methods properly differentiated pomegranate cultivars in terms of tolerance level to artificial freezing treatments. Furthermore, the LT_{50} values based on all these indices revealed that the FT of the pomegranate tree changed as a function of yearly weather conditions. It seems that lower minimum temperatures in autumn, especially subfreezing temperature in December (−1.7 °C), may act as a substantial signal to trigger further acclimation and enhance the FT in the mid-winter of the second year. Winter thaw and increasing temperature above 0.0 °C in January probably was another reason that resulted in precocious deacclimation and lower LT_{50} values (less negative) in the first year [19,21]. Meanwhile, survival percentage assessment has been considered the most accurate injury index [24], and its results have a more realistic reflection of the plant response to freezing stress. In line with this fact, in the current study, this method could distinguish all four pomegranate cultivars based on FT degree and divided them into tolerant, semi-tolerant/semi-sensitive, and sensitive to freezing treatments (**Table 2**). Similarly, survival percentage was applied as the most precise method to compare the FT degree of grape [34], olive [35], and pomegranate [24] cultivars. However, the plant material limitations and the time-

taking nature of this method makes its use impossible in some situations. Therefore, it seems that choosing a compatible approach with the results of the mentioned index has an effective role in accelerating FT estimation in a variety of plants, including pomegranate. In our study, survival percentage- LT_{50} values were at the highest correlation with electrolyte leakage- LT_{50} values (r = 0.93***) (**Table 3**), as the loss of normal plasma membrane activity and degree of damage to the cell [36]. Balanian et al. [24] and Ershadi et al. [37] also showed a significant and higher correlation between survival percentage and electrolyte leakage in pomegranate (r = 0.98) and grape (r = 0.90) cultivars, respectively.

Acclimation to the surrounding environment is associated with extensive metabolic changes at the cellular level in plants, which ultimately determinates their tolerance and/or vulnerability threshold to minimum survival temperatures during cold seasons [21]. Accumulation of soluble carbohydrates is considered as one of the most consistent mechanisms of FT in a wide range of plant species [38]. Nasrabadi et al. [25] stated that starch hydrolysis to soluble carbohydrates during the cold acclimation is highly associated with improved FT of pomegranate cultivars by mid-winter. In current study also negative and significant correlation was observed between soluble carbohydrate concentrations and LT_{50} values calculated based on all four injury indices (**Figure 2a**). Furthermore, the carbohydrate concentration was the highest in 'Poost Syah' as the cold hardiest cultivar as well as in the second year with lower minimum temperature (**Figure 1a**). These results confirmed the cryoprotectant functions of soluble carbohydrates in response to low temperature demonstrated in numerous preceding studies. It was unrevealed that soluble carbohydrates, specially sucrose, and its α-galactosyl derivatives, including raffinose and stachyose, were increased in early acclimation and remained high during the winter based on the seasonal studies on pomegranate [19], pear [39], peach [40], and blueberry [41]. Soluble carbohydrates notably reduce the freezing point and its damage, via playing major multiple roles in scavenging free radicals, energizing the respiratory cycles, strengthening the membrane structure, and regulating the osmotic potential [8,42].

As a non-toxic molecule with high solubility, proline plays a vital role in maintaining the function of proteins, antioxidant enzymes, and osmotic regulation in a wide range of abiotic stresses [43,44]. In spite of weaker correlation as compared to soluble carbohydrates with FT (**Figure 2a** and **b**), higher content of free proline in 'Poost Syah' as the most freezing-tolerant cultivar in both years confirmed the osmoprotectant role of this compound under freezing circumstances in pomegranate tree (**Figure 1b**). According to results, it seems that in second year, the increasing proline concentration in response to lower minimum temperatures of the late autumn and early winter during acclimation process enhanced the FT level in studied pomegranate cultivars in mid-winter (**Figure 1b**). These results were consistent with preceding reports on stress tolerance response in pomegranate [45], olive [36], and grape [46]. No significant correlations were observed between proline content and the survival percentage as well as electrolyte leakage LT_{50} values (**Figure 2b**), which were not in line with the previous reports on pomegranate [20,25]. The contradiction in the results of different research might be raised from different identity of studied cultivars, time of sampling, and other factors.

The phenolics content was strongly influenced by the interaction of cultivar and year. As shown in **Figure 1c**, the concentration of phenolics was higher at lower temperatures in the second year, and the increase of FT in 'Poost Syah'. It has been demonstrated that the increase of phenolics synthesis is another antioxidative response to abiotic stresses, for example, low temperature. Also, accumulation of these secondary metabolites is generally considered to be a prominent strategy in more hardy species and cultivars, enhancing plant tolerance via neutralization of the reactive oxygen species generated by lipid peroxidation under stress condition [15,47]. Present findings revealed that the level of phenolics content in 'Poost Syah' was significantly higher than 'Alak Torsh' and 'Tabestaneh Torsh' in both years (**Figure 1c**). This was in accordance with a previous report by Nasrabadi et al. [25] on pomegranate, who showed that the phenolics content was higher in severe stress and a more tolerant cultivar. A significant and high correlation was also observed between LT_{50} values measured by all injury indices and phenolics content in pomegranate cultivars (**Figure 2c**). Previous reports on different herbaceous and woody plants attributed the phenolics content to low-temperature tolerance. As instance, Saadati et al. [36] also reported a high correlation between phenolics accumulation, antioxidant capacity, and acclimation to low temperature in olive.

DPPH is a stable-free radical at room temperature, which accepts an electron/hydrogen radical to change into a stable molecule. Hence, DPPH is frequently applied as a substrate to examine the antioxidant activity [48]. The results indicated that a lower antioxidant activity was detected in 'Alak Torsh' and 'Tabestaneh Torsh', as more sensitive cultivars in the first year, and enhanced by decreasing the temperature in the second year, as a defensive response. However, 'Poost Syah' and 'Poost Sefid' showed the highest activity in both years (**Figure 1d**). Oxidative studies have shown that abiotic stresses are the main source of ROS causing damage to the cell membrane and exposing the plant to destruction [49]. High antioxidant components are necessary to compensate for the stress damage and to improve stress tolerance [50]. Based on a previous report, the enzymatic antioxidant activity system at early acclimation had a crucial role in eliminating low-temperature induced ROS in studied pomegranate cultivars [19]. Providing better conditions for the start and deepening of the acclimation process in the second year of the current experiment increased the antioxidant activity and decreased MDA in more sensitive cultivars, especially 'Alak Torsh' (**Figure 1d**). Moreover, higher correlations were observed between antioxidant activity and estimated LT_{50} values based on survival percentage and electrolyte leakage (**Figure 1d**). These results suggest that scavenging ROS is a crucial factor that determines the survival and tolerance of pomegranates under adverse environmental conditions. These data alongside the results reported previously regarding pomegranate [25] and olive [36,51] explain that the high level of DPPH scavenging capacity, at least in subtropical fruit trees, could be assumed as the main defensive reaction to keep cell integrity under freezing stress.

In parallel to the severity of oxidative stress, lipid peroxidation of membranes is intensified and the low molecular weight aldehydic carbohydrates are produced [52]. In this present experiment, the concentration of MDA as one of the membrane peroxidation products and an efficient indicator to assess the intensity of oxidative stress-cell integrity was measured. MDA concentration was significantly different

among four pomegranate cultivars and between two years. The highest MDA accumulation in 'Alak Torsh' and 'Tabestaneh Torsh' in the first year exhibited their most vulnerability to destructive effects of low temperature-induced oxidative stress, as a result of weaker development of the acclimation process in warmer autumn. Whereas the lowest level of MDA in 'Poost Syah' and 'Poost Sefid' was related to better FT of these cultivars due to better biochemical adaptation during autumn and mid-winter, especially in the second year (**Figure 1e**). Saadati et al. [36] also successfully used this biomarker for dividing olive cultivars based on sensitivity degree to low temperature. Similarly, Wang et al. [53] reported 2-fold higher MDA in 'M9', as a low-temperature sensitive apple rootstock compared to 'G256', as a low-temperature tolerant rootstock. In the current research, a closer correlation between MDA and FT was observed by survival percentage and electrolyte leakage confirmed the above observation and demonstrated the direct relationship between MDA concentration and the intensity of damage and/or sensitivity under freezing treatments (**Figure 2e**).

5. Conclusion

Finding a suitable climatic zone to fit the chilling requirements and FT of specific pomegranate cultivars can be considered a crucial step for reducing freezing damages, especially under high temperature fluctuations in mid-winters. In this present experiment, recording a higher correlation than 90% between electrolyte leakage and survival percentage ($r = 0.93$***) showed that this index can be the most practical and reliable method for determining the minimum tolerable temperature in pomegranate cultivars for fitting to climatic conditions. However, current results showed that FT in pomegranate cultivars changed as a function of yearly air temperature, affecting the accumulation of cryoprotectants (such as soluble carbohydrates, proline, and phenolic compounds) and antioxidant activity during the acclimation process. A lower minimum temperature in the second year, as a stronger promotor, stimulated the biochemical signaling chains and resulted in a higher FT in all four pomegranate cultivars. The lowest LT_{50} values were observed in 'Poost Syah', which represented that this cultivar is the most promising one to grow in areas with a higher risk of freezing indices and could also be considered to be involved in conventional and modern breeding programs to develop novel commercial cultivars. Although grafting is not a prevalent method in pomegranate propagation worldwide, it is worth mentioning that 'Poost Syah' can be used as a reliable cold-tolerant rootstock for propagating the sensitive commercial cultivars in most-at-risk cold regions.

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