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

Screening Pomegranate Cultivars for Freezing Tolerance by Reliable Methods

Ali Akbar Ghasemi-Soloklui[1], Ahmad Ershadi[2]*

1 Nuclear Agriculture Research School, Nuclear Science and Technology Research Institute (NSTRI), Karaj, P.O. Box 31485498, Iran. E-mail address: Aliakbarghasemisoloklui@gmail.com.[https://orcid.org/0000-0002-3565-8051]

2 Department of Horticultural Science, Faculty of Agriculture, University of Bu-Ali Sina, Hamedan, Iran.

ARTICLE INFO

Article history:

Received: 30 January 2022,
Received in revised form: 3 May 2022,
Accepted: 15 July 2022

Article type:

Research paper

Keywords:

Acclimation,
Cold hardiness,
Osmoregulants,
Proline

ABSTRACT

Freezing damage is a significant environmental challenge that limits both the geographic distribution and production of pomegranates in the world. The objective of this research was to search for useful correlations between freezing tolerance and soluble carbohydrate and proline content, as well as stomatal density in different parts of the leaves, to allow indirect selection of winter hardiness in pomegranate cultivars. Our results showed that freezing tolerance at the non-acclimated stage (August) was not strongly correlated with freezing tolerance during deep winter dormancy in January. Cold tolerance in summer was strongly correlated with leaf stomatal density; however, a moderate correlation was observed between cold tolerance in winter and leaf stomatal density. The results showed that 'Alak', the most cold-tolerant cultivar in summer and winter, had the highest leaf stomatal density. The 'Agha Mohammad Ali' cultivar had the lowest leaf stomatal density and cold tolerance in summer; however, this cultivar showed considerable cold tolerance in winter. Moreover, except for 'Agha Mohammad Ali', stomatal density in summer was related to soluble carbohydrate concentration in the stems, which could justify the correlation between stomatal density and winter hardiness in pomegranate trees. It was found that LT_{50} values in January were negatively related to soluble carbohydrate concentration in the stems. However, there was no statistical correlation between winter hardiness in January and proline content in the stems. These results suggested that soluble carbohydrates and stomatal density are suitable indices for predicting freezing-tolerance of pomegranate cultivars throughout the year and growing season, respectively.

Introduction

Pomegranate (*Punica granatum* L.) is one of the most important fruit trees of Iran, which is also commercially cultivated in many areas including the Mediterranean Basin, South Asia and several countries in North and South America. Iran is one of the largest pomegranate-producing countries and has an enormous genetic diversity of pomegranates with a germplasm collection of over 700 cultivars and accessions (Ghasemi-Soloklui et al., 2019). Lack of cold tolerance is a major constraint to the spread and productivity of subtropical fruits, so freezing damage causes significant economic losses in many regions of the world. The degree of freezing resistance and acclimation varies among species, cultivars, and ecotypes, showing genetic variability in cold tolerance and genetic adaptation to location (Reyes-Díaz et al., 2005; Rowland et al., 2005; Kalberer et al., 2007; Pagter et al., 2008). Determination of freezing damage after artificial subzero temperatures in the laboratory is a useful tool for comparing the freezing tolerance of different pomegranate genotypes and cultivars

*Corresponding author's email: ershadi@basu.ac.ir

(Ghasemi-Soloklui et al., 2012). After exposure to freezing temperatures, the electrolyte leakage test is a useful tool to distinguish between cold hardy, and susceptible pomegranate cultivars (Ghasemi-Soloklui et al., 2012). In addition, determining the winter hardiness of plants is usually very tedious, time-consuming, and not always easy, so simple screening methods have been developed for comparative studies (Mittelstädt 1965; Fischer 1992; Hummer et al., 1995). Most methods for determining the freezing tolerance of plants are based on controlled freezing tests. However, indirect indicators for determining winter hardiness without freezing tests have also been sought for many years (Lindén 2002). Cold hardiness is related to anatomical and morphological traits of plants (Palta et al., 1979). Stomatal density index in plant leaves is a numerical trait that is genetically determined (Gailing 2008). Therefore, plant breeders can use stomatal density to make rapid progress in their breeding programs (Falconer et al., 1996). Numerous researchers found a relationship between small cell size (Levitt et al., 1936), stomatal density index (Roselli et al., 1989), stomatal size (Nassuth et al., 2021), and degree of cold hardiness. The correlation between stomatal density index and cold tolerance of olive (Soleimani et al., 2002), pecan (Sagaram et al., 2007), walnut (Aslamarz et al., 2009) and rhododendron (Wang et al., 2008) has been previously reported. During cold acclimation, several metabolic changes occur in plants before they adapt to the environment. In addition, strong correlations have been found between cold stress tolerance and the content of osmoregulants such as soluble carbohydrates (Morin et al., 2007), proline (Patton et al., 2007), glycine betaine (Ashraf et al., 2007), and proteins (Guy 1990). The selection and identification of cold-hardy cultivars are necessary for the increasing establishment of pomegranate crops and for the success of any breeding program to select winter-hardy cultivars. This will ensure that more cultivars can be evaluated without increasing costs and the more cultivars that are evaluated, the greater the chances of selecting winter-hardy cultivars in pomegranates. The objective of this study was to search for useful correlations between freezing hardiness and stomatal density in different parts of the leaves, soluble carbohydrates, and proline content for indirect selection of cold hardiness in pomegranate cultivars.

Material and Methods

Plant materials

One-year-old cuttings in winter (20 January) and fully developed leaves in summer (02 August) of six pomegranate cultivars, including 'Alak', 'Agha Mohammad Ali', 'Tabestaneh', 'Shirin Nar Paveh', 'Malas Torosh Saveh' and 'Golmakhane Orumieh', obtained from ~26-year-old trees at the Saveh Pomegranate Research Center in Arak. During transportation from the orchard to the laboratory for the freezing tests, samples (stems and leaves) were wrapped in damp paper, sealed in plastic bags, and stored at 5 °C. Samples of shoots (one centimeter-long) and leaves (1×1 cm^2) were cleaned in deionized water, chopped, and placed in 50 ml plastic tubes per replicate. To start ice formation, one ml of deionized water was added to each tube. To expose the tubes to low temperatures, they were placed in a freezing chamber (Kimia Rahavard, Tehran, Iran). The starting temperature was 5°C and the cooling rate was 2°C per hour. The treatment temperatures in two stages were:
Stage one (summer): 0°C, -4°C, -8°C, -12°C.
Stage two (winter): -12°C, -15°C, -18°C, -21°C, -24°C.

Electrolyte leakage

Before the first electrical conductivity evaluation (EC1), each tube was filled with 20 ml of deionized water, spun at 23°C (250 rpm) for one hour, and kept at room temperature for 24 hours. The samples were then autoclaved at 120°C for 20 minutes to allow maximum ion leakage. They were then cooled at room temperature for 2 hours and electrical conductivity (EC2) was measured again. Relative electrolyte leakage (REL) was calculated using the following formula: REL = (EC1 /EC2) ×100. Cold tolerance was expressed as LT_{50} (the lethal temperature at which 50% of the total ion leakage occurs) by fitting the response curves with the following logistic sigmoid function (Mancuso et al., 2000).

Stomatal density

Fully developed leaves were randomly taken from the middle part of the current season's shoots. The number of stomata was determined using the replica method (Soleimani et al., 2002). The stellate hairs were removed from the underside of each leaf using adhesive tape. A thin film of cellulose acetate was applied directly to the lower epidermis of the leaves and allowed to dry at room temperature before removal. Sections were taken from the center of each leaf and placed on coded slides. A binocular microscope with 40x magnification was used to count stomata. Stomatal density was determined over an area of 1 mm^2.

Soluble carbohydrates

The anthrone method was used to determine soluble carbohydrates (Yemm et al., 1954). Stem samples were pulverized with liquid nitrogen. Soluble carbohydrates were extracted three times from 1 g of powdered tissue with 5 ml of 80% ethanol and centrifuged at 1500 gn for 15 min. To 100 ml of the ethanolic extract, one ml of the 0.2% anthrone reagent (2 g of anthrone in 1 L of 72% sulfuric acid) was added. The reaction mixture was incubated in a boiling water bath for 10 minutes before being quickly cooled on ice. The absorbance at 620 nm was determined spectrophotometrically. Finally, using a calibration curve, the concentration of soluble carbohydrates was determined and expressed as mg soluble carbohydrates g-1 dry weight (DW).

Proline content

The concentration of free proline in the stem samples was determined as described by Bates et al. (1973). Stem samples were crushed in liquid nitrogen, and 0.5 g of the powdered tissue was homogenized in 10 ml of aqueous sulfosalicylic acid (3% (w/v)). The homogenate was then filtered using a No. 1 Whatman filter paper. Two milliliters of the filtered extract were obtained for analysis along with two milliliters of ninhydrin and two milliliters of glacial acetic acid. The reaction mixture was incubated in a boiling water bath for one hour before being stopped in an ice bath. The organic phase was extracted with four milliliters of toluene added to the mixture. A UV-visible spectrophotometer (Bel Engineering Srl, Monza, Italy) was used to measure the absorbance of the extract at 520 nm, while toluene served as a blank. Finally, using a calibration curve, the proline concentration was determined and expressed as µM proline g-1 fresh weight (FW).

Statistical analyses

All data were analyzed using (ANOVA) (PROC GLM, SAS Institute). Duncan's multiple range tests were used to differentiate means at $P \leq 0.05$. Pearson's correlation coefficient (PROC CORR) in SAS was also used to measure the correlation between pairs of attributes.

Results

Soluble carbohydrates

The amounts of soluble carbohydrates differed significantly ($p \leq 0.01$) among cultivars (Fig. 1). The highest amounts were found in 'Agha Mohammad Ali' (152.73 mg/g DW) and 'Alak' (134.4 mg/g DW), while the remaining cultivars did not show significant differences.

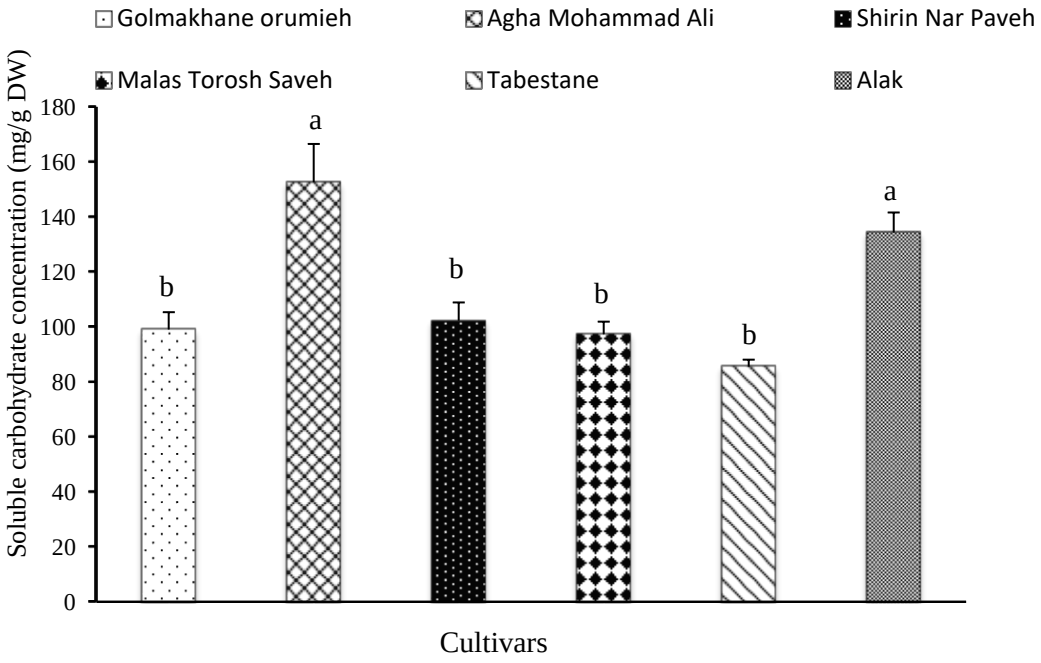

Fig. 1. Concentrations of soluble carbohydrates in stem samples of six Iranian pomegranate cultivars. Values are means ± standard errors of three replicates. Similar letters at the top of columns indicate non-significant differences among cultivars at $P \leq 0.05$.

Proline content

Proline content varied significantly ($p \leq 0.01$) within cultivars, ranging from 0.46 to 2.41 µM/g FW (Fig. 2). Stem samples of 'Agha Mohammad Ali' and 'Shirin Nar Paveh' contained the highest proline concentrations; 'Tabestaneh' had the lowest values. Other cultivars ('Alak', 'Malas Torosh Saveh', and 'Golmakhane Orumieh') had intermediate proline concentrations.

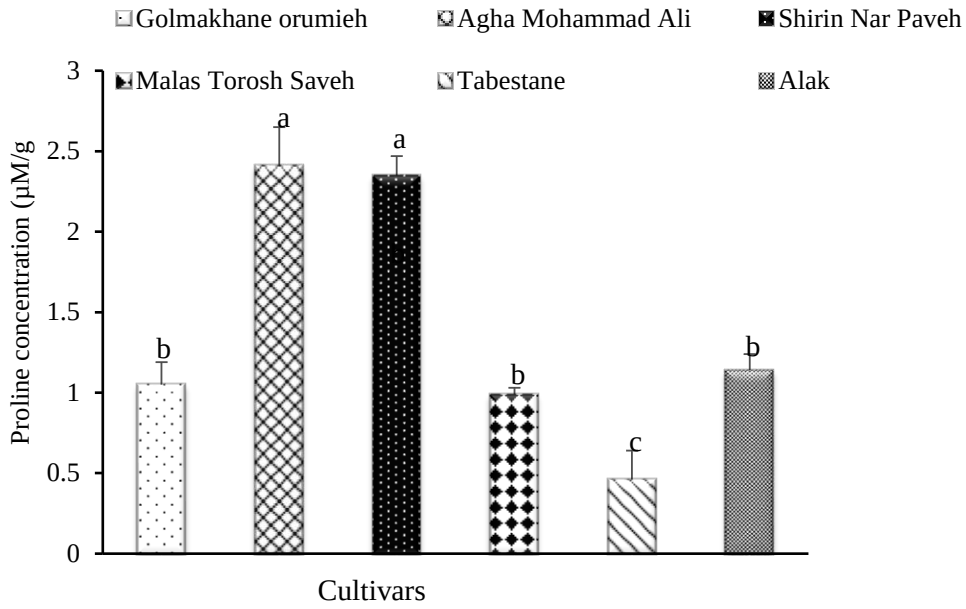

Fig. 2. Concentrations of proline in stem samples of six Iranian pomegranate cultivars. Values are means ± standard errors of three replicates. Similar letters at the top of columns indicate non-significant differences among cultivars at $P \leq 0.05$.

Stomatal density

As shown in Table 1, stomatal density on the three-leaf halves varied significantly among cultivars. In addition, stomatal density was not the same in the different parts of the leaves. Overall, the highest densities were observed in the middle part (91.72) compared to the apex (90.07) and base (86.25). 'Alak' and 'Agha Mohammad Ali' showed the highest and lowest stomatal densities in the basal parts of the leaves, respectively, while the other cultivars did not show significant differences. The highest variation in stomatal density was found in the middle part of the leaves. Based on the stomatal density in the middle part of the leaves, cultivars could be classified into three groups: 1. 'Alak' (maximum stomatal density), 2. 'Shirin Nar Paveh', 'Tabestaneh' and 'Malas Torosh Saveh' (medium stomatal density), and 3. 'Agha Mohammad Ali' and 'Golmakhane Orumieh' (minimum stomatal density). Concerning the apical part of the leaves, the cultivars showed similar rankings in stomatal density as in the middle part of the leaves, except for 'Tabestaneh', which showed a lower stomatal density in the leaf apex. In addition, cultivars had similar rankings for average stomatal density in the three parts of the leaves as in the middle of the leaves (Table 1).

Freezing tolerance

LT_{50} values differed significantly among cultivars in summer and winter (Table 2). In August, 'Alak' and 'Malas Torosh Saveh' showed the highest cold hardiness and 'Agha Mohammad Ali' was the least hardy cultivar, while 'Shirin Nar Paveh', 'Golmakhane Orumieh' and 'Tabestaneh' showed intermediate winter hardiness.

Winter freezing tolerance of all pomegranate cultivars increased drastically compared to the non-acclimation stage. On the other hand, pomegranate cultivars did not show a similar acclimation rate in winter. For example, the cultivar 'Agha Mohammad Ali', which was most sensitive to cold in summer, showed relatively high freezing tolerance in winter. Pomegranate cultivars can be divided into three groups based on their LT_{50} values in winter: 1) the cultivar with the highest freezing hardiness, 'Alak' (-23.50 °C), 2) the freezing-sensitive cultivar, 'Shirin Nar Paveh' (-18.78 °C) and 3) 'Agha Mohammad Ali', 'Golmakhane Orumieh', 'Tabestaneh' and 'Malas Torosh Saveh' with intermediate winter hardiness.

Table 1. Stomatal density ± standard errors, of six pomegranate cultivars at three parts of leaves (apex, center, and base) and means three parts per mm^2.

Cultivars	Base	Center	Apex	Means
Alak	109.95±0.92a [Z]	120.79±0.75a	116.73±0.15a	115.82±0.15a
Agha Mohammad Ali	62.65±0.42c	65.29±0.38d	67.34±0.43e	65.09±0.33d
GolmakhaneOrumieh	82.52±0.69b	82.75±0.38c	74.84±0.43d	80.04±0.42c
MalasToroshSaveh	89.20±0.46b	95.08±0.38b	94.91±0.35b	93.06±0.36b
Shirin Nar Paveh	89.40±0.37b	93.05±0.59b	97.54±0.47b	93.33±0.47b
Tabestane	83.76±0.37b	93.37±0.48b	89.08±0.36c	88.74±0.36b
Overall means	86.25	91.72	90.07	89.35
P values	≤0.001	≤0.001	≤0.001	≤0.001
CV (%)	6.67	5.62	3.84	3.45

[Z]: Similar letters in each column indicate non-significant differences among cultivars ($P \leq 0.05$).

Table 2. LT_{50} ± standard errors, estimated by electrolyte leakage measurement, of six pomegranate cultivars in August and January.

Cultivars	LT_{50}	
	August	January
Alak	-10.0±0.43 d	-23.50±0.18c
Agha Mohammad Ali	-4.16±0.23a	-21.05±0.20b [Z]
Golmakhane Orumieh	-4.66±0.28 a	-20.22±0.18b
Malas ToroshSaveh	-8.50±0.31c	-19.93±0.19ab
Shirin Nar Paveh	-5.73±0.35ab	-18.78±0.10a
Tabestaneh	-6±0.41ab	-20±0.23ab
Overall means	-6.13	-20.58
P values	≤0.001	≤0.001
CV (%)	15.44	2.52

[Z]: Similar letters at each column indicate non-significant differences among cultivars ($P \leq 0.05$).

Winter freezing tolerance of all pomegranate cultivars increased drastically compared to the non-acclimation stage. On the other hand, pomegranate cultivars did not show a similar acclimation rate in winter. For example, the cultivar 'Agha Mohammad Ali', which was most sensitive to cold in summer, showed relatively high freezing tolerance in winter. Pomegranate cultivars can be divided into three groups based on their LT_{50} values in winter: 1) the cultivar with the highest freezing hardiness, 'Alak' (-23.50 °C), 2) the freezing-sensitive cultivar, 'Shirin Nar Paveh' (-18.78 °C) and 3) 'Agha Mohammad Ali', 'Golmakhane Orumieh', 'Tabestaneh' and 'Malas Torosh Saveh' with intermediate winter hardiness.

Relationships between cold hardiness, stomatal density, and biochemical indices

High correlations were found between stomatal density in different parts of the leaves and cold hardiness in summer (Fig. 3). The correlation coefficients between summer LT_{50} values and stomatal density varied significantly among different parts of the leaves (Fig. 3). The highest correlation was observed between cold hardiness in summer and stomatal density in the middle of the leaf (r = -0.72) and in the leaf apex (r = -0.70), respectively, while stomatal density in the basal region had the lowest correlation with LT_{50} value in summer (r = -0.66) (Fig. 3). The highest correlation between stomatal density and winter hardiness (LT_{50}) was observed for the leaf middle part (r = -0.42) and leaf apex (r = -0.38),

respectively; the lowest correlation was observed between the stomatal density of the leaf basal part and winter hardiness (r = -0.35) (Fig. 4). Our results showed that freezing tolerance at the non-acclimated stage (August) was not strongly correlated with freezing tolerance during deep dormancy in January. In addition, a positive correlation was found between soluble carbohydrate content and stomatal density (r = 0.54) (Fig. 5). A high correlation (r = -0.56) was found between LT_{50} values and soluble carbohydrates in January; however, no significant correlation was observed between winter hardiness and proline content of stem samples.

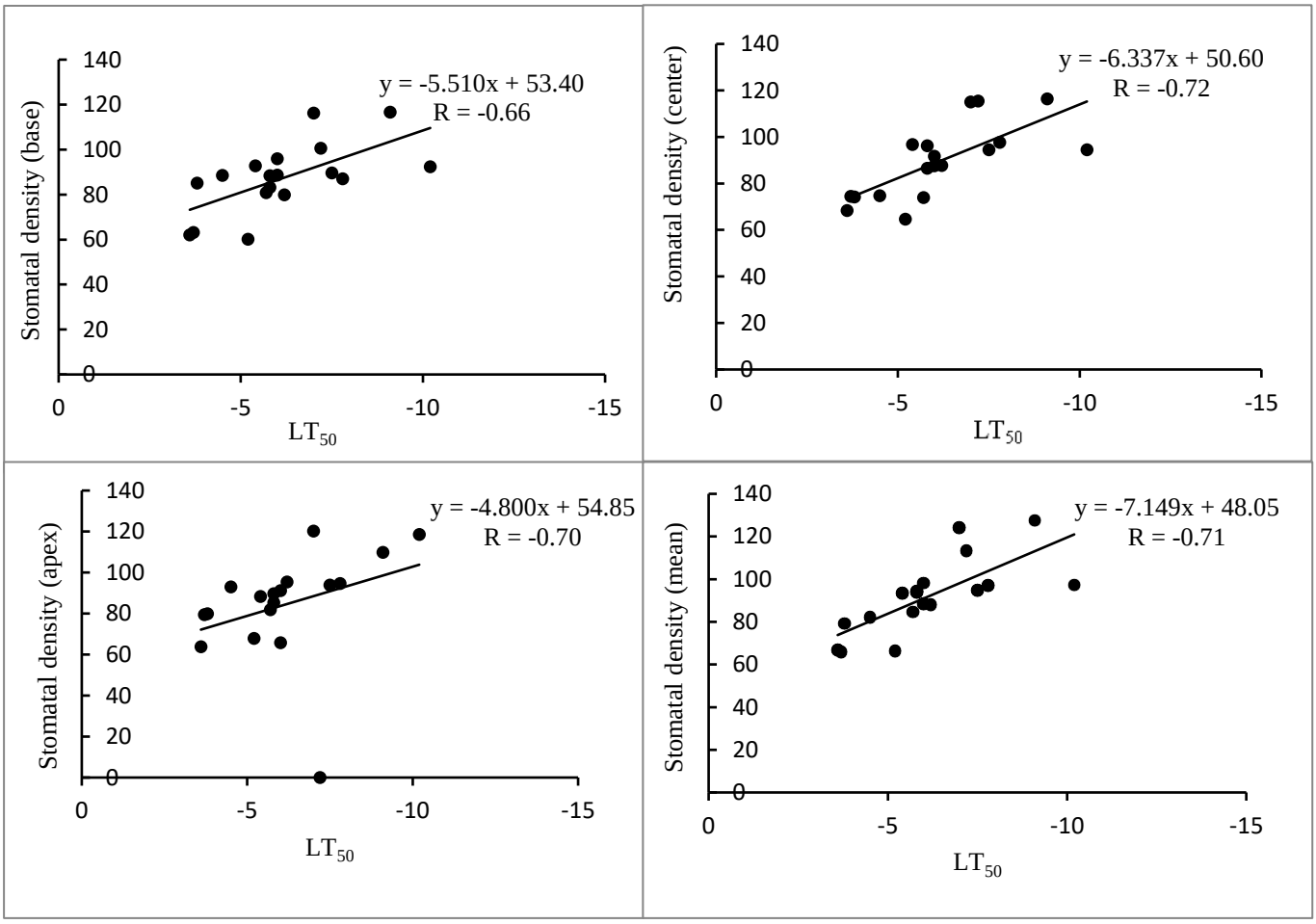

Fig. 3. Correlation coefficients between LT_{50}values of leaves in summer and stomatal density in different parts of leaves (apex, center, base and average of three parts) in pomegranate cultivars.

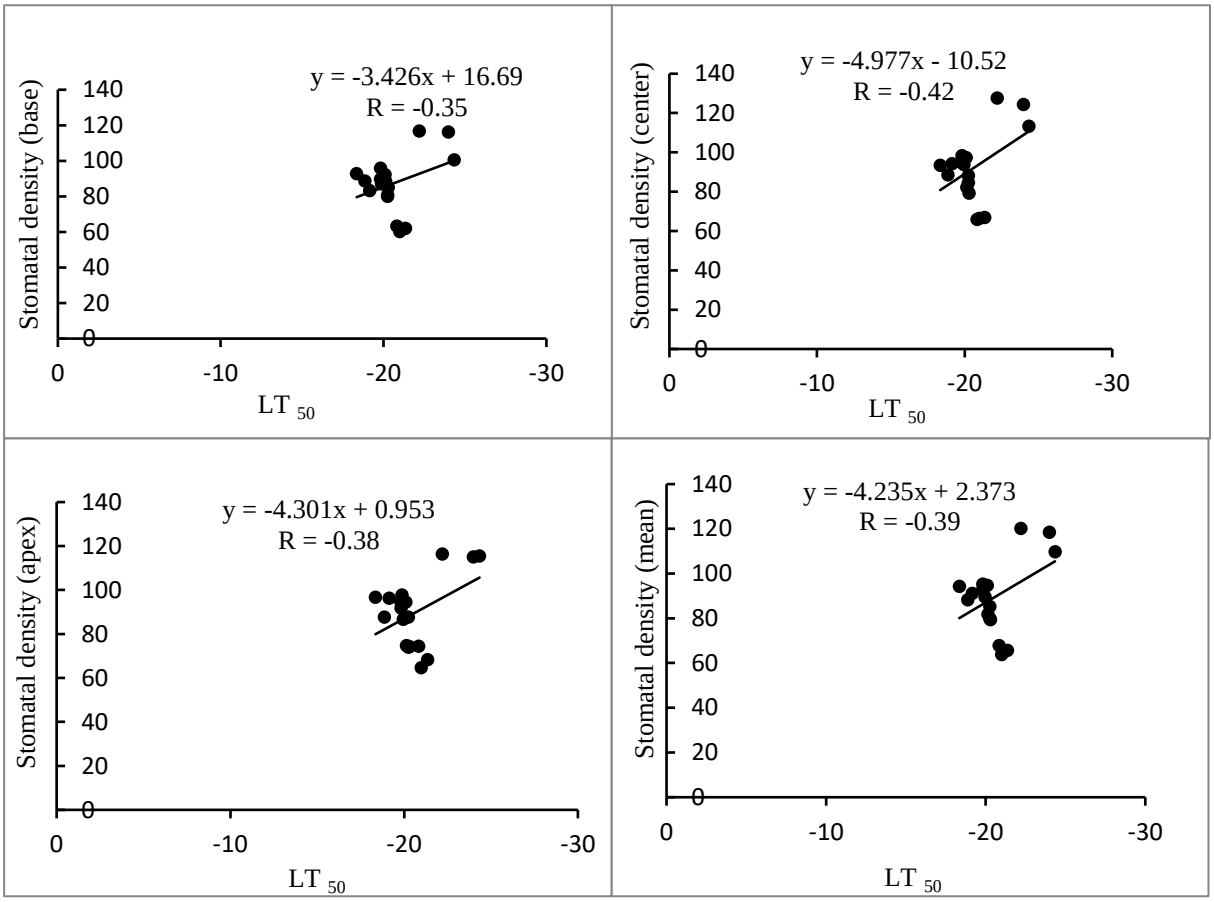

Fig. 4. Correlation coefficients between LT_{50} values of stems in winter and stomatal density in different parts of leaves (apex, center, base, and average three parts) of pomegranate cultivars.

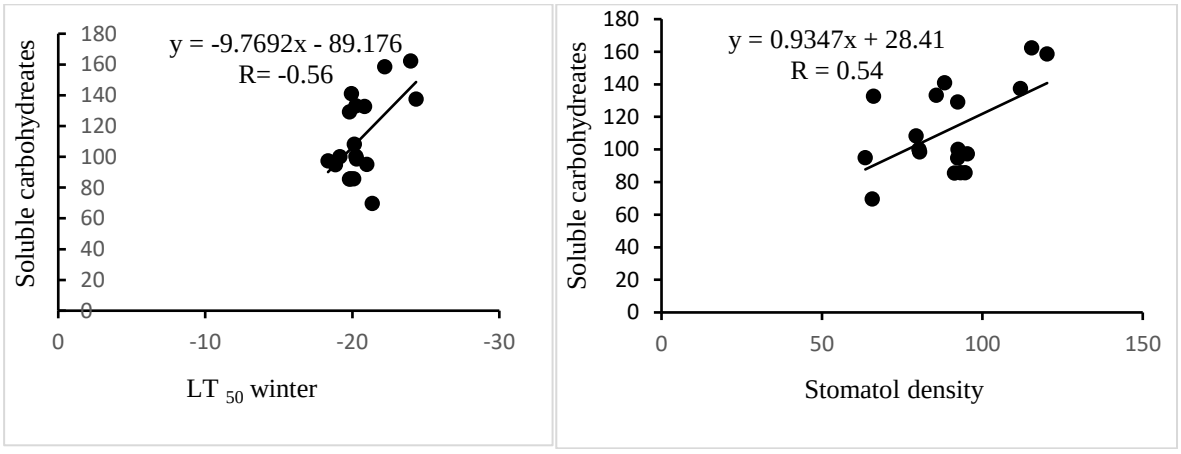

Fig. 5. Correlation coefficients between LT_{50}values in winter and stomatal density (average three parts) with soluble carbohydrates of pomegranate cultivars.

Discussion

Measurement of electrolyte leakage after controlled freezing allowed us to distinguish between different pomegranate cultivars in terms of their cold resistance. 'Alak' was the most freezing tolerant cultivar at both stages (August= -10°C; January= -23.50°C), while 'Agha Mohammad Ali' had the lowest and intermediate cold hardiness in August (-4.16°C) and January (-21.05°C), respectively. This cultivar had the lowest stomatal density in August, but the highest value of proline and soluble carbohydrates in January. The cold tolerance of non-acclimated pomegranates assessed in August was not reliable enough to predict the freezing tolerance of cold-acclimated plants in January. Assessment of the freezing tolerance of *Hydrangea macrophylla* (cold sensitive) and *H. puniculata* (cold resistant) revealed that the freezing tolerance of both species was -5 °C in September and October, but reached a maximum of -17 and -30 °C, respectively, in January (Pagter et al., 2008). The weak correlation between the cold tolerance of the different cultivars in August and January suggests that freezing tolerance and adaptation to low temperatures are not necessarily due to similar physiological and biochemical characteristics. Previous studies on commercial Iranian pomegranate cultivars have shown that the most cold-tolerant cultivars could withstand freezing temperatures of -22°C in January, while the less winter-hardy cultivars reached LT50 at -20 to -20.5°C (Ghasemi-Soloklui et al., 2012). In this study, the cold tolerant cultivars withstood -23.5°C. Also, Sirooeinejad et al. (2020) reported that LT50 values for some Iranian pomegranate cultivars were around -17 ºC in January.

Winter hardiness was positively associated with soluble carbohydrate concentration in January. There are numerous reports on the close correlation between winter freezing tolerance and soluble carbohydrate content in plants (Morin et al., 2007; Ghasemi-Soloklui et al., 2012). Soluble carbohydrates are thought to have a positive effect on cell membrane integrity and play an adaptive role in mediating osmotic adjustment in plants exposed to low temperatures. However, some researchers considered that the increase in soluble carbohydrates and winter hardiness are likely the result of weather changes and their synchrony may be only a coincidental relationship (Palonen 1999). Researchers discovered a significant negative relationship between LT50 levels of pomegranate cultivars in January and soluble carbohydrate content. (Ghasemi-Soloklui et al., 2012; Nasrabadi et al., 2019; Sirooeinejad et al., 2020).

Winter hardiness was not related to proline content in January. Proline was found to accumulate in several plant species in response to abiotic conditions such as cold (Dörffling et al., 1997), salinity (Ahmad et al., 1981), and drought (Hsu et al., 2003). In evaluating the cold tolerance of seven pomegranate cultivars, Ghasemi-Soloklui et al. (2012) found that a high proline concentration does not always mean a high level of cold tolerance, as proline content remained high even after trees were acclimatized in March. Sirooeinejad et al. (2020) also showed significant changes in proline concentration in pomegranate cultivars ('Tabestaneh Torosh') during adaptation and deadaptation, but proline did not correlate with cold tolerance. Under abiotic stress, the increase in proline content may be a product rather than an adaptive response to stress and the role of proline under stressful conditions may differ among species (Ashraf et al., 2007). Therefore, proline content was not good enough to use for selecting pomegranate cultivars with high freezing tolerance in January.

Stomatal density varied among different parts of pomegranate leaves and ranged from 86 to 90 stomata/mm2, with the highest stomatal density observed at the center (91.72 mm^2), apex (90.07 mm^2), and base (86.25 mm^2). 'Alak' showed the highest significant differences in stomatal density at the leaf apex, middle, and base. Sagaram et al. (2007) discovered that there were no changes in stomatal density within the same pecan genotype grown at different locations. Thus, the stomatal density index, as a quantitative attribute, is genetically determined (Gailing 2008). According to a previous study, stomatal density in leaves of Iranian pomegranate cultivars varied from 46 to 108 mm^2 (Ghasemi-Soloklui et al., 2017). Drogoudi et al. (2012) reported that the stomatal density in pomegranate leaves ranged from 68 to 149.9 stomata/mm^2 in Greek cultivars, while Meena et al. (2011) reported that the maximum stomatal density in pomegranates was 130.67 per mm^2. The stomatal density of pomegranate cultivars is lower than that of pecans (Sagaram et al., 2007) (363- 463 stomata/mm^2), olives (604 and 713 stomata/mm^2) (Pérez López et al., 2010) and walnuts (Muradoğlu et al., 2011) (183- 335 stomata/mm^2). In subtropical forests, stomatal density varied from 11.16 to 1403.27 pores mm^{-2}, with an average of 255.12 pores mm^{-2}. (Liu et al., 2019). Our results showed that the highest stomatal density was observed in the middle of the leaf. These results are consistent with a previous study on walnuts (Aslamarz et al., 2009). Roselli et al. (1989) showed that there are significant differences between leaf positions in

olive cultivars. They reported similar stomatal density in the leaf apex and leaf base of olive cultivar Moraiolo, while the middle part had a slightly higher number of stomata.

In our studies, a strong correlation was found between cold hardiness in summer and winter and stomatal density, with the highest correlation, which was observed in the middle part of the leaves. The results of our study showed that the winter hardy cultivar 'Alak' had the highest leaf stomatal density, while the cultivar 'Agha Mohammad Ali' had the lowest stomatal density and cold tolerance in summer, but showed considerable cold tolerance in winter.

Stomatal density has been reported to be associated with tolerance to abiotic stresses such as cold (Pérez López et al., 2010) and drought (Van Rensburg et al., 1999). Roselli et al. (1989) indicated that the stomatal density index is a suitable parameter for evaluating the freezing hardiness of olive trees. In addition, Nassuth et al. (2021) showed that leaves of cold-tolerant *V. riparia* had higher stomatal density than leaves of less cold-tolerant *V. vinifera*; however, cold treatment in growth chambers appeared to increase the number of stomata in both. The evaluation of six olive cultivars by Pérez López et al. (2010) also showed that stomatal density was positively related to winter hardiness. However, some reports showed a negative correlation between the stomatal density and freezing tolerance in olives (Roselli 1989; Roselli and Venora 1989) and walnuts (Aslamarz and Vahdati, 2009).

Our results showed that the stomatal density of pomegranate leaves was correlated with the carbohydrate concentration in the stems ($r= 0.54$, $p \leq 0.01$). However, 'Agha Mohammad Ali' had the lowest leaf stomatal density but the highest soluble carbohydrate concentration in winter. This result suggests that several other factors may affect plant carbohydrate reservoirs. Meng and Yao (2015) suggested that stomatal density is not a factor that directly affects the cold-hardiness of plants. However, a previous study has shown that a higher stomatal density of olive leaves may help reduce CO_2 diffusion resistance and allow efficient photosynthesis under drought stress; it has also been reported that higher stomatal density increases the ability to regulate leaf transpiration (Bosabalidis et al., 2002). This may explain the higher correlations between stomatal density and freezing tolerance in summer compared to winter.

Conclusion

The cold tolerance of trees in the non-acclimation stage determined in August was not a good predictor of the freezing tolerance of plants in the cold acclimation stage in January. In January, cold tolerance was positively related to soluble carbohydrate concentration, while proline content was not sufficient to predict winter freezing tolerance of the selected pomegranate cultivars. The results of this study revealed a high and a moderate relationship between leaf stomatal density and cold tolerance in summer and winter, respectively. Although stomatal density was generally correlated with the concentration of soluble carbohydrates in the stems, a contradictory result was found for the cultivar 'Agha Mohammad Ali'.

Acknowledgments

The authors thank Ziya Eddin Tabatabaei (R.I.P.) for providing plant samples and Bu-Ali Sina University for their financial support of this study.

Conflict of Interests

The authors indicate no conflict of interest for this work.

References

Ahmad I, Wainwright S, Stewart G. 1981. The solute and water relations of Agrostis stolonifera ecotypes differing in their salt tolerance. New Phytologist 87(3), 615-629.

Ashraf M, Foolad MR. 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environmental and experimental botany 59(2), 206-216.

Aslamarz AA, Vahdati K. 2010. Stomatal density and ion leakage as indicators of cold hardiness in walnut. Acta Horticulturae 861, 321-324.

Bates LS, Waldren RP, Teare I. 1973. Rapid determination of free proline for water-stress studies. Plant and soil 39(1), 205-207.

Bosabalidis AM, Kofidis G. 2002. Comparative effects of drought stress on leaf anatomy of two olive cultivars. Plant Science 163(2), 375-379.

Boussiba S, Rikin AE. 1975. The role of abscisic acid in cross-adaptation of tobacco. Plant Physiology 56, 337-339.

Dörffling K, Dörffling H, Lesselich G, Luck E, Zimmermann C, Melz G, Jürgens H. 1997. Heritable improvement of frost tolerance in winter wheat by in vitro-selection of hydroxyproline-resistant proline overproducing mutants. Euphytica 93(1), 1-10.

Drogoudi P, Pantelidis G, Manganaris A. 2012. Morphological and physiological characteristics in pomegranate cultivars with different yields. Options Méditerranéennes 103, 67-69.

Falconer D, Mackay T. 1996. Introduction to

quantitative genetics (4th ed.). Prentice Hall, Longman Press.

Fischer M. 1992. Frostresistenzuntersuchungen an Malus-Artbastard-Unterlagen. Erwerbsobstbau 34, 194-197.

Gailing O. 2008. QTL analysis of leaf morphological characters in a *Quercus robur full-sib family* (*Q. robur*× *Q. robur ssp. slavonica*). Plant Biology 10(5), 624-634.

Ghasemi-Soloklui AA, Ershadi A, Fallahi E. 2012. Evaluation of cold hardiness in seven Iranian commercial pomegranate (*Punica granatum* L.) cultivars. HortScience 47(12), 1821-1825.

Ghasemi-Soloklui AA, Gharaghani A, Oraguzie N, Eshghi S, Vazifeshenas M. 2017. Chilling and heat requirements of 20 Iranian pomegranate cultivars and their correlations with geographical and climatic parameters, as well as tree and fruit characteristics. HortScience 52(4), 560-565.

Ghasemi-Soloklui AA, Gharaghani A, Oraguzie N, Saed-Moucheshi A, Vazifeshenas M. 2019. Genetic diversity, heritability and inter-relationships of fruit quality and taste attributes among Iranian pomegranate (Punica granatum L.) cultivars using multivariate statistical analysis. Fruits 74(6), 303–318.

Guy CL. 1990. Cold acclimation and freezing stress tolerance: Role of protein metabolism. Annual review of plant biology 41(1), 187-223.

Hsu S, Hsu Y, Kao C. 2003. The effect of polyethylene glycol on proline accumulation in rice leaves. Biologia Plantarum 46(1), 73-78.

Hummer K, Fuchigami LH, Peters V, Bell N. 1995. Cold hardiness in Rubus. Fruit varieties journal 49, 52-58.

Kalberer SR, Leyva-Estrada N, Krebs SL, Arora R. 2007. Frost dehardening and rehardening of floral buds of deciduous azaleas are influenced by genotypic biogeography. Environmental and Experimental Botany 59(3), 264-275.

Levitt J, Scarth GW. 1936. Frost-hardening studies with living cells: I. Osmotic and bound water changes in relation to frost resistance and the seasonal cycle. Canadian Journal of Research 14(7), 267-284.

Li PH, Weise CJ. 1971. Increasing cold resistance of stem section of *Cornus stonlonifera* by artificial dehydration: a preliminary report. Cryobiology 8, 108-111.

Lindén L. 2002. Measuring cold hardiness in woody plants. PhD thesis, University of Helsinki, Finland.

Liu C, Li Y, Xu L, Chen Z, He N. 2019. Variation in leaf morphological, stomatal, and anatomical traits and their relationships in temperate and subtropical forests. Scientific reports 9(1), 1-8.

Mancuso S, Fiorino P. 2000. Differential thermal analysis, supercooling and cell viability in organs of *Olea europaea* at subzero temperatures. Advances in horticultural science 14, 23-27.

Meena K, Singh R, Pareek S, Kashyap P, Sheikh M, Mokashi A, Rokhade A. 2011. Evaluation of pomegranate (*Punica granatum* L.) genotypes for morphological and flowering characteristics under semi-arid climate. Acta Horticulturae 890, 233-238.

Meng LS, Yao SQ. 2015. Transcription co-activator Arabidopsis Angostifolia3 (AN3) regulates water-use efficiency and drought tolerance by modulating stomatal density and improving root architecture by the transrepression of YODA (YDA). Plant Biotechnology Journal 13, 893-902.

Mittelstädt H. 1965. Beiträge zur Züchtungsforschung beim Apfel. Der Züchter 35(7), 311-327.

Morin X, Améglio T, Ahas R, Kurz-Besson C, Lanta V, Lebourgeois F, Miglietta F, Chuine I. 2007. Variation in cold hardiness and carbohydrate concentration from dormancy induction to bud burst among provenances of three European oak species. Tree Physiology 27(6), 817-825.

Muradoğlu F, Gündoğdu M. 2011. Stomata size and frequency in some walnut (*Juglans regia*) cultivars. International Journal of Agriculture & Biology 13, 1011–1015.

Nasrabadi M, Ramezanian A, Eshghi S, Kamgar-Haghighi AA, Vazifeshenas MR, Valero D. 2019. Biochemical changes and winter hardiness in pomegranate (*Punica granatum* L.) trees grown under deficit irrigation. Scientia Horticulturae 251, 39-47.

Nassuth A, Rahman MA, Nguyen T, Ebadi A, Lee C. 2021. Leaves of more cold hardy grapes have a higher density of small, sunken stomata. Vitis 60(2), 63-67.

Pagter M, Jensen CR, Petersen KK, Liu F, Arora R. 2008. Changes in carbohydrates, ABA and bark proteins during seasonal cold acclimation and deacclimation in *Hydrangea* species differing in cold hardiness. Physiologia Plantarum 134(3), 473-485.

Palonen P. 1999. Relationship of seasonal changes in carbohydrates and cold hardiness in canes and buds of three red raspberry cultivars. Journal of the American Society for Horticultural Science 124(5), 507-513.

Palta JP, Li PH. 1979. Frost-hardiness in relation to leaf anatomy and natural distribution of several solanum species. Crop Science 19(5), 665-671.

Patton AJ, Cunningham SM, Volenec JJ, Reicher ZJ. 2007. Differences in freeze tolerance of zoysiagrasses: II. Carbohydrate and proline accumulation. Crop Science 47(5), 2170-2181.

Pérez López D, Moriana Elvira A, Mariño Barnechea J, Gijón López MDC. 2010. Water relation response to soil chilling of six olive (*Olea europaea* L.) cultivars with different frost resistance. Spanish Journal of Agricultural Research, 8, 780-789.

Reyes-Díaz M, Alberdi M, Piper F, Bravo LA, Corcuera LJ. 2005. Low temperature responses of Nothofagus dombeyi and Nothofagus nitida, two evergreen species from south central Chile. Tree physiology 25(11), 1389-1398.

Roselli G, Venora G. 990. Relationship between stomatal size and winter hardiness in the olive. Acta Horticulturae 286, 89-92.

Roselli G, Benelli G, Morelli D. 1989. Relationship between stomatal density and winter hardiness in olive (*Olea europaea* L.). Journal of Horticultural Science 64(2), 199-203.

Rowland LJ, Ogden EL, Ehlenfeldt MK, Vinyard B. 2005. Cold hardiness, deacclimation kinetics, and bud development among 12 diverse blueberry genotypes under field conditions. Journal of the American Society for Horticultural Science 130(4), 508-514.

Sagaram M, Lombardini L, Grauke L. 2007. Variation in leaf anatomy of pecan cultivars from three ecogeographic locations. Journal of the American Society for Horticultural Science 132(5), 592-596.

Sheidai M, Khandan M, Nasre-Esfahani S. 2005. Cytogenetical study of some Iranian pomegranate (*Punica granatum* L.) cultivars. Caryologia 58(2), 132-139.

Sirooeinejad B, Zamani Z, Fatahi MR. 2020. Study of physiological and biochemical responses to freezing stress in pomegranate (*Punica granatum* L.) trees during acclimation and deaclimation cycle. The Journal of Horticultural Science and Biotechnology 95(3), 341-355.

Soleimani A, Lessani H, Talaie A. 2002. Relationship between stomatal density and ionic leakage as indicators of cold hardiness in olive (*Olea europaea* L.). Acta Horticulturae 618, 521-525.

Van Rensburg L, Peacock J, Krueger G. 1999. Boundary layer, stomatal geometry and-spacing in relation to drought tolerance in four *Nicotiana tabacum* L. cultivars. South African Journal of Plant and Soil 16(1), 44-49.

Wang X, Arora R, Horner HT, Krebs SL. 2008. Structural adaptations in overwintering leaves of thermonastic and nonthermonastic Rhododendron species. Journal of the American Society for Horticultural Science 133(6), 768-776.

Yemm EW, Willis AJ. 1954. The estimation of carbohydrates in plant extracts by anthrone. Biochemical journal 57(3), 508-514.