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Cold Hardiness and its Relationship with Proline Content in Persian Walnut

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Summary

The study of cold hardiness is important because freeze injury is a leading cause of walnut yield losses in many cold regions of Europe and Asia. In this study, three methods of determining cold acclimation in walnut cultivars/genotypes were compared: 1) electrolyte leakage measurement (EL); 2) triphenyl tetrazolium chloride assay (TTC) and; 3) proline analysis. Stems of eight walnut cultivars and genotypes were cut and collected every month from November 2007 to April 2008. EL was conducted using uniform pieces inserted into plastic vials programmed to decrease their temperature by 2 °C h^{-1} to freezing temperatures of −5, −10, −15, −20, −25 and −30 °C. The electrolyte leakage from frozen stem tissues was captured in solution and measured using an electrical conductivity meter. Tissue injury was also assessed by TTC analysis using stem segments cooled under controlled temperatures at 2 °C h^{-1} to set temperatures ranging from 5 to −30 °C and results were quantified using a spectrophotometer. Proline content of fresh stem tissues was determined using the Bates method. Cold hardiness of samples increased with accumulated seasonal chilling and decreased as they approached spring bud break. A 1.4 to 2.7 fold increase in proline content was observed in stems from November to midwinter. LT_{50s} (temperature causing 50 % lethal injury) ranged from −5.8 to −10 °C in November, −12.5 to −19.3 °C in midwinter and −5.8 to −9.2 °C in early spring. Using both the EL and TTC methods, cultivars and genotypes were classified into three groups based on their cold hardiness: sensitive ('Z_{30}' and 'Serr'), semi-hardy ('Z_{53}', 'Z_{67}', and 'Hartley') and hardy ('Lara', 'Z_{63}' and 'Pedro').

Key words. cold hardy – frost injury – ion leakage – proline – temperature

Introduction

Cold hardiness is defined as resistance to diverse types of injury caused by low and changeable temperatures during the winter (Quamme 1978). During acclimation to cold several environmentally induced changes take place in plant tissues. These changes are associated with hardy plants undergoing the biochemical and physiological changes needed to withstand injurious temperatures (George and Burke 1977). In hardy plants, a decrease in photoperiod and day and night temperatures results in fluctuations of plant cell materials such as proline (Lalk and Dorffing 1985). According to previous investigations, proline can act as an osmoprotectant (Csonka 1989; Morita et al. 2002) with a cryoprotective activity nearly equal to that of glycerol or trehalose (Takagi et al. 1977) and can, therefore, play an important role in plant resistance to freezing injury.

Cold hardiness of plant tissue is usually estimated by subjecting the whole plant or samples of plant tissues to a range of freezing temperatures and evaluating whether the treated tissues are dead or alive. The temperature that is estimated to kill 50 % of samples is called the LT_{50}. Several methods have been proposed to measure cold hardiness. Two of these are the triphenyl tetrazolium chloride (TTC) assay and measurement of electrolyte leakage (Stergios and Howel 1973; Timmis 1976).

The triphenyl tetrazolium chloride (TTC) test has been used primarily as a qualitative estimation of tissue viability (Parker 1951; Larcher and Eggarter 1960). Living plant cells reduce TTC in their mitochondria, producing red formazan, but dead cells do not. Thus, formazan can be extracted and quantified with a spectrophotometer (Steponkus and Lanphear 1967; Towill and Mazur 1974).

The electrolyte leakage method was reported by Dexter et al. (1930) and since that time has been widely applied to assess freezing injury (Levitt 1980). Electrolyte leakage analysis is based on the fact that injured cells are unable to maintain membrane regulation and therefore release electrolytes through damaged membranes (Lyons et al. 1979). This method is a very widely utilized viability test and relatively quick and cheap method of for determination of cold injury (Eldridge et al. 1983).

Persian walnut (*Juglans regia* L.) is a woody plant sensitive to winter cold injury and subsequent economic losses in many European and Asian countries. This species, originated in Central Asia, the West Himalayan chain and Kyrgyzstan (Fernandez-Lopez et al. 2000), and was being

cultivated in southern Europe by 1000 BC (Ducci et al. 1997). The most promising genotypes are adapted to these climates (Aleta and Ninot 1997). This genetic variability is useful to breeding programs desiring late bud break, resistance to frost injury and late spring cold (Germain 1997; Zeneli et al. 2005).

There are a few studies in walnut of cold hardiness mechanisms (Ewers et al. 2001; Améglio et al. 2004; Poirier et al. 2010) and methods of adaptation to cold (Améglio et al. 2001; Davarnejad et al. 2009; Aslani Aslamarz et al. 2010) but reported data on cold tolerance estimation by TTC and ion leakage methods and on seasonal fluctuation in proline content of walnut are scarce. Therefore, the objectives of this study were to 1) screen walnut cultivars and genotypes for cold hardiness using both the electrolyte leakage (EL) and triphenyl tetrazolium chloride (TTC) methods, 2) examine changes in the acclimation of walnut stems before, during and after winter hardening, 3) determine the proline concentration of acclimated and deacclimated stems and, 4) examine the relationship between cold acclimation and proline concentration.

Materials and Methods

Source of plant material

One year old stems were collected at random from east, south, west, north and centre portions of ten 14-year-old trees of the commercial walnut cultivars 'Serr', 'Pedro', 'Hartley', and 'Lara' and the promising Iranian genotypes 'Z_{63}', 'Z_{53}', 'Z_{30}' and 'Z_{67}' grown in the Kamal Shahr experimental orchard of the Horticulture Department of the Seed and Plant Improvement Institute at Karaj, Iran. All the trees were grafted on *Juglans regia* rootstocks. The orchard is located 1.300 m above sea level at 35.51° N, 50.51° E and is characterized by 240 mm average annual rainfall, a relatively short (about 2 months) dry period in the summer, cold winters (min. temp −21 °C), and 6 months of frost danger (November to April). The trees have been furrow irrigated four times a month, fertilized with NO_3 nitrogen compounds (400 g per tree) and micro elements during the growing period, and treated rarely for pests and diseases.

Electrolyte leakage (EL) measurement

Stems cut every month from November 2007 to April 2008 were put in plastic bags and placed in insulated containers partially filled with crushed ice for transportation to the laboratory and then placed in refrigerators at 5 ± 1 °C. Previous studies showed that measurement of electrical conductivity is affected by the size of the samples (Prasil and Zamecnik 1998) so stems were cut into uniform subsamples (1.5 g) before insertion into plastic film vials. The vials were programmed to decrease in temperature by 2 °C h^{-1} to freezing temperatures of −5, −10, −15, −20, −25 and −30 °C and samples were held at each final temperature for 6 h. Samples were removed during the course of freezing at 5 °C intervals and thawed at 4 °C h^{-1}. Vials with thawed samples were then filled with 20 ml of double distilled water, covered, and shaken in a horizontal shaker (150 cycles min^{-1}) at 20 °C for 24 h. The conductivity of each solution was measured by an electrical conductivity meter (HI 8633). Samples were then killed completely by refreezing each vial at −80 °C for 24 h. After re-thawing, the samples were re-shaken and the conductivity was determined a second time. The conductivity percent was calculated for each sample by using the ratio of the initial to the final measurements × 100.

Triphenyl tetrazolium chloride (TTC) assay

Stems collected from November 2007 to April 2008 were transferred to the laboratory as previously described, cut into uniform pieces (100 ± 10 mg), and inserted in 17×120 mm test tubes. These were subjected to a programmed temperature decrease of 2 °C h^{-1} to temperatures of 5 °C (control) −5, −10, −15, −20, −25 and −30 °C and then held at each final temperature for 6 h. For each cultivar and genotype, test tubes were removed at 5 °C intervals and frozen samples were then thawed at 4 °C h^{-1}. Refined TTC was then determined according to the method of Steponkus and Lanphear (1967) as follows: 1) stem samples were placed in 10 ml graduated test tubes with 3 ml of 0.6 (w/v) TTC in 0.05 M Na_2HPO_4~KH_2PO_4 buffer (pH 7.4) +0.05 % (v/v) wetting agent and samples were then infiltrated under a vacuum. A 0.06 % TTC solution was used to insure that TTC was in excess. The wetting agent and infiltration under vacuum ensured uniform uptake by the tissue. 2) Samples were incubated at 30 °C for 15 h. 3) The TTC solution was drained and the sample tissue rinsed once with distilled water. Samples were extracted with 7 ml of 95 % (v/v) ethanol in a boiling water bath. Resulting water-insoluble formazan, a marker of living cells, was extracted after 10 min. The extract was cooled, the volume was brought up to a 10 ml volume with 95 % ethanol, and then the absorbance of the red phases was read at 530 nm using a Lambda 25 UV-visible spectrophotometer (Perkin Elmer Co.). The $LT_{50,}$ the lethal temperature at which 50 % of formazan production occurs, was calculated for each sample using the ratio of formazan production at the tested temperature to the formazan production at the control condition × 100.

Proline analysis

Free proline accumulation was determined using the method of Bates et al. (1973). Fresh tissue samples (0.5 g) of stems collected between November 2007 and April 2008 were homogenized with 3 % sulfosalicylic acid. After 72 h the homogenates were centrifuged at 3000 g for 20 min. The supernatants were treated with acetic acid and ninhydrin and boiled for 1 h. Absorbance of the coloured phases was read at 520 nm using Lambda 25 UV-visible spectrophotometer (Perkin Elmer Co.). Proline content was expressed as mg g^{-1} FW.

Statistical analysis

The experiments were conducted using a completely randomized design with five replications. Data were analyzed using SAS Software (Sas Institute 2002). Correlation coefficients were determined using Pierson ranked-order correlation. Means with significant differences were compared using Duncan's multiple range tests at P≤0.01.

Results

Maximum and minimum daily temperature and bud break time of cultivars and genotypes

The air temperatures at the experimental orchard were recorded from November 2007 to April 2008 with an automatic temperature recorder. Minimum daily temperatures decreased rapidly on two occasions. The first large temperature drop occurred during the late autumn in December and the second occurred during January. The lowest recorded mid-winter minimum air temperature was −17 °C. Subsequently temperatures gradually increased through March and April (Fig. 1).

The dormant-season chilling and heat requirements of the cultivars and genotypes used in this study were estimated previously using excised stems (Aslani Aslamarz et al. 2009). According to that data (Table 1), the cultivars and genotypes can be categorized into three groups: those with a low chilling and heat requirement ('Z_{30}' and 'Serr'), a medium ('Z_{53}', 'Z_{67}', 'Lara' and 'Pedro ') and a high requirement ('Hartley' and 'Z_{63}').

Table 1. Estimated chilling (CR) and heat requirements (HR) of walnut cultivars and genotypes as calculated based on the Richardson et al. (1974) model using excised stems in two successive years (Aslani Aslamarz et al. 2009).

Cultivar/ genotype	CR (h < 7 °C)	HR (GDH°C)
'Hartley'	1000 ± 20	14322 ± 136
'Lara'	900 ± 30	12957 ± 214
'Pedro'	750 ± 20	13625 ± 124
'Serr'	650 ± 25	10934 ± 211
'Z_{30}'	650 ± 30	11503 ± 215
'Z_{53}'	800 ± 20	11064 ± 125
'Z_{63}'	900 ± 40	15186 ± 124
'Z_{67}'	750 ± 25	12743 ± 325

h < 7 °C: hours below 7 °C; GDH°C: growth degree hours.

Electrolyte leakage(EL) measurement

Changes in LT_{50} were determined using the electrolyte leakage method. Data for the LT_{50}s of cultivars (A) and Iranian selections (B) of Persian walnut are shown in Fig. 2. 'Serr' and 'Z_{30}' were the most cold-sensitive genotypes whereas 'Lara' and 'Pedro' were the most resistant. Other cultivars and genotypes tested showed intermediate cold hardiness. This result is consistent with long-term field observations from a previous study in Iran (Aslani Aslamarz et al. 2010). LT_{50}s of stems samples sharply decreased after the first large decrease in air temperature in December and the cultivars and genotypes with the greatest freezing resistance exhibited LT_{50}s below −12.5 °C at that time. LT_{50}s reached their lowest levels, −10.3 to −10.7 °C for sensitive and −14.5 to −15.4 °C for resistant cultivars and genotypes, in February after a second rapid decrease in air temperature in January. In March and April, the LT_{50}s increased dramatically as air temperatures raised, ranging from −4.4 to −8.1 °C for sensitive and −8 to −13.1 °C for resistant cultivars and genotypes.

Triphenyl tetrazolium chloride(TTC) assay

Results of a TTC test are presented in Fig. 3. Data show the LT_{50}s of cultivars (A) and Iranian selections (B) of Persian walnut. The capacity for production of formazan at lower temperatures increased in all cultivars and genotypes with the overall trend of declining temperatures. Therefore the LT_{50} (the lethal temperature at which 50 % of formazan production occurs) ranged from −5.8 to −10 °C in November, −12.5 to −19.3 °C in mid-winter and from −5.8 to −9.2 °C in early spring. Also based on the LT_{50}, the cultivars and genotypes studied were classified as sensitive ('Serr' and 'Z_{30}'), semi-hardy ('Hartley', 'Z_{67}' and 'Z_{53}') or hardy ('Lara', 'Z_{63}' and 'Pedro'). These data confirmed the results of the EL test.

Correlation between LT_{50}s of EL and TTC methods

A positive significant correlation was observed between the LT_{50}s calculated using the TTC and EL methods during the late fall and early spring but not during mid-winter.

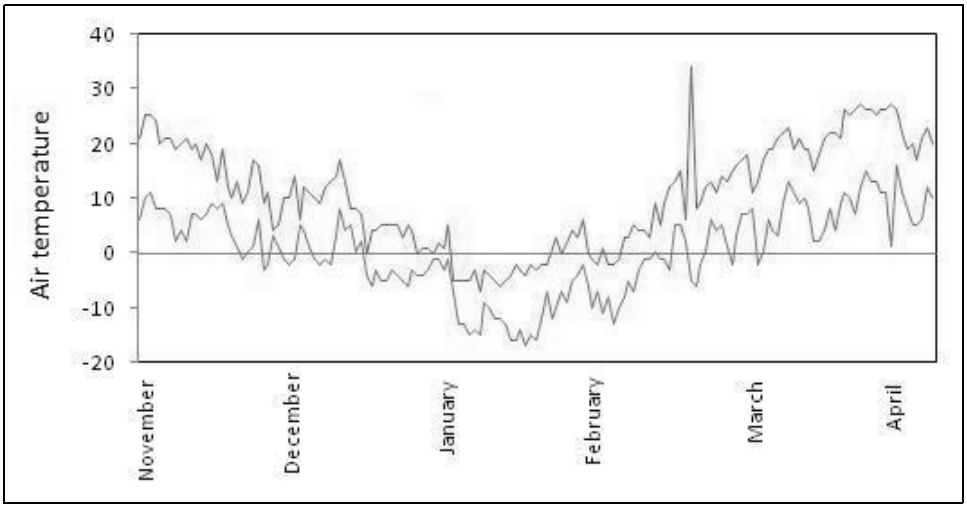

Fig. 1. Minimum and maximum daily air temperatures recorded from November 2007 to April 2008 at the experimental orchard in Kamal Shahr, a city 50 km west of Tehran at 1312 m altitude. Minimum temperatures decreased rapidly at two times. The first was during late autumn in December and the second was during an unprecedented freeze in January. Cultivars and genotypes indicated by the same letter are not significantly different from each other (P≤0.01).

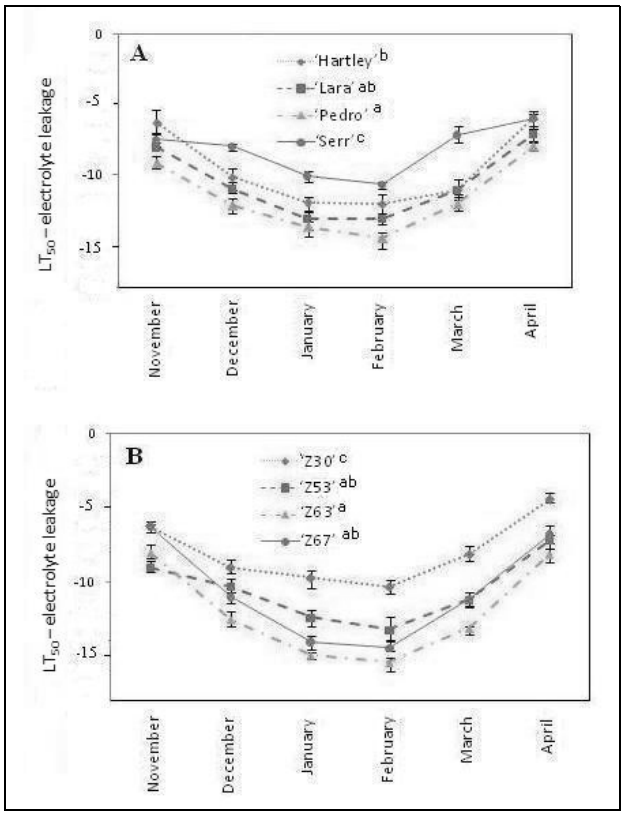

Fig. 2. Changes in LT_{50} determined using the electrolyte leakage method. Data show the LT_{50}s of cultivars (A) and Iranian selections (B) of Persian walnut decreased rapidly following the first sharp drop in air temperature in December and exhibited the greatest freeze resistance in February after a hard January freeze. During March and April the LT_{50}s increased dramatically as air temperatures rose. Tissue samples were collected from November 2007 to April 2008. Cultivars and genotypes indicated by the same letter are not significantly different from each other ($P \leq 0.01$). Each bar represents the mean ± SE in the cultivars/genotype.

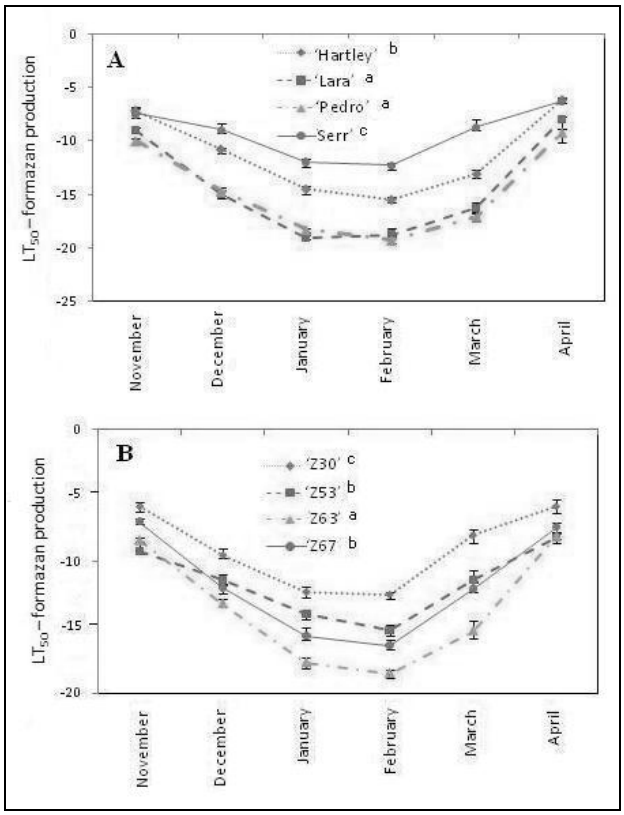

Fig. 3. Changes in LT_{50} determined using the triphenyl tetrazolium chloride method (formazan production). Data show the LT_{50}s of cultivars (A) and Iranian selections (B) of Persian walnut decreased rapidly following the first sharp drop in air temperature in December and exhibited the greatest freeze resistance in February following a hard January freeze. During March and April the LT_{50}s increased dramatically as air temperatures rose. Stem samples were collected from November 2007 to April 2008. Cultivars and genotypes indicated by the same letter are not significantly different from each other ($P \leq 0.01$). Each bar represents the mean ± SE in the cultivars/genotype.

During the first evaluation period (November and December) the correlation between LT_{50}s was highly significant but in January–February the correlation coefficients were low and not significant. Then during the March–April period there was again a significant correlation between the LT_{50}s of the two methods (Table 2).

Proline concentration

The free proline concentration in stems of cultivars (A) and Iranian selections (B) of Persian walnut as determined by the Bates method (Bates et al. 1973) is shown in Fig. 4. The mean concentration of proline varied among the tested cultivars and genotypes in all months. The cold-resistant cultivars ('Lara' and 'Pedro') accumulated the greatest amounts of free proline, 13.2 and 12.9 mg g^{-1} respectively, while the sensitive cultivars and genotypes ('Serr' and 'Z_{30}') with 7.9 and 7.8 mg g^{-1} respectively contained the least (Table 3). During cold acclimation the concentrations of proline increased soon after a sharp air temperature drop in December and reached their maximum in January and February after a second rapid decrease in air temperature. Proline concentrations then decreased with the rise of air temperature in March and April (Fig. 4). Proline concentration was highly correlated with the LT_{50}s obtained in both the EL and TTC tests (Table 3). Coefficients were negative and high for all genotypes except 'Lara'. The observed increase in proline as temperatures declined and cold hardening increased indicated proline may play an important role in walnut cold tolerance. The proline concentration increase in hardened stems was more than 2.7 fold greater than that in non-hardened stems. The degree of increase in proline concentration in a genotype had a close correlation with its cold resistance. For example in 'Z_{30}', a sensitive type, the proline concentration increase seen in hardened stems was 2.7 fold that of non-hardened ones while in hardened stems of 'Lara', a cold tolerant cultivar, the proline concentration increased only 1.4 fold more than in non-hardened stems. However, total proline content is related to the degree of cold tolerance. Tolerant cultivars and genotypes had the highest concentration of proline.

Table 2. Correlation coefficients between LT_{50}s (temperatures producing 50 % lethal damage) determined using either the triphenyl tetrazolium chloride or the electrolyte leakage method at three time periods between November 2007 and April 2008.

	First period		Second period		Third period	
Month	November	December	January	February	March	April
Correlation coefficient	0.93**	0.99**	0.05^{NS}	0.17^{NS}	0.97**	0.91**

**, ^{NS}: Significant at P<0.01, or not significant, respectively.

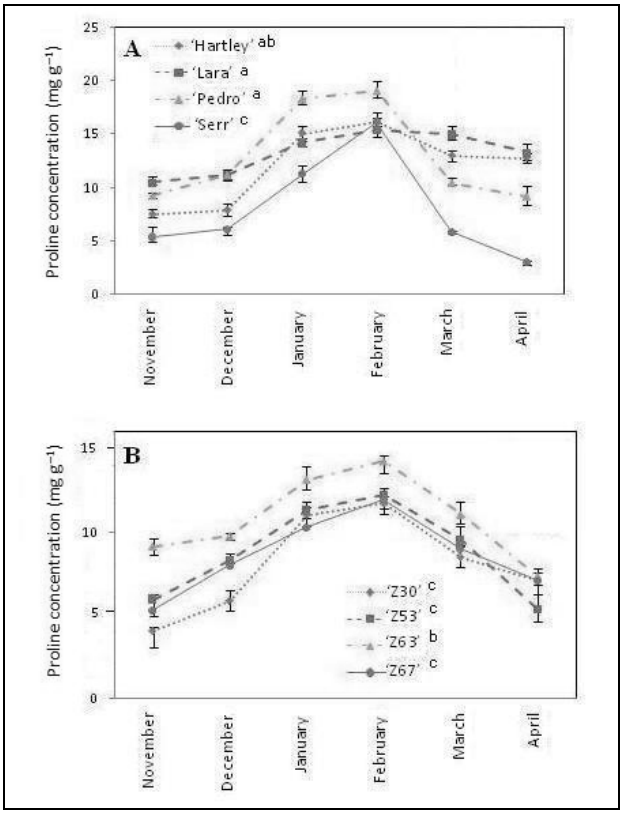

Fig. 4. Free proline concentration in stems of cultivars (A) and Iranian selections (B) of Persian walnut as determined by the Bates method (Bates et al. 1973). The concentration of proline increased soon after a rapid temperature drop in December and peaked in February following a hard freeze in January. Proline concentrations then decreased as air temperatures rose again in March and April. Samples were collected from November 2007 to April 2008. Genotypes and cultivars indicated by the same letter are not significantly different from each other (P≤0.01). Each bar represents the mean ± SE in the cultivars/genotype.

Thus, in walnut stems, the absolute amount rather than the relative increase in proline concentration is a more reliable marker for predicting cold hardiness.

Discussion

Membrane leakage has become a common method for evaluating cell membrane stability, and measuring the degree of injury or acclimation of plants. Electrolyte leakage and associated lethality (LT_{50}s) of walnut stems decreased in all cultivars and genotypes as cold acclimation progressed between November and January. Acclimation has been attributed to altered pit membrane activity which plays an important role in preventing intracellular ice formation (Wisniewski and Davis 1989). The membrane porosity of plants can influence the leakage of electrolytes and concentration of intracellular substances responsible for cold injury (Lyons and Haig 1995). The pectic component of the pit membrane appears to play a key role in regulating membrane porosity but unique arabinogalactan-rich glycoprotein has been identified which also may be involved during the acclimation (Wisniewski and Davis 1995). A possible role of glycoprotein is suggested by observations that water within small diameter pores freezes at a lower temperature than bulk water or water in cell walls with low concentrations of this material and consequently broader pores (George and Burke 1977; Ashworth and Abeles 1984). The effect of frost injury on walnut (Améglio et al. 2005; Davarnejad et al. 2009) and almond (Imani et al. 2011) on ion leakage and its use as an indicator of cold hardiness has been demonstrated.

Previous studies have confirmed the significant role of cell water content in frost hardiness (Chen and Gusta 1978; Tanino et al. 1990; Gusta et al. 2004; Aslani Aslamarz et al. 2010; Charrier and Améglio 2011). After autumn leaf drop, the decreasing air-soil temperature results in reduced cell water content in stems (Levitt 1980), allowing efficient frost hardening. Then in the spring (March–April) as air-soil temperature increases water absorption picks up again (Turcotte et al. 2009). Améglio et al. (2002) showed that water absorption in walnut trees occurred when soil temperature exceeded 8 °C. Our results confirmed previous studies regarding the effect of water content on cold hardiness. Furthermore we found that when the buds of cultivars and genotypes began to swell and break bud in March, hardening was suddenly lost in comparison to the previous month. It has been shown that the moisture content of buds in several species is related closely to cold hardiness (Andrews and Proebsting 1987; Dunner and Gianfagan 1991).

After leaf senescence, the stems begin to accumulate materials such as proline in their cell membranes (Cai et al. 2004). A series of investigations of the relationship between proline and freezing tolerance in plants has been reported. For example Xin and Browse (1998) demonstrated that in *Arabidopsis*, cold hardiness increased with increased proline concentration. Murelli et al. (1995) and Wanner and Junttila (1999), however, found no direct correlation between cold acclimation and proline

Table 3. Mean concentration of proline (method of BATES et al. 1973) and correlation coefficients between LT_{50}s (lethal temperatures to 50 % damage) and amount of free proline concentration in electrolyte leakage (EL) and triphenyl tetrazolium chloride (TTC) method in eight Persian walnut cultivars and genotypes from November 2007 to April 2008.

Cultivar/genotype	Proline concentration (mg g^{-1})	Correlation coefficient between proline concentration and LT_{50}s in:	
		EL method	TTC method
'Hartley'	12.04 b+	−0.70**	−0.71**
'Lara'	13.26 a	−0.53ns	−0.66**
'Pedro'	12.93 a	0.65**	−0.83**
'Serr'	7.98 e	−0.97**	−0.96**
'Z_{30}'	7.88 e	−0.79**	−0.82**
'Z_{53}'	9.32 d	−0.91**	−0.93**
'Z_{63}'	10.73 c	−0.83**	−0.94**
'Z_{67}'	8.62 ed	−0.90**	−0.92**

+ Values followed by a common letter in a row for each cultivar are not significantly different at P<0.01 according to Duncan's multiple range test.
**, NS: Significant at P<0.01, or not significant, respectively.

concentration in either cold acclimated or deacclimated samples. Our results show that an increase in proline concentration in acclimated walnut stems was correlated with resistance to lower temperatures, suggesting a stabilizing effect of proline and other macromolecules on cell membranes structure (RHODES and SAMARAS 1994).

An increase in proline concentration can also increase the osmotic potential and enhance the stability of colloidal protoplasm which could further contribute to cold acclimation (CHEN et al. 1996; CAI et al. 2004). However, YASHIDA and UEMURA (1984) found that cultivars and genotypes with the greatest amounts of extracellular proline showed less change in water potential than cultivars and genotypes with less proline. KUSHAD and YELENOSKY (1987) further suggest that proline increase during cold acclimation plays a role in protection of cellular membranes during freezing stress.

The LT_{50}s determine using the EL and TTC methods are highly correlated in fall and spring but were not significantly correlated during mid-winter (Table 2). This may reflect the fact that electrolyte leakage can be affected by seasonal changes in the permeability of living cells (WILNER 1959) or may indicate that the EL test did not accurately assess stem survival in midwinter due to difficulty in interpreting stem viability. The TTC method was able to more reliably predict the temperature causing death during the mid-winter period. For this reason the correlation coefficients for these two methods decreased in midwinter.

In conclusion, results from the EL and TTC studies showed that 'Lara', 'Pedro' and 'Z_{63}' may be appropriate for cultivation in regions where mid-winter cold is a concern. 'Serr' and 'Z_{30}' exhibited the least cold hardiness among cultivars and genotypes tested and appeared to be less suitable for such climates. Cold hardiness of walnut is correlated with stem proline concentration. The absolute amount rather than the relative increase in proline concentration seems to be the more reliable marker for predicting cold hardiness. Breeding programs seeking walnut cultivars adapted to cold climates should consider utilizing cultivars and genotypes with a high degree of mid-winter hardening such as 'Pedro', 'Lara' and 'Z_{63}' as parents and in the region where late spring frost is an important problem, cultivars and genotypes that leaf-out late enough to escape the frost('Hartley' and 'Z_{63}') should be selected.

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References

ALETA, N. and A. NINOT 1997: Field evaluation of *Juglans regia* selected clones from seedling populations of Mediterranean and Atlantic Spanish coast. Acta Hort. **442**, 63–67.
AMÉGLIO, T., H. COCHARD and F.W. EWERS 2001: Stem diameter variations and cold hardiness in walnut trees. J. Exp. Bot. **52**, 2135–2142.
AMÉGLIO, T., C. BODET, A. LACOINTE and H. COCHARD 2002: Winter embolism, mechanisms of xylem hydraulic conductivity recovery and springtime growth patterns in walnut and peach trees. Tree Physiol. **22**, 1211–1220.
AMÉGLIO, T., M. DECOURTEIX, G. ALVES, V. VALENTIN, S. SAKR, J.L. JULIEN, G. PETEL, A. GUILLIOT and A. LACOINTE 2004: Temperature effects on xylem sap osmolarity in walnut trees: evidence for a vitalistic model of winter embolism repair. Tree Physiol. **24**, 785–793.
AMÉGLIO, T., G. ALVES, M. DECOURTEIX, M.M. POIRIER, M. BONHOME, A. GUILLIOT, S. SAKR, N. BRUNEL, G. PETEL, R. RAGEAU, H. COCHARD, J.L.J. JULIEN and A. LACOINTE 2005: Winter biology in walnut tree: Freezing tolerance by cold acclimation and embolism repair. Acta Hort. **705**, 241–249.
ANDREWS, P.K. and E.L. PROEBSTING 1987: Effects of temperature on the deep supercooling characteristics of dormant and deacclimating sweet cherry flower buds. J. Amer. Soc. Hort. Sci. **1123**, 334–340.
ASHWORTH, E.N. and F.B. ABELES 1984: Freezing behavior of water in small pores and the possible role in the freezing of plant tissue. Plant Physiol. **76**, 201–204.
ASLANI ASLAMARZ, A., K. VAHDATI, M. RAHEMI and D. HASSANI 2009: Estimation of chilling and heat requirement of some Persian walnut cultivars. HortScience **44**, 697–701.
ASLANI ASLAMARZ, A., K. VAHDATI, M. RAHEMI, D. HASSANI and C. LESLIE 2010: Supercooling and cold-hardiness of acclimated and deacclimated buds and stems of Persian walnut cultivars and selections. HortScience **45**, 1662–1667.

Bates, L.S., R.P. Waldron and I.D. Teare 1973: Rapid determination of free proline for water stress studies. Plant Soil **39**, 205–208.
Cai, Q., S. Wang, Z. Gui, J. Sun and Y. Ishii 2004: Change in freezing tolerance and its relationship with the contents of carbohydrate and proline in overwintering centipede grass (*Eremochloa ophiuroides* (Munro) Hack.). Plant Prod. Sci. **7**, 421–426.
Charrier, G. and T. Améglio 2011: The timing of leaf fall affects cold acclimation by interactions with air temperature through water and carbohydrate contents. Env. Exp. Bot.
Chen, P.M. and L.V. Gusta 1978: Role of water in cold hardiness of winter cereals. In: Lip, H. and A. Sakai (eds.): Plant Cold Hardiness and Freezing Stress. Academic Press, New York, pp. 165–174.
Chen, Y.J., G.W. Cui and X.Q. Fu 1996: The effect of low temperature on free proline content in different alfalfa seedling. Grassland of China **6**, 46–50.
Csonka, L.N. 1989: Physical and genetic responses of bacteria to osmotic stress. Microbiol. Rev, **53**, 121–147.
Davarnejad, G., Z. Aryanpooya, A. Fahadan and E. Davarnejad 2009: Evaluation of susceptibility of walnut genotypes to sudden cold and frost injury. Hort. Environ. Biotechnol. **50**, 497–501.
Dexter, S.T., W.E. Tottingham and L.F. Graber 1930: Preliminary results in measuring the hardiness of plant. Plant Physiol. **5**, 215–223.
Ducci, F., A. Rogatis and R. Proietti 1997: Protezione delle risorse genetiche di *Juglans regia* L.. Ann. Inst. Sper. Selv. **25/26**, 35–55 (In Italian).
Dunner, E.F. and T.J. Gianfagna 1991: Peach pistil carbohydrate and moisture contents and growth during controlled deacclimation following ethephon application. J. Amer. Soc. Hort. Sci. **116**, 507–511.
Eldridge, K.G., J.V. Owen, A.R. Griffin and C.E. Hardwood 1983: Development of a method for assessing frost resistance of *Eucalyptus*. In: Colloques internationaux sur les eucalyptus resistants au froid. Bordeaux, France, 26–30 Sept. 1983. Association Foret-Cellulose, Nangis, France, pp. 145–151.
Ewers, F.W., T. Améglio, H. Cochard, F. Beaujard, M. Martignac, M. Vandame, C. Bodet and P. Cruiziat 2001: Seasonal variation in xylem pressure of walnut trees: root and stem pressures. Tree Physiol. **21**, 1123–1132.
Fernandez-Lopez, J., N. Aleta and R. Alia 2000: Forest genetic resources conservation of Juglans regia L. IPGRI, Rome, Italy.
George, M.F. and M.J. Burke 1977: Cold hardiness and deep supercooling in xylem of shagbark hickory. Plant Physiol. **59**, 319–325.
Germain, E. 1997: Genetic improvement of the Persian walnut (*Juglans regia*). Acta Hort. **442**, 21–30.
Gusta, L.V., M. Wisniewski, N.T. Nesbitt and M.L. Gusta 2004: The effect of water, sugars proteins on the pattern of ice nucleation and propagation in acclimated and non acclimated canola leaves. Plant Physiol. **135**, 1642–1653.
Imani, A., K. Barzegar and S. Piripireivatlou 2011: Relationship between frost injury and ion leakage as an indicator of cold hardiness in 60 almond selections. Int. J. Nut. Rel. Sci. **2**, 22–26.
Kushad, M.M. and G. Yelenosky 1987: Evaluation of polyamine and proline levels during low temperature acclimation of citrus. Plant Physiol. **84**, 692–695.
Lalk, I. and K. Dorffing 1985: Hardening, abscisic acid, proline and freezing resistance in two winter wheat varieties. Plant Physiol. **63**, 287–292.
Larcher, W. and H. Eggarter 1960: Anwendung des Triphenyltetrazoliumchlorids zur Beurteilung von Frostschäden in verschiedene Achsengeweben bei Pyrus-Arten, und Jahresgang der Resistenz. Protoplasma **51**, 595–619.
Levitt, U. 1980: Responses of plants to environmental stresses. In: Kozlowski, T.T (ed.): Vol. I. Chilling, Freezing and High temperature Stress. Academic Press, New York, pp. 256–258.
Lyons, J.M., D. Graham and J.K. Raison 1979: The plant membrane in response to low temperature: an overview. In: Lyons, J.M., D. Graham and J.K. Raison (eds.): Low Temperature stress in Crop Plant. Academic Press, New York, pp. 4–24.
Lyons, J.M. and S.M. Haig 1995: Estimation of lean and lipid mass in shorebirds using total-body electrical conductivity. The Auk. **112**, 590–602.
Morita, Y., S. Nakamori and H. Takagi 2002: Effect of proline and arginine metabolism on freezing stress of *Saccharomyces cerevisiae.* J. Biosci. Bioeng. **5**, 390–394.
Murelli, C., F. Rizza and F.M. Albini 1995: Metabolic changes associated with cold-acclimation in contrasting cultivars of barely. Plant Physiol. **94**, 87–93.
Parker, J. 1951: Moisture retention in leaves of conifers of the northern Rocky Mountains. Botan. Gaz. **113**, 210–216.
Poirier, M., A. Lacointe and T. Améglio 2010: A semi-physiological model of cold hardening and de-hardening in walnut stem. TreePhysiol. **30**, 1555–1569.
Prasil, I. and J. Zamecnik 1998: The use of a conductivity measurement method for assessing freezing injury. Influence of leakage time, segment number, size and shape in a sample on evaluation of degree of injury. Env. Exp. Bot. **40**, 1–10.
Quamme, H.A. 1978: Mechanism of supercooling in overwintering peach flower buds. J. Amer. Soc. Hort. Sci. **103**, 57–61.
Rhodes, D. and Y. Samaras 1994: Genetic control of osmoregulation in plants. In: Strange, S.K. (ed.): Cellular and molecular physiology of cell volume regulation. CRC Press, Boca Raton, pp. 374–361.
Richardson, E.A., S.D. Seeley and D.R. Walker 1974: A model for estimating the completion of rest of 'Redhaven' and 'Elberta' peach trees. HortScience **9**, 331–332.
Sas Institue, Inc. 2002: SAS User's guide: Statistics. Version **9**. 1. Gray, N.C.
Steponkus, P.L. and F.O. Lanphear 1967: Refinement of the Triphenyl Tetrazolium Chloride method of determining cold injury. Plant Physiol. **42**, 1423–1426.
Stergios, B.G. and G.S. Howel 1973: Evaluation of viability tests for cold stressed plants. J. Amer. Soc. Hort. Sci. **98**, 325–330.
Takagi, H., F. Wamoto and S. Nakamori 1977: Isolation of freeze-tolerant laboratory strains of *Saccharomyces cerevisiae* from proline-analogue-resistant mutants. Appl. Microbiol. Biotechnol. **47**, 405–411.
Tanino, K., C.J. Weiser, L.H. Fuchigami and T.H.H. Chen 1990: Water-content during abscisic-acid induced freezing tolerance in brome grass cells. Plant Physiol. **93**, 460–464.
Timmis, R. 1976: Methods of screening tree seedlings for frost hardiness. In: Cannell, M.G.R. and F.T. Last: Tree physiology and Yield Improvement. Academic Press, New York, pp. 421–435.
Towill, L.E. and P. Mazur 1974: Studies on the reduction of 2, 3, 5-triphenyltetrazolium chloride as a viability assay for plant tissue culture. Can. J. Bot. **53**, 1097–1102.
Turcotte, A., H. Morin, C. Krause, A. Deslauriers and M. Thibeault-Martel 2009: The timing of spring rehydration and its relation with the onset of wood formation in black-spruce. Agr. For. Met. **149**, 1403–1409.
Wanner, L.A. and O. Junttila 1999: Cold-induced freezing tolerance in *Arabidopsis*. Plant Physiol. **120**, 391–399.
Wilner, J. 1959: Note on an electrolyte procedure for differentiating between frost injury of roots and shoots in woody plants. Can. J. Plant Sci. **39**, 512–513.
Wisniewski, M. and G. Davis 1989: Evidence for the involvement of specific cell wall layer in the regulation of deep supercooling of xylem parenchyma. Plant Physiol. **139**, 105–116.
Wisniewski, M. and G. Davis 1995: Immunogold localization of pectin and glycoprotein in tissues of peach with reference to deep supercooling. Trees **9**, 253–260.
Xin, Z. and J. Browse 1998: Eskimole mutants of *Arabidopsis* are constitutively freezing-tolerant. Proc. Natl. Acad. Sci. USA **95**, 7799–7804.
Yashida, S. and M. Uemura 1984: Protein and lipid composition of isolated plasma membranes from orchard grass and changes during cold acclimation. Plant Physiol. **75**, 31–37.
Zeneli, G., H. Kola and M. Dida 2005: Phenotypic variation in native walnut populations of Northern Albania Sci. Hort. **105**, 91–100.

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