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FROST TOLERANCE OF HUNGARIAN-BRED WALNUT CULTIVARS BASED ON CLIMATE CHAMBER AND BIOCHEMICAL INVESTIGATIONS

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1. BACKGROUND AND AIMS

According to an old Hungarian saying, people who plant walnut trees trust in the future. Although we eat far fewer walnuts than we do other fruit, they nevertheless have an important place in our everyday diet.

For people aiming to keep to what current knowledge considers to be a healthy diet, walnuts are a useful food supplement. It is worth including walnuts in the diet on a daily basis, as they have proved to help in preventing a number of chronic diseases.

In addition to a significant amount of protein and omega-3 fatty acid, walnuts are also rich in vitamins B1, B2, B6, C and E and in minerals. The latter include magnesium, iron, copper, zinc and manganese, all of which make a substantial contribution to the proper functioning of enzymes essential for the metabolism.

Among the secondary metabolic products, the consumption of polyphenolic compounds makes a powerful contribution to the long-term maintenance of good health and the strengthening of the immune system thanks to their antioxidant and reducing properties.

Global climate change has a considerable influence on the yield quantity and quality of cultivated crops. In recent years there has been an increase in the frequency of extreme weather events. The weather problems encountered most frequently in the Carpathian Basin are low and high temperatures and fluctuations in the distribution of precipitation. Abiotic stress tolerance has thus become a precondition for safe crop production (Veisz, 2005).

Walnut (*Juglans regia* L.) has poor ecological adaptability. The choice of a suitable growing site is of key importance for the achievement of optimum yield safety. The introduction of new walnut cultivars is often problematic, as the high yield potential of the cultivars will not be manifested if the trees are unable to adapt to the Hungarian climate. From this point of view, one of the major obstacles is the frequency of frosts in early spring. It is thus of prime importance for the cultivars to have late bud break. For this reason, the aim of the present work was to investigate the frost tolerance of walnut for the first time in Hungary. Investigations of this type have been carried out regularly in recent years on a number of stone fruit species, on the basis of which recommendations can be made on which cultivars can be safely cultivated on new growing areas.

With this end in view, changes in biochemical parameters that are related in some way to the frost tolerance of walnut were monitored using methods that could be performed easily, rapidly and cheaply in laboratories with limited facilities. On this basis, in addition to the determination of the extent of frost damage, spectrophotometric measurements were chosen for the analysis of biochemical parameters such as the activity of the peroxidase enzyme, the total polyphenol content and the reducing capacity.

The first aim was to perform the following investigations:

- frost tolerance tests on walnut cultivars, based on the frost damage recorded in the buds in a climate chamber and its relationship with weather parameters;
- measurements on changes in the activity of the peroxidase enzyme in the buds of the tested cultivars in order to determine whether the method was suitable for use in further experiments;
- in the case of positive results, measurements on the peroxidase enzyme activity in various phenophases in the buds, phloem, leaves and shoots of five cultivars (the two cultivars most frequently cultivated at present, one frost-sensitive and one frost-tolerant hybrid, and a foreign cultivar);
- determination of the total quantity of polyphenolic components produced in various plant organs via secondary metabolic processes in the plant defence system, after several types of extraction (FRAP, TPC).

Based on the biochemical results, the next aim was to reveal how the parameters tested correlated with the results of frost tolerance tests and the weather parameters.

Finally, the results were statistically evaluated to find explanations, to draw conclusions and to make recommendations on the usefulness of the methods.

The present work was novel, as experiments of this type have not previously been performed on walnut in Hungary.

2. MATERIALS AND METHODS

2.1. Experimental varieties

The varieties included int he study are listed in table 1. The table also indicates which variety was included in which study.

Table 1. The varieties studied

Variety name	Abbreviated designation	Artifical freezing (Lengyeltóti)	Preliminary experiment 2015. (POD) (Lengyeltóti)	Expariments 2016. (POD,TPC,FRAP) (Érd- Elvira major)
’Alsószentiváni 117’	A117	*	*	*
’Bonifác’	A117-15		*	
’Alsószentiváni kései’	A117-31	*	*	*
’Milotai 10’	M10	*	*	*
’Milotai bőtermő’	M10-9	*	*	
’Milotai kései’	M10-14	*	*	
’Milotai intenzív’	M10-37	*	*	*
’Tiszacsécsi 83’	T83	*	*	
’Chandler’	CH		*	
’Fernor’	FE		*	
’Pedro’	P	*	*	*

Our experiments from October 2013 to January 2017, using several plant parts and variois studies, were very diverse. Experimental dates and types are shown table 2.

Table 2. Collection time of the studied varieties, examined plant parts and examination methods (October 2013- January 2017; Lengyeltóti, Érd-Elvira major)

Experimental marking	Date of examination	Varieties	Plant part	Experiment
A	2013. October-2014. March; Lengyeltóti	Milotai 10, Milotai bőtermő, Milotai kései, Milotai intenzív, Tiszacsécsi 83, Alsószentiváni 117, Alsószentiváni kései, Pedro, Fernor	shoot	frost tolerance
B	2014. October-2015. March; Lengyeltóti	Milotai 10, Milotai bőtermő, Milotai kései, Milotai intenzív, Tiszacsécsi 83, Alsószentiváni 117, Alsószentiváni kései, Pedro	shoot	frost tolerance
C	2015. October-2016. March; Lengyeltóti	Milotai 10, Milotai bőtermő, Milotai kései, Milotai intenzív, Tiszacsécsi 83, Alsószentiváni 117, Alsószentiváni kései, Pedro	shoot	frost tolerance
D	2015. February-March; Lengyeltóti	Milotai 10, Milotai bőtermő, Milotai kései, Milotai intenzív, Tiszacsécsi 83, Alsószentiváni 117, Alsószentiváni kései, Pedro, Fernor	bud	POD
E	2015. September; Érd- Elvira major	Milotai 10, Milotai bőtermő, Milotai kései, Milotai intenzív, Tiszacsécsi 83, Alsószentiváni 117, Alsószentiváni kései, Bonifác, Pedro, Fernor	leaf	POD
F	2015. September- 2016. March Érd, Elvira major		phloem	POD
G	2015. September-2016. March; Érd- Elvira major	Milotai 10, Milotai intenzív, Alsószentiváni 117, Alsószentiváni kései, Pedro	bud	POD
H	2016. May; Érd- Elvira major	Milotai 10, Milotai intenzív, Alsószentiváni 117, Alsószentiváni kései, Pedro	leaf, F, K, A	POD
I	2016. November-2017. January; Érd, Elvira- major	Milotai 10, Milotai intenzív, Alsószentiváni 117, Alsószentiváni kései, Pedro	shoot, F, K, A	TPC, FRAP

2.2. Experimental location

The ***frost tolerance tests*** (A, B, C) were carried out from October 2013 to March 2014, from October 2014 to March 2015 and from October 2015 to March 2016. One-year-old shoots were collected in the orchards maintained by the Juglans Hungária Kft. in Lengyeltóti, where the number of sunshine hours a year averaged 2098 hours, the annual mean temperature was 11.2°C and the annual mean rainfall quantity was 550 mm. The soil was a chernozem (upper limit of plasticity, $K_A \equiv 38$, pH=8, total lime content in the upper 60 cm soil layer 5%, humus content 1.9%) and the height above sea level was 150–160 m (Bujdosó et al., 2019). During the first week of each month 10–12 one-year-old shoots each measuring 50 cm were collected from each cultivar in Lengyeltóti and sent to the Department of Fruit-Bearing Plants, Faculty of Horticulture, Hungarian University of Agricultural and Life Sciences for frost tolerance tests.

The plant material used for the ***analytical tests*** (D, E, F, G, H, I) was collected from trees in the central stock plantation of the Fruitculture Research Institute of the Hungarian University of Agricultural and Life Sciences in Elvira-major, Érd. These samples were tested using biochemical methods in the university's Department of Applied Chemistry. The number of sunshine hours in this orchard averaged 2079 hours a year, the annual mean temperature was 11.4°C, the mean temperature during the growing season (Apr.–Sept.) was 18.4°C, the minimum temperature in spring (Mar.–May) averaged 5.3°C, the number of frost days in spring averaged 4.8 days a year, and the annual mean rainfall quantity in the three years of the experiment averaged 552 mm. The soil was a chernozem with lime deposits (upper limit of plasticity K_A=40, pH=8, total lime content in the upper 60 cm soil layer 5%, humus content 2.3–2.5%) (Makay, 2013).

2.3. Experimental protocols

2.3.1. Artificial freezing tests (A, B, C)

The cultivars tested were 'Milotai 10', 'Milotai bőtermő'. 'Milotai kései', 'Milotai intenzív', Alsószentiváni 117'. Álsószentiváni kései' and 'Tiszacsécsi 83', all bred in Hungary, and 'Pedro', bred in California. One-year-old shoots, collected once a month from October to March, were used in the experiment. Ten to twelve shoots were collected for each cultivar, depending on their thickness and on the number of mixed buds. The laboratory analyses were performed in the Department of Fruit-Bearing Plants, Faculty of Agriculture, Hungarian University of Agricultural and Life Sciences.

The ***artificial freezing tests*** were carried out in a Rumed 3301 climate chamber (Rubarth Apparate GmbH). On each occasion tests were made at three freezing temperatures, chosen based on trends in the external temperature. While the lowest temperature applied in October was –16°C, in January buds that had already undergone a hardening process were tested at –26°C. The rate of cooling and warming was 2°C an hour. The shoots were kept at the freezing temperature for a period of four hours. At the end of the treatment the samples were left at room temperature for 12 hours, before dissecting the buds and determining the degree of frost damage on the basis of tissue discoloration, green tissues being regarded as healthy and brown tissues as frost-damaged. The aim of the analysis was to determine the LT_{50} value (mean frost tolerance value), i.e. the temperature that caused 50% frost damage in a given cultivar at a given sampling date. The IBM

PASW Statistics 18 program package was used for the statistical analysis and the LT_{50} values were determined by means of linear regression.

2.3.2. Preparatory and experimental steps in biochemical analysis

Buds, phloem, shoots and leaves were tested, all of which may be involved in frost tolerance. Before carrying out the comprehensive analysis, a preliminary experiment was performed to determine whether there was any correlation between the results and the frost tolerance of the cultivars, which would justify further investigations. The analysis of peroxidase enzyme activity, performed as a ***preliminary experiment*** (D), was begun in 2015 on the Hungarian cultivars ‘Milotai 10’, ‘Milotai bőtermő’. ‘Milotai kései’, ‘Milotai intenzív’, Álsószentiváni 117’. Álsószentiváni kései’, ‘Bonifác’ and ‘Tiszacsécsi 83’, and on the foreign cultivars ‘Pedro’, ‘Chandler’ and ‘Fernor’.

2.3.2.1. Preparation of bud samples (D, G)

The buds were cut off the shoots and rubbed in liquid nitrogen, after which a 250 mg/ml extract was prepared by rubbing them with quartz sand in Na phosphate buffer, pH7.0. After centrifugation (1300 rpm, 20 min, 10°C) the clear supernatant was stored at –32°C until required for analysis.

2.3.2.2. Preparation of phloem and leaf samples (E, F)

After removing the phloem from the shoots, the protocol described for buds was followed, except that a 300 mg/ml extract was prepared. This procedure was also followed for leaf samples taken in September 2015.

2.3.2.3. Preparation of leaf samples (H)

Samples were taken on three occasions in May 2016 (May 2nd, 12th, 20th) during *the leaf bud bursting stage* and measurements were made on the uppermost (youngest) leaves in three replications. Freshly collected leaf samples were rubbed in liquid nitrogen and a 300 mg/ml solution was prepared in Na phosphate buffer (pH 7.0) by rubbing with quartz sand. After centrifugation (1300 rpm, 20 min, 10°C) the clear supernatant was stored at –32°C until required for analysis.

2.3.2.4. Preparation of shoot samples (I)

The tests were made in November and December 2016 and in January 2017. One-year-old shoots were collected and divided into three parts, upper (U), middle (M) and lower (L). After separately grating the individual internodes, 300 mg of each was used to prepare a solution in 10 ml 20% alcohol. These were then placed in an ultrasonic water bath for an hour to achieve better dissolution. After centrifugation (1300 rpm, 20 min, 10°C) the clear supernatant was stored at –32°C until required for analysis.

2.3.2.5. Measurement of peroxidase enzyme activity (D, E, F, G)

The peroxidase enzyme activity of the leaves was recorded spectrophotometrically (Hitachi U-2880A) at $\lambda = 460$ nm in the presence of H_2O_2 substrate and orthodianisidine chromogenic reagent ($\varepsilon = 11.3$) (Shannon et al., 1966). The results were expressed as U/g wet mass.

2.3.2.6. Determination of total polyphenol content (H, I)

The polyphenol content of the upper node of each shoot was determined spectrophotometrically at $\lambda = 760$ nm with Folin-Ciocalteu reagent (Merck 109001) according to the method described by Singleton and Rossi (1965), with the help of a gallic acid (GA) calibration curve. The results were expressed as gallic acid equivalents (µm GA/g wet mass).

2.3.2.7. Determination of antioxidant capacity (H, I)

The antioxidant capacity was determined spectrophotometrically at $\lambda = 593$ nm using the FRAP (ferric reducing antioxidant power) method as described by Benzie and Strain (1966) with the help of an ascorbic acid (AA) calibration curve. The results were expressed as ascorbic acid equivalents (µm AA/g wet mass).

The data were statistically analysed using the ANOVA model. Means were compared and significant differences were determined using the Duncan test at the 95% significance level. The SPSS 25.0 (Chicago, USA) program package was used for the statistical evaluation.

3. RESULTS AND DISCUSSION

3.1. Frost tolerance experiments

3.1.1. Experiment A, Okt. 2013–Mar. 2014, artificial freezing, samples from Lengyeltóti

Artificial freezing experiments were begun during the 2013/2014 dormancy period. The standard cultivars tested were ‘Milotai 10’, ‘Alsószentiváni 117’ and ‘Tiszacsécsi 83’, all state registered genotypes originating from selection on local varieties in Hungary, and ‘Pedro’, a cultivar bred in California. The greatest frost tolerance was recorded for ‘Tiszacsécsi 83’. ‘Milotai 10’ and ‘Pedro’ were frost-sensitive during the dormancy period, while ‘Alsószentiváni 117’ and ‘Tiszacsécsi 83’ were shown to be frost-tolerant in comparison with the other walnut cultivars. The cultivars ‘Milotai bőtermő’, ‘Milotai kései’ and ‘Milotai intenzív’ were also included in the experiments. The following order was obtained on the basis of the results: ‘Milotai kései’ was the most frost-tolerant, followed by ‘Milotai bőtermő’ and ‘Milotai 10’, while ‘Milotai intenzív’ was the most frost-sensitive. Very similar LT_{50} values were detected for ‘Alsószentiváni 117’ and ‘Alsószentiváni kései’ in the artificial freezing tests at the beginning of dormancy, and the LT_{50} values of ‘Alsószentiváni kései’ remained low in January and March.

3.1.2. Experiment B, Okt. 2014–Mar. 2015, artificial freezing, samples from Lengyeltóti

During the 2014/2015 dormancy period the lowest LT_{50} values in January were recorded for ‘Tiszacsécsi 83’. This was at least two degrees lower than that of ‘Alsószentiváni 117’ and at least three degrees lower than that of ‘Milotai 10’. During this period ‘Pedro’ proved to be the most frost-sensitive. As the weather warmed up in March the LT_{50} values of the cultivars also rose.

The cultivar ‘Milotai 10’ and its hybrids were tested again in this season, and the results were similar to those obtained in the previous year. The frost tolerance of ‘Alsószentiváni 117’ and ‘Alsószentiváni kései’ in the 2014/2015 dormancy period were also similar to those recorded in the previous year.

The mean frost tolerance values of five cultivars were selected based on the two-year results. It was important for the group selected to include frost-sensitive and frost-tolerant genotypes with an important role in cultivation (‘Milotai 10’ and ‘Alsószentiváni 117’), one further frost-sensitive (‘Milotai intenzív’) and one frost-tolerant hybrid (‘Alsószentiváni kései’) and the foreign cultivar ‘Pedro’, important as the male parent used in the crosses. In both years ‘Pedro’ had the highest LT_{50} values. The next most sensitive cultivar was ‘Milotai intenzív’, followed by ‘Milotai 10’. ‘Alsószentiváni 117’ had lower values than these cultivars, while the most frost-tolerant genotype was ‘Alsószentiváni kései’.

3.1.3. Experiment C, Okt. 2015–Mar. 2016, artificial freezing, samples from Lengyeltóti

In the winter of 2015/2016 ‘Tiszacsécsi 83’ was found to have the best frost tolerance and ‘Pedro’ the worst throughout the test period. The lowest mean frost tolerance values were recorded for all the cultivars in this winter, as the outside temperature was the lowest in the January of this year. It is thus clear that the frost tolerance of the cultivars followed the outside temperature within a certain temperature range. During February and March a significant 2–3 degree difference was observed between the frost tolerance levels of ‘Milotai 10’ and ‘Alsószentiváni 117’.

3.1.4. Comparison of the three years of artificial freezing tests (Experiments A, B, C) and evaluation of the frost tolerance of the cultivars

No significant differences were revealed by statistical analysis between the results obtained in the three years, so the results were averaged and these averages and their significant differences were used to evaluate the dynamics of frost resistance in the mixed buds and the differences between the cultivars. The frost resistance of the mixed buds gradually increased in the first half of the winter. The hardening process started well before the first sampling time, as LT_{50} values of below –10°C were regularly recorded in mid-October. The first stage of hardening took place when the external temperature was still well above freezing point. By the time longer periods of frost occurred, the LT_{50} values of the mixed buds of some genotypes were already close to –20°C. The hardening process continued until January, when the lowest LT_{50} values were recorded in all three years. This was followed by the dehardening period, when the frost tolerance of the mixed buds gradually decreased, parallel with the gradual rise in the external temperature.

In all three dormancy periods the mixed buds of the tested genotypes were the most frost-tolerant in January. As the LT_{50} values of individual cultivars did not differ significantly over the years, it was concluded that they had reached the maximum genetically possible level of frost resistance, though this needs to be confirmed by investigations in further years. Differences between the winter frost resistance levels of the mixed buds of the cultivars were analysed on the basis of results recorded in January.

Based on the results obtained in the three-year series of experiments, the cultivars were ranked for frost sensitivity and three homogeneous groups were distinguished. The cultivar ‘Pedro’ was the most frost-sensitive, ‘Milotai kései’, ‘Milotai 10’, ‘Milotai bőtermő’ and ‘Milotai intenzív’ were moderately frost-tolerant, while ‘Alsószentiváni 117’, ‘Alsószentiváni kései’ and ‘Tiszacsécsi 83’ were classified as frost-tolerant in this comparison.

3.2. Results of biochemical analysis

3.2.1. Experiment D, Feb.–Mar. 2015, peroxidase enzyme activity of bud samples from Lengyeltóti

Analysis of the buds showed that in February there was a correlation between frost tolerance and the peroxidase enzyme activity in the buds for all the genotypes. It was observed that the peroxidase enzyme activity of the frost-sensitive genotypes was higher at this time, at the end of the dormancy period, than that of the frost-tolerant genotypes, while the enzyme activity declined in all the cultivars in March. As the external temperature rose, leading to a reduction in the stress caused by low winter temperatures, there was a gradual decrease in the activity of the peroxidase enzyme. These observations confirmed those reported in the literature (Lotfi et al., 2010), so other plant organs were included in the experiments during the next dormancy period.

3.2.2. Experiment E, Sept. 2015, peroxidase enzyme activity of leaf samples from Érd-Elvira major

Investigations on 10 genotypes detected similar values for ‘Milotai bőtermő’ and ‘Milotai kései’ and also for ‘Milotai 10’ and ‘Milotai intenzív’. These similarities were reflected in the frost tolerance of the genotypes. The cultivar ‘Alsószentiváni kései’ had the lowest enzyme activity at the beginning of the dormancy period.

3.2.3. Experiment F, Sept. 2015–Mar. 2016, peroxidase enzyme activity of phloem samples from Érd-Elvira major

In the case of frost-tolerant genotypes the peroxidase enzyme quantity was lower than that of frost-sensitive cultivars. Lower values of enzyme activity were recorded in both leaves and phloem for cultivars with greater frost tolerance. A genotype effect was observed between the hybrids and the parental cultivars, as the hybrids always had intermediate values.

The peroxidase enzyme activity values recorded over a lengthy phenological period, from September to March, revealed a cyclic movement in both buds and phloem in response to the hardening process and during bud break (Németh, 2012).

3.2.4. Experiment G, Sept. 2015–Mar. 2016, peroxidase enzyme activity of bud samples from Érd-Elvira major

Based on results obtained in the 2015/2016 dormancy period, changes in the peroxidase enzyme activity in the buds indicated a rise in September and October, parallel with the drop in the external temperature, which peaked in December, when the genotypes achieved maximum hardiness appropriate to the weather conditions. The decrease in the temperature continued in January and February. The rise in the March values correlated with bud break, when early spring frosts may cause damage to more sensitive plant organs, so the plants protect themselves with a rise in peroxidase enzyme activity.

3.2.5. Experiment H, May 2016, peroxidase enzyme activity of leaf samples from Érd-Elvira major during leaf burst

When the leaves burst in May the enzyme activity recorded in the youngest leaves exhibited a trend differing from that recorded in older plant organs. The enzyme activity in the younger leaves of frost-tolerant genotypes was greater, indicating that they were better prepared to cope with stress conditions than the leaves of sensitive genotypes, which had lower enzyme activity. It is therefore probable that young leaves with greater enzyme activity would survive a short period of colder weather better, while the lower enzyme activity of frost-sensitive genotypes would correspond to poorer cold tolerance, so a sudden drop in temperature would be more likely to result in frost damage to young shoots.

3.2.6. Experiment I, Nov. 2016–Jan. 2017, polyphenol content of shoot samples from Érd/Elvira major

In experiments designed to investigate the polyphenol content and reducing capacity of the shoots, it was found that the outstanding polyphenol content of ‘Alsószentiváni 117’ resulted in greater frost tolerance, while less polyphenol was accumulated by the frost-sensitive cultivar ‘Milotai 10’. ‘Alsószentiváni kései’ shifted from the endodormancy state to the ecodormancy state. The sensitivity of ‘Milotai intenzív’ became very obvious, with significant differences throughout the period tested. The values recorded for ‘Pedro’ were medium. As it originates from a warmer climate, it needs a higher polyphenol content if it is to survive frosts than cultivars selected and bred under Hungarian climatic conditions, despite having approximately the same or poorer frost tolerance.

4. CONCLUSIONS AND RECOMMENDATIONS

4.1. Frost tolerance investigations

Walnut is not one of the most frost-sensitive fruit species in the temperate zone, as the overwintering organs are capable of surviving temperatures as low as minus 28–29°C during December and January (Westwood, 1993; Szentiványi and Kállayné 2006; Kállayné, 2014). In spring, however, after the buds burst, the young shoots and the flower organs are often damaged when the orchards are exposed to recurring cold spells. It is therefore important for breeders to select for genotypes with late bud break (Szentiványi and Kállayné, 2006; Kállayné, 2014). If long-term yield safety is to be achieved, however, it is essential to investigate the frost tolerance of cultivated walnut varieties during the winter dormancy period. Detailed studies were thus initiated on the winter frost tolerance of the Hungarian-bred cultivars planted most frequently in Hungary, using the Californian cultivar ‘Pedro’ as a control.

The LT_{50} values of the cultivars were determined during the dormancy period in three consecutive years. The results obtained in the three years were very similar, so no significant year effect could be detected. During these three years the cultivar ‘Tiszacsécse 83’ exhibited the greatest frost tolerance, while the control cultivar, ‘Pedro’, was the most sensitive. The frost tolerance values of ‘Alsószentiváni kései’ were similar to those of ‘Tiszacsécse 83’, while those of ‘Milotai 10’ were close to those of ‘Pedro’. Differences were observed between the hybrids. ‘Milotai intenzív’ proved to be frost-sensitive, ‘Milotai bőtermő’ and ‘Milotai kései’ moderately frost-tolerant and ‘Alsószentiváni 117’ frost-tolerant. The mixed buds had the greatest frost tolerance during the month of January. It is probable that the values recorded in January represented the best frost tolerance levels genetically possible for these cultivars, though further studies will be required to confirm this. In experiments performed by Charrier et al. (2011) a genotype effect was observed from January to the bud burst stage, which was found to play a greater role during this period than the environmental effect.

As regards the weather in the years of the experiment, the minimum temperatures in the first week of October were slightly below average, but from then on the temperature followed the normal trend. Sudden drops in temperature were observed in mid-December, after which the mixed buds entered the second stage of hardening. An intense fall in the temperature was experienced again in mid-January, but at this time the mixed buds of walnut have attained their maximum level of hardiness, so even the most frost-sensitive cultivar had a mean frost tolerance value averaging –23.8°C. The continuous rise in temperature recorded from January onwards was interrupted by one or two very cold mornings in February, but as warming did not take place at a fast rate, these cold spells caused no problems and the mixed buds retained their frost tolerance. In March, as the weather continued to warm up, higher LT_{50} values were detected. In the three years tested, the winter weather was very similar at the experimental location, so no significant year effects could be demonstrated. In longer-term experiments differences would no doubt be observed, as the effect of climatic factors on the frost resistance of overwintering organs has been reported for many temperate zone fruit species (Faust, 1989; Tromp, 2005; Szalay et al., 2010; Szalay et al., 2016; Szalay et al., 2017). No relevant data on walnut have been found, however, in the literature.

The experiments were begun during the 2013/2014 dormancy period, when ‘Tiszacsécse 83’ was found to be the most frost-tolerant cultivar. Among the locally selected cultivars, ‘Alsószentiváni 117’ proved to be frost-tolerant and ‘Milotai 10’ frost-sensitive compared to the other cultivars tested. The most frost-tolerant of the hybrids was ‘Alsószentiváni kései’, followed by ‘Milotai kései’ and ‘Milotai bőtermő’, while ‘Milotai intenzív’ proved to be frost-sensitive. The LT_{50} values recorded for the cultivar ‘Pedro’ during the first two months of the dormancy period differed substantially from those of the other cultivars. This trend continued in the second year. In the third year, however, a considerable change was observed for ‘Alsószentiváni kései’, revealing not only the importance of the year effect, but also the role played by the condition of the tree at the start of the dormancy period. As the external temperature dropped there was a parallel rise in the frost tolerance values of the cultivars, which declined again in response to warming. The walnut cultivars grown in Hungary can be safely grown under Hungarian climatic conditions.

4.2. Biochemical analysis

The biochemical analysis was performed not only on the buds, which are the most sensitive overwintering organs of walnut, but also on the leaves at the beginning of the dormancy period, on the shoots during the dormancy period and on young leaves at the leaf burst stage. Changes in the peroxidase enzyme activity and polyphenol content were monitored in the various plant organs.

At the beginning of the endodormancy period lower levels of enzyme activity were detected in the leaves of more frost-tolerant genotypes than in more frost-sensitive types. In the phloem the enzyme activity was first observed to rise, followed by a decline. As the external temperature decreased (cold stress) there was an increase in peroxidase enzyme activity, but once the plant organs acquired a certain level of hardiness the enzyme activity stagnated or declined, with the reduction in the stress effect. This could be attributed to the fact that the frost tolerance of deciduous trees develops in several stage (Tromp, 2005). In the course of the analyses, 'Tiszacsécse 83' exhibited the lowest values, the sensitive genotypes all having higher values. The reason for this could be that, in the course of development, free radicals may be formed in response to a variety of external or internal effects, in response to which the enzymes involved in eliminating these effects may express enhanced activity. This process could only be studied in detail if new plants of varieties with different levels of sensitivity could be germinated under identical conditions and their enzyme activity could be compared in an identical stage of development. In the present case, the trees are in the process of development, which means that rises in enzyme activity may be due to the aging process as well as to the response to various stress effects.

The comparison of enzyme activity in the selected genotypes revealed a stress response to a change in temperature: at the beginning of October there was an unusually warm period, with a maximum daily temperature of over 20°C for several days, after which the temperature dropped to the 13–16°C level typical of this period. This could have been responsible for the change observed in enzyme activity.

The changes in peroxidase enzyme activity in the buds of the tested genotypes during the 2015/2016 dormancy period reflected the hardening process induced by the external temperature and the transition from endodormancy to ecodormancy. Enzyme activity was low in autumn (in September), with a slight rise in October as the external temperature declined. By December the genotypes had achieved maximum hardiness, as the enzyme activity was the highest in this month. The value dropped again by January, and the lowest values were recorded in February. This is the period when the cultivars pass from the endodormancy to the ecodormancy period, when bud burst begins in response to warming. After the buds have attained maximum frost tolerance, which protects them from damaging effects, a gradual decrease in enzyme activity can be observed. The increase in enzyme activity in

March may have occurred in response to sudden cold spells in early spring. These are special mechanisms that induce the start and finish of dormancy (Westwood 1993). The March measurements proved that, in response to re-hardening in February, the genotypes had regained maximum hardiness to frost by March. This is necessary because spring frosts cause the greatest damage in the annual yield.

It could also be seen from the results that, though opposing enzyme activity values were observed in the buds and phloem in frost-tolerant and frost-sensitive genotypes at the beginning of the dormancy period, by the end of dormancy, i.e. in the ecodormancy period, the level of enzyme activity was lower in frost-tolerant genotypes and higher in frost-sensitive types.

A comparison of the various plant organs investigated in the analysis of peroxidase enzyme activity showed that the highest enzyme activity levels in any given period were recorded in the buds. In all the genotypes the lowest enzyme activity (in terms of wet matter) was detected in the leaves, followed by the phloem and the buds. The question arises of why these values were not converted into dry matter content, but it was thought that these values gave the best illustration of the results observed at sampling, being more expressive of the differences in the individual samples and genotypes. All in all, it can be said that the initial hypothesis was confirmed, as differences in enzyme activity could be demonstrated in all three plant organs. These differences could be attributed partly to the genetic background and partly to adaptation to the weather conditions.

During leaf burst in spring it was observed that the enzyme activity of frost-tolerant genotypes was high, allowing the young leaves to survive minor drops in temperature. The opposite was observed for the frost-sensitive genotypes, i.e. the reduction in enzyme activity was paralleled by lower cold tolerance, leading to a greater possibility that a sudden drop in temperature could cause frost damage to the shoots. In the case of older leaves, however, higher enzyme activity was observed for the frost-sensitive genotypes. It was thus concluded that the lower enzyme activity in the younger organs resulted in lower resistance, making them less able to protect themselves against early spring frosts.

In the experiment designed to investigate the polyphenol content and reducing capacity of the shoots, it was found that the outstandingly high polyphenol content of 'Alsószentiváni 117' resulted in better frost tolerance, while 'Milotai 10' accumulated less polyphenol and was thus more sensitive. These results were in agreement with those of Farokhzad et al. (2018), who reported that higher polyphenol content was associated with better stress tolerance. The results obtained for ‘Alsószentiváni 117’, together with its high level of frost tolerance, suggested that the transition from endodormancy to ecodormancy took place later in this cultivar. Significant differences were detected for ‘Milotai intenzív’ throughout the experimental period, clearly indicating its sensitivity. The values recorded for ‘Pedro’ were consistently intermediate between those of the other four genotypes. It would appear that, as it originates from a warmer climate, it requires a much higher polyphenol content if it is to survive frosts than is necessary for cultivars selected and bred under Hungarian climatic conditions, even though it has a similar or poorer level of frost tolerance. Similar tendencies were detected for the alcoholic extracts, but this method appeared to be less suitable for the demonstration of stress tolerance, suggesting that it is sufficient to analyse the polyphenol content. Further studies will be required, however, to decide whether the cheap aqueous or the more expensive alcoholic extract should be used.

5. NOVEL SCIENTIFIC RESULTS

1. The frost tolerance of seven Hungarian walnut cultivars and one foreign cultivar was determined by artificial freezing during the dormancy period, with the help of a linear regression model.

2. Measurements proved that the peroxidase enzyme activity and polyphenol content in various plant organs of walnut in certain phenophases were suitable parameters for characterising the frost tolerance of the cultivars.

3. Among the plant organs, the peroxidase enzyme activity measured in the buds was found to be most closely correlated with the frost tolerance of the cultivars in the course of the hardening process.

4. A close correlation was detected between the frost tolerance of the cultivars and the total polyphenol content recorded in the shoots.

6. PUBLICATIONS RELATED TO THE SUBJECT OF THE PhD THESIS

Scientific articles in reviewed journals with impact facot (IF):

1. **KRISZTINA SZÜGYI-B.**, GÉZA BUJDOSÓ, VERONIKA FROEMEL-H., SÁNDOR SZÜGYI, ÉVA STEFANOVITS-B.‚ LÁSZLÓ SZALAY (2021): Evaluation of the frost tolerance of Hungarian-bred walnut cultivars. Acta Biologica Szegediensis. Volume 65 (2) "in press"

Scientific articles in reviewed journals without impact facot (non-IF):

2. **SZÜGYINÉ BARTHA,** K., BUJDOSÓ, G., HAJNAL, V., SZALAY, L. (2014): Magyar nemesítésű diófajták vegyesrügyeinek fagytűrő képessége, Kertgazdaság, 46(1): 31-37 p.

3. **SZÜGYINÉ BARTHA,** K., Froemel- Hajnal, V., Szalay, L., Stefanovitsné Dr. Bányai É., Bujdosó G., (2019): Hazai nemesítésű diófajták fagytűrésének értékelése, Kertgazdaság 51(1) 25-31.p.

Other journals atricle:

4. **SZÜGYINÉ BARTHA,** K., BUJDOSÓ, G., HAJNAL, V., SZALAY, L. (2015): A dió fagytűrése. Agrofórum, 58. extra: 74-75 p. E

5. **SZÜGYINÉ BARTHA**, K., IZSÉPI, F., BUJDOSÓ, G., HAJNAL, V., SZALAY, L. (2015): A dió alkalmazkodóképessége. Kertészet és Szőlészet, 10 (64): 16.p.

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8. **SZÜGYI**, K. **B**., BUJDOSÓ, G., HAJNAL, V., SZALAY., L. (2017): Examination of Hungarian bred Persian walnut cultivars' resistance to frost. In: *XIV EUCARPIA Symposium on Fruit Breeding and Genetics 1172*. 2015. p. 391-394. DOI: 10.17660/ActaHortic.2017.1172.74

9. **SZÜGYI-BARTHA**, K., HAJNAL, V., SZALAY, L., BUJDOSÓ, G. (2020): Frost behavior of Hungarian bred Persian walnut cultivars. In: *XXX International Horticultural Congress IHC2018: International Symposium on Nuts and Mediterranean Climate Fruits, Carob and X 1280*. 2018. p. 85-88. DOI: 10.17660/ActaHortic.2020.1280.12

Conference proceeding abstract:

10. **SZÜGYINÉ BARTHA,** K., HAJNAL, V., SZALAY, L., IZSÉPI, F., BUJDOSÓ, G. (2015): Magyar és külföldi nemesítésű diófajták fagytűrő képességének vizsgálata, XXI. Növénynemesítési Tudományos Napok, 126 p.

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