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ORIGINAL ARTICLE / ORIGINALBEITRAG

Biochemical Methods for the Characterisation of Walnut Genotypes with Different Levels of Frost Tolerance

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Abstract

In plants, stress induces changes in peroxidase enzymes, which play various physiological roles, including involvement in the development of resistance. Experiments were performed at the Elvira major Experimental Station of the NARIC Research Institute for Fruitgrowing and Ornamentals in Érd, Hungary, on two genotypes selected in Hungary ('Alsószentiváni 117' and 'Milotai 10'), on the Californian-bred cultivar 'Pedro', and two genotypes bred in Hungary by crossing 'Pedro' with the two Hungarian selections: 'Milotai intenzív' and 'Alsószentiváni kései'. These genotypes were chosen on the basis of frost tolerance. Measurements were made on the peroxidase activity and total polyphenol content in the leaves during sprouting (May 2nd, 12th and 20th 2016) and in the uppermost internode of the shoots (November, December 2016; January 2017). Higher peroxidase enzyme activity and polyphenol content in the uppermost internode were good indicators of the frost tolerance of the genotypes and of the stress level to which they were exposed.

Keywords Peroxidase enzyme · Polyphenol content · Human health · Nut tree species · One-year-old shoots

Introduction

The fruit production as part of agriculture faces major challenges to ensure annual production. While the fruit site conditions are more or less stable, the temperature data show a great interannual variability, which is likely the most initiative factors of year effects in phenological phases (Archibald et al. 2009; Hereford et al. 2006; Zaitchik et al. 2007).

Many in situ studies were published about behaviours of pome (Bergamaschi et al. 2008; Chmielewski et al. 2011; Legave et al. 2013; Kunz and Blanke 2016) and stone fruit species (Bergamaschi et al. 2008, Aliman and Drkenda 2009; Kaufmann and Blanke 2017) for variations in temperature during winters and springs, but there were just a handful on how the nut tree species respond to these factors. Warm winters may delay the spring phenology, and the warm springs increased the "speed" of flowering in California (Luedeling and Gassner 2012).

Fu et al. (2015) reported on the declining spring phenology response of trees to the warming springs over the past 30 years. This decline is sensitive, response of the early spring species probably correlates with cooling trend during late winter in western Central Europe (Fu et al. 2014) and the reason for it may be the decreased chilling during the warm years (Fu et al. 2015). Walnut (*Juglans regia* L.) is a widespread deciduous tree species of great economic importance. According to Vavilov (1951) this walnut species originated in Persia, its primary gene centre. Its native range extends from Iran, northern Turkey, the Carpathian Mountains of Eastern Europe to the Southern Caucasus (Aradhya et al. 2010). Walnuts are very valuable fruit with high energy content (630 kcal/100 g). They are rich in saturated and unsaturated fatty acids, which have a very favourable composition, including a substantial quantity of omega-3. Walnuts are good sources of vitamin E, folic acid, calcium, magnesium and potassium (Lavardine et al. 1997; Ruggeri et al. 1998) and are also very rich in polyphenolic components (Amaral et al. 2004; Anderson et al. 2001; Fukuda et al. 2003; Pereira et al. 2007). Thanks to the presence of these endogenous compounds, which are important for antioxidant protection, epidemiological analyses detected

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a correlation between walnut consumption and a decline in deaths caused by heart and circulation diseases (Feldman 2002). Negi et al. (2001) also reported that regular walnut consumption reduced the risk of coronary diseases.

It is generally considered that polyphenolic compounds have a positive effect on human health as they reduce the risk of degenerative diseases, mitigate oxidative stress and inhibit macromolecular oxidation (Silva et al. 2004; Pulido et al. 2000). These compounds are the products of secondary plant metabolism and have strong antioxidant characteristics, thus protecting both plants and their human consumers from stress (Grace and Logan 2000; Haminiuk et al. 2012; Manach et al. 2004; Michalak 2006). One possible response to stress is the neutralisation of reactive oxygen species (ROS) through the activation of enzymatic (superoxide dismutase, peroxidases, catalase, etc.) or non-enzymatic (vitamins C and E, polyphenols, carotenoids, etc.) defence systems (Asada 2006; Mittler 2002). Changes in peroxidase enzymes and phenolic compounds were reported in plants in response to stress (Pereira et al. 2000; Amaral et al. 2004; Cosmulescu and Trandafir 2011). These enzymes are thought to play various physiological roles, for example in the development of resistance (Böddi 2002). Solar et al. (2006) detected a correlation between enhanced polyphenol content in the shoots and infection with *Xanthomonas campestris pv. juglandis*. The accumulation of phenolic compounds lowered sensitivity to the pathogen. The aim of the present work was to detect possible correlations between the development of cold stress and changes in polyphenol concentration or peroxidase enzyme activity, as these could characterise the physiological and biochemical processes taking place in the plants during various phenophases.

Materials and Methods

Biological Material

The experiments were performed on two varieties selected in Hungary at the Elvira major Station of the Research Institute for Fruitgrowing and Ornamentals, National Agricultural Research and Innovation Centre in Érd: 'Alsószentiváni 117' and 'Milotai 10', on the 'Pedro' variety bred in California, and on genotypes made in Hungary by crossing 'Pedro' with the two Hungarian varieties: 'Milotai intenzív' ('Milotai 10' × 'Pedro') and 'Alsószentiváni kései' ('Alsószentiváni 117' × 'Pedro'). These genotypes were chosen based on freezing experiments conducted between 2013 and 2015, which allowed them to be classified as frost-sensitive, moderately frost-sensitive or frost-tolerant. 'Alsószentiváni kései' proved to be the most frost-tolerant, followed by 'Alsószentiváni 117', 'Milotai 10', 'Milotai intenzív' and 'Pedro', which was the most frost-sensitive under Hungarian conditions.

Leaf Sample Preparation

Samples were taken from the uppermost leaves in three replications on three occasions during the sprouting period in May (on May 2nd, 12th and 20th 2016). The freshly picked leaves were ground with quartz sand in liquid nitrogen and a 300 mg/ml solution was prepared using Na phosphate (pH 7.0) buffer. After centrifugation (1300 rpm for 20 min at 10 °C) the clear supernatant was stored at –32 °C until required for analytical measurements.

Shoot Sample Preparation

The upper internodes of shoots were collected in November and December 2016 and January 2017, and the analyses were made in two replications. A 300 mg/10 ml 20% alcohol solution was made from the grated internodes, and this was placed in a UH water bath for 1 h to achieve better dissolution. After centrifugation (1300 rpm for 20 min at 10 °C) the clear supernatant was stored at –32 °C until required for analytical measurements.

Measurement of Peroxidase Enzyme Activity

The peroxidase enzyme activity of the leaves was determined in the presence of H_2O_2 substrate and ortho-dianisidine chromogenic reagent ($\varepsilon = 11.3$) using a spectrophotometer (Hitachi U-2880A) at $\lambda = 460$ nm (Shannoon et al. 1966). The results were given as U/g wet weight.

Determination of Total Polyphenol Content

The polyphenol content of the upper internodes of the shoots was determined spectrophotometrically according to the method of Singleton and Rossi (1965), using Folin-Ciocalteu reagent (Merck 109001) at $\lambda = 760$ nm with the help of a calibration curve for gallic acid. The results were given as mM gallic acid equivalent (GAE)/g wet weight.

Results

A comparison of the peroxidase enzyme activity in the upper, youngest leaf samples revealed an increase in this activity from the beginning to the end of May (Fig. 1). The differences between the initial and final values, expressed in round numbers (U/g), were 244 for 'Alsószentiváni kései', 166 for 'Alsószentiváni 117', 367 for 'Pedro', 490 for 'Milotai 10' and 430 for 'Milotai intenzív', which exhibited a correlation with the resistance of the genotypes to

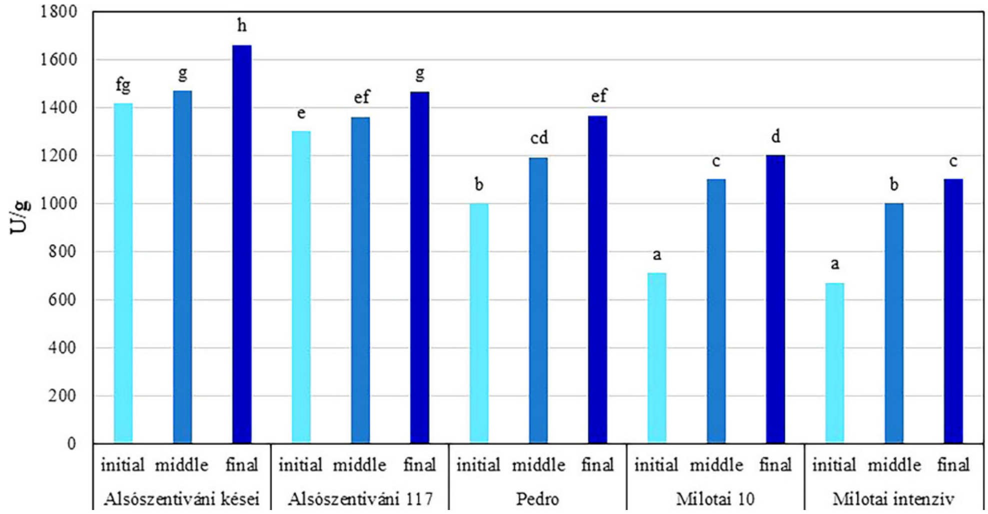

Fig. 1 Peroxidase enzyme activity (U/g) in the uppermost (juvenile) leaves during spring sprouting (different small letters represent significant differences at $P=0.05$)

a sudden drop in temperature. The difference in enzyme activity could be attributed to two factors: the aging of the leaves (Sárdi and Stefanovits-Bányai 2006) and the stress effects indicated by the meteorological data (Fig. 2; Pereira et al. 2000). The initial levels of enzyme activity differed for the individual genotypes, with higher values for frost-tolerant varieties and lower values for those sensitive to frost. The differences between the initial and final values were smaller for the resistant varieties and greater for the sensitive ones, indicating their response to cold stress (Sárdi and Stefanovits Bányai 2006). A decline of almost five degrees in the lowest daily temperature was observed between the first and last measuring dates.

It is a well-known fact that the content of polyphenol compounds is closely correlated to the stress tolerance of individual varieties (Melo et al. 2006). The data in Fig. 3 show that the polyphenol content in the upper internodes increased over the months, as the trees prepared for the winter. The differences already observed in the initial values in November for varieties with different levels of frost sensitivity became more pronounced by January. Again, the values recorded for more resistant varieties were higher than those of their sensitive counterparts both in November and January. Here too the increase could be related in part to the aging of the leaves, but was also influenced by the cold stress, to which the sensitive varieties gave a greater response. While a temperature minimum of around –5 °C was characteristic of early November, this dropped to almost –15 °C in January (Fig. 4). A lower polyphenol content was recorded in the frost-sensitive Hungarian varieties, making the buds more likely to suffer frost damage, while 'Pedro' was found to have a polyphenol content similar to

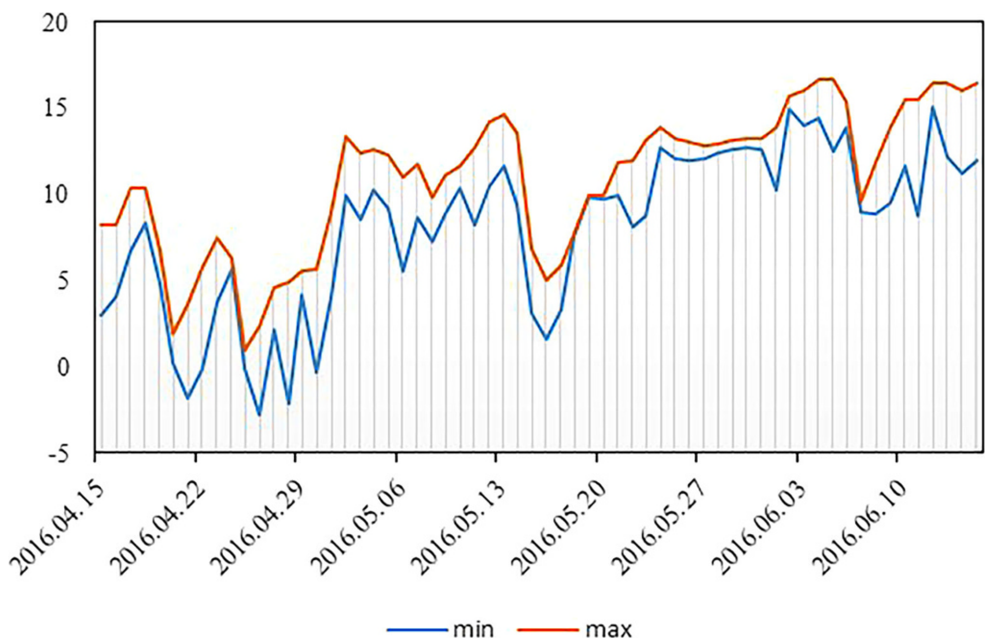

Fig. 2 Changes in temperature during spring sprouting at the Experimental Station in Elvira major, Érd (April–June 2016)

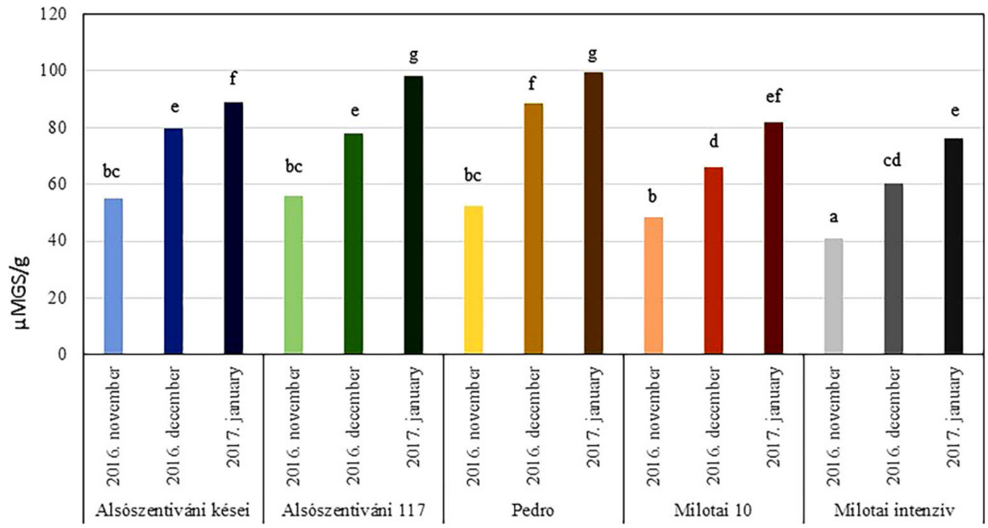

Fig. 3 Changes in the polyphenol content (mMGS/g) in the uppermost internodes of the shoots during the dormancy period (different small letters represents significant differences at $P=0.05$)

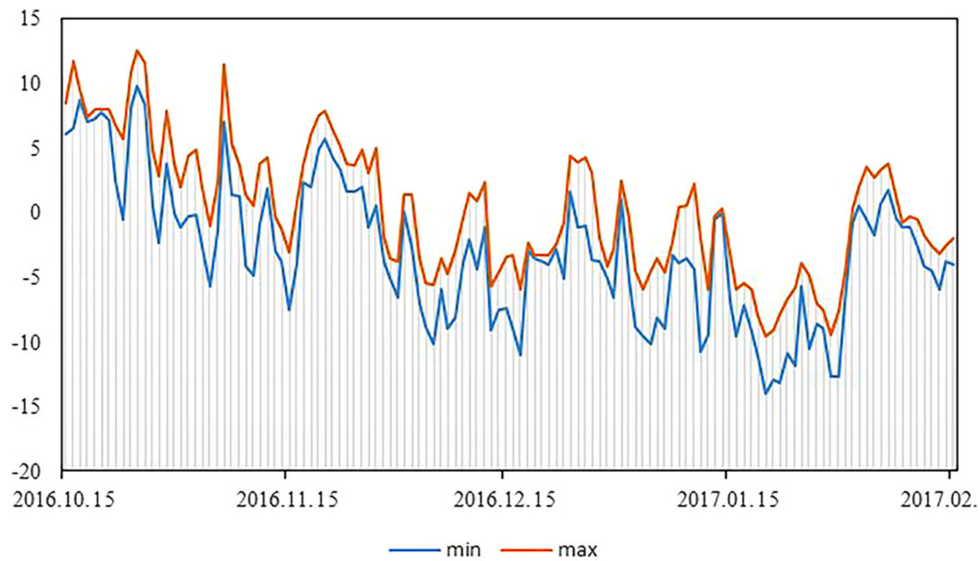

Fig. 4 Temperature trends during the dormancy period at the Experimental Station in Elvira major, Érd (October 2016–February 2017)

that of the frost-tolerant varieties. This could be explained by the fact that, having been bred in a warmer climate, this variety responded to cold stress by the overproduction of antioxidant polyphenol compounds, thus allowing the buds to survive the winter months. Therefore, although the polyphenol content was high, the frost resistance of the shoots was similar to that of the frost-sensitive varieties.

Conclusions

In summary, it can be said that the peroxidase enzyme activity in the leaves increased in accordance with the frost sensitivity of the genotypes both as the vegetation period proceeded and in response to the cold stress caused by a decline in temperature. The polyphenols recorded in the shoots during the winter dormancy period also exhibited a rise both over time and parallel to the drop in the external temperature. It could be seen that both the rise in the peroxidase enzyme activity in the leaves and the accumulation of polyphenolic compounds in the uppermost internode could be good indicators of the diverse frost tolerance of varieties and of the stress effects to which they are exposed.

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Conflict of interest K.S. Bartha, G. Bujdosó and É. Stefanovits-Bányai declare that they have no competing interests.

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