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Effects of Green Husks of Hungarian-Bred Cultivars on Their Walnut Bacterial Blight Susceptivity

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RESEARCH ARTICLE

Abstract

The Persian walnut (*Juglans regia* L.) is one of Central Europe's most grown nut trees. In the green husks of walnuts, among the most important bioactive compounds are phenolics (hydroxybenzoic acids, hydroxycinnamic acids, flavonoids, naphthoquinones), which play an important role in the plant defense mechanisms against stress caused by various pathogens e.g., *Xanthomonas arboricola* pv. *juglandis*, furthermore mentioned as *Xaj*. The study examined the full Hungarian walnut assortment to detect the difference in phenolic profiles of their green husk and the relationship between the concentration of phenolic compounds, as well as their effects on tolerance against *Xaj*. All the phenological stages were recorded by Ctifl scheme. The phenolic compounds were determined by HPLC-DAD-ESI-QDA method, after which susceptibility test was carried out. The results show that concentration of the phenolic compounds varied by cultivars; it was higher in the locally-bred cultivars compared to 'Chandler', a more widespread cultivar. Phenolic compounds inhibited the artificial *Xaj* infection in the green husk. This effect had a strong correlation with the high phenols' concentration, except for 'Bonifác'. It is likely that the phenolics' concentration in the walnut green husk has an important role in their defense against *Xaj* infection.

Keywords: phenolic compounds; Hungary; fruit development; state-approved cultivars; walnut blight immunity test; walnut phenology

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INTRODUCTION

The Persian walnut (*Juglans regia* L.) is Central Europe's most grown nut tree crop; its planting area and harvested yield are increasing annually in this region. All parts of the walnut tree, such as leaves, green husk (mesocarp), pellicle, septum, and kernel are rich in phytochemicals (Rahimipanah et al., 2010; Al-Nadaf et al., 2018; Marius et al., 2018; Acquaviva et al., 2021). The fruit is surrounded by a green husk that contains many bioactive compounds, such as polyphenols, which include phenolics (Oliveira et al., 2008; Carvalho et al., 2010; Croitoru et al., 2019; La Torre et al., 2021), pectin and glucans (Acquaviva et al., 2021) and chlorophyll (Popovici et al., 2012). The main groups of phenolics are hydroxybenzoic acids, hydroxycinnamic acids, flavonoids, and naphthoquinones (Jakopic et al., 2008; Jahanban-Esfahlan et al., 2019). Dihydroxybenzoic acid, gallic acid, and syringic acid, together with their derivatives, are hydroxybenzoic acids, while coumaric acid, chlorogenic acid, caffeic acid, ferulic acid and its esters, are hydroxycinnamic acids. Flavonoids include myricetin, quercetin and

naringenin and their derivative, while naphthoquinones include juglone, with its different derivatives (Jakopic et al., 2008; Jahanban-Esfahlan et al., 2019). The phytochemicals have antimicrobial, antifungal and antioxidant activities (Oliveira et al., 2008; Ding et al., 2013; Salejda et al., 2015; Panth et al., 2016; Wianowska et al., 2016; Sadeghi-Kiakhani et al., 2019; Wianowska et al., 2020; Dehghani et al., 2020).

The phenolic compounds play an important role in the plant defense mechanisms against stress caused by many environmental factors and various pathogens (Medic et al., 2022). The phenolic content and its profile vary in all walnut organs (Kamran et al., 2011; Hama et al., 2016; Colarič et al., 2020). The quantity depends on the soil and climatic conditions (Cosmulescu et al., 2011; La Torre et al., 2021), cultivar (Oliveira et al., 2008; Jahanban-Esfahlan et al., 2019; Cosmulesscu et al., 2010, 2011; Akbari et al., 2012; Rahmani et al., 2018), sampling period (Jakopic et al., 2008), extraction solvent (Singleton et al., 1999; Jakopic et al., 2007; Jakopic et al., 2009; Fernández et al., 2013; Wianowska et al., 2020; Oluwaseun et al., 2021), including the cultivation technology used in the orchard.

The phenolic compounds in husks play an important role in the defense against walnut blight caused by *Xanthomonas arboricola* pv. *juglandis* (Xaj). The Xaj can attack all green parts of the walnut, and thus destroy the attacked organ(s).
The aim of the study was to examine the full Hungarian walnut assortment, having a distinct early spring phenology, in order to detect the difference in phenolic profiles of their green husks. Furthermore, the relationship between the concentration of phenolic compounds and tolerance/resistance of the observed cultivars to walnut bacterial blight at the maturity stage of fruits was investigated.

MATERIALS AND METHODS

Plant Material

The trial was conducted at the Experimental Fields of the Hungarian University of Agriculture and Life Sciences, Research Centre for Fruit Growing (GPS coordinates: N 47°20′11,44" E 18°51′53,42"). The orchard was established in 1990 with a plant spacing of 10 × 10 m. The trees were trained as a central leader canopy. The orchard was not irrigated. Sampling was done in 2020 and the meteorological conditions were as shown in Table 1 below.

Table 1. Meteorological data during the data collection 2020

Parameters	Value
Average yearly temperature	11.4 ℃
Average yearly temperature during the growing season (March–September)	16.1 ℃
Average yearly luminous flux	1015 l/m^2/day
Average yearly precipitation	434.1 mm
Annual average of sunshine hours	2065

The entire Hungarian walnut assortment namely 'Alsószentiváni 117′ (A117), 'Milotai 10 (M10) ′, 'Tiszacsécsi 83′ (T83), 'Milotai intenzív' (M. intenziv), 'Milotai kései' (M. kései') 'Alsószentiváni kései' (A. kései') 'Bonifác®'were examined. Other cultivars examined were 'Köpcös' and 'Chandler', a US-bred cultivar.

Sample Collection and Preparation

Samples were collected in late June, on the 181st day of 2020, during the lignification of the nutshell. Ten fruits of each cultivar were picked from the trees and were immediately delivered to the laboratory. The fruits were peeled and the green husk was lyophilized. The dried samples were ground to a fine powder and stored hermetically at 4 °C until analysis.

Chemicals

We applied standards for quantification as follows: rutin (quercetin-3-O-rutinoside) and juglone (Merck, Darmstadt, Germany). HPLC/MS grade acetonitrile and formic acid, analytical grade methanol, acetic acid and Folin–Ciocalteu reagent were purchased from Avantor (Radnor, PA, USA).

Total phenolic content and determination of phenolic compounds

Total phenolic content was determined by Folin–Ciocalteu's photometric method after extraction by 80% methanol and the colour reaction, carried out by Folin–Ciocalteu reagent, whereas the absorbance was measured at 750 nm, and results are given in gallic acid equivalent (Singleton et al., 1999).

One hundred mg of dried green husks were placed in a test tube and 10 mL of the extraction solvent (bidistilled water/2%, acetic acid in methanol, 30/70) added. Mixtures were sonicated for 20 min at room temperature. After centrifugation (5000 rpm, 20 min), the supernatant was filtered through a 0.45 μm PVDF syringe filter before HPLC injection. A Waters Alliance system (Waters, Milford, USA) was used, consisting of a Model e2695 separation module, with Model 2998 photodiode array (PDA) detector, operated by Empower software (Waters, Milford, USA).

The separation was carried out using Sphinx 5 μm 250x4.6 mm column (Macherey-Nagel, Düren, Germany) by gradient elution.

The mobile phase was (A) 0.1% formic acid in bidistilled water and (B) 0.1% formic acid in acetonitrile. The detection wavelengths were at 280, 320, 355 and 420 nm. The HPLC system was coupled to a Model Acquity Mass (QDa) detector (Waters, Milford, USA). The mass spectrometry conditions were set as both negative and positive modes: electrospray ionization (ESI) was used as a source, mass spectra in the m/z range from 100 to 1000 were obtained and probe temperature was adjusted to 600 °C (default). In positive ion mode the cone voltage was set to 15 V and capillary voltage was 1.5 kV. In negative ion mode those were 50 V and 0.8 kV, respectively. Identification of phenolic compounds was achieved by comparing their spectral characteristic, retention times, measured mass (m/z) and fragmentation pattern. In addition, the previously published literature (Nour et al., 2012, Huo et al., 2018,) and internet databases (Phenol-Explorer, PubChem) were applied. The quantification of the identified phenolic compounds was based on calibration curve of rutin at 280, 320 and 355 nm, as rutin gives an easily measurable signal at these wavelengths, and juglone at 420 nm.

The results were given in sum of phenolic components related to different groups, so we used rutin equivalent in quantification to carry out better comparison, except for naphthoquinones, which was calculated in juglone equivalent.

Walnut Blight Immunity Test

The susceptibility test was carried out based on Ozaktan et al. (2008) and Solar et al. (2012) methods, using 10 immature green fruits from each cultivar. A mixture of 3 Xaj strains isolated from naturally infected walnuts were inoculated into the intercellular tissue of either tobacco leaf ('White Burley') or fruits, to test and confirm their virulence. The immature fruits samples were disinfected with alcohol. Two inoculations of 20 μL bacterial suspension were performed in the exocarp of each fruit. Sterile distilled water (SDW) was also injected into each fruit as control treatment. After infection, the fruits were incubated in transparent plastic boxes for 7 days at 26–28 °C with 90–95% relative humidity. A microsensor was used to monitor the temperature and relative humidity. The disease severity was recorded on a scale from 0 to 4 Figure 1 based on the diameter and depth of necrosis reached on the 7th day.

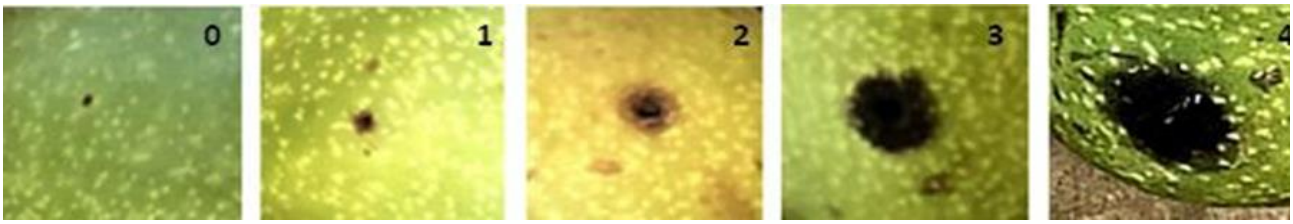

Figure 1. Susceptibility scale (0–4): 0-no symptoms; 1-less than 2.0 mm, superficial and small spots on the inoculation point; 2-from 2.1 mm to 2.6 mm, blackening on the inoculation point; 3-from 2.7 mm to 3.1 mm, blackening on the inoculation point; 4-blackening on the inoculation point of fruit more than 3.1 mm.

Statistical Analysis

The data was analyzed using the SPSS software (IBM SPSS 27.0, Chicago, IL, USA). Discriminant analysis was carried out based on the results of analytical measurements. Relationships between the observed traits were calculated by Pearson correlation methods. For the immunity test, the statistical analyses were determined based on the sample size, distribution analysis (Kolmogorov–Smirnov test) and t-test.

RESULTS AND DISCUSSIONS

The 'Alsószentiváni 117', 'Milotai 10', 'Tiszacsécsi 83' and 'Köpcös' cultivars started their budburst and pistillate flowers' receptivity almost at the same time as the control cultivar 'Chandler'. The budburst of 'Milotai intenzív' was similar to the control, but it needed the longest period among the observed cultivars for the pistillate flowers to appear, meaning 7 to 12 days after budburst. The budburst and pistillate flowers' receptivity of 'Bonifác', 'Milotai kései' and 'Alsószentiváni kései' were of 3 to 12 days and 7 to 17 days later than the control, respectively. During sample collection, the following periods were taken from the pistillate flowers' receptivity until the sample collection: for 'Alsószentiváni 117', 'Milotai 10', 'Tiszacsécsi 83', 'Köpcös' 63 days, for 'Chandler' 62 days, for Milotai

intenzív' 53 days, for 'Alsószentiváni kései' and 'Bonifác' 48 days, as well as 45 days for 'Milotai kései'. The data are recorded in Table 2 below.

Table 2. The time of budburst and pistillate flowers' receptivity in calendar days starting from 1st January 2020

Budburst (Days)	Pistillate Flowers'	Days after Receptivity before Sampling	Receptivity (Days)
Alsószentiváni 117	104 a	118 a	63 a
Milotai 10	107 ab	118 a	63 a
Tiszacsécsi 83	103 a	118 a	63 a
Köpcös	108 ab	118 a	63 a
Chandler	109 ab	119 a	62 a
Milotai intenzív	107 ab	128 b	53 b
Bonifác	116 b	133 c	48 c
Milotai kései	120 c	135 c	45 c
Alsószentiváni kései	120 c	133 c	48 c

Note: SD (5%) [1] = 2.3; SD (5%) [2] = 3.1; SD (5%) [3] = 2.9

The total phenolic content of the samples ranged between 4,420 and 5,740 GAE mg/100 g dry matter (d.m.). The results are in line with the findings of Soto-Madrid and co-workers (2021) and Oliveira and co-workers (2008) whose phenolic content ranged from 3,117 - 10,601 GAE mg /100 g d.m. and 32.61 - 74.08 GAE mg/g d.m. However, the results of this study were relatively higher than Soto-Maldonado (2019) who obtained 1,862.9 ± 72.4 GAE mg /100 g d.m.

The compositions of phenolic compound groups detected are as shown in Figure 2 below. The figure shows significant differences across samples. All compounds rutin equivalent (RE), except for the naphthoquinones calculated in the juglone equivalent (JE).

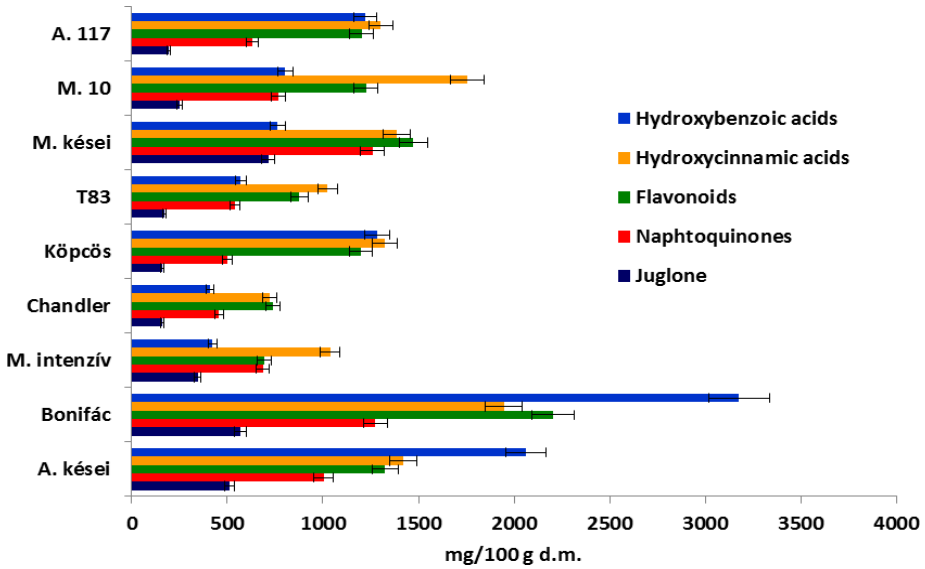

Figure 2. Composition of phenolic compounds green husk of walnut samples

Figure 2 depicts that the green husk of 'Bonifác', had the highest concentrations of hydroxybenzoic acids, hydroxycinnamic acids, flavonoids, and naphthoquinones at 3,176 RE mg/100 g d.m, 1,947 RE mg/100 g d.m, 2,203 RE mg/100 g d.m and 1,275 JE mg/100 g d.m, respectively. The green husks of 'Bonifác' had about 3.7 times of phenolic compounds than 'Chandler'.

Hydroxybenzoic acids varied between 407 and 3,176 mg/100 g d.m, with 'Bonifác' having the highest Concentration, followed by 'A. kései' (2,063 RE mg/100 g d.m), A117 (1283 RE mg/100 g d.m), and 'Köpcös', 1220 RE mg/100 g d.m. 'Chandler' had the lowest hydroxybenzoic acids content, estimated at 407 RE mg/100 g d.m. The content of hydroxycinnamic acids varied between 723 and 1,947 RE mg/100 g d.m, whereas flavonoids ranged from 741 RE mg/100 g d.m to 2,203 mg/100 g d.m. With respect to naphthoquinones, 'Bonifác' (1275 mg/100 g d.m) and

'M. kései' (1260 mg/100 g d.m) had higher concentrations than other cultivars. The lowest concentration of this compound group was detected in 'Köpcös' (499 mg/100 g d.m).

DISCUSSION

Phenolic Compounds and Early Spring Phenology

There were strong significant correlations between the budburst, as well as the pistillate flowers' receptivity, and the concentration of juglone, where R2 was 0.80 and 0.92, respectively Figure 3. We suppose that concentration of juglone is decreasing after the blossom. Reason of this decrease might be either the big increase of fruits during the fruit development or a real decrease of the juglone concentration due to stopping the juglone synthesis after the blossom.

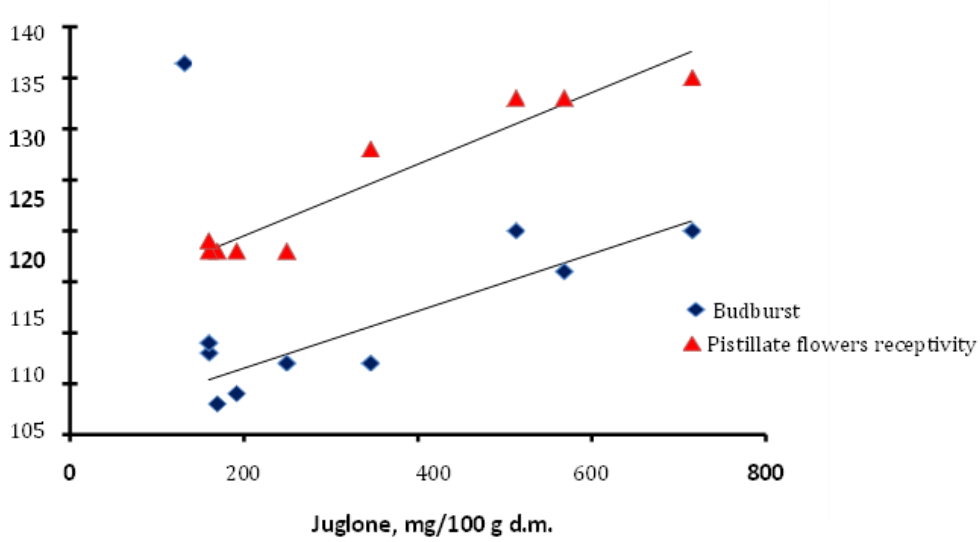

Figure 3. Correlation between the budburst and pistillate flowers' receptivity and the concentration of juglone

Table 3 contains the correlations between early spring phenology and the observed phenolic compounds. The naphthoquinones correlated well to the budburst (0.70) and the blossom time (0.78). This phenomenon can confirm our hypothesis, that the juglone concentration is decreasing after the blossom. Interestingly, there was astrong correlation between the budburst and the blossom of the pistillate flowers (0.79), as without a shoot, it is not possible for the female flower to develop.

Table 3. Correlation matrix of measured parameters and observed characteristics (R2) *

	Budburst	Blossom Time
Hydroxybenzoic acids	0.27	0.27
Hydroxycinnamic acids	0.15	0.14
Flavonoids	0.37	0.31
Naphthoquinones	0.70	0.78
Total phenolic acids	0.40	0.35
Budburst		0.79

Note: * Samples were collected during lignification of the nuts

Tolerance/Susceptivity to Xanthomonas Arboricola pv. Juglandis

The dendrogram, containing the diameter of the necrotic spots and the disease rate values, differentiated the cultivars belonging to susceptibility groups. In 2020, the susceptibility/resistance of cultivars showed significant differences. Based on the statistical evaluation of the data, 'Milotai intenzív' proved to have a high susceptibility (hS), while susceptibility (S) was detected for 'Bonifác', 'Tiszacsécsi 83', 'Alsószentiváni kései', 'Milotai 10' and

'Chandler'. Moderately susceptible cultivars were 'Milotai kései' and 'Alsószentiváni 117'. The origin of the Hungarian walnut cultivars did not relate with their susceptibility to walnut blight Figure 4.

In previous research a linkage between the phenolics content and resistance to walnut bacterial blight was determined (Jakopic et al., 2009). The current results confirmed this statement, except in the case of cultivar 'Bonifác'. This cultivar contained the highest concentration of phenolic compounds, but its fruits were susceptible to walnut blight (Figure 4) as described in the cultivars' description (Szentiványi and Kállay, 2006). Based on the artificial infections, the varieties with late budburst usually had better resistance to walnut blight, due to their higher phenolic compounds (Figures 2 and 4). However, a high concentration of phenolic compounds only link to resistance. Some cultivars with an early budburst can also have good resistance, such as 'BD6' (Bujdosó et al. 2020). Evaluating the results, it is concluded that the concentration of phenolic compounds of the most susceptible cultivars, 'Milotai intenzív' 'Tiszacsécsi 83' and 'Chandler', was the lowest during the measurements.

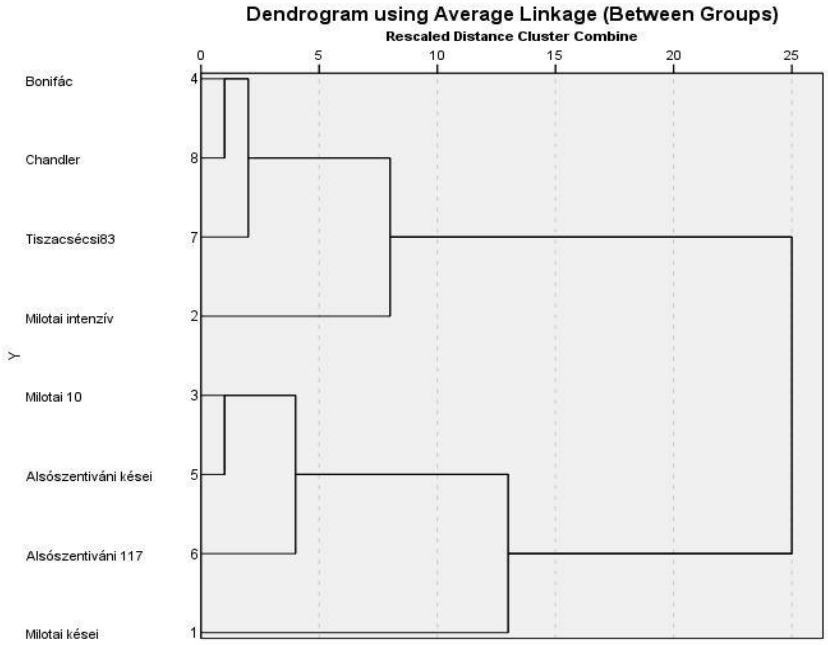

Figure 4. Dendrogram of Hungarian-bred walnut cultivars from the point of view of their susceptibility to walnut blight

CONCLUSIONS

The concentration of phenolic compounds varied by cultivars; it was higher in the locally-bred cultivars compared to the foreign-bred 'Chandler', grown and collected among Hungarian climatic conditions. The phenolic compounds inhibited the artificial *Xaj* infection on the surface of the green husk. This effect had a strong correlation with the high phenolics' concentration, except on 'Bonifác'. Naphthoquinones showed strong correlations between budburst and blossom time, which are related to their yearly fluctuation; the earlier the budburst and blossom, the earlier their peak concentration can be reached (Solar et al., 2006). Other reasons of the decrease in juglone concentration can be either the big increase of fruits during the fruit development with stabil juglone content or real decrease of the juglone concentration due to stopping the juglone synthesis after the blossom. The local-bred cultivars with late budburst and blossom time are the best for growers, due to their lower susceptibility to walnut blight, as described in some previous papers (Solar and Stampar, 2005; Botu et al., 2010; Bujdosó et al., 2020). Fortunately, there is a strong correlation between the budburst and blossom time (0.79), as described by breeders.

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Supervision, G.B. and N.A.; Project administration, G.B.; Funding acquisition, G.B. and A.V. All Authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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