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Phenolic response in green walnut husk after the infection with bacteria *Xanthomonas arboricola pv. juglandis*

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ABSTRACT

The research was performed on healthy green walnut fruits and on fruits infected with *Xanthomonas arboricola* pv. *juglandis* (*Xaj*). Fruits of cultivars 'Cisco', 'Sampion', 'Fernette', 'Seiferdorfer' and genotypes 'Zdole' and 'Erjavec' were sampled at phenophases Gf, Gf + 30 and Gf + 45. In the green husk tissue the content level of gallic acid, three hydroxycinnamic acids, catechin and five quercetin glycosides was determined with the high-performance liquid chromatograph coupled with mass spectrometer (HPLC-MS). During the growing season, the content of phenolic compounds decreased and was related to the physiological stage of the fruits and cultivar analyzed. The cumulative content of ten determined polyphenols in healthy walnuts was cultivar dependent, and weakly correlated to the blight susceptibility observed in the orchard. In comparison to healthy husk tissue, the infected husks contained up to 5 fold more hydroxycinnamic acids, up to 3 fold more gallic acid, up to 4.3 fold more quercetins and up to 23 fold more catechin. The cultivars 'Cisco' and 'Zdole' showed the strongest post-infectional accumulation of the phenolic compounds. An essential influence of quercetin-3-*O*-rhamnoside, as well as 4-*O*-*p*-coumaroylquinic, 3-*O*-caffeoylquinic and 3-*O*-*p*-coumaroylquinic acid on the walnut blight severity was confirmed and points out to the role of these phenolic compounds in the walnut resistance against bacterial blight.

1. Introduction

Persian walnut (*Juglans regia* L.) is cultivated commercially throughout southern Europe, northern Africa, eastern Asia, the USA, western South America and Australia. The tree is not affected by many pests and diseases, but walnut blight (*Xanthomonas arboricola* pv. *juglandis* (Pierce) Vauterin et al.) (*Xaj*) may cause economically important reductions in fruit yield and tree vigor in all production areas, especially those with high spring and summer rainfall [1]. The disease causes severe damage to all current-season green tissue, such as leaves, twigs, buds, petioles, rachides, male and female catkins, nutlets and kernels [2,3]. Host susceptibility depends on the cultivar, plant organ, weather conditions, leafing date, and previous history of the disease in the orchard [2,4–7].

Preventive copper bactericides are applied in order to control the disease, although they are not always effective, in spite of frequent treatments and complete spray tree-coverage. Moreover, copper resistant populations of the bacterial pathogen are often evolved; copper accumulates in the soil, and consequently disturbs the metabolism and performance of walnut trees [2,8].

Insight into the physiological response of walnut tree to the infection with *Xaj* may explain an incidence and severity of bacterial blight in different cultivars during their ontogenetic development. Resistance to blight may be related to phenolic compounds, as it is reported for some economically important pests and diseases of plants in general and also in several fruit species [9–11]. Phenolic compounds are toxic to pathogens and many of them, such as flavanols and hydroxycinnamic acids can act as passive or inducible barriers against herbivores or microbial pathogens. In response to the pathogen attack, the content and composition of polyphenols can change, playing an active role in induced resistance to the pathogens [9,12].

Phenolic compounds may also clarify the genetically determined resistance of different cultivars to a certain pest and/or disease. As assumed Michalek et al. [10], the resistance genes of a resistant cultivar act as regulatory genes of phenol synthesis. The accumulated defense phenols in the resistant cultivars may account for a more efficient response against the pathogen [9]. Up to date, such constitutive resistance has most frequently been confirmed in apple fruits and *Venturia inaequalis* [9,11,13], and also in walnut shoots and *X. arboricola* pv. *juglandis* [7].

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In addition to annual shoots, phenolic compounds have also been determined in walnut husks [14], leaves [15], kernels [16,17], and alcoholic extracts [18]. Several groups of polyphenols, hydroxybenzoic and hydroxycinnamic acids, flavonoids, tannins and quinones were found in the extracts of different walnut tissues.

Walnut blight is the most important disease on walnuts causing severe economic loss. Therefore, the mechanisms of plant response to the infection should be further investigated, since the application of pesticides is not desired or has low efficiency. Seasonal specific phenolic synthesis, and the relationship between the infection of walnut fruits with bacterial blight and phenolic metabolism have been poorly investigated to our knowledge. The aims of our study were thus to determine the seasonal content of major polyphenols, synthesized in both healthy and infected fruits of different walnut cultivars, and to investigate, how the infection with bacterial blight changed the metabolism of phenolic compounds. Based on previous studies, we hypothesized that the infected tissue would have a higher content of certain phenolic compound compared to healthy tissue, contributing to plant response mechanisms. We also expected that a relationship between the content of some phenols in walnut fruits and the disease severity will be determined.

2. Materials and methods

2.1. Materials

2.1.1. Plant material and growing conditions

The study was conducted in 2008. Phenolic compounds were analyzed in six walnut cultivars which were grown under the same agricultural, geographical and climatic conditions at the experimental orchard of Biotechnical Faculty, located in Maribor (NE Slovenia). In Slovenian climatic conditions, fruits of 'Fernette' and 'Erjavec' cultivars show very low susceptibility to walnut blight (*Xaj*), 'Cisco' and 'Zdole' cultivars are moderately susceptible, and the losses of crop due to blight exceed 80% in 'Sampion' and 'Seiferdorfer' cultivars. The experimental trees were of adult age, between 14 and 19 years-old and grown at a flat level, on moderately acid, shallow soil, at a spacing of 10 × 10 m. Floor management included regular grass mowing between the rows, and two herbicide treatments per year within the rows. No other pesticide, such as bactericide or fungicide was applied in the experimental orchard.

During the April–June period, which is critical for the walnut blight progress, only 37–96% of the long-term average rainfall was recorded [19]. This resulted in weak disease severity, in particular at the initial developmental stages of the fruits.

Fruits were sampled at three phenophases: Gf (brown stigmas or nut set), Gf + 30 (30 days after Gf – walnut fruits the size of olive fruits), and Gf + 45 (45 days after Gf – shell hardening). At the Gf stage, no symptoms of blight were detected on the tissue, therefore only healthy fruits were sampled. At the Gf + 30 and Gf + 45 phenophases, both healthy and symptomatic fruits were collected. For individual cultivar, 3 trees were selected. From each tree 10 healthy and 10 infected fruits were sampled. Six fruits were combined to one sample. Five repetitions for polyphenol analyses were prepared per stage for each individual cultivar. After the sampling the fruits were immediately immersed in liquid nitrogen and stored at −20 °C until further analysis.

2.1.2. Chemicals

The following standards were used for the quantification of phenolic compounds: chlorogenic acid (3-*O*-caffeoylquinic acid) from Sigma (St. Louis, MO, USA), quercitrin (quercetin-3-*O*-rhamnoside), quercetin-3-*O*-glucoside and *p*-coumaric acid from Fluka Chemie GmBH (Buchs, Switzerland), gallic acid from Merck (Darmstadt, Germany), quercetin-3-*O*-arabinoside and quercetin-3-*O*-xyloside from Apin Chemicals (Abingdon, UK) and (+)-catechin from Roth (Karlsruhe, Germany). Methanol for the extraction of phenolic compounds was acquired from Sigma. The chemicals for the mobile phases were HPLC-MS grade acetonitrile and formic acid from Fluka Chemie GmbH. Water for the mobile phase was bi-distilled and purified with the Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Extraction and determination of phenolic compounds

For the extraction, healthy and infected green walnut fruits were used. 0.3 g healthy fruit tissue was taken, and only infected tissue with a surrounding narrow zone of healthy cells (1–2 mm) was taken from the infected husks to comprise 0.3 g. The frozen tissue was ground to a fine powder in a mortar chilled with liquid nitrogen. Extraction with some modification was performed as described by Mikulic-Petkovsek et al. [11]. The fine powder (0.3 g) was extracted with methanol (3 ml) containing 1% 2.6-di-tert-butyl-4-methylphenol (BHT) for 30 min in a cooled water bath using sonification. BHT was added to the samples to prevent oxidation during the extraction. There was no interference with the extracted phenols during the subsequent HPLC analysis, because it was eluted at the end of the gradient or in the equilibration delay between the two analyses.

After the extraction, the walnut extracts were centrifuged for 10 min at 10,000 rpm. The supernatant was filtered through a Chromafil AO-45/25 polyamide filter produced by Macherey–Nagel (Düren, Germany) and transferred to a vial prior to injection into the HPLC (high-performance liquid chromatography) system.

The phenolic compounds were analyzed on a Thermo Finnigan Surveyor HPLC system (Thermo Scientific, San Jose, USA) with a diode array detector. The hydroxybenzoic acid (gallic acid), hydroxycinnamic acids (3-*O*-caffeoylquinic, 3-*O*-*p*-coumaroylquinic and 4-*O*-*p*-coumaroylquinic acid), the monomeric flavan 3-ols (catechin) were detected at 280 nm, whereas quercetin-3-*O*-arabinoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-xyloside, and myricetin-3-*O*-xyloside were estimated at 350 nm. Spectra of the compounds were recorded between 200 and 600 nm. The column was a Gemini C_{18} (150 × 4.6 mm 3 μm; Phenomenex, USA) operated at 25 °C. The elution solvents were aqueous 1% formic acid (A) and 100% acetonitrile (B). Samples were eluted according to the linear gradient described by Marks et al. [20], with the injection amount of 20 μl and flow rate of 1 ml/min.

The identification of compounds was achieved by comparing retention times and spectra as well as by adding the standard solution to the samples. All phenolic compounds were also confirmed using a mass spectrometer (Thermo Scientific, LCQ Deca XP MAX) with an electrospray interface (ESI) operating in negative ion mode. Analysis was carried out using MS^2 scanning from m/z 115 to 1000.

Concentrations of phenolic compounds were calculated from the peak areas of the sample and the corresponding standards. The concentrations were expressed in mg/kg fresh weight (FW). For compounds lacking standards, quantification was carried out using similar compounds as standards. Thus, 3- and 4-*O*-*p*-coumaroylquinic acid were quantified in equivalents of *p*-coumaric acid and myricetin-3-*O*-xyloside in equivalents of myricetin.

2.3. Field evaluation of walnut blight severity

Parallel with the analysis of phenolic compounds, the external severity of the disease was observed on the fruits in the orchard.