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BRIEF COMMUNICATION

Phenolic compounds in apple leaves after infection with apple scab

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Abstract

Leaves of the scab-susceptible apple (*Malus domestica*) cultivar Golden Delicious were harvested from May to August 2008 and 2009. Some leaves were healthy and some infected with fungus *Venturia inaequalis*. The phenolic compounds were analysed in healthy leaves, infected leaves and in the scab spot tissue. In comparison to healthy leaves, the infected leaves showed higher contents of hydroxycinnamic acid, flavanols and phloridzin, while lower contents on procyanidins, quercetins and phloretin. The total amount of phenolic compounds in the infected tissue was 10 to 20 % higher than in the healthy leaves. Accumulation of phenolic compounds is a post-infection response, and probably their further transformation is a prerequisite for plant resistance.

Additional key words: *Venturia inaequalis*, *Malus domestica*, hydroxycinnamic acids, dihydrochalcones, flavonoids.

Apple scab, which is caused by the ascomycetous fungus *Venturia inaequalis* (Cooke) Wint., is the most problematic disease of apple and the massive use of fungicides may pollute the environment (Benaouf and Parisi 1998). Researchers studying the apple resistance mechanism against *Venturia inaequalis* often found high concentrations of phenolic compounds (*e.g.*, chlorogenic, ferulic and *p*-coumaric acids) in apple tissue (Usenik *et al.* 2004, Mikulic Petkovsek *et al.* 2008). Treutter and Feucht (1990 a,b) showed that the amount of flavanols (catechins and proanthocyanidins) in apple leaves of different cultivars correlated positively with resistance. A positive correlation between procyanidins and scab resistance was also shown by Picinelli *et al.* (1995) and Mayr *et al.* (1997). Lattanzio *et al.* (2001) indicated that the oxidation products of phloridzin, which is degraded at first to phloretin and then to *o*-quinones, are very important in the scab resistance. In contrast, Sierotski and Gessler (1993) reported that there is no positive correlation between resistance and pre-formed flavanols in the connection between *Malus domestica* and *Venturia inaequalis*.

It has been proposed that the first stage of the defence mechanism involves a rapid accumulation of polyphenols at the infection site. These slow down the pathogen growth. Phenolic derivatives can react with proteins, thus causing a loss of enzyme function and restricting the viability of aggressors, or they can be deposited inside the cell wall as an important first defence line against infection (Schwalb and Feucht 1999). Previous studies (Mayr *et al.* 1997, Michalek *et al.* 1999) established that for successful protection, rapid biosynthesis of flavanols starting from phenylalanine was necessary. The inhibition of the enzyme phenylalanineammonia lyase (PAL) resulted in severe sporulation symptoms.

This paper deals with the defence reactions of apple leaves against fungal attack. We studied the polyphenol pattern in healthy and scab infected apple leaves to find a relation between scab resistance and the levels of some phenolic compounds present in leaves. Several previously published papers address this subject only for selected polyphenols, while our research covers all major phenolic compounds present in apple leaves.

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Abbreviations: BHT - 2.6-di-tert-butyl-4-methylphenol; DM - dry mass; GAE - gallic acid equivalent; HPLC-MS - high-performance liquid chromatography and tandem mass spectrometry; PAL - phenylalanine ammonia lyase.

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The experiment was carried out during the growing seasons of 2008 and 2009. Leaf samples were taken from 9-year-old apple (*Malus domestica* Borkh., scab-susceptible cv. Golden Delicious) trees grafted on M9 rootstock growing at the Ljubljana location (latitude: 46° 2' N, longitude: 14° 28' E). The trees were planted at a distance of 1.2 × 3.5 m, the tree training system was slender spindle, winter pruning was applied, while summer pruning was not necessary. The orchard had no irrigation system. Ten trees were sprayed with fungicides, while another ten were not. Because of weather conditions favourable for apple scab development, numerous spots were present on unsprayed leaves. The scabbed surface was scored using the grading scale described by Parisi *et al.* (1993): score 0 = no visible symptoms, score 1 = 0 - 1 % scabbed leaf surface (sls), score 2 = 1 - 5 % sls, score 3 = 5 - 10 % sls; score 4 = 10 - 25 % sls; score 5 = 25 - 50 % sls, score 6 = 50 - 75 % sls and score 7 = sls > 75 %. Healthy leaves showed no visible symptoms of scab infection, while the infection rate for infected samples during the growing season quickly increased.

Twenty fully developed healthy or infected leaves from annual shoots were collected from each tree. Sampling began on May 26th (35 days after full bloom). The leaves were sampled three times at 14-day intervals, and the last two samplings were made at 25-day intervals. In the trial, three treatments were determined: healthy leaves, scab infected leaves and scab lesion on leaves. For scab lesion treatment, only infected tissue was taken. Before extraction, the leaves were frozen in liquid nitrogen and stored at -20 °C.

The following standards were used for the quantification of phenolic compounds: chlorogenic acid (5-caffeoylquinic acid), phloretin and rutin (quercetin-3-*O*-rutinoside) from *Sigma-Aldrich* (Steinheim, Germany), ferulic acid, (+)-epicatechin, quercitrin (quercetin-3-*O*-rhamnoside), quercetin-3-*O*-galactoside, quercetin 3-*O*-glucoside, *p*-coumaric acid, procyanidin B2 and phloridzin dihydrate from *Fluka* (Buchs, Switzerland), quercetin-3-*O*-arabinofuranoside and quercetin-3-*O*-xyloside from *Apin Chemicals* (Abingdon, UK) and (+)-epicatechin from *Roth* (Karlsruhe, Germany). BHT (2.6-di-tert-butyl-4-methylphenol) and methanol for extraction of polyphenols was acquired from *Sigma-Aldrich*. The chemicals for mobile phases were HPLC-MS grade acetonitrile and formic acid from *Fluka*. Water for the mobile phase was bidistilled and purified with the *Milli-Q* system (*Millipore*, Bedford, MA, USA). For the total phenolic content, Folin-Ciocalteu phenol reagent (*Fluka*), sodium carbonate (*Merck*, Darmstadt, Germany), gallic acid and ethanol (*Sigma-Aldrich*) were used.

The frozen leaves were lyophilized and ground into fine powder with liquid nitrogen. Extraction with some modification was done as described by Mikulic Petkovsek *et al.* (2008). The fine powder (40 mg) was extracted with methanol (3 cm^3) containing 1 % 2.6-di-tert-butyl-4-methylphenol (BHT) for 30 min in a cooled water bath, using sonification. The leaf extracts were centrifuged at 10 000 *g* for 10 min at 4 °C, and the supernatant was filtered through a 0.45 μm membrane filter (*Macherey-Nagel*, Düren, Germany) prior to injection into the *Thermo Finnigan Surveyor* HPLC system (*Thermo Scientific*, San Jose, USA) with a diode array detector, at 280 and 350 nm. The hydroxycinnamic acids (chlorogenic, *p*-coumaric, ferulic and 4'-*O*-*p*-coumaroylquinic acid), the monomeric flavan 3-ols (catechin, epicatechin), oligomeric flavanol (procyanidin B2) and dihydrohalcone (phloretin) were detected at 280 nm, whereas phloridzin (phloretin 2'-*O*-glucoside), quercetin-3-*O*-rutinoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-arabinofuranoside, quercetin-3-*O*-arabinpyranoside and quercetin-hexoside were estimated at 350 nm. Spectra of the compounds were recorded between 200 and 400 nm. The column was a *Gemini* C_{18} (150 × 4.6 mm; 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 as described by Marks *et al.* (2007) with an injection amount of 0.02 cm^3 and a flow rate of 1 cm^3 min^{-1}. All phenolic compounds were also confirmed using a mass spectrometer (*LCQ Deca XP MAX*, *Thermo Scientific,* San Jose, USA) with an electrospray interface operating in negative ion mode.

The contents of phenolic compounds were calculated from the peak areas of samples and the corresponding standards.

For the determination of total phenolic content, the extraction of leaf samples was made according to the same protocol as for phenolic compounds, with the difference that no BHT was added. The total phenolic content (TPC) of extracts was assessed by using the Folin-Ciocalteau phenol reagent method (Singleton and Rossi 1965). To 0.1 cm^3 of the sample extracts (diluted 1:5 with MeOH), 6 cm^3 of bidistilled water and 0.5 cm^3 of Folin-Ciocalteau reagent were added; after resting between 8 s and 8 min at room temperature, 1.5 cm^3 of sodium carbonate (20 %; m/v) was added. The extracts were mixed and allowed to stand for 30 min at 40 °C before measuring the absorbance on a spectrophotometer (*UV/VIS Lambada Bio 20*, *Perkin Elmer*, Waltham, USA) at 765 nm.

The data were analyzed by using the *Statgraphics Plus 4.0* program (*Manugistics*, Rockville, Maryland, USA). The significance of the infection with *Venturia inaequalis* on individual and total phenolic content was tested using the one-way *ANOVA*. Differences between treatments were tested with the LSD test at a significance level of 0.05.

Based on the weather parameters in the growing seasons of 2008 and 2009, it is evident that the two years differ mainly in the quantity of precipitates. The amount of precipitates was lower in the year 2009 compared to

2008. In both years there was less precipitations in May and also in August 2009, as compared to the long-term average. Both years had temperatures above the long-term average. It is known that environmental conditions influence the synthesis of phenolic compounds (Treutter 2001, Hamauzu 2006), and therefore the individual year had a significant influence on the amount of polyphenols. Although the environmental conditions appeared to be more stressful in 2009, the amounts of most phenolic groups were higher in 2008 (Figs. 1 and 2). Only total phenolics seem to be higher in 2009. We concluded from these results that the accumulation of phenolic compounds in apple tissues is – apart from apple scab infection – also influenced by the growing season. Similar results were reported by Veberic *et al.* (2007).

The dihydrochalcones and, within this group, phloridzin in particular, are the main polyphenols, representing more than 90 % of the extractable phenolic compounds in the leaves (Gosch *et al.* 2009). Phloridzin and the corresponding aglycon phloretin are often

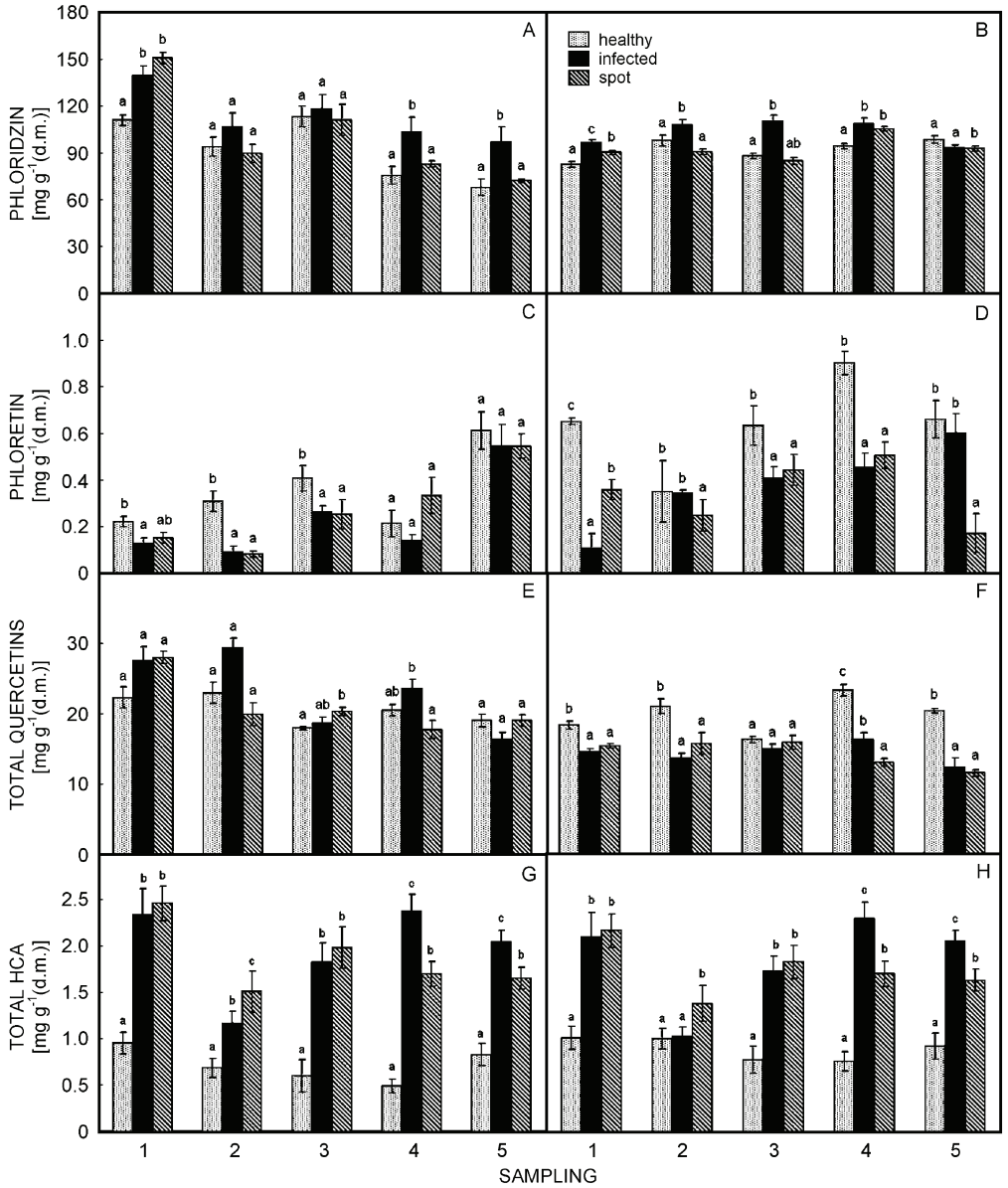

Fig. 1. The content of single phenolic compounds in healthy and in infected apple leaves (separately for the whole scab infected leaf and the scab spots). Means ± SE, n = 5, different letters denote statistically significant differences between treatments within one sampling in a particular year (50, 64, 78, 103 and 128 DAFB). The left (*A*,*C*,*E*,*G*) and the right (*B*, *D*, *F*, *H*) columns show year 2008 and year 2009, respectively. Total quercetins represent the sum of quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-arabinopyranoside, quercetin-3-*O*-arabinofuranoside, quercetin-3-*O*-rhamnoside and quercetin-hexoside. Total hydroxycinnamic acids (HCA) represent the sum of chlorogenic, 4'-*O*-*p*-coumaroylquinic, ferulic and *p*-coumaric acids.

implicated in resistance to various diseases. The scab infected leaves had phloridzin content 1.1 to 1.4 times higher than healthy leaves (Fig. 1*A*,*B*). This finding was in agreement with results of Leser and Treutter (2005) and Lattanzio *et al.* (2001). Phloridzin and the ratio flavanol/phloridzin are often discussed with regard to resistance against apple scab (Picinelli *et al.* 1995). In the case of phloretin, the response of the plant to infection was different. Healthy leaves had significantly higher amounts of phloretin than either infected leaves or the scab spot (Fig. 1*C*,*D*). This can be explained by defence reactions triggered by the attack of the pathogen. It is known that phloretin is degraded into phloroglucinol, phloretic acid and *p*-hydroxybenzoic acid, which inhibit the development of the fungus (Lattanzio *et al.* 2001). It can be concluded that, in infected tissue, phloretin was an active compound against the fungi attack.

In quantity, quercetins are the second major phenol group in apple leaves. The sum of their content in leaves represents only one fifth of the quantity of phloridzin. In apple leaves, several quercetin glycosides were detected: quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-arabinopyranoside, quercetin-3-*O*-arabinofuranoside, quercetin-3-*O*-rhamnoside and quercetin-hexoside. The changes in quercetin content after scab infection differed between the two years. In 2008 there were almost no significant differences, while in 2009 the healthy apple leaves contained, on the majority of dates, significantly higher amount of total quercetins than the infected leaves and scab spots (Fig. 1*E*,*F*). Varying response of quercetins between the years may be attributed to different weather

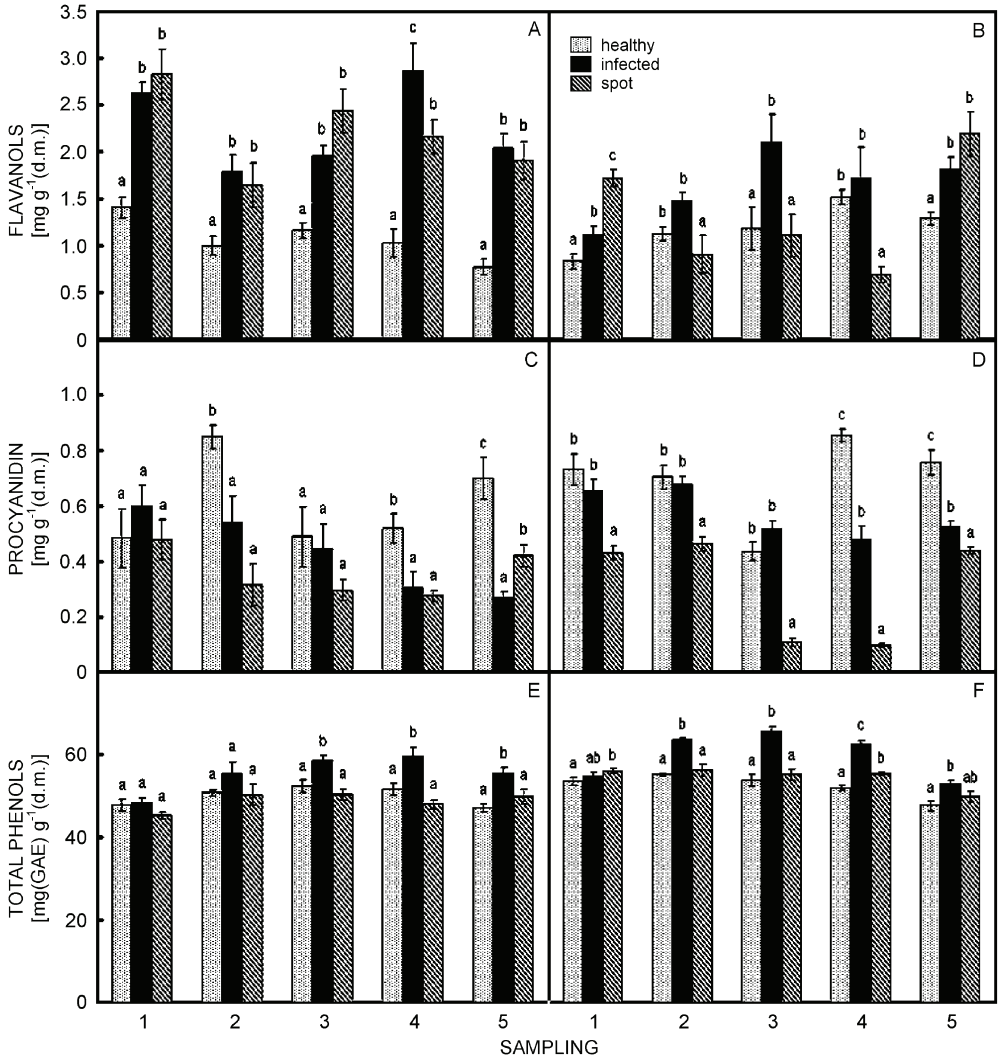

Fig. 2. The content of single and total phenolic compounds in healthy and in infected apple leaves (separately for the whole scab infected leaf and the scab spot). Means ± SE, n = 5, different letters denote statistically significant differences between treatments within one sampling in a particular year (50, 64, 78, 103 and 128 DAFB). The left (*A*,*C*,*E*) and the right (*B*, *D*, *F*) columns show year 2008 and year 2009, respectively. Flavanols represent the sum of catechin and epicatechin. Total phenols were measured spectrophotometrically with the Folin-Ciocalteu method.

parameters. In contrast to our results, Feucht (1994) and Mikulic Petkovsek *et al.* (2008) reported that scab infection led to an increased synthesis of flavonols. Nevertheless, Picinelli *et al.* (1995) did not find a clear connection between the flavonol content and resistance to apple scab.

The greatest share the total content of hydroxycinnamic acids belongs to chlorogenic acid (*ca.* 50 - 65 %), followed by 4'-*O*-*p*-coumaroylquinic acid (*ca.* 20 - 35 %), and the smallest share belongs to the ferulic and *p*-coumaric acids (5 -15 %). The scab spot had the highest content of total hydroxycinnamic acids at the first three sampling dates, while in the last two dates in both years, the leaves sprinkled with numerous scab spots (Fig. 1*G*,*H*) containing the highest amount of these acids. These results may be explained by the fact that the phenylpropanoid pathway in spots was probably less active in these dates, since the spots had already started to synthesize cork. It is well-known that the most intensive defence reactions, *i.e.*, the synthesis of phenolic substances, occupy the borderline between the spot and the healthy tissue (Feucht *et al.* 1992, Mikulic Petkovsek *et al.* 2009). We have also noted similar results in our previous studies (Mikulic Petkovsek *et al.* 2008, 2009). Lattanzio and co-workers (2001) reported that chlorogenic acid inhibited the spore germination or the mycelial growth. Scab resistant cultivars contained significantly greater amounts of chlorogenic acid in comparison with susceptible ones (Rat-Morisse *et al.* 1996, Mikulic Petkovsek *et al.* 2007). The increase in all analyzed hydroxycinnamic acids after the infection may also be a consequence of the stimulated activity of phenylalanine ammonia lyase (PAL).

We also analyzed the flavanols catechin, epicatechin and procyanidin B2. It became evident that the infection with apple scab in both years caused higher synthesis of catechin and epicatechin on the entire infected leaf and on the spot itself. In the healthy leaves contents of monomeric flavanols (catechin and epicatechin) were 1.3 to 2.8 times lower than in infected leaves (Fig. 2*A*,*B*). Treutter and Feucht (1990b) reported contents of flavanols in pear leaves infected by *Gymnosporangium sabinae* even 6 times higher than those in healthy leaves. Treutter and Feucht (1990a), Picinelli *et al.* (1995) and Mikulic Petkovsek *et al.* (2009) also reported an increased synthesis of flavanols after the *Venturia inaequalis* attack. In contrast, healthy leaves contained significantly higher content of procyanidin B2 than the infected leaves or spot on a majority of the sampling dates (Fig. 2*C*,*D*) in both growing seasons. Scab infection caused changes in the polyphenol metabolism of apple leaves. Catechin and epicatechin are probably involved in the defence reaction, and their content in infected tissue increases, which causes consequently lower content of procyanidin B2 in the spot or infected leaf. This may be explained in terms of the biosynthetic pathway of flavonoids, in which catechin and epicatechin represent a precursors of the procyanidins (Roemmelt *et al.* 2003).

The response of the plant to scab infection was thus reflected in the content of total phenols. The infected leaves showed values 10 - 20 % higher in comparison with healthy ones on the majority of dates (Fig. 2*E*,*F*). The values of total phenols in the spot differed significantly from healthy tissue on only two dates. This may be explained by the fact that in the spot itself there is no active synthesis of phenols, since this occurs on the border-line between the spot and the healthy leaf tissue. Higher accumulations of phenolic compounds in the surrounding cells between healthy and infected tissue were also observed by Treutter and Feucht (1990b), Mikulic Petkovsek *et al.* (2009) and Anand *et al.* (2009).

In conclusion, the accumulation of different phenolic compounds as well as the possible activation of the phenylpropanoid pathway, which has been established by previous experiments (Treutter and Feucht 1990a,b) may explain the broad and unspecific prevention of plant diseases such as apple scab. However, there still remains the question of whether the constitutive level of some phenolic compounds is really involved in resistance mechanisms, or if it represents only the capacity of the tissue to produce large amounts of these compounds (Treutter 2001). Our results demonstrate that scab infection caused a significant increase in hydroxycinnamic acids, catechin, epicatechin and phloridzin in infected tissue. These results support the hypothesis that phenolic compounds play an important role in host resistance in infected tissue and that the mechanism of resistance may be influenced by responses linked to the host-pathogen interaction. Detailed knowledge of the production of resistance-related secondary metabolites may contribute to a better understanding of plant defence against pathogenic organisms. The conclusion available from these data is that the constitutively present polyphenols are not necessarily the cause of resistance. Accumulation of phenolic compounds could be a post-infection event, and their further transformation is a prerequisite for higher resistance.

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