

## INCREASED PHENOLIC CONTENT IN APPLE LEAVES INFECTED WITH THE APPLE SCAB PATHOGEN

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### SUMMARY

Phenolic compounds were measured in leaf tissues of apple cvs Jonagold and Golden Delicious, healthy and infected by *Venturia inaequalis*. Leaves were sampled from May to September 2005 and analyzed by high performance liquid chromatography. Hydroxycinnamic acids detected were chlorogenic, caffeic, ferulic and *p*-coumaric. In addition, the presence was ascertained of the dihydrochalcone phloridzin and the flavonoids epicatechin, catechin, rutin and quercitrin. Total phenolics were determined with the Folin-Ciocalteu method. Infection by *V. inaequalis* caused an accumulation of phenolic compounds in infected leaves with a 1.4 to 6.2-fold increase of flavanols, a 2 to 6-fold increase of chlorogenic acid and a 1.4 to 2.4-fold increase of the Folin-Ciocalteu values.

*Key words:* apple scab, *Malus domestica*, phenolic compounds, *Venturia inaequalis*.

### INTRODUCTION

Scab caused by the fungus *Venturia inaequalis* (Cooke) G. Winter is a widespread disease of apple (*Malus domestica* Borkh.). Growers try to counteract the impact of this disease with fungicide treatments, the use of resistant or tolerant genotypes, or through the induction of defense mechanisms by treating plants with suitable triggering agents (e.g. prohexadione-Ca) (Mayr *et al.*, 1995; Hrazdina *et al.*, 1997; Rademacher *et al.*, 1999).

Defense strategies of plants against pathogens are several, including the production of antifungal chemicals, which are either pre-formed (i.e. already present in plant tissue in different amounts) or induced following infection (e.g. *de novo* synthesized phytoalexins) (Grayer and Kokubun, 2001). In both groups of chemicals, those synthesized in secondary metabolism are especially interesting. Many of the phytoalexins or pre-formed

chemicals belong to the phenolic group (Grayer and Kokubun, 2001), which are involved in the natural defense reactions of apples against various diseases, being toxic to pathogens. These compounds are produced and accumulate at a faster rate after infection (Picinelli *et al.*, 1995; Usenik *et al.*, 2004; Treutter, 2005).

Phenolics, flavanols in particular, play a role in the resistance of apples to *V. inaequalis* (Treutter and Feucht, 1990a, 1990b). The ability to accumulate flavanols after infection in the tissue surrounding infected sites is a differential property of susceptible and resistant cultivars (Treutter and Feucht, 1990b), and constitutes additional evidence that these compounds are involved in the defense mechanism against apple scab (Mayr *et al.*, 1995). In resistant cultivars, phenolics, such as chlorogenic, caffeic and ferulic acids (Mikulic Petkovšek *et al.*, 2003), accumulate at a faster rate than in the susceptible ones (Picinelli *et al.*, 1995; Usenik *et al.*, 2004; Treutter, 2005).

As to the connection between resistance and phenolic content, the information is somewhat contradictory. Picinelli *et al.* (1995) reported that the content of chlorogenic acid, coumaric acid derivatives and flavanols was generally higher in the resistant than in the susceptible apple varieties. However, Sierotzki and Gessler (1993) came to the conclusion that there is no positive correlation between resistance and pre-formed flavan-3-ols in the relationship between *Malus x domestica* and *Venturia inaequalis*.

Phloridzin was shown to play an important role in host resistance to *V. inaequalis*. It was suggested that phloridzin is hydrolysed *in vivo* by various fungi (*V. inaequalis* included) to create phloretin, which, in turn, is degraded to phloroglucinol, phloretic acid and *p*-hydroxybenzoic acid, which inhibit the development of *V. inaequalis* (Hamazu, 2006).

The phenolic derivatives can react and oxidize proteins, thus causing the loss of enzyme function and restricting the viability of aggressors. They can also be deposited inside cell walls as an important first line of defense against infection (Schwalb and Feucht, 1999). Previous studies (Mayr *et al.*, 1997; Michalek *et al.*, 1999) showed that, for a successful protection, rapid biosynthesis of flavanols starting from phenylalanine was nec-

essary. The inhibition of the enzyme phenylalanine-ammonia-lyase (PAL) led to severe scab symptoms because there was a reduced flavonol accumulation. Furthermore, earlier investigations had shown that growth-promoting N nutrition reduce flavonoid accumulation in the leaves, thus increasing susceptibility to scab (Rühmann *et al.*, 2002; Leser and Treutter, 2005; Strissel *et al.*, 2005).

The way in which orchards are managed can influence the amount of phenolics, as shown by Veberic *et al.* (2005), who reported that organically grown apples had somewhat higher amounts of phenolics as compared with traditionally grown apples. These authors concluded that this is probably because organically grown apples face more stressing conditions, for synthetic fertilizers and pesticides are not used.

According to previous studies, we hypothesized that infected leaves have a higher content of certain phenolic compounds compared with healthy leaves. As already mentioned, several previously published papers addressed this subject only in part. Therefore, we tried to compare the differences in the content of single phenolic compounds, as well as of total phenolics in healthy leaves and in *V. inaequalis*-infected leaves of cvs Jonagold and Golden Delicious over a whole growing season. Golden Delicious was grown in two different locations.

## MATERIALS AND METHODS

**Plant material and growing conditions.** Experiment was carried out in 2005 using leaves from six-year-old apple trees grafted on M9 rootstocks, growing in Ljubljana (central Slovenia) and Maribor (north-eastern Slovenia). The orchards were managed following the guidelines for integrated production. Despite the use of fungicides, weak scab symptoms were present on the leaves, consisting of small velvety lesions with olive green colour, and undefined margins. Samples were collected from cv. Golden Delicious in both locations and cv. Jonagold in Maribor.

In 2005, weather parameters (temperatures and rainfall) were favourable to the development of scab. Average temperatures during the growing season were approximately 1 to 1.5°C higher than the long term average (1961-1990). Rainfalls in May and June were somewhat lower than the long term average but, in other months, the limit of long term rainfall average was widely exceeded in both locations.

Ten fully developed healthy or infected leaves were collected from each tree. The leaves were sampled from five trees at different dates for each cultivar. The 3<sup>rd</sup> or the 4<sup>th</sup> fully developed leaf from annual shoots was taken for analysis. Infected leaves showed young green sporulating lesions, which were excised together with a narrow strip (1–2 mm) of surrounding tissues. Before

extraction, healthy or infected leaves were frozen in liquid nitrogen and stored at –20 °C.

Leaf sampling was made every 20 days. Sampling at Ljubljana began on June 2<sup>nd</sup> (25 days after full bloom), at Maribor on May 27<sup>th</sup> (24 days after full bloom) and was completed on September 12<sup>th</sup>.

**Extraction and determination of phenolic compounds.** Frozen leaves were lyophilized and ground in a mortar. For each treatment, 5 groups of 10 leaves per group were extracted as described by Colaric *et al.* (2005) with some modifications. The fine powder (50 mg) was extracted with methanol (20 ml) containing 1% 2,6-di-tert-butyl-4-methylphenol (BHT) for 30 min in a cooled water bath using sonication. BHT was added to the samples to prevent oxidation. It did not interfere with the extracted phenols during the subsequent HPLC analysis, because it was eluted at the end of the gradient or in the equilibration phase between the two analyses. After centrifuging at 10,000 rpm for 10 min at 4°C, the supernatant was filtered through a 0.45 µm membrane filter (Macherey-Nagel, Düren, Germany) prior to injection into HPLC.

Phenolic compounds were analyzed using the Thermo Finnigan Surveyor HPLC system with a diode array detector at 280 and 350 nm. Hydroxycinnamic acids (chlorogenic, *p*-coumaric, ferulic and caffeic) and the monomeric flavan 3-ols (catechin, epicatechin) were detected at 280 nm, whereas rutin, phloridzin and quercetin-3-rhamnoside (quercitrin) were estimated at 350 nm. Spectra were also recorded between 200 and 400 nm. The column used was Phenomenex Gemini C<sub>18</sub> (150x4.60 mm) operated at 25°C. The elution solvents were A (aqueous 0.01 M phosphoric acid) and B (100% methanol). The samples were eluted according to the linear gradient described by Escarpa and Gonzales (2000) with slight changes: 5% B initially; 50% B for 10 min; 70% B for 5 min; 80% B for 5 min and, finally, 100% B for 10 min. The injection amount was 10 µl, and the flow-rate was 1 ml/min. Identification of compounds was achieved through a comparison of retention times and spectra, as well as with spiking by standards. Concentrations of phenolic compounds were calculated from the peak areas and expressed as mg/100 g dry weight or mg/g dry weight (d. wt).

**Determination of total phenolic content.** Extraction of leaf samples for determining total phenolics was done as described above, but without BHT addition. The total phenolic content (TPC) of extracts was assessed using the Folin-Ciocalteu phenol reagent method (Singleton and Rossi, 1965). To 100 µml of extracts [diluted 1: 5 (v/v) with MeOH], 6 ml of bidistilled water and 500 µml of Folin-Ciocalteu reagent were added; after resting between 8 sec and 8 min at room temperature, 1.5 ml of sodium carbonate (20% w/v) was added. The extracts

were mixed and left to stand for 30 min at 40°C before measuring the absorbance with the spectrophotometer (Perkin Elmer, UV/VIS Lambda Bio 20) at 765 nm. A mixture of water and reagents was used as a blank. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram d. wt of leaf tissue. Absorptions were measured in three replicates.

**Chemicals.** The following standards were used for quantification of phenolic compounds: chlorogenic acid (5-caffeoylquinic acid) and rutin (quercetin-3-rutinoside) were obtained from Sigma (St. Louis, MO, USA), (+)-catechin from Roth (Karlsruhe, Germany), *p*-coumaric acid, (-)-epicatechin, quercetin-3-rhamnoside, phloridzin dihydrate, caffeic acid and ferulic acid from Fluka (Buchs, Switzerland). Methanol was from Sigma (St. Louis, MO, USA). Water was bidistilled and purified with the Milli-Q system (Millipore, Bedford, MA, USA). For the total phenolic content, Folin-Ciocalteu's reagent, sodium carbonate, gallic acid and ethanol from Sigma (St. Louis, MO, USA) were used.

**Statistical analysis.** The significance of treatments (healthy and infected leaves) was assessed using the one-way analysis of variance (ANOVA). Differences between treatments were tested with the LSD test at the 0.05 significance level. Data were analyzed by using the Statgraphics Plus 4.0 program (Manugistics, Rockville, Maryland, MD, USA).

## RESULTS AND DISCUSSION

In this investigation, the following phenolic substances were identified in the leaves of apple trees: chlorogenic acid, *p*-coumaric acid, ferulic acid, caffeic acid, phloridzin, (-)-epicatechin, rutin, (+)-catechin, quercetin-3-rhamnoside and phloretin.

Chlorogenic acid was the most widely represented among hydroxycinnamic acids. Its content ranged from 26.4 to 67.1 mg/100 g d. wt in healthy leaves (Table 1, 2, 3), which tallies with the results obtained by Picinelli *et al.* (1995). Small variations were found owing to the different varieties used in the analysis and different locations of growth. Infected leaves contained 2 to 6 times more chlorogenic acid (Table 1, 2, 3) than the healthy ones. The content of the remaining hydroxycinnamic acids analyzed was lower than that of chlorogenic acid. At the beginning of June, healthy leaves contained from 20.7 to 28.6 mg caffeic, 12.4 to 15.5 mg *p*-coumaric (Table 1, 2, 3) and 17.5 to 35.5 mg ferulic acids/100 g d. wt of tissues (data not shown). During the vegetation period, the values varied only slightly. With scab infection, the content of caffeic, *p*-coumaric and ferulic acids increased by 1.5 to 2.8 times compared with healthy controls. These data confirm the results of Leser and Treutter (2005), who re-

**Table 1.** Content of phenolic compounds [mean  $\pm$  SE in mg/100g or in mg/g dry weight (rutin, phloridzin, quercetin-3-rhamnoside)] in healthy and infected leaves of cv. Golden Delicious at various times in Ljubljana.

Date	Treatment	Chlorogenic acid	<i>p</i> -Coumaric acid	Caffeic acid	Catechin	Epicatechin	Rutin	Phloridzin	Quercetin-3-rhamnoside
June 2	healthy	30.9 $\pm$ 6.1a	12.4 $\pm$ 0.4a	20.7 $\pm$ 0.7a	45.4 $\pm$ 1.4a	75.3 $\pm$ 2.6a	6.7 $\pm$ 0.6a	139.3 $\pm$ 8.2a	10.1 $\pm$ 0.6a
	infected	145.7 $\pm$ 7.3b	23.8 $\pm$ 1.2b	46.8 $\pm$ 2.2b	104.2 $\pm$ 5.5b	156.8 $\pm$ 10.8b	20.3 $\pm$ 0.7b	309.1 $\pm$ 15.7b	23.7 $\pm$ 0.9b
June 23	healthy	50.7 $\pm$ 2.9a	12.2 $\pm$ 0.7a	19.5 $\pm$ 1.3a	49.4 $\pm$ 3.3a	82.4 $\pm$ 17.6a	11.2 $\pm$ 0.7a	83.3 $\pm$ 7.6a	13.3 $\pm$ 0.7a
	infected	193.4 $\pm$ 29.2b	30.4 $\pm$ 1.1b	46.3 $\pm$ 2.1b	117.6 $\pm$ 6.7b	168.9 $\pm$ 12.4b	19.8 $\pm$ 1.6b	285.7 $\pm$ 12.9b	21.9 $\pm$ 1.4b
July 13	healthy	33.3 $\pm$ 6.2a	12.5 $\pm$ 0.6a	23.8 $\pm$ 1.5a	36.3 $\pm$ 3.1a	120.8 $\pm$ 22.3a	3.8 $\pm$ 0.4a	122.1 $\pm$ 18.1a	6.8 $\pm$ 2.5a
	infected	197.4 $\pm$ 31.7b	32.8 $\pm$ 2.3b	54.5 $\pm$ 4.1b	97.2 $\pm$ 8.0b	309.1 $\pm$ 18.8b	23.6 $\pm$ 2.4b	327.5 $\pm$ 23.3b	28.8 $\pm$ 1.1b
July 25	healthy	35.1 $\pm$ 7.1a	11.3 $\pm$ 2.0a	18.5 $\pm$ 3.5a	36.1 $\pm$ 9.0a	92.6 $\pm$ 28.2	4.2 $\pm$ 0.9a	100.9 $\pm$ 13.6a	7.1 $\pm$ 1.1a
	infected	220.2 $\pm$ 34.2b	25.8 $\pm$ 3.1b	42.6 $\pm$ 5.5b	91.9 $\pm$ 7.5b	233.5 $\pm$ 47.0	18.5 $\pm$ 2.1b	201.4 $\pm$ 22.5b	15.6 $\pm$ 1.3b
August 2	healthy	26.9 $\pm$ 2.1a	13.1 $\pm$ 2.7a	22.6 $\pm$ 1.4a	26.5 $\pm$ 6.6	116.9 $\pm$ 9.7a	3.9 $\pm$ 0.4a	92.6 $\pm$ 8.3a	5.5 $\pm$ 0.5a
	infected	132.2 $\pm$ 34.4b	30.8 $\pm$ 1.9b	51.2 $\pm$ 5.5b	55.7 $\pm$ 12.4	365.5 $\pm$ 40.8b	15.5 $\pm$ 1.8b	198.9 $\pm$ 10.1b	13.1 $\pm$ 1.3b
August 22	healthy	43.4 $\pm$ 6.2a	15.7 $\pm$ 2.0a	22.2 $\pm$ 1.7a	37.4 $\pm$ 4.1ba	162.1 $\pm$ 20.8a	5.9 $\pm$ 0.5a	112.4 $\pm$ 18.7a	7.2 $\pm$ 0.9a
	infected	267.6 $\pm$ 30.1b	31.2 $\pm$ 2.9b	49.5 $\pm$ 1.8b	101.2 $\pm$ 5.2ab	376.8 $\pm$ 19.8b	25.1 $\pm$ 2.3b	199.7 $\pm$ 10.2b	18.5 $\pm$ 0.7b
September 12	healthy	40.9 $\pm$ 13.3a	17.2 $\pm$ 1.1a	22.6 $\pm$ 1.6a	28.5 $\pm$ 4.1ba	148.9 $\pm$ 16.4a	5.9 $\pm$ 0.4a	81.7 $\pm$ 5.5a	5.8 $\pm$ 0.6a
	infected	194.4 $\pm$ 28.4b	35.9 $\pm$ 1.3b	42.4 $\pm$ 1.2b	77.8 $\pm$ 10.0b	347.9 $\pm$ 36.5b	20.9 $\pm$ 2.1b	153.4 $\pm$ 28.4b	15.1 $\pm$ 1.2b

Different letters denote significant differences (LSD test,  $p < 0.05$ ). Comparison was made between healthy and infected leaves on the same date.

**Table 2.** Content of phenolic compounds (mean  $\pm$  SE in mg/100g or in mg/g dry weight (rutin, phloridzin, quercetin-3-rhamnoside) in healthy and infected leaves of the cv. *Golden Delicious* at various times in Maribor.

Date	Treatment	Chlorogenic acid	<i>p</i> -Coumaric acid	Caffeic acid	Catechin	Epicatechin	Rutin	Phloridzin	Quercetin-3-rhamnoside
May 27	healthy	26.4 $\pm$ 3.4	14.4 $\pm$ 0.4a	28.6 $\pm$ 0.9a	62.2 $\pm$ 2.5a	99.2 $\pm$ 2.5a	5.7 $\pm$ 0.2a	93.8 $\pm$ 3.7a	7.1 $\pm$ 0.3a
	infected	66.8 $\pm$ 19.6	27.1 $\pm$ 1.2b	57.4 $\pm$ 2.4b	99.8 $\pm$ 5.1b	175.6 $\pm$ 6.2b	11.1 $\pm$ 0.5b	209.9 $\pm$ 11.4b	13.6 $\pm$ 0.4b
June 2	healthy	30.8 $\pm$ 1.5a	18.3 $\pm$ 2	29.6 $\pm$ 1.4a	68.2 $\pm$ 4.1a	98.8 $\pm$ 5.9a	6.2 $\pm$ 0.4a	114.5 $\pm$ 4.8a	10.8 $\pm$ 0.6a
	infected	162.4 $\pm$ 12.1b	25.1 $\pm$ 0.8	55.9 $\pm$ 2.1b	126.5 $\pm$ 5.3b	215.4 $\pm$ 8.8b	10.7 $\pm$ 1.2b	299.2 $\pm$ 9.4b	19.7 $\pm$ 0.6b
June 23	healthy	67.1 $\pm$ 3.7a	25.2 $\pm$ 0.9	30.3 $\pm$ 1.5a	82.5 $\pm$ 6.6	156.9 $\pm$ 5.7	6.1 $\pm$ 0.2a	88.4 $\pm$ 6.2a	8.4 $\pm$ 0.4a
	infected	108.4 $\pm$ 16.3b	25.4 $\pm$ 1.6	45.2 $\pm$ 5.4b	100.7 $\pm$ 12.8	201.5 $\pm$ 14.4	8.9 $\pm$ 0.8b	222.2 $\pm$ 22.4b	13.9 $\pm$ 1.3b
July 25	healthy	57.4 $\pm$ 16.2a	17.1 $\pm$ 3.1	29.4 $\pm$ 2.7a	54.8 $\pm$ 11.9a	162.2 $\pm$ 22.7	7.4 $\pm$ 1.1	109.2 $\pm$ 11.8a	6.6 $\pm$ 0.8a
	infected	133.8 $\pm$ 7.2b	23.3 $\pm$ 3.1	48.1 $\pm$ 3.8b	97.1 $\pm$ 12.7b	225.8 $\pm$ 17.2	12.9 $\pm$ 1.2	213.4 $\pm$ 27.4b	16.5 $\pm$ 1.9b
August 22	healthy	41.5 $\pm$ 2.8a	16.2 $\pm$ 0.4a	26.1 $\pm$ 0.8a	38.7 $\pm$ 4.5a	166.5 $\pm$ 21.7a	10.4 $\pm$ 1.1a	121.3 $\pm$ 10.2a	10.2 $\pm$ 0.6a
	infected	173.3 $\pm$ 21.3b	28.5 $\pm$ 2.4b	50.2 $\pm$ 3.4b	76.4 $\pm$ 7.8b	269.8 $\pm$ 25.7b	17.4 $\pm$ 1.2b	247.3 $\pm$ 20.0b	16.4 $\pm$ 1.3b
September 12	healthy	28.7 $\pm$ 4.4a	14.1 $\pm$ 0.9a	21.7 $\pm$ 1.4a	32.4 $\pm$ 4.6ba	136.9 $\pm$ 11.3a	7.1 $\pm$ 0.6a	87.5 $\pm$ 12.8a	6.3 $\pm$ 0.7a
	infected	129.5 $\pm$ 10.6b	27.8 $\pm$ 1.9b	41.6 $\pm$ 1.2b	70.2 $\pm$ 8.8b	261.4 $\pm$ 10.9b	13.6 $\pm$ 0.6b	176.4 $\pm$ 11.7b	12.8 $\pm$ 0.8b

Different letters denote significant differences (LSD test.  $p < 0.05$ ). Comparison was made between healthy and infected leaves on the same date.

**Table 3.** Content of phenolic compounds (mean  $\pm$  SE in mg/100g or in mg/g dry weight (rutin, phloridzin, quercetin-3-rhamnoside) in healthy and infected leaves of the cv. *Jonagold* at various times in Maribor.

Date	Treatment	Chlorogenic acid	<i>p</i> -Coumaric acid	Caffeic acid	Catechin	Epicatechin	Rutin	Phloridzin	Quercetin-3-rhamnoside
June 2	healthy	36.7 $\pm$ 6.0a	15.5 $\pm$ 1.1a	28.5 $\pm$ 1.2a	43.9 $\pm$ 4.1a	116.6 $\pm$ 16.3a	4.4 $\pm$ 0.5a	105.0 $\pm$ 6.5a	8.5 $\pm$ 0.6a
	infected	102.3 $\pm$ 5.5b	22.1 $\pm$ 1.8b	48.7 $\pm$ 1.9b	98.7 $\pm$ 7.8b	167.3 $\pm$ 10.6b	6.3 $\pm$ 0.3b	200.4 $\pm$ 10.8b	12.6 $\pm$ 0.8b
June 23	healthy	32.4 $\pm$ 2.3a	11.9 $\pm$ 0.6a	27.7 $\pm$ 1.0a	43.9 $\pm$ 5.9a	83.9 $\pm$ 10.9a	3.5 $\pm$ 0.3a	101.8 $\pm$ 4.6	5.7 $\pm$ 0.3
	infected	124.5 $\pm$ 12.6b	30.7 $\pm$ 2.9b	49.5 $\pm$ 4.6b	130.8 $\pm$ 12.1b	165.0 $\pm$ 19.1b	6.6 $\pm$ 0.7b	166.7 $\pm$ 14.4	8.6 $\pm$ 0.7
July 25	healthy	28.4 $\pm$ 3.0a	15.5 $\pm$ 1.1a	27.0 $\pm$ 2.0a	53.6 $\pm$ 6.1a	128.2 $\pm$ 11.7a	3.2 $\pm$ 0.2a	125.8 $\pm$ 13.3a	7.2 $\pm$ 0.8a
	infected	159.2 $\pm$ 10.0b	28.5 $\pm$ 1.4b	55.4 $\pm$ 3.1b	121.0 $\pm$ 5.3b	348.98 $\pm$ 14.7b	9.6 $\pm$ 0.6b	249.3 $\pm$ 12.9b	12.4 $\pm$ 0.8b
August 22	healthy	31.2 $\pm$ 3.3a	15.7 $\pm$ 1.2a	24.0 $\pm$ 1.3a	28.6 $\pm$ 3.8a	131.3 $\pm$ 11.2a	6.2 $\pm$ 1.0a	108.1 $\pm$ 7.5a	6.1 $\pm$ 0.4a
	infected	156.8 $\pm$ 5.7b	26.5 $\pm$ 1.0b	51.6 $\pm$ 2.1b	77.2 $\pm$ 2.7b	308.7 $\pm$ 10.9b	11.3 $\pm$ 0.4b	236.6 $\pm$ 13.7b	11.4 $\pm$ 0.9b
September 12	healthy	27.5 $\pm$ 1.7a	16.3 $\pm$ 2.5a	23.8 $\pm$ 0.2a	17.1 $\pm$ 4.3a	124.0 $\pm$ 2.7a	4.7 $\pm$ 0.4a	60.7 $\pm$ 3.4a	3.7 $\pm$ 0.4a
	infected	100.9 $\pm$ 13.2b	24.6 $\pm$ 1.3b	37.2 $\pm$ 1.3b	51.2 $\pm$ 7.9b	214.0 $\pm$ 24.7b	6.7 $\pm$ 0.4b	146.9 $\pm$ 8.4b	6.2 $\pm$ 0.4b

Different letters denote significant differences (LSD test.  $p < 0.05$ ). Comparison was made between healthy and infected leaves on the same date.

ported that hydroxycinnamic acids increased in the leaves inoculated with *V. inaequalis*. Their total content was up to 350 mg/100 g d.wt in infected leaves.

Williams and Kuc (1969) reported that hydroxycinnamic acids hinder the growth and sporulation of *V. inaequalis*. Bennet and Wallsgrove (1994) found that, in some cases, there was an accumulation of derivatives of chlorogenic and coumaric acids after infection of apples with *V. inaequalis*. Picinelli *et al.* (1995) discovered that resistant varieties contained more *p*-coumaric acid in comparison with susceptible varieties.

Flavan-3-ols, i.e. epicatechin and catechin, were also found in the analyzed leaves. In particular, healthy leaves contained from 17.1 to 82.5 mg/100g d. wt of catechin and 75.3 to 166.5 mg/100g d. wt of epicatechin (Table 1, 2, 3). These values tally with those reported by Leser and Treutter (2005) for catechin (20 to 75 mg/100g d. wt) and by Mayr *et al.* (1996) for epicatechin (30-160 mg/100g d. wt).

However, we found that infected leaves contained higher concentrations of epicatechin (1.3 to 3.1 times more) and catechin (1.2 to 3 times more) than healthy leaves. Treutter and Feucht (1990b) reported a 6-fold increase of extractable flavan-3-ols in the boundary zones of pear leaves infected by *Gymnosporangium sabinae* in comparison with healthy tissues, and accumulation of flavan-3-ols in apple trees tissues infected by *V. inaequalis* was also observed by Treutter and Feucht (1990a), Picinelli *et al.* (1995) and Mayr *et al.* (1997). In cherry leaves infected by *Blumeriella jaapii*, higher concentrations of catechin, epicatechin and the procyanidins B2, B5 and C1 were discovered, compared with healthy tissues (Niederleitner *et al.*, 1994).

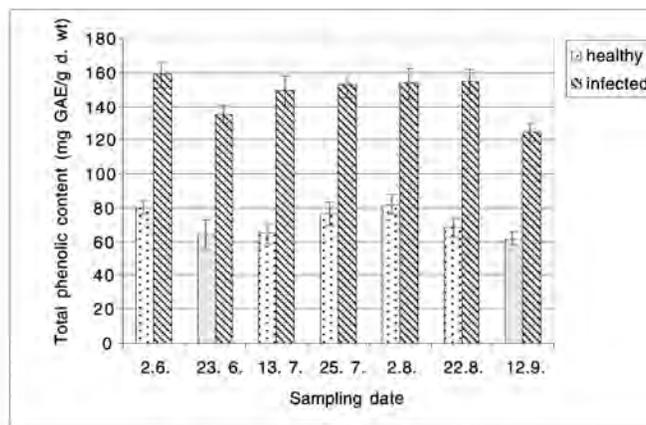
In terms of quantity, rutin (quercetin-3-*O*-rutinoside) and quercetin-3-rhamnoside were the two main flavonols found in apple leaves. In particular, in our analyses the rutin content of healthy leaves ranged from 3.18 to 11.19 mg/g d. wt (Table 1, 2, 3). Treutter (2001) reported a higher total content of hyperin (quercetin-3-*O*-galactoside), isoquercitrin (quercetin-3-*O*-glucoside) and rutin, in the range of 8.31 to 21.02 mg/g d. wt of tissues, whereas in our study the content of rutin in scab-infected leaves increased by 1.4 to 6.2 times in comparison with healthy leaves.

Determination of quercitrin content disclosed that healthy leaves contained a significantly lower amount of this compound (from 3.8 to 10.8 mg/g d. wt, Table 1, 2, 3) than infected leaves (1.5 to 4.2 times more quercitrin), which agrees with the data obtained by Treutter (2001) and Picinelli *et al.* (1995).

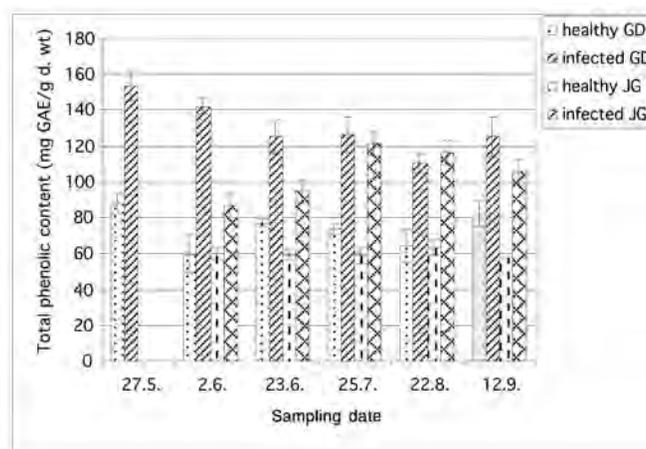
Scab infection caused an increased synthesis of the flavanols rutin and quercetrin, as reported by Feucht (1994), who found that *V. inaequalis*-infected apple leaves accumulate flavonols, i.e. compounds that can precipitate proteins, which may explain the presence of catechins in the defense mechanisms of plants (Treutter, 1989).

Concentration of the dihydrochalcone phloridzin in the leaves of apple trees was the highest of all phenols. Its content in healthy leaves ranged from 60.7 to 139.4 mg/g d. wt of tissues (Table 1, 2, 3), as reported by Treutter (2001) and Leser and Treutter (2005). However, infection by *V. inaequalis* caused a higher accumulation of phloridzin in infected leaves, which contained 1.6 to 3.4 times more phloridzin than healthy leaves, the same as found by Leser and Treutter (2005). Phloridzin is oxidized to 3-hydroxyphloridzin and further to the corresponding *o*-quinone (Williams and Kuc, 1969), which are toxic to the fungus (Misagi, 1982).

If the content of total phenols is considered, healthy apple leaves contained significantly less phenols than the scab-infected leaves. This is true for both locations and all dates of leaf collection. Readings of total phenols ranged from 60.4 to 87.6 mg GAE/g d. wt in healthy leaves, with an increase of 1.4 to 2.4 times in infected leaves (86.4 to 158.7 mg GAE/g d. wt) (Figs. 1, 2).



**Fig. 1.** Content of total phenolics (mean values and standard error bars expressed as mg GAE/g dry weight of leaf tissue) in healthy and in infected leaves of cv. Golden Delicious determined at various times in Ljubljana.



**Fig. 2.** Content of total phenolics (mean values and standard error bars expressed as mg GAE/g dry weight of leaf tissue) in healthy and in infected leaves of cv. Golden Delicious (GD) and cv. Jonagold (JG) determined at various times in Maribor.

In this study, the change of phenolic profiles in apple leaves throughout the growing season was investigated. When the content of phenolic substances in the leaves of healthy apple trees is compared with that of scab-infected leaves, it emerges that infection by *V. inaequalis* changed the synthesis of phenolic substances.

Generally, when a plant is infected, its phenolic content increases, as a consequence of a defense reaction to infection. Gessler *et al.* (2006) give a plausible reason for the greater or lesser extent of resistance to scab stating that one type of resistance is probably polygenic horizontal resistance, in which the combined action of many factors, for example, fungitoxic compounds, provides a modest contribution to overall resistance. Phenolics are probably not the only compounds that contribute to resistance of apples to scab but, as it could be seen from our results, their synthesis is increased by the infection.

The results achieved represent an important contribution to understanding plant-pathogen interaction and may be useful for further investigations. For further studies, other specific phenolics as well as other compounds should be studied in more detail. In particular, the time scale in which they are synthesized could be of great importance as a mechanism of quick response to apple scab infection, which can lead to a higher degree of resistance.

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