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Response of the phenylpropanoid pathway to *Venturia inaequalis* infection in maturing fruit of 'Braeburn' apple

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SUMMARY
This study investigated the activities of key enzymes in the phenylpropanoid pathway and the accumulation of phenolic products in apple peel in response to infection with *Venturia inaequalis* [(Cooke) G. Wint.]. We compared healthy apple peel with apple peel showing symptomatic scab lesions, and with peel tissue from 1 – 2 mm around the scab lesions in fruit 1 month before maturity [140 d after full bloom (DAFB)] and at physiological maturity (175 DAFB). Infection with *V. inaequalis* enhanced the synthesis of some phenolic compounds. Compared to healthy peel, scab lesion tissue had ≤ 3.1-times higher hydroxycinnamic acid content, ≤ 1.3-times higher dihydrochalcone content, and ≤ 3.9-times higher flavan-3-ol content. Scab lesions showed slightly higher phenylalanine ammonia-lyase, chalcone synthase, chalcone isomerase, flavonol synthase, and dihydroflavonol 4-reductase activities. The total amount of phenolics remained relatively stable between the two sampling dates, except for epicatechin and caffeic acid which decreased in amount toward fruit maturity, and levels of catechin which increased in more mature fruit. During fruit maturation, only chalcone synthase and chalcone isomerase activities decreased in all tissues examined. This study showed that the phenylpropanoid pathway in apple fruit peel changed significantly following infection with the apple scab pathogen.

Apple scab, caused by the fungus *Venturia inaequalis* [(Cooke) G. Wint.], is the most important disease present in all apple-growing areas with high Spring and Summer rainfall. Disease control in commercial orchards can require several fungicide treatments per year. An alternative approach is the use of resistant apple cultivars, or treatment with chemical agents which can induce higher resistance to the apple scab pathogen (Brun *et al*., 2008).

Phenolic compounds have been shown to be involved in plant defences against biotic and abiotic stresses (Geibel *et al*., 1994). A large number of different phenolic compounds have been reported to occur in various apple tissues (Treutter, 2001; Bazzi *et al*., 2003). Phenolic compounds in apple, particularly flavonoids, have been claimed to play a role in scab resistance (Treutter and Feucht, 1990). Other phenolics, such as phenolic acids, also contribute to healing through increased lignification of damaged areas. Furthermore, they possess anti-microbial properties, and their concentrations may increase after infection (Bostock *et al*., 1999, Treutter, 2005).

Secondary metabolism is part of the dynamic biochemical processes that are involved in plant growth and differentiation. Interconnections between many different metabolic processes are responsible for the variety of responses of plant tissues to eliciting agents, and highly specialised pathogens are adapted to the specific physiology of the host plant. Research into *Erwinia amylovora* has shown that this bacterium, which causes fire blight disease in apple, is able to decrease accumulation of the natural anti-bacterial compound, phloretin, to below bactericidal levels (Pontais *et al*., 2008).

Previous studies (Mayr *et al*., 1997; Michalek *et al*., 1999a) revealed that the rapid biosynthesis of flavanols from phenylalanine was necessary for protection against pathogens. Inhibition of the enzyme phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) resulted in severe symptoms of sporulation. PAL provides the link between primary plant metabolism and the phenylpropanoid pathway. PAL catalyses the conversion of phenylalanine to *trans*-cinnamic acid, which is a precursor of many different phenolic compounds. The key enzyme in the flavanoid pathway is chalcone synthase (CHS; EC 3.2.1.74). CHS and chalcone isomerase (CHI; EC 3.2.1.14) together lead to the formation of flavanones. Naringenin is converted by flavanone 3-hydroxylase (FHT; EC 1.14.11.9) and dihydroflavonol 4-reductase (DFR; EC 1.1.1.219) to leuco-anthocyanidins (flavanols), which are the precursors of anthocyanidins and flavan-3-ols (e.g., catechins and procyanidins; Treutter, 2001). The latter, in particular, also play an important role in plant disease resistance mechanisms. Flavonol synthase (FLS; EC 1.14.11.23) catalyses the dehydrogenation of dihydroflavonols to flavonols and therefore competes with flavanol-forming enzymes for their common precursors. Dihydrochalcone 2'-*O*-glucosyltransferase (D2'GT; EC 2.4.1.17) catalyses the formation of phloridzin, which is also present in high concentrations in apple peel.

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In addition to its structural and defence-related functions, the phenylpropanoid pathway also produces a variety of phenolics that are closely-associated with fruit quality. However, there are few reports on phenlypropanoid enzyme activities in apple fruit during development, particularly in their responses to infection by pathogens. The objective of this study was to evaluate the biochemical responses of various zones of apple fruit skin to infection with *V. inaequalis*. This was investigated at the level of individual phenolic compounds related to key enzyme activities in the phenylpropanoid pathway. The activities of PAL, CHS/CHI, FHT, DFR, FLS, and D2'GT, and the accumulation of various phenolic compounds (hydroxycinnamic acids, flavan-3-ols, dihydrochalcones, and flavonols) were also investigated. Three different areas of peel tissue on the same fruit (i.e., symptomatic lesions, tissue around lesions, and healthy peel tissue) were analysed at two different stages of fruit maturity (i.e., 1 month before commercial maturity, and at commercial maturity). No comparable research has been reported in the literature.

MATERIALS AND METHODS

Plant material and growth conditions

This study was performed on apple trees (*Malus* × *domestica* Borkh.), cultivar 'Braeburn', which is a scab-susceptible cultivar. Fruits were taken from 9-year-old trees that had been grafted onto M.9 rootstock and were growing at the University Experimental Orchard in Ljubljana. Trees were cultivated according to commercial guidelines for integrated fruit production.

Fruit samples (n = 5) were picked 1 month before commercial maturity, on 15 September 2008 (140 d after full bloom; DAFB), and at commercial maturity on 20 October 2008 (175 DAFB). The apples were peeled using a fruit peeler and the peel was divided into three parts: symptomatic scab lesions, peel tissue 1 – 2 mm around the scab lesions, and healthy fruit peel. All samples were shock-frozen immediately in liquid nitrogen and stored at –80°C until analysis.

Extraction and HPLC determination of phenolic compounds

Extractions were carried out as described by Mikulic Petkovsek *et al*. (2007) with some modifications. Each finely-powdered apple peel sample (0.5 g) was extracted with 3 ml 100% (v/v) methanol containing 1% (w/v) 2,6-di-*tert*-butyl-4-methylphenol (BHT) and homogenised using a T-25 Ultra-Turrax (Ika-Labortechnik, Staufen, Germany). BHT was added to the samples to prevent oxidation during the extraction. All samples were placed in a Sonis 4 (Iskra pio, Ljubljana, Slovenia) ultrasonic bath for 1 h, then centrifuged at 15,550 × *g* for 7 min at 4°C. Each supernatant was filtered through a Chromafil A0-45/25 polyamide filter (Macherey-Nagel, Düren, Germany). HPLC analysis was performed using a Surveyor HPLC system with a PDA detector (Thermo-Finnigan, San Jose, CA, USA). The column was a Gemini C_{18} column (150 mm × 4.6 mm; particle size 3 µm; Phenomenex, Torrance, CA, USA) operated at 25°C. The elution solvents were 1% (v/v) formic acid in double-distilled water (solvent A) and 100% (v/v) acetonitrile (solvent B). Samples were eluted using the linear gradient described by Marks *et al*. (2007), with a sample injection volume of 20 µl and a flow rate of 1.0 ml min^{-1}. Phenolic compounds were identified by comparing their UV-VIS spectra from 220 – 550 nm, and their retention times, with standards. Hydroxycinnamic acids (chlorogenic, *p*-coumaric, ferulic, and caffeic acid), flavan 3-ols (catechin, epicatechin, and procyanidin B2), dihydrochalcone (phloretin), and phloroglucinol were detected at 280 nm; whereas phloridzin, quercetin-3-*O*-rutinoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glucoside, and quercetin-3-*O*-galactoside were detected at 350 nm. The concentration of each phenolic compound was calculated by comparing the peak area of the samples with those of the corresponding standard compound. All concentrations were expressed as mg 100 g^{-1} FW, except in the case of phloretin which was expressed as µg 100 g^{-1} FW.

Enzyme preparation

Each fruit peel sample (0.5 g), plus 0.25 g quartz sand and 0.25 g Polyclar AT was homogenised in 3 ml 0.1 M Tris-HCl pH 7.5 containing 0.4% (w/v) Na-ascorbate in a pre-cooled mortar and pestle. The homogenate was then centrifuged at 10,000 × g for 10 min at 4°C. To remove low molecular compounds, 400 µl of the supernatant was passed through a Sephadex G25 medium chromatography column. The eluate from the column ("crude extract") was used for all enzyme assays. Protein contents were quantified by a modified Lowry procedure (Sandermann *et al*., 1972) with bovine serum albumin as a standard.

Buffers

The following buffers were used for the various enzyme assays, Buffer A (PAL assays), 0.1 M H_3BO_3 containing 0.4% (w/v) Na-ascorbate, pH 8.5; Buffer B (CHS/CHI assays), 0.1 M potassium phosphate, pH 7.0 containing 0.4% (w/v) Na-ascorbate, pH 7.0; Buffer C (FHT assays), 0.1 M Tris-HCl, pH 7.25 containing 0.4% (w/v) Na-ascorbate; Buffer D (FLS, D2'GT assays), 0.1 M Tris-HCl, pH 7.5 containing 0.4% (w/v) Na-ascorbate, pH 7.5; and Buffer E (DFR assay), 0.1 M potassium phosphate, pH 6.8 containing 0.4% (w/v) Na-ascorbate.

Enzyme assays

PAL assays (100 µl final volume) contained 40 µl "crude extract", 5 µl [^{14}C]-phenylalanine (0.063 nmol) and 55 µl Buffer A. The CHS/CHI assays (100 µl final volume) contained 40 µl "crude extract", 5 µl [^{14}C]-malonyl-CoA (1.5 nmol), 5 µl [^{14}C]-*p*-coumaroyl-CoA (1.0 nmol) and 50 µl Buffer B. The FHT assays (100 µl final volume) contained 8.2 µl [^{14}C]-naringenin (0.036 nmol), 30 µl "crude extract", 5 µl 2-oxoglutarate (1.46 mg ml^{-1}), 5 µl $FeSO_4.7H_2O$ (0.56 mg ml^{-1}) and 60 µl Buffer C. The FLS assays (100 µl final volume) contained 2.9 µl [^{14}C]-dihydrokaempferol (0.036 nmol), 30 µl "crude extract", 5 µl 2-oxoglutarate (1.46 mg ml^{-1}1), 5 µl $FeSO_4.7H_2O$ (0.56 mg ml^{-1}) and 60 µl Buffer D. The DFR assays (50 µl final volume) contained 19 µl (0.036 nmol) [^{14}C]-dihydroquercetin, 20 µl "crude extract", 5 µl NADPH (4.18 mg 100 µl $^{-1}$) and 25 µl Buffer E. The D2'GT assays (50 µl final volume) contained 20 µl "crude extract", 2.5 µl phloretin (300 µM), 2.5 µl [^{14}C]-UDP-glucose and 25 µl Buffer D.

All assays were incubated for 30 min at 30°C, with the exception of the DFR assays which were incubated for 15 min at 30°C. The PAL and CHS/CHI assays were stopped with 200 µl 100% (v/v) ethyl acetate and 10 µl glacial acetic acid. The amounts of the products formed were determined using a WinSpectral 1414 liquid scintillation counter (Wallac, Turku, Finland). The FHT, DFR, and FLS assays were terminated by adding 70 µl 100% (v/v) ethyl acetate and 10 µl glacial acetic acid. Ten µl of 0.1 M EDTA were also added to the FLS assays. The organic phases were transferred to pre-coated cellulose plates (Merck, Darmstadt, Germany) for thin-layer chromatography (TLC) using a 10:9:1 (v/v/v) mix of chloroform/acetic acid/H_2O for the FHT and DFR assays, and 30% (v/v) glacial acetic acid for the FLS assays. Conversion rates were then determined using a TLC linear analyser (Berthold, BadWildbad, Germany). The D2'GT assays were terminated by adding 10 µl glacial acetic acid and 25 µl 100% (v/v) methanol. The mixture was then chromatographed on Schleicher and Schüll 2043b paper using water as solvent. Zones containing the labelled products were cut out, and the radioactivity was quantified using a Winspectral 1414 scintillation counter. All products were identified, as described above, using authentic substances (Fischer *et al*., 2003). All enzyme activities were expressed as nkat mg^{-1} FW.

Chemicals

The standards used to identify and determine the levels of phenolic compounds in each sample were chlorogenic acid, quercetin-3-*O*-rutinoside, (-)-epicatechin, phloretin, phloroglucinol, and phloridzin from Sigma-Aldrich (Steinheim, Germany), (+)- catechin from Roth (Karlsruhe, Germany), and caffeic acid, *p*-coumaric acid, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-galactoside, procyanidin B2, and ferulic acid from Fluka Chemie GmbH (Buchs, Switzerland). The chemicals used for the mobile phases were HPLC-grade acetonitrile and formic acid from Fluka. Water for the mobile phase was double-distilled and purified using a Milli-Q system (Millipore, Bedford, MA, USA). The following ^{14}C-labelled substrates were used for determining enzyme activities L-[U-^{14}C] phenylalanine and [2-^{14}C]-malonyl-coenzyme A, (Amersham International, Freiburg, Germany). [^{14}C]-Naringenin, [^{14}C]-dihydrokaempferol, and [^{14}C]-dihydroquercetin were prepared as described (Fischer *et al*., 2003).

Statistical analysis

The results were analysed using Statgraphics Plus Version 4.0 (Manugistics Inc., Rockville, MD, USA). Data from all chemical analyses were tested for spatial or temporal differences between the samples using one-way analysis of variance (ANOVA). Differences between apple peel tissues were analysed independently for each time point using Duncan's multiple range test with a significance level of $P \leq 0.05$. A one-way analysis of variance (ANOVA) was also carried out to determine differences in the contents of phenolic compounds between the different sampling dates followed by the LSD test with a significance level of $P \leq 0.05$. Means and standard errors of the means (means + SE) are reported.

RESULTS AND DISCUSSION

Analysis of phenolic compounds

Hydroxycinnamic acids represented the largest group of products in the biosynthetic pathways for phenolic compounds. Chlorogenic acid contents were higher than those of the other hydroxycinnamic acids analysed and were comparable on both sampling dates. On both sampling dates, tissues infected with the apple scab fungus contained statistically higher quantities of chlorogenic acid compared with tissues around the lesions or healthy apple peel (Figure 1C). The chlorogenic acid contents of healthy peel varied from 0.04 – 1.24 mg 100 g^{-1} FW. Ferulic acid contents were at least 17-times lower, but showed a similar distribution (Figure 1A). The amount of caffeic acid was statistically lower at 175 DAFB than at 140 DAFB (Table I). Peel tissue surrounding the scab lesions accumulated *p*-coumaric and caffeic acid acid (Figure 1B, D). Compared to healthy peel, apple scab lesions contained 1.4- to 1.7-times higher concentrations of caffeic acid, and 1.6- to 3.1-times more *p*-coumaric acid. This agreed with previous reports on the accumulation of hydroxycinnamic acids in the response of apple leaves to fungus infection. Mikulic Petkovsek *et al*. (2008) and Williams and Kuç (1969) found that hydroxycinnamic acids inhibited the growth and sporulation of *V. inaequalis*.

Dihydrochalcone phloridzin, a phloretin 2' *O*-glucoside, was the major phenolic compound in apple fruit extracts (Iwashina, 2000). Phloridzin and its corresponding aglycone, phloretin, have often been linked to resistance to numerous diseases. Studies on apple have shown that resistance to *V. inaequalis* was related to the presence of this compound (Hamauzu, 2006). The fungus degrades phloridzin to its toxic aglycone, phloretin. In our experiments, healthy apple peel and tissues around the symptomatic lesions contained significantly lower levels of phloridzin than the scab lesion itself. Phloridzin concentrations in scab lesions ranged from 114.7 – 238.8 mg 100 g^{-1} FW (Figure 1F), which was 6.5-times higher than in healthy peel. It was therefore evident that phloridzin accumulated when apple peel tissue was infected with *V. inaequalis*. This effect was also reported by Mikulic Petkovsek *et al*. (2008) and by Leser and Treutter (2005). The aglycone, phloretin, was only found in small amounts in scab lesion tissues, while surrounding

TABLE I

Differences in the levels of selected phenolic compounds in the peel of Malus × domestica *'Braeburn' between two sampling dates (140 DAFB and 175 DAFB) by one-way ANOVA*

Compound	Concentration (mg 100 g^{-1} FW) 140 DAFB	175 DAFB	ANOVA‡
Total quercetin	81.56 ± 7.78+	63.15 ± 8.60	NS
Epicatechin	24.11 ± 1.26	20.02 ± 1.33	*
Catechin	2.16 ± 0.32	4.64 ± 0.37	***
Ferulic acid	0.13 ± 0.03	0.16 ± 0.02	NS
Phloridzin	52.83 ± 5.8	66.05 ± 8.82	NS
Phloroglucinol	112.64 ± 11.6	139.22 ± 13.2	NS
Caffeic acid	0.95 ± 0.09	0.65 ± 0.07	**
Chlorogenic acid	1.82 ± 0.42	2.74 ± 0.53	NS
p-Coumaric acid	1.55 ± 0.13	1.16 ± 0.16	NS
Procyanidin B2	28.89 ± 2.05	26.54 ± 2.15	NS
Phloretin†	0.70 ± 0.08	0.77 ± 0.08	NS

+Values are means ± SD (n=5).

‡NS,*, **, ***; non-significant, significant at $P < 0.05$, $P < 0.01$, or $P < 0.001$ respectively.

†Phloroglucinol in µg 100 g^{-1} FW.

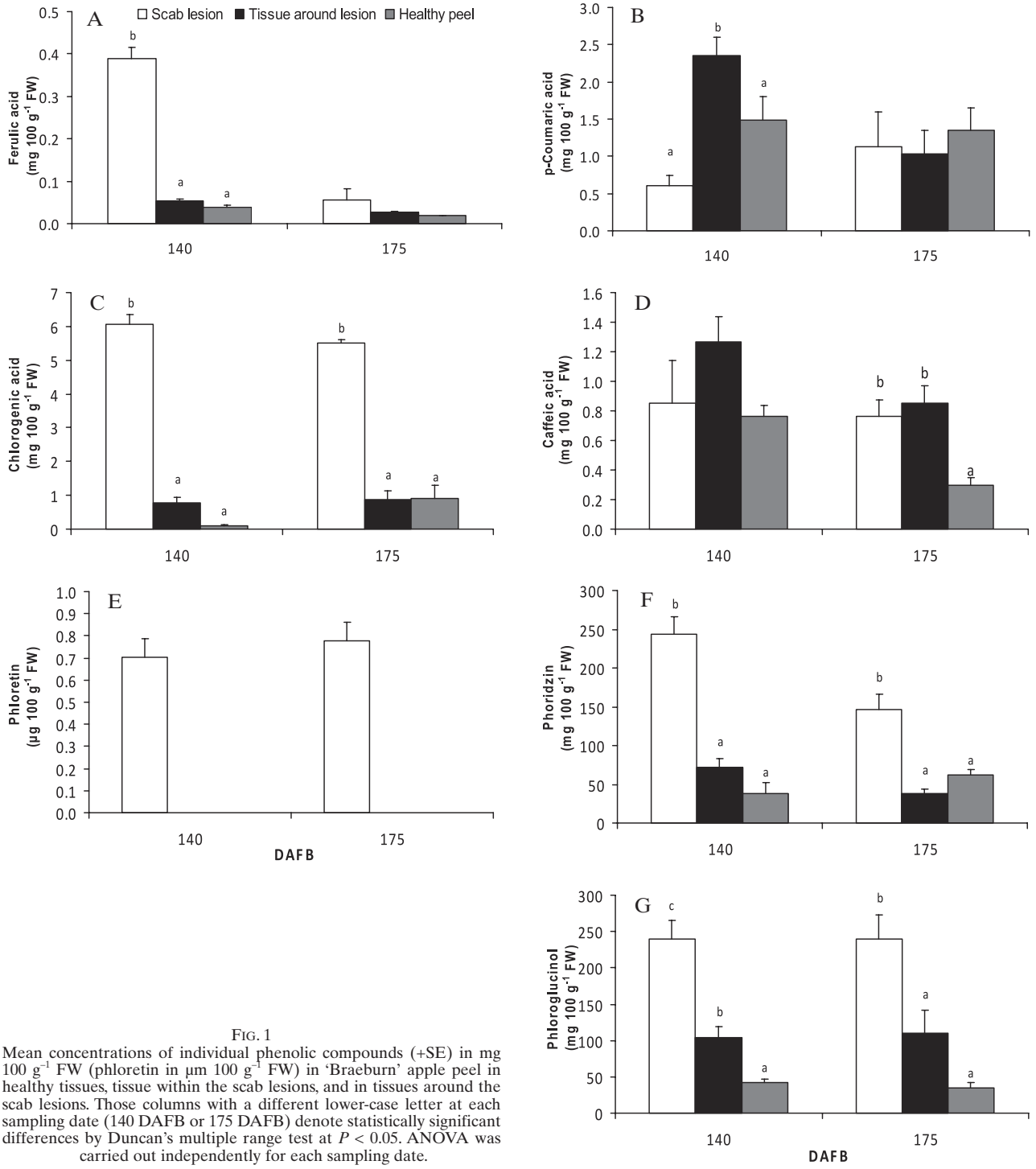

FIG. 1
Mean concentrations of individual phenolic compounds (+SE) in mg 100 g^{-1} FW (phloretin in µm 100 g^{-1} FW) in 'Braeburn' apple peel in healthy tissues, tissue within the scab lesions, and in tissues around the scab lesions. Those columns with a different lower-case letter at each sampling date (140 DAFB or 175 DAFB) denote statistically significant differences by Duncan's multiple range test at $P < 0.05$. ANOVA was carried out independently for each sampling date.

tissues and healthy apple peel contained none (Figure 1E). However, phloretin rather than phloridzin has been claimed to be the key agent in defence reactions, related to formation of the highly reactive *o*-quinone (Elstner *et al.*, 1996). In addition, phloretin can be degraded to form phloroglucinol, which inhibited development of the fungus (Hamauzu, 2006). In our experiments, apple scab-infected tissues contained statistically greater quantities of phloroglucinol on both sampling dates, compared to tissues around the lesions or healthy peel. Phloroglucinol concentrations in the lesions ranged from 196.2 – 292.0 mg 100 g^{-1} FW (Figure 1G), which was 6.9-times higher than in healthy apple peel.

Flavan-3-ols are capable of precipitating proteins, which may explain the presence of catechins in the defence mechanisms of plants (Treutter, 1989). Mayr *et al.* (1997) related the rapid accumulation of catechins and oligomeric pro-anthocyanidins at the sites of infection to the restriction of fungal spread. Feucht *et al.* (1998) reported that epicatechin was the main flavanol synthesised during fruit infection by *V. inaequalis*. In our study, the highest levels of flavanols were found for procyanidin B2, compared to epicatechin and catechin.

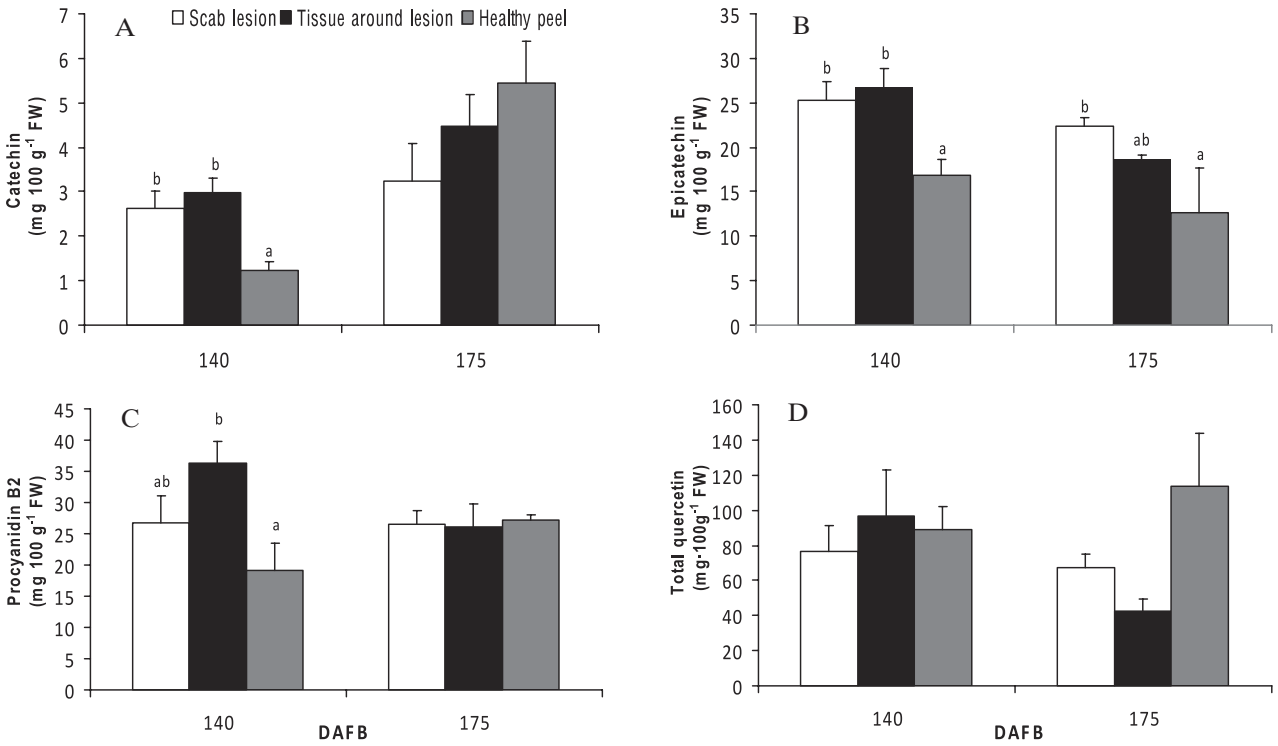

FIG. 2
Mean concentrations of flavanols and flavonols (+SE) in mg 100 g^{-1} FW in 'Braeburn' apple peel in healthy tissues, tissue within the scab lesions, and in tissues around the scab lesions. Those columns with a different lower-case letter at each sampling date (140 DAFB or 175 DAFB) denote statistically significant differences by Duncan's multiple range test at $P < 0.05$. ANOVA was carried out independently for each sampling date.

The amounts of epicatechin differed significantly between treatments (Table I) and appeared lower at 175 DAFB (Figure 2B). This was also noted in healthy fruit during maturation by Mayr *et al.* (1994). Tissues around the scab lesions contained the highest amounts of epicatechin compared to lesion tissue or healthy peel (Figure 2B). Treutter and Feucht (1990) and Feucht *et al.* (1994) also observed a dramatic increase in the levels of catechins and their polymers in the boundary zones around the sites of infection by *V. inaequalis* in apple leaves. In our case, statistically higher concentrations of catechins were observed at 175 DAFB, regardless of the tissue sample (Figure 2A; Table I).

Mikulic *et al.* (2009) reported the highest concentrations of flavonols in the zone between scab lesions and healthy skin tissue. In our experiments, the concentrations of individual phenolic compounds (data not shown) and total quercetin-*O*-glucosides (i.e., the

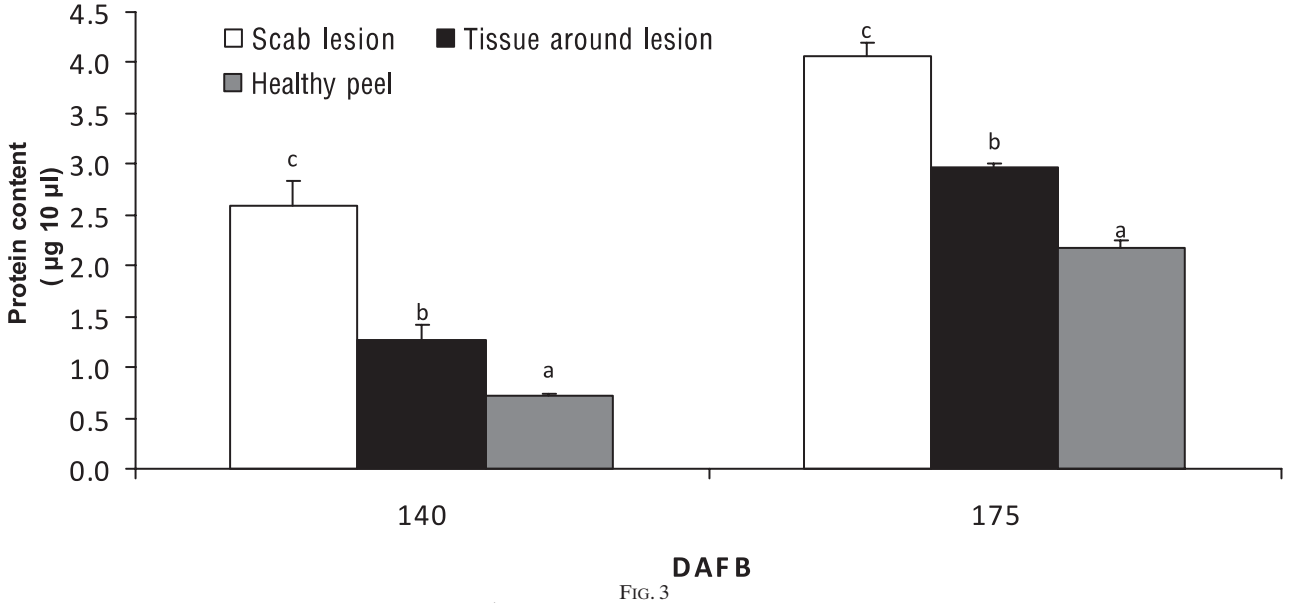

FIG. 3
Mean concentrations of total proteins (+SE) in µg 10 μl^{-1} "crude tissue" extract from 'Braeburn' apple peel in healthy tissues, tissues within the scab lesions, or in tissues around the scab lesions. Columns with a different lower-case letter at each sampling date (140 DAFB or 175 DAFB) denote statistically significant differences by Duncan's multiple range test at $P < 0.05$. ANOVA was carried out independently for each sampling date.

sum of quercetin-3-*O*-rutinoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glucoside, and quercetin-3-*O*-galactoside) showed no statistically significant differences between the different tissues (Figure 2D) on either fruit maturity date (140 DAFB and 175 DAFB). This also agreed with the results of Picinelli *et al*. (1995), who found no relationship between the level of flavonols and scab resistance in apple leaves.

Analyses of enzyme activities

Protein contents were highest in scab lesion tissue and lowest in healthy apple peel (Figure 3). This could be due to increased protein production in response to fungal infection and/or to the presence of added fungal proteins in infected tissues. All enzyme activities were therefore expressed as mg^{-1} FW, rather than per total protein content (specific activity), in order to reduce the impact of additional fungal proteins. Figure 4 shows a comparison of the activities of the five key enzymes examined in the different apple peel samples on the two sampling dates.

PAL, which catalyses deamination of the amino acid phenylalanine, is a key enzyme and represents the interface between primary and secondary metabolism. The resulting product, cinnamic acid, is a precursor for the biosynthesis of flavonoids, lignin, and alkaloids. In our analysis, PAL activity could only be measured in symptomatic scab lesions and in the tissues around the lesions on both sampling dates. By 175 DAFB, PAL activities had increased, particularly around the symptomatic lesions, but remained almost unchanged within the scab lesions (Figure 4A). Previous studies (Mayr *et al*., 1997; Michalek *et al*., 1999b) revealed that the rapid biosynthesis of flavanols from phenylalanine was necessary for successful plant protection. Inhibition of PAL activity resulted in severe symptoms of fungal sporulation in a resistant cultivar. Saunders and Olechno (1988) reported that differences in PAL mRNA levels in plants occurred as a response to fungal inoculation. Saunders and O'Neill (2004) reported that alfalfa plants responded to inoculation with an avirulent fungus by developing induced resistance that enhanced *PAL* gene expression, which increased the activity of PAL for flavonoid metabolism.

CHS is the key enzyme for the formation of flavonoids because it produces the first C_{15}-structure which is an intermediate for the formation of all further classes of flavonoids. The CHS reaction leads to the formation of naringenin chalcone, which is converted by CHI into naringenin, the first true flavonoid structure. At 175 DAFB, statistically higher CHS/CHI activities were detected in symptomatic lesion tissues (Figure 4B) than

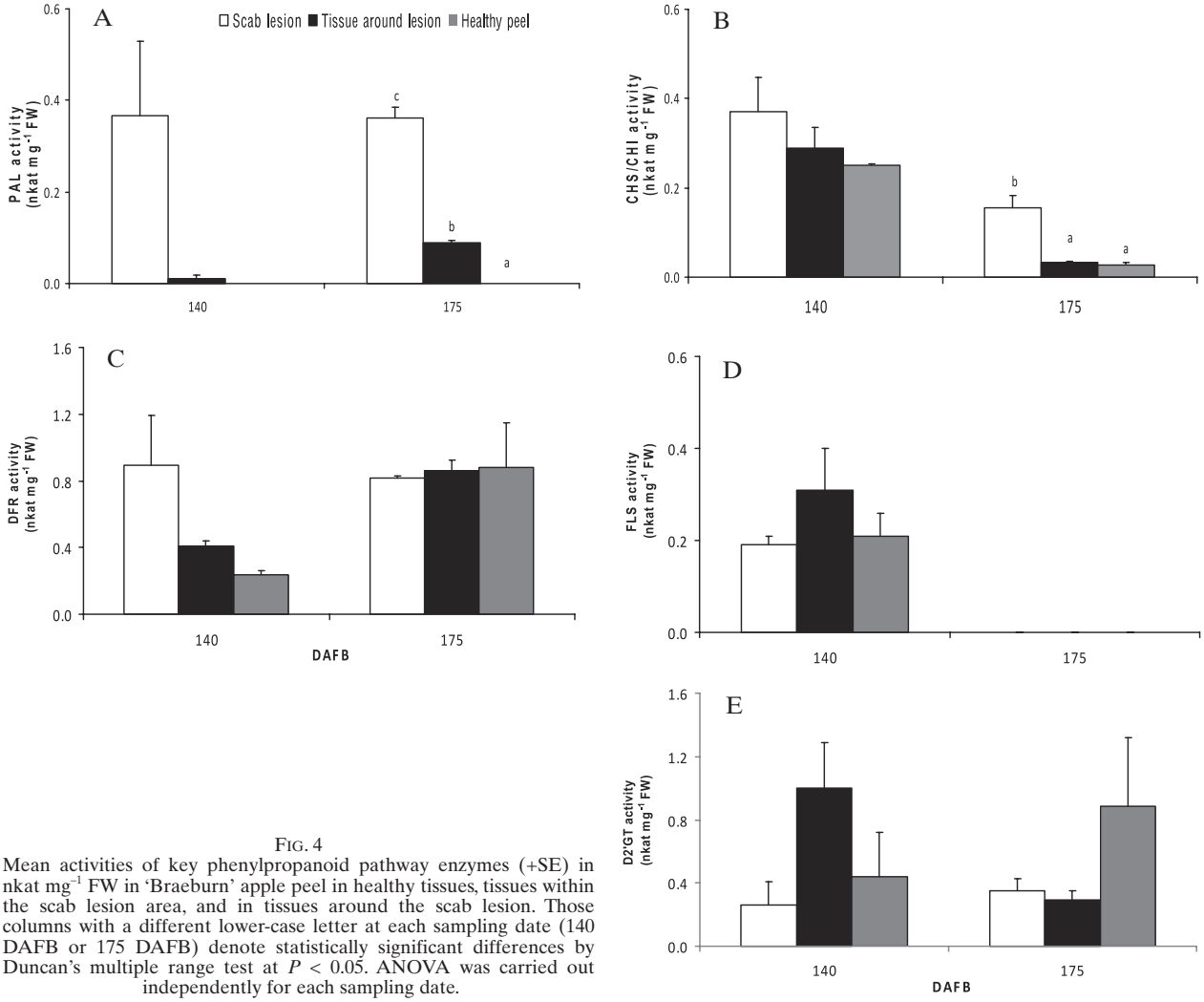

FIG. 4
Mean activities of key phenylpropanoid pathway enzymes (+SE) in nkat mg^{-1} FW in 'Braeburn' apple peel in healthy tissues, tissues within the scab lesion area, and in tissues around the scab lesion. Those columns with a different lower-case letter at each sampling date (140 DAFB or 175 DAFB) denote statistically significant differences by Duncan's multiple range test at $P < 0.05$. ANOVA was carried out independently for each sampling date.

in healthy apple peel tissue, or in tissues surrounding the scab lesions, which showed no differences in activity. Between 140 DAFB and 175 DAFB, CHS/CHI activities decreased in all tissues, indicating that the processes of fruit maturation lowered the levels of enzyme activity (Figure 4B).

DFR is an NADPH-dependent oxido-reductase which reduces the oxo-group at position-4 to provide flavan-3,4-diols; precursors for the formation of catechins, anthocyanidins, and epicatechins (Forkmann and Heller, 1999). DFR activities showed no statistically significant differences between any of the peel zones studied. By 175 DAFB, DFR activities had increased around the scab lesions and in healthy apple peel, but had decreased in lesion tissues (Figure 4C).

FHT and FLS are di-oxygenases which require 2-oxoglutarate, Fe^{2+}, and ascorbate as co-factors, and catalyse the formation of flavanones and flavonols. FHT activity was undetectable on either sampling date. FLS activity could only be measured at 140 DAFB (Figure 4D). However, those flavonols that are the products of this enzyme showed higher concentrations in scab-infected tissues and in surrounding tissues than in healthy apple peel.

D2'GT converts phloretin to phloridzin. In contrast to the other enzymes examined, symptomatic lesions showed lower D2'GT activities than healthy peel tissue on both sampling dates. The decrease in D2'GT enzyme activity in infected peel tissues could be explained by the fact that the aglycone, not the 2'-*O*-glucoside, is considered to be the active compound against fungal attack. In fact, as can be seen in Figure 4E, the aglycone (phloretin) was only detected in scab lesions. The levels of phloridzin in lesion tissues were also statistically higher. At 175 DAFB, D2'GT activities were higher in lesions and in healthy peel tissue than in immature fruit at 140 DAFB. D2'GT activities around the lesions decreased between the two sampling dates (Figure 4E).

CONCLUSIONS

Knowledge of the phenolic compounds and enzyme activities present in fruit tissues is an indispensable precondition for future studies on complex host-pathogen interactions and polyphenol-mediated disease resistance. We have shown that phenolic compounds accumulate in apple peel in response to infection by *V. inaequalis*. This results from the higher activities of key phenylpropanoid pathway enzymes in scab lesions and in surrounding tissues. All key enzyme activities, except DFR and G2'TP, were lower at 175 DAFB which indicates that these enzyme activities decreased with fruit maturity. Nevertheless, only decreases in caffeic acid and epicatechin levels were noticed, while the levels of all other phenolic compounds remained constant. Our results represent an important contribution to increase our knowledge of the activity of the phenlypropanoid pathway in apple fruit. Future work should focus on differences in this defense scheme in apple varieties having different levels of susceptibility to scab, and on the responses to infection at the early stages of fruit development which are crucial for successful resistance to the apple scab pathogen.

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REFERENCES

Bazzi, C., Messina, C., Tortoreto, L., Stefani, E., Bini, F., Brunelli, A., Andreotti, C., Sabatini, E., Spinelli, F., Costa, G., Hauptmann, S., Stammler, G., Doerr, S., Marr, J. and Rademacher, W. (2003). Control of pathogen incidence in pome fruits and other horticultural crop plants with prohexadione-Ca. *European Journal of Horticultural Science*, **68,** 108–114.

Bostock, R. M., Wilcox, S. M., Wang, G. and Adaskaveg, J. E. (1999). Suppression of *Monilinia fruticola* cutinase production by peach fruit surface phenolic acid. *Physiological and Molecular Plant Pathology*, **54,** 37–50.

Brun, L., Didelot, F. and Parisi, L. (2008). Effects of apple cultivar susceptibility to *Venturia inaequalis* on scab epidemics in apple orchards. *Crop Protection*, **27,** 1009–1019.

Elstner, E. F., Oßwald, W. and Schneider, I. (1996). Phytopathologie. *Allgemeine und biochemische Grundlagen*. Spektrum Akademischer Verlag, Heidelberg, Germany. 208–209.

Feucht, W. (1994). The localization of phenols at the cellular and tissue level. *Acta Horticulturae*, **381,** 803–815.

Fischer, T. C., Halbwirth, H., Meisel, B., Stich, K. and Forkmann, G. (2003). Molecular cloning, substrate specificity of the functionally expressed dihydroflavonol 4-reductases from *Malus domestica* and *Pyrus communis* cultivars and the consequences for flavonoid metabolism. *Archives of Biochemistry and Biophysics*, **412,** 223–230.

Forkmann, G. and Heller, W. (1999). Biosynthesis of flavonoids. In: *Comprehensive Natural Products Chemistry*. Volume 2. (Barton, D., Nakanishi, K. and Meth-Cohn, O., Eds.). Elsevier, Oxford, UK. 713–748.

Geibel, M., Treutter, D. and Feucht, W. (1994). Activity of phenylalanine ammonia lyase in apple leaves. *Acta Horticulturae*, **381,** 474–478.

Hamauzu, Y. (2006). Role and evolution of fruit phenolic compounds during ripening. *Stewart Postharvest Review*, **2,** 1–7.

Iwashina, T. (2000). The structure and distribution of the flavonoids in plants. *Journal of Plant Research*, **113,** 287–299.

Leser, C. and Treutter, D. (2005). Effects of nitrogen supply on growth, contents of phenolic compounds and pathogen (scab) resistance of apple trees. *Plant Physiology*, **123,** 49–56.

Marks, S. C., Mullen, W. and Crozier, A. (2007). Flavonoid and chlorogenic acid profiles of English cider apples. *Journal of the Science of Food and Agriculture*, **87,** 719–728.

Mayr, U., Treutter, D., Santos-Buelga, C., Bauer, H. and Feucht, W. (1994). Developmental changes in the phenol concentrations of 'Golden Delicious' apple fruits and leaves. *Phytochemistry*, **38,** 1151–1155.

Mayr, U., Michalek, S., Treutter, D. and Feucht, W. (1997). Phenolic compounds of apple and their relationship to scab resistance. *Journal of Phytopathology*, **145,** 69–75.

MICHALEK, S., KLEBEL, C., TREUTTER, D. and FEUCHT, W. (1999a). Stimulation of flavonol biosynthesis in apple (*Malus domestica*) by abiotic elicitors. *Acta Horticulturae*, **484,** 535–539.

MICHALEK, S., MAYER, U., TREUTTER, D., LUX-ENDRICH, A., GUTMANN, M., FEUCHT, W. and GEIBEL, M. (1999b). Role of flavan-3-ols in resistance of apple trees to *Venturia inaequalis*. *Acta Horticulturae*, **484,** 535–539.

MIKULIC PETKOVSEK, M., STAMPAR, F. and VEBERIC, R. (2007). Parameters of inner quality of the apple scab resistant and susceptible apple cultivars (*Malus domestica* Borkh.). *Scientia Horticulturae*, **144,** 37–44.

MIKULIC PETKOVSEK, M., STAMPAR, F. and VEBERIC, R. (2008). Increased phenolic content in apple leaves infected with the apple scab pathogen. *Journal of Plant Pathology*, **90,** 49–55.

MIKULIC PETKOVSEK, M., STAMPAR, F. and VEBERIC, R. (2009). Accumulation of phenolic compounds in apple in response to infection by the scab pathogen, *Venturia inaequalis*. *Physiological and Molecular Plant Pathology*, **74,** 60–67.

PICINELLI, A., DAPENA, E. and MANGAS, J. J. (1995). Polyphenolic pattern in apple tree leaves in relation to scab resistance. A preliminary study. *Journal of Agricultural and Food Chemistry*, **43,** 2273–2278.

PONTAIS, I., TREUTTER, D., PAULIN, J. P. and BRISSET, M. N. (2008). *Erwinia amylovora* modifies phenolic profiles of susceptible and resistant apple through its type II secretion system. *Plant Physiology*, **132,** 262–271.

SANDERMANN, H. and STROMINGER, L. (1972). Purification and properties of C_{55}-isoprenoid alcohol phosphokinase from *Staphylococcus aureus*. *Journal of Biological Chemistry*, **247,** 5123–5131.

SAUNDERS, J. A. and OLECHNO, J. D. (1988). Radioisotope detectors for investigations on phenolic biosynthesis. In: *HPLC: Flow-Through Radioactivity Detection in HPLC Progress*. Volume 3. (Parvez, H., Reich, A., Lucas-Reich, S. and Parves, S., Eds.). VSP Press, Utrecht, The Netherlands. 167–189.

SAUNDERS, J. A. and O'NEILL, N. R. (2004). The characterization of defence responses to fungal infection in alfalfa. *BioControl*, **49,** 715–728.

TREUTTER, D. (1989). Chemical reaction detection of catechins and proanthocyanidins with 4-dimethylaminocinnamaldehyde. *Journal of Chromatography*, **467,** 185–193.

TREUTTER, D. (2001). Biosynthesis of phenolic compounds and its regulation in apple. *Plant Growth Regulation*, **34,** 71–89.

TREUTTER, D. (2005). Significance of flavanoids in plant resistance and enhancement of their biosynthesis. *Plant Biology*, **7,** 581–591.

TREUTTER, D. and FEUCHT, W. (1990). Accumulation of flavan-3-ols in fungus-infected leaves of Rosaceae. *Zeitschrift für Pflanzenkrankheiten Pflanzenpathologie und Pflanzenschutz*, **97,** 634–641.

WILLIAMS, E. B. and KUĆ, J. (1969). Resistance in *Malus* to *Venturia inaequalis*. *Annual Review of Phytopathology*, **7,** 223–246.