Defensive reactions of apple cultivars Angold and HL 1834 after fungal infection

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Abstract

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Apple cultivars (*Malus domestica* Borkh.) were inoculated with a significant apple fruit fungal pathogen, *Monilinia fructigena* Honey ex Whetzel. Defensive reactions, especially enzyme activity and production of phenolic compounds, were compared. Changes of phenolic content and activity of phenylalanine-ammonia lyase (PAL) were determined 7, 14 and 21 days after the inoculation. Progress of rotting was similar for both cultivars at first but defensive reactions were different. The increase of phenolic compounds was observed; their concentration and composition were influenced by location in the fruit. A very good correlation was found between the activity of phenylalanine-ammonia lyase and total phenolic content.

Keywords: apples; *Monilinia fructigena*; phenolic compounds; phenylalanine-ammonia lyase

Postharvest losses in apples vary each year according to weather, fruit condition, and time in storage. Weather-related problems such as hail increase decay by allowing decay-causing fungi easy entry to the fruit. Rain at harvest allows for increased contamination and infection of the fruit by certain fungi. The length of storage influences decay by extending the time fruit may be infected while at the same time the fruit loses its natural resistance to infection. Generally, high quality fruit is stored in controlled atmosphere storage and is less prone to decay due to slower fungi growth in the low oxygen atmosphere. On the other hand, the poorer quality fruit used for processing is stored in air where considerably more fruit is lost to decay by fungi. Losses of up to 10% are possible in storage, and knowledge of the fungi that cause decay and their control is important (Meheriuk, McPhee

1984). *Sclerotinia fructigena*, *Penicillium* spp., *Gloeosporium* spp., *Alternaria* spp., *Fusarium* spp., *Botrytis cinerea*, *Trichothecium roseum* etc., are the main organisms causing postharvest decay.

The conidial stage of *Sclerotinia fructigena* (Pers.:Fr.) J. Schröt. is *Monilinia fructigena* Honey ex Whetzel. This species is a wound pathogen and is capable of attacking apple and pear fruits before and after harvest and may attack woody tissue to form a spur canker (Dennis 1983). Dispersal of *M. fructigena* conidia can occur by wind, water, insects, birds and man (Holb 2008). It causes lesions that are firm, irregular in shape, spread rapidly, and in the orchard produce masses of brown oval conidial from which the term "brown rot" is derived (Dennis 1983).

It is well-known that plants protect themselves from fungal attack; one part of the defensive mech-
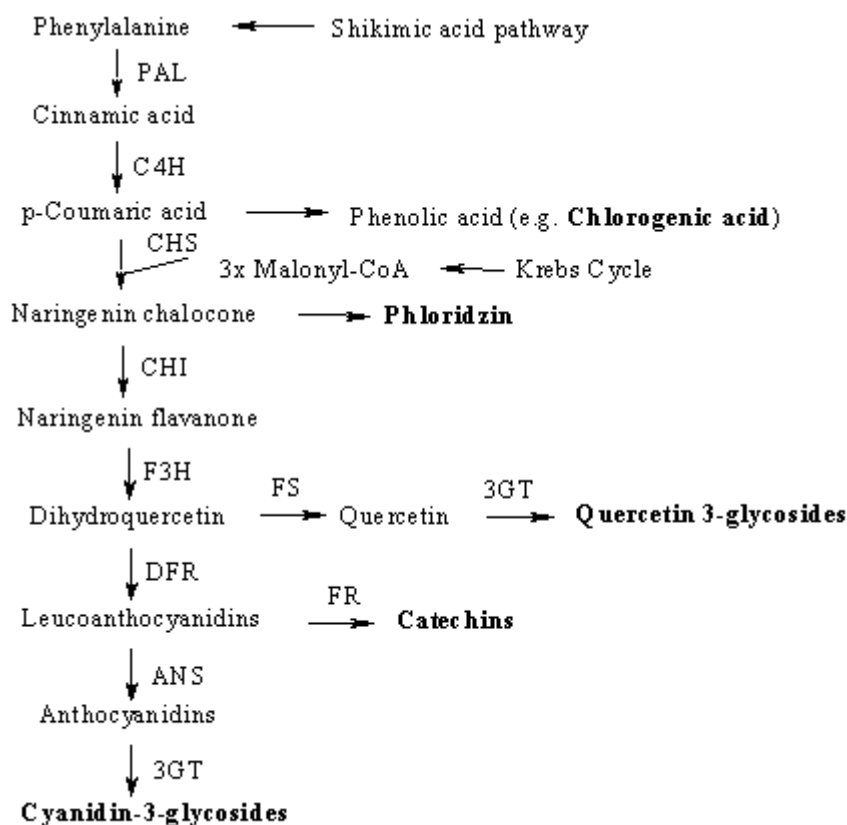
Fig. 1. Putative biosynthesis route of chlorogenic acid, phloridzin, quercetin, glycosides, catechins and cyanidin glycosides in apple skin

PAL (phenylalanine ammonia-lyase); C4H – cinnamate hydroxylase; 4CL – 4-coumarate CoA ligase; CHS – chalcone synthase; CHI – chalcone flavanone isomerase; F3H – flavanone-3-hydroxylase; FS – flavonol synthase; DFR – dihydroflavonol-4-reductase; FR – flavan-3, 4-cis-diol-4-reductase; ANS – anthocyanidin synthase; 3GT – cyanidin/flavonol 3-*O*-glucosyl transferase (Awad et al. 2001)

anism is production of phenolic compounds. They respond to phytopathogenic infections and promote healing processes in forming wound barriers such as lignin (Bennett, Wallsgrove 1994; Oh et al. 1999; Nagy et al. 2004) and suberin (Ikediobi et al. 1989; Klaiber et al. 2003). Phenylalanine ammonia-lyase (PAL) is considered to be the principal enzyme of the phenylpropanoid pathway, catalysing the transformation by deamination of L-phenylalanine into *trans*-cinnamic acid, which is the prime intermediary in the biosynthesis of phenolics (Rivero et al. 2001; Fig. 1). PAL activity is induced not only by wounding and/or exposure to ethylene but also by other stresses such as temperature and fungal infection (Pereyra et al. 2005).

In the frame of investigation of natural apple resistance to postharvest decay, the defensive reactions of long-term stored fruit were studied. The behaviour of two different cultivars inoculated with *Monilinia fructigena* were compared: Angold was chosen as a well-known resistant cultivar and HL 1834 as a new cultivar.

MATERIAL AND METHODS

Plant material

Apple cultivars Angold and HL 1834 (Fantasia × (Lord Lambourne × Spartan)), harvested on the single date in October at the stage of harvest maturity and stored at 2–3°C in a commercial cold storage, were used for experiments. The fruit was provided by the Research and Breeding Institute of Pomology at Holovousy, Czech Republic. The apples were inoculated with the pathogen *Monilinia fructigena* in April (after 6 months of storage in commercial cold storage).

The fungi and inoculation

Monilinia fructigena Honey ex Whetzel was isolated from decayed apples and maintained on GTK agar (plate-count agar) at 8°C. The organism was identified by the Department of Botany, Charles University in Prague, Czech Republic. The apples were inoculated with 2.9×10^3 spores of the pathogen into two holes made by a sterile syringe needle on one side of each fruit. The diameter of each hole was 4 mm wide and 3 mm in depth. Three apple fruits were inoculated in parallel. After the inoculation, the fruits were stored at 25°C for 4 days and then in an experimental cool chamber at 4°C. The apples were analysed on days 7, 14 and 21 after the inoculation. A fresh fruit (day 0) was used as a control for changes in evaluations after the inoculation. All monitored parameters are changing over the long term storage, but the apples were stocked out at the same time and immediately inoculated, therefore the significant differences of phenolic composition over 21 days were not expected and the control was evaluated only at the beginning of the study.

Analysis of phenolic compounds

Extraction and quantification of phenols were performed as described by Escarpa and González (2001) with some modifications. Apple peel (1.5 g), pulp (2.5 g) and whole apples (2.5 g, mixture of pulp and peel) from 3 fruits were extracted by 80% methanol with BHT (butylated hydroxytoluene) in an ultrasonic bath for 2 h. The extracts were stored at –20°C until analysis.

The spectrophotometric method with Folin-Ciocalteu phenol reagent was used for determination of total phenolic content. The extract was mixed with 0.5 ml of the reagent and 10 ml of 1M sodium carbonate and the absorbance at 750 nm was measured after 1 h. Gallic acid was used for calibration. Tree replicates of the same extract were performed for each determination.

Liquid chromatography was used for a single phenolic compound determination. HPLC analyses were performed with a Gynkotek system (Dionex GmbH, Idstein, Germany), equipped with UV detector. A Zorbax C8 column (150 × 4 mm, 5 µm) was used. The elution solvents were 0.01M phosphoric acid (A) and 0.01M phosphoric acid in acetonitrile (B) with elution profile 5–20% B for 0–15 min, 20–40% B for 15–20 min, 40–80% B for 20–22 min, 80% B

for 22–28 min, flow rate 1 ml/min. Three replicates were performed for each determination.

Activity of phenylalanine-ammonia lyase

PAL activity was assayed as described by Morelló et al. (2005) with some modifications. Twenty-five grams of sample (3 whole apples – mixture of pulp and peel) with 50 ml of 0.1M phosphate buffer (pH 6.5) and 1.875 g of polyvinylpyrrolidone were homogenized. An aliquot of 0.4 ml of supernatant was mixed with 4.1 ml of sodium borate buffer (pH 8.5) and with 1ml of L-phenylalanine (10 mg/ml). The absorbance was measured after incubation at 35°C for 1 h. The enzyme activity was expressed in µmol of cinnamic acid liberated per gram of fresh weight of sample per hour. Three replicates of the same extract were performed for each determination.

Statistical analysis

The software STATISTICA 9.0 (StatSoft CR, Ltd., Prague, Czech Republic) was used for the data analyses. The increase or decrease of the monitored parameters were tested using linear regression and one-way ANOVA ($P < 0.05$), Tukey's HSD test at the significance level of 0.05 and Spearman's correlation coefficients were used for testing differences between parameters. Dunnett's test was used for comparison of the control and inoculated samples.

RESULTS AND DISCUSSION

The only known characteristic from the literature for both cultivars is skin thickness, 69.6 µm for cv. Angold and 59.6 µm for cv. HL 1834. Assuming that the skin thickness influences penetration of fungal pathogens into fruits (Homutová, Blažek 2006; Blažek et al. 2007), this parameter is considered to be very important. The apple cultivar Angold is

Table 1. Content (mg/kg) of total phenolic compounds in fresh apples

	Angold	HL 1834
Pulp	1,608 ± 15	1,273 ± 21
Peel	5,483 ± 36	3,582 ± 28

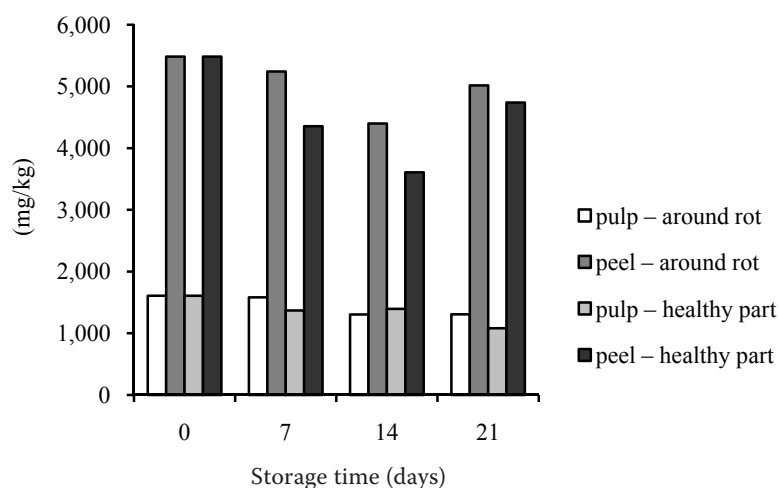All values are expressed as mean ± standard deviation

Fig. 2. Content of total phenolic compounds (mg/kg) in apple cultivar Angold inoculated with *Monilinia fructigena*
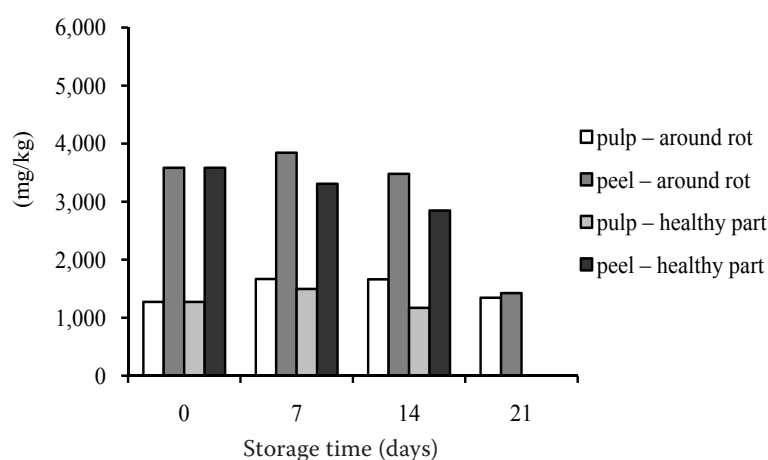

Fig. 3. Content of total phenolic compounds (mg/kg) in apple cultivar HL 1834 inoculated with *Monilinia fructigena*

considered to be resistant against fungal infection (BLAŽEK et al. 2007), and the thickness of the skin may be one of its causes.

In our previous unpublished study, the amount of phenolic compounds in several apple cultivars was measured and cv. Angold was found to contain the highest concentration of phenolics; contrariwise, HL 1834 belonged to the cultivars with the lowest content. Significant differences were also found in the polyphenoloxidase (PPO) and catalase activity.

Contrariwise, visual comparison after the inoculation showed a very similar progression of decay for both cultivars at first. The diameter of the decayed zone after 7 days from inoculation was 3.5–4.5 cm. Finally, the progress of decay was more intensive in HL 1834 and the healthy tissue was not possible to analyse after 21 days there.

Total phenolic content

The content of total phenols in fresh pulp and peel is presented in Table 1. The most significant differences ($P < 0.05$) between cultivars were found in the content of phenolics both in the peel and pulp (Table 1), whereas the amount in the pulp was low in comparison with the peel for both cultivars. The results are similar to the findings of ESCARPA and GONZÁLEZ (1998) and TSAO et al. (2003).

Diverse biochemical defensive reactions were observed after the inoculation. The content of total phenolics in cultivar Angold decreased in comparison to uninoculated control, both in the tissues surrounding decay ($P < 0.05$) and in the healthy part ($P < 0.05$) (Fig. 2). In contrast to Angold, the synthesis or release of new phenolics was initiated in HL 1834 (Fig. 3). The increase of phenolics ($P < 0.05$) in comparison with control was observed in the pulp and peel around the decay and in the healthy part of pulp 7 days after the inoculation. The surprising difference between cultivars in the defensive reaction could probably be ascribed to a high initial concentration of phenolics in Angold, which was probably the reason why the enzyme phenylalanine-ammonia lyase and phenylpropanoid metabolism were not activated, in spite of the decay. In
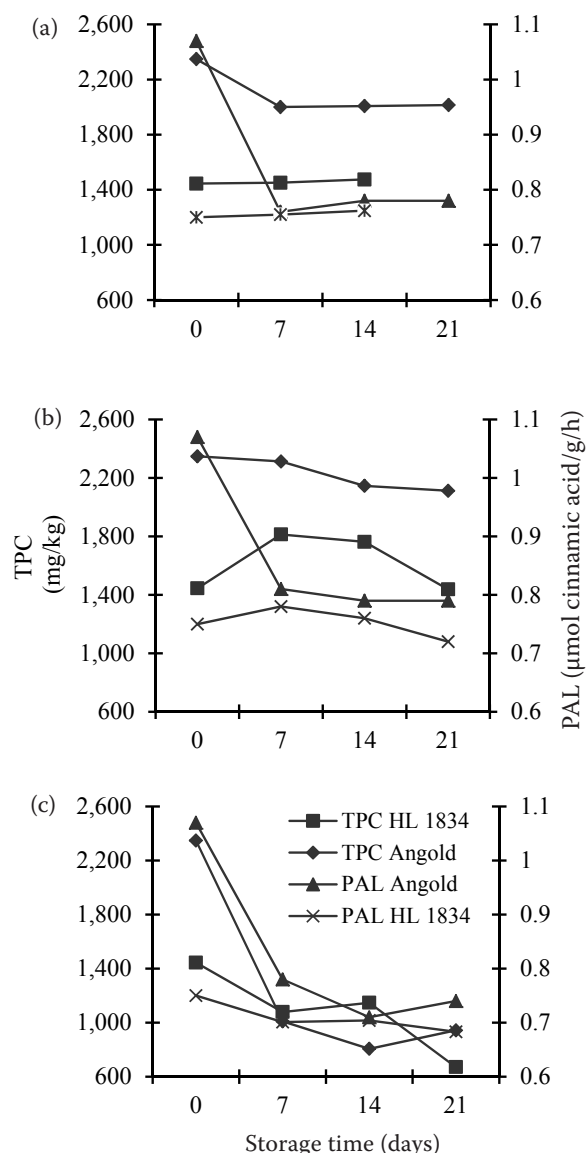
Fig. 4. Activity of PAL (μmol cinnamic acid/g/h) and total phenol content (TPC) in the healthy part (a), in the surrounding part (b) and in the decayed zone (c) in apple cultivars HL 1834 and Angold

almost all cases, the level of phenolics was higher in the part surrounding decay than in the healthy part. Lattanzio et al. (2001) reported similar results.

No healthy pulp of HL 1834 could be analysed after 21 days, because the tissues under peel were decayed. The total phenolic content was also determined in the decayed zone; the concentration decreased in this part gradually after inoculation.

Activity of phenylalanine-ammonia lyase (PAL)

Whole apples (the mixture of peel and pulp) were used for the PAL analyses. The enzyme activity was significantly different ($P < 0.05$) for both cultivars. In fresh fruits of Angold, the PAL activity was 1.07 μmol cinnamic acid/g/h and in HL 1834 the corresponding value was 0.75 μmol cinnamic acid/g/h. The enzyme activity was associated with total phenolic content as well as with concentration of selected single phenolic compounds. Fig. 4 shows the relationship between activity of PAL and total phenolic content. Simultaneously with a decrease of total phenolic content, the decrease of PAL activity ($P < 0.05$) in apple cultivar Angold was noted (Fig. 4). In cultivar HL 1834 the enzyme activity increased at first ($P < 0.05$), the highest activity being in the part around decay (Fig. 4b), but after 7 days the activity slowly decreased. In the healthy part, the activity of PAL slightly increased all the time (Fig. 4a), presumably under the influence of the decay progress and induction of defensive reactions. In the decayed tissues, both total phenolic content and PAL activity decreased ($P < 0.05$) all the time (Fig. 4c).

The results indicate a very good association between activity of PAL and total phenolic content. In all parts of inoculated fruit, the changes of enzyme activity were associated with changes of total phenolic content ($r = 0.71–0.99$).

Concentration of single phenolic compounds

Chlorogenic acid, catechin, epicatechin, caffeic acid, and phloridzin are important phenolic

Table 2. Content (mg/kg) of monitored phenolic compounds in fresh samples

		Catechin	Chlorogenic acid	Epicatechin	Caffeic acid	Phloridzin
Angold	pulp	15.8 ± 0.6	582.3 ± 2.7	54.7 ± 2.4	1.9 ± 0.1	9.2 ± 0.1
	peel	77.4 ± 7.1	835.9 ± 66.4	253.7 ± 8.4	9.6 ± 0.8	99.1 ± 8.4
HL 1834	pulp	16.7 ± 1.1	104.4 ± 7.2	111.4 ± 2.4	1.2 ± 0.1	12.4 ± 0.7
	peel	258.2 ± 0.7	98.4 ± 2.9	322.0 ± 0.1	2.5 ± 0.0	94.2 ± 0.5

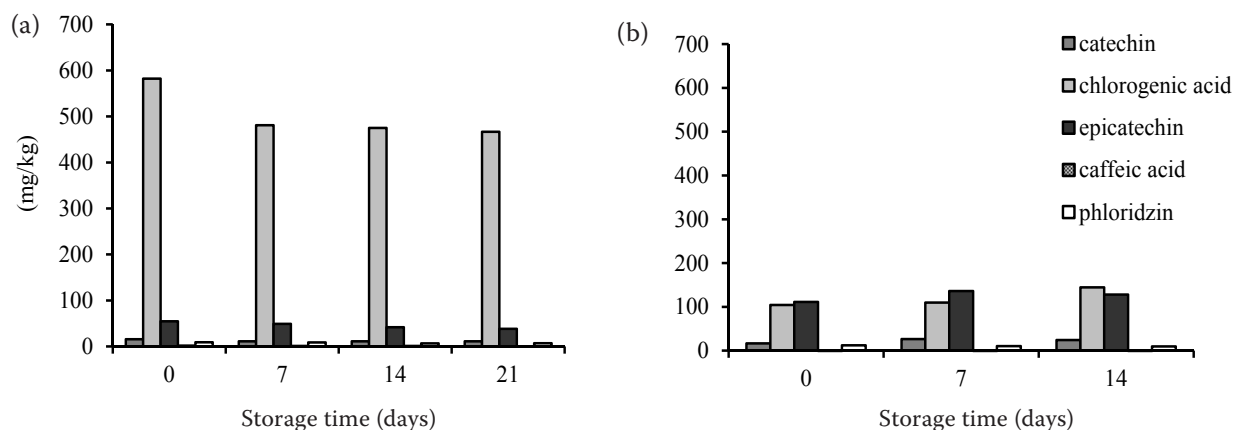All values are expressed as mean ± standard deviation

Fig. 5. The concentration of phenolic compounds after the inoculation in the healthy pulp in cv. Angold (a) and HL 1834 (b)
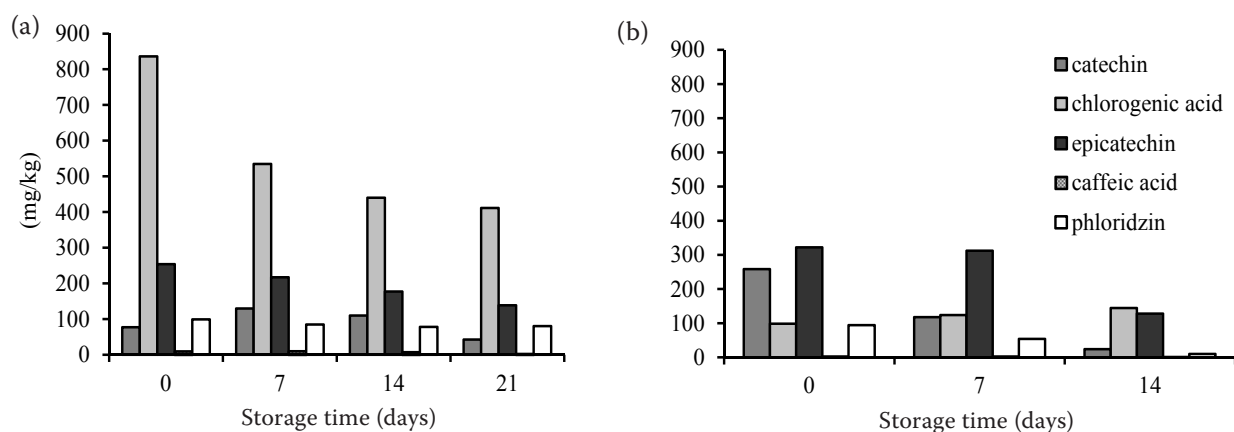

Fig. 6. The concentration of phenolic compounds after the inoculation in the healthy peel in cv. Angold (a) and HL 1834 (b)
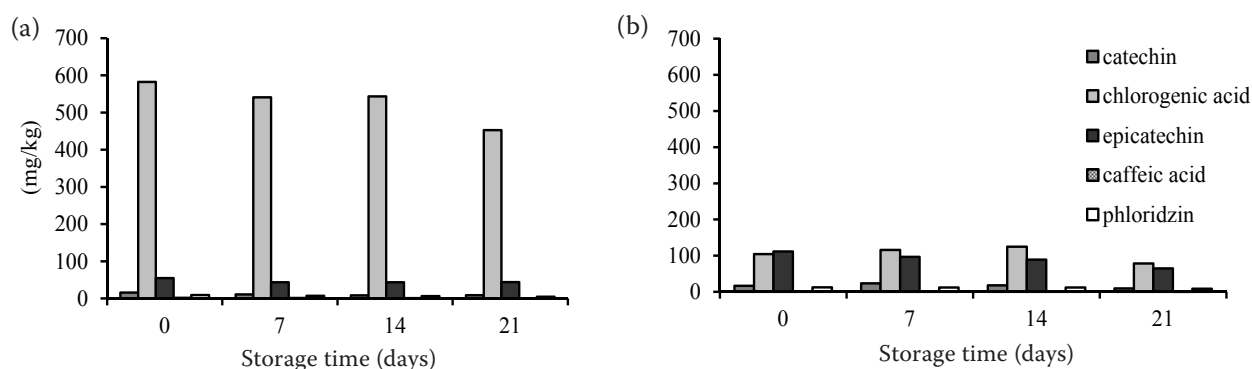

Fig. 7. The concentration of phenolic compounds after the inoculation in the pulp around the decay in cv. Angold (a) and HL 1834 (b)

compounds contained in apples. Many of these compounds were tested for antifungal properties and their roles in defensive mechanism of apples were confirmed (Dennis 1983; Lattanzio et al. 2001; Mikulič Petkovšek et al. 2003). Table 2 shows the amount of phenolic compounds in the fresh uninoculated peel and pulp. Among the determined phenolics, chlorogenic acid was the predominant compound in Angold, whereas epicatechin, chlorogenic acid, and catechin are typical for
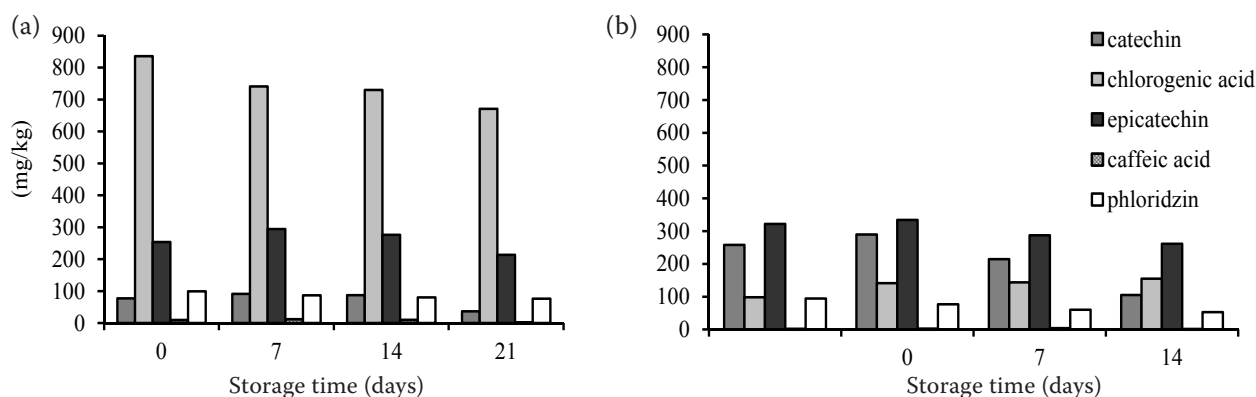
(a)

(b)

Fig. 8. The concentration of phenolic compounds after the inoculation in the peel around the decay in cv. Angold (a) and HL 1834 (b)

HL 1834. With the exception of chlorogenic acid in HL 1834, the concentration of individual phenolic compounds was higher in the peel than in the pulp ($P < 0.05$). With respect to the different phenol profile for each cultivar, the activation of different enzymes of the phenylpropanoid metabolism can be expected.

Increased concentration of different phenolic compounds was observed after the inoculation (Figs 5–8). The apple of cv. Angold, which contained a high level of phenolics, showed only a small production of compounds such as caffeic acid, catechin and epicatechin 7 days after the inoculation (Figs 5–8). If other substances were synthesized, they were probably immediately changed to other derivatives. In cultivar HL 1834 (Figs 5–8), major compounds such as chlorogenic acid, catechin, and epicatechin were predominantly synthesized.

The differences between cultivars could be due to various activities of enzymes in the phenylpropanoid pathway. L-phenylalanine-ammonia lyase catalyses are only the first step in the biosynthesis of plant phenylpropanoid compounds (Morelló et al. 2005; Jones 1984). Other enzymes involved in phenylpropanoid metabolism are shown in Fig. 1.

When comparing the results, a close correlation ($r = 0.98–0.99$) between PAL and chlorogenic acid in the healthy part of both cultivars was found (Fig. 9). In the tissues surrounding decay, the relationships between PAL and catechin ($r = 0.92$), epicatechin ($r = 0.80$), caffeic acid ($r = 0.77$) were found for cv. HL 1834 only. We can conclude that the activity of PAL is not an indicator of production or nonproduction of specific phenolic compounds. Phenylpropanoid metabolism is a very complex process and it is probable that many chemical reactions take place simultaneously and many compounds are produced and immediately converted to other products. Phenols are also present in conjugated forms, usually with glycosidic attachment. They may be released in the free form during the fungal infection or insect feeding or oviposition through enzymatic or other hydrolysis (Mikulič Petkovšek et al. 2003). Therefore it is very difficult to find a direct relationship between PAL activity and the concentration of individual phenols.
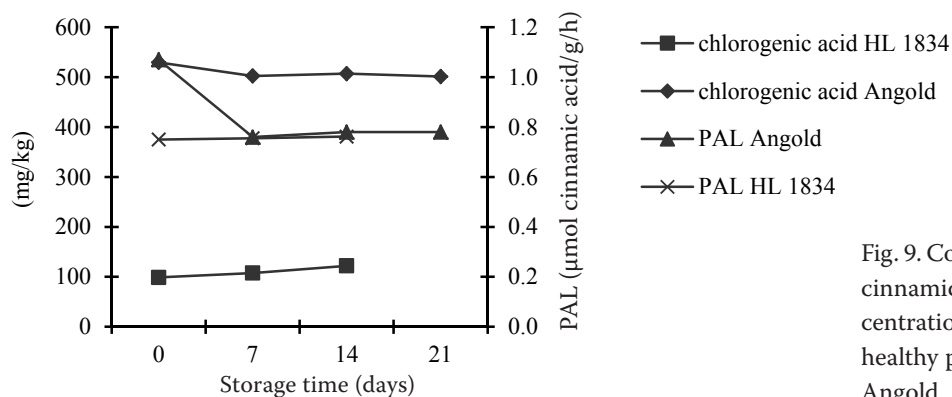

Fig. 9. Correlation between PAL (µmol cinnamic acid/g/h) activity and concentration of chlorogenic acid in the healthy part in cultivars HL 1834 and Angold

CONCLUSION

The chemical composition, especially the content of phenolic compounds, and activity of enzyme involved in the phenylpropanoid metabolism influence the defensive reactions of the infected fruit.

The experiments were carried out with apples in a consumer stage of maturity (not with overmature fruit) with the aim to examine the defensive reactions of long-stored fruit. Defensive reactions of immature fruits can be different. Results of our preliminary study with apple infection showed that some cultivars (e.g. Angold) after harvest are resistant to the pathogen attack but after a 4-month storage the fruit is not capable to limit the infection. It is generally assumed that an increasing number of *Monilinia*-infected fruit is due to ripening of the fruits and, perhaps, due to the increasing sugar content of the fruits (Holb 2004).

This study is a good example of variation in the defensive reactions of different cultivars after inoculation by *Monilinia fructigena.* On the basis of our results, we suggest that the apple cultivar HL 1834 is less suitable for organic production with limited antifungal treatments. Nevertheless, in spite of the fact that this cultivar contains lower levels of phenolic compounds in comparison with Angold, its phenolic profile is different and its PAL activity is lower, its defensive system is operating. The defensive system was activated and the diameter of lesions was similar to resistant cv. Angold the first week after the inoculation. However, the defensive reaction was insufficient to limit the further progress of pathogen. It is necessary to note that apples were purposely wounded and inoculated. It is probable that without injury of the fruit the rot would not establish or expand so easily (Blažek et al. 2007). Also, *Monilinia fructigena* is a very aggressive pathogen in comparison with e.g. *Penicillium expansum* and *Gloeosporium* spp. as showed our unpublished results.

Differences in the synthesis of selected single phenolics in the pulp and peel can be explained by the location of responsible enzymes, as described by Jones (1984). Compartmentation of the relevant enzymes at the cellular and subcellular level is an important factor in determining which branches of a pathway predominate and what metabolic end product is synthesized.

The correlation between PAL and total phenolic content was confirmed, but explicit dependence between PAL and particular phenolic compounds for both cultivars was not found, with the exception of chlorogenic acid. The controversial results of correlation between PAL activity and phenolic compounds are published in the literature, but the relationship between PAL and chlorogenic acid was also confirmed in potato and transgenic tobacco (Cantos et al. 2002; Klaiber et al. 2003). We can conclude that phenylalanine-ammonia lyase, its activity or *de novo* synthesis, is influenced by the level of phenolic compounds, as illustrated by the response of Angold to the inoculation. Probably due to the high level of phenolic compounds in this cultivar, the PAL was not activated after the pathogen attack and the main defensive role was overtaken by the polyphenoloxidase (PPO), oxidating present phenolics to antifungal quinones. The similar reaction we found in wounded uninoculated apples, where the lower activity of PAL was accompanied by higher activity of PPO involved in the lignification process.

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