Changes in phenols composition and activity of phenylalanine-ammonia lyase in apples after fungal infections

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Abstract

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The defensive reaction of apple cultivar Idared (*Malus domestica* Borkh.) was studied after inoculation with three different pathogens (*Penicillium expansum*, *Monilinia fructigena*, and *Gloeosporium* spp.). Changes in phenolic content and activity of phenylalanine-ammonia lyase were determined after 7, 14, and 21 days after the inoculation. The significant differences were discovered in the progress of rotting after the inoculation. The increase in phenols concentration and in phenylalanine-ammonia lyase activity varied in the place of fungal attack, in the tissues around rotten zone and in the healthy part. The response to the infection was different in the fruit peel and flesh. Very good correlation was found between the activity of phenylalanine-ammonia lyase and total phenol content (r = 0.76–0.98).

Keywords: apples; postharvest rot; fungal pathogens; phenolic compounds; phenylalanine-ammonia lyase

Postharvest diseases of fruit represent a very important source of wastage and mainly economic losses. Roughly 70% of all the major crop diseases are caused by fungi (Deacon 2006). Fungal diseases play a major role in the wastage of long-stored apples and many fungicides are used in production and storage for the apple rot control. However, an increased consumer concern for healthy products prefers biological production with limited fungicides application and natural resistance is favoured. Therefore the investigation of natural resistance of many apple varieties (Dennis 1983; Lattanzio et al. 2001; Mikulič Petkovšek et al. 2003; Torres et al. 2003; Veberic et al. 2005; Lattanzio et al. 2006; Mikulič Petkovšek et al. 2008; Mikulič Petkovšek et al. 2009) was conducted.

There are many devastating pathogens inducing fungal diseases, in particular *Penicillium expansum*, *Monilinia fructigena*, and *Gloeosporium* spp. The spores of pathogens arrive at the surface of the fruit during the growing period when the natural resistance is high. The decay is initiated after a mechanical damage or decrease of apple resistance due to ripening (Dennis 1983; Spotts 1985; Prusky 1996; Torres et al. 2003; Adámková et al. 2006; Blažek et al. 2006).

Spores of *Penicillium* species are present in the soil, on the fruit surface, in the air of the store etc. Its preferred hosts are pomiferous fruits. This species is responsible for blue mould rot, a major postharvest disease of apples worldwide (Baert et al. 2007). Pathogen penetrates into the fruit due to

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mechanical damages, cuttings, and punctures and is able to produce toxic metabolite as patulin. Up to 61% of rotten apples contain patulin; the highest concentration can be found in the brown rotten parts of apples (Jesenská 1993).

Monilinia fructigena is spread in apple orchards through wounds caused by insects or mechanically. Fallen fruit left on the ground is the source of infection for the next season. This pathogen attacks apple before and after harvest and causes brown mould.

Gloeosporium spp. (*Phlyctaena vagabunda*, *Pezicula alba*, *Colletotrichum gleosporiodes*) is a very important pathogen responsible for apples rotting in stores. Conidia are produced by small infections on the wood of the tree throughout the year and are spread by rain and dew on the fruit, which is thus exposed to infection during the whole of the growing season (Lattanzio et al. 2001).

A plant can respond to fungal infection by synthesis of antifungal agent at the site of attack (Mikulič Petkovšek et al. 2003, 2008, 2009). Phenolic compounds are considered to be important antimicrobial substances. It is well-known that apple fruit contain, besides quercetin glycosides, catechins and chlorogenic acid, phloridzin (dihydrochalcon-O-glucoside), whose oxidation products may be involved in the defense mechanism of apple leaves against the scab fungus *Venturia inaequalis* (Lattanzio et al. 2001; Mikulič Petkovšek et al. 2008, 2009). Many other phenols were tested *in vitro* against pathogenic fungi.

Phenolic compounds have their origin in the general phenylpropanoid metabolism, which consists of three early steps in conversion of L-phenylalanine to various hydroxycinnamic acids. The enzymes catalysing the individual step in this sequence are respectively: phenylalanine-ammonia lyase (PAL, EC 4.3.1.5), cinnamate-4-hydroxylase (EC 1.14.13.11), and 4-coumarate: CoA ligase (EC 6.2.1.12). L-phenylalanine-ammonia lyase is considered a key enzyme of phenolic biosynthesis since it catalyses the reductive deamination of L-phenylalanine resulting in trans-cinnamic acid, the first step in the biosynthesis of plant phenylpropanoid compounds such as lignin, flavonoids, and hydroxycinnamic acid. L-phenylalanine-ammonia lyase activity varies during the plant development and cell differentiation. Stress conditions as irradiation, wounding, nutrient deficiencies, herbicide treatment and viral, fungal and insect attacks, increase PAL synthesis or PAL activity in various plants (Camm, Towers 1973; Jones 1984; Morelló et al. 2005; Slatnar et al. 2010). An increase of PAL activity could be also induced by exposure to ethylene. Lattanzio et al. (2001) reported the coincidence of ethylene production and increase in PAL activity. It was suggested that ethylene produced by the host specifically at ripening may act as a signal to terminate pathogen quiescence on the fruit surface.

Although many studies of the natural apple resistance were carried out, majority of them were aimed to the apple resistance to pathogen *Venturia inaequalis*. Our study is focused on natural resistance to the most typical storage disease pathogens (*Penicillium expansum*, *Monilinia fructigena*, and *Gloeosporium* spp.). The most typical apple cultivars grown in the Czech Republic are cv. Golden Delicious and cv. Idared, and Idared was chosen for our experiments. The objective of the study was to observe the defensive reaction of cv. Idared after inoculation with these pathogens. In particular the phenolics changes and activity of phenylalanine-ammonia lyase were monitored after inoculation in the healthy part, the surrounding and the decayed part of the apple tissue to find some differences among pathogen attacks and to find the relationship between PAL activity and phenol synthesis.

MATERIAL AND METHODS

Plant material

Apple cultivar Idared (*Malus domestica* Borkh.), harvested at the beginning of October 2004 (at the optimal stage of maturity) and stored at 2–3°C in a commercial cold store without controlled atmosphere was used for the experiments. The trees were grafted on M 9 rootstock and grown in the Holovousy experimental orchards (Eastern Bohemia, Czech Republic). The location is characterised by an average annual temperature of 8.1°C, average rainfall about 650 mm, and altitude about 300 m a.s.l. (Homutová, Blažek 2006). The weather conditions were favourable, only because of local hailstorms the damage and fungal attack of fruit (mainly apple scab) occurred. Fruit was provided by the Research and Breeding Institute of Pomology at Holovousy, Czech Republic. The apples were inoculated after 6-months storage (in April) with three different pathogens and subsequently stored for 4 days at 25°C and then in cooling chamber in normal atmosphere at 4°C. Apples were analysed 7, 14, and 21 days after inoculation.

The fungi

Penicillium expansum and *Monilinia fructigena* were isolated from apples and maintained on Chloramphenicol Yeast Glucose Agar at 8°C. *Gloeosporium* spp. was provided by the Research and Breeding Institute of Pomology at Holovousy, Czech Republic and stored under the same conditions. The apples were inoculated with 2.9×10^3 spores of pathogen into each of two holes on one side of apple. The holes into the apples were made by the sterile syringe needle. The diameter of each hole was 4 mm wide and 3 mm deep. Three apple fruits were inoculated every time.

Analysis of phenol compounds

Extraction, spectrophotometric determination, and HPLC method 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) were extracted in several steps by 80% methanol (total volume 25 ml) with BHT (butylated hydroxytoluene) in ultrasonic bath (in dark) for 2 h. The extracts were stored at –20°C until analysis.

The spectrophotometric method with the Folin-Ciocalteu agent was used for determination of total phenol content. The extract was mixed with 0.5 ml of Folin-Ciocalteu agent and 10 ml of 1M sodium carbonate and the absorbance at 750 nm was measured after 1 h. The gallic acid (GAE – gallic acid equivalents) was used for calibration. Three replicates were performed for each determination.

Phenolic compounds were analysed by HPLC: Gynkotek unit (Dionex, Sunnyvale, USA) equipped with UV detector, Zorbax C8 column 150 × 4 mm, 5 µm particle size, elution solvents 0.01M phosphoric acid (A), and 0.01M phosphoric acid in acetonitril (B). The elution profile was 5–20% B for 0–15 min, 20–40% B for 15–20 min, and 40–80% B for 20–22 min, 80% B for 22–28 min. The flow rate was always 1 ml/min. Three replicates were performed for each determination.

Activity of phenylalanine-ammonia lyase

PAL activity was determined as described by Morelló et al. (2005) with some modifications. Twenty-five g of whole apples with 50 ml of 0.1M phosphate buffer (pH 6.5) and 1.875 g of polyvinylpyrolidon were homogenised, 0.4 ml of supernatant was mixed with 4.1 ml of sodium borate buffer (pH 8.5) and 1ml of L-phenylalanine (10 mg/ml). The absorbance at 270 nm was measured after incubation at 35°C for 1 h. Enzymatic activity was expressed in µmol cinnamic acid liberated per g of fresh weight of sample per h. Three replicates were performed for each determination.

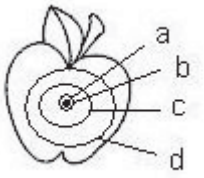

Fig. 1. The decayed area of cv. Idared after 7 days after inoculation: (a) needle penetration with *Gloeosporium* spp. (b), *Penicillium expansum* (c), and *Monilinia fructigena* (d)

Statistical analysis

The software STATISTICA 9.0 (StatSoft, Czech Republic) was used for data analyses. One-way ANOVA was used ($P < 0.05$) and the differences between pathogens were tested using the Tukey HSD test at the significant level 0.05.

RESULTS AND DISCUSSION

Idared is a late winter cultivar with long-time of ripening. This cultivar is very resistant to mechanical injury but susceptible to apple scab, powdery mildew, fire blight, and slightly subject to mouldy core.

Apples were inoculated into two holes on one side of fruit. The rotten part, part surrounding rotten zone, and the healthy tissue were analysed. The important differences among ranges of rotting were found after 7 days from inoculation (Fig. 1).

Very intensive progress of decay was initiated after inoculation with *Monilinia fructigena*. The rot development was so fast that no healthy tissue remained after 14 days after inoculation. By contrast to this rapid effect, the development of rot in fruit infected by *Gloeosporium* spp. was initiated later on, after 14 days only. The difference of decay intensity affected defensive reactions of inoculated fruits.

Total phenol content (TP)

Phenolic compounds are important antifungal substances. Apples contain a high amount of differ-

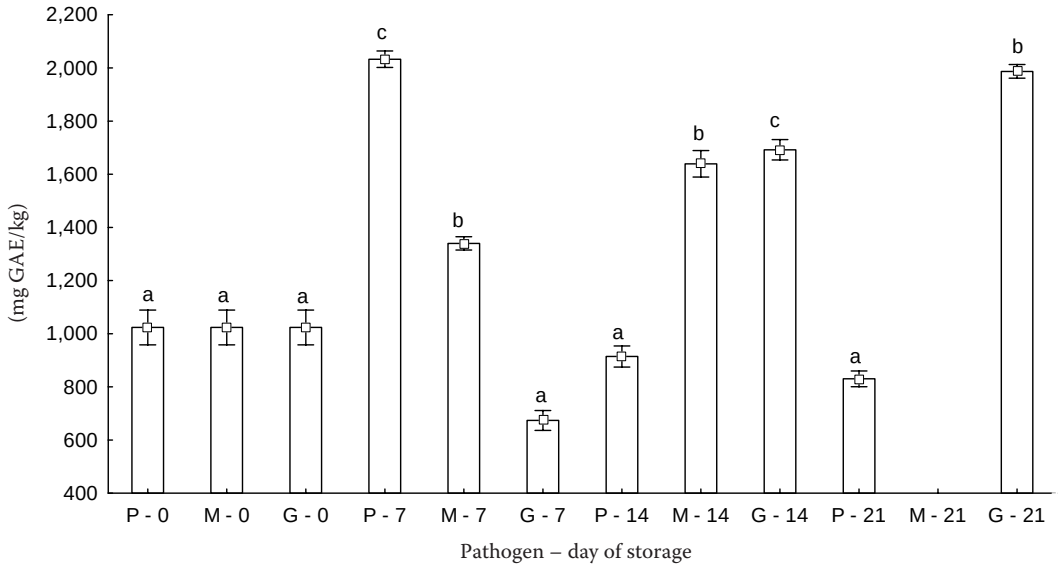

Fig. 2. Content of total phenolic compounds (mg GAE/kg) in the pulp around decay inoculated with *Penicillium expansum* (P), *Monilinia fructigena* (M), and *Gloeosporium* spp. (G). Different letters mean statistical differences (Tukey HSD test, $P < 0.05$) among pathogens in the same storage day

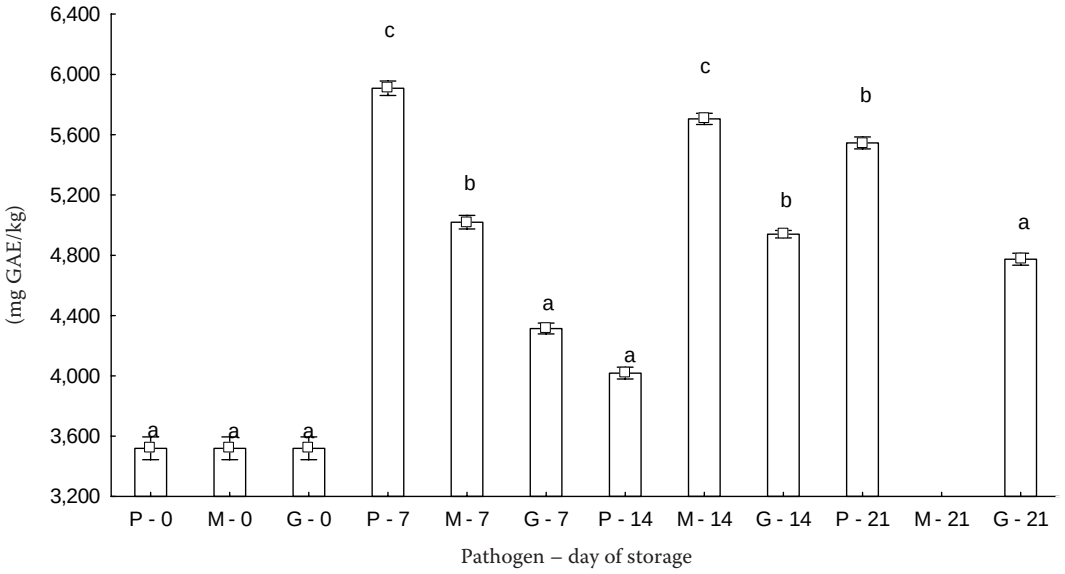

Fig. 3. Content of total phenolic compounds (mg GAE/kg) in the peel around decay inoculated with *Penicillium expansum* (P), *Monilinia fructigena* (M), and *Gloeosporium* spp. (G). Different letters mean statistical differences (Tukey HSD test, $P < 0.05$) among pathogens in the same storage day

ent phenolics, therefore the content of total phenols is considerable in determination of apple resistance. However, it is more difficult to assign a specific antifungal role to a particular monitored compound. The significant difference ($P < 0.05$) in the content of total phenols was found between fresh pulp (936 mg GAE/kg) and peel (2,482 mg GAE/kg). Total phenol content was in comparison with Tsao et al. (2003) higher both in the peel (1,478.8 mg GAE/kg) and in the pulp (413.1 mg GAE/kg) but it could be due to different climatic conditions during growing in the orchards. The defensive response was observed after the inoculation. The content of phenolic compounds increased after 7 days not only around the rotten part but also in the healthy tissue except in pulp of fruit inoculated with *Gloeo-*

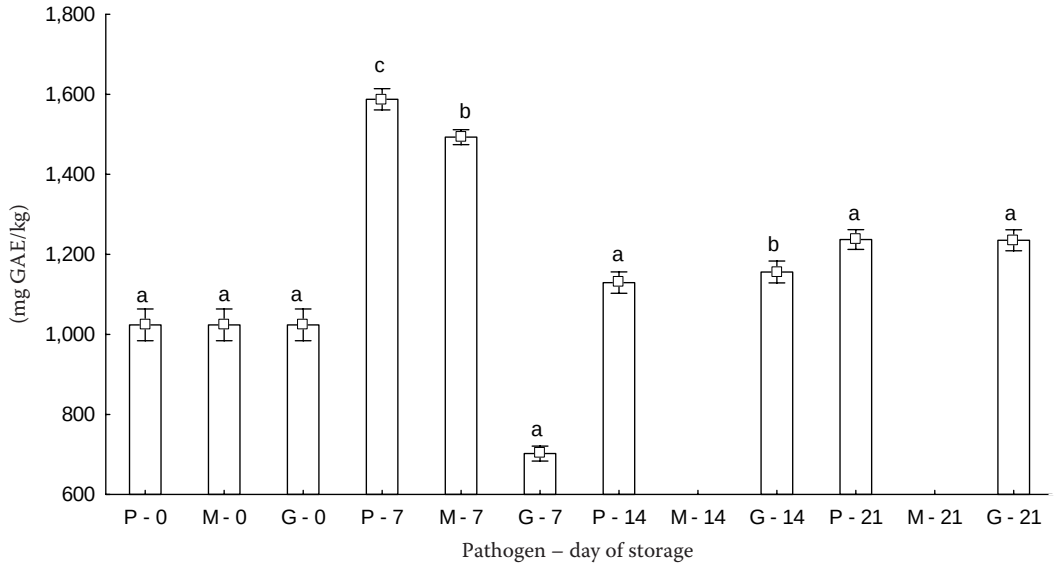

Fig. 4. Content of total phenolic compounds (mg GAE/kg) in the healthy pulp of fruit inoculated with *Penicillium expansum* (P), *Monilinia fructigena* (M), and *Gloeosporium* spp. (G). Different letters mean statistical differences (Tukey HSD test, $P < 0.05$) among pathogens in the same storage day

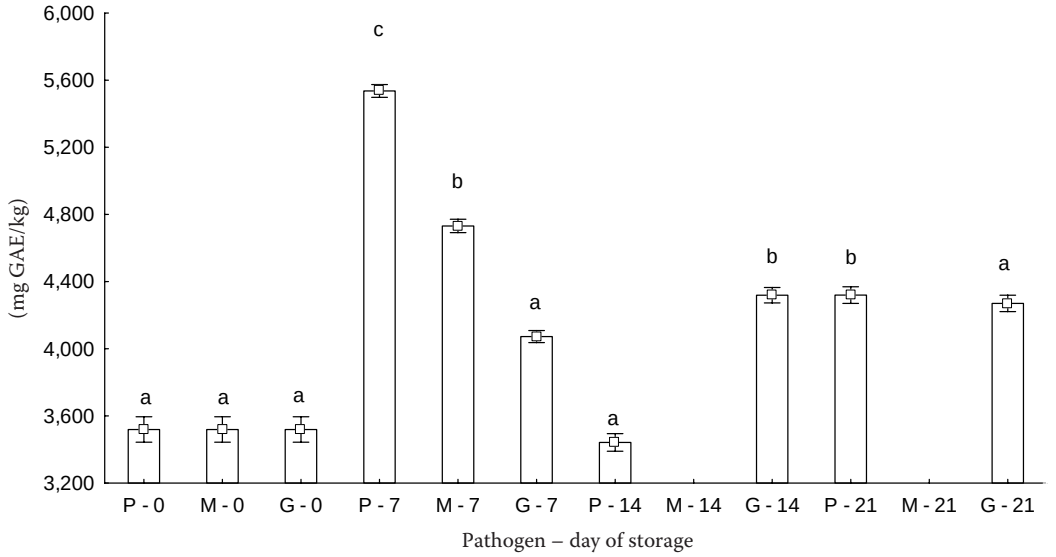

Fig. 5. Content of total phenolic compounds (mg GAE/kg) in the healthy peel of fruit inoculated with *Penicillium expansum* (P), *Monilinia fructigena* (M), and *Gloeosporium* spp. (G). Different letters mean statistical differences (Tukey HSD test, $P < 0.05$) among pathogens in the same storage day

sporium spp. However, in all cases the content of total phenols was higher in the peel around the rotten zone than in the healthy peel (Figs 2–5). Similar results reported Lattanzio et al. (2001), who mentioned that when rot appears, an increase of phenolic levels can be observed in infected tissue surrounding the rotten zone, as compared to healthy tissue of the same fruit.

The elevation of phenols in the fruit inoculated with *Penicillium expansum* appeared mainly after 7 days (Figs 2–5). Phenols were synthetized or released from glycosidic attachments (Tsao et al. 2003; Lattanzio et al. 2006) under influence of decays progress, primarily in the part around rottenness, but their level rapidly declined beside the healthy pulp. The defensive response of the fruit inoculated with this pathogen

Table 1. Total phenol content (TP; mg gallic acid equivalents (GAE)/kg) and phenylalanine-ammonia lyase (PAL) activity (μmol cinnamic acid/g/h) after fungal infection)

Pathogen	Apple part	Time (day)	TP	PAL activity
Penicillium expansum	fresh tissue	0	1,891.68 ± 11.13[a]	0.80 ± 0.02[a]
Monilinia fructigena	fresh tissue	0	1,891.68 ± 11.13[a]	0.80 ± 0.02[a]
Gloeosporium spp.	fresh tissue	0	1,891.68 ± 11.13[a]	0.80 ± 0.02[a]
Penicillium expansum	healthy part	7	1,853.38 ± 12.15[b]	0.76 ± 0.01[b]
Monilinia fructigena	healthy part	7	2,024.99 ± 18.33[c]	0.82 ± 0.01[c]
Gloeosporium spp.	healthy part	7	1,640.54 ± 11.86[a]	0.79 ± 0.01[a]
Penicillium expansum	around decay	7	2,300.98 ± 27.20[b]	0.97 ± 0.01[c]
Monilinia fructigena	around decay	7	2,324.51 ± 14.45[b]	0.81 ± 0.01[b]
Gloeosporium spp.	around decay	7	1,786.74 ± 10.76[a]	0.70 ± 0.02[a]
Penicillium expansum	decayed part	7	3,554.97 ± 12.00[b]	2.08 ± 0.08[b]
Monilinia fructigena	decayed part	7	1,222.71 ± 12.33[a]	0.78 ± 0.03[a]
Gloeosporium spp.	decayed part	7	–	–
Penicillium expansum	healthy part	14	1,793.89 ± 26.40[b]	0.63 ± 0.02[a]
Monilinia fructigena	healthy part	14	–	–
Gloeosporium spp.	healthy part	14	1,550.57 ± 18.95[a]	0.79 ± 0.03[b]
Penicillium expansum	around decay	14	1,637.92 ± 14.55[a]	0.72 ± 0.01[a]
Monilinia fructigena	around decay	14	1,949.53 ± 27.02[b]	0.80 ± 0.01[c]
Gloeosporium spp.	around decay	14	1,948.73 ± 17.57[b]	0.79 ± 0.01[b]
Penicillium expansum	decayed part	14	1,293.28 ± 23.55[b]	0.77 ± 0.04[b]
Monilinia fructigena	decayed part	14	1,067.67 ± 13.82[a]	0.71 ± 0.01[a]
Gloeosporium spp.	decayed part	14	2,369.61 ± 35.73[c]	0.88 ± 0.01[c]
Penicillium expansum	healthy part	21	1,544.43 ± 19.44[b]	0.50 ± 0.06[a]
Monilinia fructigena	healthy part	21	–	–
Gloeosporium spp.	healthy part	21	1,501.60 ± 15.95[a]	0.75 ± 0.02[b]
Penicillium expansum	around decay	21	801.91 ±13.59[a]	0.71 ± 0.02[a]
Monilinia fructigena	around decay	21	–	–
Gloeosporium spp.	around decay	21	2,210.59 ± 28.12[b]	0.96 ± 0.01[b]
Penicillium expansum	decayed part	21	1,022.93 ± 16.90[b]	0.48 ± 0.02[a]
Monilinia fructigena	decayed part	21	491.15 ± 18.55[a]	0.51 ± 0.02[a]
Gloeosporium spp.	decayed part	21	2,226.72 ± 35.70[c]	0.81 ±0.01[b]

Different letters mean statistical differences (Tukey HSD test, $P < 0.05$) among pathogens in the same storage day

was very rapid, the phenols elevation was the most intensive among the fruit infested by other pathogens (Figs 2–5). The increase in concentration of phenols was observed after 7 days in the infected part and then the amount decreased, but the decrease in the peel was not consistent. The activity of PAL was not measured separately in peel therefore is not possible to compare the activity of PAL with phenolics content and to define a further explanation.

The apples inoculated with *Monilinia fructigena* demonstrated higher concentration of total phenols in the healthy pulp than in the area surround-

Table 2. Concentration (mg/kg) of phenolic compounds in examined cv. Idared

	Catechin	Chlorogenic acid	Epicatechin	Caffeic acid	Phloridzin
Pulp	33.7 ± 0.8	278.0 ± 3.6	157.1 ± 2.0	11.6 ± 0.2	16.4 ± 0.8
Peel	73.4 ± 1.2	210.4 ± 2.5	276.8 ± 3.4	4.0 ± 0.1	139.5 ± 1.2

Table 3. Concentration (mg/kg) of phenolic compounds cv. Idared [Tsao et al. (2003)]

	Catechin	Chlorogenic acid	Epicatechin	Caffeic acid	Phloridzin
Pulp	21	232	52	–	14
Peel	79	195	290	–	79

ing the rotten part. The progress of decay in fruit infected by *M. fructigena* was so intensive that the defensive reaction was initiated rapidly not only in the surrounding pulp but also in the healthy tissue (Figs 2–5). The content of phenols subsequently declined in the decayed part. No healthy tissue remained after two weeks after inoculation.

Gloeosporium spp. as the least progressive mould initiated phenol rising mainly in the tissue around the rotten zone. The elevation was slower than in presence of other pathogens but the concentration was maintained at relatively stable level (Figs 2–5).

An increase of enzyme activity was observed mainly in the area surrounding the rotten zone *(Penicillium expansum)* and in the healthy part (*Monilinia fructigena*, *Gloeosporium* spp.). Our results confirmed by other authors (Morelló et al. 2005; Pereyra et al. 2005), demonstrated that PAL is induced by stress such as wounding and fungal attack. Comparing the results, a very good correlation was found between PAL activity and total phenol content. The changes of enzyme activity (Table 1) correlated with changes of total phenol content in all parts of inoculated fruit (r = 0.76–0.98).

Activity of phenylalanine-ammonia lyase (PAL)

Activity of PAL was determined in the mix of pulp and peel and compared with total phenol content and concentration of selected phenolic compounds in the same tissue. The activity of enzyme in the fresh tissue was quite high (0.801 μmol cinnamic acid/g/h) in comparison with other cultivars (HL 1834, HL 447, Golden Delicious, Angold).

Concentration of selected phenolic compounds

The phenolic profile of cv. Idared is presented in Table 2. Chlorogenic acid and epicatechin were the significant phenolic compounds in the pulp. In the peel predominated chlorogenic acid, epicatechin, and phloridzin.

The obtained results indicate higher phenols content in the peel than in the flesh (Escarpa,

Table 4. Correlation coefficients between phenylalanine-ammonia lyase (PAL) activity and single phenolic compounds concentration

	Gloeosporium spp.	*Penicillium expansum*	*Monilinia fructigena*	*Penicillium expansum*
	healthy part		around rot	
Catechin	0.01	0.62	0.92	0.36
Chlorogenic acid	0.96	0.90	0.93	0.25
Epicatechin	0.44	0.95	0.65	0.19
Caffeic acid	0.61	0.63	0.47	0.00
Phloridzin	–0.46	0.46	0.94	0.77

Table 5. Elevation of phenols after inoculation

	Gloeosporium spp.	*Monilinia fructigena*	*Penicillium expansum*
Healthy part of pulp	–	–	phloridzin
Around rotten zone of pulp	phloridzin	–	–
Decayed part of pulp	–	–	–
Healthy part of peel	chlorogenic acid, epicatechin, phloridzin	chlorogenic acid, epicatechin	chlorogenic acid, epicatechin
Around rotten zone of peel	chlorogenic acid, epicatechin, phloridzin	chlorogenic acid, epicatechin	chlorogenic acid, epicatechin, phloridzin
Decayed part of peel	–	–	–

González 1998; Łata 2007; Mikulič Petkovšek et al. 2008, 2009, 2010). The finding confirms that apple phytoprotective compounds are mainly localized in the skin, which is the first barrier to pathogen penetration. Similar results of phenol composition were found by Tsao et al. (2003), (Table 3).

The accumulation of phenols was observed after inoculation with pathogens. The production was very intensive the first week after seeding; then concentration of monitored phenols gradually decreased. The decline of phenols could be due to participation of these substances in the reactions of enzymatic browning to produce fungitoxic quinones (Lattanzio et al. 2001). Although excellent correlation between total phenol content and activity of PAL was not defined, an explicit correlation between PAL and all monitored phenolic compounds was observed. The correlation was found between PAL and chlorogenic acid, epicatechin, and caffeic acid in the healthy part of apples inoculated with *Gloeosporium* spp. and *Penicillium expansum*. Relationship between PAL and phloridzin was found in the tissue surrounding rotten zone in the apples inoculated with *Monilinia fructigena* and *Penicillium expansum* (Table 4).

The PAL was the most active in tissues around decay but possibly other phenolic compounds were synthesized, not only the monitored ones. For example Zhi-Guo et al. (1995) found a positive correlation between PAL and anthocyanin synthesis in apples. Furthermore, monitored phenols could be converted to other substances. As Jones (1984) indicated, an increase in PAL activity cannot always been correlated with the production of specific phenylpropanoid compounds. Where a correlation was shown, in only few instances the accumulation of a specific product was exactly related to the integrated values of PAL activity with time. It is possible that new phenols are rapidly oxidized by polyphenol oxidase or hydrolyzed by fungi. An increase in PAL activity results in increase in concentration of phenolic compounds, which are substrates for oxidative enzymes such as polyphenol oxidase and peroxidase (Cantos et al. 2002; Slatnar et al. 2010).

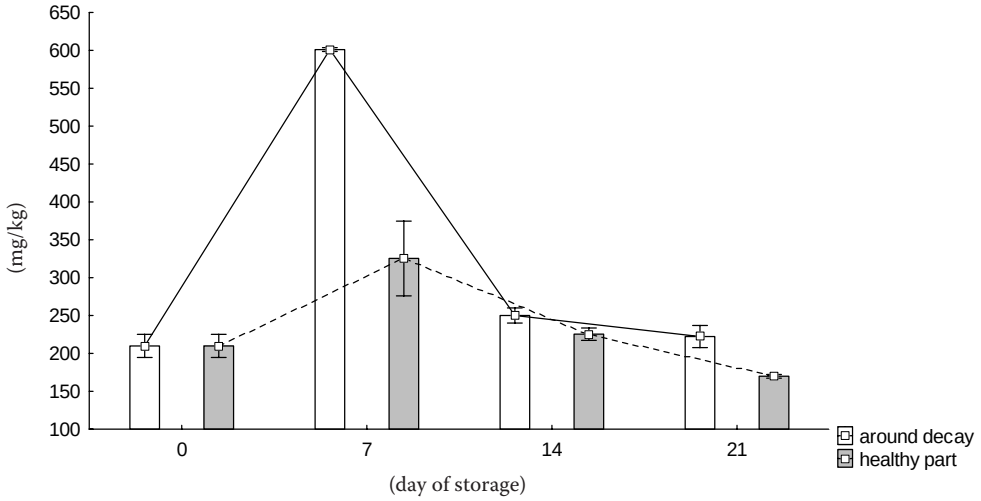

Fig. 6. Changes of the concentration (mg/kg) of chlorogenic acid in the peel after the inoculation with *Penicillium expansum*

When the concentration of monitored phenols was evaluated separately in the pulp and in the peel of inoculated apples, the results showed an increase in phloridzin in the pulp only (Table 5). The elevation of all major phenolic compounds – chlorogenic acid (Fig. 6), epicatechin and phloridzin was initiated in apple peel after the inoculation. The increase was more intensive in the part surrounding the rotten zone. The results demonstrate the important role of monitored compounds in defensive reaction and their antifungal activity. The antifungal activity of chlorogenic acid and phloridzin were studied and confirmed for other plant pathogens as well (Lattanzio 2001; Mikulič Petkovšek et al. 2003, 2007, 2008, 2009).

CONCLUSIONS

From the results observed in this work we can conclude that after infection the phenylpropanoid metabolism is activated in the whole fruit, and mainly in tissues surrounding rotten zone. The accumulation of especially dominant phenols were monitored; it signifies that explicit part of phenylpropanoid metabolism pathway is activated or these phenols are preformed and sequestered in conjugated form (Lattanzio et al. 2006). The elevation of phenols was localized mainly in the apple peel which is the first barrier against fungal attack. The progress of rotting depends also on the type of pathogen; in our study with stored apples the rate of decay increased in order *Gloeosporium* spp., *Penicillium expansum*, and *Monilinia fructigena*. Chlorogenic acid and phloridzin confirmed their important role in defensive mechanism. The correlation between activity of PAL and total phenol content showed that other phenols are produced besides compounds monitored in our experiments.

This study confirmed that defensive mechanism is active in spite of the advanced level of ripeness. It is presumable that less mature fruits are more efficient in their defensive reactions; e.g. in limiting progress of decay due to pathogen *Gloeosporium* spp. which was the least aggressive.

Acknowledgement

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