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Response of scab-susceptible (McIntosh) and scab-resistant (Liberty) apple tissues to treatment with yeast extract and *Venturia inaequalis*

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Yeast extract and *Venturia inaequalis* treated intact scab-susceptible (McIntosh) and scab-resistant (Liberty) apple plants and their organs were analyzed for phenolic metabolites. The major phenolic compounds found in both non-treated and treated leaves were phloridzin and phloretin which accumulated in mM concentrations. Untreated and treated stems and roots contained only phloridzin as the major detectable metabolite during the course of the investigation. The accumulation of phloridzin and phloretin was not developmentally regulated, since they were present in both young and old leaves, and also in the intercellular washings of both scab-susceptible and scab-resistant plants. The major metabolites of both McIntosh and Liberty fruits were cinnamyl glucose and *p*-coumarylquinic acid, which increased 20-fold in Liberty fruit upon yeast extract treatment. The same compounds increased only 2-fold in McIntosh fruits. Minor compounds in the fruits of both cultivars were *p*-coumaric acid, phloridzin and phloretin, the latter compound being present at the threshold of detection. Biphenyl and dibenzofuran compounds, the major metabolites of elicitor treated Liberty cell suspension cultures, could not be detected in the intact plants. These results indicate differential response of plant organs and cell suspension cultures to elicitor treatment or pathogen invasion.

Keywords: Scab resistance; *Malus domestica*; cv Liberty; cv McIntosh; Rosaceae; Elicitation; *Venturia inaequalis*; Yeast extract; Phytoalexin; Phloretin; Phloridzin; Cinnamyl glucose; *p*-Coumaric acid; *p*-Coumarylquinic acid; HPLC

1. Introduction

Apple scab, a fruit disease restricted to the genus *Malus* caused by the pathogen *Venturia inaequalis*, presents an economically significant problem to growers. The disease attacks leaves, stems, fruits and roots of apple trees and results in the production of scabby, inferior fruit. Present measures against apple scab include frequent chemical treatment of scab susceptible trees and development of scab-resistant apple cultivars by conventional breeding (McHardy, 1996). Resistance against scab in most commercial apple cultivars is derived from the ornamental apple *Malus floribunda* 821, referred to as *Vf*, which provides broad protection against the different races of *V. inaequalis* (Williams and Kuc, 1969).

Although intensively belabored in the past 50 years by chemical (Müller et al., 1994), physiological (McHardy, 1996) and genetic approaches (Williams et al., 1966), the biochemical principle of scab-resistance is not known. Marker assisted analysis of scab-resistance against different pathovars of *V. inaequalis* indicates a multigenic trait with individual genes located on different chromosomes (Gianfranceschi et al., 1994; Hemmat et al., 1994; Hemmat et al., 1998; Koller et al., 1994). As this is the case of pathogen-resistance in other plants, scab-resistance in apples is also likely to be a multi-component system, involving primary, secondary and tertiary mechanisms, among them possibly the production of phytoalexins.

We have started recently to investigate the response of scab-susceptible and scab-resistant apple cell cultures to elicitor treatment (Hrazdina et al., 1997; Borejsza-Wysocki et al., 1999). This model system is convenient to use, not seasonally limited, and unlike plant organs

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and tissues, provides an amplified response. However, cells in suspension cultures are de-differentiated, and their response may differ from those in differentiated tissues and organs. Therefore, we have extended our investigation to whole leaves, stems, roots and fruits using both elicitor treatment and infection with *V. inaequalis*.

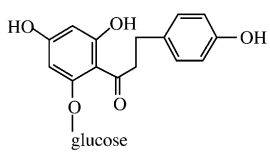
2. Results and discussion

2.1. Phenolic metabolism in yeast extract treated plants

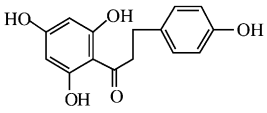
In previous papers (Hrazdina et al., 1997; Hrazdina, 1998; Borejsza-Wysocki et al., 1999) the accumulation of biphenyl and dibenzofuran phytoalexins in yeast extract treated cell suspension cultures of the scab-resistant cultivar Liberty, but not in the scab-susceptible cultivar McIntosh, was reported. Here, similar investigations with in vitro propagated plants and shoot cultures of McIntosh and Liberty and with apple fruits obtained from field grown trees were carried out. Plants were grown under sterile conditions and high humidity, which causes them to have a thin cuticle and consequently become sensitive to stress, including treatment with yeast extract. For comparison with the cell cultures, the phenolic profile of the leaves of rooted plants were determined 96 h after yeast extract treatment because at this time point the end products of the phytoalexin pathway, 2-*O*-glucosyloxyaucuparin and malusfuran, reached maximal concentrations and were easy to detect. The 96-h time point was also considered important for the invading fungus' strategy. At approximately this time after the original infection, the cuticle has been penetrated, hyphal growth is substantial and biserial stroma extend laterally over two to six epidermal cells from a single infection point (McHardy, 1996).

There were no qualitative differences in the observed phenolic profile of water-treated and yeast extract treated McIntosh leaves [Fig. 1(A), (B)], both of which have phloridzin (compound #3, $R_t \sim 12$ min) and phloretin (compound #4, $R_t \sim 18$ min) present. The major compounds accumulating in the yeast extract treated McIntosh leaves were *p*-coumarylquinic acid (compound #1, $R_t \sim 7$ min) and cinnamylglucose (compound #2, $R_t \sim 10$ min). Essentially the same compounds were present in both the control and the yeast extract treated leaves of Liberty [Fig. 1(C), (D)]. Biphenyl or dibenzofuran phytoalexins could not be detected in either the control and yeast extract treated McIntosh or Liberty leaves, similar to what was found in water and yeast extract treated apple shoot cultures (data not shown). The absence of biphenyl and dibenzofuran phytoalexins in apple leaves confirms earlier reports on (*Maloideae Rosaceae*), in which phytoalexins were not detected in leaves of 130 representative species treated with both biotic and abiotic elicitors (Kokubun and Harborne, 1994, 1995). However, the compounds were found in the fungus-infected sapwood of *Malus* species and in the sapwood of other members of the *Rosaceae* (Grayer and Kokubun, 2001; Kokubun and Harborne, 1995). The presence of these compounds in the leaves (Miyakodo et al., 1985; Kokubun and Harborne, 1994) and roots (Kokubun et al., 1994) of plants belonging to the *Rosaceae* were reported on two occasions only, but these reports did not involve *Malus* species. Since *p*-coumarylquinic acid and cinnamylglucose accumulated briefly in the yeast extract treated leaves, this is considered to be the result of elicitor response by the plants.

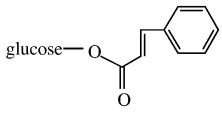
The accumulation of phloridzin and phloretin in leaves, stems and roots of both susceptible and resistant plants were analyzed as a function of time. As shown in Table 1, the highest amount of phloretin and phloridzin accumulation was in the leaves followed by stems and roots. Phloretin could not be detected in stems and roots, but accumulated nearly equally in McIntosh and Liberty leaves; however, phloridzin, the end product of the pathway, accumulated at higher concentrations in the leaves of both plants.



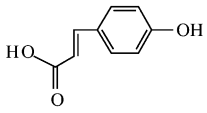
Phloridzin



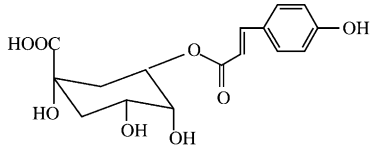
Phloretin



Cinnamyl glucose



p-Coumaric acid



p-Coumarylquinic acid

2.2. Phenolic metabolism in V. inaequalis infected leaves

The specificity underlying the determination of resistance or susceptibility of plants occurs in many cases at the level of different host cultivars exhibiting differential responses to distinct physiological races of the pathogen. Therefore, to check the possibility whether the phytoalexin response in the scab-resistant cultivar is due to the presence of the pathogen *V. inaequalis*, the leaves of both McIntosh and Liberty plants were infected with a conidia-spore suspension of the fungus. Leaves in

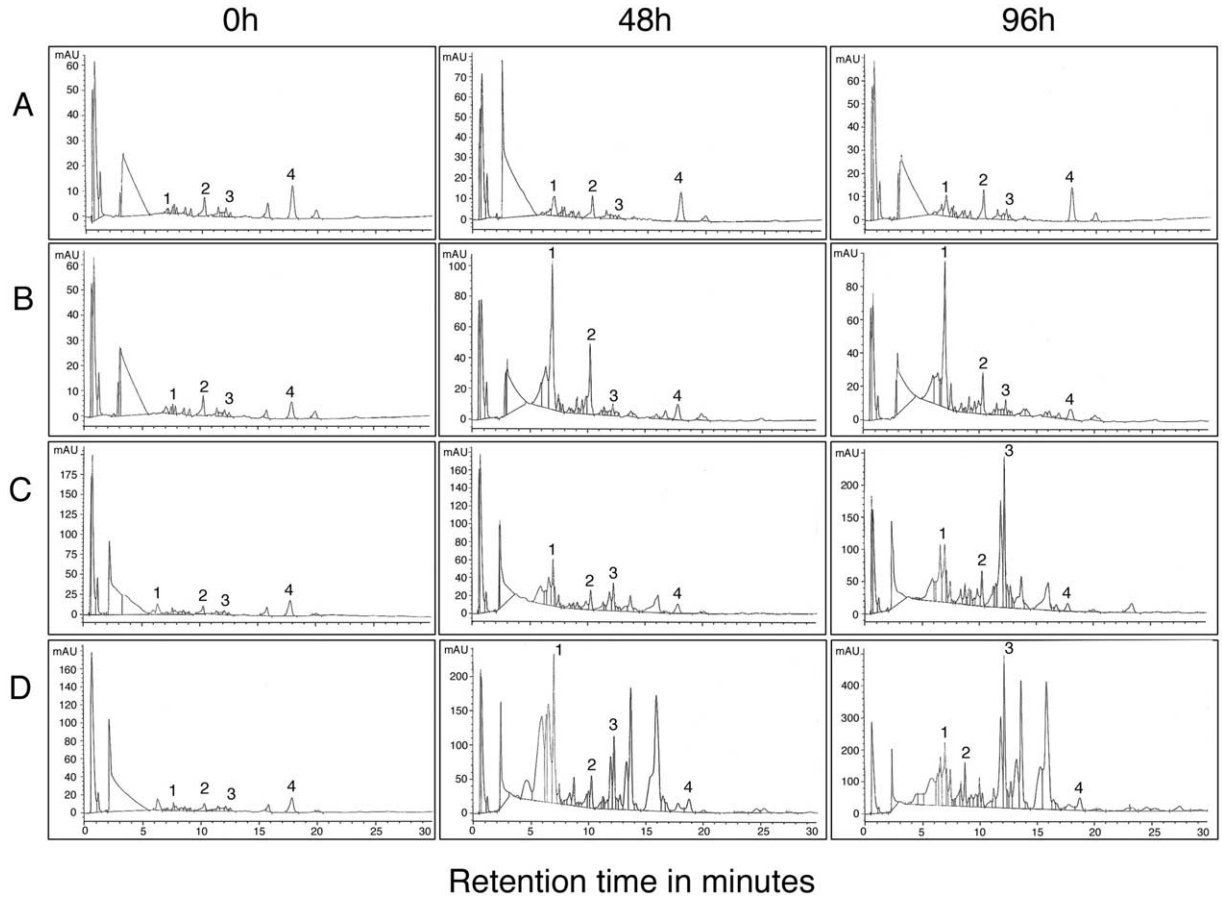


Fig. 1. HPLC profile of apple leaves 96 h after treatment. McIntosh treated with water (A) or yeast extract (B). Liberty leaves treated with water (C) or yeast extract (D). Note quantitative differences in response between A, B and C, D. Compound 1: *p*-coumaryl-quinic acid; compound 2: cinnamylglucose; compound 3: phloridzin; compound 4: phloretin.

Table 1
Changes in phloretin and phloridzin content in leaves, stems and roots of yeast extract treated scab-susceptible (McIntosh) and scab-resistant (Liberty) micropropagated apple plants 96 h after treatment[a] (μmol g^{-1} fresh wt.)

Plant parts	Time	McIntosh				Liberty			
		Phloretin		Phloridzin		Phloretin		Phloridzin	
		Control	Treated	Control	Treated	Control	Treated	Control	Treated
Leaves	0	9.8±1.0	8.1±0.8	7.5±0.7	8.1±0.8	10.3±1.0	7.2±0.7	2.1±0.2	1.1±0.1
	24	7.9±0.8	8.2±0.8	6.2±0.6	5.2±0.5	9.8±1.0	10.1±1.0	2.2±0.2	2.2±0.2
	48	9.1±0.9	12.7±1.0	8.5±0.8	6.4±0.6	8.6±0.9	10.9±1.0	2.5±0.2	2.5±0.2
	96	7.6±0.8	10.6±1.0	13.4±1.0	7.2±0.7	8.7±0.9	9.5±0.9	2.3±0.2	4.6±0.5
Stems	0	n.d.	n.d.	1.7±0.2	2.6±0.3	n.d.	n.d.	0.9±0.1	1.2±0.1
	24	n.d.	n.d.	2.9±0.3	2.6±0.3	n.d.	n.d.	1.2±0.1	1.3±0.1
	48	n.d.	n.d.	2.6±0.3	2.5±0.2	n.d.	n.d.	2.5±0.2	1.3±0.1
	96	n.d.	n.d.	2.6±0.3	3.7±0.4	n.d.	n.d.	0.9±0.1	1.4±0.1
Roots	0	n.d.	n.d.	0.1±0.0	0.1±0.0	n.d.	n.d.	0.1±0.0	0.1±0.0
	24	n.d.	n.d.	0.1±0.0	0.1±0.0	n.d.	n.d.	0.2±0.0	0.2±0.0
	48	n.d.	n.d.	0.3±0.0	0.2±0.0	n.d.	n.d.	0.1±0.0	0.1±0.0
	96	n.d.	n.d.	0.1±0.0	0.3±0.0	n.d.	n.d.	0.1±0.0	0.1±0.0

[a] n.d. not detected; ± standard error.

three developmental stages were harvested 8 days after inoculation and were analyzed for their phenolic profile. As in the previous experiment, only phloretin and phloridzin were detected and there were no biphenyl or dibenzofuran phytoalexins present. The young expanding leaves accumulated the highest amount of phloretin and phloridzin, the latter being present at approximately 10-fold higher concentrations (Table 2). There was no significant difference in phloridzin and phloretin accumulation between the control and infected leaves in McIntosh. Leaves of Liberty plants showed a similar tendency to McIntosh, an exception being a decrease in phloretin and phloridzin accumulation in the median age leaves. However, scab-susceptibility, or resistance cannot be explained by this observation. These results are consistent with data published earlier for other apple cultivars (Hunter and Hull, 1993; Picinelli et al., 1995), where both phloretin and phloridzin content apparently varied with leaf age and cultivar of the plants.

Elicited cell suspension cultures of Liberty transported the largest amounts of phytoalexins into the cell suspension medium (Hrazdina, et al., 1997; Borejsza-Wysocki et al., 1999), perhaps an indication that in differentiated tissues of the intact plant these compounds accumulate in the cell wall or the intercellular spaces. Therefore, intercellular space washings of control and scab-infected McIntosh and Liberty leaves were analyzed for the presence of phenolic compounds. As shown in Table 2, phloretin and phloridzin were the major compounds present in intercellular space washings from the leaves of both apple cultivars, and biphenyl or dibenzofuran phytoalexins could not be detected. The latter compounds could not be detected either in the intercellular spaces of leaves infected with spores of *V. inaequalis*. Young leaves of Liberty apple plants contained larger levels of phloretin. It is not clear at this point whether this resulted from increased phloretin synthesis by the challenged plants, increased transport to the cell walls/intercellular spaces, or increased phloridzin production, transport and deglucosylation by a ß-glucosidase produced by the fungus. These data are consistent with observations of others questioning the role of phloridzin and phloretin in scab-resistance of apple plants (Nicholson, 1977).

2.3. Phenolic metabolism in treated apple fruits

HPLC analysis of the exudate from water or yeast extract treated McIntosh apple tissue showed no major differences in phenolic metabolite accumulation between treated and untreated fruit, both of which contain cinnamyl glucose, *p*-coumaric acid, *p*-coumarylquinic acid and phloridzin. Cinnamyl glucose, the major compound in both the exudate and fruit tissue of McIntosh apples, showed no significant changes in accumulation upon yeast extract treatment (Table 3) whereas *p*-coumaric acid, *p*-coumarylquinic acid and phloridzin were present at significantly lower concentrations.

The scab-resistant Liberty fruit tissue showed major differences in the accumulation of phenolic metabolites upon yeast extract treatment (Fig. 2). The major phenolic metabolites accumulating in yeast extract treated Liberty fruit tissue were cinnamyl glucose and *p*-coumarylquinic acid. These compounds showed an over 20-fold increase from the water-treated tissue and were the major metabolites in the fruit cells (Fig. 2, Table 3; see also Whiting and Coggins, 1975a,b). In McIntosh fruits the increase was only approximately two-fold (Table 3). In the exudate of Liberty fruit tissue treated with water, the metabolites cinnamyl glucose, *p*-coumarylquinic acid and phloridzin could be detected, although at low concentrations, resembling phenolic metabolism in water treated McIntosh fruit. *p*-Coumaric acid was produced as the major metabolite in the exudate upon yeast extract treatment (Table 3).

The presence of biphenyl- or dibenzofuran compounds, which accumulate in *V. inaequalis* infected cell suspension cultures of scab resistant apple cultivars

Table 2
Phloretin and phloridzin in control and *Venturia inaequalis* infected leaves of McIntosh (scab-susceptible) and Liberty (scab-resistant) apple plants 8 days after inoculation[a] [μmol g^{-1} fresh wt.]

Source	McIntosh				Liberty			
	Phloretin		Phloridzin		Phloretin		Phloridzin	
	Control	Inoculated	Control	Inoculated	Control	Inoculated	Control	Inoculated
Leaf extract								
Expanding leaf	6.5±3.3	7.4±0.4	63.1±3.1	57.3±2.9	5.0±0.3	4.3±0.2	52.7±2.6	61.1±3.0
Median leaf	6.6±0.3	5.3±0.3	55.5±2.8	55.2±2.8	2.1±0.1	2.5±0.1	28.9±1.4	42.6±2.1
Old leaf	9.9±0.5	8.2±0.4	32.3±1.6	34.7±1.8	6.8±0.3	2.9±0.1	56.8±2.8	48.4±2.4
Intercellular space washing								
Expanding leaf	14.9±0.0	29.7±8.9	0.0±0.0	0.3±0.0	22.2±4.4	25.9±3.8	0.0±0.0	12.2±2.4
Median leaf	8.9±1.2	6.6±1.3	14.2±0.6	1.3±0.0	13.5±1.2	7.7±0.7	8.1±0.0	16.9±0.5
Old leaf	3.0±0.8	2.5±0.3	13.4±2.4	3.2±0.7	2.3±0.7	2.6±0.8	9.1±2.0	7.0±0.4

[a] ±standard error.

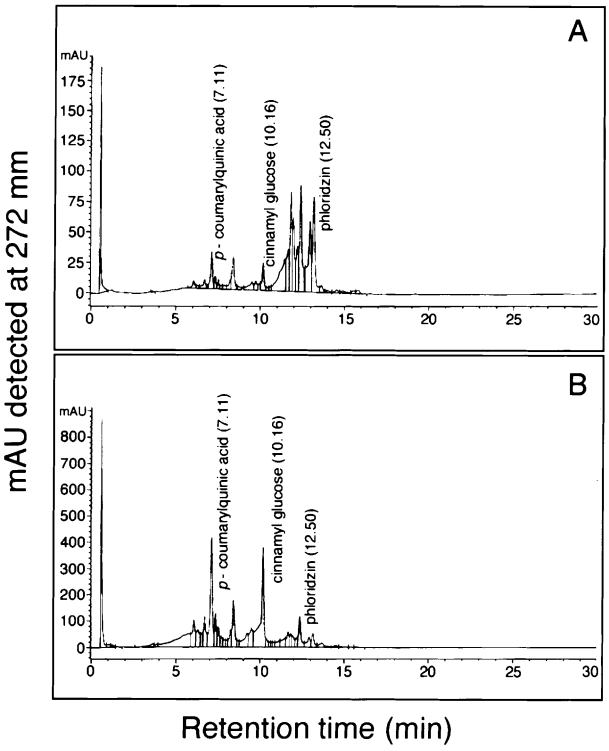


Fig. 2. HPLC profile of water (A) and yeast extract (B) treated Liberty apple fruit tissue 96 h after treatment. Note quantitative differences in response between A and B.

(Hrazdina et al., 1997; Borejsza-Wysocki et al., 1999), and in cell cultures of *M. floribunda* from which scab resistance of commercial apple cultivars derives, could not be detected (data not shown).

In precursor feeding experiments using elicited Liberty cell cultures the incorporation of cinnamic and benzoic acids into both biphenyl and dibenzofuran derivatives were observed, but not that of salicylic acid (data not shown). These data indicate that both aucuparin and malusfuran are synthesized from cinnamic acid derivatives most likely via benzoic and syringic acids (see Fig. 3 for the proposed biochemical pathway). Therefore, accumulation of cinnamic acid derivatives, the putative precursors of benzoic and *p*-hydroxybenzoic acids, and accumulation of *p*-coumaric acid indicates activation of the first enzymes of the phenylpropanoid pathway. That of *p*-coumarylquinic acid indicates that further metabolism of *p*-coumaric acid into phytoalexins is not taking place, and that excess *p*-coumaric acid is removed from the pool by esterification with quinic acid. Lack of detection of benzoic acid, malusfuran (Hrazdina et al., 1997) and aucuparin (Borejsza-Wysocki et al., 1999) suggests that a signal in the fruit tissue cells for the activation of the specific pathway leading to the formation of phytoalexins was not sent or received, or that the specific pathway is blocked.

3. Experimental

3.1. Materials

The $CHCl_3$ and EtOAc used for extraction of phenolic compounds, and MeOH employed as solvent for HPLC were from Fisher (Fair Lawn, NJ). Cinnamic and *p*-coumaric acids, phloridzin and phloretin were obtained from Sigma (St. Louis, MO), whereas yeast extract was from Difco Laboratories (Detroit, MI).

3.2. Plant material

Rooted apple plants and micro-propagated apple cultures were received from Dr. H.S. Aldwinckle, Department of Plant Pathology, Cornell University, NYSAES, Geneva, NY. Rooted plants were grown in a controlled environment chamber, whereas micro-propagated plant and shoot cultures were grown under sterile conditions.

3.2.1. Rooted plants

In vitro propagated apple plants were transplanted to soil and grown in a controlled environment chamber (Bolar et al., 1998b) with a 16 h photo period, 23/20 °C day/night temperature, 80% relative humidity. Plants were watered daily and fertilized weekly with Miracle-Grow fertilizer (15 N–30 P–15 K). Young plants having 7–10 fully developed leaves were sprayed either with water

Table 3
Phenolic compounds of yeast extract treated scab-susceptible (McIntosh) and scab-resistant (Liberty) apple fruits (nmol g^{-1} fresh wt.)

Source	Cinnamyl glucose		*p*-Coumarylquinic acid		*p*-Coumaric acid		Phloridzin	
	McIntosh	Liberty	McIntosh	Liberty	McIntosh	Liberty	McIntosh	Liberty
Exudate								
Control	2.0±0.2	1.6±0.2	0.2±0.0	0.8±0.1	n.d.	n.d.	0.2±0.0	0.1±0.0
Treated	2.6±0.3	1.4±0.1	2.5±0.2	1.8±0.2	1.6±0.2	6.7±0.7	0.2±0.0	0.1±0.0
In fruit tissue								
Control	4.0±0.4	2.2±0.2	2.1±0.2	2.8±0.3	n.d.	n.d.	1.0±0.1	1.5±0.1
Treated	5.3±0.5	43.6±4.4	5.6±0.6	47.1±4.7	n.d.	n.d.	0.5±0.0	1.1±0.1

n.d. not detected; ±standard error.

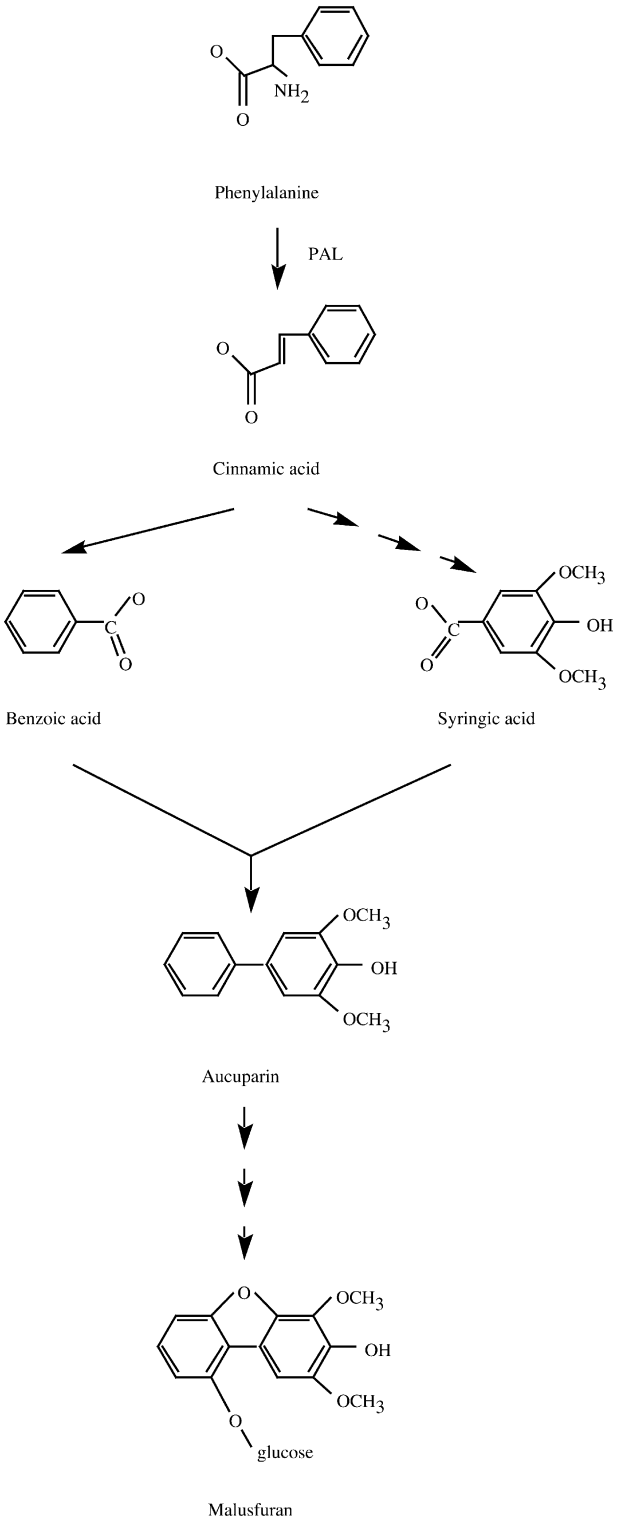


Fig. 3. Putative biochemical scheme of aucuparin and malusfuran formation.

(control) or with an aqueous conidia-spore suspension (270,000 spores/ml) of *V. inaequalis*. The youngest expanding leaf on each plant was marked. Plants were incubated for 48 h in a chamber at 20 °C and 100% relative humidity and returned to the controlled environment growth chamber under normal growth conditions as described above. Leaves of different ages (top—youngest, middle and lowest—oldest) were harvested 8 days after inoculation. Leaves were weighed, infiltrated with distilled water for 60 min under vacuum (637 mm Hg), removed from the water and dried with a paper towel. Leaves were rolled and placed in a centrifuge tube. Intercellular space washings were removed by centrifugation at 800 *g* for 10 min. The supernatant was collected, lyophilized, and residue dissolved in MeOH (500 μl) which was then used for HPLC analysis.

3.2.2. Micropropagated plants

McIntosh and Liberty plants were micro-propagated in vitro and grown under sterile condition on MS medium supplemented with growth regulators for shoot proliferation or shoot rooting (Bolar et al., 1998a). Four-week-old plants with developed root systems were sprayed with a 0.3% autoclaved aqueous solution of yeast extract. Control plants were treated identically with sterile water. Plant material (leaves, stems, roots) was harvested after 0, 24, 48 and 96 h intervals, cut into small pieces, frozen in liquid nitrogen and stored at −80 °C for analysis.

3.2.3. Micropropagated shoot cultures

Two-month-old McIntosh and Liberty shoot cultures were grown in glass jars on MS medium supplemented with growth regulators for stimulation of shoot proliferation (Bolar et al., 1998a). Whole multiple shoots were transferred from the glass jars to an 0.5% autoclaved aqueous solution of yeast extract and submerged at normal atmospheric pressure or under vacuum (637 mm Hg) for 5 min. Control shoots were treated with sterile water under identical conditions. Elicitor treated and control plant material was transferred back to glass jars and kept at 25 °C with a 16 h photoperiod. Leaves and stems were harvested 96 h after treatment, cut into small pieces, frozen in liquid nitrogen and stored at −80 °C prior to analysis.

3.2.4. Fruits

Commercial ripe-stage Liberty and McIntosh fruits were washed, dried, sterilized in 70% EtOH for 5 min, and washed 3×5 min in sterile distilled water. Endocarp and seeds were removed; fruits were sliced in 15 mm thick slices and cut into 20×20 mm pieces. Fruit pieces (mesocarp and exocarp) were weighed and from each cultivar a 100 g sample was transferred to an Erlenmeyer flask (1000 ml) and covered with either 250 ml sterile distilled water or aqueous yeast extract solution (3 g/l). Flasks containing the apple tissues were shaken at 120 rpm for 96 h in the dark at 25 °C. Apple pieces were removed from the solution by filtration, frozen in liquid nitrogen and stored at −80 °C. The filtrate containing

apple tissue exudate was also frozen and stored at −80 °C.

3.3. Extraction of phenolic compounds

3.3.1. Leaves, stems and roots

The cut leaves, stems and roots of apple plants were collected, cut into small pieces and extracted with MeOH (10:1, v/w) in the dark for 4 days at 4 °C. Aliquots of the methanolic extract (250 μl) were used for sample preparation. Water (250 μl) was added to each methanolic extract (250 μl) and chlorophyll was removed by extracting for 5 min with $CHCl_3$ (250 μl). After centrifugation (1 min at 12,000 *g*) the lower phase containing chlorophyll was discarded. Volume of upper phase (400 μl) was reduced to approximately 200 μl in a SpeedVac concentrator and after addition of glacial acetic acid (20 μl) compounds were extracted from the solution with EtOAc (3×250 μl). EtoAc extracts were separated from the aq. phase by centrifugation (1 min at 12,000 *g*), dried (Na_2S0_4) and evaporated to dryness in vacuo at room temp. The residue was dissolved in MeOH (100 μl) and used for HPLC analysis.

3.3.2. Fruits

Apple pieces were removed from the aq. sol. by filtration, and compounds were extracted separately from the liquid and from apple tissue.

The pH of the liquid was adjusted to 4.5 by addition of glacial acetic acid and phenolic compounds were extracted with EtOAc (3×250 ml) as reported earlier (Borejsza-Wysocki et al., 1999). EtOAc was evaporated, at room temp. in a SpeedVac evaporator, to dryness and the residue was dissolved in MeOH (1.0 ml) and used for HPLC analysis. Apple tissue (50 g) was homogenized with MeOH (100 ml) in a commercial blender for 5 min and compounds were extracted for 1 h at 0 °C. The homogenate was centrifuged at 20,000 *g* for 20 min; the pellet was discarded and the supernatant was concentrated in a rotary evaporator. Phenolic compounds were extracted from the supernatant with three volumes of EtOAc as reported earlier (Borejsza-Wysocki et al., 1999). The residue after evaporation of EtOAc was dissolved in MeOH (1 ml) and analyzed by HPLC.

3.4. High pressure liquid chromatography (HPLC)

Aliquots (25 μl) of extracts were separated using a HP1100 HPLC system (Hewlett Packard) on an Eclipse XDB-C18 column (4.6 mm×50 mm; particle size 3–5 μm). Samples were separated with an aq. phosphoric acid (0.1%): MeOH gradient from 0 to 40% MeOH (0–10 min) and 40–50% MeOH (10–30 min). Separation was monitored in a DAD at 272 nm. Compounds were identified by retention time and UV-spectra of authentic reference compounds. Quantitation of the individual compounds was from standard curves established with reference material from our laboratory collection. Standard error was calculated from three replicates.

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