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PHLORIDZIN AND APPLE SCAB

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Key Word Index—*Malus* spp.; Rosaceae; apple scab; *Venturia inaequalis*; phloridzin; sieboldin; trilobatin; disease resistance.

Abstract—Phloridzin, sieboldin, trilobatin, phloretin and 3-hydroxyphloretin can all be used as carbon sources by *Venturia inaequalis* in culture. Resistance to apple scab was not linked with inheritance of sieboldin or trilobatin in seedlings. There is no direct connection between phloridzin or its breakdown products and scab resistance.

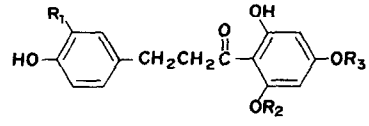
INTRODUCTION

Phloridzin (**1**), the 2′-glucoside of phloretin (**2**), is the major phenolic compound in commercially grown varieties of apple (*Malus pumila* Mill.) [1,2]; it represents from 3 to 7% of the dry weight of leaves, and occurs in bark and roots but not in fruit [3]. No relationship has been found between the concentration of phloridzin in leaves and resistance to the apple scab organism *Venturia inaequalis* Cke. Wint [1,2]. In *M. trilobata*, phloridzin is replaced by the isomeric trilobatin (**3**) and in those *Malus* species included by Rehder [4] in the Sieboldianae some sieboldin (**4**) occurs [3,5].

RESULTS

Dihydrochalcones and scab resistance

That neither **3** nor **4** is directly responsible for scab resistance was demonstrated by examination of crosses between the scab-susceptible apple cultivar Cox's Orange Pippin and either *M. floribunda* [6,7] (scab immune and containing **4**) or *M. trilobata* (scab immune and containing **3**). The seedlings were inoculated with conidia to test their reaction to scab and leaves were also tested by TLC for their glucoside content. The *M. floribunda* cross produced 45 seedlings and 23 of those were scab-resistant. Sieboldin and phloridzin were found in nine of the resistant plants and 12 of the susceptible ones. The other 24 plants contained only phloridzin. The *M. trilobata* cross produced 48 seedlings, 31 of which were scab-susceptible. All the susceptible plants and eight of the resistant ones contained phloridzin only. The other nine resistant plants, which had inherited the trilobed leaf of *M. trilobata*, contained both trilobatin and phloridzin.



	R_1	R_2	R_3
(1) Phloridzin	H	Glc	H
(2) Phloretin	H	H	H
(3) Trilobatin	H	H	Glc
(4) Sieboldin	OH	H	Glc
(5) 3-Hydroxyphloridzin	OH	Glc	H

Further evidence that sieboldin is not involved in scab resistance was provided by a natural infection of *M. atrosanguinea* trees at East Malling with a virulent race 5 isolate of *V. inaequalis* [8]; leaves of *M. atrosanguinea* contain sieboldin as well as phloridzin.

Metabolism of dihydrochalcones by V. inaequalis

Phloridzin has been shown to possess antifungal properties [9] and it has been claimed that 3-hydroxyphloridzin (**5**) (produced by partial oxidation of phloridzin) is important in resistance

Table 1. Metabolites detected in cultures of *V. inaequalis*

Substrate	Phloretin	Hydroxy-phloretin	Dihydrocaffeic acid	Phloretic acid	Phloro-glucinol
Phloridzin	+			+	+
Phloretin	+			+	+
Trilobatin	+			+	+
Sieboldin		+	+		+
3-Hydroxy-phloridzin		+	+		+
3-Hydroxy-phloretin		+	+		+

Traces of quinone were found with sieboldin, 3-hydroxyphloridzin and 3-hydroxyphloretin as substrates.

to apple scab [1]. Phloretin is bactericidal [10] and at high concentration inhibits the germination and subsequent growth of conidia of *V. inaequalis* [11]. Studies on the metabolism of phloridzin by various micro-organisms have shown that two enzymes are normally responsible for its breakdown [12,13]. Initially, β-glucosidase hydrolyses the molecule giving phloretin and glucose. The phloretin is then broken down to phloretic acid and phloroglucinol by phloretin hydrolase [13].

The *in vitro* metabolism by *V. inaequalis* of phloridzin, sieboldin and 3-hydroxyphloridzin and the aglycones phloretin and 3-hydroxyphloretin was studied. Alcoholic solutions were added to 3-week-old filter paper cylinders covered with *V. inaequalis* mycelium [14] and suspended in pH 5·6 phosphate buffer at 20°. Samples taken at $\frac{1}{2}$ hr intervals were examined by TLC and PC, and compared with standards. Enzyme activity ceased after 4–6 hr. The results (Table 1) show that β-glucosidase and phloretin hydrolase were present and were responsible for phloridzin metabolism. Sieboldin and trilobatin were also found to be metabolized by enzymes of *V. inaequalis* in a similar way. As expected, 3-hydroxyphloridzin and 3-hydroxyphloretin gave dihydrocaffeic acid as product rather than phloretic acid. With phloridzin as substrate, no hydroxylated derivatives were produced by *V. inaequalis* indicating that there was no cresolase (hydroxylating activity of phenolase) present. With dihydroxylated substrates (sieboldin, hydroxyphloretin), however, some quinone formation was observed indicating that the mycelium does have a catecholase (oxidative activity of phenolase).

Effects of dihydrochalcones on the growth of V. inaequalis

The effects of adding the various dihydrochalcones (see Table 2) to cultures of *V. inaequalis* was examined. Alcoholic solutions, sterilized by passage through Millipore filters, were added to Roux bottles, containing glucose/salts medium, with the inoculum [15]; growth of the cultures at 18° was compared by taking dry weight yields after 21 days. All the compounds added gave increased yields over cultures having glucose as the

Table 2. Growth of *V. inaequalis* in culture

Treatment	Dry wt yields (mg) of mycelium	Increase over control (%)
Control	21·6	
Phloridzin	36·5	68
Trilobatin	34·9	62
Phloretin + glucose	28·3	31
Phloroglucinol + phloretic acid + glucose	29·9	38

Figures are means of eight replicates for each treatment. The control had 4% (W/V) glucose as sole carbon source. The amounts of chemicals added to 50 ml cultures were: phloridzin or trilobatin 50 mg, phloretin 29 mg, glucose 19 mg, phloroglucinol 13·2 mg and phloretic acid 17·6 mg. All treatments differed significantly from the controls at the $P < 0{\cdot}01$ level. The phloridzin and trilobatin treatments differed at the $P < 0{\cdot}001$ level.

Table 3. Metabolites detected when apple enzymes and apple phenolics were incubated together

Starting materials	Products* Phloretin	3-Hydroxy-phloretin	3-Hydroxy-phloridzin	Phloroglucinol†	Quinones
Phloridzin	t(t)	−(+)	−(+)	t(t)	+(−)
Phloretin	t(+)	−(+)	−(−)	t(t)	+(−)
Trilobatin	t(t)	−(t)	−(−)	t(t)	+(−)
Sieboldin	−(−)	t(+)	−(−)	t(t)	+(−)
3-Hydroxy-phloridzin	−(−)	t(+)	+(+)	t(t)	+(−)
3-Hydroxy-phloretin	−(−)	+(+)	−(−)	t(t)	+(−)

* Key: + = present; t = trace amount; − = absent. Results with ascorbate added in parentheses; others are without ascorbate.

† Phloretic acid or dihydrocaffeic acid were detected in trace amounts with phloroglucinol.

sole carbon source. Analyses of culture fluids by TLC showed that only phloroglucinol remained. In another experiment, sieboldin was added to cultures grown on an orbital shaker and growth compared with a similar culture containing phloridzin. Although dry weight yields were not reproducible, sieboldin and phloridzin gave visibly greater yields than were obtained with shake cultures containing only glucose. These results contrast with those of previous work [16] on the growth, in culture, of *V. inaequalis* which indicated that "oxidation products" of phloridzin inhibited spore germination whilst phloridzin and phloretin, even when supplemented with 4% glucose, barely sustained growth of the fungus in mineral medium.

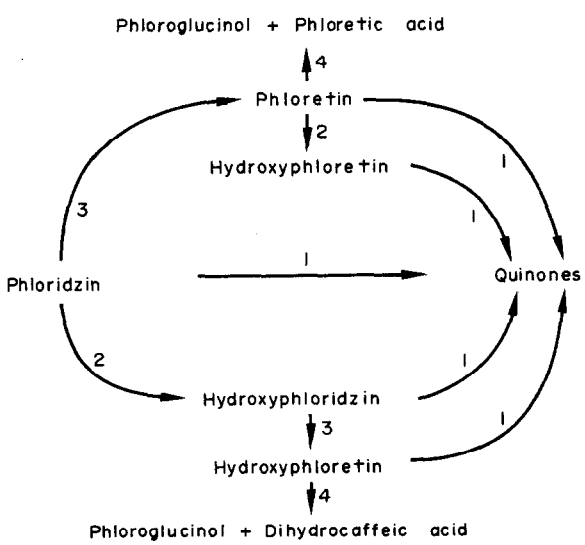


Fig. 1. Metabolism of phloridzin by apple enzymes. Enzyme key: 1 = catecholase; 2 = cresolase; 3 = β-glucosidase; and 4 = phloretin hydrolase.

Metabolism of phloridzin by apple enzymes

When leaves are damaged, either mechanically or following infection by a pathogen such as scab, disruption of membranes releases substrates onto enzymes. Therefore the metabolism of phloridzin by apple enzymes was followed by incubating macerated leaves in buffer, or by incubating apple phenolics with enzymes obtained from leaves or immature fruit. Aliquots of enzyme preparation, either particulate or "soluble", were suspended in phosphate buffer of the required pH. Ascorbic acid, a co-factor for phenolase, neutralized with alkali was added to some of the treatments. The dihydrochalcone substrates were added and the whole was kept mixed by a stream of moist air. Samples taken at intervals were examined by TLC and PC. The substances detected on TLC after metabolism of phloridzin with apple enzymes in the presence and absence of ascorbate are shown in Table 3. The enzymes β-glucosidase and phloretin hydrolase, found in *V. inaequalis*, were present but the enzyme responsible for most of the metabolism of phloridzin was a phenolase. With ascorbate present, *o*-diphenolic compounds (3-hydroxyphloridzin and 3-hydroxyphloretin) were produced. In the absence of ascorbate, oxidation proceeded directly to quinones which polymerized or conjugated with proteins and were thus precipitated from the reaction mixture. The overall metabolism of phloridzin is shown in Fig. 1.

Trilobatin was metabolized in the same way as phloridzin, and sieboldin was found to behave similarly to 3-hydroxyphloridzin [5]. Apple phenolase is similar to the phenolase complex described by Conn [17] in having both cresolase (hydroxylating) and catecholase (oxidizing) functions, the former acting in the presence of ascorbate, the latter being inhibited by it. The catecholase differs from the classical enzyme [18] in not having a lag phase. Oxygen uptake started immediately the enzyme and phloridzin were mixed.

A number of workers have studied the metabolism of phloridzin in apple enzymes and obtained varying results [1,5,16]. Most identified the products of β-glucosidase and phloretin hydrolase activity. Some mention quinone formation whilst others, by the use of reducing agents, deliberately prevented quinone production. Although "oxidation products of phloridzin" were reported to inhibit spore germination and phloridzin and phloretin were reported to inhibit growth, we found that, with the exception of the quinones which we were unable to test, all the metabolites of phloridzin and its naturally occurring analogues enhanced the growth of *V. inaequalis* in culture. Phloridzin can therefore be disregarded as a direct factor in the resistance of apples to apple scab.

EXPERIMENTAL

Apple leaves and fruit were obtained from trees grown at East Malling. Phloridzin, phloroglucinol and phloretic acid were recrystallized commercial samples. Phloretin was obtained by acid hydrolysis of phloridzin. Sieboldin and 3-hydroxyphloretin were gifts from A. H. Williams and J. C. Overeem respectively. 3-Hydroxyphloridzin was produced by incubation of phloridzin with apple enzymes in buffer containing 10% ascorbic acid.

Enzyme preparations were made by homogenizing leaves or fruits in pH 5·6 phosphate buffer (0·01 M) [18]. The homogenate was filtered through a double thickness of muslin and the particles were washed several times with buffer and then cold Me_2CO to remove endogenous phenols. The particles were then stored at 4° in 1% aq. KCl. Anti-oxidants such as Dieca or cysteine hydrochloride were not normally added during maceration as they prevented oxidation by inhibiting the phenolase.

A "soluble" enzyme could be prepared by maceration or ultrasonic treatment of the particles with detergents or sodium deoxycholate present in the buffer. This was not a true soln as the activity precipitated in the ultracentrifuge. The "soluble" enzyme was very active but "browned" and lost activity extremely quickly.

Enzyme studies were carried out in 0·1 M phosphate buffer of the required pH. Ascorbic acid, neutralized with aq. NaOH, was then added to some expts at 10% w/v [1].

Cultures were grown at 18° in Roux bottles or at the same temp. on an orbital shaker (200 throws per min—65 mm throw). Mineral medium [15] containing 4% glucose was used for cultures with additions of phloridzin etc. as required. The filter paper tubes were grown on 5% malt as described previously [14] but the tubes were secured with cotton and autoclaved after the malt was added.

Paper chromatography was on Whatman No. 1, No. 20 or 3 MM paper with BuOH–HOAc–H_2O (63:10:27), PrOH–EtOAc–H_2O (6:1:7) or 2% HOAc as solvents. TLC was on 0·25 mm plates of Si gel H (Merck) or cellulose powder No. 144 (Schleicher and Schull). Solvents were $CHCl_3$–MeOH, (5:1) containing 1% HOAc, or 2% HOAc.

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