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PHYTOALEXIN INDUCTION IN THE SAPWOOD OF PLANTS OF THE MALOIDEAE (ROSACEAE): BIPHENYLS OR DIBENZOFURANS

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IN HONOUR OF PROFESSOR ANTONIO G. GONZALEZ

Key Word Index—*Malus*; *Pyrus*; *Sorbus*; Rosaceae; Maloideae; phytoalexins; biphenyls; dibenzofurans; antifungal agents; acetovanillone; 5,7-dihydroxychromone.

Abstract—Following fungal inoculation or natural infection, five biphenyl phytoalexins (aucuparin and its 2′ and 4′ oxygenated derivatives) were induced variously in the sapwood of *Aronia*, *Chaenomeles*, *Eriobotrya*, *Malus* (three spp.) and of *Sorbus aucuparia*. By contrast, 14 dibenzofuran phytoalexins were induced variously in sapwood of *Cotoneaster* (7 spp.), *Crateagus*, *Cydonia*, *Mespilus*, *Photinia*, *Pseudocydonia*, *Pyracantha*, *Pyrus* and two *Sorbus* spp. (*S. chamaemespilum* and *S. domestica*). These were five cotonefurans, three eriobofurans, five pyrufurans and a 2,3,4,7,8-pentaoxygenated dibenzofuran trimethyl ether. No plant has yet been found to produce both types of phytoalexin, although *o*-hydroxybiphenyls are theoretically precursors of the dibenzofurans. The ability to synthesize either biphenyls or dibenzofurans appears to be genus-specific, except in the case of *Sorbus*. In 18 of the 38 species tested, these phytoalexins were accompanied by constitutive antifungal phenolics, most of which appeared to be released from bound (glycosidic) forms during the infection process. These were identified variously as hydroquinone, *p*-hydroxyacetophenone, acetovanillone, 5,7-dihydroxychromone, chrysin, sakuranetin and naringenin. Woody members of the subfamilies Prunoideae and Spiraeoideae failed to yield any phytoalexins on induction, but did contain constitutive antifungal compounds. The limited frequency of the phytoalexin response within the family as a whole is considered in relation to the accumulation of constitutive antifungal agents in these plants.

INTRODUCTION

Considering its large size (100 genera, 3000 spp.) and its economic importance (many fruit crops), relatively little is known of the disease resistance mechanisms present in the family Rosaceae. The first phytoalexin to be reported was benzoic acid, formed in apple fruit after infection by *Nectria galligena* [1]. Since then, biphenyl or dibenzofuran phytoalexins have been characterized in six species: from sapwood of *Cotoneaster* [2], *Malus* [3] and *Pyrus* [4] and leaves of *Eriobotrya* [5], *Photinia* [6] and *Rhaphiolepis* [7].

Our first phytoalexin survey of the family was concentrated on the leaves of 130 representative species, using both biotic and abiotic elicitation, but we failed to detect any *de novo* response, except in the case of *Sorbus aucuparia*, which produced the well known biphenyl, aucuparin [8]. Our results, however, suggested that about one third of the species examined contained constitutive catechin-like constituents, probably in glycosidic form, in line with earlier work on Rosaceous fruit trees. A similar study of root tissues of some more herbaceous members of the family was also unfruitful and revealed only one species *Sanguisorba minor* with a true phytoalexin response. This plant produced 2′,6′-dihydroxy-4′-methoxyacetophenone as a phytoalexin, but only in the underground tissues [9]. Finally, we turned to the sapwood of woody members of the family and were successful in eliciting biphenyls or dibenzofurans, many of novel structure, in species of *Cotoneaster* [10], *Mespilus* [11], *Photinia* [12] and *Sorbus* [13]. These plants all belong to the subfamily Maloideae and we here report the results of surveying further species of the Maloideae in the sapwood for their phytoalexins. We describe for the first time the occurrence of a number of constitutive antifungal phenolics, including 5,7-dihydroxychromone, in these woody tissues. We summarize the taxonomic and pathological findings of our various surveys.

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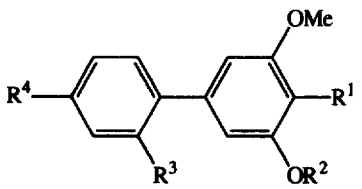
RESULTS AND DISCUSSION

The sapwoods of 29 species of the Maloideae produced a considerable range of mainly novel phytoalexins, often in large quantities (Tables 1 and 2). The phytoalexins were five biphenyls **1–5** and 14 dibenzofurans, compounds **7–14** and **16–21**. Their characterization has already been described [10–13]. It is a significant result, considering that the leaves of these same plants generally



	Biphenyl	R1	R2	R3	R4
1	Aucuparin	OH	Me	H	H
2	2'-Hydroxyaucuparin	OH	Me	OH	H
3	2'-Methoxyaucuparin	OH	Me	OMe	H
4	4'-Methoxyaucuparin	OH	Me	H	OMe
5	Isoaucuparin	H	Me	OH	H
6	Rhaphiolepsin	OH	H	H	OMe

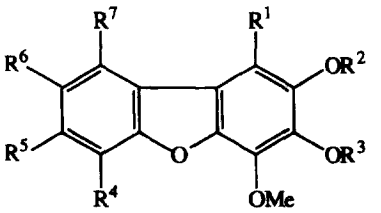
Table 1. Species of Rosaceae producing biphenyls as phytoalexins in the sapwood

Species	Biphenyls 1	3	4	2	5	Other antifungal phenolics
Amelanchier alnifolia Nuttal						5,7-Dihydroxychromone
A. ovalis Medickus						*p*-Hydroxyacetophenone, acetovanillone
Aronia arbutifolia (L.) Elliott	+	+	+			—
Chaenomeles cathayensis (Hemsley) Schneid.	+	+	+			—
C. japonica (Mast.) Lavalée		+	+			—
Eriobotrya japonica						—
Malus baccata (L.) Borkh.						*p*-Hydroxyacetophenone, flavanones
M. coronaria (L.) Mill.						5,7-Dihydroxychromone
M. domestica Borkh.	+	+				5,7-Dihydroxychromone
M. fusca (Raf.) Schneid.						Chrysin
M. orientalis (Thunb.) Lindl.						5,7-Dihydroxychromone
M. seiboldii (Regel) Rehder						Chrysin, flavanones
M. sieversii (Ledeb.) Roem.	+	+				*p*-Hydroxyacetophenone
M. silvestris Miller	+	+	+			5,7-Dihydroxychromone
M. toringoides (Rehder) Hughes						5,7-Dihydroxychromone
Sorbus aucuparia L.	+	+	+	+	+	—

failed to produce any phytoalexin [8]. Among the phytoalexins described by other workers prior to our survey, rhaphiolepsin (**6**) and α-pyrufuran (**15**) could not be detected, although closely related species to those originally examined were surveyed during the present work. Of the 29 species listed in Tables 1 and 2, nine failed to produce detectable phytoalexin in the sapwood after elicitation. Nevertheless, sapwood of these and some positive species contained constitutive antifungal agents, as will be described below.

A simple antifungal constituent was isolated during the survey from six species and identified as 5,7-dihydroxychromone (see Experimental). This chromone was detected in sapwood of *Amelanchier alnifolia* and five *Malus* species (Table 1) and seemed at first to be a new class of phytoalexin in the family. Thus, this chromone could not be detected in the healthy control tissue and nor was it released by acid treatment, assuming that a glycoside conjugate was present. However, it may not be a true phytoalexin, since it could arise by peroxidase-mediated degradation of 5,7,4'-trihydroxyflavanones [14], which are constitutively present in the wood of many Rosaceous plants [15]. Thus, we cannot be sure that this chromone is a genuine phytoalexin and it might be more suitably described as a phytoanticipin [16] until its biosynthetic origin has been established.

Turning now to the biphenyls and dibenzofurans, either one or other class are formed and their production within the Rosaceae does appear to be taxonomically significant. In general, plants in the same genus give the same type of phytoalexin, but there are two exceptions. In *Eriobotrya japonica*, there is surprising variation in the phytoalexin response of different tissues. Thus, the cortex produces the biphenyl aucuparin, while the leaf produces a dibenzofuran [5, 17]. In our survey of sapwood, however, we could not detect any phytoalexin response (Table 1). The other exception is in the genus *Sorbus*, where different species vary in their phytoalexin production, as will be discussed later. The fact that no single plant tissue so far investigated produces both biphenyls



	Dibenzofuran	R^1	R^2	R^3	R^4	R^5	R^6	R^7
7	α-Cotonefuran	H	H	Me	OMe	OH	H	H
8	β-Cotonefuran	H	Me	Me	OMe	OH	H	H
9	γ-Cotonefuran	H	H	Me	H	OH	H	H
10	δ-Cotonefuran	H	H	Me	H	OMe	H	H
11	ε-Cotonefuran	H	H	Me	OH	OMe	H	H
12	Eriobofuran	H	Me	H	H	H	H	H
13	7-Methoxyeriobofuran	H	Me	H	H	OMe	H	H
14	9-Hydroxyeriobofuran	H	Me	H	H	H	H	OH
15	α-Pyrufuran	OMe	H	Me	H	H	H	H
16	6-Hydroxy-α-pyrufuran	OMe	H	Me	OH	H	H	H
17	6-Methoxy-α-pyrufuran	OMe	H	Me	OMe	H	H	H
18	7-Hydroxy-6-Methoxy-α-pyrufuran	OMe	H	Me	OMe	OH	H	H
19	β-Pyrufuran	OMe	Me	H	H	H	H	H
20	γ-Pyrufuran	OMe	H	Me	H	OH	H	H
21	2,8-Dihydroxy-3,4,7-trimethoxy-dibenzofuran	H	Me	Me	H	OMe	O H	H

Table 2. Species of the Rosaceae producing dibenzofurans as phytoalexins in the sapwood

Plant species	Dibenzofurans															Other antifungal phenolics
	7	8	9	10	11	12	13	14	16	17	18	15	19	20	21	
Cotoneaster acutifolius Turcz.	+	+	+	+	+											
C. divaricatus Rehd. &. Wils.	+	+	+													
C. henryanus (Schneid.) Rehd. & Wils	+	+	+													
C. horizontalis Decne	+	+	+		+											
C. lactea W. W. Sm.	+															
C. splendens Flink & Hylmo	+		+													
*C. veitchii**†			+													
*Crataegus monogyna**	+		+													
*C. pontica**																
Cydonia oblonga Miller*‡				+	+										+	
Mespilus germanica L.	+								+	+	+					
Photinia davidiana§						+	+									Acetovanillone
*P. davidiana**†¶						+	+									Acetovanillone
*Pseudocydonia sinensis**					+											
Pyracantha coccinea Roemer						+		+								
Pyrus communis L.*													+	+	+	Hydroquinone
P. elaeagrifolia Pallus																Hydroquinone
P. nivalis Jacq.			+												+	Hydroquinone
P. pyraster Burgsd.															+	Hydroquinone
P. ussuriensis Maxim			+												+	Hydroquinone
*Sorbus chamaemespilus**†			+													
*S. domestica**†			+													

*Naturally infected wood tissue.
‡Plant from Royal Botanic Gardens, Kew (Accession No. 1949–16402).
†Plant from Ness Botanic Gardens, The Wirral.
¶*Photima davidiana* denote different individual samples.

and dibenzofurans suggests that, although closely related, these two classes of phytoalexin are formed by parallel, rather than by sequential, pathways.

In *Malus* sapwood, the occurrence of 5,7-dihydroxychromone does not show any correlation with either biphenyl phytoalexins or with constitutive flavanones [18]. In addition to the results reported in Table 1, a few naturally diseased wood tissues were also analysed. *Malus silvestris* infected with *Nectria galligena*, gave three biphenyls, aucuparin, 2'- and 4'-methoxyaucuparins. A similar result was obtained with the same plant inoculated with *Phomopsis perniciosa*. Diseased *M. domestica*, infected with *N. galligena*, on the contrary, failed to give even a trace of a biphenyl. *Malus baccata* var. *mandshurica*, *M. fusca* and *M. sieboldii* did not produce any of the antifungal compounds mentioned above. The latter two, however, released an antifungal flavone chrysin upon either inoculation or other stress treatment. The occurrence of chrysin, as its 5-glucoside (toringin), in *M. fusca* was described earlier by Williams [19] and the result obtained here agrees with this. As for *M. baccata*, a trace of *p*-hydroxyacetophenone was detected after examining the bands on TLC plates with UV absorption spectra. However, the amount was too low for detection by means of direct bioassay on the developed TLC plates. *p*-Hydroxyacetophenone was also detected in *M. sieversii*. It should be noted that the dihydrochalcone phloridzin was detected in all *Malus* species examined as a strong constitutive antifungal component. Above all, *Malus* is always a biphenyl producer and distinction can be made with *Pyrus*, which always produces dibenzofuran phytoalexins. These two genera are also distinguished by differences in the phenolic pattern, i.e. dihydrochalcones in *Malus* and hydroquinone in *Pyrus* [20].

The genera *Chaenomeles* and *Cydonia*, treated as '*Cydonia* group' by Kalkman, are the source of considerable taxonomic and nomenclatural confusion [21]. However, they have different phytoalexin patterns. *Chaenomeles* gives biphenyls while *Cydonia* produces dibenzofurans. *Pseudocydonia sinensis*, now often included in *Chaenomeles* as *C. sinensis* (Dum.-Cours.) Koehne, produced dibenzofurans, and shares ε-cotonefuran with *Cydonia oblonga* (Table 2).

Biphenyls are produced by *Aronia* and this is perhaps a good marker of the affinity between this genus and *Sorbus*. Thus, a phytogeographical hypothesis that *Crataegus* (North America) diverted to *Aronia* and *Malacomeles* (Central America) and *Hesperomeles* (South America) while migrating southward [22] cannot be supported. The parental stock of *Aronia* is more likely *Sorbus* in view of the phytoalexin pattern. *Aronia* is closely allied to *Sorbus* and sometimes included in it [23, 24]. It is interesting to note that *Crataegus* and *Hesperomeles* produce two flavone-*C*-glycosides (vitexin and orientin) while *Sorbus*, *Aronia* and *Malacomeles* produce vitexin only, in leaves [18].

The diversity of the genus *Sorbus* (*sensu lato*) is perhaps worth mentioning. Prior to our systematic survey, biphenyls were isolated from only a single subgenus *Sorbus* (*S. aucuparia*, *S. decora*, *S. americana* and *S. scopulina*) either as the constitutive compounds or as the phytoalexins [13, 15–27]. However, as shown in Table 1, *S. domestica* (subgenus *Cormus*) and *S. chamaemespilus* (subgenus *Chamaemespilus*) give the dibenzofuran γ-cotonefuran (Table 2). An analysis on a few naturally diseased wood tissues gave further interesting results, although complete structural determination is yet to be done. The UV absorption spectra of the semi-purified, most prominent fungitoxins in the sapwood of *S. intermedia* (subgenus *Aria*), that could not be detected in the healthy tissue, somewhat resemble those of a dibenzofuran (data not shown). On the other hand, *S. torminalis* (subgenus *Torminaria*) produced two phytoalexins whose structures were unassignable to either biphenyl or dibenzofuran from their UV spectra. Their chemical identities are now being investigated. It has been suggested that these subgenera of *Sorbus* species should be separated and raised to the rank of genera, as treated by some authors, in recognition of their morphological and chemical diversity [28] and possible polyphyletic origin [29]. The phytoalexin patterns found here favour the separation of *Sorbus* into smaller genera.

So far, no plants have been observed to produce both types of phytoalexin, biphenyls or dibenzofurans, in a single infection, despite the similarity in chemical structures. This apparent dichotomy can be tested, since the plants of the Maloideae often give rise to sexual hybrids of biphenyl producers and dibenzofuran producers [28]. Analysis of naturally infected or damaged tissue of two such trihybrid species (*Sorbus commixta* × *Pyracantha* × *Photinia*, *Sorbus commixta* × *Pyracantha* × *Sorbus*) produced only eriobofuran, a dibenzofuran that has not hitherto been observed in the subgenus *Sorbus*. It seems likely that a factor (or factors) required in the biosynthesis of dibenzofurans was carried through in the hybrids from *Pyracantha* which regularly produces dibenzofurans [12].

It seemed interesting from the phylogenetical point of view, to see if Prunoideae and Spiraeoideae, possible parental subfamilies to the Maloideae, produces similar phytoalexins. However, these subfamilies (16 and 7 species were examined, respectively, see Experimental), in contrast to the Maloideae, gave no sign of phytoalexin production although extensive necrosis was observed after infection. The Prunoideae all possess strong antifungal compounds constitutively, and they are released from bound forms when attacked by microorganisms. Most of them are flavanones such as naringenin and sakuranetin, isoflavonoids such as biochanin A and prunetin and coumarins [30]. Scopoletin produced in the diseased *Prunus* species [31] may now be regarded as a post-inhibitin. Strong blue fluorescence was often observed on our chromatograms, especially in fungus-challenged wood. However, the same fluorescent spots were present in the healthy wood extract, although the fungitoxicity was not apparent in the bioassay. Wood tissue of *Prunus* is generally rich in flavanones [15] but all failed to produce 5,7-dihydroxychromone upon the same treatment. Therefore, this compound is not

a fungal degradation product; the difference lies in the peroxidase system or the flavonoid pattern in the sapwood tissue.

The members of the Spiraeoideae, by contrast, gave very little fungitoxin in the methanol extracts, acid-treated tissue, or in naturally diseased stem tissue. A re-examination of these plants was thus carried out by means of UV absorption spectra-orientated fractionation in a search for biphenyl or dibenzofuran-looking bands, as the direct bioassay on the TLC plates requires rather a large amount of compound. However, this resulted in no trace of biphenyl or dibenzofuran-like spectra; nor were those of a chromone obtained. No chemical link could be found to support the allopolyploid origin of the Maloideae, in terms of phytoalexin production. The available data positively indicate the lack of phytoalexin synthesis in these subfamilies, since exactly the same procedures were regularly used to induce phytoalexins as with the Maloideae plants. Hence, the ability to produce phytoalexins, biphenyls or dibenzofurans, is confined in the Maloideae, and has been acquired after the Maloideae arose from the ancestral Rosaceous stock.

Catechins (flavan-3-ols), a series of moderately antifungal compounds, are the most frequently observed constituents from the Rosaceae in connection with disease resistance mechanisms [32–34]. They occur constitutively but the production may be boosted in various tissues, when attacked by microorganisms. They are also present in the wood tissue [35]. When it is considered that the time required in the production of biphenyls and dibenzofurans is long, it seems that catechins in large amounts provide an immediate barrier and the phytoalexins constitute a secondary line of defence. Several other antifungal compounds, phenolic in nature, were found in members of the family, including acetovanillone, hydroquinone and phloretin (Tables 1 and 2). Their activities as isolated compounds may be low but they co-occur and the sum effect of them on the invading microorganisms may be significant.

In summary, our surveys of leaf, root and sapwood tissues of representative members of the Rosaceae indicate that the production of biphenyl and dibenzofuran phytoalexins, with hydroxy/methoxy substitution, is a characteristic feature and unique so far to this family. Nevertheless, it is clear that the phytoalexin response is of relatively minor importance in protecting these plants from microbial infection. Instead, the presence of constitutive low-molecular-weight antifungal phenolics appears to have a significant role in disease protection. This is in sharp contrast to other angiosperm plant families such as the Leguminosae and Solanaceae where the phytoalexin response can be regularly elicited. In the Leguminosae, well over 500 species have been induced to produce isoflavonoid or related phytoalexins [30]. Macromolecular barriers to microbial infection may also be present in many of these plants and it is yet to be determined how much low-molecular-weight constituents, either as constitutive agents or as phytoalexins, contribute to overall disease resistance.

EXPERIMENTAL

Plant materials examined were all raised from seed and were collected mostly from the Harris Garden, and the same plants were used as those used in the survey on leaves [8]. The mature stem tissue [36] of three subfamilies, Maloideae, Prunoideae and Spiraeoideae were surveyed. In the experiment involving artificial inoculation and examination of intact tissue, the material was carefully chosen to use only healthy, non wounded wood. A precaution to remove all the leaves from the branch in advance of cutting the branch off was taken in order to avoid water deficiency stress, and the wood harvested was processed and inoculated immediately.

Fungal spores (*Phomopsis perniciosa* and *Nectria cinnabarina*) were used as the phytoalexin inducer. The spores of *P. perniciosa* (Accession No. 321467, International Mycological Institute, Surrey, U.K.) were obtained by flooding 10-day-old cultures growing on PDA medium in a Petri dish. Occasionally, *N. cinnabarina*, the coral spot pathogen of *Acer*, *Betula* and other hardwood species, was used as the inducer. In this case, actively growing fruiting bodies on ornamental *Cotoneaster* sp. were mechanically removed and suspended in deionized water for 1 hr. An even suspension of spores was obtained by grinding the fruiting body with a mortar and pestle. For both fungal species, the spore suspensions were filtered to remove undesired mycelia or debris of unground fruiting bodies. The spore density was kept at more than 10^5 ml^{-1}. The stem harvested was immediately washed briefly to remove mosses and other potential sources of contamination, and the cambium and/or xylem was exposed by scraping off the bark. The spore suspension was placed on the exposed xylem tissue, and incubated at 20–24° for different periods in 60 ml jars containing *ca* 5 ml of water to sustain moisture.

Incubation was terminated at 1 or 2 months after inoculation [11–13]. The wood tissue was then cut into small pieces with secateurs and subsequently extracted with MeOH in the dark for 1 week. The extract was filtered and concentrated *in vacuo*, and the EtOAc-soluble fraction was examined for possible phytoalexins. The healthy tissue was also extracted with MeOH to synchronize with the fungus-treated materials. Biologically active compounds were detected with *Cladosporium herbarum* as the test fungus in the methods described by Homans and Fuchs [37].

After the initial survey, scaling up was practised to obtain large amounts of pure phytoalexins sufficient to characterize them chemically. Basically, the same procedures were used and, in general, a treatment on 1 kg of wood tissue gave *ca* 50 g of necrotic sapwood tissue (wet wt) affording maximum 10–60 mg of pure compounds.

The plants subjected to the survey are listed in Tables 1 and 2 (Maloideae). In addition to this, the sapwood of the following species were also investigated: Spiraeoideae: *Neillia thibetica*, *Physocarpus malvaceus*, *Sibiraea altaiensis*, *Spiraea bella*, *S. betulifolia*, *S. nipponica* and *S. pubescens*; Prunoideae: *Osmaronia cerasiformis*, *Prinsepia uniflora*, *Prunus armeniaca*, *P. autumnalis*, *P.*

avium, *P. divaricatus*, *P. domestica*, *P. lusitanica*, *P. padus*, *P. persica*, *P. rufa*, *P. serrulata*, *P. spinosa*, *P. tenella*, *P.* 'Ichiyo' and *P.* 'Taihaku'.

Flavonoids (chrysin, naringenin, sakuranetin, biochanin A) were confirmed on the basis of UV spectroscopic measurement and the direct comparison with authentic samples by means of co-chromatography. 5,7-Dihydroxychromone was isolated from the shell of common peanuts, *Arachis hypogaea*, with the modification of the method described by Pendse *et al.* [38], replacing column chromatography with prep. TLC.

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