Sitosterol and Quercetin 3-Galactoside, Obscure Root Weevil Feeding Stimulants from *Rhododendron*

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Compounds that stimulate feeding by the obscure root weevil (*Sciopithes obscurus* Horn) on membrane filters have been identified in fractions chromographically separated from ethanolic and chloroform–methanolic extracts of *Rhododendron*, cv. Cynthia, leaves. The most prominent polar (ethanol-soluble) phagostimulant obtained by paper chromatography was quercetin 3-galactoside. The principal nonpolar (lipid fraction) phagostimulant was identified as sitosterol.

The obscure root weevil (*Sciopithes obscurus* Horn; family Curculionidae) is a serious pest of *Rhododendron* and a number of other ornamental plants in the Pacific Northwest. Adult weevils cause unsightly damage by feeding on leaves, and larval feeding below ground can damage plant roots. Doss (1980) found that several chromatographically distinct components extracted from *Rhododendron* Cynthia leaves could promote obscure root weevil feeding on membrane filter disks. One of the phagostimulatory components was sucrose. This paper describes work that resulted in isolation and characterization of two additional obscure root weevil phagostimulants.

MATERIALS AND METHODS

Plant Material and Insects. Current year *Rhododendron* cv. Cynthia (*Rhododendron catawbiense* Michaux. × *Rhododendron griffithianum* Wight.) leaves were obtained from a planting at Western Washington Research and Extension Center, Puyallup, WA. Leaves were cut into pieces and extracted immediately after collection.

Adult obscure root weevils were collected from the field. Colonies of weevils were fed *Rhododendron* leaves and kept at 10–15 °C in continuous darkness. Under these conditions colonies could be maintained for up to 1 year.

Extraction. Leaf pieces were extracted with ethanol in a Soxhlet apparatus, and the extract was partitioned 4 times against hexane. The ethanol layer was reduced in volume under vacuum at 30 °C. The ethanolic extract had a final concentration of 500 mg fresh weight equiv/mL. (One milligram fresh weight equivalent is the amount extracted from 1 mg of fresh tissue.)

A lipid extract was obtained by homogenizing leaves in chloroform–methanol (2:1 v/v). The extract was filtered through a pad of Celite, and the residue was washed with the homogenizing medium. Extract and washings were combined and taken to near dryness under vacuum at 30 °C. The residue was suspended in water, and the aqueous suspension was partitioned 4 times against hexane. The combined hexane fractions were taken to dryness under vacuum at 30 °C, and the oily residue was dissolved in chloroform. The concentration of this crude lipid extract was 1250 mg fresh weight equiv/mL.

Paper Chromatography. A portion of the ethanolic fraction was streaked onto Whatman 3MM chromatography papers. A 1-butanol–acetic acid–water (4:1:5 v/v/v, upper layer) solvent system was used for development. Sections corresponding to specific R_f regions were then cut from the papers and extracted exhaustively with 95% ethanol. The ethanolic fractions were concentrated under vacuum and bioassayed (see below). The fractions containing phagostimulatory materials were further separated by chromatography in 15% (v/v) acetic acid. After development, papers were examined under ultraviolet light and cut into sections corresponding to specific bands. These sections were extracted with ethanol, and the concentrated extracts were bioassayed.

Column and Thin-Layer Chromatography. The crude lipid extract was subjected to column chromatography using 38 g of acid-washed Florisil in a 2.5-cm diameter (i.d.) column (Carroll, 1963; Christie, 1973). Simple lipids were eluted with chloroform and complex lipids with methanol. Several subfractions of simple and complex lipids were collected. Concentration under vacuum, and bioassay, revealed a phagostimulatory simple lipid fraction that was dissolved in hexane and subjected to chromatography on 10 g of Florisil (rehydrated to 5%, w/v) using a 1.0-cm diameter (i.d.) column (Carroll, 1961; Christie, 1973). Hexane, with increasing proportions of diethyl ether, diethyl ether, and 2% acetic acid in diethyl ether were used as eluents. Eluates were collected in five column volume fractions.

These fractions, obtained from chromatography on Florisil, were concentrated and bioassayed. A phagostimulatory fraction was then subjected to preparative thin-layer chromatography on Whatman $KC_{18}F$, reverse-phase thin-layer plates using a 1-butanol–water (80:20 v/v) solvent system. After development the plate edges were removed, sprayed with sulfuric acid–methanol (1:1 v/v), and heated at 110 °C until bands became visible. Sections of adsorbent, corresponding in location to visualized bands, were scraped from the plates, extracted with chloroform–methanol (1:1 v/v), and taken to near dryness under vacuum at 30 °C. Chloroform-reconstituted fractions were then bioassayed.

Liquid and Gas Chromatography. Analysis of the purified ethanol-soluble fraction employed a liquid chromatographic procedure modified after that of Stewart et al. (1980). A Waters 30 cm (long) × 3.9 mm (i.d.) μBondapak C_{18} column was used with a linear solvent gradient ranging from 10 to 30% acetonitrile in 2% acetic acid; a flow rate of 2.0 mL/min and an ultraviolet absorbance detector set at 340 nm.

Gas chromatographic analysis of the purified lipid phagostimulant was carried out by using a 0.91 m (long) × 2 mm (i.d.) glass column packed with 3% SP 2250 on 100/120 Supelcoport. Conditions were as follows: injector temperature of 270 °C, column temperature of 260 °C, flame ionization detector temperature of 270 °C, and he-

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lium carrier gas, at a flow rate of 40 mL/min.

Bioassay. Fractions were bioassayed for their ability to stimulate obscure root weevil feeding by using the membrane filter disk procedure of Bristow et al. (1979). Each bioassay arena contained as many disks as were needed to evaluate all the fractions derived from any given chromatographic step (e.g., if chromatography yielded six fractions, each arena would contain six disks, each bearing an aliquot of a different fraction). For statistical analysis each bioassay arena was treated as a block.

For bioassays of the ethanol-soluble fractions obtained from paper chromatography, each membrane filter disk bore 50 mg fresh weight equiv. Disks bore approximately 146, 219, and 438 mg fresh weight equiv for bioassay of fractions obtained from chromatography on acid-washed Florisil (Carroll, 1963; Christie, 1973), Florisil (Carroll, 1961), and reverse-phase thin-layer plates, respectively. These figures assume 100% recovery.

Reference Compound Synthesis. Quercetin 3-galactoside and quercetin 3-glucoside were synthesized by using the method of Ice and Wender (1952). Paper chromatography in butanol–acetic acid–water (4:1:5 v/v/v, upper layer) and 15% (v/v) acetic acids was used to isolate the quercetin 3-glycosides from the other reaction products.

Additional Information. Solvents were reagent grade and, except for diethyl ether, were redistilled from glass. All solvents used in preparation of the lipid phagostimulant contained 1 ppm of the antioxidant, 2,6-di-*tert*-butyl-4-methylphenol (BHT). When lipid fractions were concentrated in vacuo, the vacuum was broken with nitrogen. Nitrogen was layered over lipid fractions during storage at −10 °C. Also, membrane filters used for bioassay of lipid fractions were pretreated with 50 μg of BHT antioxidant.

Commercial preparations of sitosterol and stigmasterol (ICN Pharmaceuticals) were recrystallized from methanol after clarification with Norite. A sample of quercetin 3-galactoside, obtained from B. A. Bohm, University of British Columbia, and commercially prepared quercetin 3-rhamnoside (ICN Pharmaceuticals) were used without further purification.

Chromatographic fractions used for the bioassays discussed herein were chosen on the basis of preliminary chromatographic runs and bioassays. All portions of the study were repeated at least twice, with qualitatively identical results.

RESULTS AND DISCUSSION

The variability in amounts eaten from membrane filters from bioassay to bioassay results, in part, from differences in the average age of the weevils used for each bioassay. Younger weevils tend to eat more from membrane filters than older weevils. Hence, bioassays conducted shortly after collection of the weevil colonies, in late spring and summer, exhibit more feeding than later bioassays run with older colonies. Other, as yet undefined, factors also influence weevil feeding.

Paper chromatography of an ethanolic extract of *Rhododendron* Cynthia leaves using butanol–acetic acid–water yielded two crude fractions that were prominently active as obscure root weevil phagostimulants (Figure 1). When the most active fraction, extracted from the R_f region 0.5–0.7, was subjected to further paper chromatography in 15% acetic acid, at least 12 bands visible under ultraviolet light were resolved. Bioassay of these bands (some as single bands, others as multiple band composites) showed that maximum phagostimulatory activity was associated with a single band at $R_f \sim 0.44$ (Figure 2). This

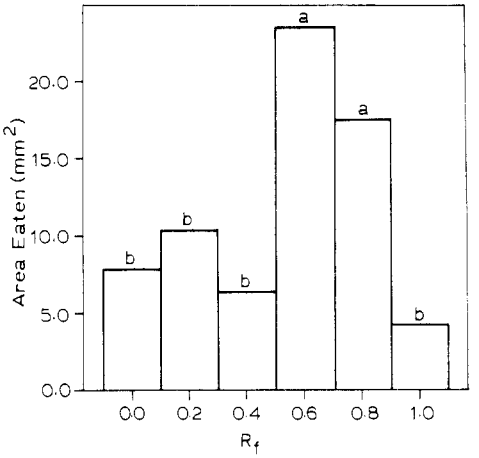

Figure 1. Area eaten by obscure root weevils from membrane filters bearing fractions obtained through paper chromatography of an ethanolic extract of *Rhododendron* Cynthia leaves using butanol–acetic acid–water. Each bar represents the mean for five replicates. Bars bearing different letters represent significantly different amounts of feeding (95% level) as determined with Duncan's multiple range test.

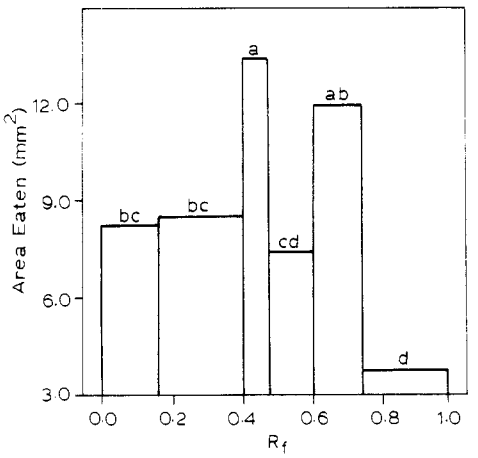

Figure 2. Area eaten by obscure root weevils from membrane filters bearing fractions obtained through paper chromatography of fractions 4 (see Figure 1) using 15% acetic acid; see the caption of Figure 1 for statistical information.

material had the ultraviolet spectral characteristics of quercetin 3-galactoside (Mabry et al., 1970) and exhibited a retention time identical with that of authentic quercetin 3-galactoside when subjected to HPLC. An earlier preparation had yielded a material identical in retention time and in ultraviolet spectral characteristics with quercetin 3-galactoside but it also contained a second component having the liquid chromatographic behavior of quercetin 3-glucoside (Figure 3). With the earlier preparation the behavior upon chromatography in 15% acetic acid was typical of quercetin 3-galactoside (R_f = 0.35; Harborne, 1973). The atypical R_f value noted above (i.e., $R_f \sim 0.44$) was undoubtedly due to the poor technique used in the chromatographic run; i.e., failure to ensure chamber saturation and failure to equilibrate papers.

If the molar absorption coefficient of quercetin 3-galactoside is assumed to be 2.04×10^4 at 259 nm, and if it is assumed that the compound exists as the 2.5 hydrate (Stecher, 1968), the amount applied to the filters in the bioassay described in Figure 2 was about 175 μg. One hundred fifty micrograms of authentic quercetin 3-galactoside applied to membrane filters stimulated obscure root weevil feeding. The 3-glucoside and 3-rhamoside of quercetin also stimulated feeding.

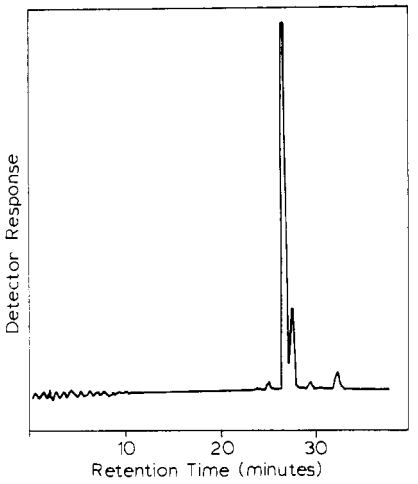

Figure 3. Liquid chromatography of an ethanol-soluble phagostimulant. The major peak represents quercetin 3-galactoside; the shoulder represents quercetin 3-glucoside.

Table I. Phagostimulatory Activity of Fractions Obtained from Acid-Washed Florisil Chromatography of a Lipid Extract of *Rhododendron* Cynthia Leaves

fraction[a]	eluting solvent	area eaten,[b] mm²
1 (0-90)	chloroform	4.1 c
2 (90-360)	chloroform	12.7 b
3 (360-790)	chloroform	40.5 a
4 (0-60)	methanol	6.2 bc
5 (60-100)	methanol	11.6 bc
6 (100-300)	methanol	12.3 bc

[a] Values in parentheses indicate the order of elution of fractions and volumes (milliliters) of eluent used. For example, fraction 2 was eluted with 270 mL of chloroform with collection beginning after 90 mL had passed through the column and terminating after 360 mL had passed through the column. [b] Each value is a mean for five membrane filters. Means bearing different letters are significantly different (95% level) as determined with Duncan's multiple range test.

The fact that the 3-glucoside and 3-rhamoside of quercetin, as well as the 3-galactoside, can stimulate weevil feeding suggests that the quercetin 3-glycosides, or, perhaps, the flavonol 3-glycosides, as a group are phagostimulatory. It is possible that quercetin 3-galactoside was identified as the principal phagostimulant because it was present at the highest concentration.

Silkworm larvae (*Bombys mori* L.), like the obscure root weevil, are stimulated to feed by quercetin 3-glucoside (Hamamura et al., 1962). It is not known whether quercetin 3-galactoside can stimulate feeding of silkworm or any insect other than the obscure root weevils, nor is it known to what extent the phagostimulatory activity of flavanol glycosides is due to the presence of the sugar moiety in the molecule. Both glucose and galactose can stimulate obscure root weevil feeding (Doss, 1980).

Six crude fractions were obtained from chromatography of a lipid extract of *Rhododendron* "Cynthia" leaves on acid-washed Florisil (Table I). A simple lipid fraction (fraction 3) stimulated large amounts of weevil feeding.

Chromatography of fraction 3 on a Florisil column yielded a single phagostimulatory fraction (fraction 4) that was eluted with 15% diethyl ether in hexane (Table II). Five distinct bands were visualized when this fraction was subjected to preparative thin-layer chromatography and the plate edges were removed and charred. A single, prominent phagostimulatory band at the R_f range 0.18–0.27 was obtained when six regions from the plate were eluted

Table II. Phagostimulatory Activity of Fractions Obtained from Chromatography on Florisil

fraction	eluting solvent	column volumes of eluent	area eaten,[a] mm²
1	hexane	5	7.1 bcd
2	5% diethyl ether in hexane	5	6.0 d
3	5% diethyl ether in hexane 15% diethyl ether in hexane	2.5 2.5	9.8 bc
4	15% diethyl ether in hexane	5	30.7 a
5	30% diethyl ether in hexane	5	8.3 bcd
6	30% diethyl ether in hexane	5	10.1 b
7	30% diethyl ether in hexane	5	7.5 bcd
8	100% diethyl ether	5	6.4 cd
9	2% acetic acid in diethyl ether	5	5.2 d

[a] Each value is a mean for five membrane filters. Means bearing different letters are significantly different (95% level) as determined with Duncan's multiple range test.

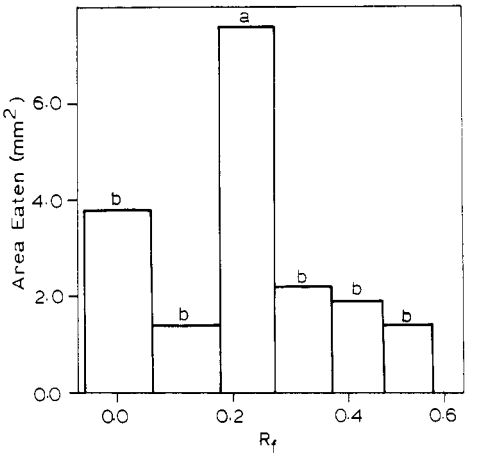

Figure 4. Area eaten by obscure root weevils from membrane filters bearing fractions obtained by thin-layer chromatography of fraction 4 (see Table II); see the caption of Figure 1 for statistical information.

and subjected to bioassy (Figure 4). This fraction (fraction 3) had a faint yellow color, probably due to slight contamination with a xanthophyll. The phagostimulatory material chromatographed to the same R_f as sitosterol and stigmasterol in several thin-layer chromatographic solvent systems. Gas chromatography of this material (from the R_f range 0.18–0.27) indicated the presence of a single component with retention time identical with that of authentic sitosterol and distinct from those of stigmasterol or campesterol. The amount of sitosterol applied to the membrane filter disks in the bioassay (Figure 4) was estimated to be 188 μg. In a test of the phagostimulatory activity of authentic sitosterol, 100 μg stimulated obscure root weevil feeding. Stigmasterol also stimulated feeding.

Insects require an exogeneous source of phytosterols for normal growth and development (Robbins et al., 1971). Thus, sitosterol, like sucrose, which is an obscure root weevil phagostimulant (Doss, 1980), is both a phagostimulant and a nutrient. Ito et al. (1964) has shown that sitosterol stimulates feeding of silkworm larva.

Considering the polyphagous habit of the obscure root weevil (van Dyke, 1935; Bell and Clarke, 1978), it is not surprising that compounds ubiquitous in the higher plants would act as phagostimulants. The fact that only a few of these common phytochemicals are prominently active is consistent with the idea that polyphagous insects, like

oligophagous ones, depend on a few distinct chemical cues for host-plant selection (Thorsteinson, 1960).

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Gas–Liquid Chromatographic Methods for the Determination of Disulfoton, Phorate, Oxydemeton-methyl, and Their Toxic Metabolites in Asparagus Tissue and Soil

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GLC methods were developed for the determination of individual insecticide residues in asparagus tissue and soil for three groups of compounds: (I) disulfoton and (II) phorate and their sulfoxides, sulfones, oxons, oxon sulfoxides, and oxon sulfones and (III) oxydemeton-methyl and its sulfone. Residues were extracted by blending with ethyl acetate, except soil samples fortified with III, which were extracted with a mixture of 20% methanol in ethyl acetate. Cleanup and separation of individual compounds within each group were achieved by column chromatography using a 2:5 (w/w) mixture of Nuchar C charcoal and Whatman CF-11 cellulose. After separation, disulfoton sulfoxide, disulfoton oxon sulfoxide, and oxydemeton-methyl were oxidized with aqueous $KMnO_4$ to their corresponding sulfones. Gas chromatographic detection was accomplished by using an alkali flame ionization detector and a GLC column (75 cm × 2 mm i.d.) packed with 2% OV-101 on 80–100-mesh Ultra-Bond 20M. Recoveries of the individual compounds ranged from 83.7 to 110%.

Disulfoton [*O,O*-diethyl *S*-[2-(ethylthio)ethyl] phosphorodithioate] is a systemic insecticide and acaricide marketed in North America by the Mobay Chemical Corp. under the trade name Di-Syston. It is used mainly as emulsifiable concentrates for foliage treatment and as granules for soil application to protect plants from insect attack. Currently disulfoton and two other structurally related systematic insecticides, phorate [*O,O*-diethyl *S*-(ethylthio)methyl phosphorodithioate, Thimet] and oxydemeton-methyl [*S*-[2-(ethylsulfinyl)ethyl] *O,O*-dimethyl phosphorothioate, Metasystox-R], are under evaluation as control agents against the asparagus aphid, *Brachycolus asparagi*, in British Columbia.

In order to investigate the degradation, metabolism, and persistence of the three pesticides following their applications for the control of asparagus aphid, sensitive analytical methods for these chemicals and their toxic metabolites were essential. Using gas–liquid chromatography (GLC) and flame photometry, Bowman et al. (1969a) individually determined disulfoton and oxydemeton-methyl with their toxic metabolites, after these had been separated by liquid chromatography on silica gel. The same methods with minor modifications were successfully applied in the determination of phorate and five of its metabolites in corn (Bowman et al., 1969b). The major disadvantage of these methods was the poor chromatographic response of the metabolites, especially of the two sulfoxides. Reproducible response was difficult to obtain even after conditioning of the GLC column by injecting successively several plant extracts. So that this disadvantage could be overcome, residues of these compounds were all oxidized to the more stable oxygen analogue sulfone with *m*-chloroperbenzoic acid (Bowman and Beroza, 1969) or to their corresponding sulfones with aqueous $KMnO_4$ (Thornton and Anderson,

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