Oviposition Responses of *Coleomegilla maculata lengi* (Coleoptera: Coccinellidae) to the Wood and Extracts of *Juniperus virginiana* and to Various Chemicals[1]

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ABSTRACT

Methods are described to determine the effects of different substances on the oviposition behavior of *Coleomegilla maculata lengi* Timberlake. The preparation, bioassay, and chemistry of various materials from the wood of *Juniperus virginiana* L. are reported. Two preparations (A and B) acted as oviposition stimulants whereas surfaces treated with another fraction (C) of the extract were avoided. Materials A and B were high-molecular-weight polyphenols and showed general similarities to phlobaphenes from other sources. Material A caused apterous adult *Acyrthosiphon pisum* (Harris) to form aggregates on treated filter paper. *O*-Coumaric, salicylic, and protocatechuic acids, fluorescein, tannin, and widdrol at concentrations of 2.0–10.0 mg/50 cm² influenced *C. m. lengi* to lay eggs on or near treated surfaces. Contact with fluorescein (2.0 mg/50 cm²) increased the proportion of laying *C. m. lengi* females.

Coccinellids form an important part of a whole complex of natural controls that may be used to advantage by man in the biological and integrated control of aphids, scales, and mites (Hodek 1970). Coccinellid density may be augmented by spraying eggs on aphid-infested crops (Shands et al. 1972), or by spraying crops with materials that are preferred by coccinellids for oviposition, thereby increasing the local density of naturally occurring populations of these predators. We are concerned here with the latter problem. Previously it was established that females of 4 coccinellid species preferred the wood of *Juniperus virginiana* L., to other woods and even to aphid-infested plants for oviposition (Boldyrev et al. 1969). This report describes the bioassay methods and the isolation and chemical nature of active components from *J. virginiana* wood. Other biological effects and active substances also are reported.

MATERIALS AND METHODS

Bioassay of Materials.—Methods were developed to determine the effects of various materials extracted from *J. virginiana* on the oviposition behavior of *Coleomegilla maculata lengi* Timberlake adults confined singly in small cages. Larvae were reared in the laboratory on live pea aphids, *Acyrthosiphon pisum* (Harris) (Smith 1965), and females also were collected as mated adults from marsh marigold, *Caltha palustris* L., and corn, *Zea mays* L. Laboratory-reared females were mated and all insects were fed on live *A. pisum* for 2 weeks before use in the bioassay. Using specially designed cages, tests were conducted for the following: (1) to determine if female *C. m. lengi* prefer a selected material (wood panel or treated glass panel) for oviposition, to a blank of glass, (2) to test materials that might serve as ovipositional stimulants or deterrents (Dethier et al. 1960), (3) to determine if the magnitude of a response is related to the concentration of a material, and (4) to determine if some materials affect the number of eggs laid per female per day. The tests were done in total darkness at 25±1°C and 60±10% RH. Between tests the insects were kept singly in dishes with live *A. pisum* at 20±1°C and in fluorescent light with a light:dark cycle of 16:8 hr.

The cage has been illustrated (Nicholls 1970). It was constructed of plastic and the inside dimensions were ca. 5×10×10 cm. The two 5×10-cm end walls were removable panels with inner surfaces of glass. The 10×10-cm side walls were perforated for ventilation. The inner glass surface of one end panel was spread with 0.4 ml of a solution containing the test material while the other end panel was treated with the solvent (usually ethanol) alone. The concentration was expressed as milligrams of test material per 50 cm² of surface. After the solvent had evaporated 1 ♀ and its food (10 adult *A. pisum*) were released into a cage. A test lasted for 24 hr and 12 such tests were conducted at the same time. Each material was assayed 3 times, usually with the same 12 ♀, yielding a maximum of 36 egg batches. The locations and numbers of all *C. m. lengi* eggs in the cages were recorded. To determine whether contact is necessary for a material to affect oviposition, females were tested in cages with and without screens over both end panels. The screens were of fine-mesh cotton and were located ca. 6 mm from the panels. To determine whether an aggregation of aphids affects the egg distribution of *C. m. lengi*, tests were conducted with 10 *A. pisum* confined to one end of a cage between the end panel and a screen. Tests with only glass blanks and with only blanks and screens at the cage ends were done to show that cages without treated panels were free from bias (Table 1).

The results of tests on a material were averaged and are summarized as percent of females ovipositing, the ratio usually expressed as a quotient of egg batches and eggs in the cage half adjacent to the material to those in the other half (response ratio (Byrne 1969)), and the mean number of eggs per female per day (Table 1). A material was con-

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sidered to have affected the selection of oviposition sites and the response to it was considered to be positive when the ratios for batches and eggs were ≥ 2 and negative when the ratios were ≤ 0.5 in 24 of 36 tests.

Materials A, B, and C, prepared from extracts of *J. virginiana* and the chemicals listed in Table 1 were tested. The latter compounds were mainly phenolic and most were naturally occurring compounds that possibly could be utilized by coccinellids in the selection of oviposition sites. In addition, oil of cedarwood and 2 of its constituents, cedrol and widdrol, were tested. Panels of the wood of *J. virginiana* were made to replace one of the glass end panels of the cages and the response to this wood was used as a standard for comparison with other materials. Two-choice tests were conducted to compare the effect of the wood panels with fluorescein or *o*-coumaric acid applied to the glass panels.

Tests were conducted to see if fluorescein at a concentration of 2 mg/50 cm^2 affected either the percent of insects ovipositing or the number of eggs per female per day. The same 12 ♀ were tested first in cages with untreated panels and then with panels which had been treated with fluorescein. Using the same insects, each pair of tests was repeated 3 times at weekly intervals.

Material A was included in a water formulation at a concentration of 0.5% with Tween® 80 as an emulsifying agent at 0.12 ml/liter. Tests were conducted to confirm the observation that the distribution of *A. pisum* is affected by Material A. Half the surface of a 9.0-cm (diam) filter paper in a petri dish containing apterous adults of *A. pisum* was treated with 0.4 ml of water formulation. Four observations were made on 3 such groups ($n = 120$) and the locations of the aphids were classified as on or over the treated and untreated halves of the paper. This procedure was repeated using water with Tween 80.

Isolation of Active Components of Juniperus Wood.—One hundred g of sawdust of *J. virginiana* (kiln-dried) were shaken for 72 hr at room temperature in 1.5 liter of absolute ethanol. The filtrate was taken to near dryness on a rotary evaporator with the temperature not exceeding 30°C. The red, viscous concentrate was dissolved in ca. 10 ml of ethanol and one liter of chloroform was added. A finely divided, red, flocculent precipitate formed on standing. It was collected by filtration, washed with chloroform, and air-dried overnight. The yield was 0.95 g or ca. 1% of the weight of the starting material and is referred to as Material A. Evaporation of the solvent from the filtrate yielded 1.86 g of a viscous oil (Material C).

Based on the properties of Material A, a 2nd procedure was developed for the isolation of active material from *Juniperus* wood. One hundred g of sawdust were shaken with 2 liters of 0.1 N NaOH for 72 hr, filtered twice, and neutralized with ca. 200 ml of 1.0 N HCl slowly with stirring. A voluminous, finely divided, red-brown precipitate was formed. This precipitate, referred to as Material B, was removed by filtration, washed with water, and air-dried.

Chemical Nature of Materials A and B from J. virginiana.—Tests were conducted to define the chemical nature of the active preparations obtained from *J. virginiana*. The UV spectra of materials dissolved in alcohol were recorded using a Beckman DK spectrophotometer and IR spectra were obtained on a Perkin-Elmer model 21 spectrometer. The microanalysis was perforemd by Dr. C. Daessle, Montreal.

Materials A and B were chromatographed 2-dimensionally on 20×20-cm plates coated with Avicel using methanol:concn HCl:water (190:1:10) for the 1st direction and acetic acid:concn HCl:water (30:3:10) for the 2nd direction. The following solvent systems were used for the 2-dimensional chromatographic separation on Avicel of phenolic substances produced by KOH treatment and for the comparison of these substances with standards for identification purposes; *n*-butanol:acetic acid:water (4:1:5) and water:acetic acid (1:49). For confirmation of the presence of phloroglucinol, Kieselgel (Camag) plates were developed with the following solvent combinations: benezene:formic acid:ethyl acetate (5:2:5) and *n*-butanol:pyridine:water (14:3:3). Spots were made visible by spraying with a solution of diazotized 4-nitroaniline; plates were then oversprayed with 20% sodium carbonate. The sucrose:concn HCl:absolute ethanol spray of Roux (1951) was also used for the detection and comparison of phenols on Avicel plates.

A part of Material A was chromatographed over Sephadex LH-20 using methanol for elution and 78 fractions (5 ml each) were collected. Material contained in fractions 1–38 and 39–78 (22 and 13 mg respectively) was assayed (Table 1). A small amount of Material B (49 mg) was placed on a column (9.5×2.5 cm) of Polyclar AT and eluted with 95% ethanol. After removal of the solvent in vacuo the recovered material (23 mg) was assayed (Table 1).

Material A (199 mg) and 50% aqueous KOH were heated under reflux for 30 min in a nitrogen atmosphere. After cooling the mixture was neutralized with dilute H_2SO_4 and extracted with ether. Material B (113 mg) was similarly treated. Part of the fraction extracted with ether was transferred to a sublimation tube and distilled at 60°C in vacuo (0.4 mm Hg).

RESULTS AND DISCUSSION

Two procedures were developed for the extraction and preparation of material from *J. virginiana* wood which influenced *C. m. lengi* to lay eggs on or near treated surfaces. These preparations have been called Materials A and B. There was no response to Material A at a concentration of 0.04 mg/50 cm^2 but activity comparable to that exhibited in tests with the panels of *J. virginiana* was shown at a concentration of 0.08 mg/50 cm^2. While still influencing ovipositional behavior, higher concentrations did not

evoke a proportionately higher response (Table 1). There was no response to a panel treated with Material A which was covered with a cotton screen. This result showed that this material was not an attractant because contact was necessary for activity.

Material A exhibited no melting point and the fraction distilling or subliming when heated in high vacuum in a sublimation tube was negligible. It was very soluble in methanol, ethanol, and acetone and insoluble in water. It gave a green color when treated with ethanolic ferric chloride, in concn H_2SO_4 it showed a dark bluish green color, and it rapidly discolored neutral potassium permanganate solution. The properties of Material B were very similar. The following microanalytical results were obtained for one preparation of Material A: C, 61.51%; H, 4.99% O, 33.41%.

The UV spectrum of one preparation of Material A showed λ_{max} 277, 418, 535 nm and λ_{inf} 556 nm. Addition of alkali caused a disappearance of defined peaks and produced a spectrum similar to that observed for Material B. Acidification (dilute HCl) did not reverse this change. The UV spectrum of other preparations differed in detail. The IR spectrum of Material A showed strong bands at 3340 and 1620 cm^{-1}.

Many chromatographic systems were tried and in most the material contained in the extracts either stayed at the origin or streaked, giving no distinct spots. However, for Material A, a solvent combination used by Mullick (1969) for separation of pigments in the secondary periderm tissues of conifers resulted in the separation of several colored substances from the bulk of the sample which ran as a large brown spot nearer the solvent front. These minor components of our mixture seem quite similar to the pigments described by Mullick (1969). They were not present in Material B.

When Material A was chromatographed over Sephadex LH-40 using methanol as eluent, brown material was eluted first. Later fractions contained small amounts of reddish material. The UV spectrum

Table 1.—Oviposition responses of *C. m. lengi* to the wood and various fractions extracted from the wood of *J. virginiana* and to various chemicals.

Material	Amount (mg/50 cm^2)	Proportion ovipositing (%)	Response ratio[a]		Eggs/♀ per day
			Egg batches	Eggs	
Wood, and various extracts from the wood, of J. virginiana					
Controls					
Glass		73.3	0.9	1.1	6.3
Glass (S)[b]		75.0	1.0	0.8	5.8
A. pisum (S)		70.0	1.3	1.5	4.0
Test materials					
Wood		86.8	3.4	4.2	6.4
Material A	0.08	72.6	3.6	5.6	4.3
Material A	.24	63.7	2.5	3.3	7.0
Material A	.60	55.6	4.0	2.1	5.9
Material A	2.4	63.6	3.2	2.6	5.3
Material A	10.0	66.6	2.0	10.0	3.6
Material A (S)	10.0	66.7	1.2	1.0	5.6
Material A					
Fractions 1–38	22.0	93.9	6.7	9.1	9.8
Fraction 39–78	13.0	96.8	2.3	2.4	8.0
Material B	10.0	93.7	3.3	5.8	10.9
Fractions eluted from Polyclar AT column	10.0	75.0	.3	.5	7.1
Material C	10.0	73.3	.3	.5	7.5
Various chemicals					
Caffeic acid	2.0	71.4	1.8	1.7	4.9
o-Coumaric acid	2.0	88.9	2.2	4.5	7.0
p-Coumaric acid	2.0	91.4	1.7	1.8	7.3
Ferulic acid	2.0	80.5	1.9	1.9	6.2
Gallic acid	3.6	66.7	1.7	1.6	5.5
Salicylic acid	2.0	80.5	3.1	2.0	4.8
Protocatechuic acid	2.0	71.4	2.6	2.6	5.8
Cedrol	.4	80.0	2.6	2.7	7.6
Oil of cedarwood	1.9	77.1	1.1	1.5	5.9
Coumarin	2.0	63.7	0.4	.4	8.3
Fluorescein	2.0	97.2	10.1	16.6	9.8
Phloroglucinol	2.0	57.1	.5	.7	8.1
Tannin	10.0	55.9	2.1	2.3	3.8
Thymol	2.0	75.0	1.7	1.9	5.6
Widdrol	4.0	79.5	2.5	3.5	5.4

[a] Ratio expressed as quotient of egg batches and eggs in the cage-half adjacent to the material to those in the other half.
[b] With cotton screens.

of one of these fractions was measured and found to be similar to that reported hereinbefore. Addition of aluminum chloride caused a shift of the long wavelength band from 535 to 575 nm characteristic of anthocyanidins with adjacent phenolic hydroxyl groups (Morgan and Orsler 1967). Activity was observed for the early fractions which presumably contain the higher-molecular-weight components as well as for the latter fractions (Table 1).

Only about half of a sample of Material B could be removed from a column of Polyclar AT with alcohol. Assay showed that the recovered material was not active (Table 1).

This brief investigation of the chemical nature of these materials indicates that they consist of high-molecular-weight polyphenols. They show general similarities to phlobaphenes from other sources (Hergert and Kurth 1953, Kurth and Becker 1953, Swan 1963). In the present case, a large proportion of phlobaphenic material may have arisen by further condensation and polymerization during kiln drying of tannins and other polyphenols present in the wood of *J. virginiana*.

Further information about the nature of these extracts was obtained by KOH treatment and investigation of the fraction extracted by ether. Phloroglucinol was identified by TLC as one of the several ether-soluble phenolic substances from both Materials A and B. Swan (1963) identified this product as one obtained when a phlobaphene from western red cedar bark was treated similarly. Much of the ether-soluble fraction from both Materials A and B was nonphenolic, smelled like oil of cedarwood, and could be distilled giving a mixture of an oil and crystals. Slow release of these substances could account for the smell of Materials A and B which is characteristic of *J. virginiana* wood.

The activity shown by the juniper phlobaphenes prompted the assay of similar phenols for effects on oviposition. It is possible that the activity may result in part at least, from a compound arising from but not present in the original juniper extract. For instance, tannins from oak and eucalyptus produced resorcinol when treated with cold alcoholic solvents (Seikel and Hillis 1970).

Several phenolic substances influenced *C. m. lengi* to lay egg on or near a treated surface; the most effective were: *o*-coumaric acid, salicylic acid, protocatechuic acid, tannin, and fluorescein (Table 1). Phloroglucinol and coumarin, the cyclization product of *o*-coumaric acid, acted as ovipositional deterrents. Fluorescein was a very effective ovipositional stimulant; in a 2-choice test, glass panels treated with fluorescein at a concentration of 2.0 mg/50 cm^2 were clearly preferred to panels of *J. virginiana* wood. The ratios of egg batches and eggs in the cage half adjacent to fluorescein to those adjacent to the wood were 4:0:1 and 4:5:1 respectively. In a similar test, the activity of *o*-coumaric acid at 2.0 mg/50 cm^2 was about equal to the wood.

Fluorescein was selected for studies on egg lay. At a concentration of 2.0 mg/50 cm^2, 91.7% of the insects oviposited compared to 69.4% when the same group of insects was tested in the absence of fluorescein. However, the number of eggs per female per day was unaffected (t-test); numbers of eggs were 9.6±0.88 in the presence and 8.4±0.89 in the absence of fluorescein. In this test, 71% of the eggs were on the panels which had been treated with fluorescein compared with 27% in the absence of fluorescein.

There were significantly more occurrences of *A. pisum* on or over areas of filter paper treated with Material A than untreated areas (χ^2 12.1, $P<0.01$). Tween alone did not affect distribution. It is improbable that this effect on aphid distribution had much influence on the egg distribution of *C. m. lengi* as it was shown that aphids are not attractive (Table 1), and *C. m. lengi* preferred the wood of *J. virginiana* to aphid-infested plants for oviposition (Boldyrev et al. 1969).

Material C, the chloroform-soluble fraction from the ethanol extract, caused the female *C. m. lengi* to avoid treated surfaces (Table 1) and caused over 25% mortality[5] at the concentration tested. It was an oil and was quite similar to oil of cedarwood, the commercially available volatile oil from wood of *J. virginiana*. Oil of cedarwood at a concentration of 1.9 mg/50 cm^2 showed no significant effect on the ovipositional response of *C. m. lengi* but 2 of its constituents, widdrol (4 mg/50 cm^2) and cedrol (0.4 mg/50 cm^2) showed activity. However, cedrol caused over 50% mortality in this test. Further work is needed to determine if cedrol is nontoxic and active at lower concentrations.

This study has shown that more than one class of compound can influence the ovipositional behavior of *C. m. lengi*. However, additional work is required to determine if plant extracts, their active components, or other chemicals can be used to increase the density of coccinellid predators in the field.

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[5] Mortality of *C. m. lengi* was generally negligible in this work.

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