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A Bioassay for Detecting Compounds Which Stimulate or Deter Feeding by the Sweetclover Weevil[1, 2]

W. R. AKESON, G. R. MANGLITZ, H. J. GORZ, and F. A. HASKINS[3]

ABSTRACT

A bioassay employing sweetclover root disks impregnated with various plant extracts has been developed. The bioassay has been used to demonstrate the distribution of substances influencing feeding in fractionated water-methanol-chloroform extracts of *Melilotus infesta* Guss. and *M. officinalis* L. Lam. leaves. Indications are that substances responsible for the resistance of *M. infesta* or the susceptibility of *M. officinalis* to feeding by adult sweetclover weevils, *Sitona cylindricollis* Fåhraeus, reside in the water-methanol fraction.

Among 19 *Melilotus* species screened by Manglitz and Gorz (1964), *M. infesta* Guss. was the only species not fed upon to an appreciable extent by the adult sweetclover weevil, *Sitona cylindricollis* (Fåhraeus). The weevil resistance of *M. infesta* was confirmed by Gross and Stevenson (1964) and Radcliffe and Holdaway (1964).

Since the resistance of *M. infesta* is manifested by a refusal of the adult weevils to feed extensively on this species, it appears that resistance may result from either a lack of attractiveness or the presence of deterrent substances. Thus, studies to determine the chemical nature of this resistance should focus first on detecting substances which would stimulate or deter weevil feeding.

One prerequisite for a study of the chemical nature of resistance is a suitable bioassay for selecting biologically active fractions in various plant extracts. This paper describes such a bioassay and gives results obtained from preliminary experiments using the bioassay.

MATERIALS AND METHODS.—One of the first requirements in the development of an effective bioassay is a suitable means of presenting the extracts or fractions to the weevils for feeding. For a bioassay involving the sweetclover weevil, which feeds exclusively from the leaf margins, the requirements for the feeding medium are: (a) the medium must be thin enough to allow feeding on the edges but rigid enough to support the weight of the weevils; (b) the consistency of the medium must permit chewing by the weevil; (c) the medium must be relatively inert so as to prevent appreciable feeding on the untreated material and to avoid the presence of substances which alter the feeding response of the weevil to the active chemical components; and (d) except for changes caused by feeding, the material should remain constant in size and shape so that the extent of feeding can be readily determined. Many materials, including pith from the Japanese elder as used by Thorsteinson (1955), agar blocks, and disks of potato tuber, carrot, sweet potato roots, cabbage, and celery hearts were tested as media for the bioasay, but all were unsuitable with respect to one or more of the foregoing requirements. Disks cut from sweetclover roots and subjected to certain pretreatments are the most satisfactory of the bioassay media thus far tested.

First-year plants of field-grown Evergreen sweetclover (*M. alba* Desr.) provided the roots used in preparing the bioassay disks. The plants were dug in November 1965, and fleshy branch roots from 12 to 15 mm in diam were selected. Sections approximately 20 mm long, cut from the central portion of these roots with a 9-mm cork borer, were sliced into disks 0.1 mm thick with a hand microtome. The disks were washed several times in distilled water, suspended in water (50 ml/300 disks) in a 250-ml flask, frozen, and lyophilized. To inactivate enzymes and remove alcohol and chloroform-soluble constituents, the lyophilized disks were refluxed for 4 hr with boiling 100% ethanol followed by 4 hr of extraction with chloroform and 4 hr with methanol in a Soxhlet thimble. After treatment, the disks were stored under methanol at −20°C until used.

Prior to use in the bioassay, disks were attached to no. 1 stainless-steel insect pins cut to a length of 13 mm. The 5 disks used for each treatment were placed in a 50-mm watch glass containing 0.15 to 1 ml of plant extract which had been evaporated nearly to dryness under vacuum. Sufficient water was added to nonaqueous extracts or fractions so that the disks were still damp after the organic solvents had evaporated. Petri plates 15 mm deep and 95 or 145

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Table 1.—Feeding preference of the sweetclover weevil on sweetclover root disks treated with water-methanol or chloroform phases of a methanol-chloroform-water extract of sweetclover leaves.

Treatment	Percent disk area consumed in each test[a] I	II	III	IV	Avg[b]
A. Comparison of same phase from 2 different species					
Water-methanol phase (Group I)					
Solvent check[c]	3.0	5.4	8.2	5.2	5.5 a
M. infesta[d]	8.8	7.0	6.0	3.0	6.3 a
M. officinalis[d]	26.7	52.4	40.6	61.3	45.3 b
Chloroform phase (Group II)					
Solvent check[e]	0.3	1.2	1.0	4.7	1.8 a
M. infesta[f]	31.3	23.7	30.3	19.4	26.2 b
M. officinalis[f]	28.0	24.3	21.0	20.4	23.4 b
B. Comparison of the 2 phases from same species					
M. infesta *extract (Group III)*					
Solvent check[e]	8.0	2.3	1.7		4.0 a
Water-methanol phase[d]	1.7	0.7	2.0		1.5 a
Chloroform phase[f]	49.3	62.3	40.7		50.7 b
M. officinalis *extract (Group IV)*					
Solvent check[e]	4.3	2.5	0.0		2.3 a
Water-methanol phase[d]	16.7	23.0	10.3		16.7 b
Chloroform phase[f]	46.3	64.7	39.0		50.0 c

[a] Values for each test are averages of 5 disks.
[b] Values with different letters differ significantly at the 1% level according to Duncan's multiple range test. Calculations for significance were made only within groups.
[c] Water-methanol (1:1), 0.5 ml.
[d] Water-methanol phase (0.5 ml) representing 0.125 g fresh plant material.
[e] Chloroform-methanol-water (5:5:1), 0.5 ml.
[f] Chloroform phase (0.25 ml) representing 0.125 g fresh plant material diluted with 0.25 ml of methanol and 0.05 ml water.

mm in diam were used in the bioassay. A 3-mm layer of melted paraffin was poured into each dish and allowed to harden. This layer was covered with water-moistened Whatman no. 3 filter paper, and the pins holding the disks were mounted upright in the paraffin.

Plant extracts used in the development of the bioassay were obtained from fresh young leaves of greenhouse-grown plants of the susceptible species, *M. officinalis* L. Lam. (variety Goldtop), and the species resistant to sweetclover weevil feeding, *M. infesta*. Chloroform-methanol-water extracts were found to give the best feeding response among the various types of extracts prepared and thus were used in bioassay development. In preparing these extracts, a modification of the method of Bligh and Dyer (1959) was used. Fifty g of fresh young leaflets were homogenized for 5 mins under a nitrogen atmosphere with 150 ml of chloroform-methanol (1:2, v/v). After addition of 50 ml of chloroform, the preparation was homogenized for another 30 sec. The mixture was homogenized for a final 30-sec period after addition of 50 ml of water. The homogenate was filtered with a slight vacuum until the residue was nearly dry. The water-methanol phase (upper) was separated from the chloroform phase and washed with 100 ml of chloroform, while the chloroform phase (lower) was washed with 180 ml of water-methanol (4:5, v/v). Washings were added to similar fractions from the original separation. The phases were concentrated under vacuum so that 2 ml of the chloroform phase and 4 ml of the water-methanol phase each represented 1 g of fresh plant material.

Weevils used in the assay had been collected in September and October of 1965 and stored in tightly closed cardboard containers at 2°C. Weevils taken out of cold storage were held for 2 days without food and then allowed to feed for 24 hr on root disks treated with crude plant extract to condition them for the bioassay. Weevils not conditioned in this manner often would not feed satisfactorily during the bioassay. Once conditioned to eating the treated root disks, the weevils could be used repeatedly. All bioassays were done at room temperature under fluorescent lights with a population of 2 weevils/disk. The duration of each test was 6 hr.

After the bioassay was completed, pins were removed and the remaining portions of disks were mounted on the adhesive side of Scotch Magic Transparent Tape.[4] Dehydrated disks were moistened with water before being mounted, to restore them to their original size. The tape was applied to graph paper (20 grids/inch) and the original area of the disk was marked. The area consumed was then measured by counting squares on the graph paper. Data were expressed as percent of the total disk area consumed. This was essentially the method used by Calkins[5] (1964) for measuring weevil feeding on leaves.

RESULTS AND DISCUSSION.—The results of 4 different groups of sweetclover weevil feeding trials are presented in Table 1. Each group consisted of 3 or 4 successive feeding tests with 3 treatments or comparisons. The water-methanol phases of *M. infesta* and

[4] Mention of a proprietary product does not necessarily imply its endorsement by the USDA.
[5] C. O. Calkins. 1964. Factors affecting the activity rhythms and feeding rates of the sweetclover weevil. M. S. thesis. University of Nebraska.

Table 2.—Feeding by sweetclover weevils on sweetclover root disks treated with chloroform or combined chloroform and watermethanol phases of *M. infesta* and *M. officinalis* extracts.

Treatment	Percent disk area consumed in each test[a] I	II	Avg[b]
1. Solvent check[c]	0.0	4.0	2.0 a
2. *M. infesta*-chloroform phase[d]	11.7	12.0	11.9 ab
3. *M. officinalis*-chloroform phase[d]	21.6	13.7	17.7 b
4. *M. infesta*-combined chloroform and water-methanol phases[e]	23.0	16.0	19.5 b
5. *M. officinalis*-combined chloroform and water-methanol phases[e]	52.3	52.6	52.4 c

[a] Values for each test are averages of 5 disks.
[b] Values with different letters differ significantly at the 1% level according to Duncan's multiple range test. Calculations for significance were made only within groups.
[c] Chloroform-methanol-water (5/5/1), 0.5 ml.
[d] Chloroform phase (0.05 ml) representing 0.025 g fresh plant material diluted with 0.10 ml water and 0.35 ml methanol.
[e] Chloroform phase (0.05 ml) and water-methanol phase (0.10 ml) combined to represent 0.025 g fresh plant material and diluted with 0.35 ml methanol.

M. officinalis were compared in Group I while the chloroform phases of the 2 species were compared in Group II. Disks treated with the water-methanol phase of *M. officinalis* leaf extracts were fed upon to a significantly (1% level) greater extent than disks treated with a comparable solution from *M. infesta* leaves or with the water-methanol solvent alone. Disks treated with the chloroform phase (Group II) from *M. officinalis* and *M. infesta* leaf extracts were essentially alike, but this feeding was significantly greater than that observed on the blank disks treated only with the chloroform-methanol-water solvent. These data provide evidence that the compounds responsible for differences in weevil resistance between the 2 species exist in the water-methanol phase, and therefore the compounds are probably hydrophilic in nature.

Direct comparisons of the water-methanol and chloroform phases of extracts from each species also are given in Table 1 (Group III and IV). Each of the 2 groups consisted of 3 feeding tests with 3 treatments or comparisons. In a comparison of the water-methanol and chloroform phases from *M. officinalis* leaf extracts (Group IV) the weevils fed upon disks treated with both phases, but they showed a decided preference for the disks treated with the chloroform phase. In the case of *M. infesta* leaf extracts (Group III), the weevils fed appreciably only on the chloroform phase. No significant difference was noted between the water-methanol phase of *M. infesta* extracts and the solvent check. Although the weevils prefer the chloroform phase to the water-methanol phase in a direct comparison, the chemical difference(s) responsible for resistance or susceptibility in the 2 species resides in the water-methanol phase.

Since the weevils showed a decided preference for the chloroform phases, these were compared with the combined chloroform and water-methanol phases from each species. The data in Table 2 are the summary of results from feeding trials involving the following treatments: (1) chloroform-methanol-water blank, (2) chloroform phase from *M. infesta*, (3) chloroform phase from *M. officinalis*, (4) combined water-methanol and chloroform phases from *M. infesta*, and (5) combined phases from *M. officinalis*. In the direct comparison of chloroform phases and combined chloroform and water-methanol phases for the 2 species, disks impregnated with the chloroform phase from *M. infesta* did not differ significantly from disks treated with the chloroform phase from *M. officinalis* or with the combined phases from *M. infesta*. Disks treated with the combined chloroform and water-methanol phases from *M. officinalis*, on the other hand, were fed upon significantly (1% level) more than any other disks.

Thus, despite the weevils' apparent preference for the chloroform phase over the water-methanol phase (Table 1, Group IV), the combined phases from *M. officinalis* leaves were much preferred to the chloroform phase alone. A synergistic stimulation of feeding, involving constituents of both phases, seems possible.

The foregoing data demonstrate that a workable and reliable bioassay has been developed for detecting chemicals in sweetclover extracts that stimulate or deter feeding by adult sweetclover weevils. Use of this method in preliminary studies has shown that chemical differences do exist between the weevil-susceptible species, *M. officinalis*, and the resistant species, *M. infesta*.

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