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Sweetclover Weevil Feeding Stimulants: Isolation and Identification of Glucose, Fructose, and Sucrose[1]

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ABSTRACT

The water-soluble fraction of *Melilotus* leaves previously referred to as Stimulant A was further fractionated by preparative paper chromatography into three factors, each having a stimulatory effect on sweetclover weevil feeding. The three factors, designated Stimulant A_1, A_2, and A_3, were identified as sucrose, glucose, and fructose, respectively, by co-chromatography with known sugars on cellulose-coated thin-layer plates. Silver nitrate, anisidine phthalate, and anthrone were employed as detection agents. The isolated compounds and corresponding reagent grade sugars were identical in chromatographic and chemical behavior, as well as in feeding stimulant activity. Sucrose stimulated the greatest amount of feeding when the three sugars were compared at equal concentration in the same bioassay. Disks treated with glucose and fructose were fed upon equally at the three concentrations tested. Although these sugars probably play a significant role in the mechanism of resistance and susceptibility of *Melilotus* plants to weevil feeding, it is not likely that they are wholly responsible for the host specificity displayed by the sweetclover weevil.

Additional index words: Sitona cylindricollis, Melilotus officinalis, Melilotus infesta.

A water-soluble factor derived from *Melilotus* leaves stimulates feeding by the adult sweetclover weevil, *Sitona cylindricollis* (Fahraeus) (1, 3). The active fraction was separated by preparative paper chromatography of hot water extracts and detected by means of a bioassay (4) which permit determination of the extent of weevil feeding on washed disks of sweetclover root tissue impregnated with the fraction to be tested. The stimulant, designated Stimulant A, was found to occur in plants of *M. infesta* Guss. as well as in *M. officinalis* (L.) Lam., species that are resistant and susceptible, respectively, to weevil feeding (7). Results obtained with the semi-quantitative bioassay suggested that young leaves of the two species were sufficiently similar in content of Stimulant A to rule out the possibility that the resistance of *M. infesta* was due primarily to lack of this feeding stimulant (3). The further separation of Stimulant A into three distinct components, each with feeding stimulant activity, and their subsequent isolation and identification, are described in the present report.

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MATERIALS AND METHODS

Freshly harvested young leaves of field-grown *M. officinalis* var. 'Goldtop' plants were weighed, washed with distilled water, dropped into boiling water (5 ml/g of fresh tissue), and autoclaved for 20 min at 1.1 kg/sq cm. After removal of the leaf material, the extract was frozen and lyophilized to dryness. The powder obtained was stored at −20 C until used.

Solutions for chromatography were prepared by dissolving the dried powder in water such that approximately 0.75 ml of the final solution represented 1 g of the original fresh tissue. Ascending chromatography for the separation of feeding stimulants was accomplished essentially as described by Akeson et al. (1, 3), except that different solvents were used. Chromatograms were cut into horizontal strips at widths previously determined to be suitable for separating the stimulant factors, and each strip was eluted with water. Eluates were tested for stimulant activity by means of the previously described bioassay procedure (4).

Partial purification of crude extracts was effected by chromatographing with Solvent I (isopropyl alcohol, ammonium hydroxide, water, 8:1:2, v/v/v). The eluate from the single band containing most of the stimulant activity was rechromatographed with Solvent II (chloroform, acetic acid, water, 4:4:1, v/v/v). Eluates from the two bands containing stimulant activity were applied to separate chromatograms and developed with five successive passes of Solvent III (ethyl acetate, isopropyl alcohol, water, 65:38:17, v/v/v). Chromatograms were air dried between solvent passes. Finally eluates containing the three partially purified feeding stimulants were separately rechromatographed with five passes of Solvent III. On each of these final chromatograms, activity was confined essentially to a single band. Elution of the active bands yielded the three purified feeding stimulants, designated Stimulants A_1, A_2, and A_3 (in order of increasing Rf in Solvent III), which were used in further studies.

Stimulants A_1, A_2, and A_3 were tentatively identified as sugars on the basis of their reaction to the phenol sulfuric acid and anthrone tests (6). The phenol sulfuric acid test was used to determine the sugar content of the final eluates and concentrations were adjusted as needed for subsequent tests.

The identity and homogeneity of the three purified stimulants were determined by co-chromatography with known mono- and disaccharides on 20 × 20 cm cellulose-coated thin-layer plates. The chromatograms consisted of a 0.3-mm layer of MN cellulose powder applied to glass plates as a water slurry (15 g cellulose in 90 ml water). Spots of the solutions to be chromatographed were applied to the air-dried plates along a line 2.5 cm from one edge of the plate. Concentration of all solutions was 1% and aliquots of approximately 2 µl were chromatographed. Solvent ascent was 15 cm above the base line. Silver nitrate, anisidine phthalate, and anthrone reagents were used for the detection of sugars on the chromatograms. Chromatograms dipped for 1 min in silver nitrate solution (5 ml saturated silver nitrate solution in 25 ml water added to 1000 ml acetone) were subsequently sprayed with ethanolic 0.5 *N* sodium hydroxide and then with aqueous 5% sodium thiosulfate for the detection of reducing compounds (8). Other chromatograms sprayed with anisidine phthalate {*p*-anisidine (0.1 *M*) and phthalic acid (0.1 *M*) in 96% ethanol} were heated for 5 min at 100 C for the identification of aldopentoses (red) and aldohexoses (yellow). Ketohexoses developed a yellow color after being heated at 100 C for 20 min (8). Anthrone reagent (0.3 g anthrone in 10 ml glacial acetic acid, 20 ml ethanol, 3 ml 85% phosphoric acid, and 1 ml water) was used to detect ketohexoses or mixtures of sugars containing ketohexoses (dark yellow), and sugar alcohols (white on light-yellow background) (10). Colors were developed by heating the anthrone-treated chromatograms at 100 C for 12 min.

After each of the three stimulants had been identified, the biological activity of the isolated compound was compared with that of the corresponding reagent grade sugar by means of the bioassay procedure (4). Separate comparisons were made of each stimulant and the corresponding sugar at three concentrations

(0.1, 0.3, and 0.9%, w/v with water as a control. Each comparison consisted of five disks per treatment, seven treatments (two compounds, three concentrations of each, plus a control) in each feeding chamber, a population of three weevils per disk, and four replications. A mixture of 0.1 ml of the sugar solution being tested plus 0.05 ml of water was applied to the five disks of each treatment.

In another test equal concentrations of the reagent grade sugars corresponding to Stimulants A_1, A_2, and A_3 were compared by the procedure described above except that each comparison consisted of four treatments (three sugars at equal concentration plus a water control). Three concentration levels were assayed in three separate comparisons.

RESULTS AND DISCUSSION

The association of feeding stimulant activity with various bands during the stepwise chromatographic purification of Stimulants A_1, A_2, and A_3 is shown in Table 1. Initial fractionation of the crude hot water extract with Solvent I gave a single eluate (from Band 3) which had significant stimulant activity (Table 1, Step A). Rechromatography of the eluate from Band 3 with Solvent II resulted in the appearance of activity in Bands 3 and 4 (Step B). The feeding stimulant activity in Band 3 was about twice as great as that in Band 4. The location of the two active bands coincided with two separated bands that became visible when chromatograms developed with Solvent II were treated with silver nitrate or anisidine phthalate. These observations clearly indicated that the factor designated as Stimulant A in previous work (1, 3) actually consisted of two or more factors.

When the eluate from Band 3 (Step B) was rechromatographed with Solvent III, the activity was again fractioned into two major bands (Step C, Bands 2 and 3). Although there was appreciable activity in Band 4 (Step C), additional work revealed that the activity was due to a mixture of Stimulants A_2 and A_3. Therefore, no further work was done with this fraction. The eluates from Band 2 (designated Stimulant A_1) and Band 3 (designated Stimulant A_2) were chromatographed a second time with Solvent III and in each case, the major activity was now confined to the respective band from which the eluate originated (Band 2 in Step E; Band 3 in Step F).

Rechromatography of the eluate from Band 4 (Step B) with Solvent III resulted in the appearance of most of the stimulant activity in Band 4 (Step D), with some activity also present in Bands 2 and 3. The latter two bands were subsequently found to contain small amounts of Stimulants A_1, A_2, and A_3; therefore, no further work was done with these bands. When the eluate from Band 4 (Step D), which was designated Stimulant A_3, was chromatographed a second time with Solvent III, the activity was essentially confined to Band 4 (Step G).

Thin-layer chromatography of Stimulants A_1, A_2, and A_3 on cellulose-coated plates gave single spots for each stimulant with the nine solvent systems listed in Table 2. These data provide additional evidence that each of the three stimulants is a single compound, distinct from the other two stimulants.

The three stimulants were identified by co-chromatography with known sugars on cellulose-coated thin-layer plates (Table 2.) Nine different solvent systems and three detection agents were used. Stimulant A_1 had R_f values identical to those of sucrose. Neither compound reacted with silver nitrate or anisidine pthalate; both turned yellow when treated with anthrone reagent, which is the characteristic reaction of ketoses and ketose-containing compounds. Stimulant A_2 and glucose had identical R_f values. Both reacted with silver nitrate, as is characteristic of reducing compounds. Neither gave a positive reaction with anthrone reagent, but anisidine phthalate treatment produced a yellow color after heating for 5 min, indicating the presence of an aldohexose. Stimulant A_3 had R_f values corresponding to those of fructose, as well as identical reactions with silver nitrate (positive), anthrone reagent (positive), and anisidine phthalate (negative with 5 min heating but positive with 20 min heating). Based on the data presented in Table 2, Stimulant A_1 was identified as sucrose, Stimulant A_2 as glucose, and Stimulant A_3 as fructose.

Comparisons of the feeding-stimulant activities of the isolated compounds with those of the corresponding reagent grade sugars are presented in Table 3. The solutions assayed contained 0.1, 0.3, and 0.9% (w/v) of sugar and provided approximately 20, 60, and 180 μg, respectively, of sugar in each bioassay disk. At each concentration, the feeding value of the isolated compound was nearly identical to that of the corresponding known sugar. These results provide critical evidence that the isolated stimulants and corresponding reagent grade sugars are identical in biological activity as well as in chromatographic and chemical behavior. Under the conditions employed in the bioassays, the response of the weevils to concentration differences was most sensitive for fructose and least sensitive for glucose.

When a direct comparison was made of the relative feeding stimulant activities of glucose, fructose, and

Table 1. Chromatographic purification of three feeding stimulants from a hot-water extract of *M. officinalis* leaves.

Step	Extract or eluate chromatographed*	Solvent no.†	Band no.	Band limits‡ (R_f)	% disk area consumed§ Mean ± SE	Stimulant
A	Crude	I	1	0.00-0.17	1.2 ± 0.6	
			2	0.17-03.7	7.1 ± 2.2	
			3	0.37-0.40	42.4 ± 3.0	
			4	0.50-0.67	1.6 ± 0.7	
			5	0.67-0.83	0.8 ± 0.7	
			6	0.83-1.00	0.3 ± 0.3	
B	A-3	II	1	0.00-0.13	0.2 ± 0.1	
			2	0.13-0.25	0.0 ± 0.0	
			3	0.25-0.40	53.9 ± 5.8	
			4	0.40-0.52	27.4 ± 2.2	
			5	0.52-0.75	0.5 ± 0.3	
			6	0.75-1.00	1.0 ± 0.5	
C	B-3	III	1	0.00-0.13	5.0 ± 1.5	
			2	0.13-02.6	63.5 ± 4.7	A_1
			3	0.26-0.37	40.8 ± 4.9	A_2
			4	0.37-0.47	27.7 ± 4.6	
			5	0.47-0.74	6.3 ± 2.9	
			6	0.74-1.00	3.2 ± 1.6	
D	B-4	III	1	0.00-0.13	4.2 ± 2.6	
			2	0.13-0.26	12.0 ± 2.8	
			3	0.26-0.35	20.5 ± 3.8	
			4	0.35-0.47	47.0 ± 5.8	A_3
			5	0.47-0.74	2.5 ± 1.7	
			6	0.74-1.00	3.8 ± 1.8	
E	C-2	III	2	0.13-0.27	49.8 ± 5.9	A_1
			3	0.27-0.37	10.2 ± 2.4	
			4	0.37-0.50	7.2 ± 3.6	
F	C-3	III	2	0.13-0.27	2.0 ± 0.7	
			3	0.27-0.37	29.7 ± 3.0	A_2
			4	0.37-0.50	4.7 ± 2.1	
G	D-4	III	2	0.13-0.27	5.3 ± 2.4	
			3	0.27-0.37	10.0 ± 3.8	
			4	0.37-0.50	42.8 ± 5.1	A_3

* Refers to the step and band, respectively, from which the eluate was obtained for rechromatography. † Solvent I: isopropyl alcohol, ammonium hydroxide, water (8:1:2, v/v/v); Solvent II: chloroform, acetic acid, water (4:4:1, v/v/v); Solvent III: ethyl acetate, isopropyl alcohol, water (65:38:17, v/v/v, five solvent passes). ‡ Solvent ascent of 15 cm on 18.5 × 28.5 cm sheets of Whatman No. 3 filter paper. § Bands from two chromatograms were eluted. Five disks were treated with each eluate; thus each mean is the average of 10 observations. A population of three weevils per disk was used. Feeding comparisons were made within chromatrographic steps, not between steps.

Table 2. Chromatography of Stimulants A_1, A_2, and A_3 and reagent grade sugars on 20 × 20 cm cellulose-coated thin-layer plates in nine solvent systems, and reaction of spots with three detection agents.

	Rf values (15-cm ascent) in nine solvent systems*									Reaction with detection agent†			
											Anisidine phthalate		
											Heat, min		
Sugar	Water-sat. phenol	8 Chl 8 MeOH 1 H_2O	16 Chl 16 HAc 3 H_2O	8 EtAc 2 Pyr 1 H_2O	65 EtAc 23 i-PrOH 12 H_2O (5)‡	2 n-BuOH 1 HAc 1 H_2O	3 n-BuOH 1 Pyr 1 H_2O	4 n-BuOH 5 acetone 1 H_2O (3)‡	5 n-BuOH 3 i-PrOH 2 H_2O	Silver nitrate	5	20	Anthrone reagent
Stim. A_1	0.58	0.20	0.35	0.03	0.14	0.49	0.30	0.27	0.27	-	-	-	Y
Stim. A_2	0.53	0.31	0.34	0.10	0.29	0.55	0.37	0.36	0.33	+	Y	Y	-
Stim. A_3	0.63	0.40	0.46	0.12	0.37	0.61	0.43	0.49	0.37	+	-	Y	Y
Ribose	0.71	0.55	0.51	0.30	0.69	0.66	0.52	0.65	0.45	+	R	R	-
Xylose	0.58	0.48	0.41	0.20	0.50	0.63	0.49	0.53	0.40	+	R	R	-
Arabinose	0.66	0.44	0.46	0.15	0.43	0.61	0.42	0.48	0.37	+	R	R	-
Mannose	0.58	0.36	0.36	0.12	0.37	0.59	0.44	0.43	0.39	+	Y	Y	-
Galactose	0.57	0.28	0.31	0.07	0.25	0.53	0.33	0.27	0.33	+	Y	Y	-
Glucose	0.53	0.31	0.33	0.09	0.29	0.55	0.37	0.36	0.33	+	Y	Y	-
Sorbose	0.55	0.36	0.39	0.13	0.35	0.59	0.41	0.44	0.35	+	-	Y	Y
Fructose	0.64	0.40	0.47	0.12	0.37	0.61	0.43	0.49	0.37	+	-	Y	Y
Sucrose	0.58	0.20	0.35	0.03	0.14	0.49	0.30	0.27	0.27	-	-	-	Y
Maltose	0.51	0.12	0.14	0.02	0.08	0.41	0.24	0.19	0.17	+	Y	Y	-
Xylitol	0.66	0.41	0.57	0.11	0.40	0.65	0.41	0.47	0.39	+	-	-	W†
Arabitol	0.67	0.62	0.55	0.13	0.42	0.66	0.43	0.51	0.40	+	-	-	W
Mannitol	0.57	0.29	0.48	0.08	0.31	0.59	0.35	0.32	0.36		-	-	W

* Abbreviations: Chl, chloroform; EtAc, ethyl acetate; HAc, acetic acid; i-PrOH, isopropyl alcohol; MeOH, methanol; n-BuOH, n-butyl alcohol; Pyr, pyridine. † R = red, Y = yellow, W = white. Xylitol treated with anthrone produced a white spot with a light yellow background. ‡ Number of solvent passes.

Table 3. Comparison of the feeding stimulant activity of Stimulants A_1, A_2, and A_3 with reagent grade sucrose, glucose and fructose, respectively. Each comparison consisted of five disks per treatment, seven treatments in each replication, a population of three weevils per disk, and four replications.

Solutions compared*		Percent disk area consumed when comparing		
Conc.	Type	Sucrose and stim. A_1	Glucose and stim. A_2	Fructose and stim A_3
% (w/v)		mean ± SE	mean ± SE	mean ± SE
0.0	Water	0.1 ± 0.0	2.0 ± 0.6	1.8 ± 0.5
0.1	Known sugar	11.9 ± 1.9	19.8 ± 3.0	5.6 ± 1.2
0.1	Isolated stim.	11.4 ± 2.0	18.4 ± 2.8	5.6 ± 1.4
0.3	Known sugar	20.9 ± 2.5	29.7 ± 4.3	13.8 ± 3.1
0.3	Isolated stim.	19.0 ± 2.6	27.4 ± 3.3	12.1 ± 3.0
0.9	Known sugar	30.9 ± 4.7	30.9 ± 2.5	30.2 ± 3.8
0.9	Isolated stim.	34.5 ± 4.4	34.4 ± 4.6	34.1 ± 4.0

* A mixture of 0.1 ml of the sugar solution being tested plus 0.05 ml of water applied to the five disks of each treatment.

Table 4. Comparison of sucrose, glucose, and fructose as sweetclover weevil feeding stimulants. Equal concentrations of the three sugars were assayed together in the same chamber with a population of three weevils per disk. Each comparison consisted of five disks per treatment, four treatments in each replication and four replications.

Solution compared*		% disk area consumed, mean ± SE
Conc., %	Sugar	
---	Water	4.4 ± 1.7
0.9	Sucrose	43.9 ± 4.4
0.9	Glucose	28.5 ± 3.8
0.9	Fructose	29.6 ± 3.8
---	Water	2.2 ± 0.5
0.3	Sucrose	24.3 ± 5.2
0.3	Glucose	17.3 ± 3.7
0.3	Fructose	16.8 ± 2.7
---	Water	3.0 ± 1.1
0.1	Sucrose	15.4 ± 3.7
0.1	Glucose	9.6 ± 2.1
0.1	Fructose	9.9 ± 1.1

* A mixture of 0.1 ml of the sugar solution being tested plus 0.05 ml of water was applied to the five disks of each treatment.

sucrose at equal concentrations in the same bioassay feeding chamber, sucrose stimulated the greatest feeding at each of the three concentrations tested (Table 4). Disks treated with glucose and fructose were fed upon equally at all concentrations.

The reviews of Beck (5) and Thorsteinson (9) cite several instances in which sugars served as feeding stimulants for phytophagous insects. Although sugars may play a significant role in determining the resistance or susceptibility of plants to certain insects, it is unlikely that they are wholly responsible for the host specificity of the sweetclover weevil, whose feeding appears to be limited to certain species within the genera *Melilotus, Trigonella,* and *Medicago.* Other constituents of *Melilotus* leaves which influence sweetclover weevil feeding include unidentified chloroform-soluble feeding stimulant(s) (4), unidentified Deterrent A (3) and Deterrent B which has been identified as nitrate (2). A full understanding of the resistance and susceptibility of *Melilotus* plants to weevil feeding must take into account these constituents, and possibly others, as well as sucrose, glucose, and fructose.

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