Appl. Entomol. Zool. 39 (3): 411–416 (2004)
http://odokon.ac.affrc.go.jp/

Feeding stimulants of Solanaceae-feeding lady beetle, *Epilachna vigintioctomaculata* (Coleoptera: Coccinellidae) from potato leaves

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(Received 17 October 2003; Accepted 9 March 2004)

Abstract
A phytophagous lady beetle, *Epilachna vigintioctomaculata*, feeds mainly on potato (*Solanum tuberosum*) leaves. The methanol extracts of potato leaves showed feeding stimulative effects on the adult of the beetle. The feeding stimulants were isolated and identified as methyl linolenate from a lipid-soluble fraction, and glucose and fructose from an aqueous fraction. Although methyl linolenate alone was inactive, it acted synergistically with sugars. Methyl linolenate maximized the feeding activity of sugars at the concentration contained in the potato leaves. It is suggested that methyl linolenate plays an important role in the host selection of *E. vigintioctomaculata*.

Key words: *Epilachna vigintioctomaculata*; Solanaceae; host plant selection; feeding stimulants; methyl linolenate

INTRODUCTION

In host plant selection by phytophagous insects, the chemical and physical properties of plants are important. In particular, gustatory and olfactory response by herbivorous insects to plant chemicals are considered to be important factors when finding and accepting a plant host (Hirano, 1960; Hsiao, 1985). In *Epilachna* beetles, limited studies of the feeding stimulants have been investigated. Linamarin, lotaustrin, and phaseolunatin were feeding stimulants of *Epilachna varivestis* (Nayar and Fraenkel, 1963). Cucurbitacins, contained specifically in cucurbitaceous plants, acted as feeding stimulants for *E. admirabilis*, *E. boisduvali*, *E. vigintioctopunctata* and *E. vigintioctomaculata* (Abe and Matsuda, 2000). *E. vigintioctomaculata*, one of the solanaceous feeding lady beetles, is known to be a noxious insect for potato plants (*Solanum tuberosum*). Until the present, only cucurbitacins, contained specifically in cucurbitaceous plants, have been reported as feeding stimulants for the beetle. However, except for one cucurbitaceous plant, *Schizopepon bryoniaefolius* (Katakura, 1975), host plants of *E. vigintioctomaculata* are primarily restricted to solanaceous plants. Therefore, other substances in solanaceous plants should stimulate the feeding of the beetle.

It is important to identify the feeding stimulants contained in solanaceous plants to facilitate our understanding of the host selection process of this lady beetle. In this study, we investigated the feeding stimulants of *E. vigintioctomaculata* contained in potato leaves.

MATERIALS AND METHODS

Insects and plants. *E. vigintioctomaculata* adults were collected at Natori City, Miyagi Prefecture and their progeny were reared at 24±1°C, 16L–8D photoregime. Leaves of potato, tomato (*Lycopericon esculentum*), and *Lycium chinense* were provided as food. These plants were cultivated in the field, except for the winter season in the greenhouse of the Faculty of Agriculture, Tohoku University.

Bioassay. Feeding tests were conducted with a filter paper assay (Abe and Matsuda, 2000). Test extracts were dissolved in each extracted solvent (1 g fresh leaf equivalent/ml), and a square piece of

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DOI: 10.1303/aez.2004.411

filter paper (Toyo Roshi, No. 50, 2×2 cm) was treated with 75 μl of the test solution and allowed to dry at room temperature. Three filter papers (7 cm i.d.) were placed on the bottom of a plastic petri dish (1.7×9 cm i.d.) and moistened with 3ml distilled water. A doughnut-like plastic disc (2 cm i.d. and 7 cm o.d.) was set on the filter papers. Two treated and two control (treated with solvent only) filter papers were placed equidistantly on the plastic disc. Distilled water (75 μl) was added to the filter paper prior to the test. In bioassays, the water portion (1 g leaf equivalent/ml) was added to each chloroform fraction and the chloroform portion (1 g leaf equivalent/ml) was added to each water fraction, respectively. An overview of the testing method was described in Abe and Matsuda (2000). Five adults, starved for 24 h, during 4 to 11 days after eclosion were used for the bioassay. The petri dish was placed in an incubator (24±1°C, 16L–8D photoregime) for 24 h. After the test was finished, each filter paper square was divided into 100 sections (2×2 mm each), and the number of sections with bites in them was counted. Each feeding test was replicated five times. The feeding activity is shown as feeding index (maximum is 200) and was evaluated by Mann-Whitney's *U*-test.

Extraction of potato leaves. Fresh leaves of Dansyaku potato (2,634.7 g) were extracted with methanol (15 *l*×3) for 24 h and the extracts were filtered. After removal of methanol *in vacuo* below 40°C, the extracts were successively partitioned into chloroform, *n*-butanol (1 *l*×3 each) and water-soluble (1 *l*) portions. The extracts were evaporated to dryness.

Isolation of a feeding stimulant from the chloroform fraction. The chloroform portion was dissolved in 80% methanol in water (1 *l*), partitioned with hexane (1 *l*×3) and the solvent was removed *in vacuo* below 40°C. The aliquot of the hexane portion was chromatographed on a silica gel column (Wako-gel® C-200, 100–200 mesh, 200 g, 225×45 mm i.d.) and successively eluted with hexane:ethyl acetate (9 : 1, 8 : 2, 5 : 5; 1 *l* each) and ethyl acetate:methanol (1 : 1; 1 *l*). Eluates were collected (500 ml each) and analyzed by TLC (Silica gel 60 F_{254}, 5×10 cm, Merck). Eluates with the same *Rf* value on TLC were combined. The active fraction was chromatographed on a silica gel column (Wako-gel® C-200, 100–200 mesh, 50 g, 304×21 mm i.d.). The column was eluted with hexane : ethyl acetate (97 : 3, 95 : 5, 90 : 10, 50 : 50; 250 ml each). Eluates were collected (50 ml each) and fractionated by the same manner mentioned above. Further purification of the biologically active fraction was conducted with the preparative TLC silica gel plate (Silica gel 60 F_{254}, 20×20 cm, 2 mm. Merck) using a solvent system of hexane : ethyl acetate=9 : 1. After development, compounds were scraped from the plate and extracted with chloroform. The isolated stimulant was analyzed with the following instruments.

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ with TMS as an internal standard with a Varian Unity INOVA 500 spectrometer. EI mass spectra was measured on a JEOL JMS-700 spectrometer, programmed from 60°C for 2 min to 250°C at 15°C/min.

Fractionation and identification of feeding stimulants from the water portion. The water portion was chromatographed on an ODS column (YMC-gel® ODS-A 60-60/30, 100 g, 315×26 mm i.d.) and successively eluted with each 500 ml of water, 25%, 50%, 75% methanol in water and methanol, and the solvent was evaporated to dryness. The active fraction was further chromatographed on a cation (Dowex® 50W, 100–200 mesh, H^+ form) and anion (Dowex®-1, 100–200 mesh, OH^- form) ion-exchange column into acidic, basic and neutral fractions. The acidic fraction and basic fraction were neutralized by 1 N-NH_4OH and 1 N-HCl (50 ml each), respectively, and then used for bioassay.

The neutral fraction was further analyzed by HPLC using TSKgel® Amide-80 (Toso, 250×4.6 mm i.d.) at 60°C by a column oven (Jasco Co. Ltd.). The HPLC system was CCPD pump, RI-8010 refractive index detector (Toso Co. Ltd.), and the eluate was 70% acetonitrile in water at a flow rate of 1 ml/min.

Quantification of feeding stimulants. The quantity of methyl linolenate contained in flesh potato leaves was calculated by quantitative GC-MS. GC analyses were carried out on a HITACHI G-5000M equipped with DB-5 (30 m×0.25 mm) operated at 40°C for 5 min, and then increased to 270°C at a rate of 10°C/min and held at this temperature for 1 min. MS was carried out with HITACHI M-7200. The peak area was measured and the concentrations of methyl linolenate were calculated using the calibration curve obtained from the peak area of synthetic methyl linolenate.

Fructose and glucose contained in flesh potato leaves were quantitated by HPLC in the same system as described above and calculated by the same method used for methyl linolenate using authentic sugars.

Chemicals. Methyl linolenate, fructose and glucose were purchased from Wako Pure Chemicals Industries, Ltd.

RESULTS

The methanol extracts of potato leaves acted as a feeding stimulant to *E. vigintioctomaculata*. After fractionation of the methanol extract, feeding activity of each portion became weaker (Fig. 1a). Feeding activity was recovered when two, in any combination, or all portions were combined (Fig. 1b). The highest feeding index was recorded when the chloroform and water portions were combined. Therefore, we investigated feeding stimulants in both chloroform and water portions. The isolation procedure of the chloroform and water portions and the feeding activity of each fraction is summarized in Fig. 2.

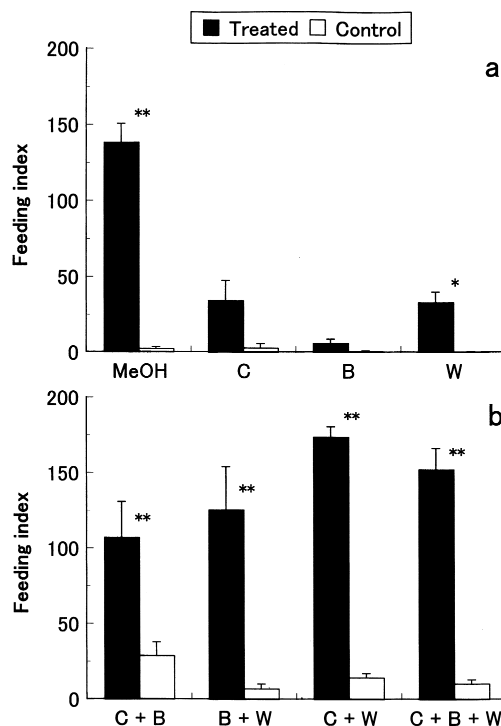

Fig. 1. Feeding responses (mean±SE) of *E. vigintioctomaculata* to each portion (a) and mixture of portions (b). Significant difference between the control and the treated filter papers is represented by an asterisk (Mann-Whitney's *U*-test: * $0.01 \leq p < 0.05$, ** $p < 0.01$). C: chloroform portion, B: butanol portion, W: water portion.

Feeding stimulant in the chloroform portion

Both the hexane and the 80% methanol portions stimulated feeding. Because one purpose of this study was to identify the main feeding stimulant

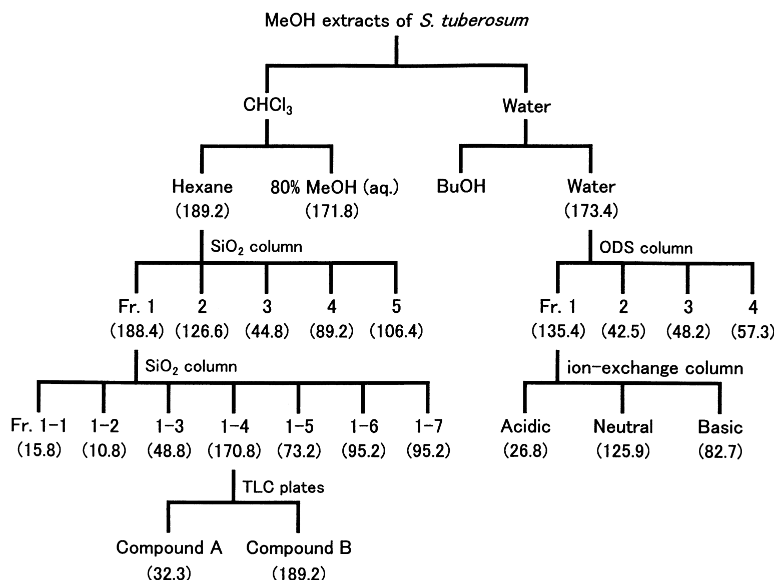

Fig. 2. Separation scheme and feeding stimulant activity of *S. tuberosum* to *E. vigintioctomaculata*. The chloroform and the water portion is added to each water and chloroform portion, respectively. Figures in parenthesis indicate the average of feeding index.

for this beetle, we continued to separate the fraction which showed the highest feeding index. The hexane portion was separated into five fractions with silica gel column chromatography, and Fr. 1 scored the highest feeding index (188.4±4.0) of the five fractions. Fr. 1 was further fractionated with a silica gel column, and the main stimulative activity existed in Fr. 1-4 (170.8±10.9). The analysis of Fr. 1-4 showed two main spots on TLC plate and Fr. 1-4 was thus separated into compounds A and B. Finally, compound B with the water portion showed strong feeding activity (feeding index was 189.2±8.4) to *E. vigintioctomaculata*.

EI-MS of compound B gave the molecular ions at *m/z* 292$(M)^+$ and from the fragmentation pattern, this compound was estimated to be a long-chain ester. The ^{1}H NMR spectrum of compound B showed a methyl triplet (δ 0.98, J=7.5 Hz), ten methylene groups (δ 1.25–1.38, 8H; 1.59–1.65, 2H; 2.03–2.11, 4H; 2.29–2.32, 2H; 2.80–2.82, 4H), a methyl singlet attached to oxygen (δ 3.67) and olefinic proton groups (δ 5.29–5.43, 6H). The ^{13}C NMR spectrum showed a methyl (δ 14.3), ten methylenes (δ 20.5, 24.9, 25.5, 25.6, 27.2, 29.1, 29.1, 29.2, 29.6, 34.1), a methyl attached to oxygen (δ 51.5), six olefinic (δ 127.1, 127.7, 128.2, 128.3, 130.3, 132.0) and a carbonyl (δ 174.3) carbons.

Based on these data, compound B was identified as methyl linolenate ($C_{19}H_{32}O_2$), a methyl ester of linolenic acid, and the NMR data was in good agreement with that of authentic methyl linolenate. The quantity of methyl linolenate contained in flesh potato leaves was calculated as 0.171 mg/g.

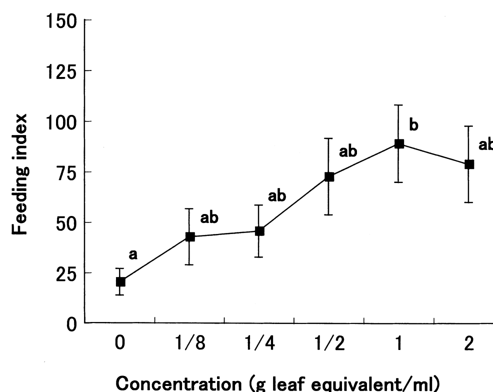

Fig. 3. Feeding responses of *E. vigintioctomaculata* to different concentrations of methyl linolenate (1 g leaf equivalent=0.171 mg) with the water portion. Each point indicates the mean±SE of five replicates. Different letters indicate significant differences (Tukey-Kramer test: $p<0.05$).

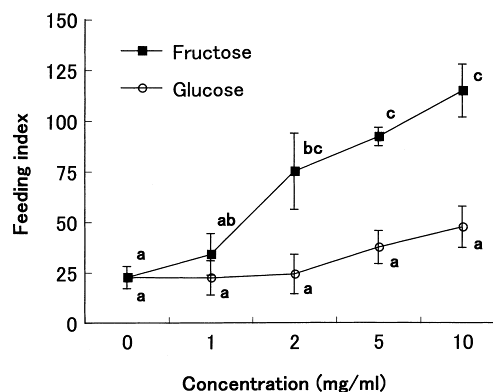

Fig. 4. Feeding responses of *E. vigintioctomaculata* to different concentrations of fructose and glucose with the hexane portion. Each point indicates the mean±SE of five replicates. Different letters indicate significant differences among concentrations (Tukey-Kramer test: $p<0.05$).

Feeding stimulant in the water portion

Fr. 1 separated by ODS column showed the highest feeding index (135.4±15.9) among the four fractions. Fr. 1 was further fractionated by an ion-exchange column and the main stimulative activity existed in the neutral fraction (feeding index was 125.9±14.8).

The neutral fraction showed two major peaks on HPLC at retention times of 5.53 and 6.26 min and, compared with authentic sugars, each peak was identical to fructose and glucose, respectively. By quantitative HPLC, the amount of fructose and glucose contained in fresh potato leaves was calculated as 5.00 and 4.77 mg/g, respectively.

Feeding response to authentic samples

Feeding response to authentic methyl linolenate combined with the water portion is shown in Fig. 3. The feeding index of methyl linolenate with the water portion was increased at all tested concentrations; significant increases of feeding activity were observed only at the concentration of 1 g leaf equivalent (0.171 mg/ml) compared to the water portion alone (ANOVA and Tukey-Kramer test, $p<0.05$).

The results of the feeding response to fructose and glucose combined with the hexane portion are shown in Fig. 4. Feeding activity of fructose with the hexane portion was significantly higher than the hexane portion alone at concentrations of 2, 5, 10 mg/ml (ANOVA and Tukey-Kramer test, $p<0.05$). The feeding index of glucose with the

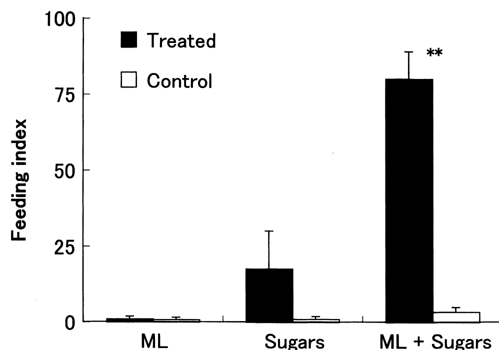

Fig. 5. Feeding responses (mean±SE) of *E. vigintioctomaculata* to authentic samples at 1 g leaf equivalent/ml. Significant difference between the control and the treated filter papers is represented by an asterisk (Mann-Whitney's *U*-test: $**p<0.01$). ML: methyl linolenate. Sugars: mixture of fructose and glucose.

hexane portion was increased at concentrations of 5, 10 mg/ml, but no significant difference was observed compared to the hexane portion alone (ANOVA, $p>0.05$). The comparison of feeding activity with the hexane portion showed that fructose was clearly stronger than glucose (Two-way ANOVA and Tukey-Kramer test, $p<0.001$).

Finally, we performed feeding tests using only these authentic samples at the concentration of 1 g leaf equivalent/ml. Neither methyl linolenate nor sugars alone stimulated feeding (Mann-Whitney's *U*-test, $p>0.05$), but the activity was induced when these substances were combined (Mann-Whitney's *U*-test, $p<0.01$) (Fig. 5).

DISCUSSION

For phytophagous insects, plant chemical components play an important role in host selection. A solanaceous feeding lady beetle, *E. vigintioctomaculata*, was stimulated by the methanol extract of potato leaves. Final fractions which showed the feeding stimulative activity contained methyl linolenate in the chloroform portion, and fructose and glucose in the water portion. A mixture of authentic methyl linolenate and sugars induced the feeding stimulative activity from *E. vigintioctomaculata*. These results reveal that this species is stimulated to feed by the mixture of lipid-soluble methyl linolenate and water-soluble sugars which have quite different chemical natures. Some substances contained in potato leaves such as chlorogenic acid and sucrose have been reported as feeding stimulants for solanaceous feeding insects, such as the Colorado potato beetle (Hsiao and Fraenkel, 1968). This is the first report demonstrating that methyl linolenate, a methyl ester of unsaturated fatty acid, acts as an insect feeding stimulant. An unsaturated fatty acid, linolenic acid is an essential fatty acid for insects, and a component of the cell membrane. Also, linolenic acid is a precursor of jasmonic acid (Hamberg and Gardner, 1992) which affects insect resistance (Farmer et al., 1992), thus methyl linolenate may be related to the insect and plant interactions.

Feeding response to authentic methyl linolenate plus sugars was lower than that of methanol extracts. It seems that there are still other minor compounds which affect the feeding of *E. vigintioctomaculata* in other fractions. In authentic sugar tests, because activity of fructose was clearly higher than glucose at the same concentration, fructose is thought to be a main feeding stimulant in the water portion.

Sugars have been known as feeding stimulants for many insects (Bernays and Simpson, 1982). Solanine and tomatine, contained specifically in potato and tomato respectively, did not stimulate the feeding of *E. vigintioctomaculata* (Abe and Matsuda, 2000). Though methyl linolenate is not thought to be a peculiar component in the Solanaceae, it was especially rich in potato leaves among seven species of solanaceous and cucurbitaceous plants (N. Endo, unpublished data). Furthermore, the strongest feeding activity was induced by the amount of methyl linolenate contained in the potato leaves. It is suggest that methyl linolenate plays an important role in the host selection of *E. vigintioctomaculata*.

ACKNOWLEDGEMENTS

We thank Dr. T. Wada, National Agricultural Research Center for Kyusyu Okinawa Region and W. Sugeno of our laboratory for reading and improving our manuscript. We also thank two anonymous reviewers for their valuable comments.

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