JOURNAL OF APPLIED ENTOMOLOGY

J. Appl. Entomol.

Chemicals affecting the feeding preference of the Solanaceae-feeding lady beetle *Henosepilachna vigintioctomaculata* (Coleoptera: Coccinellidae)

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Keywords
alkaloids, feeding stimulatory activity, host selection, methyl linoleate, methyl linolenate, Solanaceae

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Received: September 3, 2009; accepted: February 9, 2010.

doi: 10.1111/j.1439-0418.2010.01519.x

Abstract

The phytophagous lady beetle, *Henosepilachna vigintioctomaculata*, feeds mainly on potato, tomato, and eggplant leaves. The methanol extracts of tomato and eggplant leaves stimulated feeding activity in the adult beetles. The feeding stimulants from the lipid-soluble fractions of tomato and eggplant leaves were found to be same compounds, identified as methyl linoleate and methyl linolenate. The feeding stimulants in the aqueous fractions of tomato leaves were identified as three sugars – fructose, glucose, and sucrose – and in eggplant leaves, the feeding stimulant was one sugar, sucrose. Although methyl linoleate and methyl linolenate were inactive without sugars, they acted synergistically with sugars, and the amounts of methyl linoleate, methyl linolenate, and sugars contained in tomato and eggplant leaves were adequate to stimulate feeding activity in the beetles. It is suggested that the synergisms of methyl esters of unsaturated fatty acid and sugars play important roles in host selection of this insect. Solanaceae species are often rich in alkaloids, which act as feeding deterrents for many insect species. Leaves of potato, Chinese wolfberry, and black nightshade, which are suitable hosts of *H. vigintioctomaculata*, contain α-solanine and α-chaconine as the main alkaloids. A mixture of α-solanine and α-chaconine showed neither feeding stimulant nor inhibitory activity at a concentration of 1 g leaf equivalents of the above plants. α-Solanine and tomatine contained in tomato did not inhibit the beetle's feeding at a concentration of 0.5%. On the contrary, nicotine and capsaicin contained in non-hosts (tobacco and red pepper, respectively) showed feeding deterrent activities at a concentration of 0.1%. It is thought that adaptation of the beetles to alkaloids contained in solanaceous hosts also plays an important role in their host selection.

Introduction

Henosepilachna vigintioctomaculata Motschulsky, belonging to Epilachninae, is a phytophagous lady beetle known as an important pest of potato *Solanum tuberosum* L., tomato *Solanum lycopersicum* L., and eggplant *Solanum melongena* L. Among wild solanaceous plants, Chinese wolfberry (*Lycium chinense* Miller) and black nightshade (*Solanum nigrum* L.) are suitable hosts for this insect. Regarding the feeding stimulants of Epilachninae, the following information is known. *Epilachna varivestis* Mulsant is stimulated to feed by sucrose, fructose, glucose (Augustine et al. 1964), phaseolunatin, linamarin, and lotaustraline (Nayer and Fraenkel 1963) contained in lima bean *Phaseolus lunatus* L. The feeding of *Henosepilachna boisduvali* (Mulsant) is stimulated by cucurbitacin B (Abe et al. 2000), and *Epilachna*

admirabilis Crotch is stimulated by cucurbitacin E, B, I, and E-glucoside (Abe and Matsuda 2000). *H. vigintioctomaculata* and its related species, *H. vigintioctopunctata*, are stimulated to feed by sugars, fructose, maltose, and sucrose (Hori et al. 2005b). *H. vigintioctomaculata* is also stimulated by alanine, phenylalanine, tryptophan, ascorbic acid, linoleic acid, and linolenic acid. Luteolin 7-*O*-glucoside contained in the leaves of *Physalis alkekengi* L. acts as a feeding stimulant for *H. vigintioctomaculata* and *H. vigintioctopunctata* when this compound is combined with the water portion of *P. alkekengi* (Hori et al. 2005a). However, *H. vigintioctopunctata* feeds on *P. alkekengi*, whereas *H. vigintioctomaculata* cannot feed on this plant. Feeding deterrents contained in *P. alkekengi* cause its rejection by *H. vigintioctomaculata*; for *H. vigintioctomaculata*, the activities of feeding deterrents in *P. alkekengi* overcome the activities of feeding stimulants such as luteolin 7-*O*-glucoside.

The feeding stimulants of *H. vigintioctomaculata* contained in potato leaves have already been identified as methyl linolenate and the sugars glucose and fructose (Endo et al. 2004). Although methyl linolenate alone is inactive, it acts synergistically with sugars. However, the feeding stimulants for the other main host crops, i.e. tomato and eggplant, remain unclear. Therefore, we tried to isolate the feeding stimulants from tomato and eggplant leaves that showed the highest activity to *H. vigintioctomaculata*. We then investigated whether the main chemical factors of feeding stimulation were common among solanaceous plant species.

Solanaceous plants are often rich in alkaloids, which are toxic to many animal species. Therefore, it is necessary to investigate not only feeding stimulants but also the effect of solanaceous alkaloids on beetles' feeding in order to clarify the host-selection mechanism of this insect. However, the effects of alkaloids have not been clarified as yet. It is only known that solanine and tomatine did not stimulate the feeding of *H. vigintioctomaculata* at concentrations of 0.1 and 1.0 mg/ml (Abe and Matsuda 2000). α-Solanine and α-chaconine are major glycoalkaloids in solanaceous plants and are the main alkaloids in the most suitable host crop, potato, and a suitable host wild plant, black nightshade (Lyytinen et al. 2007; Ganguly et al. 2009). It is thought that wild solanaceous plants, such as black nightshade and Chinese wolfberry, had been the main host plants of *H. vigintioctomaculata* before solanaceous crops, such as potato and tomato, began to be cultivated in Japan. Therefore, it is thought that *H. vigintioctomaculata* was already adapted to solanaceous glycoalkaloids when cultivation of solanaceous crops started. Therefore, the effects of α-solanine and α-chaconine contained in potato, black nightshade, and Chinese wolfberry on the feeding of *H. vigintioctomaculata* were investigated in order to clarify the adaptation of the beetles to solanaceous alkaloids during host selection. In addition, the antifeeding activities of major solanaceous crop alkaloids contained in the host plants, potato and tomato, and non-host plants, tobacco and red pepper, were compared to investigate the influence of alkaloids on the feeding preference of *H. vigintioctomaculata*.

Materials and Methods

Insects and plants

Henosepilachna vigintioctomaculata adults were collected from cultivated potato fields at Natori City, Miyagi Prefecture, Japan, and their progeny were reared at 24 ± 1°C under a photoperiod of 16L:8D. Leaves of potato, tomato, and Chinese wolfberry were provided as food for *H. vigintioctomaculata*. Unsexed 4- to 11-day-old adults were used for all bioassays. The tomato, eggplant, and potato used for the tests were cultivated in the experimental fields at Tohoku University. Chinese wolfberry and black nightshade used for the tests were collected from the experimental fields at Tohoku University.

Chemicals

Methyl linolenate, methyl palmitate, methyl stearate, α-tocopherol, sucrose, fructose, glucose, capsaicin, and nicotine were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Methyl linoleate and tomatine were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). α-Solanine and α-chaconine were purchased from Sigma-Aldrich Co. (St. Louis, MO 63103).

Feeding stimulants contained in the leaves of tomato and eggplant

Bioassay

Feeding tests were conducted with a filter paper assay (Abe and Matsuda 2000). Three sheets of filter paper (ADVANTEC®, No. 1, diameter 70 mm; Toyo Roshi Kaisha, Ltd., Tokyo, Japan) were placed on the bottom of a plastic Petri dish (diameter 90 mm; height 20 mm) and moistened with 3 ml of distilled water. A doughnut-like plastic disc (inner diameter 20 mm; outer diameter 70 mm) was set on the filter

paper sheets. Two treated and two control filter papers (ADVANTEC®, No. 50, 2 × 2 cm; Toyo Roshi Kaisha, Ltd., Tokyo, Japan) were placed equidistantly on the plastic disc. Two control filter papers or two treated filter papers were placed facing each other. As the exceptions, the activity of mixtures of methyl esters of unsaturated fatty acid and sugars, and dose–response were assayed without control filter papers. Five adult beetles that were starved for 24 h were released in the Petri dish and the dish was placed in an incubator (24 ± 1°C, 16L:8D photoregime) for 24 h.

The piece of treated filter paper was impregnated with 75 μl of the test extracts dissolved in each extracted solvent (1 g fresh leaf equivalent/ml) and allowed to dry at room temperature. Control filter paper was treated with 75 μl of each solvent. Distilled water (75 μl) was added to the filter paper prior to the test.

In the bioassays of the mixture of two different portions, the filter paper was first treated with one of the two portions of the mixture; after the paper was dried, it was treated with the other portion. After the paper dried, distilled water (75 μl) was added. The results of these bioassays showed that neither the chloroform portion nor the water portion acted individually as a feeding stimulant, but the mixture of these two portions together acted as a feeding stimulant. Therefore, in the following bioassays, the water portion (1 g leaf equivalent/ml) was added to each fraction of the chloroform portion, and the hexane portion (1 g leaf equivalent/ml), which was the active portion of the chloroform portion, was added to each fraction of the water portion, respectively. Control filter paper was treated with solvents only. As the exceptions, the activity of methyl esters of fatty acid and α-tocopherol were compared with that of the water-portion control, and the activity of sugars was compared with that of the hexane-portion control.

Dose–responses to the authentic compounds were assayed at following concentrations. Unsaturated fatty acids were assayed at the concentrations of 0.125, 0.25, 0.5, 1, 2, 4, 10 and 50 g leaf equivalent of tomato/ml (1 g leaf equivalent of tomato: mixture of 38.02 μg of methyl linoleate and 88.59 μg of methyl linolenate), and 0.25, 0.5, 2, 4 and 10 g leaf equivalent of eggplant/ml (1 g leaf equivalent of eggplant: mixture of 114.61 μg of methyl linoleate and 141.72 μg of methyl linolenate). Sugars were assayed at the concentrations of 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 10 and 50 g leaf equivalent of tomato/ml (1 g leaf equivalent of tomato: mixture of 6.53 mg of fructose, 5.05 mg of sucrose, and 5.31 mg of glucose), and 0.01, 0.1, 0.25, 0.5, 1, 2, 10 and 100 g leaf equivalent of eggplant/ml (1 g leaf equivalent of eggplant: 15.58 mg of sucrose).

After the completion of the test, each filter paper square was divided into 100 sections (2 × 2 mm each), and the number of sections with bites in them were counted. The feeding activity is shown as a feeding index (maximum 200) and was evaluated by a paired *t*-test.

Extraction of tomato and eggplant leaves

Fresh leaves of tomato and eggplant (1000 g each) were treated with methanol (4 l and 6 l, respectively) for 24 h to obtain the extracts; both extracts were filtered. Extractions were conducted six times in each plant. After removal of methanol *in vacuo* below 40°C, the extracts were successively partitioned into chloroform, *n*-butanol, and water-soluble portions (1 l each). Partitions were replicated three times in each solvent. The extracts were evaporated to dryness.

Fractionation and identification of the feeding stimulants in the chloroform portion

The chloroform portion was dissolved in 80% methanol in water (1 l), partitioned with hexane (1 l), and the solvent was removed *in vacuo* at temperature below 40°C. Partitions were replicated three times. The hexane portion was chromatographed on a silica gel column (Wako-gel® C-200, 100–200 mesh, 200 g, 250 × 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 each). Eluates were collected (500 ml each) and analyzed by thin layer chromatography (TLC, Silica gel 60 F_{254}, 5 × 10 cm, Merck, Darmstadt, Land Hessen, Germany). Eluates with the same R_f value on TLC were combined. In eggplant leaves, the most active fraction was analyzed by gas chromatography-mass spectrometry (GC-MS), and the components were identified by comparing the gas chromatograph (GC) retention times and mass spectra with those of the authentic standards of methyl linolenate, methyl linoleate, methyl palmitate and methyl stearate (see 'Chemicals'). GC-MS was conducted with a Shimadzu GCMS-QP2010 equipped with a DB-5MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness, J&W). The mass data were analyzed by a Shimadzu GCMS Solution with a NIST mass spectral database. Helium was used as the carrier gas at a column head pressure of 100 kPa. GC was set for split injection (split ratio 100 : 1). The temperature program of the column oven was as follows: isotherm for 5 min at 40°C,

10°C/min gradient to 280°C, and isotherm for 30 min at 280°C. The injector and detector temperatures were 200°C and 220°C, respectively. In the tomato leaves, the most active fraction was further chromatographed on a silica gel column (Wako-gel® C-200, 100–200 mesh, 50 g, 350 × 21 mm i.d.). The column was eluted with hexane : ethyl acetate (70 : 1, 97 : 3, 93 : 7, 0 : 100; 300 ml each). Eluates were collected (50 ml each) and fractionated in the manner described above. The active fractions were analyzed by GC-MS, and the components were identified the same manner as the eggplant leaves, with authentic standards of methyl linolenate, methyl linoleate, methyl palmitate, and α-tocopherol (see 'Chemicals').

Fractionation and identification of feeding stimulants in the water portion

The water portion was chromatographed on an ODS column (YMC-gel® ODS-AQ, S-50 μm, 12 nm, 45 g, 470 × 15.5 mm i.d.) and successively eluted with water : methanol (1 : 0, 3 : 1, 1 : 1, 0 : 1; 200 ml each). Eluates were collected (100 ml each) and analyzed by TLC (Silica gel 60 F_{254}, 5 × 10 cm, Merck). Eluates with the same R_f value on TLC were combined. The most active fraction was further chromatographed by high performance liquid chromatography (HPLC) using a YMC-Pack® Polyamine II column (250 × 10.0 mm i.d., particle size 5 μm) at 30°C by a column oven. The HPLC system (Shimadzu) was composed of a pump LC-20AT (×2), refractive index detector RID-10A, column oven CTO-20AC, system controller CBM-20A, online degasser DGU-20A3, autosampler SIL-10AF, fraction collector FRC-10A, chromatography workstation, and data processing system LCsolution. In the tomato leaves, the eluent was 60% acetonitrile in water at a flow rate of 2 ml/min. In the eggplant leaves, the eluent was 75% acetonitrile in water at a flow rate of 5 ml/min. The components of the active fractions were identified by comparison of HPLC retention times to those of authentic compounds.

Quantification of feeding stimulants

The quantity of each component contained in the hexane portion of fresh tomato and eggplant leaves was calculated by using GC-MS as described above. The peak area of total ion chromatogram was measured and the concentration of each component was calculated using the calibration curve obtained from the peak area of authentic standards of methyl linolenate, methyl palmitate, methyl stearate, α-tocopherol, and methyl linoleate (see 'Chemicals'). Each component was analyzed three times in each plant, except for methyl palmitate in tomato which was analyzed four times. Sugars contained in the water portion of fresh tomato and eggplant leaves were quantitated by HPLC as described above and calculated by the same method used for the hexane portion using authentic sugars. Authentic standards of sucrose, fructose, and glucose (see 'Chemicals') were used for confirmation of identity. Each sugar was analyzed four times and three times in tomato and eggplant leaves, respectively.

Effects of alkaloids contained in solanaceous plants on feeding

Feeding stimulatory activity of α-solanine and α-chaconine, main alkaloids in the leaves of potato, Chinese wolfberry, and black nightshade

Feeding stimulatory activities of α-solanine and α-chaconine to *H. vigintioctomaculata* were evaluated with the same filter paper assay as described in section 3.1. Two treated and two control filter papers were impregnated with methanol solution (75 μl) of authentic alkaloids (1 g leaf equivalent/ml) and methanol solvents, respectively. After the paper was dried, distilled water (75 μl) was added to both treated and control filter papers. Furthermore, the effects of these alkaloids on feeding stimulatory activity of sucrose were also evaluated. In these assays, 0.1 M sucrose solution (75 μl), which exhibited feeding stimulatory activity in a preliminary test, was added to the filter papers instead of distilled water.

Quantification of α-solanine and α-chaconine contained in the leaves of potato, Chinese wolfberry, and black nightshade

α-Solanine and α-chaconine in leaves of each plant were quantitated by the same manner of Shindo et al. (2004) with the HPLC system described above, with the exception of the column, detector, and fraction collector. A YMC-Pack ODS-AM column (250 × 4.6 mm i.d., particle size 5 μm) and photodiode array detector SPD-M20A (Shimadzu) were used, and a fraction collector was not used. Fresh leaves of potato, Chinese wolfberry, and black nightshade were extracted with methanol for 24 h (20 ml methanol per gram fresh leaves) and the extracts were evaporated below 40°C. Extractions were conducted three times in each plant. A 100-mg aliquot of methanol extract of each plant was dissolved in 5 ml of methanol. Each extract solution was mixed with 12 ml of distilled water and developed based on solid-phase extraction using a Sep-Pak® Plus C18 cartridge (Waters Corp., Milford, MA 01757) conditioned with 10 ml of methanol and 10 ml of distilled

water prior to development. Each sample developed on the cartridge was washed in 5 ml of 30% methanol and successively eluted with 15 ml of methanol. Each eluate was evaporated to dryness and analyzed by HPLC. The mixture of acetonitrile in water (72.2%) and 0.1 M sodium phosphate buffer (pH 7.6) (mixture ratio was 9 : 1) was used as the eluent at a flow rate of 0.5 ml/min. Column oven temperature was 40°C. The wave length of the photo-diode array detector was adjusted to 202 nm. The peak area was measured and the concentration of each alkaloid was calculated using the calibration curve obtained from the peak area of the authentic alkaloid. Each alkaloid was analyzed two times in each plant.

Feeding deterrent activities of solanaceous alkaloids

Feeding deterrent activities of solanaceous alkaloids, α-solanine, tomatine, capsaicin, and nicotine, were investigated with a leaf-disc assay. A 25-μl aliquot of ethanol solution of each alkaloid (concentration: 0.5% in α-solanine and tomatine, 0.1% in capsaicin and nicotine) was applied to the underside of a tomato-leaf disc (20 mm) and was dried to remove the ethanol. The control disc was treated with only ethanol. A treated or control leaf disc was put at the centre of the filter paper (ADVANTEC®, No. 1, diameter 55 mm; Toyo Roshi Kaisha, Ltd., Tokyo, Japan) moistened with 0.5 ml of distilled water in a glass Petri dish (diameter 55 mm; height 15 mm). Five adults starved for 24 h were released in the Petri dish and the dish was placed in an incubator (24 ± 1°C) for 2 h under light. Males and females were tested separately. After the test was finished, the feeding area of each leaf disc tested was measured by using sectional tracing paper. Feeding inhibitory activity of each alkaloid was evaluated by Dunnett's test for comparison of feeding area in each alkaloid with that in the control. These data were analyzed separately for males and females. Five replications were conducted.

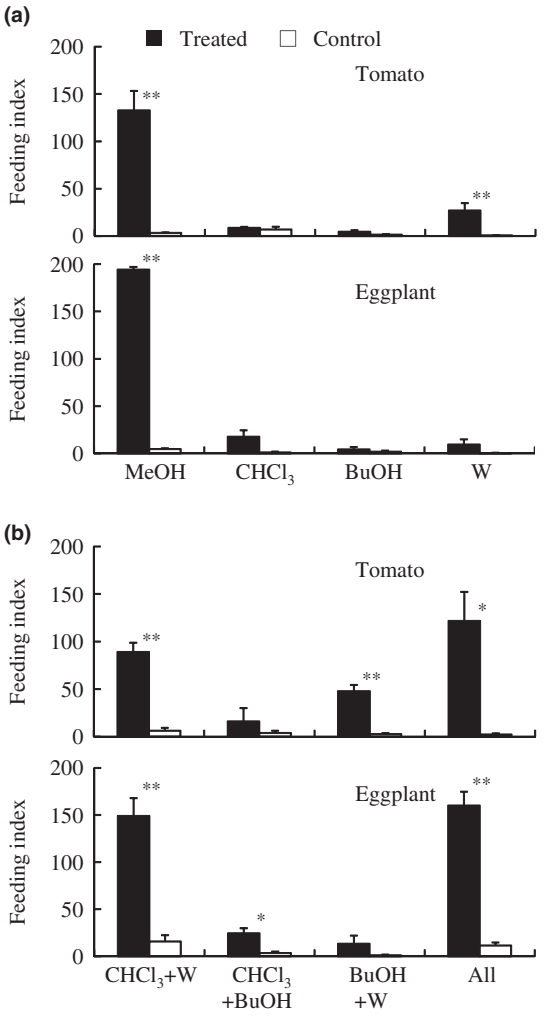

Fig. 1 Feeding responses (mean ± SE) of *H. vigintioctomaculata* to each portion (a) and mixture of portions (b). Significant difference between the control and the treated filter papers is represented by an asterisk [paired *t*-test: *P < 0.05, **P < 0.01, n = 5 except for MeOH (n = 10) and W (n = 20) in tomato]. MeOH: methanol extract; $CHCl_3$: chloroform portion; BuOH: butanol portion; W: water portion; All: mixture of chloroform, butanol, and water portions. Control filter papers were impregnated with only distilled water.

Results

Feeding stimulatory activities of methanol extracts and each solvent portion of tomato and eggplant leaves

The methanol extracts of tomato and eggplant leaves stimulated the feeding of *H. vigintioctomaculata* (fig. 1a). The water portion of tomato leaves significantly stimulated feeding activity, but the feeding stimulatory activity was not so high. The other portions of tomato leaves and all portions of eggplant leaves did not stimulate beetle feeding. In both plants, feeding stimulatory activities were induced when all portions were combined (fig. 1b). In the tomato leaves, the mixture of the water portion and chloroform or butanol portion also showed significant feeding stimulatory activity. The activity of the mixture of the chloroform and water portions was higher than that of the mixture of the butanol and water portions. In the eggplant leaves, feeding stimulatory activity was also induced when the chloroform and water portions were combined. Although the mixture of the chloroform and butanol portions

stimulated feeding significantly, the activity was not so high. Therefore, we investigated feeding stimulants in both the chloroform and water portions.

Feeding stimulants in the chloroform portion of tomato and eggplant leaves

In both tomato and eggplant leaves, although both the hexane and the 80% methanol portions stimulated beetle feeding, the feeding stimulatory activities of the hexane portion were higher than those of the 80% methanol portion (fig. 2). Therefore, the hexane portions were fractionated with silica gel column chromatography.

In tomato leaves, the hexane portion was separated into six fractions, and Fr-1 showed the highest feeding stimulatory activity. Fr-1 was further separated into seven fractions with silica gel column chromatography, and Fr-1-3 showed the highest feeding stimulatory activity, followed by Fr-1-4. The combination of Fr-1-2, Fr-1-3, and Fr-1-4 induced the highest activity among the various combinations of the seven fractions. Therefore, the components contained in Fr-1-2, Fr-1-3, and Fr-1-4 were identified by GC-MS analysis. Fr-1-2 and Fr-1-4 were identified as methyl palmitate and α-tocopherol, respectively, and Fr-1-3 was composed of methyl linoleate and methyl linolenate.

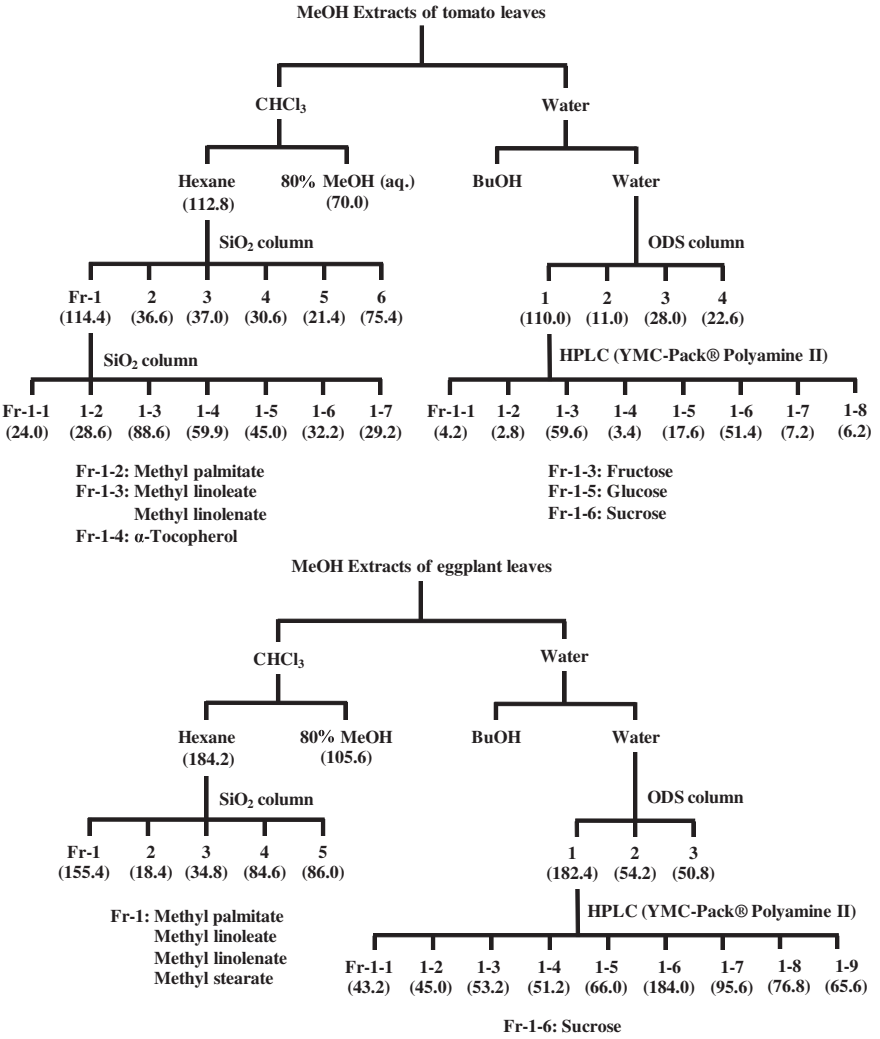

Fig. 2 Separation scheme and feeding stimulant activity of tomato and eggplant leaves to *H. vigintioctomaculata*. The chloroform and the water portions were added to water and hexane portions, respectively. Control filter papers were impregnated with only distilled water. Values in parentheses indicate the average feeding index. Five replications were conducted, except for the hexane portion and its fractions, Fr-1-2, 1-3, 1-4 (n = 10).

In the eggplant leaves, the hexane portion was separated into five fractions, and Fr-1 showed the highest feeding stimulatory activity. GC-MS analysis showed that Fr-1 was mainly composed of four compounds, methyl palmitate, methyl linoleate, methyl linolenate, and methyl stearate. The quantity of each compound contained in the 1 g of fresh leaves of each plant is shown in table 1.

Feeding stimulants in the water portion of tomato and eggplant leaves

In both tomato and eggplant leaves, Fr-1 separated by using an ODS column showed the highest feeding stimulatory activity among the fractions (fig. 2). Therefore, Fr-1 was further fractionated by HPLC.

In the tomato leaves, Fr-1-3 induced the highest feeding stimulatory activity among the eight fractions, followed by Fr-1-6 and Fr-1-5. HPLC analysis showed that Fr-1-3, Fr-1-6, and Fr-1-5 were fructose, sucrose, and glucose, respectively. In the eggplant leaves, Fr-1-6 showed the highest activity among nine fractions. Fr-1-6 was identified as sucrose by HPLC analysis. The quantity of each sugar contained in 1 g of fresh leaves of each plant is shown in table 2.

Feeding responses to authentic compounds contained in tomato and eggplant leaves

In both tomato and eggplant leaves, methyl linoleate and methyl linolenate combined with the water portion significantly stimulated beetle feeding at a concentration of 1 g leaf equivalent (fig. 3). Methyl palmitate, methyl stearate, and α-tocopherol did not show feeding stimulatory activities at a concentration of 1 g leaf equivalent of tomato or eggplant. Fructose, glucose, and sucrose with the hexane portion significantly stimulated feeding at a concentration of 1 g leaf equivalent of tomato or eggplant (fig. 4). The feeding stimulatory activities of methyl esters of unsaturated fatty acid or sugars alone were low (fig. 5). However, the activities were enhanced when methyl esters of unsaturated fatty acid and sugars were combined. The feeding index of methyl esters of unsaturated fatty acid with the water portion was the highest at 2–4 g leaf equivalent and 1 g leaf equivalent of tomato and eggplant leaves, respectively (fig. 6). The feeding index of sugars with the hexane portion was the highest at 4 g leaf equivalent and 0.5–2 g leaf equivalent in tomato and eggplant leaves, respectively (fig. 7). Sugars at 50–100 g leaf equivalent did not show feeding stimulatory activity.

Table 1 The compounds in the active fraction of chloroform portion of tomato and eggplant leaves

Plant compounds	Mean quantity in the 1 g of fresh leaves (μg)	SD	N
Tomato			
Methyl palmitate	3.69	0.16	4
Methyl linoleate	38.02	3.79	3
Methyl linolenate	88.59	2.18	3
α-Tocopherol	42.36	16.44	3
Eggplant			
Methyl palmitate	62.03	3.05	3
Methyl linoleate	114.61	6.50	3
Methyl linolenate	141.72	26.28	3
Methyl stearate	19.16	1.87	3

Table 2 The compounds in the active fraction of water portion of tomato and eggplant leaves

Plant compounds	Mean quantity in the 1 g of fresh leaves (mg)	SD	N
Tomato			
Fructose	6.53	0.14	4
Sucrose	5.05	0.12	4
Glucose	5.31	0.16	4
Eggplant			
Sucrose	15.58	0.50	3

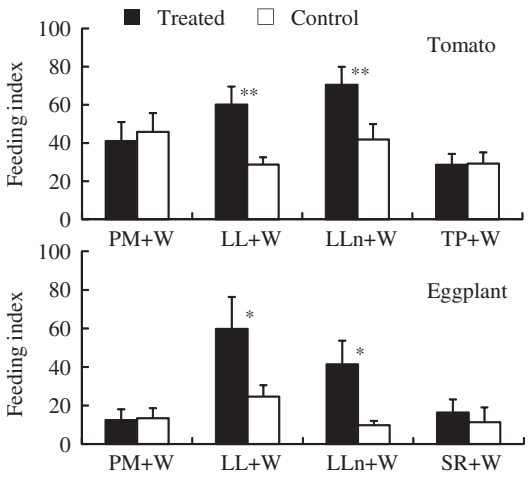

Fig. 3 Feeding responses (mean ± SE) of *H. vigintioctomaculata* to each authentic chemical contained in the hexane portion with the water portion at 1 g leaf equivalent/ml. Significant difference between the control and the treated filter papers is represented by an asterisk (paired *t*-test: *P < 0.05, **P < 0.01, n = 10 in tomato except for LLn + W (n = 15), n = 5 in eggplant). PM: methyl palmitate, LL: methyl linoleate, LLn: methyl linolenate, TP: α-tocopherol, SR: methyl stearate, W: water portion. Control filter paper was treated with the water portion of each plant.

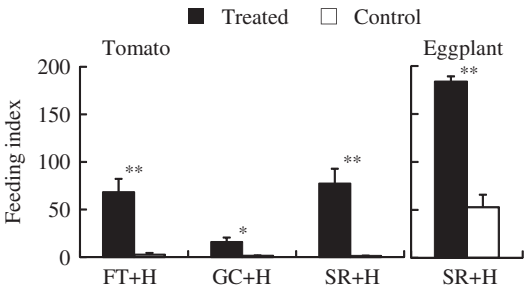

Fig. 4 Feeding responses (mean ± SE) of *H. vigintioctomaculata* to each authentic sugar contained in the water portion with the hexane portion at 1 g leaf equivalent/ml. Significant difference between the control and the treated filter papers is represented by an asterisk (paired *t*-test: *P < 0.05, **P < 0.01, n = 10 in tomato, n = 5 in eggplant). FT: fructose, GC: glucose, SR: sucrose, H: hexane portion. Control filter paper was treated with the hexane portion of each plant.

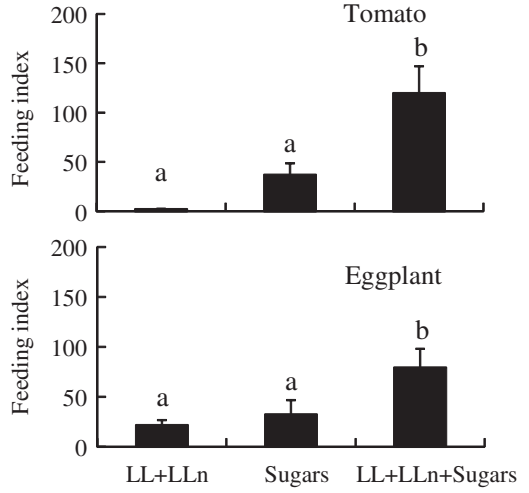

Fig. 5 Feeding responses (mean ± SE) of *H. vigintioctomaculata* to the mixtures of authentic methyl esters of unsaturated fatty acid and sugars at 1 g leaf equivalent/ml. Different letters above bars indicate significant differences among treatments at P < 0.05 (Tukey–Kramer test, n = 5). LL: methyl linoleate; LLn: methyl linolenate; Sugars: mixture of fructose, glucose, and sucrose (tomato), and sucrose (eggplant). The activities were assayed without control filter papers.

Effects of alkaloids contained in solanaceous plants on feeding activity

The quantities of α-solanine and α-chaconine contained in the 1 g of fresh potato, Chinese wolfberry, and black nightshade leaves are shown in table 3. The mixture of α-solanine and α-chaconine without sucrose did not stimulate beetle feeding at 1 g leaf equivalent of these plants. The mixtures neither enhanced nor inhibited the feeding stimulatory activity of sucrose at 1 g leaf equivalent of these three plants (fig. 8). That is, the mixture of

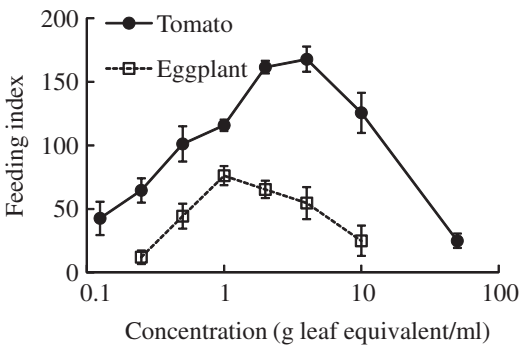

Fig. 6 Feeding responses of *H. vigintioctomaculata* to different concentrations of the mixture of methyl linoleate and methyl linolenate with the water portion of 1 g leaf equivalent. Each point indicates the mean ± SE of five replications. The activities were assayed without control filter papers.

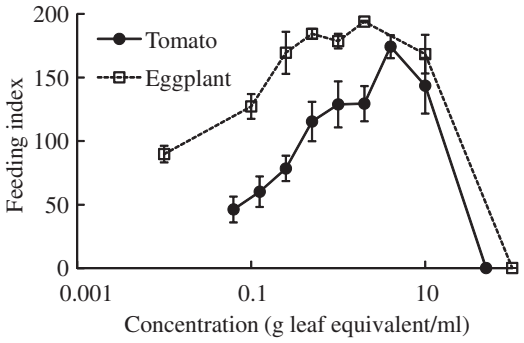

Fig. 7 Feeding responses of *H. vigintioctomaculata* to different concentrations of sugars contained in tomato or eggplant leaves with the hexane portion of 1 g leaf equivalent. Each point indicates the mean SE of five replications. The activities were assayed without control filter papers.

α-solanine and α-chaconine did not affect the feeding of *H. vigintioctomaculata* at 1 g leaf equivalent of these plants. Host-plant alkaloids, α-solanine and tomatine contained in tomato leaves, did not inhibit the beetles from feeding at a concentration of 0.5%, whereas non-host plant alkaloids, nicotine contained in tobacco leaves and capsaicin contained in red pepper leaves, strongly inhibited feeding even at a concentration of 0.1%, regardless of sex (fig. 9).

Discussion

A previous study (Endo et al. 2004) and the present study show that feeding stimulants contained in the host-plants potato, tomato, and eggplant play an important role in the host selection of *H. vigintioctomaculata*.

Table 3 The quantities of α-solanine and α-chaconine contained in 1 g of fresh potato, Chinese wolfberry and black nightshade leaves

Plant alkaloids	Mean quantity (μg)	SD	N
Potato			
α-Solanine	83.50	4.75	2
α-Chaconine	372.56	35.30	2
Chinese wolfberry			
α-Solanine	14.37	1.02	2
α-Chaconine	217.91	32.12	2
Black nightshade			
α-Solanine	31.83	6.39	2
α-Chaconine	198.15	18.20	2

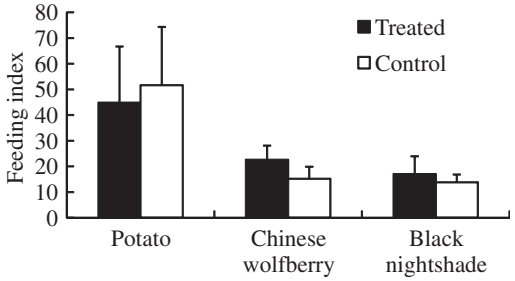

Fig. 8 Feeding responses (mean ± SE) of *H. vigintioctomaculata* to the mixtures of α-solanine and α-chaconine, main alkaloids of potato, Chinese wolfberry, and black nightshade at 1 g leaf equivalent/ml with 0.1 M sucrose. No significant difference between the control and the treated filter papers was obtained (paired *t*-test: P > 0.05, n = 5). Control filter papers were treated with only 0.1 M sucrose.

Although the methanol extracts of tomato and eggplant leaves strongly stimulated feeding, none of the individual portions fractionated from methanol extracts stimulated feeding. However, in both tomato and eggplant leaves, a combination of the chloroform and water portions exhibited high feeding stimulatory activity in the beetles. In addition, the combination of butanol and water portions in tomato leaves, and also the combination of chloroform and butanol portions in eggplant leaves, significantly stimulated feeding. However, these activities were much lower than those of combinations of the chloroform and water portions. Therefore, it is thought that the feeding stimulatory activities of both plant leaves are mainly caused by the synergism of the chloroform and water portions. In leaves of both plants, the main active components in the chloroform and water portions were identified as two methyl esters of unsaturated fatty acid, methyl linoleate and methyl linolenate, and sugars, respectively. Endo et al. (2004) reported that synergism of methyl linolenate and sugars of potato leaves were one of the important factors of feeding

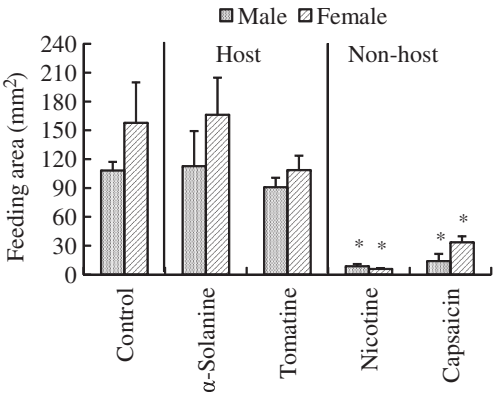

Fig. 9 Feeding deterrent activities of each solanaceous alkaloid to *H. vigintioctomaculata*. Significant difference from control is represented by an asterisk (Dunnett's test: *P < 0.05, n = 5). Value of feeding area is represented as the mean ± SE. Control leaf disc was treated with only ethanol. α-Solanine and tomatine are contained in host plant leaves, and nicotine and capsaicin are contained in non-host plant leaves. Concentration of α-solanine and tomatine was 0.5%, and concentration of nicotine and capsaicin was 0.1%.

stimulatory activity. The present study showed that the main chemical factors of feeding stimulation to *H. vigintioctomaculata* are common among the main host crops, potato, tomato, and eggplant. That is, synergism of the methyl esters of unsaturated fatty acid and sugars plays an important role in the preference of *H. vigintioctomaculata* for solanaceous plants.

In some insect species, it is known that lipid nutrient chemicals act as feeding stimulants (Hsiao 1985). *Diabrotica virgifera virgifera* LeConte is attracted to a combination of linoleic acid or oleic acid and carbon dioxide (Hibbard et al. 1994). Linolenic acid and linoleic acid are essential nutrients for almost all insect species and act as feeding stimulants for *D. v. virgifera* (Lin and Mullin 1999) and also *H. vigintioctomaculata* (Hori et al. 2005b). However, no report has demonstrated that methyl esters of unsaturated fatty acid, such as methyl linoleate and methyl linolenate, act as feeding stimulants, except the previous study by Endo et al. (2004). The feeding stimulatory activity of methyl esters of unsaturated fatty acid may be related to nutritional requirements as fatty acids.

Although methyl esters of unsaturated fatty acid showed feeding stimulatory activities, methyl esters of saturated fatty acid, methyl palmitate, and methyl stearate did not show such activities. *H. vigintioctomaculata* is stimulated to feed by methyl oleate, a methyl ester of unsaturated fatty acid (N. Endo and K. Matsuda, unpublished data). It is thought that *H. vigintioctomaculata* recognize the slight difference caused by the presence of double bonds on the structures of methyl esters of fatty acid.

Feeding stimulants in the water portions of tomato leaves were identified as three sugars, fructose, glucose, and sucrose, and in eggplant leaves only one sugar was identified, i.e. sucrose. Approximately 5 mg of each sugar was contained in 1 g of fresh tomato leaves, while 1 g of fresh eggplant leaves contained 15 mg of sucrose and negligible other sugars. In the present study, sucrose contained in 0.5 g of fresh eggplant leaves (sucrose: ca. 7.5 mg) exhibited sufficient feeding stimulatory activity. In Endo et al.'s previous study (2004), fructose significantly stimulated the feeding of *H. vigintioctomaculata* but glucose did not. The total amount of sucrose and fructose may be important in feeding stimulatory activity for *H. vigintioctomaculata*.

In the eggplant leaves, the mixture of methyl linoleate and methyl linolenate showed the highest feeding stimulatory activity at a concentration of 1 g leaf equivalent, and showed negligible activity at a concentration of 10 g leaf equivalent. In the tomato leaves, the mixture of methyl linoleate and methyl linolenate showed relatively high feeding stimulatory activity at a concentration of 0.5–10 g leaf equivalents and showed negligible activity at concentrations above 50 g or below 0.25 g leaf equivalent. In addition, sugars showed relatively high feeding stimulatory activity at concentrations of 0.5–10 g and 0.25–10 g leaf equivalents of tomato and eggplant, respectively. However, sugars at concentrations of 50 or 100 g leaf equivalents had no activity. These results show that the amounts of methyl esters of both unsaturated fatty acids and sugars contained in tomato or eggplant leaves are adequate for feeding stimulatory activities to *H. vigintioctomaculata*.

In the dose–response tests, feeding stimulatory activities of unsaturated fatty acids or sugars were different between the compositions based on tomato and eggplant leaves. Total amount of methyl linoleate and methyl linolenate in eggplant leaves was approximately twofold amount in tomato leaves. However, the feeding stimulatory activity of unsaturated fatty acids at a concentration of 2 g leaf equivalent of tomato was much higher than that of 1 g leaf equivalent of eggplant. It is thought that ratio of methyl linoleate and methyl linolenate is important for the feeding stimulatory activity of unsaturated fatty acids rather than total amount. Also in sugars, although total amounts of sugars were similar between tomato and eggplant leaves, sugar composition based on eggplant leaves showed higher activity than that on tomato leaves. Sucrose, which is the only sugar identified from eggplant leaves, may be most important to the stimulatory activity of sugars.

Methyl linoleate, methyl linolenate, and sugars are general compounds that are contained in broad species of plant. Food plants of *H. vigintioctomaculata* are relatively broad and the beetles can feed on 59 species belonging to Solanaceae, Cucurbitaceae, Leguminosae, Brassicaceae, and so on (Hoshikawa 1983). *H. vigintioctomaculata* may be able to feed on a broad variety of plant species because they utilize general compounds as feeding stimulants.

On the contrary, solanaceous plants are often rich in alkaloids. Steroid alkaloids contained in solanaceous plants play an important role in enabling the Colorado potato beetle *Leptinotarsa decemlineata* Say to recognize host plants (Hsiao and Fraenkel 1968a; b,c; Buhr et al. 1958; Schreiber 1958). However, the feeding of *H. vigintioctomaculata* was not affected by α-solanine and α-chaconine at a 1 g leaf equivalent of each host plant. α-Solanine and α-chaconine may play no role in enabling *H. vigintioctomaculata* to recognize host plants. α-Solanine, α-chaconine, and tomatine inhibit the larvae of spruce budworm *Choristoneura fumiferana* (Clemens) from feeding (Bentley et al. 1984). Tomatine exhibits feeding deterrent activity against many species of insects, such as the Colorado potato beetle (Kuhn et al. 1950; Buhr et al. 1958; Schreiber 1958; Stürckow and Löw 1961), melon thrips *Thrips palmi* Karny (Hirano et al. 1994), and potato leafhopper *Empoasca fabae* (Harris) (Dahlman and Hibbs 1967). However, α-solanine and tomatine, host-plant alkaloids, did not show feeding deterrent activities to *H. vigintioctomaculata* at a concentration of 0.5%, whereas nicotine and capsaicin, non-host plant alkaloids, inhibited the beetles from feeding even at a concentration of 0.1%. Although in this study the activities of these alkaloids were evaluated at only one concentration, these results at least show that the beetles are better adapted to α-solanine and tomatine than to nicotine and capsaicin.

It is thought that *H. vigintioctomaculata* can utilize solanaceous plants, such as potato and tomato, as host plants because they are not inhibited from feeding by host-plant alkaloids such as α-solanine, α-chaconine, and tomatine. On the contrary, they cannot utilize some of the solanaceous plants, such as tobacco and red pepper, because their alkaloids act as feeding deterrents against *H. vigintioctomaculata*. That is, the non-effect of alkaloids contained in host plants on their feeding might play an important role in their host selection. It might be advantageous for food acquisition of *H. vigintioctomaculata* to be able to utilize solanaceous plants as hosts because many insect species do not prefer solanaceous plants

because of the feeding deterrent activity of alkaloids. It is necessary to investigate the dose–response of beetles to each alkaloid and content of each alkaloid in leaves of each plant in order to further clarify the role of alkaloids in host preference of the beetles.

It is thought that they can feed on solanaceous plants such as potato, tomato, and eggplant because their feeding is not deterred by these plant alkaloids and is stimulated by synergistic activity of methyl esters of unsaturated fatty acids and sugars.

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