# Ovipositional Deterrent on Mature Stage of Sweet Pepper, *Capsicum annuum*, against *Liriomyza trifolii* (Burgess)

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*Liriomyza trifolii* (Burgess), the American serpentine leafminer fly, is a well-known serious pest in the world. This insect species attacks plants of more than 21 families including Solanaceae plants. A sweet pepper, *Capsicum annuum* (Solanaceae), on mature stage, however, shows resistance to this leafminer fly. This resistance is based on the ovipositional deterrent in the sweet pepper leaf against the fly species. Based on the bioassay guided fractionation, phytol [(2*E*)-3,7,11,15-tetramethyl-2-hexadecen-1-ol] was isolated and identified as an ovipositional deterrent against this insect species. The yield of this compound was 815 $\mu$g/g fresh leaf of *C. annuum.* This compound completely deterred the females from laying their eggs on host plant leaves treated at 35.2 $\mu$g/cm$^2$.

*Key words: Liriomyza trifolii*, Ovipositional Deterrent, *Capsicum annuum*, Phytol

## Introduction

*Liriomyza trifolii* (Burgess), the American serpentine leafminer fly, originated from North America, is a well-known serious pest to many vegetables and crops in the world. It has a broad host range and can attack over 120 plant species of more than 21 families (Minkenberg and van Lenteren, 1986). Females of *L. trifolii* puncture the leaf surface for feeding and oviposition by using the ovipositor. The larvae, after hatching from the eggs, feed on the mesophyll tissue in the leaf and form a serpentine mine, which can reduce significantly the photosynthetic capacity of the plant (Chandler and Thomas, 1983; Johnson *et al.*, 1983). In addition to the broad host range, because the leafminers show resistance to most insecticides and can develop all year round in a greenhouse (Saito, 1992, 1993), their distribution area has quickly spread out to the world including Japan in 1990 (Saito, 1992; Sasakawa, 1993; Isawa *et al.*, 1999) and the pest control of the leafminer has become more difficult. Therefore, development of integrated pest control method against this pest species is necessary.

A sweet pepper, *Capsicum annuum* (Solanaceae) has been reported along with other Solanaceae plants as a host plant for this insect (Saito *et al.*, 1995). In fact, this insect species fiercely attacks *C. annuum* in young stage. However, we observed that *L. trifolii* seldom attacked and laid eggs on it at the maturity stage in our greenhouse.

Thus, in this paper we report the factors that contribute to the resistance of matured *C. annuum* against *L. trifolii.*

## Materials and Methods

### *Instruments*

GC-MS data was recorded on a JEOL JMS600 mass spectrometer with a capillary column (HP-5, closslinked 5% PH ME Siloxane, 30 m × 0.32 mm i.d., 0.25 $\mu$m thickness; carrier gas: He; temperature program: 70 °C with 2 min hold to 250 °C with 30 min hold at 10 °C/min). $^1$H NMR and $^{13}$C NMR spectra were measured on a JEOL JNM-AL 400 (400 MHz) instrument. TMS was used as the internal standard. The letters (br)s, d, t, q, and m represent (broad)singlet, doublet, triplet, quartet, and multiplet, respectively, and coupling constants are given in Hz. The solvent used was $CDCl_3$.

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*Insect and plant*

*Insect:* Stoke colonies of *L. trifolii* were initially provided by Kochi Pref. Agricultural Center, and then were successively reared on 10- to 14-day-old seedlings of kidney bean, *Phaseolus vulgaris* L., at (27 ± 2) °C and relative humidity of 60–70% with a 16 h:8 h (L:D) illumination. After emerging, 1-day-old females were used for bioassay.

*Plant:* Forty seeds of *C. annuum* var. *angulosum* (Wase) were sown in a plastic tray (20 cm × 27 cm length, 1 cm depth) containing nursery soil in a plant growth incubator at (27 ± 2) °C. After four weeks, the seedlings were transplanted in an individual pot (6.6 cm height, 5.0 cm i.d.) containing nursery soil and grown in a greenhouse without any application of insecticides. Plants on twenty leaves (approx. 17-week-old) stage were used for extract. 10- to 14-day-old seedlings of *P. vulgaris* (Daikintoki) were used for bioassay.

*Preparation of plant extract*

Fresh sweet pepper leaves (420 g) were twice extracted with 80% methanol in water (1 l) for 3 d under darkness. After concentrating under vacuum, the residues (33.9 g) were dissolved at a concentration of 1 g of fresh leaf equivalent/ml in methanol.

*Bioassay for plant extract*

A fresh leaf of kidney bean was dipped in the test solution (1 g of fresh leaf equivalent/ml in methanol) for 30 s. After removing the solvent, the leaf was put on a moistened filter paper in a petri dish (10 mm hight, 90 mm i.d) to maintain humidity. Five adult female flies, at least 24 h old, were released into a screw vial (28 mm ϕ) and placed on treated and control leaves. They were allowed to oviposit for 24 h at 27 °C with a 16 h:8 h (L:D) illumination. The number of ovipositional marks was counted on the leaf under a microscope. Each bioassay was replicated five times. Control leaves were treated in the same manner with methanol only.

*Isolation of compound **1***

The methanol extract (17.8 g, 320 g of fresh leaf equivalent) was dissolved in water (430 ml), then successively extracted with hexane (300 ml × 4, 3.80 g), diethyl ether (300 ml × 4, 0.45 g), ethyl acetate (300 ml × 4, 0.53 g) and water-saturated butanol (300 ml × 4, 3.1 g), respectively. The hexane layer (0.278 g, 200 g of fresh leaf equivalent) was applied on a silica gel open column (56 g), successively eluted with 100% hexane, 10% ethyl acetate in hexane, 30% ethyl acetate in hexane, 100% ethyl acetate and 100% methanol (560 ml each) to get hexane (9.0 mg), 10% ethyl acetate in hexane (310.1 mg), 30% ethyl acetate in hexane (142.7 mg), 100% ethyl acetate (103.5 mg) and 100% methanol (40.2 mg) fractions. The 10% ethyl acetate in hexane fraction (230 mg, 140 g of fresh leaf equivalent) was separated into three fractions, fr. A ($R_t$ = 0–10.0 min, 82.4 mg), fr. B ($R_t$ = 10.0–12.0 min, 114.0 mg) and fr. C ($R_t$ = 12.0–40.0 min, 32.0 mg), by using HPLC (column: YMC Packed Column SIL, 300 mm × 10 mm i.d.) eluting with 10% ethyl acetate in hexane at a flow rate of 3 ml/min with detection at 254 nm. Compound **1** was isolated at $R_t$ = 10.8 min from fr. B. The yield of compound **1** was 815 $\mu$g/g of fresh leaf equivalent.

Compound **1** [(2*E*)-3,7,11,15-tetramethyl-2-hexadecen-1-ol]: FT-IR (liquid film): $\nu_{max}$ = 3100–3400 (OH), 1500 $cm^{-1}$ (double bond). – EI-MS (30 eV): *m/z* (%) = 296 ($M^+$, 3.8), 279 (6.8), 278 (26.1), 179 (10.6), 138 (16.4), 137 (20.3), 126 (22.2), 125 (17.4), 124 (47.6), 123 (100), 111 (28.5), 109 (28.4), 97 (33.0). – $^1$H NMR: $\delta$ = 0.85 (3H, d, *J* = 2.4 Hz), 0.86 (3H, d, *J* = 6.0 Hz), 0.87 (3H, d, *J* = 4.0 Hz), 1.0 ~ 1.45 (19H, m), 1.52 (1H,7, *J* = 6.4 Hz), 1.66 (3H, d, *J* = 0.8 Hz), 1.98 (2H, t, *J* = 8.0 Hz), 4.14 (2H, d, *J* = 6.8 Hz), 5.40 (1H, tq, *J* = 6.8 Hz, 0.8). – $^{13}$C NMR: see Table I.

Reagent: [(2*E*)-3,7,11,15-tetramethyl-2-hexadecen-1-ol]: was purchased from Wako Chemical Ltd, Japan. – EI-MS (30 eV): *m/z* (%) = 296 ($M^+$, 3.8), 279 (6.8), 278 (26.1), 179 (10.6), 138 (16.4), 137 (20.3), 126 (822.2), 125 (17.4), 124 (47.6), 123 (100), 111 (28.5), 109 (28.4), 97 (33.0). – $^1$H NMR: $\delta$ = 0.85 (3H, d, *J* = 2.4 Hz), 0.86 (3H, d, *J* = 6.0 Hz), 0.87 (3H, d, *J* = 4.0 Hz), 1.0 ~ 1.45 (19H, m), 1.52 (1H, 7, *J* = 6.4 Hz), 1.66 (3H, d, *J* = 0.8 Hz), 1.98 (2H, t, *J* = 8.0 Hz), 4.14 (2H, d, *J* = 6.8 Hz), 5.40 (1H, tq, *J* = 6.8 Hz, 0.8). – $^{13}$C NMR: see Table I.

## Results and Discussion

A fresh kidney bean leaf was soaked in the methanol solution of matured *Capsicum annuum* (1 g of fresh leaf equivalent/ml) and then submit-

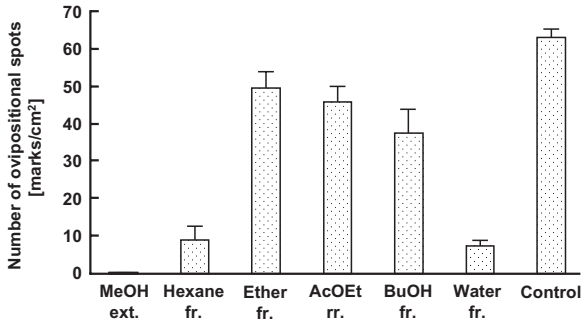


Fig. 1. Activities of the crude methanol extract and the several fractions separated by liquid/liquid partition.

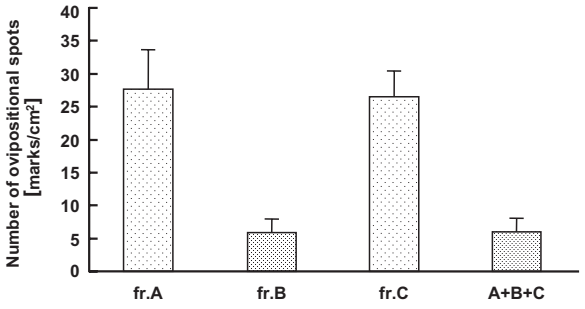


Fig. 2. Activities of the three fractions separated by HPLC.

ted to the bioassay. As shown in Fig. 1, only a few ovipositional marks (1.85 marks/cm$^2$ ± 0.72; mean ± S.E.) were observed on the treated leaves. On the other hand, a significant number of marks (64.83 marks/cm$^2$ ± 2.85) was observed on the control leaves. There was no larve at hatching from the treated leaves after keeping them in the incubator for a while. These results were almost similar to our observation in the greenhouse and clearly showed that the leaves of *C. annuum* at the mature stage had some ovipositional deterrent against this insect species

The active methanol extract was separated into the hexane, diethyl ether, ethyl acetate, water-saturated *n*-butanol and water fractions by liquid/liquid partition. Of these fractions, the hexane (9.03 marks/cm$^2$ ± 1.88) and water fraction (7.31 marks/cm$^2$ ± 0.13) showed intense activity. The other fractions (diethyl ether: 49.35 marks/cm$^2$ ± 4.27; ethyl acetate: 45.71 marks/cm$^2$ ± 4.04; water-saturated *n*-butanol: 37.53 marks/cm$^2$ ± 6.38) did not have any activity against this insect species (Fig. 1). Of active two fractions, the hexane fraction was applied on silica gel open column to get 100% hexane, 10% ethyl acetate in hexane, 30% ethyl acetate in hexane, 100% ethyl acetate, 100% methanol fractions. The obtained fractions were submitted to the bioassay. The bioassay results clearly exposed the active fraction. The 10% ethyl acetate in hexane fraction only showed activity (14.00 marks/cm$^2$ ± 5.99). The other fractions did not show any activity as well as that of the control (hexane: 48.68 marks/cm$^2$ ± 8.08; 30% ethyl acetate in hexane: 36.75 marks/cm$^2$ ± 6.90; 100% ethyl acetate: 42.41 marks/cm$^2$ ± 3.26; 100% methanol: 41.56 marks/cm$^2$ ± 7.57). The 10% ethyl acetate in hexane fraction was separated into three fractions (frs. A, B and C) by using HPLC. Of these three fractions, only fr. B showed a high activity (frs. A: 27.69 marks/cm$^2$ ± 5.99; fr. B: 5.91 marks/cm$^2$ ± 2.04; fr. C 26.55 marks/cm$^2$ ± 3.88; frs. A+B+C: 5.98 marks/cm$^2$ ± 2.09) as shown in Fig. 2. This Fr. B is an oily liquid and gave a single peak (compound **1**) by GC analysis.

From GC-MS data (EI *m*/*z* 296 [M$^+$]), the relative molar mass of compound **1** was found to be 296. In the $^{13}$C NMR spectrum (Table I), 20 carbon atoms were observed. Of those, two carbon atoms (140.0 ppm and 122.9 ppm) were based on a double bond and one carbon atom (59.4 ppm) was attached to a hydroxyl group according to their chemical shift values, respectively. FT-IR

Table I. List of the chemical shift values for compound **1** and phytol in $^{13}$C NMR spectra.

| C | **1** | Phytol |
|---|---|---|
| 1 | 59.4 | 59.3 |
| 2 | 122.9 | 123.2 |
| 3 | 140.0 | 140.0 |
| 4 | 39.9 | 39.9 |
| 5 | 25.2 | 25.1 |
| 6 | 36.7 | 36.7 |
| 7 | 32.9 | 32.8 |
| 8 | 37.4 | 37.4 |
| 9 | 24.6 | 24.5 |
| 10 | 37.5 | 37.4 |
| 11 | 32.8 | 32.7 |
| 12 | 37.3 | 37.3 |
| 13 | 24.9 | 24.8 |
| 14 | 39.4 | 39.4 |
| 15 | 28.0 | 28.0 |
| 16 | 22.7 | 22.6 |
| 17 | 22.8 | 22.7 |
| 18 | 19.8 | 19.7 |
| 19 | 19.8 | 19.7 |
| 20 | 16.3 | 16.1 |

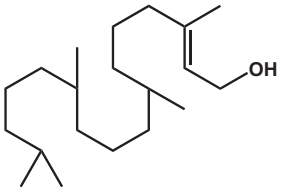


Fig. 3. Structure of active compound (*E*)-phytol (**1**).

data (3100–3400 $cm^{-1}$: -OH, 1500 $cm^{-1}$: double bond) also confirmed these results. Therefore, the molecular formula of this compound was determined as $C_{20}H_{40}O$. Comparing $^{1}H$, $^{13}C$ NMR and GC-MS spectra of this compound with that of phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol), both data were closely coincided. Therefore, this compound was determined as (2*E*)-3,7,11,15-tetramethyl-2-hexadecen-1-ol (Fig. 3). However the absolute configuration is still not confirmed. This compound completely deterred the flies from laying their eggs on the leaves treated at 35.2 $\mu g/cm^2$. The activity was almost similar to that of the original hexane fraction. Therefore, the activity of the hexane fraction was based on this compound.

This compound is known as phytol, which is generated as the result of the decomposition of chlorophyll. Antimycobacterial activity against *Mycobacterium tuberculosis* was reported (Rajab *et al.*, 1998).

This compound may be a by-product generated during the extraction procedure by using methanol. Even if it is true, it is very beneficial for people to find out the activity of phytol in this study because phytol is contained in daily food and we eat it every, that is to say, safe phytol may contribute as one of development of integrated pest control methods against this serious pest in the world.

From now on, the active compounds in the water fraction need to be investigated. Then, the mechanism for resistance of *C. annuum* changing with growth stage also must be elucidated.

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