Studies on Biological Properties of a Novel Repellent, Acetylated Glyceride, against Adult Sweet Potato Whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae)

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CONTENTS

Chapter 1

General Introduction

According to the Food and Agriculture Organization (FAO) of the United Nations, more than 870 million people were undernourished in 2010–2011, with a central focus on Asia, the Pacific, and Africa, despite a three-fold increase in global crop production in the past 50 years (Alexandratos and Bruinsma, 2012). In addition, the global population is expected to rise from approximately 7 billion today to more than 9 billion by 2050. At that time, 370 million people will be undernourished. Crop demands will increase due to population growth, expanded biofuel production, and changes in consumer diets (e.g., meat demand), particularly in developing countries. It is estimated that agricultural production must increase an average of 70% and by almost 100% in developing countries over the same period to feed the expected world population. Without enforcement of crop productivity, 50–100% more land will be required to cultivate crops compared to that needed in 1960. Worldwide, 1,592 million ha of arable land existed in 2005–2007; however, the potential available farmland is expected to increase by < 5% (70 million ha) to reach 1,661 million ha by 2050.

Additional increases in agricultural productivity per unit land are needed under these conditions to ensure future food security, to adjust for the imbalance between supply and demand, and to protect humans, animals, and the environment. Most fruit and vegetable yields will fall 50–90% without crop protection. The total theoretical yield could decrease, and 40% of the world's food would cease to exist. For example, according to the Japan Plant Protection Association (JPPA), the estimated reductions in yield without the use of pesticides

are: tomatoes, 36%; cabbage, 67%; eggplant, 48%; rice, 24%; wheat, 36%; and soybean, 30%.

Tomato (*Lycopersicon esculentum*), which originated in South America, is one of the most important vegetable crops in the world. Production was approximately 161 million tons per 4.7 million ha from all tropical and subtropical regions in 2012 (FAOSTAT). The risk of viral transmission presents a serious threat to tomato production. Tomato yellow leaf curl virus (TYLCV) (Fig. 1-1) is of particular concern, as it is the main tomato production limiting factor worldwide (Czosnek and Laterrot, 1997). TYLCV is believed to be a phloem-restricted virus transmitted by sweet potato whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) vector (Fig. 1-1). An integrated pest management (IPM) strategy is necessary to effectively control insecticide-resistant populations of *B. tabaci*, as no method is available to recover TYLCV-infected plants.

Natural products, including plant-derived oils and seed extracts, are becoming increasingly important as an alternative pest control strategy intended to be compatible with IPM, as conceived by Stern et al. in 1959. However, commercially applicable and environmentally safe substances to treat pests are limited in number, and some are poor performers with occasional incidents of phytotoxicity and harmful effects on beneficial organisms. We have been developing agents composed of food and food additives that have been scientifically demonstrated to be safe and whose safety is empirically supported by a long history of dietary intake under the concept of Safe and Friendly to the Environment (SaFE). I have been interested in identifying a target substance that is highly repellent against adult *B. tabaci*.

The comprehensive objective of this study was to conduct screening tests to find the best substance for repellent, then to elucidate its biological properties against *B. tabaci*, plant

virus infection, and beneficial organisms in order to propose its appropriate use in grape tomato and tomato cultivation.

In Chapter 2, as a result of screening, acetylated glyceride (acetic and fatty acid esters of glycerol), food additive, was found to possess strong repellent activity against *B. tabaci* adults. The basic repellent activity of acetylated glyceride treatment was clarified against adult *B. tabaci* and *Trialeurodes vaporariorum*, and the impact of the treatment on *B. tabaci* progeny on host leaves was evaluated.

In Chapter 3, the number of *B. tabaci* adult courting pairs was found to decrease significantly after treatment with acetylated glyceride. The effect of acetylated glyceride treatment was evaluated on *B. tabaci* and *T. vaporariorum* courtship behaviors and *B. tabaci* progeny. Moreover, the effects of direct and contact treatment at different *B. tabaci* developmental stages were assessed under laboratory conditions.

In Chapter 4, the biological mechanism underlying the disrupted courtship caused by acetylated glyceride in adult *B. tabaci* was elucidated from the bio-acoustic's viewpoint.

In Chapter 5, the efficacy of acetylated glyceride treatment for controlling *B tabaci* and its phyototoxicity to grape tomato under practical conditions were evaluated. In addition, the effect of the treatment on natural enemies was assessed under laboratory conditions.

In Chapter 6, the suppressive activity of single and mixed treatments with conventional chemical insecticides on TYLCV transmission was investigated. In addition, a biological mechanism for the effects of acetylated glyceride on TYLCV transmission is proposed.

In the final Chapter, the significance of the research of acetylated glyceride and its appropriate use in IPM during tomato cultivation were discussed.

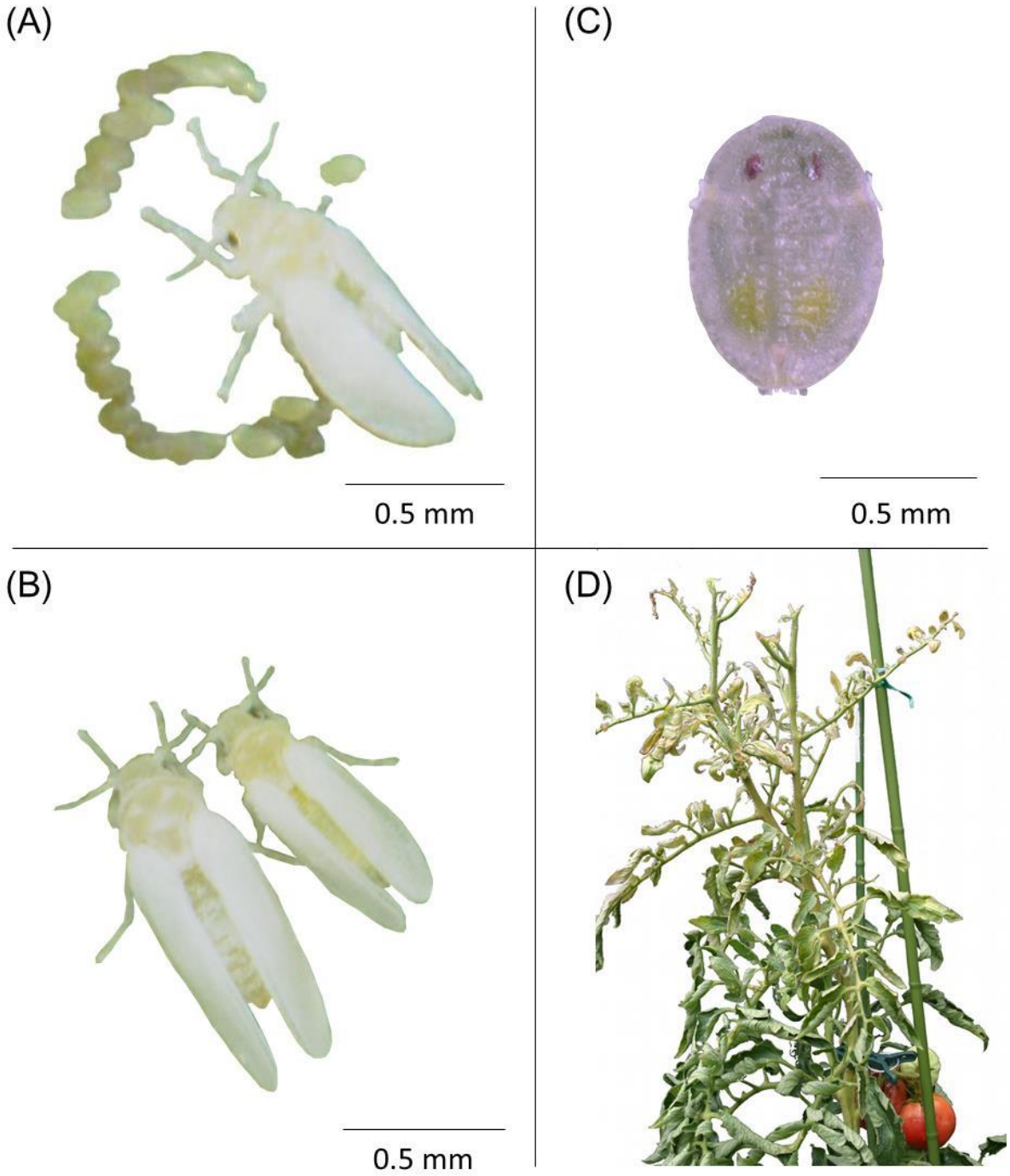

Fig. 1-1 Photographs of different developmental stages of sweet potato whitefly *Bemisia tabaci* and disease symptom of tomato yellow leaf curl virus (TYLCV)-infected tomato.
(A) Eggs laid by female of *B. tabaci* Q, (B) Adults pair of *B. tabaci* Q, (C) Fourth instar nymph of *B. tabaci* B, (D) Severe disease symptom of TYLCV-infected tomato.

Chapter 2

Screening Tests, Observation of Repellent Behaviors and Impact on Oviposition

1 Introduction

The sweet potato whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), was described as *Aleyrodes tabaci* in Greece (Gennadius, 1889). *Bemisia tabaci* B (known as the B-biotype, now referred to as the Middle East-Asia Minor 1 (MEAM1) species) originated in the northeast Africa-Middle East-Arabian peninsular region (De Barro et al., 2011) and was transported to Florida, USA in associated with a poinsettia strain in the mid-1980s (Brown et al., 1995).Very high economic loss is caused annually by whiteflies. For instance, in the early 1990s in Arizona, California, Florida, and Texas, farm revenues loss estimated at about 200–500 millions of dollars (Oliveira et al., 2001). In Brazil, almost all vegetable fields are seriously damaged by *B. tabaci*, incurring a cumulative economic loss of US $5 billion or greater since 1995 (Oliveira, 2001). *Bemisia tabaci* B has developed resistance against a broad range of chemical insecticides including permethrin, methyl parathion and diafenthiuron (Prabhaker et al., 1985; Shadmany et al., 2015). *Bemisia tabaci* Q (commonly known as the Q-biotype, now referred to as the Mediterranean) (De Barro et al., 2011; Tay et al., 2012) is thought to have originated on the Iberian Peninsula (Horowitz et al., 2005) and currently distributes in Japan (Ueda and Brown, 2006), China (Chu et al., 2006; Zhang et al., 2005), USA (Boykin et al., 2007) and several other areas with the exception of Antarctica (Martin et al., 2000). Q of *B. tabaci* not only is highly resistant to several kinds of insecticides (Horowitz et al., 2005), but also exhibits cross-resistance to insecticides such as

chloronicotinyl (Prabhaker et al., 2005), which plays a pivotal role in control programs for important insect pests. Therefore, Q of *B. tabaci* has very rapidly supplanted B in certain areas of China, where the different susceptibility of B and Q to chemicals (e.g. neonicotinoid insecticides: imidacloprid, acetamiprid and thiamethoxam) usage is an intense driver promoting this displacement (Luo et al., 2010). With the aim of controlling them effectively, chemical susceptibility must initially assess both B and Q as world major biotypes, and in addition to the greenhouse whitefly, *Trialeurodes vaporariorum* as other species that has become an important pest in certain areas of Japan.

In this Chapter 2, chemical susceptibility of acetylated glyceride treatment as a repellent on B and Q of adults *B. tabaci* and *T. vaporariorum* was evaluated, and the impact in the number of oviposition of *B. tabaci* was assessed. Furthermore, a process of repellent behaviors of adult *B.tabaci* was circumstantially observed.

2 Materials and Methods

2-1 Insects tested

Bemisia tabaci (B and Q) were reared on potted cabbage plants (*Brassica oleracea* L., Takii & Co., Ltd., cv. Shikidori) in individual growth chambers at 25°C ± 1°C, with approximately 60% ± 5% relative humidity (RH), and with a photoperiod cycle of 16:8 hr light:dark. Under these rearing conditions, the development time from egg to adult was 23 ± 1 days.

Trialeurodes vaporariorum were grown on potted kidney bean (*Phaseolus vulgaris* L., Takii & Co., Ltd., cv. Shinedogawana) in the growth chamber at 22°C ± 1°C, 60% ± 5% RH,

16L/8D lighting schedule. All of the insects tested in this study were derived from these colonies.

2-2 Screening tests

Screening was conducted based on repellency against adult *B. tabaci* B of 18 food additives and 10 plant-derived oils on cucumber leaves (*Cucumis satirus* L, Takii & Co., Ltd., cv. Hokushin) in plastic pots (6.5 cm in diameter and 7.5 cm in height) under a non-choice condition. The food additives were from nos. 1–18 and 29 and the plant-derived oils were from nos. 19–28 (Table 2-1). All food additives were obtained from Riken Vitamin Co., Ltd. (Tokyo, Japan). The plant-derived oils (canola oil, corn oil, extra virgin olive oil, refined olive oil, sunflower oil, and tea tree oil) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and other oils were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

All test substances were formulated at 90% (active ingredient: AI) and 10% inert ingredients, except for DL-100 (70% AI), C-251 (50% AI), C-250 (50% AI), and L-71-D (70 % AI). A 0.9% (v/v) test substance concentration, 0.5% (v/v) inert ingredients, and water were sprayed onto the first-true-leaf stage seedlings for the first screening test using a hand sprayer with run-off. Then, 0.18% (v/v) test substance, 0.1% (v/v) inert ingredients, and water were sprayed for the second screening test. After drying for a few hours, two seedlings per treatment were placed in a clear plastic cage (26 cm in length, 34 cm in width, and 34 cm in height) made of polyvinyl chloride. Three sides were covered with a finely woven polyethylene mesh to allow ventilation. A small piece of cucumber leaf bearing 250 ± 25 *B. tabaci* B adults (mixed sex) was placed upside down in the cage as an inoculation source. The test was conducted at 25°C ± 2°C and 60% ± 10% RH. Adults that settled on the leaf were counted 1 day after release (DAR) for the first screening test and 2 DAR in the second screening test.

The experiments were performed with six replicates.

Table 2-1. Test substances for repellency.

No*	Short name	Main component
1	C-7031H	Decaglycerol monocaprylate
2	DL-100	Diglycerol monolaurate
3	L-71-D	Diglycerol mono and dilaurate
4	C-7032	Diglycerol mono and dicaprylate
5	GM-4	Glycerol diacetomonocaprate
6	PL-009	Glycerol diacetomonocaprylate
7	PL-019	Glycerol diacetomonocaprylate and caprate
8	PL-004	Glycerol diacetomonolaurate
9	M-100	Glycerol monocaprylate
10	M-107FR	Glycerol tricaprylate, caprate and laurate
11	LO-1	Glycerol trioleate
12	C-251	Sorbitan monocaprate
13	C-250	Sorbitan monocaprylate
14	OR-85	Sorbitan trioleate
15	PL-100	Propyleneglycol monolaurate
16	PC-8100	Propyleneglycol monocaprylate
17	PC-8200	Propyleneglycol mono and dilcaprylate
18	PC-0100	Propyleneglycol monocaprate
19		Canola oil**
20		Corn oil**
21		Cotton seed oil
22		Olive oil (extra virgin)**
23		Olive oil (refined)**
24		Rape seed oil
25		Soybean oil
26		Sunflower oil**
27		Sunflower seed oil
28		Tea tree oil**
29		Inert ingredient
30		Non-treated control

* The food additives were from nos. 1–18 and 29 and the plant-derived oils were from nos. 19–28. All food additives were obtained from Riken Vitamin Co., Ltd. (Tokyo, Japan). The plant-derived oils (**) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and other oils were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

2-3 Acetylated glyceride

After screening several plant-derived oils and food additives, acetylated glyceride (Fig. 2-1; glycerol diacetomonolaurate is the main component) had the a strongest repellency against adult *B. tabaci* B. Acetylated glyceride has long been used and consumed as a plasticizer for chewing gum. Acetylated glyceride was formulated as an 80% emulsifiable concentrate (EC) solution (under the developmental code name IKR-001EC); the remaining 20% of inert ingredients were optimized from a series of screening trials consisting of safe substances listed on the EPA inert lists 4A and 4B. Basically, the treatment used in this study employed acetylated glyceride as an 80% EC diluted to 0.2% (v/v) for testing. No spreader was used to prepare the spray. Water was sprayed as a control in all experiments.

2-4 Observation of adult repellency

Upon landing on preferred host plants, most adult whiteflies move to and settle on the undersides of leaves, where they feed on phloem sap, rest, grow, and reproduce. Based on this behavior, the repellency of acetylated glyceride treatment was evaluated by comparing the number of whiteflies on the undersides of leaves treated and non-treated with acetylated glyceride.

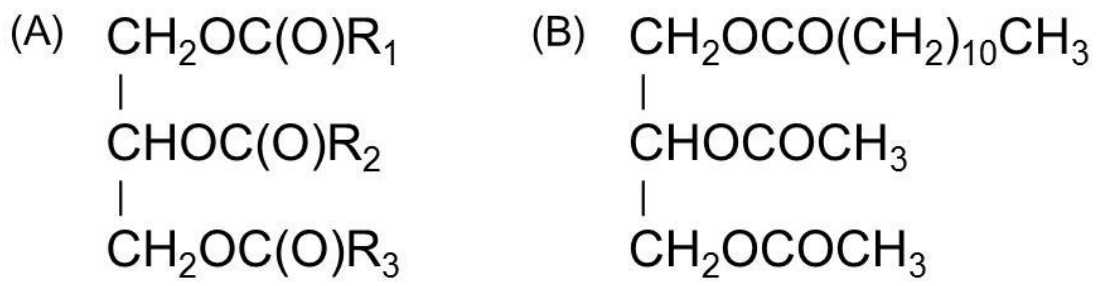

Fig. 2-1 (A) Chemical structure of the active ingredient (acetylated glyceride = acetic and fatty acid esters of glycerol). R_1, R_2, R_3 = $(CH_2)_n CH_3$ (n = 6, 8, 10, 12, 14, 16). One or two of R_1, R_2, and R_3 are CH_3. (B) Glycerol diacetomonolaurate as a representative compound.

2-5 Repellency of active ingredient against adult *B. tabaci* B

A multiple-choice bioassay was employed to reveal repellency of both active ingredient and inert ingredients of the formulation against adult *B. tabaci* B on cucumber leaves (cv. Hokushin) grown at 25°C ± 2°C and 60% ± 10% RH in plastic pots (6.5 cm in diameter, 7.5 cm in height). Solutions of acetylated glyceride (0.2%, v/v), inert ingredients (0.04%, v/v) and water were sprayed onto the seedlings at the first-true-leaf stage, using a hand sprayer with run-off. After drying for a few hours in the dark, the seedlings were placed side by side in the clear plastic cage (26 cm in length, 34 cm in width, 34 cm in height). Furthermore, a small piece of cucumber leaf bearing 200 ± 20 individuals *B. tabaci* adults (sex-mixed; 3–6 days after emergence) was placed upside down as an inoculation source at the same distance and height from test pots in a cage. The adults naturally flew to determine a suitable host over time. The remaining adults settled on the leaves were counted one DAR. The experiment was performed with four replicates.

2-6 Spectrum of repellency

Experiment 1: *Bemisia tabaci* B and Q adults

Grape tomato seedlings (*Solanum lycopersicum*, Takii & Co., Ltd., cv. Yellow-pear) were raised until the three-true-leaf stages in the plastic pot. To obtain seedlings with only the third-true-leaf, other leaves, new shoots, and seed leaves were removed, and the plants were left for one day. The solution of acetylated glyceride (0.2%, v/v) and water was treated with plants according to same procedures in the previous Subsection 2-5. A cucumber leaf (cv. Hokushin) bearing 150 ± 15 individuals *B. tabaci* adults (B and Q; sex-mixed; 3–6 days after emergence) was placed upside down at the same distance and height from both treated and non-treated (water) pots in the cage. One DAR, the remaining adults settled on the leaves were counted. Three replicates were performed.

Experiment 2: *Trialeurodes vaporariorum* adults

A dual-choice bioassay was conducted to assess the repellency of acetylated glyceride (0.2%, v/v) and water against the adults *T. vaporariorum* (sex-mixed) on cucumber leaves (cv. Hokushin) at the first-true-leaf stage according to the same procedures in EXP. 1 of Subsection 2-6. The remaining adults settled on the leaves were counted 16 hr after releasing 100–120 individuals. Three replicates were performed.

2-7 Adult repellent behaviors and impact on oviposition

2-7-1 Observation of repellent processes

The choice test using grape tomato seedlings (cv. Yellow-pear) at three-true-leaf stage was conducted according to same procedures in EXP.1 of Subsection 2-6. Five replicates were performed. All adult behaviors were recorded for 1 hr using a video camera (Panasonic, model

NV-DJ100) after the first individual landed on the upper sides of leaves. After carefully watching the recorded video tapes, the numbers of adults on the leaves were classified according to the following four groups: 1, an adult landed on the upper side of leaves; 2, an adult flew away after landing on the upper side of leaves; 3, an adult moved to the underside of leaves and settled after landing; 4, an adult remained on the underside of leaves at the end of the observation. The latter value included the numbers of adults that directly flew to and settled on the underside of leaves. In addition, the required response time was measured for all individuals between groups 1 and 2 (from landing to flying away) and between groups 1 and 3 (from landing to the initiation of movement).

2-7-2 Impact on oviposition by the repellent effect

Grape tomato seedlings (cv. Yellow-pear) at the two-true-leaf stages were cut back to remove a new shoot and the two seed leaves to retain both the first and second true leaves before transferring into small plastic cups (2 cm in diameter, 4 cm in height) filled with water. The tops of the cups were sealed with parafilm. A solution of acetylated glyceride (0.2%, v/v) was carefully sprayed onto either the first or second leaf using a handy sprayer; the remaining leaf that was not sprayed was retained as a non-treated control. After drying, the seedlings in the cup were placed into glass test tubes (4 cm in diameter, 13 cm in height). One female of *B. tabaci* B aged 3–5 days after emergence were released into an individual test tube for 7 days. The numbers of settled adults on the treated leaf, non-treated leaf, and places other than the leaf (e.g., the stem and the side of the tube) were counted each day. At 7 DAR, the numbers of eggs laid were counted on both leaves. In total, 21 test tubes were tested with one replicate.

2-8 Data analysis

One-way ANOVA was employed to analyze remaining adults settled on cucumber leaves in screening tests (Subsection 2-2), to assess the effect of treatment of acetylated glyceride, inert ingredients, and water in the numbers of settled adults on cucumber leaves in the multiple choice test (Subsection 2-5). Differences among means were compared with Tukey's HSD-test at $P = 0.05$. The numbers of adults of *B. tabaci* B, Q, and *T. vaporariorum* that settled on the treated and non-treated leaves (Subsection 2-6), the numbers of adults that landed and settled on leaves (Subsubsection 2-7-1), of eggs laid (Subsubsection 2-7-2) were analyzed using t-tests at $\alpha = 0.05$.

3 Results

3-1 Screening tests

The number of adults that settled on the cucumber leaf treated with PL-004 (0.9%, v/v) during the first screening test was 73.2% lower than on the non-treated control at 1 DAR (Table 2-2; $F = 3.28$, df = 29, 150, $P < 0.05$). Basic repellency against *B. tabaci* B of PL-004 was superior to that of the other test substances.

The PL-004 and L-71-D treatments significantly reduced the number of adults that settled on leaves at 2 DAR ($F = 3.17$, df = 10, 55, $P < 0.05$) during the second screening test. In particular, the activity of PL-004 was long-lasting compared with the other test substances, as determined during the first screening test. No differences were observed between the inert ingredients and the non-treated control during the first and second screening tests. PL-004 was considered the best substance to repel adult *B. tabaci* B.

3-2 Repellency of active ingredient against adult *B. tabaci* B

Table 2-3 shows the repellency of acetylated glyceride treatment evaluated by the number of settled adults *B. tabaci* B in the multiple-choice bioassay tests. The treatment of 0.2% (v/v) acetylated glyceride resulted in a significant reduction in the number of settled adults, whereas those of 0.04 % (v/v) inert ingredients was not significant. The number of adults settled on leaves treated acetylated glyceride was 72% lower than those on non-treated leaves (F = 8.34; df = 2, 9; P = 0.009).

3-3 Spectrum of repellency

Experiment 1: *Bemisia tabaci* B and Q adults

The number of settled adults of *B. tabaci* B and Q on the underside of treated leaves were significantly reduced by 45% and 43%, respectively, compare with those on non-treated leaves (Table 2-4; B: T = 2.56; df = 4; P =0.031; Q: T = 4.14; df = 4; P < 0.001). Susceptibility of acetylated glyceride treatment is not significantly different between B and Q.

Experiment 2: *Trialeurodes vaporariorum* adults

The number of adults settled on the underside of treated leaves 16 hr after release was significantly reduced by 77% compare with those on non-treated leaves (Table 2-5; T = 2.59; df = 4; P = 0.030).

Table 2-2. Repellent activity of test substances against adult *B. tabaci* B on cucumber leaves.

No	Short name	Main component	No. of adults settled on the leaf*			
			0.9% (v/v)		0.18% (v/v)	
1	C-7031H	Decaglycerol monocaprylate	104 ± 16	cd	-	
2	DL-100	Diglycerol monolaurate	58 ± 9	abcd	65 ± 6	abc
3	L-71-D	Diglycerol mono and dilaurate	64 ± 7	abcd	63 ± 7	ab
4	C-7032	Diglycerol mono and dicaprylate	97 ± 21	abcd	-	
5	GM-4	Glycerol diacetomonocaprate	73 ± 16	abcd	-	
6	PL-009	Glycerol diacetomonocaprylate	61 ± 7	abcd	65 ± 9	abc
7	PL-019	Glycerol diacetomonocaprylate and capra	51 ± 13	abcd	67 ± 4	abc
8	PL-004	Glycerol diacetomonolaurate	26 ± 5	a	46 ± 4	a
9	M-100	Glycerol monocaprylate	72 ± 12	abcd	73 ± 6	abc
10	M-107FR	Glycerol tricaprylate, caprate and laurate	75 ± 11	abcd	-	
11	LO-1	Glycerol trioleate	36 ± 5	ab	76 ± 6	abc
12	C-251	Sorbitan monocaprate	51 ± 4	abcd	86 ± 16	abc
13	C-250	Sorbitan monocaprylate	77 ± 6	abcd	-	
14	OR-85	Sorbitan trioleate	113 ± 14	d	-	
15	PL-100	Propyleneglycol monolaurate	67 ± 11	abcd	-	
16	PC-8100	Propyleneglycol monocaprylate	73 ± 8	abcd	-	
17	PC-8200	Propyleneglycol mono and dilcaprylate	70 ± 8	abcd	-	
18	PC-0100	Propyleneglycol monocaprate	73 ± 13	abcd	-	
19		Canola oil	97 ± 14	bcd	-	
20		Corn oil	68 ± 12	abcd	-	
21		Cotton seed oil	93 ± 10	bcd	-	
22		Olive oil (extra virgin)	47 ± 8	abc	82 ± 22	abc
23		Olive oil (refined)	72 ± 11	abcd	-	
24		Rape seed oil	98 ± 12	bcd	-	
25		Soybean oil	72 ± 13	abcd	-	
26		Sunflower oil	91 ± 11	bcd	-	
27		Sunflower seed oil	72 ± 17	abcd	-	
28		Tea tree oil	108 ± 15	cd	-	
29		Inert ingredient	102 ± 12	cd	99 ± 11	bc
30		Non-treated control	98 ± 14	bcd	114 ± 8	c

*Mean number (±S.E.) of remaining adults settled on the cucumber leaf was counted one day (at 0.9%, v/v) and two day (at 0.18%, v/v) after release. Different letters in the same column indicate significant differences among treatments ($P < 0.05$; One-way ANOVA followed Tukey's HSD-test).

Table 2-3. Repellency of treatment with acetylated glyceride (0.2%, v/v) and inert ingredients (0.04%, v/v) on settling of *Bemisia tabaci* B adults on cucumber leaves in a multiple-choice bioassay.

Treatment	Number of settled adults per leaf
Acetylated glyceride-treated	23.0 ± 3.2[a]
Inert ingredients-treated	71.8 ± 11.3[b]
Non-treated	81.3 ± 14.6[b]

Each value indicates the mean ± standard error (S.E.) of four replicates one day after releasing 200 ± 20 individuals. Different letters indicate significant differences among treatments ($P < 0.05$, One-way ANOVA followed by Tukey's HSD-test).

Table 2-4. Repellency of treatment with acetylated glyceride (0.2%, v/v) against adults *Bemisia tabaci* B and Q on grape tomato leaves in a choice test.

Treatment	Number of settled adults per leaf	
	B	Q
Acetylated glyceride-treated	12.8 ± 3.6	10.6 ± 3.7
Non-treated	27.9 ± 4.8	24.7 ± 1.4

Each value is the mean ± standard error (S.E.) based on three replicates one day after releasing 150 ± 15 individuals. The result in the same column is significantly different from those obtained with non-treated leaves ($P < 0.05$, *t*-test).

Table 2-5. Repellency of treatment with acetylated glyceride (0.2%, v/v) against *Trialeurodes vaporariorum* adults on cucumber leaves in a choice test.

Treatment	Number of settled adults per leaf
Acetylated glyceride-treated	14.3 ± 3.3
Non-treated	63.0 ± 18.5

Each value is the mean ± standard error (S.E.) based on three replicates 16 hr after releasing 100–120 individuals. The result in the same column is significantly different from those obtained with non-treated leaves ($P < 0.05$, *t*-test).

3-4 Adult repellent behaviors and impact on oviposition

3-4-1 Observation of repellent processes

The mean (± standard error, S.E.) numbers of *B. tabaci* B adults that landed on the upper sides of acetylated glyceride-treated and non-treated leaves were 20.0 ± 4.6 and 18.8 ±2.5, respectively (Fig. 2-2) (T = 0.23; df = 8; P < 0.41). In contrast, 1 hr after releasing the adults, the numbers (mean ± S.E.) of adults that settled on the undersides of treated leaves (5.2±1.7) were approximately 82% lower than those on the non-treated controls (28.2±3.2) (T = 6.22; df = 10; P < 0.001).

In these choice tests, the total numbers of adults that landed on the treated and non-treated leaves were 99 and 94, respectively (Fig. 2-3). After landing, the numbers of adults that flew away from the treated and non-treated leaves were 72/99 (72.7%) and 5/94 (5.3%), respectively. Of the adults that flew away after landing on the leaves, 46/72 individuals (63.9%) flew away from the treated leaves within 5 sec, whereas 0/5 individuals flew away from the non-treated leaves within 5 sec. Of the adults that moved to the underside of the treated leaves after landing, 25/27 individuals (92.6%) for treated and 87/89 individuals (97.8%) for the non-treated control spent 5 sec or more moving on the underside of the leaf.

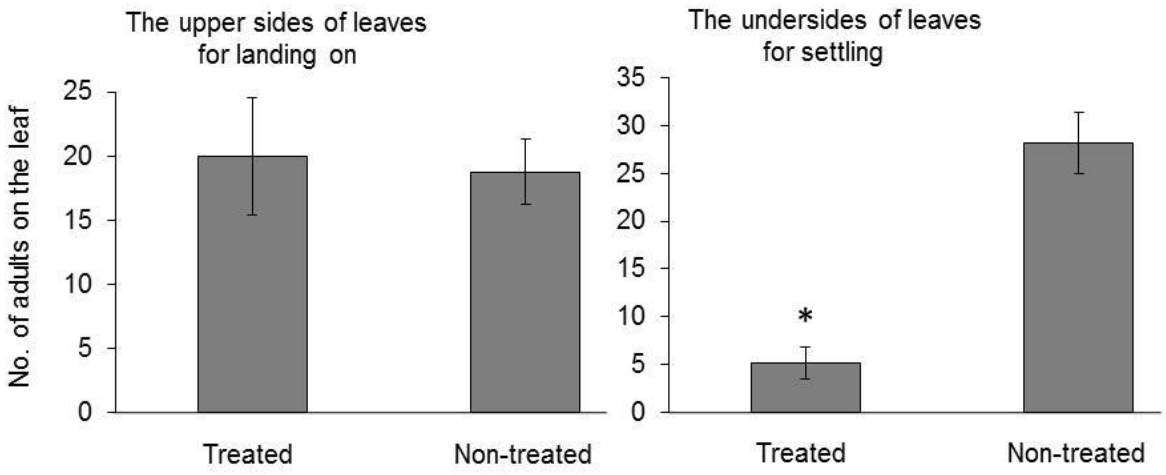

Fig. 2-2 Effects of treatment with acetylated glyceride (0.2%, v/v) on the landing and settling of *Bemisia tabaci* B adults on grape tomato leaves. Each value is the mean ± standard error (S.E.) based on five replicates. Asterisks (*) denote values that significantly differed from those of non-treated leaves ($P < 0.05$, *t*-test).

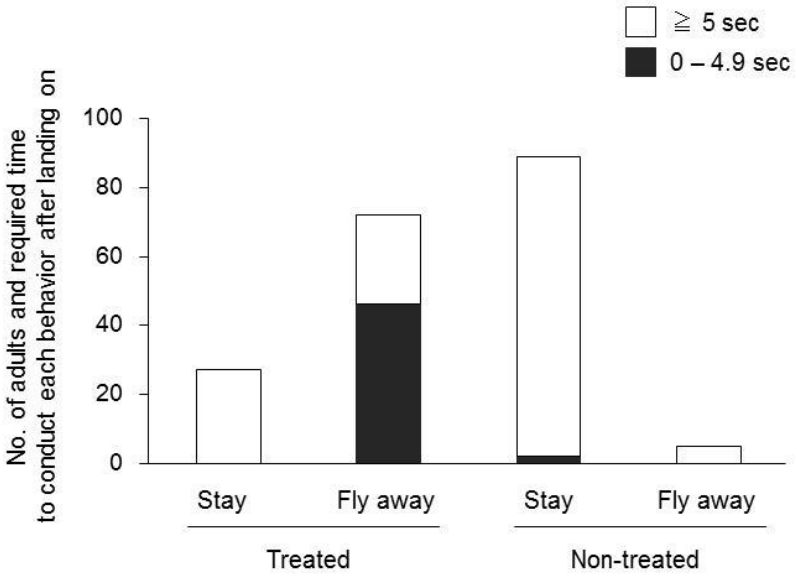

Fig. 2-3 Effects of treatment with acetylated glyceride (0.2%, v/v) on the behaviors of *Bemisia tabaci* B adults and on the response time required for conducting subsequent actions after landing on grape tomato leaves. Each value is the total number of adults observed from five replicates.

3-4-2 Impact on oviposition by the repellent effect

One DAR, no females settled on the underside of acetylated glyceride-treated leaves (Fig. 2-4). Two DAR, all females that settled on either treated (14%) or non-treated leaves (86%) were selected. Most of these adults did not change their positions from 2 to 5 DAR, except one female that changed position from treated leaves to non-treated leaves at 4 DAR. From 6 to 7 DAR, some females gradually changed their positions from non-treated leaves to treated leaves.

Seven DAR, Figure 2-5 shows that the mean (± S.E.) numbers of eggs laid on treated leaves (4.1 ± 1.4) were significantly reduced by 85.6% as compared with those on non-treated leaves (28.4 ± 2.5) (T = 8.59; df = 40; P < 0.001). No dead females were observed during the 7 days. The reduction of egg numbers was caused by lower numbers of female adults settling on the treated leaves.

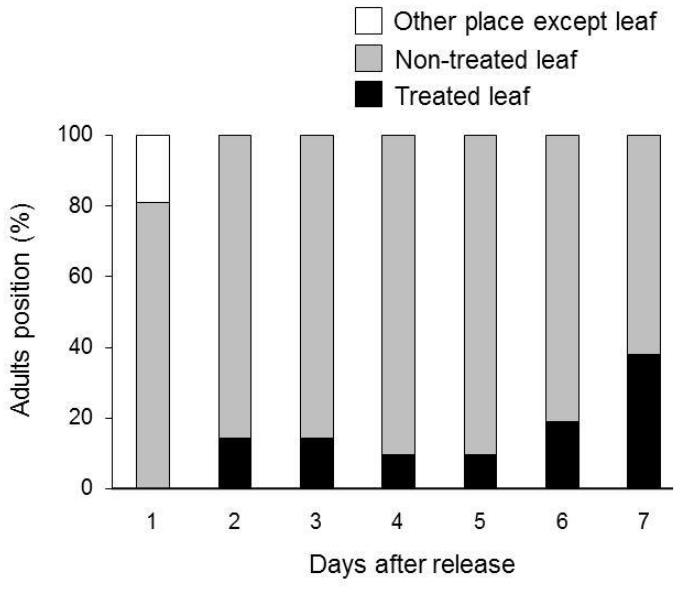

Fig. 2-4 The effect of treatment with acetylated glyceride (0.2%, v/v) on the settling of *Bemisia tabaci* B adults on grape tomato leaves in a choice test. Twenty-one test tubes were tested with one replicate.

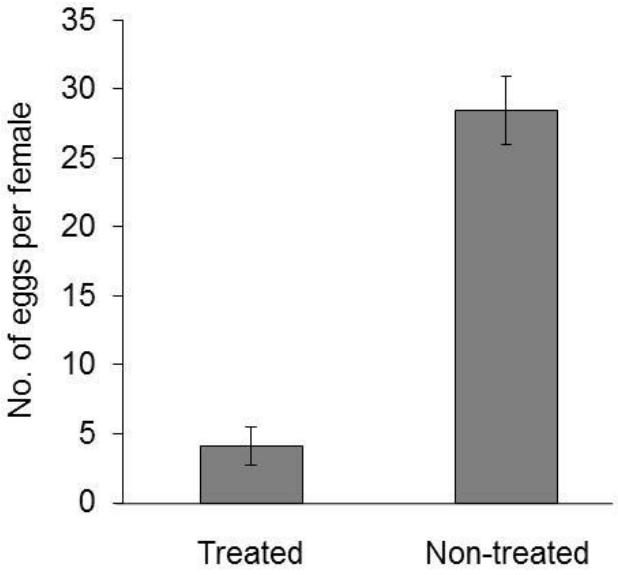

Fig. 2-5 The effect of treatment with acetylated glyceride (0.2%, v/v) on oviposition by *Bemisia tabaci* B adults on grape tomato leaves in a choice test. Each value is the mean ± standard error (S.E.) based on 21 test tubes with one replicate. The result is significantly different from those obtained with non-treated leaves ($P < 0.05$, *t*-test).

4 Discussion

A treatment with acetylated glyceride as an active ingredient show repellent effect against *B. tabaci* B adults though only inert ingredients did not show any repellency (Table 2-3). Moreover, the number of settled adults of Q of *B. tabaci* (Table 2-4) and *T. vaporariorum* (Table 2-5) on the host leaf were reduced by acetylated glyceride treatment, with the activity being as high as that against the B of *B. tabaci*.

Observations of adult behaviors in the choice test showed that the numbers of adults that landed on treated and non-treated leaves were almost the same (Fig. 2-2). Of the adults that

landed on a leaf, 64% (46/72) adults immediately flew away from the treated leaves, whereas no (0/5) adults flew away from non-treated leaves (Fig. 2-3). As a preliminary observation, adults extended their labia was observed to touch the leaf surface within 5 sec after landing on both treated and non-treated leaves. In agreement with my observations, the adult bayberry whitefly *Parabemisia myricae* (Walker, 1987) and the greenhouse whitefly *Trialeurodes vaporariorum* (van Vianen et al., 1988) are known to touch, rub, and tap the apex of their labia on a plant surface immediately before inserting their stylets into the plant tissues. Electrophysiological measurements obtained using an electronic monitoring system showed that a specific waveform lasting 3–15 sec was recorded before they inserted their stylets, which occurred during the establishment of electrical contact between the *B. tabaci* B adult's mouthparts and the plant tissues (Jiang et al., 1999). I guess that the adult behaviors exhibited immediately after landing may be related to the acquisition of plant surface information regarding potential hosts. Several terpenoids, including sesquiterpenes and monoterpenes, are observed in tomato accessions (Bleeker et al., 2009) and ginger oil (Zhang et al., 2004), which have putative repellency on *B. tabaci* adults in the vapor phase. However, the repellency of treatment with acetylated glyceride seems not to be caused by the vapor phase but to be primarily attributable to interference with cues associated with host recognition immediately after adults land on plant surfaces.

After adult whiteflies settle on a host plant that is suitable for both feeding and mating, they rarely fly away from the host plant unless host deterioration occurs or an external stimulus, such as bumping, is received (Fig. 2-4). Therefore, the large reduction in the egg numbers in the choice test was mainly caused by the reduced numbers of settling adults due to the repellency (Fig. 2-5).

In conclusion, the repellency by acetylated glyceride treatment is effective against adults

of B and Q of *B.tabaci*, and *T. vaporariorum*. The repellency of acetylated glyceride treatment on *B. tabaci* adults was induced immediately after they landed on the plant surface, resulting in a large reduction in the number of eggs on leaves.

Chapter 3

Observation of Courtship Behaviors and Impact on the Sex Ratio of Adults, and Direct and Contact Treatment at Different Developmental Stages of *B. tabaci*

1 Introduction

The sweet potato whitefly is one of the most destructive insect pests of vegetables and ornamental plants in the tropics and subtropics (Brown et al., 1995; Denholm et al., 1998). Its host range is very broad and covers approximately 600 plant species including many economically important vegetable corps (Mound and Halsey, 1978). Phytotoxic damage is directly caused by large numbers of adult whiteflies feeding on the phloem sap of squash (Yokomi et al., 1990), pumpkin (Costa and Brown, 1991), Brassica spp. (Brown and Bird, 1992), and tomato (Schuster et al., 1990), as well as indirectly by the production of large amounts of honeydew excreta, which induces the growth of saprophytic fungi on foliage and fruit.

Bemisia tabaci can complete 16–17 generations each year in favorable climate conditions (Verma et al., 1990). In addition, the numbers of eggs laid by a female vary from 48 to 394 eggs on cotton in Egypt, depending on the temperature and host species (Byrne and Bellows, 1991). This high reproductive potential partly explains the rapid development of pesticide resistance in whiteflies (Byrne and Bellows, 1991). To avoid causing of plant damage and the development of further insecticidal resistance in *B. tabaci*, it is important to suppress increases in the progeny population, particularly in crops that are cultivated for long periods, such as tomatoes and eggplants.

Bemisia tabaci B is known to have a stronger propensity by adjusting sex ratio in reproductive competition with non-B of *B. tabaci* (Luan and Liu, 2012; Zang and Liu, 2007), leading to displacement of the other populations in a field (Liu et al., 2007; Zang et al., 2006). Considering these findings from a different angle, controlling reproductive behaviors of *B. tabaci* has the possibility to become an important tactic to suppress its progeny in IPM strategies, as well as to disrupt mating other insect species.

In the previous Chapter, the treatment of acetylated glyceride was demonstrated to induce high repellency against adult *B. tabaci* and *T. vaporariorum* on host leaves. In this Chapter 3, courting disruption effect of acetylated glyceride treatment on B and Q of *B. tabaci* and *T. vaporariorum* was assessed to determine its chemical susceptibility. Moreover, the effect of treatment with acetylated glyceride was observed on the courtship behavior of adult *B. tabaci* to determine its impact on their progeny under laboratory conditions. In addition, the impact of the direct and contact treatment at different developmental stages of *B.tabaci* was demonstrated.

2 Materials and Methods

2-1 Observation of the courtship behaviors

Adult whiteflies are known to settle on the undersides of leaves, where they feed on phloem sap, rest, and find suitable mating partners for reproduction. Based on these behaviors, the effect of acetylated glyceride treatment on *B. tabaci* courting pair formation was evaluated on the underside of leaves. The courting pairs were counted based on the number of female adults, irrespective of the number of males that paired with a single female;

a single female and a single male, as well as a single female and several males were considered as one pair.

2-2 Effect of active ingredient on courting pair formation of adults *B. tabaci* B

One day after releasing adults of *B. tabaci*, courting pairs were counted on underside of cucumber leaves (cv. Hokushin), which were treated with acetylated glyceride (0.2 %, v/v), inert ingredients (0.04%, v/v), and water as described in Subsection 2-5 of Chapter 2. A multiple-choice bioassay was performed with four replicates.

2-3 Spectrum of courting disruption effect

Experiment 1: *Bemisia tabaci* B and Q adults

Immediately after counting the settled adults in the dual-choice tests described in Subsection 2-6 (Exp.1) of Chapter 2, courting pairs out of all settled adults on grape tomato leaves (cv. Yellow-pear) treated and non-treated with acetylated glyceride (0.2%, v/v) were counted. The experiment was performed with three replicates.

Experiment 2: *Trialeurodes vaporariorum* adults

Courting pairs were counted on cucumber leaves (cv. Hokushin) treated and non-treated with acetylated glyceride (0.2%, v/v) in Subsection 2-6 (Exp. 2) of Chapter 2, just after counting settled adults in the dual-choice tests. Three replicates were performed.

2-4 Courtship behaviors and impact on the progeny

2-4-1 Observation of the courting disruption effect

Grape tomato seedlings (cv. Yellow-pear) at the two-true-leaf stages were cut back,

removing the young third-stage leaves and the pairs of seed leaves and retaining only the second true leaves, before transferring the seedlings into small plastic cups (2 cm in diameter, 4 cm in height) filled with water. The tops of the cups were sealed with Parafilm. A solution of acetylated glyceride (0.2%, v/v) or water was sprayed onto the seedlings using a hand sprayer until run-off. The seedlings were then dried and maintained at 25°C ± 1°C for 3 days to allow adult settling as the residual repellency of the treatment decreased. Then, the seedlings in the plastic cups were placed into glass test tubes (4 cm in diameter, 13 cm in height). Non-mated adult *B. tabaci* B, collected 2–5 days after emergence, were released into the test tubes. Tests were conducted under the following two conditions: 1, a pair of adults (male and female) were released together onto an acetylated glyceride-treated leaf and a non-treated leaf in the test tube (both sexes release condition = BR); 2, a male and female were released separately onto a non-treated leaf in an individual test tube (individual release condition = IR). Five test tubes were set up for each condition, with five replicates. The top of the test tube was covered with tissue paper that was held in place with a rubber band to prevent the adult(s) from escaping while allowing ventilation.

The behaviors of the adults on the undersides of the leaves were recorded using a video camera positioned outside and below the test tube for 60 min immediately after both sexes (under the BR condition) or each sex (under the IR condition) was observed on the underside of the leaves. After carefully and repeatedly viewing the recorded videos, the behaviors were classified into the following three categories: 1) resting behaviors, in which the adults were in a quiescent state, including feeding or resting in a row; 2) movement behaviors, in which the adults moved randomly over the leaves (for males, the definition also included the courtship behavior immediately before detecting a female for courtship; for females, it included avoidance or rejection behaviors during male courtship); and 3) courtship and mating

behaviors, i.e., the series of behaviors exhibited by both sexes between the initiation of courtship (immediately after a male detects a female) and mating (the end of successful or unsuccessful mating behaviors). In addition, the last category included antennal drumming, male abdominal undulation, male positioning, mating, and post-mating behaviors (i.e., guarding behaviors to protect mated females from being approached by other males). The adult behaviors observed after uncoupling were classified into one of the three categories.

2-4-2 Impact on the sex ratio of adults of second generation resulting from the courtship disruption effect

Grape tomato seedlings (cv. Yellow-pear) at the two-true-leaf stages were prepared in test tubes according to the method described in Subsubsection 2-4-1. Non-mated *B. tabaci* Q adults, collected 1 day after emergence, were used in tests conducted under the following two release conditions: 1, three adults (one female and two males) were released together onto both treated and non-treated leaves in individual test tubes for 4 or 7 days (BR); 2, a single female was released onto a non-treated leaf for 4 days (female release condition = FR). The top of the test tube was covered with tissue paper secured with a rubber band. At end of the day, at least one adult of each sex (one male and one female) under the BR condition and one female under the FR condition survived on the grape tomato leaves, which were retransplanted into plastic pots for evaluation. A total of 11 to 16 test tubes were analyzed per condition, each with four replicates. The potted grape tomatoes were placed in a growth chamber at 25°C ± 1°C. From 19 to 31 days after retransplantation, the number of newly emerged second-generation adults was carefully counted. The adults were subsequently captured to determine their sexes under a stereoscopic microscope.

2-5 Impact of acetylated glyceride at different developmental stages of *B. tabaci* B

2-5-1 Direct treatment on settled adults

Insecticidal activity against *B. tabaci* B (sex-mixed) adults was assessed following direct foliar treatment with acetylated glyceride. Cucumbers (cv. Hokushin) were grown in plastic pots to the first-true-leaf stage. They were then placed in a rearing cage where adult whiteflies were released for 24 hr at 25°C ± 1°C to ensure that the adults settled on leaves. The mean (± S.E.) numbers of adults that settled on treated and non-treated leaves was 107 ± 14 and 88 ± 5, respectively during pretreatment. After the settled adults were counted, the plants were placed individually in a whitefly-free cage. Approximately 0.2 ml of acetylated glyceride solution (0.2%, v/v) or water was applied once to each leaf using a hand sprayer. Immediately after treatment, the non-treated cucumber plants were placed in the cage as shelter for adult *B. tabaci*. The numbers of dead adults on the treated and non-treated plants were counted 24 hr after treatment. The experiments were performed with 6–7 replicates.

2-5-2 Direct treatment on eggs and first, third, and fourth instar nymphs on eggplant

Eggplant (*Solanum melongena*, Takii & Co., Ltd., cv. Senryo Ni Gou) plants were grown to the first-true-leaf stage in plastic pots. The plants were placed in the clear plastic cage (26 cm in length, 34 cm in width, 34 cm in height), and approximately 20 *B. tabaci* B adults per plant were released into the case. *Bemisia tabaci* were allowed to lay eggs freely for 2 days. After all adults *B. tabaci* were removed, the eggplants were placed in a whitefly-free chamber at 25°C ± 1°C and 60% ± 5% RH to promote egg growth. Egg stage (1–2 days after oviposition), early instar stage nymphs (first (main stage) and second instar nymphs 10–11 days after oviposition), and late instar nymph stage (third (main stage) and fourth instar nymphs; 15–16 days after oviposition) *B. tabaci* were dipped into an acetylated

glyceride solution (0.2%, v/v) or water for 15 sec. The insecticidal activity of the treatment was evaluated 8, 10, 15, 17, and 28 days after treatment (DAT) until adult emergence. Hatchability was evaluated by counting the number of eggs laid and egg shells 8 DAT. The number of eggs laid per replicate was 48–97. The experiments were performed with 3–4 replicates per stage.

2-5-3 Contact treatment on first instar nymphs

Cucumbers (cv. Hokushin) were grown in plastic pots until the first-true-leaf stage. A small plastic container covered with a fine mesh for ventilation (15 mm in diameter and height) was attached to the underside of the leaves. Approximately 20 *B. tabaci* B females per plant were released into the case and allowed to lay eggs freely for 15 hr. Five days after oviposition, an acetylated glyceride solution (0.2%, v/v) or water was applied using a brush to all leaves, except those beneath the container (oviposition site). After hatching, the first instar nymph generally walks around the leaf to find a suitable settling site. About 31–38 target individuals were selected for evaluation and were settled in treated areas outside the oviposition sites. The experiments were performed with three (treated) and four (non-treated) replicates. Stereoscopic microscopic observations were made at the early instar nymph stage (primarily second instar nymphs 14 DAT), the late instar nymph stage (primarily fourth instar nymphs 22 DAT), and exuviae 26 DAT.

2-6 Data analysis

One-way ANOVA was employed to assess the effect of treatment of acetylated glyceride and inert ingredients on the numbers of courtship pairs on cucumber leaves in the multiple choice test of laboratory experiment (Subsection 2-2). Differences among means

were compared with Tukey's HSD-test at $P = 0.05$.

The percentage of courting pairs of *B. tabaci* B, Q, and *T. vaporariorum* that settled on the treated and non-treated leaves (Subsection 2-3), the numbers of dead adults (Subsubsection 2-5-1), and of various developmental stage of *B. tabaci* on the treated leaves following direct foliar treatment (Subsubsection 2-5-2), and of dead first-instar nymphs by contact treatment (Subsubsection 2-5-3) were analyzed using t-tests at $\alpha = 0.05$.

The proportion of each adult behavior on the treatments (treated leaf in BR and non-treated leaves in BR and IR) (Subsubsection 2-4-1) were analyzed with a generalized linear mixed model (GLMM) using SAS proc Glimmix. Binominal errors distribution was used for each behavior. The proportion of each adult behavior on the treatments was as response variable. In this model, different pairs of adult whitefly used for observation were considered as a repeated replication and therefore taken into account by a random effect, while treatments, sexes, and both test conditions (BR and IR) were fixed effects. The differences among means were compared using Tukey's HSD-test.

The sex ratio of newly emerged adults (Subsubsection 2-4-2) on the treated and non-treated leaves in BR under 4 and 7 days releasing conditions were treated as binominal errors distribution and analyzed with a GLMM. The sex ratio of newly emerged adult numbers was as response variable. The treatments were fixed effects, while releasing time is random effect. In addition, since different pairs used for test were considered as a repeated observation, they should be recognized as a random effect. The differences among means were compared using Tukey's HSD-test.

3 Results

3-1 Effect of active ingredient on courting pair formation of adults *B. tabaci* B

Table 3-1 shows the effect of acetylated glyceride treatment evaluated by the number of courting pairs of adults *B. tabaci* B in the multiple-choice bioassay tests; the treatment significantly reduced in the number of courting pairs on the underside of cucumber leaves ($F = 30.15$; df = 2, 9; $P < 0.001$). In contrast, the treatment of cucumber leaves with inert ingredients did not show any efficacy on courting pair formation.

Table 3-1. Effect of treatment with acetylated glyceride (0.2%, v/v) and inert ingredients (0.04%, v/v) on courting of *Bemisia tabaci* B adults released onto cucumber leaves in a multiple-choice bioassay.

Treatment	Courting pair (%)
Acetylated glyceride-treated	3.6[a]
Inert ingredients-treated	20.2[b]
Non-treated	20.8[b]

Each value indicates the mean of four replicates one day after releasing 200 ± 20 individuals. Different letters indicate significant differences among treatments (arcsine transformation before analysis; $P < 0.05$; Tukey's HSD-test).

3-2 Spectrum of courting disruption effect

Experiment 1: *Bemisia tabaci* B and Q adults

One DAT with 0.2% (v/v) of acetylated glyceride, no courting pair formation of both *B. tabaci* B and Q was observed on the treated grape tomato leaves, when the percentage of courting pair of B and Q on non-treated leaves were 12.9% and 13.2%, respectively (Table

3-2; B: T =4.71; df = 4; P = 0.004; Q: T = 3.03; df = 4; P = 0.019).

Table 3-2. Effect of treatment with acetylated glyceride (0.2%, v/v) on courting of adults *Bemisia tabaci* B and Q on grape tomato leaves in a choice test.

Treatment	Courting pair (%)	
	B	Q
Acetylated glyceride-treated	0	0
Non-treated	12.9	13.2

Each value is the mean based on three replicates one day after releasing 150 ± 15 individuals. The result in the same column is significantly different from those obtained with non-treated leaves (arcsine transformation before analysis; $P < 0.05$; *t*-test).

Experiment 2: *Trialeurodes vaporariorum* adults

The treatment of acetylated glyceride moderately reduced in the number of courting pairs compare with non-treated cucumber leaves (4.6% for treated, 7.8% for non-treated), but there was no significant difference between treatments (Table 3-3; T = 1.07; df = 4; P = 0.172).

Table 3-3. Effect of treatment with acetylated glyceride (0.2%, v/v) on courting of *Trialeurodes vaporariorum* adults on cucumber leaves in a choice test.

Treatment	Courting pair (%)
Acetylated glyceride-treated	4.6
Non-treated	7.8

Each value is the mean of three replicates 16 hr after releasing 100–120 individuals. The result in the same column is not significantly different from those obtained with non-treated leaves (arcsine transformation before analysis; $P > 0.05$; *t*-test).

3-3 Courtship behaviors and impact on the progeny

3-3-1 Observation of the courting disruption effect

Figure 3-1 shows that the male movement behaviors on the acetylated glyceride-treated (2.1%) under the BR condition and non-treated leaves (1%) under the IR condition were significantly lower than that on the non-treated leaves (26.5%) under the BR condition ($P < 0.05$, Tukey's HSD-test). Most of the males showed slightly altered settling positions on the treated leaves. The typical male searching behaviors for courting females were observed only twice in five replicates, although the searches rapidly ended. Similarly, male movement behaviors were rarely observed on the non-treated leaves under the IR condition. The females rarely exhibited movement behaviors across all treatments. The few female movement behaviors were observed to only relate to the avoidance and rejection of male courtship behaviors. No courtship or mating behaviors were observed on the treated leaves under the BR condition and on the non-treated leaves under the IR condition during the observations. In contrast, these behaviors accounted for 37.8% of the observation time in both sexes on the non-treated leaves under the BR condition ($P < 0.05$, Tukey's HSD-test).

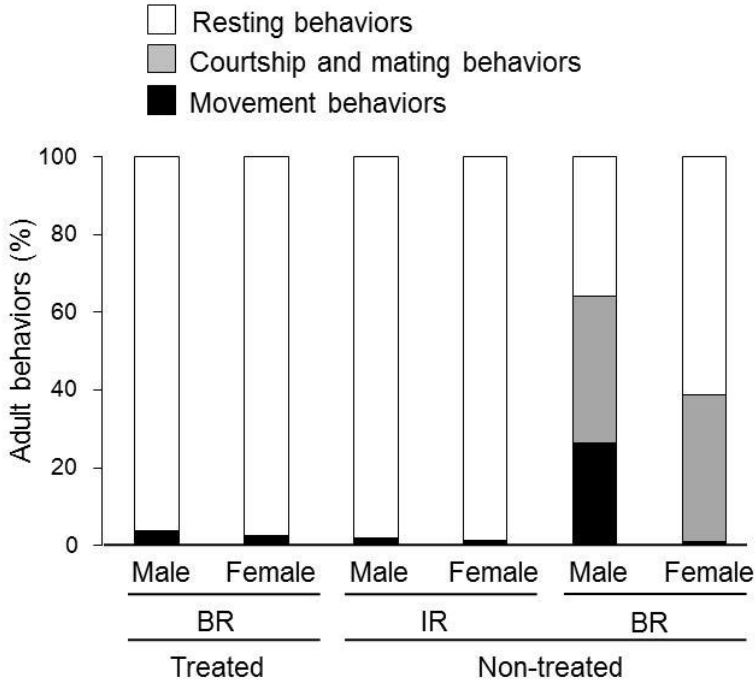

Fig. 3-1 Effect of treatment with acetylated glyceride (0.2%, v/v) on the behaviors of *Bemisia tabaci* B adults on grape tomato leaves. Each value is the mean based on five test tubes per condition with five replicates. BR indicates that a pair of adults was released together into the test tube. IR indicates that an adult of each sex was released separately into the test tube. The values corresponding to the male movement behaviors on the acetylated glyceride-treated under the BR condition and non-treated leaves under the IR condition were significantly lower than that on the non-treated leaves under the BR condition. ($P < 0.05$; generalized linear mixed model with a binominal errors distribution followed by Tukey's HSD-test).

3-3-2 Impact on the sex ratio of adults of the second generation resulting from the courting disruption effect

Figure 3-2 shows that all of the newly emerged second-generation adults were recognized as male progeny under the FR condition because of arrhenotokous parthenogenesis by virgin *B. tabaci* females (Byrne and Bellows 1991). After four days under

the BR condition, the sex ratio (male/female) of the newly emerged adults on treated leaves (2.4) was significantly different from that on the non-treated control leaves (0.9) ($P < 0.05$, Tukey's HSD-test). At seven days under the BR condition, the sex ratios of the newly emerged adults differed only moderately (1.6 for the treated leaves and 1.2 for the non-treated control leaves; $P > 0.05$, Tukey's HSD-test).

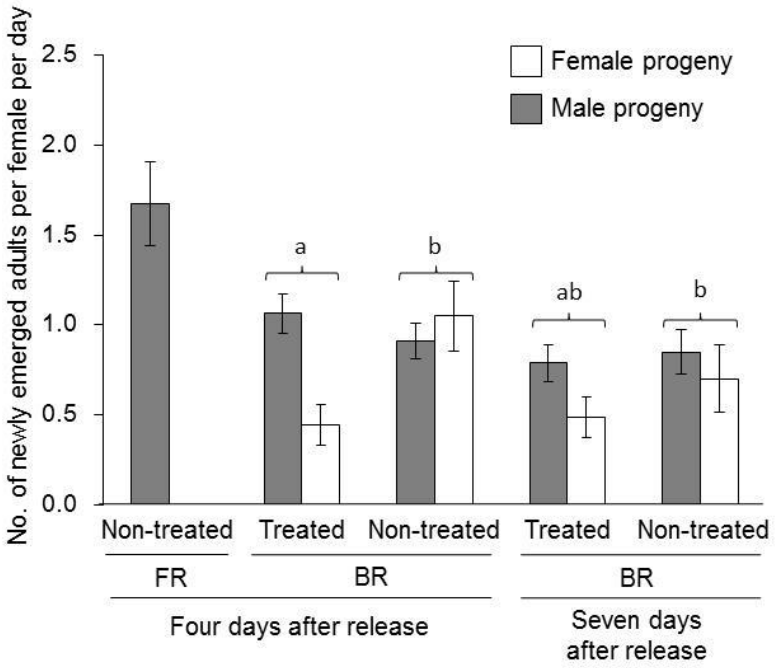

Fig. 3-2 Effect of treatment with acetylated glyceride (0.2%, v/v) on the sex ratio of *Bemisia tabaci* Q progeny on grape tomato leaves. Each value is the mean ± standard error (S.E.) based on 11–16 test tubes per condition with four replicates. BR indicates that a female and two males were released together into the test tube. FR indicates that a single female was released into the test tube. Different letters denote significantly different values in the progeny of a given sex ratio between treatments ($P < 0.05$, generalized linear mixed model with a binominal errors distribution followed by Tukey's HSD-test).

3-4 Impact of acetylated glyceride at different developmental stages of *B. tabaci* B.

3-4-1 Direct treatment of settled adults

No insecticidal activity was observed 24 hr after direct acetylated glyceride foliar treatment against *B. tabaci* B adults (Table 3-4; $T = 1.13$; df = 11; $P = 0.140$)

3-4-2 Direct treatment of eggs and first, third, and fourth instar nymphs on eggplant

No significant difference in hatchability was observed between the acetylated glyceride treated (100%) and non-treated (99%) groups (data not shown) ($T = 1.71$; df = 6; $P > 0.05$). when eggs were dipped into an acetylated glyceride solution. Moreover, no insecticidal activity was detected when eggs and late instar nymphs were treated (Tables 3-5 and 3-7) ($P > 0.05$, *t*-test). Treatment-related mortality from the first and second to third instar nymphs (61%) was significantly higher than that of the non-treated control (21%) (Table 3-6; $T = 10.3$; df = 6; $P < 0.05$) when first and second instar nymphs were dipped into the acetylated glyceride solution.

3-4-3 Contact treatment of first instar nymphs

The mean mortality rates of the acetylated glyceride-treated and non-treated control were l4.4% and 11.9%, respectively, at 26 DAT (Table 3-8) ($P > 0.05$, *t*-test)

Table 3-4. Effect of direct acetylated glyceride treatment (0.2%, v/v) on *Bemisia tabaci* B adults settled on cucumber leaves.

Treatment	Percent mortality
Acetylated glyceride-treated	5.3
Non-treated	4.4

The values are the means of 6–7 replicates 24 hr after treatment. The numbers (mean ± S.E.) of adults settled on treated and non-treated leaves were 107 ± 14 and 88 ± 5, respectively, at pretreatment. No significant difference was observed between treatments (arcsine transformation before analysis; $P > 0.05$; *t*-test).

Table 3-5. Insecticidal effect of direct acetylated glyceride treatment (0.2%, v/v) on *Bemisia tabaci* B eggs laid on eggplant leaves.

Treatment	Percent mortality for each period				
	Egg–1st	1st–2nd	2nd–3rd	3rd–4th	4th–adult
Acetylated glyceride-treated	13.5	9.0	0.5	1.0	0.2
Non-treated	6.0	5.4	5.8	0.9	0.0

Each value indicates the mean of 3–4 replicates. No significant difference was observed between treatments in the same column (arcsine transformation before analysis; $P > 0.05$; *t*-test).

Table 3-6. Insecticidal effect of direct acetylated glyceride treatment (0.2%, v/v) on early instar nymphs of *Bemisia tabaci* B on eggplant leaves.

Treatment	Percent mortality for each period			
	1st, 2nd–3rd		3rd–4th	4th–adult
Acetylated glyceride-treated	61.4	*	4.2	0.0
Non-treated	21.3		0.3	2.9

Each value indicates the mean of 3–4 replicates. The asterisk indicates that the values are significantly different between treatments in the same column (arcsine transformation before analysis; $P < 0.05$; *t*-test).

Table 3-7. Insecticidal effect of acetylated glyceride direct treatment (0.2%, v/v) on late instar nymphs of *Bemisia tabaci* B on eggplant leaves.

Treatment	Percent mortality for each period	
	3rd–4th	4th–adult
Acetylated glyceride-treated	5.9	18.5
Non-treated	1.0	2.7

Each value indicates the mean of 3–4 replicates. No significant difference was observed between treatments in the same column (arcsine transformation before analysis; $P > 0.05$; *t*-test).

Table 3-8. Effect of acetylated glyceride contact treatment (0.2%, v/v) on first instar nymphs of *Bemisia tabaci* B on cucumber leaves.

Treatment	Percent mortality		
	14 DAT	22 DAT	26 DAT
Acetylated glyceride-treated	5.9	11.0	14.4
Non-treated	8.0	11.9	11.9

The values are the means of 3–4 replicates. No significant differences were observed between treatments in the same column (arcsine transformation before analysis; $P > 0.05$; *t*-test). DAT, days after treatment.

4 Discussion

The number of courting pairs of adults of *B. tabaci* Q and *T. vaporariorum* were clearly reduced by acetylated glyceride treatment, with the activity being as high as that against the B of *B. tabaci* (Tables 3-2 and 3-3). In addition, a treatment with only inert ingredients did not show courting disruption effect on adult whiteflies (Table 3-1). Therefore, acetylated

glyceride as active ingredients provided courting disruption effect.

Observations of the courtship behavior of pairs of *B. tabaci* adults showed that a male would mate repeatedly with the same female on non-treated leaves, even after successful mating followed by the pairs dissolving. *B. tabaci* A (Li et al., 1989) (now referred to as *New world*) and *Bemisia argentifolii* (*B. tabaci* B; Zang and Liu, 2007) pairs are capable of mating several times. Multiple mating is necessary for sustaining the female progeny of *B. tabaci* B (Liu et al., 2007). Therefore, the courting disruption effect of acetylated glyceride treatment is expected to affect not only non-mated but also mated females. In addition, under normal conditions, when pairs of *B. tabaci* adults meet, the percentage of initial courtships that result in mating is low: 7.7% for the A *B.tabaci* (Li et al., 1989) and 12% for the B *B.tabaci* (Perring and Symmes, 2006). *Bemisia tabaci* B shows a stronger propensity to mate under reproductive competition with non-B whiteflies, leading to a reduction in the number of female progeny from non-B whiteflies. Consequently, the proportion of non-B whiteflies has been decreasing gradually in field surveys (Liu et al., 2007; Luan and Liu, 2012). *Bemisia tabaci* has an arrhenotokous parthenogenetic reproduction in which males are produced from unfertilized eggs (haploid) and females are produced from fertilized eggs (diploid) (Byrne and Bellows, 1991) (Fig. 3-2). Therefore, given the impact of acetylated glyceride treatment on reproductive behavior, the use of this agent might be an important tactic for suppressing of *B. tabaci*, in addition to mating disruption techniques based on the release of pheromones and pheromone analogs used in other insect species (Cardé and Minks, 1995; Koppenhöfer et al., 2005; Polavarapu et al., 2002; Walton et al., 2006). The repellency of acetylated glyceride treatment on adult whiteflies is considered to last for two or three DAT. After adult whiteflies landed on the treated host leaves, they were not able to conduct normal mating behaviors during the remaining four days. The total residual efficacy of acetylated glyceride treatment

may have lasted for approximately seven DAT.

No insecticidal activity was observed after the direct acetylated glyceride foliar treatment of adults or after the contact treatment of first instar nymphs (Tables 3-4 and 3-8). Only early instar nymphs decreased significantly, by approximately 51% of those in the non-treated control when acetylated glyceride was applied directly to eggs and early and late instar nymps of *B. tabaci* (Tables 3-5, 3-6 and 3-7). Acetylated glyceride seemed to act as a spiracle-blocking substance in early instar nymphs, partly because its active ingredient is classified with a fatty acid ester of glycerol, which is used in spiracle-blocking insecticides.

In conclusion, acetylated glyceride treatment significantly reduced the number of courting adult pairs of B and Q of *B. tabaci*, and *T. vaporariorum*. The disruption of courtship behaviors by acetylated glyceride treatment significantly reduced the number of female progeny. No insecticidal activity against any developmental stages of *B. tabaci* was detected, except for early instar nymphs.

Chapter 4

Courtship Behavior and Vibratory Signals

1 Introduction

Insects in the order Hemiptera produce sounds via stridulation, and wing vibrations are associated with the production of sound (Ossiannilsson, 1949). It is now known that insects in 16 orders, including Hemiptera, produce substrate-borne signals to identify the location of mating partners (Miyatake, 2011). These findings imply that stridulation is a form of communication among Hemiptera.

Bemisia tabaci is a cryptic species complex (De Barro et al., 2011; Dinsdale et al., 2010; Frohlich et al., 1999) based on mitochondrial CO1 and ribosomal ITS1 DNA sequences (Boykin et al., 2007), cross-test and allozymic frequency analyses (Perring et al., 1993). There are distinct differences in the courtship signals produced by *B. tabaci* B and JpL (Kanmiya, 2006, 2011) and between *B. tabaci* B and Q (Kanmiya, 2011) in terms of the burst duration and fundamental frequency on a host plant. The sounds produced by adult whiteflies are intricate, and the insect is believed to use them to discriminate the sex, species, and species of potential mates.

In the Chapter 2 and 3, adult whiteflies were observed to repel on the acetylated glyceride-treated host leaf. Although adults of *B. tabaci* B will eventually settle on an acetylated glyceride-treated host leaf due to the decrease in residual repellency with time, the number of courting pairs was significantly reduced on treated leaves. Courting pair formation is known to be necessary process for adult whiteflies to mate (Perring and Symmes, 2006).

In this Chapter 4, the mechanism of courting disruption of acetylated glyceride treatment was elucidated to validate a hypothesis that the substrate-borne signals produced by adults are affected by the treatment.

2 Materials and Methods

2-1 Acoustic-based analysis of the courting disruption effect

Sexually mature *B. tabaci* males frequently produce vibratory sounds to attract females during the processes of mating and mate finding (Kanmiya, 2006, 2011). Males perform abdominal undulations that contact the leaf surface with each undulation. Therefore, the frequency of the vibratory sounds produced by males can be used as a critical index of the mating interactions between the sexes.

A solution of acetylated glyceride (1.0%, v/v) or distilled water was sprayed onto a small piece of cabbage leaf (cv. Shikidori) using a hand sprayer until run-off. To reduce the repellency of the acetylated glyceride, the cabbage leaves were left for 3–5 days at 25°C ± 1°C after treatment. Thereafter, adult whiteflies were released onto the leaves in test tubes. Acoustic recordings were collected for 25 min in an anechoic chamber (a 35-mm film case) at the Institute of Comparative Studies of International Cultures and Societies of Kurume University during 2011–12; the chamber was maintained at room temperature (25°C ± 2°C). A sheet of cellophane paper in the film case was used to pick up the substrate-borne sounds produced by the whitefly via contact with the piece of cabbage leaf where the whitefly was located. The top of the plastic film case was sealed with a polyethylene sheet after releasing the sampled pair of whiteflies. A small hole was made in the bottom of the case (in the case

cap) to insert the microphone head and record any sounds that were produced. The best sounds were recorded when the male was located on the polyethylene roof of the cage, but whitefly mating usually occurred beneath the leaflet on the cellophane. The vibratory signal transducer was a one-inch condenser microphone (Type 4165, Brüel & Kjaer, Denmark) with a preamplifier (Type 2645, Brüel & Kjaer). The output was amplified using a measuring amplifier (Type 2610, Brüel & Kjaer) and fed into a digital audio tape recorder (Model DA-P1, TASCAM, Japan). Acoustic analysis of the waveforms reproduced from the data recorder was conducted using an FFT digital spectrum analyzer (TR-9305, Advantest, Japan), and time- and frequency-domain parameters were obtained. Time-domain analyses of long sequences were conducted using a memory recorder (Type 8830, Hioki, Japan).

Experimental 1: Release of different pairs onto treated and non-treated leaves

A pair of *B. tabaci* B adults (24 hr after emergence) was released into a 35-mm film case (a small cylindrical plastic case) containing a small piece of either an acetylated glyceride-treated or non-treated cabbage leaf, to measure the vibratory sounds produced by the male. After collecting the measurements, a new pair of adults was subjected to sound measurements with a new leaf. For the treated and non-treated leaves, sound measurements were collected for 40 and 19 replicates, respectively.

Experimental 2: Release of the same pair onto treated and non-treated leaves

First, one pair of *B. tabaci* B adults (24 hr after emergence) was released onto an acetylated glyceride-treated cabbage leaf in a 35-mm film case. After measuring the vibratory sounds produced by the adults on the treated leaf, the same pair was immediately released

onto a non-treated leaf, and the measurements were performed again. Both tests comprised 14 replicates.

2-2 Data analysis

The frequencies of the vibratory sounds produced by males on the leaves in both experimental plots were analyzed with the Mann–Whitney U-test at $\alpha = 0.05$.

3 Results

3-1 Acoustic-based analysis of the courting disruption effect

Experimental 1: Release of different pairs onto treated and non-treated leaves

The mean (± S.E.) frequencies of the vibratory sounds produced by males on acetylated glyceride-treated and non-treated leaves were 6.4 ± 1.4 and 18.8 ± 1.7, respectively (Fig. 4-1). The frequency of the male vibratory sounds produced was significantly lower (by 66%) on the treated leaves than on the non-treated leaves (Mann–Whitney: $U = 101.0$; $P < 0.01$).

Experimental 2: Release of the same pair onto treated and non-treated leaves

Figure 4-2 shows that the mean frequency of the vibratory sounds produced by males on acetylated glyceride-treated leaves was lower, at approximately 81% of the frequency recorded on non-treated leaves (Mann–Whitney: $U = 9.0$; $P < 0.01$). Figure 4-3 shows a typical oscillogram of the acoustic signals produced to mediate courtship by both sexes on the treated and non-treated leaves. The acoustic signals produced by each sex were exchanged rhythmically on non-treated leaves. In contrast, the signals produced by males

were irregular on treated leaves, preventing signal communication between males and females in most cases.

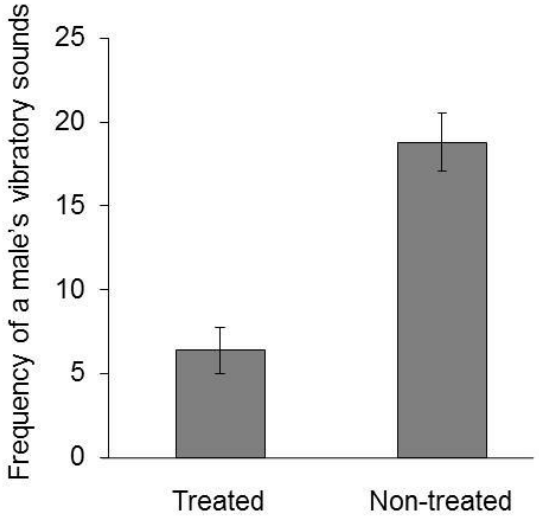

Fig. 4-1 Effect of treatment with acetylated glyceride (1.0%, v/v) on the vibratory sounds produced by males when different pairs of *Bemisia tabaci* B adults were released onto treated and non-treated cabbage leaves. Each value is the mean ± standard error (S.E.) of 40 replicates (treated) and 19 replicates (non-treated). Significant differences were found between treatments ($P < 0.01$, Mann–Whitney U-test).

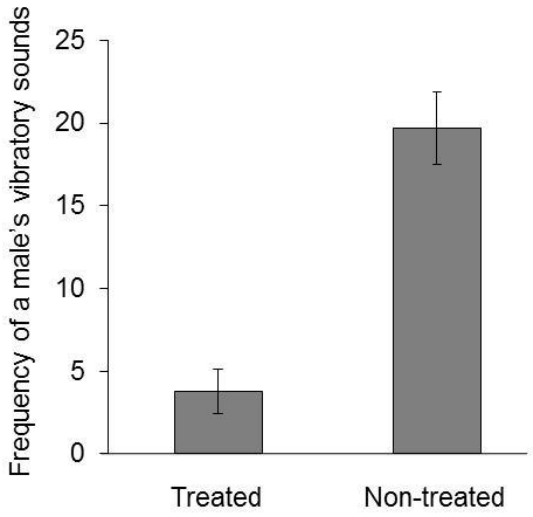

Fig. 4-2 Effect of treatment with acetylated glyceride (1.0%, v/v) on the vibratory sounds produced by males when the same pair of *Bemisia tabaci* B adults was released onto treated and non-treated cabbage leaves. Each value is the mean ± standard error (S.E.) of 14 replicates. Significant differences were found between treatments ($P < 0.01$, Mann–Whitney U-test).

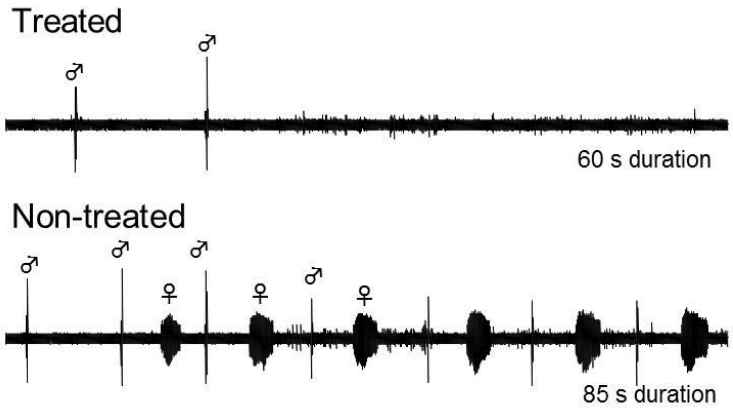

Fig. 4-3 Typical oscillograms of the acoustic signals produced during courtship when the same pair of *Bemisia tabaci* B adults was released onto cabbage leaves that were either treated with acetylated glyceride (1.0%, v/v) or non-treated.

4 Discussion

The courting disruption mechanism of acetylated glyceride treatment in adult whiteflies was reproted in view of acoustic study in this Chapter.

In the previous Chapter, adult whiteflies were able to land on grape tomato leaves a few days after treatment, when the residual repellency had dissipated. Almost no male courtship behavior in terms of searching for sexually mature females was observed throughout the observation period (60 min) under conditions in which adults of both sexes remained on a treated leaf or those in which an adult of either sex remained on a non-treated leaf. By contrast, these behaviors accounted for 26.5% of the observation time under conditions in which both sexes remained on a non-treated leaf. In cross-tests performed for 4 days using one non-mated female and two non-mated males, the sex ratio (male/female) of the newly emerged adults on treated leaves (2.4) was 63.6% lower than that of the non-treated controls (0.9) due to their arrhenotokous parthenogenetic reproduction.

When seeking mates on a host leaf, sexually mature *B. tabaci* B males produce vibrations to communicate with nearby females, allowing the males to orient to the females (Kanmiya, 2006). The leaf substrate transmits the sounds produced by males. The females produce short vibratory sounds in response to the male sounds (Kanmiya, 2006). The frequency of the female response increases as the pair progresses toward mating (Kanmiya,2006). Within the *B. tabaci* species complex, various levels of reproductive incompatibility are apparent, even when the species engage in courtship behavior (De Barro and Hart, 2000; Kanmiya, 2011; Perring et al., 1993; Zang and Liu, 2007). One reason for this situation is that cross-tests indicate that most mating signals are common to most *B. tabaci* species, although several variants lack the signals necessary for successful mating.

In the observational study of previous Chapter, only males on non-treated leaves initiated walking to search for females. In general, males of *B. tabaci* B search for females (Perring and Symmes, 2006; Zang and Liu, 2007), although A females have sometimes been observed to approach males (Li et al., 1989). In the acoustic analysis, the males rarely produced vibratory sounds on treated leaves, even when sexually mature females of the same species were present nearby (Figs. 4-1 and 4-2). In a few cases, females responded to the male sounds briefly, but the female sounds stopped immediately because the male sounds were weak and irregular (Fig. 4-3). Given the lack of response sounds by females on the leaves treated with acetylated glyceride, the males did not initiate typical movement behaviors; i.e., no searching for females was observed.

In conclusion, there was a marked reduction in the frequency of male vibratory sounds, which serve as a trigger of acoustic-based communication, during the courtship process on acetylated glyceride-treated leaves.

Chapter 5

Recommended Treatment Conditions in Practical and Impact on Beneficial Organisms

1 Introduction

Several attempts have been made to develop effective control measures for whiteflies. Among these measures, the most widely used are insecticides. However, available insecticide is limited number, partly because continuous field applications occasionally harm natural enemies and pollinators and deposit pesticide residues on crops.

As an alternative to pesticides, biological control has been extensively studied using various natural enemies. However, it is not easily applicable in fields, partly because growers are required to have deep technical knowledge of the economic injury level (EIL) and economic threshold (ET), which are essential for efficiently deploying natural enemies.

Another example in cultural control is that most of *B. tabaci* adults are not able to pass through fine insect proof net (≦0.4 mm) in greenhouse openings (Matsuura et al., 2005; Oida, 2007), though high temperature damage to tomatoes would be a concern especially in summer (Mihara and Ishida, 2005).

Under these circumstances, plant-derived oils and seed extracts are growing in importance as alternative pest controls, because they intend to be not only compatible with IPM strategies, but also not necessary to have an extensive knowledge for use compare to biological control. The effect of plant extracts and vegetable oils as repellents, antifeedants, and toxicants has been evaluated against whiteflies *B. tabaci* (Aslan et al., 2004; Butler et al.,

1988, 1989; Butler and Henneberry, 1990, 1991; Fenigstein et al., 2001; Kim et al., 2011; Prabhaker et al., 1999; Schuster et al., 2009; Yang et al., 2010; Zhang et al., 2004) and *Trialeurodes vaporariorum* (Camarillo et al., 2009; Choi et al., 2003; Moreau and Isman, 2012; Simmonds et al., 2002). However, continuous field application of insecticides is not always feasible as described above, because of the deposition of pesticide residues on crops during the harvest season, harmful effects on beneficial organisms, and the development of insecticide-resistant populations, such as Q of *B. tabaci* (Fernández et al., 2009; Koyama et al., 2008; Roditakis et al., 2009; Tokumaru and Hayashida, 2010). In addition, the effective application of insecticides may be difficult because nymphal stages prefer to settle on the undersides of the host leaves (Simmons, 1994).

Chapter 5 introduces recommended treatment conditions of acetylated glyceride that are satisfactory and well-balance among efficacy on *B. tabaci*, phytotoxicity on leaves and fruits of grape tomato, and impacts on beneficial organisms.

2 Materials and Methods

2-1 Repellency

Experiment 1: Treatment dose rates in pot tests in a glass greenhouse

Tomato (*Lycopersicon esculentum* Mill. Takii & Co., Ltd., cv. Kyouryoku-Beiju) plants were grown in 15-cm plastic pots in a whitefly-free greenhouse at Ishihara Sangyo Kaisha, Ltd in Kusatsu City, Shiga prefecture. When seedlings reached true-leaf stages 8–9 on August 1, 2007, they were treated with acetylated glyceride (0.2%, 0.125%, and 0.1%, v/v). Four treated and four non-treated potted plants were placed in two rows on a greenhouse

bench, alternately arranged at equidistant intervals (approximately 50 cm). In the middle of the two plant rows, four potted cabbage plants with numerous *B. tabaci* B nymphs and adults were placed as an adult migration source. At 1, 2, 5, and 7 days after plant placement, the settled adults on all leaves of the treated and non-treated tomato plants were counted. The experiment was performed with one replicate.

Experiment 2: Treatment numbers in plastic greenhouse test

Tomato (cv. Kyouryoku-Beiju) at true–leaf stages 5–5.5 planted in a plastic greenhouse (5.5×12 m = 66 m^2) near Kusatsu City from May 25, 2006, to June 22, 2006. Five plants per plot were planted in duplicate. The plants were treated with acetylated glyceride (0.2%, v/v) one, two and three times at 7-day intervals. Settled adults of *B. tabaci* B on all leaves were counted before treatment, 4, 7, 11, 14, 18, and 21 days after planting. At 21 days after planting, the third–fourth instar nymphs and exuviae on leaves were counted as progeny. Unless otherwise indicated, counting included only the third and fourth instar nymphs, easily visible to the naked eye.

Experiment 3: Recommended treatment conditions in plastic greenhouse test

Eggplants (cv. Senryo Ni Gou) at true-leaf stages 7–9 with *B. tabaci* B eggs, first instar nymphs, and adults were treated with acetylated glyceride (0.2%, v/v). Seven to nine plants per plot were planted in duplicate. The plants were treated three times at 7–8-day intervals. The test was conducted in the plastic greenhouse from April 4, 2006, to May 8, 2006. Settled adults on 20 randomly selected leaves were counted 2, 6, 7, 9, 12, 15, 19, 23, and 33 days after the first treatment. At 33 days, the third and fourth instar nymphs on leaves were counted as progeny.

2-2 Effect on courting pair formation

Experiment 1: Courting pairs were counted in the greenhouse 1, 2, 5, and 7 days after treatment (DAT) with acetylated glyceride (0.2%, 0.125%, and 0.1%, v/v) on tomato leaves (cv. Kyouryoku-Beiju) at true-leaf stages 8–9 as described in set of "EXP.1 Treatment dose rates in pot tests in a glass greenhouse". The experiment was performed using four treated and four non-treated potted plants, with one replicate.

Experiment 2: As described in set of "EXP. 2 Treatment numbers in plastic greenhouse test", courting pairs on tomato (cv. Kyouryoku-Beiju) were counted in the greenhouse before treatment, 4, 7, 11, 14, 18, and 21 days after planting. The plants were treated with acetylated glyceride (0.2%, v/v) one, two, and three times at 7-day intervals. The experiment was performed in duplicate using five plants per plot.

Experiment 3: As described in set of "EXP. 3 Recommended treatment conditions in plastic greenhouse test", courting pairs were counted in the greenhouse 2, 6, 7, 9, 12, 15, 19, 23, and 33 days after the first treatment with acetylated glyceride (0.2%, v/v) on eggplants (cv. Senryo Ni Gou) at true-leaf stages 7–9 that were parasitized by eggs, nymphs, and adults. The plants were treated three times at 7–8-day intervals. The experiment was performed in duplicate using 7–9 plants per plot.

2-3 Effect on nymphs remaining on leaves

Three greenhouse experiments were performed to decide recommended treatment conditions of acetylated glyceride against B of *B. tabaci* progeny*:* nymphs and exuviae on tomato, grape tomato, and eggplant. Fourth instar nymphs were discriminated from third

instar nymphs by the presence of developing eyespots.

Experiment 1: Treatment dose rates in a plastic greenhouse test

The aim of this study is to clarify the best treatment dose rates of acetylated glyceride. Grape tomato (cv. Yellow-pear) at true-leaf stages 9–10 with *B. tabaci* B eggs, nymphs, and adults were treated with acetylated glyceride (0.2 and 0.125%, v/v) on August 17, 2007. The plants were treated three times at 7-day intervals in a plastic greenhouse (5.5×12 m = 66 m^2) near Kusatsu City. Five plants per treatment were planted in duplicate. Twenty-three days after the first treatment, nymphs and exuviae on all leaves were counted as progeny.

Experiment 2: As described in set of "EXP. 2 Treatment numbers in plastic greenhouse test", Tomato (cv. Kyouryoku-Beiju) parasitized by eggs, nymphs, and adults of *B. tabaci* B were treated with acetylated glyceride (0.2%, v/v), first at true-leaf stages 5–5.5 and, subsequently, one, two, and three times at 7-day intervals. Five plants were used for each plot in duplicate. The number of nymphs and exuviae on all leaves were counted 21 days after the first treatment.

Experiment 3: As described in set of "EXP. 3 Recommended treatment conditions in plastic greenhouse test", eggplants (cv. Senryo Ni Gou) with *B. tabaci* B at mixed developmental stages were treated with acetylated glyceride (0.2%, v/v), first at true-leaf stages 7–9 and, subsequently, three times at 7–8-day intervals. Seven to nine plants were used for each plot in duplicate. Thirty-three days after the first treatment, nymphs on all leaves and exuviae on 20 randomly selected leaves were counted. The experiment was performed in duplicate using 7–9 plants per plot.

2-4 Phytotoxicity

Grape tomato (*Solanum lycopersicum*, Takii & Co., Ltd., cv. Minicarol) plants at the 4th flowering stage were treated with acetylated glyceride (0.4% and 0.2%, v/v) three times at 7-day intervals. Seven days after the third treatment, phytotoxicity induced on leaves and fruits of tomato was examined and assigned to one of the following four classes: N, no phytotoxicity; L, light phytotoxicity below the commercially acceptable level (e.g., dirt spots easily cleaned off by wiping or covering with fruit coloring); M, moderate phytotoxicity above the commercially acceptable level (e.g., epidermal injuries with well-defined rings); H, heavy phytotoxicity far above the commercially acceptable level (e.g., full rings with raised borders). The experiment was performed from November 27, 2007 to March 25, 2008 in the greenhouse near Kusatsu city using four plants per plot with one replicate.

2-5 Mortality of natural enemies

With the aim of evaluating the side effects of acetylated glyceride treatment, mortality was evaluated with three replicates for different developmental stages of natural enemies of whiteflies 5–15 days after direct and/or contact treatment with acetylated glyceride (0.2%, v/v). The four species of natural enemies examined are all commercially available products from the Arysta LifeScience Corporation: the predacious mite *Phytoseiulus persimilis* (SPIDEX®), the predacious mite *Amblyseius californicus* (SPICAL EX®), the predatory bug *Orius strigicollis* (TAIRIKU®), and the parasitoid *Encarsia formosa* (EN-STRIP®).

Mortality was corrected before analysis using Abbott's formula: % corrected mortality = (% mortality in treated − % mortality in non-treated)/(100 − % mortality in non-treated) × 100. The corrected mortality was assigned to one of the four classes: class I, harmless (<30% mortality); class II, slightly harmful (30%–79%); class III, moderately harmful (80%–99%),

and class IV, harmful (>99%), following the toxicity ratings of the International Organization for Biological and Integrated Control of Noxious Animals and Plants (IOBC) (Hassan et al., 1994).

2-6 Data analysis

One-way ANOVA was employed to assess the effect of acetylated glyceride treatment in the numbers of nymphs and exuviae on host plants in Subsection 2-3 (EXP. 1 and EXP. 2). Differences among means were compared with Tukey's HSD-test at $P = 0.05$. The numbers of *B. tabaci* nymphs remaining on eggplant leaves after treatment with acetylated glyceride (0.2%, v/v) in Subsection 2-3 (EXP. 3) were analyzed using t-tests at $\alpha = 0.05$.

3 Results

3-1 Repellency

Experiment 1: Treatment dose rates in pot tests in a glass greenhouse

Figure 5-1 shows the repellency of acetylated glyceride treatment evaluated by the number of *B. tabaci* settled adults on potted tomato plants in the glass greenhouse. In the 7-day time-course experiment, the number of settled adults on treated tomato leaves remained lower as compared to the number on non-treated leaves. One DAT with 0.2%, 0.125%, and 0.1% (v/v) acetylated glyceride, the numbers of settled adults were 85%, 82%, and 72% lower, respectively, compared with the numbers on non-treated plants, respectively. Treatment at the highest concentration (0.2%, v/v) tended to show a stable repellency on adults compared to treatment with other concentrations for 7 days.

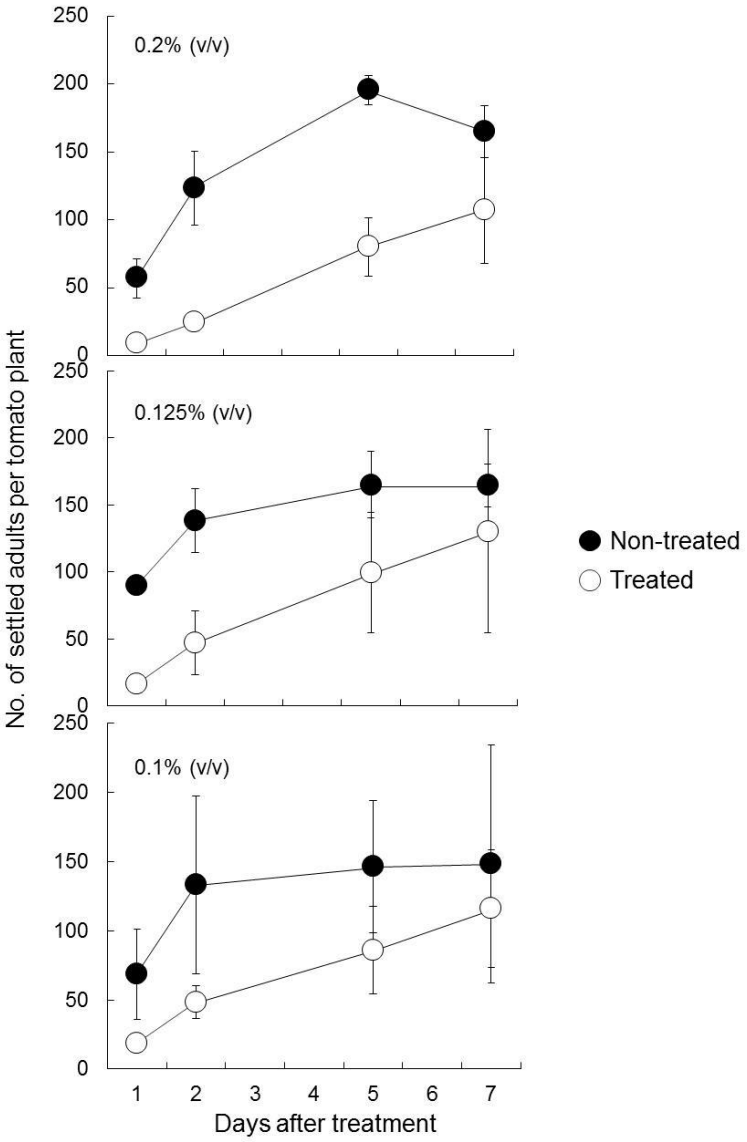

Fig. 5-1 Effect of acetylated glyceride treatment (0.2%, 0.125%, and 0.1%, v/v) one time treatment on the settling of *Bemisia tabaci* B adults on potted tomato leaves. Each value is the mean ± standard error (S.E.) of four plants with one replicate.

Experiment 2: Treatment numbers in plastic greenhouse test

The large reduction in the number of settled adults on tomato plants was correlated closely with increasing the number of treatment times of acetylated glyceride (Fig. 5-2). In particular, the treatment of acetylated glyceride three consecutive times with 7-day intervals was stably suppress the settled adult populations compare to other treatments throughout the experimental period of 21 days.

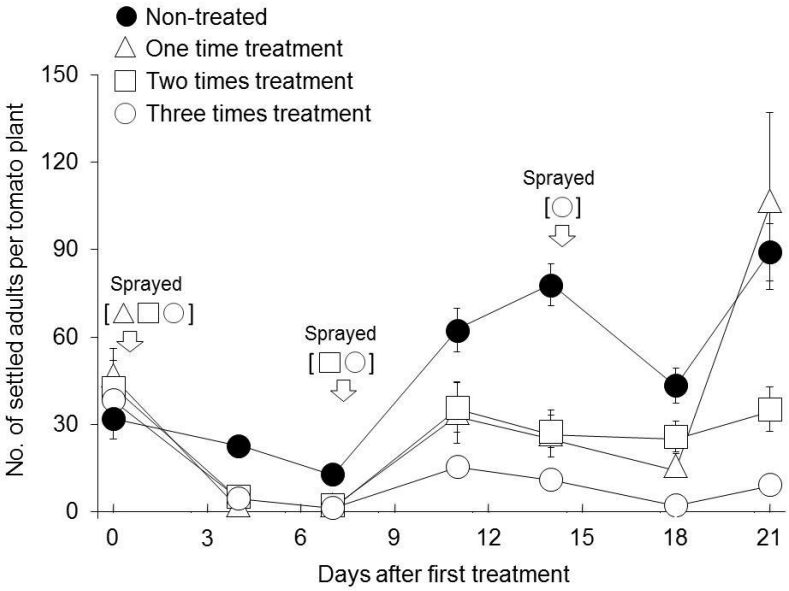

Fig. 5-2 Effect of acetylated glyceride treatment (0.2%, v/v) one, two, and three times treatment on the settling of *Bemisia tabaci* B adults on tomato plants. Each value is the mean ± standard error (S.E.) of five plants in duplicates.

Experiment 3: Recommended treatment conditions in plastic greenhouse test

Figure 5-3 shows the effect of successive foliar treatment with 0.2% (v/v) acetylated glyceride on the settling of whiteflies on eggplant leaves in the plastic greenhouse. On non-treated leaves, the number of settled adults increased during the first 6 days and gradually decreased with time. In contrast, whitefly populations on treated leaves remained below approximately 45% of those on non-treated plants. Two days after the second treatment, the number of settled adults per 20 leaves decreased to approximately 84% per leaf, corresponding to 1.8 per leaf, on average. This low population level persisted until the end of the experimental period, which was 33 days after the first treatment.

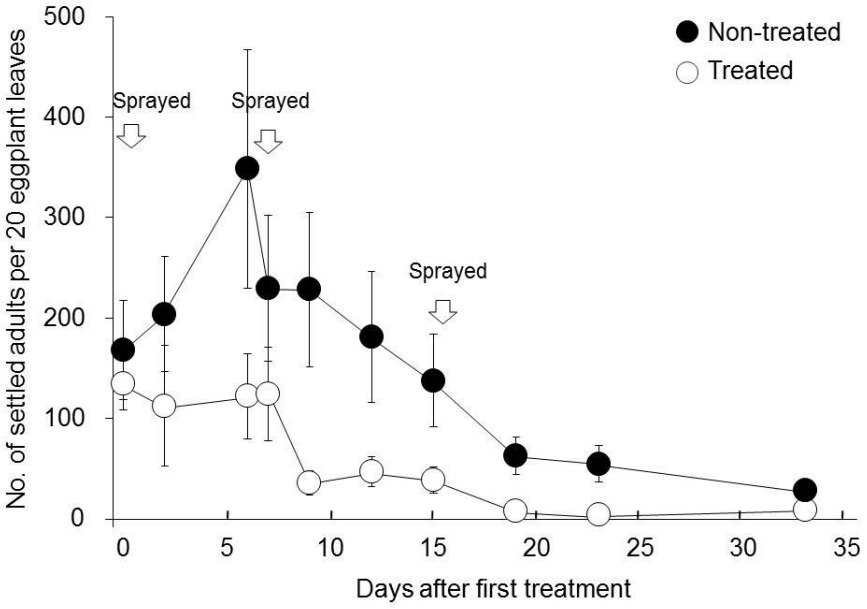

Fig. 5-3 Effect of acetylated glyceride treatment (0.2%, v/v) three times treatment on the settling of *Bemisia tabaci* B adults on eggplant leaves. Each value is the mean ± standard error (S.E.) of seven to nine plants in duplicates.

3-2 Effect on courting pair formation

Experiment 1: Treatment dose rates in pot tests in a glass greenhouse

The effect of acetylated glyceride treatment on *B. tabaci* courting pair formation on potted tomato plants is shown in Figure 5-4. One DAT with 0.2%, 0.125%, and 0.1% (v/v) of acetylated glyceride, the proportions of adults showing courting behavior were 0% (20% for the non-treated control), 9% (23%), and 8% (16%), respectively. Courting pair formation tended to be more strongly inhibited by acetylated glyceride treatment at the highest concentration (0.2 %, v/v); the percentage of courting pairs on the treated leaves was lower than 6%–20% of that on non-treated leaves throughout the experimental period of 7 days in the glass greenhouse.

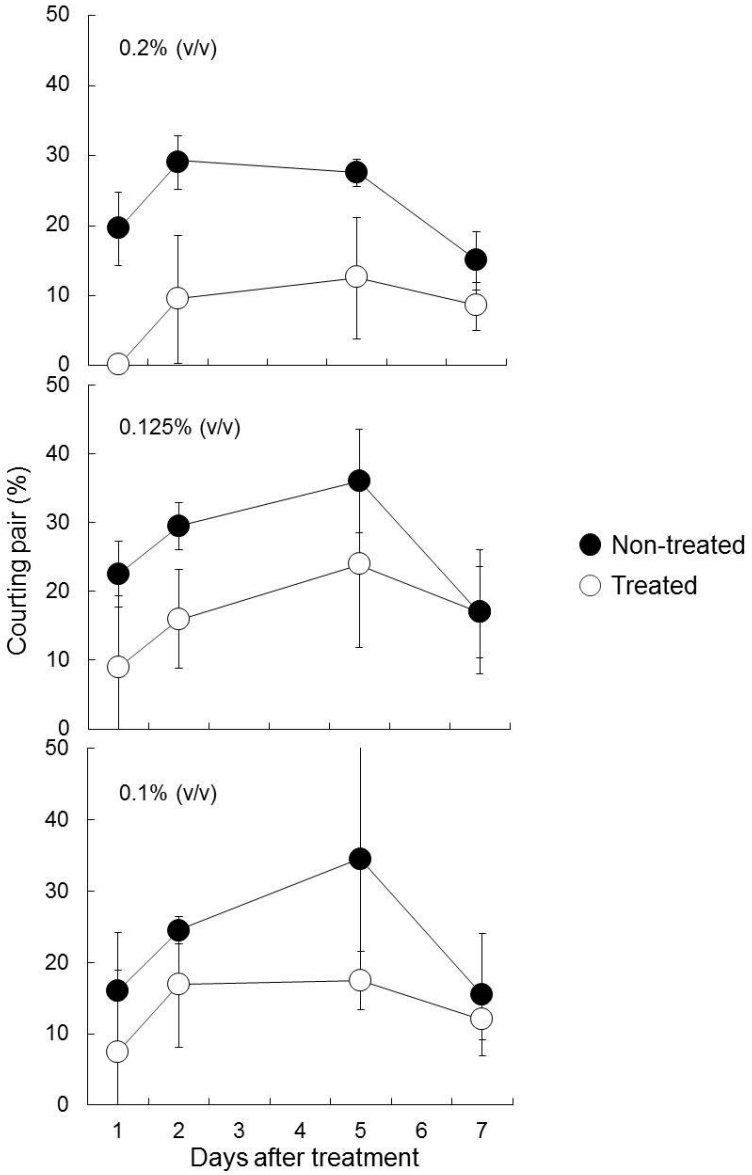

Fig. 5-4 Effect of acetylated glyceride treatment (0.2%, 0.125%, and 0.1%, v/v) one time treatment on *Bemisia tabaci* B adults courting on potted tomato plant leaves. Each value is the mean ± standard error (S.E.) of four plants with one replicate.

Experiment 2: Treatment numbers in plastic greenhouse test

Figure 5-5 shows that the treatment of acetylated glyceride (0.2%, v/v) with three times reduced by 50% and less in the percentage of courtship pairs as compare with non-treated control for all test periods of 21 days. The efficacy of the treatment of acetylated glyceride with one and two times was inferior to those with three times treatment, but they were slightly superior to non-treated.

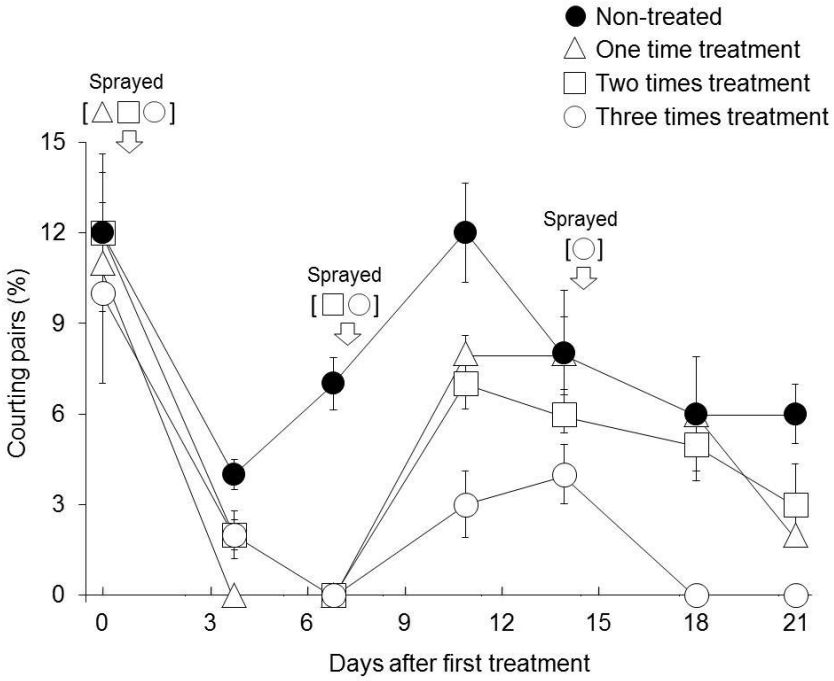

Fig. 5-5 Effect of acetylated glyceride treatment (0.2%, v/v) one, two, and three times treatment on *Bemisia tabaci* B adults courting on tomato plants. Each value is the mean ± standard error (S.E.) of five plants in duplicates.

Experiment 3: Recommended treatment conditions in plastic greenhouse test

Figure 5-6 shows the effect of successive treatments of acetylated glyceride on *B. tabaci* B courting pair formation on eggplants in the plastic greenhouse. The percentage of courting pairs per settled adult on treated leaves was reduced to ≤60% throughout the test period, except 12 days after the first treatment.

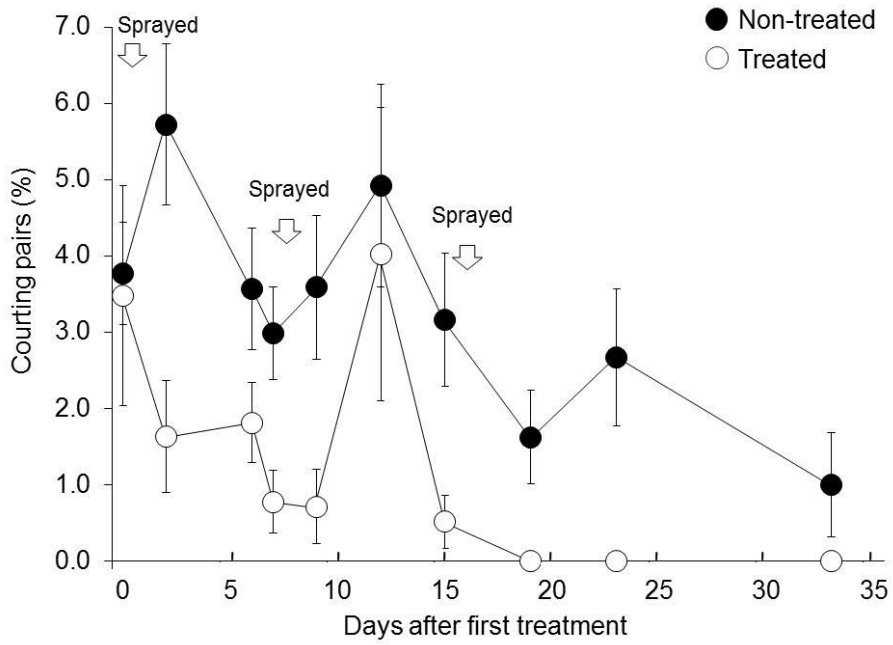

Fig. 5-6 Effect of acetylated glyceride treatment (0.2%, v/v) three times treatment on *Bemisia tabaci* B adults courting on eggplant leaves. Each value is the mean ± standard error (S.E.) of seven to nine plants in duplicates.

3-3 Effect on nymphs remaining on leaves

Experiment 1: Treatment dose rates in a plastic greenhouse test

As shown in Figure 5-7, there was a significant difference in the numbers of third and fourth instar nymphs and exuviae among the treatments (Nymphs: $F = 47.84$; df = 2, 24; $P < 0.001$; Exuvia: $F = 11.09$; df = 2, 24; $P < 0.001$). The nymphs and exuviae showed a dose-dependent response to acetylated glyceride treatment; the numbers of nymphs remaining on the grape tomato leaves after treatment with 0.2% acetylated glyceride, 0.125% acetylated glyceride, and 0% (water) were 7, 17, and 82, respectively. For all treatments, the numbers of remaining exuviae were greater than those of nymphs.

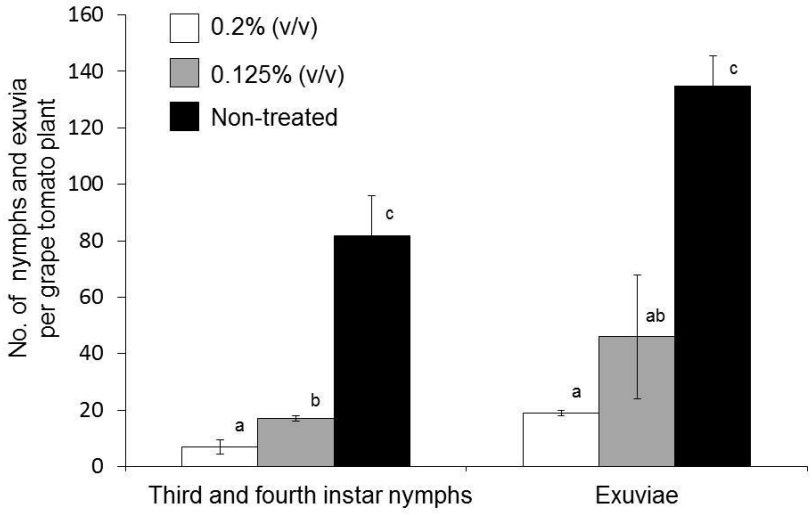

Fig.5-7 Effect of acetylated glyceride treatment (0.2 and 0.125%, v/v) three times treatment on the population size of *Bemisia tabaci* B nymphs and exuviae remaining on grape tomato leaves. Each value is the mean ± standard error (S.E.) of five plants in duplicate. Different letters indicate significant differences among treatments ($P < 0.05$, Tukey's HSD-test).

Experiment 2: Treatment numbers in plastic greenhouse test

After treatment with 0.2% (v/v) acetylated glyceride at different time treatment, the number of nymphs remaining on the tomato plants one, two, and three times treatment were 419, 291, and 90, respectively, when non-treated was observed 592 individuals (Fig. 5-8; Nymphs: $F = 7.55$; df = 3, 8; $P < 0.001$; Exuvia: $F = 11.46$; df = 3, 8; $P < 0.001$). The number of exuviae remaining on the plants observed a treatment time-dependent response to acetylated glyceride as with those of nymphs.

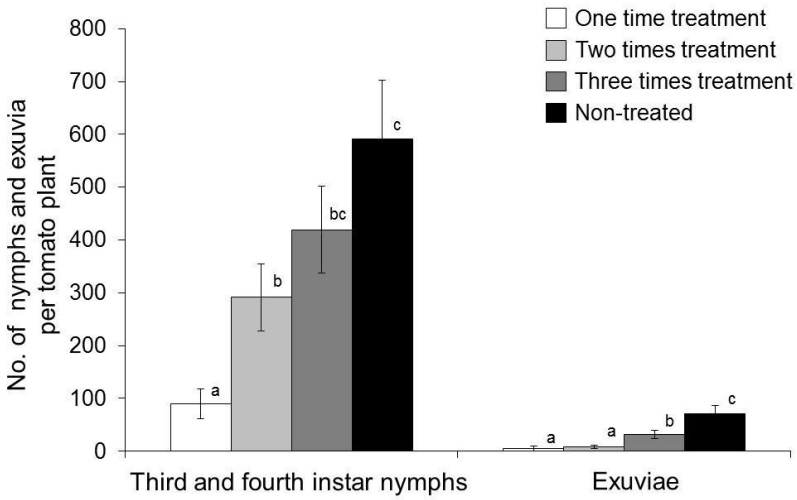

Fig. 5-8 Effect of acetylated glyceride treatment (0.2%, v/v) one, two, and three times treatment on the population size of *Bemisia tabaci* B nymphs and exuviae remaining on tomato plants. Each value is the mean ± standard error (S.E.) of five plants in duplicate. Different letters indicate significant differences among treatments ($P < 0.05$, Tukey's HSD-test).

Experiment 3: Recommended treatment conditions in plastic greenhouse test

Figure 5-9 shows the effect of acetylated glyceride treatment on the population size of *B. tabaci* nymphs remaining on eggplant leaves. The total numbers of third and fourth instar nymphs were significantly reduced after treatment with acetylated glyceride (0.2%, v/v) three times (T = 2.80; df = 14; P = 0.006). Thirty-three DAT, the average numbers of nymphs remaining on treated and non-treated leaves were 76 and 744 per 20 leaves, corresponding to 3.8 and 37.2 per leaf, respectively.

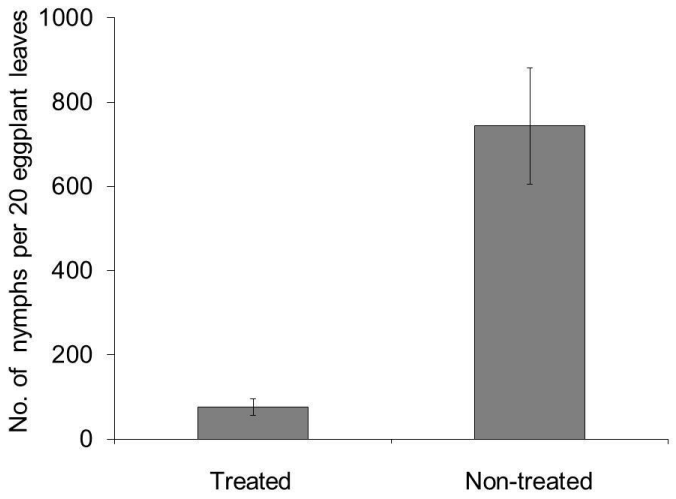

Fig. 5-9 Effect of acetylated glyceride treatment (0.2%, v/v) three times treatment on the population size of *Bemisia tabaci* B nymphs remaining on eggplant leaves. Each value is the mean ± standard error (S.E.) of seven to nine plants in duplicate. The result is significantly different from those obtained with non-treated leaves (P < 0.05, t-test).

3-4 Phytotoxicity

Table 5-1 shows the phytotoxicity induced on grape tomato leaves and fruits 7 days after treatment of acetylated glyceride (0.4% and 0.2%, v/v) three times at 7-day intervals. No phytotoxicity was observed on the leaves. However, on the fruits, acetylated glyceride treatment induced light and moderate phytotoxicity at 0.4% (v/v) but only light phytotoxicity at 0.2% (v/v). The typical slight symptom induced on fruits was indistinct light oil rings originating from dried spray spots. These spots were easily cleaned off by wiping and so were below the commercially acceptable level. The moderate symptom observed on a few fruits after acetylated glyceride (0.4%, v/v) treatment was epidermal injuries that were above the acceptable level.

Table 5-1. Induced phytotoxicity on leaves and fruits of grape tomato (cv. Minicarol) seven days after acetylated glyceride three times treatment.

Treatment	Concentration (%, v/v)	No. of plants examined				No. of fruits examined			
		N	L	M	H	N	L	M	H
Acetylated glyceride	0.4	4	0	0	0	30	6	3	0
-treated	0.2	4	0	0	0	34	3	0	0
Non-treated	-	4	0	0	0	37	0	0	0

Four plants per treatment were used with one replicate. Plants were treated three times at 7-day intervals. N, No phytotoxicity; L, light phytotoxicity; M, moderate phytotoxicity; and H, heavy phytotoxicity

3-5 Mortality of natural enemies

Direct and/or contact treatment with acetylated glyceride (0.2%, v/v) did not cause significant mortality at the various developmental stages of the natural enemies examined (*P. persimilis*, *A. californicus*, *O. strigicollis*, and *E. Formosa*; Table 5-2). The corrected mortality ranged from 0% to 20.5% and, hence, fell in toxicity rating I of the IOBC.

Table 5-2. Effect of acetylated glyceride treatment (0.2%, v/v) on mortality of natural enemies.

Natural enemies	Treatment Methods	Stages	Day after treatment	Corrected mortality
Phytoseiulus persimilis	Direct spray	Adult	5	6.7
		Nymph	5	15.9
Amblyseius californicus	Direct spray	Adult	5	7.9
		Nymph	5	10.4
		Egg	5	4.0
Orius strigicollis	Direct spray	Adult	5	3.4
		Nymph	5	20.5
		Egg	6	11.4
	Contact	Adult	5	12.9
Encarsia Formosa	Direct spray	Adult	7	14.5
		Pupa	15	9.1
	Contact	Adult	7	0

Each treatment was applied with three replicates.

4 Discussion

With the aim of developing a broad-based approach to controlling *B. tabaci*, a rational combination of several control measures, including selective chemical pesticides, natural enemies (Gabarra et al., 2006), virus-resistant or virus-tolerant varieties (Toda et al., 2010; Saito, 2006), yellow sticky traps (Gerling and Horowitz, 1984; Yaobin et al., 2012), insect-proof screens (Berlinger et al., 2002), and UV-controlling films (Antignus et al., 1996, 1998) have been selectively used to keep pest density below acceptable economic loss levels in Integrated Pest Management (IPM) strategies. To date, the principal control strategy in practice for several cultivated crops is treatment by the judicious use of selective insecticides, based on not only their mode of action but also their impact on natural enemies (Desneux et al., 2007) and pollinators (Desneux et al., 2007), with the aim of avoiding pest resurgence (Devine et al., 1998) and the development of cross-resistance (Prabhaker et al., 2005). Under these circumstances, natural products, including plant-derived oils and seed extracts, are becoming importance alternative control measures intended to be compatible with IPM strategies.

As first factors in practical use, natural products may not show sufficient control of target pests under practical conditions, partly because of their lower basic and residual activities, as well as lower systemic properties in comparison with chemical pesticides. For substances intended for practical control of *B. tabaci*, the optimization of both treatment frequency and concentration is very important for pest control. In the study of treatment frequency, acetylated glyceride (0.2%, v/v) treatment two or three times showed more residual activity on *B. tabaci* B adults than did a single treatment, resulting in a large reduction of adults settling and nymphs remaining on tomato plants in a plastic greenhouse

test (Figs. 5-2 and 5-8). In the study of treatment dose rate, the highest concentration of acetylated glyceride (0.2%, v/v) exhibited stronger activity against *B. tabaci* B adults as compared with lower concentrations (0.125% and 0.1%, v/v) in both glass and plastic greenhouse tests (Figs. 5-1 and 5-7).

In the next step toward practical use, treatment conditions that allow natural products to exert practical control on a target pest may lead to phytotoxicity on crops (Butler and Henneberry, 1990, 1991; Zhang et al., 2004) and side effects on beneficial insects (Beattie et al., 2000). The acetylated glyceride treatment conditions defined here were commercially acceptable in terms of both grape tomato phytotoxicity (Table 5-1) and side effects on natural enemies (Table 5-2). In addition, acute oral and contact toxicities of acetylated glyceride for the pollinator *Apis mellifera L.* (Hymenoptera, Apidae) (honeybee) were evaluated in accordance with EPPO Bulletin No.170 (1992) and OECD, JMAFF, and EPA guidelines. There was no mortality at the upper limit of 100 μg AI/bee in both a 48-hr contact test and a 48-hr oral test (data not shown).

Courting pair formation among adult whiteflies was significantly reduced with acetylated glyceride treatment in practical conditions. The efficacy of courting disruption exhibited a dose-dependent and a time-dependent response to acetylated glyceride treatment as with those of adults settled on the plants (Figs. 5-4 and 5-5). To the best of our knowledge, no mating disruption or courting disruption among *B. tabaci* adults on oil-treated leaves has been reported, although pheromone or pheromone analogs for a variety of insect species (Cardé et al., 1995; Koppenhöfer et al., 2005; Polavarapu et al., 2002; Walton et al., 2006) for release over wide areas as described in Chapter 3, and sprayable microencapsulated sex pheromone formulation sprays (Il'ichev; 2006) are available.

In conclusion, for best results, the observed residual efficacy of acetylated glyceride

was consistent with the finding that high control of *B. tabaci* B was achieved in field studies by applying foliar treatment (0.2%, v/v) two or three consecutive times at 7-day intervals (Figs. 5-3, 5-6 and 5-9), when *B. tabaci* adults have not appeared or are at a low density in the greenhouse.

Chapter 6

Interference with Tomato Yellow Leaf Curl Virus Acquisition and Its Transmission

1 Introduction

Tomato yellow leaf curl virus (TYLCV) is a devastating virus that reduces tomato production worldwide (Czosnek and Laterrot, 1997). TYLCV was first described in the Middle East during the 1960s (Cohen and Harpaz, 1964), and it spread rapidly across the region and then to Japan and the United States (Czosnek and Laterrot, 1997; Kato et al., 1998; Lefeuvre et al., 2010; Moriones and Navas-Castillo, 2000; Polston et al., 1999). The typical symptoms of TYLCV-infected grape tomato plants include leaf curling, reduction in leaflet size, chlorotic spots on leaves, shoot erection, irregular ripening of fruits, and the dropping of flowers (Peterschmitt et al., 1999). In particular, the losses of fruits due to viral infection can have major economic impacts by causing lower incomes for growers and higher prices for consumers. Due to the devastating damage of TYLCV, the tomato yield sharply dropped from 21.6 t/ha in 1989 to 11.3 t/ha in 1993 in Azua valley (Hilje et al., 2001).

Four whitefly species (*Bemisia tabaci, Trialeurodes vaporariorum, T. abutilonia, and T. ricini*) have been found to transmit plant viruses, though approximately 1300 whitefly species have been reported (Jones, 2003). *Bemisia tabaci* transmits 18% of the plant viruses among approximately 700 plant viruses (Hogenhout et al., 2008); TYLCV belongs to the genus *Begomovirus,* family Geminiviridae (Czosnek and Laterrot, 1997); its virus has a genome composed of circular single-standarded DNA molecules of approximately 2800 kb (Czosnek

et al., 1988).

The virus is transmitted systematically by the only known natural vector sweet potato whitefly, *B. tabaci* (Harrison et al., 1985; Hogenhout et al., 2008*)*, not transmissible through seeds, soil and mechanical action. Thus, controlling *B. tabaci* populations with insecticides is currently the main method used to prevent the spread of TYLCV.

In this Chapter 6, the efficacy of acetylated glyceride foliar treatment was evaluated against TYLCV acquisition and its transmission by *B. tabaci* adults, and the main biological mechanism of TYLCV suppression was speculated under laboratory conditions.

2 Materials and Methods

2-1 Virus sources

The strains of TYLCV used in the tests described in Subsubsections 2-3-2, 2-3-3, 2-3-4, 2-4-1, and 2-4-2 were TYLCV-Israel (TYLCV-IL) and TYLCV-Mild (TYLCV-Mld), respectively.

2-2 Test locations

The tests described in Subsubsections 2-3-1, 2-3-2, 2-3-3, 2-3-4 and 2-4-1 were conducted at the Physical and Chemical Research Institute (RIKEN) in Wako, Saitama Prefecture, Japan, the test described in Subsubsection 2-3-5 was done at Ishihara Sangyo Kaisha (ISK), Ltd in Shiga Prefecture, Japan, and the tests described in Subsubsection 2-4-2 were conducted at the National Agriculture and Food Research Organization (NARO) Institute of Vegetable and Tea Science in Mie Prefecture, Japan.

2-3 Mechanism of controlling TYLCV

2-3-1 Repellent effect in no-choice test

Grape tomato seedlings (cv. Yellow-pear) were raised until the second-true-leaf stage in plastic pots (6.5 cm in diameter, 7.5 cm in height). To obtain seedlings that retained only the second-true-leaf, the other leaves, new shoots, and seed leaves were cut 1 day before starting the test. Then, the plants were transferred into small plastic cups (2 cm in width, 4 cm in height) that were half filled with water, and the tops of the cups were sealed with parafilm. Solutions of acetylated glyceride (0.2%, v/v) or water were used to treat the plants with a hand sprayer until run-off. After the seedlings were dried for a few hours at 25°C ± 1°C, they were placed in glass test tubes (4 cm in diameter, 13 cm in height). Adult whiteflies were individually observed with a stereoscopic microscope to sex them before releasing them into the test tubes. Thirteen to sixteen *B. tabaci* B adults of each sex, aged 2–5 days after emergence, were released separately for 7 days in individual glass test tubes. The numbers of treated and non-treated (water) test tubes were 12 and 10, respectively. The treatment was conducted with five replicates. The top of the glass test tube was covered with tissue paper and secured with a rubber band. The adults that remained on the leaves were counted at 3, 6, and 12 hr and 1, 2, 3, 4, 5, 6, and 7 day after release (DAR) of the adult whiteflies. The number of insects that had died since the previous count was counted simultaneously.

2-3-2 TYLCV acquisition

The leaflet of a TYLCV-infected grape tomato plant (cv. Yellow-pear) that exhibited severe disease symptoms was transferred to a small plastic cup and then placed in the glass test tubes according to the methods described in Subsubsection 2-3-1. Solutions of acetylated glyceride (0.2%, v/v) or water were used to treat the plants with a hand sprayer until run-off.

After drying, 10 to 16 *B. tabaci* B adults of each sex, aged 2–5 days after emergence, were released for 6, 12, 24, 48, 72, and 96 hr in the individual glass test tubes. Three replicates were performed for each trial period. All of the released adults were captured at the end of each release period, and the adults were stored at −80°C until determining the presence or absence of TYLCV in their bodies using polymerase chain reaction (PCR) as described in Subsection 2-5.

2-3-3 TYLCV transmission

Adults of *B. tabaci* B aged 0–2 days after emergence were released for 3 days onto grape tomato seedlings (cv. Yellow-pear) with severe disease symptoms to obtain viruliferous individuals. Non-infected grape tomato seedlings that retained only the second leaf were treated with a solution of acetylated glyceride (0.2%, v/v) or water according to the methods described in Subsubsection 2-3-1. A viruliferous adult of each sex was separately released for 7 days into the individual glass test tubes. The top of the glass test tube was covered with tissue paper, which was secured with a rubber band. Sixteen to 18 glass test tubes per treatment were used with three replicates. All adult whiteflies were taken away from the tomato seedlings. Next, the tomato seedlings were grown in a whitefly-free chamber for 46 DAR, and the plants were then carefully inspected to detect the typical disease symptoms of TYLCV infection. The presence or absence of TYLCV in all the plant leaves was confirmed by PCR, as described in Subsection 2-5.

2-3-4 Inhibitory effect of the active ingredient against TYLCV transmission

A bioassay was employed to reveal the inhibitory effect of the active ingredient and inert ingredients of the formulation against TYLCV transmitted by female *B. tabaci* B.

Non-infected grape tomato seedlings with only the second leaf were treated with a solution of acetylated glyceride (0.2%, v/v), inert ingredients (0.04%, v/v), and water according to the methods described in Subsubsection 2-3-1. Viruliferous *B. tabaci* B females were obtained using the methods described in Subsubsection 2-3-3. A female was separately released for 7 days in the individual glass test tubes. Thirteen to 16 glass test tubes per treatment were used with three replicates. All other procedures are the same as described in Subsubsection 2-3-3, except the test plants were placed in a whitefly-free chamber for 28 DAR.

2-3-5 Antifeeding activity

Plant sap is the common diet of hemipteran insects such as aphids and whiteflies (Douglas, 2006). After the digestion and absorption of plant sap, *B. tabaci* excrete honeydew, the quantity of which was used as a criterion to assess plant sap ingestion.

A piece of water-sensitive paper (Spraying Systems Co., Tokyo, Japan) was placed in the bottom of a petri dish (2.5 cm in diameter and 1.0 cm in height). A black piece of paper (3 cm square), in which a circular hole (1.0 cm in diameter) was cut from the center portion, was placed on the petri dish. Thinly extended parafilm was sealed to the upper side of small petri dishes (1.2 cm in diameter and 1.0 cm in height) and filled with one of three test solutions: a mixed solution of acetylated glyceride (0.2%, v/v) plus sucrose (30%, w/v; Nacalai Tesque, Inc.), a mixed solution of inert ingredients (0.04%, v/v) plus sucrose, and sucrose solution as the non-treated control. Then, the parafilm side of the small petri dish was placed over the circular hole in the black piece of paper. A 4-hr starved female adult *B. tabaci* Q was released into the petri dish for 14 hr. Nineteen to 20 replicates per test solution were conducted. The number of honeydew excretions on the paper was counted under a digital microscope (VHK–1000, Keyence Corporation, Osaka, Japan). Additionally, 50–60 spots of

honeydew excreta per treatment were measured individually to determine their areas.

2-3-6 Honeydew excreta

As described in Subsubsection 2-3-1, grape tomato seedlings (cv. Yellow-pear) that retained only the second leaf were treated with solutions of acetylated glyceride (0.2% v/v) or water. Viruliferous *B. tabaci* B females aged 2–5 days after emergence were obtained using the methods described in Subsubsection 2-3-3. A female was separately released for 7 days in the individual glass test tubes. The small piece of water-sensitive paper was placed directly underneath the second-true-leaf to monitor the honeydew excreted by the female. The paper was frequently rotated to present an unused area to avoid the overlap of honeydew excreta. The paper was also replaced with a new one every 6, 8 and 12 hr for 7 DAR depending on the frequency of honeydew excreta. Eleven to 12 test tubes per treatment were used with three replicates. The number of honeydew excretions on the paper was counted by using a digital microscope (KH–3000VD, Hirox Company Ltd.). In addition, all of the spots of honeydew excreta on the paper were measured to determine their areas at 1 and 2 DAR, whereas 100–120 excreta spots were individually analyzed from 3 to 7 DAR. To determine the estimated cumulative honeydew excreta per adult, the values were computed as the total number of honeydew excreta multiplied by the mean area of honeydew excreta per day.

2-4 Control of TYLCV transmission

The suppressive activity of a single foliar treatment with acetylated glyceride and mixed foliar treatments with conventional chemical insecticides was evaluated to prevent the spread of TYLCV infection via adult *B. tabaci* in a chamber under greenhouse conditions.

2-4-1 Small chamber test in a glass greenhouse

Grape tomato seedlings (cv. Yellow-pear) were reared until the second-true-leaf stage in plastic pots in the whitefly-free chamber. A series of tests was conducted in winter (from December to February) from 2010 to 2012. After treatment with acetylated glyceride solution or water, 16 pots (6.5 cm in diameter, 7.5 cm in height per pot) for each treatment were evenly arranged (four pots per row in a square) in a small chamber (69 cm in length, 44 cm in width, and 82 cm in height) (Fig. 6-1) in a glass greenhouse. The chamber frame consisted of aluminum, and all sides, including the top, were covered with fine woven polyethylene mesh for ventilation. Based on previous studies of the insecticide sensitivity on *B. tabaci* Q, four commercial insecticide products used widely to control *B. tabaci* Q were selected as follows: nitenpyram (100 mg L^{-1}), pyridaben (200 mg L^{-1}), milbemycin (10 mg L^{-1}), and a mixture of fenpyroximate (40 mg L^{-1}) and buprofezin (200 mg L^{-1}) (fenpyroximate–buprofezin). Four types of treatment were tested in individual chambers: single solutions of each of the four chemical pesticides, acetylated glyceride, mixed solutions of each of the four separate pesticides plus acetylated glyceride, and water as the non-treated control. Therefore, the total number of treatments was 10.

Viruliferous *B. tabaci* Q were obtained using the methods described in Subsubsection 2-3-3. Furthermore, small pieces of grape tomato leaves with several *B. tabaci* adults (mixed sex, aged 3–5 days after emergence) were placed upside down as an inoculation source on two stands, which were diagonally located in two corners of the chamber. The numbers of adults released in the chamber were 1.0 and 2.5 individuals per seedling on average in the two corners. The experiments comprised three replicates for both conditions. At 7 DAR, the seedlings were completely sprayed with a mixed solution of nitenpyram (mg L^{-1}), pyridaben (200 mg L^{-1}), and sodium oleate (2000 mg L^{-1}) to immediately kill the adults, eggs, and

nymphs. The frequency of TYLCV-infected seedlings was determined at approximately 4 weeks after inoculation in the whitefly-free greenhouse. The virus infection was detected in the seedlings by observing disease symptom and diagnostic assays as same methods described in Subsubsection 2-3-3.

Fig. 6-1 Small aluminum chamber (69 cm in length, 44 cm in width, and 82 cm in height) for TYLCV transmission tests. (A) Whole picture of chambers, (B) Arrangement of potted grape tomato seedlings and inoculation source of adult whiteflies.

2-4-2 Semi-practical tests in a plastic greenhouse

Four isolation chambers (each 180 cm in length, width, and height) that were individually covered with 0.4-mm mesh were arranged uniformly in a plastic greenhouse. Grape tomatoes (*Solanum lycopersicum*, Takii & Co., Ltd., cv. Chika) were reared in a 15-cm plastic pot until the fifth-true-leaf stages, and 16 potted plants (four pots in rows in a square) were uniformly arranged in each chamber. Four foliar treatments were applied, as follows: Two treatments (a single treatment of fenpyroximate-buprofezin and a mixed treatment of fenpyroximate–buprofezin and acetylated glyceride) were applied once at the beginning of the test. The other two treatments (acetylated glyceride and water) were applied three times at 7-day intervals. After each plant was treated with 62 mL of the test solution, the plants were allowed to dry for a few hours after the first spraying. The average number of viruliferous *B. tabaci* B adults released into the chamber was 2.0 individuals per plant. The tests comprised one replicate in two different periods: Exp. 1 (winter: from December 13, 2011, to February 8, 2012) and Exp. 2 (spring: from April 9 to May 8, 2012). At the end of each trial, the frequency of TYLCV-infected grape tomato plants was assessed by PCR at 57 days after the first treatment in Exp. 1 and at 29 days after the first treatment in Exp. 2.

2-5 Diagnostic assays

An adult whitefly or a piece of individually cut young grape tomato leaf was transferred to a 1.5 mL micro tube with 100 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The samples were thawed and homogenized manually using a sterilized needle for use as PCR templates. The KOD Kit (TOYOBO, Osaka, Japan) protocol and reagents were used to detect the virus. The PCR primers used for TYLCV-IL were 5′-CTCGAAGGTTCGCCGAAGG-3′ and 5′-TTGAAAAATTGG (G/A) CTCTCAA-3′,

which were designed by Onuki (2000), and those for TYLCV-MId were 5′-CAATTTATTTGGAAGCGCTTAGG-3′ and 5′-CTCGTAAGTTTCCTCAACGGACTG-3′. The reaction mixture had a total volume of 50 μL, comprising 25 μL of 2× PCR buffer, 10 μL of dNTPs (2 mM), 1.5 μL of each primer (10 μM), 10 μL of distilled water, 1 μL of PCR template, and 1 μL of KODFX, and the reaction was performed using a thermal cycler (MyCycler ™ thermal cycler, Bio-Rad Laboratories Inc., CA, USA). The PCR program on the thermal cycler used a temperature profile of 94°C for 3 min (denaturation), followed by 30 cycles at 98°C for 10 sec (amplification), 60°C for 30 sec (annealing), and 68°C for 90 sec (extension), with a final extension at 72°C for 7 min. The loading samples comprised 8 μL of PCR products and 2 μL of dye. For electrophoresis, 1% acrylamide gels were used to run TAE buffer (40 mM Tris-acetate, pH 8.3, 1 mM EDTA) at 100 V for 13 min, which were stained with ethidium bromide and visualized using a High-Performance Ultraviolet Transilluminator (Ultra-violet Products Ltd., CA, USA).

2-6 Data analysis

The percentage of settled adults out of the adults released between treatments at 7 DAR (Subsubsection 2-3-1), the percentages of individuals that acquired TYLCV on treated and non-treated grape tomato leaflets by sex for each day (Subsubsection 2-3-2), the frequency of TYLCV-infected grape tomato plants between treatments in the no-choice test (Subsubsection 2-3-3), and the number and area of honeydew excreta each day and the estimated cumulative honeydew excreta area between treatments at 7 DAR (Subsubsection 2-3-6) were compared using a *t*-test. One-way ANOVA was employed to assess the effect of treatment of acetylated glyceride and inert ingredients in the incidence of TYLCV-infected grape tomato seedlings (Subsubsection 2-3-4) and in the number and area of honeydew

excreta (Subsubsection 2-3-5). Differences among means were compared with Tukey's HSD-test at $P = 0.05$. The comparison between single solution of each of four chemical pesticides and mixed solution of each of four chemical pesticides plus acetylated glyceride, between single solution of acetylated glyceride and non-treated control were done by the chi-square test using a significantly level of $P = 0.05$ (Subsubsection 2-4-1). In addition, a chi-square test at $P = 0.05$ was used to compare the in the proportion of individuals started feeding 14 hr after release (Subsubsection 2-3-5) and to compare the incidence of infected and non-infected plants among treatments in semi-practical tests in a plastic greenhouse (Subsubsection 2-4-2).

3 Results

3-1 Mechanism of controlling TYLCV

3-1-1 Repellent effect in the no-choice test

As shown in Fig. 6-2, the number of individuals that settled on the underside of the grape tomato leaves treated with acetylated glyceride was always lower than that on non-treated leaves up to 7 DAR in males ($T = 3.63$; df = 98; $P < 0.001$), whereas differences between treatments were only detected up to 2 DAR in females (7DAR; $T = 1.15$; df = 100; $P = 0.1313$).

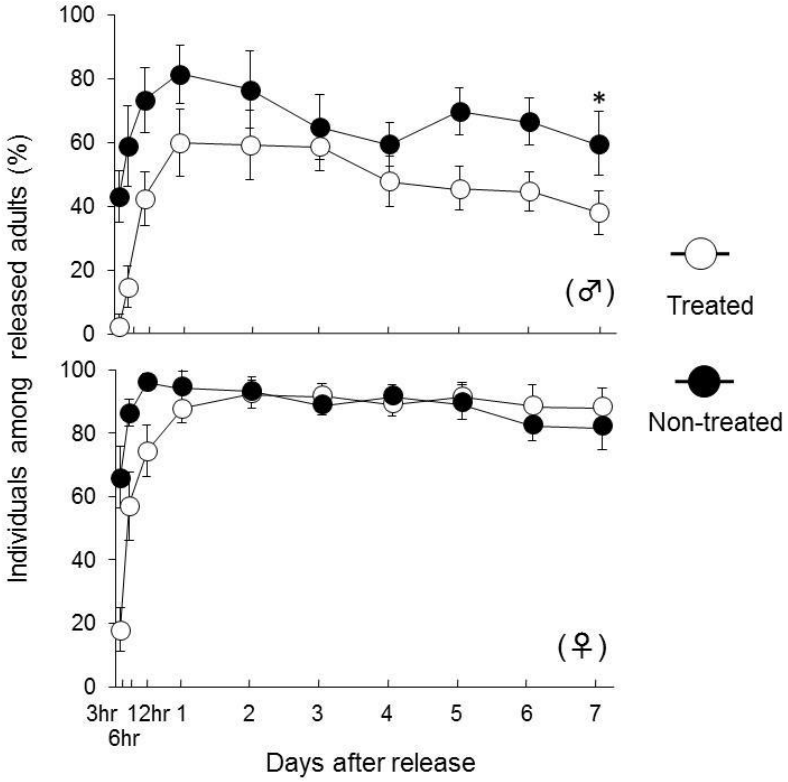

Fig. 6-2 Effect of treatment with acetylated glyceride on the numbers of settled *Bemisia tabaci* B adults of each sex on grape tomato seedlings in a no-choice test. Each value represents the mean ± standard error (S.E.) based on 10 to 12 test tubes per treatment with five replicates. The asterisk (*) indicates significant differences between treatments of each sex at 7 days after release (arcsine log transformation before analysis; $P < 0.05$; *t*-test).

3-1-2 TYLCV acquisition

At 6 hr and 12 hr after release, the numbers of individuals that acquired TYLCV on both treated and non-treated grape tomato leaflets comprised ≤40% of the total adults released in both sexes (Fig. 6-3). At 1 DAR, the proportions of viruliferous individuals on the treated leaflets were 44% for females and 21% for males, whereas those on the non-treated

control leaflets were 83% for females and 87% for males. There were significance between treatments (for females: $T = 9.89$; df = 4; $P = 0.002$; for males: $T = 6.36$; df = 4; $P < 0.001$). Subsequently, the number of viruliferous individuals in both sexes increased gradually on both types of leaflets at 2 DAR (for females: $T = 47.2$; df = 4; $P < 0.001$; for males: $T = 3.75$; df = 4; $P = 0.010$), and almost all of the adults had acquired the virus in all treatments at 3 DAR.

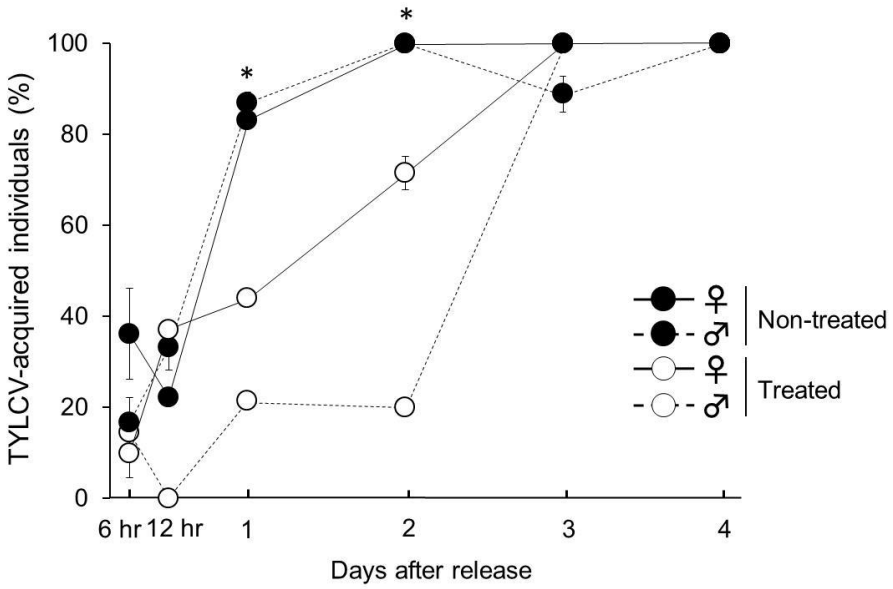

Fig. 6-3 Effect of treatment with acetylated glyceride on the percentage of TYLCV acquisition (mean ± S.E.) by *Bemisia tabaci* B adults on grape tomato seedlings in a no-choice test. Each value represents the result based on 10 to 16 adults per treatment with three replicates. The asterisk (*) indicates significant differences between treatments of each sex on the same day (arcsine transformation before analysis; $P < 0.05$; *t*-test).

3-1-3 TYLCV transmission

The proportions of TYLCV-infected grape tomato seedlings (number of seedlings infected/plants tested) after the treatments with acetylated glyceride and water (non-treated

control) were 65% and 94% for females and 33% and 44% for males, respectively. There was a significant difference between the treatments for females (T = 2.94; df = 4; P = 0.021) but not for males (Fig. 6-4) (T = 1.47; df = 4; P = 0.107).

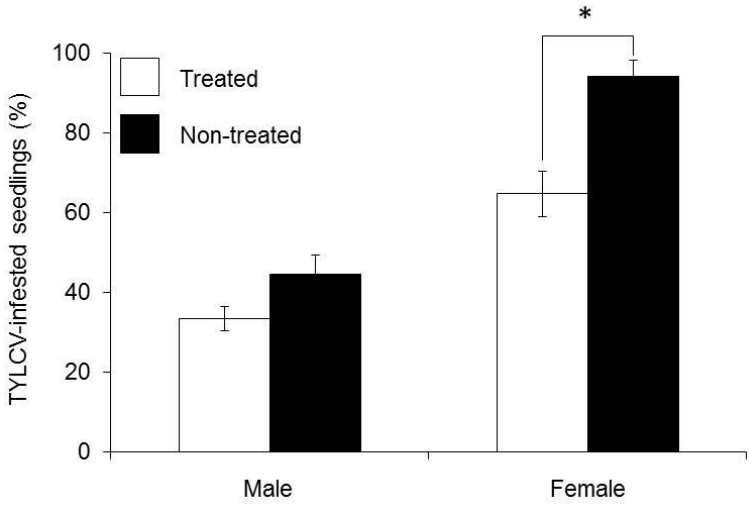

Fig. 6-4 Effect of treatment with acetylated glyceride on the percentage of TYLCV transmission (mean ± S.E.) by *Bemisia tabaci* B adults on grape tomato seedling in a no-choice test. Each value represents the result based on 16 to 18 test tubes per treatment with three replicates. The asterisk (*) indicates significant differences between treatments (arcsine log transformation before analysis; $P < 0.05$; *t*-test).

3-1-4 Inhibitory effect of the active ingredient against TYLCV transmission

Table 6-1 shows that the percentage of TYLCV-infected plants treated with acetylated glyceride (44.6%) was significantly lower than those treated with the inert ingredients (66.9%) or non-treated (74.6%) (F = 12.8; df = 2, 6; $P < 0.05$). The treatment of acetylated glyceride as the active ingredient suppressed TYLCV transmission. No significant

differences were observed between plants treated with the inert ingredients and the non-treated controls.

Table 6-1. Inhibitory effect of treatment with acetylated glyceride (0.2%, v/v) and inert ingredients (0.04%, v/v) against TYLCV transmitted by female *Bemisia tabaci* B to grape tomato seedlings.

Treatment	Percentage of infected plants
Acetylated glyceride-treated	44.6[a]
Inert ingredients-treated	66.9[b]
Non-treated	74.6[b]

Each value is the mean percentage of 13 to 16 test tubes per treatment with three replicates 28 days after releasing a viruliferous adult. Different letters indicate significant differences among treatments ($P < 0.05$; one-way ANOVA followed by Tukey's HSD-test).

3-1-5 Antifeeding activity

Most females of *B. tabaci* started feeding by 14 hr after release in the inert ingredients plus sucrose (75.0%) and sucrose (78.9%) treatments. In contrast, individuals started feeding accounted for 31.6% of the total number of individuals tested in the treatment of acetylated glyceride plus sucrose (Table 6-2; $\chi^2 = 11.2$; $P < 0.01$).

The number of honeydew excreted by females treated with acetylated glyceride plus sucrose was 72–76% lower than the number excreted by females treated with the inert ingredients plus sucrose and sucrose alone (Table 6-3; $F = 5.6$; df = 2, 55; $P < 0.05$). No difference in mean area of honeydew excreta was observed among treatments (Table 6-3; $F = 0.42$; df = 2, 165; $P > 0.05$).

Table 6-2. Antifeeding activity of the acetylated glyceride (0.2%, v/v) and inert ingredients (0.04%, v/v) treatments to start feeding of females *Bemisia tabaci* Q.

Treatment	Percentage of adult feeding
Acetylated glyceride plus sucrose	31.6 *
Inert ingredients plus sucrose	75.0
Sucrose	78.9

Each value indicates the mean of 19–20 replicates 14 hr after release of *B. tabaci*. The asterisk (*) indicate significant differences among treatments ($P < 0.01$, Chi-square test).

Table 6-3. Antifeeding activity of the acetylated glyceride (0.2%, v/v) and inert ingredients (0.04%, v/v) treatments against females *Bemisia tabaci* Q.

Treatment	Number of honeydew excreted by a female	Area of honeydew excretion (μm^2)
Acetylated glyceride plus sucrose	6.4 ± 3.0[a]	4736.8 ± 213.8[a]
Inert ingredients plus sucrose	23.1 ± 4.6[b]	4723.6 ± 130.0[a]
Sucrose	26.8 ± 5.7[b]	4542.7 ± 144.0[a]

Each value indicates mean ± standard error (S.E.) of 19–20 replicates per treatment for counting honeydew excreta, of 50–60 spots of honeydew excreta per treatment for measuring area 14 hr after release of *B. tabaci*. Different letters in the same column indicate significant differences among treatments ($P < 0.05$; one-way ANOVA followed by Tukey's HSD-test).

3-1-6 Honeydew excreta

The number of honeydew excretions deposited by females on the grape tomato leaves treated with acetylated glyceride was 37–96% lower than the number of excretions on the non-treated control on all days. There was a significant difference between treatments at all days ($T_{1\ DAR} = 8.07$; df = 66; $P < 0.001$; $T_{2\ DAR} = 3.66$; df = 65; $P < 0.001$; $T_{3\ DAR} = 1.76$; df =

65; $P < 0.001$; $T_{4\,DAR} = 1.24$; df = 64; $P = 0.006$; $T_{5\,DAR} = 2.83$; df = 64; $P < 0.001$; $T_{6\,DAR} = 1.86$; df = 64; $P < 0.001$; $T_{7\,DAR} = 1.12$; df = 64; $P = 0.034$). The mean area of honeydew excreta on the treated leaves was reduced significantly by 9%–38% compared with the non-treated control at 1, 2, and 4 DAR ($T_{1\,DAR} = 1.35$; df = 74; $P < 0.001$; $T_{2\,DAR} = 1.02$; df = 238; $P < 0.007$; $T_{4\,DAR} = 1.46$; df = 238; $P < 0.001$) but not on the other days (Fig. 6-5).

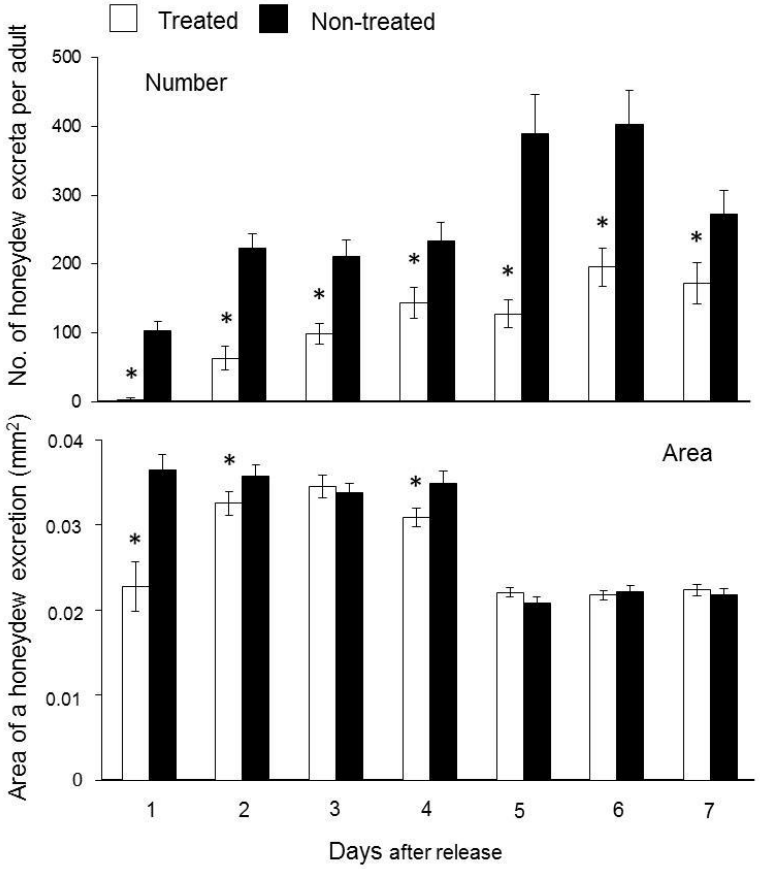

Fig. 6-5 Effect of treatment with acetylated glyceride on the numbers and areas of honeydew excreted by *Bemisia tabaci* B adults on grape tomato seedlings. Each value represents the mean ± S.E. based on 11 to 12 test tubes with three replicates. The asterisk (*) indicates a significant difference between treatments on each day ($P < 0.05$, *t*-test).

3-1-7 The estimated area of honeydew excreta

As shown in Fig. 6-6, the amount of cumulative honeydew excreta per adult was estimated to be reduced by 55% with the acetylated glyceride treatment compared to the control at 7 DAR (T = 6.56; df = 63; P < 0.001).

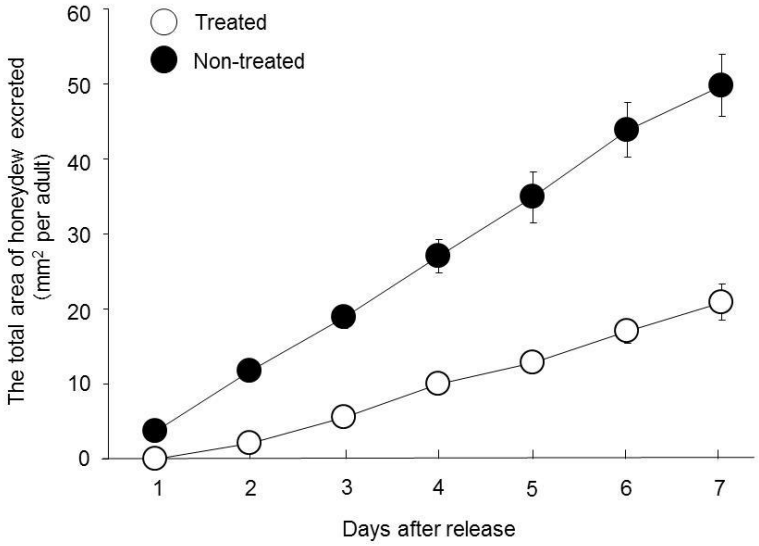

Fig. 6-6 Effect of acetylated glyceride application on the amount of cumulative honeydew excreted by *Bemisia tabaci* B adults on grape tomato seedlings. Each value represents the mean ± S.E. based on 11 to 12 test tubes with three replicates. The results differed significantly from those obtained with non-treated leaves at 7 days after release (P < 0.05, *t*-test).

3-2 Control of TYLCV transmission

3-2-1 Small chamber test in a glass greenhouse

After the release of one individual *B. tabaci* per plant, the TYLCV infection rates of grape tomato plants sprayed with acetylated glyceride and water treatments were 38% and 75%, respectively (Table 6-4; $\chi^2 = 11.3$; $P < 0.05$). Almost no TYLCV-infected grape tomato plants were observed after treatment with both the separate solutions and mixed solutions of nitenpyram and pyridaben plus acetylated glyceride. However, with 2.5 individuals per plant, the frequency of virus-infected plants after treatments with mixed solutions of nitenpyram (13%) or pyridaben (6%) plus acetylated glyceride were significantly lower than those with separate solutions of nitenpyram (44%) or pyridaben (31%), in which the level in the non-treated control was 56% (Nitenpyram; $\chi^2 = 11.7$; $P < 0.05$; Pyridaben: $\chi^2 = 8.8$; $P < 0.05$). The average reduction in the frequency of infection after treatment with a mixed solution of milbemycin solution plus acetylated glyceride was 45% (range, 27–63%) compared with the milbemycin single solution in both release conditions. Similarly, treatment using a mixed solution with fenpyroximate–buprofezin plus acetylated glyceride reduced the infection rate by 63%–81%, with an average of 72%, compared with a separate fenpyroximate–buprofezin single solution in both release conditions. Based on Colby's formula (Colby, 1967), adult release conditions that were detected a synergistic effect of a mixed solution of acetylated glyceride with milbemycin and with three other insecticides were 1.0 and 2.5 individuals per plant, respectively. In summary, the frequency of infected plants in treatment with a mixed solution of each chemical pesticide plus acetylated glyceride was reduced synergistically compared with each single solution.

Table 6-4. Effects of treatment with acetylated glyceride (0.2% v/v) on TYLCV transmission by Q of *Bemisia tabaci* adults on potted grape tomato seedlings in chambers of a glass greenhouse.

Treatment		TYLCV-infected plants			
		1.0 individual per seedling		2.5 individuals per seedling	
Nitenpyram		2 ± 3	ns	44 ± 4	*
Nitenpyram	plus Acetylated glyceride	4 ± 3		13 ± 4	
Pyridaben		2 ± 3	ns	31 ± 4	*
Pyridaben	plus Acetylated glyceride	0 ± 0		6 ± 4	
Milbemycin		75 ± 8	*	69 ± 8	*
Milbemycin	plus Acetylated glyceride	13 ± 4		42 ± 9	
Fenpyroximate–buprofezin mixture		65 ± 7	*	94 ± 8	*
Fenpyroximate–buprofezin mixture plus Acetylated glyceride		2 ± 3		13 ± 4	
Acetylated glyceride		38 ± 4	*	50 ± 4	ns
Non-treated control		75 ± 4		56 ± 11	

Mean percentage (± S.E.) of TYLCV-infected grape tomato plants. The asterisk (*) indicate significant differences between single solution of each chemical pesticide and mixed solution of each chemical pesticide plus acetylated glyceride, between single solution of acetylated glyceride and non-treated control ($P < 0.05$, Chi-square test). ns, not significant difference.

3-2-2 Semi-practical tests in a plastic greenhouse

Table 6-5 shows that the frequency of TYLCV-infected grape tomatoes in the treatment with acetylated glyceride (single solution) was 6% during both test seasons, whereas those with the non-treated control varied from 31% (winter) to 13% (spring). Thus, the treatment was highly effective in suppressing virus transmission. The average frequency of infected plants after treatment with the fenpyroximate–buprofezin plus acetylated glyceride mixed solution (3%) was better than that with the fenpyroximate–buprofezin single solution (10%). No significant differences, however, were observed between treatments in each season.

Table 6-5. Effects of treatment with acetylated glyceride (0.2%, v/v) on TYLCV transmission by *Bemisia tabaci* B adults on potted grape tomatoes in chambers in a plastic greenhouse.

Treatment	TYLCV-infected plants					
	Exp. 1			Exp. 2		
Fenpyroximate–buprofezin mixture	13%	(2/16)	[a]	6%	(1/16)	[a]
Fenpyroximate–buprofezin mixture plus Acetylated glyceride	6%	(1/16)	[a]	0%	(0/16)	[a]
Acetylated glyceride	6%	(1/16)	[a]	6%	(1/16)	[a]
Non-treated control	31%	(5/16)	[a]	13%	(2/16)	[a]

Percentage of TYLCV-infected grape tomato plants. The number of infected plants per total plants tested is given in parenthesis. Tests were conducted in the winter season (Exp. 1: from December 10, 2011, to February 10, 2012) and spring season (Exp. 2: from April 10 to June 10, 2012). No significant differences were detected by Chi-square test in each column.

4 Discussion

The aim of this study was to evaluate the suppressive effect of a foliar treatment with acetylated glyceride on TYLCV acquisition and transmission by *B. tabaci* adults on host plants and to elucidate the mechanism of suppression.

In the test tube test, the repellent effect of the acetylated glyceride treatment on both *B. tabaci* sexes lasted for approximately 2 days in the no-choice test (Fig. 6-2), whereas the effect on females lasted for at least 7 days in the choice test (acetylated glyceride solution was sprayed onto either the first or the second leaf of a grape tomato seedling) in previous study (Fig. 2-4 of Chapter 2). This large difference between the tests was presumably caused by the presence or absence of a refuge area for settling after being repelled. In a similar study, 7-epizingiberene purified from *Solanum habrochaites* exhibited a strong repellent effect on *B. tabaci* adults in a choice test, whereas all of the individuals were observed to settle on a host plant within 2 hr after release in a no-choice test, even though the substance had adulticidal activity (Bleeker et al., 2011). The mature glandular trichome exudates of tomato plants are crucial mechanisms for resisting adult whitefly settlement via repulsion and entrapment (Muigai et al., 2002). The large difference in the repellent effect between sexes was observed primarily because most dead males were observed to be trapped by a tiny hooked trichome on the tomato seedlings, whereas no females were dead at 7 DAR; this result might have occurred because the body size of males is generally smaller than that of females.

The minimum feeding period required to acquire TYLCV successfully from infected grape tomato plants has been reported as 0–15 min (range: 0–3 hr) immediately after the release of *B. tabaci* adults (Caciagli et al., 1995; Cohen and Nitzany, 1966; Kitamura et al., 2009; Mehta et al., 1994). The number of *B. tabaci* adults that acquired TYLCV gradually

increased within 12–24 hr after release before reaching a maximum value (Caciagli et al., 1995; Cohen and Nitzany, 1966; Kitamura et al., 2009; Mehta et al., 1994). Based on the characteristics of the virus–vector relationship, it is considered that the almost 2-day delay in the TYLCV acquisition with the acetylated glyceride treatment in the no-choice test was caused by the 2-day delay in adult settlement before feeding on the plant due to the repellent effect (Fig. 6-3).

The minimum feeding period required to successfully transmit TYLCV to tomato plants for adult *B. tabaci* is 0–15 min (range: 0–30 min) (Caciagli et al., 1995; Cohen and Nitzany, 1966; Mehta et al., 1994). In a study using an electronic feeding monitoring system, TYLCV transmission was positively correlated with virus titer of saliva and frequency of phloem sap ingestion (Jiang et al., 2000, 1999). However, the TYLCV infection was significantly lower in the acetylated glyceride treatment than in the non-treated control for females (Fig. 6-4). Acetylated glyceride as active ingredient was demonstrated to prevent TYLCV transmission (Table 6-1). A treatment with olive oil, which also has a repellent effect on *B. tabaci* adults, was reported to suppress the TYLCV infection simply by reducing the number of settled adults (Schuster et al., 2009). Mineral oil is also effective in reducing the spread of non-persistent viruses that are transmitted by aphids (Asjes, 1991; Bradley et al., 1962; Loebenstein et al., 1964; Martin et al., 2004; Wang and Pirone, 1996). Based on the high reduction of honeydew excreta in the acetylated glyceride treatment, a major mechanism for suppression might be interference with adult feeding behavior (Tables 6-2, 6-3, Figs. 6-5 and 6-6).

Concerning the suppression of the TYLCV infection, acetylated glyceride was effective as a single foliar treatment but also in the mixed foliar treatments with each of four chemical insecticides in no-choice tests for 7 days in small chamber test (Table 6-4) and semi-practical

test (Table 6-5). These results may suggest additional options for growers to facilitate the flexible application of treatments with acetylated glyceride depending on the adult densities and specific cultivation contexts (*e.g.*, harvesting periods). Further experiments are required to elucidate the synergistic effect between each chemical insecticide and acetylated glyceride.

In the present study, it is demonstrated that a single foliar treatment with acetylated glyceride had a moderate suppressive effect on TYLCV infection, regardless of its lack of an adulticidal effect on *B. tabaci* adults. Acetylated glyceride apparently caused two types of feeding interference on adult whiteflies in a no-choice condition. The first function was the delayed initiation of plant sap ingestion caused by the repellent effect in the first 2 days. The second function was constant interference with adult feeding behavior, which resulted in a major reduction in honeydew excreta during the last 5 days of 7-day observation periods. The cause for the discrepancy in the difference between honeydew number and the honeydew excretion area on 5–7 DAR, however, requires further investigation. It is speculated that these functions allowed the acetylated glyceride treatment to reduce the injection of virus particles into phloem tissues by adults, thereby suppressing TYLCV transmission.

Chapter 7

General Conclusion

1 Introduction

Traditional crop protection has long focused on utilizing the best products, as many chemical pesticides were developed in the mid-20th century. Growers faced an increasing number of problems in complex environments, where single-product solutions were no longer sufficient to address pests, diseases, and weeds. In particular, broad-spectrum insecticides, which kill both target pests and beneficial organisms, were used until they induced a resurgence of pests and increased insecticide resistance became a major issue. It was finally determined that the best yields were obtained using an integrated approach, including a rational combination of biologically selective chemicals and other control methods to suppress pest populations to levels below acceptable economic target crop losses. Under these circumstances, a wide range of non-mutually contradicting choices were needed by growers depending on various crop protection situations for many different crops.

Tomato is regarded as the second most important vegetable crop next to potato. The deepest impact a tomato grower faces is tomato yellow leaf curl virus (TYLCV) transmission between crops. A new type of adult repellent insecticide with practical effects on tomato/grape tomato production was produced in this study to solve this problem practically. The biological properties of the novel repellent acetylated glyceride were evaluated on Q of *Bemisia tabaci* adult, whose chemical-resistant populations are found worldwide. In addition, well-balanced treatment conditions to control *B. tabaci* and to limit both phytotoxicity to target crops and the impact on beneficial organisms were determined.

2 The significance of the study

Five important results were obtained in this study. First, almost the same number of adults landed on treated and non-treated grape tomato leaves during the choice test. Thereafter, repellent behavior was observed within 5 sec of an adult landing on a treated plant surface, and no insecticidal activity occurred when adults settled on non-treated leaves. Acetylated glyceride treatment potentially acted as a contact repellent against adult whiteflies, although several terpenoids are effective in tomato accessions, and ginger oil has a putative repellent effect on *B. tabaci* adults in the vapor phase.

Second, almost no courtship pair formation was observed on treated host plant leaves even as the residual repellency of the treatment decreased over time. In general, immediately after males landed on host plant leaves, they initiated a search for a mating partner among the sexually mature females in the vicinity. However, males on treated leaves did not exhibit searching behavior, leading to a significant reduction in the number of female offspring due to arrhenotokous parthenogenesis in virgin *B. tabaci* females. This is the first study to investigate measures to control the formation of courting pairs in adult *B. tabaci*, although commercial products that disrupt mating after the release of a sex pheromone as a cue to find a mating partner over wide areas are available.

Third, the relationship between acoustic signals, courtship pair formation and its disruption was elucidated. The acoustic signals produced by *B. tabaci* B males induce mating communication between adults. During courtship, the acoustic signals generated by both sexes were exchanged rhythmically on non-treated leaves, whereas the signals on treated leaves were irregular.

Fourth, single foliar acetylated glyceride treatment had a moderate suppressive effect on infection by TYLCV, even after most adults settled and survived on treated leaves due to

the decrease in residual repellency. Olive oil treatment, which also has repellent activity toward *B. tabaci* adults, has been reported to suppress TYLCV infection by reducing the number of settled adults. Mineral oil is also effective for reducing the spread of non-persistent viruses transmitted by aphids, as virus retention time on the aphid stylet is lower in treated than in non-treated aphids. In this study, acetylated glyceride firstly indicated to have a suppressive effect on TYLCV transmission, as a reduced number of virus particles were injected based on results of the reduction of honeydew excreta by antifeedant activity.

Finally, the recommended acetylated glyceride treatment conditions were optimized for practical use. The best control effect against adult whiteflies was attained with three foliar treatments (0.2%, v/v) at 7-day intervals; this was not toxic to grape tomato plants above a commercially acceptable level. Acetylated glyceride had no effect on four natural enemy species tested. Plant extracts and vegetable oils have been tested as repellents, antifeedants, and toxicants to control *B. tabaci* and *T. vaporariorum*. However, commercially applicable substances are limited due to poor performance against whiteflies and severe toxicity to crops. Acetylated glyceride is officially approved a novel repellent pesticide (Registration No. 23731) on November 11th, 2015 in Japan.

3 Application of acetylated glyceride in IPM during tomato cultivation

In tomato/grape tomato cultivation in the greenhouse, hormones for pollination are dispersed by spraying, to compensate for the lack of wind dispersal. This is a time-consuming process but it must be completed during the short time interval between blooming and bud drop. The use of pollinating insects (bumble bee) is less labor-intensive than pollination spraying and results in larger and more numerous fruits. Given these considerations, in the

establishment of an IPM program the side effects on pollinators must be taken into account. In addition, the pre-harvest interval (PHI) is an important factor in pesticide selection since growers generally harvest tomato fruits every 1–2 days for commercial sale.

Viruliferous *B. tabaci* frequently invades tomato greenhouses when the outside air temperature is relatively high, which coincides with the tomato seedlings rearing period of forced cultivation (September–October) (Honda, 2006). At that time, the tomato seeding is small and easily infected by TYLCV. Infection of the tomato seedling by this virus prevents fruit development and therefore causes serious economic damage for growers.

As described in the previous chapter, the integrated use of several control measures, such as pre-planting (e.g., pricking-in hole treatment) and post-planting (e.g., plant foot treatment) chemical pesticide treatments and the introduction of natural enemies (Gabarra et al., 2006), yellow sticky traps (Gerling and Horowitz, 1984; Yaobin et al., 2012), insect-proof screens (Berlinger et al., 2002), and UV-controlling films (Antignus et al., 1996, 1998), is effective in pest management. Foliar spraying of adulticidal insecticides suppresses TYLCV infection and offers a further approach. In the selection of an insecticide, there are three important points that must be considered: 1. minimal impact on beneficial organisms, 2. short PHI, and 3. adulticidal activity against insecticide-resistant populations of Q biotype *B. tabaci*. Insecticides such as pyridaben and nitenpyram meet these three conditions. Therefore, both acetylated glyceride treatment alone and in combination with an adulticidal insecticide depending on the whitefly invasion pressure are expected to be effective methods to control TYLCV infection during the rearing period. Further study is needed to propose other systematic methods in combination with acetylated glyceride.

During pollinator use in forced tomato cultivation, greenhouse invasion pressure by whiteflies is low due to the low outside air temperature. However, this means that the

whitefly population has increased by the time it invades the greenhouse. To control this population increase, the minimum requirements of insecticides (e.g., milbemycin, a mixture of fenpyroximate and buprofezin) used in this period need to meet the first two of the above-described requirements (minimal impact on beneficial organisms and short PHI). Understandably, growers could select the way of acetylated glyceride use (alone or combination with conventional insecticides) depending on the cultivated situation. But, during this period, main use of acetylated glyceride should consist of a single continuous foliar application to prevent further TYLCV infection and suppress the whitefly population by repellency and courtship disruption. The introduction during this period of natural enemies simultaneously with single foliar acetylated glyceride applications can achieve long-lasted whitefly control in the greenhouse.

In this study, TYLCV was chosen as a representative plant disease virus transmitted by *B. tabaci*. However, acetylated glyceride is also expected to be effective against other viruses, such as cucurbit chlorotic yellow virus (CCYV), which causes severe disease and economic loss in cucurbit cultivation. This virus was first reported in the Kumamoto prefecture of Japan in 2004.

IPM has been used in many countries for several decades. To add a control measure to an already implemented IPM program for crops requires that the benefits of its application, the methods, and the timing of the control measure are well-defined to allow its appropriate use together with established technologies. Therefore, the seasonal prevalence in pest emergence, widespread on-the-spot use of the pest management technique, and the crop system must all be considered.

SUMMARY

Tomato yellow leaf curl virus (TYLCV) transmitted by only sweet potato whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae), is of particular concern, as it is the main tomato production limiting factor worldwide. Commercially applicable insecticide in tomato production are limited in number because of three main factors as follow: 1. Appearance of insecticide-resistant population, Q of *B. tabaci*. 2. Side effect of insecticides against pollinating insect. 3. Insufficient number of insecticides with short Pre-Harvest Interval (PHI) due to frequency harvest during long cultivated periods. Under these circumference, natural products, including plant-derived oils and seed extracts, are becoming more important as an alternative pest control strategy intended to be compatible with Integrated Pest Management (IPM). However, these alternative control measures to control pests are limited in number because of poor efficacy againt target pests, problematic incidents of phytotoxicity on crop, and harmful effects on beneficial organisms.

With the aim of identifying safe control agents for whiteflies repellent, I screened foods and food additives, then observed that acetylated glyceride (acetic and fatty acid esters of glycerol) showed strong repellent effect against adult whitefly. The acetylated glyceride has been used as food additives (a plasticizer for chewing gum) worldwide. This study evaluated the effect of the foliar treatment of formulated acetylated glyceride (80% Emulsifiable Concentrate) to elucidate the basic activity and the best treatment conditions against sweet potato whitefly under laboratory and practical conditions.

In a choice test between treated and non-treated grape tomato leaves, it was observed that almost the same number of *B. tabaci* B adults landed on the leaves, but the retention rates of adults settling under the leaves were 27% and 95%, respectively. Repellent behavior was

induced within 5 sec of the adult's landing on the plant surface. The repellent led to a large reduction in the number of eggs laid on grape tomato leaves as compared to non-treated leaves. On the other hand, no courting pair formation of *B. tabaci* B was observed on the treated grape tomato leaves, when the percentage of courting pair on non-treated leaves were 12.9%. In addition, the treatment of inert ingredients has no repellent effect and courting disruption effect against *B. tabaci* B. No significant difference of repellent and courting disruption was observed B, Q of *B. tabaci*, and greenhouse whitefly, *Trialeurodes vaporariorum*.

Only early instar nymphs decreased significantly by approximately 51% of those in the non-treated control when acetylated glyceride was applied directly to eggs, early instar nymphs, late instar nymphs, and adults of *B. tabaci*, respectively. No insecticidal activity was observed after the contact treatment of first instar nymphs. Therefore, acetylated glyceride seemed to act as a spiracle-blocking substance in early instar nymphs, because its active ingredient is classified with a fatty acid ester of glycerol, one of which is commercially used in spiracle-blocking insecticides.

The effect of acetylated glyceride treatment on acoustic-based communication was evaluated, as this type of communication is known to play a pivotal role in the search for mating partners on host-plant leaves in adult whiteflies. Adults of *B. tabaci* B were able to land on grape tomato leaves a few days after treatment, when the residual repellency had dissipated. Almost no male courtship behavior in terms of searching for sexually mature females was observed throughout the observation period (60 min) under conditions in which adults of both sexes remained on a treated leaf or those in which an adult of either sex remained on a non-treated leaf. By contrast, these behaviors accounted for 26.5% of the observation time under conditions in which both sexes remained on a non-treated leaf. In cross-tests performed for 4 days using one non-mated female and two non-mated males of *B.*

tabaci Q, the sex ratio (male/female) of the newly emerged adults on treated leaves (2.4) was 63.6% lower than that of the non-treated controls (0.9). During courtship, acoustic signals produced by both sexes were exchanged rhythmically on non-treated leaves, whereas the signals on treated leaves were irregular. The frequency of the vibratory sound produced by males was 66–81% lower on the treated leaves than on the non-treated leaves. A large reduction in the acoustic signals produced by males on treated leaves caused a decrease in courting pair formation, leading to a significant reduction in the number of female progeny due to arrhenotokous parthenogenesis in virgin *B. tabaci* females.

Under tomato and eggplant greenhouse conditions, the numbers of settled adults, courting pairs, and nymphs on host plant leaves were significantly reduced by acetylated glyceride treatment with increasing dose rate and treatment times. The highest control was attained with foliar treatment (0.2%, v/v) at 7-day intervals with three times. Phytotoxicity to grape tomato plants was below the commercially acceptable level. The acetylated glyceride was harmless against four species of natural enemies examined.

The effect of the foliar treatment of acetylated glyceride on TYLCV acquisition and its transmission was evaluated. In a no-choice test, fewer adult females settled on grape tomato leaves treated with acetylated glyceride than non-treated leaves up to 2 day after release (DAR) during 7-day observation periods, reducing the frequency of individuals that acquired TYLCV only for 2 DAR. Notably, in the transmission test, the treatment significantly reduced the incidence of TYLCV infection in plants during the 7-day observation. At the time, the amount of cumulative honeydew excreta per adult was reduced by 55% in the treatment compared to the control at 7 DAR. Similarly, the number of honeydew excreted by females fed acetylated glyceride plus sucrose through parafilm was 72 and 76% lower than the number excreted by females fed the inert ingredients plus sucrose and sucrose alone,

respectively. In the chamber tests, the frequency of TYLCV-infected plants was decreased significantly by mixed treatments with acetylated glyceride and each of four pesticides (nitenpyram, pyridaben, milbemycin, and a fenpyroximate–buprofezin mixture) compared to treatment with each pesticide alone. The main functional mechanism for suppressed TYLCV transmission by acetylated glyceride might be the delayed settlement of *B. tabaci* on the host plant caused by repellency in the first 2 days, and interference with the feeding behavior of *B. tabaci* during the last 5 days of 7-day observation periods.

Acetylated glyceride is officially approved a novel repellent pesticide (Registration No. 23731) on November 11th, 2015 in Japan; its registration contents are extremely growers-consumer friendly good agricultural practice (GAP) compared with general chemical insecticide as follows: “Target crops: tomato/grape tomato at 0.2% (v/v); PHI: Not specified; A total number of application times per crop cultivation: Not specified”. Acetylated glyceride certainly regulates adult whitefly behavior and will help growers by providing a flexible control measure against not *B. tabaci* itself but virus transmission in practical.

SUMMARY in Japanese

トマト黄化葉巻病は，世界中で発生報告があり，トマト黄化葉巻ウイルス（TYLCV）の感染より引き起こされるトマト栽培での最重要病害である．TYLCV はタバココナジラミを唯一のベクターとして半永続的に媒介され，移動性の高い本種成虫の発生抑制が，最も有効な TYLCV 感染抑制方法となっている．その中で，化学殺虫剤は，防除効果が高くかつ経済的に優れているため，基幹防除を担っていたが，以下の要因により，現場で使用可能な種類が限定されている．1. 薬剤抵抗性個体群（Q）の出現，2. トマト栽培期間中に使用必須である訪花昆虫への化学農薬の悪

影響, 3. 長期栽培でかつ頻繁な収穫を行う栽培体系に不適合な農薬使用条件. このような状況下, 近年，総合的病害虫管理（IPM）での害虫管理の必要性が高まり，それに適合した農薬開発が求められている．これまで植物油，植物抽出物等の天然物のコナジラミ類に対する成虫忌避効果，殺卵，殺幼虫効果などが報告されているが，薬効不足，薬害発生や有用昆虫類への影響等から，実際，現場で使用可能な性能を具備するものは限定される．

タバココナジラミ成虫防除を目的として各種植物油や食品添加物の処理効果を調べた結果，アセチル化グリセリド（グリセリン酢酸脂肪酸エステル）に強い忌避効果が認められた. 本物質は，世界中でチューイングガムやケーキミックス等で長年利用されている安全な食品添加物であり，アセチル化グリセリド 80%乳剤として研究を行った．アセチル化グリセリド乳剤は，成虫忌避効果，配偶行動阻害効果，TYLCV 感染抑制効果の 3 種類の作用があり，製品中の助剤にはこれらの効果は確認されなかった．本剤は，タバココナジラミ（B と Q），オンシツコナジラミに対して種間差は認められなかった.

タバココナジラミ各発育ステージ（卵，若齢幼虫，老齢幼虫，成虫）への本剤の影響を評価した. 若齢幼虫に直接撒布した場合にのみ約 51%の殺虫効果が生じたが, 接触殺虫効果はなかった. それ以外の発育ステージでは有意な影響は認められなかった．アセチル化グリセリドは，気門封鎖剤として一般的に市販されているグリセリン脂肪酸エステルに分類されることから，弱い気門封鎖効果を有していると考えられた.

成虫忌避効果の行動観察では，キュウリ処理葉（0.2%, v/v）と無処理葉（水）の選択試験では，ほぼ同数の成虫が着地したが，処理葉と無処理葉での最終的な葉裏定着数は，それぞれ飛来個体数の 27%と 95%であった．処理葉上での行動観察では，成虫が口吻にて植物体表面を接触した直後に忌避行動を示した．本剤は，コナジラミ成虫に本来の宿主植物を非宿主植物として誤認識させることにより，忌避効果を発揮していると考えられた．この忌避行動により，処理葉での産卵数は無処理葉の 9%に抑制された.

タバココナジラミ成虫の一連の交尾行動は，宿主植物体上で雄が発する基質振動波に対し，雌

が振動波で応答することで，雄の探索行動が開始されることが知られている．本剤の配偶行動阻害効果は，処理葉を数日間静置して忌避効果の残効を低下させた葉面上で観察された．音響解析では，処理葉上に雌雄 1 対が定着した状態では，雄成虫が発する基質振動波の大幅な減少が確認され，それに伴い雌成虫の応答信号もほとんど検出されなかった．一方，無処理葉上では雄成虫の頻繁な基質振動波と雌成虫の応答が規則的に行なわれていた．行動観察においても，処理葉では雄成虫の雌探索行動は全く認められなかったが，無処理葉では観察時間（60 分間）の 26.5%で探索行動が観察された．未交尾雌を用いた交尾試験では，処理葉上での次世代雌成虫割合は，無処理葉の 63.4%に減少し配偶行動を抑制した．

ビニールハウスでのトマトとナスの圃場試験において，アセチル化グリセリド乳剤（0.1%，0.125%， 0.2%，v/v）を寄主植物に茎葉散布すると，葉裏上の成虫数，交尾ペア形成数，ならびに散布後の産生幼虫数は有意に減少した．防除効果は散布濃度や散布回数に依存して効果が高くなった．最大の防除効果は，0.2%水溶液の 7 日間隔 3 回散布で得られた．ミニトマトに対する薬害は軽微で，実用上問題となる薬害は認められなかった．IOBC に準拠した試験方法において，天敵 4 種に対する悪影響は認められなかった．

TYLCV 感染抑制効果を，処理ミニトマトを全面に配置した隔離網室内で評価した．株当たり 1 頭の保毒成虫を放飼した場合，アセチル化グリセリド処理株の感染株率は，無処理の約半分に抑制された．本剤と慣行防除化学農薬 4 種類（ニテンピラム水溶剤，ピリダベンフロアブル，ミルベメクチン乳剤，フェンピロキシメート＋ブプロフェジンフロアブル）との混用では，株当たり 2.5 頭の条件でも，各化学農薬単用と比較して有意に高い感染抑制効果があり，コナジラミ発生密度に応じて散布方法を選択できることを明らかとした．

小型ガラス容器内に処理ミニトマト幼苗のみを配置し, 株当たり 1 頭の保毒成虫を 7 日間放飼した試験条件にて，TYLCV 感染抑制作用機構の解明を行った．雌成虫では，放飼 2 日以内で処理検体に全個体が定着し，試験期間 7 日後でも死亡個体は観察されなかった．しかし，その際の TYLCV 感染抑制は無処理の約半数に抑制されており，甘露排泄量も約半数に抑制されていた．

また，本種成虫にパラフィルムを通して，スクロース水溶液（単用区）またはスクロースとアセチル化グリセリドの混合水溶液（混用区）を吸汁させると，混合区の甘露排泄量は単用区の 76%に減少した．吸汁性害虫であるコナジラミは師部を吸汁し，余分な物質を甘露として排泄することが知られている．TYLCV は師部局在性であり，コナジラミ成虫の師部吸汁時の際に行われる唾液吐出とともに植物体に感染することが知られている．以上のことから，本剤の感染抑制効果のメカニズムは，散布初期（散布 2 日間）では，成虫忌避効果により植物体への定着を遅延させ，散布後期（散布 3 日後から 7 日後）では，宿主に定着したコナジラミ成虫の篩部の摂食吸汁活動を抑制することで，複合的に TYLCV 感染抑制効果が生じたと考えられた．

アセチル化グリセリド乳剤は 2015 年 11 月 11 日に日本で初めてコナジラミ類成虫忌避剤として農薬登録認可（登録番号 23731）された．農薬登録要件としては，対象作物はトマト・ミニトマト，散布濃度は 0.2%，本物質の薬効薬害や毒性面等の安全性が認められ，「薬剤散布直後から収穫可能であり，散布回数の制限がない」という農家に使用しやすい登録内容となった．本剤はコナジラミ類成虫の行動制御作用を有する TYLCV 感染抑制可能な新規忌避剤になると考えられ，現場のトマト栽培状況やウイルス感染圧に応じて使用方法が選択可能な IPM 適合農薬になると考えられる．

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