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Development of Ethyl Formate Disinfestation Treatment Methods for the Prevention of the Introduction and Establishment of Exotic Insect Pests in Greenhouse Cultivation

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Abstract: Globalization has increased international trade and caused an annual increase in the number of non-native insect pest species found in quarantine sites in Korea. Since over 80% of Korean farms use greenhouses with internal conditions conducive to growing crops and hospitable to exotic pests, efficient pest control is crucial. This study evaluated the efficacy of ethyl formate (EF) against three major exotic insect pests (*Aphis gossypii*, *Frankliniella occidentalis*, and *Tetranychus urticae*) and beneficial organisms (earthworms, honeybees, and silkworms) via EF fumigation experiments at two concentrations. The lethal concentration–time $(LCt)_{99}$ values of *A. gossypii*, *F. occidentalis*, and *T. urticae* were 8.96, 14.00, and 19.07 g h/m^3, respectively. Four hours of fumigation of EF at 15 g/m^3 left no residue on the crops or soil after a maximum of 3 h. The beneficial organisms exhibited higher tolerance levels than *A. gossypii* with regard to LCt_{99} value, but the higher EF treatment dosage needed to control *F. occidentalis* and *T. urticae* could be highly lethal to honeybees. The lower EF dose (4 g/m^3) effectively controlled *A. gossypii*, but 15 g/m^3 was needed for *F. occidentalis* and *T. urticae*. Phytotoxicity varied in severity with EF concentration. These insights can help in developing a refined disinfestation strategy for greenhouses.

Keywords: pest management; *Aphis gossypii*; *Frankliniella occidentalis*; *Tetranychus urticae*; greenhouse fumigation; soil residue; beneficial organisms

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1. Introduction

Increases in international agricultural commodity trade have resulted in an increased risk of exotic insect pest invasion and establishment in several countries, including the United States, China, and South Korea [1–4]. With the introduction of non-native insect species during international and/or interstate trades, fumigation has been employed for a long time to completely eradicate insect pests in every country [5–9]. It is also used to control insect pests and nematodes in stored grains and soil by injecting gaseous insecticides into a tightly sealed space [5–7]. Methyl bromide (MB) fumigation has been prohibited due to ozone layer depletion and worker safety issues and has led to ongoing research into the use and effectiveness of phosphine (PH_3) and ethyl formate (EF) as MB alternatives to suppress insect pest populations during trade [9,10]. Although PH_3 resistance due to a dihydrolipoamide dehydrogenase mutation in insect pests has been reported [11], resistance to EF has never been reported in insect pests. Regulated EF disinfestation treatment standards have been established and are widely used on various commodities,

including many different types of fruits, vegetables, nursery plants, nonfood commodities, and greenhouse applications [12–17].

South Korea has 378,000 ha dedicated to domestic fruit and vegetable cultivation, with about 80% of farms utilizing greenhouses [18]. Pest management is more complicated in the temperature and humidity conditions of greenhouses than it is in general cultivation methods; thus, specific disinfestation treatment methods are required [17,19]. The current plant protection law calls for the incineration and disposal of crops when quarantine insect pests are detected, which causes significant economic losses for farmers. Since the invasion and establishment of exotic insect pests can damage many greenhouse crops, a disinfestation treatment method is needed to prevent the establishment of quarantine insect pests in South Korea. Furthermore, traditional insecticides are used to control insect pests in greenhouses, but pesticide use is limited to a pesticide residue of less than 0.01 mg/kg for various crops according to the positive list system and to reduce insecticide resistance [20]. Thus, an effective alternative to pesticide use is also needed.

The cotton aphid (*Aphis gossypii* Glover), a widespread pest found in a variety of cut flowers, fruits, and vegetables, feeds on crops from 88 plant families and is a major destructive pest in the U.S., causing significant crop damage and quality reduction through its piercing–sucking mouthpart and virus transmission [19,21,22]. It is particularly problematic in orchard crops and greenhouses, damaging crops like cucumbers, peppers, and tomatoes [23]. However, its strong resistance to common insecticides like dimethoate and cypermethrin poses a challenge in controlling it [24]. And the Western flower thrips (*Frankliniella occidentalis* Pergande), first identified in Korea in 1993, infests various vegetable crops, having a high reproduction rate and spreading capability [25–27]. It lays eggs in plant tissues, with its larval and adult stages inhabiting flowers, yet current insecticides are not fully effective against it due to its developed resistance [28]. The species has also developed insecticide resistance to neonicotinoid-containing insecticides. Failures in *F. occidentalis* control have been reported all over the world, including China, Egypt, and Korea [29–31]. Two-spotted spider mites (*Tetranychus urticae* Koch) are considered a serious, major pest in a variety of agricultural crops and ornamental plants [32,33]. This pest causes severe global economic losses due to the heavy damage it causes to plants, such as leaf defoliation and leaf burning [34–36]. This mite is barely controlled in both greenhouse and outdoor environments with current acaricides due to its developed resistance [37,38]. *T. urticae*, a major pest in agricultural and ornamental plants, causes global economic losses through severe plant damage like leaf defoliation and burning. Its resistance to acaricides, including mechanisms like insensitive acetylcholinesterase and detoxifying enzyme upregulation, necessitates the development of new acaricides with novel modes of action for effective control. The escalating resistance levels among pests highlight the critical need for innovative control strategies in greenhouses, emphasizing the integration of biological and physical control methods. Consequently, the development of a multifaceted control approach for agricultural greenhouses which effectively combines various control methods, including fumigation techniques, is becoming increasingly essential.

The main purpose of this study was to determine the effectiveness of EF in controlling *A. gossypii*, *F. occidentalis*, and *T. urticae*, which are problematic at quarantine sites and in greenhouses, by establishing a disinfestation scenario to prevent the establishment of potential exotic insect pests that cause crop damage. We performed the following experiments: (1) efficacy of EF against adults of *A. gossypii*, *F. occidentalis*, and *T. urticae* for 4 h at 20 °C; (2) field trials and phytotoxic assessments in greenhouse cultivation on melon (*Cucumis melo*), zucchini (*Cucucrbita moschata* Duchesne), and watermelon (*Citrullus lanatus*); (3) residue analysis of EF in a greenhouse after field trials; and (4) mortality confirmation of beneficial terrestrial organisms in the EF-treated greenhouse using earthworms (*Eisenia fetida*), adult honeybees (*Apis mellifera*), and silkworm (*Bombyx mori*) larvae.

2. Materials and Methods

2.1. Fumigants

Liquid EF (99%, Fumate^{TM}) was supplied by Safefume Inc. (Daegu, Republic of Korea).

2.2. Organisms

A. gossypii, *F. occidentalis*, and *T. urticae* were obtained from the Rural Development Administration (RDA; Jeonju, Republic of Korea) and reared in a laboratory maintaining conditions at 25 °C ± 1 °C with 60% relative humidity (RH) and a 16:8 h light/dark (L:D) cycle in Kyungpook National University (Daegu, Republic of Korea). Tobacco leaves were provided as a food source. Earthworms (*E. fetida*) and honeybees (*A. mellifera*) were purchased from the market. The earthworms were kept in a soil blend of earthworm excreta and biomedium (Hungnong Seed Co., Seoul, Republic of Korea) for growing prior to experimentation at 23 °C ± 2 °C and 65 ± 5% RH. The biomedium was made up of 20% cocopeat, 60% peat moss, and 20% perlite. The honeybees were fed and bred at a temperature of 25 °C and 35% RH. Silkworm (*B. mori*) eggs were acquired from the Edible Insect Research Institute of Gyeongsangnam-do Agricultural Research & Extension Services (Jinju, Republic of Korea). The eggs were incubated at 15 °C and 75% RH. The silkworm larvae were maintained at 24 °C ± 1 °C and 80 ± 5% RH post-incubation until they became 3rd-instar larva.

2.3. Efficacy of EF on A. gossypii, F. occidentalis, and T. urticae

Fumigation with EF on adult *A. gossypii*, *F. occidentalis*, and *T. urticae* was conducted in airtight, 6.9 L desiccators (Duran®, Wertheim, Germany) with a magnetic mini fan for air circulation. Briefly, the insects were inoculated in insect breeding dishes (ø = 4.5 cm, SPL Life Science Inc., Pocheon, Republic of Korea) and placed inside the desiccators, which were sealed with grease. Liquid EF in concentrations of 1, 2, 4, 8, 10, and 15 g/m^3 was injected using a gas-tight syringe (SGE Analytical Science, Victoria, Australia) at 20 °C for 4 h. The fumigated insect pests were transferred to an insect-rearing room after fumigation and were kept at 25 °C ± 2 °C and 75 ± 5% RH. Adult mortality was determined 72 h after fumigation with mortality criteria. For each species, 30 adults were used per replication. Mortality was determined if an insect did not move when touched. All experiments and controls were replicated three times.

2.4. Residue Analysis of EF on Crops and Soil

Residue analysis of EF was performed on the leaves and soil using headspace autosampler–gas chromatography–mass spectrometry (HS-GC–MS, Agilent 5973N with Agilent G1888, Agilent Technologies Inc., Santa Clara, CA, USA), following the procedure of a previous study [17]. Briefly, HS analysis occurred at an oven temperature of 70 °C, a loop temperature of 75 °C, a transfer line temperature of 80 °C, an equilibration time of 0.2 min, and an injection time of 0.5 min. A mass of 1 g of each leaf or soil sample was placed in a headspace vial right after fumigation to prevent EF loss, and the analysis was conducted immediately. A standard curve reflecting the matrix effect was established for each crop and soil at 10, 50, 100, 250, 500, 1000, and 2000 µg/g, and the limit of detection (LOD) value was set. Three individual plants at the flowering stage for each crop were placed in separate 0.275 m^3 fumigation chambers and fumigated for 4 h at the highest concentration (15 g/m^3 EF) for EF residue evaluation in the lab. After opening, 1 g of soil or leaves was immediately sampled into the headspace vial before the vial cap was sealed using a crimper. Soil samples were collected from the soil surface of both pots in the greenhouse for residue assessment with 0.275 m^3 considering the permeability of EF. The residual amount of EF was determined via HS-GC–MS at 0, 0.5, 1, 2, and 3 h post-fumigation (hpf).

2.5. Greenhouse Cultivation Field Trials with Efficacy and Phytotoxic Assessments

Greenhouse fumigation trials on melon, zucchini, and watermelon using EF were conducted in a 340 m^3 greenhouse (25 m × 4 m × 3.4 m) located in Sancheong-gun, Republic of Korea, at 4 g/m^3 for 4 h at 30 °C. Briefly, adult *A. gossypii*, *F. occidentalis*, and *T. urticae* were inoculated in insect breeding dishes (ø = 4.5 cm), which were located at various positions in the greenhouse. Gas sampling was conducted at 3 points (top, middle, and bottom) of the greenhouse to check EF concentration and distribution. Gas sampling occurred at 0, 1, 2, and 4 h. EF concentrations were measured using gas chromatography with a flame ionization detector (GC-FID, Agilent 6890N; Agilent, Santa Clara, CA, USA). An HP-1 column (30 m in length, 250 μm internal diameter, and 0.25 μm film) was used, and the temperatures of the injector, oven, and detector were set at 250 °C, 100 °C, and 290 °C, respectively. Based on GC-FID results, the concentration–time (Ct) value of the greenhouse fumigation was calculated. During fumigation, 4 g/m^3 EF was applied using an airless pump, which is a new technology that sprays EF using a microfine nozzle (0.3 mm nozzle hole, Safefume Inc., Daegu, Republic of Korea) for 4 h at 30 °C ± 3.5 °C and 70–99% RH. To ensure effective evaporation and uniform distribution of EF in the greenhouse, fans (SGT-350S, SGT Industrial Co. Ltd., Gyeonggi-do, Republic of Korea) were installed on both sides of the greenhouse and turned on for 10 min after EF treatment using the airless pump.

For the phytotoxic assessments, each crop was differentiated into seedlings, flowering plants, and fruiting plants. Next, we evaluated the damage index as follows: 0 (no damage); 1 (<5% of total leaves dropped, browned, and shriveled); 2 (5–25% leaf damage); 3 (25–50% leaf damage); and 4 (>50% leaf damage). The chlorophyll contents of 10 leaves from each crop were evaluated using a chlorophyll meter (SPAD-502 Plus, Minolta, Tokyo, Japan). The leaf color was measured for 10 leaves from each crop as Hunter L (i.e., a, b values using a colorimeter and expressed as hue values based on hue = $[L^2 + a^2 + b^2]^{1/2}$ (TES 135A, Electrical & Electronic Corp., Taipei, Taiwan).

2.6. Acute Toxicity Testing of EF on Non-Target Organisms

Adult earthworms with a well-developed annulus and a weight of 300–600 mg were selected. The acute toxicity test was performed on the earthworms following OECD guideline 207. Briefly, artificial soil was prepared as a mixture of 1 kg of cocopeat, 7 kg of industrial sand, 2 kg of kaolin, and 20 g of $CaCO_3$. The soil's pH value was maintained in the 6.0 ± 0.5 range. The moisture content was set at 35% of the soil's dry weight. Each beaker (500 mL) contained 400 g of this artificial soil, with 10 earthworms inoculated in triplicate. Mortality rates were determined at 14 days post-fumigation. The conditions during this period were 23 °C ± 2 °C, >50% RH, and total darkness. For the acute toxicity testing on honeybees, 40 adult bees per treatment concentration in three groups were individually placed in insect breeding dishes (SPL Life Science Inc., Pocheon, Republic of Korea). For silkworms, 30 silkworms (3rd instar) were used per treatment concentration in triplicate. After EF fumigation, they were kept at 24 °C ± 1 °C and 80 ± 5% RH.

2.7. Statistical Analysis

The lethal concentration-time (LCt) values of EF on various organisms were analyzed using a Probit analysis, following the procedure of a previous study [17]. All quality parameter data related to the phytotoxic assessments (e.g., damage index, chlorophyll contents, and hue value) were calculated using Proc Univariate in SAS (ver. 9.4; SAS Institute Inc., Cary, NC, USA). To analyze the phytotoxic damage of EF fumigation on crops, a *t*-test was performed using SAS.

3. Results

3.1. Efficacy of EF on A. gossypi, F. occidentalis, and T. urticae

The LCt_{50} and LCt_{99} values for EF on *A. gossypii* were 3.12 and 8.96 g h/m^3 in adults, respectively, as determined from the fitted slopes, and 3.54 ± 0.6 for 4 h fumigation at

20 °C (Table 1). The values in *F. occidentalis* and *T. urticae* were 3.71 and 5.34 g h/m^3 for LCt_{50} and 14.00 and 19.07 g h/m^3 for LCt_{99}. *A. gossypii* exhibited a greater sensitivity to EF fumigation compared to *F. occidentalis* and *T. urticae*, with a fold change of 1.56 and 2.13 in LCt_{99}, respectively (Table 1).

Table 1. Efficacy of 4 h ethyl formate (EF) fumigation in targeting *Aphis gossypii*, *Frankliniella occidentalis*, and *Tetranychus urticae* adults at 20 °C.

Insect Pest	[a] LCt_{50} (g h/m^3, 95% CI [b])	LCt_{99} (g h/m^3, 95% CI)	Slope ± SE	df	X^2
A. gossypii	3.12 (2.63–3.48)	8.96 (6.58–12.2)	3.54 ± 0.60	7	32.4
F. occidentalis	3.71 (3.17–4.24)	14.0 (11.4–18.7)	4.03 ± 0.38	7	27.5
T. urticae	5.34 (4.77–5.89)	19.1 (15.9–24.6)	4.21 ± 0.39	7	14.8

[a] LCt: lethal concentration–time; [b] CI: confidence interval.

3.2. Residue Analysis of EF on Crops and Soil

We selected a concentration of 15 g/m^3, the highest EF concentration for controlling target insect pests, for a 4 h fumigation period to predict the patterns of EF residue in both leaves and the soil in a time-dependent manner (Figure 1).

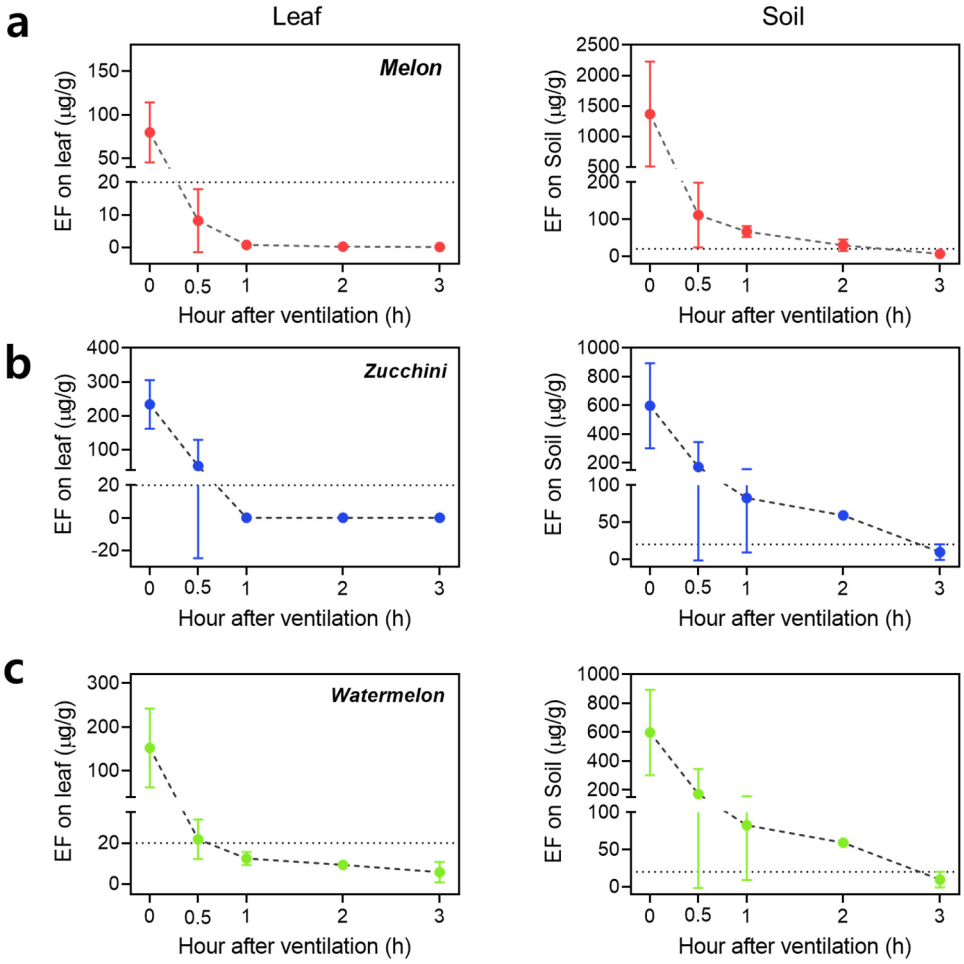

Figure 1. Time-dependent residue evaluation of ethyl formate (EF) on cucurbit crop leaves and soil after 15 g/m^3 EF fumigation for 4 h (0.275 m^3). Each batch was conducted in three independent replications, with 1 g of leaves or soil collected at 0 (immediately), 0.5, 1, 2, and 3 h of ventilation from (**a**) melon, (**b**) zucchini, and (**c**) watermelon. The concentration–time (Ct) values were 24.4 ± 0.78 g h/m^3 for melon, 46.4 ± 3.34 g h/m^3 for zucchini, and 47.0 ± 7.03 g h/m^3 for watermelon. The dotted line represents 20 μg/g as the limit of detection (LOD) for EF residue analysis.

This decision was based on the rationale that lower fumigation concentrations would likely lead to reduced EF residue. The EF residue patterns in the leaves were notably similar across the three cucurbit crops (Figure 1). In melon leaves, the EF residue dropped below the LOD by 0.5 h after ventilation (Figure 1a). This decrease in zucchini and watermelon occurred 1 h after ventilation (Figure 1b,c). The initial EF residue in the soil immediately after ventilation varied widely, ranging from 300 to 2300 μg/g soil among the three distinct soil samples. This residue consistently diminished after the first 0.5 h and ultimately fell below the LOD after 3 h of ventilation (Figure 1). Thus, EF residue remained on leaves for a maximum of 1 h and in soil for up to 3 h, after which it became largely nonresidual.

3.3. Field Trials in Greenhouse Cultivation

The EF concentrations at specific time intervals during the 4 h of fumigation are provided in Table 2. It was confirmed that the EF concentrations for the three sample points in the greenhouse (top, middle, and bottom) were uniform. The EF concentration decreased steadily due to the absorption of EF into the crops, soil, and other surfaces. The final EF concentration was 30% of the initial dose after 4 h of fumigation. The accumulated Ct products of EF were 8.8 ± 0.1, 9.2 ± 0.1, and 9.5 ± 0.1 g h/m^3 at the three points (top, middle, and bottom), respectively. The *A. gossypii* adult mortality rate was 100% (Table 2). Thus, the applied dose (4 g/m^3 EF) effectively controlled *A. gossypii* and reached the target Ct product (>8.96 g h/m^3, equivalent to the LCt_{99} value of *A. gossypii*). The applied dose (15 g/m^3 EF) effectively controlled *F. occidentalis* and *T. urticae* with 100% mortality and reached the target Ct product (>19.07 g h/m^3, equivalent to the LCt_{99} value of *T. urticae*).

Table 2. The point-specific concentration and accumulated concentration–time (Ct) products of ethyl formate (EF) in greenhouse cultivation and efficacy of EF against three target insect pests (4 h fumigation, 340 m^3, 30 °C ± 3.5 °C, RH: 70–99%).

Applied Dose (g/m^3)	Exposure Time (h)	EF Concentration (Mean ± SE, g/m^3)			Target Insect (Mortality, %)		
		Top	Middle	Bottom	*A. gossypii*	*F. occidentalis*	*T. urticae*
0	**0**	**0**	**0**	**0**	**1.6 ± 0.7**	**0.4 ± 0.4**	**1.2 ± 0.5**
4	0.1	3.6 ± 0.1	3.8 ± 0.1	3.9 ± 0.1	100 ± 0.0	N/A *	N/A
	1.0	2.6 ± 0.1	2.8 ± 0.1	2.9 ± 0.1			
	2.0	2.0 ± 0.2	2.2 ± 0.1	2.2 ± 0.1			
	4.0	1.7 ± 0.1	1.7 ± 0.0	1.7 ± 0.1			
	Ct products (g h/m^3)	8.8 ± 0.1	9.2 ± 0.1	9.5 ± 0.1			
15	0.1	14.5 ± 0.1	14.5 ± 0.1	14.6 ± 0.1	N/A	100 ± 0.0	100 ± 0.0
	1.0	9.0 ± 0.1	9.2 ± 0.0	9.4 ± 0.0			
	2.0	7.0 ± 0.1	7.1 ± 0.0	7.3 ± 0.1			
	4.0	5.1 ± 0.0	5.6 ± 0.0	5.7 ± 0.0			
	Ct products (g h/m^3)	31.3 ± 0.1	32.2 ± 0.0	32.9 ± 0.0			

* Not applicable.

During the phytotoxic assessments, each crop was separated into seedling, flowering, and fruiting periods. No phytotoxic damage was observed in melons or zucchini when treated with 4 g/m^3 EF for 4 h at 30 °C ± 3.5 °C and 70–99% RH in greenhouse cultivation (Table 3). However, the damage index in watermelons was confirmed to be at level 1 of phytotoxic damage (Table 3). It was confirmed that there was no significant difference in the chlorophyll contents or hue values in melons, zucchinis, and watermelon (Table 3). Application with 15 g/m^3 EF for 4 h evoked severe phytotoxicity in all crop developmental stages (Table 3). No EF residue on the leaves or in the soil was found, even in the treatment with the highest concentration (15 g/m^3 EF; Table 4).

Table 3. Phytotoxic assessments of various crop developmental stages (melon, zucchini, and watermelon) after ethyl formate (EF) fumigation and treatment with 4 or 15 g/m^3 EF for 4 h in greenhouse cultivation at 30 °C ± 3.5 °C (concentration–time (Ct) products: 9.2 g h/m^3 for 4 g/m^3 EF and 32.2 g h/m^3 for 15 g/m^3 EF).

Applied Dose (g/m^3)	Crops	Developmental Stage	Damage Index [a]		Chlorophyll Content		Hue Value [b]	
			Before	After	Before	After	Before	After
4	Melon	Seedling	0.0 ± 0.0	0.0 ± 0.0 ^{ns}	44.0 ± 2.3	42.6 ± 0.6 ^{ns}	63.6 ± 2.1	58.1 ± 3.0 ^{ns}
		Flowering	0.0 ± 0.0	0.0 ± 0.0 ^{ns}	41.5 ± 0.6	43.0 ± 0.9 ^{ns}	61.0 ± 2.3	57.5 ± 1.0 ^{ns}
		Fruiting	0.0 ± 0.0	0.0 ± 0.0 ^{ns}	44.1 ± 0.8	45.6 ± 0.7 ^{ns}	55.3 ± 2.4	55.3 ± 0.7 ^{ns}
	Zucchini	Seedling	0.0 ± 0.0	0.0 ± 0.0 ^{ns}	41.6 ± 0.4	43.3 ± 1.3 ^{ns}	57.9 ± 1.7	62.9 ± 2.4 ^{ns}
		Flowering	0.0 ± 0.0	0.0 ± 0.0 ^{ns}	42.7 ± 1.1	43.5 ± 0.7 ^{ns}	63.6 ± 1.4	61.8 ± 1.6 ^{ns}
		Fruiting	0.0 ± 0.0	0.0 ± 0.0 ^{ns}	44.1 ± 1.0	45.4 ± 0.4 ^{ns}	58.2 ± 1.8	59.9 ± 1.2 ^{ns}
	Watermelon	Seedling	0.0 ± 0.0	1.0 ± 0.0 *	42.8 ± 1.0	43.8 ± 1.1 ^{ns}	59.6 ± 2.4	60.5 ± 3.5 ^{ns}
		Flowering	0.0 ± 0.0	1.0 ± 0.0 *	43.8 ± 1.4	44.1 ± 1.8 ^{ns}	60.7 ± 4.9	58.9 ± 1.8 ^{ns}
		Fruiting	0.0 ± 0.0	1.0 ± 0.0 *	44.5 ± 0.5	44.5 ± 0.4 ^{ns}	57.4 ± 1.0	62.3 ± 1.2 ^{ns}
15	Melon	Seedling	0.0 ± 0.0	5.0 ± 0.0 ****	44.5 ± 1.2	- [c]	60.6 ± 2.4	-
		Flowering	0.0 ± 0.0	5.0 ± 0.0 ****	42.6 ± 1.1	-	61.2 ± 2.3	-
		Fruiting	0.0 ± 0.0	4.0 ± 0.0 ****	44.7 ± 0.7	-	61.9 ± 2.2	-
	Zucchini	Seedling	0.0 ± 0.0	5.0 ± 0.0 ****	45.1 ± 0.9	-	60.6 ± 2.9	-
		Flowering	0.0 ± 0.0	4.0 ± 0.0 ****	46.6 ± 0.8	-	61.6 ± 2.4	-
		Fruiting	0.0 ± 0.0	4.0 ± 0.0 ****	45.4 ± 1.0	-	63.3 ± 2.4	-
	Watermelon	Seedling	0.0 ± 0.0	5.0 ± 0.0 ****	41.5 ± 1.3	-	58.8 ± 1.5	-
		Flowering	0.0 ± 0.0	5.0 ± 0.0 ****	42.9 ± 1.1	-	59.1 ± 0.8	-
		Fruiting	0.0 ± 0.0	4.0 ± 0.0 ****	44.8 ± 0.8	-	58.9 ± 1.9	-

[a] 0.0 (no damage); 1.0 (<5% of total leaves dropped, browned, and shriveled); 2.0 (5–25% leaf damage); 3.0 (25–50% leaves damage); 4.0 (>50% leaf damage); [b] [color L^2 + color a^2 + color b^2]$^{1/2}$; [c] not detected on dead leaves. Statistical analysis was conducted using a *t*-test compared to the data before and after. *ns*, not significant; *, p <0.05; ****, p <0.0001.

Table 4. The residue analysis of ethyl formate (EF) at different developmental stages of three crops and soils after EF fumigation (treated with 15 g/m^3 EF for 4 h) in greenhouse cultivation at 30 °C ± 3.5 °C (concentration–time (Ct) products: 32.2 g h/m^3).

Crops or Soil	Developmental Stage	Control		EF-Treated	
		Sample (g)	EF (μg/g)	Sample (g)	EF (μg/g)
Melon	Seedling	1.04 ± 0.01	ND *	1.01 ± 0.01	ND
	Flowering			1.01 ± 0.03	ND
	Fruiting			1.03 ± 0.02	ND
Zucchini	Seedling	1.04 ± 0.01	ND	1.02 ± 0.03	ND
	Flowering			1.01 ± 0.02	ND
	Fruiting			1.02 ± 0.01	ND
Soil		1.00 ± 0.02	ND	1.03 ± 0.02	ND

* Not detected (ND): below the limit of detection.

3.4. Acute EF Toxicity toward Agriculturally Beneficial Organisms

To evaluate the effect of EF fumigation on agriculturally useful organisms, LCt values were calculated and compared with those of target pests (Table 5). The EF LCt_{50} values in honeybees, silkworms, and earthworms were 15.1, 26.5, and 48.9 g h/m^3, respectively (Table 5). Similarly, the LCt_{90} values in honeybees, silkworms, and earthworms were 20.1, 41.9, and 198 g h/m^3, respectively. The LCt_{99} values in honeybees, silkworms, and earthworms were 27.8, 68.9, and 910 g h/m^3, respectively. Even though their existence in greenhouse cultivation was unclear, these LCt values were high enough to completely control *A. gossypii* at 8.96 g h/m^3 inside the greenhouse. However, the Ct product applied for the complete control of *F. occidentalis* and *T. urticae* had an average range of 32.2 g h/m^3. This suggested that the Ct product could induce high mortality in honeybees at the LCt_{99} value and in silkworms at the LCt_{50} value.

Table 5. Lethal concentration–time (LCt) values after ethyl formate (EF) fumigation for 4 h against beneficial organisms, including honeybees, silkworms, and earthworms at 20 °C.

Organism	LCtx (g h/m^3, 95% CI [a])				Slope ± SE	df	χ^2
	10	50	90	99			
Honeybee	11.3 (7.31–13.3)	15.1 (12.6–17.4)	20.1 (17.4–28.7)	27.8 (22.0–55.8)	−20.7 ± 4.56	25	351
Silkworm	16.8 (11.0–20.4)	26.5 (22.4–31.3)	41.9 (34.7–62.9)	68.9 (50.2–150)	−15.8 ± 3.41	22	167
Earthworm	12.1 (2.97–22.2)	48.9 (29.2–66.4)	198 (137–414)	910 (429–5290)	−6.11 ± 1.43	34	48

[a] CI: confidence interval.

4. Discussion

4.1. Lab-Scale and Field Efficacy of EF against A. gossypii, F. occidentalis, and T. urticae

A previous study found EF efficacy values at LCt_{99} in adult and nymph *A. gossypii* to be 4.44 and 3.80 g h/m^3, respectively, at 5 °C after 2 h of exposure and LCt_{99} values of 4.42 and 3.73 g h/m^3 at 20 °C after 2 h of exposure [39]. The LCt_{50} values were 1.71 and 1.37 g h/m^3, respectively, at 5 °C after 2 h of exposure and 2.49 and 1.48 g h/m^3, respectively, at 20 °C after 2 h of exposure [39]. These results indicate that EF had a stronger fumigant efficacy against *A. gossypii* adults and nymphs when treated at a lower temperature if the fumigant was similarly vaporized. In our study, EF led to LCt_{50} and LCt_{99} values of 3.12 and 8.96 g h/m^3, respectively, at 20 °C after 4 h of fumigation. The different LCt values between these two studies could be due to the synergy effect with low-temperature damage and the different fumigation conditions, such as differing fumigation durations and treatment doses against *A. gossypii* adults.

The LCt_{50} and LCt_{99} values of *F. occidentalis* were higher than those of *A. gossypii* (Table 1). EF fumigation is used for the postharvest control of this species. In a previous study, *F. occidentalis* individuals were placed in a 9.6 L fumigation jar with grapes at 24 °C for 1 h of EF fumigation, and the LCt_{99} values were determined to be 1.99, 0.60, 0.87, and 0.33% for eggs, second-instar insects, prepupae, and adults, respectively [12]. Another similar EF fumigation study on adults fed navel orange at 4 °C showed an LCt_{99} value of 5.0 g/m^3 [40]. However, there is no direct comparison for EF fumigation to control *F. occidentalis*, since no other study has been performed in a greenhouse setting. In a previous greenhouse field trial, there was a similar pattern where thrips (*Thrips palmi* Karny) required approximately twice the LCt value of EF for control compared to aphids (*Myzus persicae* Sulzer) [17]. Although the species are different, insects belonging to the same family seem to show similar EF tolerance. Despite species differences, insects from the same family appear to exhibit similar tolerances to EF. For instance, Eucalyptus weevils (*Gonipterus platensis* Marelli), which have markedly different morphologies from thrips and aphids, were effectively controlled with 25–30 g/m^3 EF over 24 h of fumigation, indicating a Ct value that was much higher than expected [15]. The varied efficacy of EF across different pest species may be linked to morphological and physiological differences. Aspects such as respiratory system structure or cuticle thickness could influence fumigant penetration and overall effectiveness, as highlighted by Subramanyam and Hagstrum [41]. Understanding these distinct reactions to EF fumigation is crucial for developing precise and effective pest management strategies in agriculture. In general, greenhouse pests are found to be relatively susceptible to EF, underscoring its potential as an effective agricultural pesticide.

Other agricultural pests, including *T. palmi*, *Bemisia tabaci*, and *M. persicae*, have been controlled by EF with more than 93.3% mortality without phytotoxicities on seedlings of yellow melon, cucumber, tomatoes, and peppers under the fumigation condition of 1.5 g/m^3 for 12 h [42]. Only *T. urticae* was not properly controlled under the same fumigation conditions (except for the treated dose of 2.0 g/m^3) and exhibited only a 20% lethal effect [42]. These results indicate that *T. urticae* populations may be more resistant to EF fumigation than the other three insect pests. A further study demonstrated that *T. urticae* nymphs had LCt_{50} and LCt_{99} values of 1.51 and 4.71 g h/m^3 for EF fumigation at 5 °C and 0.61 and 3.93 g h/m^3 for EF fumigation at 20 °C, respectively [43]. Similarly, *T. urticae*

adults possessed LCt_{50} and LCt_{99} values of 1.72 and 5.01 g h/m^3 for EF fumigation at 5 °C and 0.88 and 3.53 g h/m^3 for EF fumigation at 20 °C. In our study, the *T. urticae* adults were better able to resist EF fumigation toxicity compared to the other two species.

Monitoring the concentration of fumigants during application is critical in fumigation treatment methods, and this importance has led to the generalization of the Ct concept [9,17,39,42,43]. This concept is particularly relevant due to various factors affecting fumigant concentration, such as the airtightness of enclosed spaces (e.g., containers, polyvinyl chloride (PVC) tarpaulin fumigation chambers, desiccators, and greenhouses), adsorption on commodities, temperature, and humidity [17,43]. The Ct value represents the actual fumigant concentration during application time, serving as a comprehensive metric for comparing the effectiveness against insect pests in diverse fumigation scenarios. Our field trials were carried out in greenhouse environments with melon, zucchini, and watermelon crops at an average temperature of 30 ± 3.5 °C. In greenhouses, we observed a faster decrease in Ct values due to higher humidity and less airtight conditions compared to desiccators used for laboratory-scale efficacy testing (refer to Figure 1 and Table 3).

In the original experimental design, the complete control of the target insect pests should have been reached at the LCt_{99} values of 8.96, 14.0, and 19.07 g h/m^3 for *A. gossypii*, *F. occidentalis*, and *T. urticae* adults, respectively (Table 1). Thus, it was essential to obtain EF Ct products of 8.96 g h/m^3 inside the greenhouse for the successful control of *A. gossypii*. The average Ct values for all three locations within the greenhouse were about 9.17 g h/m^3 (Table 2). This Ct value indicates that the complete control of *A. gossypii* adults was successfully achieved beyond any doubt. Furthermore, EF fumigation at 15 g/m^3 resulted in Ct products at the top, middle, and bottom of the greenhouse that were >31.3 g h/m^3, which was high enough to completely control *F. occidentalis* and *T. urticae* adults. These results indicate that EF fumigation can be used to eradicate these three pest species in greenhouses.

4.2. Miscellaneous Considerations for EF Fumigation

The EF residue measured in the soils and crops of the EF-fumigated greenhouse after ventilation was a critical point in this study. A longer duration of EF residue on crop leaves and in soil after ventilation might decrease EF for insect pest control use in greenhouse cultivation; however, EF residue was not found 1 h after ventilation on crop leaves or 3 h after ventilation in soils (Figure 1). Thus, no residue remained on the leaves or in soils after EF fumigation and subsequent ventilation. These results indicate that EF fumigation might be effective for the control of insect pests in greenhouses while maintaining a high safety level for agricultural workers and consumers.

In phytotoxic assessments of EF fumigation on various commodities (e.g., fruits, vegetables, nursery plants, and cut flowers), the degree of phytotoxic damage differs according to the characteristics of each commodity and the EF dose administered. There are many reports of the effective application of EF on fruits, such as apples, oranges, and pears [15,44,45], and phytotoxic assessments have been conducted on various nursery plants. In our study, a phytotoxic damage index of level 1 appeared in watermelons when EF was dosed up to 4 g/m^3 to control *A. gossypii*. However, after the increase to 15 g/m^3 to control *F. occidentalis* and *T. urticae* adults, the phytotoxic level reached 4 or 5, which indicates complete inhibition of plant growth. Thus, the success of EF fumigation is dependent on the target exotic insect species, since species requiring a high dosage might evoke phytotoxic effects on the agricultural crops grown.

In this experiment, an airless pump vaporizer was used for EF fumigation in a large-scale greenhouse under various conditions and crops. Previously, this airless pump vaporizer has been used to conduct EF fumigation in a greenhouse with cucurbit crops, with the finding that the EF concentration was uniformly distributed in the greenhouse [17]. Based on these data, it can be inferred that an airless pump vaporizer can be used to apply EF in greenhouses. In this study, an airless pump system was utilized to spray liquid EF through a microfine nozzle with a 0.3 mm hole. The high temperatures within the greenhouse, often surpassing 30 °C, facilitated the rapid vaporization of the sprayed EF. This method enabled

a uniform distribution of the EF concentration across the top, middle, and bottom parts of the greenhouse during both EF treatment scenarios (Table 2).

Of the three beneficial insects, the honeybee was the most sensitive to EF fumigation, whereas the earthworm exhibited a reduced sensitivity, likely due to the protective effects of the soil. After 4 h of fumigation at 4 g/m^3 EF in the greenhouse, the target Ct value exceeded 8.96 g h/m^3, which corresponded to the LCt_{99} value of *A. gossypii* (Table 2). Notably, this target Ct value is below the LCt_{10} value for honeybees, silkworms, and earthworms. This suggests that a 4 h fumigation period with 4 g/m^3 EF would likely not adversely affect these beneficial organisms (Table 5). However, applying 15 g/m^3 EF for 4 h in a greenhouse derived a high Ct product exceeding 32 g h/m^3, which implies a significant risk to not only crops but also honeybees (Tables 3 and 5). To mitigate this risk, it is recommended that EF fumigation be performed during non-daylight hours when honeybees are less active.

5. Conclusions

In this study, a schematic procedure was set up to apply EF, a quarantine fumigant, for the eradication of exotic insect pests (i.e., *A. gossypii*, *F. occidentalis*, and *T. urticae*) in a greenhouse. This study demonstrated that 4 g/m^3 EF fumigation for 4 h effectively controlled *A. gossypii* on melon, zucchini, and watermelon without damaging the plants, but a higher dosage of 15 g/m^3 for *F. occidentalis* and *T. urticae* caused significant damage, although no EF residue was detected post-ventilation. We suggest that new disinfestation treatment methods be developed to use EF fumigation in greenhouses, especially since EF is currently successfully replacing MB in quarantine sites. EF has shown its efficacy in the disinfestation of *A. gossypii* adults. Based on these results, an effective control strategy is expected to be established using EF fumigation when exotic insect pests are introduced and established in greenhouse cultivation.

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