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Addition of cinnamon oil improves toxicity of rotenone to *Spodoptera litura* (Lepidoptera: Noctuidae) larvae

Zihao Li[1], Rilin Huang[1], Weisheng Li[1], Dongmei Cheng[2], Runqian Mao[3], and Zhixiang Zhang[1,4]*

Abstract

Although rotenone is widely used as a pesticide, it has a low level of insecticidal activity on *Spodoptera litura* (F.) (Lepidoptera: Noctuidae). To gain a better understanding of the high tolerance to rotenone, a synergist (cinnamon oil) was added, and the comparative physiological impacts were assessed. After rotenone treatment, a considerable amount of rotenone was discharged in excreta, but extremely low levels were found in the ventral nerve cord and brain. By contrast, the rotenone + cinnamon oil treatment group showed an increased amount of rotenone in the ventral nerve cord and brain. The co-toxicity coefficient for rotenone + cinnamon was 213, indicating synergism. The midgut cells from insects treated with rotenone alone, and the controls, exhibited no significant differences, whereas those of the rotenone + cinnamon oil group had larger intercellular spaces. These findings suggest that rotenone alone could not effectively penetrate the midgut, perhaps accounting for its low toxicity to *S. litura*. The rotenone + cinnamon oil mixture apparently affected midgut cell spacing and membrane permeability, thus effectively increasing rotenone toxicity.

Key Words: midgut penetration; botanical insecticide; synergism; paraffin section

Resumen

Aunque la rotenona es ampliamente utilizada como pesticida, tiene un bajo nivel de actividad insecticida sobre *Spodoptera litura* (F.) (Lepidoptera: Noctuidae). Para obtener una mejor comprensión de la alta tolerancia a la rotenona, se añadió un sinergista (aceite de canela) y se evaluaron los impactos fisiológicos comparativos. Después del tratamiento con rotenona, se descargó una cantidad considerable de rotenona en el excremento, pero se encontraron niveles extremadamente bajos en el cordón nervioso ventral y en el cerebro. Por el contrario, el grupo de tratamiento con aceite de rotenona + canela mostró una mayor cantidad de rotenona en el cordón nervioso ventral y en el cerebro. El coeficiente de co-toxicidad para rotenona + canela fue de 213, lo que indica sinergismo. Las células del intestino medio de los insectos tratados con la rotenona sola, y los controles, no mostraron diferencias significativas, mientras que las del grupo de rotenona + aceite de canela tenían mayores espacios intercelulares. Estos hallazgos sugieren que la rotenona por sí sola no podría penetrar eficazmente en el intestino medio, tal vez debido a su baja toxicidad para *S. litura*. La mezcla de rotenona + aceite de canela aparentemente afectó el espaciamiento de las células del intestino medio y la permeabilidad de la membrana, aumentando así efectivamente la toxicidad de la rotenona.

Palabras Clave: penetración del intestino medio; insecticida botánico; sinergismo; sección de parafina

Spodoptera litura (F.) (Lepidoptera: Noctuidae), 1 of the most serious crop pests in the world, infests more than 180 plant species (Arumugam et al. 2015). *Spodoptera litura* is also known as the cotton leaf worm, tobacco cutworm, and tropical armyworm (Li et al. 2014b). It affects the yield of various cultivated crops, vegetables, weeds, and ornamental plants by feeding gregariously on leaves, and causing large economic losses (Kaur et al. 2014; Ahmad &Mehmood 2015). *Spodoptera litura* is widely distributed throughout the Middle East, East Asia, Oceania, and the Pacific islands, and is found in climates ranging from tropical to temperate (Fu et al. 2015). In China, India, and Japan, its larval stages cause up to 30% damage in several crops, including tobacco, castor, groundnut, tomato, cabbage, cauliflower, cotton, and other crucifers (Kumar et al. 2014).

Chemical control is the most common method of *S. litura* management because of its ease of use and reliability (Zhou et al. 2011). However, *S. litura* is capable of developing resistance to various classes of insecticides (Su et al. 2012; Muthusamy et al. 2014). The intensive use of insecticides for the control of this pest has resulted in high levels of resistance to almost all commercial insecticides available for its control worldwide (Rehan and Freed 2014; Babu et al. 2015). Therefore, identification of effective insecticides to control *S. litura* is a continuing need.

Rotenone is a common agricultural pesticide, as well as a pisciside (Grefte et al. 2015). This compound is a natural toxin derived from the roots and stems of several plants of the family Leguminosae (Rohan et al. 2015). Rotenone possesses significant activity against many taxa, including the mite *Panonychus citri* (McGregor) (Prostigmata: Tetranychidae), the nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer)

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Nickle (Aphelenchida: Parasitaphelenchidae), and the insect *Phyllotreta vittata* (Fabricius) (Coleoptera: Chrysomelidae) (Zeng et al. 2002; Hu et al. 2005; Zeng et al. 2009). In general, rotenone has high insecticidal activity (Xu et al. 2010), although it is susceptible to rapid photodegradation (Cabizza et al. 2004).

Cinnamon oil extracted from the cinnamon tree also is used widely (Rao & Gan 2014; Xing et al. 2014), although it is mostly known for its antimicrobial activity (Echegoyen & Nerin 2015). For example, Al-Othman et al. (2013) found that cinnamon oil had significant inhibitory effect on the fungi *Aspergillus flavus* Link (Trichocomaceae). Wang et al. (2014) reported that a cinnamon oil micro-emulsion is an alternative approach to control gray mold on pears without a negative influence on fruit quality. Todd et al. (2013) revealed that cinnamon oil has the potential for use as an alternative treatment for washing organic baby and mature spinach, as well as iceberg and romaine lettuce.

Rotenone has low insecticidal activity to *S. litura* (Akhtar et al. 2008). The biochemical mechanism of the high tolerance of *S. litura* to rotenone remains unclear, and determining an effective synergist to improve its insecticidal activity against *S. litura* is desirable. This study investigated the mechanisms underlying the low toxicity of rotenone to *S. litura* larvae. Rotenone or cinnamon oil were used alone, or mixed together, to evaluate their toxicity against *S. litura*. The possible mechanisms that lead to cinnamon oil increasing the insecticidal activity of rotenone against *S. litura* also were investigated.

Materials and Methods

INSECTS

Spodoptera litura larvae were collected from Guangzhou, China. The larvae were reared in rectangular plastic jars (30 × 20 × 18 cm); the lids were cut (3 × 3 cm) and replaced with a mesh cloth for aeration. Larvae were reared in an environmentally controlled room (25 ± 1 °C, 14:10 h L:D, and 75% relative humidity [RH]) (Baskar et al. 2011) and fed with an artificial diet adapted from Gahloth et al. (2011). The diet consisted of soy flour (100.0 g), oatmeal (80.0 g), dried brewer yeast (26.0 g), sorbic acid (2.0 g), casein (8.0 g), vitamin C (8.0 g), agar (7.5 g), choline chloride (1.0 g), inositol (0.2 g), cholesterol (0.2 g), formaldehyde (2.0 mL), and distilled water (500.0 mL). The food was changed daily until pupation, and jars were regularly cleaned to avoid any type of infection. Third, fourth, or fifth instar larvae were used in the experiments.

CHEMICALS

Rotenone certified reference standard (97.0%) was purchased from Sigma Chemical Company, Germany. Cinnamon oil, obtained from Yunan Kingtide Notoginseng Industry Co., Ltd., China, was analyzed by gas chromatography–mass spectrometry and its chemical composition is shown in Table 1. Trypsin–ethylenediaminetetraacetic acid digestive juice was acquired from Beijing Solarbio, China. All other general chemicals used were of the highest purity grade that was available commercially. Phosphate buffer solution (pH 7.2–7.4) consisted of distilled water (800.00 mL), sodium chloride (8.00 g), potassium chloride (0.20 g), disodium phosphate (1.15 g), and monopotassium phosphate (0.20 g). The chromogenic agent was comprised of 1% Fast Blue B salt solution and 5% sodium dodecyl sulfate solution (2:5, v/v).

INSECTICIDES

The leaf dip method (Sun et al. 2015) was used to study the toxicity of rotenone and rotenone + cinnamon oil to *S. litura* third-instar larvae. Insecticide solutions were diluted in a series of concentrations in acetone (5–7 doses), and acetone alone was used as the control. Equal-sized tapioca (*Manihot esculenta* Crantz; Euphorbiaceae) leaf discs (1 cm diameter) were cut and dipped into the test solutions for 3 s and air dried at room temperature for 1 h. Larvae were placed on the treated leaves in a Petri dish (9 cm diameter). Three replicates were maintained for each treatment with 15 larvae per replicate (N = 45). The experiment was performed at a controlled room (25 ± 1 °C, 14:10 h L:D, and 75% RH). Larval mortality was recorded 24, 48, and 72 h after treatment. Bioassays that showed mortality higher than 10% in the untreated control were discarded, and the entire replicate was repeated.

The LC_{50} value of each insecticide was determined with probit analysis (Finney 1971). The co-toxicity coefficient of rotenone and cinnamon oil mixture was analyzed by the Sun method (Wen et al. 2013). Co-toxicity coefficients (CTC) <80 are considered antagonistic, CTC >80 or <120 is additive, and CTC >120 is synergistic (Islam et al. 2010).

Table 1. Chemical composition of cinnamon oil.

Peak number	Compound	RRT (min)	%
1	Benzaldehyde	6.714	4.59
2	Phenol	7.357	0.13
3	Phenylacetaldehyde	9.345	0.40
4	Phenyl methyl ketone	10.095	0.17
5	Benzyl formate	10.486	0.40
6	Benzoic acid	13.917	0.99
7	Cinnamaldehyde	15.410	0.31
8	Phenylacetic acid	16.741	0.31
9	Trans-Cinnamaldehyde	17.349	74.70
10	Cinnamic acid	22.955	15.47
11	Cinnamic acid trimethylsilyl ester	25.870	0.17
12	1,4-Diphenyl-1,3-butadiene	35.332	0.13
13	trans,trans-1,4-Diphenyl-1,3-butadiene	38.291	0.21
14	Benzyl cinnamate	40.090	0.47
15	1,2-Dibenzoylethane	42.150	0.38
16	3-cyclopenten-1-one	42.385	0.51
17	Acetyl tributyl citrate	43.613	0.34
18	2-Methylphenanthro[3,4-d][1,3]oxaz ol-10-ol	44.153	0.10

RRT = relative retention time.

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ROTENONE CONTENT IN LARVAE

Larvae were fed on leaves treated with rotenone, or rotenone + cinnamon oil, and water was used as control. Fifth-instar larvae, which were starved for 24 h before the tests, were released on tapioca leaf discs soaked in test solutions (1,000 µg/mL rotenone and 1,000 µg/mL rotenone + 35,000 µg/mL cinnamon oil) in a Petri dish, and covered with a lid. One larva was released in each dish. The larvae were fed with treated leaves at various times. At 3, 9, 12, 18, and 24 h post treatment the larvae and their excreta were collected in new dishes.

The hemolymph was collected using the method of Li et al. (2014a); the larvae were dissected, and the midgut, brain, and ventral nerve cord were collected (Zhao et al. 2015). All samples, including excreta, were stored at −20 °C until use.

DETECTION OF ROTENONE

A stock solution of rotenone (1,000 mg/kg) was prepared in acetone. Working standard solutions were prepared daily by dilution with the mobile phase (acetonitrile/water; 64:36, v/v).

A high-performance liquid chromatography system (Shimadzu LC-20A, Japan) with an ultraviolet visible detector was used for rotenone analysis. Chromatography was performed using an Agilent Zorbax TC-C18 column (4.6 mm × 250.0 mm × 5.0 µm) (Zhou et al. 2014). Isocratic elution was performed with acetonitrile and water (64:36, v/v) for 15 min. The injection volume was 10 µL, and the flow was 1 mL/min. The detection wavelength was set at 299 nm.

Previously homogenized tissue sample was weighed and placed into a 10 mL centrifuge tube with 0.5 g of protease digestive juices. The mixture was agitated in a shaker and heated in a thermostatically controlled water bath (37 °C) until it was colorless. The mixture was cooled to room temperature, and then 1.5 mL of methanol was added. The mixture was mixed end-over-end for 10 min. Subsequently, the organic solvent phase was separated through centrifugation at 3,500 rpm for 5 min, and about 1 mL of the organic extract was used with 1 mL of the mobile phase and injected onto a high-performance liquid chromatography system for analysis.

Recovery assays were performed for rotenone using standards to reach 0.5, 1.0, and 2.0 mg/kg concentrations in midgut, brain, ventral nerve cord, hemolymph, and excreta. Five replicates of each concentration were analyzed.

INFLUENCE OF ROTENONE, AND ROTENONE + CINNAMON OIL, ON THE MIDGUT

Approximately 1,000 µg/mL concentration of rotenone and 1,000 µg/mL rotenone + 35,000 µg/mL cinnamon oil was used to treat larvae, by feeding, for 24 h. The surviving larvae were washed with cold distilled water and then transferred on ice for dissection. The midgut was fixed with Bouin fixative, dehydrated with an ascending ethanol series, cleared with xylene, and infiltrated with paraffin; afterward, the midgut attached to the slide glass was sectioned, deparaffined, and stained (Kim et al. 2015).

Morphological alterations of the midgut cell structure and organization of each *S. litura* were recorded and compared with the tissues obtained from the control group. Pictures were taken using a photomicroscope (E200 type, Nikon Corporation, Japan) coupled to a microcamera connected to a computer fitted with an image capture card and ImageLab software (NIS-Elements, Nikon Corporation, Japan).

DATA ANALYSES

Data are presented as means ± standard errors for 3 independent experiments. Charts were constructed using Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, Washington). A 2-way ANOVA was performed to compare the effects of the chemical and exposure period using SPSS 16.0 (International Business Machines Corporation, North Castle, New York). The means were compared using the Tukey honest significant difference test, with $P < 0.05$ considered statistically significant.

Results

DETERMINATION OF LC_{50} AND CO-TOXICITY COEFFICIENT

The comparative toxicity of rotenone, cinnamon oil, and rotenone + cinnamon oil (1:35, m/m) on *S. litura* is shown in Table 2. Toxicity varied with concentration and exposure period, with the greatest toxicity attained with rotenone + cinnamon oil after 72 h of exposure. The 72 h LC_{50} values of rotenone, cinnamon oil, and rotenone + cinnamon oil to larvae were 1,081 mg/L, > 10,000 mg/L, and 506 mg/L, respectively. The co-toxicity coefficient of rotenone + cinnamon oil was 213.52.

RECOVERY RATE OF ROTENONE

The retention time of rotenone was approximately 7.51 min (Fig. 1). An external calibration was provided, and the standard calibration curve was constructed by plotting concentration against the peak area. Good linearities were achieved for all active ingredients between 0.01 and 5.00 mg/L, with a correlation coefficient of 0.999. The detection limit was 6.2 µg/kg.

Recovery experiments were carried out at different levels to establish the reliability and validity of the analytical method and determine the efficiency of extraction and clean-up procedures for each larval tissue. The control samples of each tissue were spiked at 0.5, 1.0, and 2.0 mg/kg, and processed by following the methodology described above. Table 3 shows the recoveries and relative standard deviations of fortified samples. The mean recoveries of rotenone in the hemolymph, midgut, ventral nerve cord, brain, and excreta were within the ranges of 80.15 to 110.46%, 87.66 to 113.09%, 86.97 to 111.67%, 84.30 to 92.35%, and 77.76 to 104.31%, respectively; the relative standard deviations of hemolymph, midgut, ventral nerve cord, brain, and excreta were within 3.43 to 7.21%, 3.00 to 12.79%, 4.01 to 11.40%, 5.35 to 9.55%, and 2.02 to 12.56%, respectively.

Table 2. The insecticidal activity of rotenone, with and without cinnamon oil, on third instar larvae of *S. litura*.

Treatment	Exposure time (h)	Regression equation	LC_{50} (mg/L)	95% confidence limit (mg/kg)	Correlation coefficient	CTC
Rotenone	48	$y = 3.5624x - 8.1903$	5,043	3,624–7,017	0.9882	
Rotenone	72	$y = 1.7934x - 0.4410$	1,081	613–1,907	0.9914	
Cinnamon oil	72		>10,000			
Rotenone + cinnamon oil (1:35)	72	$y = 1.3062x + 1.4674$	506	232–1,104	0.9872	213.52

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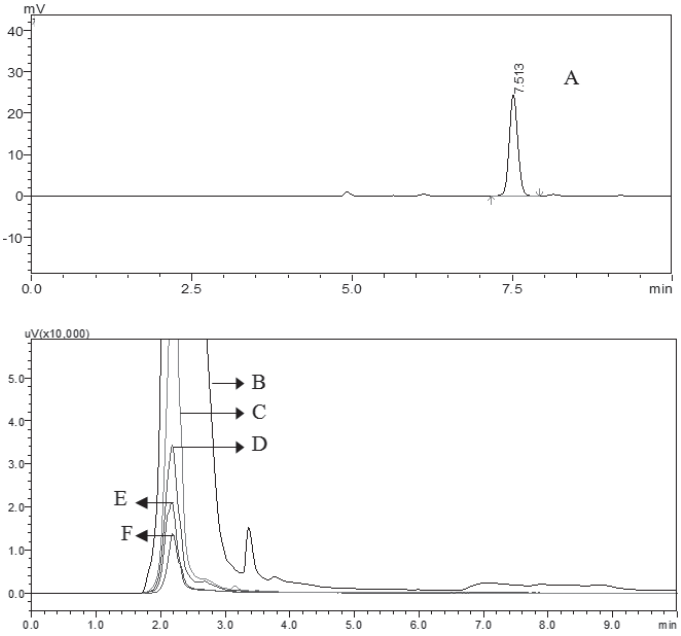


Fig. 1. Liquid chromatogram (A: rotenone standard, B: excreta, C: hemolymph, D: brain, E: ventral nerve cord, F: midgut).

CONCENTRATION OF ROTENONE

Each fifth-instar larva of *S. litura* in the testing period was treated with 1,000 µg/mL rotenone and 1,000 µg/mL rotenone + 35,000 µg/mL cinnamon oil. The larvae and their excreta were collected after treatment for 3, 9, 12, 18, and 24 h, and then dissected. The concentration of rotenone in the excreta, midgut, hemolymph, ventral nerve cord, and brain of larvae are shown in Figures 2 to 6. A considerable amount of rotenone was discharged in excreta, and the discharge rate from the larvae treated with rotenone alone was higher than from those treated with rotenone + cinnamon oil. In the group treated with rotenone, the highest rotenone content in the excreta was 177.36 mg/kg. The highest concentrations of rotenone in the midgut, hemolymph, and ventral nerve cord were 29.44, 4.86, and 1.20 mg/kg, respectively.

Table 3. Recoveries and relative standard deviation of rotenone from fortified samples.

Substrate	Fortification level (mg/kg)	Mean recovery (%)	Relative standard deviation (%)
Hemolymph	0.5	91.77	6.52
	1.0	80.15	3.43
	2.0	110.46	7.21
Midgut	0.5	113.09	3.00
	1.0	87.66	12.79
	2.0	102.32	7.22
Ventral nerve cord	0.5	100.23	4.01
	1.0	111.67	11.40
	2.0	86.97	7.44
Brain	0.5	92.35	7.44
	1.0	91.88	5.35
	2.0	84.30	9.55
Excreta	0.5	104.31	2.02
	1.0	77.76	12.56
	2.0	92.62	3.49

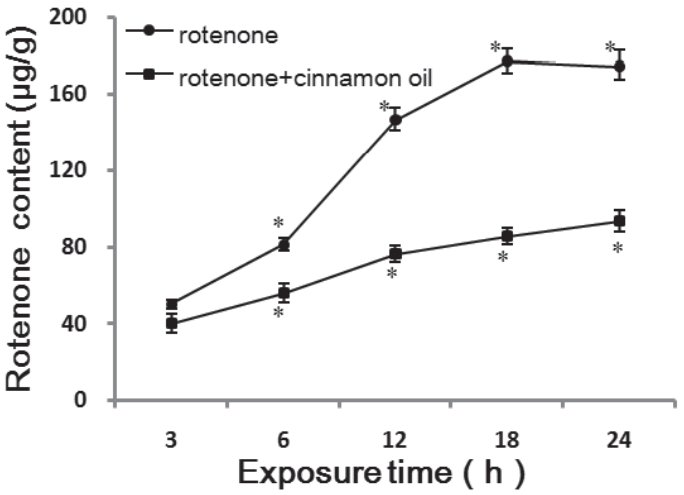


Fig. 2. The concentration of rotenone in excreta after treatment.* indicates significant difference between the 2 treatments at the same point in time ($P < 0.05$, Tukey honest significant difference tests).

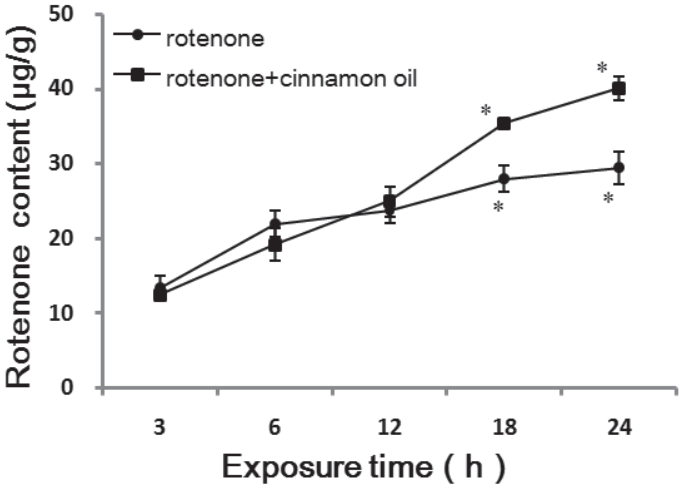


Fig. 3. The concentration of rotenone in midgut tissue after treatment.* indicates significant difference between the 2 treatments at the same point in time ($P < 0.05$, Tukey honest significant difference tests).

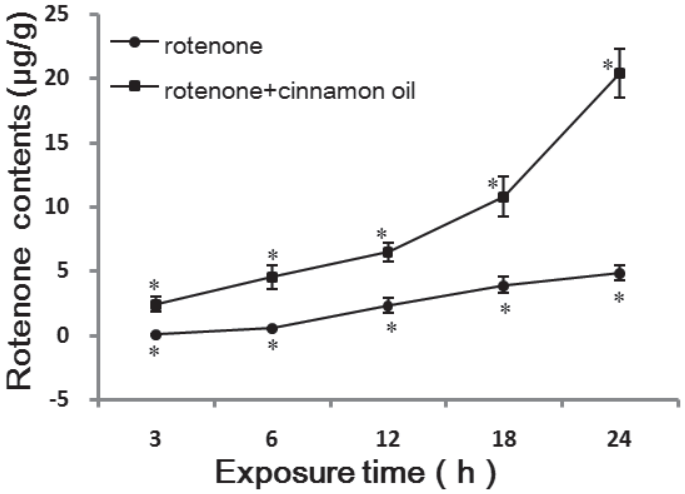


Fig. 4. The concentration of rotenone in hemolymph after treatment.* indicates significant difference between the 2 treatments at the same point in time ($P < 0.05$, Tukey honest significant difference tests).

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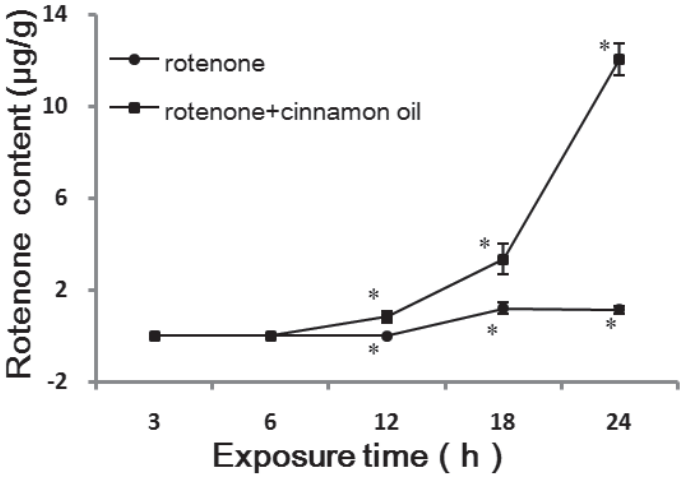


Fig. 5. The concentration of rotenone in ventral nerve cord tissue after treatment.* indicates significant difference between the 2 treatments at the same point in time ($P < 0.05$, Tukey honest significant difference tests).

The rotenone contents in the brain were below the detection limit. In strong contrast, the rotenone contents in the ventral nerve cord and brain of larvae treated with rotenone + cinnamon oil were significantly higher. The rotenone contents in the larval tissues treated with 1,000 µg/mL rotenone + cinnamon oil were (in descending order): excreta (93.77 mg/kg), midgut (40.11 mg/kg), hemolymph (20.40 mg/kg), ventral nerve cord (12.07 mg/kg), and brain (8.74 mg/kg).

STRUCTURE AND ORGANIZATION OF MIDGUT CELLS

Larval midgut cell structure and organization from the control, rotenone, and rotenone + cinnamon oil treatment groups were examined for differences. Figure 7 shows the images of the treated and untreated *S. litura* peritrophic membrane. The cells of *S. litura* peritrophic membrane in the control group were single, tightly packed, and clearly visible (Fig. 7A). Other than the slightly wider cell spacing, the cells of the peritrophic membrane in the rotenone treatment group (Fig. 7B) were similar to those in the control. However, the cells in the rotenone + cinnamon oil treatment group were different; cell spacing was wider, and the cell membranes were abnormal (Fig. 7C).

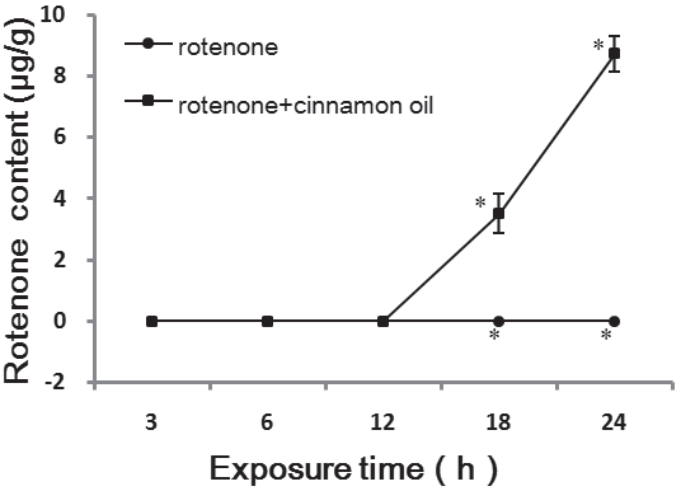


Fig. 6. The concentration of rotenone in brain tissue after treatment.* indicates significant difference between the 2 treatments at the same point in time ($P < 0.05$, Tukey honest significant difference tests).

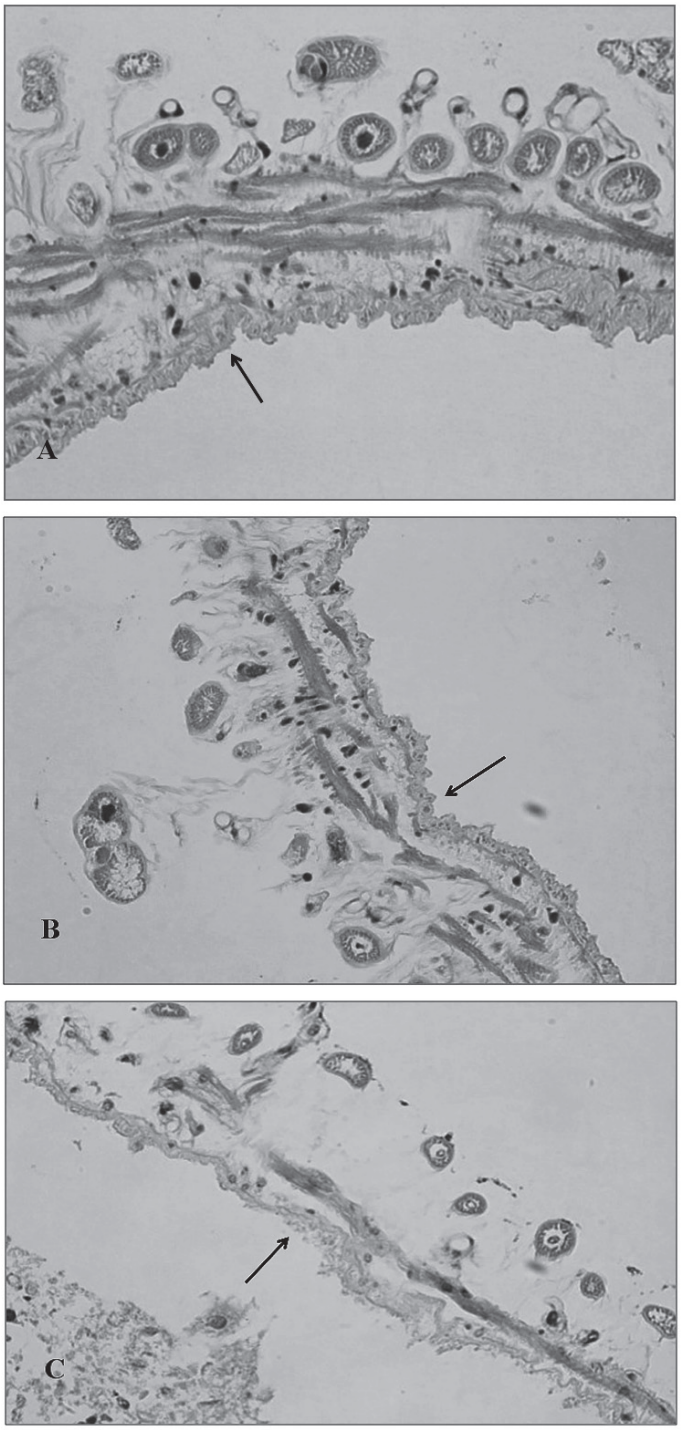


Fig. 7. Cells of *Spodoptera litura* midgut peritrophic membrane (A: control, B: rotenone, C: rotenone + cinnamon oil). The arrows show the change in cell structure in response to treatment. Note that in A the cells are single, packed, and clearly visible, whereas in B the cell spacing is wider, and in C there is slightly wider cell spacing, and abnormality of the membrane.

Discussion

Similarly to Zhu et al. (2006), this study showed the inefficiency of rotenone, when used alone, to poison the larvae of *S. litura*. Toxicity of stomach poisons may be due to 1 or more of the following: low effective intake, inefficient penetration or inactivation of midgut cells followed by excretion, and detoxification of related enzymes (Wilson

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2001; Aizoun et al. 2013). Rotenone is not known to be repellent to larvae of *S. litura*. In this study, we found that rotenone alone was not very toxic to larvae of *S. litura.* Rotenone, as a mitochondrial complex I inhibitor, could induce oxidative stress and cell death (Shao et al. 2015). In this case, the low toxicity of rotenone to *S. litura* is attributed to the inability of the chemical to penetrate the midgut cells, or the occurrence of chemical degradation before reaching the target site.

This study developed a method to monitor rotenone content in several larval tissues. A considerable amount of rotenone was discharged in excreta; low levels were observed in hemolymph, and extremely low levels were found in the ventral nerve cord and brain after rotenone treatment. This suggests that rotenone, applied alone, was poorly absorbed by the midgut of *S. litura*, and explains the low toxicity of rotenone to *S. litura*.

Insecticide synergists play a significant role in enhancing the insect control potential of active ingredients by broadening their bioactivity spectrum, countering resistance development, increasing effective commercial lives, and mitigating the residual effects of persistent and highly toxic products by reducing application dosage (Walia et al. 2004). Synergists, including mixed function oxidase, diethyl maleate, piperonylbutoxide, triphenyl phosphate, and S,S,S-tributylphosphorotrithioate, have long been used with insecticides to control pests (Pasay et al. 2009; Sun et al. 2012). In this study, cinnamon oil obtained from plants was used as a synergist to enhance the insect control potential of rotenone.

We demonstrated that the LC_{50} of rotenone + cinnamon oil was significantly lower than that of rotenone or cinnamon oil used alone. This result was similar to that of Tong and Bloomquist (2013), who found that several plant essential oils show significant synergistic effects with carbaryl. Cinnamon oil has been used in several toothpastes as an antimicrobial substitute (Kalia et al. 2015). Thus, this oil is relatively safe for human consumption.

Cinnamon oil appears to have increased the insecticidal activity of rotenone by increasing the penetrability of the midgut cells and causing increased retention of rotenone in the hemolymph, ventral nerve cord, and brain. Optical microscopy showed disruption of the midgut cell structure when cinnamon oil was added to rotenone, increasing the insecticidal activity of rotenone to *S. litura*.

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