Bioassay and expression alterations of *acetyl cholinesterase enzyme* gene to spinosad (bio-insecticides) on nontarget silkworm, *Bombyx mori* (Lepidoptera: Bombycidae)

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Background
Bombyx mori, the mulberry silkworm, feeds entirely on mulberry leaves and is extremely sensitive to agrochemicals, even in low doses. The mulberry plantations must be insecticide-free. However, contamination by pesticides from neighboring crops occurs indirectly and harms silkworm breeding. Spinosad, a neurotoxic insecticide that acts on the nervous system of insects through contact or feeding, is the most environmentally friendly suitable bioinsecticide. It has been used to control pests in field crops.

Objective
In this study, the insecticide, spinosad formulation was first tested for bioassay, utilizing three different concentrations of spinosad on *B. mori* larvae. Second, the total RNA was isolated (isolation of total RNA) from silkworm, *B. mori* larvae to study the spinosad effect on *acetylcholinesterase* (*Ace*) gene expression.

Materials and methods
The type of insecticide used in this study is the spinosad formulation. Spinosad is available under the commercial name, Biosad 22.8% SC; the recommended concentration is 0.1 ppm. Bioassay test was done with three different concentrations of spinosad (0.1, 0.05, and 0.025 ppm). Determination of the LC values of the toxicity of three concentrations of spinosad on the fifth instar larvae of *B. mori* was evaluated using the mulberry leaves dipping technique. The treated mulberry leaves were offered once on the first day of the fifth instar after morning feeds, then the fresh leaves were offered during the remaining days. After 24 h of treatment, the mortality counts were recorded. LC_{25}, LC_{50}, and LC_{90} values for spinosad were calculated by probit analysis using the Ldp line software. Total RNA was isolated from entire tissues of the fifth instar larvae of the silkworm, *B. mori* by the standard TRIzol reagent extraction method. The complete Poly $(A)^+$RNA isolated from insect tissues was reverse transcribed into cDNA. The sequence of primers of apoptosis is used in real-time quantitative PCR reactions to determine the expression levels of *Ace*-related gene.

Results and conclusion
Spinosad is the most economically and ecologically recommended insecticide to be used to control the agricultural pests that attack different field crops in Egypt. The toxicological effects of spinosad and its effect on the *Ace* gene of mulberry silkworm, *B. mori* were studied in this study. The results showed that treatment with 0.1 ppm of spinosad caused the highest mortality (88.9%) to the fifth instar larvae of *B. mori*, followed by the spinosad concentrations 0.05 and 0.025 ppm. The results showed a significant difference in LC values of spinosad on the fifth instar of *B. mori*. LC_{25}, LC_{50}, LC_{75}, and LC_{90} values were recorded to be 0.008, 0.0217, 0.0536, and 0.1969 ppm, respectively. The expression levels of *Ace* gene in the *B. mori* group treated with low (0.025 ppm) and medium doses (0.05 ppm) of spinosad were increased by 141 and 396%, respectively. However, the expression level of *Ace* gene was increased by 657% for the group exposed to high doses (0.1 ppm) of spinosad compared with the control group with highly significant differences ($P<0.01$). This study confirmed that using spinosad with the lowest concentrations and caused damage occurs to silkworms when feeding on sprayed mulberry leaves with a minimum of spinosad compound, so it is a better solution to not spray spinosad on mulberry trees and any neighboring fields.

Keywords:
acetylcholinesterase *gene* expression, insecticides, silkworm, spinosad

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Introduction

Bombyx mori is the scientific name of the mulberry silkworm that feeds entirely on mulberry leaves and is extremely sensitive to agrochemicals, even in low doses. The mulberry plantations must be insecticide free. However, contamination by pesticides from neighboring crops occurs indirectly and harms silkworm breeding. Spinosad, a neurotoxic insecticide that acts on the nervous system of insects through contact or feeding, is the most environmentally friendly suitable bioinsecticide. It has been used to control pests in field crops. Spinosyns are fermentation byproducts of one or more chemical mutants of the naturally occurring actinomycetes soil bacterium *Saccharopolyspora spinosa* [1]. Spinosad has been used on more than 200 different crops. It has been used to control caterpillars in cotton plants, apple leafrollers, leaf miners in various plants, cabbage loopers, citrus thrips, and other pests [2]. Spinosad disrupts the nicotinic acetylcholine receptors and GABA-gated ion channels, causing rapid nervous system excitation and muscle contraction, which cause paralysis and finally death of insects [3]. In insect brains, acetylcholine (Ach) is the most prevalent excitatory neurotransmitter, while nicotinic receptors are the most prevalent cholinergic receptors (nAChRs). Insect nicotinic receptors (nAChRs) are a target for different classes of insecticides [4].

The *acetylcholinesterase* (*Ace*) gene provides instructions for making the angiotensin-converting enzyme. The Ace plays important roles in many vital functions in the insect body such as physiological processes. The function of Ace-like proteins is largely unknown. After the first cloning of the insect *AChE* gene (*Ace*) from *Drosophila melanogaster* [5], extensive research using the ace from different insects to more than 30 insects and acarus species have been sequenced. The second ace has been found in various insects, such as mosquitoes [6], aphids [7], and lepidopteran [1]. The role of two AChEs arrives more complicated in insects, because some insects have only one type of ace in their genomes, while many other insects have two types of ace and also, the overlapping or nonoverlapping and mutations responsible to insecticides such as resistance to organophosphorus (OP) and carbamate compounds.

The silkworm is the third insect to have its genome sequenced after the genome of *D. melanogaster* and *Anopheles gambiae*. It is a model for lepidopteran genomics, addition to an important economical insect to the production of silk. AChE activity in silkworm is affected by different insecticides such as organophosphorus insecticides [8].

The present work dealt with the alterations of *Ace* gene expression and some bioassay of mulberry silkworm, *B. mori* with different treated concentrations of spinosad.

The current study examined *Ace* gene expression changes and some bioassays of mulberry silkworm, *B. mori* tissue as a result of treatment with the recommended concentration of spinosad, or its half and quarter concentrations. It was conducted in the laboratories of the Sericulture Research Department, Plant Protection Research Institute Sharkia Branch, ARC, and Microbial Genetics, NRC, Egypt.

Materials and methods

The mulberry silkworm, *B. mori* eggs were obtained from the Sericulture Research Department of Plant Protection Research Institute (PPRI), Agriculture Research Center (ARC), Giza, Egypt, and kept under laboratory conditions of 25±2°C and 75±5% RH according to the techniques of Krishnaswami [9]. Fresh clean mulberry leaves were offered to the larvae from hatching to pupation four times/day. The fifth instar larvae were used in this study and after the fourth molting the larvae were divided into control and three groups treated with three concentrations of spinosad; each group contained 10 larvae. Three replicates were used for each treatment.

(1) Insecticides used in this study: the spinosad formulation used under the commercial name, Biosad 22.8% SC, at a recommended concentration of 0.1 ppm.
(2) Bioassay test: three different concentrations of spinosad were used (0.1, 0.05, and 0.025 ppm). The mortality was corrected by Abbott formula [10].
(3) Determination of LC values: the toxicity of three concentrations of spinosad on the fourth instar larvae of *B. mori* was measured using the mulberry leaves dipping technique according to Shepard [11]. The treated mulberry leaves were offered once on the first day of the fifth instar after morning feeds and then the fresh leaves were offered during the remaining days. After 24 h of treatment, the mortality counts were recorded. LC_{25}, LC_{50}, and LC_{90} values for spinosad were calculated by probit analysis using the Ldp line software according to Finney [12].

Isolation of total RNA of larvae

The conventional TRIzol reagent extraction procedure was used to separate total RNA from all the tissues of *B. mori*, a silkworm larval in its fifth instar (Invitrogen,

Germany). Briefly, 50–100 mg of tissue samples were homogenized in 1 ml of TRIzol reagent. The homogenized material was then incubated at room temperature for 15 min. For every 1 ml of TRIzol reagent, 0.2 ml of chloroform was added. The samples were then violently vortexed for 15 s and incubated for 3 min at room temperature. The samples were centrifuged at 4°C for 15 min at a maximum of 12.000*g*. After centrifugation, the mixture was divided into a colorless upper aqueous phase and a lower red, phenol–chloroform phase. Only RNA continued to exist in the aqueous phase. As a result, the upper aqueous phase was carefully moved into a new tube without affecting the interphase. By combining isopropyl alcohol with the aqueous phase, the RNA was precipitated. For every 1 ml of the TRIzol reagent that was used for the first homogenization, 0.5 ml of isopropyl alcohol was added. Following a 15-s vigorous vortex, the samples were incubated for 3 min at room temperature. The samples were centrifuged for no longer than 15 min at a speed of no more than 12.000*g*. After centrifugation, the mixture was divided into a colorless upper aqueous phase, a lower red phenol–chloroform phase, an interphase, and a phenol–chloroform phase. Only the aqueous phase continued to contain RNA. To avoid affecting the interphase, the upper aqueous phase was carefully transferred into a new tube. By combining with isopropyl alcohol, the RNA was precipitated from the aqueous phase. Each 1 ml of TRIzol reagent used for the initial homogenization received an addition of 0.5 ml of isopropyl alcohol. To digest DNA leftovers, total RNA was treated with 1 U of RQ1 RNAse-free DNAse from Invitrogen, then resuspended in DEPC-treated water. The 260/280 nm ratio was used to determine how pure the total RNA was (between 1.8 and 2.1). Ethidium bromide-stain examination of the 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis further ensured integrity. Reverse transcription (RT) aliquots were used right away; otherwise, they were kept at −80°C.

Reverse transcription reaction

RevertAidTM First Strand cDNA Synthesis Kit was used to reverse transcribe the whole Poly(A)+ RNA recovered from insect tissues into cDNA in a total volume of 20 l (MBI Fermentas, Germany). A reaction combination known as the master mix was used with a quantity of total RNA (5 g) (MM). The MM contained 50 mM $MgCl_2$, 5× RT buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3), 10 mM of each dNTP, 50 M of oligo-dT primer, 20 U ribonuclease inhibitor (50 kD recombinant enzyme to block RNase activity), and 50 U M-MuLV reverse transcriptase. The mixture of each sample was placed in a thermocycler (Biometra GmbH, Göttingen, Germany) and centrifuged at 1000*g* for 30 s. The RT reaction was run for 10 min at 25°C, for 1 h at 42°C, then for 5 min at 99°C to terminate the reaction. Before being used for real-time PCR-based DNA amplification, the reaction tubes holding the RT preparations were flash-cooled in an ice chamber (RT-PCR).

Real time-PCR

Step One Thermo Fisher Scientific, Waltham, Massachusetts, USA real-time PCR system was utilized to ascertain the insect's cDNA copy number. The following ingredients were used to set up the PCR reactions: 12.5 µl of 1 SYBR Premix Ex TaqTM (TaKaRa; Biotech. Co Ltd, Shiga, Japan), 0.5 µl of 0.2 M sense primer, 0.5 µl of 0.2 M antisense primer, 6.5 µl of distilled water, and 5 µl of cDNA template.

The reaction program has three steps assigned to it. The first phase lasted for 3 min at 95.0°C. The second phase was made up of 40 cycles, with each cycle having three steps: (a) 15 s at 95°C, (b) 30 s at 55°C, and (c) 30 s at 72.0°C. The third stage was made up of 71 cycles that began at 60.0°C and grew by roughly 0.5°C every 10 s until reaching 95.0°C. A melting curve analysis was carried out at 95.0°C at the conclusion of each qRT-PCR to evaluate the effectiveness of the primers utilized. There was a distilled water control in each experiment. The sequences of specific primers for the genes used, as per Macours *et al.* [13], are presented in Table 1. To evaluate the quality of the employed primers, a melting curve analysis was carried out at 95.0°C at the conclusion of each qPCR. Using the $2^{-\Delta\Delta CT}$ method, the following relative quantification of the target to the reference was established:

$$\Delta C_{Ttest=}C_T(\text{target, test})^{-}C_T(\text{reference, test}).$$

$$\Delta CT_{(\text{calibrator})=}C_T(\text{target, calibrator})^{-}C_T(\text{reference, calibrator}).$$

$$\Delta\Delta CT=\Delta C_{T(\text{Test})}{}^{-}\Delta C_{T(\text{calibrator})}.$$

Table 1 Sequence of primers used in real-time quantitative PCR reactions to determine the expression levels of *Acetylcholinesterase*-related genes

Primer name	Abbreviation	Primer sequence (5'–3')
ß-actin forward	ß-ActF	AGG AGA TGG CTA CTG CTG CA
ß-actin reverse	ß-ActR	GAA CAG TGC CTC AGG TAC C
Ace forward	Ace F	CCGGTACTTTTGCTCGATCTTG
Ace reverse	Ace R	ATGCCATTATATCTGCTTTATATAG

Ace, acetylcholinesterase.

Statistical analysis

All data obtained from biochemical and molecular genetic studies were expressed as means±SEM. The data were investigated with the Statistical Package for the Social Sciences (SPSS 0.26 for Windows, Barkley Co., United States of America). Outcomes were dissected utilizing one route investigation of difference (analysis of variance) trailed by Duncan's test for examination between various treatment gatherings, and the statistical significance was set at *P* value less than 0.05.

Results

Bioassay studies

The data in Table 2 and Fig. 1 showed that the highest mortality was 88.9% with 0.1 ppm of spinosad on the fifth instar of *B. mori*, while half and quarter concentrations of spinosad were 69.8 and 56.1%, respectively. The slope was 1.7167±0.3343.

LC_{25}, LC_{50}, LC_{75}, and LC_{90} values of spinosad on the fifth instar of *B. mori* were 0.008, 0.0217, 0.0536, and 0.1969 ppm %, respectively (Table 3). These results show a significant difference in LC values.

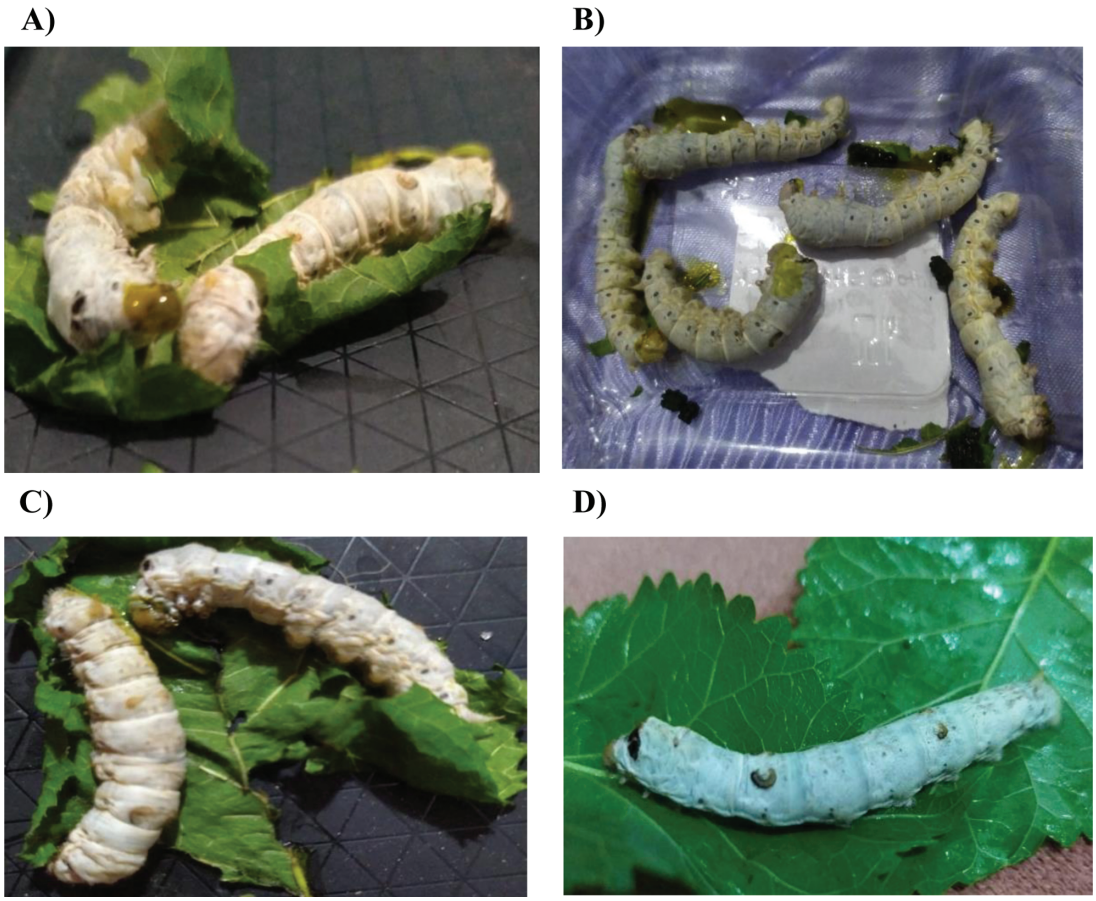
Symptomology

Studying the toxic effects of the bioinsecticide, spinosad on the model silkworm *B. mori* can give us a good reference for environmental special hazards. After spinosad treatment with different concentrations the larvae of *B. mori* show some morphological symptoms, such as vomiting green fluids from their

Table 2 Toxicity of three concentrations of spinosad on the fifth instar of *Bombyx mori*

Concentrations (ppm)	Log (conc.)	Mortality%	Linear response %	Linear probit	Slope±SE
0.025	0.3979	56.1	54.228	5.1062	1.7167±0.3343
0.05	0.699	69.8	73.3367	5.6231	
0.1	1	88.9	87.2759	6.1398	

Figure 1



(A) Larvae after 3 hours treatment with 0.1% spinosad, (B) Larvae after 6 hours treatment with 0.1% spinosad, (C) Larvae after 9 hours treatment with 0.1% spinosad, (D) Control larva without treatment.

mouths, lying on the side of their bodies, and finally body shrinking occurs due to contraction of the muscles or loss of their body fluids.

Ace gene expression changes in insect tissues after exposure to various biological pesticide dosages (spinosad)

Ace gene expression changes in insect tissues after exposure to various biological pesticide dosages (spinosad) are analyzed in Fig. 2. *Ace* gene is a key enzyme in the insect nervous system (terminates nerve impulse by catalyzing the hydrolysis of the neurotransmitter acetyl choline). Results showed that the treatment of *B. mori* with a low dose of spinosad (0.025 ppm) increased the expression levels of *Ace gene* (by 141%) compared with the control group but without significant differences. Using a medium dose (0.05 ppm) of spinosad increased the expression levels of *Ace gene* (by 396%) significantly ($P<0.05$) as compared with the control group. Using high doses (0.1 ppm) of spinosad increased the expression levels of *Ace* gene (by 657%) significantly ($P<0.01$) as compared with the control group.

According to the obtained results from the treatment of spinosad on silkworm larvae, we can say that its use negatively affected the larvae of the mulberry silkworm even with the lowest concentration that was used, and led to large mortality rates. *Ace* levels, the key enzyme in the insect nervous system, increased dramatically, and it is necessary not to expand using the spinosad as a safe alternative to pesticides because of its deadly effect on the mulberry silkworm, as it is considered a model and any negative response from it toward any of the substances or pesticides will be indicative that the substance is dangerous for humans if they are exposed to it, whether by contact or ingestion of foodstuffs exposed to this biocidal compound. Therefore, it is necessary to conduct research in the future to study the negative impact of commercial spinosad preparations on humans, beneficial organisms, and the surrounding environment.

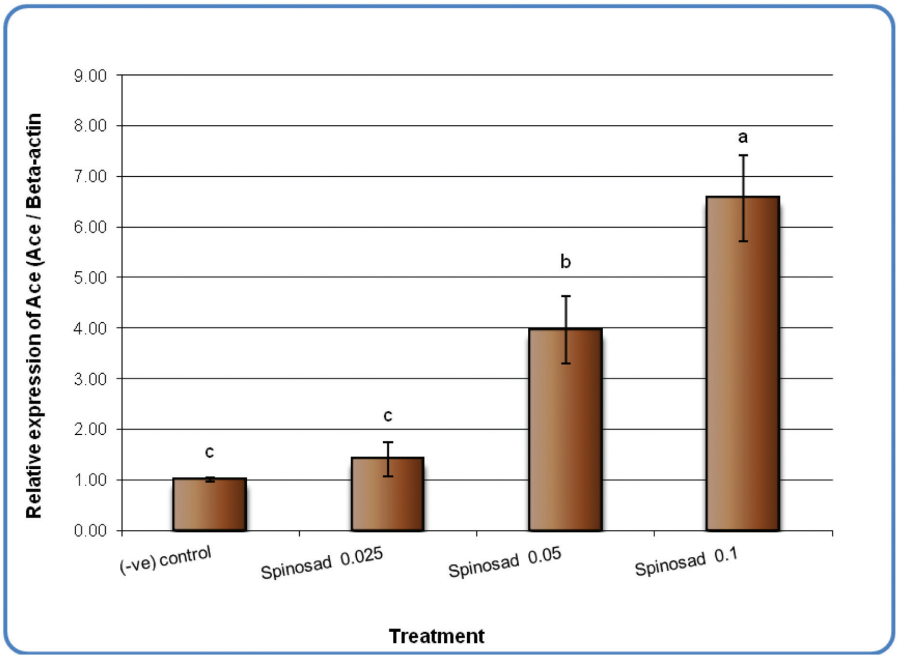
Table 3 Lethal concentrations of spinosad on the fifth instar of *Bombyx mori*

LC (values)	Conc. (ppm)	Lower limit (ppm)	Upper limit (ppm)
25	0.0088	0.0031	0.0143
50	0.0217	0.0129	0.0285
75	0.0536	0.0434	0.0702
90	0.1969	0.0871	0.2353

Discussion

Based on the above results, spinosad caused high mortality for silkworm, *B. mori* which are similar to the finding of Johnson and Gnanadhas [14], who stated that the silkworms were affected by pesticides in many different ways, acute toxicity, or sublethal

Figure 2



Ace gene expression changes in insect tissues after exposure to various biological pesticide dosages (spinosad). Data are displayed as mean ±SEM. [a,b,c]Mean values within tissues and mean values with dissimilar superscript letters showed a statistically significant difference (a: $P=0.01$, b: $P=0.05$).

effects leading to the impairment of silk production and quality. Kuwana and Sugiyama [15] concluded to avoid mortality in silkworm; the applications of insecticides must be at the minutest doses and this is reliable to the current study.

The results were in harmony with Xu *et al.* [16], who reported that the toxicity of 25 g/l spinosad SC to silkworms was very high. Also, Nagat and Doaa [17] mentioned that the fifth instar larval mortality percentages increased, where data ranged between 18.30% in Cymbush and 10.21% in Tracer compared with 9.93% in control.

The high doses of spinosad 48 SC to silkworm with 100 g a.i/ha caused 45 and 43.4% mortality of silkworm larvae, while the lowest doses caused less mortality to silkworms [18].

Satish [19] studied the selective toxicity of some insecticides to silkworm under laboratory conditions; first and third larval instars were used in the study. Regarding the toxicity of eight insecticides they noticed that spinosad 45 SC caused mortality (89.96%) after 24 h for first and third instar larvae. Those results agreed with the present study.

The silkworm, *B. mori*, is an important model organism, which shows relatively weak resistance to circumstances and disease, and it is especially sensitive to chemical pesticides, heavy metals, and any harmful substances [20].

Results in this study revealed that the treatment of *B. mori* with a low dose of spinosad (0.025 ppm)increased the expression levels of *Ace gene* (by 141%) compared with the control group but without significant differences. Using a medium dose (0.05 ppm) of spinosad, increased the expression levels of *Ace* gene (by 396%) significantly ($P<0.05$) compared with the control group. However, using a high dose (0.1 ppm) of spinosad increased the expression levels of *Ace* gene (by 657%) significantly ($P<0.01$) compared with the control group after 24 h.

The expression levels of *Ace* gene in the group treated with a low dose (0.025 ppm) of spinosad were increased as the concentration increased. Similar results were recorded by Megahed *et al.* [21], who observed the highest level of change percentage of *Ace* activity after 24 h to leaf worm, *Spodoptera littoralis* by spinosad, while the lowest level after 72 h using doses of 0.17, 0.23, and 38 ppm. The natural pesticide spinosad is considered an alternative biocide; however, it is still unclear what causes sublethal spinosad exposure to have developmental impacts. Shuangyan *et al.* [22] explored the process using an insect model of *Helicoverpa armigera* and found that exposure to sublethal spinosad doses decreased larval wet weight, prolonged the larval developmental stage, made molting difficult, and resulted in malformed pupae. Further research revealed that sublethal spinosad exposure significantly altered the expression levels of hormone receptor 3 (HR3) and Krüppel homolog 1 (Kr-h1) by causing a decline in 20E titer and an increase in JH titer. This resulted in the discordance between 20E and JH titers. The nAChR α6 subunit was confirmed as the target of spinosyns in *P*lutella *xylostella* [23,24], *D. melanogaster* [25], *H. armigera* [23,24], and *S*podoptera *exigua* [26] using CRISPR/Cas9-mediated genome editing technology.

Conclusion

It has been found that the spinosad caused high mortality for silkworm, *B. mori* and the treatment of *B. mori* with the lowest dose of spinosad increased the expression levels of *Ace gene*. *Ace* levels, the key enzyme in the insect nervous system, cause serious damage to silkworm when feeding on sprayed mulberry leaves with a minimum of spinosad compound, so it is better not to spray spinosad on mulberry trees and any neighboring fields.

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Conflicts of interest

There are no conflicts of interest.

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