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Resistance selection and biochemical characterization of spinosad resistance in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae)

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ABSTRACT

A *Helicoverpa armigera* population was collected from Shandong province, China. After 15 generations of selection in the laboratory, the *H. armigera* strain developed more than 20-fold resistance to spinosad. At LD_{50} level, no significant cross-resistance was found between spinosad and chlorpyrifos, methomyl, avermectin and chlorfenapyr except for fenvalerate with a low cross-resistance of 2.4-fold. However, LD_{99} values of fenvalerate against the parental and resistant strains were not different significantly. After inhibitors were used, spinosad resistance could be partially suppressed by piperonylbutoxide (PBO) and triphenylphosphate (TPP), but not by diethylmaleate (DEM). Activities of *p*-nitroanisole *O*-demethylase (ODM) developed to 8.26-fold compared with the parental strain, but no obvious changes were found in activities of carboxyl esterase (CarE) and glutathione-*S*-transferase (GST). The results indicated that resistance to spinosad in the cotton bollworm might be associated with an increase in cytochrome P450 monooxygenase.

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1. Introduction

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is the world insect pests on a wide range of agricultural, ornamental, vegetable, fruit and storage crops [1]. In China, it has achieved major pest status in cotton, maize and greenhouse crops like tomato, pepper and so on because of high polyphagy, wide geographical range, migratory potential and high fecundity [2]. Since its outbreak in 1980, abusage and overuse of pesticides have resulted in high resistance of *H. armigera* against many conventional insecticides such as organochlorine, organophosphate, carbamates and pyrethroid insecticides [3–6]. Some insecticides with novel modes of action have been introduced for controlling the pest effectively including spinosad.

Spinosad, a mixture of spinosyns A and D, is derived from the naturally occurring soil actinomycete *Saccharopolyspora spinosa* [7]. Because of its unique action mechanism, spinosad has strong insecticidal activity especially against Lepidoptera larvae with low levels of mammalian toxicity and relatively little toxicity to non-target insects [8,9]. However, any insecticide can develop resistance in target insects from the insight of organic evolution. At present, several insects have exhibited ascending resistance to spinosad in field populations [10–14]. Cross-resistance to spinosad has been documented in *Spodoptera litura*, *Plutella xylostella* and *Lucilia cuprina* [15–17]. However, information about spinosad resistance mechanism in *H. armigera* is almost absent despite a low level of spinosad resistance existed in some field populations [10,18,19].

In order to explore strategies for resistance management and prolong the useful life of spinosad, we selected a laboratory strain of the cotton bollworm, *H. armigera*, with topically applied technical spinosad. We herein present the results of our characterization of resistance level of the selected strain, cross-resistance against other pesticides and possible mechanisms of resistance.

2. Materials and methods

2.1. Insects

The *H. armigera* larvae were collected from Shandong Province, China in 2007, and maintained in the laboratory under insecticide-free conditions. The larvae were kept in an insectarium at 27 ± 1 °C and about 60% RH with a 14 h photoperiod until pupation. After emergence, adult males and females were collected and released into a 40 × 40 × 40 cm cage for mating and egg-laying under the same condition and supplied with a 10% honey solution.

2.2. Chemicals

Technical grade of 90.4% spinosad was from DowAgro Sciences. Methomyl (95.2%), fenvalerate (95%) and chlorpyrifos (95.9%) were

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from huayang pesticide factory (Shandong, China). Chlorfenapyr (96.4%) and avermectin (98%) were given by shuangxing pesticide factory (Shandong, China). Bovine serum albumin (BSA), triphenylphosphate (TPP), 98.5% diethylmaleate (DEM) and 95% piperonylbutoxide (PBO) were purchased from Shanghai Bio Life Science & Technology Co., Ltd., China. About 97% 4-nitroanisole (PNA) was from Sigma, Germany. α-Naphthol was purchased from Tianjin Chemical Factory, China. NADPH and 98% glutathione (GSH) were from Beijing Solarbio Science & Technology Co., Ltd., China. *p*-Nitrophenol (PNP), α-naphthyl acetate (α-NA), 2,4-dinitrochlorobenzene (CDNB) and coomassie brilliant blue G-250 were purchased from the Shanghai Chemical Factory, China.

2.3. Establishment of the resistant strain

For selecting the resistant strain of *H. armigera*, technical grade spinosad was dissolved in acetone and applied topically on the thoracic notum of fourth-instar larvae with selection pressure to kill about 50% individuals of the population. The survival larvae were transferred into the plastic box with an artificial diet, consisting of wheat germ (150 g), corn meal (150 g), soybean flour (100 g), cotton leaf meal (17.5 g), yeast (40 g), ascorbic acid (5 g), sodium benzoate (1.5 g), sorbic acid (2.5 g), streptomycin sulfate (1 g), 10% formalin (1.5 ml), cottonseed oil (5 ml) and agar (17.5 g). After several generations of treatment, the concentration of spinosad should be increased, so as to maintain the selection pressure at about 50%. In order to understand the trend of spinosad resistance, toxicity test was carried out every two generations to calculate its LD_{50} (50% lethal concentration) at 72 h after treatment due to the slower acting nature.

2.4. Bioassay of insecticides

The topical application recommended by the United Nations Food and Agriculture Organization (FAO) was adopted for bioassay method [20]. Insecticides for bioassay including spinosad, chlorpyrifos, methomyl, fenvalerate, avermectin and chlorfenapyr were applied with 1.0 μl per drop to the thoracic notum of fourth-instar larvae. For each treatment, three replicates with 30 individuals per replication were set up. Larvae were maintained in a 10-well culture plate singly in an insectarium at 27 ± 1 °C with a 14:10 (L:D) photoperiod. Mortality was assessed after 48 h, except for spinosad, which was assessed after 72 h due to the slower acting nature. Larvae were considered dead if unable to move in a coordinated way when prodded with a fine-haired brush. The data were analyzed by probit analysis, using the SPSS program.

2.5. Synergism studies

Triphenylphosphate (TPP), diethylmaleate (DEM) or piperonylbutoxide (PBO) was applied at 1.0 μg per larva to the thoracic notum of fourth-instar larva 1 h before application of spinosad. Mortalities were assessed after 48 and 72 h, respectively. LD_{50} values were determined by probit analysis, using the SPSS program.

2.6. Enzyme assays

2.6.1. Carboxyl esterase (CarE)

CarE activity was measured using α-NA as substrate based on the method described by Li et al. (2007) [21]. Fourth-instar larvae were collected and homogenized in 10 ml phosphate buffer (40 mM, pH 7.0) on ice. The homogenate was centrifuged at 10,000 rpm for 20 min at 4 °C and the supernatant was collected as enzyme source. Substrate solution containing 3×10^{-4} α-NA and 3×10^{-6} physostigmine, an inhibitor of acetylcholinesterase, was incubated for 5 min at 25 °C. The mixture was incubated with shaking for 30 min at 30 °C after 1 ml enzymes were added. The reaction was stopped by addition of 1 ml distilled water containing 0.01 mg fast blue B salt. Absorbance at 600 nm was read against blanks after 30 min. The activity of CarE was obtained using production α-naphthol according to an experimentally determined standard curve.

2.6.2. Glutathione-S-transferase (GST)

GST activity was measured using CDNB as substrate by the method of Habig et al. (1974) [22]. Fourth-instar larvae were homogenized in 10 ml phosphate buffer (66 mM, pH 7.0) on ice. After centrifugalization of homogenate, the supernatant was collected as enzyme solution. The reaction of mixture including enzyme solution, CDNB and PBS buffer was initiated by adding GSH. Enzyme activity was measured in a spectrophotometer (UV 2201) at 340 nm and 25 °C using the kinetic mode for 5 min. The activity of GST was determined using the extinction coefficient of 9.6 mM^{-1} cm^{-1} for CDNB.

2.6.3. p-Nitroanisole O-demethylase (ODM)

ODM activity was assayed according to the method of Qiu et al. (2003) [23]. Considering that the monooxygenase activities mostly distributed in midgut and fat body and need more biomass to make enough enzyme preparation, final instar larvae were dissected in 1.15% KCl solution on ice. The midguts were removed and their contents were rinsed in ice-cold sodium phosphate buffer (0.1 M, PH 7.8). Then midguts were homogenized in homogenization buffer on ice. After centrifugalization of homogenate, the supernatant was taken as enzymes. Reactions of mixture including enzyme solution, sodium phosphate buffer and NADPH were initiated by adding *p*-nitroanisole (200 mM) and terminated by the addition of 1 ml HCl (1 M) after incubating with shaking at 25 °C for 30 min. Then the product *p*-nitrophenol was extracted with $CHCl_3$ and centrifuged at 3000 rpm for 15 min. The $CHCl_3$ fraction was back-extracted with NaOH. Absorbance of the NaOH solution at 400 nm was recorded. The activity of ODM was obtained by an experimentally determined standard curve.

2.6.4. Protein assay

Protein content was determined by the method of Bradford (1976) using coomassie brilliant blue G-250 with bovine serum albumin as a standard [24]. The 0.1 ml working enzyme solution was mixed with coomassie blue and then incubated at 25 °C for 2 min. The OD values were measured at 595 nm and converted to protein concentration based on the standard curve.

2.6.5. Data analysis

Data were analyzed as the mean ± standard error (SE) of at least three separate experiments using *t*-test of SPSS program. Non-overlap of 95% confidence limits was the criterion for significance of differences.

3. Results

3.1. Selection of the spinosad-resistant strain

The selection process of the spinosad-resistant strain was shown in Table 1. The development of resistance was slow till the G_7 (seventh generation), which was just about 2.73-fold compared with the G_0 (parental generation). Exponential increase of resistance was found from the G_9 (ninth generation) (Fig. 1). After 15 generations of selection, 72-LD_{50} value of spinosad against the fourth-instar larvae increased significantly, which was from 1.26 to 30.3 μg g^{-1}. The resistance level to spinosad in the selected

Table 1
Selection of resistance to spinosad in *H. armigera.*

Generation	Concentration for resistance selection (mg L^{-1})	No. of larvae for resistance selection	Survival percent (%)	72 h after treatment		
				Slope (±SE)	LD_{50} (μg g^{-1}) (95% FL[a])	RR[b]
G_0	55	1500	43.5	2.83 ± 0.38	1.26 (1.01–1.55)	1.00
G_1	60	1800	40.8	2.84 ± 0.39	1.23 (0.989–1.51)	0.97
G_3	80	1500	43.7	2.13 ± 0.31	1.62 (1.25–2.10)	1.29
G_5	120	1500	45.3	2.43 ± 0.36	2.85 (2.26–3.77)	2.27
G_7	150	1800	48.6	2.56 ± 0.35	3.40 (2.72–4.28)	2.70
G_9	300	2000	46.4	3.84 ± 0.53	9.75 (8.17–11.7)	7.76
G_{11}	400	2000	38.0	3.55 ± 0.49	11.5 (9.58–13.2)	9.17
G_{13}	600	1800	47.2	2.86 ± 0.39	19.3 (15.7–24.0)	15.4
G_{15}	1000	2000	44.8	2.79 ± 0.44	30.3 (24.7–38.2)	24.1

[a] Fiducial limits (from probit analysis).
[b] Resistance ratio = LD_{50} of the resistant strain/LD_{50} of the parental strain.

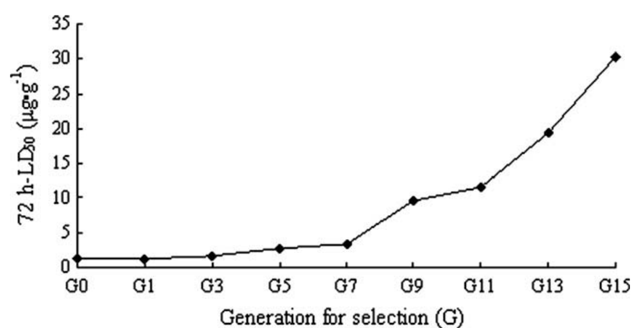

Fig. 1. The development of spinosad resistance in *H. armigera.*

strain increased more than 20-fold compared with the parental strain (Table 1).

3.2. Cross-resistance

There was no cross-resistance existed between spinosad and other pesticides including chlorpyrifos, methomyl, avermectin and chlorfenapyr after toxicity tests by topical application to fourth-instar larvae (Table 2) (chlorpyrifos: $F = 0.487$; df = 4; $p = 0.059$; methomyl: $F = 0.411$; df = 4; $p = 0.055$; avermectin: $F = 0.015$; df = 4; $p = 0.121$; chlorfenapyr: $F = 4.660$; df = 4; $p = 0.405$). At LD_{50} level, low cross-resistance was found between spinosad and fenvalerate with resistance ratio was just 2.40 ($F = 0.018$; df = 4; $p < 0.0001$). However, at LD_{99} level, no statistically difference was found on toxicity of fenvalerate to the parental and resistant strains ($F = 4.738$; df = 4; $p = 0.095$).

3.3. Synergism of TPP, DEM and PBO to spinosad

Pretreatment with TPP and PBO, toxicity of spinosad against the resistant strain increased significantly (Table 3). Take the data obtained at 72 h as examples, LD_{50} values of spinosad decreased from 30.3 to 17.6 μg g^{-1} after treatment with TPP ($F = 0.165$; df = 4; $p = 0.005$). Compared with TPP, PBO increased spinosad toxicity against the resistant strain to a greater extent, with LD_{50} values reducing from 30.3 to 3.97 μg g^{-1}. Significant decrease of resistance ratio was observed in PBO treatment, ranging from 24.1 to 7.66 ($F = 6.985$; df = 4; $p < 0.0001$). However, DEM exhibited no significant effects on toxicity of spinosad ($F = 8.916$; df = 4; $p = 0.396$).

3.4. Enzyme assay

Activities of CarE, GST and ODM in the parental and resistant strains of *H. armigera* were shown in Table 4. No significant change was detected on activities of CarE and GST between both strains (CarE: $F = 3.659$; df = 4; $p = 0.371$; GST: $F = 0.006$; df = 4; $p = 0.124$). Compared with CarE or GST activity, increasing activity of ODM was found in the resistant strain with difference at significant level ($F = 2.985$; df = 4; $p < 0.0001$), which was 8.26 times of that in the parental strain.

4. Discussion

Although spinosad is effective on *H. armigera* larvae [8], its resistance has been documented in this pest of some areas [10,18,19]. Clarification of the resistant mechanism of *H. armigera* to spinosad is important to Integrated Pest Management (IPM) and Insect Resistance Management (IRM). We selected a spinosad-resistant strain of *H. armigera* with topical application in the laboratory. The results showed that the level of resistance increased more than 20-fold compared with the parental strain after 15 generations of selection.

Cross-resistance is a very serious problem associated with management of pests in the IPM program. Knowledge about cross-resistance of spinosad with other pesticides should greatly improve methods of managing resistance and effectively

Table 2
Toxicities of spinosad and other insecticides to the parental and resistant strains of *H. armigera.*

Insecticide	Parental strain		Resistant strain		
	Slope (±SE)	LD_{50} (μg g^{-1}) (95% FL[a])	Slope (±SE)	LD_{50} (μg g^{-1}) (95% FL[a])	RR[b]
Spinosad	2.83 ± 0.38	1.26 A[c] (1.01–1.55)	2.79 ± 0.44	30.3 B (24.7–38.2)	24.1
Chlorpyrifos	2.58 ± 0.35	14.1 A (11.3–17.8)	2.70 ± 0.37	20.7 A (16.7–25.9)	1.46
Methomyl	2.09 ± 0.32	10.6 A (8.20–14.5)	2.58 ± 0.42	16.4 A (13.1–21.7)	1.55
Fenvalerate	3.45 ± 0.45	120 A (101–145)	2.58 ± 0.40	289 B (230–386)	2.40
Avermectin	1.92 ± 0.31	4.47 A (3.40–6.13)	2.09 ± 0.32	6.02 A (4.66–8.07)	1.35
Chlorfenapyr	3.10 ± 0.41	2.90 A (2.37–3.54)	2.54 ± 0.36	2.81 A (2.12–3.60)	0.96

[a] Fiducial limits (from probit analysis).
[b] Resistance ratio = LD_{50} of the resistant strain/LD_{50} of the parental strain.
[c] Means in the same line followed by different letters are significantly different ($p < 0.05$, *t*-test).

Table 3
Toxicity of spinosad to the fourth-instar larvae of *H. armigera* in both strains after synergism.

Compound	Strain	48 h after treatment			72 h after treatment		
		Slope (±SE)	LD_{50} (μg g^{-1}) (95% FL[a])	RR[b]	Slope (±SE)	LD_{50} (μg g^{-1}) (95% FL[a])	RR[b]
Spinosad	S[c]	2.95 ± 0.40	1.39 (1.12–1.70)	1.00	2.83 ± 0.38	1.26 (1.01–1.55)	1.00
	R[d]	2.99 ± 0.45	32.7 (28.1–45.6)	23.6	2.79 ± 0.44	30.3 (24.7–38.2)	24.1
Spinosad + TPP	S	2.78 ± 0.63	1.17 (0.835–1.48)	1.00	2.93 ± 0.66	1.08 (0.765–1.35)	1.00
	R	2.88 ± 0.63	20.3 (16.1–27.1)	17.4	3.23 ± 0.64	17.6 (14.2–22.2)	16.4
Spinosad + DEM	S	2.44 ± 0.60	1.38 (0.990–1.82)	1.00	2.54 ± 0.61	1.18 (0.817–1.53)	1.00
	R	2.81 ± 0.68	29.4 (22.8–47.5)	21.3	2.75 ± 0.67	28.7 (22.2–46.2)	24.2
Spinosad + PBO	S	2.15 ± 0.59	0.592 (0.367–0.797)	1.00	2.49 ± 0.62	0.518 (0.328–0.674)	1.00
	R	2.21 ± 0.65	4.11 (1.70–5.71)	6.94	3.08 ± 0.81	3.97 (2.19–5.16)	7.66

[a] Fiducial limits (from probit analysis).
[b] Resistance ratio = LD_{50} of the resistant strain/LD_{50} of the parental strain.
[c] The parental strain.
[d] The resistant strain.

Table 4
Activities of detoxification enzymes in the parental and resistant strains of *H. armigera*.

Strain	Activity of CarE (mmol min^{-1} mg^{-1} Pr.)	Ratio[a] (R/S)	Activity of GST (μmol min^{-1} mg^{-1} Pr.)	Ratio[a] (R/S)	Activity of ODM (μmol min^{-1} mg^{-1} Pr.)	Ratio[a] (R/S)
S[c]	0.4926 A[b] (±0.01092)	1.00	0.5478 A (±0.008093)	1.00	0.1400 A (±0.009290)	1.00
R[d]	0.5041 A (±0.003167)	1.02	0.5699 A (±0.008018)	1.04	1.157 B (±0.07485)	8.26

[a] Enzymic activity of the resistant strain/enzymic activity of the parental strain.
[b] Means in the same column followed by different letters are significantly different ($p < 0.05$, *t*-test).
[c] The parental strain.
[d] The resistant strain.

controlling the target pests. Our current study showed that selection with spinosad did not increase the resistance to chlorpyrifos, methomyl, avermectin and chlorfenapyr. Similarly, spinosad-resistance strains of *Spodoptera exigua* and housefly have no cross-resistance to abamectin, methomyl and so on [25,26]. Although cross-resistance existed between spinosad and fenvalerate at LD_{50} level, it was marginable which was just 2.4-fold. No statistically significant difference of LD_{99} had been observed on toxicity of fenvalerate to both strains. These results imply that cross-resistance is not a significant factor that could prevent the effective use of spinosad against *H. armigera*. However, spinosad was found cross-resistance existed to indoxacarb and imidacloprid in other insects [15–17]. Similarly, the field population of *Cydia pomonella* had increasing resistance against spinosad in the south of France where spinosad had never been used [27]. These results seem to suggest that reasonable pesticides should not be recommended for rotational use for spinosad resistance management.

Synergisms by PBO and TPP not DEM indicate that rational applications of PBO and TPP may increase the efficacy of spinosad in control of *H. armigera*. Higher synergistic ratio by PBO than TPP was observed, reflecting that cytochrome P450 monooxygenase could play a major role in the resistance of *H. armigera* against spinosad. In our further study, this hypothesis was supported by the fact that significant increase was observed in ODM activity in the resistant strain. However, our results showed that CarE and GST activities in *H. armigera* did not change significantly after 15 generations of selection. Similarly, Wang et al. (2006) documented that PBO had stronger synergism on toxicity of spinosad than TPP and DEM in the resistant strain of *Spodoptera exigua* and the activity of microsomal *O*-demethylase increased 5.2-fold after 5 generations of selection [25]. Liu and Yue (2000) found that PBO increased the spinosad toxicity to housefly of both permethrin-resistance and susceptible strains [28]. In contrast, the spinosad LD_{50} values for spinosad resistant strains of *Frankliniella occidentalis* [29,30] and housefly [26] were unchanged by pretreatment with PBO, DEF and DEM. The synergists PBO and DEF did not show any synergism for spinosad in the resistant colony of *Plutella xylostella* (L.) [13]. These results imply that resistant mechanism against spinosad is probably related with the species of pests.

The present investigation suggests that the resistance to spinosad in the cotton bollworm is associated with an increase in cytochrome P450 monooxygenase as shown by the fact that resistance was suppressed by PBO pre-treatment and that ODM activity was higher in resistant larvae. However, the enzyme inhibitors could partly but not completely eliminate resistance against spinosad in *H. armigera* which demonstrated the formation of resistance to spinosad could also involve other mechanisms other than the increase in detoxification enzymes activities, such as target resistant mechanisms [31]. The further study seems to be necessary to determinate other resistant mechanisms about target-insensitivity of *H. armigera* against spinosad.

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