Research Article

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Effects of spinosad on *Helicoverpa armigera* (Lepidoptera: Noctuidae) from China: tolerance status, synergism and enzymatic responses

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Abstract

BACKGROUND: Spinosad is increasingly used in pest management programmes, and resistance to it has been detected in recent years. However, there is no report on the susceptibilities of field populations of *Helicoverpa armigera* (Hübner) from China. Furthermore, the impact of spinosad on metabolic enzymes in this pest remains unknown.

RESULTS: Four populations of *H. armigera* from different locations in China displayed less than 6.5-fold difference in LC_{50} to spinosad, the highest being in the Xinjiang population, followed by Xiajin, Taian and Hubei populations, while there was no significant difference at LC_{99} level among the four populations. The toxicity of spinosad could be synergised by piperonyl butoxide (PBO) and triphenylphosphate (TPP), but not by diethyl maleate (DEM). Spinosad exposure for 48 h significantly increased the activities of *p*-nitroanisole *O*-demethylase (ODM), while no significant changes in glutathione-*S*-transferase (GST) and carboxyl esterase (CarE) were observed.

CONCLUSION: Field populations of *H. armigera* from China displayed marginally different susceptibilities to spinosad and had a relatively low LC_{50}. Cytochrome P450 monooxygenase might be involved in the metabolism of, and hence resistance to, spinosad in this pest in China.
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Keywords: *Helicoverpa armigera*; spinosad; susceptibility status; synergism; carboxyl esterase; glutathione-*S*-transferase; *p*-nitroanisole *O*-demethylase

1 INTRODUCTION

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is one of the most important lepidopteran pests on a wide range of crops throughout Asia, Europe, Africa and Australia.[1] It is a major pest in cotton, legumes and more than 100 other plant species.[2] In China, *H. armigera* is distributed mainly in three major cotton-growing regions: the Yellow River Region, the Yangtze River Region and the Xinjiang Region.[3] Owing to the overuse of pesticides over the past three decades, *H. armigera* has exhibited high resistance to many conventional insecticides such as organochlorines, organophosphates, carbamates and pyrethroids.[4–7] To control this pest effectively and sustain agricultural productivity, many insecticides with novel modes of action have been introduced, including spinosad.

Spinosad is a naturally derived fermentation product of soil bacteria *Saccharopolyspora spinosa* Mertz & Yao, and consists mainly of a mixture of spinosyns A and D.[8] As a biorational insecticide, the action mode of spinosad appears to be unique, with the primary site of attack being the nicotinic acetylcholine receptor (nAChR), and a secondary site being γ-aminobutyric acid (GABA) receptors.[9] Spinosad is effective against several classes of insects, especially Lepidoptera larvae, and is registered for uses on over 150 various crops in 37 countries including China.[10,11] However, resistance[12–14] or cross-resistance[15–17] to spinosad has been documented in several pests. Unfortunately, to date, the tolerance status of field populations of *H. armigera* from China against spinosad has not been well investigated.

Detoxification enzymes such as cytochrome P450-dependent monooxygenases, glutathione-*S*-transferases and hydrolases play important roles in the metabolism of insecticides in insects.[18] These enzymes can be induced in response to chemical stress. Enzymatic response represents an adaptive mechanism of living organisms to a changing environment.[19–21] Induction or inhibition of detoxification enzymes can alter the metabolism of insecticides, which may cause induced resistance or cross-resistance to insecticides and thus affect efficacy of pest management practices.

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Studies on the interaction of detoxification enzymes and insecticides may provide a better understanding of the toxic effects of insecticides and the responses of living organisms to them.[22] However, to the authors' knowledge, there is no report regarding enzymatic response to spinosad exposure in *H. armigera*.

Against this background, the current status of spinosad susceptibilities of field populations of *H. armigera* from China was analysed in the present study. Also, the potential influences of commonly used synergists on the toxicity of spinosad and the enzymatic responses of *H. armigera* to spinosad treatment were evaluated.

2 MATERIALS AND METHODS

2.1 Insects

Fifth- or sixth-instar larvae of *H. armigera* were collected from cotton fields in Xinjiang (Shihezi: N 44.18°, E 86.00°), Shandong (Xiajin: N 36.95°, E 116.00°; Taian: N 36.18°, E 117.13°) and Hubei (Tianmen: N 60.39°, E 113.10°) provinces, China, in 2007. Shandong Province is located in the Yellow River Basin, and Hubei Province belongs to the Yangtze River Basin. The larvae were reared in an insectary maintained at 27 ± 1 °C with a 14 : 10 h light : dark photoperiod on an artificial diet consisting of wheat germ 94 g, tomato paste 45 g, yeast 35 g, ascorbic acid 2.5 g, methyl *para*hydroxybenzoate 2.0 g, sorbic acid 1.0 g, linoleic acid 0.6 mL and agar 11 g.[23] Agar was dissolved in 300 mL of boiling water and added to the other constituents premixed in 350 mL of water. Adult males and females were collected and released into 40 × 40 cm cages for mating and egg laying after eclosion. Adults were held under the same temperature and light conditions at 60% RH and supplied with a 10% honey solution.

2.2 Chemicals

Spinosad 480 g L^{-1} SC (Tracer) was obtained from DowAgro Sciences, and 4-nitroanisole (PNA) from Sigma, Germany. 2,4-Dinitrochlorobenzene (CDNB), α-naphthyl acetate (α-NA), Coomassie Brilliant Blue G-250 and *p*-nitrophenol (PNP) were purchased from the Shanghai Chemical Factory, China. Bovine serum albumin (BSA), diethyl maleate (DEM), triphenyl phosphate (TPP) and piperonyl butoxide (PBO) were purchased from Shanghai Bio Life Science and Technology Co., Ltd, China. Glutathione (GSH) and NADPH were purchased from Beijing Solarbio Science and Technology Co., Ltd, China, and α-naphthol was purchased from Tianjin Chemical Factory, China.

2.3 Leaf-dip bioassays

Newly moulted third-instar larvae were exposed to spinosad using the leaf-dip technique recommended by the Insecticide Resistance Action Committee (IRAC).[24] Serial dilutions of spinosad were prepared using distilled water, and 5 cm cotton leaf discs were cut and dipped into the test solutions for 10 s with gentle agitation. They were then allowed to dry on paper towel on both sides. Larvae were released on to each leaf disc, and the mortalities were recorded after 48 h and 72 h of exposure. Larvae were considered dead if unable to move in a coordinated way when prodded with a fine-haired brush. Three replicates with 20 individuals per replication were set up. The same number of leaf discs per treatment were dipped into distilled water as an untreated control. Before and after the treatment, larvae were maintained at a constant temperature of 27 ± 1 °C with a 14 : 10 h light : dark photoperiod.

2.4 Effects of synergists on toxicity of spinosad

DEM, TPP or PBO was applied to the pronotum of fourth-instar larvae at a dose of 1 μg $larva^{-1}$ 1 h prior to treatment with spinosad. Mortalities were assessed after exposure for 48 and 72 h.

2.5 *In vivo* effects of spinosad exposure on detoxification enzymes

Doses of spinosad used for larval pre-exposure were chosen according to the results of bioassay with topical application (data not shown). Third-instar larvae exposed to spinosad at LD_{28} (28% lethal concentration, Xinjiang: 0.011 μg $larva^{-1}$; Taian: 0.032 μg $larva^{-1}$) and LD_{50} (50% lethal concentration, Xinjiang: 0.020 μg $larva^{-1}$; Taian: 0.063 μg $larva^{-1}$) were used for CarE or GST activity assay *in vivo*. Considering that the monooxygenase activities are mostly distributed in the midgut and fat body and need more biomass to make sufficient enzyme preparation, final-instar larvae exposed at doses of LD_9 (9% lethal concentration, Xinjiang: 0.17 μg $larva^{-1}$; Taian: 0.25 μg $larva^{-1}$) and LD_{50} (Xinjiang: 0.74 μg $larva^{-1}$; Taian: 1.00 μg $larva^{-1}$) were used to make enzyme preparations for ODM activity assay.

2.6 Enzyme assays

2.6.1 *Carboxyl esterase (CarE) assay*

CarE activity was measured using α-NA as substrate according to the method described by Li *et al*.[25] A total of 20 third-instar larvae were homogenised in 10 mL of 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. A quantity of 5 mL of 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, then 1 mL of enzyme was added and the mixture was incubated with shaking for 30 min at 30 °C. The reaction was stopped by the addition of 1 mL of distilled water containing 0.01 mg of fast blue B salt. Absorbance at 600 nm was read against blanks after 30 min. The activity of CarE was determined from the α-naphthol production according to an experimentally determined standard curve.

2.6.2 *Glutathione-S-transferase (GST) assay*

GST activity was measured using CDNB as substrate by the method of Habig *et al*.[26] A total of 25 third-instar larvae were homogenised in 10 mL of phosphate buffer (66 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 solution. Enzyme solution (0.2 mL) was mixed with CDNB (0.1 mL, 30 mM) and phosphate-buffered saline (PBS; 2.4 mL, 66 mM, pH 7.0), and the reaction was initiated by adding GSH (0.3 mL, 50 mM). 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) assay*

The *O*-demethylation of *p*-nitroanisole was assayed using the methods of Qiu *et al*.[27] Final-instar larvae were dissected in 1.15% potassium chloride solution on ice. The midguts were removed, and their contents were washed and rinsed in ice-cold sodium phosphate buffer (0.1 M, pH 7.8). The midguts

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were then homogenised in homogenisation buffer on ice, the homogenates were centrifuged at 4 °C for 20 min at 750 rpm and the supernatant was taken as the enzyme preparation. The mixture, in a total volume of 2 mL, contained 1 mL of enzyme preparation and sodium phosphate (0.1 M, pH 7.8) and 0.36 mM NADPH. Reactions were initiated by adding *p*-nitroanisole (200 mM) and terminated by the addition of 1 mL of 1 M hydrochloric acid after incubating with shaking at 25 °C for 30 min. The product *p*-nitrophenol was then extracted with chloroform, and the chloroform fraction was back-extracted with 0.5 M sodium hydroxide. The absorbance of the sodium hydroxide extract was recorded at 400 nm. The activity of ODM was determined according to a standard curve.

2.7 Protein assay

Protein content was determined by the method of Bradford[28] using Coomassie Brilliant Blue G-250 with bovine serum albumin as a standard.

2.8 Data analysis

LC_{50} values and slopes were determined by probit analysis using the SPSS program. Data are presented as the mean ± standard error (SE) of at least three separate experiments, with at least three replicates for each experiment. Data were statistically analysed using one-way analysis of variance (ANOVA) followed by Fisher's LSD test and *t*-test ($P < 0.05$). Non-overlap of 95% confidence limits was the criterion for significance of difference between the control and treatment groups.

3 RESULTS

3.1 Toxicity of spinosad to four field populations of *Helicoverpa armigera* from China

LC_{50} and LC_{99} values of spinosad for four populations are presented in Table 1. The LC_{50} values were lower after 72 h exposure than after 48 h, and therefore the values after 72 h will be used in the following discussion. The LC_{50} and LC_{99} values ranged from 3.69 to 24.10 mg L^{-1} and from 198.5 to 575.3 mg L^{-1} respectively. At LC_{50} level, differences among the four populations were significant ($F = 121.4$, df = 11, $P < 0.0001$). The lowest LC value was detected in the Xinjiang population, and the highest in the Hubei population. Relative to the Xinjiang population, 2.4–6.5-fold higher LC_{50} values were obtained in the Taian/Xiajin and Hubei populations respectively. No significant difference in spinosad susceptibility was observed between the Taian and Xiajin populations, which originated from the Yellow River Basin of China ($F = 0.228$, df = 4, $P = 0.658$). At LC_{99} level, no statistical difference was found among the four populations.

3.2 Synergism of DEM, TPP or PBO to spinosad

The effects of synergists on the susceptibility of larvae to spinosad are presented in Table 2. After pretreatment with PBO, the toxicity of spinosad to the Xinjiang and Taian populations increased significantly. Taking the data obtained at 72 h as examples, PBO increased the spinosad toxicity to the Taian population to a greater extent than to the Xinjiang population, with synergistic ratios of 2.0 and 4.7, respectively, in the Xinjiang and Taian populations (Xinjiang: $F = 1.103$, df = 4, $P = 0.002$; Taian: $F = 0.389$, df = 4, $P < 0.0001$). Significant synergism was also observed in TPP treatment, ranging from 1.65- to 1.95-fold (Xinjiang: $F = 0.132$, df = 4, $P = 0.014$; Taian: $F = 0.296$, df = 4, $P = 0.002$). However, DEM exhibited no significant effects on the toxicity of spinosad (Xinjiang: $F = 2.717$, df = 4, $P = 0.424$; Taian: $F = 3.125$, df = 4, $P = 0.843$).

3.3 *In vivo* effects of spinosad on detoxification enzymes

No significant change in CarE activity was detected in the Xinjiang and Taian populations (Figs 1A and B) (Xinjiang, 12 h: $F = 0.039$, df = 8, $P = 0.962$; 24 h: $F = 0.300$, df = 8, $P = 0.751$; 48 h: $F = 0.274$, df = 8, $P = 0.770$; Taian, 12 h: $F = 0.0420$, df = 8, $P = 0.959$; 24 h: $F = 0.957$, df = 8, $P = 0.436$; 48 h: $F = 0.002$, df = 8, $P = 0.998$). Similarly, exposures to spinosad did not affect significantly GST activity in either population (Figs 2A and B) (Xinjiang, 12 h: $F = 0.211$, df = 8, $P = 0.808$; 24 h: $F = 1.951$, df = 8, $P = 0.222$; 48 h: $F = 2.197$, df = 8, $P = 0.192$; Taian, 12 h: $F = 1.016$, df = 8, $P = 0.417$; 24 h: $F = 3.075$, df = 8, $P = 0.120$; 48 h: $F = 2.965$, df = 8, $P = 0.127$).

Compared with CarE or GST activity, spinosad had stronger and more significant effects on the ODM activity (Xinjiang, 12 h: $F = 6.851$, df = 8, $P = 0.028$; 24 h: $F = 15.364$, df = 8, $P = 0.004$; 48 h: $F = 148.59$, df = 8, $P < 0.0001$; Taian, 12 h: $F = 18.563$, df = 8, $P = 0.003$; 24 h: $F = 28.778$, df = 8, $P = 0.001$; 48 h:

Table 1. Toxicities of spinosad to four field populations of *Helicoverpa armigera* from China[a]

	48 h after treatment			72 h after treatment		
Population	Slope (± SE)	LC_{50}(mg L^{-1}) (95% FL[b])	LC_{99}(mg L^{-1}) (95% FL[b])	Slope (± SE)	LC_{50}(mg L^{-1}) (95% FL [b])	LC_{99}(mg L^{-1}) (95% FL[b])
Xinjiang	1.23(± 0.19)	5.796 a (4.303–7.726)	453.543 a (164.711–2849.164)	1.34(± 0.19)	3.690 a (2.646–4.804)	198.514 a (87.645–827.839)
Taian	1.41(± 0.19)	16.011 b (11.859–20.551)	708.648 a (328.668–2628.707)	1.49(± 0.21)	10.069 b (6.913–13.173)	367.543 a (187.811–1163.941)
Xiajin	2.60(± 0.36)	12.781 b (9.460–16.232)	751.970 a (333.382–3043.795)	2.85(± 0.41)	8.977 b (6.385–11.481)	366.997 a (179.172–1280.001)
Hubei	1.95(± 0.22)	56.347 c (44.917–68.450)	882.587 a (543.434–1866.806)	1.69(± 0.17)	24.095 c (19.410–29.485)	575.254 a (341.582–1226.031)

[a] Mortality was recorded 48 and 72 h after the larvae had been treated with spinosad. Results are means ± standard error (SE) of three separate experiments. Means in the same column followed by different letters differ significantly ($P < 0.05$) on the basis of the least significant difference (LSD) test.
[b] Fiducial limits (from probit analysis).

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Table 2. Toxicities of spinosad to the fourth-instar larvae of *Helicoverpa armigera* after synergism[a]

Population	Compound	48 h after treatment			72 h after treatment		
		Slope (± SE)	LD_{50}(μg larva^{-1}) (95% FL[b])	SR[c]	Slope (± SE)	LD_{50}(μg larva^{-1}) (95% FL[b])	SR[c]
Xinjiang	Spinosad	2.05(± 0.22)	0.109 a (0.089–0.129)	1.00	2.24(± 0.28)	0.086 a (0.076–0.097)	1.00
	Spinosad + DEM	1.73(± 0.26)	0.102 a (0.080–1.125)	1.07	1.70(± 0.27)	0.091 a (0.057–0.125)	0.95
	Spinosad + TPP	1.90(± 0.22)	0.057 b (0.048–0.64)	1.91	1.84(± 0.22)	0.052 b (0.050–0.053)	1.65
	Spinosad + PBO	2.31(± 0.28)	0.053 b (0.049–0.057)	2.06	2.28(± 0.27)	0.043 b (0.036–0.054)	2.00
Taian	Spinosad	1.62(± 0.28)	0.376 a (0.275–0.558)	1.00	2.15(± 0.32)	0.218 a (0.168–0.281)	1.00
	Spinosad + DEM	1.58(± 0.27)	0.364 a (0.306–0.422)	1.03	1.54(± 0.28)	0.216 a (0.192–0.239)	1.01
	Spinosad + TPP	1.59(± 0.28)	0.141 b (0.113–0.169)	2.67	1.44(± 0.27)	0.112 b (0.109–0.126)	1.95
	Spinosad + PBO	1.93(± 0.31)	0.070 c (0.065–0.071)	5.37	1.70(± 0.32)	0.046 c (0.034–0.058)	4.74

[a] Mortality was recorded 48 and 72 h after the larvae had been treated with spinosad. Results are means ± standard error (SE) of three separate experiments. Means in the same column followed by different letters differ significantly ($P < 0.05$) on the basis of the least significant difference (LSD) test.
[b] Fiducial limits (from probit analysis).
[c] Synergistic ratio (SR) = LD_{50} of spinosad to fourth-instar larvae/LD_{50} of spinosad + synergist to fourth-instar larvae.

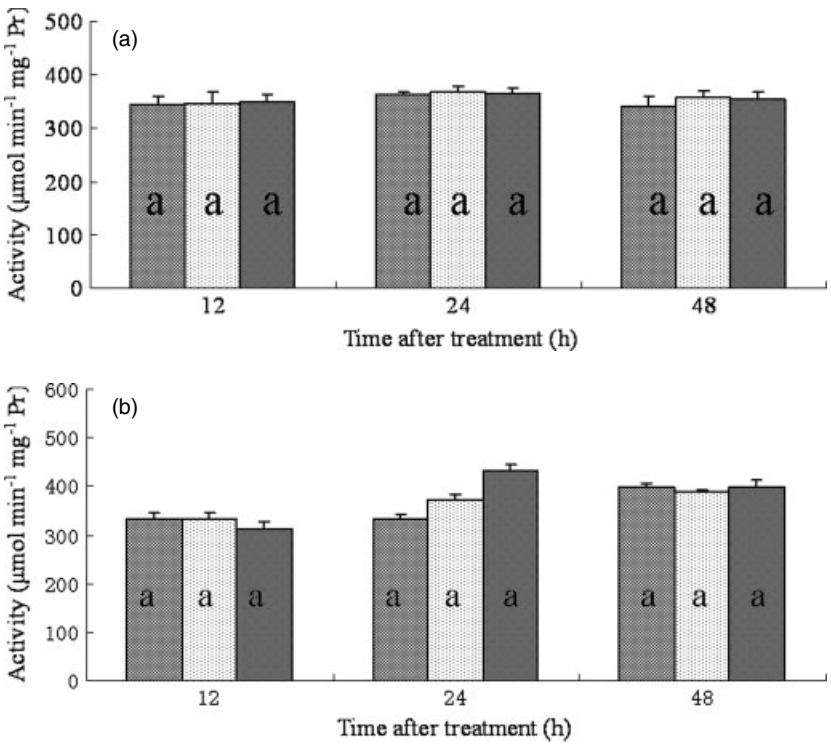

Figure 1. Effects of spinosad on CarE activity (μmol min^{-1} mg^{-1} Pr) of (a) the Xinjiang population and (b) the Taian population *in vivo* after third-instar larvae had been exposed to spinosad (control, LD_{28}, LD_{50}). Results are means ± standard error (SE) of three separate replicates. Data marked with different letters differ significantly ($P < 0.05$) on the basis of the least significant difference (LSD) multiple comparison test.

$F = 147.943$, df = 8, $P < 0.0001$). The ODM activity enhancement was in a time-, dose- and population-specific manner (Figs 3A and B). Greater induction was found in the Taian population (3.4–5.8-fold) than in the Xinjiang population (1.6–2.1-fold) after exposure to spinosad for 48 h (Xinjiang: $F = 148.59$, df = 8, $P < 0.0001$; Taian: $F = 147.94$, df = 8, $P < 0.0001$).

4 DISCUSSION AND CONCLUSIONS

Although spinosad has a unique mechanism of action, its resistance has been documented in several pests.[12–14,29–31] It has also been reported that insects have the potential to evolve high levels of resistance to spinosad in a short time in a laboratory.[32–34] To develop efficient pest management strategies, it is useful to know the spinosad susceptibility of field populations of *H. armigera*. The present bioassay results showed that LC_{50} values of the four populations, collected from cotton fields of China, fall within the range 3.69–24.10 mg L^{-1}, indicating that resistance to spinosad remains at a low level even if resistance exists. The greatest differences in LC_{50} among the four populations of *H. armigera* are 6.5-fold. No statistically significant difference in LC_{99} has been observed among the four populations. These results indicate

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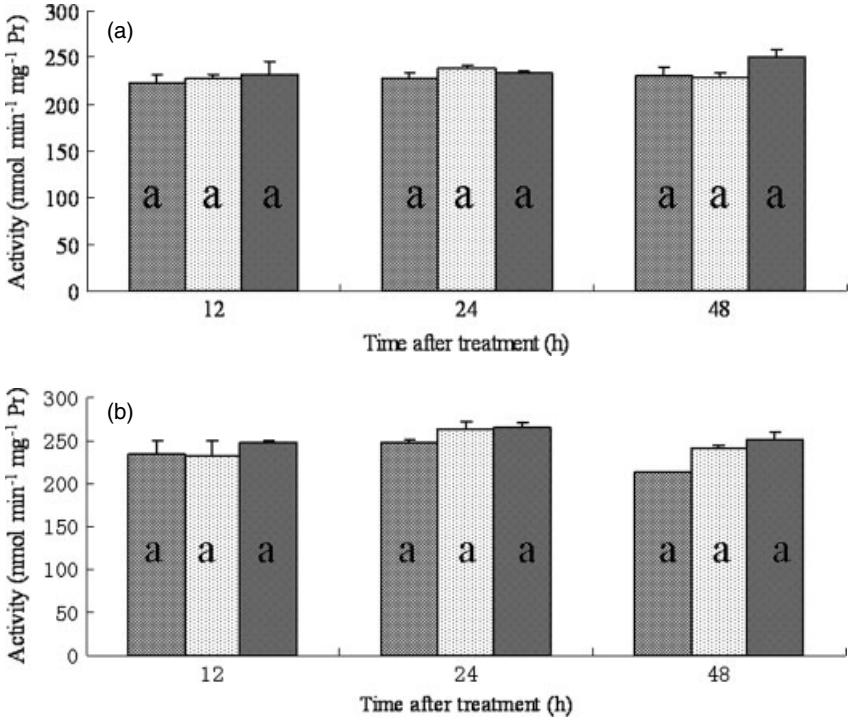

Figure 2. Effects of spinosad on GST activity (nmol min^{-1} mg^{-1} Pr) of (a) the Xinjiang population and (b) the Taian population *in vivo* after third-instar larvae had been exposed to spinosad (control, LD_{28}, LD_{50}). Results are means ± standard error (SE) of three separate replicates. Data marked with different letters differ significantly ($P < 0.05$) on the basis of the least significant difference (LSD) multiple comparison test.

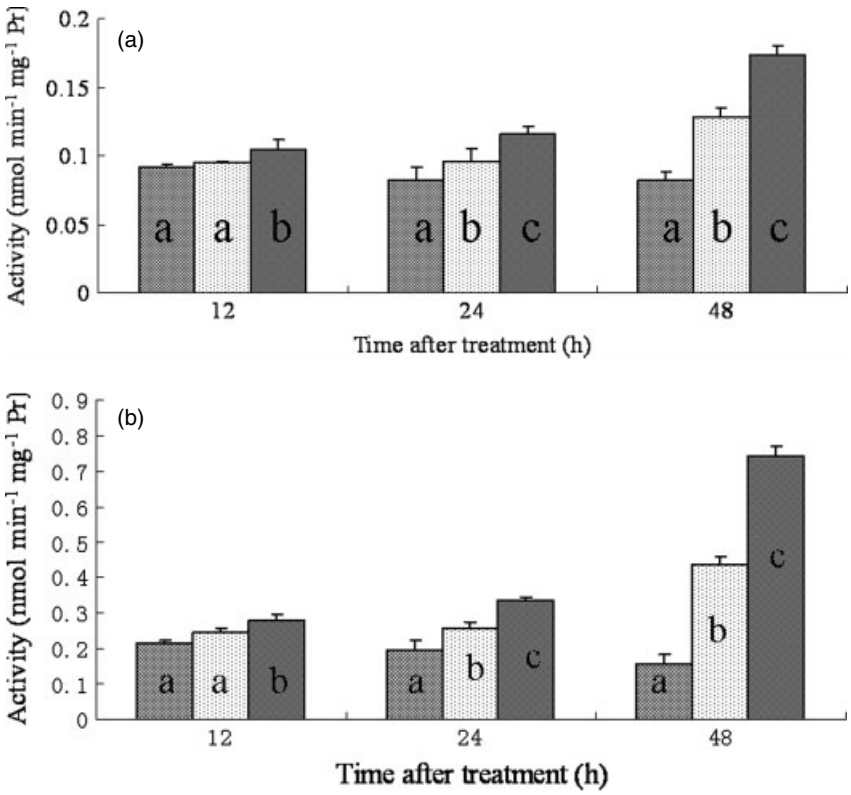

Figure 3. Effects of spinosad on ODM activity (nmol min^{-1} mg^{-1} Pr) of (a) the Xinjiang population and (b) the Taian population *in vivo* after final-instar larvae had been exposed to spinosad (control, LD_{28}, LD_{50}). Results are means ± standard error (SE) of three separate replicates. Data marked with different letters differ significantly ($P < 0.05$) on the basis of the least significant difference (LSD) multiple comparison test.

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that susceptibilities among the four populations are similar, and the population difference is probably due to natural variability. Similarly, low LC_{50} values and a low level of spinosad resistance have been recorded in *H. armigera* in Pakistan,[12] India[35] and Australia.[36]

To explore the potential role of detoxification enzymes in the tolerance of field insects to spinosad, a synergism test was conducted. Results of synergism bioassays showed that PBO and TPP, but not DEM, had a significant effect on the toxicity of spinosad to both Xinjiang and Taian populations. A higher synergistic ratio was observed in the Taian population than in the Xinjiang population, reflecting that the Xinjinag population is more susceptible. Synergism by PBO and TPP indicated that rational applications of PBO and TPP might increase the efficacy of spinosad in control of this pest. Similarly, Liu and Yue[37] found that PBO increased spinosad toxicity to houseflies of both permethrin-resistant and permethrin-susceptible strains. Wang *et al*.[34] documented that PBO had stronger synergism for spinosad than TPP and DEM. In contrast, the synergists PBO, DEF and DEM did not show any synergism on the toxicity of spinosad in the resistant strain of *Frankliniella occidentalis* Perg.,[31,38] and the spinosad LD_{50} for a spinosad-resistant housefly strain was unchanged by pretreatment with PBO, DEF and DEM.[33] Zhao *et al*.[29] showed that the synergists DEF and PBO did not enhance the toxicity of spinosad to a resistant colony of *Plutella xylostella* (L.). These results imply that synergism might be species specific.

To the authors' knowledge, very little is known about the effect of spinosad on detoxification enzymes. Wang *et al*.[20] reported that CarE activity was significantly increased when third-instar *Spodoptera exigua* (Hübner) were fed with leaves treated with 0.05 mg L^{-1} of spinosad. The present results showed that dietary spinosad had no effect on CarE and GST activity in *H. armigera* (Figs 1 and 2), but induced (1.6–5.8-fold) microsomal *O*-demethylase activity. It is unclear whether ODM induction will change the toxicity of spinosad and other insecticides, and this is one of the further subjects to be addressed.

The findings that spinosad is synergised by PBO and that microsomal *O*-demethylase activity is inducible by spinosad exposure suggest that cytochrome P450 monooxygenase may be involved in the metabolism of spinosad and hence in resistance in the cotton bollworm. However, there is still no direct evidence, and more studies such as biochemical analysis of spinosad metabolism in this pest are needed. The suggestion that monooxygenases are involved in the detoxification of spinosad has been made on the basis of data from synergist experiments in insect pests such as the housefly[37] and the beet armyworm.[34]

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