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Original Article

Cypermethrin resistance in *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) from three locations in Thailand and detoxification enzyme activities

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ABSTRACT

The tobacco cutworm *Spodoptera litura* is one of the key insect pests of vegetables in Thailand. This study evaluated cypermethrin resistance in populations of *S. litura* collected from vegetable crops in Bang Len and Kamphaeng Saen district, Nakhon Pathom province and from Wang Nam Khiao district, Nakhon Ratchasima province, Thailand. Resistance of *S. litura* was determined using the topical application method and biochemical assays, and all three field populations exhibited a trend of increasing resistance to cypermethrin compared to the susceptible laboratory population, with median lethal dose (LD_{50}) values varying from 10.98 parts per million (ppm) to 15.74 ppm. The activities of cytochrome P450 monooxygenase and carboxylesterase in field populations of *S. litura* were significantly increased compared to those in susceptible insects, but glutathione *S*-transferase activity was significantly increased only in the Kamphaeng Saen population. These results indicated that the three field populations of *S. litura* had developed resistance to cypermethrin and it is suggested that continuous application of cypermethrin to control tobacco cutworm should be avoided to prevent the development of high cypermethrin resistance in this pest.

Introduction

The tobacco cutworm, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae), is a polyphagous insect pest, damaging many vegetables belonging to 40 families around the world and causing yield losses of 26–100% (Dhir et al., 1992). In Thailand, it is one of the key pests in cultivated areas and is usually found on tobacco, cotton, cabbage and soybean, with various insecticide groups being used to control *S. litura* including organophosphates, carbamates and pyrethroids (Saleem et al., 2016).

Carbofuran, chlorpyrifos, chlorothalonil, cypermethrin, dimethoate, metalaxyl and profenofos are synthetic pyrethroids that are widely used as insecticides in crop protection to minimize yield loss and maximize yield quality in many countries, especially Thailand (Ahmad and McCaffery, 1999; Pothikasikorn et al., 2007; Wanwimolruk et al., 2017). Synthetic pyrethroids are modified derivatives of pyrethrins, which are natural substances extracted from the flowers of *Chrysanthemum cinerariaefolum* (Khazri et al., 2016), and are known to affect the nervous system, specifically by delating the closure of sodium channels, which causes repetitive after-discharges that lead to hyperexcitation of the nervous system (Narahashi et al., 1992). Cypermethrin has low mammalian toxicity (Xu and Huang, 2017) and high insecticidal properties that are effective against insects from several orders including the Coleoptera, Diptera, Hemiptera and Lepidoptera (Parsaeyan et al., 2017).

Unfortunately, the continuous application of cypermethrin has caused resistance to develop in *S. litura*, which has resulted in widespread failure of insect control. Three primary pyrethroid-resistance mechanisms exhibited by insects are decreased penetration, altered target site sensitivity and increased activity of detoxification enzymes, including cytochrome P450 monooxygenase (P450), carboxylesterase (CarE) and glutathione *S*-transferase (GST) (Ahmad and McCaffery, 1999; Dong et al., 2016). High levels of resistance to synthetic pyrethroid insecticides has been reported in countries around the world, including India (Singh et al., 2014), Pakistan (Rasool et al., 2014), China (Qiu et al., 2017),

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Iran (Ziapour et al., 2017), Brazil (Klafke et al., 2017), Niger (Soumaila et al., 2017), Mexico (Rodriguez-Vivas et al., 2012), the Americas, South Africa and Australia (Lovis et al., 2012).

In Thailand, the resistance of *S. litura* to cypermethrin has not been studied, even though farmers have complained of control failure. The objective of this study was to investigate the status of cypermethrin resistance in field populations of *S. litura* as well as to evaluate the enzyme activities of P450, GST and CarE to determine the potential mechanisms involved in the variation of cypermethrin susceptibility.

Materials and methods

Ethics statement

This study was approved by the ethics committee of Kasetsart University, Bangkok, Thailand.

Insects

The susceptible strain (Lab-SS) of *S. litura* came from the National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand, where it has been reared on an artificial diet in the laboratory without exposure to any insecticide for more than 5 yr. Three field populations of *S. litura* were collected during the 2017 summer season from vegetable crops in three different locations (Bang Len and Kamphaeng Saen districts, Nakhon Pathom province and Wang Nam Khiao district, Nakhon Ratchasima province) in Thailand (Table 1). The host plant for *S. litura* is widely cropped in all three areas, and cypermethrin is used to control this insect pest. The collected insects were reared in the laboratory, and the second-instar larvae of the first and second generations were used for susceptibility and enzyme assays.

Larvae of *S. litura* were reared on an artificial diet (a mixture of 240 g of green bean, 25 g of agar, 40 ml of mixed vitamin solution, 5 g of ascorbic acid, 40 ml of amoxicillin solution, 3 g of sorbic acid, 5 g of methylparaben, 20 g of yeast, 4 ml of 40% formalin and 1.41 L of water) at 25 ± 1 °C and 65 ± 5% relative humidity with a 16 h light:8 h dark photoperiod. Adults were fed a 20% honey solution under the same conditions in a cage (30 cm × 30 cm × 30 cm). The populations were maintained in an environmental test chamber (Versatile Environmental Test Chamber MLK 352H; Panasonic Corp.; Osaka, Japan).

Chemicals

Cypermethrin solution (100 μg/ml in acetonitrile, PESTANAL®, analytical standard) was obtained from Sigma-Aldrich (Singapore). The insecticide was diluted in acetone (AR, ≥99.5%) in a range of 0–30 ppm for topical application to determine the toxicity of the different concentrations.

Bioassays

Second-instar *S. litura* larvae were used for the topical application. Serial dilutions of cypermethrin (AR grade) in analytical grade acetone were prepared, and a 1 μL drop was applied to the thoracic dorsum of individual larvae using a micro-applicator. Control larvae were treated with acetone alone. Five replicates of 30 larvae were used for each treatment (total $n = 150$). After treatment, larvae were provided an artificial diet. Treated larvae were placed in a sealed plastic box and kept under controlled conditions in the environmental test chamber. Mortality was recorded 24 h after treatment.

Enzyme extraction method

To measure P450 activities, 30 s-instar larvae were pooled and homogenized in buffer A (100 mM phosphate buffer (pH 7.2) containing 1 M dithiothreitol, 100 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and 0.5 M ethylenediaminetetraacetic acid) and then centrifuged at 10,000×*g* for 5 min at 4 °C. The resulting supernatant was further centrifuged at 100,000×*g* for 1 h at 4 °C. Finally, the pellet was resuspended in buffer B (buffer A + 10% glycerol) and used to determine P450 activities. For CarE and GST *in vivo* assays, 30 s-instar larvae were homogenized in 0.5 mL of homogenized buffer (100 mM phosphate buffer (pH 7.2) and 1% Triton X-100] and centrifuged at 10,000×*g* for 15 min at 4 °C); the supernatant was then used as an enzyme source.

Cytochrome P450 monooxygenase activities

P450 activities were evaluated by measuring *p*-nitroanisole (PNOD) activities according to the modified method of Chang and Hodgson (1975). The assay mixture contained 100 μL of microsomal proteins, 25 mM *p*-nitroanisole and 100 mM potassium phosphate buffer (pH 7.2). The reaction was initiated by the addition of 100 mM D-glucose-6-phosphate sodium salt, 100 U/mL glucose-6-phosphate dehydrogenase and 5 mM β-nicotinamide adenine dinucleotide phosphate. After incubation for 10 min at 30 °C, the reaction was stopped by the addition of acetone and 2 mM glycine and 2 U/mL sodium hydroxide. The homogenate was centrifuged at 12,000×*g* for 1 min at 4 °C, and the resulting supernatant was used for further analysis. Next, 200 μL of supernatant was added to each well of a transparent 96-well microplate, and the optical density at 405 nm was immediately recorded at intervals of 25 s for 10 min using a microplate reader (PowerWave XS microplate spectrophotometer; Biotek; Winooski, VT, USA). The optical density value was recorded and the quantity of the product was determined from a *p*-nitrophenol standard curve.

Carboxylesterase activities

CarE activities were determined using *p*-nitrophenylacetate (pNPA) according to the modified method of Bullangpoti et al. (2012). Enzyme solution (40 μL) was mixed with 10 mM pNPA in dimethyl sulfoxide and 50 mM phosphate buffer (pH 7.4), and enzyme activity was measured at 410 nm and 37 °C for 90 s using a microplate reader (PowerWave XS microplate spectrophotometer; Biotek; Winooski, VT, US) in the kinetic mode. The CE activity was

Table 1
Sampling sites, collection dates and developmental stages of *Spodoptera litura* collected from field sampling.

Location	Collection date	Co-ordinates	Insect stage	Host plant
Bang Len, Nakhon Pathom	26 Feb 2017	13.58°N, 100.12°E	Larva	*Colocasia esculenta*
Kamphaeng Saen, Nakhon Pathom	14 May 2017	13.98°N, 99.96°E	Larva	*Colocasia esculenta*
Wang Nam Khiao, Nakhon Ratchasima	24 April 2017	14.27°N, 101.49°E	Larva	*Zizyphus mauritiana*

determined using the extinction coefficient of 176.4705 for pNPA. Five biological replicates were estimated per treatment.

Glutathione S-transferase activities

GST activities were measured using 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma-Aldrich; Darmstadt, Germany) as a substrate using the protocol of Oppenoorth et al. (1979). The reaction solution contained 100 μL of enzyme solution, 50 mM potassium phosphate buffer (pH 7.3) and 150 mM CDNB. Optical density was recorded at 30 s intervals for 3 min at 37 °C and 340 nm using a microplate reader (PowerWave XS microplate spectrophotometer; Biotek; Winooski, VT, US). The GST activity was determined from the extinction coefficient of 0.0096 for CDNB. Five biological replicates were estimated per treatment.

Protein concentration determination

The protein content of each fraction used as an enzyme source was determined using the method of Bradford (1976) with a Bradford Kit (Bio-Rad Laboratories; Hercules, CA, USA) before measuring the enzyme activities.

Statistical analysis

Probit analysis was used to determine the LD_{50} (median lethal dose) and the confidence limit (CL) in the program Stat Plus® (v.Pro 6.2.2.0.; AnalystSoft,; Walnut, CA, USA). Resistance ratios (RRs) were calculated as the LD_{50} value of the field-collected population/ LD_{50} value of the susceptible strain (Lab-SS). Statistical comparisons of P450, CarE and GST activities were made using one-way analysis of variance followed by Tukey's multiple comparison test. Statistical differences were tested at $p < 0.05$.

Results

Resistance levels of field populations

The Lab-SS exhibited relatively low LD_{50} values to cypermethrin (2.32 ppm, Table 2), whereas other LD_{50} values were higher, indicating possible resistance to cypermethrin (LD_{50} = 10.98–15.74 ppm, Table 2).

The data in Table 2 indicate that the field population of *S. litura* tended to develop resistance to cypermethrin with RRs ranging from 4.73 to 6.78. The population collected from Kamphaeng Saen, Nakhon Pathom province exhibited the highest RRs (6.78), while the lowest resistance (4.73) was observed in Bang Len, Nakhon Pathom province.

Detoxification enzyme activities

As shown in Fig. 1, P450 and CarE activities were significantly higher in the second-instar larvae from all three field populations of *S. litura* compared to the Lab-SS strain. The population from Kamphaeng Saen, Nakhon Pathom Province showed the highest P450 (1.69 times) and CarE (2.69 times) activities, but GST activity was only significantly higher in the Kamphaeng Saen population (1.24 times).

Discussion

Monitoring resistance in an important insect pest is increasingly important for formulating suitable insecticides and tracking changes in resistance (Muthusamy and Shivakumar, 2015). The current work is the first report of a trend toward cypermethrin resistance, which has now been confirmed in *S. litura* in three locations in Thailand. These data could be used for future resistance monitoring and management.

The current results showed that *S. litura* populations in three locations of Thailand expressed a level of resistance to cypermethrin (Table 2). However, it is not obvious that cypermethrin had previously been widely used by farmers, especially as a prophylactic in early crop stages. In Thailand, pesticides are used extensively but not applied according to the manufacturer's instructions, which has led to the development of pesticide resistance in insect pests (Wanwimolruk et al., 2017).

The detoxification enzymes CarE, P450 and GST are known to be actively involved in the metabolism of and resistance to insecticides (Dauterman, 1985; Soderlund and Bloomquist, 1990). It is highly likely that increased activities of these detoxifying enzymes in field populations play an important role in the observed resistance (Denholm and Rowland, 1992).

Enhanced activities of P450, CarE, and GST enzymes have been found to be related to cypermethrin resistance (Yonggyun et al., 1998; Martin et al., 2002; Zhang et al., 2010; Muthusamy and Shivakumar, 2015; Dong et al., 2016). In the current research, significantly high activities of P450 and CarE were observed in all three field populations compared with Lab-SS (Fig. 1). The P450, CarE and GST enzyme activities of these three field populations were 1.3–1.7, 1.9–2.7 and 1.1–1.2 times, respectively, that of the Lab-SS of *S. litura*.

The enzyme results from the current research were the same as those in the reports of Yonggyun et al. (1998) and Karuppaiah et al. (2017), which concluded that insecticide resistance in *S. litura* was caused by the overexpression of detoxification enzymes. Similarly, it has been reported that P450, CarE and GST play a key role in the development of cypermethrin resistance in other insect species such as *Oedaleus asiaticus* (Dong et al., 2016), *Amsacta albistriga* (Muthusamy and Shivakumar, 2015) and *Musca domestica* (Zhang et al., 2010). Sawicki (1985) described the importance of CarE-mediated detoxification or P450-mediated detoxification or both being induced by pyrethroids in the cross-resistance of insects to organophosphates and carbamates. The current results in Fig. 1 are same as those of Karuppaiah et al. (2017), who found that pyrethroid resistance in *S. litura* was due to the over-production of CarE and P450. Thus, both detoxification enzymes might be the major

Table 2
Cypermethrin resistance levels of field populations of *S. litura*.

Source	LD_{50} (ppm)	SE	LCL	UCL	RR	χ^2 (degrees of freedom)
Lab-SS[4]	2.32	0.89	0.85	3.73	–	0.22 (3)
Bang Len, Nakhon Pathom	10.98	3.14	6.51	19.66	4.73	0.65 (4)
Wang Nam Khiao, Nakhon Ratchasima	11.82	2.93	7.49	19.60	5.09	0.89 (4)
Kamphaeng Saen, Nakhon Pathom	15.74	3.61	10.33	25.17	6.78	0.51 (3)

LD_{50} = lethal dose at which half the population is killed and the active ingredient concentration is expressed in parts per million (ppm); LCL = lower confidence limit; UCL = upper confidence limit; RR = resistance ratio; Lab-SS = susceptible strain.

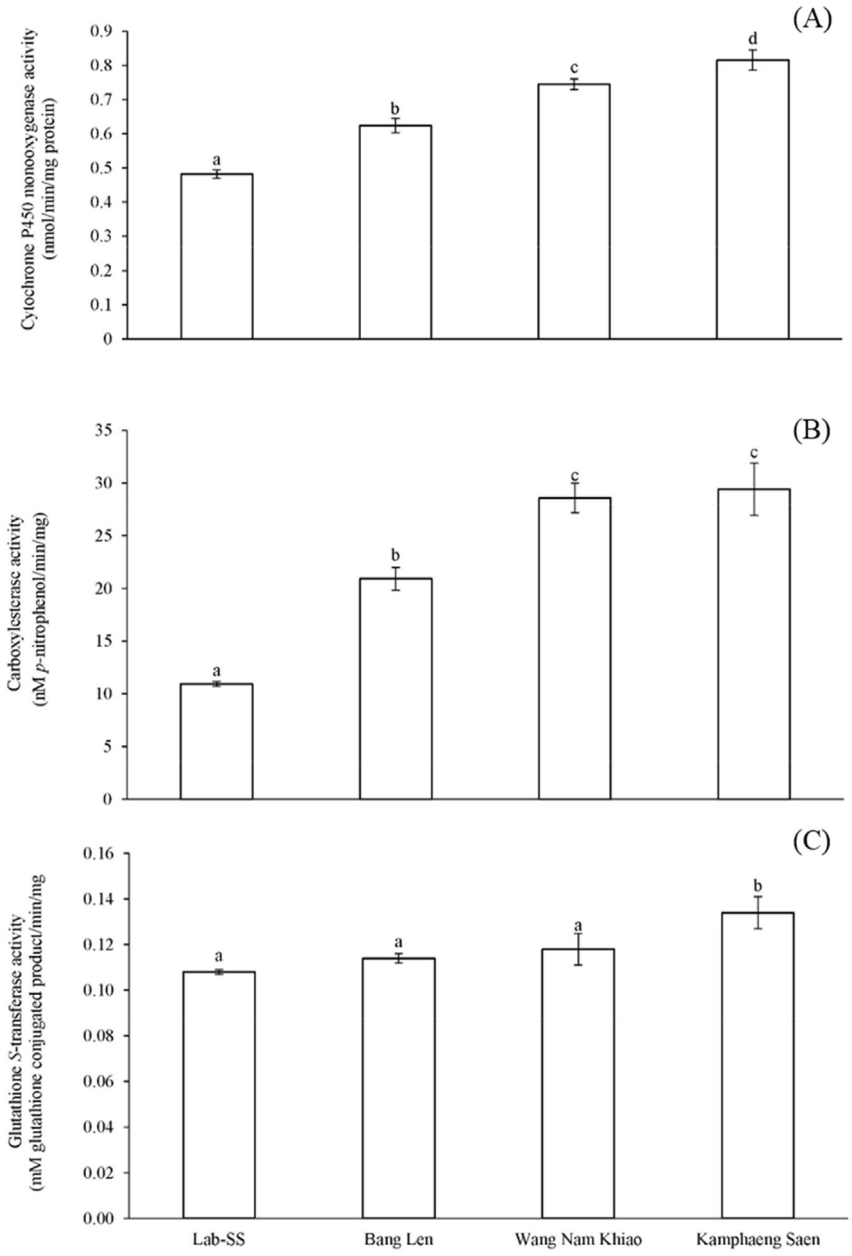

Fig. 1. Detoxification enzyme activities of field populations of *S. litura* collected from three locations in Thailand compared with the susceptible strain (Lab-SS), where different lowercase letters above the bars indicate significant differences according to Tukey's honestly significant difference test ($p < 0.05$): (A) cytochrome P450 monooxygenase activity; (B) carboxylesterase activity; (C) glutathione *S*-transferase activity.

mechanism underlying enhanced tolerance of cypermethrin toxicity.

However, only the population from Kamphaeng Saen, Nakhon Pathom province showed significantly higher GST activity compared to the Lab-SS. GST consists of several isozymes that comprise a family of multifunctional enzymes catalyzing the conjugation of glutathione to electrophilic compounds (Grant et al., 1991). In *Spodoptera littoralis*, at least two forms of isozymes were previously found according to substrate preferences (Lagadic et al., 1993). In the current GST activity experiment, only CDNB was used as a substrate, and this may partially represent the involvement of GST in the insecticide resistance mechanism of *S. litura*. Grant et al. (1991) suggested the relative importance of GST isozymes in a xenobiotic metabolism.

S. litura from the fields in Kamphaeng Saen and Bang Len (both in the same province), exhibited significantly different resistance ratios, so it is possible that the farmer in Kamphaeng Saen may have applied cypermethrin more frequently to control insect pests than in other areas. Farmers around the Wang Nam Khiao field habitually use cypermethrin for the control of multiple pests, which is also causing increasing detoxification enzyme activity.

The data on the development of resistance to cypermethrin in *S. litura* obtained from the current study can be used to guide and improve insecticide resistance management (IRM) strategies in Thailand. Furthermore, the results suggest that new IPM resistance management strategies should be developed to control *S. litura* in Thailand. For example, slow-release pheromone formulations have been shown to disrupt mating (Wei and Du, 2004), and some

simple IPM techniques have been shown to control vegetable pests (Chuachin et al., 2012.). The preservation of the predators, parasitoids and microbial parasites of *S. litura* (Nguyen et al., 2005) or the use of plant extracts are additional control alternatives to reduce applications of synthetic pesticides and could help to slow resistance development.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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