Research Article

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Evaluation and possible mechanism of beet armyworm (*Spodoptera exigua* Hubner) resistance to chlorpyrifos and their sensitivity to neem oil insecticides

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Abstract: The uncontrolled and excessive use of insecticides on *Spodoptera exigua* can cause resistance. The aim of this study is to test resistance of *S. exigua* to chlorpyrifos and determine the possible mechanism of resistance to *S. exigua*. The resistance assay was carried out on chlorpyrifos by determining the level of resistance by the comparison of LC50 between the field samples and the standard samples. The resistivity of *S. exigua* was correlated with the activity of acetylcholinesterase (AChE), esterase, and glutathione S-transferase (GST) enzymes. The samples of *S. exigua* were also tested for their sensitivity to neem oil insecticides. The results showed that *S. exigua* samples from Brebes and Cipanas had a resistance ratio (RR) of 5.50 and 3.26, respectively. The results of the present study indicate that the insensitivity of the AChE and the high activity of the GST play a significant role in the mechanism of *S. exigua* resistance to chlorpyrifos. However, the esterase has fewer roles in the *S. exigua* resistance mechanism for both samples. In addition, the results of neem oil insecticides test showed that *S. exigua* from Brebes and Cipanas samples is sensitive to the insecticide with the RR value less than 1; therefore, this biopesticide has the opportunity to manage resistant pests. A novel mechanism for insecticide resistance by insect was proposed.

Keywords: *Spodoptera exigua*, resistance, chlorpyrifos, enzyme, neem oil insecticides

1 Introduction

It is common when farmers use synthetic insecticides to overcome the invasion of pests. However, an excessive application of the insecticides can cause various adverse effects, including pest resistance. One of the pests *Spodoptera exigua* has been reported resistant to various insecticide including tebufenozide, metaflumizone, and chlorpyrifos (Jia et al. 2009; Che et al. 2013; Su and Sun 2014; Ahmad et al. 2018).

In Indonesia, *S. exigua* is claimed to be resistant to several types of insecticides such as chlorfluazuron (Negara 2005), spinosad, chlorpyrifos, trizophos, methomyl, betasiflutrin, siromazin, carbosufan, thiodikarb, and abamectin (Moekasan and Dan 2007). Wibisono et al. (2007) found that *S. exigua* from several locations in East Java and Central Java was resistant to methoxyphenozides.

The resistance mechanism of insects to insecticidal compounds can be explained by various mechanisms, including biochemical, physiological, and biomolecular mechanism. Yu and Huang (2000) showed that resistance in *Blattella germanica* involves the increasing glutathione S-transferase (GST) activity and the increasing activities of hydrolase and acetylcholinesterase. Furthermore, Nan-nan et al. (2006) found that the resistance of the pests occurred due to the inhibition of penetration of toxic substances into the body. The resistance mechanisms in a particular pest population can be different from other population because of the different types of insecticides it exposed to. In the present report, our research group aimed to determine the status of resistance and resistance mechanism and also to evaluate the sensitivity of the resistant insects to neem oil as an alternative insecticide. The latter step was applied because neem oil insecticides have different mechanism with chlorpyrifos insecticides in the way to manage the pest resistance. Oil insecticides have different mechanisms with chlorpyrifos insecticides in the way to manage the pest resistance.

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2 Materials and methods

The evaluation of *S. exigua* resistance to chlorpyrifos was carried out in several stages, including determination of the level of reference sensitivity, diagnosis of resistance, and determination of the level of resistance to chlorpyrifos. Then, a biochemical analysis of resistance mechanism was carried out on highly resistant *S. exigua* samples. The analysis of resistance was investigated by examination of AChE, esterase, and GST activities of the insects.

This study was carried out using various concentrations of chlorpyrifos with 200 g/L formula. The test was carried out at six stages of concentration including control with three repetitions. The concentrations were selected based on preliminary tests that give $0 < X < 100\%$ mortality. The concentration of the insecticide solution was made by diluting the insecticide formulation in water and adding the emulsifier of alkylaryl polyglycol ether (400 mg/L) (Agristik R) 0.1% (Dono et al. 2010).

The larvae of *S. exigua* used consisted of two groups. The first group is a group of laboratory susceptible larvae (standard) obtained from organic crops that are kept in a pesticide-free state and have passed several generations. The second group consisted of field larvae from Brebes (Central Java) and Cipanas (West Java), Indonesia, which have been using insecticides continuously. The field larvae used the second instar larvae of the F3 generation. The maintenance of the test insects followed the procedures described by Negara (2005) and Wibisono et al. (2007).

2.1 Determination of reference sensitivity

2.1.1 Feed residue test

Leaves that have been cut with a length of 4 cm were dipped into the insecticide solutions for 10 sec, and then, they were dried. The leaves were placed into a petri dish (9 cm in diameter), and 10 s-instar larvae was placed into the container. The experiment was performed for 24 h. After 24 h, the larvae were fed with fresh leaves (without treatment). Observations to calculate the dead larvae were made at 24, 48, and 72 h.

2.1.2 Topical test

In the first step, the movement of larvae was reduced by putting the larvae in the ice bath for 3 min. After the larvae activity decreased, the insecticide was tested by dropping 1 µL of the insecticide solution with particular concentration into the dorsal part of the test insect thorax. Observations to calculate the dead larvae were made at 24, 48, and 72 h.

2.1.3 Determination of resistance level

Mortality data obtained from the feed residue and topical tests were used to determine the resistance level. The data were analyzed using the probit analysis with Polo Plus version 1.0 to obtain LC_{50} and LC_{95} values. The level of resistance was determined by calculating the resistance ratio (RR) from the comparison of the LC50 field sample with the LC50 standard sample. Insect resistance was categorized into three levels: low resistance ($1 < RR < 5$), moderate resistance ($5 < RR < 10$), high resistance ($RR > 10$) (Rodriguez et al. 2007).

2.2 Analysis of resistance mechanisms

Measurements of protein levels of *S. exigua* larvae extract of the standard sample and field sample were carried out using the Lowry method (Kresze 1988). For the enzyme activity assay, third instar larvae homogenates from standard samples and F1 generation field samples were prepared. The same insects were chosen and cleaned from the surface dirt. The number of insect larvae needed for each milling was 10 (10 mg larvae/mL). The larvae were crushed using a glass homogenizer (1 mL capacity) using a 0.1 M phosphate buffer pH 7.5 at 4°C. The homogenate was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was used as a source of enzymes

2.2.1 Acetylcholinesterase assay

The acetylcholinesterase activity assay was carried out according to the method described by Ellman et al. (1961). Light absorption was measured by a spectrophotometer at $\lambda = 412$ nm. The acetylcholinesterase activity is expressed as light absorption per minute per mg of protein. The percentage inhibition of acetylcholinesterase activity by certain insecticides is calculated by equation (1).

$$\%\ \text{Inhibition} = (A_0 - A_1)/A_0 \times 100\%, \quad (1)$$

where A_0 is acetyl cholinesterase specific activity in control (without insecticide) (M substrate/min/mg protein)

and A_1: acetyl cholinesterase specific activity in insecticide treatment.

Then, the relationship between the concentration of the insecticide and the inhibition of the activity of the AChE enzyme was analyzed by using the probit analysis using Polo Plus Version 1.0. From this relationship, I_{50} (inhibitions 50) was obtained, and it showed a 50% reduction in the AChE activity.

2.2.2 Esterase assay

The activity of α-naphthyl acetate esterase (total esterase activity) and α-naphthyl carboxylesterase was determined by the method suggested by Yu et al. (2003). Light absorption was measured by a spectrophotometer at λ = 490 nm. Total esterase activity and carboxylesterase were expressed as light absorption per minute per mg of protein.

2.2.3 Glutathione S-transferase assay

The evaluation of glutathione S-transferase activity was carried out using 1-chloro-2,4-dinitro benzene (CDNB) as substrate. Into the test tube containing 0.9 mL of 0.1 M phosphate buffer pH 7.5, 20 µL *S. exigua* homogenates, 100 µL glutathione 0.001 M and 10 µL CDNB were added. Light absorption was measured by a spectrophotometer at λ = 340 nm at 30°C (Habig et al. 1974; Dono et al. 2010). The glutathione S-transferase activity was expressed as light absorption per minute per mg of protein.

The enzyme activity of AChE, esterase, and GST was calculated using the formula:

$$\mathrm{AU} = (A_s - A_k)/(0.001 \times t_i \times V_e), \quad (2)$$

where AU is the unit activity, A_s is absorption of the sample tested, A_k is control absorption (which is simmer), t_i is the incubation time (30 min), V_e is the volume of the enzyme tested (AchE, 0.2 mL), esterase (0.05 mL), and GST (0.02 mL).

The unit activity value that has been obtained is then calculated, so that the enzyme specific activity value is obtained using the following formula:

$$\mathrm{Asp} = \mathrm{AU}/\mathrm{KP}, \quad (3)$$

where ASp is the specific activity, AU is the unit activity, and KP is the protein content of the sample.

2.3 Insect sensitivity assay to neem oil insecticides

The resistant *S. exigua* sample was tested for their sensitivity to biopesticides from neem oil, which was already in the formula of 50 EC. The test was carried out using the residue method on the feed leaves. The assay was carried out using similar protocol for the determination of reference sensitivity level (sub 2.1).

3 Results and discussion

3.1 Resistance ratio of *S. exigua*

LC_{50} values of chlorpyrifos against *S. exigua* of standard, Brebes, and Cipanas samples in the feed residue test were as follows: 0.034, 1.87, and 0.111 mL/L, respectively. The resistance ratio (RR) values of *S. exigua* of Brebes and Cipanas samples were 5.50- and 3.26-folds, respectively, to the standard samples (Table 1). The feed residue test showed that *S. exigua* of the Brebes samples had moderate resistance, while the Cipanas samples had low resistance against the insecticide based on the classification described by Rodriguez et al. (2007).

LC_{50} values of chlorpyrifos against *S. exigua* of susceptible, Brebes, and Cipanas samples in the topical test were 1.289, 2,860; and 2.081 mL/L, respectively. RR values of *S. exigua* of Brebes and Cipanas populations were 2.22- and 1.61-folds, respectively, to the standard

Table 1: Probe analysis results of chlorpyrifos toxicity test in the feed residue test

Samples	*a* ± SE	*b* ± SE	LC_{50}	$CI_{95\%}$	LC_{95}	$CI_{95\%}$	RR
Standard	4.563 ± 0.518	3.108 ± 0.340	0.034	0.025–0.048	0.115	0.072–0.353	
Brebes	2.188 ± 0.237	3.002 ± 0.300	0.187	0.140–0.248	0.659	0.442–1.397	5.50
Cipanas	1.853 ± 0.247	1.941 ± 0.237	0.111	0.050–0.259	0.781	0.307–57.096	3.26

a: intercept, *b*: slope, SE: standard error, LC: lethal concentration (mL/L), CI: confidence interval, RR: resistance ratio, IC: inhibition concentration (mL/L), IR: inhibition ratio, r^2: coefficient of variation.

samples (Table 2). These values were categorized as low resistance (Rodriguez et al. 2007). The topical test was carried out to determine the mechanism of resistance that is affected by the inhibition of the penetration rate of the insect integument. The RR values of the two populations were low; however, it still indicated the resistance. This resistance can be affected by the penetration inhibition toward the insect integument. Nan-nan et al. (2006) showed that cuticle of the resistant strains are thicker than the cuticle of the susceptible strains. In addition, the cuticle of the resistant strains has waxy, chitin layers, and epidermal cells between cells that were thicker than that of the susceptible strains. The concentration value needed for chlorpyrifos in the topical method is higher than the leaf dyeing method. This allows *S. exigua* from all three samples to have developed mechanisms to reduce insecticide penetration, so that higher concentrations are needed to enter the body of the test insect. However, when viewed from the value of the resistance ratio, the RR value in the leaf dye method is higher and indicates the occurrence of a resistance mechanism that is influenced by the biochemical activity in the insect's body (Tables 1 and 2). Gong et al. (2013) state that pesticide resistance is caused by several factors such as different regions, genetics, and environment in each population.

3.2 Analysis of acetylcholinesterase, esterase, and glutathione S-transferase activity

3.2.1 Acetycholinesterase assay

The results of the probit analysis of inhibition of enzyme activity showed that the concentration required to inhibit 50% of the AChE enzyme activity (IC_{50}) was 0.028 mL/L in the standard samples, 0.189 mL/L in the Brebes samples, and 0.087 mL/L in the Cipanas samples. The ratio of the insensitivity of the AChE enzyme to chlorpyrifos in the Brebes and Cipanas samples to the standard sample was 6.75- and 3.11-folds, respectively (Table 3). This indicates that the main mechanism of chlorpyrifos resistance is the activity of AChE enzyme.

Hastutiek and Fitri (2002) stated that resistance to organophosphate is caused by the mutation of the carboxylesterase gene, so that AChE becomes insensitive to the insecticide. The insensitivity of AChE increases in resistant population according to the inhibition of AChE with the increasing inhibition value of 50 (IC_{50}) (Baek et al. 2005). Acetylcholinesterase from field strains can reach 2- to 85-folds less sensitive than from strains that are susceptible to organophosphate inhibition (Yu et al. 2003).

The increase of AChE production contributes to insect resistance, where enzymes are not sensitive to insecticide inhibition (Charpentier and Fournier 2001). AChE activity has a correlation with the level of resistance to insecticides by inhibiting AChE (Stankovic and Rahovic 2017 July).

3.2.2 Esterase assay

The specific activity of esterase from Brebes (621.35 units/mg) and Cipanas (788.22 units/mg) samples is lower than the standard samples (804.00 units/mg) (Figure 1a). This shows that the possibility of the esterase enzyme plays fewer roles in the mechanism of resistance of *S. exigua* against exposure to chlorpyrifos insecticides.

This is different from the results of other studies that the esterase activity in the standard samples is lower than the field samples in cases of resistance to organophosphate insecticides (Tiwari et al. 2012; Darvishzadeh and Sharifian 2015; Mulyaningsih et al. 2017). This can result from the differences in the area of origin affecting the esterase activity so that its activity can be low, moderate, or high depending on the chemical reactions that exist in the body of the insect against insecticides (Parmar and Patel 2018).

The increase of esterase activity is a general resistance mechanism that occurs in *Anopheles stephensi*, *Amsacta albistriga*, *Anisopteromalus calandrae*, *Myzus persicae*, and *Plutella xylostela* against organophosphate insecticides (Damayanthi and Karunaratne 2005; Muthusamy et al. 2012;

Table 2: Probe analysis results of chlorpyrifos toxicity test in topical test

Sample	$a \pm$ SE	$b \pm$ SE	LC_{50}	$CI_{95\%}$	LC_{95}	$CI_{95\%}$	RR
Standard	−0.460 ± 0.102	4.172 ± 0.447	1.289	1.163–1.437	3.195	2.637–4.233	
Brebes	−2.226 ± 0.280	4.878 ± 0.549	2.860	2.218–3.560	6.216	4.630–13.445	2.22
Cipanas	−2.143 ± 0.257	6.736 ± 0.56	2.081	1.801–2.411	3.651	2.967–5.961	1.61

a: intercept, *b*: slope, SE: standard error, LC: lethal concentration (mL/L), CI: confidence interval, RR: resistance ratio, IC: inhibition concentration (mL/L), IR: inhibition ratio, r^2: coefficient of variation.

Table 3: The level of sensitivity of AChE in *S. exigua* to chlorpyrifos

Sample	$a \pm SE$	$b \pm SE$	IC_{50}	$CI_{95\%}$	IC_{95}	$CI_{95\%}$	IR	r^2
Standard	4.950 ± 0.388	2.190 ± 0.249	0.028	0.025–0.032	0.092	0.077–0.116		0.985
Brebes	1.941 ± 0.156	2.679 ± 0.198	0.189	0.139–0.254	0.775	0.498–1.824	6.75	0.965
Cipanas	2.151 ± 0.178	2.032 ± 0.165	0.087	0.045–0.153	0.563	0.267–5.307	3.11	0.968

a: intercept, *b*: slope, SE: standard error, LC: lethal concentration (mL/L), CI: confidence interval, RR: resistance ratio, IC: inhibition concentration (mL/L), IR: inhibition ratio, r^2: coefficient of variation.

Prasad et al. 2017). However, in this study, the esterase activity cannot be used to explain the differences in the level of *S. exigua* resistance to chlorpyrifos insecticides. Tarwotjo and Rahadian state that the activity of the esterase enzyme cannot always be used to explain the differences in resistance levels because the differences in enzyme activity are likely to occur by the differences in genes in each population (Tarwotjo and Rahadian 2018). Such a mechanism of resistance to emamectin benzoate is not influenced by detoxification enzyme inhibitors and may be given by other mechanisms (Su and Sun 2014).

3.2.3 Glutathione S-transferase assay

The test results showed that the highest GST enzyme specific activity value in *S. exigua* Brebes' samples (5703.93 units/mg) was followed by Cipanas' samples (4191.50 units/mg). Both field samples have higher GST specific activity values than the standard samples (805.10 units/mg) (Figure 1b). So, it can be stated that the GST enzyme plays a role in the mechanism of *S. exigua* resistance to chlorpyrifos insecticides.

This is supported by the results of other studies that higher GST levels have been linked to detoxification and insect resistance to organophosphate insecticides such as in some lepidopteran pests, including *Helicoverpa armigera* (Yu and Huang 2000), *S. frugiferda* (Yu et al. 2003), and *H. longicornis* (Hernandez et al. 2018). GST plays an important role in the intestines of *Spodoptera litura* to protect insects from the toxic effects (Xu et al. 2015). Thus, it can be stated that GST plays a role in the mechanism of resistance of *S. exigua* against chlorpyrifos, which are organophosphate groups.

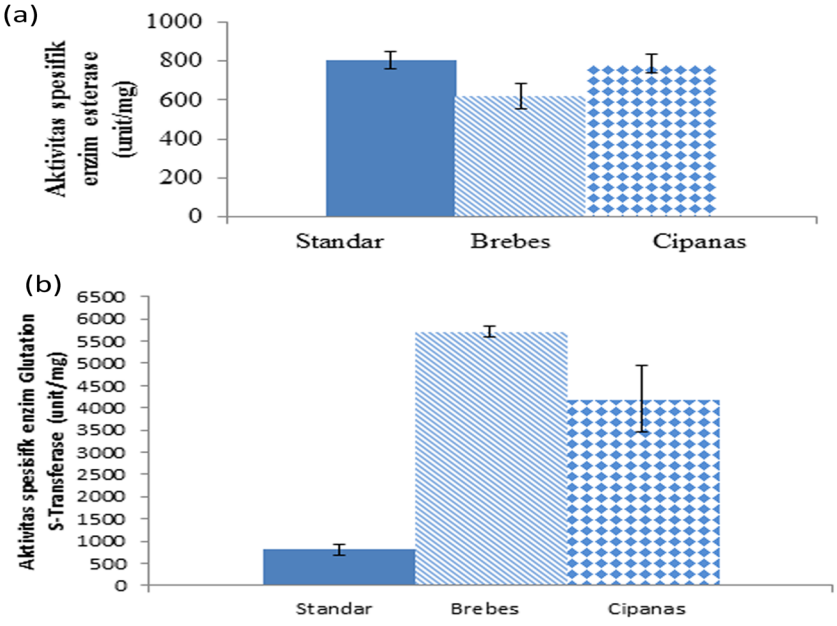

Figure 1: (a) Specific activity of esterase and (b) specific activity of GTS.

Table 4: The result of test probit analysis of the neem biopesticide test on the *S. exigua*

Sample	$a \pm$ SE	$b \pm$ SE	LC_{50}	$CI_{95\%}$	LC_{95}	$CI_{95\%}$	RR
Standard	−6.156 ± 0.695	5.187 ± 0.566	15.374	14.076–16.689	31.907	27.757–39.099	
Brebes	−5.508 ± 0.642	4.801 ± 0.33	14.036	12.723–15.325	30.893	26.687–38.281	0.913
Cipanas	−5.584 ± 0.650	4.811 ± 0.536	14.477	13.145–15.796	31.810	27.442–39.525	0.941

a: intercept, *b*: slope, SE: standard error, LC: lethal concentration (mL/L), CI: confidence interval, RR: resistance ratio, IC: inhibition concentration (mL/L), IR: inhibition ratio, r^2: coefficient of variation.

3.3 *S. exigua* sensitivity to neem oil insecticides by feed residue test

Neem oil insecticide test results show that *S. exigua* from Brebes and Cipanas samples are still sensitive to these insecticides. This can be seen from the RR values of the two samples (Brebes and Cipanas), which are smaller than one (RR < 1) to the standard samples. LC50 values of neem oil insecticide for *S. exigua* samples of standard, Brebes, and Cipanas were 15.374, 14.036, and 14.477 mL/L, respectively. *S. exigua* samples from Brebes has a higher sensitivity than that from Cipanas (Table 4).

4 Conclusion

The level of resistance of *S. exigua* of the Brebes and Cipanas samples in the feed residue and the topical test showed RR values of 5.50 and 3.26; 2.22 and 1.61, respectively. The insensitivity values of AChE in the Brebes and Cipanas samples were 6.75- and 3.11-folds higher than the laboratory susceptible samples, respectively. The test results show that the mechanism that plays a role in the resistance of *S. exigua* Brebes and Cipanas samples is the insensitivity of the acetylcholinesterase enzyme and the high activity of the GST detoxification enzyme. The esterase has fewer roles in the *S. exigua* resistance mechanism of the two samples. The possible mechanism of biochemical resistance to chlorpyrifos can be demonstrated by the increased activity of the enzyme acetylcholinesterase, GTS, or esterase. The results of neem oil insecticide test showed that *S. exigua* from Brebes and Cipanas samples were still sensitive to the insecticide with the RR value less than 1; therefore, this biopesticide is likely to be used to control *S. exigua*, which is resistant to chlorpyrifos.

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