

Resistance of *Nilaparvata lugens* to Fenobucarb and Imidacloprid and Susceptibility to Neem Oil Insecticides

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ABSTRACT

This study aims to monitor and determine the level of resistance of *N. lugens* to fenobucarb and imidacloprid, to analyse of the specific activities of acetylcholinesterase, Glutathione S-Transferase, esterase, and to determine sensitivity of resistant *N. lugens* to the neem oil. Resistance tests were conducted on a field population from Cipunagara, Subang, West Java, Indonesia, and a standard population originating from Banyuwangi, East Java, that was obtained from the Indonesian Center for Rice Research. Synthetic and neem Insecticides were applied using the leaf-stem dipping method. The resistance ratio obtained by comparing the LC_{50} value of the field population with the standard. The test insects were considered to be resistant to synthetic insecticide if they had a resistance ratio (RR) of more than 4, indicated resistance if $RR \geq 1$, and susceptible to neem if has $RR < 1$. The results of the research showed that the *N. lugens* population of Cipunagara indicated resistance to fenobucarb ($RR = 2.43$), while it was clearly resistance to imidacloprid ($RR = 13.95$). Specific activities of the acetylcholinesterase and Glutathione S-Transferase of the *N. lugens* Cipunagara populations were higher than the standard, while the esterase specific activity was lower. These two enzymes are responsible for the resistance of *N. lugens* to fenobucarb and imidacloprid. Nevertheless, the *N. lugens* population of Cipunagara was susceptible to neem ($RR < 1$) that never been reported before. Therefore, it is important to know the change in the sensitivity of the target and the metabolic pathway of the insecticide used to manage insect resistance.

1. Introduction

In general, intensive use of synthetic insecticides can cause the development of a resistant population of *Nilaparvata lugens* Stal. (Hemiptera: Delphacidae). Resistance to insecticides defined as 'a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of an insecticide product to achieve the expected level of pests control when used according to the label recommendation doses for that pest species (Insecticides Resistance Action Committee (IRAC) 2013, 2021; Baehaki *et al.* 2016). Resistant insects are formed because of the selection process, which causes an increase in the resistant population through the development of the

biochemical detoxification mechanism (Dono *et al.* 2010; Khan *et al.* 2020). Cases of *Nilaparvata lugens* resistance led to the banning of some insecticides for use with rice plants in 1986 and more recently in 2015 by the Indonesian government. The active ingredients prohibited are included in the group of organophosphates (24 active ingredients) and carbamate (carbaryl, methomyl and kartap hydrochloride). Suggest that insect resistance to insecticides is a common problem in many countries (Liu *et al.* 2005). Study resistance of *N. lugens* to insecticides in China and Bangladesh indicate that *N. lugens* has been resistance to several insecticides that are imidacloprid (resistance ratio, $RR = 2491.0-8078.5$ -fold), thiamethoxam ($RR = 303.6-1253.6$ -fold), buprofezin ($RR = 175.7-365.1$ -fold) and dinotefuran ($RR = 49.9-596.0$ -fold), low to moderate levels of resistance to nitenpyram ($RR = 12.1-67.3$ -

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fold) and sulfoxaflor (RR = 3.8–25.5-fold) (Datta *et al.* 2021). According to the latest record in Arthropod Pesticide Resistance Database (APRD), it has been reported that there were 445 cases of *N. lugens* that were resistant to insecticides throughout the world (APRD 2021).

Insecticides with active ingredient of fenobucarb (carbamate) and imidacloprid (neonicotinoid) are registered in Indonesia to control *N. lugens* (Directorate of Fertilizers and Pesticides 2016). Fenobucarb (carbamate) is an insecticide that inhibits the action of the enzyme Acetylcholinesterase (AChE) (Casida and Durkin 2013), while imidacloprid (neonicotinoid) has a neuroexcitatory effect and irreversibly blocks the acetylcholine receptor (Cox 2001; Tan *et al.* 2008).

Cases of *N. lugens* resistance to both insecticides have been widely reported. *N. lugens* from populations in Serang, Karawang, Subang, Indramayu, Purbalingga and Pasuruan (Indonesia) had resistance ratio (RR) values to imidacloprid of 4.8–108.1 times. The levels of resistance to fenobucarb were 6.6 in Indramayu, while in Karawang, Subang, Purbalingga and Pasuruan the RR values were 2.5–3.00 (Surahmat *et al.* 2016). The populations of Sukamandi and Juwirig were resistant to imidacloprid (RR = 12.7, RR = 7.0), but susceptible to fenobucarb (RR = 1.8, RR = 3.3) (Baehaki *et al.* 2016). Unfortunately, no information about biochemical mechanism of *N. lugens* resistance reported from these areas of studies, besides never yet tested the botanical insecticide against the resistant insect that will be evaluated in this research. An effective handling of resistance involves the initial detection of problems, the combination of information on resistant insects, and determination of resistance ratio data, so that appropriate selection of insecticides can be made (Haque *et al.* 2002).

There are several strategies to manage *N. lugens* resistance, one of which is the rotation of the applied insecticides with those that have different modes of action (Hastutiek and Fitri 2002). The use of a broad spectrum of botanical insecticides with various modes of action, such as neem (*Azadiracta indica*) seed oil, is one alternative. Neem oil, which acts by hormonal disruption, can inhibit or delay the development of instars, disrupt the moulting process, and disrupt ovarian development (Suryaningsih and Hadisoeganda 2007; Pathak and Tiwari 2017). Based on this background, the study aims to 1) monitor and determine the level of resistance of *N. lugens* from Cipunagara (Subang, West Java, Indonesia)

to fenobucarb and imidacloprid, 2) to evaluate the specific enzyme activities of AChE, Glutathione S-Transferase (GST) and esterase to determine the biochemical mechanism of *N. lugens* resistance, and 3) to determine sensitivity of resistant *N. lugens* to the botanical insecticide formulation of neem oil that never been studied before.

2. Materials and Methods

2.1. Plants, Insects, and Insecticides

The test insects used were *N. lugens*, which were obtained from Banyuwangi (the standard population) and Cipunagara (the field population). The standard population of *N. lugens* was obtained from the Indonesian Center for Rice Research (ICRR) and had been reared in a greenhouse for 35 generations without exposure to insecticides. The field population was taken from Padamulya Village, Cipunagara District, Subang Regency in July 2018. The rice plants used for the maintenance and testing of *N. lugens* were IR64 varieties.

The test insecticides used contained the active ingredient fenobucarb (BPMC) 500 gL⁻¹ in the form of an emulsifiable concentrate (EC) formulation, and imidacloprid 200 gL⁻¹ in the form of a soluble concentrate (SC). Fenobucarb has mode of action as acetylcholine inhibitor (class 1A: carbamate), while imidacloprid as Nicotinic acetylcholine receptor (nAChR) competitive modulators (class 4A: neonicotinoid) (Directorate of Fertilizers and Pesticides 2016; IRAC 2021). The botanical insecticide used was neem oil 500 gL⁻¹ in the form of an EC formulation (azadirachtin content 133.80±24.91 mgL⁻¹) (Dono *et al.* 2017). Five series of concentrations and controls were tested for each insecticide. Distilled water was mixed with an emulsifier (alkyl aryl polyglycol ether 400 mg.L⁻¹) (Agristic®) at a concentration of 0.5 mL.L⁻¹ (Dono *et al.* 2010). The control *N. lugens* was treated only with distilled water with the addition of emulsifiers at the same concentration.

2.2. Resistance Diagnosis

Resistance diagnosis was conducted to detect the *N. lugens* of the Cipunagara population (field population) that were resistant to insecticides. From this population, 200 and 60 *N. lugens* were taken as test insects and control respectively. The concentration of the insecticide tested was obtained from the LC₉₅ of the Banyuwangi population (standard population). The tests were performed using the residual method (leaf dipping method) that will do as described in

testing the *N. lugens* resistance level to the insecticide. Furthermore, the number of individuals that survived over the expected proportion (5%) was calculated using a one-way Z test (Roush and Miller 1986) (Equation 1).

$$Z = \frac{(s - ng) - 0.5}{\sqrt{ng(1 - g)}} \quad \text{Eq.1}$$

Where:

- s = number of individuals which survive
- n = sample size (namely 200 individuals)
- g = percentage of individuals surviving expectations in standard strains (0.05)

Furthermore, the minimum number of individuals that survived to reach the significant level was calculated using Equation 2:

$$s = Z\sqrt{ng(1 - g)} + ng + 0.5 \quad \text{Eq.2}$$

2.3. Testing of the Resistance Level of *N. lugens*

The *N. lugens* population from Cipunagara was exposed to fenobucarb and imidacloprid to determine its resistance level. The test used five levels of concentration and a control, which were repeated at least three times on 20 *N. lugens* adult female test insects per repetition (Heong *et al.* 2011). The experiment employed the leaf dipping method (residual method in plants /contacts).

The method used refers to Surahmat *et al.* (2016) and was based on IRAC test method number 005. Ten–twelve day old rice seedlings were cleaned with water to remove any soil. A total of 10 rice plants were wrapped in two sheets of wet tissue paper, put in a plastic tube (5 cm in diameter, 5.5 cm in height), given a 1% (w/v) water-agar solution 37 to cover the roots, and left until settled. Subsequently, all parts of the rice were dipped for 10 seconds into the test insecticide solution, dried for 15 minutes, and placed into a cylindrical plastic cage with a diameter of 5 cm and a height of 20 cm. Then 10 adult *N. lugens* females were put into the cage. Mortality due to fenobucarb exposure was recorded at 24 and 48 hours, while for imidacloprid exposure was recorded at 24, 48, and 72 hours (IRAC 2013). The correlation of concentration and mortality data were analyzed using the Polo Plus Version 1.0 application to obtain the LC₅₀ and LC₉₅ values.

The value of the resistance level was calculated using Equation 3 (Georghiou 1962; Dono *et al.* 2010):

$$\text{Resistance Ratio (RR)} = \frac{\text{LC}_{50} \text{ field population}}{\text{LC}_{50} \text{ standard population}} \quad \text{Eq.3}$$

Insect populations originating from the field were considered to be resistant if they have an RR ≥ 4 (Winteringham 1969; Surahmat *et al.* 2016), with indications that resistance had occurred if the RR ≥ 1 (Surahmat *et al.* 2016).

2.4. Biochemical *N. lugens* Resistance Mechanism Analysis

Samples of adult female test insects were homogenized at low temperature with a concentration of 100 mg/ml in 0.1 M phosphate buffer pH 7.0 using Potter-Elvehjem homogenizer. This was used as the stock solution. The homogenate was centrifuged at 10,000 rpm for 1 minute and the supernatant taken quickly so that the sediment did not dissolve again. For enzyme activity assay and protein determination, the stock solution was diluted when it was required. For control of enzyme activity assay, the sample were heated at 90°C for 10 minutes (Dono *et al.* 2010; Burden 2012).

2.4.1 Asetilkolinesterase Activity (AChE) Assay

The method for AChE assay was based on the method proposed by Ellman *et al.* (1961) and Dono *et al.* (2010). 200 μ l of the sample was added into a 10 ml test tube with 2 ml of 0.1 M phosphate buffer pH 7.5 and 150 μ l of 0.0011 M DTNB in the same buffer. 100 μ l of insecticide with varying concentrations (buffer solution used as control) was then added to the tube and homogenized, followed by 10 minutes of incubation at 37°C. 100 μ l of 0.0105 M acetylcholine iodide was then added to the mixture. Subsequently, the mixture was incubated for 30 minutes at 37°C. The absorbance was measured using a spectrophotometer at $\lambda = 412$ nm. Enzyme activity was determined based on the absorbance increase at a wavelength of 412 nm, and was defined as an increase in absorption of 0.001 units of absorbance above the control, per ml of the enzyme tested per minute. The activity is calculated using equation (5). The percentage inhibition of acetylcholinesterase activity by the insecticide was then calculated by Equation 4.

$$\% \text{ Inhibition} = \frac{A_0 - A_1}{A_0} \times 100\% \quad \text{Eq.4}$$

Where:

- A_0 = control of acetyl cholinesterase
(M substrate/minute/mg protein) control
(without insecticide)
 A_1 = acetyl cholinesterase activity with
insecticide treatment

Furthermore, the relationship between insecticide concentration and inhibition of AChE enzyme activity was analyzed using probit analysis Polo Plus Version 1.0. From this relationship, IC_{50} (inhibition concentration at 50%) was obtained, which showed a 50% reduction in AChE activity.

2.4.2. Esterase Activity Assay

The esterase activity was measured using α -naphthyl acetate as substrate (Yu *et al.* 2003; Dono *et al.* 2010). 0.5 ml of 0.1 M phosphate buffer solution pH 7.0, and then 15 μ l α -naphthyl acetate 0.02 M (dissolved in methanol) and 2 ml fast blue solution 0.25 mg/ml in distilled water was then added to 50 μ l of the sample. The mixture was then incubated at 37°C for 30 minutes and the absorption measured at $\lambda = 490$ nm and used to calculate the activity of the enzyme. As a control, the same sample, which had been heated at a temperature of 90°C for 10 minutes and centrifuged, was used. Enzyme activity was calculated using Equation (5). The enzyme activity was determined based on increased absorbance at 490 nm and was defined as an increase in absorption of 0.001 units of absorbance above the control, per ml of the enzyme tested per minute.

$$AU = \frac{As - Ak}{0.001 \times ti \times Ve} \quad \text{Eq.5}$$

Where:

- AU = activities Unit
As = absorption of samples tested
Ak = aontrol absorption (Boiled samples)
ti = incubation time (30 minutes)
Ve = volume of enzyme tested: AchE (0.2 ml),
esterase (0.05 ml) and GST (0.02 ml)

2.4.3. Glutathione S-Transferase Activity Assay

Glutathione S-Transferase activity used a 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. 0.86 ml of 0.1 M phosphate buffer pH 6.5, 20 μ l homogenate, 100 μ l 10 mM glutathione in pH buffer 6.5, and 10 μ l 1 mM CDNB (in ethanol) were mixed in a test tube and incubated at 37°C. Next, light absorption was measured by a spectrophotometer at $\lambda = 340$ nm and

enzyme activity calculated using Equation (5). This was determined based on the increased absorbance at 340 nm and defined as an increase in absorption of 0.001 units of absorbance above the control, per ml of the enzyme tested per minute (Habig *et al.* 1974; Dono *et al.* 2010).

2.5. Testing of *N. lugens* Susceptibility to Botanical Insecticide

The *N. lugens* populations from Cipunagara and Banyuwangi were tested for their susceptibility to neem oil insecticide (formulation 50 EC). The experiment used the food dipping method (residual method on plants), as explained in the testing of the level of fenobucarb and imidacloprid insecticide resistance. In the susceptibility testing of *N. lugens* to neem oil insecticide, nymph 4th instar was used, with observations of death made at 24-hour intervals for 10 days. The data were analyzed using the Polo Plus Version 1.0 program to obtain the LC_{50} and LC_{95} values. In this susceptibility test, a resistance ratio (RR) was used as an indicator for insect susceptibility to neem oil insecticide. If $RR < 1$, this means that the insects are sensitive to the insecticide (Dono *et al.* 2014).

3. Results

3.1. Detection and Resistance Level

3.1.1. Resistance Detection

Resistance diagnosis was performed as the first step to find *N. lugens* resistance from the Cipunagara population. The proportion of individuals that survived after the application of fenobucarb (carbamate) and imidacloprid (neonicotinoid) was 0.0786 and 0.1419 respectively (Table 1). Both figures are greater than the minimum proportion of surviving individuals, which is 0.0778. This indicates that *N. lugens* from Cipunagara has developed resistance to fenobucarb and imidacloprid. Therefore, further investigation of the resistance level is needed to determine the resistance ratio (RR) against the test insecticide.

3.1.2. Level of Resistance

The results of the fenobucarb and imidacloprid insecticide toxicity tests indicated a relationship between the concentration of insecticide and the mortality of the insects. The treatment with fenobucarb insecticide of the *N. lugens* population from Cipunagara (the field) showed that the LC_{50}

and LC₉₅ values were 0.85 ml/L and 2.73 ml/L respectively. These toxicity value were higher than for the Banyuwangi population (standard) (LC₅₀ 0.35 ml/L and LC₉₅ 2.01 ml/L). The results of the toxicity test of imidacloprid insecticides on *N. lugens* from the Cipunagara population (field) showed that this insecticide had LC₅₀ and LC₉₅ value of 7.95 ml/L and 54.11 ml/L respectively. The toxicity of imidacloprid insecticides on the *N. lugens* population from Banyuwangi (standard) was much lower, namely 0.57 ml/L and 4.07 ml/L for LC₅₀ and LC₉₅ respectively (Table 2).

Based on the calculation of the resistance ratio, the population of Cipunagara was already resistant to imidacloprid insecticides, with a ratio of 13.95, and it has also showed indications of resistance to fenobucarb insecticides, with a resistance ratio of 2.43 (Table 2). The high resistance ratio to imidacloprid was probably caused by the intensive use of this insecticide by farmers in the sampling location. The low level of resistance to fenobucarb was because farmers rarely used this insecticide. They usually rotate insecticides if any are found to be ineffective.

3.2. Analysis of Enzyme Activity

3.2.1. Acetylcholinesterase Activity Analysis

The activity of the acetylcholinesterase enzyme (AChE) was tested to observe the effect of fenobucarb (carbamate) insecticide on the activity of the AChE

enzyme and its resistance to the insecticide. The test results showed that the activity of the AChE from the Cipunagara population was higher than that of the Banyuwangi population, at 4.67 and 2.76 units/mg respectively (Figure 1).

When fenobucarb was applied, AChE-specific activity decreased in line with the increasing concentration of the insecticide used. The linear regression between insecticide concentration and enzyme specific activity is presented by equation $y = -5.5486x + 3.4275$ and $y = -0.9254x + 5.7858$, for AChE-specific activity from the Banyuwangi and Cipunagara populations respectively. The correlation coefficient in both populations was $r = 0.98$, which indicates a very strong relationship between the insecticides inhibition and enzyme activity (Figure 1). The decrease in enzyme activity is also shown by the inhibition of the AChE by the insecticides (Figure 2).

The effect of the fenobucarb insecticide on the inhibition activity of AChE showed that the resistance level of *N. lugens* to the fenobucarb insecticide was related to the insensitivity of the AChE enzyme. The level of AChE enzyme insensitivity of the *N. lugens* from the Cipunagara (field) population to fenobucarb was 2.61 times that of Banyuwangi (standard) population (Table 3). This value of insensitivity of the enzyme was similar to the level resistance to fenobucarb (Table 2), which indicates that the main mechanism of fenobucarb insecticide resistance was related to the AChE.

Table 1. Proportion of survival of *N. lugens* after treatment with LC₉₅ of the Banyuwangi standard population

Population	Insecticides	Proportion of individuals surviving in LC ₉₅ insecticide treatment
<i>N. lugens</i> population of Cipunagara	Fenobucarb	0.0786
	Imidakloprid	0.1419

Significantly different from the expectation at the 95% real level (the minimum proportion to reach the real level is ≥ 0.0778)

3.2.2. Esterase Activity

An esterase enzyme activity test was conducted to determine its role as one of the detoxification enzymes of *N. lugens*. The enzyme test results show that the esterase-specific activity in the Banyuwangi population (standard) was higher than that of the Cipunagara (field) population. Specific esterase activity from the *N. lugens* population of Banyuwangi was 187.20 units/mg, while for that of Cipunagara it was 168.41 units/mg. The difference

Table 2. The resistance ratio of 500 g/L fenobucarb insecticide and 200 g/L imidacloprid against *N. lugens* originated from Cipunagara population (field) and Banyuwangi (standard)

Insecticide	Population	a±SE	b±SE	LC ₅₀	CI _{95%}	LC ₉₅	CI _{95%}	RR
Fenobucarb (carbamate)	Banyuwangi	0.96±0.12	2.12±0.21	0.35	0.29 - 0.42	2.10	1.52 - 3.32	2.43
	Cipunagara	0.23±0.09	3.24±0.32	0.85	0.74 - 0.96	2.73	2.22 - 3.64	
Imidacloprid (neonicotinoid)	Banyuwangi	0.47±0.10	1.92±0.18	0.57	0.46 - 0.70	4.07	2.84 - 6.71	13.96
	Cipunagara	-1.78±0.21	1.98±0.19	7.95	6.45 - 9.72	54.11	38.41 - 87.49	

a: intercept, b: slope, SE: standard error, LC: lethal concentration (ml/l), CI: confidence interval, RR: resistance

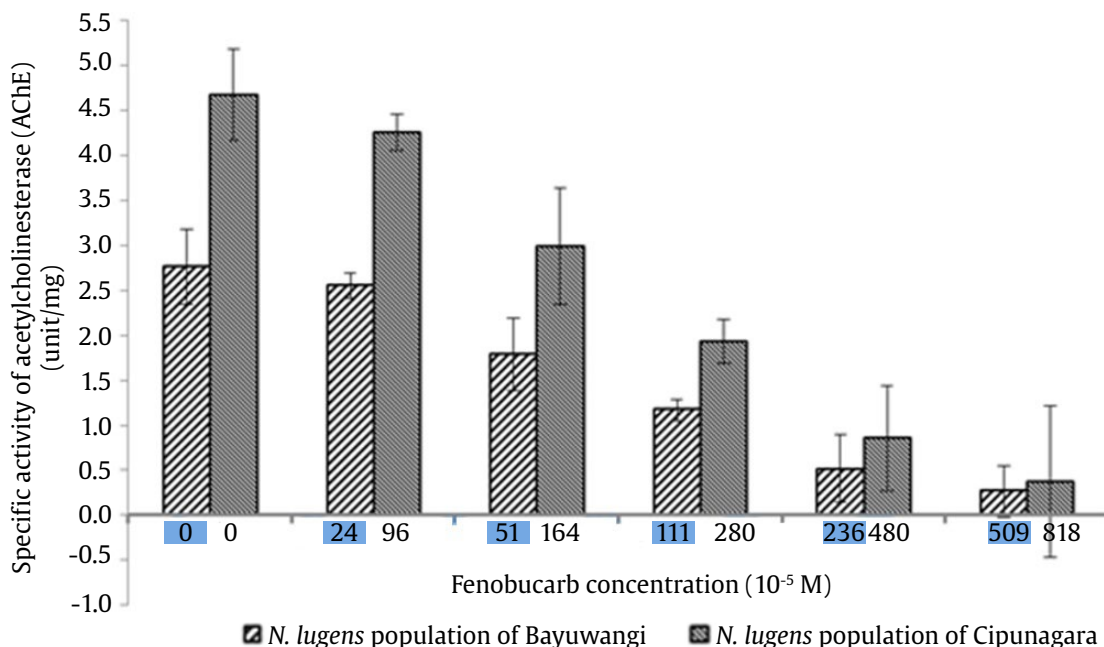


Figure 1. Specific activity of *N. lugens* AChE of Banyuwangi and Cipunagara population

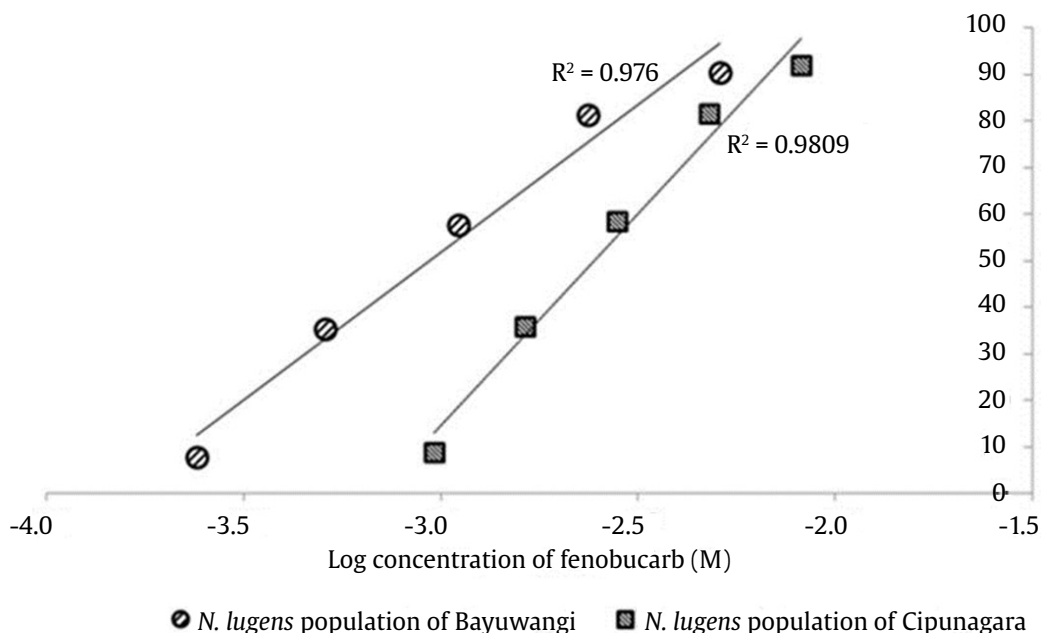


Figure 2. Inhibition of AChE by fenobucarb

Table 3. Acetylcholinesterase (AChE) enzyme susceptibility level *N. lugens* population of Banyuwangi and Cipunagara against fenobucarb.

Population	a±SE	b±SE	IC ₅₀ (10 ⁻⁵ M)	CI _{95%}	IC ₉₅ (10 ⁻⁵ M)	CI _{95%}	IR
Banyuwangi (standard)	-3.83±0.32	1.95±0.15	91.57	68.98 – 119.45	636.50	402.56 – 1353.49	2.61
Cipunagara (field)	-6.89±0.55	2.89±0.23	239.20	215.14 – 264.88	883.64	731.50 – 1132.57	

a: intercept, b: slope (slope), SE: standard error, IC: inhibition concentration (10⁻⁵ M), CI: confidence interval, IR: insensitivity ratio (IC₅₀ Cipunagara/IC₅₀ Banyuwangi)

in esterase enzyme specific activity between the two populations was 18.79 units/mg (Figure 3). That was indicated that esterase enzyme did not play in role of *N. lugens* resistance to fenobucarb. May be *N. lugens* develop another biochemical mechanism of resistance to this insecticide. The results show that the differences in esterase activity between the two populations cannot be used to explain the differences in the level of resistance of *N. lugens* to fenobucarb.

3.2.3. Activity of the Glutathione S-Transferase

Glutathione S-transferase (GST) activity was tested to determine the role of the enzyme in the resistance of *N. lugens* to insecticide. The test result of the specific GST enzyme activity in the *N. lugens* population from Cipunagara (field) was 1010.02 units/mg, higher than the specific activity of the GST *N. lugens* population of Banyuwangi (standard) (909.10 units/mg). The difference in GST-specific activity between the two populations was 100.92 units/mg (Figure 4). The results of the GST specific activity test show that GST was one of the resistance mechanisms that occurred in the *N. lugens* of the Cipunagara population.

3.3. *N. lugens* Susceptibility to Neem Oil Insecticide

The results of the toxicity test of the neem oil insecticide using the leaf-stem dipping method gave LC_{50} values of 3.68 ml/L and 3.61 ml/L against the *N. lugens* populations of Banyuwangi and Cipunagara. The results of the resistance ratios indicate that the

N. lugens Cipunagara population is susceptible to neem insecticide, with a resistance ratio of <1 (Table 4).

4. Discussion

In many countries indicates that Intensive using of insecticide causes insect resistance. Study resistance of *N. lugens* to insecticides in China indicate that *N. lugens* has develop high levels of resistance to imidacloprid (resistance ratio, RR = 2491.0 - 8078.5-fold), while in Bangladesh 3680.1 - 4430.8-fold) because of intensive using of insecticides (Datta *et al.* 2021). Differences in the toxicity values of insecticide can be caused by the excretion and metabolic rate (Park *et al.* 1991). The *N. lugens* population of Ciasem and Sukamandi, Subang Regency is still susceptible to fenobucarb insecticides, with resistance ratios of 2.7 and 1.8 (Baehaki *et al.* 2016; Surahmat *et al.* 2016). The *N. lugens* population of Cipunagara was resistant to imidacloprid insecticide, which is virtually in line with the report of Baehaki *et al.* (2016), that the population of Sukamandi is resistant, with a ratio of 12.7. Increased resistance due to exposure to imidacloprid insecticides can occur if continuous selection is carried out (Zhang *et al.* 2014). Selection using imidacloprid causes the resistance ratio of *N. lugens* to increase by 11.35 times after 25 generations, and 14 times after 27 generations (Zewen *et al.* 2003; Wang *et al.* 2008).

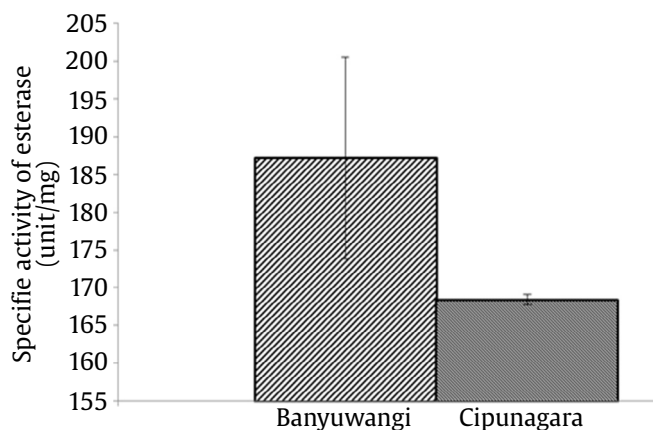


Figure 3. The specific activity of *N. lugens* esterase of Banyuwangi and Cipunagara population

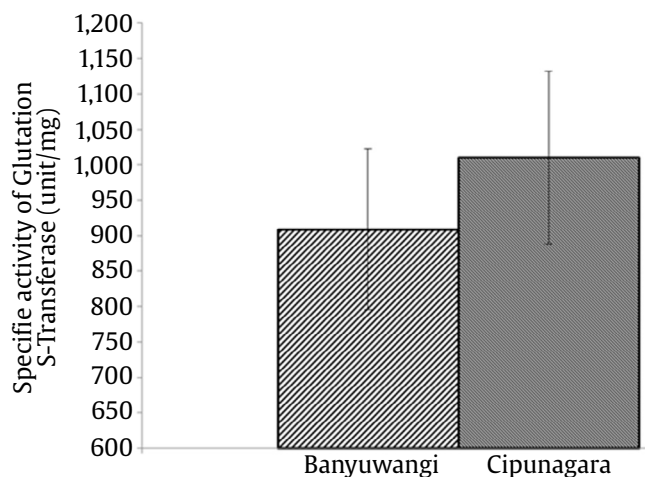


Figure 4. Specific activity of Glutathione S-Transferase in *N. lugens* population of Banyuwangi and Cipunagara

Table 4. Susceptibility of *N. lugens* to neem oil insecticides

Population	a±SE	b±SE	LC ₅₀ (ml/l)	CI _{95%}	LC ₉₅ (ml/l)	CI _{95%}	RR
Banyuwangi (standard)	-3.48±0.37	3.79±0.38	8.31	7.48 – 9.19	22.56	18.78 – 29.30	0.99
Cipunagara (field)	-3.81±0.39	4.17±0.41	8.19	7.43 – 9.01	20.32	17.23 – 25.59	

a: intercept, b: slope (slope), SE: standard error, LC: lethal concentration (ml/l), CI: confidence interval, RR: resistance ratio

However, if the selection is stopped, this can cause a rapid decline in resistance, from 759 times to 114 times after 17 generations, until it finally stabilizes, but will increase again when selection is continued (Wang *et al.* 2009). Insect migration also significantly increases the level of resistance, because of the use of imidacloprid extensively and intensively in the field (Wang *et al.* 2008). Similar result showed by research of Diptaningsari *et al.* (2020) that is continuous selections with imidacloprid during five generations of selection increased the resistance ratio (RR) from 46.20-fold to 150.39-fold. The resistance will be decrease if the selection pressure removed. Therefore, the unstable resistance insect can be managed by using insecticide with different mode of action.

AChE acts as a terminator in the transmission of impulses at the cholinergic synapse by hydrolyzing the neurotransmitter acetylcholine (ACh) (Dvir *et al.* 2010). Molecule target of inhibition of insecticide Carbamate group like fenobucarb is AChE (IRAC 2013, 2021). The values of acetylcholinesterase (AChE) insensitivity and fenobucarb resistance level are almost the similar (Tables 2 and 3). This indicates that the main mechanism for fenobucarb insecticide resistance is possibly in the insensitivity of AChE (Yoo *et al.* 2002; Toumi *et al.* 2016). According to study with chlorpyrifos-oxon treatment showed that AChE activity of earwigs collected from conventional practice higher than in organic and IPM orchards (Navenant *et al.* 2019). This indicating the insect from organic and IPM orchard were more sensitive than from conventional ones. AChE activity in resistant strains is higher than susceptible strains (Taşkin *et al.* 2007). It has been shown that the *N. lugens* population from Cipunagara (field) had AChE enzyme insensitivity 2.67 times greater than that of the *N. lugens* from Banyuwangi (standard).

Esterase activity can be associated with insect resistance. It can be detected using a substrate such as the original ester; for example, α - or β -naphthyl acetate. Resistance can be associated with an increase or decrease in naphthyl esterase activity (Devonshire 1991). Naphthyl esterase is a product formed from

the reaction of α -naphthyl acetate substrates, an enzyme source, and fast blue dye (used in this study). In Asperen's (1962) study, diazo blue-sodium lauryl sulphate was used to estimate the formation of naphthyl products. The use of insecticides is strongly correlated with increased naphthyl esterase activity (Taşkin *et al.* 2007). Based on this explanation, the esterase-specific activity in the *N. lugens* of the Cipunagara population should be higher than that of Banyuwangi (standard). However, in this study, the *N. lugens* field population did not show higher esterase-specific activity than standard.

Increased esterase activity does not always correlate with resistance status (Siegfried and Scott 1992). Differences in enzyme activity are likely to occur because of by differences in the genes in each population (Tarwotjo and Rahadian 2018). There is no correlation between changes in esterase activity between strains with imidacloprid resistance (Wang *et al.* 2009). Significantly lower levels of ali-esterase activity have not been found to be associated with carbamate resistance in *Culex tarsalis* (Plapp *et al.* 1961). The absence of the role of specific esterase enzymes in fenobucarb and imidacloprid insecticide resistance may also be caused by the development of other enzyme detoxification systems in the *N. lugens* population of Cipunagara. The main route of carbamate insecticide metabolism in animals is oxidative and is generally associated with the MFO enzyme (Futuko 1990). In addition, increased monooxygenase cytochrome P450 activity is the main mechanism of imidacloprid resistance in insects (Zewen *et al.* 2003; Wang *et al.* 2009; Puinean *et al.* 2010).

The GST activity of the *N. lugens* population of Cipunagara (field) was higher than that of Banyuwangi (standard). It was found that GST acts as one of the mechanisms of *N. lugens* resistance to fenobucarb (carbamates) and imidacloprid (neonicotinoid) insecticides. Increased GST activity has been associated with resistance to several major classes of insecticide and is associated with an increase in the number of one or more GSTs (Vontas *et al.* 2001; Nan-nan *et al.*

2006; Datta and Banik 2021). Moreover, Insects can be resistant because of selection toward of insect having effective detoxification mechanism. The difference may be because the samples tested develop a different detoxification system when exposed to different insecticides (Dono *et al.* 2010). This indicates that in a population exposed to different insecticides, organisms will develop different detoxification systems. In the *N. lugens* population of Cipunagara, one detoxification mechanism that contributes to the resistance to insecticides is GST enzyme activity. The result of resistance study in China and Bangladesh indicate that an increase GST activities and other detoxification enzymes (EST and P450) were found in multiple-resistant FY19 field population (Datta *et al.* 2021). GST is induced by neonicotinoid insecticide (Smina *et al.* 2016). *Anopheles stephensi*, which has been resistant to deltamethrin (pyrethroid), DDT (Dichloro-Diphenyl-Trichloroethane) and propoxur (carbamate) shows high GST activity (Sanil *et al.* 2014). High activity of GST also indicated in *Aphis gossypii* and *Aphis spiraecola* populations against Imidacloprid, Dimethoate, Acephate, Quinalphos, Chlorpiriphos, Spinosad, and Thiamethoxam (George *et al.* 2019). High GST activity in providing resistance by *N. lugens* is a result of protecting tissues from oxidative damage (Vontas *et al.* 2001; Maran *et al.* 2010). Therefore, study of sensitivity change of insecticide molecule target and activity of detoxification enzyme are important in strategy to control of insect resistance.

The results of the insecticide susceptibility test of the *N. lugens* population of Cipunagara against neem oil showed an RR value of <1 (Table 4). The RR values indicate that the *N. lugens* of the Cipunagara population (field) are still susceptible to the insecticides. This result shows that an insecticide which works with a different mechanism to the insecticide applied can be used as a solution for handling resistance to certain insecticides. In this study, neem oil insecticides can be used on the *N. lugens* population of Cipunagara against imidacloprid (neonicotinoid) and fenobucarb (carbamate) insecticides. Neem plays a role in resistance management because it can reduce the rate of detoxification enzymes, making the insecticide more effective (Lowery and Smirle 2000; Mordue (Luntz) and Nisbet 2000). Besides causing growth disruption (Govindachari *et al.* 2000), neem oil can disrupt the central nervous system, repel insects and inhibit feed (Mordue *et al.* 1998; Mordue (Luntz) and Nisbet 2000; Mardiningsih *et al.* 2010; Qiao *et al.* 2014). Principally, other botanical insecticides that have different action

mechanisms or different modes of detoxification to the insecticides applied can be used to manage resistant populations. Plant extract may be used as an insecticide for controlling insect resistance, such as the bufadienolides group (i.e. daigremontianins, LD₅₀ 0.9 µg/g of diet), extracted from *Kalanchoe pinnata* and *K. daigremontiana* (Hidayat *et al.* 2014). Further research is needed to explore the potency of botanicals insecticides to control of insect resistance to synthetic insecticides.

In conclusions, the *N. lugens* population of Cipunagara (field) was indicated resistant to fenobucarb insecticide (resistance ratio (RR) = 2.43) but resistant to imidacloprid insecticides (RR = 13.95). The insensitive of the acetylcholinesterase (ACHE) of the *N. lugens* field population was considered to play in resistance the insect to the fenobucarb, and Glutathione S-Transferase (GST) against fenobucarb and imidacloprid. Thus, it is important to know the change of target sensitivity and metabolism pathway of insecticide in management resistance of insect. The field population of *N. lugens* is susceptible to neem oil insecticides, with a value of RR <1 (LC₅₀ 3.61 ml/l). Neem oil could be used as a solution for *N. lugens* resistance management against fenobucarb and imidacloprid insecticides.

Conflicts of Interest

All author writers who play a role in research, funding, and writing this manuscript actually state that they have no conflict of interest.

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