Original Article

Negatively Correlated Cross-Resistance between *N*-Methyl Carbamate and Monocrotophos in Green Rice Leafhopper, *Nephotettix cincticeps* Uhler

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(Received February 1, 1999; Accepted May 12, 1999)

Although monocrotophos showed strong inhibition to *N*-methyl carbamate resistant acetylcholinesterase in the green rice leafhopper, *Nephotettix cincticeps*, the toxicity was not so high to Nakagawara populations of the leafhopper. The different results between *in vitro* and *in vivo* experiments were thought to be caused by an increase of aliesterase activity in Nakagawara strains which are resistant to both organophosphorus insecticides and *N*-methyl carbamates. Thus, MC-R strain which has only *N*-methyl carbamate resistance gene was bred by introducing *N*-methyl carbamate resistance gene of Nakagawara-3-C strain to susceptible Shiraoka strain. Then monocrotophos showed negatively correlated cross resistance against *N*-methyl carbamates in Shiraoka and MC-R strains under both *in vitro* and *in vivo* conditions.

Key words: acetylcholinesterase, aliesterase, negatively correlated cross-resistance, *Nephotettix cincticeps*, methyl carbamate, monocrotophos.

INTRODUCTION

The green rice leafhopper (GRLH), *Nephotettix cincticeps* Uhler, is one of the most vital pests of rice in Japan. Intensive use of organophosphorus and carbamate insecticides has resulted in high level of resistance to these insecticides in GRLH populations. The GRLH was first observed to have developed resistance to malathion in Kochi Prefecture, Shikoku district, Japan.^{1,2)} By early 1960s several populations of the insect had been reported to have been resistant to malathion and methyl parathion in various parts of Japan. And several years after the introduction of carbaryl and other *N*-methyl carbamates, GRLH populations were found to show resistance to these carbamate insecticides.^{3,4)}

Major mechanisms of GRLH resistance to organophosphorus insecticides are increased degradation of insecticides, which is associated with a high aliesterase (AliE) activity,^{1,3,5,6)} and a reduced sensitivity of acetylcholinesterase (AChE) to organophosphorus insecticides.^{3,4)} A major mechanism of GRLH resistance to carbamate insecticides is a reduced sensitivity of AChE to insecticides. There is a strong correlation between I_{50} and LD_{50} values.^{7,8)} To avoid or retard the development of insecticide resistance, some papers have suggested to

use a mixture of insecticides or to alternate between insecticides which shows negatively correlated cross-resistance.^{9–12)} Several cases of negatively correlated cross-resistance were reported in tobacco budworm (*Heliothis virescens*),¹³⁾ small brown planthopper (*Laodelphax striatellus*),¹⁴⁾ brown rice planthopper (*Nilaparvata lugens*),¹⁴⁾ and GRLH.^{7,15–17)}

In this paper we report the mechanism of negatively correlated cross resistance between methyl carbamate and monocrotophos in Nakagawara populations of GRLH.

MATERIALS AND METHODS

1. Insect

Shiraoka population of GRLH, which is susceptible to organophosphorous and carbamate insecticides, was collected in Shiraoka, Saitama Pref in 1961. Nakagawara-2 and Nakagawara-3 populations, which are moderately and highly resistant to organophosphorous and carbamate insecticides respectively, were collected in Nakagawara, Ehime Pref in 1980 and 1987. These three populations were maintained free from insecticide pressure on rice seedlings in a glass container (diameter 8.5 cm height 18 cm) under conditions of $25 \pm 1^\circ\text{C}$ and 16L: 8D. Shiraoka, Nakagawara-2-B and Nakagawara-3-C strains were bred by pair mating and

by selecting based on the bivariate plot from each of three populations (Shiraoka, Nakagawara-2 and Nakagawara-3) until it displayed an AChE phenotype homogeneously. These three strains of GRLH were used in the following experiments.

2. Chemicals

MTMC (96.8%) and *N*-propyl MTMC (MTMC-Z) (97.8%) were obtained from Mitubishi Chemical Co., Ltd. (Tokyo, Japan). Propoxur (98.2%) was obtained from Nihon Bayer Agrochem K.K. (Tokyo, Japan). Monocrotophos (99.0%) was purchased from Chem Service, Inc. (PA, USA). Propaphos (99.5%) was obtained from Nippon Kayaku Co., Ltd. (Tokyo, Japan). Methomyl (91.8%) was obtained from Du Pont Japan Co., Ltd. (Tokyo, Japan). All inhibitors were dissolved in acetone and all chemicals used in this study were analytical or spectral grade.

3. AChE Inhibition Test

Heads of female adults were used as sources of AChE. Five heads were homogenized in 500 μ l of 0.1 M MOPS buffer (pH7.5) using an ultrasonic disrupter (UV-200, Tomy Co., Tokyo, Japan) and supernatant from centrifugation at 15,000 g for 2 min was used as the enzyme source of AChE assay. Ten μ l of enzyme was preinhibited by addition of various concentrations of 10 μ l inhibitor. Ten min after incubation at room temperature, 200 μ l of reaction buffer containing 5×10^{-3} M ATCh, 7×10^{-4} M, sodium bicarbonate and 4×10^{-7} M DTNB were added. Ten min after, absorbance (405 nm) was read with a microtiter plate-reader (Model450, Japan Bio-Rad Laboratories, Tokyo, Japan). Only acetone was used as control. Three replications were made. I_{50} was calculated by the probit analysis.

4. Toxicity Test

Topical application was performed. Ten female GRLH adults were used in each concentration. A half μ l of insecticide acetone solution was topically applied by a microapplicator (Kiya Industries, Ltd., Tokyo,

Japan). Treated adults were kept in a glass tube (diameter 3 cm and height 10 cm) containing rice seedlings under conditions of $25 \pm 1^\circ\text{C}$ and 16L: 8D. Twenty-four hr after treatment, the number of dead insects were counted. Three replications were made over four concentrations excluding 0 and 100% mortalities.

5. Assay of AliE Activity

The method of van Asperen¹⁸⁾ was modified for measuring AliE activity. Female adults were homogenized in 0.05 M PIPES-NaOH buffer (pH7.0), and 3×10^{-4} M α -NA was used as a substrate. Ten min after incubation at 25°C , the reaction was stopped by addition of staining solution, which was a mixture of 1% naphthanil diazo blue B solution and 5% SDS (2 : 5, vol/vol). Fifteen min after, absorbance of reaction mixture (200 μ l per well) was measured by a microtiter plate reader at 595 nm. Alpha-naphthol was used to construct the standard curve.

6. Protein Assay

A protein assay kit (Japan Bio-Rad Laboratories) was used in the following recommended protocol.¹⁹⁾ Bovine serum albumin was used as a standard and stored at -20°C until use.

7. Breeding of Susceptible and *N*-methyl Carbamate Resistant (MC-R) Strains

This experiment was performed by the selective pair mating. First, the Shiraoka strain was mated with the Nakagawara-3-C strain. The resultant F1 progenies, which were heterogeneous and moderately resistant in sensitivity of AChE to *N*-methyl carbamates by the bivariate plot method, were back crossed with Shiraoka strain. Phenotypes of B1 of AChE sensitivity to *N*-methyl carbamates were susceptible and moderately resistant. From B1 population, moderately resistant individuals were selected by the bivariate plot method and they were back crossed with Shiraoka strain. This procedure was repeated for four generations. Progenies of B4 which showed the moderately resistant AChE phenotype

Table 1 *In vitro* inhibition of AChE in three strains of GRLH by various insecticides.

Insecticides	I_{50} value (μM) (95% c.i.) ^{b)}				R/S ^{a)}	
	MTMC	MTMC-Z	propoxur	monocrotophos	methomyl	propaphos
Shiraoka	10.4 (8.91–12.0)	156 (109–311)	3.73 (3.20–4.52)	32.0 (27.0–39.1)	0.52 (0.41–0.63)	893 (504–1210)
Nakagawara-2-B	57.1 5.5 (44.3–74.6)	10.4 1/15 (8.94–12.3)	144 39 (121–172)	0.71 1/45 (0.30–3.51)	0.63 1.2 (0.54–0.71)	121 1/7.4 (97.9–175)
Nakagawara-3-C	138 13 (96.1–207)	3.60 1/43 (3.22–4.14)	275 74 (201–426)	0.21 1/152 (0.12–0.80)	0.8 1.5 (0.72–0.94)	33.5 1/27 (25.5–45.7)

^{a)} Figures in R/S indicate resistance ratio values. ^{b)} Figures in parenthesis indicate 95% confidence interval.

were mated. AChE phenotypes of resulting families were homogeneously either susceptible, moderately resistant or resistant. Only the phenotypes, which were either susceptible or resistant, were maintained as S strain and MC-R strain, respectively.

RESULTS AND DISCUSSION

1. Correlation between I_{50} and LD_{50} in Shiraoka, Nakagawara-2-B and Nakagawara-3-C Strains

As a result of the screening based on AChE sensitivity to several insecticides, cross resistance was found between MTMC and propoxur, and negatively correlated cross resistance was found between MTMC and monocrotophos (Nomura *et al.*, unpublished data). The *in vitro* inhibition of AChE to several insecticides was estimated by I_{50} (Table 1). Cross resistance was found between propoxur and MTMC and negatively correlated cross resistance was found between methyl carbamates (propoxur, MTMC and methomyl) and MTMC-Z, propaphos, or monocrotophos. So far in GRLH a negatively correlated cross resistance was found between *N*-methyl and *N*-propyl carbamate insecticide, and between *N*-methyl carbamate insecticides and propaphos, diazinon and pyridafenthion. These results confirmed the data obtained by Iwata and Hama⁷⁾ and Yamamoto *et al.*¹⁵⁻¹⁷⁾ To confirm the relationship of negatively correlated cross resistance of these combinations of insecticides for AChE inhibition, bioassay by topical application to GRLH adults was performed. The resistant ratio showed also negatively correlated cross resistance between *N*-methyl carbamate and MTMC-Z, but did not show negatively correlated cross resistance between *N*-methyl carbamates and monocrotophos (Table 2). LD_{50} values of MTMC-Z were 2.7- and 3.5-fold lower in the Nakagawara-2-B and Nakagawara-3-C strains than the Shiraoka strain, respectively. LD_{50} values of monocrotophos were 4.8- and 1.8-fold higher in the Nakagawara-2-B and Nakagawara-3-C strains than the Shiraoka strain, respectively. Cross resistance was observed between propoxur and monocrotophos. Organophosphorus insecticides are known to be hydrolyzed or sequestered by AliE in GRLH.⁶⁾ However, this factor was not taken into account in these data. The AliE activity was 19-fold higher in Nakagawara-3-C strain than that of the Shiraoka strain (Table 3). Thus a high AliE activity in Nakagawara strains might negate the negatively correlated cross resistance between monocrotophos and *N*-methyl carbamates.

2. LD_{50} , I_{50} and AliE Activity of S and MC-R Strains

I_{50} values of S and MC-R were similar to these of Shiraoka and Nakagawara-3-C strains for propoxur and monocrotophos, respectively. The resistant ratio of I_{50} values in S and MC-R strains also showed a negatively correlated cross resistance to monocrotophos (Table 4).

Table 2 Susceptibility of three strains of GRLH to several insecticides at 24 hr after topical application.

Strain	MTMC			Propoxur			MTMC-Z			Monocrotophos		
	$LD_{50}^{a)}$ (95% i.i.)	SRE ^{b)}	R/S ^{c)}	LD_{50} (95% i.i.)	SRE	R/S	LD_{50} (95% i.i.)	SRE	R/S	LD_{50} (95% i.i.)	SRE	R/S
Shiraoka	3.56 (3.13-4.20)	4.1		1.50 (1.12-2.10)	3.3		75.0 (63.1-84.8)	4.3		1.11 (0.92-1.23)	5.3	
Nakagawara-2-B	6.92 (7.91-14.2)	2.2	1.9	22.4 (18.9-30.4)	4.5	15	28.0 (24.0-32.1)	4.2	1/2.7	5.10 (4.10-7.71)	4.4	4.6
Nakagawara-3-C	114 (96.3-132)	3.5	32	84.6 (59.6-116)	3.3	56	21.5 (18.1-26.3)	3.3	1/3.6	2.02 (1.05-2.83)	2.8	1.8

^{a)} $\mu\text{g/g}$. ^{b)} Slope of regression equation. ^{c,d)} See Table 1.

Table 3 AliE activity of three strains of GRLH.

Strain	AliE activity ^{a)} $\mu\text{mol}/\text{min}/\text{mg}$ protein	$R/S^{b)}$
S	$0.12 \pm 0.01^{c)}$	
Nakagawara-3-C	2.31 ± 0.32	19
MC-R	0.15 ± 0.03	1.3

^{a)} α -Naphthyl acetate was used as substrate. ^{b)} See Table 1.^{c)} Mean $\pm$ SE of three replications.Table 4 *In vitro* inhibition of AChE in S and MC-R strains of GRLH by propoxur and monocrotophos.

Insecticides	I_{50} value (μM) (95% c.i.) ^{b)}		$R/S^{a)}$	
	propoxur		monocrotophos	
S	4.21 (3.41–5.62)		30.1 (21.5–31.6)	
MC-R	269 (192–389)	64	0.14 (0.062–0.24)	1/215

^{a,b)} See Table 1.

Table 5 Susceptibility of S and MC-R strains of GRLH to propoxur and monocrotophos.

Strain	propoxur		monocrotophos		
	$LD_{50}^{a)}$ (95% c.i.) ^{d)}	SRE ^{b)} $R/S^{c)}$	LD_{50} (95% c.i.)	SRE	R/S
S	1.12 (0.83–1.75)	3.1	1.44 (1.25–1.70)	5.3	
MC-R	82.4 (78.1–90.8)	3.4 74	0.049 (0.011–0.082)	2.7	1/29

^{a)} See Table 2. ^{b,c,d)} See Table 1.

LD_{50} values of S and MC-R strains were shown in Table 5. LD_{50} of propoxur was 74-fold higher in the MC-R strain than the S strain. This result was similar to that for the Shiraoka and the Nakagawara-3-C strains. However, LD_{50} value of monocrotophos was 29-fold lower in the MC-R strain than the S strain. And AliE activity of the MC-R strain was reduced compared to that of the Nakagawara-3-C strain (Table 3). As a result, the most important factor of resistance was considered to be the resistance-associated esterase negating the effect of a negatively correlated cross resistance in AChE sensitivity.

A mixture of insecticides which shows negatively correlated cross resistance is effective to avoid or retard the development of insecticide resistance. Thus rotational use of insecticides which shows a negatively correlated cross resistance in the sensitivity of AChE, even if it is not observed in toxicity tests, is likely to reduce the rate of the resistance in the GRLH population. Two factors involving the resistance: insensitivity of AChE and highly active AliE could be separated by producing the

MC-R strain. This strain, absent AliE, was then much more susceptible to monocrotophos due to its hypersensitive AChE which was no longer masked by the detoxicative mechanism.

ACKNOWLEDGMENTS

We thank Dr. T. M. Brown, Department of Entomology, Clemson University for helpful suggestion of this study and reviewing the draft of this manuscript. Thanks are due to Drs. T. Tanaka and T. Itioka of our laboratory. We also thank Mr. O. Mimori, Nissan Chemical Co., Ltd., Mr. K. Imai, Japan Agricultural Cooperatives, Mr. N. Yasudomi, Otsuka Chemical Co., Ltd., for supplying green rice leafhopper populations and the pesticide manufacturing companies which provided insecticides used for this study. A part of this work was supported by Grant-in-Aid for Scientific Research (A) No. 07556014, (A) No. 11356002, (B) No. 11460019 and (C) No. 09660046 by the Ministry of Education, Science, Sports and Culture, Japan.

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要 約

ツマグロヨコバイにおける *N*-メチルカーバメートとモノクロトホスとの負相関交差抵抗性

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感受性系統および、有機リン剤・カーバメート剤抵抗性系統を用い負相関交差抵抗性を示す薬剤の組み合わせのス

クリーニングの結果、複数の *N*-メチルカーバメート剤とモノクロトホスとの間に負相関交差抵抗性のあることが明らかとなった。しかし、これらの薬剤の LD_{50} 値の抵抗性比と AChE の感受性比を比較したところ、 LD_{50} 値の抵抗性比では、負相関交差抵抗性の関係は認められなかった。そこで、感受性系統に抵抗性系統の遺伝子を導入した MC-R 系統を育成し、殺虫試験をおこなうとともに AChE の感受性比を検討したところ、両系統間において、いずれも明瞭な負相関の関係が認められた。さらに感受性、抵抗性およ

び MC-R 系統のアリエステラーゼ (AliE) 活性を調べたところ、抵抗性系統の AliE 活性は感受性系統に比べ 19 倍高かったが、MC-R 系統の AliE 活性は低く感受性系統と違いが認められなかった。以上の結果より、感受性および抵抗性系統において *N*-メチルカーバメートとモノクロトホスの LD_{50} 値で負相関交差抵抗性が認められなかった要因は、抵抗性系統の高い AliE 活性によりモノクロトホスが解毒分解されるためと考えられた。