Mechanism of Malathion Resistance in the Green Rice Leafhopper, *Nephotettix cincticeps* Uhler (Hemiptera: Deltocephalidae)*

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LC₅₀ values of five strains of the green rice leafhopper, Odawara, Koga, Nangoku, Kagami and Geisei, against malathion were 21.1, 311, 1,760, 2,740 and 2,140 ppm, respectively. Eight esterase bands were detected by thin layer agar gel electrophoresis, and malathion resistant strains showed higher β -naphthyl acetate hydrolyzing activity of E₁, E₂, E₃ and E₄ bands than susceptible one. *In vitro* degradation of ¹⁴C-methyl malathion by the homogenate of leafhoppers was 5.6, 16.0 and 21.2 times higher in Koga, Nangoku and Kagami strains than in Odawara strain. *In vitro* degradation of ¹⁴C-methyl malathion by the enzymes separated by thin layer agar gel electrophoresis showed that E₁, E₂ and E₃ bands showed the degradation activity with the highest activity on the E₂ band. Susceptibility of the leafhopper head cholinesterase against malaoxon was 1/1.9 and 1/9.2 in Koga and Nangoku strains to Odawara strain, respectively. The combination of two resistance factors, *in vitro* degradation of ¹⁴C-methyl malathion and cholinesterase susceptibility to malaoxon were $5.6 \times 1.9 = 10.6$ and $16.0 \times 9.2 = 147.2$ in Koga and Nangoku strains, respectively.

INTRODUCTION

Kojima *et al.*¹⁾ studied on the resistance mechanism of the green rice leafhopper, *Nephotettix cincticeps* Uhler, against malathion which was first found in Kochi Prefecture in 1961. They reported that there were no differences in cholinesterase activity and susceptibility of this enzyme to malaoxon between susceptible and resistant strains, and that malathion resistance was due to the enhanced carboxylesterase activity degrading malathion. On the other hand, Ozaki and Koike²⁾ reported that β -naphthyl acetate hydrolyzing activity of the malathion resistant leafhopper was significantly higher than that of the susceptible one. Kasai and Ogita³⁾ and Ozaki *et al.*⁴⁾ separated β -naphthyl acetate

hydrolyzing esterases of the leafhopper by thin layer agar gel electrophoresis and studied the relation between malathion resistance and non-specific esterase activity. There was a good correlation between activities of certain esterase bands and malathion resistance. The similar phenomenon was observed in the malathion resistance of the smaller brown planthopper, *Laodelphax striatellus* Fallén, and there was also a good correlation between malathion resistance and esterase activity of the E₇ band separated by electrophoresis.⁵⁾ Further, Miyata *et al.*⁶⁾ observed ¹⁴C-methyl malathion degrading activity on the E₇ band.

On the carbamates insecticide resistance of the green rice leafhopper found in the western parts of Japan since 1969, the resistance mechanism was thought to be reduced susceptibility of cholinesterase against some carbamates insecticides.⁷⁻⁹⁾

In this paper, *in vitro* metabolism of ¹⁴C-

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methyl malathion by the crude homogenate of the leafhopper, correlation between β -naphthyl acetate hydrolyzing activity and ^{14}C -methyl malathion degrading activity of the enzyme separated by thin layer agar gel electrophoresis, and susceptibility of cholinesterase to malaoxon were studied to clarify the mechanism of malathion resistance in the leafhopper.

MATERIALS AND METHODS

1. Insect

Female adults of the green rice leafhopper were used throughout this study. The leafhopper was reared at 25°C, 16 hr illumination per day with renewing rice seedlings weekly. Nangoku, Kagami and Geisei strains were used after rearing 3-5 generations in the laboratory. Five strains of the leafhopper used are shown in Table 1.

Table 1 The origin of the green rice leafhoppers used in this study.

Strain	Place of collection	Time of collection
Odawara	Odawara-City, Kanagawa-Prefecture	April, 1960
Koga	Koga-Town, Koga-District, Shiga-Prefecture	Summer, 1970
Nangoku	Nangoku-City, Kochi-Prefecture	July, 1973
Kagami	Kagami-Town, Kami-District, Kochi-Prefecture	July, 1973
Geisei	Geisei-Village, Aki-District, Kochi-Prefecture	July, 1973

2. Insecticide

Malathion and *O*-sec-buthylphenyl methylcarbamate (BASSA®) were obtained from Sumitomo Chem. Co. Ltd. Malaoxon was prepared by the oxidation of malathion with bromine. Pyridafenthion, *O*, *O*-diethyl-*O*-(3-oxo-2-phenyl-2H-pyridazine-6-yl) phosphorothioate, and pyridafenthion-oxon, *O*, *O*-diethyl-*O*-(3-oxo-2-phenyl-2H-pyridazine-6-yl) phosphorate, were obtained from Mitsui-toatsu Chemicals Inc. ^{14}C -Methyl malathion used here has been described previously.⁶⁾ The specific activity was 1,500 dpm/ μg determined by an Aloka LSC-653 liquid scintillation spectrometer.

3. Insecticidal Test

Insecticidal test was performed according to the method described by Miyata *et al.*⁶⁾ Malathion, pyridafenthion and BASSA® solutions of 40, 10 and 10%, respectively, were prepared with a mixture of acetone:benzene:Newcol 863 (3:3:1, v/v). The insecticide solution was diluted with water and 3 ml of the solution was sprayed at 280 mmHg with a rotary type spray tower (Mizuho Scientific Co., Nagoya). Treated leafhoppers were transferred to a (2.5×20 cm, ten leafhoppers/tube) with rice seedlings and kept at 25°C. The mortality was observed at 24 hr after treatment. Thirty to fifty leafhoppers were used at each concentration. From these results, LC_{50} values were calculated.¹⁰⁾

4. Electrophoresis

Thin layer agar gel electrophoresis used was slightly modified from the methods described by Ozaki *et al.*⁴⁾ and Miyata *et al.*⁶⁾ Each leafhopper was crushed with 40 μl of distilled water by a glass rod, and the brei was absorbed to Toyo filter paper No. 2 (2×20 mm). Electrophoresis was performed at the constant current of 2 mA/cm width for about 90 min. To detect non-specific esterase activity, 0.5% acetone solution of β -naphthyl acetate was sprayed on the plate, followed after 15 to 20 min incubation at 37°C by spraying 0.4% of naphthanil diazoblu B. To study ^{14}C -methyl malathion degradation by the enzyme separated by thin layer agar gel electrophoresis, 40 (Odawara and Koga strains) to 20 (Nangoku and Kagami strains) leafhoppers were crushed with 40 μl of distilled water and a strip of filter paper (20×60 mm) was immersed to the brei. After electrophoresis the agar plate was first transversely sectioned into a strip of 5×50 mm each to test ^{14}C -methyl malathion degradation, the other was stained for esterases with β -naphthyl acetate.

5. In Vitro Metabolism of ^{14}C -methyl Malathion

Female leafhoppers were homogenized with 1/15M phosphate buffer at pH 7.4 by using a Potter-Elvehjem glass homogenizer. The homogenate was briefly centrifuged at 1,500 $\times g$ for 10 min, and the resulting supernatant was used as the enzyme source. To study

the direct degradation activity of the leafhopper homogenate, a 10 μ l aliquot of 14 C-methyl malathion (10^{-9} M) in absolute ethyl alcohol solution was added to the reaction mixture containing 0.5ml each of the enzyme solution (homogenate containing 10 (Odawara strain), 5 (Koga strain) and 2.5% (Nangoku and Kagami strains) weight by volume of the leafhopper and phosphate buffer (1/15M, pH 7.4)). The reaction mixture was kept at 37°C with shaking for 60 min. The reaction mixture was first extracted with an equal volume of chloroform twice, and then 0.3 ml of 2.5% trichloro acetic acid was added. The mixture was again extracted with an equal volume of chloroform twice.¹¹⁾

For degradation study of 14 C-methyl malathion on the electrophoretically separated enzyme bands, the agar piece (5×50 mm) was incubated with 1.5 ml of buffer solution containing 10^{-5} M 14 C-methyl malathion. After 2 hr incubation at 37°C the mixture was extracted with an equal volume of chloroform twice. The radioactivity in the aqueous phase was determined for the degradative products with Bray's counting solution¹²⁾ by an Aloka LSC-653 liquid scintillation spectrometer.

6. Measurement of Anticholinesterase Activity

Micro measurement of Hesterin's colori-

metric method described by Motoyama and Saito¹³⁾ was slightly modified as follows: Twenty heads of the leafhopper were homogenized with 1 ml of phosphate buffer (1/15M, pH 7.4), and the homogenate was filtrate through tetron gauze. The filtrate was used as enzyme source. To a small test tube, acetone solution of insecticide was added. After evaporating acetone, 0.1 ml of enzyme solution was added and kept at 37°C for 30 min. Then, 0.1 ml of 0.008M acetylcholine chloride (AChCl) solution was added and again kept at 37°C for additional 30 min. Remained AChCl was colorimetrically detected by Shimazu Bausch and Lomb Spectronic 20 colorimeter with a micro adapter.

RESULTS

1. Susceptibilities of the Green Rice Leafhopper to Malathion, Pyridafenthion and BASSA®

LC₅₀ values of five strains of the leafhopper to malathion, pyridafenthion and BASSA® are shown in Table 2. Among the leafhopper strains, Odawara strain was the most sensitive to these three insecticides, and was thought to be a susceptible strain. Nangoku, Kagami and Geisei strains of Kochi Prefecture were insensitive to these insecticides,

Table 2 Toxicities of malathion, pyridafenthion and BASSA® to Odawara, Koga, Nangoku, Kagami and Geisei strains of the green rice leafhopper.

Insecticide	Strain	Dorsage-mortality* regression line	LC ₅₀ **	Resistant ratio
Malathion	Odawara	$Y=5+1.705(X-1.324)$	21.1	1
	Koga	$Y=5+1.910(X-2.492)$	311	14.7
	Nangoku	$Y=5+6.913(X-3.245)$	1760	83.4
	Kagami	$Y=5+3.234(X-3.437)$	2740	129.9
	Geisei	$Y=5+4.266(X-3.329)$	2140	101.4
Pyridafenthion	Odawara	$Y=5+2.778(X-1.548)$	35.3	1
	Koga	$Y=5+3.202(X-2.150)$	141	4.0
	Nangoku	$Y=5+4.187(X-2.733)$	541	15.3
	Kagami	$Y=5+3.302(X-2.721)$	527	14.9
BASSA®	Odawara	$Y=5+3.233(X-1.676)$	47.5	1
	Koga	$Y=5+2.542(X-2.227)$	169	3.6
	Nangoku	$Y=5+4.990(X-2.359)$	229	4.8

* Y=probit of death rate, and X=insecticide concentration (ppm).

** determined at 24 hr after spraying.

Table 3 Frequencies of individual leafhoppers with low (L), middle (M) or high (H) esterase activities of E₁, E₂, E₃ and E₄ bands (%).

Strain*	E ₁			E ₂			E ₃			E ₄		
	L	M	H	L	M	H	L	M	H	L	M	H
Odawara	100			100			100			100		
Koga	98	2		23	21	56	78	14	8	90	10	
Nangoku	59	24	17	2		98	31	28	41	55	31	14
Kagami	58	22	20		6	94	57	25	18	58	23	24
Geisei	70	8	22			100	18	54	28	64	27	9

* One hundred leafhoppers of each strain were used.

especially LC₅₀ values to malathion were about 2,000 ppm. Koga strain showed the intermediate susceptibility between Odawara and Kochi strains. Resistance ratios of Kochi strains to Odawara strain were 80 to 130, 15 and 5 in malathion, pyridafenthion and BASSA®, respectively.

2. Separation of β -naphthyl Acetate Hydrolyzing Esterases by Thin Layer Agar Gel Electrophoresis

Using thin layer agar gel electrophoretic method, eight esterase bands hydrolyzing β -naphthyl acetate were detected. Table 3 shows frequencies of the esterase activity of E₁, E₂, E₃ and E₄ bands, which were frequently observed in the leafhopper. β -Naphthyl acetate hydrolyzing activities of E₁, E₂, E₃ and E₄ bands were higher in the resistant strain than in the susceptible one.

3. In Vitro Metabolism of ¹⁴C-methyl Malathion

In vitro metabolism of ¹⁴C-methyl malathion by the leafhopper homogenate is shown in

Table 4. Degradation activities of Kagami, Nangoku and Koga strains were 21.2, 16.0 and 5.6 fold to that of Odawara strain, respectively. Most degradation products were carboxylesterase products and little amount of phosphatase products were observed.

Results of *in vitro* degradation of ¹⁴C-methyl malathion by the enzyme separated by thin layer agar gel electrophoresis are shown in Figs. 1-3. The ¹⁴C-methyl malathion degrading activity was observed on E₁, E₂ and E₃ bands with the highest activity on the E₂ band. The highest degrading enzyme activity was found in Kagami and Nangoku strains, which were highly resistant to malathion, and the small degradation activity was found in Koga strain, which was moderately resistant. No degradation was detected in Odawara strain which was susceptible to malathion.

4. Cholinesterase Sensitivity of Odawara, Koga and Nangoku Strains to Insecticides

Sensitivities of leafhopper head choline-

Table 4 In vitro metabolism of ¹⁴C-methyl malathion in Odawara, Koga, Nangoku and Kagami strains of the green rice leafhopper.

Enzyme source*	Degradation activity (%)			Degradative ratio
	Carboxylesterase products	Phosphatase products	Total	
Buffer only	1.2	2.4	3.6	—
Odawara	12.5	0.6	13.1	1
Koga	29.6	1.0	30.6	5.6
Nangoku	40.7	0.8	41.5	16.0
Kagami	50.4	1.1	51.5	21.2

* Odawara: 5% final enzyme solution, Koga: 2.5% final enzyme solution, Nangoku and Kagami: 1.25% final enzyme solution.

Fig. 1 Degradation of ^{14}C -methyl malathion by the green rice leafhopper enzymes of Koga strain separated by thin layer agar gel electrophoresis. The agar plate was sliced into 5×50 mm pieces and each agar piece was incubated at 37°C for 2 hr with 10^{-5}M of ^{14}C -methyl malathion. The corresponding zymogram for β -naphthyl acetate indicates the relative positions of esteratic bands against malathion degrading enzyme.

Fig. 2 Degradation of ^{14}C -methyl malathion by the green rice leafhopper enzymes of Nangoku strain separated by thin layer agar gel electrophoresis.

terase in Odawara, Koga and Nangoku strains are shown in Table 5. Cholinesterase sensitivities of Nangoku strain against malaoxon, pyridafenthion-oxon and BASSA® were reduced 1/9.2, 1/3.7 and 1/2.7 to those

Fig. 3 Degradation of ^{14}C -methyl malathion by the green rice leafhopper enzymes of Kagami strain separated by thin layer agar gel electrophoresis.

Table 5 Anticholinesterase activity in the head homogenate of Odawara, Koga and Nangoku strains of the green rice leafhopper.

Enzyme source	I_{50} (M)		
	Malaoxon	Pyridafenthion-oxon	BASSA®
Odawara	1.3×10^{-7}	1.4×10^{-6}	3.5×10^{-5}
Koga	2.5×10^{-7}	1.6×10^{-6}	4.8×10^{-5}
Nangoku	1.2×10^{-6}	5.2×10^{-6}	9.3×10^{-5}

of Odawara strain, respectively.

DISCUSSIONS

Kasai and Ogita³⁾ studied the relationship between malathion resistance and non-specific esterase activity of the leafhopper separated by thin layer agar gel electrophoresis and found that β -naphthyl acetate hydrolyzing activity of E_9 band (correspond to E_2 band in this paper) in resistant populations was significantly higher than a susceptible strain. Ozaki *et al.*⁴⁾ also reported that non-specific esterase activities in malathion resistant populations were significantly higher than that in susceptible one, and that both malathion resistance and non-specific esterase activity were controlled by the genes located on autosomal chromosome(s). They also reported that the leafhoppers having higher

activity of the non-specific esterase bands designated as E_2 , E_3 and/or E_4 were more numerous in the resistant populations than in susceptible one. Among the leafhopper strains used here, it was also shown that non-specific esterase activities of E_1 , E_2 , E_3 and E_4 bands increased with increase of resistance to malathion.

Mechanism of malathion resistance in the leafhopper found in Kochi Prefecture in 1961 was thought to be enhanced malathion degrading carboxylesterase activity by Kojima *et al.*¹⁾ and was thought to be enhanced degradation activity of carbophenoxy malathion by Ohkawa *et al.*¹⁴⁾ From this experiment, enhanced degradative activity of ^{14}C -methyl malathion by carboxylesterase was again confirmed to be one of the resistance mechanisms, as already reported in other insects.^{6,14-17)}

It is reported by Ozaki and Kassai⁵⁾ that malathion resistant smaller brown planthopper, *Laodelphax striatellus* Fallén, had higher non-specific esterase activity of E_7 band separated by thin layer agar gel electrophoresis than a susceptible one, and that non-specific esterase activity of E_7 band and malathion resistance was thought to be controlled by the same genetic factor. The enhanced carboxylesterase activity degrading ^{14}C -methyl malathion was thought to be one of the most important resistance mechanisms, and ^{14}C -methyl malathion degrading activity was detected only on the E_7 band.⁶⁾

^{14}C -Methyl malathion degrading activity of the leafhopper enzymes separated by thin layer agar gel electrophoresis was studied, the degradation activity was found on E_1 , E_2 and E_3 bands which showed high activities in hydrolyzing β -naphthyl acetate.

Hayashi and Hayakawa¹⁸⁾ and Kojima *et al.*¹⁾ reported that there were no differences in cholinesterase sensitivity to malaoxon between malathion resistant leafhopper of Kochi and a susceptible strain in the time of 1961. In 1972, Iwata and Hama⁸⁾ reported that sensitivity of cholinesterase to carbamates insecticides were reduced in some carbamates insecticides resistant leafhoppers, and they also reported cholinesterase sensitivity to malaoxon of this carbamate resistant

strain was slightly reduced. Cholinesterase sensitivity to malaoxon was reduced to 1/9.2 in Nangoku strain in comparison to that of Odawara strain. It needs further studies to conclude whether this difference is due to malathion resistance or strain differences. Considering the results of Hayashi and Hayakawa¹⁸⁾ and Kojima *et al.*,¹⁾ however, the reduced sensitivity of cholinesterase to malaoxon seemed to be one of the non-specific malathion resistance mechanisms in the leafhopper.

From the genetic analysis of insecticide resistance, Sawicki and Farnham,¹⁹⁾ Plapp,²⁰⁾ Lewis and Sawicki²¹⁾ and Georgiou²²⁾ showed that the effect of two resistance genes in combination was multicative, not additive. In malathion resistance of the leafhopper, the combination of two resistance factors, *in vitro* degradation of ^{14}C -methyl malathion and cholinesterase sensitivity to malaoxon, were $5.9 \times 1.9 = 10.6$ in Koga strain and $16.0 \times 9.2 = 147.2$ in Nangoku strain.

According to Kojima *et al.*²³⁾ a subcolony from Kochi strain, reared without any insecticide pressure, showed gradual decrease of resistance to malathion, while another subcolony selected with LD_{50} of malathion kept the resistance to this compound consistently. LD_{50} values to malathion of the leafhopper of Nakagawara, Ehime Prefecture in 1963 and 1970 were $12.41 \mu\text{g/g}$ ²⁴⁾ and $330 \mu\text{g/g}$,²⁵⁾ respectively. Even though the testing methods were different, malathion resistance ratios of Kochi strains (Nangoku, Kagami and Geisei) were much higher than that of Kojima *et al.*¹⁾ As malathion has never been used for pest control of rice pests since malathion resistant leafhoppers were found at these districts, these elevated malathion resistant ratios of the leafhopper probably due to other insecticides used to control rice pests.

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要 約

ツマグロヨコバイ 5 系統 (小田原, 甲賀, 南国, 香我美, 芸西) のマラチオンに対する LC_{50} 値は, それぞれ 21.1, 311, 1,760, 2,740, 2,140 ppm であった。寒天ゲル電気泳動法によりツマグロヨコバイのエステラーゼを分離したところ, 8 本の泳動帯が検出され, 感受性系統に比べマラチオンに対して抵抗性の系統では E_1 , E_2 , E_3 , E_4 泳動帯のエステラーゼ活性が高かった。ツマグロヨコバイまい液による *in vitro* での ^{14}C -メチル マラチオンの分解は小田原系に比べ, 甲賀, 南国, 香我美系統ではそれぞれ 5.6, 16.0, 21.2 倍であった。寒天ゲル電気泳動法により分離された酵素による *in vitro* での ^{14}C -メチル マラチオンの分解作用をしらべたところ, E_2 泳動帯を中心に E_1 , E_2 , E_3 泳動帯で分解作用が認められた。甲賀, 南国系ツマグロヨコバイ頭部コリンエステラーゼのマラオクソンに対する感受性は小田原系統のそれぞれ 1/1.9, 1/9.2 であった。ツマグロヨコバイにおけるマラチオン抵抗性について, *in vitro* での ^{14}C -メチル マラチオンの分解およびマラオクソンに対するコリンエステラーゼの感受性の二つの抵抗性要因を考慮すると, 甲賀および南国系統の抵抗性比はそれぞれ $5.6 \times 1.9 = 10.6$, $16.0 \times 9.2 = 147.2$ となった。

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