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Note

Permethrin Resistance Mechanisms in the Beet Armyworm (*Spodoptera exigua* (Hübner))

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The toxicity of pyrethroids was evaluated in a permethrin-susceptible (TS) and a permethrin-resistant (TR) strain of the beet armyworm, *Spodoptera exigua* (Hübner). The TR-strain showed 92-fold more resistance to permethrin and higher cross-resistance (97- and 130-fold, respectively) to cypermethrin and fenvalerate than the TS-strain. Moreover, all larval instars exhibited greater susceptibility to permethrin in the TS-strain than TR-strain. There was very little difference in susceptibility between the two strains with respect to chlorphenapyr. The effect of piperonyl butoxide on the toxicity of permethrin indicated that the resistance of the TR-strain is due to enhanced metabolic detoxification by cytochrome P450 monooxygenase.

Keywords: *Spodoptera exigua*, insecticide resistance, permethrin, cytochrome P450 monooxygenase.

INTRODUCTION

The beet armyworm, *Spodoptera exigua* (Hübner), feeds on various agricultural crops, including cotton, soybeans, peanuts, and several other crops in the Southeastern United States,[1] Northern United States,[2,3] Europe[4] and Asia.[5,6] In Japan, the beet armyworm has threatened production of various vegetables and ornamental crops since the 1980's.[7,8] Many insecticides were certainly effective against the beet armyworm in the early 1980's, but due to a heavy reliance on insecticides for its control the pest rapidly developed resistance to almost all kinds of insecticides.[9] Resistance of the beet armyworm to insecticides such as DDT,[10] endrin,[11] organophosphates,[12] carbamates,[13–15] pyrethroids,[16,17] chitin synthesis inhibitors,[18,19] and *Bacillus thuringiensis* ssp. *kurstaki*[20] has been documented in the last 40 years. This phenomenon has made it difficult to control this insect pest using insecticides alone, and other approaches such as mating disruption using a synthetic sex pheromone, and the use of a nucleopolyhedrovirus (SeNPV)-based insecticide have had to be considered.[20–26] Many studies showed that when conditions were right, mating disruption using a synthetic sex pheromone significantly reduced the population of beet armyworm in the field.[27] However, the use of SeNPV has no immediate effect on the control of the beet armyworm. While there are still limitations in the use of other control measures to manage populations of beet armyworm, the development of more effective insecticides is necessary. Hence, understanding the mechanisms of resistance to insecticides in this pest is considered essential to its management. In response to this need, we investigated the mechanisms involved in the resistance of the beet armyworm to pyrethroids, particularly permethrin. It has been recognized that resistance to pyrethroids in insects is due to decreased cuticle penetration, target site insensitivity (kdr), enhanced metabolic detoxification and/or other factors.[28] In this study, we evaluated the toxicity of several pyrethroids in 5th instar larvae of a permethrin-susceptible and a permethrin-resistant strain of the beet armyworm. Susceptibility to permethrin at all larval stages in both strains was determined as well. Synergism with piperonyl butoxide was studied to assess the possible involvement of cytochrome P450 monooxygenase in the resistance of the beet armyworm to permethrin.

MATERIALS AND METHODS

1. *Insects*

The beet armyworm was reared at 25±1°C with a 16 : 8H (L : D) cycle on an artificial diet (Insecta LF®, Nihon Nosan Kogyo, Japan). Adults were maintained on a 5% sucrose solution. The susceptible strain of the beet armyworm (TS) was obtained from Kochi Experimental Station, Japan Plant Protection Association in 1998, and has been cultured without exposure to any insecticides in the laboratory. The resistant strain (TR) was obtained by selecting a sub-colony of the TS-strain with a commercial formulation of permethrin. The TR-strain has been maintained by selection every two generations since 1998.

2. *Chemicals*

Formulated permethrin (Adion® 20% EC, Agros, Japan) was used to establish the TR-strain. The following technical grade pyrethroid insecticides were gifts from Sumitomo Chemical Co., Ltd., Japan. Technical grade pyrethroid racemic permethrin (94.5%), racemic cypermethrin (96.0%) and Aα-fenvalerate[29] (99.0%), and formulated chlorphenapyr (Nisso Kotetsu® FL 10%, Nippon Soda Co., Ltd., Japan) were used for the toxicity tests. The following synergists were purchased: piperonyl butoxide (PBO, >90.0%) and diethylmaleate (DEM, >98%) from Tokyo Kasei Kogyo Co., Ltd., Japan; S,S,S-tributylphosphorotrithioate (DEF, >98%) from Chem Service, USA, and acetone (99.5%) from Wako Pure Chemical, Japan.

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3. Bioassays

Topical application, feeding, and dipping methods were employed in the toxicity tests. The toxicity of permethrin, cypermethrin and fenvalerate in 0-day old 5th instar larvae of both susceptible (TS) and resistant (TR) strains was determined using the topical application technique. Acetone (1 μl) containing different concentrations of each insecticide was applied onto the larvae using a topical applicator (Kiya Co., Ltd., Japan). Larvae in the control set-up were treated with 1 μl of acetone only. The chlorphenapyr assay in the same larval stage was conducted by dipping the larvae for 3 sec in different test concentrations prepared with distilled water. Untreated larvae were dipped in distilled water only. The susceptibility of each larval instar of the test strains to permethrin (Adion®) alone was also determined using the dipping method. The procedure was similar to that of the chlorphenapyr assay described earlier. Each synergist was also administered to 5th instar larvae of the TS- and TR-strains by topical application and feeding. A similar procedure to that described previously was followed for the topical application. However, a topical application of 100 μg synergist in 1 μl acetone per larva was made 1 hr before the treatment with insecticide treatments. In the feeding experiment, four to five doses of permethrin in 1 μl of acetone were mixed into 1 mg of artificial diet, and offered to larvae. Acetone alone was used as a control. Three replications were made for each test concentration used in the bioassay. The bioassay set-up was kept at 25±1°C and mortality was assessed 24 hrs after exposure to insecticides. Larvae which showed no response when touched with forceps were considered dead. Control mortality was 0% with acetone, water or synergist only. LD_{50} and LC_{50} values for each insecticide were calculated using a log-probit mortality regression analysis.[30] The resistance ratio was calculated by dividing the LD_{50} and LC_{50} values of the TR-strain by those of the TS-strain. The synergistic ratio was obtained by dividing the LD_{50} of unsynergized treatment with the LD_{50} of synergized treatment.

RESULTS AND DISCUSSION

On topical application to 5th instar larvae, LD_{50} values of permethrin in the TS- and TR-strains were 0.382 and 35.3 μg/larva, respectively. The TR-strain showed 92-fold more resistance to permethrin than the TS-strain (Table 1). It was reported that beet armyworm from Kochi prefecture showed an LD_{50} value of 25.4 μg/larva for permethrin.[31] In the test for synergism, pretreatment with PBO (cytochrome P450 inhibitor) significantly increased susceptibility to permethrin in the TR-strain, with a synergistic ratio (SR) of 12 (Table 1). Thus, PBO significantly reduced the resistance ratio (RR), from 92- to 14-fold. In contrast, DEF (esterase inhibitor) and DEM (glutathione *S*-transferase inhibitor) had no significant effect on the toxicity of permethrin in the TR-strain, with a SR of 1.5 and 0.84, respectively (Table 1). Bioassays and synergism studies showed that the major mechanism of resistance in the TR-strain is cytochrome P450 monooxygenase-mediated detoxification, and that one or more additional mechanisms are responsible for the remaining 14-fold resistance. The role of other mechanisms in the resistance of the beet armyworm to permethrin was indicated in the study of Oomori *et al.*[32] on target site sensitivity. Relative to the TS-strain, decreased target site sensitivity to permethrin was observed in the TR-strain as revealed by electrophysiologic analysis.

In the feeding experiments, LD_{50} values of permethrin in the TS- and TR-strains were 0.914 and 5.60 μg/larva, respectively. The TR-strain showed 6.1-fold resistance to permethrin when fed the compound (Table 1). Pre-treatment with PBO increased the toxicity of permethrin in both strains, but the RR value was not reduced by PBO treatment. The RR value for permethrin in the TR-strain was higher after the topical application than feeding method. This result is indicative of the possible involvement of cuticular penetration in the observed resistance. It was previously reported that resistance to deltamethrin in beet armyworm from Guatemala was caused by a delayed cuticular penetration and cleavage of deltamethrin at the ester bond.[33]

Slopes of 1.66 and 3.09 were obtained for the TS- and TR-strains, respectively and suggest that the TR-strain is more homogeneous in its response to permethrin than the TS-strain (Table 1). This phenomenon was not observed in the feeding experiment which may be caused by the difference in the method of selection used.

Based on LD_{50} and LC_{50} values, 5th instar larvae of the TR-strain demonstrated high cross-resistance to cypermethrin and fenvalerate (Table 2). The topical LD_{50} values of these pyrethroids were 1.01 and 2.60 μg/larva, respectively, in the TS-strain and 98.4 and 336 μg/larva, respectively, in the TR-strain. The resistance of the TR-strain to these insecticides was 97- and 130-fold, respectively, that of the TS-strain. Chlorphenapyr, applied to the TS- and TR-strains by the dipping method, afforded LC_{50} values of 34.8 and 33.0 ppm, respectively (Table 2). Very little difference in susceptibility was found between the two strains with respect to chlorphenapyr. The slope for the TS- and TR-strains was 11.6 and 4.65, respectively (Table 2). This result showed that the TS-strain is more homogeneous in its response to chlorphenapyr than the TR-strain. The slope is reduced to 4.65 in the TR-strain which is probably the result of previous exposure to permethrin, even though the LC_{50} value did not differ between the strains. Chlorphenapyr is a pyrrole insecticide which exerts its toxicity through mitochondrial uncoupling. Cross-resistance would not occur between chlorphenapyr and pyrethroids because the active site of chlorphenapyr is different from that of pyrethroids such as permethrin.[34] In fact, the TR-strain showed no resistance to chlorphenapyr. Chlorphenapyr is a propesticide that is bioactivated to a higher insecticidal metabolite[35] by the oxidative removal of the N-ethoxymethyl group in a process that is likely catalyzed by cytochrome P450 monooxygenase.[36] It is possible that the cytochrome P450 monooxygenase that metabolizes permethrin is different from the cytochrome P450 monooxygenase that activates chlorphenapyr. Based on the results discussed above, chlorphenapyr should still provide effective control of the beet armyworm.

The toxicity of permethrin to beet armyworm larvae at different stages in their development is presented in Table 3. At every

Table 1. Toxicity of permethrin and permethrin with PBO, DEF and DEM in 5th instar larvae of the TS- and TR-strains of the beet armyworm 24 hours after treatment by topical application and feeding

Method	Strain	Insecticide	n[a]	LD_{50}[b] (95% CL)[c]	Slope	SR[d]	RR[e]
Topical application							
	TS-strain	Permethrin	420	0.382 (0.321–0.462)	1.66		
		Permethrin +PBO[f]	360	0.214 (0.137–0.253)	1.98	1.8	
		Permethrin +DEF[g]	180	0.218 (0.178–0.245)	2.62	1.8	
		Permethrin +DEM[h]	180	0.461 (0.381–0.505)	3.02	0.83	
	TR-strain	Permethrin	125	35.3 (28.6–42.5)	3.09		92
		Permethrin +PBO[f]	125	3.04 (2.55–3.56)	3.91	12	14
		Permethrin +DEF[g]	180	24.2 (16.8–44.1)	1.52	1.5	9.0
		Permethrin +DEM[h]	180	42.1 (23.9–63.6)	1.66	0.84	91
Feeding							
	TS-strain	Permethrin	109	0.914 (0.582–1.165)	1.64		
		Permethrin +PBO[f]	57	0.073 (0.048–0.156)	1.65	13	
	TR-strain	Permethrin	130	5.60 (4.01–9.20)	1.23		6.1
		Permethrin +PBO[f]	70	0.659 (0.481–0.856)	2.49	8.5	9.0

[a] Number of larvae tested. [b] μg/larva. [c] 95% confidence limit. [d] Synergism ratio; LD_{50} without synergist/LD_{50} with synergist. [e] Resistance ratio; LD_{50} of the TR-strain/LD_{50} of the TS-strain. [f] PBO; Piperonyl butoxide, applied at 100 μg/insect. [g] DEF; S,S,S-tributylphosphorotrithioate, applied at 100 μg/insect. [h] DEM; Diethyl maleate, applied at 100 μg/insect.

larval stage, susceptibility to permethrin is greater in the TS-strain than TR-strain. LC_{50} values of permethrin for the TS-strain gradually increased from 27.3 (1st instar) to 120 ppm (5th instar). On the other hand, LC_{50} values for the TR-strain drastically increased from 80.7 (1st instar) to more than 57,200 ppm (5th instar) as the larvae matured. The mortality obtained by treating 5th instar larvae of the TR-strain with 4000 ppm was 1.7% (1 death/ 60 insects), thus the LC_{50} value was not calculated. As a result, RR values increased from 3.0 (1st instar) to more than 480 (5th instar). These results implied difficulty in controlling the later instar of the TR-strain using permethrin. The relatively high resistance observed in 4th and 5th instar larvae may be due to the presence of more developed organs (such as a midgut and fatbody) which metabolize insecticides. On the other hand, 1st instar of both strains showed greater susceptibility to permethrin than did other instars. This result is consistent with the report of Takai[9] on 1st instar of the beet armyworm exhibiting greater susceptibility to certain kinds of organophosphate and pyrethroid insecticides. Based on the results presented above, permethrin can be considered an effective compound for the control of 1st instar larvae of the beet armyworm.

Bioassays and synergism studies showed that resistance to permethrin in the TR-strain is due to decreased cuticle penetration, and target site insensitivity (kdr) to some extent, but enhanced metabolic detoxification by cytochrome P450 monooxygenase is the major mechanism involved. On the other hand, it was reported that susceptibility to permethrin after phenobarbital (cytochrome P450 inducer) treatment was reduced in the TS-

Table 2. Toxicity of permethrin, cypermethrin, fenvalerate and chlorphenapyr in 5th instar larvae of the TS- and TR-strains of the beet armyworm 24 hours after treatment by topical application or dipping

Strain	Insecticide	n[a)]	LD_{50}[b)] or LC_{50} (95% CL)[c)]	Slope	RR[d)]
TS-strain	Permethrin	420	0.382 (0.321–0.462)	1.66	
	Cypermethrin	200	1.01 (0.62–1.38)	2.87	
	Fenvalerate	100	2.60 (1.43–3.61)	1.52	
	Chlorphenapyr[e)]	130	34.8[f)] (32.9–37.0)	11.6	
TR-strain	Permethrin	125	35.3 (28.6–42.5)	3.09	92
	Cypermethrin	105	98.4 (86.0–110)	7.23	97
	Fenvalerate	100	336 (141–356)	3.66	130
	Chlorphenapyr[e)]	160	33.0[f)] (29.8–37.3)	4.65	0.95

[a)] Number of larvae tested. [b)] μg/larva. [c)] 95% confidence limit. [d)] Resistance ratio; LD_{50} or LC_{50} of the TR-strain/LD_{50} or LC_{50} of the TS-strain. [e)] Dipping method. [f)] LC_{50} values.

strain.[37)] The effect of induction by phenobarbital was almost completely overcome by the PBO treatment.[37)] This result showed that cytochrome P450 monooxygenase plays an important role in the resistance to permethrin of the TS-strain too. The pyrethroid resistance in *Musca domestica*,[38,39)] *Drosophila melanogaster*,[40)] *Blatella germanica*,[41)] *Heliothis virescens*,[42)] *Helicoverpa armigera*,[43)] and *Culex quinquefasciatus*[44)] was reported to be due to enhanced metabolic detoxification by cy-

Table 3. Toxicity test of permethrin against different larval stages of the TS- and TR-strains of the beet armyworm 24 hours after treatment by the dipping method

Larval stage	TS-strain		TR-strain		RR[b)]
	LC_{50} (ppm) (95% CL)[a)]	Slope±SE	LC_{50} (ppm) (95% CL)[a)]	Slope±SE	LC_{50}
1st instar	27.3 (22.4–37.7)	3.4±0.6	80.7 (67.4–94.1)	2.7±0.5	3.0
2nd instar	140 (120–168)	2.2±0.2	1090 (854–1390)	2.3±0.2	7.8
3rd instar	157 (42–1078)	3.3±0.7	6870 (898–9710)	1.2±0.4	44
4th instar	112 (94–132)	3.4±0.2	57200 (3660–102000)	0.8±0.2	510
5th instar	120 (82–123)	3.6±0.4	>57200[c)]	—	>480

[a)] 95% confidence limit. [b)] Resistance ratio; LC_{50} of the TR-strain/LC_{50} of the TS-strain. [c)] The death rate with 4000 ppm of formulated permethrin was 1.7%.

tochrome P450 monooxygenase. The cytochrome P450 monooxygenase is extremely important in the metabolism of endogenous compounds and xenobiotics including insecticides. The large number of substrates metabolized is due to a number of P450 isoforms and the broad substrate specificity of some isoforms.[45,46] *CYP6D1* was reported as the P450 involved in pyrethroid resistance in the LPR strain of *Musca domestica*,[47] and *CYP9A1* as that involved in thiodicarb resistance in the Hebert strain of *Heliothis virescens*.[42] To date, few investigations have been conducted on P450 isoforms implicated in insecticide resistance of the beet armyworm, and further research on the function and gene regulation of this important multigene family of enzymes in this economically important agricultural insect pest is warranted.

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