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Resistance selection and molecular mechanisms of cypermethrin resistance in red hairy caterpillar (*Amsacta albistriga* walker)

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ABSTRACT

Amsacta albistriga is one of the important pests of oilseed crops in India. This pest has developed high resistance to organophosphate (OP) insecticide in field. Therefore, cypermethrin insecticide was used as an alternative for this pest. After 20 generations of selection with cypermethrin, the LD_{50} value for *A. albistriga* was increased by 21.5-folds. The synergism ratio of piperonyl butoxide (PBO) and triphenyl phosphate (TPP) was increased by 10- and 9.6-fold in resistant strains and comparatively, 3.9 and 4.2-fold in susceptible strains. Detoxification enzyme analysis and native PAGE electrophoresis of esterase isoenzyme further revealed that esterase and mixed function oxidase may be involved in cypermethrin resistance in CypRes strain. In addition to enzyme analysis overexpression of CYP4M44, CYP9A77 and CYP6B47 (ortholog) can confer metabolic resistance in the CypRes strain. These data provide a foundation for further study of cypermethrin resistance mechanism observed in *A. albistriga*.

1. Introduction

The red hairy caterpillar, *Amsacta albistriga*, is an important pest of oil seed crops in India. The larvae attack flowers and leaves of many crops, such as groundnut, castor and cotton [1]. In the past couple of years chemical insecticides has been implemented to minimize the problem. However, the development of insecticide resistance has posed a major challenge in the control of *A. albistriga* [2].

Insecticide synergist has long been used as a tool to examine the ability of a particular insecticide and its resistance mechanisms under laboratory conditions. Synergistic compounds have the ability to inhibit specific metabolic pathways, thus altering the way insects respond to the pesticides [3]. Preliminary evidence of the involvement of detoxification enzymes in insecticide resistance mechanisms has been obtained by using synergists [4]. Some researchers have focused on the use of synergists in combination with or rotation of insecticides for disrupting resistance mechanisms or delaying the development of insecticide resistance [5,6].

There are several ways that insects can become resistant to insecticides, which may involve more than one mechanism at the same time. These include decreased penetration, altered target site insensitivity and metabolic resistance mechanism mediated by detoxifying enzymes [7]. Decreased penetration, plays little role in most cases and target site insensitivity always makes useless a kind of insecticide with the same mode of action [8,9]. Metabolic resistance involving an array of detoxification enzymes, like cytochrome P450 monooxygenase, esterase and glutathione S-transferase are primary modes of insecticide detoxification [10,11].

Among metabolic enzymes, esterase and cytochrome P450 monooxygenases are mainly involved in resistance development to pyrethriod insecticides in many insect species [12,13]. Esterases are a large group of enzymes involved in the metabolism of organophosphates and pyrethroids by sequestration, hydrolysis [11,14] and cleavage of functional groups of toxicants [15]. Resistance to pyrethroid owing to elevated activity of esterase is reported in Indian strains of *Helicoverpa armigera, Spodoptera litura, Plutella xylostella, H. armigera, Bemisia tabaci* and mosquitoes [16–21].

Microsomal cytochrome P450 monooxygenase is another important metabolic enzyme involved in the detoxification of wide classes of chemical insecticides including pyrethroids and organophosphates [22–24]. Cytochrome P450s are a complex family of heme containing enzymes, which catalyze a variety of oxidative reaction with a significant diversity of endogenous, exogenous substrates such as pesticides, plant toxins and drugs [25,26]. Many cytochrome P450s genes belonging to the families of microsomal Cyp4, Cyp6 are responsible for pyrethroid and insecticide resistance mechanism [27–30].

In the previous study we found that the field population of *A. albistriga* had the ability to develop resistance to dichlorvos an organophosphate insecticide, but not for pyrethroid resistance, which was revealed by biochemical studies and esterase isoenzyme banding patterns. Therefore, in the present study an effort was taken to study

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the possible resistance mechanism to cypermethrin in the red hairy caterpillar, *Amsacta albistriga*.

2. Materials and methods

2.1. Insects

A laboratory strain of *A. albistriga* was obtained from the institute's molecular entomology laboratory originated from the groundnut fields in India (June–August 2009 and 2011). The colony was reared using young castor leaf diet (*Ricinus communis*). The larvae were reared under laboratory conditions for more than 20 generations and used as a susceptible strain (Sus).

2.2. Insecticides and synergists

Insecticides used in this study were of commercial grade cypermethrin 10% EC, imidacloprid 17.8% SL and monocrotophos 36% SL. Piperonyl butoxide (PBO, 90% purity) was purchased from Himedia Chemical Company Inc., diethyl maleate (DEM, 90% purity) was purchased from Himedia Chemical Company, and triphenyl phosphate (TPP, 90% purity) was purchased from Sigma-Aldrich.

2.3. Toxicity bioassay

Insecticide bioassay was performed using the leaf dipping method adapted from IRAC method No. 7 [31]. Each insecticide was dissolved in deionized water and diluted into six serial concentrations. Triton X-100 was added at the rate of 0.54 ml/l of water as a spreading and sticking agent [32]. Using castor leaves a number of small leaf discs was prepared and dipped in to the different concentrations of insecticide for 30 s and then air dried. Early third instar larvae were transferred in to plastic cups containing insecticide dipped leaf discs. The control used acetone alone. For synergism assays, the synergist (PBO, DEM and TPP) were dissolved in acetone and 1 µl of each synergist were treated with the dose of 23 µg/larvae (PBO), 23 µg/larvae (DEM), and 18 µg/larvae (TPP) 1 h before insecticide application. These synergist concentrations did not cause mortality in preliminary assays using *A. albistriga*. All the tests above were with three replications. The mortality was checked in 48 h.

2.4. Selection for cypermethrin resistance strain

Based on the results of initial bioassays, the third instar larvae of the laboratory colony were subjected to resistance selection with cypermethrin (CypRes) using the leaf-dip method. Unselected controls were treated identically, without exposure to insecticides. The concentration of insecticides used for resistance selection in each generation was based on the results of bioassays LD_{50} from the previous generation. Surviving larvae were reared in the chamber on castor leaf diet under the control conditions at 25 °C with a 16:8 L:D photoperiod.

2.5. Preparation of enzymes

Whole body of third instar larvae and the midgut and fatbody dissected from 30 third instar larvae (35–40 mg) were used for enzyme preparation. The whole body, midgut and fatbody were individually homogenized with 1.5 ml of 0.1 M ice cold sodium phosphate buffer (pH 7.2). After centrifugation at 10,000 rpm for 30 min, the supernatant was re-centrifuged at 12,000 *g* for 30 min. Then, the clear supernatant was collected and used as enzyme resources for analysis of the activity of MFO, GST and esterase. All operation was carried out on ice and centrifugation at 4 °C to minimize loss of enzyme activity.

2.6. Total protein

Total protein content of the enzyme solution was determined by the Lowry et al. method [33] using bovine serum albumin (BSA) as the standard.

2.7. Detoxification enzymes assay

2.7.1. Mixed-function oxidase

The MFO activity was determined using peroxidation of Tetramethylbenzidine (TMBZ) assay according to Brogdon [34] with slight modifications. Two-fifty microliters of 0.05 M potassium phosphate buffer (pH 7.0) were added to 50 µl microfuged supernatant and 500 µl tetramethyl benzidine solution (0.05% 3,3',5,5' Tetramethyl Benzidine, i.e. TMBZ + 5 ml methanol + 15 ml sodium acetate buffer 0.25 M pH 5.0). Two-hundred microliter of 3% hydrogen peroxide was added and the mixture was incubated for 30 min at room temperature. Absorbance was read at 630 nm and values calculated from a standard curve of cytochrome C.

2.7.2. Glutathione S-transferase

Glutathione S-transferase assay were performed according to the method developed by Kao et al. [35] using 1-chloro 2, 4-dinitrobenzene and reduced glutathione (GSH) as substrate. The total reaction solution contained 2.79 ml of phosphate buffer saline 0.1 M pH-6.5, 10 µl of diluted enzyme supernatant (the stock solution was diluted 10-fold with 0.1 M pH-6.5, sodium phosphate buffer), 50 µl of 50 mM CDNB (dissolved in the 0.1% (v/v) ethanol), and 150 µl of reduced glutathione in Tris–HCL (0.05 M, pH 7.5). The changes in absorbance were measured continuously for 5 min at 340 nm using the time scan mode of UV–Visible spectrophotometer.

2.7.3. Esterase activity

Esterase activity was determined using the method described by Kranthi [36]. The reaction mixture contains 0.1 ml of supernatant (10 µl of enzyme sample with 99 µl of 40 Mm PBS pH-6.8) was added to the 15 ml clean test tube, containing 5 ml of substrate (5 mg of 30 mM α-napthyl acetate/ml of acetone dissolved in 99 ml of 40 mM PBS), tubes were incubated in dark for 20 min at 30 °C. Then 1 ml of staining solution (5% SDS and 1% fast blue BB salt dissolved in 16 ml of sodium phosphate buffer pH 6.8) were added to the reaction tube in the ratio of 1:5. The esterase activity was measured continuously at 590 nm for 10 min. The esterase activity was calculated as µmol/min/mg protein by using 1-napthyl as the standard.

2.7.4. Data analysis

For bioassay data the LD_{50} and their confidence interval were estimated by Probit Analysis using the SPSS software (version 16.0). Resistance ratio (RR) were estimated at the LD_{50} level as RR = LD_{50} of collected field (or resistance) strain/LD_{50} of susceptible strain. The synergism ratios (SR) were calculated as follows: SR = LD_{50} value of insecticide alone/LD_{50} value of insecticide after synergist. All the enzyme assays above were with three replications. The data obtained from enzyme assays were subjected to analysis of variance followed by Bonferroni multiple comparison post-hoc test using PRISM 5 software (Graph Pad Software Inc., La Jolla, CA, USA).

2.8. Esterase isoenzyme analysis

Non-denaturing PAGE was carried out following the method of Kranthi [36] on vertical polyacrylamide gel electrophoresis using the 10% separating gel and 4% stacking gel with continuous Tris-glycine buffer running system (50 mM, pH 8.3). Ten microliters of each midgut sample containing 20 µg protein was diluted with 8 µl