MATERIALS & METHODS

4 MATERIALS AND METHODS

4.1 Study area

West Bengal is a state of the Indian union located in the eastern part of the country bounded by countries like Bangladesh, Bhutan and Nepal and shares boundary with five Indian fellow states of Assam, Bihar, Jharkhand, Odisha and Sikkim. The state is a major producer of agricultural goods and is the sixth largest contributor to the net domestic product of India (Rajkumar, 2014). Agriculture is the leading employment generating sector in the state. Tea is an important agricultural product of the state besides rice and jute. About ¼ of the total Indian tea produced is contributed by the state with the world renowned 'Darjeeling Tea' as one of the finest products. In West Bengal, cultivation of tea is traditionally confined to Darjeeling, Terai and the Dooars in the northern region, covering an area of about 103,431 hectares (www.teaboard.gov.in; accessed on 05.07.2016) mostly in the two districts of Darjeeling and Jalpaiguri. Recently farmers of North Dinajpur district are also venturing into the profitable cultivation of the foliage crop, tea. The present study encompasses the area of tea plantations spread across the flat Indo-Gangetic lowlands of Terai and the Dooars and the foothills of Darjeeling Himalayas in northern part of West Bengal (Figure 4.1 A and B).

FIGURE 4.1:

A) Map showing position of West Bengal in the Indian union and the two major tea growing districts, Darjeeling and Jalpaiguri therein

B) Map showing sampling sites from the two districts, Darjeeling and Jalpaiguri in northern part of West Bengal

FIGURE-4.1A

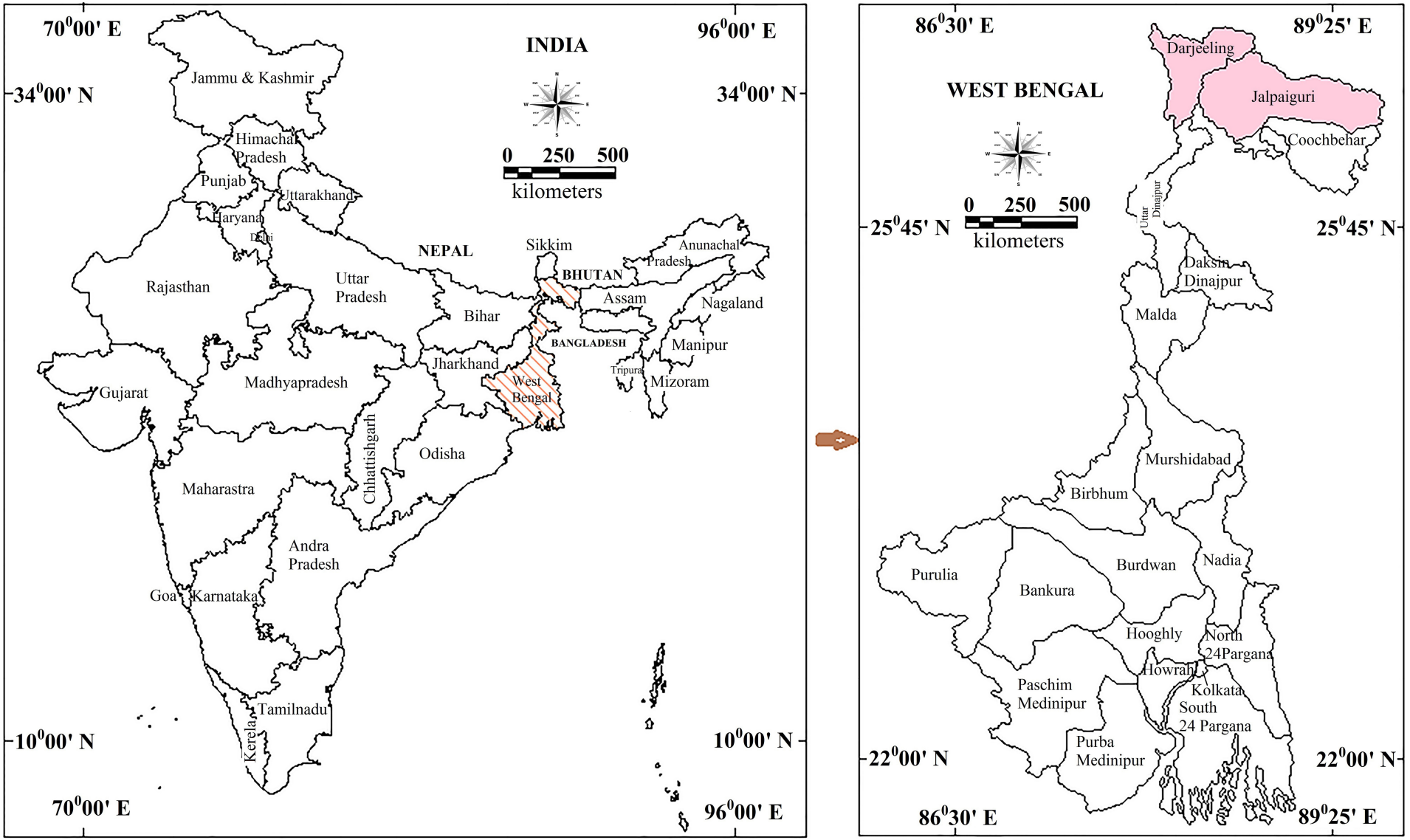
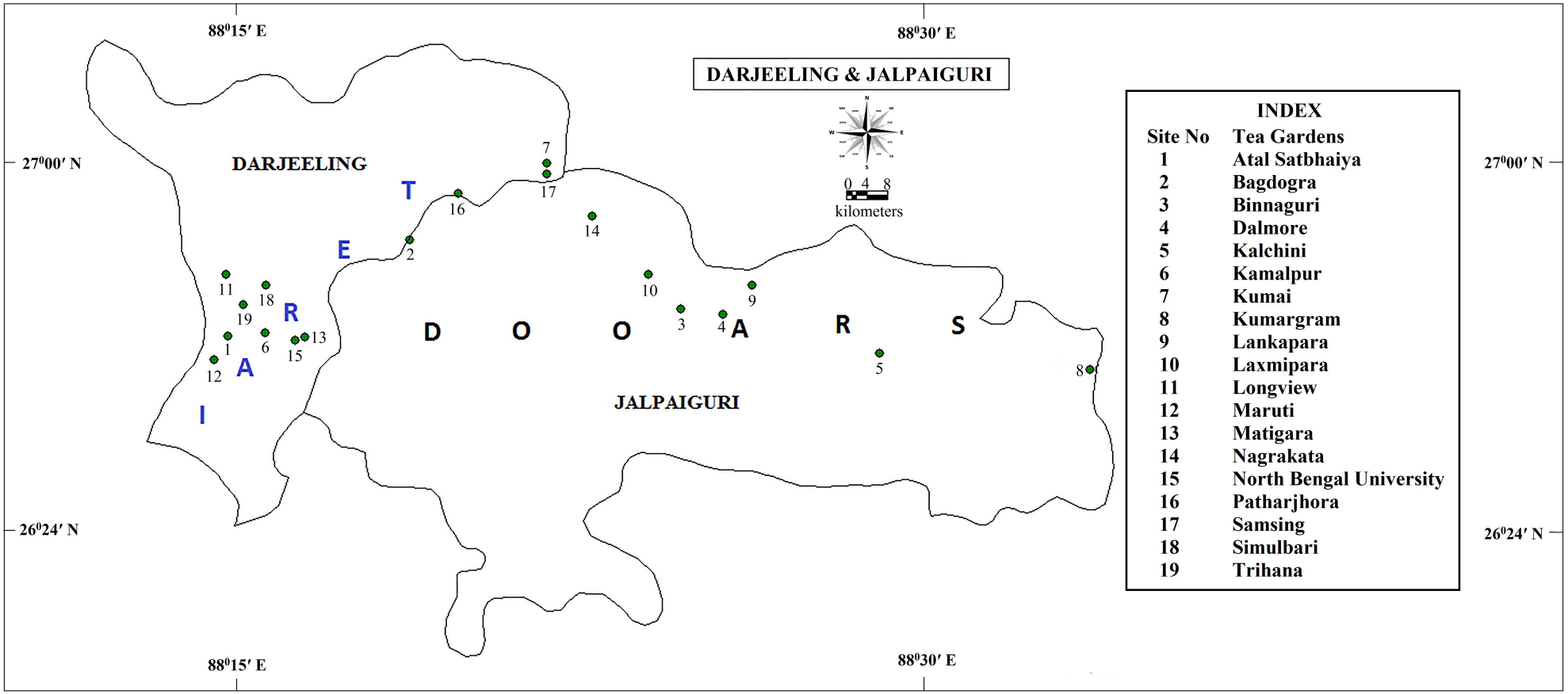

INDIA
70⁰00' E
96⁰00' E
34⁰00' N
10⁰00' N
0 250 500
kilometers
Jammu & Kashmir
Himachal Pradesh
Punjab
Uttarakhand
Haryana
Delhi
NEPAL
Sikkim
BHUTAN
Anunachal Pradesh
Assam
Nagaland
Manipur
Tripura
Mizoram
BANGLADESH
Rajasthan
Uttar Pradesh
Bihar
Jharkhand
West Bengal
Madhyapradesh
Gujarat
Chhattishgarh
Odisha
Maharastra
Andra Pradesh
Goa
Karnataka
Tamilnadu
Kerela
WEST BENGAL
86⁰30' E
89⁰25' E
25⁰45' N
22⁰00' N
Darjeeling
Jalpaiguri
Coochbehar
Uttar Dinajpur
Daksin Dinajpur
Malda
Murshidabad
Birbhum
Burdwan
Nadia
Purulia
Bankura
Hooghly
North 24Pargana
Howrah
Kolkata
Paschim Medinipur
South 24 Pargana
Purba Medinipur

FIGURE-4.1B


88⁰15′ E
88⁰30′ E
27⁰00′ N
26⁰24′ N
DARJEELING & JALPAIGURI
DARJEELING
JALPAIGURI
T E R A I
D O O A R S
0 4 8
kilometers
INDEX
Site No Tea Gardens
1 Atal Satbhaiya
2 Bagdogra
3 Binnaguri
4 Dalmore
5 Kalchini
6 Kamalpur
7 Kumai
8 Kumargram
9 Lankapara
10 Laxmipara
11 Longview
12 Maruti
13 Matigara
14 Nagrakata
15 North Bengal University
16 Patharjhora
17 Samsing
18 Simulbari
19 Trihana

4.2 Sampling

Both the adults and nymphal stages of the sucking pest, *Helopeltis theivora* were collected from conventionally and bio-organically managed tea plantations across Darjeeling foothills, Terai and the Dooars plains, using hand picking, aspirator and vacuum sampler (Plate 4.1). The collected specimens were divided into two sets. One was used for determining the pesticide tolerance level in terms of lethal concentration (LC) and the other was reared in the laboratory condition following a standard established method described in next section. The laboratory-reared specimens were used as the control and for further studies and analyses.

Nymphs were easier to collect. Little activity during day time along with their greenish body colour blending and camouflaging with tea bushes compounded the difficulty in procuring adults. Weather and time of collection were important factors for the successful collection. During sunny days, adults were found hiding in the shades of tea bushes, escaping eyes of the collector. Dawn and dusk were found to be the optimum time for collection. Overcast days were also found to be suitable for the collection of adults *H. theivora*. Unlike adults, nymphs could be collected at any time of the day. It was found that *H. theivora* prefers tea bushes of shady and moist areas in a tea estate.

4.3 Rearing of *Helopeltis theivora*

Tube method of Sudhakaran (2000) was modified for rearing of the pest species. Adult specimens collected from the field were reared and mass cultured on pesticide-free tea leaves under laboratory conditions for generations to conduct experiments. They were reared in plastic jars, glass hurricane lamp chimneys and insect rearing cages with dimension of 60×30×30 cm, sleeved by metal mesh of 2 mm^2 with 12 L: 12 D photoperiod at 25 ± 2 °C and 85 – 90% RH. Tea shoots with buds and young leaves were served to them every 24 hours in a conical flask or centrifuge tubes with cut end dipped in water to maintain the turgidity of leaves (Plate 4.2). They fed and oviposited on the young and turgid twigs provided. Females laid eggs on the midribs, buds and young stems. Shoots used for oviposition and bearing eggs were kept for incubation at room temperature (25 ± 2 °C). Dead insects at the bottom of the rearing cage or the glass chimney were removed using a long forceps (Jackson, 1974) and camel hair painting brush. Nymphs were also reared in a similar setup.

PLATE 4.1: Sampling of *Helopeltis theivora* by Vacuum sampler

PLATE - 4.1

PLATE 4.2: Rearing technique of *Helopeltis theivora*

A) In glass hurricane chimney

B) In wooden insect rearing cag

C) Tea twig supplying technique for adult

D) Tea twig supplying technique for nymph

PLATE - 4.2

PLATE - 4.2

4.4 Bioassay for determining tolerance level

To determine their tolerance level expressed in lethal concentration (LC) values of the tested pesticides, bioassays of *H. theivora* populations obtained from culture as well as collected from the field were done by 'leaf dip technique' (Anonymous, 1990). The leaf dip technique is recommended by 'Pesticide Resistance Action Committee of the International Group of National Association of Manufacturers of Agrochemical Products'. Taking a clue from the results obtained in a series of trials conducted, five concentrations of pesticide belonging to organophosphate and synthetic pyrethroid were prepared in distilled water for bioassay. Adults of *H. theivora*, containing both females and males in equal proportion were then exposed to the freshly prepared aqueous solutions of the two commonly used pesticides to determine their lethal concentrations (Plate 4.3). They were exposed in equal proportion to negate the effect of gender-based difference in tolerance level on the cumulative result of the bioassay. The two pesticides used were monocrotophos (36% SL) and cypermethrin (10% EC), representing organophosphate and synthetic pyrethroid, respectively.

4.5 Selection of *Helopeltis theivora* by synthetic pesticides through generations

The insect pest was selected using a high dose of an organophosphate pesticide, monocrotophos (36% SL). Adults of *H. theivora* with both females and males in equal proportion, obtained from laboratory culture were exposed to $LC_{80,}$ the concentration causing 80% mortality of the exposed individuals (Plate 4.3). The field recommended dose of these organophosphate pesticides for controlling *H. theivora* ranges from 2,500 to 5,000 ppm (Anonymous, 2010; 2014b), many fold higher than the median lethal concentration (LC_{50}) (Misra, 1989). The pesticide dose of LC_{80} was chosen for artificial selection (bottlenecking) of the pest population to simulate field condition in the laboratory. The selected individuals were reared in the setups as described above in section 4.3. The tolerance levels were studied after the artificial selection for three generations of parental (P), filial-l (F1) and filial-2 (F2).

4.6 Sample preparation for assay of defence enzyme activity

Adults of *H. theivora* (n=150) from each pesticide-selected generation were starved for 2 – 3 hours to allow digestion, assimilation and clearance of the gut content (ingested tea sap).

PLATE 4.3: Setup for bioassay and selection of *Helopeltis theivora* by exposure to pesticide through generations

PLATE-4.3

The starved specimens were then homogenised in microcentrifuge tube using micropestle. Each individual was homogenised in 500 µL of ice-cold 100 mM sodium phosphate buffer (pH 7.0) in an ice bath. The whole-body homogenate was centrifuged at 11,500***g*** for 20 minutes at 4 °C (Eppendorf Centrifuge, Model: 5417R). The activities of defence enzymes and quantification of the total protein content was done using the supernatant.

4.7 Quantitative study of defence enzymes of *Helopeltis theivora*

4.7.1 General esterases

The activity of general esterases (EC.3.1.1.1) was estimated using α-naphthyl acetate (α-NA) as substrate following the method of van Asperen (1962), with minor modifications. Reactions between 200 µL of 30 mM α-NA and 20 µL of supernatant containing esterases from 150 pesticide-selected *H. theivora* per generation were carried out individually in each well of a flat bottom microplate (Tarsons with Catalogue No 941196). 50 µL of staining solution containing was added to the reaction mixture after 15 minutes of the initiation of the reaction. The staining solution was composed of 0.1% Fast Blue BB salt and 5% sodium dodecyl sulphate (SDS) in the ratio of 2:5 (v:v). After 5 minutes of the staining, optical density was measured at 570 nm in endpoint mode in a microplate reader (Dynex Technologies, USA). The three wells per microplate with all the ingredients of the reaction mixture except the supernatant, the source of esterase were used as control. The optical density was converted to the activity of general esterases with the help of a standard curve of α-naphthol (Plate 4.4 and 4.5A).

4.7.2 Cytochrome P450 monooxygenase

The total activity of cytochrome P450 monooxygenase was measured following the method of Brogdon and McAllister (1998) and Brogdon et al. (1997) with slight modifications. 200 µL of 6.3 mM 3, 3′, 5, 5′-tetramethylbenzidine (TMBZ) solution and 20 µL of supernatant from 150 pesticide-selected *H. theivora* per generation were mixed individually in a well of a flat bottom microplate having the specifications described in the preceding section. 6.3 mM TMBZ solution was prepared by dissolving 10 mg TMBZ in 5 ml absolute methanol followed by mixing with 15 ml of 250 mM sodium acetate buffer, pH 5. 80 µL of 62.5 mM potassium phosphate buffer

PLATE 4.4: Microplate reader (Opsys MRX_{TC} revelation, Dynex technologies, USA)

PLATE - 4.4

with pH 7.2 and 25 µL of 3% aqueous solution of hydrogen peroxide were added to the reaction mixture. Three wells per microplate, containing 20 µL of 100 mM sodium phosphate, pH 7.1 along with all other ingredients of the reaction mixture except the supernatant were used as control. 100 mM sodium phosphate buffer was used for homogenising the pest. Optical density was recorded at 630 nm after 30 minutes of reaction at room temperature, using a microplate reader. The absorbance was used to calculate the activity of CYP450 with the help of a standard curve of cytochrome C from horse heart (Code No. RM510, Himedia). The activity of CYP450 was expressed in nmol equivalent of cytochrome P450s per mg protein (Plate 4.4 and 4.5B).

4.7.3 Glutathione *S*-transferases

Glutathione *S*-transferases (GST) activity in *H. theivora* homogenate was assayed by its catabolic prowess to conjugate glutathione with the substrate, 1-chloro 2, 4-dinitrobenzene (CDNB) through the thiol group of the glutathione as shown in the reaction equation, GSH + CDNB → GS-DNB (conjugate) + HCL. The method of Brogdon & Barber (1990) was followed for the assay with slight modification. 10 µL of the supernatant was added to 200 µL of substrate solution (95 parts of 10.5 mM reduced glutathione prepared in 100 mM phosphate buffer, pH 6.5 plus 5 parts of 63 mM CDNB in ethanol) in a well of a UV transparent microplate (Make: Greiner bio-one; Ref 65580, Germany) (Plate 4.5C). After three minutes, optical density (OD) proportional to the increase in the amount of GS-DNB conjugate formed as a product of the reaction catalysed by the GST present in the homogenate was measured using microplate reader (Opsys MRX TC Revelation, Dynex Technologies, USA) at 340 nm, the absorption maxima of the conjugate for 8 minutes. The ΔOD_{340} $minute^{-1}$ obtained was converted to the activity of GST using the following formula. The rate of increase in absorption is directly proportional to the GST activity in the sample. The molar extinction coefficient (ε_{340}) for the GS-DNB conjugate was deduced to 5.76 mM^{-1} corrected for the path length of 0.6 cm (Perera et al., 2008), which is equal to the depth of a microplate well.

$$GST\ Activity\ (in\ mM\ min^{-1}\mu g\ protein^{-1}) = \frac{\Delta OD \times Vreaction}{\varepsilon_{340} \times T \times Vsample \times P}$$

Where:

$$\Delta OD = \frac{\text{Final OD} - \text{Initial OD}}{\text{Reaction time in minute}}$$

$V_{reaction}$ = Reaction volume

ε_{340} = 5.76 mM^{-1}(molar extinction coefficient)

T = Reaction time in minute

V_{sample} = Volume of sample

P = Protein content per sample volume in µg

4.8 Protein quantification

The total protein content per µL of the supernatant was quantified following the procedure described by Lowry et al. (1951) and calculated using a standard curve prepared with the known concentrations (0.1 – 1.0 µg μL^{-1}) of Bovine serum albumin (Appendix B).

4.9 Qualitative study of defence enzymes

4.9.1 Non-denaturing native PAGE

The native polyacrylamide gel electrophoreses (PAGE) of whole body homogenate were carried out to study isozyme profile of general esterases and cytochrome P450 in pesticide-selected generations of *H. theivora* using 8% resolving and 4% stacking gels. The gels were pre-electrophoresed at 4 °C at 80V for overnight to remove excess ammonium persulfate (APS) followed by loading of the supernatant. The supernatants of the pest's whole body homogenate were prepared by the method described in section 4.6. Samples were double diluted with sample buffer (10% glycerol, 0.002% bromophenol blue as tracking dye) prior to loading. 20 µg of total protein per well was loaded and electrophoresis was carried out using a freshly prepared tris-glycine buffer for over 4 hours at 4°C and fixed voltage of 120V in a mini gel setup (Model No. 05 – 01, Biotech, Yercauda, TN).

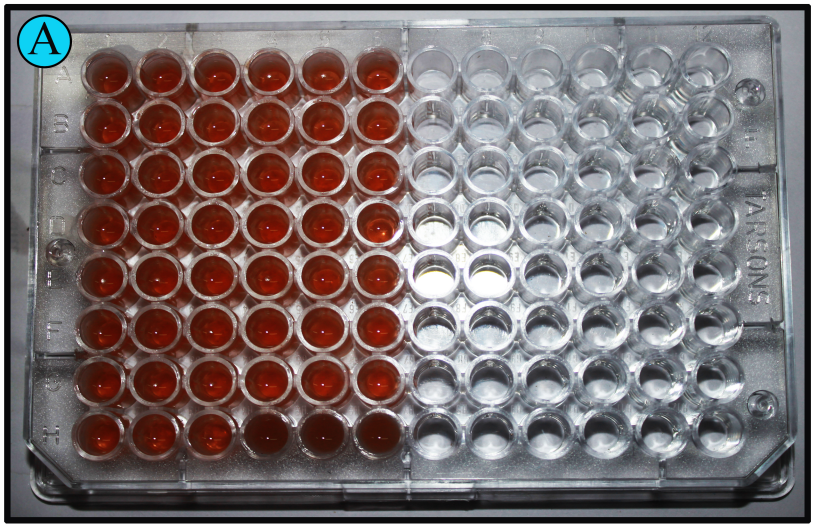
PLATE 4.5: Microplate showing reaction pattern of defence enzymes

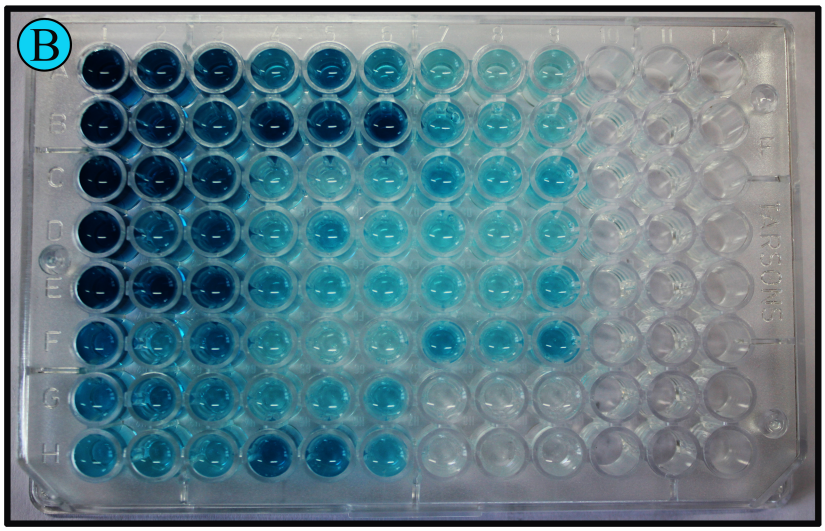
A) General esterases

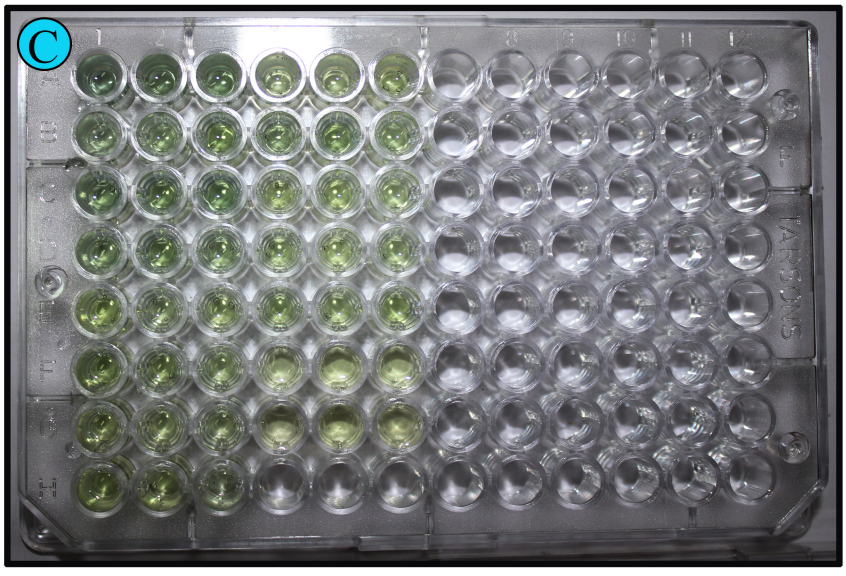
B) Cytochrome P450

C) Glutathione *S*-transferase

PLATE - 4.5







4.9.2 Visualisation and analysis of GE isozymes

For general esterases, the gels were stained following the method described by Georghiou and Pasteur (1978) with slight modifications. The gels were preincubated at room temperature for 15 minutes dipped in 100 ml of 40 mM phosphate buffer (pH 6.5) containing 0.02% α-naphthyl acetate. The gels were then incubated at room temperature submerged in staining solution consisting of 100 ml 40 mM phosphate buffer, pH 6.5, containing 0.02% α-naphthyl acetate and 0.1% fast blue BB salt for 30 minutes in dark with occasional shaking.

4.9.3 Heme staining for analysis of CYP450 isozymes

Non-denaturing native PAGE was carried out as described in the previous section. Bands of isozymes of cytochrome P450 (CYP450) with peroxidase activity was visualised following the method described by Butler and Lachance (1987). The gels were incubated at room temperature submerged in the staining solution containing 2 mM methanolic solution of TMBZ and 250 mM sodium acetate buffer (pH 5.0) in the ratio of 3:7 (v:v) in dark with occasional shaking for two hours before the addition of hydrogen peroxide to the final concentration of 0.03%.

4.10 Inhibition of GE and CYP450 isozymes by pesticide

To study the inhibitory action of organophosphate pesticide, monocrotophos (36% SL) on general esterases and cytochrome P450, gels were divided vertically into two equal halves. Each half loaded with the sample of pesticide-selected *H. theivora* containing isozymes of the defence enzymes was stained separately either for GE or CYP450. One half was stained using staining solution without the pesticide and the other containing commercially formulated pesticide at a concentration of 855.35 ppm, which is equal to the median lethal concentration (LC_{50}) value of the pesticide for the pesticide-selected F2 generation. The F2 generation was noted as the most tolerant amongst the studied generations.

4.11 Densitometric analysis

Densitometric analysis of the stained gels were carried out by using gel analysis software, ImageAide, version 3.06.04 (Spectronics corporation, New York, USA) to determine the retention factor (R_f) and the activity profiles of isozyme groups of general esterases and cytochrome P450 monooxygenases.

4.12 Reaction pattern of defence enzymes to synergists

S, S, S-tributylphosphorotrithioate, also known as tribufos (DEF) and piperonyl butoxide (PBO), the two commonly used pesticide synergists were used as the detoxifying enzyme inhibitors to assess *in vitro* inhibition and their effectiveness against the detoxifying enzymes, GE and CYP450, respectively. Technical grade DEF (100%) manufactured by Sigma-Aldrich (Supelco), was procured from Chem Service Inc., West Chester, USA. PBO was purchased from Himedia Laboratories Pvt. Ltd, Mumbai, India.

Stock solutions of the above mentioned two synergists, DEF and PBO were prepared in methanol. Aliquots of insect homogenate were incubated with the synergists at various dilutions for 30 minutes. Insect homogenate plus methanol was used as control. The activities of the detoxifying enzymes, GE and CYP450 after the incubation were determined as per the methods described in previous sections 4.7.1 and 4.7.2, respectively. Half the maximum inhibitory concentration (IC_{50}) was calculated by fitting data of enzyme activity inhibition to a linear probit regression model through transforming sigmoid concentration-inhibition commonly known as dose-response curve to a straight line.

4.13 Defence enzyme based technique to determine pesticide tolerance levels in *Helopeltis theivora*

4.13.1 Microplate assay

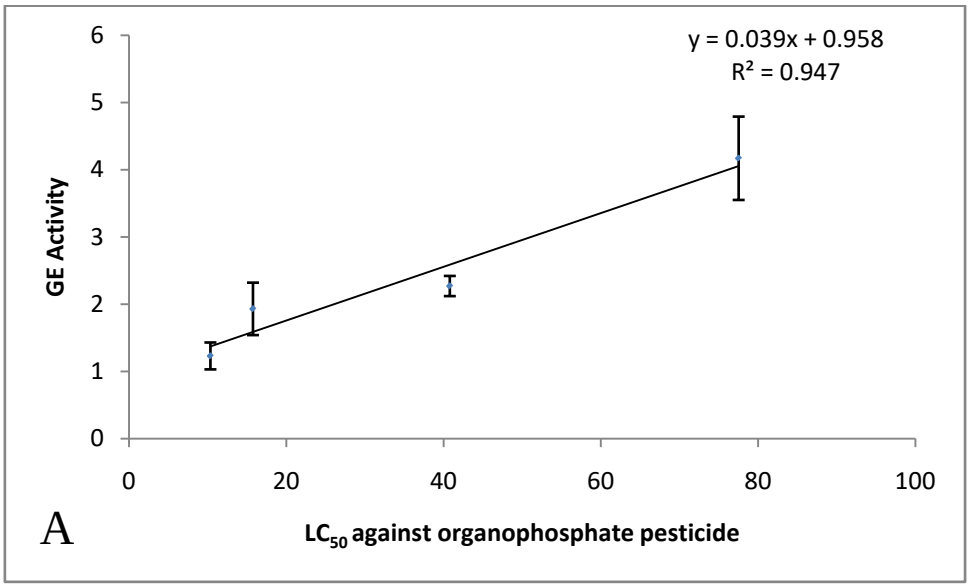
Regression curve for determining tolerance level of unknow population of *H. theivora* were prepared. The regression curves as standard were prepared based on the known tolerance levels (LC_{50}) of various population of *H. theivora* and the corresponding activity levels of the defence enzymes, GE and CYP450. To prepare the curves,

Table 4.1: Standard regression equations for determination of tolerance level (LC_{50}) of *Helopeltis theivora* based on two defence enzymes

Defence Enzymes	Organophosphate		Synthetic Pyrethroid		Expected LC_{50}
	Regression Equation	R^2	Regression Equation	R^2	
GE	Y=0.0399X+0.9582	0.94	Y=0.2560X-0.4887	0.97	Value of
CYP450	Y=0.0026X+0.0563	0.97	Y=0.0115X+0.0151	0.95	X

-X axis represents LC_{50}

-Y axis represents the activity of the defence enzyme



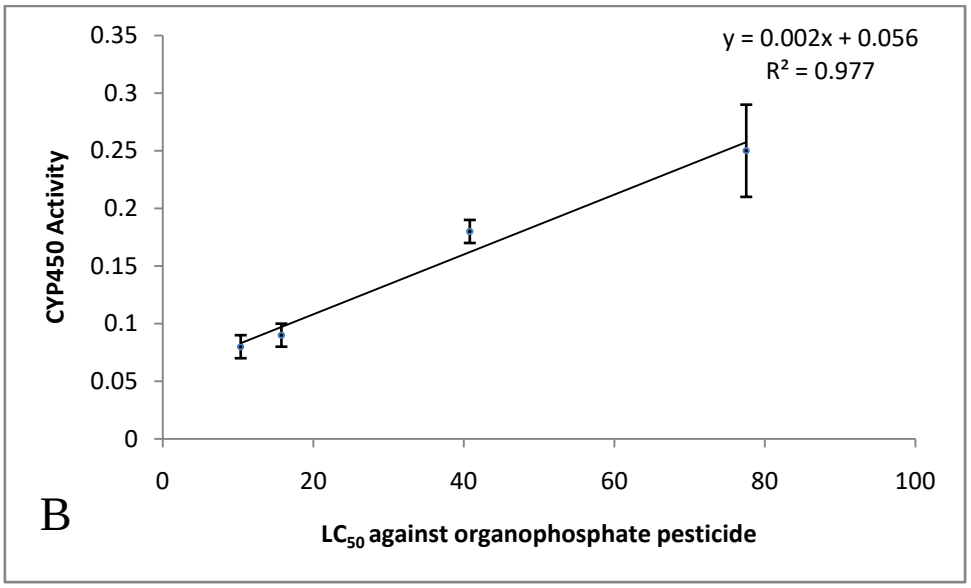


Figure 4.2: Curves based on detoxifying enzymes GE (A) and CYP450 (B), for determination of LC_{50} values against organophosphate in the field populations of *Helopeltis theivora*

-error bar represents *SD*

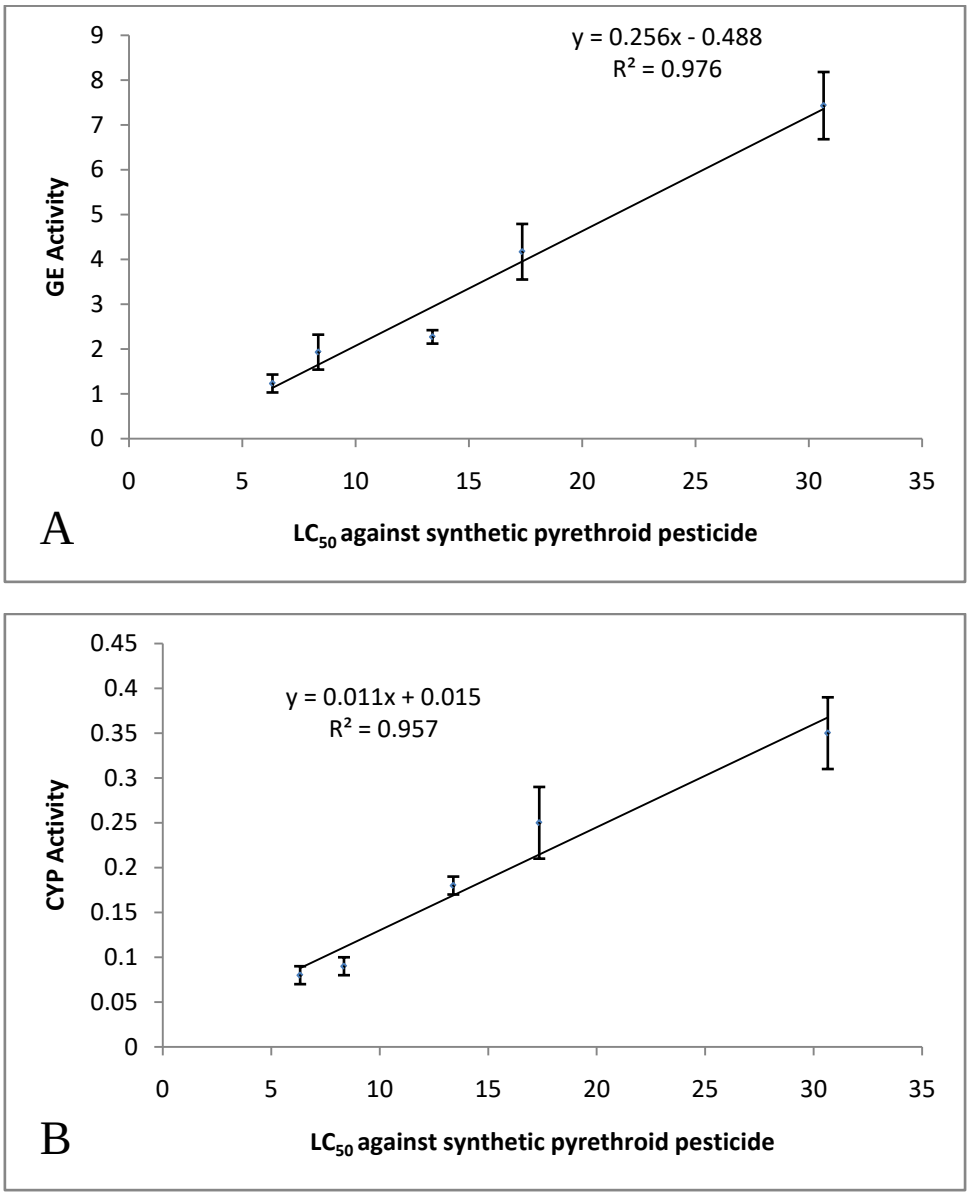


Figure 4.3: Curves based on detoxifying enzymes GE (A) and CYP450 (B), for determination of LC_{50} values against synthetic pyrethroid in the field populations of *Helopeltis theivora*

-error bar represents *SD*

tolerance levels in terms of LC_{50} values against the two commonly used pesticides belonging to organophosphate and synthetic pyrethroid were determined. The LC_{50} were then linearly regressed with the activities of the two above mentioned defence enzymes. X and Y axes of the standard curves represent the LC_{50} values and the activities of detoxifying enzyme, respectively. Figures 4.2A & B, and 4.3A & B shows the curves for determining tolerance level against organophosphate and synthetic pyrethroid pesticide, respectively. The activities of the detoxifying enzymes were assessed as per the methods described in sections 4.7.1 and 4.7.2. The regression equations and the R^2 values of the standard curves are shown in Table 4.1. Based on the standard curves prepared, the expected LC_{50} values of unknown populations of *H. theivora* were determined. The Chi-square test was performed between the expected and the observed LC_{50} values to assess the goodness of fit and the robustness of the curve for determination of LC_{50} values of unknown populations.

4.14 Study on the biology of lynx spider *Oxyopes javanus*

4.14.1 Rearing of *Oxyopes javanus*

Adults of the lynx spider, *Oxyopes javanus* were collected from the tea plantations in Terai in the northern part of West Bengal and reared in laboratory condition (at 25 ± 2^0 C; 85 – 90% RH; 12 L: 12 D photoperiod) individually in a jar to avoid any interferences like cannibalism. Depending upon the developmental stage of the spider, they were provided with nymphs and adults of *H. theivora* besides other insects found in tea plantations of Terai, as food. The rearing apparatus consisted of a wide mouth plastic jar lidded with a muslin cloth and tied by rubber bands. As suggested by Jackson (1974) a wet cotton plug was kept at the bottom of the jar as a source of water to the spider and also for maintaining moisture inside the jar.

4.14.2 Study on life history of *Oxyopes javanus*

A pair of mature male and female spider, *O. javanus* was kept in a jar for mating. Following successful mating, gravid females were separated from the males and reared individually to avoid any interference. Courtship and mating behaviour, oviposition pattern, fecundity, incubation period and life history were studied. Five replications for each of the experiments were conducted.

4.15 Predation efficacy of *Oxyopes javanus* on *Helopeltis theivora*

4.15.1 Functional Response

Tea twigs with 2 – 3 leaves and a bud were kept in an experimental arena, a large plastic jar as described above with sufficient space (10,000 cm^3) for free movement and activities of both prey and predator. Adults of *H. theivora* ranging from 1 to 15 per experimental arena were introduced, allowed 5 – 7 minutes for settling down followed by the release of either one male or female spider *O. javanus* to each of the arena. To determine the number of *H. theivora* predated by the spider, the numbers of survivors were counted in the arena after 24 hours. Based on the number of the survivors and carcasses left, the number of *H. theivora* predated by the spider was determined. The experiment was repeated for five times. Controls were maintained with similar parameters but with no spider predator.

4.15.2 Aggregative Response

The experimental setup was made similar to the one used for functional response study. Adequate number (40) of *H. theivora* adults were provided to both male and female *O. javanus*. After the introduction of *H. theivora* into the experimental arena, *O. javanus* ranging from 1 to 6 per arena were released. Either male or female but not the mixture of both to avoid any interference was released in the arena at a time. The number of live *H. theivora* was counted after 24 hours to determine their resultant death due to cooperative predation of the spider, *O. javanus*. Five replications of the experiment were carried out.

4.15.3 Prey handling and consumption time

The time required by the spider, *O. javanus* to capture, kill and consume prey is defined as 'prey handling time' T_H, which is calculated using disk equation by Holling (1959), explained in details under data analyses section. The prey consumption time i.e., the time period between the successful attack and the abandonment of the carcasses by the spider predator was recorded by using a stopwatch. For recording the prey consumption time, the experiment was carried out by introducing one *H. theivora* per arena per *O. javanus* in a similar setup as described in the section 4.15.2. The experiment was repeated for five times.

4.15.4 Data analyses

The type of functional response was determined by fitting the data in the Holling's disc equation, $N_A = \frac{a\,T_T\,N_o}{(1+aT_H N_0)}$, where N_A is the number of prey killed per spider predator, N_0 is the number of prey provided to the predator, T_T is the total time available for predation, which is equal to one day, T_H is the prey handling time and a is the searching efficiency of the predator (Holling, 1959). The searching efficiency a was calculated using a linear regression technique (Daniel, 1987) where reciprocal of N_A was regressed on the reciprocal of N_0. The reciprocal of the slope is the prey searching efficiency a and the intercept is the handling time T_H. The effectiveness of predation is determined by the ratio of a to T_H.

4.16 Statistical analyses

4.16.1 Probit analysis

To determine generation wise median lethal concentration (LC_{50}), mortality data were converted to percent mortality followed by probit analysis (Finney, 1971).

4.16.2 Preparation of standard curves and graphs

Standard curves of α-naphthol, cytochrome C, and BSA were plotted using Microsoft Excel 2007 (Microsoft Corporation, USA).

4.16.3 Statistical treatment of data

Data were checked for normality applying Shapiro-Wilk's test. Non-parametric tests such as Kruskal-Wallis and Wilcoxon Rank-Sum tests were applied when the data did not meet normality. Data of defence enzyme activities across generations were subjected to one-way analysis of variance (ANOVA) and posthoc Bonferroni separation test using a statistical package SPSS, version 20 (International Business Machine Corporation, USA). The activities of defence enzymes through generations were considered significantly different at $p \leq 0.05$. To test statistical significance between the mean predation potentials of male and female spider individually, independent samples *t*-test was performed using the statistical software SPSS, version 20.