4. Materials and Methods

4.1. Collection of pest specimens: *Buzura suppressaria*, *Eterusia magnifica*, *Helopeltis theivora* and *Oligonychus coffeae* from tea plantations

Periodic surveys were undertaken at different organic and conventional plantations of Terai, the Dooars and Darjeeling foothills located in the North Bengal, to name some of them are Singell Tea Estate (T.E.), Castleton T.E. of Darjeeling foothills, Matigara T.E., Maruti T.E., Kamalpur T.E., Chandmani T.E., Atal T.E., Gangaram T.E., Dagapur T.E. and North Bengal University campus plantation of Terai, and Debpara, Lakhipara, Dalgaon, Nagrakata and Nangdala T.E. of the Dooars. These TEs had mostly matured tea bushes of Tocklai vegetative clones that produced vigorous flush in Terai and the Dooars agro-climate. Four important tea pests of this region namely *Buzura (Biston) suppressaria* Guenee (Looper Caterpillar), *Eterusia magnifica* Butler (Red Slug Caterpillar), and Tea mosquito bug, *Helopeltis theivora* Waterhouse and Red spider mite, *Oligonychus coffeae* (Nietner) have been considered in the present study. Usually caterpillar stages and adults of loopers and red slug were collected (handpicked) from tea bushes and trunks of shade trees in the morning hours. Adult stages of tea mosquito bug and red spider mites were also collected in the morning and during dusk. The collected larvae and adults of sucking pests were brought to the laboratory in polythene packets and containers, along with the leafy twigs of tea. The specimens that were collected from conventional plantation, managed by regular spray of pesticides, have been referred to in the text as “pesticide-exposed” or “field-collected”, and the specimens maintained in laboratory without pesticide exposure have been mentioned in the text as “laboratory-reared”.

4.2. Laboratory rearing (culture) of the pests for experimentation

Natural food (tea leaves), which was provided for rearing and for various experiments was collected from the experimental tea plot of the Department of Zoology at North Bengal University campus. The plantation was about 14 years old. The Tocklai vegetative clones, TV 1, TV 18, TV 25 and TV 26. The experimental garden was mainained under usual cultural practices with organic manure and no pesticide application.

TV 1: It is one of the earliest clones released by 'Tocklai Experimental Station', Assam (India) in 1949. TV 1 is a standard clone, having high yield potential and high quality. It has a compact frame with acute branch angle (<50°). Leaves are erect, medium sized with pubescence on lower surface and sunken stomata. Surface matty in nature. Fairly draught tolerant. It is a hybrid of Assam × China origin.

TV 18: It is of Cambod hybrid origin. More or less of compact frame with glossy medium sized leaf. Leaf axil with an angle of 50° to 70°. It has a high yield potential but of average quality. Leaf has pinkish pigmentation in the petiole.

TV 25 and TV 26: These are high yield clones of Cambod type. Morphologically both the clones are similar in nature, having compact frame and glossy medium sized leaf. Both the clones are fairly drought tolerant having high yield potential but of average quality. Leaf axil being >70°.

Larvae of *B.suppressaria* and *Et.magnifica* were randomly collected from conventional plantations (maintained by synthetic pesticide spray) of eastern Dooars and western Terai and Darjeeling foothills of West Bengal State. These larvae were reared separately on the Tocklai vegetative clones, TV 1, TV 18 and TV 25, for two generations at 27 ± 2^0 C and 72 ± 2 % RH with a photoperiod of 12:12 hrs (L: D), in

transparent containers (30 x 30 cm) with a supply of fresh tea twigs, obtained from the experimental tea plot of Department.

Field populations of *Helopeltis theivora* were reared in laboratory on Tocklai vegetative clones (TV 1) for two generations at 25 ± 1^0 C; 85 ± 5 % RH with a photoperiod of 12:12 hrs (L: D) in transparent containers (20 x 20 cm) with a regular supply of fresh tea twigs.

Oligonychus coffeae populations were collected from organic and conventional tea plantations of Darjeeling foothills areas. These were maintained in laboratory on Tocklai vegetative clones (TV) at 25 ± 2^0 C with 70 ± 5 % RH.

4.3. Dissection of salivary glands, midgut and cerebral ganglia

The 5th instar larvae of *B.suppressaria* and *Et.magnifica* were under ice-cold dissection buffer in a paraffin tray. Incision was given along the mid dorsal line of the larva. Gut was also slit and the gut contents were removed by gentle stroke of brush. Fat bodies and food particles were scraped out. The salivary gland, midgut and cerebral ganglia were removed and were immediately preserved in ice-cold homogenization buffer (placed in an ice bath). In case of *Helopeltis*, the insects were placed at –20^0 C for 4 min and dissected in ice-cold phosphate-buffered saline (pH 7.2) under a dissecting microscope. The salivary gland complex, including all lobes, accessory glands and tubules was exposed by gently pulling the head and prothorax away from the abdomen with fine forceps. Subsequently the midgut and cerebral ganglia was removed by dissecting the body. The whole body of *Oligonychus coffeae* was taken for enzyme analysis, due to its minute size.

4.4. Biochemical analysis

4.4.1. Isolation of salivary and midgut enzymes of

4.4.1.1. *B.suppressaria*

Enzyme extraction was done from laboratory-reared 5th instar larvae of *B.suppressaria,* and from larvae of the same stage collected from natural populations occurring in conventionally managed plantations that were subjected to routine spraying of synthetic pesticide. Each larva was dissected and its salivary gland, midgut and cerebral ganglia were removed. Dissections were carried out in ice-cold sodium phosphate buffer, 0.1 M, pH 7.0 using sterilized scissors and needles. Salivary glands and midguts were homogenized separately in fresh sodium phosphate buffer containing 0.01 M each of EDTA (Ethylene Diamine Tetra Acetic Acid) and 0.5% Triton X-100. The volume of the buffer was adjusted to produce similar protein concentrations for the homogenates of each individual. The homogenate was centrifuged at 10,000 x g for 15 min at 4^0 C. The supernatant of this preparation was stored at –20^0 C for future use.

4.4.1.2. *Et.magnifica*

The 5th instar larvae of *Et.magnifica* were collected from laboratory colonies and from natural populations occurring in conventionally managed plantation (with synthetic pesticide spraying). Each larva was dissected and its salivary gland and midgut were collected. Dissections were carried out with the help of sterilized scissors and needle in ice-cold sodium phosphate buffer (0.1 M, pH 7.0). After removing the fat bodies and food particles from the midgut it was homogenized in fresh sodium phosphate buffer containing 0.01 M each of EDTA (Ethylene Diamine Tetra Acetic Acid) and 0.5% Triton X-100. Similarly the salivary gland was also processed. The volume of the buffer was adjusted to produce similar protein concentration for the homogenates

of each individual. Each homogenate was centrifuged at 10,000 x g for 15 min at 4^0C and the supernatant of this preparation was stored at -20^0 C for future use.

4.4.1.3. *H.theivora*

Adults were used for enzyme extraction following slightly modified method of Cohen (1993). The insects were placed for immobilization at -20^0 C for 4 min and dissected in ice-cold phosphate buffer (pH 7.2) under dissecting microscope. The salivary gland complex, including all lobes, accessory glands and tubules was exposed by gently pulling the head and prothorax away from the abdomen with fine forceps. Subsequently the midgut and cerebral ganglia were removed by dissecting the body. The salivary glands of 10 insects were removed, placed in 1 ml of phosphate buffer, homogenized and centrifuged at 12, 000 x g for 10 min at 4^0 C. The supernatant was placed in a 1.5 ml centrifuge tube and kept at -20^0 C for use (within 48 h). The midgut and cerebral ganglia of the same 10 insects were homogenized and processed in the same way as the salivary glands. The enzyme extraction was done from three separate batches of insects collected from different conventional plantations and laboratory rearing.

4.4.1.4. *O.coffeae* (whole body)

Oligonychus coffeae were colleted by using camel hair brush from the surface of the tea leaves and about 100 mg of its fresh weight were placed in a homogenizer in 1 ml of phosphate buffer. This was then homogenized and centrifuged at 15, 000 x g for 10 min at 4^0 C. The supernatant was placed in a 1.5 ml centrifuge tube and kept at -20^0 C for use (within 48 h).

4.4.2. Gel diffusion assay of salivary, midgut digestive enzymes (amylase, protease) of

4.4.2.1. *B.suppressaria*

The 5th instar larvae of *B.suppressaria* was dissected out with the help of sterilized scissors and needle in ice-cold sodium phosphate buffer (0.1 M, pH 7.0). Each pair of glands and midgut was homogenized in a glass tissue grinder separately. The homogenate was then centrifuged at 10,000 x g for 5 minutes at 4^{0}C. Aliquots of supernatants were used for tests of different digestive enzymes.

Amylase

Amylase was measured by modified Somogyi (1960) method. A solution of starch (1gm / litre) in phosphate buffer (pH 7.0) was mixed with 0.5 % agar (0.005 gm/ 10ml) and heated for 10 mins in a boiling water bath. The slurry was poured into pettry plates, and well of 2.5 mm diameter was cut into the gel. 10 µl aliquots of enzyme extract was dispensed in each well. A postitive control of commercial amylase (0.001g/ml) was used concurrently. Plates were kept at 30^{0}C for 20 hrs and developed with 0.002 N iodine in 2 % potassium iodide for 3 min. Rings of clear area (against a distinctly blue background) were considered as positive.

Protease

Proteases were determined by casein–gel method of Bjerrum (1975). 10 µl of both salivary and midgut homogenate were separately dispensed in wells of 2.5 mm diameter cut in the gel on a pettry plate. The gel was composed of 1% casein mixed with 0.5 % agar, buffered to 7.8 with phosphate. The mixture was heated for 10 mins in a boiling water bath for gelling to take place. Sterilized double distilled water with commercial protease (0.001g/ml) used as positive control. Plates were then covered with 3% acetic acid solution for 10 mins to stop the reaction and rings of diffusion

were observed for enzyme activity. Positive tests were those in which clear rings appeared against a white background.

4.4.2.2. *Et.magnifica*

Same procedure as *B.suppressaria* was followed for detecting amylase and protease of salivary and gut enzymes of *Et.magnifica.*

4.4.2.3. *H.theivora*

In case of *Helopeltis*, the salivary glands and midgut of 10 insects were removed and placed in 1 ml of phosphate buffer, homogenized and centrifuged at 12, 000 X g for 10 min at 4^0 C. The supernatant was placed in a 1.5 ml centrifuge tube and kept at -20^0 C for future testing. Same procedure as described for *B.suppressaria* was followed to test the amylase and protease of the salivary and gut of *H.theivora.*

4.4.2.4. *O.coffeae* (whole body)

100 mg of fresh weight of *O.coffeae* was placed in 1 ml of phosphate buffer. This was then homogenized and centrifuged at 15, 000 x g for 10 min at 4^0 C. The supernatant was placed in a 1.5 ml centrifuge tube and kept at -20^0 C for use (within 48 h). Same procedure as described for *B.suppressaria* was followed for conducting the tests for amylase and protease enzymes present in *O.coffeae.*

4.4.3. Quantitative assay of digestive enzymes

4.4.3.1. Amylase

Amylase activity in the salivary gland and midgut was determined after the method of Madhusudhan *et al.* (1994) combined with the method of Sadasivam and Manickam (1996) using dinitrosalicylic acid reagent. Quantification of enzyme product was deduced from a standard curve prepared using various concentration of maltose at 520 nm using UV-Vis spectrophotometer. The enzyme activity was expressed as µM. mg protein $^{-1}$. min $^{-1}$.

4.4.3.2. Protease

Proteolytic activity was assayed after methods of Kunitz (1947) subsequently modified by Jayaraman (1981). 1% (w/v) casein was used as the substrate. 1 ml of casein prepared in 0.1 N NaOH was incubated with equal volume of enzyme. After incubation for one hour, the reaction was terminated by the addition of 10% TCA and the acid-soluble peptides were quantified using the biuret reagent at 520 nm using UV-Vis spectrophotometer. The enzyme activity was expressed as µg / mg of protein.

4.4.3.3. Lipase

Lipase activity was measured following the method of Sadasivam and Manickam (1996). The enzyme activity was calculated as milliequivalent (meq) activity of free fatty acid / min/ g sample.

4.4.4. Assay of oxidoreductases

4.4.4.1. Catalase

3 ml of Hydrogen peroxide and phosphate buffer was allowed to stabilize at 25 ± 2 ^{0} C for 10 min. After incubation period, 0.2 ml of salivary gland and midgut

homogenate was separately added to cuvettes, and the change in absorbance was measured at 240 nm over a 10 sec period upto 4 min using UV-Vis spectrophotometer. Catalase activity was expressed as decrease in OD of hydrogen peroxide / min / mg of protein (Laurema and Varis, 1991).

4.4.4.2. Peroxidase

Peroxidase activity was measured by monitoring the increase in absorbance (OD) by 0.1 and noting the time required (in min) by using the slightly modified method prescribed by Hampton (1963). The enzyme reaction was started by adding 3 ml of 0.1 M phosphate buffer (pH 7.0), 0.05 ml guiacol solution, 0.1 ml enzyme extract and 0.03 ml hydrogen peroxide in a cuvette. The activity of peroxidase was determined by increase in OD / min / mg of protein at 436 nm.

4.4.4.3. Polyphenol-oxidase

Polyphenol-oxidase activity was estimated after the method of Hampton (1963) at 495 nm for 4 min after the start of the reaction. The reaction was initiated by adding 2.5 ml of phosphate buffer (pH 6.5) and 0.3 ml of catechol solution (0.01M) and then 0.2 ml of enzyme extract. Enzyme activity was calculated as the increase in the absorbance/ min / mg of protein.

4.4.5. Quantitative assay of detoxifying enzymes

4.4.5.1. General esterases

The esterase activity was determined according to the procedure of van Asperen (1962). 1 ml of tissue supernatant (salivary glands or midgut homogenate) was mixed with 5 ml of substrate solution, 3.0×10^{-4} M α-napthyl acetate [in 0.04 M, phosphate

buffer, pH 7.0, containing 1% (v/v) acetone]. After 20 min of incubation at 30^0 C with shaking, 1ml of a freshly prepared solution of the dye, containing two parts of 1% (w/v) diazo dye and five parts (w/v) of sodium laurylsulphate was added to the incubated mixture. A red colour immediately developed that quickly changed into a fairly stable blue colour, which was measured at 590 nm spectrophotometrically. The quantity of napthol produced was determined from the standard curve of α-napthol. The nonenzymatic reaction (control samples) was accounted for by mixing 1 ml of buffer with the substrate solution and incubating as above. All samples and controls were read against the blanks. The experiments were repeated five times.

4.4.5.2. Glutathione *S*-transferases

GST activity was measured according to Habig *et al.* (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB). The reaction mixture, consisting of 50µl of 50mM CDNB and 150 µl 50 mM reduced glutathione, was added to 2.77 ml phosphate buffer (100mM, pH 6.5). The enzyme solution (30 µl) was then added to the above mixture. The content was shaken gently and incubated at 25^0 C for 2-3 minutes. A blank was run concurrently in the reference slot of the spectrophotometer. Absorbance was recoded for 6-7 minutes at 340 nm ($\varepsilon_{340} = 9.6$ mM^{-1} cm^{-1}). The increase in absorbance over 5 minutes period was considered for calculation. The GST activity assay was replicated five times.

4.4.5.3. Acetylcholinesterases

Acetylcholinesterase activity was determined by the method of Ellman *et al.* (1961) with some modification. 100 µl of the enzyme solution was added to a test tube containing 2.86 ml of 0.1 M sodium phosphate buffer (pH 7.5) and the mixture was then incubated at room temperature for 5 min. To this 10 µl of 0.01 M 5,5' – dithiobis

(2-nitrobenzoic acid) (DTNB) was mixed. After 10 min incubation of the above mixture 30 μi of the acetyithiochoiine iodide in phosphate buffer was added and the change in absorbance was determined at 412 nm. The change in absorbance was taken every 1 min for a period of 12 min. The increase in absorbance over 5 minutes period was considered for calculation. The test was replicated five times.

The protein concentrations of the all the above tissue supernatants were determined by the method of Lowry *et al.* (1951). Bovine serum albumin was used as standard.

4.4.6. Qualitative analysis of detoxifying enzymes of *B.suppressaria*, *Et.magnifica*, *H.theivora* and *O.coffeae* collected from pesticide-exposed (conventional plantation) and un-exposed (laboratory-reared) populations by Native Polyacrylamide Gel Electrophoresis

4.4.6.1. Esterase isozymes of salivary, midgut, or whole body extracts

Native discontinuous polyacrylamide gel electrophoresis (5% stacking and 8% separating gels) was carried out. A continuous buffer system (Tris-glycine, pH 8.3) was used. Electrophoresis was conducted at 4^0 C with 10mA for 1.5 hrs (Davis, 1964) using vertical gel apparatus with power pack (Biotech make).

The electrophoresed gel was stained for esterase according to Murphy *et al.* (1996). The gel was first pre-incubated in 100 ml of phosphate buffer (40 mM, pH 6.5) containing 0.02% α-napthyl acetate (0.02 gm of α-napthyl acetate in 2 ml of acetone) and the gel was then transferred to 100 ml of sodium phosphate buffer (40 mM, pH 6.5). After the pre-incubation step, 2.5% (w/v) Fast Blue BB salt was added. The gel was incubated in dark at room temperature for 20-30 mins with occasional shaking. The reaction was stopped after 15-20 minutes of incubation in distilled water.The gel

was then fixed in a fixative solution (glacial acetic acid: methanol: water = 1: 2: 7) for 30 min. The fixed gel was stored in 10% glycerol solution. The relative migration of esterase bands in the zymograms was determined by the Kodak digital science 1D Image Analysis Software, version 2.0.3. Relative mobility (R_m) was calculated as: distance migrated by the specific bands (cm) / distance migrated by the marker dye (cm).

4.4.6.2. Isozymes of Glutathione *S*-transferase

The electrophoresed gel was stained according to Manchenko (1994) for GST. After electrophoresis, gels were preincubated in 20 ml of 4.5 mM reduced glutathione, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and 1 mM nitro blue tetrazolium (NBT) in 0.1 M phosphate buffer (pH 6.5). The pre-incubations were carried out at 37^0C for 10 min with gentle shaking. The gel was then transferred to a second solution and incubated for a period from 3 to 5 min with periodic agitation. The second solution consisted of Tris-HCl (pH 9.6) and 3 mM phenazine methosulphate (PMS). Blue insoluble formazan appeared on the gel surface, except in the areas with glutathione S-transferase activity. Because the superoxide dismutase also caused tetrazolium salt (i.e., NBT) reduction, a control gel was simultaneously processed with all reagents except CDNB to show the superoxide dismutase activities. Thus, glutathione S-transferase activity could be identified by comparing the control (without CDNB) and test (with CDNB) gels. The negatively stained gels were then photographed and the relative migration of glutathione *S*-transferase bands in the zymograms was determined by the Kodak digital science 1D Image Analysis Software, version 2.0.3. Relative mobility (R_m) was calculated as: distance migrated by the specific bands (cm) / distance migrated by the marker dye (cm).

4.4.6.3. Acetylcholinesterase isozymes

Native polyacrylamide gel electrophoresis was performed in a vertical electrophoresis unit by using 8% separating and 5% stacking gel with a discontinuous Tris-glycine buffer system. 15 µl of sample homogenate prepared from head region (brain) of the pests was loaded in each lane. Acetylcholiesterase activity was marked according to the method of Lewis and Shute (1966). The gel was preincubated with a mixture of 65 mi 0.1 M sodium phosphate buffer (pH 6.0) and 0.05 g acetyl thiocholine iodide. Then 5 ml 0.1 M sodium citrate was added to the gel buffer and the same was shake well. After that 10 ml of 30 mM copper sulphate ($CuSO_4$) was added. Finally 10 ml 5 mM potassium ferricyanide was added in the reaction mixture and was shaken well. The incubation was completed when the background of the gel turned a yellowish brown. After incubation, the gel was washed for 1 hr with three changes of distilled water and then the gels were photographed and the relative migration of acetylcholinesterase bands in the zymograms was determined by the Kodak digital science 1D Image Analysis Software, version 2.0.3. Relative mobility (R_m) was calculated as: distance migrated by the specific bands (cm) / distance migrated by the marker dye (cm).

4.4.6.4. Inhibition tests of general esterases and acetylcholinesterases of the pests

Electrophoregrams of general esterase and aceylcholinesterase were prepared after Davis (1964) from the midgut, cerebral ganglia or whole body homogenates. After electrophoresis the same gel slab was vertically divided into two parts for the control and for inhibition tests.

For inhibition tests of esterases and acetylcholiesterases, the gels were treated with substances regarded as inhibitors (Holmes and Masters, 1967). The inhibitors used were insecticides commonly used against tea pests i.e. organophosphate,

Quinalphos (Ekalux 25EC) (1:400) (v/v). i.e. 0.1 ml in 40 ml of water. In these tests, electrophoresed polyacrylamide gels were preincubated in dark at room temperature for 30 minutes in sodium phosphate solution containing the inhibitor and then treated with the staining solution (which also contained the inhibitor). The other half of the same gel was treated with staining solution without any inhibitor. Using these tests, the isozyme bands of esterases and acetylcholinesterases of susceptible got blocked by the pesticides and could not be visualized in the stained and differentiated gel slabs.

4.5. Statistical analysis and computer application

Documentation and analysis of zymograms were done by the software package of Kodak digital science 1D Image Analysis version 2.0.3. Relative mobility (R_m) was calculated by: distance migrated by the specific bands (cm) / distance migrated by the marker dye. Smith's statistical package was used to analyze the data where necessary. Excel and photoshop programmes were used for preparing tables and shaping photographs. The details of statistical analysis of the data have been mentioned under results and discussion.