Materials And Methods

IV. MATERIALS AND METHODS

4.1.SELECTION OF SITES AND PLANTING MATERIALS

Two sites were selected at mid (TRA, Clonal proving Station, Ging at 1200 m amsl) and high (Sungma tea estate at 1650 m amsl) elevations for the study, as difference in elevation has great influence in tea cultivation in Darjeeling. The experimental areas are located at 27^0-17/ N Latitude and 88^0-19/ E Longitude.

Two types of planting materials selected for the study were :

1. The common china seed *jat* (Fig. 7A)

2. A popular china hybrid clone – Tukdah 78 (Fig. 7 B).

The characteristics of both the materials under study are presented in table 3.

Table 3 : Characteristics of the planting materials (Singh, 1989)

Name	Genotype	Leaf type	Frame	Yield	Flavour
China seed jat	seed	Erect , small	Compact, multistemmed	Low	Very good
TRA/ Tukdah 78	Clone	Erect, medium	Widely spreading	High	Good

Fig. 7A: A bush of China seed jat

a ⟶ small size leaves
b ⟶ compact frame with multistemmed collar

Fig. 7B: A bush of T 78 clone

a ⟶ medium size leaves
b ⟶ widely spreading frame with single stemmed collar

4.2. POPULATION DYNAMICS

Initial attempt was made to study the population dynamics of four major sucking insect-pests of tea, namely Common thrips, greenfly, aphid and tea mosquito bug active in Darjeeling tea. But infestation of tea mosquito bug was not noticed in any of the two sites selected at mid and high elevations during any of the three seasons under study. Hence, later on the study was confined to three other species namely common thrips [*Mycterothrips setiventris*, Bagnal, (Thripidae: Thysanoptera)], greenfly [*Empoasca flavescens* Fabricius (Cicadellidae: Homoptera) and aphid [*Toxoptera aurantii* Boyer de Fons (Aphididae: Homoptera)] excluding tea mosquito bug.

4.2.1. IDENTIFICATION OF THRIPS SPECIES

At the beginning of the study on population dynamics, it appeared that more than one thrips species were associated with tea in Darjeeling. Hence, it became essential to identify the actual thrips species involved as the major pest of tea as an off shoot study. As all the thrips species were very minute soft bodied insect with an adult size of around 1.25 – 1.5 cm long, the specimens had to be mounted in slides to send for identification. The following procedure for processing the specimen was adopted :

1. Specimens of different thrips species encountered during initial observations in the field were collected and preserved in 70 % ethanol.

2. The specimens to be mounted were taken out from the preservative and put in 5 % KOH for 3-4 hours to make them soft and transparent by

dechitinization.

3. From KOH solution they were put in water for 10-15 minutes to remove KOH.

4. From water the specimens were put in 70 % alcohol for 15 minutes, then in 90 % alcohol for 15 minutes and finally in 100 % alcohol for 30 minutes.

5. From 100 % alcohol specimens were put in xylol for 15–20 minutes. Thereafter, they were checked whether they became transparent or not, if not, they were put back in 100 % alcohol and then Xylol again to make them transparent up to the desired level.

6. The above dehydrated specimen was put on a DPX drop singly on a clean glass slide. Antennae, wings, legs, and other structures of the specimens were oriented in proper position using needle under a binocular microscope (Wild M3). The DPX drop (with the specimen) on the glass slide was covered gently with a cover slip.

7. The ready slides with the specimen were dried adequately in oven and then used for taxonomic study and sending to the experts for identification.

The thrips species infesting tea shoot was identified by expert as *Mycterothrips setiventris* (Bagnall) and so the present study was confined to this species of thrips.

4.2.2. LAYOUT OF THE EXPERIMENTAL PLOTS AND SAMPLING METHODS

To study population dynamics, the field experiment was laid out in randomized block design with three replications. For this purpose, three plots having 100 tea bushes in each plot were randomly selected for both the

planting materials at both the sites. These plots were kept totally out of any pesticide treatment to study the population dynamics under natural condition. A similar set of plots was also selected 50 m away from the first set to study the population dynamics under pest control measures. Insecticide (Endosulfan 35 EC) was applied in these plots as soon as infestation was noticed as per garden practice.

The plots were maintained under unpruned conditions (no pruning or skiffing was done at the end of the season) and normal weekly plucking was done after collection of samples in each week. Other cultural practices like manuring, weed control etc. were followed as per garden practices recommenced by Tea Research Association.

Weekly observations were taken on the population of the three sucking insect-pests under study on insecticide treated and untreated plots separately for 3 consecutive years from 1999 to 2001. Initially the sampling method as defined by Le Pelley (1942) for thrips and other small insects on the coffee leaves was tried. But it was found to be cumbersome, time consuming and ineffective. Subsequently the following method was followed :

Since all the three sucking pests were chiefly associated with the growing shoots on the plucking table of the bushes, 10 growing shoots (Bud with 2 leaves below) were plucked randomly in each plot, replicated thrice, in each weekly observation. Each shoot immediately after plucking was put in a small polythene packet. The open end of the packet was tied with a rubber band immediately after putting the shoot inside. The packets with shoots were

carried to the laboratory of Tea Research Association, Darjeeling. Then each shoot was examined for the 3 sucking insects under study. The total number of adults and nymphs were counted for each species and tabulated for future analysis. Dissecting binocular microscope and hand lens were used to count the small immature ones. Similar sampling methods have been used in study of population abundance of insect pests by Sasidhar *et al.* (1999), Sannigrahi and Mukhopadhyaya (1993) and Atwal and Singh (1989).

4.3. ALTERNATIVE HOST

To study the alternative hosts of the above three pest species, regular observations were done on the weeds and tree species in and around the tea estates of Darjeeling. The plant species found infested by any of the pests under study were recorded.

4.4. COLONIZING AND FEEDING IMPACT

Investigation on the feeding impact of common thrips and greenfly on made tea quality was conducted at mid-elevation with the facilities available at the laboratory-cum miniature manufacturing unit of Clonal Proving Station (CPS) of Tea Research Association (TRA) at Ging, Darjeeling.

4.4.1. PREPARATION OF MADE TEA SAMPLES

Made tea samples were prepared at TRA's Miniature Factory at CPS

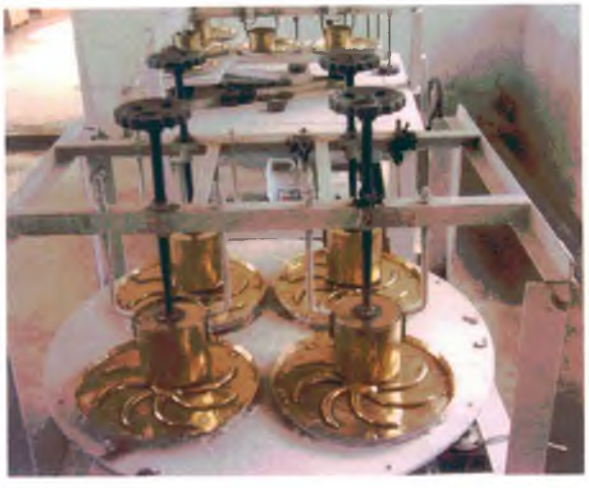
Ging (Fig. 8) during peak infestation period of the pests in the year 2002. Thrips and greenfly infested shoots of top bud and two leaves below under natural conditions in the field were plucked separately for each of these sucking pest (exclusively) with extreme care for both the planting materials. Around 600 gm green leaf was plucked for each sample. Same quantity of infestation free shoots was also plucked from the insecticide treated plots separately for each planting material and each sample under identical field conditions as control for comparison. In total, there were 4 replications of infested and uninfersted leaves each for greenflies and common thrips on both the planting materials.

In each set of manufacture, there were three replications of 200 g green leaf for each sample of 600 gm green leaf to avoid heterogeneity in manufacture. After manufacture the made tea of all the replications of each sample was mixed together and considered as one composite sample. In total 130-135 gm made tea was prepared from one green leaf sample of 600 gm with around 22-22.5 % recovery. Procedure followed to manufacture the made tea samples is given hereunder.

4.4.1.1. Withering

It was the first step of manufacture. The freshly plucked shoots were kept for 12-16 hours spread uniformly on withering trough to remove 65 – 68 % moisture from the leaves *i.e.* 100 g green leaf is reduced to 35 to 32 g withered leaf.

Withering trough



Rolling tables

Fermentation

Dryer

Fig. 8 : Miniature manufacturing factory at TRA, CPS, Ging

4.4.1.2. Rolling

In this step the cell walls were ruptured to mix polyphenol and polyphenol oxidase which were located separately in a tea leaf cell. The withered tea shoots were rolled in a miniature roller for 35 to 50 m by application of light to medium pressure.

4.4.1.3. Fermentation

In true sense it was not fermentation as no microorganism was involved, but an oxidation reaction. In this step, the oxidation reaction was completed, through conversion of polyphenol to secondary metabolites namely theaflavin (TF) and thearubigin (TR) in presence of the enzyme polyphenol oxidase, which worked as catalyst. The rolled leaves were kept spread on aluminium trays in a cool humidified room. The process started right from beginning of rolling to charging of leaf in dryer. Depending on room temperature and humidity the time required to complete fermentation was 1.5 – 3 hours.

4.4.1.4. Drying or firing

As soon as fermentation was completed the materials were dried in trays separately in small dryer maintaining an inlet temperature of 115 ^{0}C to 120 ^{0}C. The outlet temperature was 20-25 ^{0}C lesser than inlet temperature and time requirement was 20-22 minutes. The moisture content was reduced to 3-4 %. The dried leaves were called dryer mouth samples.

The tea leaf samples prepared by the above procedure were divided into two parts, one part was utilised for organoleptic taste and other for Biochemical analysis at the laboratory of Biochemistry Department, Tocklai Experimental Station (TRA), Jorhat, Assam.

4.4.2. ORGANOLEPTIC TASTE

One part of the made tea (30 g) of each sample prepared by the above method was utilized for traditional organoleptic taste (tasting by mouth). It was divided into 3 equal parts of 10 gm each and sent to three different tasters of Darjeeling for organoleptic taste with four replications. Mainly flavour, liquor, and quality of made tea were tasted to see the difference between uninfetsed and infested shoots by thrips and green fly. The volatile flavour constituents (VFC) present in made tea are known to play a crucial role in the organoleptic evaluation.

Flavour: This is the most important parameter in case of Darjeeling tea. Flavour is defined as a desirable and most apparent aroma in tea liquor perceived through the mouth and distinguished through the nose. All teas have flavour, but it varies depending on the planting material, location, plucking standards, processing procedure, pruning cycle etc. Darjeeling tea has typical pronounced rosy or muscatel flavour, which is the main characteristic of Darjeeling tea. It is more in china type (*Camellia sinensis* var. *sinensis*) and China hybrid tea than Assam type (*Camellia sinensis* var. *assamica*) tea. Higher the elevation of tea area more is the flavour in general.

Liquor: The following terms are used to express overall characteristics of tea liquor :- bakey, body, brisk, bright, character, coarse, colour, common, cream, dull, dry, flat, flavour, full, harshness, hay, heavy, high-fired, hungry, light, malty, neutral, nose, plain, pungent, quality, soft, sour, stewed, strength, thick, thin and weathery.

Quality: Quality is defined as the essential characteristic of a good tea. Subjectively "quality" is a versatile term and generally refers to consumers overall acceptability of a type of tea brew. The consumers acceptability of a tea drink can in ultimate analysis be attributed to biochemical constituent in the shoot, as well as the finished product as formed during processing through interactions with various enzymes which results in the development of desirable aroma and gradual loss of greenness (Hazarika *et al.*, 1984)

4.4.3. BIOCHEMICAL INVESTIGATIONS

One part of each made tea sample (100 gm) was utilized for biochemical analysis at Biochemistry laboratory, Tea Research Association; Tocklai. Biochemical investigations were made to see the variation in different biochemical parameters in made tea manufactured from thrips and jassid infested and uninfested tea shoots at the laboratory of Tocklai Experimental Station, Tea Research Association, Jorhat, Assam. The residual catechins, caffeine and volatile flavour compounds in made tea were estimated using HPLC and GC as per the procedure laid down below.

4.4.3.1. Estimation of individual catechin and caffeine – Method using HPLC (ISO Method)

Catechins and caffeine were extracted by refluxing 0.2 g of test sample with 70% (v/v) methanol at 70 ^{0}C. The extract was five times diluted with stabilizing agent containg 1 % acetonitrile, 0.02 % EDTA & 0.02 % l-ascorbic acid. Individual catechin and caffeine were estimated by injecting 10 µl of diluted test solution in HPLC using Luna-5µm phenyl hexyl column by HPLC. Mobile phases were : a) 2% Acetic acid, 9% Acetonitrile and b) 80% Acetonitrile.

Flow rate: 1 ml/min.

Detector: UV detector set at 278 nm.

Binary gradient conditions were 100 % Mobile Phase for 10 min, then over 15 min a linear gradient of 68 % of Mobile Phase A and 32 % of Mobile Phase B and held at this composition for 10 min.

Amount of Individual Catechins and caffeine were determined by comparing the areas given by the test sample with the areas given by individual standard Catechins under similar chromatographic conditions.

4.4.3.2. Estimation of Volatile Flavour Constituents (VFC) Method using GC

With the use of modern analytical technique like gas chromatography (GC) it is possible to detect VFC up to less than one million of a gram (10^{-6}g), which is helping enormously in understanding the role of VFC in tea quality (Mahanta and Hazarika, 1985). The following procedure was followed to study the VFC in orthodox black tea made from pest infested and uninfested tea shoots.

Fifty grams of made tea was steam distilled for 45 min in Steam Distillation Apparatus. The distillate obtained was transferred into a Separating Funnel and saturated it with NaCl. The distillate was extracted for 3-4 times with 50 ml of each of methylene chloride. The organic layer (150-200 ml) that contains VFC is concentrated under reduced pressure at 40 ^{0}C. The concentrated organic layer is transferred to a graduated eppendrops tube and further concentrated to 100 µl by purging N_2 gas.

Protocol for Extraction and Estimation of VFC by GC : The concentrate from the above were analysed for VFC profile by Varian GC, Model 3800 with FID. The instrument condition is given below:

Detector:	FID
Column:	CP Wax 52, 50m x 0.32mm and 0.22µ film thickness.
Split:	1 : 100
Inject Volume:	2 µi

Oven Temp. Program:	50 ^{0}C hold for 15 min, 2 ^{0}C /min up to 220 ^{0}C, hold for 20 min with a total runtime of 120 min.
Detector Temp.:	250 ^{0}C
Injector Temp.:	220 ^{0}C
Carrier gas flow:	1.2 ml/min & make up 30ml/min.
Air flow rate:	300 ml/min
H_2 flow rate:	30 ml/min

4.5. NATURAL ENEMIES

4.5.1. SURVEY BY SUCTION MACHINE

To study the natural enemies samplings were done during 2001 using "*D-Vac vacuum insect net*" in different tea estates of Darjeeling when the pests under study were active. "D-*Vac vacuum insect net*" is a petrol-operated machine, which works on vacuum suction principle. While in operation, air was sucked through a large flexible rubber hose of 20 cm diameter with a mouth of 33 cm diameter fitted with a nylon cloth bag. The mouth of the hose was placed on the tea bushes for few minutes and small organisms including mobile insects were sucked in along with the airflow. The insects and other organisms with dried leaves, twigs etc were collected in the nylon bag.

In total 5 bio-organic and 5 conventional tea estates were covered at different valleys to study the difference in their population between gardens

under bio-organic and conventional farming system. In each estate, 3 samples (replications) were taken at mid elevation at 1200 m above mean sea level (amsi) and 3 at high elevation at 1650 amsi to know the difference in population of natural enemies at different elevations. Each sampling was done for 15 minutes. Everything collected in nylon bag during each catch was transferred to a polythene bag of 30 cm X 60 cm size. All the catches were taken to TRA Darjeeling and kept in deep freeze for 2 to 3 hrs to kill the living organisms. Then numbers of different species observed were noted and sent for identification where necessary.

The data was subjected to "Student t " test whenever necessary to see if the difference in their population mean was statistically significant or not.

4.5.2. SURVEY BY MANUAL SEARCH

Intensive manual search was also carried out to observe the natural enemies not collected by D-Vac insect net. The sucking insects were collected at their peak level of infestation along with all other organisms associated with them in polythene bag followed by observation in the laboratory.

4.5.3. BIOLOGICAL NOTE AND IDENTIFICATION

Different stages of the natural enemies encountered in the field were collected and reared up to possible extent in the laboratory of tea Research Association, Darjeeling to make observations on the morphology of different stages, feeding capacity, style of feeding and for identification.

4.6. METEOROLOGICAL DATA

The meteorological data were collected from the nearest weather recording facilities at both the sites, and these were correlated with the population incidence of different pests. At mid elevation (TRA-CPS, Ging) meteorological data on rainfall, maximum temperature, minimum temperature, relative humidity in the morning, relative humidity in the afternoon and sunshine hours were available for the study. But, at high elevation (Sungma tea estate) met data were available only on rainfall, maximum temperature and minimum temperature.

4.7. LABORATORY USED

The laboratories of Zoology Department, University of North Bengal; Tea Research Association (TRA), Darjeeling Branch, and Biochemistry Department, Tocklai Experimental Station, TRA, Jorhat were used for different purpose during the course of study.

4.8. IDENTIFICATION OF SPECIES

Identification of insect-pests, parasites and predators collected for the present study were done using literature available at the laboratories. In cases of difficulties, preserved specimens were sent to different experts for identification as far as possible.

4.9. PHOTOGRAPH

Close up as well as microscopic photographs were taken where necessary for documentation using Ashai Pentax SLR camera and micro photo binocular attachment.

4.10. STATISTICS USED

Statistical analysis was done following the guidelines from Gomez and Gomez (1984). Two online statistical packages, "Analyse-it" and "Smith's statistical package" were used to analyse the data, where necessary. The details of statistical analysis done were explained under results and discussion of each item.