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Materials & methods

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A. Survey of major lepidopteran tea pests: a study on the occurrence of bacterial infection:

A.a Tea Estates/plantations surveyed for tea pest

The main tea plantation areas surveyed for collecting lepidopteran specimens were Terai and the Dooars region of Darjeeling foothills and adjoining plains. Samples were collected from East, West, South, North and Central areas of Terai and the Dooars. The name of the main tea estates surveyed in Terai were (Fig. 9, Fig. 10):

I. Terai region

a) Central: Kamalpur T.E., Panighata T.E., Bengdubi T.E.

b) Southern: Bidhannagar T.E., Hashkhoya T.E., Gungaram T.E.

c) Western: Sanyashi T.E., Atol T.E., Batashi T.E. Maruti T.E.

d) Northern: Matigara T.E., Nischintapur T.E., Dagapur T.E. Simulbari T.E.

II. The Dooars region

a) Eastern: Bhatkhawa, Chuapara, Kumargram and Sankosh T.E.

b) Central: Binnaguri, Mujnai and Hantapara T.E.

c) Northern: Jiti and Nagrakata T.E.

Major lepidopteran tea pests were collected from these tea estates. Infected state of larvae and cadavers were also collected. Collected active larvae were reared in the laboratory condition for observation. The larvae dying in laboratory reared population were also studied for estimating portion of natural population dying out of bacterial infection and also for isolation of the causative entomopathogens.

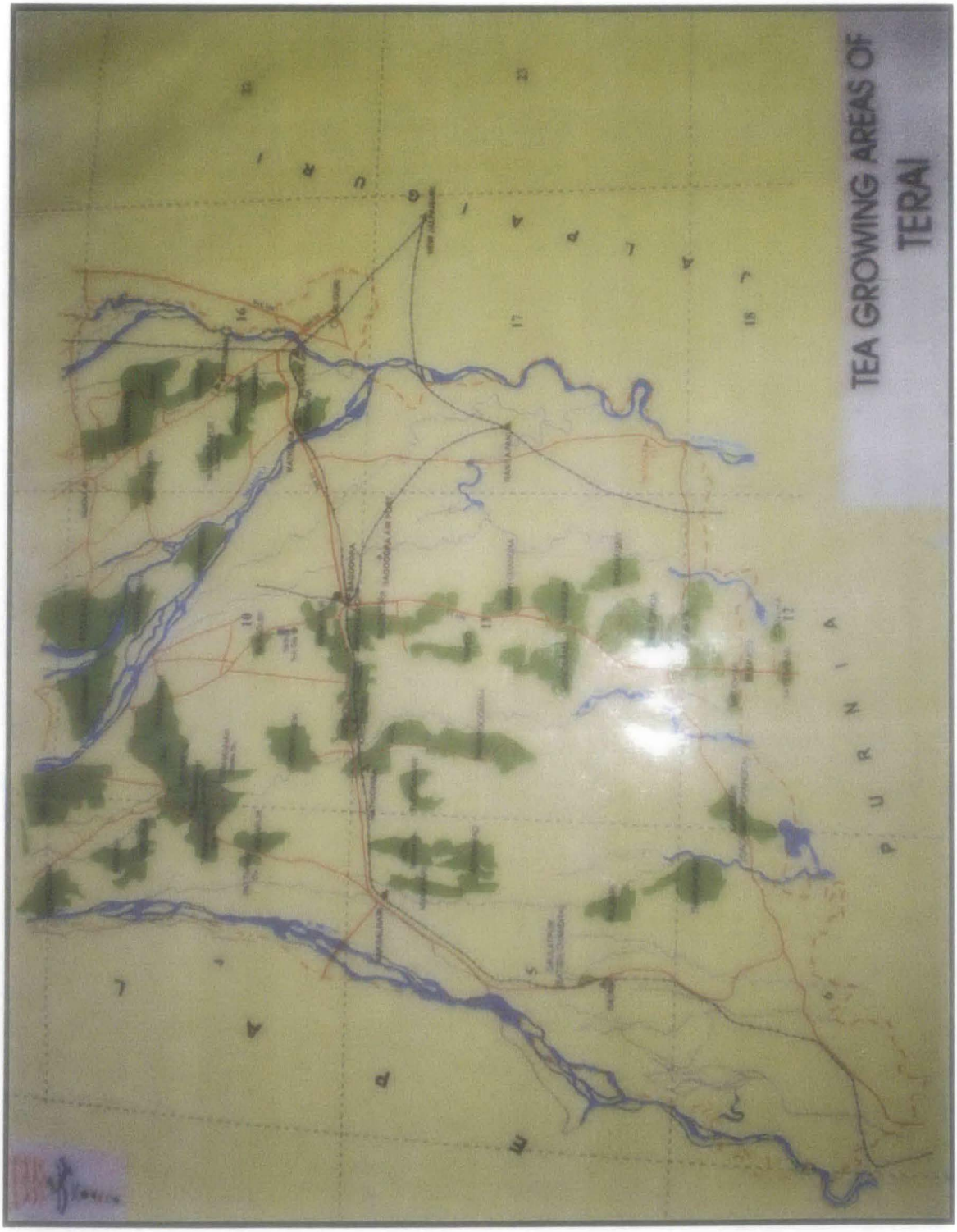

Fig.9 Map showing the tea growing areas in Terai region

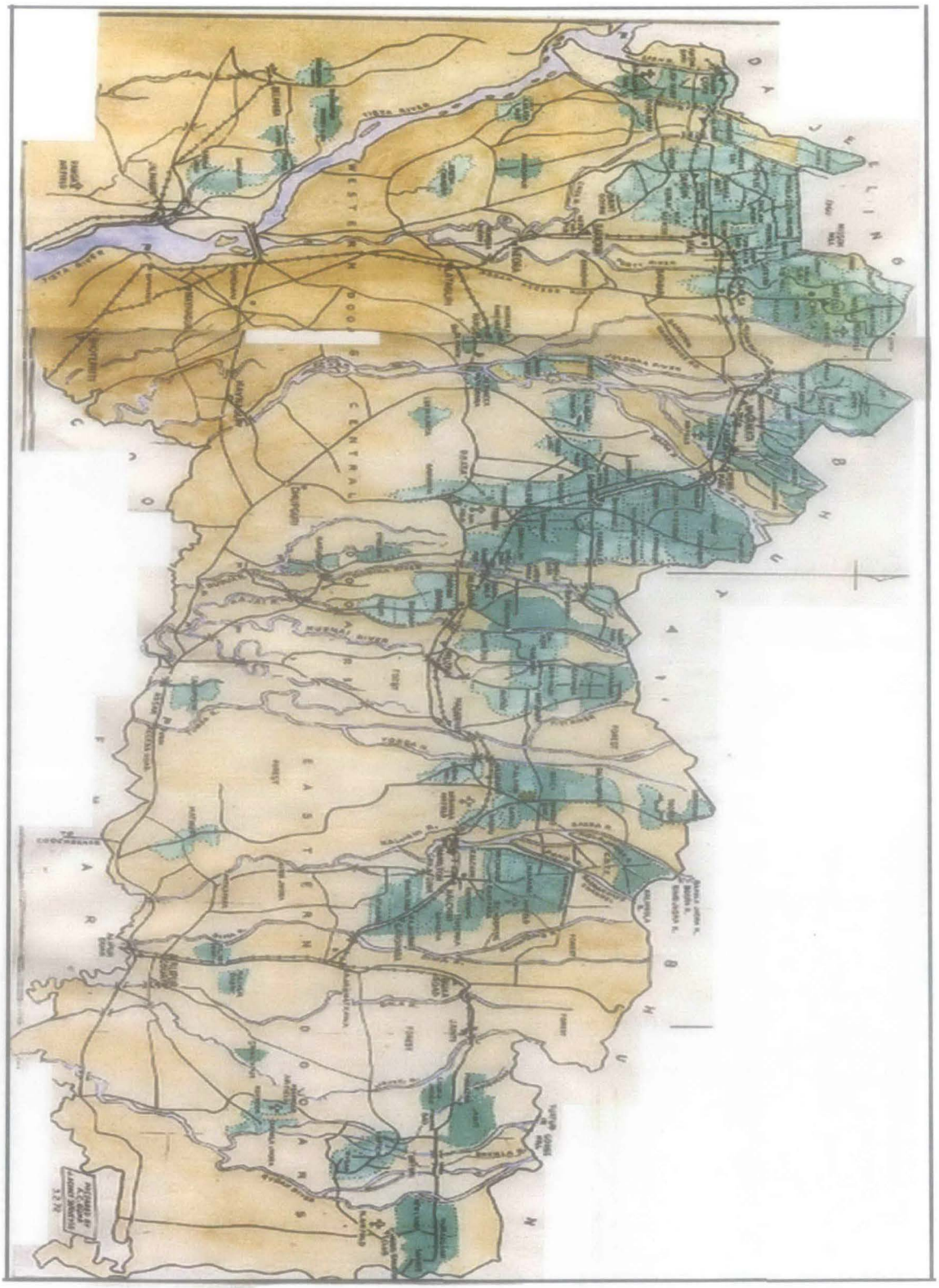

Fig.9 Map showing the tea growing areas in Dooars region

A.b Collection and processing of dead larvae

Dead and diseased larvae were collected from the field and laboratory reared population of respective tea pests and dipped in 70% alcohol for two seconds for surface sterilization. Then the dead larvae were washed three times with sterilized double distilled water and then kept in sterilized eppendorf tube with sterilized double distilled water. The tube was wrapped with parafilm and stored at –20°C (Lacey and Brooks, 1997).

A.c Isolation of Entomopathogenic Bacteria

For isolating bacteria, the stored dead larvae were taken and thoroughly macerated by glass homogenizer and the crude homogenate was sieved through fine sterilized cotton cloth. The filtrate was centrifuged at 3000 rpm for 30 min. The supernatant was discarded and the precipitate containing bacterial suspension was taken for pure culture isolation by 'dilution streak method' in nutrient agar medium (Lacey and Brooks, 1997). The infectivity of the bacteria was determined following Koch's postulates by infecting healthy first instar larvae with the aforesaid cultured bacteria. After proving the Koch's postulates the viability of the bacteria was checked by inoculating in newly prepared agar medium at weekly intervals.

B. Characterization of bacterial strains isolated from the lepidopteran pest species (viz. *Buzura suppressaria*, *Hyposidra talaca*, *Eterusia magnifica* and *Caloptilia theivora*)

B.a Morphological Characteristics

Determination of cell morphology

The morphology of the bacterial cell was determined after culturing the bacteria overnight in nutrient agar medium in a petriplate, incubated at 37°C. A smear of the overnight grown culture was taken in a glass slide and observed under phase contrast microscope (Olympus, CX31) (oil immersion, 1000X). To determine the motility of a

bacterium, a drop of culture (24 and 48 hours old) in nutrient broth medium was used to prepare a hanging drop in a cavity slide. The drop was observed using a phase contrast microscope (Olympus, CX31) (oil immersion, 1000X).

The spore and crystal protein shape of bacteria were determined after growing bacterial culture in nutrient agar medium for 4 days (up to sporulation) in incubator at 37°C. The spore-crystal mixture was suspended in 1 ml ice-cold 1M Nacl and centrifuged for 5 min at 12,000 rpm. The pellet was suspended in distilled water. The presence of spores and crystals was recorded directly by preparing a smear of this culture in a glass slide and observed under phase contrast microscope (Olympus, CX31) (oil immersion, 1000X) (Kati *et al.*, 2007).

The colony morphology and colony texture were determined in overnight grown culture prepared in glass petriplate and observed with naked eye.

Bacillus thuringiensis kurstaki (*Btk*) was used as control for comparative study, obtained from an authenticate Institutional source.

B.b Biochemical Characteristics

Several biochemical tests like indole, Voges-proskour, methyl red, citrate utilization, esculin hydrolysis, lysine decarboxylase, ornithin decarboxylase, H_2S production, nitrate reduction, fermentation of different carbohydrates, urease tests were performed using Biochemical testing kit (KB003) (Himedia) with *Btk* as control.

Enterobacteriaceae Identification Kit (KB003 Hi25) (Himedia) was also used to identify entomopathogenic *Enterobacter* sp.

Some more tests such as gram reaction, production of catalase and determination of anaerobic growth were also performed without the help of biochemical testing kit. A brief description of these biochemical tests are given below:

Gram staining

The method of Bartholomew (1962) was followed. A suspension of a 24 hours old culture on nutrient agar medium was prepared in distilled water. A drop of that suspension was smeared on a grease-free slide. The smear was air-dried, heat-fixed,

flooded with crystal violet stain for 1 minute, and washed for 5 second with water. The smear was then flooded with Burke's iodine solution, allowed to react for 1 minute, washed again for 5 second with water. The smear was then observed under compound microscope (100X) for stained bacteria.

Production of catalase

A 24 hours old slant culture was flooded with 0.5 ml of 10% hydrogen peroxide solution and observed for the production of gas bubbles, indicating the presence of catalase (Norris *et al.*, 1981).

Anaerobic growth

Anaerobic agar medium was distributed into culture tubes in amount sufficient to give 7.5 cm depth of the medium and sterilized by autoclaving at 121°C for 20 minutes. The tubes were inoculated with a small (outside diameter 1.5 mm) loopful of 2 days old culture on nutrient agar medium by stabbing up to the bottom of the column. They were incubated at 30°C for 7 days, and observed for growth along the length of the stab (anaerobic) and surface of the agar (aerobic) (Claus and Berkeley, 1986).

Biochemical testing kit

The kit materials consisted of medium for 24 biochemical tests and separate disc for oxidase test. A brief description containing, name of each test, name of reagents to be added after incubation of bacterial culture in kit medium, original colour of each medium, positive reaction of a bacterium and negative reaction was given as is mentioned in the following table (Table.1)

Table.1 Details of biochemical tests used for identifying bacterial strains.

Serial No.	Name of biochemical test	Reagents to be added after incubation	Original colour of the medium	Positive reaction	Negative reaction
1.	ONPG	-	Colourless	Yellow	Colourless
2.	Lysine decarboxylase	-	Olive green	Purple	Yellow
3.	Ornithin decarboxylase	-	Olive green	Purple	Yellow
4.	Urease	-	Organish yellow	Pink	Organish yellow
5.	Phenylalanine deamination	2-3 drops of TDA reagent	Colourless	Green	Colourless
6.	Nitrate reduction	1-2 drops of sulphanilic acid and 1-2 drops of N,N-Dimethyl-1-Napthylamine	Colourless	Pinkish red	Colourless
7.	H_2S production	-	Organish yellow	Black	Organish yellow
8.	Citrate utilization	-	Colourless	Blue	Yellowish -green
9.	Voges-Proskauer's	1-2 drops of Baritt reagent A and 1-2 drops of Baritt reagent B	Colourless	Pinkish red	Colourless/ slight copper
10.	Methyl red	1-2 drops of Methyl red reagent	Colourless	Red	Yellow
11.	Indole	1-2 drops of Kovac's red reagent	Light green	Pinkish red	Colourless
12.	Malonate	-	Cream	Blue	Light green
13.	Esculine hydrolysis	-	Red	Black	Cream
14.	Arabinose utilization	-	Red	Yellow	Red/pink
15.	Xylose utilization	-	Red	Yellow	Red/pink
16.	Adonitol utilization	-	Red	Yellow	Red/pink
17.	Rhamnose utilization	-	Red	Yellow	Red/pink
18.	Cellobiose utilization	-	Red	Yellow	Red/pink
19.	Melibiose utilization	-	Red	Yellow	Red/pink
20.	Saccharose utilization	-	Red	Yellow	Red/pink
21.	Raffinose utilization	-	Red	Yellow	Red/pink
22.	Trehalose utilization	-	Red	Yellow	Red/pink
23.	Glucose utilization	-	Red	Yellow	Red/pink
24.	Lactose utilization	-	Red	Yellow	Red/pink
25.	Oxidase test	Done on oxidase disc separately. Detects cytochrome oxidase production.	White	Deep purple within 10 sec.	White/ purple after 60 seconds

B.c Physiological Characteristics of bacterial strains

B.c.i Growth characteristics: determination of generation time

In this procedure, growth of bacterial strains was determined by turbidimetric method after Cappuccino and Sherman (1996). The growth of the bacteria was determined in nutrient broth medium (Himedia Co.). In this procedure one loopful of bacterial culture (from one day old culture) was inoculated in nutrient broth medium and incubated at 37°C in static condition in incubator. The reading of the O.D. value of the growing culture was taken at 540 nm in spectrophotometer every 30 minutes interval up to the stationary phase of growth of bacteria. Finally optical density and time interval were plotted on a graph paper and from this the generation time (doubling time) was determined. *Bacillus thuringiensis kurstaki* was used as control for comparison.

B.c.ii SDS-PAGE profile (Qualitative) of crystal protein

Bacterial strains were grown in Luria Bertani medium at 37°C without shaking. It was grown up to the phase of sporulation. Visual confirmation of crystalline bodies was done with the aid of phase contrast microscopy. The crystal was harvested in high pH buffer of sodium carbonate and 2-marcaptoethanol (Kranthi, 2005) with slight modification (2-marcaptoehtanol was used instead of Dithiothreitol (DTT)) in incubator for 2 hours at 37°C. Then the crystal was separated from spore-crystal mixture with the help of cooling centrifuge operated at high speed (10000 rpm) for 15 minutes. The supernatant containing crystal protein was separated from spores accumulated as precipitate. This crystal protein was taken for SDS-PAGE analysis.

B.c.iii Qualitative (SDS-PAGE) analysis of whole body protein of bacteria

The bacterial strains were cultured on Luria-Bertani (LB) agar for 24 hours at 37°C and the protein was extracted using 1% lysozyme solution and lysis buffer comprising: 4% SDS, 20% glycerol, 2% 2-marcaptoethanol, 70% Tris-HCL, P^H 6.8, and 4% deionized water. The extracted protein was analysed by SDS-PAGE after the method of Costas (1992). Gels were fixed in trichloroacetic acid and stained with Coomassie Brilliant blue G-250 (Neuhoff *et al.*, 1988). The molecular weights of proteins were

determined by using protein molecular weight standards [Broad range (PMW-B) from Bnagalore Genei Co.].

C. Bioassay: Determination of LC_{50} value and LT_{50} values

For bioassay about 250 larvae of lepidopteran pests were collected from tea plantations of Tocklai vegetative clone (namely TV-25). The plantations were about 20 years old mainly growing in Darjeeling Terai and the Dooars region. The larvae were reared in laboratory and passed through two generations at 27 ± 2^0 C; 72 ± 2 % R^H under a photoperiod phases (L: D) of 13:11 hrs. in aseptic conditions. Fresh tea twigs/leaves (TV-25) from the experimental tea garden organically maintained on North Bengal University campus was supplied as food. The two-day old second instar larvae were used for every bioassay test.

The efficacy of the entomopathogenic micro-organisms was determined by bioassay (Fig. 11). In case of spore and crystal producing gram positive bacteria, extraction and bioassay was done after the procedure of Dulmage *et al.*, (1970). Different concentrations of crude samples (spores and crystal) (100, 300, 500, 750 and 1000 µg/ml) were used in the LC_{50} (median lethal concentration) and LT_{50} (median lethal time) bioassay by spreading these uniformly on tea leaves offered as food according to the procedure of Unnamalai and Sekar (1995). In case of non-spore forming bacteria aqueous formulation of bacteria (vegetative body) was used for bioassay (100, 300, 500, 750 and 1000 µg/ml) after the method of Jackson *et al.*, (1992).

One hundred larval specimens of second instar were taken for treatment with each bacterial concentration (both in case of spore forming and non-spore forming bacteria). Sterile distilled water was used in control. The mortality was observed at an interval of 24 hrs from the day of inoculation (since 1^{st} day of feeding up to 9^{th} day). The mortality caused by each concentration was corrected for the control rate of mortality in each assay by using Abbott's formula (Abbott, 1925; Damgaard *et al.*, 1998). Data were subjected to probit analysis (Finney, 1954) and median lethal concentration (LC_{50}) value was calculated from the regression equation. Median lethal time (LT_{50}) value was also determined simultaneously following the method of Biever and Hostetter (1971):

$$LT_{50}=a + e\ (c-b)/D$$

Where, a = the number of hours from the initiation of the test until the reading made just before the 50% value was recorded, b = the total number of larvae dead at the reading just before 50% value was recorded, c = 50% of the total number tested, D= the number of larvae dying in 24 hr period during which the 50% mortality was reached, and e = the number of hours between mortality counts.

Fig. 11: Bioassay set up in laboratory

D. Cross infectivity testing

D.a Other lepidopteran tea pests

Cross infectivity of the isolated entomopathogenic bacteria to other lepidopteran tea pests sharing same niche were tested.

In case of spore forming and crystal producing bacteria crude spore-crystal mixture (100, 300, 500, 750 and 1000 µg/ml) were taken for cross infectivity testing. Aqueous formulations of bacterial concentrations (vegetative body) (100, 300, 500, 750 and 1000 µg/ml) were used in case of non spore-forming bacteria. One hundred larval specimens of second instar were taken for treatment with each concentration. Sterile distilled water was used in control. Tea leaves dipped in different concentrations were offered as food; only distilled water was used in control.

The mortality was observed at an interval of 24 hrs from the day of inoculation (1^{st} day feeding up to 9^{th} day). The corrected mortality was calculated using Abbott's

formula (Abbott, 1925). Data were subjected to probit analysis (Finney, 1954) and median lethal concentration (LC_{50}) value was calculated from the regression equation. Median lethal time (LT_{50}) value was also determined simultaneously following the method of Biever and Hostetter (1971).

D.b Mulberry silkworm (*Bombyx mori*) (Locally cultured multivoltine race)

As the sericulture industry is running side by side with the tea industry in North Bengal so it is necessary to determine the toxicity/infectivity of the bacterial isolate to mulberry silk worms before spraying in the tea plantations. Experiment was done for determining the infectivity in the laboratory condition. For the experiment the multivoltine silkworm (nistari variety) was taken (Fig. 12). Disease free layings (DFLs) were collected from West Bengal Sericulture Department situated at Matigara, Siliguri. Freshly hatched neonates of silkworm were given fresh sterilized mulberry leaf collected from North Bengal University campus and from West Bengal Sericulture Department, Matigara. They were reared up to second instar and were taken for the cross infectivity experiment. In case of spore and crystal protein forming bacteria crude spore-crystal mixture (100, 300, 500, 750 and 1000 µg/ml) were taken for silk worm infectivity testing. Aqueous formulation of vegetative body (100, 300, 500, 750 and 1000 µg/ml) were used in case of non spore-forming bacteria. Hundred second instar larvae of silkworms were treated with each concentration. Sterile distilled water was used in control. Silkworm larvae were kept under everyday observation upto 9^{th} day of inoculation (first day feeding) for recording any mortality.

Fig. 12: Silk worm (*Bombyx mori*) larvae feeding mulberry leaves.

E. Field trials on biocontrol efficacy

To find out the efficacy of a particular entomopathogenic bacterium in field condition, field study was conducted in the tea estates of Terai region (Fig. 13). The experiment was conducted in a randomized block design (RBD) with three replicates for each treatment as well as control. Four different concentrations of an entomopathogen was sprayed (as separate treatment) after the method of Guerrero *et al.*, (2007) and Ravi *et al.*, (2008). Atleast thirty pest infested tea bushes (TV-25 cultivar) were taken per replicate. A fairly large population (≈100/bushes) of second instar larvae were treated with the bacterial formulations at different concentrations (Salama and Salem, 1999). Water was used as carrier and high volume knapsack sprayer was used. Precount of insect larvae was taken before spraying to determine the number of larvae per bush exposed to each treatment and also in the control (Guerrero *et al.*, 2007; Ravi *et al.*, 2008; Salama and Salem, 1999). After spraying the data of live larvae recovered was taken on 3rd, 5th and finally on 7th day as per the method prescribed by Salama and Salem, (1999) and Ravi *et al.*, (2008). To evaluate the relative efficacy of each treatment the numbers of live larvae recovered in each treatment was compared with that of control plots after Guerrero *et al.*, (2007) and Hellman, (1994). The percent of live larvae on 3rd, 5th and 7th day was graphically represented (Mean±SD) after Guerrero *et al.*, (2007) and Cory *et al.*, (1994). The percentage live larvae after arcsine transformation were compared between treatments after 3$^{rd.}$ 5th and 7th day using one-way analysis of variance (ANOVA) (Guerrero *et al.*, 2007; Ravi *et al.*, 2008).

Fig. 13: Field experiment of microbial formulation

F. Statistical analysis

Quantitative results were subjected to standard statistical analysis. Software packages such as SSP, SPSS, Origin 50 and other were used. Results of bioassay and cross infectivity experiments were subjected to log Probit analysis. MS-EXCEL program was mainly used for analysis and graphics.