CHAPTER IV

MATERIALS AND METHODS
1) Selection of Host plants

Host plant (1) Som (*Machilus bombycina*), (2) Soalu (*Litsaea polyantha*), and (3) Mejankari (*Litsaea citrata*) were selected from cultivated areas in different localities. Selection was done keeping in mind the health and age of the plants. Too young and old plants were not considered for experiments. Plants were selected randomly and marked. These plants were used for all types of studies.

(2) Morphological Studies

A detail morphological study has been made on three types of host plants. The morphological features of these plants have been given in chapter—III.

(3) Biochemical studies

*Determination of moisture content in Machilus bombycina, Litsaea-polyantha and Litsaea citrata*

Samples of tender, semimature and mature leaves of Som, Soalu and Mejankori were collected separately from the field of Khanapara Muga Seed Farm; Guwahati in different rearing seasons and biochemical analysis was done. The estimations were done in duplicate and mean values were recorded for the interpretation of the results.

To determine the moister content, constant weight of a porcelain crucible was determined. In a hot air oven 5 grams of fresh leaves were placed and the temperature was maintained for 16 hours at 100 °c. On cooling, the weight of the crucible along with the oven dried leaf material was taken again. The loss in weight was measured, which was expressed as percentage basis using the following formula:

\[
\text{% of moisture content} = \frac{\text{wt of fresh leaves} - \text{wt of oven dry leaves}}{\text{wt of oven dry leaves}} \times 100
\]
Determination of mineral content of *Machilus bombycina*, *Litsaea polyantha* and *Litsaea citrata* leaves.

To determine mineral content in Soin Soalu and Mejankari leaves the method of Moiramani and Wankhade (1970) was followed.

2 grams of leaf sample was weighed into a porcelain crucible (which was previously heated to 600°C and cooled). The crucible containing the leaves was heated first over a low flame till all the material was completely charred followed by heating in a muffle furnace for about 6 hours at 600°C. It was then cooled in a dessicator and weighed. To ensure complete burning, the crucible was again heated to 600°C for an hour, cooled and weighed. This was repeated till weight become constant and the ash was almost white in colour. Percentage of ash contents was measured for each leaf type for determination of mineral contents as per the procedure of Moiramani and Wankhade (1970).

Determination of Crude Protein in the leaves of *Machilus bombycina*, *Litsaea polyantha* and *Litsaea citrata*

The total N\textsubscript{2} content in the leaf sample was determining by the micro Kjeldahl method described by Humphries (1956). 1 gm of well dried and powered leaf sample and 1 gm of digestion mixture was digested with 5 ml of con. H\textsubscript{2}SO\textsubscript{4}. The digested material was then distilled with 40% NaOH and the distillate was collected in 4% boric acid. The N\textsubscript{2} was determined by titrating the distillate with standard HCl using mixed indicator. A blank (without leaf sample) was carried out in a similar manner. The estimation was in duplicate for each sample and mean value was recorded for the interpretation of the
results. The total N$_2$ was expressed in percentage on oven dry basis of the leaf sample. The crude protein content of the leaf was determined by multiplying the percentage of N$_2$ by the constant factor 6.25.

\[
\text{Crude protein} = \text{Wt. of nitrogen} \times 6.25
\]

\[
\text{Percentage of crude protein} = \frac{\text{crude protein}}{\text{Wt. of leaf samples}} \times 100
\]

**Method of Determination of total sugar of Machilus bombycina, Litsaea polyantha and Litsaea citrata**

Fine powder of dry leaves of Som, Soalu and Mejankari were taken separately in the centrifuge tubes, a few drops of ethyl alcohol and 2 ml of distilled water were added in each tubes. The mixtures were stirred for 5 minutes with glass rods and added 10 ml of boiling ethanol (80%) in each tubes and stirred and centrifuged for 10 minutes. The supernatant was filtered into the volumetric flask carefully, keeping the residue in the test tube. The process was repeated 3 times and the total volume of the extract in the flask was made upto 100 ml with 80% ethanol. The extract was used for estimations of sugar in the samples. 0.1 ml sugar extract was taken in test tubes and the volume was made upto 2ml with distilled water. 4 ml of anthrone reagent (0.2%) was added to it keeping the tube on hot water bath in boiling condition and then cooled it to room temperature. At the time of cooling the bluish green colour was measured in spectrophotometer using the wave length of 630 nm. The amount of sugars present in the extract was calculated with the help of standard curve.

(4) **Rearing Methods**

An attempt has been made to study the comparative rearing performance of muga silkworm (*Antheraea assama*) on the food plants Som, Soalu, and Mejankari in Khanapara cultivation area near Guwahati during
Since silkworms have been domesticated for many centuries, they are by nature quite delicate and very sensitive to environmental conditions. Among the various environmental factors, the most important are atmospheric temperature, humidity and rainfall prevailing at the time of rearing. For the study of fecundity, hatching, effective rate of rearing (ERR%) disease free layings were taken for studying the rearing performance of each and every crop (Season-wise) during the year of 1998-1999 and 1999-2000. Cocoon weight (gm) and shell weight (gm) of male and female, worms were taken into consideration. The silk ratio was calculated following the equation described by Joshi and Mishra (1982) as given below.

\[
\text{Silk percent} = \frac{\text{Shell weight of the cocoon}}{\text{Cocoon weight}} \times \frac{c}{c}
\]

### (5) Location of study

Study was done at Khanapara Muga Farm of the Govt. of Assam at Guwahati during the year of 1998-99 and 1999-2000. The meteorological condition (mean of 50 years) of the district has been given in Table - 3.

<table>
<thead>
<tr>
<th>District</th>
<th>Temperature °C</th>
<th>Rainfall (mm)</th>
<th>Humidity %</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Minimum</td>
<td>Maximum</td>
<td>Total</td>
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<tr>
<td>Kamrup</td>
<td>22.8</td>
<td>26.4</td>
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(6) **Microbiological study**

1. **Isolation of fungi from the Phylloplanes of the food plants of muga silkworm - Som, Soalu and Mejankori.**

Som, Soalu and Mejankori leaves required for the present experiment were collected from the muga Farm (Khanapara, Guwahati Assam). The leaves were collected randomly during 1998-2000 in different seasons, viz-Winter, Spring, Rainy/Summer and Autumn (During the period of muga silkworm rearing). Tender, semi-mature and mature leaves were randomly collected from the cultivating areas. The methods of sampling of leaves as described by Kamal and Singh (1970) were followed.

Serial washing technique as described by Kamal and Sinha (1970) was employed. For the purpose, leaf discs were cut out from each leaf category with the help of sharp sterilized borer. Pieces from leaf category were placed separately in 20 ml of sterilized distilled water in 100 ml Erlenmeyer flasks and shaken for 20 min on a shaker (120 r.p.m). After shaking, the water was poured off and saved. A fresh volume of 20 ml of sterile water was added to the same flask containing the leaf discs which are further shaken. The process was carried out 5 times. The extent of detachable fungal propagules from the surface was determined by plating 1 ml solution from the respective washing to the agar plate containing PDA.

The washed discs were blotted dry and placed on sterile but solidified agar surface (PDA) to detect the presence of those fungi which remain still attached to the leaf surface.

For isolation of fungi from upper surface and lower surface, leaf discs were cut out after washing the leaves gently using washing bottle. The cut out leaf discs were imprinted on the surface of the agar plate. Separate plates were used for imprinting upper and lower surface of the leaf discs.
plates were incubated at 30°C ± 1°C for 4 days and then the plates were examined for development of fungal colonies. Experiments were conducted in different seasons corresponding to the different crops such as winter crop (Aghenna & Late-jarua), spring crop (Jethua), rainy/summer crop (Aherua & Bhodia), autumn crop (Kotia).

(2) **Maintenances of stock culture**

Stock cultures were maintained in semi-solid Czapek Dox agar medium at 28°C and transfers were made fortnightly.

(3) **Taxonomic studies**

Taxonomic studies on the isolated fungi were made with the help of the method described in Manual of Microbiological Methods (1957) and identified with the help of “A manual of soil fungi” by Joseph C. Gilman (1995) and “Illustrated genera of imperfect fungi” by H.L. Barnett (1960).

(4) **Preparation of inoculum**

A small portion of the actively growing mycelium from agar slant of each test fungus was aseptically transferred to a sterile Erlenmeyer flask (250 ml) containing 50 ml of medium and was incubated at 30°C ± 1°C for 70 h. After 70 h, the mycelial mat was aseptically fragmented into small pieces with the help of a waring blender. The fragmented mycelium was washed several times with sterile distilled water to remove any trace of adhering medium and then suspended in a phosphate buffer (pH 5.5) for 24 hours to overcome the shock encountered during blending. An aliquot of 1.0 ml of the mycelial cell suspension was used as inoculum.

(5) **Medium and growth condition:**

The Czapek’s medium was prepared without agar. The composition of the medium was as follows -(g %)- KH₂PO₄ -0.1; NaNO₃ - 0.2; MgSO₄ 7H₂O - 0.05; KCl - 0.05; FeSO₄ 7H₂O -0.001; Sucrose 3.0; and distilled
water 100ml. Unless otherwise stated each test fungus was grown in 50 ml of the above mentioned medium in a 250 ml Erlenmeyer flask under stationary condition at 30°C ± 1°C for a period of 70 hours.

(6) **Measurement of growth**

After the incubation periods each test fungus were harvested. The mycelial mates were removed from the medium by filtration through whatman filter paper No- 1. The filtered mycelium was washed repeatedly with distilled water to make them free from adherent medium and dried to constant weight in the oven at 60°C for 24 hours, cooled in a desicator and weighed. The dry weight of the mycelium thus obtained was taken as an index of growth.

(7) **Effect of physical and chemical environment on growth of the phylloplane fungi**

**Temperature**

The effect of temperature on growth and sporulation of the fungal isolates were studied under stationary conditions by incubating each organisms at 25°C, 28°C, 30°C and 35°C with Czapek’s synthetic solutions and measuring the dry weight of the mycelia in respective sets.

**Hydrogen –Ion concentration (pH)**

The effect of different PH on growth and sporulation of the fungal isolates were studied under stationary conditions by incubating each organisms at the pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 with Czapek’s synthetic solutions and measuring the mycelial dry weight in each case.

**Effect of Carbon sources**

The effect of carbon sources viz- Maltose, D- glucose, Sucrose, D- xylose and starch in different concentrations were studied for the
growth and sporulation under stationary condition in the liquid Czapek’s medium and measuring the dry weight of mycelial mats in each time.

**Nitrogen sources**

The effect of nitrogen sources viz- Ammonium sulphate, Ammonium nitrate, Sodium nitrate in different concentrations were studied for the growth and sporulation under stationary condition in the liquid Czapek’s medium and measuring the dry weight of mycelial mats in each time.

(8) **Study the effect of feeding spores of phylloplane fungi to silkworms.**

All the phylloplane fungi isolated from Som, Soalu, and Mejankari leaves were grown asceptically on Czapek’s liquid medium. After 7th days of growth at 30\(^o\)C \pm 1\(^o\)C the mycelial mats were harvested. The liquid remain of the medium was removed by decantation. After which sterile distilled water of 50 ml was poured to each flask containing the mycelial mat. The flasks were shaken and then the water containing the spores was decanted and stored separately. In such fashion 4 times the spores were removed from each flask containing the mycelial mat. Spores from all replica flasks and from all types of experimental fungi were stored in refrigerator for experiment.

Before feeding the spores of different types the selected healthy mature leaves were cleaned and washed using several changes of sterile water without plucking. The solution containing suspended spores of specific types were then sprayed over the leaves. The leaves were then allowed to be fed by experimental silkworms.

The weight of the experimental larvae, the weight of male and female cocoons, shell weight of male and female and the Percentages of silk obtained compared to control were recorded stage by stage

After rearing the mature worms are collected and kept for cocoon formation. Ten male and ten female cocoons are randomly selected to
study the cocoon weight (gm), shell weight (gm) and silk ratio (%) to compare with the control of ten male and ten female cocoons.