Chapter III
MATERIALS AND METHODS

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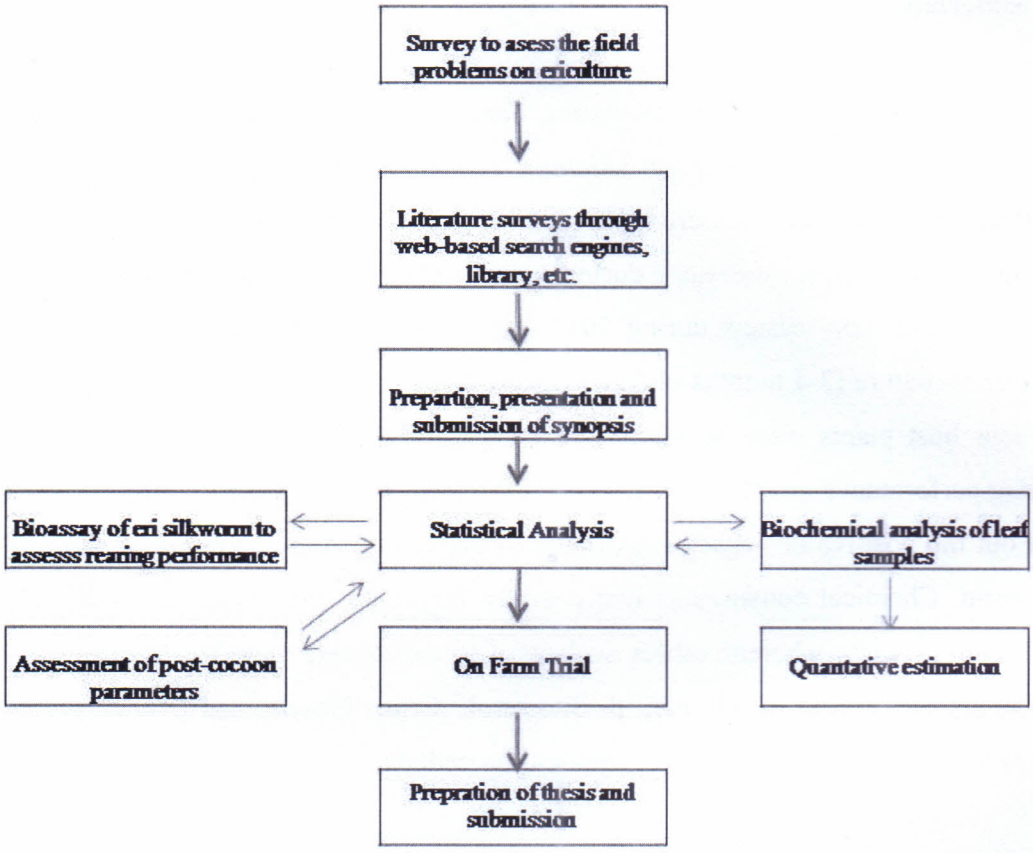
3.1 Materials

Four host plants of eri silkworm, *Samia ricini* Donovan were chosen for the experiments. These are Castor (*Ricinus communis),* Borpat (*Ailanthus grandis*), Borkesseru (*A. excelsa*), Kesseru (*Heteropanax fragrans*). To start with the experiments, rearing of eri silkworm was done during January-February, April-May, July-August and September-October seasons during 2012 and 2013. Concurrently, tender (1-2 months old), semi-mature (3-4 months old) and mature (more than 4 months old) leaves from all the four host plants were harvested for analysis of the biochemical constituents. The rearing performance and the biochemical constituents were then statistically correlated to find out the positive or negative responses of the constituents to the productivity of the silkworm. Chemical constituents like phenols and chlorogenic acid were estimated on fresh weight basis whereas others such as, Carbohydrates, Tannins, Lignins, Trypsin inhibitors, Phytic acid, Crude Fat, β- Sitosterol, Crude Protein and Crude Fibre were done on dry weight basis following standard procedures.

Most of the experiments were conducted in the laboratory of Biotechnology Division and Eri silkworm Section, Central Muga Eri Research & Training Institute, Lahdoigarh, Jorhat, Assam, India. Rearing of silkworms was conducted in the experimental field and rearing houses of Germplasm Conservation Centre, Central Muga Eri Research & Training Institute, Central Silk Board, Chenijan, Jorhat, Assam, India. Post-cocoon parameters were evaluated at Post-Cocoon Technology Section of the institute as well as at Regional Silk Technological Research Station, Khanapara, Guwahati, India.

The schemes for conducting the experiments are depicted below:



3.2 Methods

3.2.1 Test of attraction by eri silkworm *S. ricini* (Donovan) towards leaves of different host plants

Based on morphological and physiological characteristics, the leaves of the host plants were selected as tender, semi-mature/medium and mature. One thousand two

hundred newly hatched eri silkworms (3 replications with 400 numbers per replication) were placed at the centre of a circle. Castor, Borpat, Borkesseru, Kesseru twigs with 5-6 leaves of tender, medium and mature type immediately after detachment from their branches were placed one foot away from the centre. The number of larvae settled on the leaves of the different plants was recorded after 60 min. and this number is expressed as settling percent (Neog, K. 2011).

3.2.2 Biochemical analysis of leaves of host plants

Eleven biochemical constituents viz., crude fiber, crude protein, carbohydrate, crude fat, Trypsin inhibitor , β-sitosterol, total phenol, phytic acid, chlorogenic acid, tannin and lignin content of tender, medium or semi-mature and mature leaves of Castor, Borpat, Borkesseru, Kesseru leaves were analyzed for four crop seasons, i.e. January-February, April-May, July-August, September-October. The data generated are statistically analyzed. The reference standard procedures for extraction and estimation of the chemical constituents are described below.

The reference standard procedures for extraction and estimation of the chemical constituents are presented in the following chapters.

3.2.2.1 Preparation of leaf sample:

Leaf samples of three different types (tender, medium and mature) were collected separately from each host plants during January-February, April-May, July-August, and September-October of the year 2013. The leaves were properly cleaned and then dried in hot air oven at a temperature ranging between 80-90°C for several hours till dried completely. The dried leaves were ground in an electric grinder (Make: BAJAJ Mixer grinder Gx7) to make fine powder. The powdered leaf samples were kept separately in polypropylene containers which were subsequently used for analysis.

3.2.2.2 Determination of carbohydrate content

The carbohydrate content in the leaves of the selected eri silkworm host plants were estimated by Anthrone method (Sadasivam and Manickam, 2005). In this method, carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium, glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone a green colored product with an absorption maximum at 630 nm.

50 mg of the leaf sample was taken into a boiling tube and hydrolysed it by keeping in boiling water bath for 1 hour with 2.5mL of 2.5 N-HCl and cool to room temperature. Neutralized it with solid sodium carbonate until the effervescence ceases. Made up the volume to 50mL and centrifuged it. The supernatant was collected and 0.5 and 1mL aliquots were taken for analysis. Prepared the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1mL of the working standard. '0' serves as blank and make up the volume to 1mL in all the tubes including the sample tubes by adding distilled water. Then 2mL of anthrone reagent was added. Heated for ten minutes in a boiling water bath. Cooled rapidly and read the green to dark green color at 630nm.

50 mg sample + 2.5 ml of 2.5N-HCL in to a test tube

↓

Hydrolyse by keeping it in a boiling water bath for 1 hr.

↓

Neutralize it with Na2CO3 until effervescence ceases

↓

Make up the volume to 50 ml and centrifuge

↓

Collect the supernatant and take aliquots for analysis

↓

Prepare the working standard. Zero serves as blank

↓

Make up the volume to 1ml in all tubes

↓

Add 2ml of Anthrone reagent and heat for 10mins.

↓

Cool rapidly and read the absorbance at 630nm.

↓

Carbohydrates hydrolyzed in to sugar using dil. HCl. In acidic medium glucose dehydrated to hydroxymethyl furfural which forms a green colored Anthrone.

3.2.2.3 Determination of total phenol content

The total phenol content in the leaf samples was estimated by the Folin-Ciocalteau method described by Malick and Singh (1980). 0.5 g of leaf sample was weighed and extractions were done with 10 ml 80% ethanol and grind it with mortar. Centrifuge the homogenates 10,000 rpm for 20 mins and save the supernatant. Re-extract the residue with 3 times the volume of 80% ethanol. The supernatant was pooled and evaporated to dryness which was then re-dissolved with 50 ml of water. Three milliliter of the extract was taken in test tubes and volume was equalized to three milliliter with water. 0.5 ml of Folin-Ciocalteau reagent was added. After 3 minutes, 2 ml of 20% sodium carbonate solution was added to each tube and then kept in boiling water bath for exactly two minute. The tubes were then cooled and the intensity of color was read at 650nm in a PC based UV-Vis spectrophotometer (Make: Systronics, Model: 2202). The total phenol content was estimated from a standard curve prepared with known concentration of catechol and was expressed as mg phenols per 100g leaf on fresh weight basis.

0.5g sample + 10 times volume of 80% ethanol

↓

After grind it with mortar, centrifuge the homogenate at 10,000rpm for 20 mins. and save the supernatant

↓

Re-extract the residue with 3 times the volume of 80% ethanol

↓

Evaporate the supernatant to dryness and dissolve it in 50ml D/W

↓

Pipette out different aliquots and make up the volume to 3ml

↓

Add 0.5ml of Folin-Ciocalteau reagent + 2ml of 20% Na2CO3

↓

Place the tubes in a boiling water (2min.), cool and measure the absorbance at 650nm against a blank.

↓

Phenols react with phosphomolybdic acid in Folin-Ciocalteau in alkaline medium and produce Molybdenum blue complex

3.2.2.4 Determination of chlorogenic acid content

Chlorogenic acid content in the leaves of the eri silkworm host plants were estimated following the method of Michael *et al.* (1978) also known as Titanium chloride method. 1g of the leaf sample was taken in a round bottom flask and refluxed twice with 125 ml 80% ethanol (adjusted pH 4.0 with 2.5 N HCl) for 30 minutes. About 250 ml of the extract was collected. 0.5 ml of the extract was taken in test tubes which were dried in a vacuum oven at 50°C and 700 nm pressures for 2 hours. The dried extract was dissolved in 4.75 ml acetone to which 0.25 ml Titanium Chloride was added. Absorbance was measured at 450 nm against a reagent blank in a spectrophotometer (PC based UV-Vis spectrophotometer (Make: Systronics, Model: 2202). Chlorogenic acid was calculated by referring to the standard curve prepared with known concentration of chlorogenic acid.

1g sample + 80% ethanol (adjusted pH 4.0 with 2.5N HCl) reflux twice for 30mins.

↓

Discard the precipitate and collect 250ml of extract

↓

Remove 0.5ml samples and dry in a vacuum oven at 500c and 700nm for 2hr.

↓

Dissolve dried extract in 4.75ml of acetone Add 0.25ml of $TiCl_4$

↓

Similarly treat standard (Chlorogenic acid + Acetone) with $TiCl_4$

↓

Read the color intensity 450nm against a blank

↓

Chlorogenic acid extracted with alcohol and dissolved in acetone. It is reacted with Titanium ion to form colored complex.

3.2.2.5 Determination of crude fibre content

The crude fibre content was determined by the method of A.O.A.C. (1970). Four grams of moisture and fat free sample was digested with 200 ml of 1.25 per cent sulphuric acid (H_2SO_4) for 30 minutes. The acid solution was decanted and the material was washed with hot water to remove the acid. The acid free residue was treated with 200 ml of 1.25 per cent sodium hydroxide (W/V) solution for 30 minutes. After decantation of top layer, solid material was filtered through previously weighed filter paper. The residue was made free from alkali by repeated washing with hot water and then washed with alcohol and finally with ether. The material was then dried in an oven at 100°C for five hours and weighed (W_e). The material was transferred to a crucible, heated in a muffle furnace (Make: INSIF) at 600°C for three hours, cooled and weighed again (W_a). The difference in weight (W_e-W_a) represents the weight of crude fibre.

$$\text{Crude fibre content (\%)} = \frac{(W_e - W_a)}{\text{Weight of leaf sample}} \times 100$$

The crude fibre content was expressed in percentage of moisture and fat free sample on dry weight basis.

2g sample + 2.5g Con. H_2SO_4 + 200ml D/W (acidic treatment)

↓

After boiling (30mins), the samples are filtered in a muslin cloth and washed it in D/W

↓

Drying sample extract + 2.5g NaOH pellets + 200ml D/W (alkaline treatment)

↓

Again boiling (30mins), the samples are filtered in a muslin cloth and washed it in D/W.

↓

The extra water is removed by washing alcohol and kept it in oven for 24hrs at 600c.

↓

The sample is weighted note down

↓

The acid and alkali treatment, oxidative hydrolytic degradation of the cellulose and degradation of lignin occur. The loss is weight gives the crude fiber content.

3.2.2.6 Determination of crude protein content

The total nitrogen content of leaf samples were first estimated using Micro-Kjeldahl method (A.O.A.C., 1970) and protein content was estimated from the total nitrogen by multiplying the per cent nitrogen content with 6.25.

Fifty milligram powdered leaf sample was transferred into a digestion tube and one gram of catalyst mixture (95 parts of K_2SO_4, 4.1 parts of HgO and 0.9 part of $CuSO_4$.) and two milliliter of concentrated sulphuric acid (H_2SO_4) was added. Digestion was carried on at 370°C until solution turned colourless. Six milliliter of Boric acid (4% w/v) solution was taken in a 100ml Erlenmeyer flask and 2-3 drops of indicator solution (one part 0.2 per cent methyl red in ethanol and five parts of 0.2 per cent bromocresol green in ethanol) was added to it. The flask was then placed under condenser with the tip extending below surface of the solution. The digest was transferred to distillation

apparatus and flask was rinsed four times with distilled water. Eight milliliter of sodium hydroxide-sodium thiosulphate solution (50 g NaOH with 5g $Na_2S_2O_3.5H_2O$ and volume was made up to 100 milliliter with distilled water) was added steam distillation was carried out until 20 milliliter of distillate collected and liberation of ammonia ceased. A blank was prepared using the same procedure but without the sample. The distillate was then titrated against 0.02 N hydrochloric acid. The total nitrogen was calculated out from the following relationship.

$$\text{Total nitrogen content (\%)} = \frac{(a - b) \times \text{Normality of HCl} \times 14.01 \times 100}{\text{Weight of leaf sample in milligram}}$$

Where,

a = ml HCl required for titration in sample and

b = ml HCl required for titration in blank

The value of total nitrogen content was multiplied by a factor 6.25 to attain the value of crude protein content and was expressed in percentage on dry weight basis.

3.2.2.7 Determination of ß-sitosterol content

ß-sitosterol content in the leaves of the muga silkworm host plants were estimated following the method of Katayama *et al.* (1974).

Ten grams of the leaf sample was taken in a round bottom flask of Soxhlet apparatus and 100 ml chloroform and 10 ml methanol was added to it. The material is then refluxed for 2 hours. After completion of refluxing, 2-5 ml of the extract was taken in test tubes which were evaporated in a boiling water bath. Then it was cooled and 2 ml of dried acetic anhydride was added followed by 2 drops of concentrated sulphuric acid. The tubes were kept standing for thirty minutes. Absorbance was measured at 655nm against a reagent blank in a spectrophotometer (PC based UV-Vis spectrophotometer

(Make: Systronics, Model: 2202). Sterol values were calculated by referring to the standard curve prepared with known concentration of ß-sitosterol.

2g sample taken in a round bottom flask of Soxhlet apparatus
↓
10ml Chloroform and 1ml methanol added to it and refluxed for 2 hrs.
↓
2ml of the extract was taken in test tubes and evaporated in a boiling water bath for 30mins.
↓
Cooled and 2ml of dried acetic anhydride was added followed by 2drops of conc. H_2SO_4
↓
After 30mins the absorbance was measured at 655nm against a reagent blank
↓
Sterol values were calculated by referring to the standard curve prepared with known conc. of β – sitosterol.

3.2.2.8 Determination of tannin content:

The tannin content in the leaf samples was estimated by the Folin-Denis method described by Schanderl (1970).

0.5 gram of powdered leaf sample was weighed and taken in a 250ml conical flask and 75ml distilled water was added to it. The flask was gently heated and boiled for 30 minutes. The solution was then centrifuged in a centrifuge (Research Centrifuge, Make: Remi, Model: R-24) at 2000 rpm for 20 minutes and the supernatant was collected in 100ml volumetric flask and volume made up to 100ml. Then, 1ml of the sample extract was transferred to a 100 ml volumetric flask containing 75ml water to which 5ml of Folin-Denis reagent, 10ml of sodium carbonate solution and diluted to 100ml with water. It was then shaken well and absorbance was read at 700nm in a PC based UV-Vis spectrophotometer (Make: Systronics, Model: 2202) after 30 minutes. The tannin content was estimated from a standard curve prepared with known concentration of tannic acid and was expressed as percentage on dry weight basis.

0.5g sample + 37.5 ml D/W and boiled for 30mins.

↓

Centrifuge at 2,000 rpm for 20mins

↓

Collect the supernatant in volumetric flask and make up the volume to 50 ml

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Transfer 0.5 ml of sample to 50 ml volumetric flask containing 37.5ml D/W.

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Add 2.5ml of Folin-Denis reagent + 5ml Na2CO3 and dilute to 50 ml D/W.

↓

Shake well and read the absorbance at 700nm against a reagent blank

↓

Tannin reduces phosphotungstomolybdic acid in alkaline medium to produce highly blue solution, the intensity of which is proportional to the amount of tannins.

3.2.2.9 Determination of lignin content:

Acid Detergent Lignin (ADL) content in the leaf samples were estimated by the AOAC method (1975). One gram of the leaf sample was taken in a round bottom flask with 100ml of acid detergent solution (20g of cetyl methyl ammonium bromide in one litre of 1N sulphuric acid). Heated to boil for 5 to 10 minutes and the heat is reduced to avoid foaming as boiling begins. The material was refluxed for 1 hour after the onset of boiling adjusting boiling to slow, even level. The container was then removed, swirled and the content was filtered through a pre-weighed sintered glass crucible (G-2) by suction and washed with hot water twice. The filtrate was then washed till it becomes colourless and dried at 100°C for overnight. The weight of material was weight after cooling in desiccators and this is the Acid Detergent Fibre expressed in percentage i.e. W/Sx100, where W is the weight of the fibre and S is the weight of the sample.

The ADF obtained from the previous method was transferred to a 100ml beaker with 25-50 ml of 72% sulphuric acid to which one gram asbestos was added. It was then allowed to stand for 3 hours with intermittent stirring with a glass rod.

The acid was then diluted with water and filtered with preweighed Whatman No 1 filter paper. The glass rod and the residue were washed several times to get rid of acids. The filter paper was then dried at 100°C and weight was taken after cooling in a desiccator. The filter paper was transferred to a pre-weighed silica crucible and it was ashed with the content in a muffle furnace (INSIF Make) at 550 °C for about 3 hours. The crucible was cooled in a desiccator and weighed. For blank, 1 g asbestos was taken in a similar manner.

ADL content was calculated using the following equation.

$$\% \text{ ADL} = \frac{\text{Wt. of 72\% H2SO4 Washed fibre (Test-Asbestos blank) - Ash (Test-Asbestos blank) x 100}}{\text{Weight of sample}}$$

3.2.2.10. Determination of trypsin inhibitor

The trypsin inhibitor content in the leaf samples was estimated by Kakade *et. al.* (1974)..

Principle:

The trypsin inhibitor (TI) activity by inhibiting the activity of trypsin. A synthetic substrate (BAPNA) was subjected to hydrolysis by trypsin to produce yellow coloured p-nitroanilide. The degree of inhibition by the extract of the yellow colour production was measured at 410nm.

Materials:

-30%Glacial Acetic Acid (v/v)

-*Substrate*: Benzyl-DL-Arginine-paranitroanilide (BAPNA). Completely dissolved 40mg BAPNA in 0.5mL of dimethyl sulphoxide and then make up to 100mL with Tris-Hcl buffer *p*H 8.2.

-*Tris-HCl Buffer pH 8.2*

Weighed 6.05g Tris (hydroxymethyl aminomethane) and 2.94g $CaCl_2.H_2O$, dissolved in 900 mL water, adjusted to pH 8.2 with dil HCl and made up to 1000 mL with distilled water.

-*Source of TI*

Extract of 0.5g sample in 25mL water by grinding in a prechilled mortar and pestle. Extract the ground sample in a refrigerator for 2-3h with occasional shaking for complete extraction of TI. Centrifuge the homogenate at 12,000 rpm for 20 min at 4-6 ^{0}C. Dilute 1 mL of the supernatant to 10mL with distilled water and use as TI source.

Procedure:

- ✓ Pipette out 0-1ml of extract in duplicate sets of test tubes, one to serve as endogenous (E) and the other test (T).
- ✓ Make up the volume to 2ml with Tris -HCl buffer (pH8.2) in the 'E' set.
- ✓ Make up the volume to 1ml in the 'T' set.
- ✓ Add 1ml trypsin solution to each tube in the T-set.
- ✓ Pipette out into a separate test tube 1ml of buffer and 1ml of trypsin solution for standard.
- ✓ Incubate all the tubes in a water bath at 37^{0}C.
- ✓ After a few minutes, add 2.5ml of substrate (1mg BAPNA-Benzoyl-DL-Arginine-Paranitro Anilide) to each tube.
- ✓ Allow the reaction by adding 0.5ml of 30% glacial acetic acid.
- ✓ Stop the reaction by adding 0.5 ml of 30% glacial acetic acid.
- ✓ Read the absorbance at 410 nm in a spectrophotometer.

Calculation:

Defining a trypsin unit as an A410 increases of 0.01 under the condition of the assay, the trypsin inhibitory activity is expressed in trypsin units inhibited (TUI) per milligram of dry sample and calculated as follows.

$$\text{TUI/mg sample} = \frac{[(A_r\,410 - A_s\,410) \times 100] / \text{ml diluted sample extract}}{(\text{mg sample} / \text{ml diluted sample extract})}$$

3.2.2.11. Determination of Phytic acid:

The Phytic acid content in the leaf samples was estimated by the TCA method described by Wheeler and Ferrel (1971). Phytic acid (phytate) is extracted with trichloroacetic acid and precipitate as ferric salt .The iron content of the precipitate is determined spectrophotometrically and phytate phosphorus content is calculated from this value assuming a content 4Fe/6P molecular ratio in the precipitate (I).

2 g sample + 30ml 3% TCA swirling by hand for 30mins in Erlenmeyer flask.

↓

Centrifuge the suspension and transfer a 5ml aliquot of the supernatant.

↓

Add 2ml of FeCl3 by blowing rapidly and heat the content for 30mins.

↓

Add 2 drops of 3%Na2SO4 in 3% TCA and continue heating.

↓

Centrifuge 10mins and becant the clear supernatant

↓

Wash the ppt. by dispersing well in 10ml 3% TCA, heat for 10mins and centrifuge

↓

Disperse the ppt. in 0.5ml of D/W and add 1.5ml of 1.5N NaOH

↓

Bring volume to 5ml and heat in boiling water bath for 30 min.

↓

Dissolve the ppt. with 10 ml 3.2 N HNO3 in to 50 ml volumetric flask

↓

Transfer 10 ml aliquot to another 50 ml volumetric flask and dilute to 20 ml.

↓

Add 10ml of 1.5M KSCN and dilute to volume

↓

Read color immediately at 480 nm, against a blank.

↓

For standard, 433 mg Fe $(NO3)_3$ + 100 ml D/W in volumetric flask

↓

Dilute 2.5 ml stock standard and make up to 250 ml

↓

Pipette out of this working standard into a series of 100 ml volumetric flask

↓

Transfer 10ml aliquot to another 50ml volumetric flask and dilute to 20 ml.

↓

Add 10 ml of 1.5M KSCN and dilute to volume

↓

Read color immediately at 480 nm, against a blank

The Phytic acid is extracted with TCA and ppt. as ferric salt. The iron and phytate phosphorus content of the ppt. is determined calorimetrically.

3.2.2.12. Determination of crude fat:

The crude fat content in the leaf samples was estimated by the Buddhi Katangodage (2009) method. Crude fat content was determined by extracting the fat

from the sample using a solvent, then determining the weight of the fat recovered. As lipids/fats are relatively non-polar molecules, they were pulled out of a sample using relatively non-polar solvents. With a non-polar solvent, only non-polar molecules in the sample dissolved while polar ones do not.

Equipments

- Analytical balance (at least 1 mg sensitivity).
- Electrical drying oven to be operated at 105°C± 1°C.
- Soxhlet extraction unit comprising:
  - Round bottom flask, 150 mL
  - Soxhlet extractor with 60 mL siphoning capacity and condenser.
  - Cellulose extraction thimbles (28 x80 mm)
  - Heat source, either electric heating mantle, or steam bath 100 mL beaker
  - Desiccator with silica gel desiccant

Reagents: Petroleum Ether (boiling point: 40-60°C)

Method

1. Weighed 2-3g of the dried food sample into extraction thimble.
2. Placed the thimble inside the Soxhlet Apparatus.
3. Connected a dry pre-weighted solvent flask beneath the apparatus and added required quantity of solvent and connect the condenser.
4. Adjusted the heating rate to give a condensation rate of 2-3 drops and extract for about 16hours.
5. After completing the extraction, removed the thimble and reclaim ether using the apparatus.
6. Completed the removal of ether on a boiling bath and dry flask at 105°C for 30 mins.
7. Cooled in a desiccator and weighed.

Calculation:

Crude Fat (% of Dry matter) = [(weight of fat (x)/ weight of sample (w)] x 100

3.3 Rearing performance of eri silkworms on different host plants during different seasons

Eri silkworm rearing on different *Ailanthus* species conducted following standard methodology of Central Muga Eri Research & Training Institute, Lahdoigarh. Rearing will be carried out maintaining three replications per treatment and one disease free layings (300 worms) per treatment per replication.

3.3.1. Eri silkworm rearing techniques:

Depending upon the nutritional requirements and micro-climatic conditions to be maintained during rearing, eri silkworm rearing was divided into two main phases, *viz.*, i) young age silkworm rearing (1st and 2nd instar larva) and ii) late age silkworm rearing (3rd to 5th instar larva).

Young age silkworm rearing:

Young age worms reared under good rearing conditions providing them with tender and good quality tender leaves as well as water soaked foam pad and paraffin paper were put on the rearing tray.

Late age silkworm rearing:

The late age worms consumed around 90 % leaves supplied during the entire larval period. The mature and semi-matured leaves depending upon the type of food plants. The feeding of dried, yellow and diseased leaves were avaoided.

The tray rearing technique was adopted in the present study. The system was slightly modified for *Ailanthus* species and Kesseru based on the experience during the study. During late stage, the larvae were provided with entire shoot of the *Ailanthus* and

Kesseru and three cross bar sticks made up of bamboo were provided for better aeration and maintenance of hygiene which was found to very much useful to reduce larval mortality in late stage.

Feeding and its frequency:

After collection, the leaves were washed in water and preserved in the leaf preservation chamber covering with wet gunny cloth / bag all around. The castor leaves provided without petiole in tray rearing. 4-5 feedings was given per day at regular intervals during the young age rearing. In late age rearing, increased feeding frequency to 5 feedings per day. In night times, excess leaves provided to fulfill the required consumption throughout the night.

Bed cleaning:

Only one cleaning was given during first stage, in second stage, two times bed cleaning given, one after first moult and another before second moult. Three bed cleanings resorted to during third and fourth stages, first after second moult, second in the middle and the third before third moult, similarly in between third and fourth stagesThe daily bed cleaning was done in fufth stage, preferably in the morning after one or two feedings.

Matured worm collection and mounting:

Collapsible plastic mountages were used for spinning of the cocoons.

Harvesting and assessment of cocoons:

Cocoons were harvested after 5-6 days of spinning in summer and 8-9 days in winter. The cocoons were sorted out into good, double, melted, stained, dead or inferior, cut or pierced cocoons. Good commercial cocoons were shifted and dried perfectly after the harvest.

3.3.2. Treatments for experiment:

Four host plants were selected for the study i.e. castor (*Ricinus communis*), kesseru (*Heteropanax fragrans*), borpat (*Ailanthus grandis*) and borkesseru (*Ailanthus excelsa*). Except castor, all three food plants are perennial in nature. Hence, six treatments were drawn to assess performance of different perennial food plants alone as well as in combination with castor. The castor was considered only during early two instars of rearing in the case of treatments involving perennial food plants with castor. The details of the treatments are as follows:

T_1: *A. grandis* (I-V instar larva)
T_2: Castor (I-II instar larva) + A. grandis (III-V instar larva)
T_3: A. excelsa (I-V instar larva)
T_4: Castor (I-II instar larva) + A. excelsa (III-V instar larva)
T_5: Kesseru (I-V instar larva)
T_6: Castor (I-II instar larva) + Kesseru (III-V) instar larva

3.3.3. Rearing Seasons of Eri Silkworms:

Eri silkworm can be reared throughout the year in North Eastern States of India. However, due to rapid climate changes in recent years, rearing schedule is badly affected during extreme summer season (June - August) and cold season (January-February). Hence, the attempts have been envisaged to find out the best combination of food plants which can be utilized even in the extreme climatic conditions as well as in favourable conditions to make the sector sustainable. Accordingly, the rearing was carried out during following seasons to evaluate the seasonal effects on rearing performance.

S1: January-February
S2: April-May
S3: July-August

S4: September-October

Further, rearing schedule was adjusted according to the grainage activities and availability of disease free layings (dfls).

3.3.4. Observation on the rearing, grainage and post-cocoon parameter:

The following indicators of rearing, grainage and post-cocoon parameters were recorded to assess the best combination of treatment.

3.3.4.1. Indicators of rearing performance

Larval duration: It is estimated as duration from date of hatching followed by brushing of larva till spinning (maturation) and expressed in days.

Mature larval weight: The weight of fully grown larva of 5^{th} instar prior spinning is considered as mature larval weight and expressed in gram (g) which was measured with electronic balance.

Single cocoon weight: The weight of the individual cocoon at the time of harvesting which is otherwise cumulative weight of eri cocoon shell and pupa and expressed in gram (g). It is one of the most important economic parameter.

Single shell weight: The empty shell weight of individual cocoon which is estimated after removing the pupa from eri cocoon and expressed in gram (g). It is the most important economic character for assessment of productivity.

Shell Ratio (%): It is the ratio between single shell weight and single cocoon weight and expressed in percentage.

$$\text{Shell ratio (\%)} = \frac{\text{Single shell weight (g)}}{\text{Single cocoon weight (g)}} \times 100$$

Effective rate of rearing (ERR): It indicates the effectives of rearing in terms of total cocoon harvest over total larvae brushed.

$$\text{Effective rate of rearing (\%)} = \frac{\text{Number of cocoon harvested}}{\text{Number of larvae brushed}} \times 100$$

Yield per 100 dfls: It is a commercially terminology and estimated as empty shell weight of cocoons harvested out of 100 dfls and expressed in terms of kg.

Yield per 100 dfls (Kg) = Good cocoon harvested/ dfl (no.) x single shell weight (g) x 100

Waste cocoon (%): It is the number of the stained, diseased and mutilated cocoons and expressed in percentage.

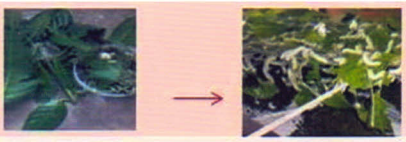
T_1	*Ailanthus grandis* (I-V)	
T_2	Castor (I-II) +*Ailanthus grandis* (III-V)	
T_3	*Ailanthus excelsa* (I-V)	
T_4	Castor (I-II) +*Ailanthus excelsa* (III-V)	
T_5	Kesseru (I-V)	
T_6	Castor (I-II) +Kesseru (III-V)	

Plate .7. Different treatments for assessment of rearing performance of eri silkworm feeding different food plants

3.3.4.2. Indicators of grainage performance

Moth emergence: Twenty five cocoons for each replication were preserved for the emergence of moths. The per cent moth emergence as reflected by the different treatments was computed by the formula.

$$\text{Moth emergence (\%)} = \frac{\text{Number of moth emerged}}{\text{Number of cocoons kept for moth emergence}} \times 100$$

The emerged, sexed moths were allowed to mate in bamboo trays. Males were separated from females after ensuring three hours of mating.

Fecundity: Moths from each treatment and replication were tied in Kharika and kept in the dark condition for egg laying. The eggs laid by individual females were counted and recorded separately.

Incubation and hatching: Incubation period was accounted for period from day of egg deposition until the day of hatching. The eggs laid by five females were collected and incubated at room temperature (24-26°C) till they hatched. Hatching percentage was calculated by the formula.

$$\text{Hatchability (\%)} = \frac{\text{Number of eggs hatched}}{\text{Total number of eggs laid by a female}} \times 100$$

Cocoon: dfls ratio: One dfl of eggs is expressed by average weight of 0.6 g which contains on an average 320-330 egg. Hence, it is estimated as number of cocoon required to produce one dfl.

3.3.4.3. Indicators of post-cocoon parameters

To assess the post-cocoon parameters and for degumming, the eri cocoons were tied in a perforated cotton cloth and boiled in a solution of mild soap and washing soda for 25 to 30 minutes with the following recipe.

Mild Soap – 2 gms/lts
Washing Soda – 5 gms/lts
Duration of Boiling- 25 to 30 minute
Temperature- Boiling temperature

After boiling is over the material was washed in plain water, then dried and made cakes individually. These dried eri silk cakes are then spun on pedel-cum- motorized spinning machine and parameters such as boil off loss, tenacity and elongation (%) were calculated.

$$\text{Boil off loss} = \frac{\text{Initial dry weight of cocoon- Final dry weight after degumming}}{\text{Initial dry weight of cocoon}} \times 100$$

The other parameters were tested at Regional Silk Technological Research Station, Khanapara, Guwaharti.

3.3.5 Statistical analysis

Data were analyzed statistically for test of significance using Fisher's method of "Analysis of variance" as outlined by Sundera Raj *et al.* (1972). The level of significance of 'F' test was tested at 5 per cent. The interpretation of the data was done using critical difference (CD) values calculated at $P < 0.05$.