Chapter - III

Materials and Methods

3. MATERIALS AND METHODS

3.1. Geographical location , soil and climate

Terai zone is situated between 25°57′and 27°N latitude and 88°25′E and 89 54 E longitude. This northern region of West Bengal is situated along the foot of Kurseong and Kalimpong hills and Bhutan hills in the north , Bihar border on the west and Assam border on the east . It includes Siliguri sub-division of Darjeeling district and entire district of Jalpaiguri and Cooch Behar and Islampur sub-division of North Dinajpur district . The total geographical area of the zone is 12025 sq.km. which is 13.5% of the state area with 9.7% of the state population . Rural population comprises more than 90% of the population of the zone.

The soil of this zone can be classified into two broad tracts (a) Old Himalayan piedment plains and (b) Teesta flood plain . Old Himalayan piedment plains spread along with northern part of the area from Kharibari- Phansidewa in the West to Kumargram in east covering of northern part of Siliguri sub-division and Jalpaiguri district. Soils are sandy loam to loam type, formed mostly from the Himalayan debris. This area has a dark brown top soil, 30cm-90cm deep, medium to strong acidic in nature and fairly rich in humus content . available N2 available P_2O_5 and available K_2O contents are low to medium. The teesta flood plain spreads along the southern portion of Siliguri sub-division and Jalpaiguri district and entire Coochbehar district. Soil texture vary from sandy to silky clay loam. Most soils of this zone have high fixing capacity for phosphorus. Soil is grilty and porous and poor in secondary micro nutrients.

The climate of the zone subtropical humid climate. Average annual rainfall, about 80% is received from south western monsoon during the rainy months of June to September. While the range of minimum temperature of the area is 7-8°C that the maximum temperature is 24-33.2°C . The relative humidity of the area at 8.30 A.M. is 58% to 87% respectively in March-July . The relative humidity in the afternoon at 17.30 hours is 48% to 81% respectively in March to November. On the whole the area has a humid and warm climate except having a short spell in winter , December to early February.

3.2.Experimental site

The experiments were conducted from 1994 to 1996 at the Sericulture Research Laboratory of Bidhan Chandra Krishi Viswavidyalaya (BCKV), North Bengal Campus , Pundibari , Dist. Cooch Behar , West Bengal .

3.3. Insect and food plant

The experimental insects were pure breeds and their hybrids as well as reciprocal of bivoltine races of the mulberry silkworm, *Bombyx mori* L. namely P5, KPGB , P5 x KPGB and KPGB x P5 eggs which were collected from Regional Sericulture Research Station (RSRS) , Central Silk Board (CSB) , Kalimpong , West Bengal , India. The experimental food plants were different varieties of mulberry , *Morus* sp., namely S1 , TR10 , TR4 , TR8 , C763, S799 , C776 and Kosen saplings which were collected from Central Sericultural Research and Training Institute (CSR&TI) , Behrampur , West Bengal , India and were raised in the Instructional Farm of BCKV , NB Campus , Pundibari . Preliminary screening of mulberry varieties was made taking 8 (eight) well-known mulberry varieties namely C776 , S779 , C763 , TR4 , TR8, TR10 , Kosen and S1 , leaves of which were fed to P5xKPGB hybrid of silkworm. Selection of superior quality mulberry varieties had undertaken among S1 , TR10 and Kosen from initial screening fed on all four silkworm breeds and hybrids. Then qualitative improvement of S1 variety using different levels of fertilizer has been undertaken , leaves of which were fed to all four silkworm breeds and hybrids. Combination of leaves as food were from S1 , TR10 and Kosen and consequent performance silkworm was from P5 x KPGB.

3.4. Rearing of Silkworm

The rearing was undertaken following the recommendation of Ullal and Narasimhanna (1987) with respect to incubation , hatching , brushing , feeding , bed cleaning , spacing and other rearing practices under natural environment in the rearing room , in three different seasons , namely spring (February-March) , Autumn (October-November) , and summer (May-June) .In each season ,eggs after hatching were reared following the recommended schedule till the

attainment of fifth instar larval stage which used as stock culture for different experiments . The experiment was conducted starting from the fifth larval instar immediately after the fourth moult.

In order to determine the consumption and utilization of mulberry leaves of different varieties and to evaluate efficiencies of conversion of consumed leaves , freshly ecdysed fifth instar larvae were taken from stock culture for both control and such of the treatment sets. Each treatment was replicated five times containing fifty larvae in each replication. Simultaneously, reserve batches of large number of larvae were maintained for both control and treatment sets in order to replace the dead larvae by healthy ones in case of necessity.

Reproductive potential of silkworm such as fecundity was recorded from the resultant twenty individuals of each of the treatments and control set.

3.4.1. Meteorological Data of Rearing Room

The maximum and minimum temperature and humidity were recorded all throughout the rearing period of different rearing seasons. Average temperature and humidity were calculated (Table 1).

Table -1 . Meteorological data of rearing room at all the rearing seasons.

Rearing season	Temp. (Max) (°C)	Temp. (Min) (°C)	Temp. Max. (Average of two yrs) (°C)	Temp. Min. Average.of two yrs. (°C)	RH. (%)	RH (%) (Average of two yrs)
Feb-Mar '94	25.42	22.21	25.54	22.73	81.63	78.34
'95	25.67	23.25			75.06	
Mar-Apr '94	26.05	24.25	26.74	24.68	70.35	69.20
'95	27.43	25.11			68.05	
May-Jun '94	29.69	27.09	28.89	26.79	85.85	84.82
'95	28.09	26.50			84.00	
Jun-Jul '94	31.37	28.71	30.89	28.34	84.34	84.15
'95	30.42	27.97			83.97	
Aug-Sep '94	30.34	28.86	27.67	25.43	82.87	82.78
'95	25.00	22.00			82.70	
Oct-Nov '94	25.25	22.21	25.22	21.99	79.49	80.30
'95	25.20	21.77			81.11	
Nov-Dec '94	19.50	17.75	19.98	17.89	76.80	77.68
'95	20.47	18.03			78.56	

3.4.2. Diet

The larvae were fed with the leaves of different varieties of mulberry , *Morus* sp. The varieties tested were S1 , TR10,Kosen , C763, C776, S779 , TR8 and TR4 grown with the recommended doses of fertilizer and culture practices . Leaves were plucked two months after each pruning.

In another experiment S1 variety was raised with the NPK fertilizer of 4 different proportions such as 40:20:20 kg (F1), 80:40:40 kg (F2) . 120:60:60 kg (F3) and 160:80:80 kg (F4) per hector. In the control field (F0) no fertilizer was applied. Each treatment was provided to the larvae right from the first instar and nutritional efficiencies were studied for the fifth instar.

In order to standardise the better performance of leaves of different varieties the larvae were fed with leaves of three varieties namely , Kosen , TR10 and S1 as mentioned above at two stages of larval growth namely chawki (up to third instar) and late stage (fourth and fifth instar) (Table 2) .

Table - 2. Combinations

	Early stage	Late stage
1.	S1	S1
2.	TR10	TR10
3.	Kosen	Kosen
4.	S1	TR10
5.	S1	Kosen
6.	Kosen	S1
7.	TR10	S1
8.	Kosen	TR10
9.	TR10	Kosen

The nutritional efficiencies at fifth instar as well as rearing performance for each case, were studied and suitable varietal combination and level of fertilizer at different stages of larval growth was determined.

3.5. Rearing Performance

Twenty five disease free laying (eggs laid by a single disease free female dfl) were reared in a mass from hatching upto the end of the third instar. After the fourth moult 1500 0-day-old fifth instar larvae were taken randomly from the mass culture for each treatment. These 1500 larvae were divided into five groups, group of 300 larvae was regarded as one replication. The larvae were reared on leaves of respective variety as well as respective nutrient levels , when the larvae became ready for spinning , these were transferred to spinning tray for the formation of cocoons. The cocoons were harvested on the sixth day from the onset of spinning , as this time gap is more than sufficient for the transformation of larvae into pupae.

From the view point of economic importance , the following parameters and their method of evaluation were considered :

1. Larval duration calculated from the date of hatching to the date of spinning (in days).
2. Weight of 10 mature larvae (g.).
3. Effective rate of rearing by number (ERR No.) which was calculated as

$$\text{ERR No} = \frac{\text{No. of cocoons harvested}}{\text{No. of larvae reared}} \times 10{,}000$$

4. Effective rate of rearing by weight (ERR wt.) which was calculated as

$$\text{ERR wt} = \frac{\text{Wt.of cocoons harvested (kg)}}{\text{No. of larvae reared}} \times 10{,}000$$

5. Single cocoon weight (g.).
6. Single shell weight (g.).
7. Shell ratio (SR%) which was determined as

$$\text{SR\%} = \frac{\text{Wt. of single shell}}{\text{Wt. of single cocoon}} \times 100$$

8. Absolute silk content (g) which was determined by
   ERR No. x Weight of single shell.

All the parameters were recorded on green weight basis. Twenty cocoons (10 males and 10 females) were taken for each of the three replications for assessment of quality of cocoon . All values of rearing results comprising rearing performance as well as quality of cocoons were subjected to suitable statistical analysis.

3.6. Consumption and Utilization of Food by the Fifth Instar Larvae

In the present investigation two bivoltine breeds and their two reciprocal hybrids namely P_5 , KPGB , P_5 x KPGB and KPGB x P_5 were the test insects. Three mulberry varieties , namely TR_{10} , S1 and Kosen were selected as superior varieties from an earlier screening of eight varieties,. In the subsequent experiment the larvae were reared on the leaves of the three selected varieties. Another experiment was carried out taking S1 as test variety where leaves of S1 variety were raised with four different doses of fertilizers (F1,F2,F3 and F4) and the control plants without fertilizer (F0).

The treatment were replicated thrice with twenty five (25) larvae in each replication. The experiment was conducted during three different seasons for standardization. Dead larvae on the rearing bed were replaced by healthy ones from stock culture on a few occasions only . The amount of dry matter ingested , digested and converted were determined by standard gravimetric methods (Waldbauer ,1968) The dry weights of the initial fifth instar larvae as wel as mature ones were estimated using the stock (larvae) culture from each treatment as per the method adopted by Horie and Watanabe (1983) . The indices used in the study were followed after Waldbauer (1968) and are given below :

3.6.1. Consumption and Growth

1. Consumption Index (C.I.) $= \frac{F}{TA}$

where , F = Dry weight of food ingested
T = Duration of feeding period (days)
A = mean dry weight of larvae during feeding period

2. Growth Rate (G.R.) $= \frac{G}{TA}$

where, G = Dry weight gain of larvae during feeding period
T = duration of feeding period (days)
A = mean dry weight of larvae during feeding period.

3.6.2. Digestibility and Efficiency of Conversion

1. Digestibility

The approximate digestibility (A.D.) was calculated as :

$$AD\ \% = \frac{\text{weight of food ingested - weight of faeces}}{\text{weight of food ingested}} \times 100$$

2.Conversion of ingested food

The efficiency of conversion of ingested food to the larval biomass (ECI %) which was calculated as --

$$ECI\ \% = \frac{\text{final wt. of larva - initial wt. of larva}}{\text{weight of food ingested}} \times 100$$

3. Conversion of digested food

The efficiency with which digested food is converted to larval biomass (ECD %) was calculated as :

$$ECD\ \% = \frac{\text{finalwt.of larva - initial wt.of larva}}{\text{weight of food ingested - weight of faeces}} \times 100$$

3.7. Reproductive Performance

The male and female pupae were kept separately . The moths after emergence in the morning (which is the usual time of emergence of *B.mori*) , were allowed to mate for three hours. Mating was arranged between the male and female moths of the same treatment . After depairing , females were alloowed to lay eggs separately on egg card covered by plastic cellule for egg laying for a period of 24 hours which was considered as the active oviposition period. The eggs of individual female moths were collected separately and counted.

3.8. Reeling characters of cocoon and filament characters of silk

The cocoons produced by the larvae of reserve batches maintained for different experiments under present investigation were used for examining the filament and reeling characters of cocoons . For each set of treatment ten (10) cocoons were collected at random and their floss (outer loose silk filaments) was removed . In order to kill the pupae and to avoide emergence of moth the cocoons were dried at $100^{o}c$ for about two hours. Furthermore , for softening the sericin coating of the fibre for easy separation from the compact shell the cocoons were treated with hot water successively in three basins maintaining different temperatures and for different durations. In the first basin , the cocoons were kept at $90^{o}C$ for 1 minute , in the second at $65^{o}C$ for 30 seconds and finally at $90^{o}C$ for 1-2 minutes (Krishnaswamy *et al* ., 1972) . After hot water treatment , the cocoons were transferred to a pot containing hot water of $50^{o}C$ and silk filament length using single cocoon reeling machine called " approvate ". The number of breaks of the filament during reeling was recorded . The entire silk filament thus collected was dried at $60^{o}C$ in an oven . The dry weight of the silk fibre and its length were considetred for determination of denier (thickness of the fibre) by gravimetric method using the formula :

$$\text{Denier} = \frac{\text{weight of the silk fibre}}{\text{total length of fibre}} \times 100$$

The data on filament length , denier and reelability were analysed statistically.These three characters were considered for assessing the quality of silk

3.9. Biochemical assay of mulberry leaves

Leaves at 4 to 8 position from the lip of a twig were taken as sample for biochemical assay.

3.9.1. Estimation of Total Protein

Estimation of protein was done following Lowry (1951) method.

Extraction of leaf tissue :

2.0 g of frozen plant tissue was ground in pre-cooled 0.2M Tris-HCl at PH 8.0 (10ml/g) in a glass morter with pestle at $0^{o}C$, centrifuged at 18,000rpm for 20 minutes at $0^{o}C$ and the supernatant was collected . Then 10 % ice-cold TCA was added to the cell free extract and made it double of its volume. The precipitate was

kept in cold (0-4^{o}C) condition overnight and then separated from supernatant fluid by centrifugation at 10,000 rpm for 20 minutes at 0^{o}C. The protein precipitate thus obtained was mixed with 20ml of 0.1(N) sodium hydroxide and was shaken until the TCA- precipitate protein was completely dissolved.

Method of estimation :

Reagent A = 2% Na_2CO_3 in 0.1N NaOH
Reagent B = 0.05% $CuSO_4$, $5H_2O$ in 1.0% Na-potassium tartarate
Reagent C = One volume of reagent B was mixed with 50 volumes of reagent A. The reagent was prepared immediately before use.
Reagent D = Folin-Ciocalteu reagent was diluted with equal volume of water.

1ml aliquot of diluted protein solution was taken in a test tube to which 4ml of reagent C was added. After 10 minutes 0.5 ml of reagent D was added and immediately mixed. After 20 minutes readings were taken in UV visible spectrophotometer at 660nm. BSA (Bovine Serum Albumin) was used as standard (1mg/ml) for the purpose of estimation.

Calculation was made in mg/ml and then converted to percentage.

3.9.2. Estimation of Total Carbohydrate

Total carbohydrate was determined by Anthrone method (Plummer ,1979)

Materials used :

1. 2.5N HCl

2. Anthrone reagent : 200mg anthrone was dissolved in 100ml of ice-cold 95% H_2SO_4 and this preparation was made before use.

3. Standard glucose : Working standard 100 ppm (10mg/10ml) [10mg of stand ard glucose was dissolved in 100ml distilled water and then stored in refrigerator after adding a few drops of toluene].

Procedure

1. 100mg of dried leaf sample was weighed in a boiling tube.

2. Leaf sample as above was hydrolysed by keeping it in a boiling water bath for three hours and 5ml of 2.5N HCl was added and cooled to room tem perature.

3. Hydrolysed sample was then neutralised in Na_2CO_3 until the effervescence ceases.

4. Volume was made upto 100ml and centrifuged.

5. Supernatant was collected and 0.5 and 1ml aliquotes were taken for analysis.

6. Working standard were prepared at 0, 0.2,0.4,0.6,0.8 and 1ml level.

7. All the tubes including sample tubes were made upto volume 1ml by adding distilled water.

8. Then 4ml of anthrone reagent was added to all the tubes.

9. All the tubes were heated for 8 minutes in a boiling water bath.

10. Rapid cooling was done till the colour changed into green to darkgreen and the content was evaluated in UV visible spectrophotometer at 630nm.

Calculation was made in mg/ml and then converted to percentage.

3.9.3. Determination of leaf moisture

This was done with a very little modification of the earlier procedure of Paul *et al*.(1992). Fresh leaves were weighed, oven-dried at 60^0C for more than 48 hours till the dry weight became constant. Percentage of moisture in leaves were calculated from the differences in the two weights.

3.10. Statistical analysis

The data of leaf yield and leaf nutrient content obtained were tabulated and analysed by using Randomised Block Design (RBD). The completely randomised design of three factors were employed for rearing parameters and cocoon yield analysis. Where as the Completely Randomised Block Design of two factors was set for cocoon quality assessment . Correlation studies were also worked out between different rearing parameters, nutritional efficiencies and leaf quality .Regression analysis of a few important parameters was carried out using the formula $Y = a + b_1 x_1 + b_2 x_2 + b_3 x_3 + \ldots + b_n x_n$. For measuring the good ness of fit and testing of linearty, the coefficient of determination (r^2) was used.