3 MATERIALS AND METHODS

3.1 SELECTION OF MATERIAL

The insect silkworm *Bombyx mori* (L.) is selected for this study due to the following reasons:

1. It is domesticated species.
2. The insects are beneficial and at the same time harmless to human being and other living organisms.
3. They complete their lifecycle in very short time of about 50 days.
4. In III, IV and V instar stages that are sufficiently large suitable for experimental studies.
5. It is remarkable for its low investment and quick and high returns.
6. Rearing of silkworm is so labour oriented, producing more employment opportunity and
7. They could be reared under laboratory conditions and the worms are bigger in size and the tissues are suitable for biochemical estimation and also for experimental purposes.

3.2 COLLECTION OF SPECIMEN

The first day of III instar silkworm larvae $L \times NB_4 D_2$ (local Biovlfine) race were collected from silkworm culture centre at Agraharam, Salem and Neyveli and Thanjavore in Tamil Nadu and they were maintained upto cocoon.
3.3 TRANSPORTATION OF THE SPECIMEN

The collected larvae were carried to the laboratory and maintained in separate aerated plastic containers along with selected mulberry leaves (MR2) dipped in known quantity (1 g solution) of natural dye, so as to avoid any damage or injury to the developing larvae (Khawaja, 1989).

The larvae transported from Salem and Neyveli and Thanjavore were transferred to bamboo baskets of size 26 cm diameter and 5 cm height as described by Govindan et al. (1981). The bamboo baskets were covered with paraffin paper and placed in an iron stand with ant wells. The larvae were reared simultaneously both in control and experimental groups separately on mulberry leaves dipped in natural dye solution in the laboratory. Proper environmental conditions provided to the silkworms with photoperiod of 12:12 h light and darkness as recommended by Krishnaswamy et al. (1973).

<table>
<thead>
<tr>
<th>Instar</th>
<th>Temperature (°C)</th>
<th>Relative humidity (%)</th>
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<tbody>
<tr>
<td>III</td>
<td>25-27</td>
<td>75-80</td>
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<tr>
<td>IV</td>
<td>25-26</td>
<td>70-75</td>
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<td>V</td>
<td>23-25</td>
<td>65-70</td>
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3.4 REARING TECHNIQUES

3.4.1 Mulberry (MR2 variety)

This is one of the varieties selected from Coonoor Sericulture Farm. Branches are simple, vertical, grayish leaves are dark green, unlobed, elliptic palmately veined, leathery/smooth/wrinkled. It has good agronomic characters like high rooting ability (80%) (Fig. 2).

3.4.2 MR2 leaves treated with natural dye

Fresh mulberry leaves were soaked in different concentration on (1 g, 2 g, 4 g, 8 g) of indigo vegetable dye dissolve in 100 ml of distilled water leaves were dipped in each concentration for 15 minutes and then were dried in air for 10 minutes. The treated leaves were used for the feeding the larvae of silkworms in Fig. 2.

3.4.3 Natural dye

Vegetable dyes are obtained from various parts of plants and herbs such as stem, wood, root, bark, leaf, flower, fruits and seeds etc. The prominent among them are indigo (*Indigofera tinctoria* L.), *Isatis tinctoria* L., turmeric (*Curcuma longa*), *Lawsonia inermis* L., Morinda (*Morinda citrifolia* L.), Maddar (*Rubia tinctorum* L.), elephant apple (*Dilhenia indica*), etc. Some of the most important dyes derived
from insect and animal sources such as lac (Laccifer lacca Kerr.), Kermes (Kermococcus ilicis Linn.), Cochneal (Dactylopins coccus Costa), lichen, etc. Some of the well-known ancient dyes include madder, a red dye made from the roots of the Rubia tinctorum L., blue indigo from the leaves of Indigofera tinctoria L., yellow from the stigmas of the saffron plant (Crocus sativus L.) and from turmeric (Curcuma longa L.).

Siva and Krishnamurthy (2005) studies an important dye-yielding plant, B. orellena for understanding the relationship between degree of genetic diversity of various populations and their pigment content. Natural dyes produce very uncommon soothing and soft shade as compared to synthetic dyes. On the other hand, synthetic dyes are widely available at an economical price and produce a wide variety of colours; these dyes however produce skin allergy, toxic wastes and other harmfulness to human body.

Saidman et al. (2005), Gulrajani et al. (1992) and mohanty have reviewed the chemistry, chemical composition and chemical based classification of natural dyes having anthroanione (Madder), alpha napthoqniones (Henna), flavones (Weld), Indigoids (Indigo and tyrian purple), carotenoids (annatto saffron), etc., which give a basic understanding of chemical nature of such colourants, natural dyes obtained from turmeric has been studied and reported by Agarwal et al. (1992).
3.5 PRECAUTIONS TO BE TAKEN DURING THE REARING OF MULBERRY SILKWORMS

i. The worms should never be kept overcrowded in a tray

ii. Dried or dusty wet leaves should never be fed to the worms

iii. No doubt, free ventilation is a must but, wind should not be allowed directly over the worms.

iv. There should be equal distribution of leaves among the worms.

v. Worms which are under process of moulting should not be disturbed, otherwise they may die or moulting may be delayed.

vi. There should be no dust at the floor of the house. For this it should be well plastered with cow dung or mud at regular intervals.

vii. It is very hot, drinking water may be sprinkled over the feeding trays.

viii. Smoking should be strictly prohibited in the rearing room.

ix. Worms should not be handled with dirty hands, otherwise they may get diseased. They should be handled only after washing the hands thoroughly with antiseptic solutions and after drying the hands.

x. One should enter the rearing house only after puffing off the shoes, chappals out.
3.6 PREPARATION OF TISSUE SAMPLES

MR$_2$ and mulberry leaves treated with natural dye fed larvae III, IV and V instarts were vivisected in insect Ringer's solution (Ephurussi and Beadle, 1936). Dissected fat body and silk gland were fixed by immersion in Bouvin’s solution or 10% formalin.

3.7 HISTOLOGY

After 24 h of fixation, the tissues were processed for dehydration using ascending grades of alcohol (Gurr, 1958). The tissues were gross stained in 70% aqueous eosin to facilitates orientation during embedding. The tissues after dehydration in absolute alcohol and acetone were cleared in xylol and finally embedded in Paraffin wax (58-62°C). Sections were cut at 6 μ thickness were deparaffinized using ascending grades of alcohol and stained with haematoxylin and counter stained with aqueous eosin for microscopical observations and microphotographs were taken.

3.8 STUDIES (SCANNING ELECTRON MICROSCOPIC STUDIES)

Scanning electron microscopy is used primarily for the study of surface topography of solid materials (Fig. A). It permits a depth of field for greater than optical and transmission electron microscopy.
The resolution of the scanning electron microscope is about 3 nm (30 Å), approximately two orders of magnitude greater than the optical microscope and one order of magnitude less than the transmission electron microscope.

The SEM uses electrons instead of light from an image. A beam of electrons is produced at the top of the microscope by heating of a metallic filament and the commonly used filament is tungsten, hairpin gun. Electron beam follow a vertical path through the column of the microscope. It makes its way through electronmagnetic lenses which focus and direct the beam down towards the sample. Once it hits the sample, other electrons (backscattered on secondary) are ejected from the sample. Detectors collect the secondary or back scattered electron and convert them to a signal that is sent to a viewing screen producing an image.

Since the SEM uses electrons to produce an image. Most conventional SEMs require that the samples be electrically conductive (Woodward, 1972). All metals are conductive and require no preparation to be viewed using a SEM. In order to view non-conductive samples such as ceramics a plastics, the sample must be covered with a thin layer of a conductive material. Using a small device called a sputter coater. It coats the sample with gold atoms. The purpose is to make non-metallic samples electrically conductive.
3.9. QUALITATIVE ESTIMATION OF AMINO ACIDS (HPLC)

Principle

The peptides with N-terminal primary amines get derivated try ortho-phthalaldehyde. The advantages of the OPA derivatives are (i) enhanced sensitivity and (ii) could be detected in fluorescence detected without interference.

Mobile phase A

A stock solution of ethylene-diaminotera acetic acid (EDTA) was made by dissolving 4.0 g EDTA, disodium salt (dehydrate) in 100 ml of water. This was stored in a refrigerator. Sodium acetate trihydrate (NaOAc) (2.72 g), EDTA stock (1 ml) and sequence grade triethyleamine (TEA) (180 ml) were added to 1000 ml of HPLC grade water. The pH was adjusted to 7.20 ± 0.05 with 1-2% acetic acid. After pH adjustment, 3.0 ml of unsterilized. UV-grade tetrahydrafuran (THF) was added and the pH was rechecked.

Mobile phase B

Sodium acetate trihydrate (2.72 g) was dissolved in 200 ml of HPLC grade water. The pH was adjusted to 7.20 ± 0.05 with 1-2% acetic acid. Methanol (400 ml) and acetonitrile (400 ml) were added and mixed well.
**OPA regent**

This reagent was prepared by dissolving O-phthaldehyde (OPA) (50 mg) in 2 ml of methanol. To this, 8 ml of borate and 50 ml of 2-mercaptoethanol was added kept in dark.

**Preparation of samples**

To analyze the amino acid composition, the samples were treated with chloroform; methanol (R: 1 v/v) to remove the lipids. The samples were transferred to test tubes and kept in a hot oven for 2 h at 40°C. The samples were then centrifuged at 3000 × g for 10 minutes. This process was repeated was added to this sample and once again kept in hot air oven for 1 h at 40°C. The solvent was decanted and the sample was allowed to precipitate. Then the samples were subjected to acid hydrolysis. The derived samples were treated with 6 N HCl at 110°C for 5 h. The acid hydrolysate was dried using speed vac concentrates (Savant, USA). The amino acid samples were dissolved in 50 ml of HPLC grade water. All the reagents used were HPLC grade.

**HPLC analysis**

Twenty microlitres of the filtered, derivated amino acid sample was infected into a C–18 reverse phase column and analyzed using sodium acetate buffer with tetrahydrofuran and triethylamine and sodium acetate with methanol as solvent systems. The amino acid were identified by comparing their retention time (RE) with the standard amino acids run at identical condition.
Procedure

Derivatization of amino acid sample

One ml of OPA reagent was added to each vial containing 200 ml of amino acid sample. The samples were mixed well and kept for 2 minutes for derivatisation. The sample were filtered and injected at the rate of 20 μl in the HPLC for amino acid analysis.

Spectral analysis

For the present research, the following instruments were used to analyze the samples. Scanning Electron Microscope (SEM of JSM –5610 series) to study the morphology and the particle size and to study the elements shown in Figure.