CHAPTER - 3

MATERIALS AND METHODS
3. MATERIALS AND METHODS

3.1 MATERIALS

The present investigation includes four non-tuberous Solanum types. The taxonomic status and distinctiveness of these Solanum types are not yet conclusively established. Therefore, these types are temporarily designated as Type-A, Type-B, Type-C, and Type-D. During the investigation, evidences from histological, cytological and palynological studies along with the classical taxonomic studies were thoroughly evaluated for their probable role in establishing the appropriate taxonomic status and distinctiveness of these Solanum types.

As already mentioned under section “Introduction and Aim of the investigation”, only the morphological features of the fruits are considered for categorization of these Solanum into four types (i.e. Type A, Type B, Type C and Type D).

3.1.1 Collection of materials

Since the fruits of the Solanum types (of the present investigation) are taken by the tribal people of the North-Eastern region of India in different forms in their diet, a thorough survey was undertaken to ascertain the availability of these Solanum types in areas inhabited by tribal folk. Consequently, plant materials were collected from those areas. The collected plant materials include both semi-ripen and ripen fruits as well as flowering twigs. Flowering twigs were made into herbarium sheets and were preserved as voucher specimens. Fruits were also kept preserved. Field notes were maintained during survey. Seeds from the collected fruits were germinated in the laboratory and subsequently seedlings and plants were raised in the experimental garden of Botany Department, Cotton College, Guwahati. These plants were utilized during further studies.

Data on ecological conditions of the habitat were also recorded. This includes types of soil, prevailing light and temperature, occurrence of rainfall, altitude etc.
During survey ethnobotanical uses of these four *Solanum* types were ascertained from the local people.

### 3.2 METHODS

#### 3.2.1 Classical taxonomic study

Morphological characters of taxonomic importance of each *Solanum* type were recorded from live specimens. Line drawings and photographs were prepared as usual. Experimental plants were classified according to Bentham and Hooker’s system.

#### 3.2.2 Micromorphological investigation

Micromorphological investigations included studies on leaf epidermis and leaf architecture.

##### 3.2.2.1 Studies on Leaf epidermis

The leaf epidermis was thoroughly investigated for details on stomata and epidermal hairs. For this purpose, temporary slides were prepared as follows: epidermal peels of both upper and lower surfaces from tip, middle and basal parts of mature leaves were taken out either mechanically or by controlled maceration using a 10% aqueous solution of nitric acid following the technique of Boulos and Beakbane (1971). The peels were stained with 1% aqueous solution of safranin and mounted in 50% glycerine. Camera lucida drawings and photographs were taken from the temporary slides.

The nature and distribution of stomatal guard cells, subsidiary cells and epidermal hairs were studied. Frequency of epidermal cells, nature of epidermal cell wall, stomatal types, stomatal frequency, stomatal index, stomatal area, absolute number of stomata, size, types and distribution of trichomes were worked out. Different parameters for calculating quantitative values of epidermal features were worked out as follows:
• **Stomatal index (I)** = \( \frac{S}{E + S} \times 100 \)

Where ‘S’ is the number of stomata per unit area and ‘E’ is the number of epidermal cells in the same unit area (Salisbury, 1927).

• **Absolute stomatal number in thousands** = \( \frac{A \times S}{1000} \)

Where ‘A’ is the total leaf area in mm\(^2\) and ‘S’ is the stomatal number per unit area (i.e. 1 sq.mm.) (Gupta, 1961).

• **Stomatal Area** = Area occupied by single stomata (in mm\(^2\))
  \( \times \) Stomatal frequency (in mm\(^2\)) \( \times \) Total leaf area (in mm\(^2\))

In order to standardise observations and measurements, the mean values of five readings were taken every time. Drawings of stomata and epidermal hairs were made from an intercostal area near the centre of the lamina.

In describing the epidermal features, terminology suggested by Stace (1984) for epidermal cells was followed. The stomata were classified and described as suggested by Rajagopal and Ramayya (1981). Microphotographs were taken with the help of Olympus B x 50 F4 Camera.

**3.2.2.2 Studies on leaf architecture**

To study the leaf architecture, slides were prepared as follows:

Mature leaves from both fresh and herbarium materials were cleared following the technique of Barsier and Bucquet (1960) with slight modifications. Materials were first treated with 5% aqueous solution of Sodium hydroxide in an oven at 37\(^\circ\)-40\(^\circ\)C for over-night. They were then transferred to a mixture of saturated aqueous solution of chloral hydrate and hydrogen peroxide (1:1) for 30 minutes to 1 hour, and then washed with distilled water. The material was then placed in gradual descending series of 75cc aqueous chloral hydrate solution + 25cc absolute ethyl alcohol for 20-30 minutes till the green colour of the leaf material was completely
removed, 50cc aqueous chloral hydrate solution + 50cc absolute ethyl alcohol for 20 minutes till the yellow colour was removed and then 25cc aqueous chloral hydrate solution + 75cc absolute ethyl alcohol for 20 minutes till the lamina became colourless. Staining was done with safranin dissolved in a mixture of absolute ethyl alcohol and xylene (1:1) for 5 minutes; then washed with absolute alcohol to remove excess safranin and finally mounted in canada balsam as usual.

Camera lucida drawings were made and micro-photographs were taken from the slides as usual. Various features of primary, secondary, tertiary and higher order venation were studied and recorded. The area of areoles and numbers of vein-endings and vein-tips were measured and counted at various places of the lamina. To standardise observations and measurements, the mean values of five readings were taken.

In describing venation pattern, the terminology suggested by Hicky (1973) have been followed with slight modification (Paliwal and Harjal, 1981). For the calculation of absolute vein-islet number and absolute vein termination number, Gupta’s formulæ (1961) were used which are as follows:

\[
\text{Absolute vein-islet number per sq. mm.} = \text{Average vein-islet number per mm}^2 \times \text{Area of the lamina in mm}^2.
\]

\[
\text{Absolute vein-let termination number per sq. mm.} = \text{Average vein-let termination number per mm}^2 \times \text{Area of the lamina in mm}^2.
\]

3.2.3. Cytological investigation

Cytological investigations were made in *Solanum* Type A, Type B, Type C, and Type D.

For chromosomal analysis of the plant materials, detail karyotypic studies were undertaken and karyotypes were prepared from somatic chromosomes.
Collection of root tips and squash preparation

For the purpose of karyotypic studies, healthy root tips of about 0.2 - 0.3 cm. long were collected between 10 AM to 11 AM from seedlings grown in earthen pots. The root tips were washed thoroughly with distilled water and then pretreated with 1% colchicine solution. The pre-treatment and fixing periods as well as treatment temperatures were previously standardized through trial and error method.

After pretreatment for an hour, root tips were washed thoroughly with distilled water to remove the residual effects of colchicine if any. Then the root tips were fixed in Carnoy's Fluid - I (1:3 glacial acetic acid and ethanol, v/v) for 2 hours and finally transferred to freshly prepared 70% ethanol for preservation. Root tips of every set of plants were then used for squash preparation.

Pretreated root tips were hydrolysed with 1N HCl at 50° - 60° C, kept for 10 - 15 minutes and then rinsed in distilled water for several times. After hydrolysis, the root tips were stained with 2% aceto-orcein and kept for 1 hour. The meristematic parts were cut out and transferred to a drop of 45% glacial acetic acid on a grease free clean slide. The meristematic cells were then pressed to form a cell suspension; covered with a clean cover glass and squashed after gentle heating and finally sealed with paraffin.

The temporary slides thus prepared were observed under compound microscope at 15x X 45x and 15x X 100x (oil immersion). Well-scattered metaphase plates were selected for karyomorphological analysis. Five such slides in each type were considered for the purpose. Perfectly stained chromosomes were photographed using Leitz microphotographic equipment. Camera Lucida diagrams of chromosomes were also made as usual. The lengths of the individual chromosomes were measured from temporary slide. Idiograms were then constructed on tracing paper.

Total chromosome length was measured by adding the length of all the chromosomes in the karyotype. Arm ratio 'R' of each chromosome was calculated as:

\[ R = \frac{\text{Length of the long arm (L)}}{\text{Length of the short arm (S)}} \]
The relative length of the chromosome represents the ratio in percentage of the length of the individual chromosome to the total chromatin length of the diploid set (Khosla and Sobati, 1985); thus:

\[
\text{Relative chromosome length} = \frac{\text{Length of the individual chromosome}}{\text{Total chromatin length of the diploid set}} \times 100
\]

On the basis of total length, the chromosomes of different types of *Solanum* understudy were classified into the following categories:

- **Category A**: Chromosomes with length 5.00 μm and above
- **Category B**: Chromosomes with length 4.00 μm to 4.99 μm
- **Category C**: Chromosomes with length 3.00 μm to 3.99 μm

Location of the centromere of the chromosome was expressed as a percentage of ratio between the arms and was calculated as a centromeric index or F% (Denver report 1960, Levan *et al.*, 1964), thus:

\[
F% = \frac{\text{Length of the short arm}}{\text{Total length of the chromosome}} \times 100
\]

Total form per cent or TF % was calculated by the formula suggested by Huziwara (1962).

\[
\text{TF} \% = \frac{\text{Total sum of short arm length}}{\text{Total sum of Chromosome length}} \times 100
\]

On the basis of centromere position, the chromosomes were classified into metacentric, submetacentric, and sub-telocentric chromosomes following the nomenclature system of Levan *et al.*, (1964).

3.2.4. Palynological studies

For palynological studies, polleniferous materials were collected from anthers of living plants. Samples for compound microscopic study were acetolysed and prepared for by following Erdtman (1952, 1960) and Nair’s (1961) method.
The procedure is described as follows:

Isolated mature anthers selected from fresh flowers were dipped in 70% ethanol for about an hour in polythene tubes. After crushing the anthers the suspension was poured through a brass metal net into centrifugal tubes and centrifuged for about 3 minutes and the alcohol was decanted. The sediment was then washed with glacial acetic acid and crushed again in glacial acetic acid and poured through net to centrifugal tubes. The centrifugal tubes containing the polleniferous material were then centrifuged and the acid was decanted off. The acetolysis mixture was prepared with 9cc. acetic anhydride and 1cc. conc. sulphuric acid. 5cc. of the freshly prepared acetolysis mixture was then added to the sediment gently. The tubes containing pollen grains then transferred to water bath and warmed up to boiling point. The tubes were kept for another 10 minutes in the water bath itself to slowly cool down. The centrifugal tubes were then placed in ice-cold water. After centrifuging again for a minute the supernatant was decanted and the sediment was washed again with glacial acetic acid and centrifuged. The glacial acetic acid was finally decanted and the residue was washed with distilled water, centrifuged and supernatant was discarded. Microscopic slides of the pollen grains were prepared in glycerin-jelly. Photomicrographs were prepared using Olympus B X 50 F4 camera. The size of the pollen grains was measured following the method proposed by Pandey (1968). Measurements were recorded with the help of ocular and stage micrometer (in \( \mu \text{m} \)) for each investigated Solanum type. An average for each type was calculated for respective length and breadth, P/E ratio was calculated.

For Scanning Electron Microscopic (SEM) studies, pollens of each Solanum type were placed on a double stick tape without chemical treatment and coated with gold palladium. The pollens were examined with a Jeol Scanning Electron microscope, JSM-35 CF at the Regional Sophisticated Instrumentation Centre (RSIC) of North Eastern Hill University, Bijni Complex, Shillong-3, Meghalaya.
3.2.5. Determination of Solasodine Content:

Solasodine of each *Solanum* type under study was extracted and its content was measured spectrophotometrically. The details of the procedure was as follows:

**Extraction and Estimation of Solasodine:**

Ripe berries of different *Solanum* types understudy were harvested and dried in a hot-air oven at a constant temperature of 60°C and also sun dried. Completely dried berries were then ground to powder using an electric mixture grinder.

**Extraction:** 2 gms of weighed powdered sample of fruits of each type were extracted with 20 ml of ethanol and left overnight, shaking at regular intervals. Then, filtered and the residue was washed with 5 ml of 70% \( \text{CH}_3 \text{COOH} \) twice and filtered again. The filtrate was taken in a conical flask and heated up to 70°C using a sand bath. The solution was evaporated to half of its volume so that ethanol content was completely removed. The solution was then defatted with petroleum ether followed by separating it from ether part, and boiled. At warm condition excess of 70% \( \text{NH}_4(\text{OH})_2 \) was added to the crude solution and left overnight in a refrigerator. After that it was filtered and the precipitate was dissolved in hot ethanol. Filtered again and evaporated to dryness to get crude Solasodine crystals.

**Purification:** Crude solasodine thus isolated was then dissolved in 3% methanolic-HCl (about 10 ml), warmed and allowed to cool (keeping for at least 3 hours) and then filtered. The precipitate, which was crude Solasodine hydrochloride was suspended in hot water and basified with 10% liquid ammonia under constant stirring, boiled and again cooled and filtered. The precipitate was again washed in distilled water (where the crystals were sparingly soluble) and dried to yield white shining orthorhombic needle like or leaflet like crystals of Solasodine. The crystals were finally dissolved in 10 ml of 4:1:5 methanol, acetic acid and distilled water solution for test of Solasodine and spectrophotometric analysis.

**Test of Solasodine:** Prior to spectrophotometric analysis the crude Solasodine was tested for its characteristic colour reaction. To 1 ml of crude solasodine solution,
2 ml of Conc. H₂SO₄ was added carefully and slowly by the side of the test tube over 2 minutes time with continuous stirring. Cooled in ice-cold water and 1 ml of 1% formaldehyde solution was added very slowly keeping continuous stirring. Left the solution for 30 - 45 minutes in ice-cold water. As a result of addition of Marquis reagent (2:1 conc. H₂SO₄ and 1% formaldehyde solution) characteristic crimson red or pink colour develops and thus confirmed the presence of Solasodine.

_Spectrophotometric analysis:_ The solution was finally analysed for its characteristic absorbances at 670 nm using ELICO SL - 171 spectrophotometer. For preparation of standard curve, authentic Solasodine collected from SIGMA was also treated in the same way using the same solvent combinations. Readings were analysed on the same day.