CHAPTER 3.

MATERIAL AND METHODS
3.1. Collection of experimental plant materials

The genus *Ipomoea* L. is ubiquitous confining mainly to the tropics and possesses medicinal properties. Some species of the genus are taken as vegetables. The present investigation included thirteen species of *Ipomoea* L. including two variants of *Ipomoea aquatica* (variant-I, with broad leaves; variant-II, with narrow leaves) and two variants of *Ipomoea carica* (variant-I, with narrow leaves; variant-II, with broad leaves). These different species were collected from various places of Assam as shown below:

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Plant species</th>
<th>Place of collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Ipomoea pes-tigridis</em> Linn.</td>
<td>Tezpur, Guwahati</td>
</tr>
<tr>
<td>2.</td>
<td><em>Ipomoea mauritiana</em> Jacq.</td>
<td>Guwahati</td>
</tr>
<tr>
<td>3.</td>
<td><em>Ipomoea fistulosa</em> Mart ex Choisy</td>
<td>Guwahati</td>
</tr>
<tr>
<td>4.</td>
<td><em>Ipomoea obscura</em> (Linn.) Ker- Gawl</td>
<td>Guwahati, Nagaon, Hojai</td>
</tr>
<tr>
<td>5.</td>
<td><em>Ipomoea nil</em> (Linn.) Roth.</td>
<td>Jorabat, Sivasagar</td>
</tr>
<tr>
<td>6.</td>
<td><em>Ipomoea indica</em> (Burm.f.) Merr.</td>
<td>Guwahati</td>
</tr>
<tr>
<td>7.</td>
<td><em>Ipomoea aquatica</em> Forsk.</td>
<td>Guwahati</td>
</tr>
<tr>
<td></td>
<td>(Variant-I; broad leaved)</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td><em>Ipomoea aquatica</em> Forsk.</td>
<td>Guwahati, Nagaon, Hojai</td>
</tr>
<tr>
<td></td>
<td>(Variant-II; narrow leaved)</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td><em>Ipomoea calycina</em> Benth.</td>
<td>Guwahati, Bongaigaon</td>
</tr>
<tr>
<td>10.</td>
<td><em>Ipomoea carica</em> (Linn.)Sweet.</td>
<td>Guwahati, Nagaon, Cachar</td>
</tr>
<tr>
<td></td>
<td>(Variant-I; narrow leaved)</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td><em>Ipomoea carica</em> (Linn.)Sweet.</td>
<td>Guwahati</td>
</tr>
<tr>
<td></td>
<td>(Variant-II; broad leaved)</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td><em>Ipomoea macrantha</em> Roem &amp; Schult.</td>
<td>Guwahati</td>
</tr>
<tr>
<td>13.</td>
<td><em>Ipomoea purpurea</em> (Linn.) Roth.</td>
<td>Guwahati, Cachar</td>
</tr>
</tbody>
</table>
Fig. No. 1. Geographical map of Assam showing the distribution of experimental species of *Ipomoea* L.

Plate I
Materials and methods

The distribution of the experimental species of *Ipomoea* L. in Assam is shown in the Fig. No. 1.

Seedlings of collected plants were raised in earthen pots to facilitate harvesting of root tips. Taxonomical investigations and identifications were carried out following Bentham and Hooker (1883), Hooker (1885), Kanjilal and Das (1939), Ghosh and Sikdar (1983), Mathew (1983). The herbaria of Department of Botany, Gauhati University, and Botanical Survey of India, Shillong, (Meghalaya, India) were consulted for the purpose of identification.

3.2. Cytological investigation

All cytological investigations pertaining to the chromosome characterization were carried out in the meristematic cells of the root tips.

3.2.1. Collection of root tips

For karyomorphological investigations, healthy root tips with active cell divisions were collected between 5.30 am to 6.30 am from the potted plants. The excised root tips were pre-treated with saturated solution of *para*-Dichloro benzene for 3 hours.

After the pre-treatment the root tips were washed thoroughly in running water and then transferred to Carnoy’s fluid-1, (1:3 glacial acetic acid and ethanol) and kept for 24 hours for fixation. After fixation the root tips were transferred to 70 per cent ethyl alcohol for preservation. Root tips were also directly fixed in Carnoy’s fluid-I without pre-treatment with *para*-Dichlorobenzene.

This procedure without pre-treatment had an advantage over the method involving pre-treatment as the cells with chromosomes were observed in a chemical free zone (i.e. they escape chemical treatment) producing clear picture in microphotographs, which were otherwise found in hazy form in pre-treated materials.
Materials and methods

Therefore, root tips without pre-treating with para-Dichlorobenzene were used throughout the investigation.

3.2.2. Preparation of slides

During the preparation of the slides the following procedures were followed:

3.2.2.1. Hydrolysis of root tip cells and staining of chromosomes

The hydrolysis of the root tip cells and staining of the chromosomes were done simultaneously with a mixture of 1N HCL and 2 percent aceto-orcein solution (1:9 v/v) for ½ to 1 hour at room temperature (30°C ± 2°C). Hydrolysis was carried out to obtain a good spreading of cells and chromosomes between the slide and coverslip at 1N HCL solution (Sharma and Sharma, 1980).

3.2.2.2. Squash preparation

From the root tips the deeply stained meristematic parts were excised out, placed in a drop of 45 per cent acetic acid on a clean grease free slide; and squashed between the slide and coverslip. To ensure uniform dissociation of cells gentle pressure with the thumb finger was applied on the coverslip without heating and finally sealed with paraffin wax. The temporary slides thus prepared were observed under a compound microscope (Model Leica, ATC 2000) at magnifications 10 x 45x and 10 x 100x oil immersion. Well scattered metaphase plates were selected for karyomorphological analysis of the chromosomes. Ten such slides from each species were prepared and the perfectly stained chromosomes were photographed by a Cosina micro photographic camera (Model No. C1s) at 10 x 100x (oil immersion) magnification.

Camera lucida diagrams of the chromosomes were drawn with the help of camera lucida apparatus at a magnification of 10 x 100x. The measurements of the chromosome lengths were taken from the slide with the help of stage and ocular chromosome lengths.
were taken from the slide with the help of stage and ocular micrometer scale.

3.2.3. Preparation of karyotype

The length and breadth of the chromosomes were taken from the prepared chromosome slide at a magnification 1000 times. The measurements were expressed in term of μm.

Total chromatin length was measured by adding the length of all the chromosomes in the karyotype. Volume of the chromosome was measured by using the formula \( \pi r^2 h \), where ‘r’ is the radius of the chromosome and ‘h’ is the length of the chromosome. 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 chromatin length of the diploid set (Khosla and Sobti, 1985); thus the

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

On the basis of the total length, the chromosomes of different species of *Ipomoea* L. were classified into the following types.

- **Type A**: Chromosomes with length 3.00 μm and above.
- **Type B**: Chromosomes with length 2.50 μm to 2.99 μm.
- **Type C**: Chromosomes with length 2.00 μm to 2.49 μm.
- **Type D**: Chromosomes with length 1.50 μm to 1.99 μm.
- **Type E**: Chromosomes with length 1.00 μm to 1.49 μm.
- **Type F**: Chromosomes with length 0.50 μm to 0.99 μm.
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The location of centromere on the chromosome was expressed as a percentage of the ratio between the arms and was calculated as a centromeric index or \( F \% \) (Denver Study Group, 1960; Levan, 1964).

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

Total Form percent or \( TF \% \) has been calculated by the formula given by Huziwara (1962).

\[
TF\% = \left( \frac{\text{Total sum of the short arm length}}{\text{Total sum of the chromosome length}} \right) \times 100
\]

Based on the centromeric position chromosomes were classified into metacentric, submetacentric, sub-telocentric and telocentric types as suggested by Laven et al., (1964). The idiograms were then prepared by arranging the chromosomes in such a way that the largest chromosome is placed on the extreme left at number 1 position and the smallest chromosome is placed on the extreme right (Stebbins, 1971).

3.2.4. Studies on meiosis

For the study of meiotic chromosome behavior including chromosome association and chiasma frequency flower buds of appropriate size from different species of *Ipomoea* L. were collected between 4:00am and 5:00am and were fixed in Carnoy’s fluid-I (Darlington and La, 1962). After 24 hours of fixation, they were transferred to 70 per cent ethanol for preservation and kept in cool dark place.

Smears were prepared from suitable anthers in a drop of 1.5 percent aceto-carmine solution on a clean grease free slide and then covered with a coverslip. The excess stains were blotted off with a blotting paper and were observed under a compound microscope (Model Leica, ATC 2000) at 10 x 45x and 10 x 100x (oil immersion)
Materials and methods

Ten slides in each species were prepared. The slides thus prepared were then screened for meiotic chromosome behavior. Data on chromosome association and chiasma frequency were recorded from diplotene and diakinesis stages. Diagrams of meiotic chromosomes were drawn with the help of a Camera lucida apparatus and their microphotographs were taken by using Cosina microphotographic camera (Model No.C1s).

In each species of Ipomoea L chiasma frequency per bivalent was calculated as follows:

$$\text{Chiasma frequency per nuclues} = \frac{\text{Number of chiasma in all the cells concerned}}{\text{Total number of cells}}$$

$$\text{Chiasma frequency per bivalent} = \frac{\text{Chiasma frequency per nuclues}}{\text{Number of bivalents per nuclues}}$$

Standard error for the observed data was calculated by using the formula

$$S.E = \frac{sd}{\sqrt{n}}$$

Where, 'S.E' is the standard error, 'n' is the number of observation and 'sd' is the standard deviation.

3.3. Studies on pollen morphology and pollen viability

For the study of pollen morphology, mature flower buds of each species of Ipomoea L. were collected one day before anthesis and were kept in 70 per cent alcohol for preservation.

3.3.1. Preparation of smears of pollens

The smear of pollen grains was prepared by tapping the anthers in a drop of 1 percent aceto-carmine stain solution in a clean grease free slide and the preparation was observed under a compound microscope at magnification 10 x 45x and 10 x 100x (oil
Materials and methods

immersion). Well stained pollen grains were selected for measuring their sizes.

Measurements were taken with stage and ocular micrometers at 10 x 100x magnification. Ten such measurements were taken in each species. Photograph of the pollen grains were taken by using a Cosina microphotographic camera (Model C1s).

3.3.2. Test for pollen viability

The viability of pollen grains of the experimental plants was determined by Tetrazolium test (Shivana and Johri, 1989). The details of the procedure were as follows:

3.3.2.1. Preparation of Triphenyltetrazolium chloride solution (TTC)

0.5gm tetrazolium salt (2,3,5-triphenyl tetrazolium chloride) was dissolved in 100ml sucrose solution in a volumetric flask and made into 0.5 per cent TTC sucrose solution.

3.3.2.2. Procedure

A small amount of pollen grains was dispersed in a drop of 0.5 per cent TTC solution on a clean grease free slide and a cover glass was lowered on to it to exclude oxygen. The slide was then put in a petri plate lined with moist filter paper and was kept in an incubator at 35°C in dark. The slide was observed after 45 minutes. Pollen grains which had turned red were scored as viable while those unstained were considered as non-viable. Ten such slides in each experimental species were screened for pollen viability.

The percentage of viability was measured by using the formula

\[
\text{Percentage of viability} = \frac{\text{Number of viable pollens observed in the microscopic field}}{\text{Total number of pollens observed in the same field}} \times 100
\]

The microphotograph of pollen grains for each species was also taken with the help of Cosina microphotographic camera (Model No. C1s) at a magnification 10 x 100x
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(oil immersion). The terminology as suggested by Nair (1965) was used for description of morphological features of the pollen grains. The features analyzed included aperture, exine ornamentation, pollen size (radius and diameter) and shape. In recording the range and mean size ($\bar{x}$), ten slides of each species under investigation containing sufficient pollens were considered. Standard error was calculated using the formula:

$$S.E = \frac{sd}{\sqrt{n}}$$

where, 'S.E' is the standard error, 'n' is the number of observation and 'sd' is the standard deviation,