Chapter 3

MATERIALS AND METHODS

3.1 Study area:

Distribution of *B. mollis* in Assam is restricted to some pockets of Karbi Anglong only. Few plants are also maintained in Khetri by NEDFi R & D Centre. The Karbi Anglong District is situated in between 25^0 33' to 26^0 35' N Latitude and 92^0 10' to 93^0 50' E Longitude covering the central part of Assam. Total area comprises of 10,434 km^2. The district comprises of low hills and flat plains and is covered with dense tropical forest. The study was carried out in different parts of the district but mainly concentrated in the three prime habitats for *B. mollisi.e.* Diphu, Manja and Lahorijan Forest Ranges.The type of the soil of the district is red loam and laterite soil soil which is red in colour found along the Karbi plateu. Average rain fall is 2416 mm. Topography shows considerable variation. This region experiences different climates in different time of the year. Temperature ranges from 6°-12°C in winter and 23°-32°C in summer. The forest area covered is about 4922.019 km^2.

3.2 Methods:

3.2.1 Field and herbarium method:

The present study is based on extensive field work undertaken during 2014 to 2017, which involved collection of specimens, observations and documentation of traditional knowledge in different parts of Karbi Anglong District, Assam. Mounted herbarium sheets are prepared by using collected specimens following the standard herbarium techniques (Jain and Rao, 1977). Important characteristic

features which cannot be retrieved from dried specimens like flower colour, position of leaf, branching pattern, habit, habitat with latitude and longitude etc. were noted down during field work. For microscopical studies specimens were collected and preserved in 4% formaldehyde solution. The field data has been incorporated on the herbarium sheets and collected specimen and deposited in the Herbarium of Botany Department, Gauhati University (GUBH).

3.2.2 Identification:

The specimens were identified with the help of relevant literature (Kurz, 1873; Bennett, 1875; Kurz, 1877; Merril, 1908, 1906, 1923; Nooteboom, 1962; Ho, 2000) and / or with the help of microfilms of herbarium specimens present in online databases of various Herbaria *viz*., Kew Royal Botanical gardens (K), Missouri Botanical garden (MO), Natural History Museum (NHM), Harvard University Herbaria, United States National Herbarium and The Linnean Collections (LINN).

3.2.3 Morphological investigation:

Detailed morphological study of the collected specimen was made during field work as well as in the laboratory. The detailed morphological characters of root, stem, leaves, inflorescence, flowers and fruits were recorded. Detail features of leaves pertaining to shape, size and texture; length of petiole; shape, size and nature of inflorescence; nature and arrangement of flowers were also recorded either from fresh or dried specimens or both. All the microscopic characters like hairs, glands, venation and the characters of the floral parts were observed in Stereo Dissecting Microscope (Olympus and Zen Digital Microscope). Both the quantitative (e.g. leaf size, plant height etc.) and qualitative characters (e.g. leaf

shape, floral morphology, presence or absence of trichomes and glands, etc.) were recorded.

3.2.4 Taxonomic enumeration:

The taxonomic treatment of the species includes the detailed description along with nomenclature acceptable in previous literature. Original citations of both accepted names and synonyms are provided.

Distributional status of the species in different countries of the world, in different states of India and different part of Assam are worked out from available literature and provided. The distribution, occurrence and phonological data are provided on the basis of observations made during field work. Photographs of herbarium specimen including type specimens were also downloaded from various herbaria (E, K, LINN and BM) and provided whenever applicable.

3.2.5 Micro morphology:

3.2.5.1 Leaf epidermis:

For leaf epidermis study, fresh leaves were fixed in formalin-acetic-ethanol (1:2:1) solution. The leaf epidermis was thoroughly investigated for detail characteristics of stomata, epidermal cells and epidermal hairs. For the preparation of temporary slides epidermal peels of both upper and lower epidermises of mature leaves were taken out either mechanically or by controlled maceration using a 10% aqueous solution of nitric acid following the technique of Boubos and Beakbane (1971). The peels were stained with 1% aqueous solution of Safranin and mounted in 50% glycerin and sealed with DPX.

The nature and distribution of stomatal guard cells, subsidiary cells and epidermal hairs were studied. Frequency, size and nature of epidermal cell wall, size, types of stomata, stomatal frequency, stomatal index, stomatal area, types and

distribution of trichomes were worked out. In order to standardize observations and measurements, the mean values of three readings were taken. Microphotographs were taken with the help of the Olympus B 50 F4 Camera.

For Scanning Electron Microscopy of the leaf epidermis, dried leaf material was dissected and mounted directly on stubs with adaxial and abaxial surfaces facing upward using double sides' adhesive tape and sputtered with a thin layer of gold. The methodology followed by the Department of SAIF, NEHU, Shillong was adopted. The Electron micrographs were obtained using a JEOL JSM -6360 system.

In describing epidermal features, terminology suggested by Stace (1984) was followed. The stomata were classified and described as suggested by Prabhakar (2004).

Different parameters for calculating quantitative values of epidermal features were worked out as follows:

Stomatal index (S.I.) = S / (S+E) × 100

Where, S = number of stomata per unit area.

E = number of epidermal cells in the same area (Salisbury, 1927)

Absolute stomatal number in thousands = (A × S) / 1000

Where, A = total leaf area in mm^2,

S = stomatal number per unit area (*i.e.* 1 mm^2) (Gupta, 1961).

Stomatal area = Area occupied by a single stomata (in mm^2) × stomatal frequency × Total leaf area in mm^2.

Stomatal Frequency (SF) = N/A

Where, N = Number of stomata per field

A = Area of the field (Salisbury, 1927).

3.2.5.2 Leaf Arcitacture:

For leaf architecture study, mature leaves from both fresh and herbarium materials were cleared following the technique of Berisar and Bocquet (1960) with slight modification.

Materials were first treated overnight with 5% aqueous solution of Sodium hydroxide in an oven at 37^0 - 40^0 C. These 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 materials were then placed in gradual descending series of a solution of 75 cc absolute Ethyl alcohol for 20 minutes till the lamina become colourless (Berisar and Bocquet, 1960).

Staining was done with Safranin dissolved in a mixture of absolute Ethyl alcohol and Xylene (1:1) for five minutes; then washed with absolute Ethyl alcohol to remove excess Safranin and finally mounted in Canada balsam as usual. Microphotographs 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 number of vein-endings were counted for various parts of the lamina. To standardize observations and measurements, the mean values of five readings were taken (Berisar and Bocquet, 1960).

In describing venation pattern, the terminology suggested by Hicky (1973), Melvile (1976) and LAWG (1999) have been followed. For the calculation of absolute vein termination number Gupta's formulae (1961) were used which are as follows:

Absolute vein-islet termination number per mm^2 = average vein-islet number per mm^2 × area of the lamina in mm^2 / 1000 (Gupta, 1961).

3.2.6 Anatomy:

For anatomical studies plant materials collected during the field work were preserved in 70% alcohol and free hand sections were made for microscopical studies. Anatomy of root, stem, petiole and mid rib of the leaf were studied following Johansen's double staining procedure. Thin hand sections were cut, stained with Safranin, subsequently dehydrated through an ethanol gradiant (70%-100%) and sections were counterstained with Fast green (Gerlach, 1977) and treated with 99.5% xylol. Finally sections were mounted on glass slides using DPX. These slides were appropriately labeled and observed under inverted light microscope Nikon Eclipse E200 (with Camera DS-Fi1C) and Leica ATC 2000.

3.2.7 Floral Biology:

The time of anthesis initiation, blooming peak (month in which more than 50% of the individuals were in flower) and the termination in flowers were observed according to methodology given by Dafni (1992) with some modifications. To describe the flowering period of an inflorescence and single floret, time of anthesis and frequency of florets per cyme 55 male and 35 female inflorescences were tagged and observed. 20 flowers from 10 inflorescences were tagged and studied flower morphology, morphological changes, anther dehiscence etc. To study the flowering dynamic florets of different growth stages were observed as suggested by Dafni with slide modification (Dafni, 1992). Three readings were taken each for male (n=55) and female (n=35) after periodic interval.

To determine the blooming peak 30 male, 30 female were tagged and observed. The month in which 50% of the individuals were in flower was recorded.

To determine the role of wind pollination Dafni's method (1992) is followed with slide modifications. Glass slides coated in sticky Vaseline were kept vertically

at 80 cm height in the canopy, and .5m, 1m, 1.5m, 2m away from canopy of the isolated trees (n=5) and counted after 24 hr (Dafni, 1992). In addition female cymes (n=5) were kept cover with paper bag to prevent the entry of insects and then monitored for fruit set.

In pollination experiment female cymes were kept cover with paper bag, which did not result in fruiting. Floral parts and leaves are observed to check the presence of pollen carried out by wind.To record the insect pollination standard procedure of observation was followed.

3.2.8 Seed germination:

Fully riped, mature seeds of *B. mollis* were collected from Diphu during 2014–2016 to study germination. Germination was studied differently for fresh seed and stored seed. Each variant had its control. All seeds were surface sterilized with 5% NaOCl (sodium hypochlorite) for 5 min to avoid fungal invasion, followed by repeated washing with distilled water (Murashige and Skoog, 1962; Hamidi *et al.,* 2013) and stored in air tight container at room temperature. Emergence of 2-5 mm radicle was taken as seed germination (ISTA, 1966). Germination was recorded regularly until the final count.

Germination percentage GP = $\frac{n}{N} X100$ (Pirasteh – Anosheh and Hamidi, 2013)

Where, n= number of seeds germinated

N= total number of seeds planted

3.2.8.1. Effect of germination methods:

Different germination methods include variation in seed maturation and medium of seed germination. Seeds were collected in March, April, May, October, November and December from 2014 to 2016. Mature ripe and unripe seeds were

collected to see the effect of ripening on germination (Keng *et al*., 2002). Germination testing methods were-

1) Sterilized fresh/ stored seeds were grown in approximately 1 cm deep in a 1:1 soil and sand mixture (Keng *et al*., 2002). The experiment was replicated three times for each germination test and each seed lot contained 10 seeds.

2) Sterilized fresh seeds were placed in Petri dishes with 2-3 layers of Whatman No.1filter paper moistened with sterilized distilled water and kept in laboratory. Then they are kept in incubator at 30±2ºC until germination (Etejere and Ajibola, 1990).

The experiment was replicated three times for each germination test and each Petri plate contained 10 seeds.

3.2.8.2 Influence of fruit wall on seed germination:

To see the influence of epicarp and mesocarp in germination methodology given by Keng *et al*., 2002 with slight modification was followed. The fruit wall was removed after the seeds were surface sterilized, again surface sterilized with 5% NaOCl (sodium hypochlorite) for 5 min followed by repeated washing with distilled water and sown along with a set of seeds with endocarp intact. 50 seeds were used for each trial and the experiment was repeated three times. Percentage of germination was recorded.

3.2.8.3 Influence of season:

Percentage of germination of seed in summer and winter was recorded after a certain interval of time.

3.2.8.4 Influence of Gibberellic acid on seed germination:

Seeds were treated with Gibberellic acid (concentrations 100 ppm, 200 ppm, 300 ppm for 2 h). 50 seeds were used for each trial and the experiment was repeated

three times. Percentage of germination was periodically recorded after a certain interval of time.

3.2.8.5 Determination of the Coefficient of Velocity of Germination:

The coefficient of velocity of germination (CVG) is computed based on Hartman and Kaster (1968) with slight modification,

$$CVG = \frac{Total\ number\ of\ germination}{A1T1 + A2T2 + \cdots + AnTn}$$

Where, A = number of fresh germination recorded at interval of 5 days

T = number of days from sowing.

The effects of different treatments in different germination methods and their interactions on the coefficient of velocity of germination were computed using ANOVA.

3.2.8.6 Determination of the seed viability:

Seed viability was evaluated using the method given by Scharpf and Parmeter (1962). *B. mollis* seeds were placed in water until they became swollen, placed on a circular piece of filter paper 9 cm in diameter and allowed to dry. Seeds were then cut in half longitudinally with a sharp razor blade. The cut seeds were placed in a 100-mm Petri dish, covered with a 0.1 percent solution of 2,3,5-triphenyltetrazolium chloride (TTC) at pH 7-8 and kept dark for 12 to 24 hours. The experiment has been replicated three times. Cut seeds were then examined for staining. Viable seeds will change the colorless TTC into an insoluble red pigment staining the embryo and endosperm. Nonviable seeds remained unstained. Later viability indicated by TTC test was compared to actual germination percentages

3.2.9 Ethnobotany:

During the field work, ethnobotanical information were collected either by personal contact with traditional practitioners, knowledgeable informants, aged persons or by personal observation on actual uses. Further verification and cross checking of the information recorded were done in different localities. Different parts used for various diseases were documented.

3.2.10 Test of significance of numerical values:

The numerical values were subjected to ANOVA test using software (MS Excel-2007) to examine the significance level. The ripe and unripe seeds, soil: sand mixture and Petri plate GA etc. are considered as treatments and months of seed collection and sowing are considered as dependent variables. The F values obtained from the test were again compared with the table values using the appropriate literature (Gomez and Gomez, 1984).