# CHAPTER 4

# MATERIALS AND METHODS

In this research, various experimental techniques were employed. Firstly, floral development and morphology of flower, flowering phenology and anthesis process were carried out to reveal the morphology and phenology of the flower. Pollen viability and germination, pollen-stigma interaction, and self-incompatibility studies were performed. Behavioral features of the visitor and pollinator-plant interaction studies were conducted. Fruit setting studies were also investigated.

## Fieldwork

Field works were carried out from June 1999 to February 2003 at Sakaerat Environmental Research Station and Phanom Rung Historical Park. Special emphasis and the main work on pollination were carried out along the main road that passes through dry dipterocarp forest, 2 kilometers in length from the entrance of Sakaerat Environmental Research Station. This rather isolated area was chosen for study in order to minimize human interference with pollinator activity.

## Methodology

### 1. Floral Development and Morphology, Flowering Phenology and Anthesis Process, and Nectary Studies

1.1 Floral development was studied from various flowering stages, starting with inflorescence-buds until the shedding of florets from the inflorescence. Floral development and morphology studies, the studies were somewhat Nopanitaya (1987), Tucker (2000a,b), and Washburn and Thomas (2000) as followed (more detail see appendix 5).

Floral buds, 1-2 cm in length, were collected from branches and fixed immediately in Karnowsky fixative at 4°C and leaved overnight. Then the specimens were rinsed twice at 25°C in 0.27 M fixing buffer pH 7.2 for ten minutes each. The specimens were post-fixed for 1 hour in 2% osmium tetroxide in 0.1 M wash buffer. After that, the specimens were washed 3 times at 25° C in 0.1 M wash

buffer. The specimens were dehydrated by gradation ethanol series: 30%, 50%, 70%, 90%, and 100% for ten minutes each, then proceeded to critical point drying, mounted on aluminum stub with adhesive tabs, and coated with gold in a sputter-coater. Finally micrographs were taken with a scanning electron microscope.

1.2 Flowering phenology and anthesis process studies were followed Dafni (1992).

Flowering phenology was conducted at three levels:

1.2.1 Events in the single inflorescence studies

1.2.1.1 One inflorescence from 5 plants at the first day of flowering time were marked with distinctive labels for future identification and observation. Flowering progress was observed every week until the opening of the last floret in the inflorescence. Number of florets and the day of inflorescence opening time were recorded.

1.2.1.2 Inflorescence length and florets number were studied in 3 inflorescences from 5 plants.

1.2.1.3 Observe flowering progress every hour, 5 inflorescences from 5 plants were observed from midnight until full blooming of all florets. Destructive technique was used to observed anthers dehiscence time.

1.2.2 Dynamics of flowering of the whole plant

Fifteen plants with 15 inflorescences each were marked. Flowering magnitude from floral bud until the last floret opening were observed every week.

1.2.3 Flowering course of the whole population

In order to obtain information on the seasonal timing of flowering, the sample units was delimited before flowering commencement and observe the flowering magnitude of the whole sample from the start of flowering time until flowering complete. In this observation 30 plants were used.

1.3 Nectar studies adapted from Bernardello et al. (2000) and Dafni (1992).

1.3.1 Studies on the floral nectary structure are as followings:

Flowers were fixed in FAA, dehydrated in an ethanol-xylol series, and embedded in Paraplast. Cross and longitudinal sections were made at 15 μm thick, mounted serially, stained with safranin-fast green series, and observed under a compound microscope.

To detect stomata in the nectariferous tissue, micrographs of gland were taken with a scanning electron microscope.

1.3.2 Nectar samples were gathered during 9.00 h, analysis of nectar composition are as followings:

The volume of nectar secreted was quantified using micropipette (2 μl), 30 flowers were used in each sample. Sugar concentration was measured in the field with pocket refractometer (Brix 0 -30%). The nectar was diluted with a known volume of distilled water, and mixed carefully before measurements.

1.3.3 The kinds of sugar were identified via HPLC, 30 flowers from 4 plants were used in this investigation.

**2. Pollen Viability and Germination, Pollen-Stigma Interaction, and Self-Incompatibility Studies**

2.1 Pollen viability and germination studies were followed Shivanna and Rangaswamy (1992) and can be describe as followed (scoring of 200-300 pollen grains and 30 pollen tubes from 10 microscopic fields for each treatment):

2.1.1 Pollen viability was investigated during anthesis at 9.00 hour. Study on pollen viability, tetrazolium test, based on the reduction of a colorless soluble tetrazolium salt to a reddish insoluble substance called formazan in the presence of dehydrogenases, were used (appendix 6). Quantitative data concerning the viability (%) were obtained.

2.1.2 Study on pollen germination was investigated during anthesis at 9.00 hour, *in vitro* germination method on cellophane membrane to raise surface cultures of pollen grains was used. A suitable sugar and its concentration were tested beforehand (appendix 7).

2.2 Pollen-stigma interaction studies were followed Shivanna and Rangaswamy (1992), and can be described as the followings:

2.2.1 Sample of 5 flowers each was taken at 16 time intervals from 9.00 hour on the day before anthesis to 9.00 hour on the day after anthesis. Cytochemical localization of esterases on stigma surface was used to determine stigma receptivity (appendix 8).

2.2.2 Pollen *in vitro* germination on cellophane membrane method was used to test pollen tube growth relate to pollen age (appendix 7).

2.2.3 Multiple staining for localizing pollen in the pistil was used to localize pollen tubes in the stigma and in the style (appendix 9).

2.3 Method for self-incompatibility studies were followed Dafni (1992). Three methods were used to obtain information on breeding systems and to check the presence of a self-incompatibility system: (1) bagging experiments, (2) aniline blue fluorescence microscopy, and (3) pollen-ovule (P/O) ratio.

2.3.1 Bagging experiment, a total of 30 inflorescences was isolated by covering them before anthesis with fine mesh nets. Special care was taken that the bags did not disturb normal flowering, compare with 30-opened inflorescence.

2.3.2 In order to confirm the results of the bagging experiments, the bags with flower were examined for pollen tube growth under a fluorescence microscope compare with selfed, crossed and opened flowers; 100 flowers were used (appendix 10).

According to no fruit production from artificial pollination. So aniline blue fluorescence method to study pollen germination and

pollen tube growth in pistil were used. Estimation of self-incompatibility rate, the index to measure self-incompatibility (ISI) was used.

ISI = pollen tube set from self pollination / pollen tube set from cross- pollination

. 2.3.3 Estimation of the out crossing level, the relation between the number of the pollen grains and ovule (P:O ratio) was observed to reflect the breeding system. The pollen-ovule ratio was determined by calculating the mean number of pollen grains produced per flower and dividing this by the mean number of ovules per flower (appendix 11).

**3. Behavioral Features of the Visitor and Pollinator-Plant Interaction Studies**

These studies were used the method described by Banzinger (1996), initially, observations on floral visitors were made both during the day– and the nighttime. Due to the apparent lack of floral visitors during the night and the fact that flowers invariably almost opened during daytime, later observations were restricted to the period between the onset of flowering, which started at about 08.00 hour, and the end of flower visits by diurnal visitors about 17.00 hour.

3.1 Study on behavioral features of the visitor and pollinator-plant interaction by recording visitor behavior on flowers, use the fixed sample method. Observation was carried out from 08.00 hour to 17.00 hour on the same sample at different times during the day and the season.

3.2 Analysis of foraging behavior; flight pattern towards the flowers, reward harvesting, and anther and stigma touch were conducted.

All insects were killed in a killing-jar prepared by adding a few drops of ethyl acetate. The specimens were then pinned with stainless steel pins in foam rubber. Manipulation was carried out before *rigor mortis* had set in. The wing were spread (or 'set') by first blowing gently from beneath while holding the tip of the pin with a pair of forceps, and then carefully bracing the wings with pins. The wings were positioned in the way that the axis of the forewings formed a straight line.

All specimens were stored in the boxes to which a ball of camphor was added in order to guard them against voracious ants.

The plant visitors were identified by Insect Taxonomy Research Group, Entomology and Zoology Division, Department of Agriculture, Ministry of Agriculture and Cooperatives with the kind help of Mr. Sura Pimpasalee and Mr. Wijit Koonthong. All insect specimens are kept at the Natural History Museum, Chulalongkorn University.

**4. Fruit Setting Studies**

These studies are the same as those described in Kaye (1999).

4.1 Study on fruit setting, a pollinator-exclusion experiment was conducted to determine whether insect pollinators were necessary for fruit and seed production.

4.2 Fruit setting in six plants, which 3 plants has the nearest other 20 meter and 3 plants has the nearest other 300 meter were also compared to determine the role of insect pollinators.

4.3 Percentage fruit set, percentage seed set, and average seed mass were determined from 30 plants.

The percentage of fruit setting was determined by selecting randomly 30 plants, counting the fruits, estimating the total florets and calculating the percentage fruit set. The percentage of seed set was determined in the same as those of percentage of fruit set, total ovules number and total seed number were used in calculation. Average seed mass was estimated from 30 seeds.