CHAPTER III
MATERIALS AND METHODS

Extensive air surveys over Paper Mill area were carried out (for two years, from October 2003 to September 2005). Sampling was done three times in a month of every year. The collected samples were identified and recorded in every month. Preparation of pollen and fungal spore calendar of different months was prepared accordingly. The phonology of the flowering such as date of blooming, flowering period, mode of pollination and prevalence were noted down for trees, shrubs and under shrubs and herbs (both wild and cultivated). For air monitoring Burkard Personal Air Sampler has been used and Petriplate Exposure was done for viable fungal spore survey of different sampling site.

A. Aeromycological survey:

Aeromycological surveys over Paper Mill Area were carried out for two years (from October 2003 to September 2005) at a regular interval of 10 days. Aeromycological studies were conducted at various sites of Paper Mill Area, Jagiroad in order to study their aeromycological population, which may cause allergic disorders in human beings.

Sampling Technique:

For air monitoring two sampling techniques have been used a) Petriplate with Sabouraud’s agar medium, b) Burkard Personal Sampler.

(a) Petri Plate exposure method:

The sampling was carried out at regular intervals of 10 days and 5 feet height above ground for 5 minutes. The exposed plates were stored properly and reaching the laboratory. The exposed plates were placed in B.O.D. incubator and maintained at a
temperature at 28° C ± 1°C for a period of 5-7 days, depending on the growth of fungal colonies. The medium was treated with an antibiotic prior to plating, to avoid the growth of bacterial colonies. After incubation the fully grown colonies were counted and identified and pure- culture of each species is maintained.

**Culture Medium:**

The Sabouraud's agar medium is very sensitive, so, it was used to trap and culture the aeromycoflora at the different sites. The compositions of the culture medium are-

**Sabouraud's Agar Medium (pH- 5.6):**

- Dextrose --------------40gm (4%)
- Peptone ---------------10gm (1%)
- Agar --------------------20gm (2%)
- Yeast extract -----------0.5gm (0.05%)
- Rose Bengal Dye -------0.03gm (0.003%)
- Distilled water ---------1000ml

**(b) Burkard Personal Sampler:**

Burkard Personal Sampler (Burkard manufacturing company Limited, England) is a portable sampler used for spot sampling. It is a compact battery/power operated device, 10cm in height and 8cm in diameter weighing 590gms for collecting airborne particles directly on to glass slide. There is a rectangular orifice at the top and a slit on the side to insert the glass slide. The sampler sucks the air at the rate of 10 litres/minute, through the orifice and the particles get impacted in the form of a band. An ‘On/Off’ switch is mounted on the under side of the sampler together with a pre-set

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PLATE-I
The sampler was placed at a height of 5 feet above the ground level. A special type of micro slide (76mm x 26mm or 3 x 1 inches) of 0.8mm thickness was used. The slide was smeared with glycerine jelly in the form of a thin film. The slide was inserted through the slit in the sampler with jelly side up and twists the lid in order to seal the inner chamber. It was operated for 5 minutes. Air monitoring was carried out at a regular interval of 10 days. Exposure was made thrice a day- morning, mid-day and evening. After each exposure, the slide was placed inside a slide box. For mounting a drop of molten jelly was placed on the slide. A cover slip was placed on the drop of molten jelly and pressed slightly. The slide was then examined under the microscope.

**Conversion Factor:**

Conversion Factor was calculated as follows-

The suction rate of Burkard Personal Sampler is 10 litres per minute.

If the sampler is operated for 10 minutes, air taken is = 0.1m$^3$

If 0.1m$^3$ has 1 fungal spore

1m$^3$ has 1/0.1 fungal spore

For 10 minutes = 10 fungal spores

For 5 minutes = 20 fungal spores

The fungal spore counts were expressed as number per m$^3$ of the air after multiplying the actual number of fungal spores with the conversion factor i.e. 20.

**Fungi Identification:**

The identification of fungal spore types was based upon Morphological characters, visual identification by comparison with reference slides preparation and by

B. Aeropalynological survey:

(a), Collection, Preservation and Identification of Herbarium:

For preparation of herbarium specimen the plants were preserved at the spot between sheets of news paper. For very small herbaceous plants, the whole plant was preserved. Preserved and dried specimens were poisoned with saturated solution of Mercuric chloride (HgCl₂) in absolute alcohol. The specimens were mounted on standard herbarium sheets. The mounted specimens were identified by consulting literatures of various authors of “Krishna Kanta Handique Library”, Gauhati University and also confirmed by herbarium sheets of earlier collection of Herbarium of Botany Department. Finally, the doubtful specimens were checked and confirmed by the Kanjilal Herbarium of Botanical Survey of India, Eastern Circle Shillong. The nomenclature used for plant species were checked by thorough verification of “Name changes in Flowering Plants of India and Adjacent Regions” (Bennet, 1987).

The flowering floras have been discussed for identification: “Flora of British India” (Hooker, 1872-1892); “Flora of Assam” (Kanjilal et al., 1934-1940); “Flora of Tripura States” (Deb, 1981-1983); “Flora of Nongpooh” (Joseph, 1968) etc.
**Preparation of Glycerine jelly:**

500 gm of gelatin powder was put into warm (50° C) water contained in a beaker. The supernatant water was removed when the gelatin was fully soaked. Equal volume of pure glycerine was added to the gelatin and the mixture was allowed to melt properly. 1 gm of phenol crystal (or liquid) was added to 1:1 gelatin and glycerine mixture. The mixture was stirred with a glass rod. The jelly was passed through a muslin cloth and collected in glass tubes. The glass tubes were placed in water and the water was boiled until the air bubbles from the jelly float to the surface. The air bubbles were skimmed off with help of spatula at convenient intervals. The jelly was finally transferred to Petri dishes and allowed to solidified and stored in a refrigerator after covering. The above solidified jelly was used for preparation of permanent slides of pollen for morphological studies.

**Preparation of reference slide:**

Pollen slides were prepared by method described by Nair (1970a). According to the method, preparation was made of both acetolysis and unacetolysis pollen grains (with slight modification).

**Pretreatment:**

The polliniferous material was collected from fresh specimens and placed in 70% alcohol. After 24 hours the material was transferred in a glass vial and crushed with a glass rod. The dispersion was sieved through a mesh into a glass centrifuge tube.

**Acetolysis method:**

The polliniferous material containing 70% ethyl alcohol was centrifuged
and decanted. Then to the sediment in the centrifuge tube, 5 cc of glacial acetic acid was added and centrifuged twice and decanted. The 5 cc of freshly prepared acetolysis mixture (acetic anhydride and concentrated sulphuric acid in the ratio 9:1) was added. The glass vials containing pollen heated in water bath till it started boiling and heating was immediately stopped. After cooling, the material was centrifuged and decanted. Now 10 cc distilled water was added and stirred thoroughly till it foamed. The foam was removed by adding few drops of acetone or alcohol. Few drops of 50% glycerine was added and left for 10 minutes. Then again centrifuged and decanted. The glass vials were inverted on a filter paper till it completely dried. Small amount of molten jelly was taken on a platinum needle. The needle was taken into the vial and touched the pollen sediment. The pollen caught on the jelly was transferred into a clean microscopic slide. The slide was slightly warmed, mounted with a circular cover slip and sealed with molten paraffin wax and kept it up side down.

Unacetolysis method:

A small amount of fresh pollen was placed on a clean micro-slide. A few drops of 30%, 70%, 90% and absolute alcohol were added one after other as it kept evaporating. The pollen was mounted in molten glycerine jelly containing saffranin. Mounting should be done in such a way that a thin film was obtained to prevent distortion of the pollen grains. It was sealed with paraffin wax and cleaned by xylol.

(b). Air Monitoring:

(i) Selection of sampling site.

From Guwahati, different workers have provided the information on the number and kinds of pollen grains in the atmosphere. Incidences of air-borne spora over
Fig No. 1: Diagram showing Sampling Sites
Kamrup district, Assam have been reported by various workers. However, no data have been provided from areas other than Kamrup and Silchar district. The pollen population varies quantitatively and qualitatively from place to place depending on botanical composition of the local flora. According to Shivpuri (1964), it is important to have records on atmospheric pollen incidence from more than one site of a locality. Therefore, to update the knowledge of pollen flora of the atmosphere, it is necessary to monitor airborne pollen at different places. Paper Mill is an industrial area and 1199 employees are there. Some pollen grains cause allergic diseases of human beings. To know the allergenically significant pollen grains present in that area, air monitoring was done. Knowledge of pollen present in an area can enhance the practice of clinical allergy. An area where there has not been extensive sampling; aeroallergen samples provide basic data on what is present that can serve as a guide for selection of specific allergy tests. Pollen calendar allows the physician to relate patients’ severity of symptoms to aeroallergen exposure and thus trace the efficacy of treatments, Sarma and Bora (1996). Considering this view in mind, air sampling was done in different sites of Paper Mill area, Jagiroad for a period of two years (1st October, 2003 to 30th September, 2005). Five sampling sites have been selected to monitor air-borne pollen grains by using Burkard Personal Sampler. The sampling sites are as follows—

**Site I: Main Gate:** Main gate is facing towards the N.H. 37. Near the main gate one security check post is present for entry into the area. On the opposite side of the Paper Mill paddy fields and some vegetation and trees are present.

**Site II: Office:** Office is the second sampling site and it is approximately 0.5 Km. away from the Main gate. In this site main office buildings are present. The surrounding of the
office buildings are more or less covered by small vegetation, trees and seasonal flowering plants.

Site III: Bamboo Storage: The Bamboo Storage is the third sampling site and it is approximately 0.5 Km. from the office complex. The Bamboo Storage site is the main dumping ground for the raw materials of the Paper Mill. It is a vast area where tonnes of bamboos are kept. Small amount of vegetation is present around this site.

Site IV: Finished Product: It is a closed area where final products i.e. papers after processing bamboo are kept. This place is approximately 1.5 Km. away from the main gate.

Site V: Guest House: This is a fully residential area surrounded by number of quarters. The open area is characterised by different plant species.

(ii) Sampling with Burkard Personal Sampler:

Burkard Personal Sampler (Burkard manufacturing company Limited, England) is a portable sampler used for spot sampling. It is a compact battery/power operated device, 10cm. in height and 8cm. in diameter weighing 590gms for collecting airborne particles directly on to glass slide. There is a rectangular orifice at the top and a slit on the side to insert the glass slide. The sampler sucks the air at the rate of 10 litres/minute through the orifice and the particles get impacted in the form of a band. An “On/Off” switch is mounted on the under side of the sampler together with a pre-set control for adjusting the through put at the orifice.

The sampler was placed at a height of 5 feet above the ground level. A special type of micro slide (76mm x 26mm or 3x1 inches) of 0.8mm thickness was used. The slide was smeared with glycerine jelly in the form of a thin film. The slide was
inserted through the slit in the sampler with jelly side up and twists the lid in order to seal the inner chamber. It was operated for 5 minutes. Air monitoring was carried out at a regular interval of 10 days. Exposure was made thrice a day: morning, mid-day and evening. After each exposure, the slide was placed inside a Slide box. For mounting, a drop of molten jelly was placed on the slide. A cover slip was placed on the drop of molten jelly and pressed slightly. The slide was then examined under the microscope.

Conversion Factor:

Conversion factor followed same as mentioned in Barkared Personal Sampler in page no. 40.

Pollen Identification:

Pollen identification was checked against reference slides made from collection and also from fresh material in flower at the time of survey. Literatures consulted for pollen identification are, “Airborne pollen, spores and other plant materials of India-A survey” (Edts. Nair et al., 1986); “Airborne Pollen and Fungal spores” (Tilak, 1989); “Biology of Airborne organisms” (Tilak, 1998); “Advances in Pollen-spore Research” (Vols. I-XVIII); “Pollen grains of Indian plants- I, II, III” (Nair, 1962a,b,c).