MATERIALS & METHODS

Ecofloristic Survey

In order to evaluate the aerobiological assemblage of Jalpaiguri town of Jalpaiguri district, West Bengal, a good knowledge of its ground flora and their pollen grains are of prime importance. Accordingly a detailed and systematic ecofloristic survey was carried out. The frequency of occurrence is based on visual observation carried out during the survey period from October 1995 to September 1997, covering forest floras, agriculture fields, scrub jungles, roadsides, barren lands etc. inside and in the periphery of the town of Jalpaiguri. Plants were identified basically in the Taxonomy and Environmental Biology laboratory of the Department of Botany, North Bengal University using available literatures and then matched at NBU – Herbarium and at CAL. The recorded flora has been enumerated with families, genera and species alphabetically as the work may find its use, mostly, among the non-taxonomists and for future reference in case of disputed identifications. For the methodology Jain and Rao (1977) has been followed.

Pollen Morphology

Pollen morphological study was done by microscopical observation of some permanent and temporary slides of common plant species. For terminology and description Erdtman (1952). Chanda (1963) and Faegri and Iversen (1975) have been followed.

Permanent Slide Preparation :

Both herbarium and fresh material collected were acetolysed and a part or the material was chlorinated after acetolysis following the method of Erdtman (1952, 1969) and Chanda (1966).

Dry polliniferous material from Herbarium sheets were carefully crushed on a finely meshed stainless steel brass sieve (0.11 sq.mm.) resting on a funnel set on hard glass centrifuge tube. After each treatment the brass sieve was exposed to a flame until it became red hot in order to avoid sample to sample contamination. Acetolysis mixture was prepared in a measuring cylinder by slowly adding one volume of concentrated sulphuric acid to nine volume of acetic acid anhydride. Then 10 ml of this acetolysis mixture was added drop by drop to test tubes containing powdered

material and stirred carefully with a glass rod. After thoroughly mixing, the mixture was heated to boiling point (100^0C) in a water bath for 2-3 minutes until it became slightly brown in colour. The mixture was allowed to stand for a few minutes, then centrifuged at 5000g. in room temperature and finally decanted. After adding a little distilled water, the sediment was shaken thoroughly, again centrifuged and decanted. The process was repeated for two to three times. Then the foam of the mixture was removed by adding a few drops of alcohol (95%) or acetone and the mixture was sieved twice through a finely meshed brass net to eliminate foreign particles and again centrifuged. After decanting, distilled water was added to the sediment. Half of the mixture was kept for chlorination and 50% glycerine was added to the other half.

To effect chlorination 5 ml of glacial acetic acid, a few drops of concentrated sodium chlorate solution and a few drops of concentrated hydrochloric acid were added and the mixture was then centrifuged, decanted and washed thoroughly to remove all traces of chlorine in it. After centrifuging and decanting, 50% glycerine was added to it. Then the two parts (non-chlorinated and chlorinated) were mixed, centrifuged and decanted. Then the tubes containing acetolysed pollen materials were kept in inverted condition on filter paper for a few hours in order to dry the material. Chlorination was avoided in case of thin walled and delicate pollen grains.

For the preparation of permanent slides, a small piece of glycerine jelly (prepared by Kisser's method) was taken on a clean platinum needle (sterilizing after each treatment burning in a flame), touched carefully to the bottom of the centrifuge tube (where the pollen are precipitated) and transferred on a clean slide. Then the slide was heated gently and after that the jelly was spread evenly by the platinum needle. A round cover glass was placed on the material and sealed off with chips of paraffin (melting point 60^0C – 62^0C). In case of air-bubble encroachment within the paraffin, the slides were kept in an incubator (temperature not more than 60^0C) for a few hours to get rid of the air-bubbles.

For fresh material, the flowers and anthers containing pollen were fixed in glacial acetic acid, then transferred directly to the brass sieve, crushed and washed down to the centrifuge tube with 70% alcohol. The mixture was then centrifuged, decanted and acetolysis mixture added to the residue and the procedure was adopted as in case of herbarium material. The delicate pollen grains (e.g. Cannaceae) which do not survive the acetolysis procedure, are refrained from such treatment. Such grains were placed in 70% alcohol, stained with safranine, and mounted in glycerine jelly.

Preparation of Safranine Stained Glycerine Jelly :

Gelatin 50 gms, glycerine 150ml, distilled water 175 ml, phenol crystals 7 gms and a few drops of safranine are the requisites for the preparation or the jelly. The first three constituents were mixed thoroughly and boiled in a water bath for one to two hours. The phenol crystals and appropriate amount of concentrated solution of safranine should be added and mixed thoroughly. While still warm and molten, the glycerine jelly should be poured on a petridish making a thin, uniform layer of about 0.5 cm thick. It was then cooled and preserved in refrigerator.

Temporary slide preparation :

For ready identification of airborne pollen, fresh and pure pollen samples were mounted on a small amount of safranine stained glycerine jelly placed on the clean glass slides. The slides were sealed in DPX by placing cover slip. This was allowed to dry and then scanned.

Aeropalynological Survey

Aeropalynological sampling methods are very diverse and vary according to individual interests in the component of airspora to be studied. Early methods of air sampling were summarized by Cunningham (1873). After a long gap the various methods of sampling have been summarized by the Committee on Aerobiology of the National Council, Washington (1941). Then the International Biological Programme (1972) also provided data on the various air sampling techniques.

This investigation deals with airborne pollen grains. The several pollen sampling devices currently used are based on some basic principles and categorized according to physical processes by which they remove the particles from the air spora are :

Sedimentation	-	Impaction by wind
Filtration	-	Impaction by suction
Centrifugal sedimentation	-	Impaction by rotation

Gravity slide sampler :

In this method sticky coated (glycerine/petroleum jelly) microslides are used for free deposition on its horizontal surface and then the slide is examined microscopically for analysis of pollen. It is a cheapest and simple routine method, which provides qualitative data for 24 hours but requires daily changing of slides.

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Rotorod Sampler :

The Rotorod sampler was originally devised by Perkins (1957) and has been somewhat modified by Harrington *et.al.* (1959). The sampler consists of a small, constant speed battery operator motor, which makes the sticky coated brass rods to whirl around its axis. The volume of air sampled can be calculated. It is very suitable for field sampling and a number of rods can be carried to effect it.

The Sampling Site and the Duration of Airborne Pollen Monitoring

One Gravitational sampler was placed on the roof of a domestic house at Jalpaiguri town, West Bengal about 4m. above ground level. Sampling was carried out for two years from October, 1995 to September, 1997. The sampler was run for 24 hours. Safranine stained glycerine jelly was smeared on two slides and exposed every 24 hours at an angle of 45^0. The trapping devise had a windvane and horizontally placed hollow tube in which the slides were mounted on a platform. The tube protected the slides from rain, which was fixed over a pivot fitted with a ball bearing so that the tube rotated in accordance with the wind direction.

Sampling Methods :

Two clean slides coated with safranine stained melted glycerine jelly were inserted. The trapped slides were removed, mounted in Canada balsam by placing a 18 mm microscopic cover slips. This was allowed to dry for 2-3 days then scanned.

Rotorod Sampler :

Air was sampled volumetrically using a Rotorod sampler which was placed 4 m above ground level in vertical position on the roof of a domestic house at Jalpaiguri district. The sampler was run for 30 mins. between 13 hr and 14hr at fortnightly intervals. The sampler was powered by a 6v battery giving a rotation speed of 2500 rpm. After sampling, the cellotape strips bearing the pollen catches were removed and remounted on clean glass slide using Canada balsam as mountant. The counts were converted to concentration m^{-3} of air by multiplying with an appropriate conversion factor (4) as suggested in the guide book of the British Aerobiological Federation (1995).

Calculation of Conversion factor (C) :

Counts were converted to concentration/m^3 using the formula :

$$C = \frac{N}{W(nw)\pi drt} = \frac{N}{0.264} = 3.79\ N = 4\ N\ \text{(Approximately)}$$

Where,

W	is breadth of the trace (2mm.)
w	is width of each scan (350 μm)
n	is number of scans (20)
t	is duration of sampling (30 minutes)
r	is speed of rotation (2500 rpm.)
d	is distance between rotorod arms (8 cm.)

Immuno-chemical technique

Collection of Pollen Sample :

The pollen samples were collected from mature anthers of relevant plants growing around the study area. The floral parts were dried at 37^{0}C, mildly crushed and passed through sieves (120, 200 and 300 mesh/cm^2) to remove the antherial tissues and other floral practicles. The purity of the isolated pollen material was checked under microscope. The batches used throughout the work contained <5% non-pollen impurities.

Preparation of Pollen Extracts :

The pollen was defatted with diethyl ether and extracted in sodium phosphate buffer (0.2 M monobasis & Na-phosphate, pH-7.4) continuous stirring at 4^{0}C for 24 hours in 1:50 (wt./vol. Ratio) by following the modified methods of Shivpuri (1962), Sheldon *et.al.* (1967) and Gupta and Chanda (1991). After centrifugation at 15,000g for 30 minutes the supernatant was taken and dialyzed in buffer and passed through 0.22 μm Millipore filter (Millipore Corp. Bedford, MA, U.S.A.). The filtrate was then lyophilized and stored at –20^{0}C in aliquots of known volume in sterile vials. The samples were sent to the Institute of Child Health, Calcutta for further study and clinical application.

Preparation of Ammonium Sulphate [$(NH_4)_2SO_4$] Cut Fractions :

The whole extract was fractionated in the range of 0% - 30%, 30% - 60% and 60% - 90% saturation of $(NH_4)_2SO_4$. 5 ml. homogenized supernatant (allergen

suspension) was taken in a beaker and $(NH_4)_2SO_4$ was added slowly as required to the supernatant with magnetic–stirer. After making homogeneous solution, the precipitate got settled and centrifuged at 12,000g rpm for 30 minutes for first cut (0% to 30%). Then more $(NH_4)_2SO_4$ (as required) was added to the supernatant for second cut (30% - 60%) and similarly for third cut (60% - 90%).

Skin Prick Test (SPT) :

Skin Prick Tests were carried out with the crude pollen extract (1:50 wt/vol) on adult respiratory allergic patients with relevant case report attending the Institute of Child Health, Calcutta. Histamine di-phosphate (1 mg./ml.) and PBS (Phosphate buffer saline) were used as positive and negative controls respectively. Tests were performed with 20 µl of aliquots of allergen solution placed on the ventral side of the forearm and each site was pricked with a disposable hypodermic (No.26) needle. The wheal responses were measured after 20 minutes and grades nil to 4^+ according to Stytis *et.al* (1982).

Gradation according to Stytis *et.al.* (1982) :

Reaction	Grade
No. wheal, no erythema	Nil
No. wheal, erythema <20 mm in diameter	1^+
No. wheal, erythema > 20 mm in diameter	2^+
Wheal and erythema	3^+
Wheal and pseudopod, erythema	4^+