CHAPTER IV

MATERIALS AND METHODS
The problem of allergy involves both botanical and clinical aspects. The first and most important step is to establish the identity and seasonal frequency of the plant allergens for carry out the clinical test and treatment of the allergenic patients. The present study mainly based on the botanical aspects which are carried out for a continuous year at this centre (starting from January 1, till December 31, 1980) under the following aspects.

I. The study of the floristic composition of the vegetation of the study site.

II. Field survey.

III. Collection of polliniferous materials – the collection of polliniferous material was done for two purposes.

(a) To prepare the index slides for reference purposes and

(b) To collect the pollen material in bulk for clinical studies (to be done by V.P. Chest Institute, Delhi).

IV. Analysis of aërospora – this includes:

(a) Description of aëroscope.

(b) Preparation of glycerine jelly.

(c) Preparation of slides for catching pollen and fungal spores.

(d) Data collection.
I. FLORISTIC COMPOSITION

Allergy is a local problem, and allergens vary both qualitatively as well as quantitatively from place to place. The first pre-requisite for the identification of pollens (one of the allergenic airborne bioparticles) of a given geographical area is to study the floristic composition, frequency, mode of pollination and to prepare a pollen calendar with their seasonal variations. It is often stressed that the local floras should be intensively studied, and now a days the suggestions made by Mukherjee (1953) and Santapau (1958 a), that Indian universities should take up an extensive exploration of restricted areas, have become more important; because of the discovery of a large number of additional species from various parts of the country from time to time, since the publication of "Flora of British India" by J.D. Hooker (1872-1897) and secondly due to successfully spreading of exotic species which have become naturalized.

Though the several remarkable contributions on the provincial and local floras have come out, the botany of Saugar is little known and except the fragmentary work by Pandeya (1949-50, 1950-51), Bhattacharya (1955), Rama Rao (1965), no concentrated exploration of the area was done.

The vegetational composition of Saugar and its surrounded areas can broadly be classified in to two types;
i.e. permanent and temporary.

**Permanent vegetation:**

The permanent vegetation of forests is quite luxuriant in suburban areas of Saugar. The tree forms are poor and in the form of discrete patches. These patches of forests are seen surrounded by grasslands, agricultural fields, wastelands and villages. The forest of the area can be described as the tropical dry deciduous (Champion 1936).

The most common trees found in the surroundings are *Butea monosperma, Diospyros melanoxylon, Acacia nilotica, A. leucophloea, Aegle marmelos, Lagerstroemia parviflora, Anona squamosa, Holoptelea integrifolia, Morus alba, Eugenia jambolana, Mangifera indica, Salmalia malabarica, Cordia dichotoma, Dalbergia sissoo, Pongamia pinnata, Allanthea excelsa* and *Cassia fistula*. Due to the biotic interference in the open area, there is a retrogressive influence on the forests, which results in the predominance of *Diospyros melanoxylon* and *Butea monosperma*. The teak is a most dominant tree; its distribution is very discontinuous. Teak plants, which found commonly on the Vindhyan slopes of Kanjargah, eastern slopes of Patharia, are not found on Vindhyan slopes of Garpahra. Teak is found associated in some patches with *Anogeissus latifolia, Terminalia tomentosa, Lannea coromandalica, Miliusa tomentosa* and *Butea monosperma*. 
The shrub layer consists of saplings and coppices of dominant trees with many spiny plants. *Butea monosperma*, *Carissa spinarum*, *Placourtia indica*, *Gardenia turcica*, *Randia dumetorum* and *Zizyphus oenoplia* are the most common plants of this layer. A number of small trees like *Anona squamosa*, *Caseria tomentosa*, *Emblica officinalis*, *Holarrhena antidysenterica*, *Morinda tinctoria*, *Nyrothea arbortristis* and *Santalum album* are also found.

The Patharia forest has been much degraded by felling of trees, because of the close proximity to human inhabitation. It has been more adversely affected and it become more open. In Garpahra the effect are less intense.

On the sites of streams and river the vegetation shows *Buchanania lanzan*, *Madhuca indica*, *Mitragyna parviflora*, *Terminalia arjuna*, *Woodfordia fruticosa* and *Mallotus philippinensis*.

The thorny and scrub forests are also found in Garpahra and dominated by *Acacia nilotica*, *Zizyphus mauritiana*, *Acacia leucophlica*, *Carissa spinarum*, and *Acacia catechu*.

**Temporary Vegetation:**

The temporary vegetation of the ground flora is mainly dependent upon the seasonal changes. With the onset of the monsoon, the whole ground becomes covered with a variety of herbs. The most common plants found in the herbaceous

In rainy season the climbers are also found growing commonly in the forests. The most common climbers are *Dioscorea bulbifera*, *Gymnema sylvestre*, *Cryptolepis buchneri*, *Hemidesmus indicus*, and *Gliricidia superba*. The other climbers are *Gissampelia pareira*, *Zizyphus oenocarpa* etc.

When the temperature becomes low during the post monsoon period the herbaceous plants of the ground flora rapidly disappear; but few winter annuals i.e. *Asphodelus tenuifolius*, *Lathyrus aphaca*, *Welwitsia alba*, *Oralis corniculata*, *Sonchus arvensis*, *S.oleraceus*, *Trigonella corniculata*, and other make their appearance. Some species such as *Acanthospermum hispidum*, *Convolvulus pluricaulis*, *Iridax procumbens*, *Vernonia cinerea* are also found growing in summer season.
A large part of Saugar flora is composed of alien species. A number of them which are accepted as natural constituents of the vegetation are mixed, grow and reproduce freely and with the result that the natural vegetation has been masked. Many introduced weeds become successful. Since they are not unaccompanied by diseases and pests which are common to their natural land. Due to interference of men, deforestation, cultivation and drainage, the natural vegetation has retrogressively much altered and created habitats which are most suitable for the appearance of a number of annual weeds. These plants grow and reproduce freely with native species and after a course of time these species are accepted as natural constituents of vegetation.

The weeds which are found on road sides are prominent in rainy season. As the winter season starts, most of the annual quickly disappear. The following are the common species met preferably along road sides: Acanthospermum hispidum, Achyranthes aspera, Amaranthus spinosus, Argemone mexicana, Bidens bidentata, Cassia tora, Echinops echinatus, Digitaria adscendens, Vertenuia annua, Lagocha aurita, Parthenium hysterophorus, Setaria glauca, Themeda quadrivalvis, Tridax procumbens, Xanthium strumarium.

Weeds are generally aggressive in nature. They produce a large number of seeds which are viable and well
equipped for dissemination. Due to these peculiarities weeds spread quickly in cultivated areas where they affect the crop growth by absorption of nourishment from soil. A number of weeds found in cultivated crops, are of tropical distribution which have been introduced together with seeds and seedlings of cultivated plants of various seasonal crops. The most common species found associated with the rainy season crops are: Aeschynomene indica, Alysicarpus tetragonolobus, Bowteria hispida, Celosia argentea, Cleome viscosa, Commelina benghalensis, Convolvulus arvensis, Corchorus trilocularis, Cyperus rotundus, Cynodon dactylon, Echinochloa colon, Euphorbia dracunculoides, Gynandropsis gynandra, Indigofera glandulosa, Leucas acora, Phyllanthus simplex, Rhynchosia capitata, Sesbania hispina, Triumphetta rhomboidea.

The weeds found common with cold season crops are: Anagallis arvensis, Asphodelus tenuifolius, Chenopodium murale, Convolvulus arvensis, Eragrostis diarhena, Leunaea asplenifolia, Orobanche cornua, Sonchus arvensis, S. oleraceus, Striga asiatica.

A number of moisture loving herbs are found growing in water and marshy areas. The trouble some weeds met in this type of habitat are: Ammania baccifera, Cassia axillaris, Colea lascrhamajobi, Echinocloa colon, Eclipta prostrata, Fimbristylis guinguanularis, Ischaemum Vagosum.
Senitaria guavarenensis, Scirpus corymbosus. It has been observed that kharif crops include greater number of weed species in comparison to that of the Rabi, however, species of latter crop represented more families than the former. It has also been observed that various families contributed differently to each crop. Graminaceae contributes maximum while Leguminosae, Compositae, Euphorbiaceae occupy next position while other families contributed only few representatives. Weeds appear with Kharif crop are represented eleven common families viz., Amaranthaceae, Capparaceae, Convolvulaceae, Cyperaceae, Euphorbiaceae, Leguminosae, Labiatae, Graminaceae, Rubiaceae and Tiliaceae, while weed species of Rabi crop are represented twelve families i.e., Cruciferae, Compositae, Caryophyllaceae, Chenopodiaceae, Convolvulaceae, Leguminosae, Liliaceae, Orobancheaceae, Graminaceae, Primulaceae, and Scrophulariaceae.

A large number of escapes which were originally considered as cultivated, are frequently met in ruderal areas, along with canal banks etc. Some of the most common plants met as escapes are: Althaea rudescii, Anethum graveolens, Capsicum annum, Catharanthus roseus, Coriandrum sativum, Cucumis melo, Cucurbita moschata, Gomphrena globosa, Linum usitatissimum, Ruellia tuberosa, Sesamum indicum, Spinacia oleracea, Trichosanthes dioica, Verbena dipinnatifida.
Several alien species have found their way in to India and have spread successfully to different parts of the country. Most of these foreign elements which coming naturalized have been brought by Portuguese, Spaniards, Dutch, the French and English people either knowingly or unknowingly. Maheshwari (1963) suggested that various factors are responsible for the spread and increase of alien plants in our country. Whenever the forests have been destroyed in connection with agricultural activities leading to open lands a mixed flora made up of weeds and weedy plants is noted.

Area around main building of the department, on which a telescope was installed, is a botanical garden. In this garden some of the important trees, shrubs, climbers and herbs are found growing. Most of them are planted and only few are growing naturally. Some important plants species are as follows:

Acalypha ciliata, Areca catechu, Casuarina equisetifolia, Delonix regia, Eucalyptus sp., Ficus benjamina, Gravillea robusta, Jacaranda mimosaefolia, Lagerstroemia indica, Mangifera indica, Azadirachta indica, Melia azedarach, Polyalthea pendula, Salmalia malabarica, Spatodea campanulata, Eugenia jamoliana, Terminalia bellierica, Pinus roxburghii, Juniperus chinensis, Ginkgo biloba, Cycas revoluta, Agathis robusta etc.
Flora of Saugar is characteristic in many ways. It shows greater correlation with that of the Gangetic Plains in order of precedence of families. The first ten dominant families of Saugar are Graminae, Leguminosae, Compositae, Cyperaceae, Euphorbiaceae, Acanthaceae, Convolvulaceae, Scrophulariaceae, Labiatae, Malvaceae. Leguminosae dominants the dicots and then is followed by Compositae. It is somewhat surprising that Compositae which is mainly temperate is so well represented in Madhya Pradesh. Amongst monocots Graminae and Cyperaceae lead the other families. Rubiaceae, Euphorbiaceae, Acanthaceae and Urticaceae are poor in their species distribution. Similarly Orchidaceae, which is the largest family of the flowering plants in India, with some 1,700 spp. is represented poorly in Saugar by only three genera. Of the total number of higher plants, dicots alone are represented roughly by 105 families and monocots by some of 20 families in compare to the entire Indian subcontinent, where there are 30,000 spp. of higher plants spread over 174 families. Thus the floristic studies of Saugar clearly reveals that it is extremely rich in its floristic endowment and is a meeting place of different geographical areas viz., Deccan, Indus plain, Gangetic plain and Malabar etc.
II. FIELD SURVEY:

For collecting the material, trips of the city and its surrounding areas (an area of 10-15 sq.km.) were made twice a week, every month. Names both botanical and vernacular, mode of pollination, flowering periods of plant growing in the area were recorded. Herbarium specimens were also prepared following the usual methods. The flowering period of the plants were recorded in the following three stages.

Stage I  - 80-90% plants are in flower - Bud stage.
Stage II - 20-30% plants are in flowering.
Stage III - 80-90% plants are in flowering.

The field observations about the average period of beginning and completion of the flowering of various plant species are given in tables 5 to 7.

III. COLLECTION OF POLLINIFEROUS MATERIALS:

The polliniferous material was collected for two purposes -

(i) to prepare reference slides and
(ii) to send the material in bulk to V.P. Chest Institute, Delhi, on their request for clinical studies to be made by them.

Polliniferous material (immature buds and flowers) was collected in the morning as in most of the plants
anthesis occurs soon after sunrise. To ensure purity of samples of polleniferous material, it was directly collected in small brown paper envelopes. Pollen from cultivated grasses and other anemophilous plants was collected by repeatedly jerking the inflorescence after bending them inside the envelopes. In other group of plants where pollen did not shed freely, flowers were picked and dried. They were than gently thresed with a pestle and mortar. As far as possible an attempt was made to collect the material, when most of the plant were flowering i.e. stage II, because at that time the yield of the pollen was maximum.

Immediately on reaching the laboratory the polleniferous material was spread properly in large enamelled trays for initial drying in diffused sunlight in a well ventilated room during summer, but during winter the drying was carried out in direct sunlight or in a oven at 35°C temperature for a couple of days. The dried material was rubbed tightly with a light pestle and mortar; and then it was passed through 100, 200 and 300 mesh sieves respectively to obtain pure pollen grains. The purity of the pollen was confirmed by microscopic examination.

For the purpose of storage the dried pollen grains were first treated with solvent ether using soxhlet apparatus to remove fats and dust particles. Next the pure defatted pollen grains were dehydrated in a vacuum desicator and
stored in air tight vials at 4°C.

PREPARATION OF REFERENCE SLIDES:

The dried pollen grains were transferred to glass vials containing 70% alcohol and allowed to be soaked for about an hour and then transferred to a plastic centrifuge tube, where it was crushed by means of a glass rod. The dispersion was passed through a sieve (48 meshes/cm), and was distributed in equal quantity in two glass centrifuge tubes marked 'A' and 'B'. The contents of these tubes were processed further following different method of pollen preparations.

(1) Unacetolysis or Alcoholic Method:

The contents of glass tube marked 'A' was centrifuged at 2500 rpm for about 3 minutes. After that the alcohol was decanted off in a separate container and water was added alongwith two or three drop of safranin (5 per cent in water) and then it was warmed slightly over a flame. To intensifies staining, tube was left aside for about 15 minutes. After that the tube was again centrifuged and the supernatant was decant off. The process of washing of material (by adding distilled water, centrifuging and decanting off the supernatant) was done repeatedly till the supernatant layer become colourless. After final washing with water dilute glycerine (50%) was added in the tube. After a few
minutes the material was finally centrifuged at the same speed, after decant off the excess glycerine, the tube was placed inverted on a filter paper till all the liquid was completely absorbed by it. The pollen material present in the tube was now ready to be used for the preparation of slides.

(ii) Acetolysis Method

The dispersion of other glass tube marked '3' was centrifuged at 2500 rpm for about 2-3 minutes. The alcohol was then decanted off and material was washed with normal glacial acetic acid. It was again centrifuged and the supernatant was again decant off. In this centrifuge tube 5 cc of acetolysis mixture (Erdtman 1952) was added and the tube was placed in a waterbath. The waterbath was heated till the water started boiling, the tube was left as such in water bath till a brown coloration appeared. The tube was again centrifuged for about a minute or so. After decanting off the supernatant layer the tube was placed inverted on a filter paper to remove the excess mixture. 5-10 cc of distilled water was added in the tube and it was again centrifuged finally. After that the water was again decant off and the tube was placed inverted on a filter paper and the material became ready for the preparation of pollen slides.
The pollen slides were made from the pollen sediments of tubes 'A' and 'B' by the following method.

Small pieces of glycerine jelly prepared by Kissner's method (Erdtman 1952) were cut by a razor blade. A pellet of jelly was carried on the hook of a needle and the jelly was passed over the flame of a spirit lamp to make the jelly melt a little. Next the jelly containing needle was inserted in the tube and was made to touched the centre of the pollen sediment. In this way some pollen adhered to the pellet, which was placed in the centre of a slightly warmed slide. It was warmed again and the pollen grains were gently and carefully spread with the help of a needle. The slide was then made permanent by keeping a coverslip and ringing with molten paraffin wax.

Large collections of pure pollen grains of the following plants were sent to V.P. Chest Institute, Delhi for clinical studies:

Helopteles integrifolia, Pongamia pinnata, Prosopis juliflora, Eucalyptus Sp., Cassia fistula, Acacia nilotica, Brassica campestris, Eragrostis tenella, Chenopodium album, Azadirachta indica, Cyperus rotundus, Terminalia arjuna, Mangifera indica, Cyndonon dactylon.
IV. ANALYSIS OF AEROSPORA OF SAUGAR

Aerobiological studies have received much attention, recently, because of application in the field of allergy, dispersal of pathogen and allied aspects of microbiology, since pollen grains and fungal spores are the common constituents of aerospora and was proved to be the major cause of certain types of human respiratory diseases.

For collection of pollen grains and fungal spores from air, an aereoscope, a pollen spore catching device was used. The aereoscope was supplied by I&BhL, Lucknow during the workshop on aerobiology project held in 1979. The details of the aereoscope i.e., description, its installation, maintenance and precautions, for handling the apparatus and method of preparation of glycerine jelly, slides are given below.

(a) DESCRIPTION OF AEREOSCOPE:

The aereoscope consists of three main parts i.e., base plate, swing cylinder and wind vane assembly.

Base Plate:

It is made up of a heavy metal and about 47 cm diameter. It is raised on rubber gromets. The plate of the aereoscope is firm enough to stand atmospheric disturbances. It has central stem with pointed apex on which swing cylinder can be fitted.
**Swing Cylinder**

It has a cylindrical pipe with two ball bearing fitted on either ends of the axle. It also has locking system at both the ends by which wind vane and base plate can be fitted on the axle.

**Wind Vane Assembly**

Complete wind vane assembly is attached by the swing cylinder at upper end by locking pin. It consists of (i) wind vane (ii) slide holder and (iii) protective cover. Wind vane is a flat plate and made up of aluminium metal, it is 28 cm width and 38 cm long; and mounted vertically i.e. at right angle to the ground level on a vertical steel rod. In slide holder three microslides can be kept. Slide holder can be pulled out from its sliding cannell fitted in the slide container. Slide holder is covered by a rectangular horizontal protective cover to avoid washing off slides due to rains. The wind vane assembly is freely rotating on a pointed shaft. The various parts of the aeroscope are shown in figure 3.

**Installation**

The aeroscope was installed on the terrace of post graduate building of Botany department, at about 10 meters above ground level. Though the base plate of aeroscope is firm enough to stand atmospheric disturbance; however, it
was permanently fixed by means of three metal holders around it. The whole aeroscope was also covered by erecting a cage around it to avoid further disturbances from birds, and other animals. The cage was made up of expanded metal wire mesh. More than 4” space was kept between aeroscope and the cage.

**Maintenance and Precautions of Aeroscope:**

Following precautions were taken for the proper maintenance of the aeroscope.

1. Aeroscope was installed on a levelled surface. A spirit level meter was used for checking vertical and horizontal level of the apparatus.

2. To avoid damage to the fan blade and slides of the slide holder, the instrument was kept covered by a metal cage.

3. To remove friction, a regular cleaning and lubrication of the bearings was done. For this first of all a locking pin was removed than the square pipe was pulled out. The two ball bearings fitted on both the ends of the pipe were first cleaned with petrol or kerosine oil; then the lubrication was done with some good quality of lubricant and it was again fitted in the aeroscope.

4. The slide holder was pulled off gently from the slide channel to avoid jerks.
(b) PREPARATION OF GLYCERINE JELLY

For the preparation of glycerine jelly, 100 gms of gelatine powder was poured in warm water (50°C) in a beaker and was left in it till the gelatin swells. After this excess water was removed from the beaker and then 100 ml of pure glycerine was added to it. The beaker was heated in a water bath. The gelatin was stirred properly and constantly with a glass rod so that the mixture became homogenous. Then two ml of phenol was added and mixture was again stirred. The air bubbles settled on the surface of the jelly were removed by spatula or spoon. When the jelly becomes transparent and free from air bubbles, the beaker, containing jelly was removed from the waterbath and jelly was filtered through a muslin cloth. Filtered jelly was collected in another beaker and immediately after this it was poured into petri plates and was allowed to solidify. These petridishes were covered to avoid contamination and were kept in a refrigerator.

(c) PREPARATION OF POLLEN SLIDES:

The gravity slide method was used for catching the airborne pollen grains and fungal spores. Daily three labelled microslides (entering the requisite information on the slide number, centre, date of exposure etc.) were smeared with a thin layer of safranin stained glycerine jelly over an area not less than that of the coverglass (25 x 50 mm).
These slides were then kept in the slits of the slide holder which is then slid into the horizontal cover of the aeroscope. The above process was done at 10:00 A.M. every day and after 24 hours the exposed slides were replaced by another set of three freshly prepared slides.

The exposed slides were brought to the laboratory and by means of hand lens, first of all large particles of inorganic matters were removed carefully, then the slides were made permanent after slight melting of jelly over the flame of lamp and covering the exposed area by rectangular (25 x 50 mm) cover glass.

For the air borne pollen identification, field observations on flowering periods, reference slides, and published palynological literature (Wodehouse, 1935; Erdtman, 1952; Durham, 1953; Hyde and Adams, 1958; Neira, 1965) were used.

(d) DATA COLLECTION

The under surface area of the coverglass was used for study as a unit area. Each slide was thoroughly studied by several sweeps, starting from one end to the another end. Quantitative analysis of pollen grains and fungal spores was done in term of the number of pollen and spore found on an area of coverglass (25 x 50 mm). The variously classified pollen grains and fungal spores trapped on the slides were counted per unit area per day, and the percentage from the total flora was calculated for the whole month.