MATERIALS AND METHODS

The South-Western part of Maharashtra State is rich in vegetation which also provides rich mycoflora. The ecological conditions, mainly high rainfall, humidity, low temperature and high altitude are favourable for the growth of various plants and fungi. These are very favourable climatic conditions required for the growth and development of parasitic as well as saprophytic fungi. All the ideal localities selected under this study are mainly confined to the Western Ghats and provide a good amount of mycological collections. These areas have been worked out sporadically by different mycologists from time to time and studied the fungal flora. But the Discomycetous fungi which have not received serious attention so far. Therefore, a systematic investigation has been undertaken here to study these fungi, which are not easily accessible due to their ephimeral nature and inaccessibility of areas during rainy seasons.

The methods have been adopted here in accordance with the florestic and taxonomic study of Discomycetous fungi. The Discomycetous fungi which were collected in the form of ascocarps or fruiting bodies i.e. the apothecia from different localities of Western Ghats particularly after the mansoon seasons.

Area under coverage : The Discomycetous fungi which have been studied here, were collected from well known localities

viz. Panhala, Radhanagari, Manjarkhind, Selap, Salvan, Patgaon, Gaganbawada, Ajara, Gawase and Amba of Kolhapur district.

Systematic collection provides a basis for taxonomic research and therefore, by a thorough field study only, one can become familier with the inherent variability of the species and the effect of the environmental factors on them. Their different habits, appearance i.e. smaller or larger, predominance of a particular group, the range of variation within the individual species within a particular area could be recognised in the field study. Workers in thefield of Mycology have to pay particular attention to the delimited habitats, parasites and their host etc. and this becomes possible only by frequent visits to a site or sites as many times of the year as possible.

The delicate, ephimeral and variously coloured apothecia and ascocarps were collected by repeated and frequent periodical visits during the mansoon. These were collected in polythene bags or in plastic bottles. A large number of Geoglossaceae forms were collected from the humus rich soils of vallies, planes, open grounds, grasslands and plant debris. Due to the high rainfall, the localities like Radhanagari, Amba and Panhala were provided a good amount of the material while other minor localities provided some of the ephimeral forms. Majority of the Geoglossaceae members were collected from these localities. The ecological features greatly influenced the mycological flora from region to region and from locality to locality. Most of the collections were collected from humus rich soil under the

shade of forests trees in the mansoon. These fungi were collected with a keen eye-sight so that even smaller and critical species were not overlooked.

Each and every selected area for the collection of fresh apothecia were screened carefully.

As soon as the fresh, and delicate ascocarps were collected in the field, the data containing the name of the locality, altitude, date of collection, colour of ascocarp, their distribution and substrata etc. were first recorded in the field note book and these materials were brought in bottles, polythene bags or specimen tubes with great care, separately in large quantity if available of various sizes and different stages of their development. After the preliminary observations of the specimens, colour photographs were taken to reflect their natural colours and habits. Specimens from the same locality were placed in separate tubes. Each tube with a specimen was labelled in the field. Tentative identification of the genera was made with the help of key characters. Fleshy ascocarps or fruit bodies of Helotiales were the easiest fungi to observe in the field. Spatulate and large ascocarps (Trichoglossum, Geoglossum, Spathularia, Thuemenidium) with black, brown and flesh coloured and their exudates were used for identification in the field.

Laboratory observations :

After the collection from a particular locality, the whole lot of tentatively labelled or unlabelled specimens were brought into the laboratory for further observations. Laboratory operations were started by clearing and washing of the material. All the specimens were sorted into different groups.

Cleaned and matured ascocarps were used to examine by usual laboratory methods. Different laboratory techniques were used for further identification of the members of the order Helotiales. The important characters like texture, colour, consistency, size, dominance and frequency were recorded. Generally, in coloured fungi, it is necessary to note the colour of ascocarp while they are fresh, as fading of colour generally occurs. Special attention also was given to the habit of the ascocarps and their position in or on the substrata, presence or absence of hairs, setae etc. The detailed external morphology of specimens was kept upto date for further use.

Laboratory techniques : After the preliminary laboratory observations were made, ascocarps were processed for further microscopic examinations. The microscopical features were mostly studied by mounting a piece of it either by sectioning or scrapping or by squashing and strained with cotton blue.

Sectioning of ascocarps : To ascertain the detailed

anatomical structure of ascocarps accurately, transverse and vertical sections are essential. All the member of Helotiales were studied by taking transverse or vertical sections of the fertile and sterile parts of the matured ascocarps.

The sectioning was made by following methods : (1) Simple hand cut method, (2) freezing microtome or routine microtome (Rotary).

1. Hand cut sectioning : Adequate T.S. or V.S. of the ascocarp was taken by hand using a single or double edged razor blade and holding the material between some tissue such as elder pith.

2. Cryostat sectioning (Freezing microtome) : Freezing microtomes form an essential part of a mycological laboratory equipment and consist of a fixed stage cooled to below the freezing point of water (either by carbon dioxide or a 'Pelcool' cooled water system). In the present investigation a Pelcool cooled water system has been used; and a movable blade calibrated to cut material frozen on the stage with a drop of dilute gum arabic in water at a range of thickness, 5,10,15 or 20 μm (10 μm thick sections are the most commonly used). The sections were collected with a fine point brush as they were cut and placed directly in a drop of mounting fluid on a slide or in water in a watch glass.

3. Squash method : This a very simple method of sectioning

and can be used to study the details of individual part of the ascocarp. Squash preparations were made with the help of single edged razor blade and mounted in different mounting media with stains. This method was used to observe and study the asci, ascospores, paraphyses, setae, hairs etc. in their free conditions so as to observe them in entire form in detail which help during the micrometry of these structures.

Staining technique :

The different stains and the staining techniques are used to study ascus layers, ascus tips, ascospores and tissues of ascocarps. The staining technique of microbes was first introduced by Garl Von Welgert in 1871. The colouring agents used in the microtechniques are of various kinds but majority of them are dyes, so called as stains. Any colouring organic matter, usually called dye, is used to stain tissues, cells, cell components or cell contents. Stains are classified according to their molecular weights or onthe basis of their chemical behaviour as, acidic, basic and neutral. Different stains have different chemical actions against the chemical compounds of a cell.

Mycological stains :

The different fungal structure such as mycelium, asci, ascospores, conidia and paraphyses etc. can be studied with the help of different colouring dyes. They are used as per the

nature of the fungus material and are called as mycological stains. In the present study different stains have been used. The unitunicate and bitunicate asci, ascus tips, hyphae, septation of ascospore etc. are very important structures used in the taxonomy of the fungi and, therefore, they were studied carefully with the help of different stains. The different stains used in the present study are as follows :

1. Melzer's Reagent : The first and most important medium other than water, is surely Melzer's Reagent (Nylander, 1869), which contains.

Iodine	..	0.5 g
Potassium iodide	..	1.5 g
Chloral hydrate	..	20.0 g
Distilled water	..	20.0 ml

This stain was prepared by dissolving 0.5 g of iodine and 1.5 g of potassium iodide in 20 ml of distilled water separately in two different containers and both the solutions were mixed in 20 ml of chloral hydrate. The mixture was filtered before use.

Melzer's Reagent is used to check the iodine reaction of the ascus wall, while chloral hydrate is added to this reagent as a clearing reagent. This reagent used both as a general differential stain and to test the iodine reaction of the ascus apex or ascus pore. A positive reaction denoted by

'J+' was usually with some shade of blue, but occassionally violet colours were also produced. The apex of the ascus may blue, or a ring of plug may blue. A negative reaction, in which no blue colour was produced, is frequently termed 'J-'. The blue reaction of the pore plug is useful in Helotiales.

2. Cotton blue : It is an acidic stain used to study the fungal structures. It is a cytoplasmic stain. The cytoplasm turns blue in colour leaving the hyphal or spore wall and septa hyaline. Cotton blue stain was prepared by dissolving 1 g of cotton blue in 100 ml of lectophenol. 1% stain is generally used in mycological study.

I) Preparation of cotton blue : 0.05 g of cotton blue in 100 ml of lectophenol

II) Preparation of Lactophenol: Mixed phenol, glycerine, lactic acid and water in equal parts by weight.

3. Lactophenol : This is used as mounting medium. The semipermanent preparations were mounted in the lactophenol and the slides were slightly warmed so as to remove the air bubbles. The mounting medium was prepared by adding 1 part of phenol, 39 parts of glycerine and 1 part of lactic acid in 9 parts of distilled water. The solution was filtered before use. The lactophenol is used because of swelling and preservative properties of lactic acid and phenol while glycerine keeps the material free from rapid dehydration.

Micropreparations : During this study, the slides were made semi-permanent by sealing them with wax or nail polish paint, while the permanent slides of micropreparations were mounted in Canada balsam or DPX. The satisfactory mounting medium for semi-permanent micropreparation was Amman's lacto-phenol including a stain cotton blue. In some cases it was found better to make permanent preparation rather than temporary mounts.

Major bulk of micropreparations were made by semipermanent techniques. Amann's lactophenol including a stain cotton blue has the advantage that the slides can be redily preserved by sealing them with wax or nail varnish. The slides in lacto-phenol wanted to preserved were warmed for an hour or so to ensured that all the air bubbles were completely eliminated and any excess mounting fluid thoroughly cleaned off. Semipermanent slides made with lactophenol were preserved by ringing with nail varnish or wax and placed in specially made wooden cabinate. Each and every semipermanent slide was labelled properly.

Measurements : Micrometry is an important and essential part in fungal taxonomical study. Various structures are to be measured and these measurements are used in the identification and classification of the different taxa. The metric units are used for macro and micromeasurements i.e. cm, mm, µm. The unit of measurement used in the descriptions of the present work is µm (micro-meter) instead of µ (micron) because most scientific journals have adopted this change over.

Measurements of asci, ascospores, paraphyses, setaes and sterile tissue from apothecia were made by occular and 10 x,15 x and 100 x objectives.

Description : All the characters which are useful in the classification and were distinctive such as colour, size, shape and cells of apothecia, types of setae, hairs, asci, ascospores, septation and paraphyses have been described as per the mycological terminology adopted by different mycologists.

Identification : The genera, species and varieties of the family Geoglossaceae were confirmed with the help of recent and upto date literature published by many workers from the field of Mycology. Keys and monographs by different mycologist were used to identify the genera and species. Confirmation of these taxa and their reports for Maharashtra and India were carried out with the help of the recent and upto date literature.

Illustration : It constitutes drawing, maps, plates and tables.

i) Camera Lucida drawings : The pencil (4 H) drawings of the micropreparations were made with the help of drawing aid (Camera lucida) attached to the microscope. The Camera Lucida pencil drawing were drawn with 5 X, 10 X and 15 X magnification eye pieces in combination with 10 X and 15 X objectives. The magnification was variable according to the size of the elements.

ii) Drawing and text plates : Sketches were drawn on plain paper with the help of black water proof Indian Ink. The thickness of each sketch was maintain uniform.

iii) Tables : Tables are valuable in the taxonomic studies for summerizing the statistical data of the taxa. Tables were made showing the comparison between original (type) species and the present collections, whenever the new variety has been proposed as well as to summerize the florestic pattern.

iv) Keys : These are the means for identification of various taxa of the different status. The adequate and upto-date keys do not exist and the taxonomists have to construct their own keys.

v) Maps : Maps are valuable additions to accounts of species as they show at a glance their known distribution. Maps were produced as accurately as possible to show the distribution of the species.

vi) Photographs : The photographs of the material show the habits and colour of fruiting body or apothecia.

vii) Plates of the photographs : Photographs of materials mounted on hard paper with the help of adhesive. They are properly numbered. ~~Photographs~~ & were mentioned on separate page.

viii) Citations and References : The references were cited by giving the list of the references or bibliography alfabetically.

ix) Preservation and deposition of the material : Preservation of the material is an important part in the fungal taxonomy. These members are fleshy. So these are preserved in 2-4% formaldehyde solution in glass tubes or containers with proper label (as class, order, family, genus, species, variety, locality and date). They were deposited in Mycological Herbarium, Department of Botany, S.U. Kolhapur under the code numbers as WIF

Preservation and deposition of Micro-preparations : The semi-permanent Micropreparations were cleaned, properly labelled with water proof black ink and arranged genuswise in wooden cabinate and were deposited in Mycological Herbarium, Botany Department, Shivaji University, Kolhapur (M.S.).