C H A P T E R - III

MATERIALS AND METHODS

Area of Coverage :

The present investigation is based on the collections collected mainly from the South-Western Maharashtra, by periodical visits to the well known botanically rich areas viz. Mahabaleshwar, Panhala, Radhanagari, Koynanagar, Kolhapur, Karad, throughout the years in different seasons. The proportion of collection recorded for a given area is directly related to the intensity of sampling. They should not be interpreted being necessarily meaningful in terms of distribution, relative abundance, or ecological amplitude of a taxon. Such information can be obtained only through a sustained, long-term collecting program that covers all parts of the region adequately.

Incidence and collection of Chlorochytrium, Synchytrium and Endogonaceae :

Some of the specimens which are included in present study were collected from variety of location (Text Fig. 1) mentioned in the text. However, the members of the genus Chlorochytrium Cohn, Synchytrium DeBary and Woronin and Endogonaceae were growing either parasitically or saprophytically as well as endophytically. The species of Chlorochytrium were to occur as endophytic, which grow

in the tissues of fresh as well as marine water plants. In rainy season Chlorochytrium species were collected on number of angiospermic plant parts which were growing in aquatic conditions in ponds, ditches, streams, tanks etc. or plant parts which were very close to damp soil surface. Infected plants parts are readily recognised by presence of green, globular or irregular elevations as green dots or as pimples. Infected plants and their parts were collected in the months of June to September and brought in to laboratory for further process.

There is a tendency for some species of Synchytrium DeBary and Woronin to infect different parts of phanerogamic plants to form galls or hypertrophied structures; such infected plants were collected in the months of June to October during rainy season. They were either kept in polythene bags or glass bottles and were brought in to laboratory for further processing and investigation.

The members of the family Endogonaceae showed variations in nature of habit and habitat (Gerde and Trappe, 1974). The species of the genus Modicella Kanouse and certain species of the genus Glomus Tulasne and Tulasne are known to occur as saprophytes on soil, litters or decaying woods. Some are known to occur hypogeously. However, not all but some may produce epigeous or hypogeous fruiting bodies or sporocarps or ectocarpic hypogeous free spores. Special technique has been used to find the hypogeous sporocarps or spores. Species with larger sporocarps were collected more or less by chance by raking and searching through

the fallen leaves or leaf litter and the upper layer of soil under shades of forest trees. As after couple of days sporocarps may vanish. It is suspected that nematods (Hall, 1977) and soil insects, rodents might be using this fungal materials as a source of food (Bhagwat and Kelker, 1974). There is also seasonal fluctuations in the total spore numbers of Endogonaceae in soil (Hayman, 1970; Majstrick, 1972; Sutton and Barren, 1972 and Bakshi, 1974). There also seem to be good and bad spore forming years (Mosse and Bowen, 1968) and there is a tendency for some species to have extremely limited distribution.

However, larger epigeous sporocarps were collected from field after first shower of rain in monsoon season and they were kept in polythene bags or bottles. Species which show ectocarpic free spores or hypogeous sporocarps in such cases, the soil samples were collected by special method (Bakshi, 1974) from various localities in the months of September to April. Then sporocarpic materials and soil samples were further processed in the laboratory.

Preservation and Storage of materials :

The specimens of Chlorochytrium and Synchytrium were collected along with their hosts and identification of hosts were done immediately, when collections were brought in to laboratory, with the help of flora. The gross changes in morphology due to infection were also simultaneously noted. However, specimens

host parts?

along with their hosts were preserved in 50 % F.A.A. (Formalin-Acetic Acid - Alcohol); and 4 % Formalin (40 % v/v) solution in glass bottles. The bottles were at the same time labelled.

The specimens of Endogonaceae which form epigeous macro-fruiting bodies i.e. sporocarps were preserved along with their substratum, in 50 % F.A.A. and 4 % Formalin (40 % v/v) solution in glass bottles. Some times fruiting bodies were also air dried and stored in glass or plastic bottles at room temperatures, for further study. Soil samples containing ectocarpic free spores of Endogonaceae were collected from rhizospheres of various forest and cultivated field plants. Soil samples were air dried and stored at room temperature in small polythene bags.

Special Methods of Extractions and Isolation of Endogonaceaeous Spores from soil :

a) Soil Sampling : Soil sampling were done by using modified technique of Bakshi (1974), in which soil samples were randomly collected at a distance of approximately one meter from the plants, in case of forest trees. While in case of field soil, the sample were randomly taken from rhizosphere of field crops. Soil samples were taken with the help of auger or iron peg, 5 cm core diameter. Five soils were sampled underneath each plant upto a depth of 10 cm, 25 samples underneath soil of each area of forest or cultivated field were collected and thoroughly mixed to yield a composite sample out of which 100 gms soil samples were then kept in cloth

or polythene bags and brought in to laboratory for further processing and analysis.

b) Wet-sieving and decanting Method : Especially for isolation and study of ectocarpic spores from soil, most common technique was used. This technique includes wet-sieving and decanting method which was first devised by nematologists and adopted by Gardemann and Nicolson (1963). Here, in this present study modified, wet-sieving and decanting methods were used for isolation of spores from soil. This involves preparation of suspension of air dried soil samples in water; shaking vigorously and allowed to settle and then decanted through a fine sieve or even Whatmann's Paper No.1 on which spores were held back. This filter paper is then spread on petridish and examined under binocular or dissecting microscope. For detailed examination, spores were picked up with the help of needles and were further cleaned or cleared in lactophenol on a grooved or ordinary glass slides. Slides were covered with cover slips and their edges were sealed with special wax sealing medium or nail polish.

c) Herbarium Deposits : Sporocarps larger than about 1 mm diam. were dried and packed. While some sporocarps and ectocarpic spores were put in small screw vials, half-filled with F.A.A. or 4 % Formalin or Lactophenol or mounted in lactophenol on microscopic slides with the cover glass edges sealed with either special wax sealing medium or nail polish.

All collections were assigned as WIF.- Fungi of Western India. Collections were also numbered and deposited in MHBDSUK. "Mycological Herberium, Botany Department, Shivaji University, Kolhapur."

Temporary Preparations :

The collections of Chlorochytrium, Synchytrium and members of Endogonaceae were examined by usual laboratory methods. For the microscopic study, slides were prepared by taking the free hand sections of fruiting bodies or infected plant parts and were stained with cotton blue and mounted in lactophenol. Slides were then covered with cover slips and their edges were sealed with special wax sealing medium or nail polish (semi-permanent preparation).

Permanent Preparation :

Sometimes it is possible to gain useful information about sporocarps, spores, hypae and infected materials, it is found to be better to prepare permanent preparations rather than usual temporary mounts. In such cases, the fresh specimens were cut into small pieces (in case of large size) and fixed in Formalin - Glacial-acetic acid - Ethyl alcohol (FAA). After fixation for 24-48 hours in fixative materials were transferred into a preservative after washing by 70 % alcohol to remove traces of fixative, (70 % ethanol) then passed through the grades of alcohol-xylol for

dehydration and then kept for infiltration in paraffin wax of 56-60°C and blocks were prepared as usual method.

The blocks were cut on rotary microtome at 5-10 μm thickness. The sections were made permanent by staining with Hematoxylin (alcoholic) and counter-stained with orange G (inclove oil). Then slides were cleared in clove oil and mounted in permanent mounting medium, that is either euporal or DPX or Canada-balasm. However, Euporal or DPX gives the better results. (A usual process for making permanent micro-preparations).

Microscopy and Drawings :

Unless otherwise stated, all microscopic examinations were made with mounts in lactophenol. Lactophenol and Cotton-blue are useful for emphasizing hyphal, spore characteristics and for interpreting some types of apparent surface ornamentation of spores. Generally, a KOH solution not used as mounting medium for Endogonaceae, as it causes extreme swellings of some spore walls, with a resulting destruction of spores (Gerdemann and Trappe, 1970).

However, all the drawings of micropreparations were drawn with the help of prism type "Erma Camera-lucida" at stage level using 10X, 45X, 100X objectives and 5X, 10X, 15X eye pieces. Obviously comibination of objectives and eye pieces were purely based upon size of sections and spores. Measurement of material

and spores, sporangia, subtending hyphae, galls or swellings were made by Ernst Letiz - Vetzlar Occular.

Photomicrography :

Photomicrography was made by using Olymphus PM-6 unit of photomicrography by 5X, 10X, 15X eye pieces and 10X, 40X, 100X objectives, which were based on the size of the specimens.

Identifications :

Identification of the species of Chlorochytrium, Synchytrium and the species of the different genera of the family Endogonaceae were confirmed by the most up-to-date literatures available as - Smith (1955), Fritsch (1961), Chapmann (1969), Venkatraman et al. (1974), David (1976), Webber (1978), Bold and Wynne (1978), Tittley (1979) and Biswas (1980).

Ainsworth, Sparrow and Susmann (1973), Karling (1964), Patil and Mahabale (1964), Patil (1975, 1978), Hawaker (1962), Gerdemann and Nicolson (1963); Mosse and Bowen (1968), Mosse (1970, 1973); Haymann (1970), Bakshi (1974), Rikkhy and Mukerji (1974); Bhattacharjee and Mukerji (1980); Mukerji et al. (1980), Gerdemann and Trappe, (1974); Gardemann and Bakshi (1976), Trappe and Gerdemann (1972), Koske (1974, 1981), Hepper and Smith (1976), Trappe (1977, 1979, 1981, 1982); Hall (1977, 1978, 1979, 1983), Becker and Gerdemann (1977), Sward, Hallam and Holland (1978), Hall and Fish (1979), Rose, Daniels and Trappe (1979), Tommerup

and Kidby (1979), Danniels (1980), Iqbal and Perveen (1980), Rose and Trappe (1980), Grand and Randall (1981), Walker and Trappe (1981), Walker (1982), Trappe (1982), Schenck and Smith (1982), Nicolson and Schenck (1979), Trappe and Schenck (1982) and Janos and Trappe (1982).

Description :

Spores and specimens were described in terms of following characteristics :-

Shape, size (mounted in lactophenol) colour (with incident lighting) appearance of the cytoplasm, wall structure, details of two structures of attachment; galls or swellings and any other relevant features.

However, it is important that a note of colour and size made before mounting, as spore colour, sporocarps colour, or galls or swellings colour and size may change in the mount and the weight and surface tension produced by a cover slip can change spore or galls dimensions. Several days or even weeks were required for adequate clearing of spores especially spore walls.

Herberium Deposits :

Sporocarps and infected plant materials were put in small screw vials or bottles half-filled with either F.A.A. or 4 % Formalin or lactophenol or their sections were mounted in

lactophenol on microslides with the cover glass edges sealed with either special wax sealing medium or nail-polish.

All collections were assigned to Fungi of Western India abbreviated as 'WIF.', numbered and deposited in the "Mycological Herberium, Botany Department, Shivaji University, Kolhapur" and abbreviated as 'MHBDSUK'.