Chapter - II

Studies in *Meliola diospyricola* Hansf.

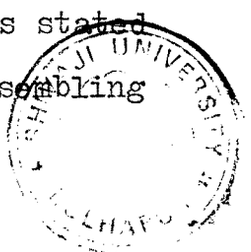
I) INTRODUCTION AND HISTORICAL REVIEW

Our knowledge of tropical fungi largely depends upon the foundations laid by the contributions of Berkeley (1857), Cooke (1872), Henning (1927), Rehm (1887) and Leveille (1845). At present with greater facilities at our disposal, closer contact with regions and better conditions for collections, the number of workers on exotic fungi have been greatly aumented. Our knowledge of tropical fungus flora is also rapidly increasing. The Meliolales have been studied extensively by Sydow (1927, 1930, 1938); Theissen (1913-a, 1913-b, 1913-c, 1916); Theissen and Sydow (1915, 1917, 1918); Stevens (1927, 1928-a, 1928-b); Hansford (1963) from the stand point of their morphology and classification. But very little work on cytology and development of the members of this order have been carried out so far. This has probably resulted from the fact that most of these fungi are being parasites on host plants lacking economic importance. As pointed out by Wellman (1972) .... "Now here are these black mildews being made a subject of major pathological study, although agriculturists who observe their crops well, know that at times these fungi are very damaging in their effects." A better understanding of the biology of these fungi is needed. With the advancement application of idea that

taxonomy should be an expression of phylogeny, more detailed studies are needed. This type of investigation needs the recognition of more fundamental facts regarding the life history of an organism.

In this way Ward (1882, 1883) studied the genus Meliola, but his conclusions regarding rudiments of sex organs were entirely hypothetical. Bornet (1883) has given the most detailed account of the development of Meliola. As regards sexuality in this genus he has stated that the original pyriform branchlets bearing the elements of fruiting body may be considered as an archicarpium; (De Bary in 1863, proposed this word to that part of the body which becomes the ascus in Podosphaera) while antheridial branch produces the antheridium and perithecial wall. Gillard (1891, 1892, 1893) believed that two types of hyphopodia can be readily recognized and distinguished in to "hyphopodies mucronees" and "hyphopodies capitees", but all hyphopodia do not produce the perithecia. Ward (1883) made no such distinction and considered them as merely hyphal branches with arrested growth. Gillard (1891, 1892, 1893) described the presence of only single protective wall about the ascocarp and without any indication of ostiole.

Thaxter (1893) studied Laboulbeniaceae and has stated that it is worthy to note that the bodies closely resembling



the typical antheridial cells, found in this family were the "hyphopodies mucronees" of Meliolae. He further concluded that the capitate hyphopodia may develop in to ascocarp while the mucronate hyphopodia do not develop further and therefore they can be considered as antheridia. Bucholtz (1897) observed well developed ostiole in the ascocarp of Meliola at the time of maturity only, though in early stages of development none was apparent. He also observed periphyses protruding through the ostiole at maturity but no paraphyses were seen. In young condition interior of ascocarp appears to be parenchymatous in nature. Von Hohnel (1907, 1917, 1918) in studing a number of species of Meliola and related forms came to the conclusion that thyriothecium is not a single structure but composed of two portions a protective shield and a true perithecium. He also found that the perithecium is not inverted but upright in its development. Thus supporting Gillard (1891, 1892) on this point. Arnaud (1918) was of the opinion that the hyphopodia were rudiments of perithecia. Thus till Graff (1932) all workers were not definite and firm about their conclusions which might be due to lack of detailed study of Meliola. Thus much of, the confusion of, the past was found to hinge upon a misconception of what constitutes a perithecium and loose use of the word in reference to outer portion of ascocarp when a stroma is present.

Graff (1932) studied Meliola circinance Earle and brought to light many facts with evidence. He showed the important events in the formation of ascocarp, asci and ascospores. He found true sex organs, antheridium and ascogonium, beneath the protection of already formed stroma. In this species he never observed the haustoria, which represents the extreme ectoparasitism. According to him Meliola should be placed in Dothidiales either on the basis of stromatic development or perithecial formation. On the other hand he is also of the opinion that this group should be excluded from Dothidiales because of well developed ostiolate - perithecium with protruding periphyses. Bessey (1965) treated the family Meliolaceae under Erysiphales because there is Pseudoparenchymatous tissue filling the perithecial cavity prior to the formation of asci. However, there is an apical opening - ostiole - with protruding periphyses and the ascospores are forcibly discharged through it, the family is better placed in the order Sphaeriales as suggested by Booth (1966) and Thite (1973). Graff (1932) has finally concluded that "further cytological studies among Meliolales including more species of Meliola and related genera are much needed, it is, therefore, obvious that it is difficult to settle the systematic, position of Meliolaceae without the cytological and developmental studies of large number of species". It is with this idea attempt is being

made to study Indian species of Meliola i.e. M.dispyricola Hansf.

II) MATERIALS AND METHODS

The leaves of Diospyros montana Roxb. infected with Meliola diospyricola Hansf. were collected from Castle-Rock in the months of October-November at an attitude of 0 to 200 meters where the average annual rainfall ranges from 250 cms. to 500 cms. and temperature from 14°C to 32°C in different places. They were then fixed on the spot and also in the laboratory in the fixative F.A.A. Later on they were passed through various grades of alcohol to paraffin. Microtome sections to the thickness 5 to 7μ were cut and stained in Heidenhein's Haematoxylin and counter stained in Orange G. for differentiation 4% Iron Alum and saturated Picric acid solutions were used which gave the satisfactory results.

III) RESULTS

i) Description of the fungus :

One of the largest genera Meliola of the family Meliolaceae was established by Fries in 1825 with Meliola amphitricha as a type species. At present this genus comprises well over thousand species parasiting various host families distributed all over the world. Stevens (1928-b); Hansford (1955); Hansford and Thirumalachar (1948);

Kapoor (1967); Kar and Maity (1971); Thite and Kulkarni (1973, 1975, 1976); Thite and Patil (1978) have reported about 85 species of Meliola from India. Meliola diospyricola was first reported by Hansford (1953) from N.S. Wales on Diospyros australis and from Philippines on Diospyros maritima. Later it was reported by Thite (1974) from Annode (Mysore State) growing on the leaves of Diospyros montana Roxb. (Ebeniaceae). This fungus was also collected by the author from Castle-Rock growing on same host (Plate-I).

The fungus is characterised by amphigenous colonies, mostly hypophyllous dense, velvety 2-3 mm diam., sometimes confluent. Hyphae substraight to undulate, cells mostly 15-25 μ long, branching opposite at wide angles, closely reticulate, capitase hyphopodia opposite, more or less antrose, straight or bent, 16-24 μ long; Stalk cell cylindric, 4-6 μ long; head cell ovate to cylindric, about slightly recurved above, entire 8-10 μ. Setae numerous, scattered straight, simple, acute, 8-10 x 650-700 μ, perithecia scattered verrucose, globose, 200-240 μ diam. Ascospores oblong, obtuse, 4-septate, slightly constricted, 16-18 x 40-46 μ (Plate-II, Fig.A, B, C, D).

ii) Relationships of the Mycelium with the Host :

The incrusting type of growth developed by the species of Meliola upon leaf surface of vast number of

tropical and sub-tropical plants raises the question as to their saprophytic or parasitic habit and relations to the host substratum. It has never been adequately shown whether they are primarily saprophytic and dependent for their food supply upon material accumulated from the air or the excretion of leaf inhabiting insects or parasitic and the amount of injury they may cause to the host leaf.

Bornet (1883) studied real parasitic nature of Meliola and was of the opinion that the lesions observed occasionally on leaves are due to the action of numerous mites whose remains were often found on leaf surface. Gillard (1892) has agreed to this and further added that, by examining numerous leaf sections, he had satisfied himself that, the Meliola is entirely superficial and do not in any way injure the tissue of host plant upon which it grows. Berkeley (1857) without direct evidence was very positive regarding the effect of Meliola on host leaf.

Ward (1883) has stated that on the underside of the hyphae there were bright spots which were the points of attachment of the hyphae to the host epidermis and might be regarded as haustoria of very rudimentary nature.

Maire (1905, 1908), Arnaud (1918), Doidge (1920), Hansford (1946, 1961) have demonstrated the presence of haustoria in sixteen species of Meliola. In two of these

haustoria pinetrate in to subepidermal cells in the same manner as that of Asterina although remaining simple and unbranched. Doidge (1920) was unable to observe the haustoria in Meliola bifida. Ward (1883) found no evidence of haustoria in an unidentified species of Meliola, though he had no difficulty in clearly demonstrating their presence in various species of Asterina. In Meliola circinance Graff (1932) had found no evidence of the presence of haustoria and the fungus appeared entirely superficial, however, the cell walls of the hyphae were seen to be markedly thinner at the point of contact.

Thite (1973) in Meliola osyridicola observed that when the growth of the mycelium takes place the head cells of capitate hyphopodia are in close contact with surface of the host cell. These head cells are provided with small circular hyaline pores which may have the function of rudimentary appresorium. Some hyphopodia continue their growth in association with stomata of the leaf and after reaching certain size, produce a 'foot' or 'stomatal plug' which fills the epidermal depression between subsidiary cells. This plug neither penetrate the stomatal opening nor produce the hyphal branch. Apparently it serves only to secure a better cohtact with the surface of the host.

It is, therefore, evident that there are three grades of parasitism in the genus Meliola. (1) Most frequently haustoria of simple type are formed and they penetrate the cuticle of the leaf; (2) There are some species which develop haustoria of Uncinula type (Smith, 1900) that penetrate upto mesophyll region; (3) Some species are entirely superficial which fail to come in direct contact with the host.

In Meliola diospyricola when the growth of the mycelium takes place the head cells of the capitate hyphopodia are in close contact with the surface of the host cells. Some hyphopodia continue their growth in association with stomata of the leaf and after reaching certain size produce a "foot" or "Stomatal plug" which fills the epidermal depression between the subsidiary cells (Plate III, Fig.1). Same type of 'stomatal plug' was observed by Thaung (1976) in Prillieuxina dipterocarpi Syd. but it does not show further growth. The 'stomatal plug' in Meliola diospyricola shows further growth through the Stomatal opening and this endophytic hypha finally reaches to the mesophyll cells (Plate III, Fig.2). Thus the fungus obtains nourishment by special hyphal branches, as described by Arnaud (1921) in Phyllactinia corylea (Pers.) Karst.

iii) Initiation and Development of the Stroma :

Considering the presence of a ascocarp as a sign of maturity it is found that the colonies of Meliola diospyricola

vary from 2-3 mm. diam.. Though ascocarps usually begin to appear on the colonies when they have reached a diameter of approximately 3 mm, they will occasionally be found on somewhat smaller colonies. The growth of ascocarp is always centrifugal. Initially their presence is not visualized on the margin of the colony but as the growth takes place in circular manner they become quite evident, when the diameter of the colony is about 3 mm. the hyphal growth ceases but the growth of ascocarp continues until they are at least found near the outer limit of the infection spot. At maturity the central portion of the colony breaks while the marginal portion remains still quite firmly applied to the host.

Besides the usual type of branches, numerous, sterile, simple, scattered hyphae or setae may be produced from various parts of vegetative mycelium measuring about 700 μ long and 8-10 μ wide at the base. At the basal region of ascocarp they are forming hyphal cushion as reported by Bornet (1883) in Meliola furcuta. In ascocarp one can differentiate three hyphal systems in two or more highly evolved groups and their origin can be summerised as follows : (1) tissue derived from the ascogonium, (2) vegetative protective tissue developed from the surrounding mycelium, (3) Secondary protective tissue formed due to the stimulus of the ascogonium.

The tissue derived from the ascogonium consists of the ascogenous hyphae and the asci. The ascogonium which may or may not be fertilized by an antheridium gives rise to ascogenous hyphae which ultimately produce the asci. The ascogenous hyphae are generally broader than the young vegetative hyphae with densely stained contents. Typically they grow out in a radiating manner to form a plate or hollow disc in lower part of fruit body, but in more primitive groups they ramify throughout the central tissue of the ascocarp.

In Meliola diospyricola stromatic initials are first seen as a short two celled lateral branches growing from the sides of vegetative hyphae and are called hyphopodia as stated by Gillard (1891). These hyphopodia which continue their further development generaly are in association with the stomata of the leaf. After reaching certain size they produce foot or a stomatal plug which fills the stomatal opening. The first division of the terminal cell of the hyphopodium takes place by the formation of a septum vertical to the plane of the mycelium as described by Ward (1883) and Graff (1932). The exposed cell walls are thickened and dark coloured. Thus in this fungus the stromatic growth is purely vegetative. Due to further growth ultimately the stroma develops in to a peltate structure (Plate IV, Fig.3 and 4) as described by Thite (1973).

iv) Initiation and Development of Perithecium :

The perithecium begins its development by the growth of hyphae from stalk cell of the ascogonium. These hyphae grow in a horizontal direction at least in their early stages, following the general ascogonial contour. Some of them may originate from the stalk cell of the antheridium but are not as numerous as the others. Since the protective cover is already present these hyphal filaments do not form intimate contact with the ascogenium and antheridium during their early development as described for the members of the Erysiphaceae by Harper (1895-a, 1895-b, 1895-c). To accomodate the growth of these sex organs the perithecium increases in size and assumes a hemispherical form. The perithecium at this stage is usually one cell in thickness, but later on becomes two celled by the formation of a new layer from within (Plate-IV, Fig.5). At maturity the sides of perithecium may be three or four celled in thickness (Plate-X, Fig.25). This extra thickening usually projects inwards towards the basal portion of the perithecium. As the growth proceeds this basal perithecial thickening extends around the entire ascocarp from within (Plate-IV, Fig.6).

The growth of comparatively large asci with their large ascospores has a marked effect upon the entire inner

structure of the perithecium. With the increased growth of the ascogenous hyphae and the attendant ascus development, the inner perithecial layer becomes much flattened until its cells become shrunken and elongated, with poor cytoplasm. The entire perithecial structure seems to serve as nurse tissue and become more and more compressed as development proceeds. The draft of the material nourishing the ascogenous hyphae and the developing asci may even extend in some degree to the innerstromatic layer. Thus all parts of ascocarp cortex except possibly the outer most protective layer of heavy walled cells may contribute to their maintenance and growth and serve as nurse cells.

Paraphyses make their appearance early during the ascus formation. They are always few in number, regular in their arrangement and scattered among the asci. They grow upwards as slender unbranched structures with thin walled cells. The nuclei in older paraphyses are elongated and appear in the state of degeneration. The younger and newly formed paraphyses have a homogenous cytoplasm and normal nuclei (Plate X, Fig.25 C). The tips of periphyses then protrude out and then the exposed ends become rounded, slightly enlarged with thick and dark walls. Thus the formation of the apical opening is similar to Meliola circinance as reported by Graff (1932), Meliola osyridicola

and Meliola jusminicola as reported by Thite (1973). Ward (1883) described in an unknown species of Meliola a slight papilla at the apex of ascocarp. He also noted that the cells of inner wall of perithecium converse towards the spot and he concluded that "this is at least a weak point through which the spores escape". Bucholtz (1897) found that in Meliola cymbasterina there is a well developed perithecial ostiole from which periphyses were seen protruding at the time of maturity.

During maturity the perithecial cells at the base remain unprotected by stroma, but in direct contact with the host. These cells become heavily thickened and dark coloured as those of exposed stromatic cells. As the growth proceeds the number of ascogenous hyphae and the asci increase and remain scattered inside the cavity of the perithecium. The paraphyses and periphyses help in dispersal of ascospores through the ostiole. The process of ostiole formation and maturity of asci are usually simultaneous as reported by Bucholtz (1897) and Graff (1932) (Plate X, Fig.25 A).

v) Initiation and Development of sex organs :

According to Ward (1882) the initiations of the sex organs takes place through a fusion or at least a contact of the first two cells formed by the hyphopodium. One of

these cells is in the nature of the "archecarpium" of De Bary (1863). Thaxter (1893) has stated that the "hyphopodia mucronees" resemble the antheridial cell in the group Laboulbeniaceae. This statement seems to carry the implication that he considers Gillard's (1891) mucronate and capitate hyphopodia to be the two sex organs or atleast to have to certain extent, similar function. In Meliola diospyricola it was found that under the protection of young peltate stroma two short stalks arise from the adjacent sides of the stalk cell of the hyphopodium. One of them is the antheridium while the other is the ascogonium. Such type of sex organs have been described by De Bary (1863) and Harper (1895-a, 1895-b, 1895-c) for Sphaerotheca, Erysiphe and Phyllactinia. The growth of these organs is usually parallel to the leaf surface, which may be due to their point of origin and relation to the protective shielding hood of stroma. Rarely they tend to assume an upright position when the curvature of the protective stromatic shield is such as to allow them sufficient space as described by Graff (1932) in Meliola circinance.

Ascogonial and antheridial development seems to take place simultaneously and they have a point of origin very close to one another. The stalk cells from which the sex organs arise are much shortened and more broad than

long. The sex organs develop as elongated, oblong-oval cells with a single nucleus and dense cytoplasm. The antheridium is slightly longer, more slender nearly cylindrical, uninucleate (Plate V, Fig.7 & 8). The sex organs of Meliola are Erysiphaceous in character but are protected by the small peltate hood. Thus the ascogenium and the antheridium are closely associated in origin as in Erysiphaceae and as young stromatic growth is attached to the vegetative hyphae by a single stalk cell, the Meliola must necessarily be homothalic.

vi) Initiation of Dikaryon and Origin of Asci :

The antheridium grows towards the ascogonium and directly presses its wall firmly as the trichogyne and the receptive haphae are absent. The intervening walls between the ascogonium and the antheridium dissolve at the point of contact allowing free passage of the cytoplasm and the male nucleus from the antheridium to the ascogonium. The male and female nuclei remain associated forming a dikaryon, resulting in a plasmogamous copulation. No nuclear fusion was observed at this stage. The antheridium is now empty and remains in close association with the binucleate ascogonium (Plate V, Fig.9). The dikaryon in the ascogenium now migrates in to the developing ascogenous hyphae after the conjugate nuclear division (Plate VI, Fig.10).

The penultimate cell of the ascogenous hyphae elongates and bends over to form the characteristic "hook" or the well-known "crozier" (Plate VI, Fig.11 & 12). The nuclei in this hook divide conjugately but mitoticaly as observed by singleton (1953) in Neurospora with a set of chromosomes passing to each pole. The spindles of these two mitotically dividing nuclei are oriented more or less parallel to one another, thus forming four daughter nuclei (Plate VII, Fig.13). Two of them which are of different sex origin lie close to each other at the apex of the bend, while the other one which is located at the tip of the hook, passes in to basal region of the penultimate cell by dissolution of the wall and lie in close association with the remaining daughter nucleus (Plate VII, Fig.14). Two septa are laid down separating the binucleate crook cell and the binucleate basal cell (Plate VII, Fig.14). The crook cell now assumes the role of ascus mother cell. This ascus mother cell now elongates and the dikaryon occupies the central position where ultimately "Karyogamy" takes place. The young asci so formed are always with a single diploid nucleus (Plate VII, Fig.15).

vii) Nuclear Events in the Ascus :

a) Introduction :

The basic knowledge regarding cytological studies in Ascomycetes is derived from the pioneer investigations of

Dangeard (1894, 1897, 1904, 1907); Blackman and Fraser (1905, 1906); Harper (1895-a, 1895-b, 1895-c); Maire (1903-a, 1903-b, 1905, 1908); Guillermond (1904, 1905, 1908, 1911) and others. According to their studies it is known that the Ascomycetes are characterised by the occurrence of a sexual phase in their life cycle which results in the production of perfect spores, the ascospores, beginning with the plasmogamy and terminating in karyogamy with the intervening dikaryophase. It is also known that in this group there is an alternation of generation as in the rust fungi. Regarding the nuclear fusion in this group two theories have been put forth. (1) Single fusion theory or "Claussen type" of nuclear behaviour originally proposed by Dangeard (1907) and Claussen (1912) in which a single fusion occurs in the ascus mother cell followed by a single reduction division in the ascus. This type of nuclear fusion has been known to be of common occurrence in the vast, majority of the Ascomycetes so far studied and have been supported by Colson (1938), Hirsch (1950), Singleton (1953), Olive (1949, 1950, 1953) and many others in recent years. (2) The other is "Double fusion" or "Harper type" according to which two fusions take place; one in the ascogonium and the other in the ascus mother cell, followed by two reductional divisions called as "Brachymeiosis" which was proposed by Harper (1895-a) and was supported by subsequent

Blackman and Fraser (1906), Fraser (1908), Cutting (1909) and others.

Recent experimental work based on a more refined cytological microtechniques with better staining methods has revealed that the "double fusion theory" no more holds good for large number of Ascomycetes so far investigated as evidenced from the critical work of Olive (1950), Hirsch (1950) and many others.

b) Historical Review :

The important cytological studies in Ascomycetes were brought out through the pioneer investigations of Harper (1895-a, 1895-b, 1895-c); Dangeard (1894) who employed such studies for determining the nuclear life cycle and the organisation of nuclear structures in Powdery Mildew fungi. Later Dodge (1927); Drayton (1934); Gwynne-Vaughan (1937); Colson (1934, 1938); Olive (1949, 1950, 1953, 1956, 1965); Singleton (1953); and recently Kowalski (1964, 1965, 1966); Berthet (1964-a, 1964-b); Rogers (1964, 1965-a, 1965-b, 1967, 1968-a, 1968-b, 1968-c, 1969-a, 1970); Uecker (1967); Furtado (1970); Kulkarni (1963); Wells (1970); Canham (1969); Woo and Partridge (1969); Muthappa (1967); Thite (1971, 1972); Parguey-Leduc, (1972); Zickler (1973); Upadhyay; and Ravgi (1973); Kimbrough (1974); Kamat and Ullasa (1974); Harris and Roth (1974); O'Donnell, et al., (1974, 1976); Rogers and Stiers

(1974); Rogers (1973, 1975, 1978); Rai and Chowdhary (1974); Ullasa and Pande (1975); Sakai (1975); Rannali and Irma (1975); Ranga Rao and Mukarji (1972); Paden and Linton (1976); Bezerra and Kimbrough (1976); Jain and Mongan-Jones (1973); Stiers (1976); Raju (1978), Rai and Saxena (1979); Patwardhan and Badhe (1979); Huang (1976); Hill (1975, 1977); Jagtap (1975, 1978); Gottwald and Cameron (1980); Ellis (1981); Jones (1981) and many others have contributed to the voluminous literature to this field. The Meliolales have been studied largely from the stand point of their morphology and taxonony, but very little contribution on cytology and development of the members of this order is to be found in the literature. However, particular mention must be made that Gaumann (1926) has studied cytology of Lanomyces sp., Hard (1883) made the developmental studies of unknown species of Meliola, while Graff (1932) made such studies in case of Melicla circinance and Thite (1973), Goos (1974, 1978), Goos and Palm (1979) have studied cytology and development of the Meliola. As compared to large number of species of this order the literature on this aspect of study is rather inadequate. The phylogenetic position of the group Meliolales is also a point of dispute and with the help of such cytological and developmental studies it will be possible to throw some light on this aspect and to fill the gap between the groups Erysiphales and Sphaeriales.

c) Nuclear Events in the Ascus :

The diploid nucleus so formed occupies the central position in the developing ascus and can be seen as densely stained mass of chromatin with intact nuclear membrane (Plate VIII, Fig.16) corresponding to the condensed nucleus reported by Rogers (1965-a) in Coniochaeta lignaria, Canham (1969) in Hypocrea ceitrina, Thite (1974), in Meliola osyridicola. Like many other Ascomycetes the first division takes place along the longitudinal aris of the ascus or in a slightly oblique manner. The two daughter nuclei so formed remain in the centre of the ascus (Plate VIII, Fig.17). The second nuclear division is also slightly oblique but four nuclei thus formed also remain in the central portion of the ascus (Plate VIII, Fig.18). As a result of the third division eight daughter nuclei are formed, which assemble in the middle part of the ascus and so make it difficult to trace their relationship to one another (Plate VIII, Fig.19).

viii) Ascosporogenesis :

Ascospore organization in the Ascomycetes is known to be brought about through the several mechanisms. This has been recently summerised by Reeves (1967). Accordingly four mechanisms of ascospore organization have been reported in this group.

(1) Gjurasin (1893) reported that the division of the nuclei in the ascus were karyokinetic and that asters were associated with the division process. In the eight-nucleate stage of the ascus he described the foldings of the rays of the asters around the individual nuclei. Harper (1897, 1900) expanded these observations of nuclear behaviour and designated it as "Free Cell Formation", a unique process that is of common occurrence in Ascomycetes. A summary of events in the process of free cell formation is as follows : (i) in the eight nucleate ascus, each of the haploid nucleus forms a beak with a persistent central body on the asteral rays at the tips of the beak. (ii) The asteral rays swing outwards and downwards and form a thin membrane which cut out young spores. (iii) The membrane around each spore separate the sporoplasm and include the nucleus, leaving epiplasm in the ascus. With few exceptions these ideas have been verified and expanded by majority of subsequent investigators. Dodge (1927) believed that the large abnormal multinucleate spores obtained in some species of Neurospora were the results of multilinear activity among the asteral rays of several nuclei although the process was similar to that described by Harper (1905) for Powdery Mildew Fungi.

(2) A second method of the ascospore organization was presented by Dangeard (1907). He was unable to find

the asteral rays described by Harper. He described that a sheet of material spreads from central part of each nucleus and gradually envelops the spore. The absence of asteral rays laid him to modified Harper's original description. Similar observations were made by Faul (1905). He considered that the spores were delimited by the double membrane of asteral rays and not through the mechanism of asteral rays.

(3) Jones (1925, 1926); Jenkins (1934); Raymond (1934); Hein (1932) and Hayman (1964) have described a third method of ascospore formation. All of these mycologists agree that the cytoplasm was divided into segments around the nuclei without the aid of asteral rays or centrioles. Jones (1925) thought that the planes of cleavage were initiated through the appearance of narrow vacuoles along certain points near the ascus wall. Raymond, Hein, Hayman were unable to determine the mechanism involved in the segmentation of cytoplasm around nuclei. However, Hein (1932) thought that the asteral rays and centrioles reported by others were either artifacts of fixation or staining or misinter-pretations of the figures of nuclear divisions.

(4) The fourth and the most radically different method of ascospore delimitation was presented by Andrus and Harter (1933, 1937), Andrus (1936) and Chadefaud (1943, 1965). These investigators believed that prior to sporo-

genesis all the eight nuclei of the ascus were enveloped in a very thin membrane (the ascus vescicle). Ascospore organization occurred through the constriction of this membrane around the individual nucleus. None of these investigators obtained any evidence in the delimitation of ascospores. Gwynne - Vaughan and Broodhead (1936) reinvestigated the fungus Ceratostomella fimbriata studied by Andrus and Harter (1933, 1937) and suggested that the reported ascus vescicle was actually a large vacuole. Chadefaud (1965) has demonstrated that the "ascus vescicle" in his material was not vacuole.

Studies on free structural aspects of higher Ascomycetes have been made by Moore (1963,1964); Ceruti et al., (1966); Wilson (1937); Becket (1966); Rudolph and Giesy (1966); Bracker and Williams (1966). These investigators found that prior to ascospore organization a double membrane was formed around the entire mass of cytoplasm in which all the eight nuclei were embeded; the ascospores were delimited from the rest of the cytoplasm of the ascus through progressive constriction of this double membrane system around the individual nucleus. Ellis (1981) in Thermoascus aurantiacus also observed that the ascospores are delimited by the progressive invagination of a pair of unit membrane and the cell wall formation takes place between these ascospore delimiting membranes and consisted of an electron transferent

endospore and an electron dense epispore. Reeves (1967) with the help of electron microscope observed that in Pyronema domesticum the "ascus vescicle" surrounding the complements of eight nuclei is composed of a two unit membranes acting as the agents by which the spore delimitation takes place. Hill (1975) in Hannizzia gypsea observed the invagination of ascus vescicle. Hill (1977) in Herpomyces Sp. (Laboulb-eniales) also observed that ascospores are delimited by membranes derived from an ascus vescicle and the wall forms between the paired prospore membrane. Dilated cisternae and epiplastic membranes are associated with wall formation. Stiers (1976) in Ceratocystis fibriata observed that the strands of double membrane forms a large ascus vescicle which encloses the eight nuclei and most of the ascus cytoplasm. The ascospores are then delimited by the formation of lobes of the ascus vescicle. According to Reeves double membrane system appeared to be essentialy the same as that described in the light of microscopic studies by Andurs and Harter (1933, 1937); Andrus (1936) and Chadefaud (1943, 1965); Thite (1973).

The process of ascosporogenesis in Keliola diospyricola based on microscopic studies essentially agree with the mechanisms proposed by Jones (1925, 1926); Jenkins (1934); Raymond (1934); Hein (1932) and Hayman (1964). No "ascus vescicle" mechanism composed of two unit membrane as reported by

Reeves (1967) was observed at any stage of ascospore organization.

Though ordinarily, only two ascospores are formed in the mature ascus of this fungus, the number of nuclei prior to the ascospore formation is usually eight (Plate-VIII, Fig.19) like that of other Ascomycetes, and all of them take part in early spore development. After the completion of the nuclear divisions they arrange themselves in pairs. Four binucleate spores are usually initiated at this stage (Plate IX, Fig.20) but only two develop further while the remaining two degenerate (Fig.21).

Ward (1883) described the process of development in an unknown species of Meliola without any reference to the mode of nuclear behaviour. The process of ascospore development in Meliola diospyricola is in accordance with Meliola circinance as reported by Graff (1932) and Meliola jasminicola as reported by Thite (1973). Graff (1932) states that even though the number of mature ascospores in each ascus is usually two, there are rare instances where they may be four. In the present investigations on the other hand there was not a single case where the number of mature ascospores is more than two. The young ascospores are single celled and binucleate. These nuclei undergo conjugate divisions and form four daughter nuclei which get arranged in a median line.

Two of these daughter nuclei remain in the centre of the spore and the other two are separated by the formation of the septa and lie on either side of the central pair, thus forming three celled spore with the binucleate central cell and uninucleate lateral or end cells (Plate IX, Fig.21 & 22). Each of these nuclei in the end cells of the ascospore divide again followed by the formation of septa. Thus the individual ascospores in Meliola diospyricola are five celled at naturity containing six nuclei, two in the central cell and one each in the other cells (Plate IX, Fig.23).

As these two spores of the ascus increase in size their adjacent sides become some what flattened by the pressure. The nuclei of the individual ascospores are arranged on a median line. With the continued growth, each cell of the ascospore become somewhat rounded with much thicker and darker walls, ultimately resulting in a slightly constricted appearance (Plate IX, Fig.24).

The ascus wall is thin throughout and as the spores mature, becomes distended and fits closely about their contours. It readily tears away near the base when spores are fully matured and often continues to invest the paired ascospores for some time. The free ascospores may also be seen in the perithecial cavity ready for discharge (Plate X, Fig.25) which are finally discharged through the ostiole.

IV) DISCUSSION

It is clear from the historical account the chief problems concerning sexuality and associated phenomenon in ascomycetes are as follows :

1) Are there definitely organized sex organs or not ?
2) If definitely organized sex organs (anthridia and ascogonia) can be recognised, Are they functional or not ?
3) If the functional anthridia and ascogonia are present; does the fusion of nuclei of opposite sex take place in the ascogonium or in ascus mother cell or in both ?
4) If the fusion of sexual nuclei does not take place in the ascogonium, Is there close pairing or not and the conjugate division of male and female nuclei in the ascogenous hyphae originating from ascogonium.
5) If the fusion occurs in ascogonium (nuclear fusion in the ascus is universal). Is there any evidence in the nuclear history of a double reduction (brachemeiosis).

In the present studies there are abundent evidences of the existence of definitely organised sex organs - the anthridia and ascogonia. They can be easily recognised as

distinct structures from the rest of the mycelium by their size, shape and position. The existence of such specialized sex organs is an agreement with the observations of Harper (1895-a, 1895-b, 1895-c, 1895-d, 1899) in Erysiphe communis, Phyllactinia corylea and Sphaerotheca castagnei. Blackman and Fraser (1905) in Sphaerotheca humuli. Graff (1932) in Meliola circinance. Thite (1973) in Meliola osyridicola and Meliola jasminicola, but differs from that of De Bary (1863), Dangeard (1907). Although they observed distinct sex organs but regarded anthridia as nonfunctional.

In Meliola diospyricola similar to Meliola osyridicola and Meliola jasminicola the intimate association of anthridia and the ascogonia with each other make it highly probable that they are functional. Contact between these two sex organs is established by the dissolution of intervening walls. There is a frequent occurence of an empty anthridium in close association with the binucleate ascogonium. This suggests that the nuclear migration took place through a small copulation canal. The actual process of nuclear migration from anthridium to ascogonium has not been demonstrated by many investigators except Harper (1897), De Bary (1868) could not observe the breaking down of the walls between anthridium and ascogonium in Erysiphe sp. and so supports the absence of the process of conjugation in it.

Harper (1897) working on Sphaerotheca castagnei was able to observe the actual migration of male nucleus through the conjugation pore and the nuclear fusion in the ascogonium. On the other hand Dangeard (1907) was unable to detect the conjugation pore between the sex organs. He claimed to have examined so much material that failure to find conjugation pore must be accepted as final and indisputable proof of its non-existence.

Blackman and Fraser (1905) working with Sphaerotheca humili had actually observed four cases in which the ascogonium and anthridium were in direct connection and they were further convinced that the fusion of male and female nuclei took place in the ascogonium.

In Erysiphe polygoni Allen (1905) observed nuclear migration directly from one hypha to the other without the indication of their fusion.

Colson (1938) working on Phyllactinia corylea was convinced that the anthridial nucleus does not migrate into the ascogonium at all, but degenerates in situ. According to her opinion the anthridium is non-functional and further development of the ascogonium is apogamous. The two nuclei of different sizes occuring in the ascogonium at an earlier stage of its development are derived from the original single

ascogonial nucleus. Though she had failed to observe the nuclear divisions which resulted the binucleate condition of the ascogonium.

In the present investigation no nuclear fusion has been observed in the ascogonium and the two nuclei remain associated as a dikaryon. The coenocytic condition of the ascogonium is thus restricted to binucleate stage only. As a result of conjugate nuclear divisions dikaryons migrate into the developing hyphae. Further conjugate nuclear divisions of the dikaryon continues in the ascogenous hyphae and immediately followed by the formation of septa resulting in the formation of row of few cells. The penmultimate cell contains two nuclei like Phyllactinia corylea, Colson (1938), Erysiphe acaciae, Tare (1956).

The phenomenon of double reduction of chromosomes number (Brachomeiosis) usually depends upon the process of double fusion. During present investigation the intra-nuclear details were not observed.

The diploid nucleus occupies the central position in the developing ascus and can be seen as densely stained mass of chromatin with intact nuclear membrane corresponding to the condensed nucleus reported by Rogers (1965-a) Coniochaeta lignaria, Canham (1969) in Hypocrea ceitrina, Thite (1973) in Meliola osyridicola. Like many other

asconycetes first division takes place along the longitudinal axis of the ascus or in slightly oblique manner. The two daughter nuclei so formed remain in the centre of the ascus as reported by Thite (1973) in Meliola osyridicola. The second nuclear division is also slightly oblique but four nuclei thus formed also remain in the central portion of the ascus as observed by Thite (1973) in Meliola jasminicola. In Meliola osyridicola at four nucleate stage the nuclei do not remain at the central portion but they lie in a single line from base to apex of the ascus. As a result of the third division eight daughter nuclei are formed, which assemble in the middle part of the ascus and so make it difficult to trace their relationship to one another.

In Meliola ascostroma initiation is generally not simultaneous with the perithecial initiation and the latter does not begin to develop till the growth of the stroma has proceeded far enough to produce protective cover beneath which the further development takes place.

In the past much confusion was found to hinge up on a misconception of what constitutes a perithecium and the loose use of the word, with reference to outer portion of an ascocarp, where the stroma is present. The genus Meliola offers a good illustration because according to the description of few species so far studied the outer fruit surface

is not designated as perithecium. Though in reality it is the surface of the stroma beneath which perithecium is hidden.

Setae appearing on the surface of this ascocarp are called as "Perithecial setae". As they are out growths from the stromatic surface they must have the same general vegetative. Origin as hyphal setae and to call them as perithecial setae is rather misleading. The setae of Meliola may be produced strictly from the vegetative hyphae, from its hyphopadia or from the stromatic surface of the ascocarp, while in some species they are developing from all the three parts. All are therefore vegetative in origin. Inspite, of the similarity in appearence the ornamentally branched setae of Meliola furcata which are found on the ascocarp have their origin in the stroma while those of Microspera develop from the true perithecial wall which originates from stalk cell of the ascoganium.

The ascocarp in Meliola diospyricola is initiated through the growth of the hyphapodium which takes place in a centrifugal manner and results in the formation of a lenticular group of cells. The exposed cell walls become thickened and dark coloured in a manner similar to the walls of regular hyphae. This peltate stromatic growth is purely vegetative structure, the formation of

which undoubtedly takes place in the manner described by De Bary (1863) as meristogenous rather than Symphogenous. This is in accordance with the observations made by Thite (1973) in Meliola osyridicola and Meliola jasminicola.

Under this protection of young peltate stroma the authridium and the ascogonium arise, which are true sex organs and are definitely of the same nature described by the De Bary (1863) for Erysiphe, Harper (1895) for Sphaerotheca, Graff (1932) for Meliola circinans and Thite (1973) for Meliola osyridicola and Meliola jasminicola.

Perithecium begins its development by the growth of hyphae from the Stalk cell immediately below the ascogonium. These hyphae grow in a horizontal manner around the ascogonium. To accomodate the growth of the anthridium, the ascogonium and the perithecium there is also an increase in the overgrowth of the Strona.

Paraphyses make their appearence during early ascus formation. They are few and usually scattered among the asci and grow upwards as slender, unbranced, slightly twisted or bent filaments of several thin walled cells. When the ascocarp increases in size and the central cavity enlarges, filaments growing in upper region from the inner wall of the perithecium, become more slender and are known as "periphyses".

They seperate from below upwards and are directed towards the apex of ascocarp. The ends of these periphyses push a part the cells of the perithecium and the stromatic hood at this point and form an ostiole through which ascospores are discharged.

The fine structure involved in the process of ascosporogenesis has been described by Moore (1963, 1964) in Cordyceps militaris. He emphasized the role of the endoplasmic reticulum in delimiting the ascospore wall. He has reported that the ascospore initials are surrounded by an outer matrix membrane seperated by plasma membrane with the development of investing wall over it. It was also shown that the epiplasm which is enucleate breaks down at the majority of ascospores. After further mitotic divisions of these nuclei in the spore initials it was seperated by the invagination of linear nuclear membrane to partitions the nucleoplasm into subunits termed as "Karyosomes" and the process as "Karyochoresis" by Moore (1963, 1964). During mitotic divisions of the spore nuclei there is also a spore elengation.

It will be observed from the results obtained that the pattern of the development of the ascus and the ascospores in this tropical fungus is very similar in many respects to that of many other temperate ascomycetes.

However, there are, differences as regards the number of ascosopores in each ascus, number of cells in each ascospore and the number and the activity of nuclei in each cell of the ascospore. In majority of the ascomycetes eight uninucleate ascospores are produced but in Meliola usually two multicellular and multinucleate ascospores are found.

Meliola diospyricola agrees with Meliola jasminicola and Meliola circinans but it differs from Meliola osyridicola in the process of ascosporagenesis. In Meliola osyridicola each ascus initially produces only two quadrinucleate ascospores while the mature ascospores are five celled and six nucleate. On the other hand in Meliola diospyricola at first four binucleate ascospores are formed in each ascus out of which only two mature and other two elegenerate as in Meliola circinans and Meliola jasminicola. The mature ascospores of this fungus are also five celled and six nucleate. The mature ascospores escape from the thin wall of the ascus and are finally discharged through the perithecial ostiole.

According to Ward (1883) the process of ascosporogenesis in Meliola is progressive one in which the first act is a longitudinal cleavage dividing the ascus contents into two halves followed by second cleavage but at right angles to the first. In Meliola diospyricola on the other

hand four spores are cut out simultaneously as four fusiform cytoplasmic bodies in the act of spore delimitation the greater portion of the cytoplasm is included within the spores and only a very small amount of residual epiplasm remains out in the ascus. Throughout the development of the ascospores the entire cytoplasmic contents of the ascus remain homogenous.

The process of delimination of the four ascospores within the ascus, instead of eight, together with cutting of this cytoplasm. So as to include two of the eight nuclei in each ascospore has also been reported in Podospora anserina by Ward (1882) and in Neurospora tetrasperma by Dodge (1929). In Keithia chamaecyparissi on the other hand usually two quadrinucleate ascospores are delimited as reported by Dodge (1928). There are instances where the number of ascospores may be less than eight e.g. Phyllactinia corylea (Colson, 1938); Laboulbenia grindarum (Faul, 1912) where some of the nuclei degenerate prior to spore delimitation and only those retaining their normal size take part in the spore formation. In Meliola diospyricola initially four fusiform ascospores are delimited in each ascus, but ultimately only two reach the maturity at the expense of other two.

There are three grades of Parasitism in the genus Meliola. (1) Most frequently haustoria of simple type are found and they penetrate the cutical of the leaf. (2) There are some species which develop haustoria of Uncinula type (Smith, 1900) that penetrate upto mesophyll region. (3) Some species are entirely superficial which fell to come in direct contact with the host.

In Meliola diospyricola when the growth of the mycelium takes place the head cells of the capitate hypopodia are in close contact with the surface of the host cell. Some hypopodia continue their growth in association with stomata of the leaf and after reaching certain size produce " a foot", or "stomatal pluge" which fills the epidermal depression between the subsidiary cells. Same type of Stomatal pluge was observed by Thite (1973) in Meliola osyridicola and Meliola jasminicola; Thaung (1976) in Prillieuxina dipterocarpi Syd. but it does not show further growth. The stomatal pluge in Meliola diospyricola shows further growth through the stomatal opening and this endophytic hypha reaches to the mesophyll cells. Thus the fungus obtains nourishment by special hyphal branches, as described by Arnaud (1921) in Phyllactinia corylea.

V) SUMMARY

The developmental pattern and cytological phenomenon of Meliola diospyricola has been studied.

(1) Mycelium is superficial and gets the nutrition by haustoria reaching to mesophyll cells of the leaf.

(2) The Stromatic initiation is not simultaneous with the perithecial initiation. The stroma is purely vegetative in origin initiated through the growth of the hypopoelium.

(3) The perithecial initiation does not beg in till the growth of the Stroma has proceeded enough to produce protective cover beneath which further development takes place.

(4) Distinctly organized and recognizable anthridia and ascogonia are found which in all probability are functional. Both of them are uninucleate.

(5) Dikaryophase is initiated by direct copulation of sex organs.

(6) The binucleate ascogonium after conjugate nuclear divisions produces the ascogneous hyphae.

(7) The hyphae originating from ascogonial and anthridial stalk cells develop to form perithecial wall.

(8) The centrally placed pseudoparenchy-matous mass disintegrates creating the space for the ascogenous hyphae.

(9) The asci develop usually from the penultimate cells of the ascogenous hyphae.

(10) The nuclear fusion is immediately followed by the three successive divisions one of them is reductional, ultimately resulting in the formation of two five celled and six nucleate ascospores in each ascus.

(11) The phenomenon of brachymeiosis has not been observed.

(12) The ascospore initials are organised through the cytoplasmic divisions into segments around the nuclei without the aid of astral says.

(13) Initially four binucleate ascospores are formed but only two mature and other two degenerate.

(14) These ascospores at maturity become five celled and six nucleate.

(15) At the time of spore maturity, perithecial ostiole is formed by the activity of Periphyses arising from the upper region of the inner wall of the Perithecium.

VI) CONCLUSION

From the above studies it appears that it is difficult to make a generalised statement regarding the sexuality and associated phenomenon in ascomycetes. It is also equally difficult to make similar statement even for a single family of this group. The outstanding phenomenon of triple division of the fusion nucleus in the ascus is however, common to all members of Ascomycetes and offers as has been pointed out by Harper (1895), a striking contrast to the double division of the nucleus of the spore mother cell of the other groups of plants. Harper (1897, 1905); Fraser (1908); Corrather (1911); Gwynne-Vaughen (1937); have undoubtedly proved the occurrence of double fusion and double reduction. However, Singleton (1953) summerising the knowledge of meiotic phenomenon have asserted that, "It is no longer possible to imagine that by taking on a third division as a brachemeiosis, two reductions in the ordinary cells can be produced." Great difficulties have been encountered by most workers in finding suitable stages of nuclear divisions anywherealse than in the ascus and in following of the events in the ascogonium which becomes ienveloped by the hyphae forming perithecial wall. The triple division in the ascus still offers a challenge to the cytologists for investigations and satisfactory theoretical explaination.

The present study has brought to light many facts with evidences, showing important steps in the formation of ascocarps, asci and ascospores with the help of these studies. It is possible to conclude that this group should be placed in Pyrenamycetes and not in Plectomycetes as treated by Dennis (1968) because there is a definite ostiole. Graff (1932) has suggested to place the family meliolaceae under the order Dothideales on the Stromatic development. Bessey (1965) and Yarwood (1973) treated it under Erysiphales because there is pseudoparenchymatic tissue filling the perithecial cavity prior to the formation of asci. However, as there is an apical opening - an ostiole - with protruding paraphyses and through which ascospores are discharged, the family is better placed in the order Sphaeriales as suggested by Booth (1966) and treated by Muller and Arx (1973). These conclusions are supported by previous studies of Graff (1932) in Meliola circinans and Meliola jasminicola.

Description of Figures

Plate-I

A twig of *Diospyros montana* Roxb. infected with *Meliola*.

Plate-II

Morphology of *Meliola*

A : Infected leaf

B : Mycelium

C : Fructification

D : Ascospore

Plate-III

Fig.1 : Section showing stomatal plug (A) x 600.

Fig.2 : Section showing Mycelial plug (B) x 600.

Plate-III A

Fig.1 A : Microphotograph of Fig.1, Plate-III.

Fig.2 A : Microphotograph of Fig.2, Plate-III.

Plate-IV

Fig.3 and Fig.4 : Hypopodium growing in to peltate stroma.

Fig.5 : Section showing young ascocarp x 600

A : Ascostroma

B : Perithecial initials

Fig.6 : Section of the ascocarp x 600.

A : Ascostroma

B : Perithecial initials.

Plate-IV A

Fig.3 A : Microphotograph of Fig.3, Plate-IV.

Fig.4 A : Microphotograph of Fig.4, Plate-IV.

Fig.5 A : Microphotograph of Fig.5, Plate-IV.

Fig.6 A : Microphotograph of Fig.6, Plate-IV.

Plate-V

Fig.7 & 8: Young perithecium with uninucleate sex organs x 600

A : Ascogonium

B : Antheridium.

Fig.9 : Perithecium with sex organs x 600.

A : Binucleate ascogonium.

B : Empty antheridium.

Plate-V A

Fig.7 A : Microphotograph of Fig.7, Plate V.

Fig.8 A : Microphotograph of Fig.8, Plate V.

Fig.9 A : Microphotograph of Fig.9, Plate V.

Plate-VI

Fig.10 : Section of Ascocarp showing Binucleate Ascogenous Cells (A) x 600.

Fig.11 : Section of the Ascocarp showing Ascogenous hyphae with crozier (A) x 600.

Fig.12 : Crozier with a pair of nuclei x 600.

Plate-VI A

Fig.10 A : Microphotograph of Fig.10, Plate VI.

Fig.11 A : Microphotograph of Fig.11, Plate VI.

Fig.12 A : Microphotograph of Fig.12, Plate VI.

Plate-VII

Fig.13 : Crozier after nuclear division x 1500

Fig.14 : Binucleate ascus mother cell x 600

Fig.15 : Ascus mother cell showing fusion nucleus x 600

Plate-VII A

Fig.13 A : Microphotograph of Fig.13, Plate VII

Fig.14 A : Microphotograph of Fig.14, Plate VII

Fig.15 A : Microphotograph of Fig.15, Plate VII.

Plate-VIII

Fig.16 : An ascus with single fusion nucleus x 600

Fig.17 : An ascus with two nuclei x 600

Fig.18 : An ascus with four nuclei x 600

Fig.19 : An ascus with eight nuclei x 600.

Plate-VIII A

Fig.16 A : Microphotograph of Fig.16, Plate VIII.

Fig.17 A : Microphotograph of Fig.17, Plate VIII.

Fig.18 A : Microphotograph of Fig.18, Plate VIII.

Fig.19 A : Microphotograph of Fig.19, Plate-VIII.

Plate-IX

Fig.20 : An ascus with four binucleate ascospores x 600

Fig.21 : An ascus with two binucleate ascospores x 600

Fig.22 : An ascus with 4 nucleated Ascospore and ascospore with binucleate central cell x 600

Fig.23 : An ascus with two Mature ascospores x 600

Fig.24 : Mature ascospore with five cells and six nuclei x 600

Plate-IX A

Fig.20 A : Microphotograph of Fig.20, Plate IX.

Fig.21 A : Microphotograph of Fig.21, Plate IX.

Fig.22 A : Microphotograph of Fig.22, Plate IX.

Fig.23 A : Microphotograph of Fig.23, Plate IX.

Fig.24 A : Microphotograph of Fig.24, Plate IX.

Plate-X

Fig.25 : Section of mature ascocarp showing

A : Ostiole

B : Periphyses arising from inner wall of perithecium x 600.

C : Paraphyses

Plate-X A

Fig.25 A and 25 B : Microphotograph of fig.25, Plate X.

PLATE I.

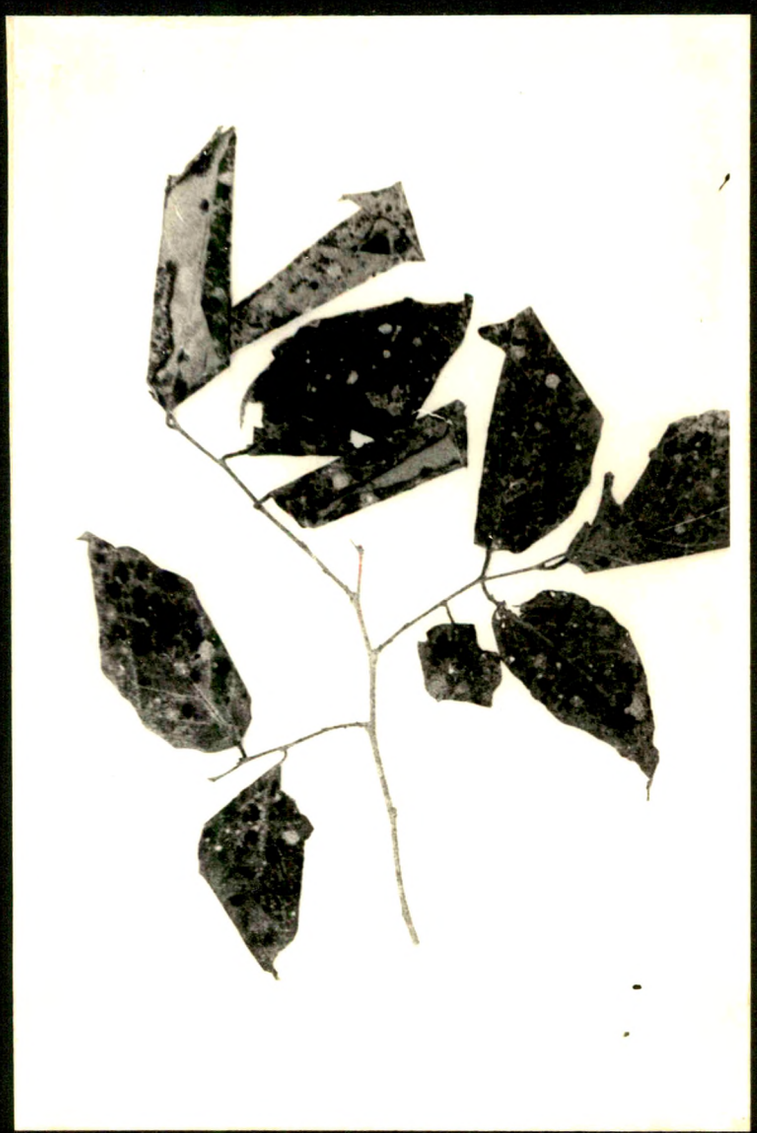
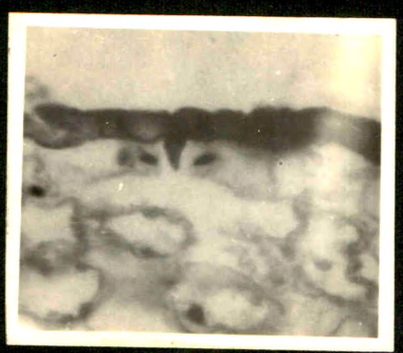


PLATE III A



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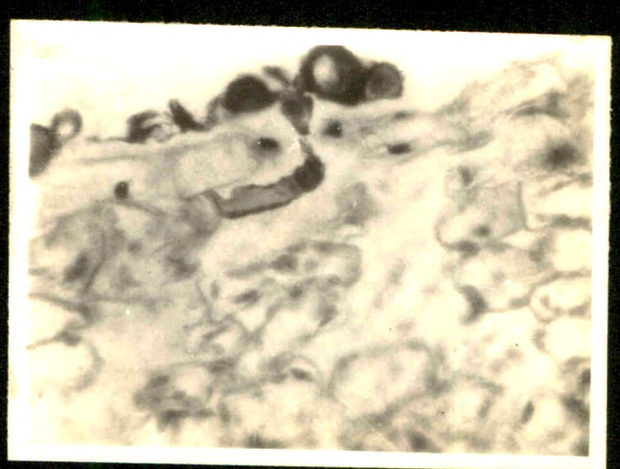
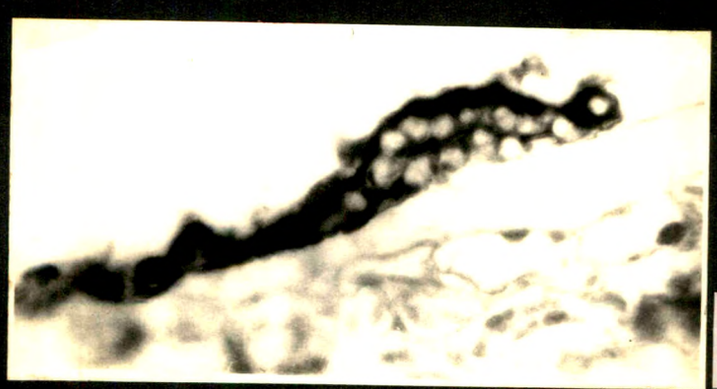


2 A

PLATE IV A

3 A

4 A



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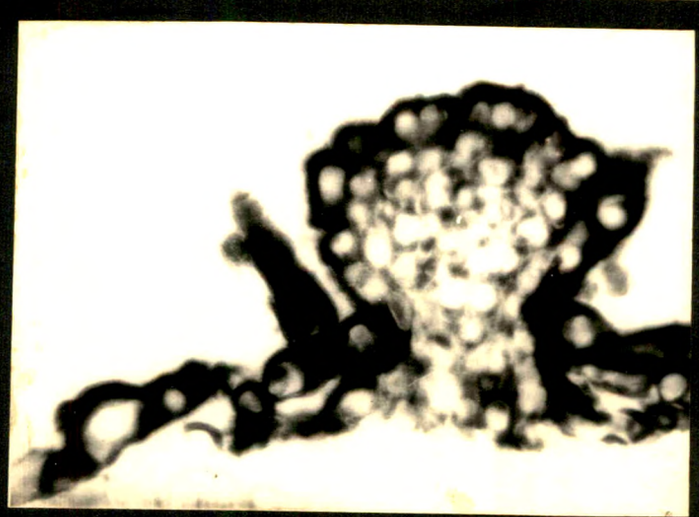

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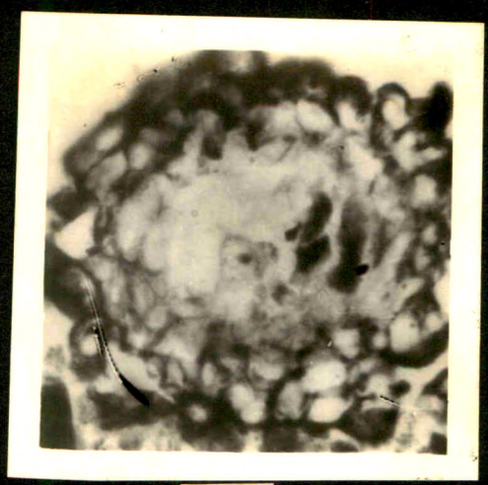
PLATE V A



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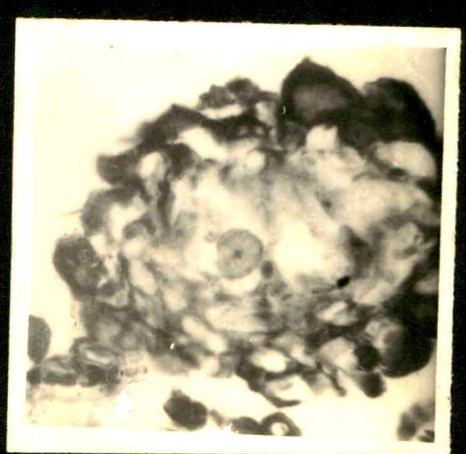

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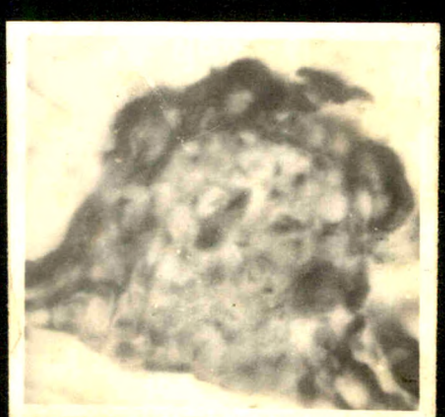

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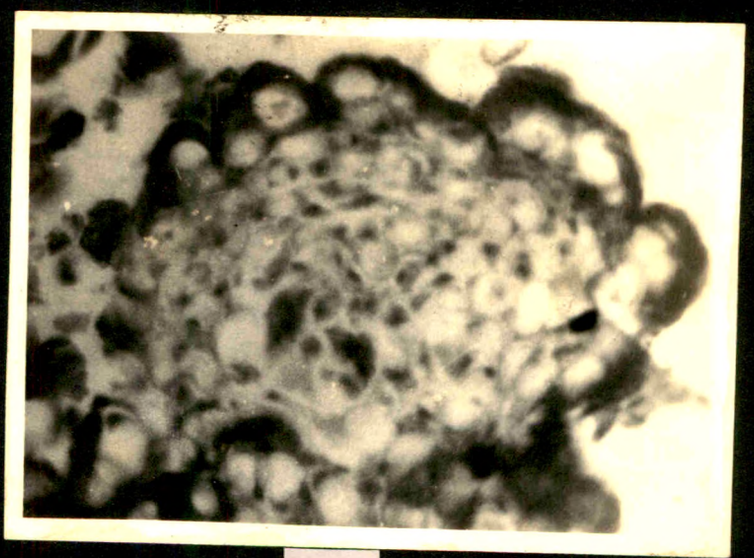
PLATE VI A



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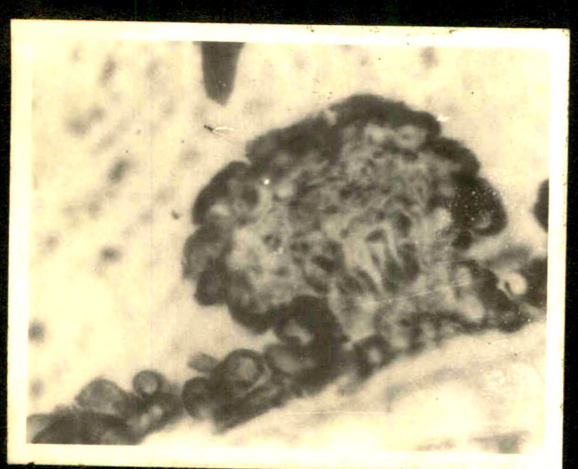

11 A

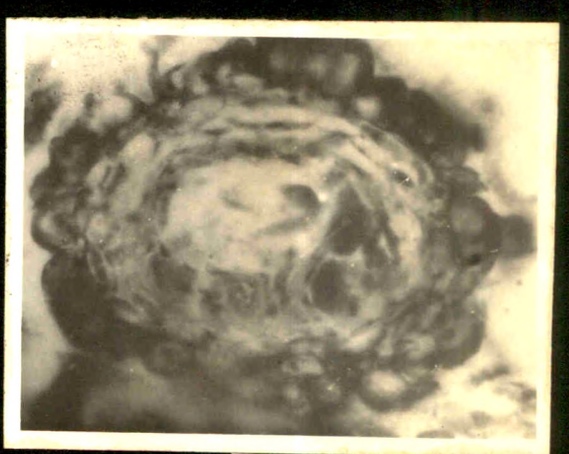

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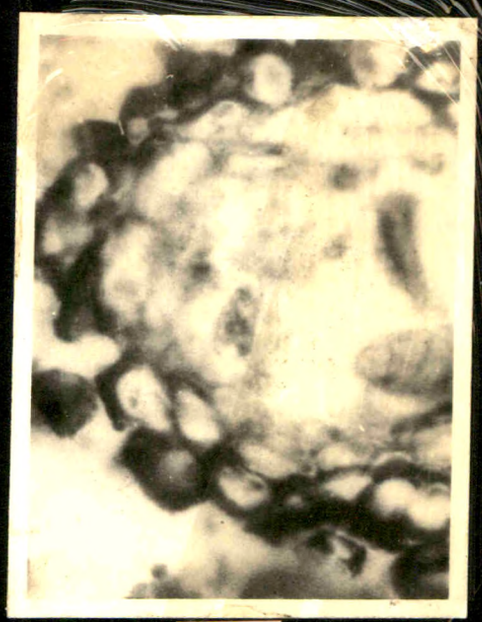
PLATE VIIA



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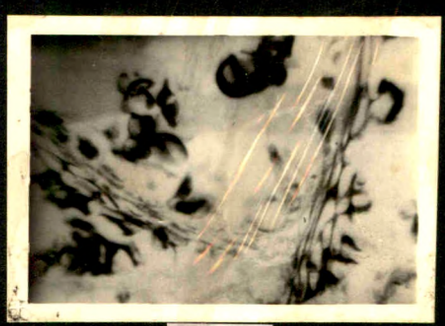

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PLATE VIIIA

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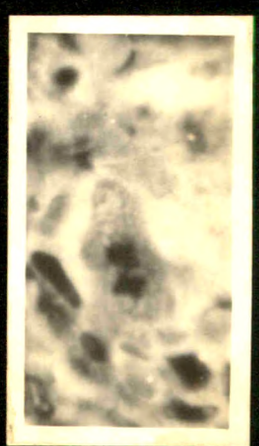

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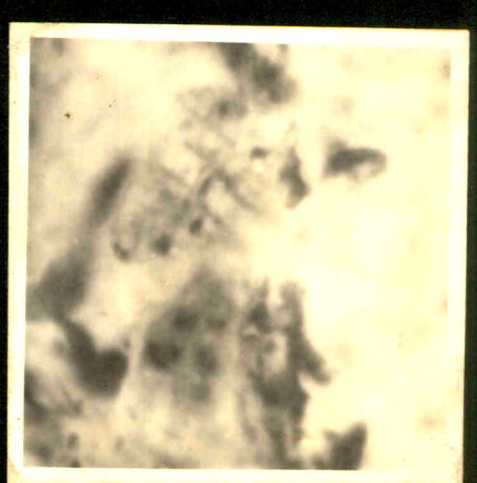

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PLATE IXA

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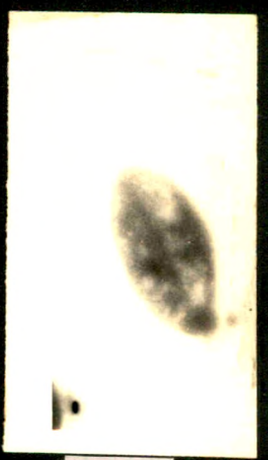

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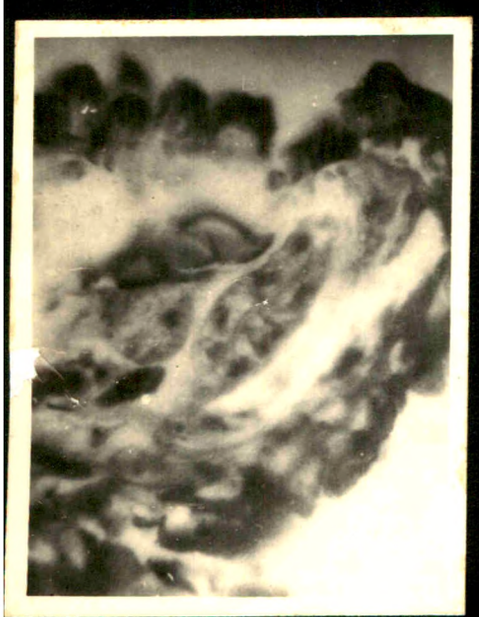

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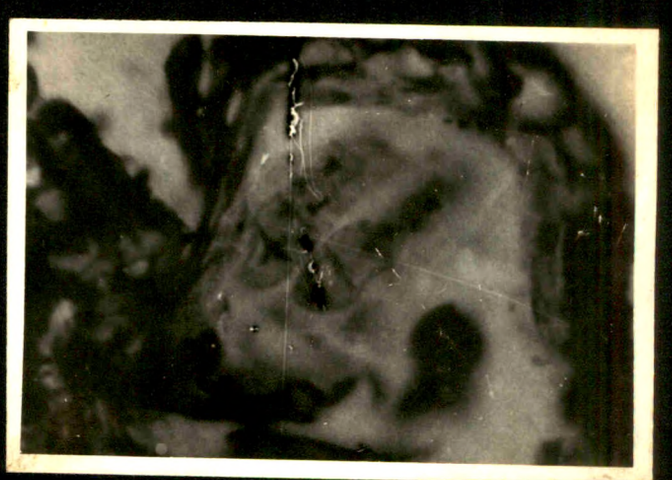

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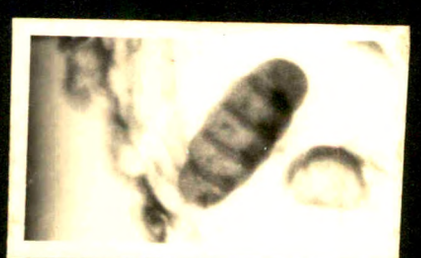

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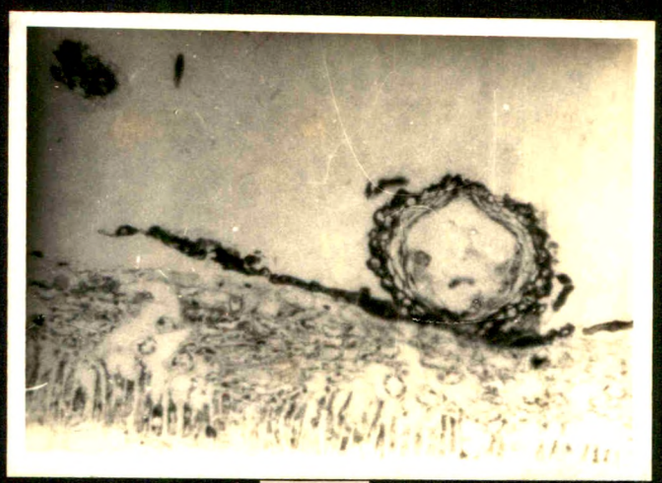
PLATE XA



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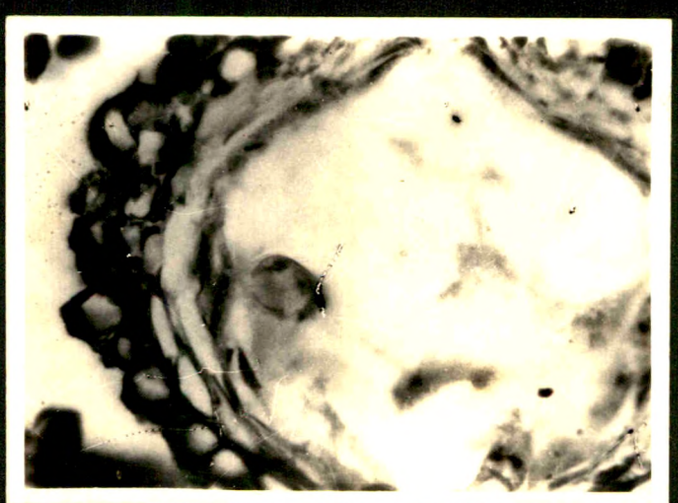

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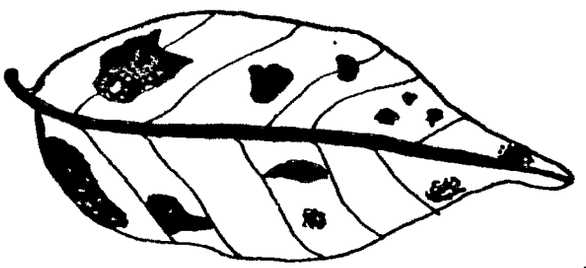

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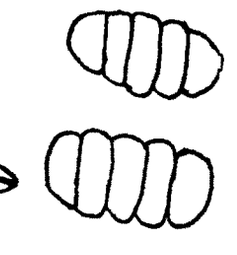

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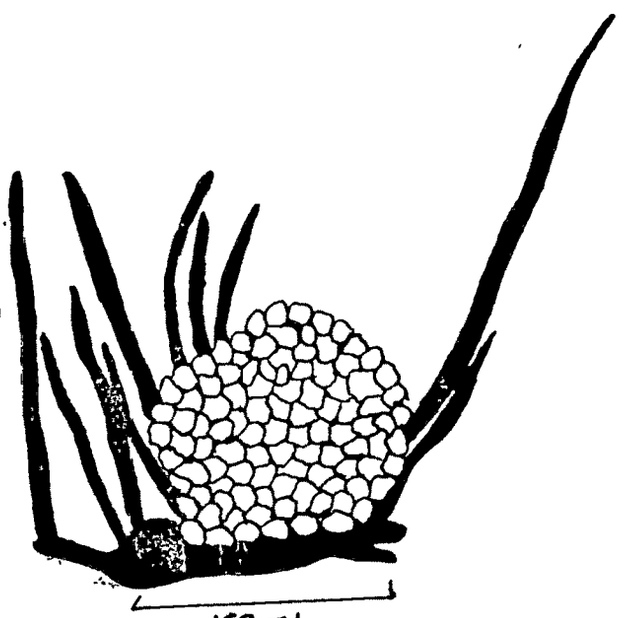

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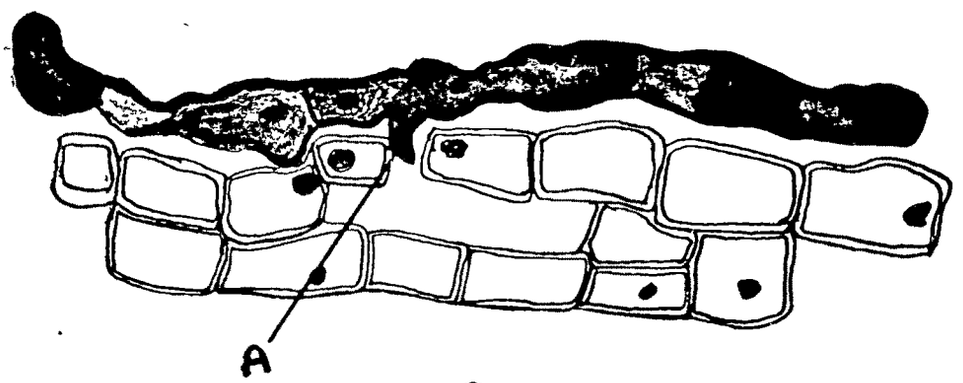

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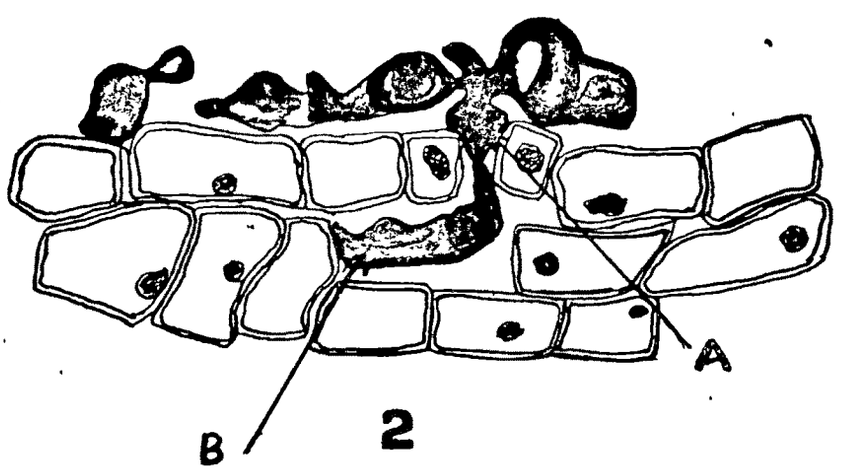

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PLATE
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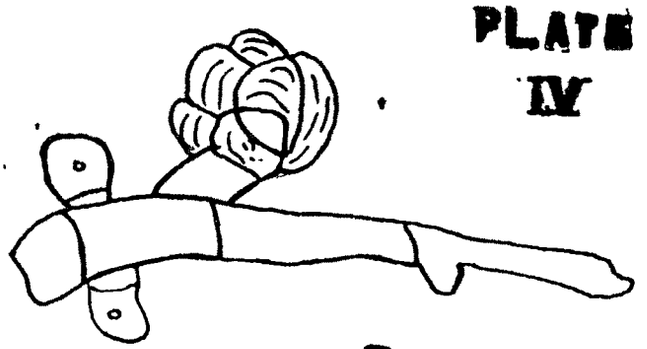

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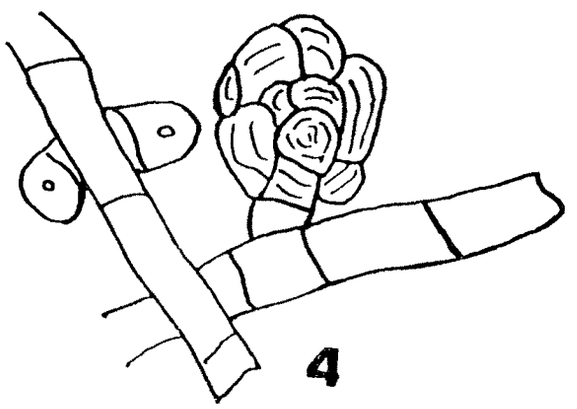

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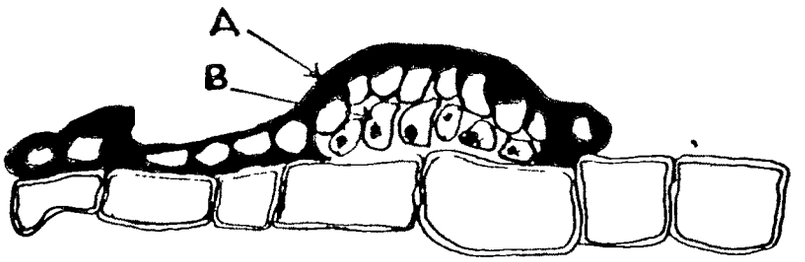

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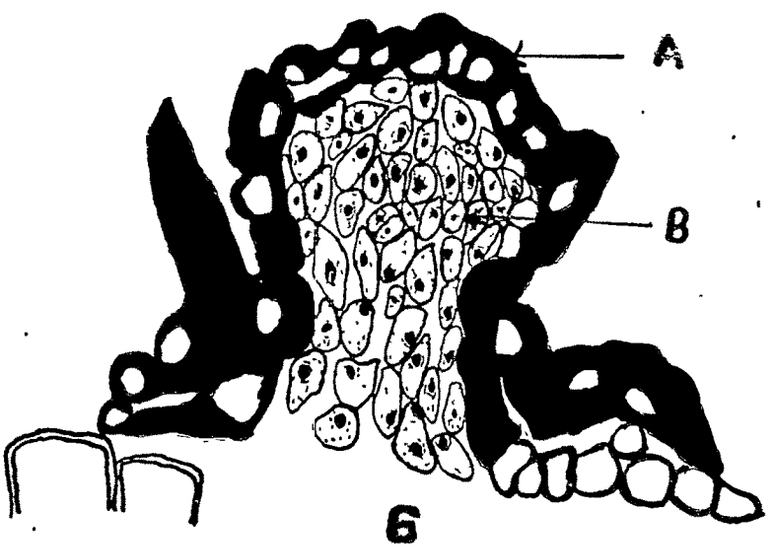

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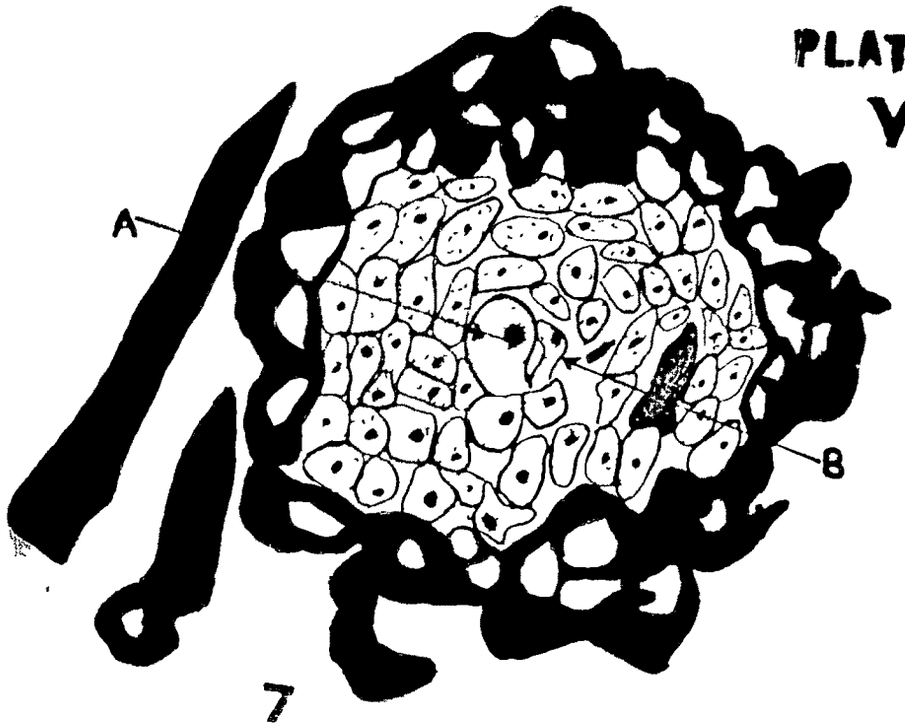

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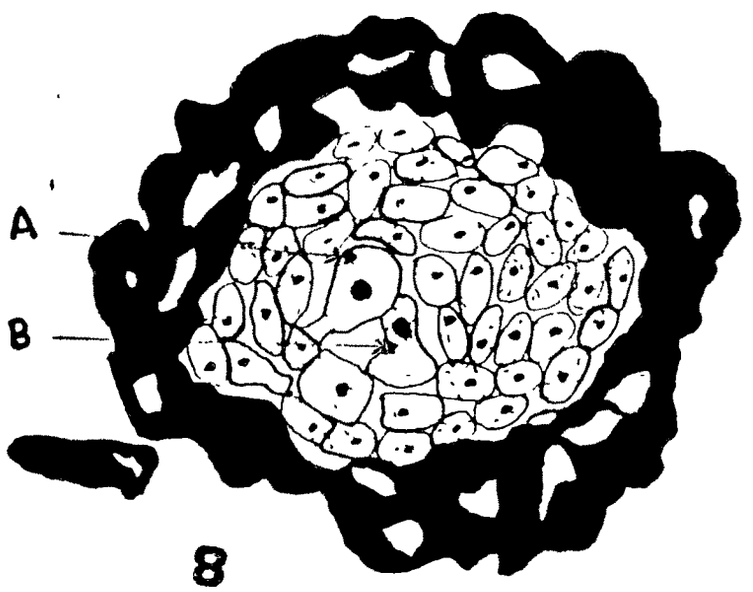

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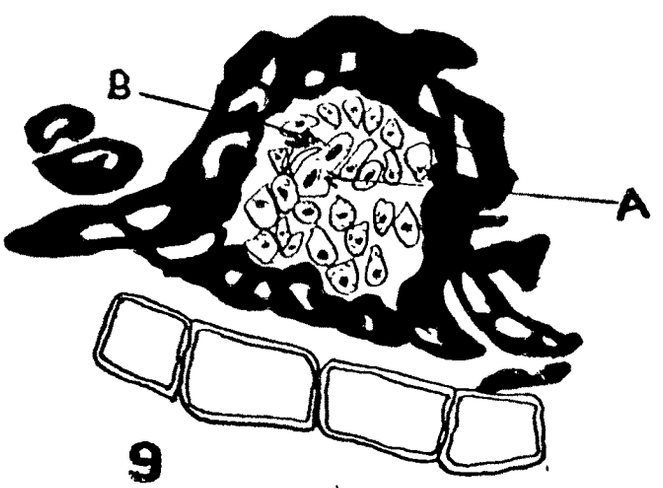

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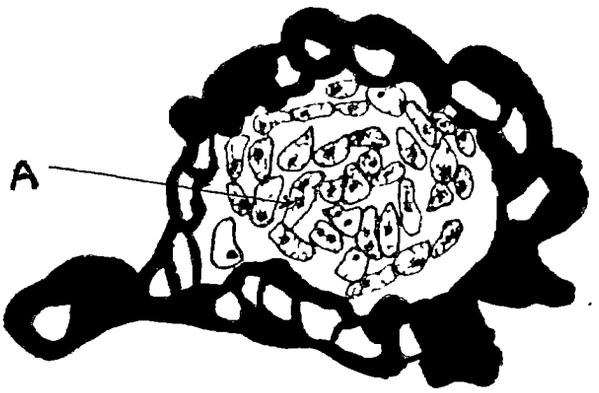

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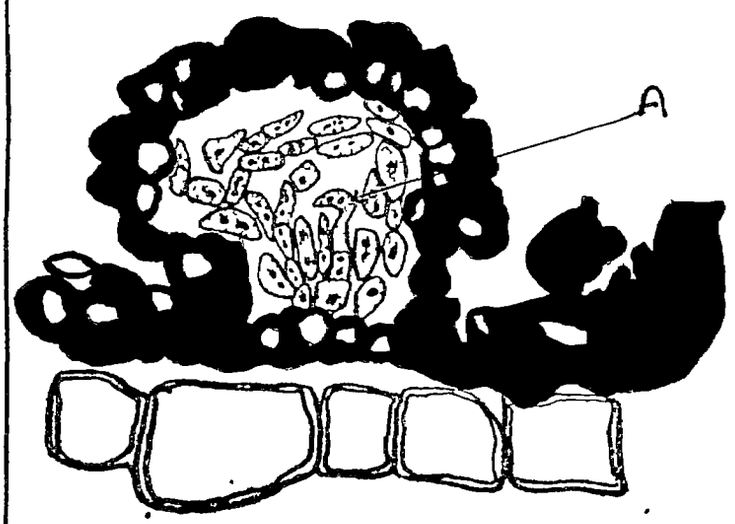

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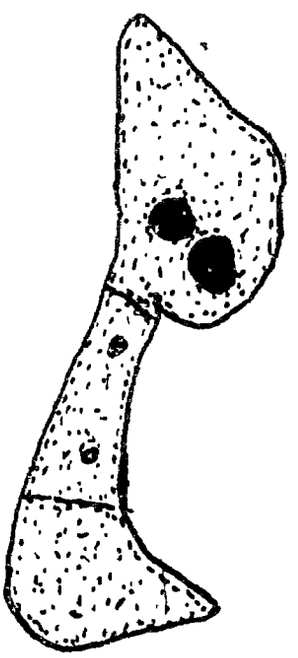

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PLATE
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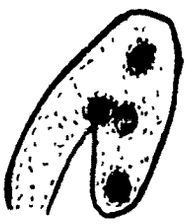

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PLATE
VII

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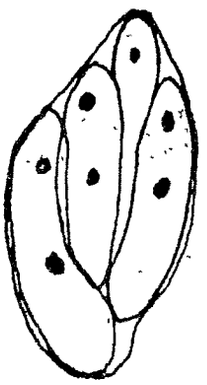
PLATE IX



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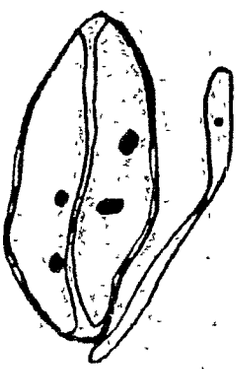

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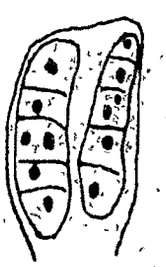

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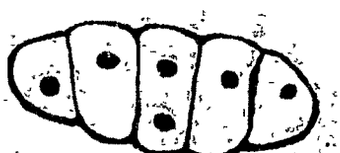

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PLATE X