CYTOLOGICAL STUDIES OF *INONOTUS TOMENTOSUS*

by

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ABSTRACT

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Cytological studies revealed irregularities in the nuclear life cycle of *Inonotus tomentosus*. The mycelia was multinucleate and the nuclei were found in groups of two to six. By the time the nuclei reached the subhymenium most of the cells were binucleate. Chlamydospores were multinucleate and most freshly shed basidiospores were uninucleate. The orientation of meiotic division in basidia was oblique. Spindle pole bodies (SPBs) were observed but microtubules were absent. Postmeiotic mitosis occurred sometimes in basidia and sometimes in basidiospores. Binucleate spores were occasionally observed.

The DNA specific dye, 4'-6-diamidino'-2-phenylindole (DAPI), was used in this study. In some hyphal cells, germ tubes, and chlamydospores, DAPI-positive particles (DPPs) were observed. The DPPs showed mostly a yellow fluorescent color. Similar observations of nuclear-like material were made with associated bacteria. The DNA material may be viruses or plasmids. Ultrastructural studies revealed some possible bacterial-like structures and mycoplasma-like organisms in hyphae and chlamydospores. The nature and function of DPPs were discussed.

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INTRODUCTION

Inonotus (Polyporus) tomentosus (Fr.) Teng, is a homobasidiomycetous fungus, in the family of Hymenochaetaceae, and occurs widely in the north temperate zone (Bondertsev, 1953; Whitney, 1976). In North America it occurs from Alaska to the southern states of Arizona, New Mexico and Alabama (Gilbertson and Ryvarden, 1986). It causes a root rot disease in natural coniferous forest stands (Whitney, 1962; Whitney and van Groenewoud, 1964) and in plantations (Can. Dep. Forest., 1966; Domanski and Dzieciolowski, 1955) where it is called stand opening disease. Severe losses due to mortality have been reported in white spruce (Whitney, 1962) and black spruce (Whitney, 1972) plantations. In some trees, the fungus grows extensively in heartwood of the butt and lower stem before attacking the sapwood and bark in the roots. In other trees, these tissues are invaded simultaneously along with the heartwood (Whitney and Bohaychuk, 1976).

Previous studies have shown that *I. tomentosus* is highly variable with regard to virulence, growth rates, polyphenol oxidase reactions, culture characteristics (Whitney, 1962), and the liberation and germination of basidiospores (Whitney, 1966; Myren, 1969; Setliff, unpublished). So far as I am aware, there are no published cytological studies on nuclear behavior and life cycle to explain

these variations. Also few cytological studies have been conducted on members of the Hymenochaetaceae.

The study of *Rigidoporus vinctus* (Berk.) Ryv.(Setliff, unpublished) has revealed that hyphae stained with the DNA specific dye 4'-6-diamidino'- 2-phenylindole (DAPI) contained large numbers of DNA positive cytoplasmic entities that varied from typical nuclei to variously sized chromatin-like particles similar to some of those reported by Russell et al. (1975) in HeLa cells deliberately inoculated with the vaccinia virus and *Mycoplasma fermentans*.

The preliminary cytological study of *I. tomentosus* nuclei with Giemsa and DAPI dyes also showed the presence of typical nuclei as well as variously sized particles in cells.

The purpose of this cytological study is to examine the nuclear life cycle of *I. tomentosus* and attempt to understand the nature of the DNA positive particles by using light and electron microscopy.

LIFE CYCLE OF A TYPICAL BASIDIOMYCETE

A wide range of nuclear behavior has been recognized within the Homobasidiomycetes during the course of vegetative and basidiomata development (Kühner, 1977). The "typical" (Raper et al., 1972) or "normal" (Boidin, 1971; Kühner, 1977) life cycle of a basidiomycete begins with a basidiospore that usually contains a single haploid or two haploid nuclei (Moore-Landecker, 1982). The spore germinates to initiate a haploid monokaryotic mycelium. The monokaryon is at first

nonseptate but later becomes divided into a number of uninucleate cells. Plasmogamy between compatible monokaryotic hyphae then may take place that allows a reciprocal exchange of nuclei between the fusing hyphae. The hyphae become dikaryotic, i.e., they form cells that contain two compatible haploid nuclei within each cell. Usually clamp connections are formed as a mechanism to maintain the dikaryotic condition and are used as morphogenetic markers for fungal compatibility within the species. The regular association of clamp connections with dikaryotic hyphae, as exemplified by the unusual fungus, *Schizophyllum commune* Fr. (Raper, 1966), has been frequently used as representative of a "typical"(Raper et al., 1972) or "normal" (Boidin, 1971; Kühner, 1977) life cycle. However clamp connections may be lacking in some basidiomycete species and this absence of clamp connections may be sporadic in some species but occurs regularly in others (Moore-Landecker, 1982).

Under favorable conditions, some hyphae of the dikaryotic mycelium change to form a basidiomata. Within the basidiomata of many species, some of the cells in sterile tissues may remain binucleate while others become multinucleate. The basidia being produced in the basidiomata are at first dikaryotic with two haploid nuclei. The nuclei then fuse(karyogamy) to form a diploid nucleus which then undergoes meiosis. Usually four presumably haploid nuclei are produced on each basidium and these then migrate through sterigmata into the basidiospores. One nucleus migrates into each basidiospore. A mitotic division may or may not occur in the basidiospore.

CYTOLOGICAL STUDIES ON SOME PATHOGENIC BASIDIOMYCETES

In studies of nuclear behavior during the life cycle of pathogenic homobasidiomycetes, atypical nuclear life cycles are seen which differ from the normal monokaryotic/dikaryotic phases found in the 'normal' cycles of most higher basidiomycetes. Unusual variations in nuclear behavior are found in *Armillaria mellea* (Vahl.) Karst. (Peabody, 1978; Raper, 1966), *Coprinus disseminatus* (Pers.:Fr.) Gray, a saprophyte fungus (Butler, 1972), *Heterobasidion (Fomes) annosum* (Fr.) Karst.(Wilson *et al.*, 1967), *Marasmius perniciosus* Stahel, a saprophyte fungus (Delgado and Cook, 1976), *Omphalia flavide* (Sequeira, 1954), *Phellinus weirii* (Murr.) Gilb. (Hansen, 1979; Furtado, 1966; Raper, 1966), *Poria latemaginata* (Dur. et Mont.) Cke. (Setliff *et al.*, 1974), and *Poria monticola* Murr. (Brushaber and Jenkins, 1971). The unusual aspects are related to the number of nuclei in the vegetative hyphae, and the irregular occurrence of clamp connections. In the case of *A. mellea* (Peabody and Peabody, 1985), and probably *Rigidosporus vinctus* (Setliff, unpublished), ploidy levels are atypical. Atypical life cycles may or may not occur in saprophytic fungi.

Nuclear patterns and numbers in vegetative mycelia and fruiting bodies

Studies of some poroid fungi have shown a multinucleate stage in mycelia or in basidioma. This has been reported for *H. annosum*

(Wilson *et al.*, 1967), *P. weirii* (Hansen 1979; Furtado, 1966; Raper, 1966), *P. latemarginata* (Setliff *et al.*, 1974), and *P. monticola* (Brushaber and Jenkins 1971). In *P. monticola*, the large number of nuclei in most cells was accounted for by the presence of clampless branches or pseudoclamps in which mitotic divisions occurred without the formation of septa. In a few hyphae, multinucleate cells were interspersed with dikaryotic cells. The greater prevalence of cells with large numbers of nuclei in certain areas of a colony was believed to be caused by some enviromental factors or genetic factors with sectoring as a result. In *P. latemaginata*, hyphae in the basidioma were simple septate, with individual cells containing two to five nuclei; terminal cells at the growing end of the basidiomata fan were multinucleate (Setliff *et al.*, 1974).

Raper(1966) pointed out that the nuclear cycle of the common agaric and tree pathogen, *A. mellea*, represented an 'aberrant' type among higher fungi. There was a lack of dikaryotic mycelium and clamp connections in rhizomorphs and in most tissues of basidioma (Korhonen and Hintikka, 1974; Motta, 1969; Peabody, 1978; Singer, 1962; Tommerup and Broadbent, 1975). The hyphal tips were uninucleate and were without clamp connections, but, in the fruit bodies, dikaryotic hyphae at the base of the basidia had clamp connections (Singer, 1962).

Other basidiomycetes such as *C. disseminatus* (Butler, 1972), *M. perniciosus* (Delgado and Cook, 1976), *P. weirii* (Hansen, 1979; Furtado, 1966; Raper, 1966) and *P. latemarginata* (Setliff *et al.*, 1974) also were reported to lack clamp connections.

Boidin (1971) analyzed the nuclear cycles of 395 species of

Aphyllophorales. Twenty six percent were holocenocytic with a multinucleate haplophase that reduced to the binucleate condition only in the basidiomata, 20% lacked clamp connections , and 12% combined these characters. The remainder (42%) had "normal" or "subnormal" life cycles.

Nuclear division in basidia

All members of the Basidiomycotina produce basidia. The basidium begins as a short binucleate cell after a conjugate mitotic division and is the site of karyogamy and meiosis. Most cytological studies of this group focus on nuclear divisions in basidia. These include *A. mellea* (Korhonen and Hintikka, 1974), *Boletus rubinellus* Peck (McLaughlin, 1970, 1971), *Collybia maculata var. scorzonerea* (Fr.) Gillet (Huffman, 1968), *Coprinus atramentarius* (Bull. ex Fr.) Fr. (Gull and Newsam, 1976; McClaren, 1967), *C. lagopus* sensu Buller (Lu, 1967; Raju and Lu, 1970, 1973), *C. radiatus* (Bolt.:Fr.) S. F. Gray (Lerbs and Thielke, 1969), *C. stercorarius* (Bull. ex St. Amans) Fr. (Rogers, 1973), *Cyathus stercoreus* (Schw.) de Toni (Lu and Brodie, 1964), *Ceratobasidium praticolum* (Kotila) Olive. (Saksena, 1961), *Marasmius spp.* (Duncan and MacDonald, 1965), *Pholiota terrestris* Overholts (Wells, 1978), *P. latemarginata* (Setliff *et al.*, 1974), *S. commune* (Sundberg, 1971), and many other species in papers reviewed earlier by Olive (1953, 1965).

Two important events take place in the basidium. These are karyogamy and meiosis. The most common situation is that in which both karyogamy and meiosis occur in essentially the same location, and

are separated only by time (Moore-Landecker, 1982). That the two successive divisions of the fusion nucleus give four nuclei are well known (Kühner, 1977). However, sometimes each of the post-meiotic nuclei divide mitotically once more to yield eight nuclei. This can give two kinds of nuclear condition in the basidiospores. In many species with eight nucleate basidia, four nuclei pass into the spores while four remain in the basidium and degenerate (residual nuclei) or all eight nuclei migrate through the sterigmata so as to form four binucleate basidiospores (Kühner, 1977; Moore-Landecker, 1982).

In *S. commune*, the original haploid nucleus in each basidiospore divides (Ehrlich and McDonough, 1949). The two nuclei are situated in the apical portion of a nearly mature basidiospore (Wells, 1965). In *P. latemarginata* (Hoch and Setliff, 1976) the four post-meiotic nuclei migrate simultaneously, one into each of the four developing basidiospores. Synchronous mitosis then follows and results in binucleate basidiospores. This same behavior has been shown in *Fomes annosus* (Wilson *et al.*, 1967), *Flammula dilepis* B.& Br. (Banerjee and Nandi 1962), *P. latemarginata* (Setliff et al., 1974), and *S. commune* (Raper 1966).

In *A. mellea*, Tommerup and Broadbent (1975) found that each of the four post-meiotic nuclei migrated to the base of a sterigma, and then appeared to move through the sterigma into the enlarging spore, where each underwent one mitotic division. Subsequently one daughter nucleus remained in the spore while the other migrated back into the basidium.

Sequeira (1954) reported that in *O. flavide*, the third nuclear

division was variable and could occur either in the basidium or in the spores. In the typical cycle, this division occurred in the basidium and, after a resting period, four nuclei migrated into the spores and four remained as residual nuclei in the basidium.

The species that have been reported to form four uninucleate spores and four residual nuclei are *A. mellea* (Tommerup and Broadbent, 1975), *O. flavide* (Sequeira, 1954), and *Stereum fuscum* (Schrad.) Quel. (Banerjee and Mukherjee, 1956).

Kühner(1977) considered the residual nuclei to be very evident in species where they remained in the apical part of the basidium (e.g. *Mycena*), but in species where they moved to the base, they more easily escaped observation.

Variation in orientation of spindles during meiosis in basidia has led us to understand two types (Kühner, 1977) -- stichobasidial, in which the spindles are longitudinal, and chiastobasidial, in which the spindles are transverse. The two orientations have been reported in both homobasidiomycetes and heterobasidiomycetes (Kühner, 1977) and are considered of taxonomic importance.

Boidin (1954) believed that at least equal importance should be given to the distance separating the dividing nucleus from the apex of the basidium. For the stichobasidial types he stated, "the first division is stituated in the medial portion of the basidium...and the second division...are never both apical, but often take place at different levels." In chiastobasidial types "the first and second divisions are apical."

Although the ultrastructure of meiosis in holobasidiomycetes has

shown some variations, the meiotic figures are very similar in the fungi studied so far (Wells, 1978).

The knowledge of nucleolar behavior in the basidiomycetes is somewhat fragmentary (Galbraith and Duncan, 1972). Maire(1902) stated that in *Hygrocybe (Godfrinia) conica* (Scop.:Fr.) Kummer, the nucleolus was expelled from the nucleus during the conjugate division preceding the development of the basidium, while Bakerspigel (1959), in his study of the vegetative hyphae of *S. commune* observed the remnants of the nucleoli lying freely in the cytoplasm during the later stages of somatic mitosis. Lu (1964) identified metaphase as being that stage of the mitotic cycle at which the nucleolus of *C. stercoreus* was freed from the chromatin and passed into the cytoplasm.

In the study of *Oudemansiella mucida* (Schrad:Fr.) v. Hoehnel and *Nolanea cetrata* (Fr.) Kummer, Galbraith and Duncan (1972) found that the nucleoli became disassociated from the parent chromatin during both mitosis and meiosis. They considered that during the final conjugate nuclear division prior to the development of the basidium, individual chromosomes cannot be detected and therefore it was impossible to state specifically the point in the division cycle at which the nucleolus was freed from the chromatin, but it was always seen as a separate entity during anaphase. Lu (1964) was of the opinion that during meiosis in *C. stercoreus*, detachment of the nucleolus from the nucleolar organizer occurred at metaphase I, whereupon it passed into the cytoplasm.

There was no evidence that the nucleolus was released to the cytoplasm during interphase, although it may move to the outside of

the nuclear envelope while still being attached. The fate of the nucleolus in *P. latemarginata* during mitosis and meiosis is unknown (Setliff *et al.*, 1974).

CYTOLOGICAL EVALUATIONS OF DNA CONTENT IN FUNGI

New techniques can often shed light on longstanding research questions. A new fluorescent compound, 4'6-diamindine-2-phenylindole (DAPI) offers such an opportunity because it is a highly fluorescent DNA binding agent, particularly for DNA with adenine-thymine (AT) rich base pairs (Williamson and Fennell, 1975, 1979). It was first developed as a trypanocide by Dann *et al.* (1971). Early studies used DAPI to bind differentially to yeast mitochondrial and nuclear DNA and thereby formed highly fluorescent complexes and enhanced the separation of the two DNAs in caesium chloride gradients (Williamson and Fennell, 1975). It continued to be used as a fluorescent stain for both nuclear and mitochondrial DNA in yeast, *Saccharomyces cerevisiae* (Williamson and Fennell, 1979; Stevens, 1981).

In 1975, Russell et al. used DAPI to stain HeLa cells infected with mycoplasmas and vaccinia viruses. The contaminated HeLa cells revealed fluorescent foci or virus 'factories' in the cytoplasm and the control HeLa cells showed only highly fluorescent nuclei and no detectable cytoplasmic fluorescence. DAPI staining has been widely used to study nuclear behavior in fungal hyphae in *Rhizoctonia solani* Kühn., *Ceratobasidium sp.* , and *Phytophthora nicotianae var.*

parasitica (Dast.) Waterhouse (Panwar et al., 1979), *Schizophyllum commune* (Runberg and Raudaskoski, 1986), *Endocronartium harknessii* (Moore) Hiratsuka (Allen et al., 1988), *Rhizoctonia spp.* (Martin, 1987), and *Uromyces appendiculatus* (Pers.) Unger. (Hoch et al. , 1987).

Cooke et al. (1987) studied the nuclei of three VA mycorrhizal fungi with DAPI staining. They observed the fluorescent nuclei in hyphae, spores, and auxiliary cells. In the study of *Loramyces*, a genus of freshwater ascomycetes, DAPI stainings of nuclei in the peridium, paraphyses, and ascospores were successfully obtained (Digby and Goos, 1987). DAPI has also been used as a specific dye to study chloroplast DNA in algae (Coleman, 1978, 1979) and higher plants (James and Jope, 1978).

One question that has arisen is that most of the organism studied with DAPI, except for the yeasts, failed to show the presence of mitochondrial DNA. Coleman (1979) suggested that yeast mitochondrial DNA may be visible only because of its extremely higher AT nucleotide content.

MATERIALS AND METHODS

FUNGAL MATERIAL

Three isolates (101H, 101I, 101J) of *Inonotus tomentosus* were prepared on 1.25% malt agar. Fruiting bodies (ZY104 and ZY105) were collected from the Jack Haggerty Forest, Lakehead University, Thunder Bay on Sept. 11, 1987.

Fungal material for staining was prepared by putting a small block of mycelia or a drop of suspension of chlamydospores onto a piece of cellophane lying on the malt agar surface and incubating it for 4-10 days. The cellophane with chlamydospores was then ready for staining.

Chlamydospores were collected from the mycelia of 6-to 12-day-old growing on cellophane overlying a 1.25% malt agar surface in a petri dish. Cultures were first examined with a light microscope to make sure that chlamydospores were present. A sterilized inoculating loop was used to collect chlamydospores by rubbing the mycelial surface and then puting the chlamydospores into a petri dish with sterile distilled water. A suspension of chlamydospores in sterile distilled water was then made. A drop of suspension was put on a coverslip and air-dried for staining and observation of the nuclear pattern.

Basidiospores were collected from freshly collected fruiting bodies by sticking a piece of basidiomata to the lid of petri dish

over a coverslip or piece of cellophane. The material on coverslips or pieces of cellophane was stained immediately. Basidiospores on cellophane were incubated for 2-4 days until many had germinated. Some of the germlings were stained and others were kept incubating for further mycelial development and staining.

All cultures and inocula were incubated at 27±1C. Giemsa stock staining solution (VWR Sci. Cherry Hill, N.J.) and DAPI (obtained from Sigma Chemical Co., St. Louis Mo.) were used for nuclear staining.

GIEMSA STAINING

Specimens were fixed in Carnoy's glacial acetic acid-ethyl alcohol (1:3) mixture for 10 min, hydrolyzed for 10 min in ca. 6N HCl at room temperature, and then washed in three changes of 0.05 M $PO_4^=$ buffer at pH 6.8 (prepared by mixing 99 ml of 1/15 M Na_2HPO_4 with 101 ml of 1/15 M KH_2PO_4 solutions) for 5 min each. Specimens then were stained in Giemsa solution (10% Giemsa stock solution in $PO_4^=$ buffer pH 6.8) for 0.5 to 1h and washed in the same buffer for 5-10 min. The cellophane pieces were put on a slide, mounted in buffer, and covered with a No.1 coverslip for observation. Semipermanant slides also were made as follows: After washing in buffer for 5-10 min following staining, the cellophane pieces were put on slides and the residual buffer on the slides was removed with a piece of filter paper. The slides were air-dried for 5-10 min and covered in immersion oil (Index

of refraction n_D1.515, obtained from R.P. Cargille Laboratories, Inc., Cedar Grove, N. J.).

Brightfield and phase observations were made with a Zeiss photomicroscope (Carl Zeiss, Ultraphot II, West Germany) equipped with Neofluar objectives. Photomicrographs were taken on Plus-X film (Kodak, Toronto, Canada) and the film was developed in Kodak D-76 developer.

DAPI STAINING

DAPI staining solution was prepared by adding 5 µl DAPI stock solution in 40 ml McIlvaine buffer. McIlvaine buffer (pH 4.4) was prepared by adding 11.25 ml (0.1 M) citric-acid solution (prepared by diluting 1.05 g citric-acid to 50 ml distilled water) to 8.75 ml (0.2 M) Na_2HPO_4 and diluted to 500 ml solution.

Samples were fixed in 70% alcohol for 30 min, stained in DAPI solution in dark for 60-90 min at room temperature. Samples then were rinsed in buffer for 5-10 min, placed on slides, mounted in buffer, and covered with a No.1 coverslip for observation. For semipermanant slides, the procedure was the same as for the Giemsa staining. Semipermanant slides were kept in the dark.

DNase treatments were conducted as a control method for determining DAPI specificity for DNA. The cellophane with hyphae was cut into two pieces. One of them was treated with DNase (Sigma Chemical Co., St. Louis, Mo.) enyzme, the other one was used as a

non-enzyme treated piece. Then they were both stained with DAPI.

Fluorescent observations and photomicrographs were made with a Zeiss photomicroscope equipped with Neofluar objectives and a UV light source emitted from a High-pressure mercury lamp HBO 200. The primary excitation light (ca. 365 nm) was obtained with UG1 exciter filter and a heat filter, GB38, was put under the UG1. The secondary filters (barrier) were filter 41 or 47. Photomicrographs were taken on Kodak Ektachrome ASA400 color slide film.

BASIDIOMA FOR LIGHT MICROSCOPIC OBSERVATIONS

The pore areas of fresh basidioma were cut in 2 mm^3 pieces. Two fixation methods were used. Some pieces were fixed in 3% glutaraldehyde in $PO_4^=$ buffer (pH 6.8) overnight. Other pieces were fixed, and hydrolyzed as in the Giemsa staining procedure described above. Then the specimens were dehydrated and embedded in glycol methacrylate (Feder and O'Brien, 1968). Both longitudinal and transverse sections (3-7 μm thick) of basidia and subhymenium were cut with glass knives mounted in a Porter-Blum, Sorvall JB-4 microtome. Individual sections were lifted from the knife with forceps and placed in a drop of water on a glass microscope slide and air-dried. The sections were stained overnight in Giemsa solution (one ml Giemsa stock solution in nine ml buffer).

BASIDIOMA FOR ELECTRON MICROSCOPIC OBSERVATIONS

TEM Preparation

Chlamydospores for TEM observation were placed on a piece of cellophane over the surface of a 1.25% malt extract + 2% agar plate and germinated at 26±1C. The chlamydospores and hyphae were fixed in 1.5% glutaraldehyde in $PO_4^=$ buffer (pH 6.8) for 2 hr, washed in the same buffer for 1 hr, and then postfixed with 1% OsO_4 in buffer for 2 hr. After washing in distilled water for 1 hr the specimen was stained in 0.5% aqueous uranyl acetate for 3 hr. The material was repeatedly rinsed in distilled water and dehydrated in a graded acetone series. Final dehydration were affected by two changes of 100% acetone. The spores and germling hyphae were embedded in Spurr's low-viscosity medium (Spurr, 1969).

Fresh fruiting bodies of *I. tomentosus* (ZY104 and ZY105) were collected and were cut in 1 mm^3 pieces and prepared as above. Thin sections were cut with a Porter-Blum Sorvall MT-26 ultramicrotome. The sections were poststained with lead citrate, and viewed with a Philips EM300 transmission electron microscope operating at 60 kv.

SEM Preparation

Basidioma were cut into 3 mm^3 pieces and fixed in 1.5% glutaraldehyde in $PO_4^=$ buffer at pH 6.8 for 4 hr. The pieces were washed in the same buffer for 1 hr and then postfixed with 1% OsO_4 in the same buffer for 2 hrs. After washing in distilled water for 1 hr,

they were dehydrated as described above. The specimens were critical-point-dried with CO_2, sputter coated with gold and then observed with a Hitachi 570 scanning electron microscope operating at 10 kv.

RESULTS

LIGHT MICROSCOPY OBSERVATIONS

Vegetative Hyphae

Young, early-formed, vegetative hyphae were simple-septate, thin-walled, and 1.5-4.3 µm in diam. Hyphal cells were multinucleate, with the nuclei tending to form into groups of 2-6 nuclei (Figs. 1-3). The grouping tendancy was particularly noticed during the formation of lateral branches and the apparent migration of nuclei into these branches(Figs. 1). Individual nuclei could often be discerned as well (Fig. 2). Groups with three nuclei (Fig. 3) were most common (Table 1).

Nuclei were mostly round to ovoid in shape. However, in some hyphae, the nuclei were elongated and apparently migrating into a branch hypha (Figs. 2 and 4). Occasionally, numerous small particles were stained with Giemsa (Fig. 5) along with nuclei. Often some cells contained no typical nuclei and were filled with particles of various sizes.

Likewise, with DAPI staining comparable particles and nuclei were observed in some hyphae (Figs. 7, 9 and 12). The particles fluoresced bright yellow and the nuclei were pale blue (Figs.12 and 13). Some particles were bluish and similar in this respect to nuclei except for size. With DAPI staining, the following three patterns of nuclei and

Table 1. Number of nuclei in groups in vegetative hyphae and their frequency.

No. of nuclei in groups	Number of observation	Percentage
1	36	22.6%
2	41	25.8%
3	64	40.3%
4	8	5.0%
5	6	3.8%
6	4	2.5%
Total	159	100%

PLATE 1. Vegetative hyphae (from isolates 101H, 101I, 101J, and ZY104) stained with Giemsa.

Fig. 1. Vegetative hyphae with a group of nuclei in branch and a chlamydospore (arrow). Bar = 7.2 μm.

Fig. 2. Six nuclei in a group and an individual nucleus in a hyphal cell (double arrow). An elongated nucleus (arrow) seems moving into a branch. Bar = 7.2 μm.

Fig. 3. Vegetative hyphae with three nuclei in group(arrow). Notice that one nucleus is separated from the other two in a group. Bar = 9.0 μm.

Fig. 4. Vegetative hyphae with elongated nuclei that seemed migrating into branches (arrows). Bar = 9.0 μm.

Fig. 5. Giemsa staining indicated nuclei and particles (arrow) mixed in a hyphal cell. Bar = 9.0 μm.

Fig. 6. Germling and bacteria (double arrows) together in culture. Notice the hyphal tip ruptures (arrow). Bar = 14.3 μm.

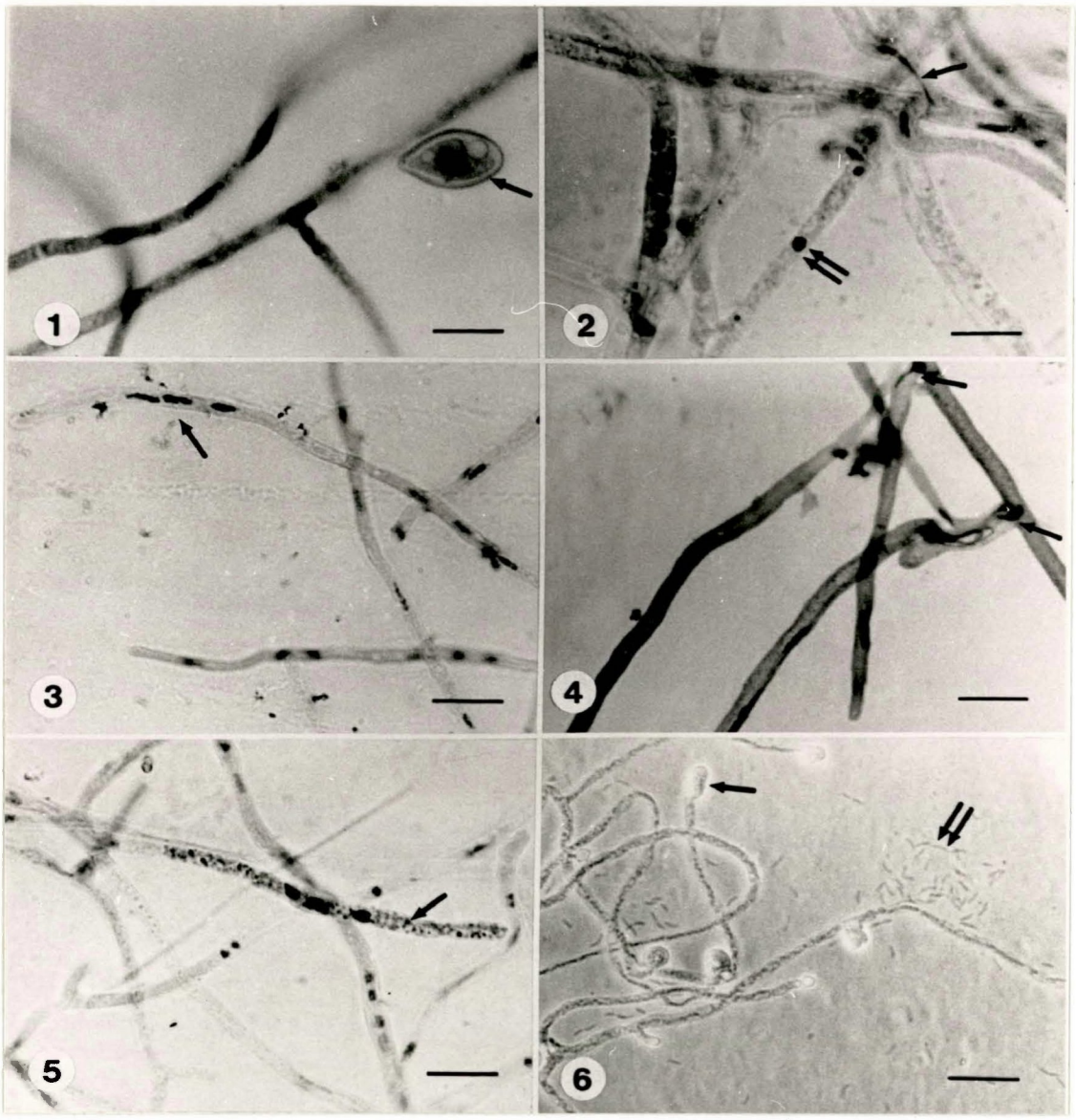

1
2
3
4
5
6

PLATE 2. Vegetative hyphae (from isolates 101I, 101H, 101J, and ZY104) stained with DAPI.

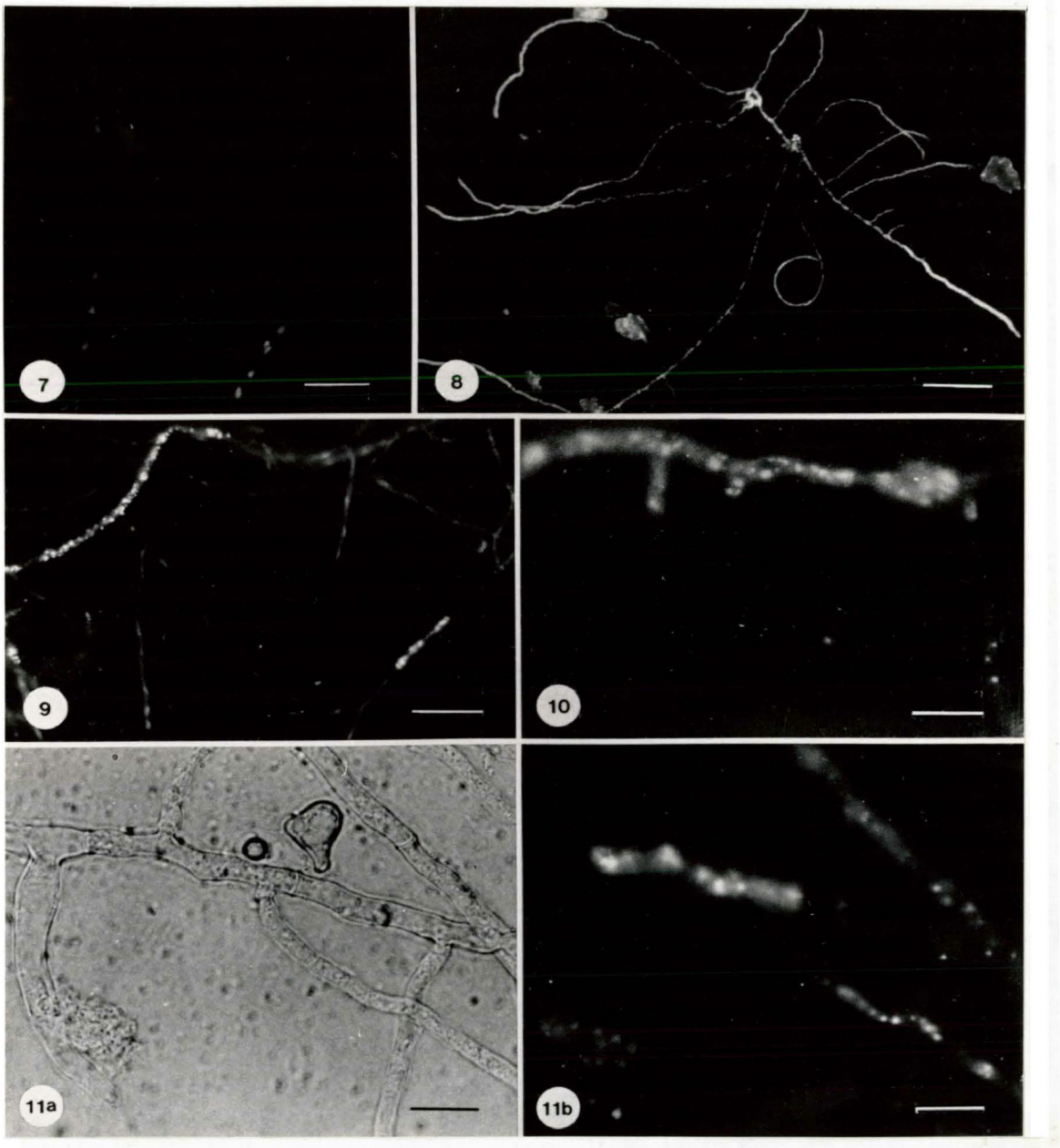
Fig. 7. Hyphae with nuclei in groups. Bar = 9.0 µm.

Fig. 8. A chlamydospore germling showing the staining pattern and DAPI-positive particles. Bar = 55 µm.

Fig. 9. DAPI staining showed the patterns of nuclei and particles changed sharply within the mycelium cell by cell. Bar = 22 µm.

Fig. 10. Variation in size of DAPI-positive particles within a cell. Bar = 9.0 µm.

Fig. 11a and b. Contrasting photgraphes of a ruptured hyphal tip as seen with phase contrast (Fig. 11a) and the nuclei with fluorescent light (Fig. 11b). The adjacent cells were with yellow particles (Fig. 11b). Bar = 9.0 µm.


7
8
9
10
11a
11b

particles in the hyphal cells were observed:

1. Cells with only pale blue nuclei. This pattern was most frequent.

2. Cells with fluorescent yellow or blue particles of various sizes.

3. Cells with DAPI positive particles (DPPs) and nuclei mixed in the same hyphal cells.

In germlings and fairly young colonies only Pattern 1 or Pattern 2 was found usually. For Pattern 2 in the young colonies, sometimes the sizes and densities of particles changed cell by cell from the hyphal tip toward the center (Fig. 8). Table 2 shows the frequencies of the three patterns in germlings of 101H, 101I, and 101J isolates.

The three patterns of nuclei and DPPs existed together among the hyphae. The pattern in each individual cell was different from its neighboring cells (Figs. 9, 12 and 16). It was common to see an individual hyphal cell full of yellow particles while neighbouring cells contained only nuclei (Fig. 9). Repeated observations of the same culture at different times showed a random pattern of DPPs that varied from infrequent to frequent. However, the most usual pattern in hyphae (at least over 60% of the hyphal cells) for each observation was Pattern 1.

The size of the particles varied from minute to the size of nuclei (1-2 μm). In some cells, the particles were similar in size and in other cells they varied (Fig. 12). Groups of nuclei were observed with DAPI staining (Figs. 13 and 17). Figure 11 shows nuclei associated with cytoplasm that burst from a hyphal tip. Neighbouring

Table 2. DAPI staining patterns in chlamydospores germlings (Isolates were on 1.25% malt agar at 26±1C for 2 days on June 28).

Isolates	DNA Patterns	No. of germlings on each pattern	Percent
101H	Particles(P)	26	26.6%
	Nuclei(N)	70	71.4%
	N & P	2	2.0%
101I	P	27	29.7%
	N	61	67.0%
	N & P	3	3.3%
101J	P	18	29.0%
	N	40	64.5%
	N & P	4	6.5%

PLATE 3. Vegetative hyphae (from isolates 101I, 101H, 101J, and ZY104) stained with DAPI.

Fig. 12a and b. DAPI-positive particles (DPPs) showed bright yellow fluorescent color with DAPI staining (Fig. 12a). Bar = 45μm. A higher magnification of a hyphal cell in Fig. 12a. with general fluorescence to indicated accummulation of particles in the two ends of the cell (Fig. 12b). Bar = 11.7μm.

Fig. 13. Hyphae with DAPI staining showed pale blue nuclei in groups. Bar = 11.7 μm.

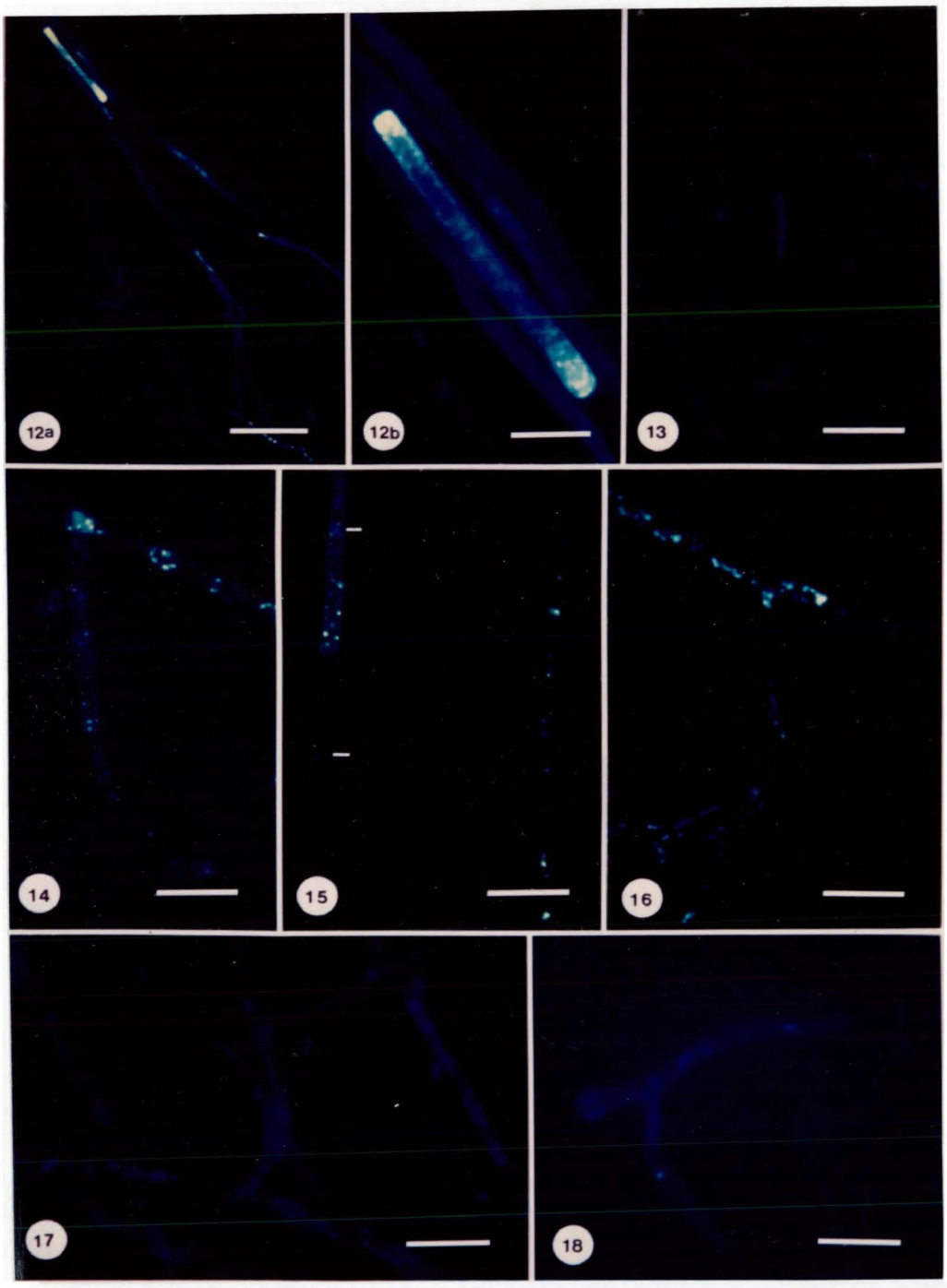
Fig. 14. Circular DAPI-positive particles were in hyphal cells. Bar = 11.7 μm.

Fig. 15. The pattern of nuclei and DAPI-positive particles mixed together in a hyphal cell. Bar = 11.7 μm.

Fig. 16. Hyphae with yellow particles. Bar = 11.7 μm.

Fig. 17. Hyphae with blue particles. Bar = 11.7 μm.

Fig. 18. A germling from basidiospore with nuclei and DAPI-positive particles. Bar = 11.7 μm.


12a
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hyphal cells also show yellow particles.

In some hyphal cells, the particles accummulated at the ends of the cell and were absent in adjacent cells (Fig. 12). Sometimes the particles formed a circular pattern (Fig. 14). Nuclei and particles were mixed together in some hyphal cells (Fig. 15). Figure 16 shows the size of the yellow particles in a branch cell was smaller than the size of those in the main hyphal cell. Blue particles were observed sometimes in hyphae (Fig. 17).

The association of nuclei and DPPs changed so randomly from cell to cell that no patterns that might result from specific influential forces was recognized, except that in a comparison of basidiospore and chlamydospore germlings, there was a pattern change (nuclei to DPPs or from DPPs to nuclei) from both basidiospores and chlamydospores to germ tubes cell by cell (Figs. 18 and 20).

Figure 19 shows an elongated pattern of DAPI-positive materials in a hyphal cell similar to that see with migrating nuclei. There were two barely visible pale blue nuclei in each of the adjacent cells on either side.

Observations of hyphae mounted in buffer and oil were the same, but hyphal nuclei and nuclear material mounted in oil were clearer than in the buffer mounted slides for both Giemsa and DAPI staining.

Chlamydospores

There were large numbers of chlamydospores formed after about 6 days on 1.25% malt agar plate (Figs. 26-28). Hyphae subtending chlamydospores were empty (Fig. 28). Chlamydospores were 5-10 x 8-14

PLATE 4. Vegetative hyphae (isolates 101I and 101J), chlalmydospores (isolate 101I), associated bacteria, and basidiospores (ZY104) with DAPI staining.

Fig. 19. Vegetative hypha with DAPI staining indicated a nuclear pattern in a cell. The nuclear material elongated in bright yellow color and particles accumulated in the two ends of the cell. The neighbour cells showed pale blue nuclei (n). Bar = 11.7 µm.

Fig. 20. A germling from chlamydospore with DAPI-positive particles. Bar = 11.7 µm.

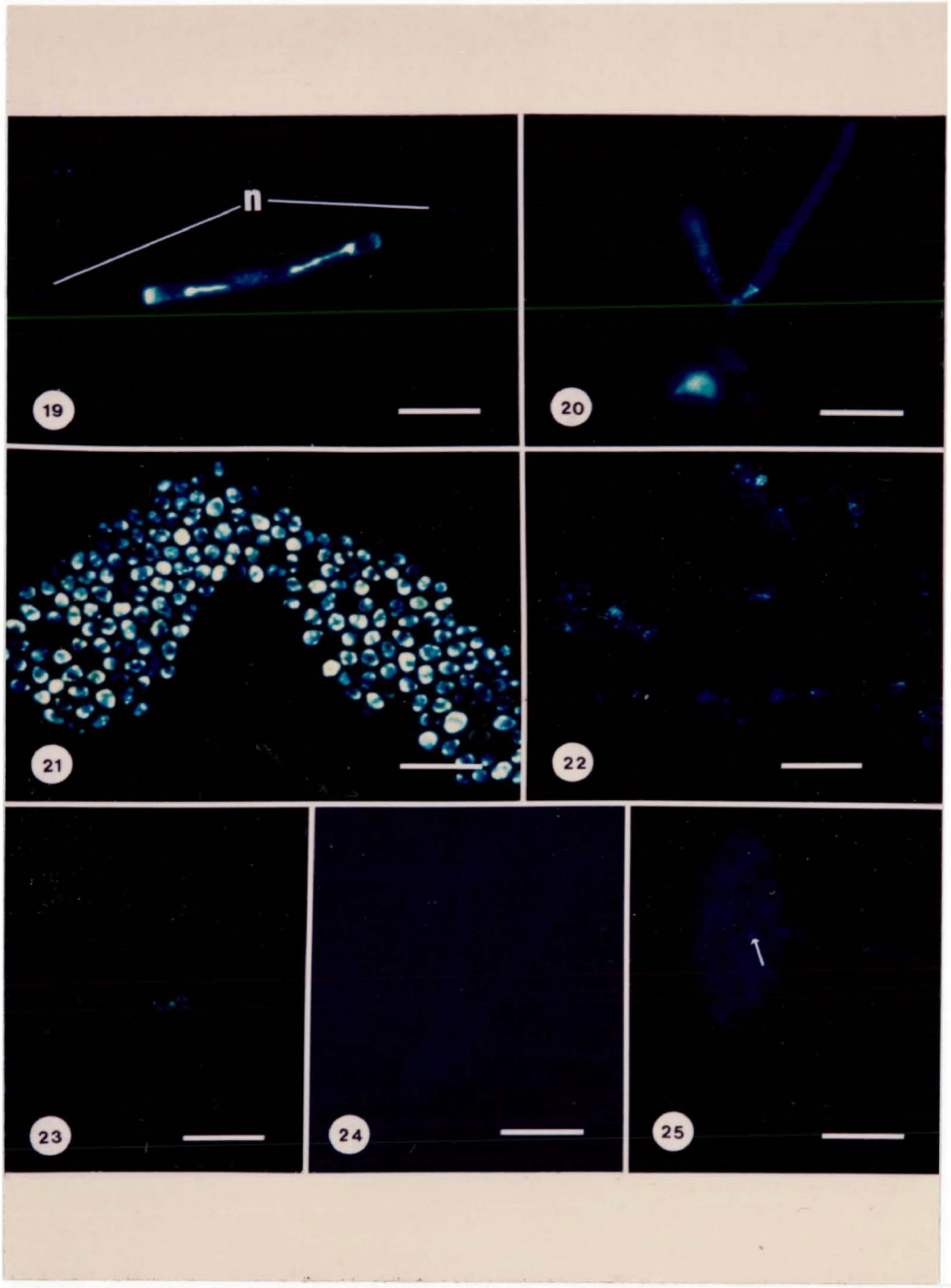
Fig. 21. Chlamydospores with DAPI staining. Bar = 28 µm.

Fig. 22. The associated bacterial species with DAPI staining showed the nuclear materials with both yellow and blue colors. Bar = 24 µm.

Fig. 23. DAPI staining of the bacterial species associated with the mycelia in culture indicated the nuclear material in the two ends of the cell in yellow color. Bar = 11.7 µm.

Fig. 24. Hyphae with DAPI staining after treatment with DNase enzyme indicated clear cells without any fluoresent materials. Bar = 11.7 µm.

Fig. 25. Basidiospores and a germling with DAPI staining. A yellow particle was found in the germinated spore (arrow). Bar = 11.7 µm.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μm in size with thick cell walls (Figs. 29 and 30). Chlamydospores stained with DAPI showed the same kind nuclear and particle staining patterns (Fig. 21) as those observed in vegetative hyphae. The frequency of each pattern in chlamydospores is indicated in Table 3. Chlamydospores always contained DPPs (Figs. 10, 20 and 21). Most of the particles fluoresced yellow color, whereas nuclei and some of the particles were pale blue (Fig. 21). In a few spores, the entire cytoplasm fluoresced yellow, but in most spores, discrete yellow particles were present. Some spores contained neither nuclei nor particles.

Giemsa staining also showed both nuclei and particles. It was common with Giemsa staining that some spores were stained whole cell while others heavily stained nuclei or particles (Fig. 27). Some spores had nuclei, some had only particles, and others had both nuclei and particles (Figs. 29 and 30). The number of nuclei in the chlamydospores varied from one to several (most of them were binucleate or trinucleate) (Figs. 27 and 29). The nucleus had a clear boundary that separated it from the cytoplasm and most of the nuclei were round. The particles in the spores varied in number and size just as in vegetative hyphae (Fig. 30). There was no clear boundary to separate the particles from the cytoplasm.

Most of the chlamydospores germinated after 0.5 - 2 days on malt agar (Figs. 31-33). The germ tubes were 2 - 4 μm in diameter with thin cell walls. A single germ tube usually originated opposite the attachment end of the spore (Fig. 31), but sometimes 1-2 germ tubes originated from the spores. Two or three nuclei were observed in the

Table 3. DAPI staining patterns in germinated chlamydospores (on 1.25% malt agar at 26±1C) based on two separate observation times.

Isolates	DNA Patterns	Number of spores observed on		Total	
		July 22 [a]	Aug. 13 [b]	Number	Percent
101H	Particles(P)	103	121	224	49.1%
	Nuclei(N)	96	124	220	48.3%
	N & P	4	8	12	2.6%
101I	P	121	65	186	42.5%
	N	186	40	226	51.6%
	N & P	5	21	26	5.9%
101J	P	117	191	308	46.5%
	N	122	222	344	51.9%
	N & P	9	3	11	1.6%

[a]: chlamydospores were on culture for two days.
[b]: chlamydospores were on culture for one day.

PLATE 5. Chlamydospores and germ tubes (101I, 101H, and 101J) and basidiospores and germinations (ZY104 and ZY105) with Giemsa staining.

Fig. 26. Chlamydospores were produced one by one in culture. Bar = 9.0 µm.

Fig. 27. Chlamydospores with multinuclei. The whole cell was lightly stained and the nuclei were heavily stained. Bar = 14 µm.

Fig. 28. Hyphal collapse after chlamydospore formation in culture. Bar = 9.0 µm.

Fig. 29. Chlamydospores with nuclei. Bar = 9.0 µm.

Fig. 30. Chlamydospores with particles. Bar = 9.0 µm.

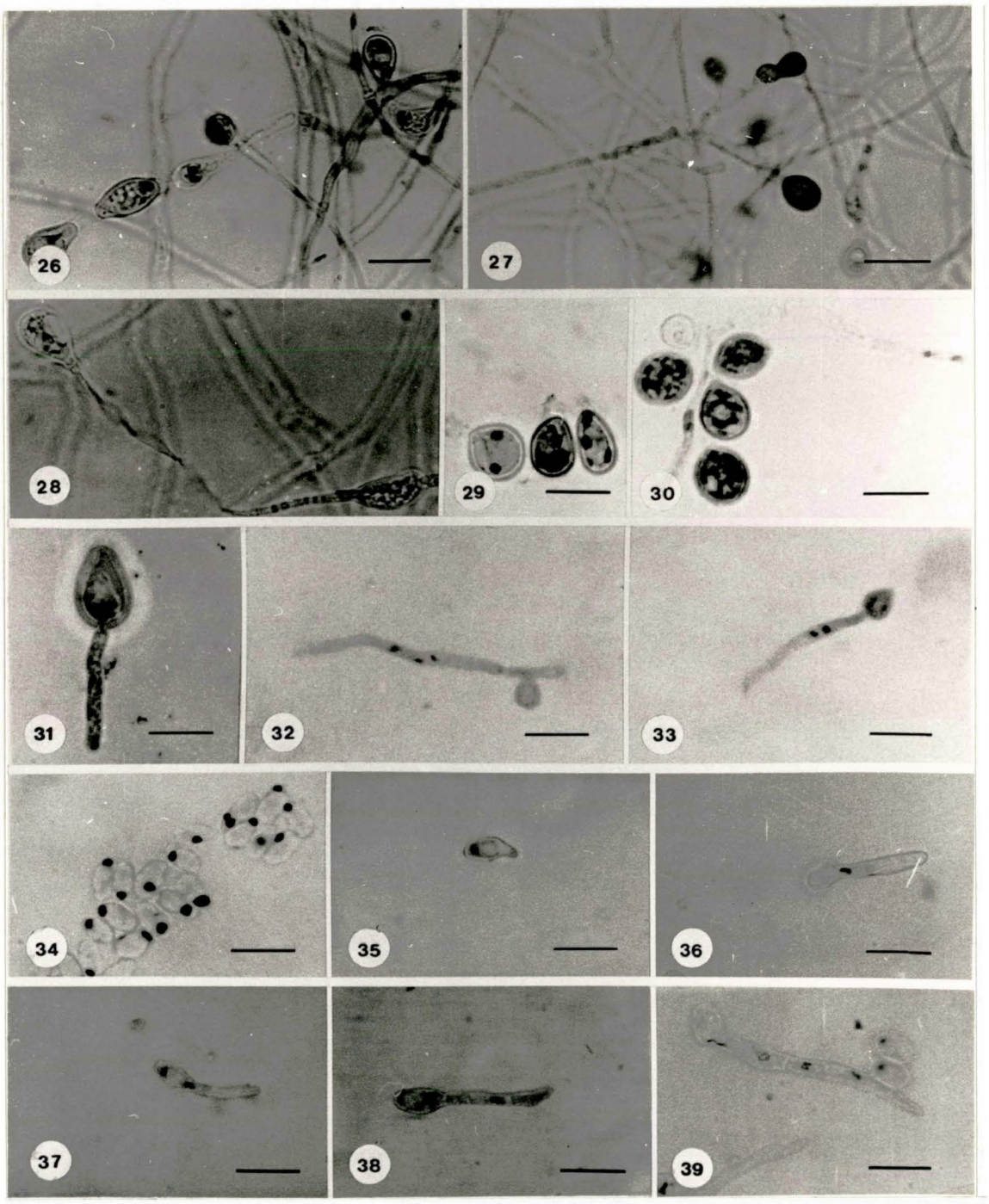
Fig. 31. Germination of chlamydospore with germ tube containing numerous particles. Bar = 7.2 µm.

Fig. 32. Chlamydospore germling with three nuclei in germ tube. Bar = 17.5 µm.

Fig. 33. Chlamydospore with two nuclei in germ tube. Bar = 15.0 µm.

Fig. 34. Nuclear patterns in basidiospores. Bar = 9.0 µm.

Figs. 35 - 39. Basidiospores germlins of isolate ZY104. Bars = 9.0 µm.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germ tubes before they branched (Figs. 32 and 33). Particles were present in some germinated spores and germ tubes (Figs. 20 and 31).

Associated Bacteria

Vegetative hyphae that grew from basidiospores cast from basidioma collected from nature were sometimes observed with rod-shaped bacteria (Fig. 6). DAPI stained bacteria showed both yellow and blue areas of DNA (Figs. 22 and 23). Individual bacteria contained either one or two areas that were blue or yellow in color. The blue areas (nucleoids) were more uniform in size, discrete, and predictably located (Fig. 22) than the yellow areas.

The ratio of yellow bacteria and blue bacteria was unequal and inconsistant with repeated observations over a period of time. Sometimes there were many bacterial cells with yellow DNA areas and at other times the bacterial cells mostly contained discrete pale blue nucleoids. However, bacteria with yellow fluorescence were never greater than 40% of the bacteria present.

DNase Enzyme

The control hyphae treated with DNase were mostly devoid of blue nuclei and yellow particles when stained with DAPI (Fig. 24). The non-DNase treated hyphae contained blue nuclei and yellow particles. A few hyphae with pale yellow particles were found in the DNase treated hyphae. The quantity and brightness of the residue pale yellow particles were greatly reduced when compared with the control hyphae.

DNase enzyme treatment completely eliminated the yellow and blue

areas of DNA in bacteria.

Basidiospores

Basidiospores obtained from fresh fruiting bodies ZY104 and ZY105, contained 1-2 nuclei. There were 94% with one nucleus and 6% with two nuclei(Fig. 34). There were no DPPs in the fresh basidiospores -- only pale blue nuclei. After culturing for 2-4 days, DPPs appeared in some germ-tubes (Fig. 25) and in some spores which failed to germinate. The patterns of DPPs and nuclei of vegetative hyphae from basidiospores were the same as with those in vegetative hyphae and hyphae originating from chlamydospores.

Basidiospores germinated from the end opposite to the distal end where the nucleus was usually located (Fig. 35); the nucleus then migrated into the germ tube (Fig. 36). Germinated spores sometimes were found with two nuclei and sometimes one nucleus was in the germ tube and the other one was in the spore (Figs. 37 and 38). Sometimes the germlings were binucleate at the beginning and maintained in this pattern for several cells (Fig. 39). The frequency of binucleate germ cells was higher than that of the binucleate fresh basidiospores observed in this study.

Most spores failed to germinate on 1.25% malt agar and the percentages of germination varied greatly among different spore casts. After culturing for 4 days, the best germination percentage was of ZY104 spores at 27%, based on a count of a total of 85 germinated and 231 ungerminated spores. For some spore casts, the germination percentage was less than 1% in the first 2 days in culture. Even

after culturing for 4-5 days, the germination was still less than 10% of total spores in culture. There was no difference in morphology between germinated and ungerminated spores as observed under bright field microscopy.

Basidia

Basidia were 12-20 µm in length and 3.5-6.5 µm in diameter (Fig. 40). Young basidia were initially binucleate (Fig. 41) and developed from swollen binucleate basal cells (Fig. 42). The two premeiotic nuclei (about 1.9-2.3 µm) were closely associated in the basidium. When the basidium reached about half of its full size, the nuclei were close together (Fig. 43). Karyogamy occurred near the middle of the basidium and the fusion nucleus was about twice (3.3-4.0 µm) the diameter of the pre-karyotic nuclei in young basidium (Figs. 44 and 45).

The fusion nucleus migrated toward the apical region of the basidium prior to division. During migration, the fusion nucleus was lightly stained and ovoid to round in shape (Fig. 45). At the top part of the basidium, at prophase I, chromosomes were visible and stained darkly (Fig. 46). At an early stage of metaphase I, the spindle pole bodies (SPBs) were visible on opposite sides with chromatin in between (Figs. 47a, b, c). Late anaphase I was observed in some basidia (Fig. 48). When the two groups of chromosomes reached their respective poles two daughter nuclei (about 2.1-2.6 µm) were formed from the first division (Figs. 46, 49, and 50).

During division II, the two daughter nuclei divided

PLATE 6. Nuclei in subhymenial basal cells and nuclear behavior in basidia of *I. tomentosus* (isolates ZY104 and ZY105) with Giemsa staining. Bar = 6.7 µm.

Fig. 40. Hymenial layer of basidia.

Fig. 41. Prekaryogamic nuclei (arrow) in a basidium.

Fig. 42. Binucleate (arrows) subhymenial basal cells. S, septum.

Fig. 43. Karyogamy occurred near the middle of the basidium (arrow).

Fig. 44. Fusion nucleus (arrows) in young basidia.

Fig. 45. Premeiotic interphase nucleus (arrow).

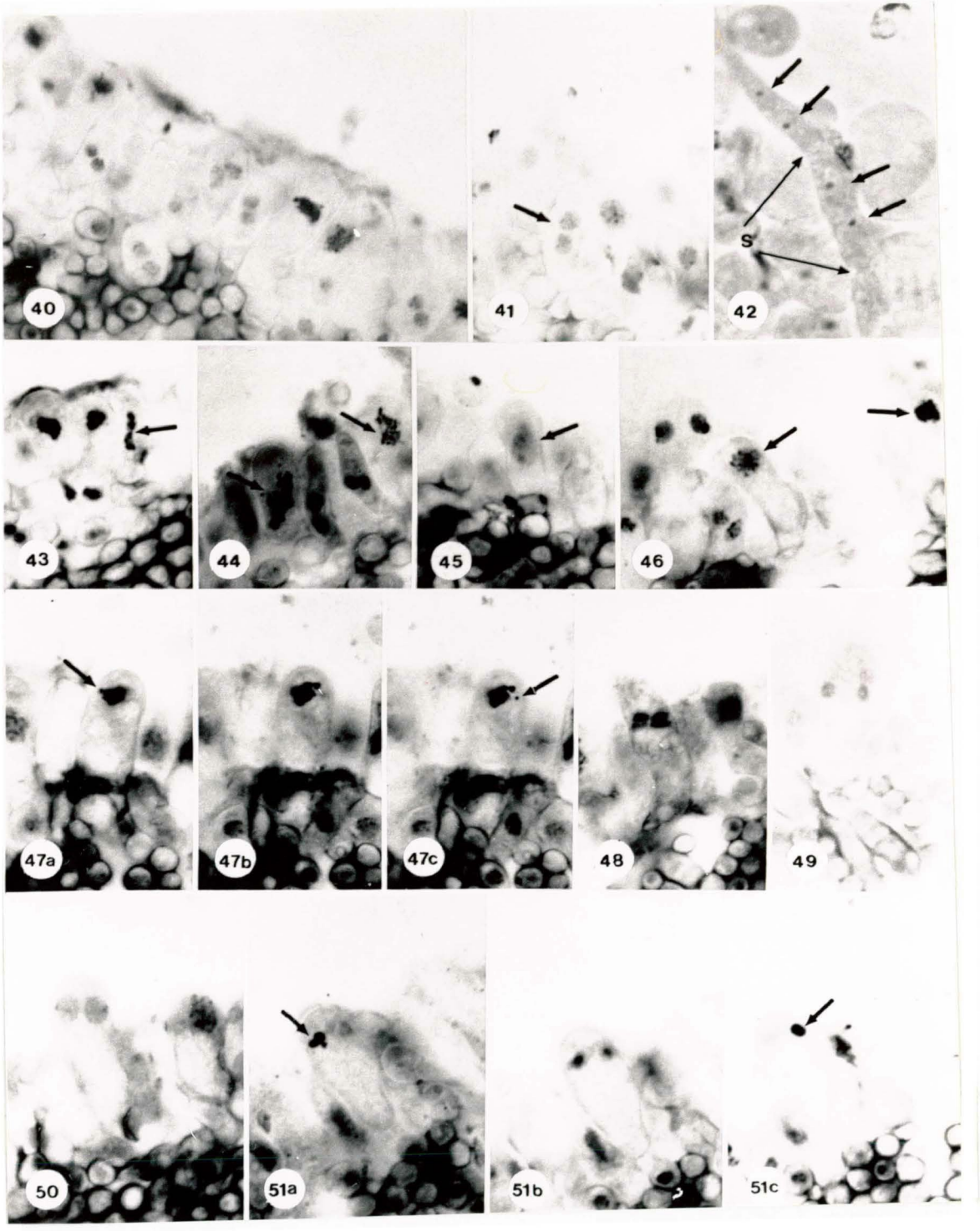
Fig. 46. Prophase I meiosis (arrows) with interwoven strands of chromosomes.

Fig. 47. Different focal planes of a basidium to show the two spindle pole bodies (SPBs) (arrows) with metaphase I.

Fig. 48. Late anaphase I nucleus.

Figs. 49 and 50. Interphase I nuclei after completion of division I meiosis.

Fig. 51. Different focal planes of a basidium to indicate the two daughter nuclei divided simultaneously in division II (arrows).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51c

simultaneously (Figs. 51a, b, c). Chromosomes condensed into two groups and moved opposite from each other (Figs. 52a, b). Division occurred at a plane oblique to the longitudinal axis of the basidium (Fig. 53a, b, c). Completion of meiosis resulted in four haploid nuclei (Fig. 54). The telophase II nuclei were small, about 1.1-1.5 μm in diam., and intensely stained. In some basidia, they became slightly larger, less intensely stained, and about 2.0-2.5 μm in diameter. Sterigmata were initiated at this time (Fig. 55).

A post-meiotic mitosis was observed in some basidia. The four nuclei first moved from the terminal end to the midregion of the basidia and condensed prior to the third division. The nuclei divided simultaneously (Figs. 56a, b, c, d). The resulting nuclei were small (about 0.9-1.3 μm) and intensely stained.

During nuclear migration into the basidiospores, the nuclei were attenuated toward the sterigmata (Figs. 57 and 58). Most of the time, there was only one nucleus in each basidiospore (Fig. 59). Four basidiospores were then formed and each received one nucleus. Sometimes another division occurred in the basidiospore and this resulted in a binucleate basidiospore (Fig. 60 and 61).

Both DAPI and Giemsa stained the nuclei effectively, but no nuclear-like particles were seen.

PLATE 7. Nuclei in subhymenial basal cells and nuclear behavior in basidia of *I. tomentosus* (isolates ZY104 and ZY105) with Giemsa staining. Bar = 6.7 μm.

Fig. 52. Different focal planes to indicate the late metaphase II nuclei (arrows).

Fig. 53. Different focus planes to indicate the anaphase II of meiosis. Notice the division plane (arrows) is oblique to the longitudinal axis of the basidiium.

Fig. 54. Telephase II of meiosis.

Fig. 55. Interphase II nuclei (arrow) after completion of division II meiosis.

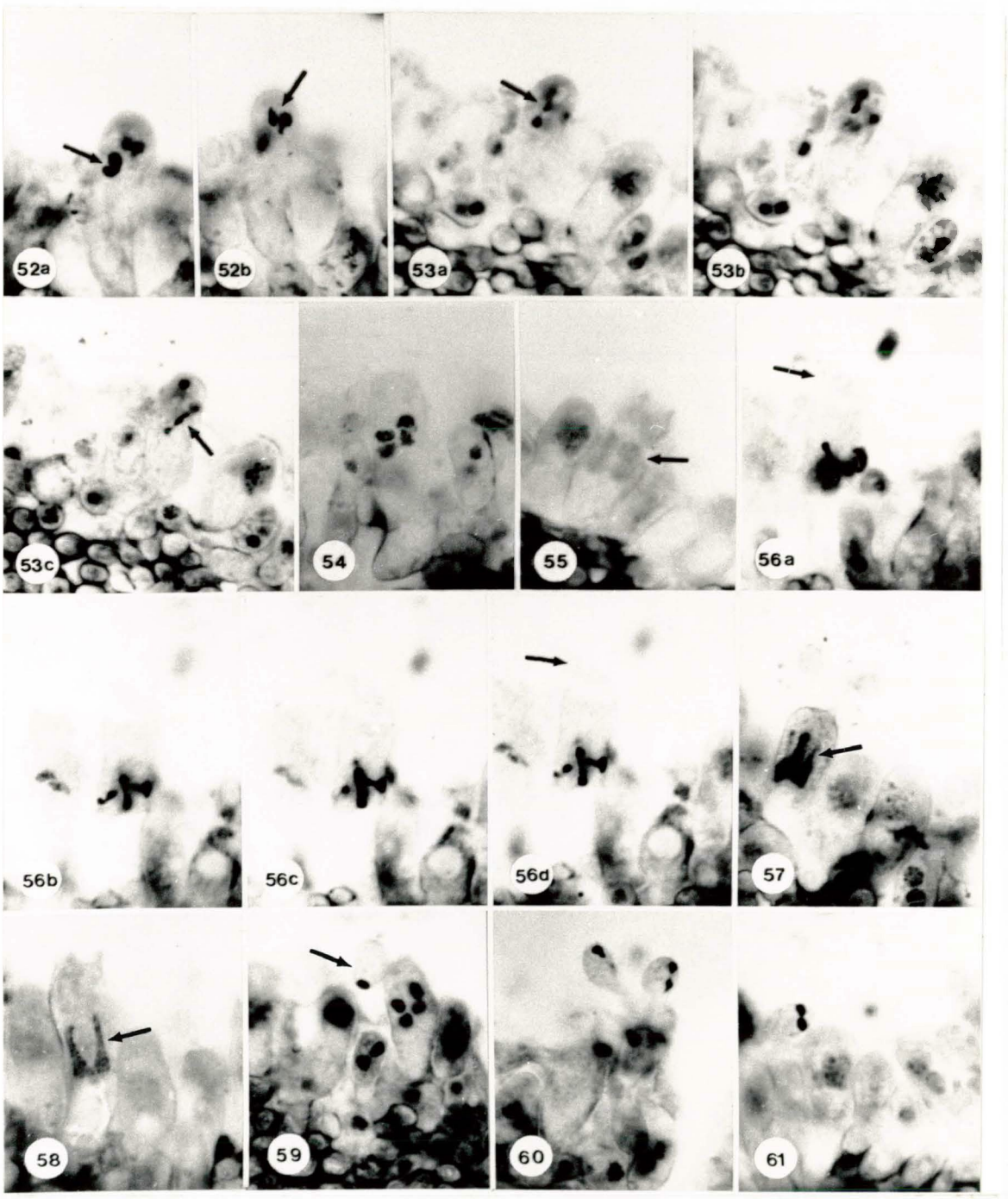
Fig. 56. Different focus planes to show a post-meiotic mitosis occuring in a basidium. Arrow in a, indicated a sterigma and arrow in d, indicated a basidiospore.

Figs. 57 and 58. Nuclei(arrows) were migrating into the basidiospores.

Fig. 59. Uninucleate basidiospore(arrow) was produced on basidium.

Fig. 60. Sometimes another division occurred in basidiospore.

Fig. 61. Binucleate basidiospore was then produced on basidium.


52a
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ELECTRON MICROSCOPY OBSERVATIONS

Chlamydospores' Hyphae

Ultrastructural studies showed vesiculated osmiophilic bodies in hyphae that originated from chlamydospores (Figs. 62-64). Most of the time these bodies were associated with lomasome configurations associated with the cell wall (Figs. 62-64). Other membranes and tubular structures usually associated with or distributed within these lomasome or multivesicle areas (Figs. 63, 64 and 65) were seen. Some electron-opaque particles were found in the vesicles (Fig. 65). Numerous vesicles were spread throughout the hyphal tip (Fig. 66).

Mitochondria were found randomly in hyphae. The internal structure of most of the mitochondria in hyphae was atypical in morphology in that the cristae were absent or poorly developed (Figs. 62, 64, 67, and 68). Some electron-dense bodies similar to 'bacterial cells' or bacteria-like structures (BLS) described by Wilson and Hanton (1979) were found in cytoplasm or inside mitochondria (Figs. 62, 64, 66-68) as were early stages of this kind of structures (Figs. 64, 68 and 69). Sometimes these latter particles were found adjacent to or inside the vesicles (Figs. 64 and 69). Some electron-dense multimembrane structures were found in vacuole or within lomasomes under the cell wall (Fig. 67 and 69).

Nuclei contained electron-dense nucleoli (Fig. 69) adjacent to the nuclear envelope. Sometimes the nuclei were elongated extensively (Fig. 69). The nuclear envelope in many nuclei was disrupted (Fig. 67 and 69) and sometimes some cytoplasmic structures were associated with

PLATE 8. TEM observations of chlamydospores hyphae (101I and 101H).

Fig. 62. Hyphae with osmiophilic bodies (Ob) associated with vacuoles. Bacterial-like structures (BLS)(double arrow) were found in an atypical mitochondrion (m). Bar = 0.6 µm.

Fig. 63. Osmiophilic bodies (Ob) associated with lomasomes under the cell wall. m, mitochondrion. Bar = 0.5 µm.

Fig. 64. Tubular structures with lomasomes associated with osmiophilic bodies (Ob) . Mitochondria (m) were with poorly developed cristae. BLS (double arrow) and their early stage structures (single arrow) were associated with mitochondria. L = lomasomes. Bar = 0.75 µm.

Fig. 65. Multivesiclular body with tubular structures. Some electron-dense particles same as the early stage of BLS were found in vesicles. L = lomasomes. Bar = 0.5 µm.

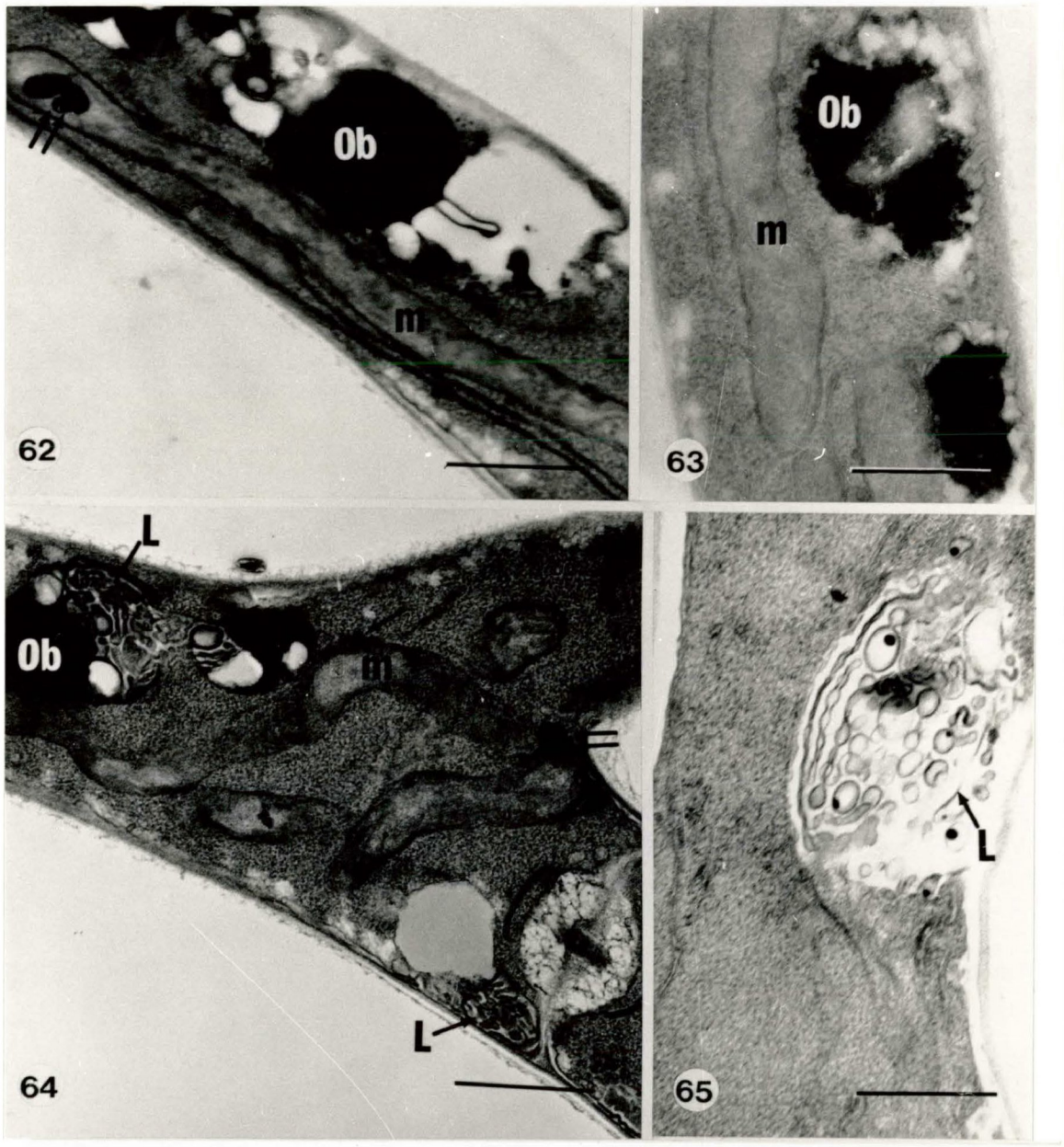
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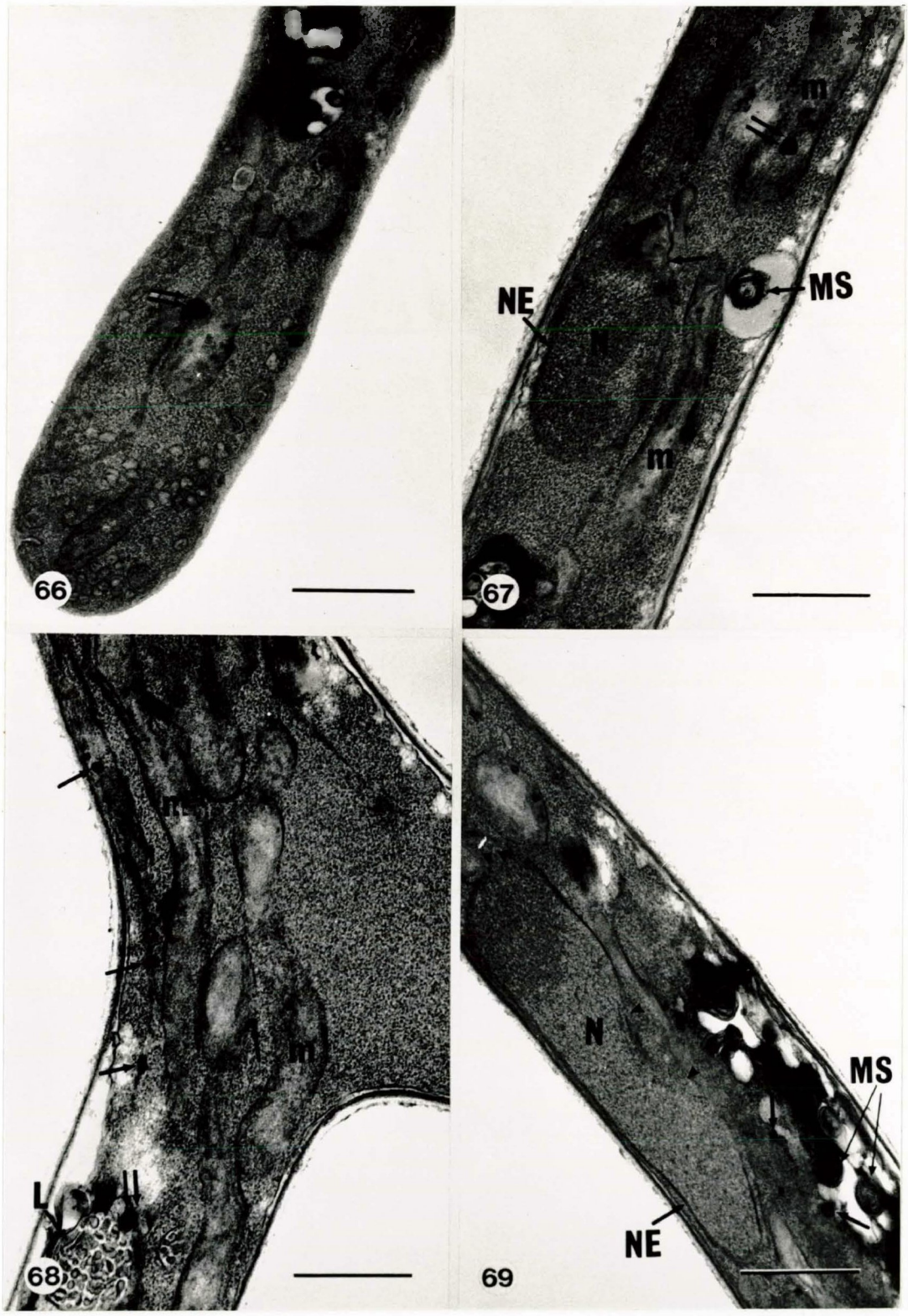
PLATE 9. Ultrastructures of chlamydospores' hyphae (101I and 101H) on TEM.

Fig. 66. Hyphal tip full of vesicles. A BLS (double arrow) was found in this area. Bar = 0.74 μm.

Fig. 67. Nuclear envelope (NE) was unclear and some cytoplasmic structures (arrow) were associated with nucleus (N). BLS (double arrow) was found in a mitochondrion and electron dense multimembrane structure (MS) was found in vacuole. Bar = 0.74 μm.

Fig. 68. Atypical mitochondria (m) in the hyphae. BLS (double arrow) was associated with multivesicle body and young structures (single arrows) of BLS were found in cytoplasm. L = lomasomes. Bar = 0.74 μm.

Fig. 69. Nucleus (N) was with electron-dense nucleolus. Nuclear envelope (NE) was abrupt in some part (arrow heads). Electron-dense multimembrane structures (MS) were associated with lomasomes under the cell wall and young structures of BLS (arrows) were found in this area. Bar = 0.74 μm.


66
NE
MS
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MS
NE
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the nucleus (Fig. 67).

Basidiospores Germlings

Vacuoles and prokaryotic-like organisms (PLOs) were noteworthy characteristics of these hyphae. The PLOs have a double complex membrane with a fibrillar network that varied from complete to empty and disintegrating. The PLOs in these hyphae were very similar to the mycoplasmalike organisms (MLOs) reported elsewhere (Schaper and Converse, 1985; Tsai et al., 1988) (Figs. 70 and 71). Tubular structures were associated with some PLOs (Figs. 71). Electron-dense bodies were found in some vacuoles and PLOs (Figs. 71 and 72). Some mitochondria were apparently partially disrupted (Figs. 71 and 72). Vesicles or multivesiclular bodies (lomasomes) usually were located close to the cell wall. Sometimes vesicles were associated with nuclei (Figs. 70 and 71). Some tubular membraneous structures were found in the cytoplasm near the septum (Fig. 73). Nuclei were typical with a double-membrane and with a nucleolus located close to the nuclear envelope (Figs. 70 and 71). Fibrillar material was seen outside some of the hyphae (Figs. 71 and 73).

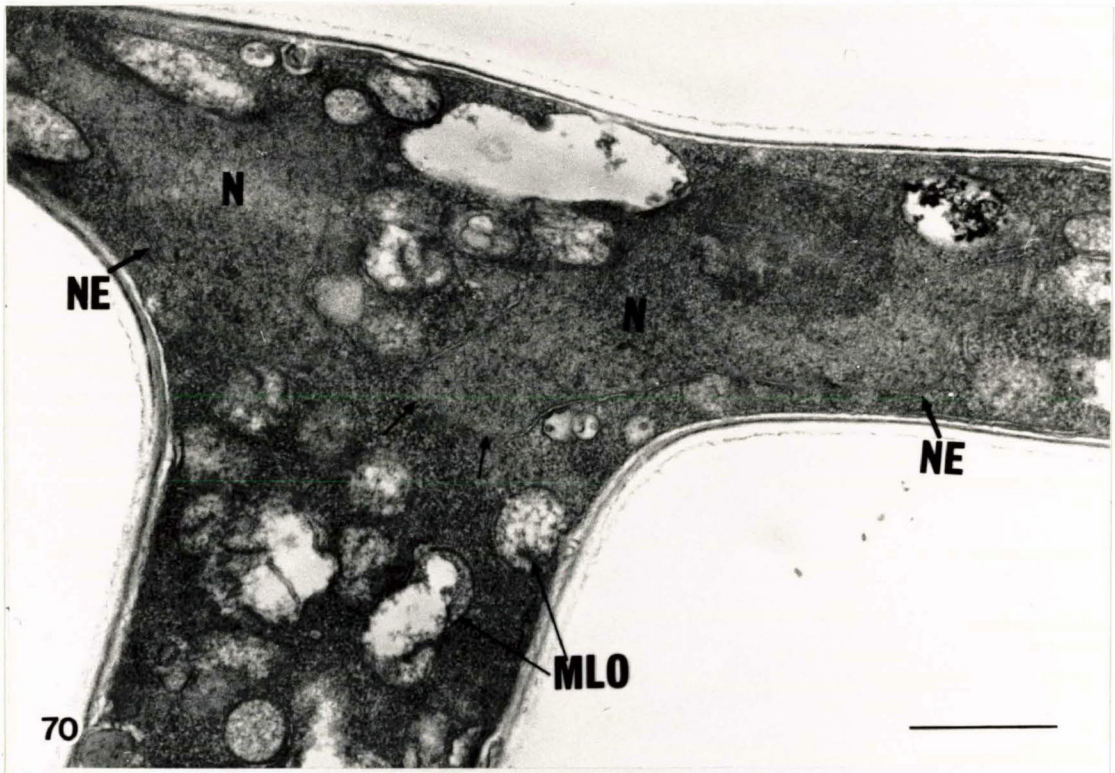
Chlamydospores

The cell wall of the chlamydospores was about 0.4 - 0.5 μm thick (Fig. 74). There were numerous vacuoles and osmiophilic bodies in the cytoplasm (Figs. 74 and 75). Some multivesicle bodies and tubular structures were associated with some osmiophilic bodies (Figs. 75 and

PLATE 10. TEM observations of basidiospores germlings (ZY104 and ZY105).

Fig. 70. Hypha showing a pair of nuclei (N). Mycoplasma-like organisms (MLOs) were distributed in the cytoplasm. Vesicle bodies were associated with nuclear envelope (NE). Some of the MLO were disintegrated. Nuclear evelope was abrupt in some parts (thin arrows). Bar = 0.7 μm.

Fig. 71. Nucleus (N) was with nuclear evelope (NE). Vesicle bodies were associated with nuclear envelope or close area (arrow heads). A mitochondrion (m) was partially destroyed. Tubular structures associated with some MLO. Electron-dense particles were found in some vacuoles. Fibrillar material was seen outside cell wall. L = lomasomes. Bar = 0.7 μm.



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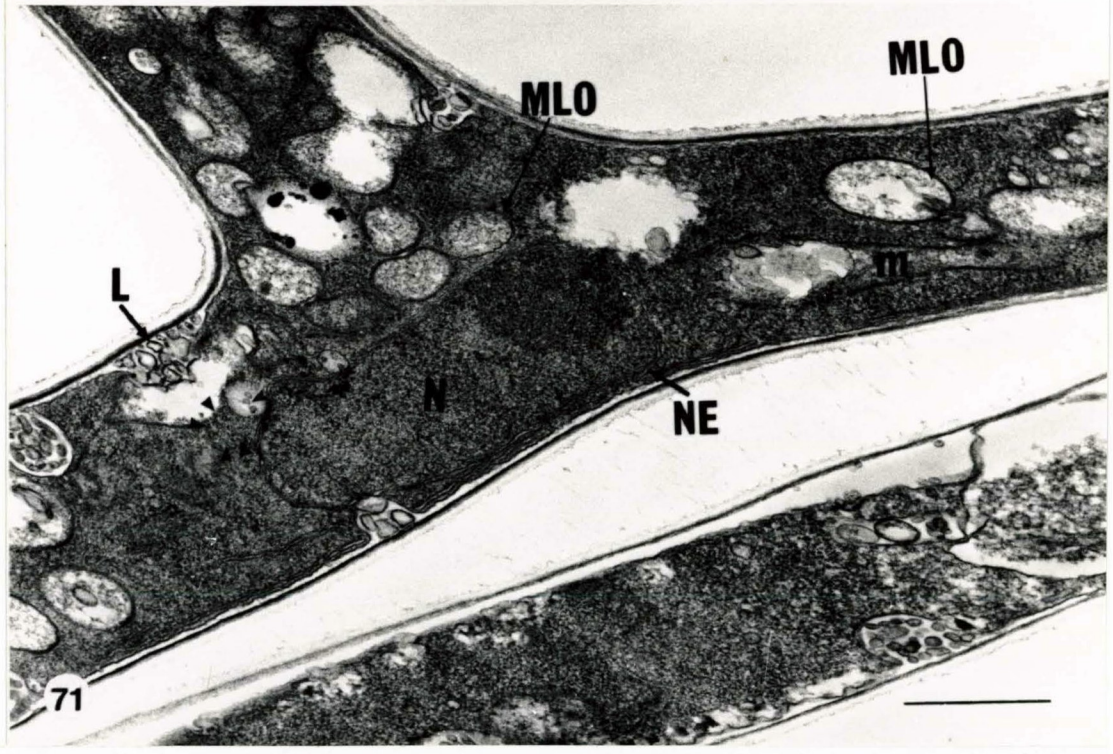

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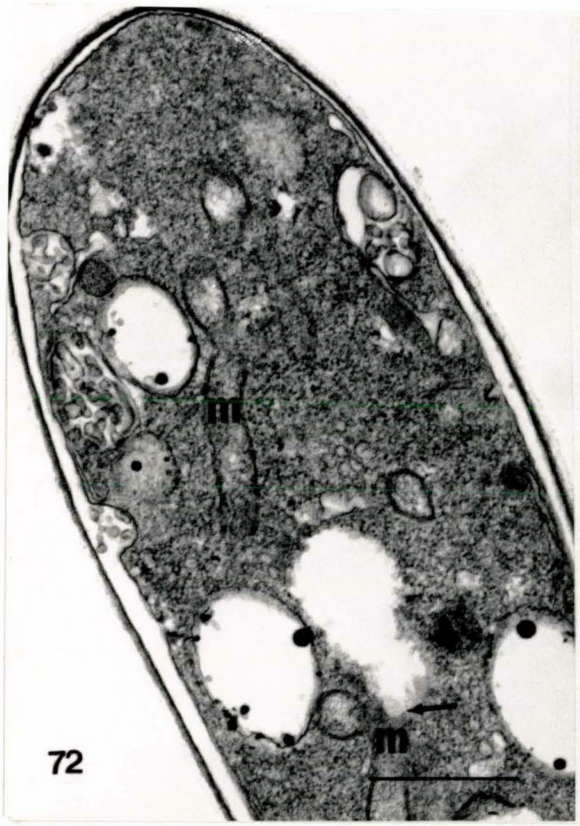
PLATE 11. TEM observations of basidiospores germlings (ZY104 and ZY105).

Fig. 72. Mitochondria (m) were atypical in morhpology and sometimes was partially disrupted (arrow). Electron dense particles were found in vacuoles. Bar = 0.7 μm.

Fig. 73. Hypha showing tubular structures (arrow) near the septum. Fibrillar material was seen outside cell wall. Bar = 0.6 μm.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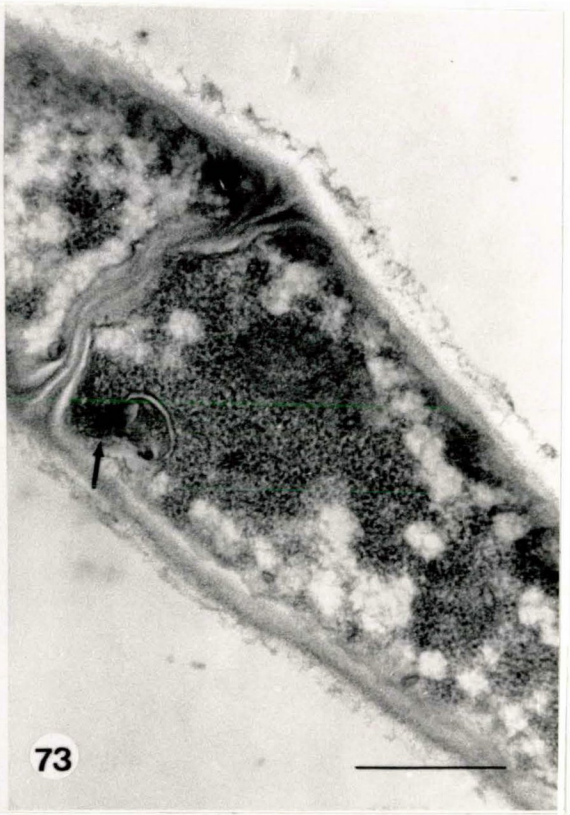
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76). These vesicles usually were rod-like in shape. Sometimes vesicles were found outside spores (Fig. 77).

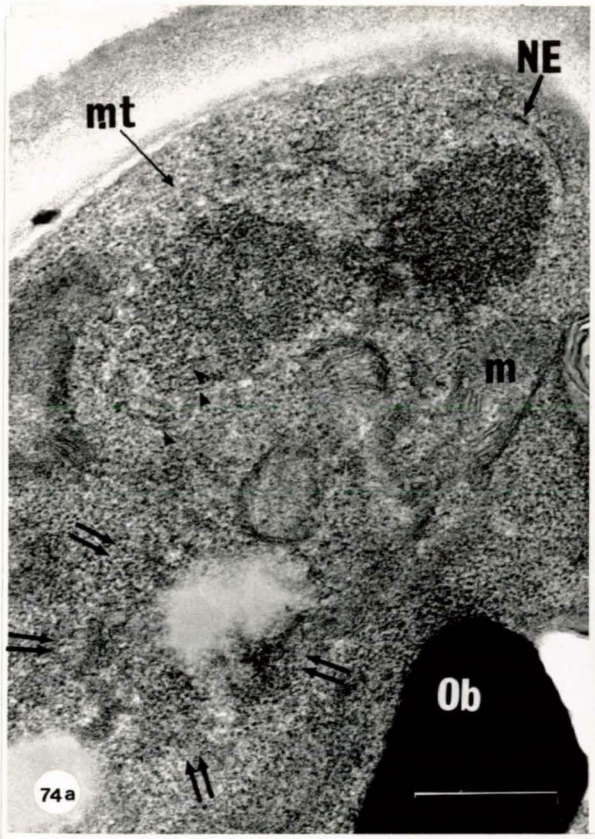
The chlamydospore germ tubes grew out through a small pore on the thick spore wall and all the organelles and nuclei migrated into germ tubes (Figs. 74, 75 and 77). The cell wall of germ tubes was initiated from the inside membrane of the spore cell wall. Some electron-opaque tubular structures were found apparently in the cell wall as well as inside the spores (Figs. 74 - 76). Typical mitochondria with clear cristae were observed in some spores (Fig. 76) whereas in others, the mitochondria structure was poorly developed (Fig. 74). Fewer vesicles were apparent in comparison to hyphae. Fibrillar material was found outside cell wall (Fig. 74 and 77).

Most of the chlamydospores contained nuclei with mostly discontinuous nuclear envelopes. Although the boundary between the nucleus and cytoplasm was unclear (Figs. 74, 75 and 78), a nucleus looked intact during its migration into the germ tube (Fig. 75). Fig. 74b shows a nucleus, at the opposite end of a germ tube in a spore, with two areas of nucleolar material with a constriction. In this spore the nuclear envelope is barely evident. Some 'V'-like structures were noticed in the nucleus. Electron-dense granules were found in the cytoplasm. It is difficult to notice the microtubules in most chlamydospores except in one case (Fig. 74). No spindle and spindle pole body were found in the division.

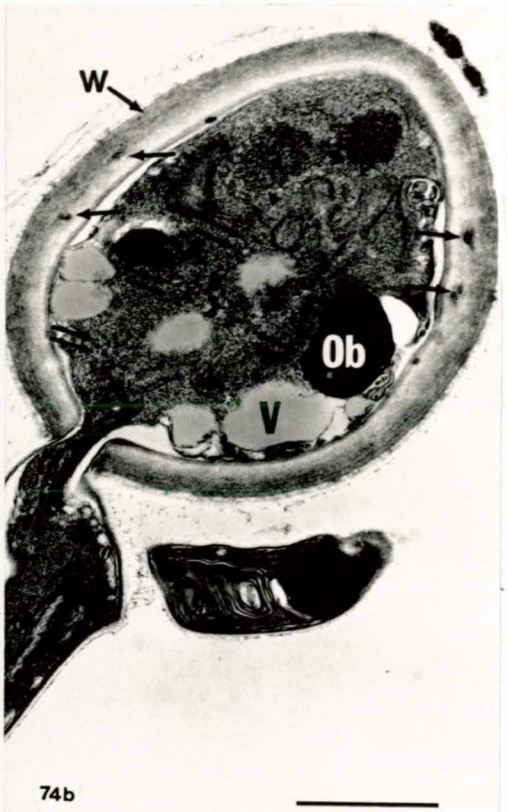
PLATE 12. TEM observations of chlamydospores (101I).

Fig. 74. A germinated chlamydospore with two areas of nucleolar material with a constriction. Nuclear envelope (NE) was completely destroyed. Mitochondria (m) were poorly developed. Electron dense granules (double arrow area) were found in cytoplasm to form a certain pattern from the nucleus to the germ tube. A microtubule (mt) was found close to the nucleus. Some 'V'-like structures (arrow heads) were found in nucleus. Electron-opaque tubular structures (arrows) were noticed in the cell wall. Ob = osmiophilic body; V = lipid; W = cell wall. a, higher magnification of nucleus area. Bar = 0.6 µm. b, overview of the spore. Bar = 1.3 µm.

Fig. 75. A germinated chlamydospore showing nuclear migration into germ tube. Nuclear envelope (NE) was disrupted. Electron-opaque tubular structures (arrows) were found inside the cell as well as in the cell wall. N = nucleus; Ob = osmiophilic body; V =lipid; W = cell wall. Bar = 1.0 µm.



74a



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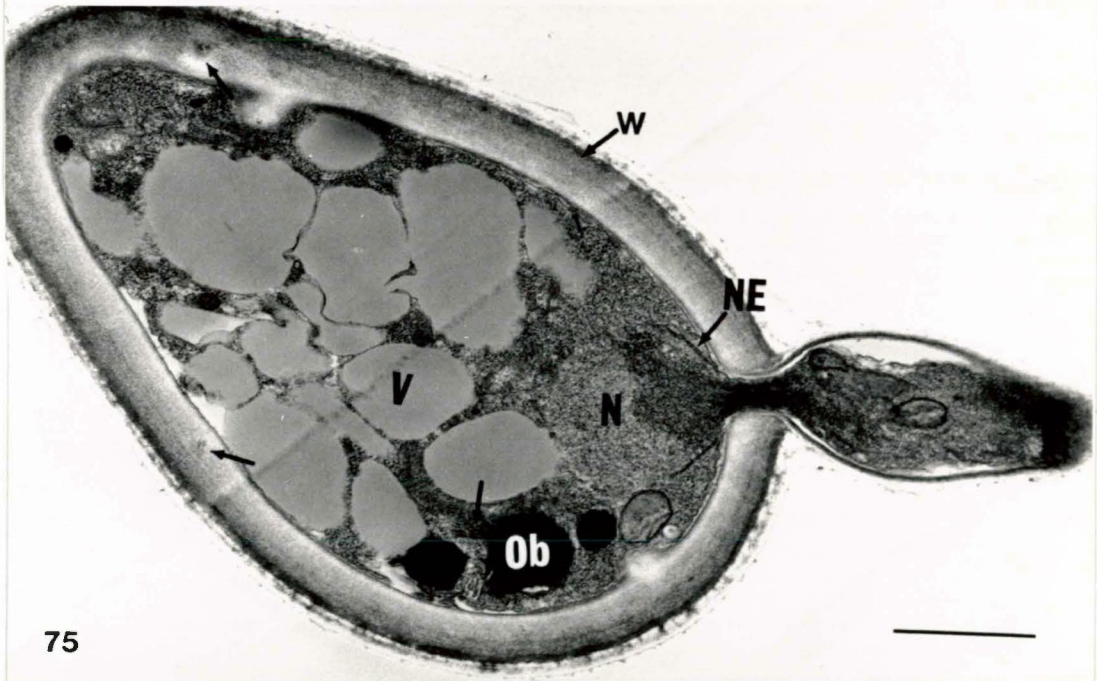

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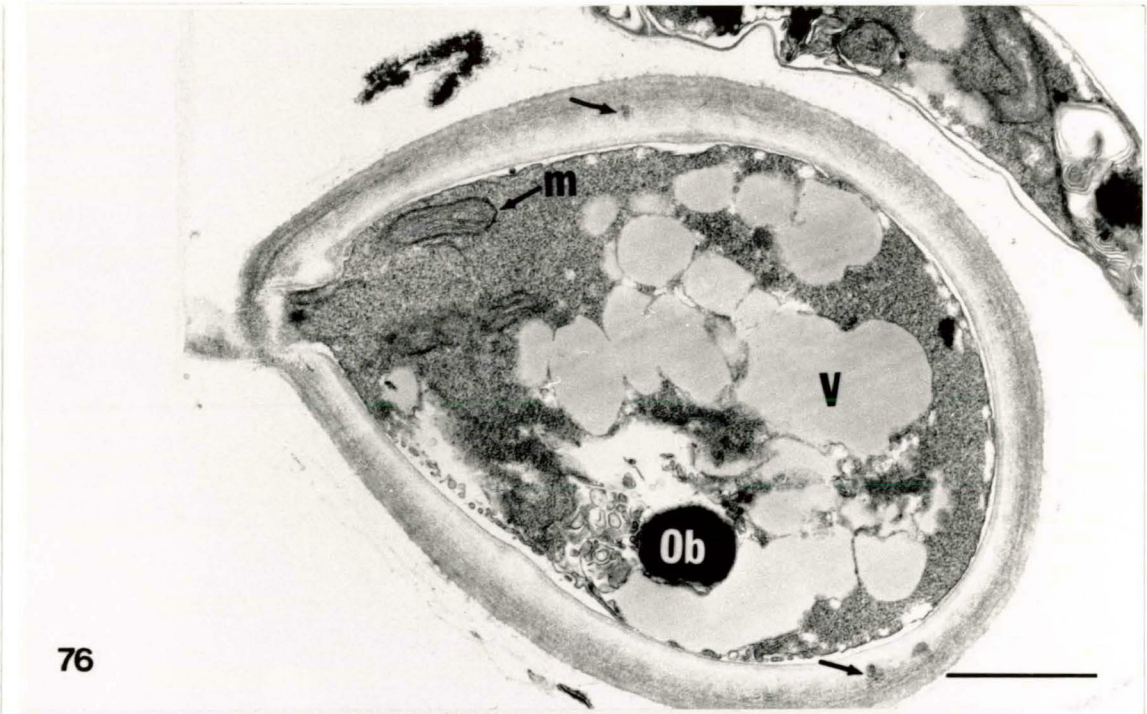
PLATE 13. TEM observations of chlamydospores (101I and 101J).

Fig. 76. A germinated spore showing a well developed mitochondrion (m). Electron-opaque tubular structures (arrows) were found in the cell wall. Ob = osmiophilic body; V = lipid. Bar = 1.0 μm.

Fig. 77. A germinated spore showing all the cytoplasmic material migrated into the germ tube. Some vesicles (arrows) were found outside the spore as well as inside spore. Ob = osmiophilic body; V = lipid. Bar = 1.2 μm.

Fig. 78. A chlamydospore showing a nucleus (N). Again nuclear envelope (NE) was scarcely apparent. m = mitochondrion; V = lipid. Bar = 0.67 μm.



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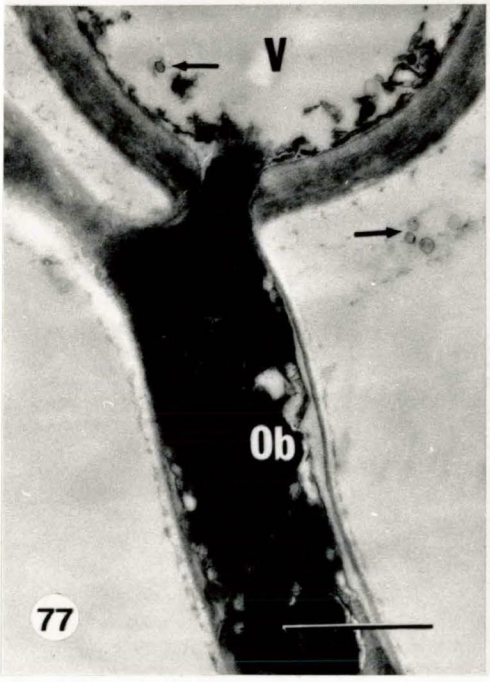

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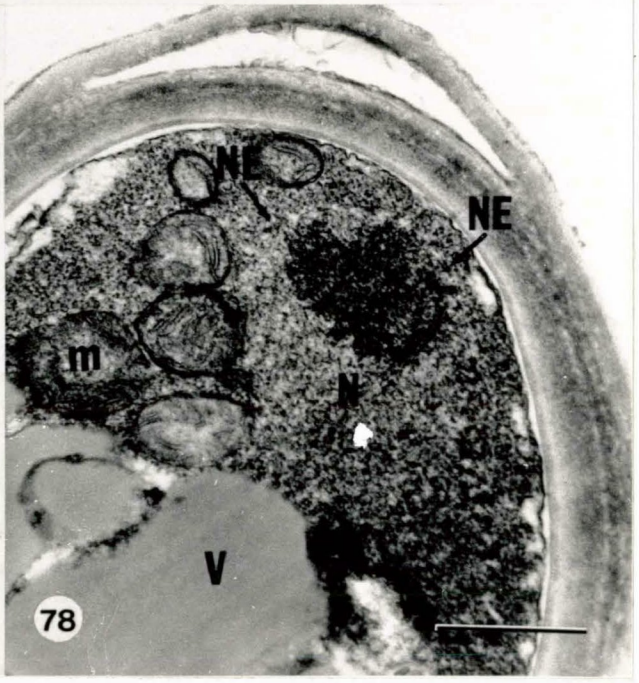


78

Basidioma

a. Transmission electron microscopic (TEM) observations

Transmission electron microscopic studies were made of the hymenial layer of basidioma collected in nature.

Mitochondria with atypical ultrastructural morphology were found in basidia (Figs. 79 and 81). Vacuoles sometimes were found with electron-dense inclusions (Fig. 79). Numerous lipid bodies were found at the base of basidia. Some vesicles and multivesiculate bodies were seen in some basidia. Basidia sometimes were initiated from within a cup-like cell wall (Fig. 80). The nuclear envelope of most nuclei was incomplete. Discontinuous nuclear envelopes were found in prefusion nuclei (Fig. 79), the fusion nucleus (Figs. 79 and 80), and postmeiotic nuclei (Fig. 81). Spindle pole bodies were observed on the nuclear envelope, but microtubules between SPBs or elsewhere were conspicuously absent (Fig. 82). Typical division figures of meiosis were unobserved.

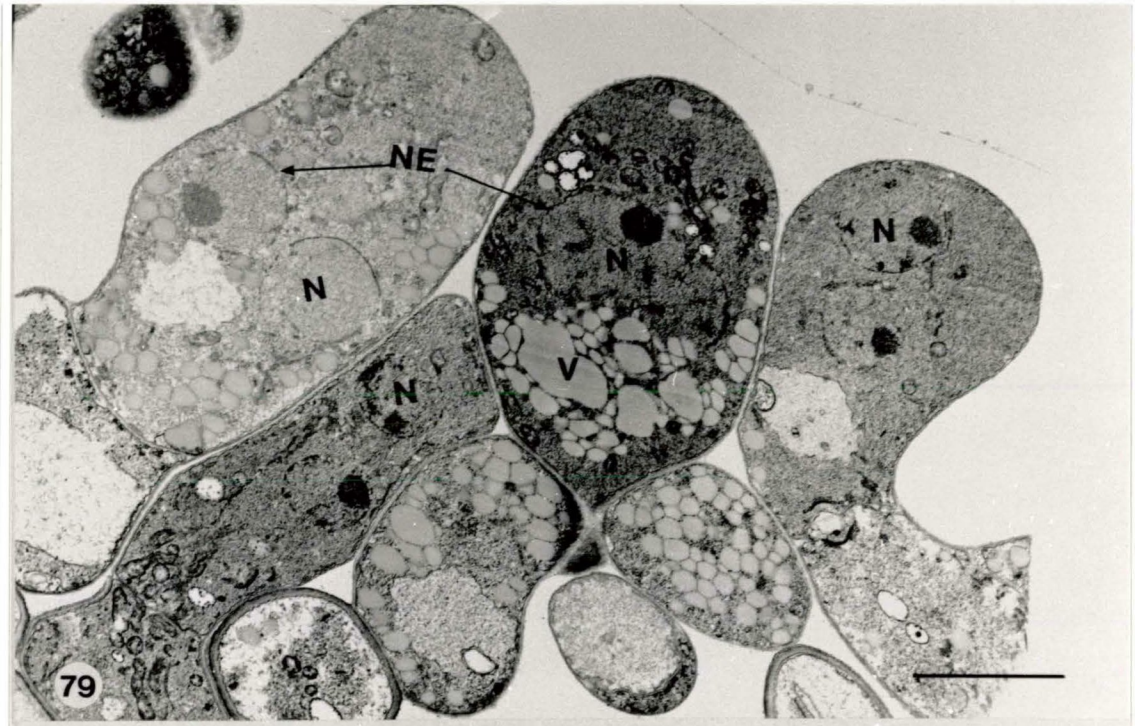
b. Scanning electron microscopic (SEM) observations

Young basidia were initiated as lateral protuberances from subhymenial hyphae. As hyphae grew forward, new basidia were initiated one after another on the same hypha (Fig. 83). The terminal hyphae and young basidia looked smooth on the surface and no outer

PLATE 14. TEM observations of basidia (ZY104 and ZY105).

Fig. 79. Young basidia with prefusion and fusion nuclei (N) in interphase and incomplete nuclear envelope (NE). m = mitochondrion; V = lipid. Bar = 2.3 μm.

Fig. 80. A basidium was initiated from a cup-like cell wall (double arrow). The fusion nucleus (N) was with a incomplete nuclear envelope (NE). Bar = 0.6 μm.


NE
N
N
N
N
V
79

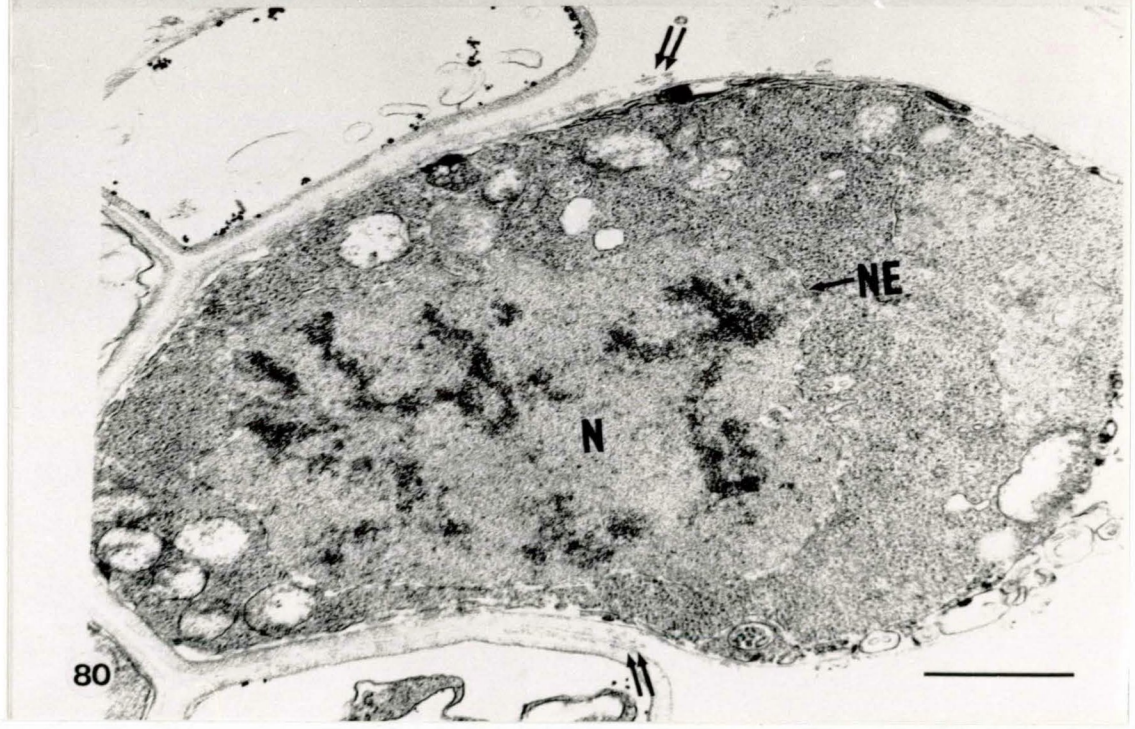
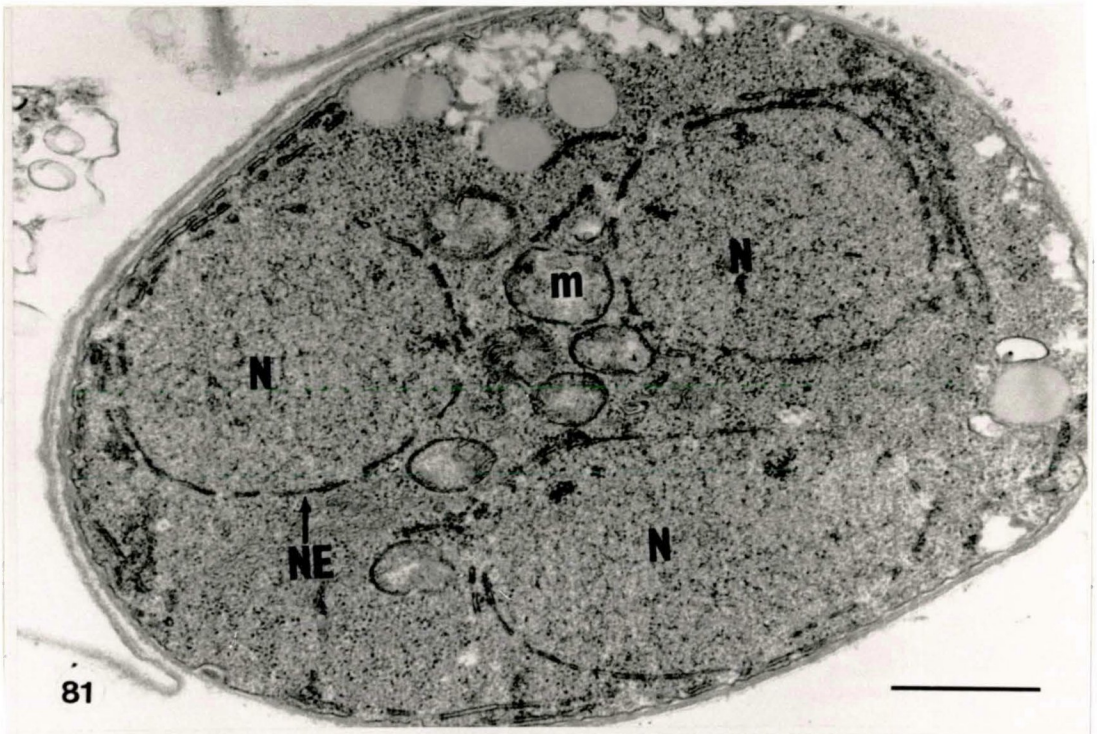

NE
N
80

PLATE 15. TEM observations of basidia (ZY104 and ZY105).

Fig. 81. Postmeiotic interphase nuclei (N) in interphase with incomplete nuclear envelope (NE). Mitochondria (m) with poorly developed cristae. Bar = 0.6 μm.

Fig. 82. A nucleus (N) with spindle pole bodies (SPBs) adjacent to the nuclear envelope (NE). Microtubules were unobserved. L = lomasomes. Bar = 0.85 μm.



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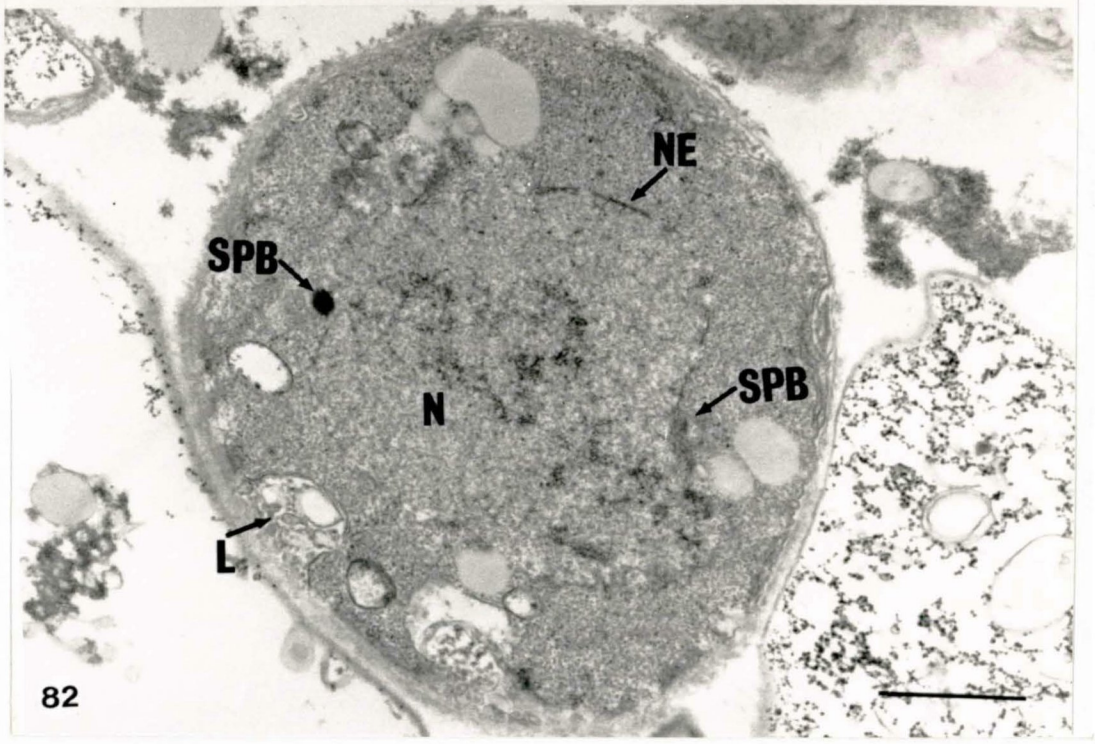


82

cup-like wall was seen. Basidia were closely arranged to form a hymenial layer on the inside pore surface of the fruiting body (Fig. 84). Most of the basidia were tetrasporic in nature (Fig. 85). Sometimes trisporic basidia were found (Fig. 86), but they occurred less than 1% of the time. Mature basidiospores were ovoid and 2.5x4.5 µm in size (Fig. 85). For some basidia, sterigmata, and basidiospores in some areas of the hymenium, there were numerous surface globules of various sizes (Fig. 87).

Setae were scattered randomly among the basidia (Fig. 84). Hyphae invariably grew up the sides of the setae (Figs. 84, 88-90) and in a few cases seemed to penetrate into the setae (Fig. 88). The hyphae and setae were linked by hair-like fibrils (Fig. 89). Sometimes a skin-like layer was found surrounding the basidia in some areas or holes from presumably collapsed basidia (Fig. 90).

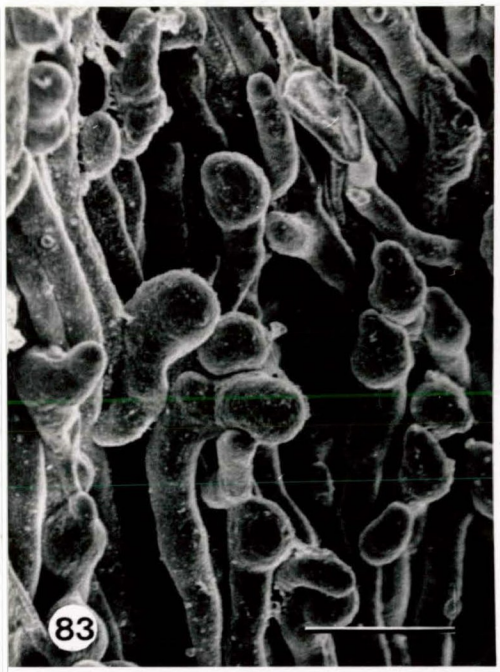
PLATE 16. SEM observations of fruiting bodies (ZY104 and ZY105).

Fig. 83. Subhymenial hyphae showed the initiation of young basidia (arrows). Bar = 7 μm.

Fig. 84. Inside surface of a pore of fruiting body showed the hymenial layer of basidia. Arrows identify setae. Bar = 33 um.

Fig. 85. Top view of a basidium that bears four basidiospores (arrows). Bar = 3 μm.

Fig. 86. A basidium with three sterigmata (arrows) was noticed here and the other one below it was with four sterigmata. Bar = 2.4 μm.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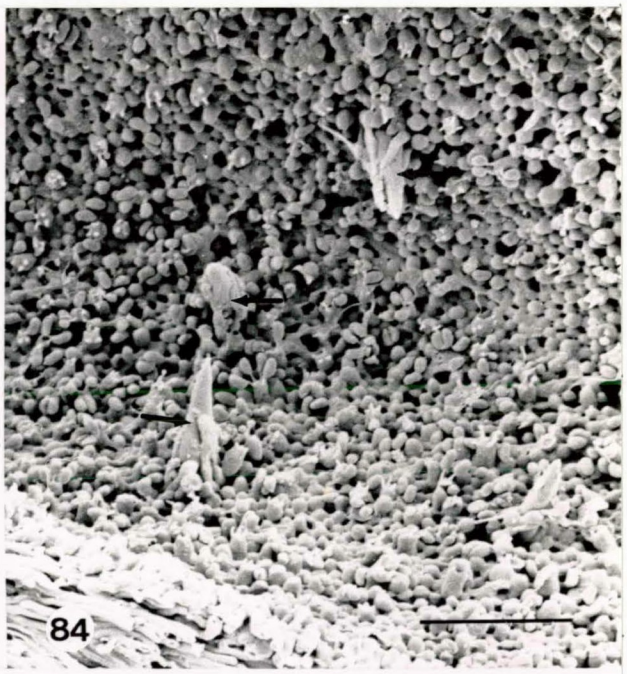
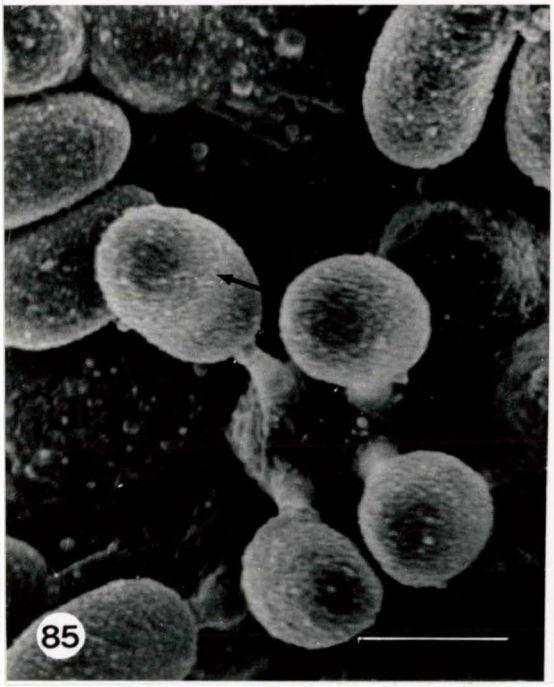

84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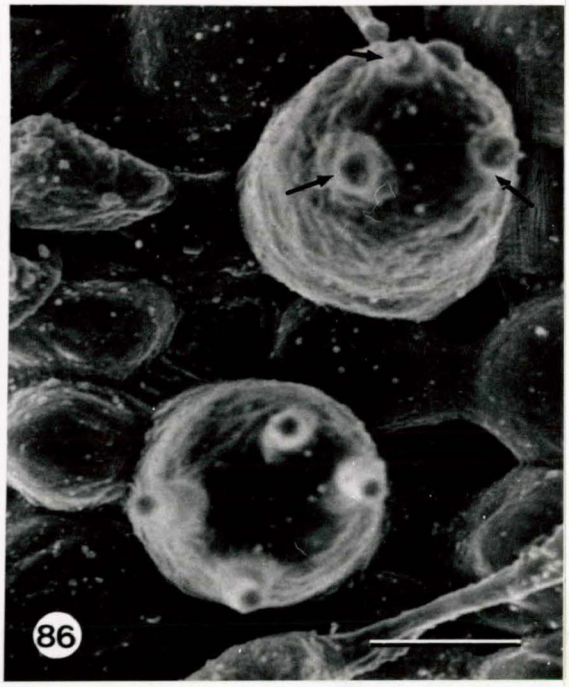

86

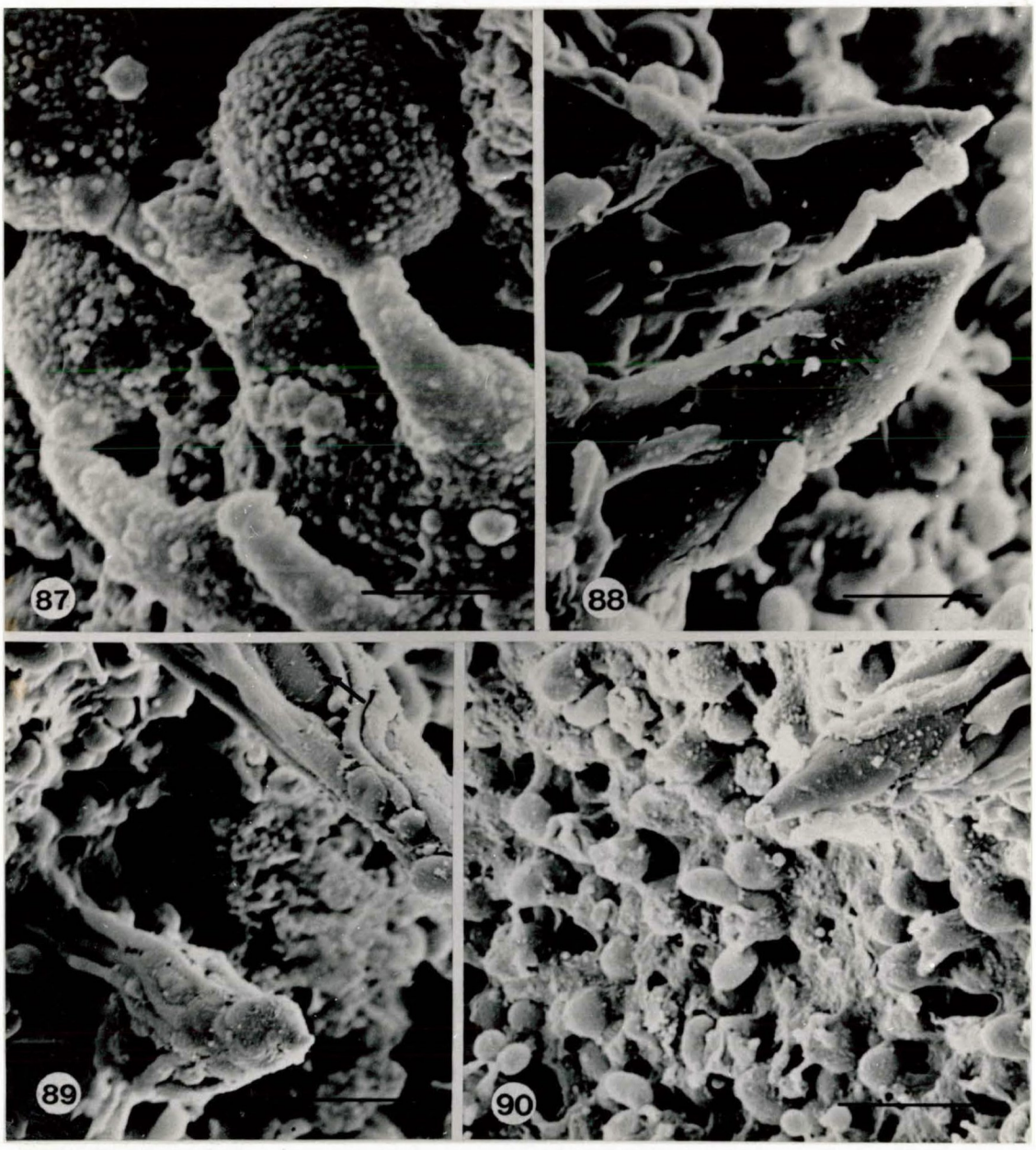
PLATE 17. SEM observations of fruiting bodies (ZY104 and ZY105).

Fig. 87. Numerous surface globules of various size were found in some basidia and basidiospores. Bar = 1.1 μm.

Fig. 88. Some hyphae that grew on the setae seemed to penetrate into the setae (arrows). Bar = 1.6 μm.

Fig. 89. Some hair-like fibrils (arrows) were noticed between the setae and hyphae. Bar = 8.4 μm.

Fig. 90. A skin-like layer was found surrounding the basidia in some areas and holes were from presumably collapsed basidia. Bar = 9.6 μm.


87
88
89
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DISCUSSION

NUCLEAR BEHAVIOR - LIGHT MICROSCOPY

The presence of multinucleate hyphae for some basidiomycetes has been noted frequently, but there are apparently no reports of a grouping tendancy among nuclei in the vegetative hyphae in the Hymenochaetaceae or other Basidiomycetes. Why these non-random groups of nuclei form is unknown, but they may represent a dikaryotic equivalent. Patterns of this kind were not reported for *Phellinus weirii* (Hanson, 1979). However in basidioma of *I. tomentosus* a definite mechanism exists that segregates the multinucleate hyphae into groups. By the time the nuclei reach the subhymenium most of the cells were binucleate so that whether the two or three nuclei in the subhymenial cells represent an equivalent condition to groups of 3-6 nuclei in vegetative hyphae is unknown, but it possible that while the dikaryotic equivalent requirement is met, other deficiencies may occur and these may explain the tendency for low germination rates. Table 1 indicated that the frequency of single nucleus was similar to that of groups with two nuclei. This result supports the idea that these groups were originally the group with three nuclei and that later, one of the nuclei becomes separated from the other two.

The pattern of nuclei and particles in chlamydospores was the same as that observed in vegetative hyphae. The number of nuclei varied in each chlamydospore (the most common number was two or three nuclei per spore). Chlamydospores of *I. tomentosus* probably serve as resting spores capable of surviving unfavorable environmental

conditions with a full nuclear complement, although they have never been found in nature.

Patterns of meiosis in *I. tomentosus* were similar in many ways to those found in other basidiomycetes (Baidwan, 1987; McClaren, 1967; Setliff et al., 1974; Wells, 1978) except meiotic and post meiotic divisions were oriented in oblique manner and although SPBs were observed both with light and electron microscopy, spindle microtubules were conspicuously absent. Basidial development was asynchronous.

Also, post-meiotic mitosis was irregular in this fungus. It is difficult to understand why the third division sometimes happened in basidiospores and sometimes in the basidium. The low percentage (6%) of binucleate basidiospores indicated that the third division (mitosis) seldom occurred in the basidiospores. It is unclear if a third division occurred either in the basidia or in the basidiospores.

Another unusual aspect in some hyphae and chlamydospores was the absence of typical nuclei. Rather, there was DNA material (DAPI positive particles) that appeared as mostly small particles. The relationship between DAPI positive particles and nuclei in hyphae is not known. However the particles only existed in a certain number of cells and the patterns of nuclei and particles have been determined the same both in Giemsa and DAPI staining. Vegetative hyphae both from basidiospores and chlamydospores indicated the same results with Giemsa and DAPI staining. All of the isolates of 101H, 101I, 101J, and vegetative hyphae from basidiospores of ZY104 and ZY105 indicated the same results of nuclear patterns.

ULTRASTRUCTURE OF *I. TOMENTOSUS*

Breaks in the nuclear envelope were a common and unexpected feature in *I. tomentosus.* The electron-dense granules in chlamydospore (Fig. 74) may be nuclear-like material released in cytoplasm, but more studies are required in relating the light and TEM observations.

Both typical and atypical mitochondria were found in chlamydospores whereas mostly atypical mitochondria were found in hyphae and the basidia. The reason for this phenomenon is unclear. However the finding of BLS, MLOs, and tubular structures in hyphae and chlamydospores leads me to think of a possible relationship between this atypical mitochondrial ultrastructure and these PLOs. However a fixation artifact of preparing the specimens probably could result in poorly preserved mitochondria, along with the disruption of nuclear envelopes. Osmophilic organelles associated with lomasomes and vesicles in chlamydospores and hyphae from chlamydospores is another unusual structure found in this fungus.

The warty globular material on the spores and basidia (Fig. 87) may represent mycoplasmas-like-structures. Another interesting phenomenon was the skin-like layer surrounding the basidia in some areas. The relationship, if any between the warty globular material on spores and basidia and the skin-like layer is unknown.

DAPI-POSTIVE PARTICLES

The DAPI-positive particles (DPPs) were found in vegetative hyphae, germlings, and hyphae that originated from both chlamydospores and basidiospores. The particles are similar to those seen in studies made of *Rigidosporus vinctus* (Setliff, unpublished) and *Flammulina velutipes* (Curt.: Fr.) Sing. (Baidwan, 1987). DNase enzyme tests confirmed that these particles were DNA. Although some DNase treated hyphae showed some pale yellow particles, they were found in intact hyphae cells. The DNase invariably worked with ruptured hyphal tips and so the chemical nature of the cell walls of this species may have slowed down the enzyme's penetration into the cells. In order to achieve adequate penetration of DNase into the yeast cells Williamson and Fennell (1975) used 1M NaOH to digest the cell wall prior to the DNase treatment. They are obviously not nuclei as based on the pattern and sizes, but they may represent end products of nuclear degeneration.

Another possibility is that DPPs represent mitochondrial DNA (mtDNA), such as those reported in yeasts (Stevens, 1981; Williamson and Fennell, 1975, 1979). However, the following evidence argues against this supposition. For instance, DPPs were only found sporadically in only certain number of vegetative hyphal cells, chlamydospores, and germinated basidiospores. Mitochondria existed in all the hyphal cells, chlamydospores, basidiospores, and basidia. Second, the pattern of DPPs indicated the sizes of particles varied and they sometimes accummulated in certain areas (say the two ends of a hyphal cell) heavily. Mitochondria were found throughout the cells observed with the TEM. Finally, the study of nuclear behavior and DNA

content with DAPI in algae (Coleman, 1978, 1979), higher plants (James and Jope, 1978), and different fungi (Allen *et al.*, 1988; Cooke et al., 1987; Digby and Goos, 1987; Martin, 1987; Panwar *et al.*, 1979; Runberg and Raudaskoski, 1986) except in the yeast *Saccharomyces cerevisiae* (Stevens, 1981; Williamson and Fennell, 1975, 1979) and in the plasmodium of *Physarum polycephalum* (Nishibahashi et al., 1987) failed to show mitochondria with mtDNA. Coleman (1979) suggested that yeast mtDNA may be visible only because of its extremely higher AT content. Also most of the DPPs in *I. tomentosus* showed yellow fluorescent color here and mtDNA that observed in the plasmodium of *Physarum polycephalum* showed 'brilliant-blue' color with DAPI staining (Nishibahashi et al., 1987).

The observation of yellow and blue nuclear materials associated with bacteria suggests the possibility of chromatin transfer or an infective agent capable of infecting both bacteria and fungi. Membrane systems apparently in the cell wall, the occurrence of vesicles, and unknown globules outside the cells become more important.

DPPs have been observed in HeLa cells deliberately infected with mycoplasmas and vaccina viruses (Russell et al., 1975), in the phloem of diseased apple shoot caused by mycoplasmalike organisms (MLO) (Seemüller, 1988), and in blueberry cultivars with detectable MLO (Schaper and Converse, 1985). Virulence of pathogenic bacteria such as *Agrobacterium tumefaciens* and *A. rhizogenes* is conferred by plasmids. The T-DNA of plasmid is inserted into the plants and is later incorporated into the host plant nucleus (Chilton et al. ,

1982). The origin of DPPs, their ultrastructural identity and the role they play in fungi and bacteria is unknown at present.

The idea that viruses and plasmids occur in fungi is widely accepted and studies of bacteria-like structures (BLS) in fungi have been reviewed by Wilson and Hanton (1979). They considered, that 'although the work done so far on BLS in fungi hardly justifies calling it a field of research, the area may be a fruitful one for the study of host-parasite relationships with the pathogenic forms' (Wilson and Hanton, 1979).

There are few, if any, satisfactory light microscopic results that indicate the existence of BLS, MLO, and VLPs in fungal hyphae. Although some TEM work has shown the ultrastructure of these parasitic organisms or particles in fungi, no corresponding light microscopic results were given (Lemke, 1979; Murrin et al., 1987; Wilson and Hanton, 1979). It is difficult to compare the TEM ultrastructures with the light microscopic results here because additional comparative studies are needed.

In higher plants, some of the variagations found in leaves and flowers are related to viral infections. In studies of maize by McClintock (1947, 1949) she realized that some patterns of inheritance could only be explained by mobile gene segments in genetic studies of the corn plant. She postulated, as these elements jumped from one place to another on the chromosome, they turned genes on and off, and provided a possible explanation for the varied gene expression that occurs during development (Roberts, 1983). The function of transposable elements within the cells and their possible role in

development and evolution still remain mysterious (Roberts, 1983). Rose and Doolittle (1983) found some sequences of DNA perform no function in the organism and yet spread through populations. The discovery of these transposable elements - 'parasitic DNA' could be thought of as viruses inside genomes (Rose and Doolittle, 1983). McClintock (1950) discovered transposable elements in the progeny of self-pollinated plants that had undergone a cycle of chromosome breakage, joining of broken ends, and rebreakage (McClintock, 1939, 1942). Transposable elements are believed to exist in an inactive form within the 'normal' maize genome and can be released under unusual circumstances (Peschke et al., 1987).

It is worthwhile to think of the possible relation between the transposable elements and the DPPs found in both a fungus and bacterium in this study. The concept of so-called "horizontal transfer" of genes and the exchanges of genetic factors at this macromolecular level have been considered in the study on *Aporpium* (Setliff, 1983). If there is genetic mobility between these two organisms that can be studied by differential coloration patterns, then this development will lead to a better understanding of evolutionary biology and 'horizontal' gene transfer. Undoubtedly, a better understanding of the nature of DPPs in pathogenic higher fungi is an important next step.

Considering a hyphal cell as a microscopic and molecular ecosystem, one should not ignore the possible involvement of mtDNA in the DPPs. The failure to observe mtDNA with DAPI staining is in itself a question that requires resolution. It is known that mtDNA

can travel within a cell and can be integrated into nucleus (Wright and Cummings, 1983; Farrelly and Butow, 1983). Fungal viruses and extrachromosomal elements in fungi, i.e. DNA plasmids and mtDNA have been found to be intermediates in the movement of transposable elements around the yeast genome (Buck, 1986).

Finally, most of pathogenic basidiomycetes have unusual nuclear life cycles. Therefore the importance of understanding the DPPs of this fungus, may apply to other pathogenic basidiomycetes. Additional studies on the origin and function of DPPs and their relationships with other cytoplasmic organelles are needed in order to explain the basic nature of pathogenic microbes.

SUMMARY

The irregularities in the nuclear life cycle of *I. tomentosus* have been revealed in the study. The mycelia were multinucleate and the nuclei were found in groups of two to six per group. Chlamydospores were multinucleate and most freshly shed basidiospores were uninucleate. Binucleate basidiospores were occasionally observed. DAPI-positive particles (DPPs) were observed in some hyphal cells, germ tubes, and chlamydospores. These DNA particles fluoresced a yellow colour whereas nuclei were pale blue. Similar observations of nuclear-like material were made on one occasion with bacteria associated with hyphae. The nature and function of the DPPs are unclear.

The Giemsa/HCl technique was used to study nuclear behaviour in the hymenium and subhymenium of basidioma that were collected from nature. The subhymenial cells were binucleate, as were young basidia. Patterns of meiosis in this fungus were similar in many ways to those found in other basidiomycetes, but meiotic and post meiotic divisions were obliquely oriented in basidia. Although SPBs were observed with both the light and electron microscopes, spindle microtubules were not observed. Breaks in the nuclear envelope were a common and unexpected feature in this fungus. A postmeiotic mitosis sometimes occurred in basidia and sometimes in basidiospores.

Ultrastructural studies revealed some prokaryotic-like structures in mycelia and chlamydospores. The ultrastructure of mitochondria was mostly atypical. Warty globular material, which may represent

mycoplasma-like structure, was observed over the surface of some basidia and basidiospores.

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