ABSTRACT

THOMAS PAUL THARP. OBSERVATIONS ON HALIPHTHOROS MILFORDENSIS, A MARINE PHYCOMYCETE. (Under the direction of Dr. Charles E. Bland) Department of Biology, May, 1976.

Light and electron microscope observations were made of the developmental morphology, life cycle, and host range of a strain of Haliphthoros milfordensis isolated from the white shrimp, Penaeus setiferus. A detailed study of each life cycle stage was conducted with the light microscope, however, ultrastructural analysis was limited to zoospores and the encystment process. Zoospores for this isolate, by virture of their variation in shape and sub-apically attached flagella, are not as described previously for this genus. However, they are biflagellate, therefore characteristic of the class Oomycetes. Encystment of spores begins by flagellar retraction and rounding-up, and terminates with the formation of an external, spined cyst wall. Germination is monopolar, producing germ tubes of various dimensions which fan out into hyphae. Vegetative growth is accompanied by multibranching at right angles and fragmentation. Sporogenesis is holocarpic but unusual in that thallus conversion into spores occurs in delimited segments non-simultaneously, and in that spore cleavage is sequential, beginning in the sporangium and ending in the discharge tube. Unique ultrastructural features of this isolate concern organelle arrangement, vesicle morphology, and the mechanism whereby the striated spines form within the external cyst wall.

OBSERVATIONS ON *HALIPHTHOROS MILFORDENSIS*, A MARINE PHYCOMYCETE

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ABBREVIATIONS

A - type A vesicle

B - type B vesicle

BB - basal body

Be - early type B vesicle

Bi - intermediate type B vesicle

C - crown area; type C vesicle

ca - constricted area

Cb - coiled body

Ce - centriole

cf - cleavage furrow

ct - cytoplasmic thread

cw - cyst wall

cwv- cell wall vesicle

Dc - dense connection

dt - discharge tube

Dy - dictyosome

ECW- early cyst wall

ER - endoplasmic reticulum

es - encysted spore

esc- empty spore cyst

f - flagellum

Fb - fiber bundle

fp - flimmer packet

Fr - retracted flagellum

Ft - tinsel flagellum

Fw - whiplash flagellum

g - gemmae

hc - hyaline cap

L - lipid

lg - longitudinal groove

Ms - mastigoneme

N - nucleus

Nb - nuclear beak

Ni - nuclear inclusion

No - nucleolus

Pb - phospholipid body

pm - plasma membrane

Pr - prop

R - rootlet

rf - retracting flagellum

rg - refractive granules

s - septum

scw- sporangial cell wall

SS - striated spine

Tp - terminal plate

INTRODUCTION

Haliphthoros milfordensis, isolated from ova of the oyster drill, Urosalpinx cinerea, was described by Vishniac (1958) as a holocarpic, filamentous, coenocytic, marine phycomycete. Vishniac placed this organism in the order Saprolegniales and in the new family, Haliphthoraceae.

Since its original discovery, H. milfordensis has been collected also on ova and larval stages of several marine crustacea (Sparrow, 1974) as well as on a few algal species (Fuller, Fowles, and McLaughlin, 1964). Additionally, Fisher, Nilson and Shleser (1975) have isolated Haliphthoros milfordensis from cultured larvae and young juveniles of the lobsters, Homarus gammarus and H. americanus. Large colonies of these lobster larvae, which were maintained in closed system aquaria, were killed by the fungus. This is the only report published to date, which describes the destructive capabilities of H. milfordensis in relation to aquaculture.

None of the previous studies of H. milfordensis detail the structure of the sexual reproductive apparatus or the pattern of sporogenesis; and, in fact, the taxonomic position of the fungus is in dispute. Dick (1973) in agreement with Vishniac (1958), placed H. milfordensis in the order Saprolegniales. However, based on its fragementation into subthalli and its unusual form of holocarpy, Sparrow (1973) placed it in the order Lagenidiales.

Although limited information is available on the developmental morphology and occurrence of Haliphthoros milfordensis, several unanswered questions remain. Among the problems yet to be resolved are taxonomic placement, life cycle, host range and infective capabilities, and

ultrastructure. The purpose of my report is to present a detailed account of the developmental morphology, life cycle, and ultrastructure of a strain of *H. milfordensis* isolated from gills of the white shrimp, *Penaeus setiferus* collected from coastal waters of North Carolina.

STRUCTURE AND DEVELOPMENT

Based on its thallus form, holocarpy, monoplanetic laterally biflagellate spores, and apparent parasitic mode of nutrition, Vishniac (1958) placed Haliphthoros milfordensis in the order Saprolegniales and the new family Haliphthoraceae. From physiological tests, Vishniac established that H. milfordensis was obligately marine but euryhaline, and capable of growth on living marine crustacean hosts as well as on synthetic sea water media. The ova and early developmental stages of the pea crab, Pinnotheres sp., were found also to be susceptible to infection with H. milfordensis, thus demonstrating clearly the parasitic nature of this organism.

In describing the morphology of this new fungus, Vishniac reported the thallus to consist of somewhat irregular, highly vacuolate, branched hyphae 10 to 25 um in diameter. Other features of H. milfordensis (Vishniac, 1958) were the formation of gemmae, fragmentation of thalli, the formation of monoplanetic, laterally biflagellate zoospores containing posterior refractive granules, the formation of long specialized, zoospore discharge tubes, and the formation of very fine germination tubes, measuring less than 0.5 um in diameter.

In a later study Fuller et al. (1964) described an isolate of H. milfordensis from the surface of the alga Enteromorpha sp., as having hyphae similar in size to species of Lagenidium, but of a more regular appearance than those of lagenidiaceous species. Fuller et al., in agreement with Vishniac, described their isolate as having a highly vacuolate thallus capable of forming multinucleate fractions. Additional features of H. milfordensis included its rather irregularly shaped

zoospores (appearing more rounded than typical laterally biflagellate planonts) and its spore cysts which swell from 7.8 um to 8.6 um in diameter prior to germination.

Sparrow's (1974) study of Haliphthoros milfordensis established the fact of early occurrence (within seventy-two hours) of fragmentation of the thallus into subthalli existing as adjacent segments of the same thallus. Zoospore formation occurred sequentially rather than simultaneously throughout the entire subthallus. This pattern, Sparrow believed, was a special form of holocarpy. In addition, Sparrow noted that cleavage of zoospores began at the distal poles of the cylindrical subthallus, producing somewhat angular individuals (of various cytoplasmic content) which escaped through long slender discharge tubes. He observed also an irregularity in the shape of zoospores along with their capability of repeated emergence from the spore cysts. One isolate of H. milfordensis described by Sparrow had subthalli with long, sparsely septate, branched rhizoids. These thalli developed in liquid culture when washed in sterile sea water and maintained at 14°C for several days.

Fisher, et al. (1975) described a method of host destruction by Haliphthoros milfordensis. During infection, the fungus attached initially the gills of the lobster larvae from which the mycelia spread throughout the muscle tissue. Host response to infection was by the formation of dark red scabs at the areas of heaviest infection. Subsequently, death occurred during the molting process when both the old exoskeleton and new skeleton adhered to the scab, preventing ecdysis. The destructive potential of H. milfordensis was demonstrated by the fact that in one aquarium containing 1000 H. americanus larvae, 46% died within 22 days of hatching; 95% of the dead animals were infected by H. milfordensis.

MATERIALS AND METHODS

The strain of *Haliphthoros milfordensis* used in the study was isolated from excised gills and eyes of the white shrimp, *Penaeus setiferus*, collected in the vicinity of Beaufort, North Carolina. Cantino's PYG agar (Difco), hydrated with sea water (PYGS) of approximately 32°/oo salinity, was used for both the isolation and subsequent early culture of *H. milfordensis*. In certain instances, it was necessary to add one gram each of penicillin-G and streptomycin sulfate to the culture medium to inhibit the growth of bacterial contaminants. The antibiotics were added to the cooled but still liquid PYGS medium following sterilization at 121°C for 15 minutes.

For isolation of fungi, shrimp gills, eyes and miscellaneous appendages were placed on the sterile antibiotic medium. After forty-eight hours, hyphal tips growing out from infected shrimp parts were transferred via a microspatula to fresh antibiotic media. Repeated transfers by this method resulted in the obtaining of a pure culture by the second or third transfer. During the summer of 1974, four isolates of *H. milfordensis* were obtained by this method. Isolate number two was used for the present study; all are being maintained in the culture collection of C. E. Bland.

In view of the fact that PYGS agar was found unsuitable for the sustained culture of *H. milfordensis*, additional artificial media were used in an attempt to obtain a medium on which optimal growth would occur. Those tested included corn meal agar and broth (Difco) with and without dextrose, hemp seed agar and broth, potato dextrose agar and broth (Difco), dextrose broth solution, shrimp dextrose broth and agar,

shrimp broth without dextrose, and peptone yeast and glucose (Difco-PYG) agar. All media were mixed with sea water of approximately 28°/oo salinity. The best growth was obtained on corn meal dextrose agar (Difco) (CMDS) made up with sea water and in shrimp dextrose broth which was prepared by autoclaving 7-10 headless adult penaeid shrimp in one liter of sea water with two grams of added dextrose. Shrimp were removed after sterilization via cheese cloth straining and the remaining broth was dispensed in 50 ml alliquots into 125 ml erlenmeyer flasks. These flasks were sterilized for 15 minutes at 15 psi and 121°C. Several fresh water media were tested also; however, growth of H. milfordensis on these were not obtained.

Propagation of broth cultures of Haliphthoros milfordensis was accomplished via the transfer of zoospores whereas excised hypal tips were used in the propagation of agar plate cultures. For broth cultures, all transfers were made initially via zoospores which were obtained by means of a hemp seed technique. For this, split sterilized hemp seeds were placed cut side down on the perimeter of a fungal colony on agar. Growth of hyphae into the hemp seeds generally occurred within 48 hours; the seeds were then transferred to Petri dishes containing 15-20 ml of sterile sea water. Sporulation usually began 48-72 hours after the seeds were placed in sea water. For transfers, 5-10 ml of sea water containing zoospores were placed on the surface of a fresh agar plate or were added to 50 ml of shrimp broth. With the excellent growth obtained in the shrimp broth medium, all subsequent transfers were made with zoospores obtained from hyphae grown in the shrimp broth. For this, hyphae from 4 day-old broth cultures were placed in sterile sea water which was changed after 24 hours. Within 48 hours, spore production began and

continued up to 72 hours. Vegetative growth and sporulation of H. milfordensis in broth media were accelerated using a mechanical shaker rotating at 100 revolutions per minute. With the use of a mechanical shaker and this method of transfer, synchronous vegetative development and sporulation of H. milfordensis was established.

Tests involving the infection of representatives of several genera of marine crustacea with H. milfordensis were carried out using six liter closed system aquaria and standard disposable Petri dishes. The six liter aquaria were used for infection tests involving adult specimens of the shrimp, Penaeus setiferus and Penaeus duorarum, and the bluecrab, Callinectes sapidus. The level of inoculum used in these infection tests varied from one million to six million spores per three liters of sea water. Larval stages of the brine shrimp, Artemia salina and ova of the blue crab, Callinectes sapidus were maintained in 15-20 ml of sea water contained in Petri dishes. These were inoculated generally with fifty thousand to five hundred thousand spores of H. milfordensis.

Light microscope observations of living and fixed material were made using a Zeiss WL research microscope equipped with phase contrast and Nomarski interference contrast optics. For fixation, specimens were either placed in 3% glutaraldehyde or exposed to the fumes of 4% osmium tetroxide for three to five minutes. All photomicrographs were taken with a Nikon AFM Microflex camera on Kodak Panatomic X film.

Although several fixation procedures were tested in the preparation of specimens for electron microscopy, only two yielded consistently good results. The first procedure (a modification of one used by Bland and Amerson, 1973) involved the sequential fixation of specimens in 3% gluteraldehyde and 2% osmium tetroxide. For this fixation, specimens

were placed in 3% gluteraldehyde in sea water for 10-15 minutes (pH 7-7.4), washed in four changes of sterile sea water for 45 minutes to one hour, and post-fixed for ten minutes in 2% osmium tetroxide in sea water. After fixation, specimens were washed in four changes of sterile sea water for 20 minutes and then dehydrated in a graded ethanol series (15 minutes each) followed by propylene oxide (two changes of 15 minutes each). Centrifugation of spores at 1470Xg for two minutes was generally necessary at each solution change. Blocks were cured at 60°C for three days.

The second fixation procedure was similar to the one described previously except that specimens were post-fixed in 2% osmium tetroxide mixed in sodium cacodylate buffer (pH 7-7.2) followed by a 20 minute wash involving two changes in 0.2 M sodium cacodylate.

Whole mounts of zoospores were prepared according to the procedure of Sharp, Eckert, Beard and Beard (1952), and shadowed with platinum-palladium.

All thin sections were cut with a Dupont diamond knife and a Reichert OmU2 ultramicrotome. Thin sections were double stained with 5% uranyl acetate and Reynolds lead citrate (Reynolds, 1963) for varying periods of time, but generally 12-20 minutes with each stain.

Transmission electron microscope observations were made using a Hitachi HS-8 electron microscope at an accelerating voltage of 50Kv. Electron micrographs were taken on Kodak Kodalith LR film.

RESULTS

Life History:

The life history of *Haliphthoros milfordensis* is summarized in figure 1.

Zoospores, the infective agents of the fungus, may remain motile up to 72 hours after release from the sporangium. On contact with a host, they retract and/or cast off their flagella, become spherical, and subsequently are surrounded by an adhesive cyst wall. Following a brief encystment period, vegetative growth begins via germination of encysted spores. Germ tubes form as outgrowths from encysted spores at points of contact between the spores and the host's surface. Subsequently, the germ tubes penetrate the host, grow into internal areas, and enlarge to form young vegetative hyphae. Further vegetative hyphal growth is accompanied by multiple branch formation and fragmentation of thallus segments. Growth continues until all host cytoplasm has been consumed by the fungus. Conversion of the vegetative thallus into zoospores occurs when segments of the thallus become delimited by septa or by cytoplasmic constrictions. Subsequently, the cytoplasm is cleaved into biflagellate zoospores. Concurrently, slender discharge tubes (1 or 2 per sporangium) of varying shapes and lengths grow out from developing sporangia. Elongation of discharge tubes continues until the surface of the host has been penetrated. Cleavage of cytoplasm within discharge tubes occurs just prior to discharge and after cleavage within the sporangia from which they grow. Spore discharge occurs shortly after cleavage of the most distally located spore. Zoospores are released one at a time until all have escaped through the discharge tubes.

Eventually, all remaining portions of the vegetative thallus become sporogenic, however, this does not occur simultaneously.

Zoospores:

Zoospores of Haliphthoros milfordensis are biflagellate, and inconsistent in size and shape (Figs. 2-5); averaging 7.73 um in length (range 3.99 to 11.97 um) and 6.01 um in width (range 3.99 to 7.98 um). Despite this inconsistency, the zoospores may be divided into four morphological categories; globose (Fig. 2), pyriform (Fig. 4), elongate (Fig. 3), and reinform (Fig. 5). These forms all occur frequently, however, the pyriform and elongate shapes are predominant.

The two heterokant flagella (anterior tinsel, 10.31 to 14.56 um in length; posterior whiplash 11.70 to 15.09 um in length) (Fig. 6) are generally attached to the spore in a raised area of a subapical, longitudinal groove (Fig. 7). However, attachment may vary slightly depending on spore shape and size. Occasional, larger than normal zoospores have four flagella attached in pairs at the anterior and posterior ends of the spore unit.

Observations of whole mounted, shadowed, zoospores, revealed the presence of small hairs on the whiplash flagellum. These "mini tinsels" are much smaller (0.36 um in length; 0.02 um in width), less complex, and have a more uniform distribution than the real mastigonemes of the tinsel flagellum (Fig. 11). Mastigonemes (1.72 um long; 0.04 um wide) terminate in small hairs measuring 0.64 um long and 0.01 um wide (Fig. 10).

Motile spores swim generally in a clockwise, helical pattern, however, a variation of this movement is noticed just prior to encystment. In this instance, the zoospore moves in a clockwise, spinning

pattern for a brief period (5 to 10 seconds), ending by rounding up, and retracting and/or casting off its flagella (Fig. 8). The tinsel flagellum is retracted by the vesicular retraction method (Koch, 1968) or cast off. The whiplash flagellum is always cast off.

Numerous refractive granules (Fig. 9) occur throughout the cytoplasm of the zoospore and may be detected with phase contrast microscopy. Similar granules may also be detected in whole mounted spores (Fig. 12).

Encysted Spore:

Zoospore encystment begins with flagellar loss or retraction and is followed by rounding-up of the zoospore. Encysting zoospores (Fig. 13) are all globose and measure initially an average 7.74 um in diameter. This diameter increases to approximately 8 um (range 5.32-10.64 um) with formation of the external cyst wall. The wall of the spore cyst is covered with numerous spines which are clearly visible in the empty cyst (Figs. 14 and 17).

During encystment the release of large globules (Fig. 16), and the discharge of minute particles from the spore surface is observed frequently. In the former, the globules (up to 3.5 to 4.0 um in diameter) are first seen on the surface of the encysting spore (Fig. 16), however, they separate eventually leaving no observable structural change. In addition to these globules, minute granules, less than 0.02 um in diameter are constantly released from the spore surface. The nature of both these granule types will be discussed more fully in the section dealing with electron microscope observations.

Duration of encystment varies depending on several conditions. Of these, nutrient availability is the most important. Generally, when nutrients are supplied to encysted spore cultures, germination of

approximately 90% of the spores occurs within 3 to 12 hours. However, in nutrients, encysted spores may remain dormant up to three days; possibly longer as tests beyond three days were not conducted. Temperature as well as culture conditions may affect also the duration of encystment.

Encysting spores are capable of adhering to almost any solid substrate. This is demonstrated in that encysting zoospores adhere readily to each other (Fig. 18). Adhesion to substrate or other spores does not appear to interfere with subsequent events such as germination or re-emergence.

Re-emergence of zoospores (Fig. 14) is evident in that empty spore cysts (Fig. 17) and amoeboid spores are observed frequently.

Germination:

In nutrient-rich media, germination of encysted spores occurs within 12 hours following encystment. Variation in size and shape of germ tubes depends on the substrate. In shrimp broth with dextrose, germ tubes are slender (approximately 0.5 um in diameter), extending 10 to 50 um in length before expanding as broad vegetative hyphae (Fig. 15 and 19). On corn meal dextrose agar, however, germ tubes are generally shorter and wider (Fig. 20), averaging 21.64 um (range 2.66-50.44 um) long by 2.8 um (range 1.3-2.6 um) wide.

Growth of germ tubes continues for 12-24 hours before young hyphae form distally (Fig. 20). During this time, cytoplasm of the encysted spore gradually migrates into the distal portion of the germ tube, leaving the spore cyst devoid of cytoplasm (Fig. 19). Close examination of germ tubes reveals that septa or dense constrictions occur at intervals along their length (Fig. 19).

Germination in a nutrient poor medium (sea water), results generally in the formation of extremely long thin germ tubes which often break off at the spore surface.

Vegetative Hyphae:

The vegetative hyphae formed at the distal end of germ tubes contain dense cytoplasm and are generally multinucleate. Branching of the vegetative hyphae may occur shortly (within 24 hours) after their formation (Fig. 21). In other instances, the hyphae may grow for a longer interval of time (48 hours) before branching occurs. Branches form at at 90^{o} angle to the primary thallus (Figs. 21 and 23). Continual branching during rapid growth results in an extensive mycelium in 3 to 4 days (Fig. 23).

Hyphae of mycelia are irregular, coenocytic, branched tubes of varying diameter. Three day old hyphae grown on CMDS average 13.74 um in diameter (range 6.65 to 26.60 um) (Fig. 23).

Mycelial fragmentation is a common occurrence when strains of H. milfordensis are grown in shrimp broth (Fig. 26). Fragmentation begins 24-48 hours after initiation of vegetative hyphal growth and continues until nutrient depletion. Fragments forming as adjacent segments of the thallus are delimited by septa or cytoplasmic constrictions (Fig. 26). Such fragments (Fig. 22), on separating from the vegetative thallus, are capable of further growth.

Occasionally, in older cultures (4-7 days), gemmae-like structures form throughout the thallus (Fig. 24). These structures may germinate while attached to or separated from the main thallus.

Sporogenesis:

Depletion of nutrients in a culture of H. milfordensis initiates sporogenesis. The following account is of sporogenesis as it occurs in hyphae grown in shrimp broth and then placed in sea water. The major events of sporogenesis are:

1) Sporogenic portions of the thallus become delimited from non-sporogenic ones (Fig. 25) via septum formation or cytoplasmic constriction.

2) Cytoplasm of the sporogenic hyphae (1-40 hours in sea water) becomes highly vacuolate (Fig. 27).

3) The cytoplasm becomes densely granular and homogeneous (42 hours in sea water) (Fig. 31).

4) Initiation of discharge tube formation occurs as pockets of light, finely granular material collect in one or two areas at the periphery of the sporogenic hyphae. Long, slender, discharge tubes containing sporogenic cytoplasm grow out at each of these localized areas (Figs. 28-30).

5) Cleavage of the sporogenic mass occurs (42-43 hours) simultaneously with discharge tube formation and lasts approximately one hour. Cleavage takes place via forrowing of the sporogenic mass (Fig. 13) and is a sequential process; beginning in areas farthest from the discharge tube and ending in the distal portions of the discharge tube itself. Spore initials are at first tightly packed and polygonal, but later become rounded (Fig. 32 and 33). That flagella are formed at this time is evident by movement of newly formed spores. Spores already cleaved in one area of a sporangium may begin active swimming or amoeboid movement before cleavage is completed in other areas of the sporangium

and in the discharge tubes (Figs. 33 and 34). Sporogenesis is completed as the last spores are cleaved in the distal end of the discharge tubes (Fig. 36).

Discharge:

Discharge tubes average 7.16 um in diameter (range 5.31 to 9.31 um), but vary in length and shape depending on the substrate. The two dominant forms, helical and straight, may measure up to 300 um in length. The helical tubes (Fig. 24 and 50) are formed primarily in shrimp broth whereas straight tubes are found on hemp seed cultures. The opening through the discharge tubes is only large enough to permit the passage of one zoospore at a time. The distal end of each discharge tube is covered by a hyaline cap; beneath which is found a highly granular cytoplasm (Fig. 35).

As the last spore cleaves, the hyaline cap at the distal end of the discharge tube breaks releasing the zoospores (Fig. 49). Movement of individual spores through the tubes occurs rapidly (Fig. 49); an entire spore mass (over 100 spores) being discharged in 20-30 seconds. Spores trapped inside the sporangium will encyst there (Fig. 25).

Cytoplasmic threads are occasionally observed connecting adjacent discharging zoospores (Fig. 37). When these zoospores are released, they hover just outside the discharge tube where they remain with limited movement for 5-25 seconds before separating and swimming slowly into the surrounding medium.

Development On Various Media:

Cultures of Haliphthoros milfordensis exhibit different growth forms on different media. Table 1 summarizes results concerning

Table 1. Development of H. milfordensis on different media.
(All measurements in microns)

Medium	Diameter 3-day hyphae	Germ tube length	Germ tube diameter	Discharge tube length	Discharge tube diameter	Hyphal form
CMDS	X=13.74 R,6.65-26.60	X=21.64 R,2.66-50.54	X=1.9 R,1.3-2.6	XXX	XXX	Twisted, irregular many gemmae
Brine Shrimp	X=15.30 R,7.60-30.00	XXX	XXX	X=155.00 R,60-280	X=7.16 R,5.32-9.31	more regular,2-4 main hyphae, many thin branches
Hemp	X=10.63	XXX	XXX	X=306.18 R,160-656	X=7.15 R,5.6-8	straight, extended hyphae
Shrimp broth	X=36.4 R,15-65	X=35.6 R,10-50	X=0.5	X=122.13 R,88-200	X=9.63 R,8-12	extensive, occurring in mycelial balls

XXX=could not be measured

development on hemp seed (Figs. 38-40), corn meal dextrose agar (Figs. 41-44), brine shrimp larvae (Artemia salina) (Figs. 45-47), and shrimp dextrose broth (Figs. 2-5,8,9,13-19,23-36).

Preliminary Infection Tests:

The host range and infectivity of H. milfordensis were tested with the following:

1) sterile and living ova of Callinectes sapidus

2) larvae, embryos, and ova of Artemia salina

3) adults of Penaeus setiferus

All of the above were susceptible to infection. For ova of Callinectes sapidus, the most noticeable effect of infection was color change. Normally brown, sterile and living ova, when infected appeared dark orange-yellow. Hyphae completely filled the ova and subsequently produced long, straight discharge tubes which protruded through the surface of the ova into the external medium (Fig. 48). Infection among ova was so rapid that zoospores from one infected egg were capable of infecting an entire egg mass within 2 days. Internal hyphae in both sterile and living ova were multibranched.

Infection of ova and larvae of brine shrimp, Artemia salina, was similar to that in crab ova. Of 300 brine shrimp larvae contained in 20 ml of sea water, all became infected with H. milfordensis and died within 5 days after inoculation with 500,000 spores. Zoospores of H. milfordensis attached not only larvae (Fig. 51) but also ova (Fig. 49) and embryonic stages (Fig. 50) which were more quickly destroyed by the fungus than were swimming larval forms.

Infection of adult shrimp, Penaeus setiferus with H. milfordensis resulted in destruction of the gill filaments and subsequent death of

the shrimp (Fig. 52). In rare instances, hyphae spread also to eyes, eye stalks, and carapace. However, hyphae were never found in muscle tissue. Of 12 P. duorarum adults, contained in closed system, 6 liter aquaria, all were infected with H. milfordensis within 2 weeks after inoculation with approximately six million zoospores.

Fine Structure of Zoospores:

Zoospores of Haliphthoros milfordensis are bound by a single plasma membrane (Figs. 53 and 54) which appears irregular because of underlying vesicles (Figs. 6,7,12). Internally, three discrete areas of spore cytoplasm may be distinquished on the basis of the arrangement and number of specific cell organelles and inclusions within each. The three areas are: 1) anterior, 2) posterior, and 3) periphery.

The anterior area contains the point of flagellar attachment, the nucleus, several mitochondria, one or two dictyosomes, sparse endoplasmic reticulum and occasionally one to several lipid bodies (Figs. 53 and 54). The subapically attached flagella, 0.20 to 0.25 um in diameter, consist of axonemal microtubules in a 9+2 arrangement and a flagellar sheath which is continuous with the plasmalemma (Figs. 54, 56,57). The proximal end of each flagellum terminates in a basal body (approxiamtely 0.67 um by 0.17 um) of typical eukaryotic structure (Figs. 57 and 90). Both basal bodies occur within a raised area (crown) of the zoospores and have an internal angle of approximately 130^{o} (Fig. 90). Dense areas occur adjacent to the terminal plate of each flagellum and appear to be connections between the plate and the plasmalemma (Figs. 57,90). Coiled structures, extending not more than 0.11 um above the terminal plate, are located around the central pair of microtubules of the axoneme (Figs. 57, 58, 90). At least two rootlets occur at the proximal ends of the basal

bodies and may extend 1.85 um beneath the plasmalemma (Figs. 57, 58, 90). Additional rootlets, in the form of one or more "props" from the two opposing basal bodies are sometimes evident (Fig. 57).

Nuclei of actively swimming spores average 3.56 um long (range 2.28-4.66 um) by 1.65 um wide (range 1.31-2.29 um) and are generally centrally located in the anterior area. They may occur in various shapes (Figs. 53 and 54) but all have a characteristic beak extending toward the crown area (Fig. 55) where the basal bodies are located. Each nucleus contains usually not more than one nucleolus, but may contain also variously shaped membranous nuclear inclusions (Figs. 55 and 60).

Dictyosomes are located on either one or on both sides of the nuclear beak (Figs. 55, 60, 91). The dictyosomes which are composed of 3 to 4 flattened, stacked lamellae are surrounded by numerous small vesicles, not more than 0.6 um in diameter.

Sausage and cup shaped mitochondria, measuring 1.26 um long (range 0.83 to 1.77 um) and 0.39 um wide (range 0.27 to 0.56 um), are located also in the anterior area (Figs. 53, 55, 56, 59, 61). These are often numerous with as many as 11-15 mitochondria being observed per thin section. Occasionally, small electron dense granules are seen in the internal mitochondrial matrix (Fig. 61). Most mitochondria are closely associated with the nucleus; however, some do occur in areas away from the nucleus.

Occasionally, lipid bodies, approximately 0.6 um in diameter are seen in the anterior zoospores area (Figs. 54, 58).

Rough endoplasmic reticulum is located throughout the zoospore in a non-uniform pattern (Figs. 54, 55, 60, 61, 91, 92). Similarly, free ribosomes and polysomes are uniformly distributed throughout the cytoplasm.

The posterior zoospore area contains two distinct vesicle types and/or inclusions. The most prominant of these is a vesicle (Type A) containing varying amounts of granular to fibrous material (Figs. 54, 62) and measuring approximately 1.47 um in diamater (range 0.53-1.92 um). The type A vesicles have an electron transparent matrix and are ovoid to spherical. These vesicles fuse occasionally with the plasmalemma (Fig. 54) and discharge their contents to the exterior.

The type B vesicles, present in some but not all zoospores, measure 2.5 to 3.0 um in diameter and contain four recognizable subunits: 1) minute spheroids, 0.08 um in diameter; 2) large spheroids, 0.2 um in diameter; 3) granules of varying electron densities; and 4) electron dense fibers. Type B vesicles (Fig. 63) appear to form via coalescence of small peripheral vesicles which accumulate in unbound multivesicular areas having dimensions comparable to the type B vesicle (Fig. 64). An intermediate stage (Fig. 65) in the formation of type B vesicles occurs in which numerous loosely packed spheroids, averaging 0.07 um are bound by a single membrane.

In addition to type B vesicles, lipid, sparse endoplasmic reticulum, and numberous free ribosomes occur in the posterior of the zoospore.

The periphery of the zoospore includes the outer 0.5 um of the spore cytoplasm (Fig. 90). Scattered in this area are numerous spherical to elongate, electron dense phospholipid bodies (Fig. 66). Although the majority of phospholipid bodies are peripheral, occasionally one or two may be centrally located. Most of these bodies are spherical, averaging 0.28 um in diameter, with profiles of 20 to 60 occurring in a single thin section.

Zoospore Modifications Associated With Encystment:

The numerous structural changes occurring during zoospore encystment may be grouped in three categories: 1) the formation of organelles and other structures; 2) movement and rearrangement of existing organelles; and 3) modification or change of existing organelles. A description of each category of change follows.

The most prominent change occurring during encystment is the formation of the external cyst wall (Figs. 69-71, 75, 91-96). The cyst wall is a three layered structure which when completely formed consists of an outer dense fibrous wall, a middle electron transparent zone containing striated spines, and an inner somewhat thickened wall of uniform electron transparency (Figs. 75 and 96). The production of the cyst wall occurs in three stages during encystment. In the first, fibers released from type A vesicles adhere to the rounding spore surface forming a dense mat around the spore periphery (Figs. 72, 92-94). In the second stage, which is concurrent with organelle rearrangement, the dense fibrous mat begins to separate from the spore surface (Fig. 73) leaving numerous bundles of fibers between the separated fibrous portion and the spore surface (Fig. 74). In the third stage the fiber bundles condense forming striate spines which are continuous between a uniform spore surface wall and an external fibrous cyst wall. Completely formed spines are always broader at the proximal end, averaging 0.11 um at the base and 0.02 um at the distal end. Spines may occur in groups of two, three, or singly. Striations of the spine are spaced uniformly, approximately 15 nm apart. The completely formed cyst wall ranges from 0.40-0.85 um in thickness.

A second group of structures, the cell wall vesicles which appear during encystement, are observed initially proximal to the nucleus

(Figs. 67, 76, 93). These vesicles contain uniformly granulate electron dense material. They are sausage, bar bell, or spherically shaped, averaging 0.45 um long (range 0.22 to 0.57) and 0.14 um wide (range 0.09-0.25 um). On several occasions, these vesicles have been observed to be continuous with the rough endoplasmic reticulum. They are usually closely associated with one or more mitochondria (Fig. 76). In later stages of encystment, cell wall vesicles move away from the nuclear region and become scattered throughout the spore cytoplasm (Fig. 77).

Packets of presumptive mastigonemes (flimmer packets) form also in proximity to the nucleus (Fig. 71). These packets when viewed in longitudianl section, are long, rectangular, membrane-bound structures which can measure up to 1.05 um long and 0.23 um wide (Fig. 71). Flimmer packets become more numerous during the later stages of encystment and occur throughout the spore cytoplasm. That flimmer packets may initially form as continuous segments of the endoplasmic reticulum is evident in that newly formed packets are often surrounded by a ribosome studded membrane which is continuous with the endoplasmic reticulum.

Large, spherical vesicles (Type C), similar to and possibly the same as type B vesicles, are observed in later stages of encystment (Fig. 78). These vesicles, 1.0 um to 2.98 um in diameter, differ from type B vesicles in that their fibers are more uniform and their internal matrix has greater electron density. Fibers, which are centrally located, average 0.31 um in length. These vesicles are generally continuous with or located near the plasmalemma.

During encystment, several pre-existing structures within the zoospores become relocated. The major changes which fall into this category include the following:

1) After flagellar loss or retraction, basal bodies of both flagella

migrate toward the centrally located nucleus where they function as centrioles (Figs. 68, 69, 79).

2) Mitochondria move to locations adjacent to and often completely surrounding the nucleus (Fig. 70).

3) Large, granulate, type A vesicles move to the periphery (Figs. 68,70) where they subsequently fuse with the plasma membrane for release of their contents to the external medium (Figs. 68, 80, 91, 92). Material from these vesicles eventually forms the cyst wall.

4) Type B vesicles move to the plasma membrane where they are eventually released, intact, to the external medium (Fig. 81).

5) Phospholipid bodies migrate from the periphery toward the center (Figs. 83, 84, 92-96).

6) Rough endoplasmic reticulum accumulates as multilayered lamellar complexes around the nucleus (Figs. 70, 91-96). Also, rough endoplasmic reticulum may occasionally be seen to surround one to several mitochondria (Fig. 70).

7) Unbound early stages of type B vesicles are often seen being released from the plasma membrane.

8) The nucleus looses its prominent beak and becomes spherical (Figs. 70, 71, 92-96).

9) Cell wall vesicles move to the periphery (Fig. 77).

10) Flimmer packets move throughout the cytoplasm with several becomming aligned adjacent to the plasmalemma (Fig. 71).

Phospholipid bodies are the only structures observed to undergo structural modification. Changes occur sequentially, starting shortly after migration toward the central zone of the spore. As phospholipids change, a scalloped fringe (Figs. 83-88, 93-96) develops around the

outside of the remaining structure.

Encysted Spore:

The fully encysted zoospore of H. milfordensis is characterized by its completely-formed, three-layered cyst wall (Figs. 69, 71, 75, 96). The final arrangement of internal organelles and structures in the encysted spore is as follows:

1) The nucleus is centrally located (Figs. 70, 71).
2) Mitochondria are scattered around the nucleus in the central zone.
3) Rough endoplasmic reticulum is complexed in many areas around the nucleus and around isolated motochondria (Figs. 70, 94-96).
4) Dictyosomes remain closely associated with the nucleus (Figs. 68, 89, 96).
5) Type A vesicles are arranged around the periphery (Fig. 96).
6) Scallop fringe phospholipid bodies occur throughout the cytoplasm in various degrees of modification (Figs. 70, 95, 96).
7) Centrioles, maintaining the 9 triplet microtubule arrangement, occur adjacent to the nucleus (Fig. 89).
8) Numerous flimmer packets are scattered throughout the cytoplasm (Figs. 71, 96).

DISCUSSION

Taxonomy:

Historically, the fungus Haliphthoros milfordensis, has been placed in two different orders; the Saprolegniales (Vishniac, 1958, and Dick, 1973) and the Lagenidiales (Sparrow, 1973). Dick (1973), in agreement with Vishniac's (1958) original placement, put H. milfordensis in the Saprolegniales on the basis of its filamentous, holocrapic, parasitic nature. Sparrow (1973); however, placed it in the Lagenidiales mainly on the basis of its fragmentation into subthalli and on its unusual form of holocarpy. From observations made in the present study, four points of potential taxonomic importance merit further consideration.

1) Zoospores of isolate number 2 (the strain investigated in the present study) differ in several ways from those described previously for H. milfordensis (Vishniac, 1958, Fuller et al., 1964, Sparrow, 1974). In contrast to the monoplanetic, laterally biflagellate planonts described by Vishniac (1958), those of isolate 2 are neither monoplanetic nor have laterally attached flagella. Since the term monoplanetic describes fungi having only one motile stage (Snell and Dick, 1971), zoospores of isolate 2 clearly do not fit the description as they are capable of repeated emergence. Similarly zoospores of isolate 2 cannot be described as monomorphic; a term used by Sparrow (1974) to describe zoospores of another isolate of H. milfordensis. Whereas monomorphic means "forming only one kind of structure" (Snell and Dick, 1971), zoospores of isolate 2 would best be described as polymorphic since they exist in a variety of shapes. Fuller, et al., (1974) described zoospores

of another strain of H. milfordensis to be "more nearly rounded." Obviously, there is great variation in zoospore shape among the various strains of H. milfordensis investigated to date.

Concerning flagellar attachment, although apical to sub-apical attachment of flagella, as found in isolate 2, is characteristic of the primary type of zoospore, zoospores of H. milfordensis have also a lateral groove which is more characteristic of the secondary zoospore type. Sparrow (1974) was unable to clearly discern the point of flagellar attachment in the strain which he studied; however, he felt that they were laterally attached. It is primarily due to the extreme variation in zoospore morphology and flagellar attachment that zoospores of H. milfordensis are difficult to categorize as either primary or secondary. Therefore, taxonomic palcement of this organism into either the Saprolegniales or the Lagenidiales on the basis of zoospore morphology is difficult.

2) The spines occurring in the cyst wall of H. milfordensis spores are basically similar to those described for the primary spore cysts of Dictyuchus sterile (Heath and Greenwood, 1970), Saprolegnia parasitica (Meier and Webster, 1954), and S. ferax (Heath and Greenwood, 1970); all members of the Saprolegniales. This is significant taxonomically in that similar spines have not been reported for any members of the Lagenidiales.

3) The discharge apparatus of H. milfordensis is basically similar to that of the genera Atkinsiella (Sparrow, 1973), Leptolegniella, Olpidiopsis, Petersenia, Sirolpidium and Pontisma (Sparrow, 1960) in that all are characterized by extended, narrow tubes which allow the passage of limited numbers of spores at a given time. With the exception of Leptolegniella, a member of the Saprolegniales, all these genera are non-vesicle forming members of the Lagenidiales. The discharge apparatus in

all saprolegniaceous genera, except Leptolegniella, is distinctly different from that occurring in H. milfordensis. In spite of these basic similarities between the discharge apparatus of H. milfordensis and members of the Lagenidiales, the unusual sequential cleavage process, first described by Sparrow (1974) for H. milfordensis, does not occur in any similar lagenidiaceous genera and has not been seen in any members of the Saprolegniales. However, H. milfordensis is still basically similar to several members of the Lagenidiales with regard to its unusual holocarpic mode, whereby thallus conversion into spores occurs in several segments non-simultaneously.

4) Two features of the vegetative thallus of H. milfordensis are of taxonomic significance. The first is the fact that its thallus structure is characteristically lagenidiaceous. This is evident in the remarkable similarities between the thallus of H. milfordensis and that of Lagenidium callinectes as described by Bland and Amerson (1973). The only observable differences between thalli of the two are that H. milfordensis is larger than L. callinectes and that gemmae occur occasionally in the former. A second vegetative feature linking H. milfordensis to lagenidiaceous forms concerns the manner in which its thallus fragments in a fashion similar to Sirolopidium bryopsidis and Pontisma (Sparrow, 1960). Fragmentation of this type does not occur in any saprolegniaceous species.

Based on these observations, I tend to agree with Sparrow's (1976) assessment that H. milfordensis should be placed in the order Lagenidiales and probably in the family Sirolpidiaceae. However, resolution of the problem concerning taxonomic placement of H. milfordensis awaits further study of it and related forms. Of special significance would be findings relative to the sexual apparatus (if any) of H. milfordensis.

Ultrastructure of Zoospores:

Ultrastructural features of H. milfordensis zoospores are somewhat different from those described previously for other oomycetous zoospores. Certainly, one reason for this is that all but one of the previous studies were of freshwater organisms. The ultrastructural features distinquishing H. milfordensis zoospores from previously described planonts include internal organelle arrangement, variation of vesicular inclusions, and modification or variation in organelle structure. Major differences between H. milfordensis and previously studied genera, including Phytophthora (Ho, Zachariah and Hickman, 1968, Hohl and Hamamota, 1967, Reichle, 1969, Desjardins, Wang and Bartnicki-Garcia, 1973, Bimpong and Hickman, 1975), Saprolegnia (Gay, Greenwood and Heath, 1971), Aphanomyces (Hoch and Mitchell, 1972), Pythium (Lunney and Bland, 1976) and Lagenidium (Bland and Amerson, 1973, Gotelli, 1974), relate to its anteriorly positioned motochondria, absence of an internal groove region, vesicular variation and arrangement of endoplasmic reticulum.

Previous studies of oomycetous zoospores showed the mitochondria to be arranged generally around the periphery of the zoospore. In H. milfordensis; however, they are positioned anteriorly around the nucleus and are often associated with rough endoplasmic reticulum (RER). A similar RER-mitochondrial association has been observed also in P. proliferum (Lunney and Bland, personal communication).

The internal groove area described for the zoospores of several saprolegniaceous freshwater fungi, including P. parasitica (Reichle, 1969), Aphanomyces euticheS (Hoch and Mitchell, 1972) and P. proliferum (Lunney and Bland, personal communication), generally lacks ribosomes, mitochondria, and RER; however, it does contain an assortment of

vesicles and usually one contractile vacuole. In contrast, a distinct groove area is not evident in zoospores of H. milfordensis. Several structural and/or environmental factors may account for the absence of this groove region. Certainly, one cannot overlook the fact that H. milfordensis zoospores do not have a pronounced groove such as is found in the majority of freshwater species, but rather only a slight depression in the anterior spore surface. Also, the fact that H. milfordensis is obligately marine may account for the absence in this organism of an internal contractile apparatus such as that described for A. euteiches (Hoch and Mitchell, 1972) and P. proliferum (Lunney and Bland, personal communication). Evidence of this is found in that L. callinectes (Bland and Amerson, 1973), also marine, lacks this same pronounced internal groove region.

Of the five vesicle types observed in zoospores of H. milfordensis, only three are similar to the five major vesicle types described by Lunney and Bland (personal communication) in their consideration of vesicles types found in fungi. These include vesicle type A (peripheral vesicles), electron dense phospholipid bodies, and cell wall vesicles. Lunney and Bland (personal communication) present the probable and proposed functions of these three vesicles; therefore, they will not be considered in this report. However, the two remaining vesicles, type B (see Figs. 62-64) and type C (see Fig. 77), are not similar to any described previously. The function of these is presently unknown; however, I believe they may serve in waste disposal or salt regulation. As they are often observed being released intact, they most probably contain products not needed by the spore. That the "flattened vesicles" and "microbodies" described by Lunney and Bland (personal communication) are absent in H. milfordensis may be an additional modification of marine zoospores. This is again supported by the absence of these same vesicles in L. callinectes (Bland and Amerson, 1973). It is possible

that these two vesicle types are needed only for freshwater organisms or that there are structures in the marine zoospores which are modified to perform similar functions or that the tasks performed by these vesicles are unnecessary in the marine environment.

The rough endoplasmic reticulum of H. milfordensis, described previously only in reference to its association with mitochondria, is quite unlike that of other fungal zoospores. Several studies (Ho et al., 1968, Hohl and Hamamoto, 1967, Hoch and Mitchell, 1972, and Lunney and Bland, 1976) describe the RER as occurring in abundant concentric layers near or around the nucleus. In contrast, the RER of H. milfordensis zoospores is sparse and swollen with a scattered arrangement more like that described by Bland and Amerson (1973) for zoospores of L. callinectes. It is possible that this arrangement of irregularly swollen RER is another unique characteristic of marine oomycetous zoospores.

The flagellar apparatus of H. milfordensis is basically similar to that of other oomycetous zoospores. The basal bodies, occurring at 130^{o} angles to each other, correspond closely to those of P. proliferum (Lunney and Bland, 1976) and A. eutiches (Hoch and Mitchell, 1972). In all of these organisms, the basal bodies are associated with the beaked portion of the nucleus; a feature of many other genera of biflagellate fungi. Other similarities between these organisms include the dense connections between the terminal plate and the plasmalemma, and the coiled fiber located just above the terminal plates. Coiled fibers have been noticed also in Ochramonas (Bouck, 1971) and Saprolegnia (Heath and Greenwood, 1970); however, in the former they were described as consisting of 3-4 subunits.

Rootlet arrangment in H. milfordensis spores remains unclear to

the author; however, it appears to be similar to the one long, one short configuration seen in L. callinectes (Bland and Amerson, 1973). Additionally, an interconnecting fiber, similar to that described for P. parasitica (Reichle, 1969) zoospores, occurs between the two basal bodies of H. milfordensis zoospores.

In H. milfordensis, microtubule complexes occur both as perpendicular arrays from the long rootlet and as extensions from the basal bodies to the nuclear beak. The presence of these complexes are common in many oomycetous zoospores. These complexes are not evident in all sections of H. milfordensis zoospores and their exact arrangement is not known at this time.

The longer whiplash flagellum of H. milfordensis is covered with fine, lateral hairs (see Fig. 11) similar to those described previously for P. parasitica (Reichle, 1969) and P. palmivora (Desjardins, Zentmeyer and Reynolds, 1969). The presence of these hairs may be of taxonomic significance in that they have never been reported for lagenidiaceous fungi.

The mastigonemes occurring in the shorter tinsel flagellum of H. milfordensis are similar to those described for several other fungi (Reichle, 1969, Desjardins et al. 1969, Heath and Greenwood, 1970, Bouck, 1971, Hoch and Mitchell, 1972, Bland and Amerson, 1973, Gotelli, 1974, and Lunney and Bland, 1976). In several of these studies, especially that of Bouck (1971), it was proposed that the mastigonemes formed within cisternae of the RER. In H. milfordensis such packets of of presumptive mastigonemes were not observed until late encystment. The fact that these packets are not found in the actively swimming spores of H. milfordensis does not preclude the possibility that they may exist in the sporangium prior to spore cleavage and; in fact, give rise to the

mastigonemes. Resolution of this question will require examination of the sporogenic stages of H. milfordensis.

Encystment:

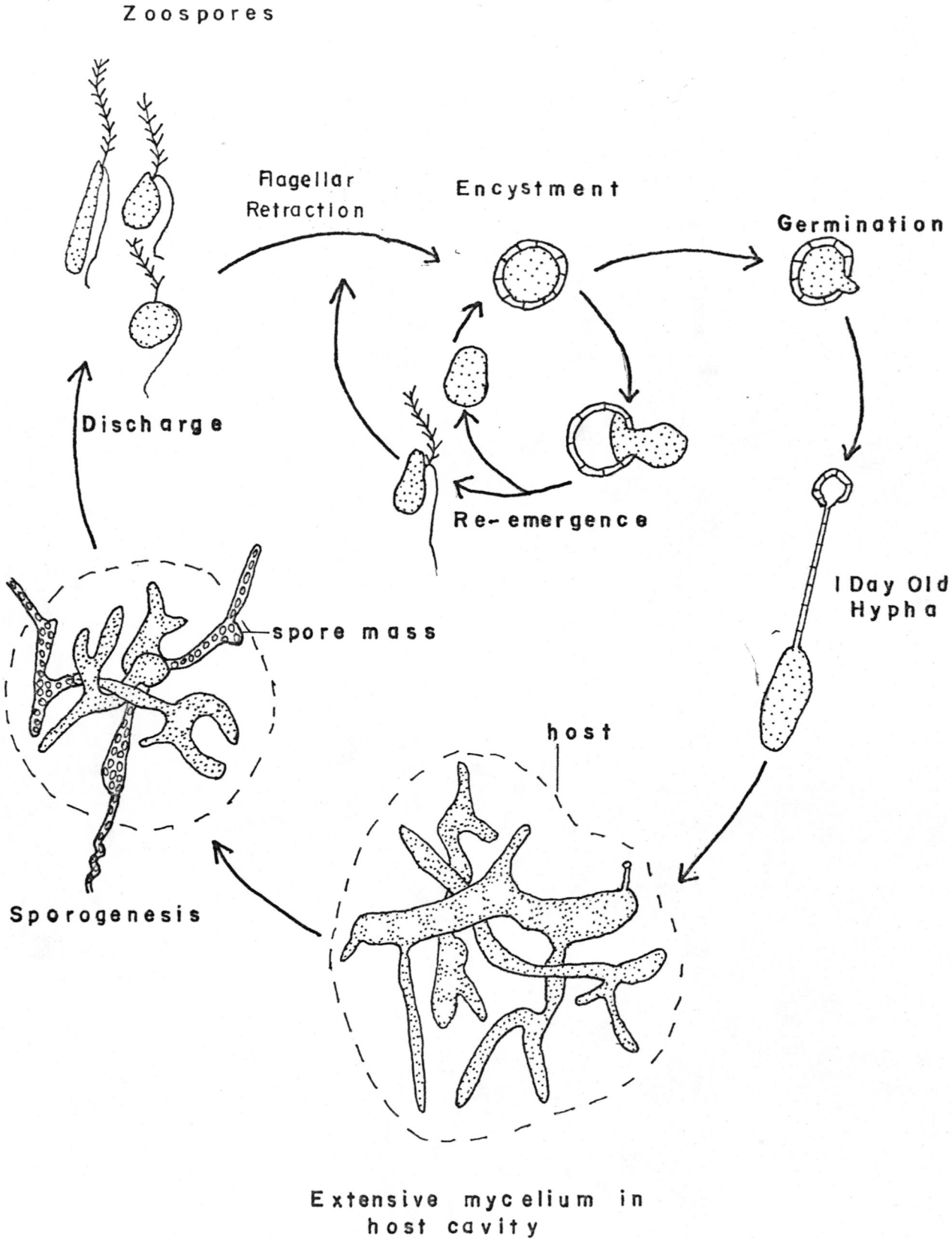
Encystment of H. milfordensis zoospores closely parallels the three stages of encystment described for P. parasitica by Hemmes and Hohl (1971). The first stage involves the retraction and/or casting off of flagella, and the release of adhesive fibrous material from type A vesicles (see Figs. 91, 92). According to Koch (1968) detachment of flagella is rare in fungal zoospores; however, detachment of at least one flagellum is common not only in H. milfordensis but L. callinectes as well (Bland and Amerson, 1973). Retraction of flagella in H. milfordensis was not studied in detail; however, that such occurs has been confirmed by the presence of the 9+2 flagellar axoneme in thin sections through the interior of encysting spores. Another part of the first stage of encystment in H. milfordensis adhesion of zoospores to solid substrate via material from type A vesicles, was described also for zoospores of P. palmivora by Sing and Bartnicki-Garcia (1975). During this process, fibers released from the vesicles may have a dual function; adhesion and formation of the external cyst wall. However, the latter of these functions is more evident in the second stage of encystment.

In H. milfordensis the second stage of encystment involves the rounding-up of the spore, the internal rearrangement of organelles, and the production of the cyst wall. Although basically similar to the events described by Hemmes and Hohl (1971) for P. parasitica, in H. milfordensis release of wall material from type A vesicles occurs simultaneously with rounding of the plasmalemma. Also occurring in the

second stage, but not observed previously, is the movement and modification of phospholipid bodies. These vesicles, which have been described previously as striate (Grove,1971), are only modified during encystment; otherwise, they are dense and homogeneous.

The third stage of encystment is characterized by the completely formed, spined cyst wall, the stable arrangement of internal organelles, and complete modification of phospholipid bodies to empty scalloped fringe vesicles. Structurally, the cyst wall of H. milfordensis resembles that described for S. ferax and D. sterile (Heath and Greenwood, 1970); however, the method of spine production in H. milfordensis is different. The claim of Heath and Greenwood (1970) that the spines form internal bar structures is not evident in H. milfordensis. In another study, Meier and Webster (1954) proposed that spines of S. ferax, S. parasitica, and Isoachlya unispora were externally produced by the adhesion of mastigonemes which had been cast off from the tinsel flagellum during retraction. In H. milfordensis; however, it appears that the spines are formed via the condensation of fiber bundles in discrete areas around the internal cyst wall.

Figure 1. Proposed asexual life cycle of *Haliphthoros milfordensis*.


Zoospores
Flagellar Retraction
Encystment
Germination
Discharge
Re-emergence
1 Day Old Hypha
spore mass
host
Sporogenesis
Extensive mycelium in host cavity

Figures 2-5. Zoospores of H. milfordensis. Note variety of shapes.

Figure 6. Whole mounted, shadowed zoospore showing anterior tinsel (Ft) and posterior whiplash (Fw) flagella.

Figure 7. Scanning electron micrograph of a zoospore showing longitudinal groove (lg) and sub-apical point of flagellar attachment. Scanning electron micrograph courtesy of C. E. Bland.

Figure 8. Flagellar retraction with vesicle forming at the distal end of the flagellum.

Figure 9. Zoospores containing spherical refractive granules (rg).

Figure 10. Whole mount, shadowed, tinsel flagellum showing arrangement of mastigonemes. Note terminal fine hairs.

Figure 11. Whole mount, shadowed, whiplash flagellum. Note the fine lateral hairs.

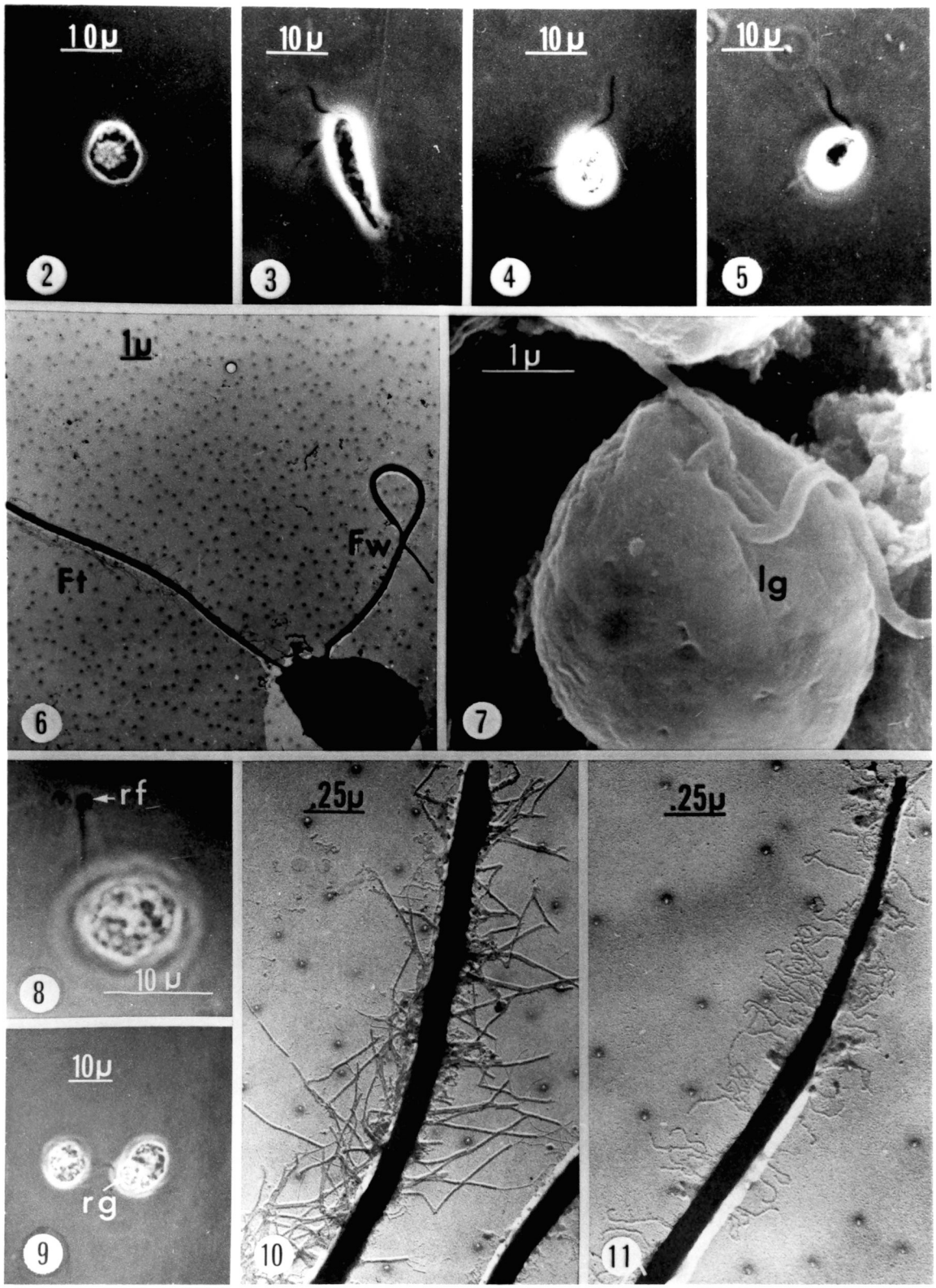

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Figure 12. Shadowed whole mount of zoospore showing presence of internal spherical, dense bodies (rg).

Figure 13. Encysted spore of H. milfordensis with thickened cyst wall.

Figure 14. Re-emerging zoospore passing through narrow opening in cyst wall. Note fine spines in cyst wall (cw).

Figure 15. Germination in liquid culture of encysted spores. Note empty spore cysts and long slender germ tubes.

Figure 16. Release of a large spherical vesicle from encysted spore.

Figure 17. Empty spore cyst showing small opening through which a re-emerging zoospore has escaped. Not thickened cyst wall.

Figure 18. Encysted zoospores which have clumped together in liquid broth culture.

Figure 19. Spore germination in liquid culture. Note the long slender germ tube, the empty spore cyst (esc), and the constricted areas (ca) occurring along the length of the germ tube.

Figure 20. Germination on solid agar medium. Note that the germ tube is much shorter and wider than in liquid culture.

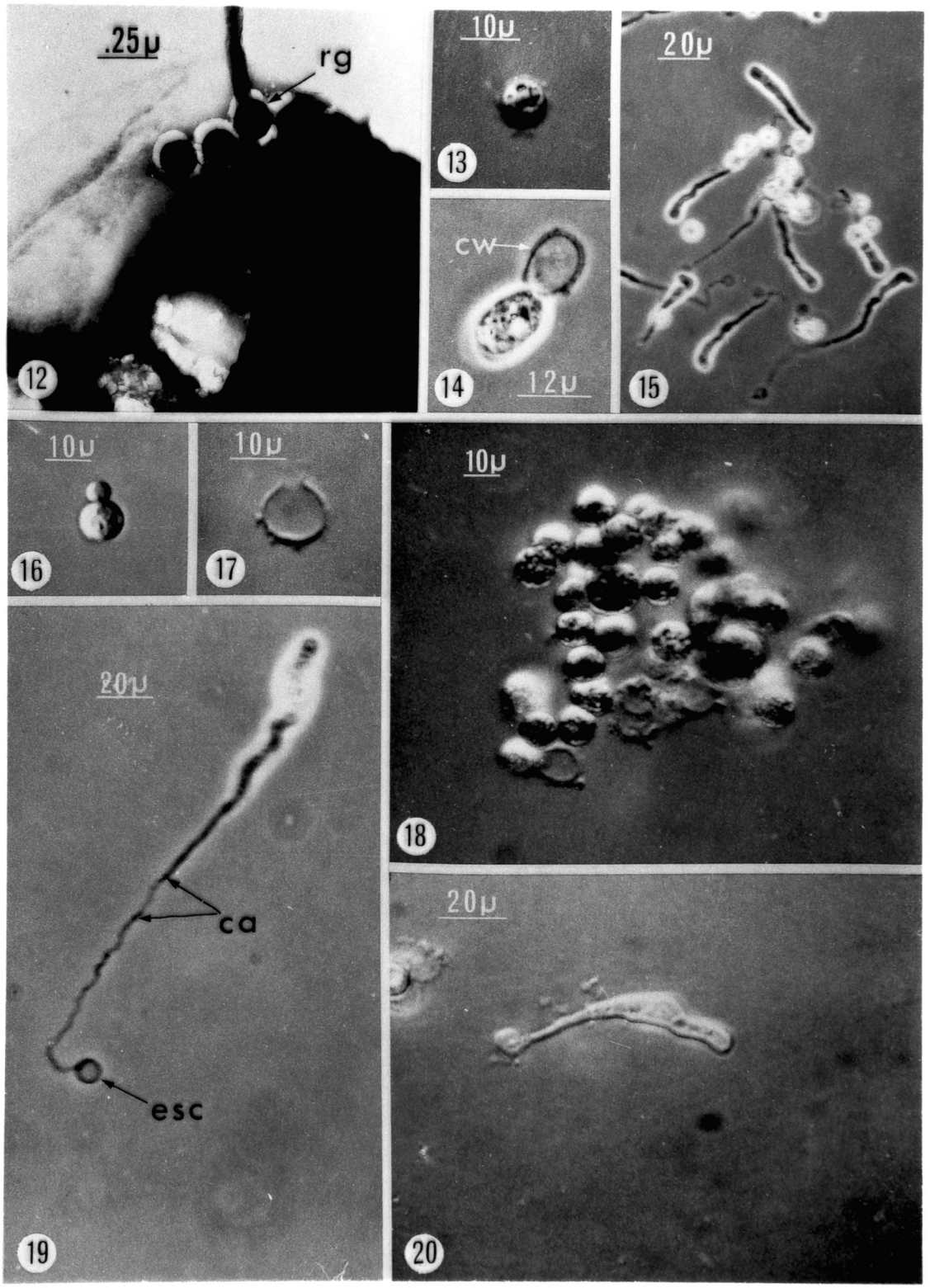
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Figure 21. Early vegetative growth showing initial branching of a newly formed hypha.

Figure 22. Hyphal fragment in liquid culture.

Figure 23. Extensive vegetative mycelium after four days of growth on agar medium.

Figure 24. Vegetative growth in liquid culture. Note large gemma-like structure (g).

Figure 25. Septum (s) delineated sporangium containing encysted spores (es).

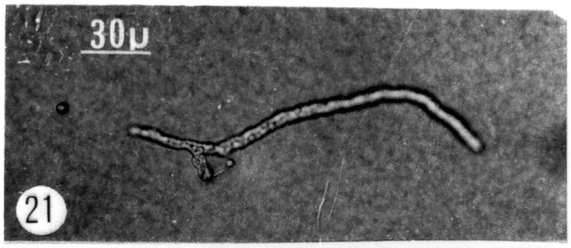
Figure 26. Fragmentation of the vegetative thallus via cytoplasmic constriction. Note constricted area (ca).

Figure 27. Highly vacuolate hypha characteristic of early sporogenesis.

Figure 28. Highly vacuolate thallus showing initial discharge tube (dt) formation.

Figure 29. Discharge tube formation from presporogenic hypha.

Figure 30. Continued growth of discharge tube from hypha.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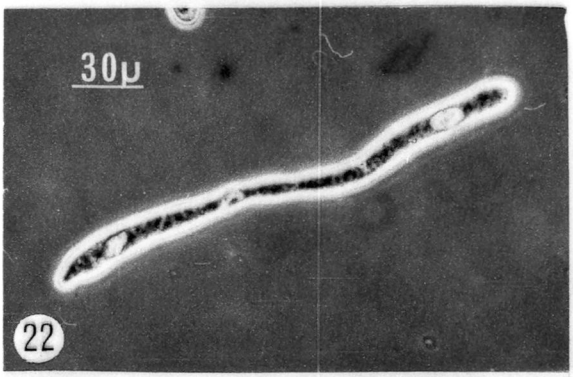
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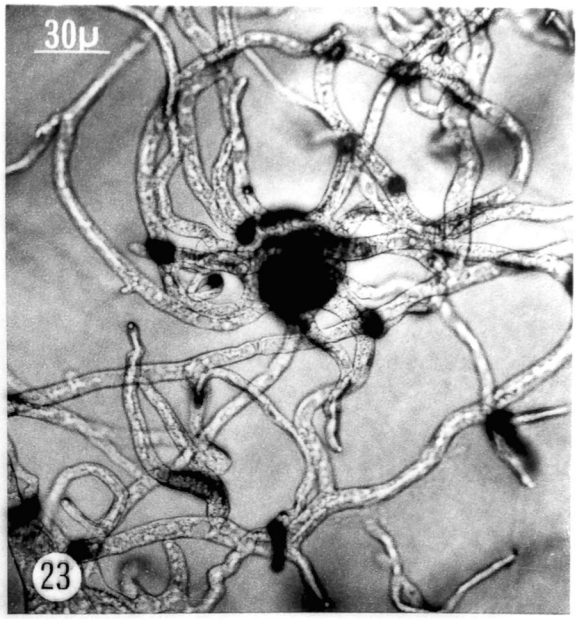
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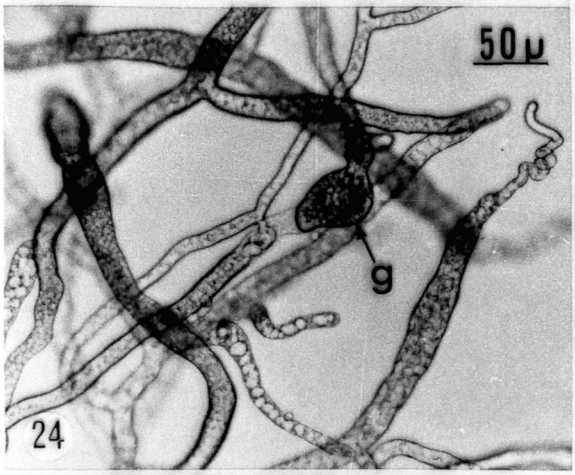
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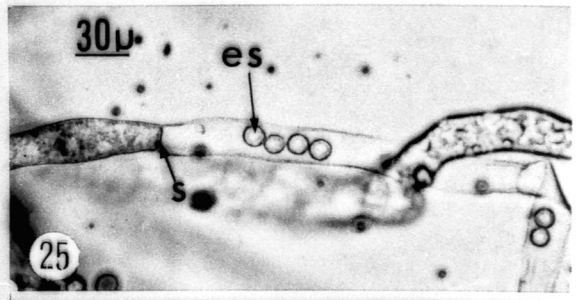
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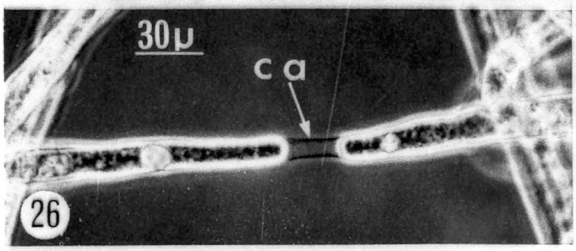
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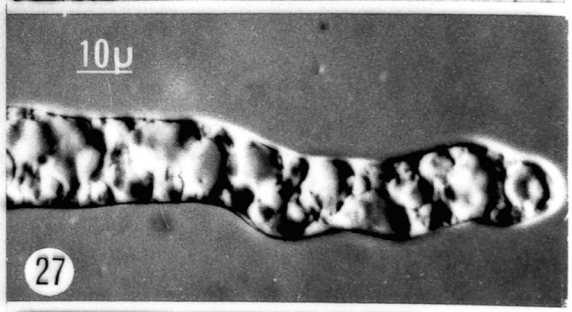
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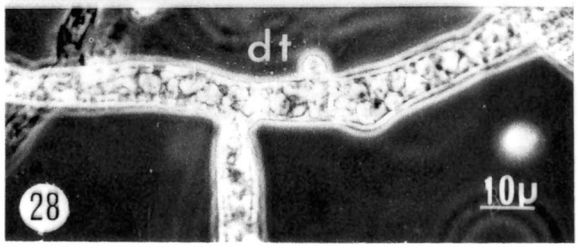
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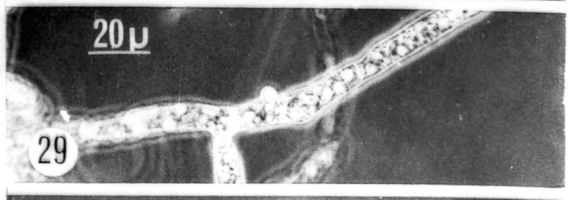
28
10µ


20µ
29

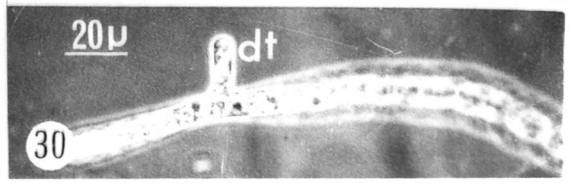

20µ
dt
30

Figure 31. Densely granulate cytoplasm in sporangium of H. milfordensis. Note cleavage furrows (cf).

Figure 32. Late sporogenesis within a sporangium. Individual spores are polygonal and flagella (f) are evident.

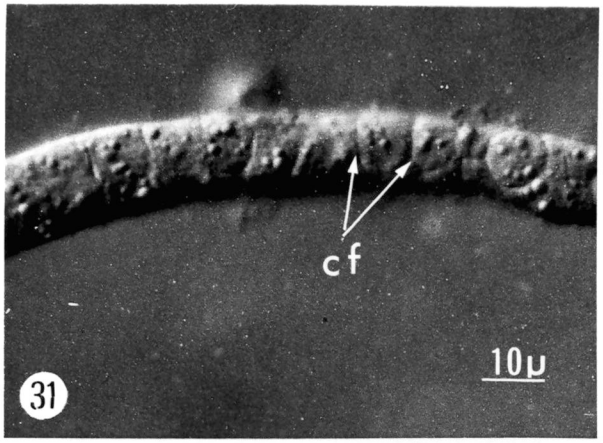
Figure 33. Spores within sporangium prior to discharge.

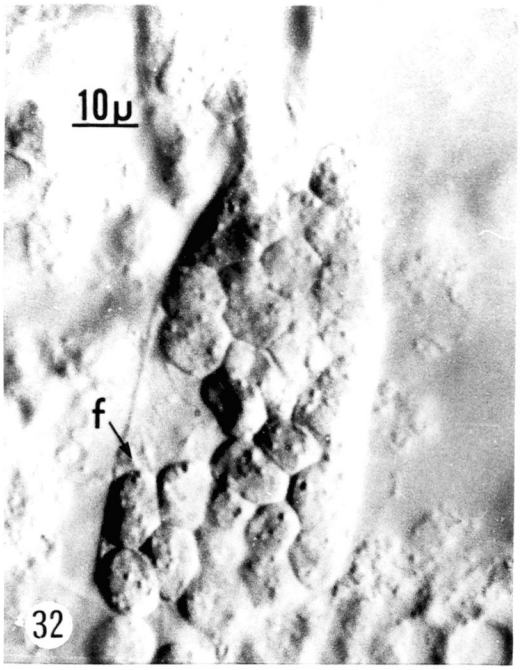
Figure 34. Zoospores within discharge tube (dt) prior to release. Note that the most distal zoospore has not completely cleaved.

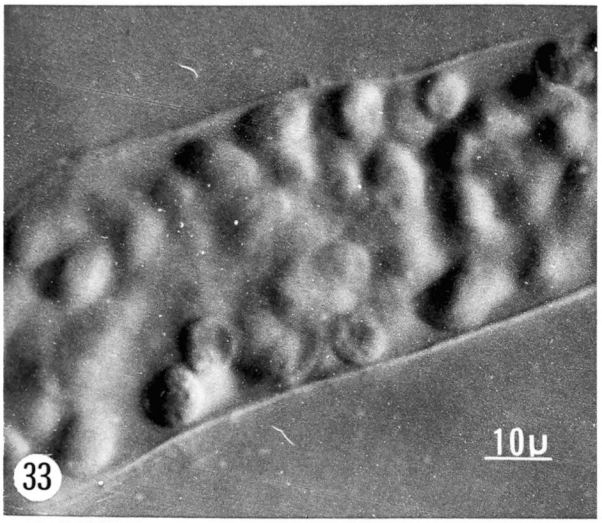
Figure 35. Densely granulate cytoplasm of discharge tube prior to spore cleavage. Note hyaline cap (hc).

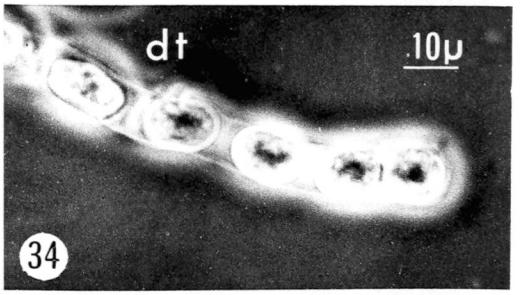
Figure 36. Zoospores within discharge tube (dt) prior to release.

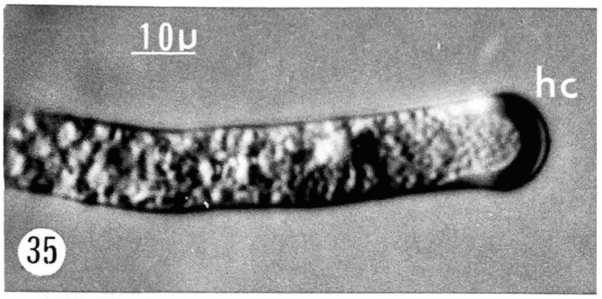
Figure 37. Cytoplasmic threads (ct) between zoospores.

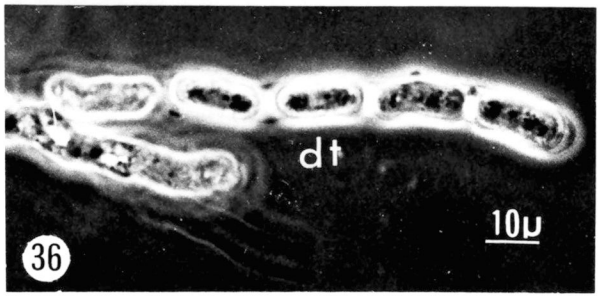

cf
10μ
31

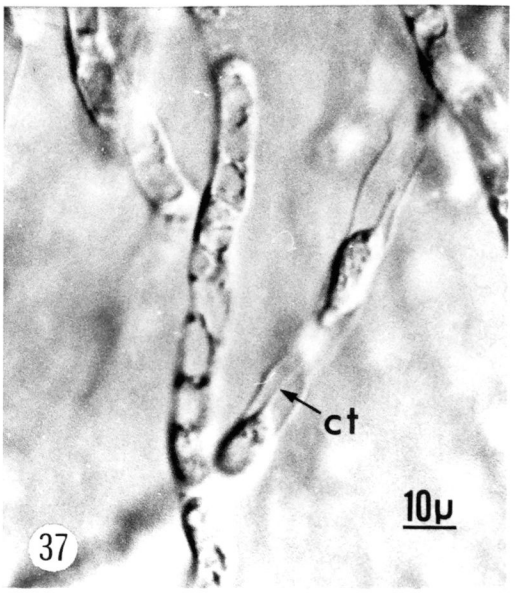

10μ
f
32


10μ
33


dt
10μ
34


10μ
hc
35


dt
10μ
36


ct
10μ
37

Figures 38-40. Vegetative growth of H. milfordensis on hemp seed; one, two and three days growth respectively.

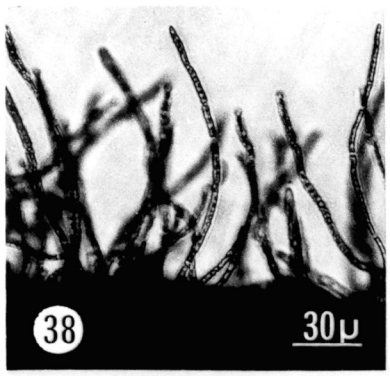
Figures 41-44. Day one through day four of vegetative growth on corn meal, dextrose agar.

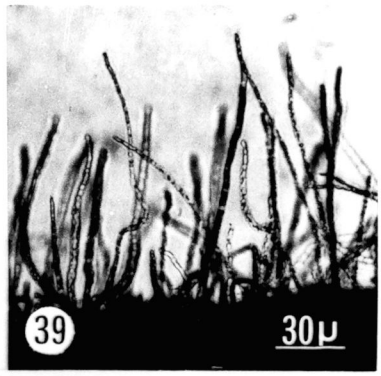
Figure 45. Penetration of brine shrimp exoskeleton by germ tubes.

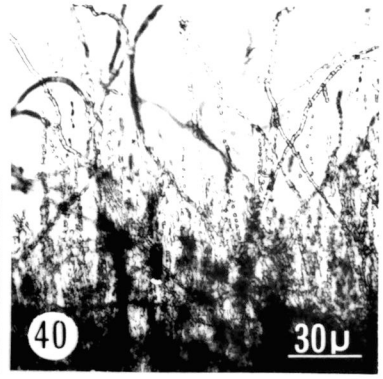
Figures 46-47. Vegetative growth within brine shrimp larva. Note that most of the host's cytoplasm has been consumed by the fungus.

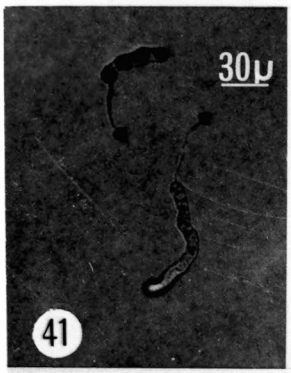
Figure 48. Discharge tubes on ova of the blue crab, Callinectes sapidus.

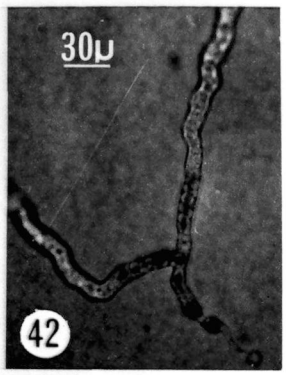
Figure 49. Discharge tubes on an ovum of the brine shrimp, Artemia salina.

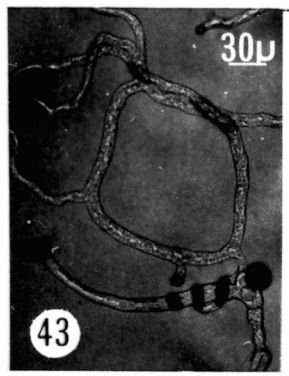

38
30µ


39
30µ

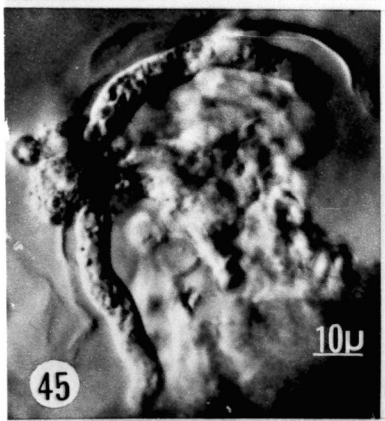

40
30µ

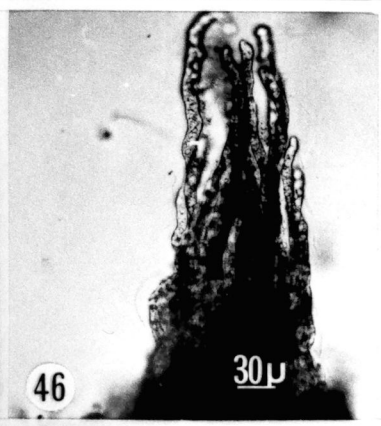

30µ
41

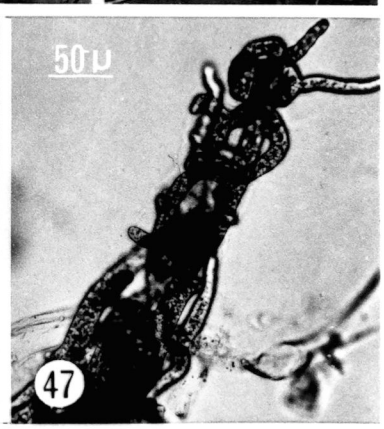

30µ
42

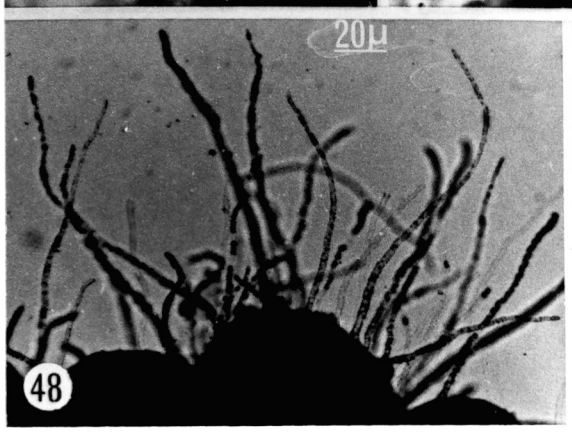

30µ
43


30µ
44


10µ
45


30µ
46


50µ
47

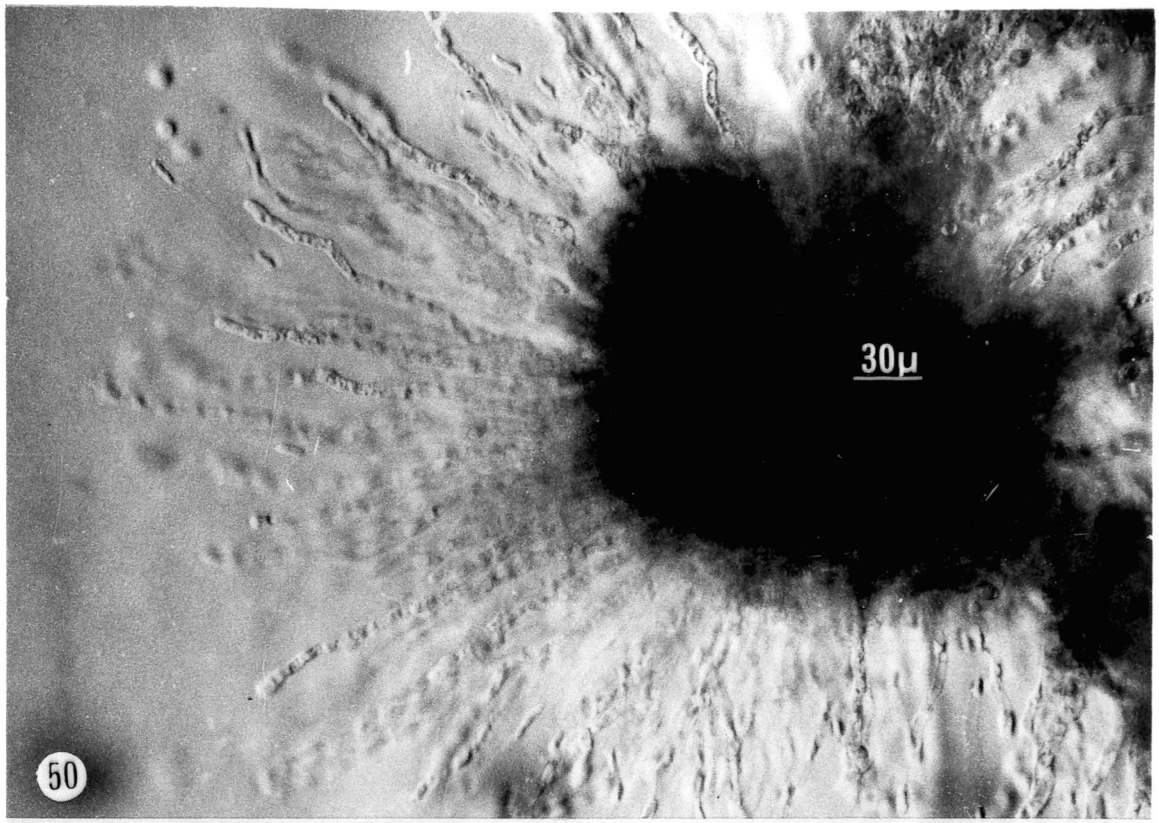

20µ
48

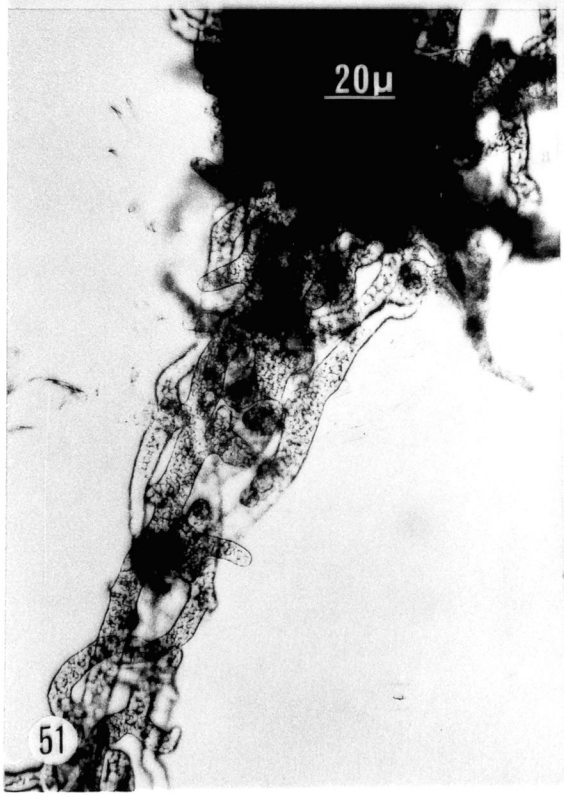

20µ
49

Figure 50. Discharge tubes radiating outward from the embryo cavity of a brine shrimp.

Figure 51. Posterior portion of brine shrimp larva which is completely filled with hyphae.

Figure 52. Shrimp gill with internal hyphae.


30μ
50


20μ
51

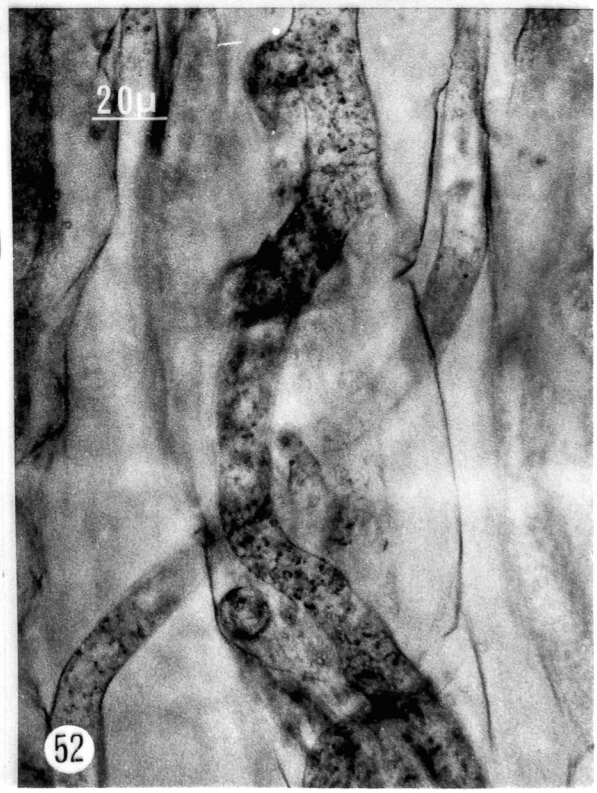

20μ
52

Figure 53. Electron micrograph of a zoospore showing internal arrangement of organelles and point of flagellar attachment. Note dense bodies located around the periphery of the spore.

Abbreviations: Pb - phospholipid bodies, pm - plasma membrane, A - type A vesicle, B - type B vesicle, N - nucleus, L - lipid

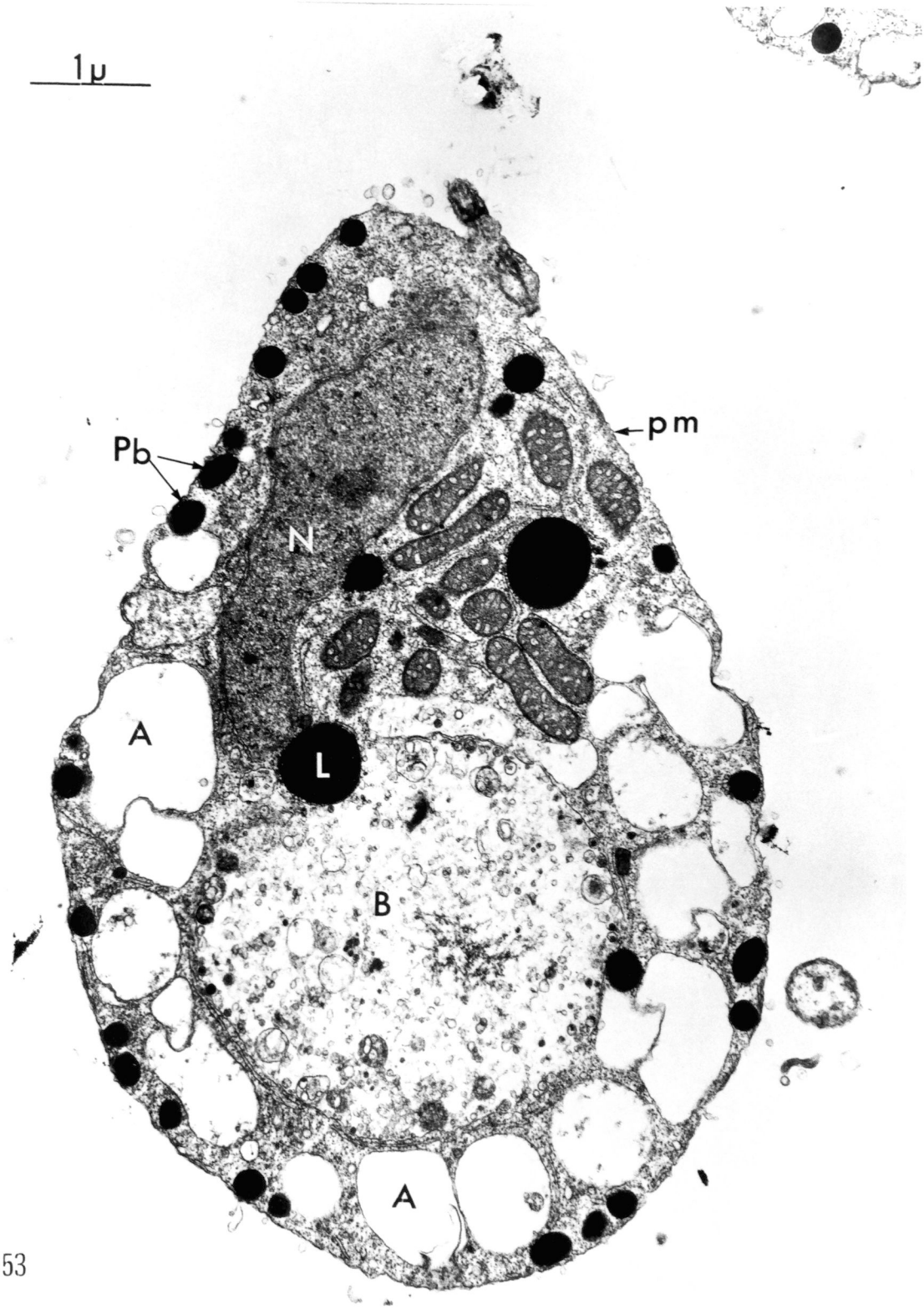

1μ
Pb
N
pm
A
L
B
A

Figure 54. Longitudinal section of zoospore showing posterior vesicles, peripheral dense bodies and cross section of a basal body.

Abbreviations: Pb - phospholipid bodies, pm - plasma membrane, A - type A vesicle, B - type B vesicle, N - nucleus, L - lipid.

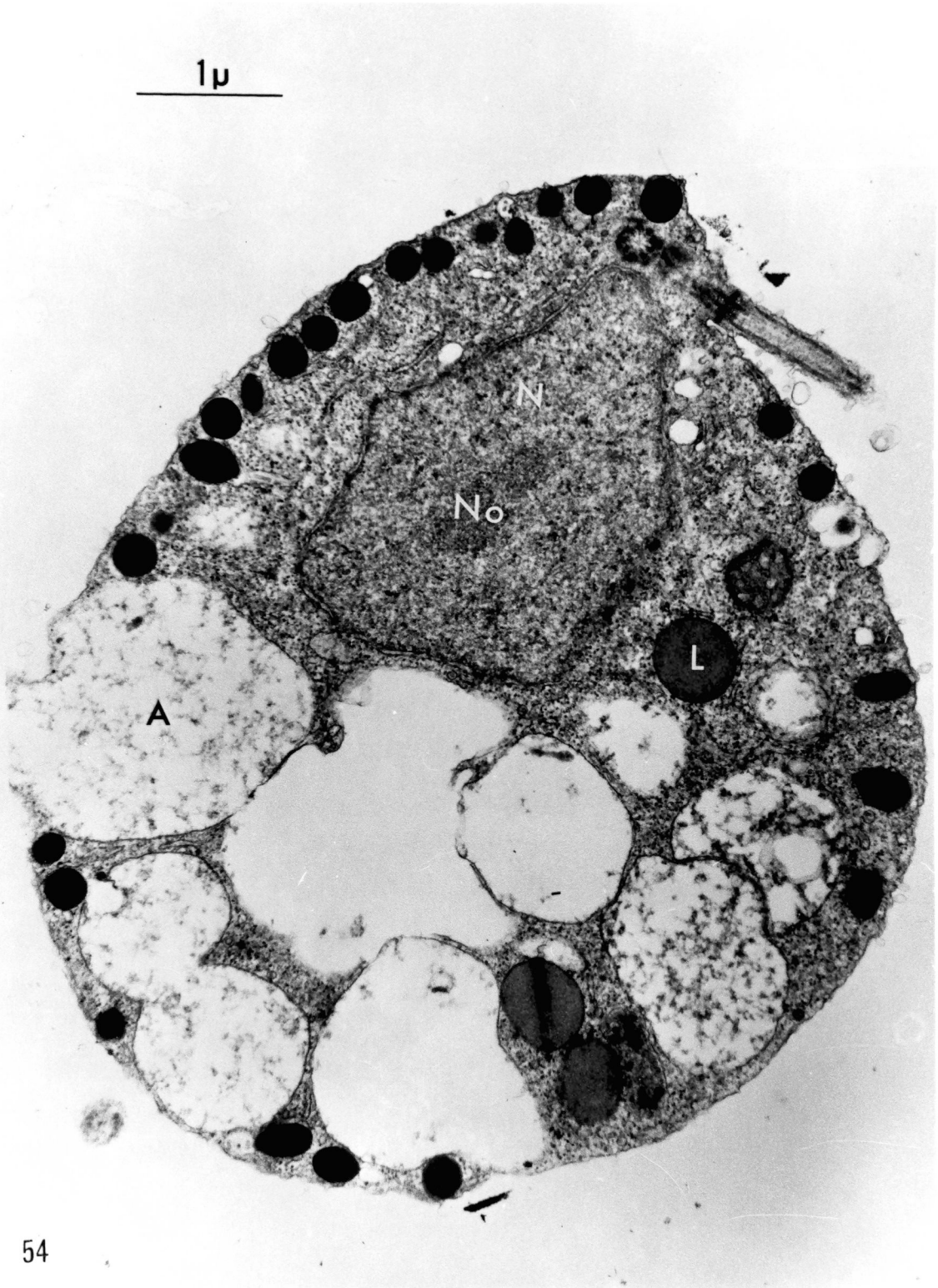
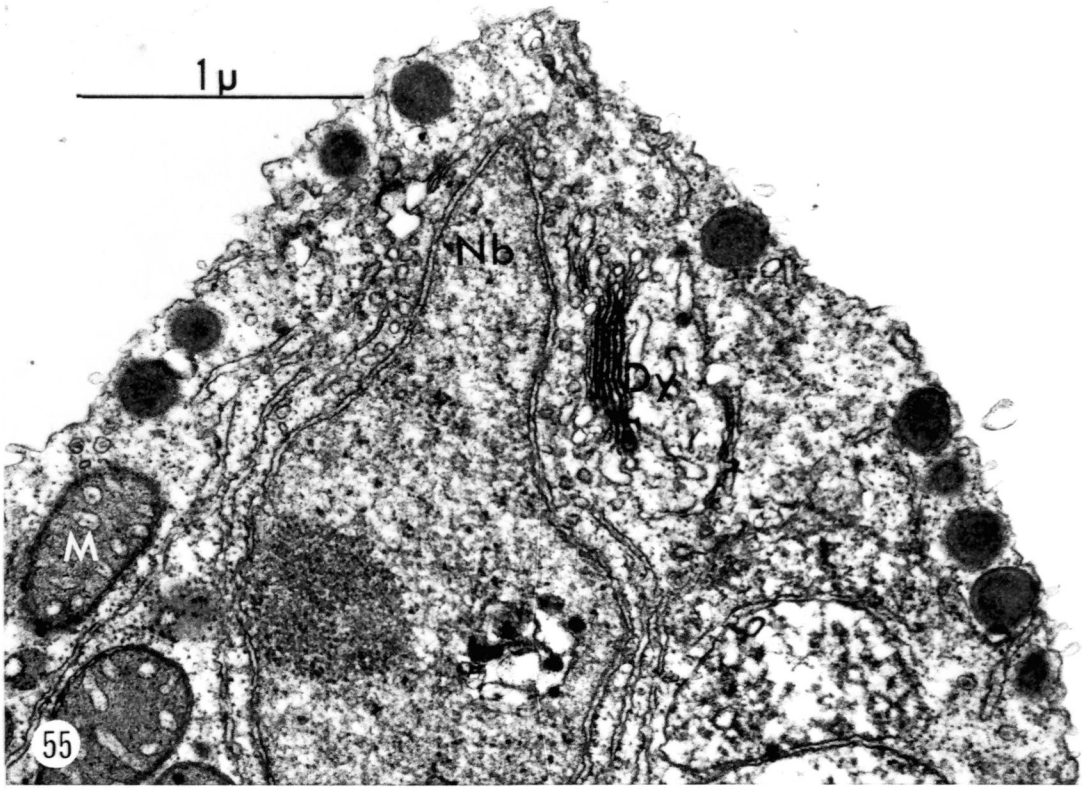

1μ
N
No
L
A

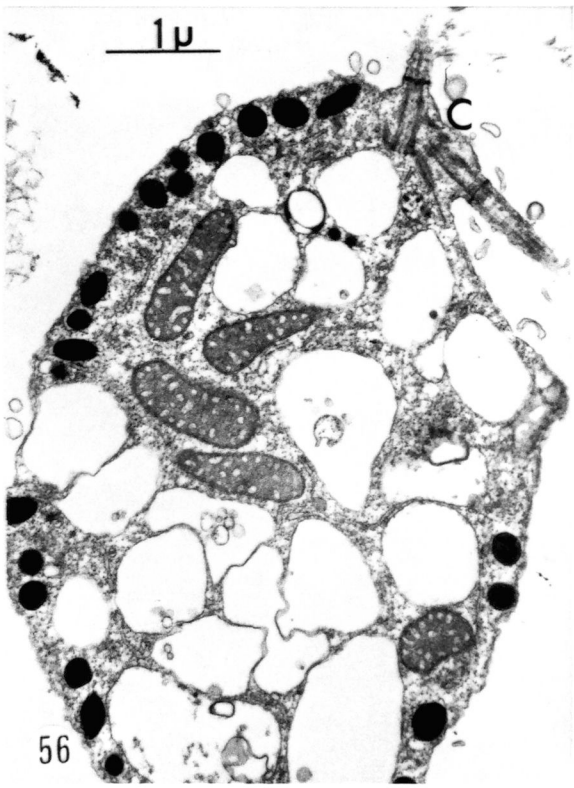
Figure 55. Anterior zoospore region showing nuclear beak with associated dictyosomes and sparse endoplasmic reticulum.

Figure 56. Longitudinal section of zoospore showing flagellar attachment and basal body structure.

Figure 57. Flagellar apparatus. Note rootlets, props, terminal plate, and dense connections.

Abbreviations: Nb - nuclear beak, M - mitochondria, C - crown area, Pb - phospholipid bodies, Ft - tinsel flagellum, Fw - whiplash flagellum, BB - basal body, Pr - prop, R - rootlet, Tp - terminal plate, Dc - dense connection.


1μ
Nb
Dy
M
55


1μ
C
56

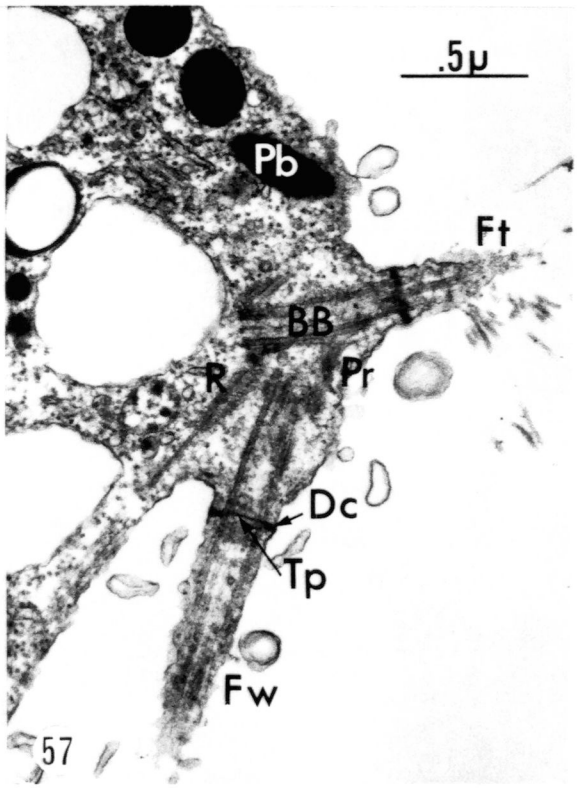

.5μ
Pb
Ft
BB
R
Pr
Dc
Tp
Fw
57

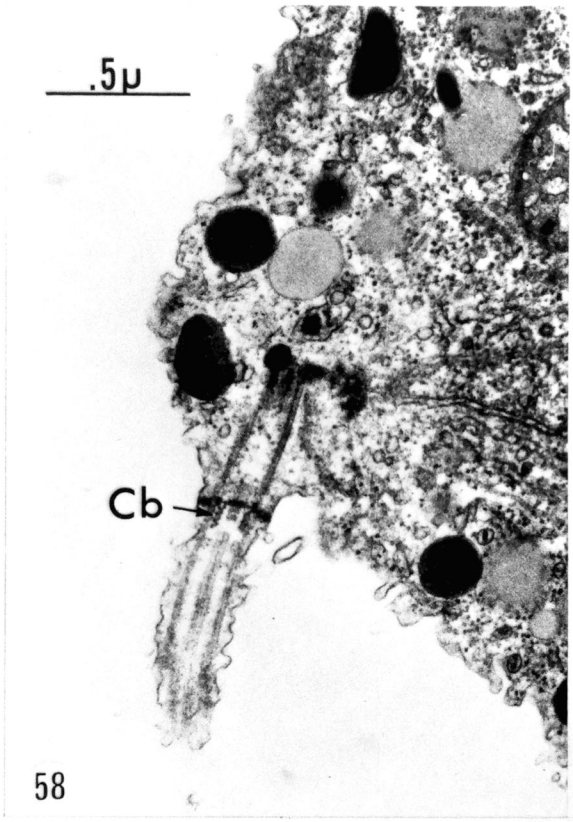
Figure 58. Proximal portion of flagellum showing coiled body just above the terminal plate.

Figure 59. Anterior protion of zoospore showing position of an extended rootlet just beneath the plasmalemma.

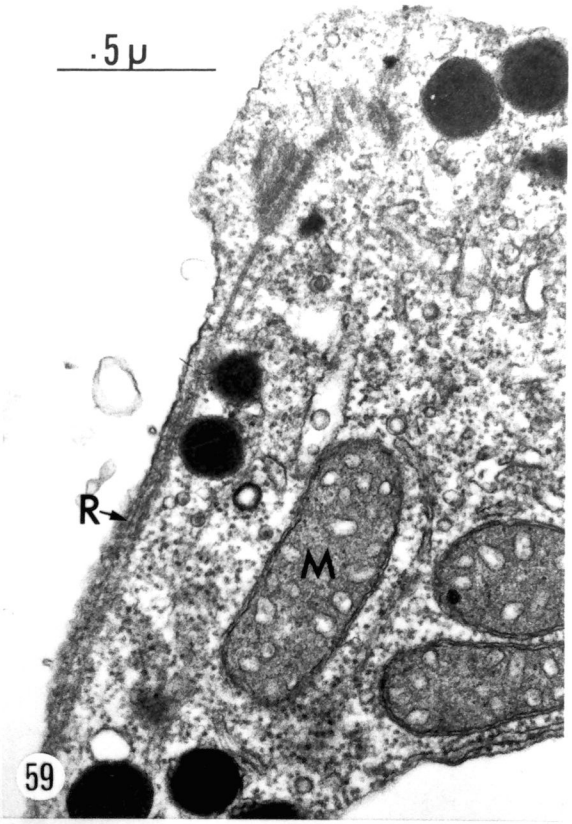
Figure 60. Nucleus of a zoospore with nuclear inclusion. Note associated dictyosome.

Figure 61. Nuclear region of a zoospore showing close association of mitochondria to nucleus.

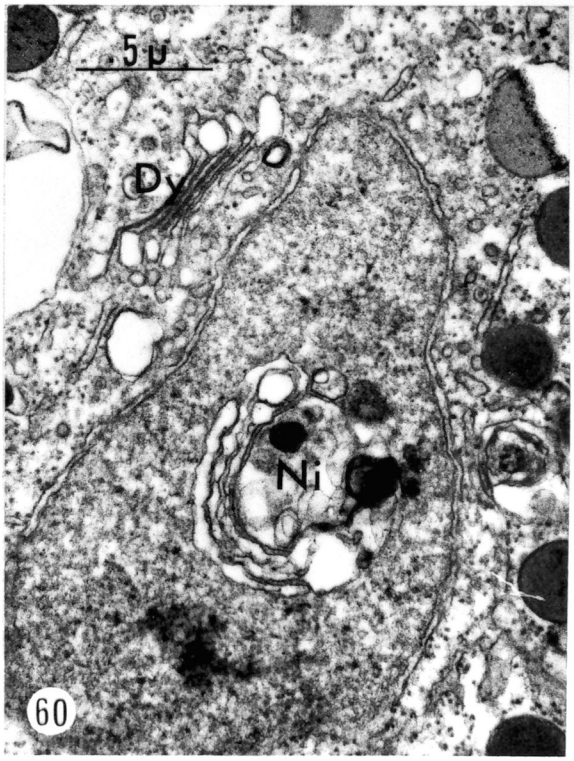
Abbreviations: Cb - coiled body, R - rootlet, M - mitochondria, Dy - dictyosome, N - nucleus, Ni - nuclear inclusion, ER - endoplasmic reticulum.



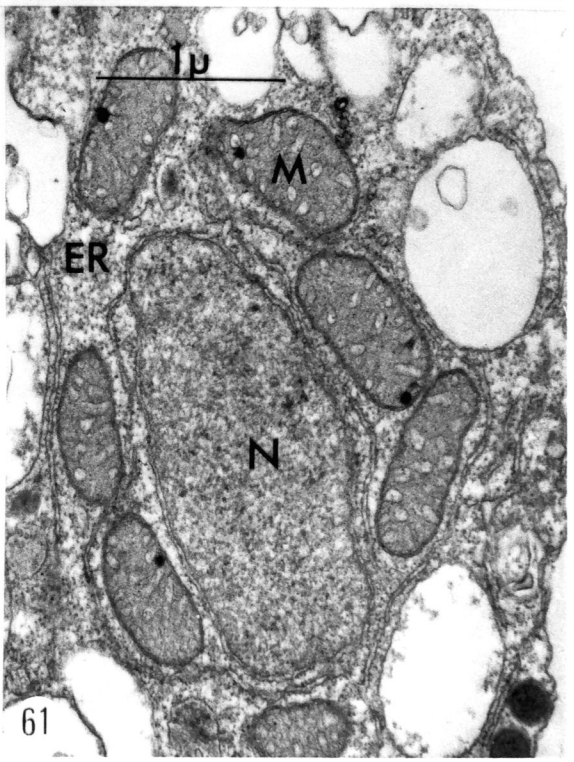
58



59



60



61

Figures 62-67. Vesicles of H. milfordensis spores. Fig. 62. Type A vesicles containing granular to fibrous material. Fig. 63. Type B vesicle. Fig. 64. Early stage in development of type B vesicle. Fig. 65. Intermediate stage of type B vesicle formation. Fig. 66. Dense phospholipid bodies. Fig. 67. Cell wall vesicles forming association with the nucleus, endoplasmic reticulum and mitochondria.

Abbreviations: A- type A vesicle vesicle, B - type B vesicle, Be - early type B vesicle, Bi - intermediate type B vesicle, Pb - phospholipid body, N - nucleus, cwv - cell wall vesicle.

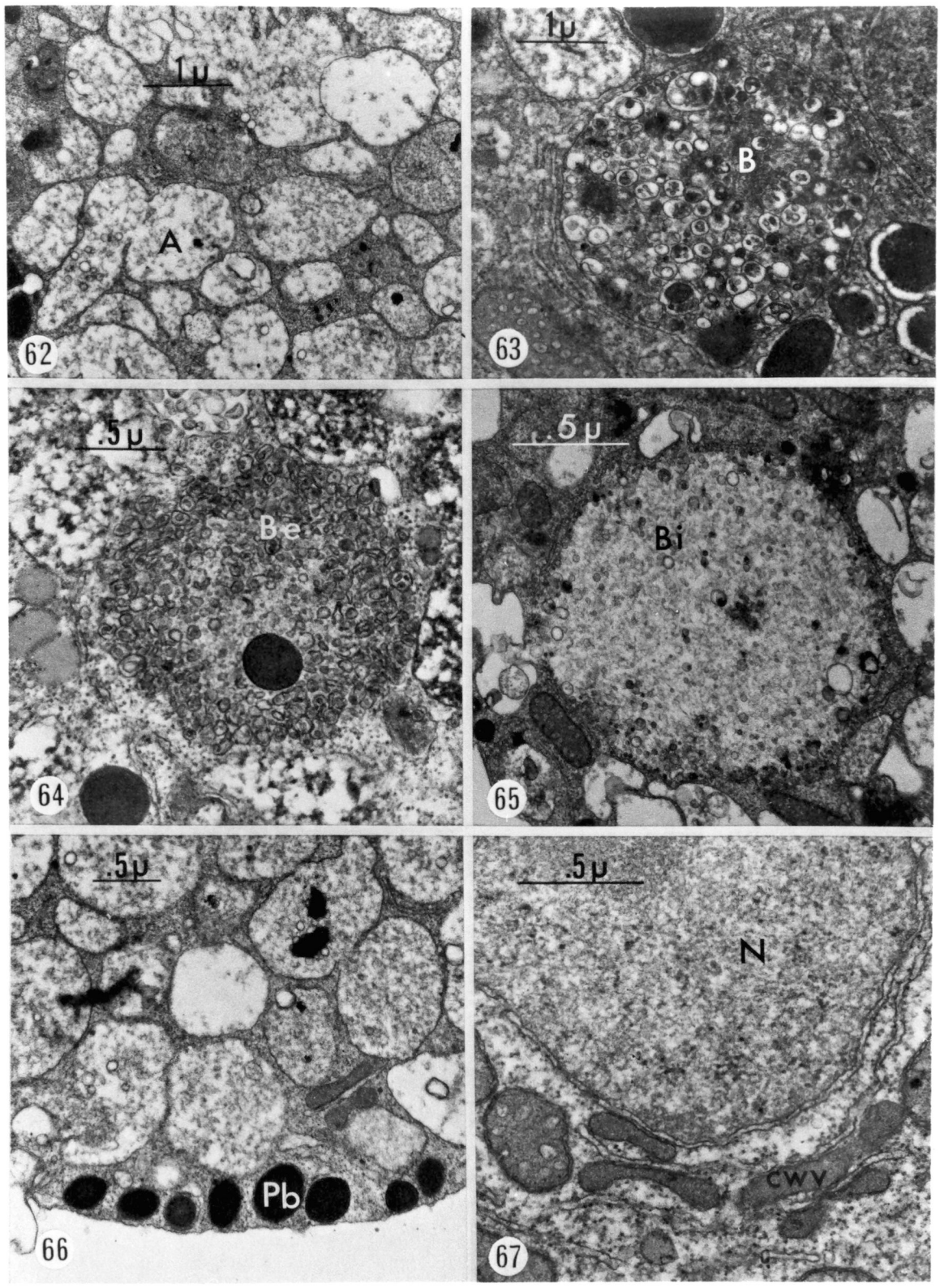
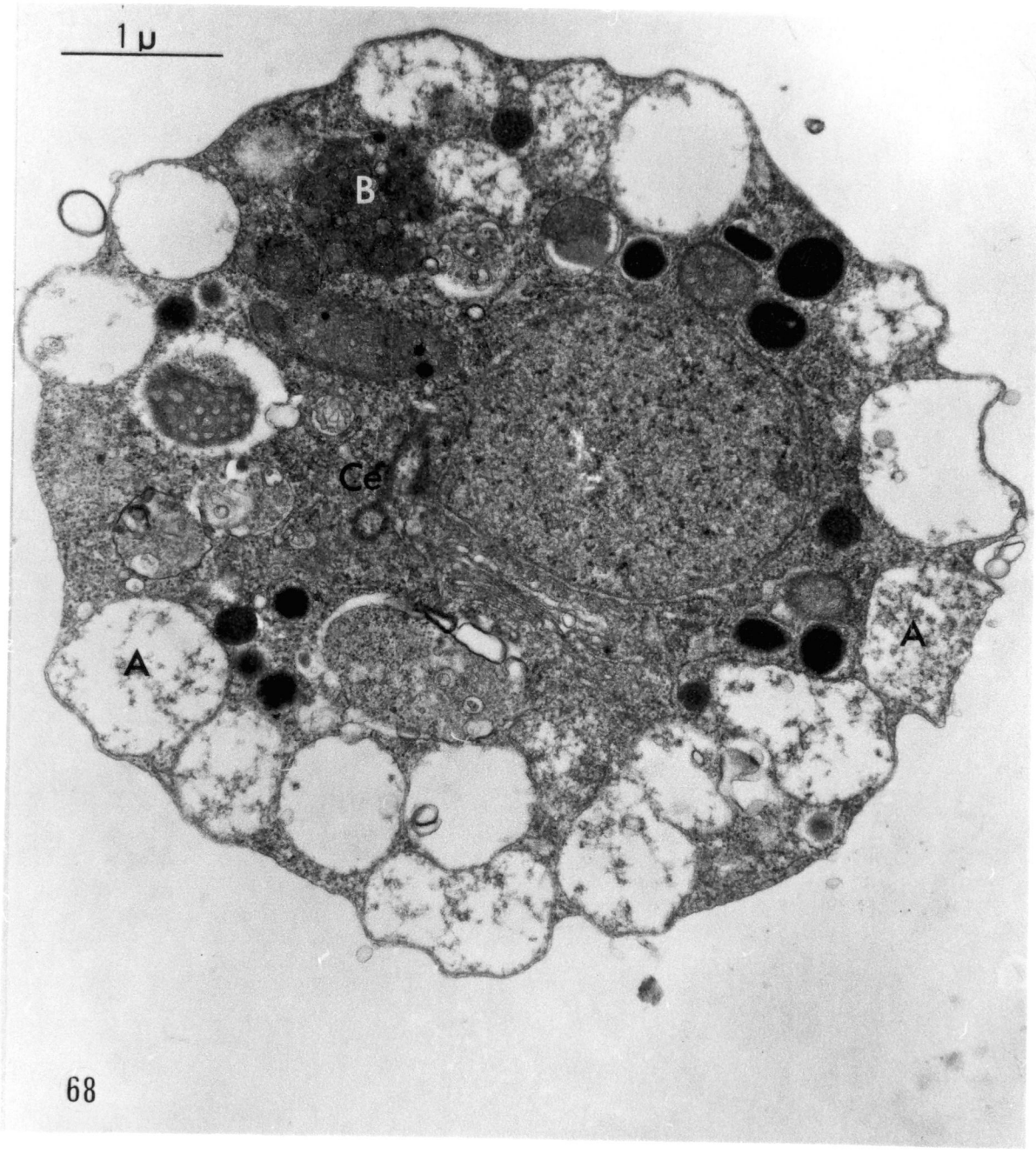

1μ
A
62
1μ
B
63
.5μ
Be
64
.5μ
Bi
65
.5μ
Pb
66
.5μ
N
CWV
67

Figure 68. Electron micrograph of an encysting spore. Note peripheral arrangement of type A vesicles which are releasing fibrous material externally. Note also central location of basal bodies.

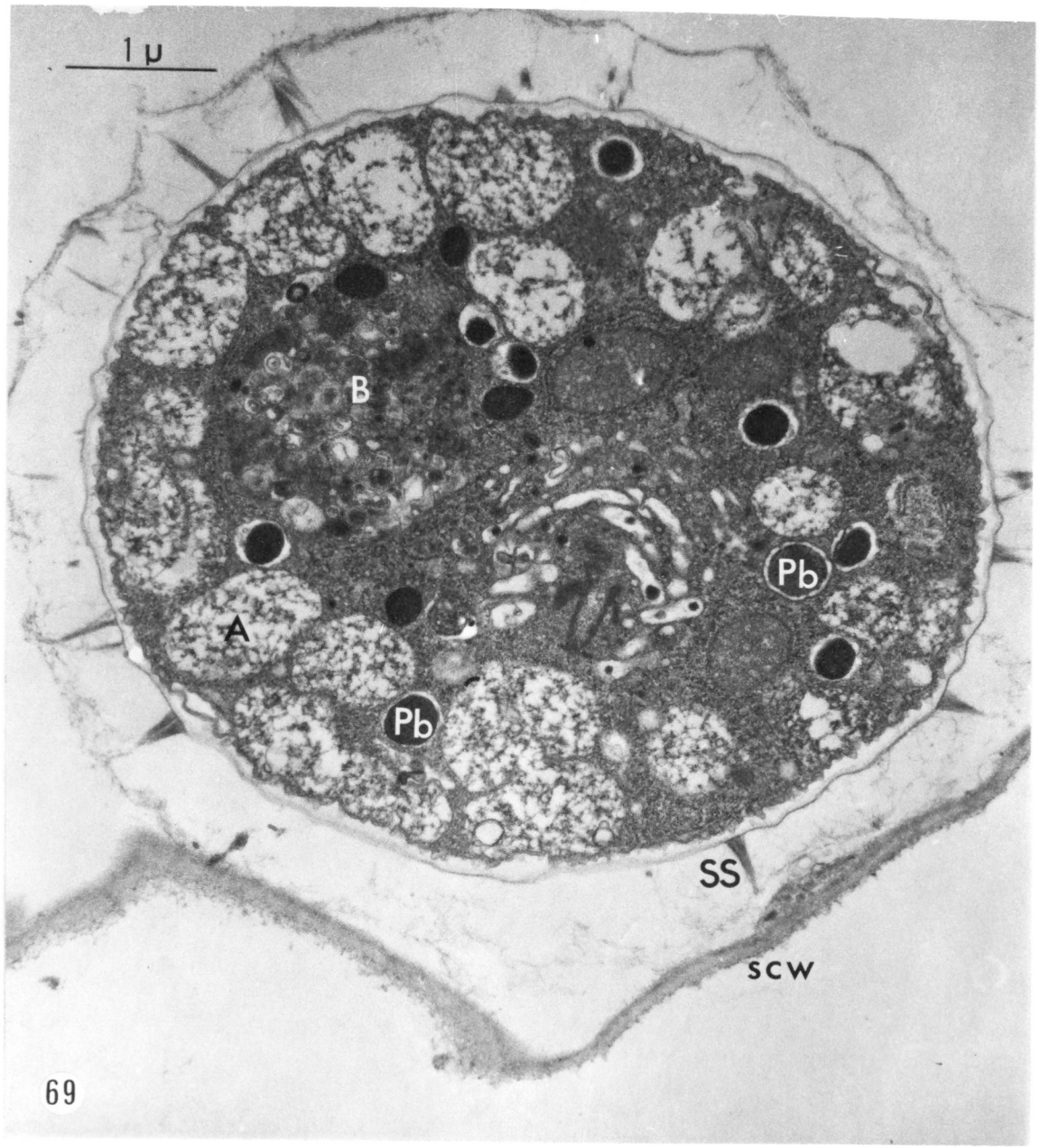
Abbreviations: B - type B vesicle, Ce - centrioles, A - type A vesicle.



68

Figure 69. Fully encysted spore showing the outer three layered cyst wall containing striated spines. Note the internal arrangement of organelles.

Abbreviations: A - type A vesicle, B - type B vesicle, PB - phospholipid bodies, SS - striated spine, scw - sporangium cell wall



69

Figure 70. Encysted zoospore showing internal organelle arrangement.

Abbreviations: A - type A vesicle, B - type B vesicle, fp - flimmer packet, L - lipid, M - mitochondria, N - nucleus.

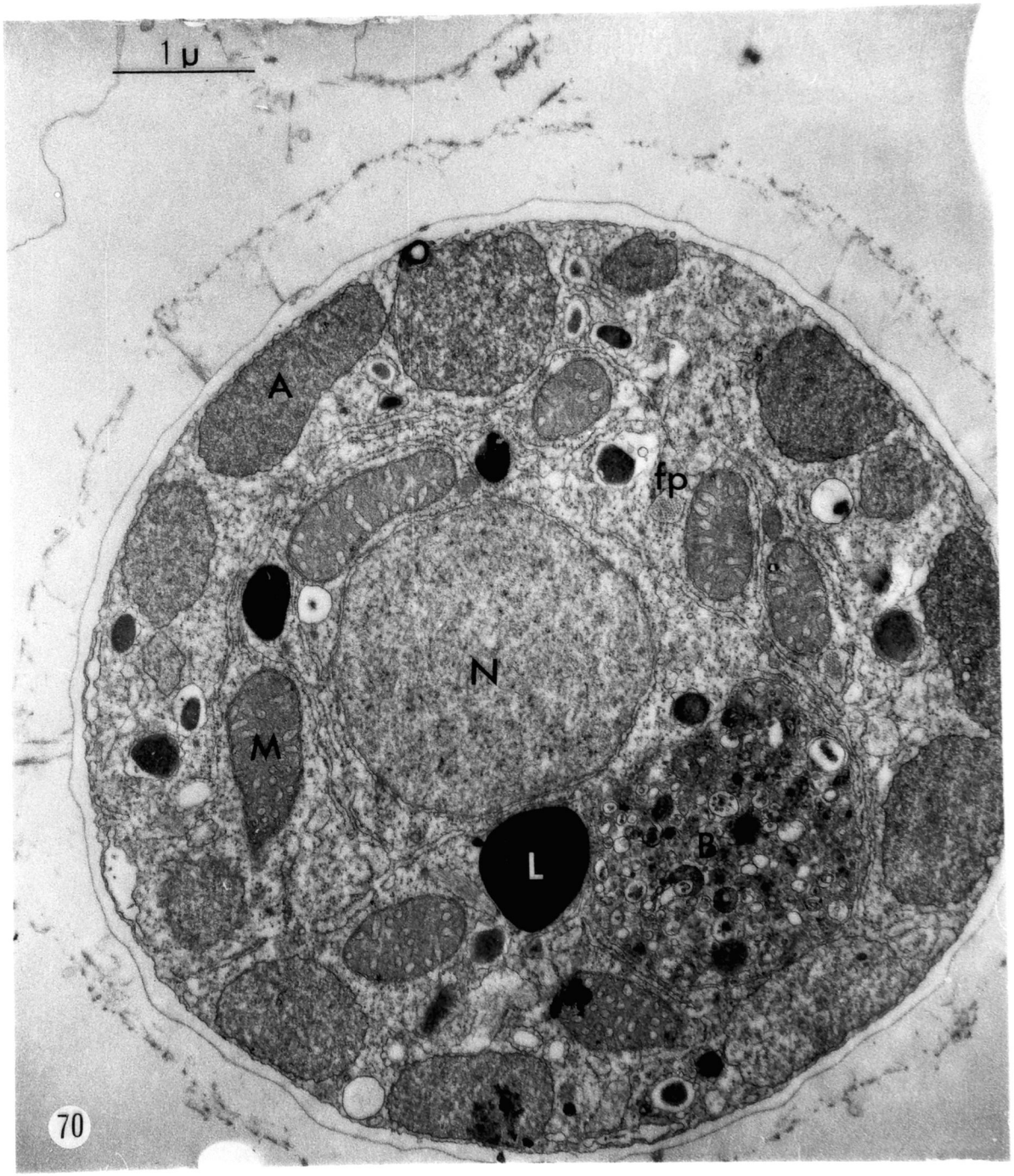
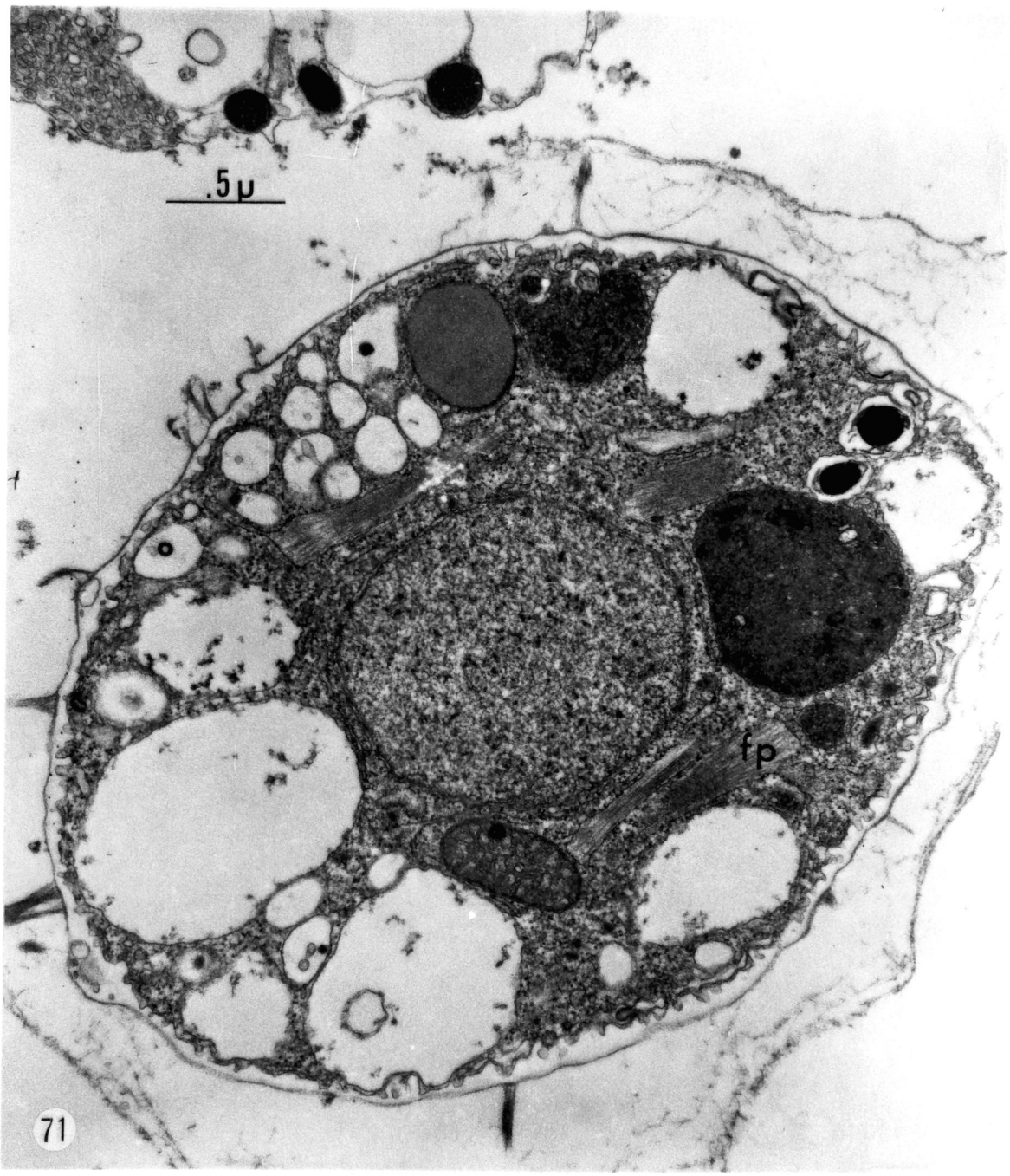

1 µ
A
fp
N
M
L
B
70

Figure 71. Encysted zoospore with abundant flimmer packets throughout cytoplasm.

Abbreviations: fp - flimmer packet


.5μ
fp
71

Figures 72-75. Cyst wall formation during encystment. Fig. 72. Fibrous mat formed from the release of material from type A vesicles. Fig. 73. Separation of the fibrous material from spore surface. Fig. 74. Fiber bundles left after separation of fibrous layer from spore surface. Fig. 75. Striated spine formed from fiber bundles.

Abbreviations: Fb - fiber bundle, fp - flimmer packet, SS - striated spine.

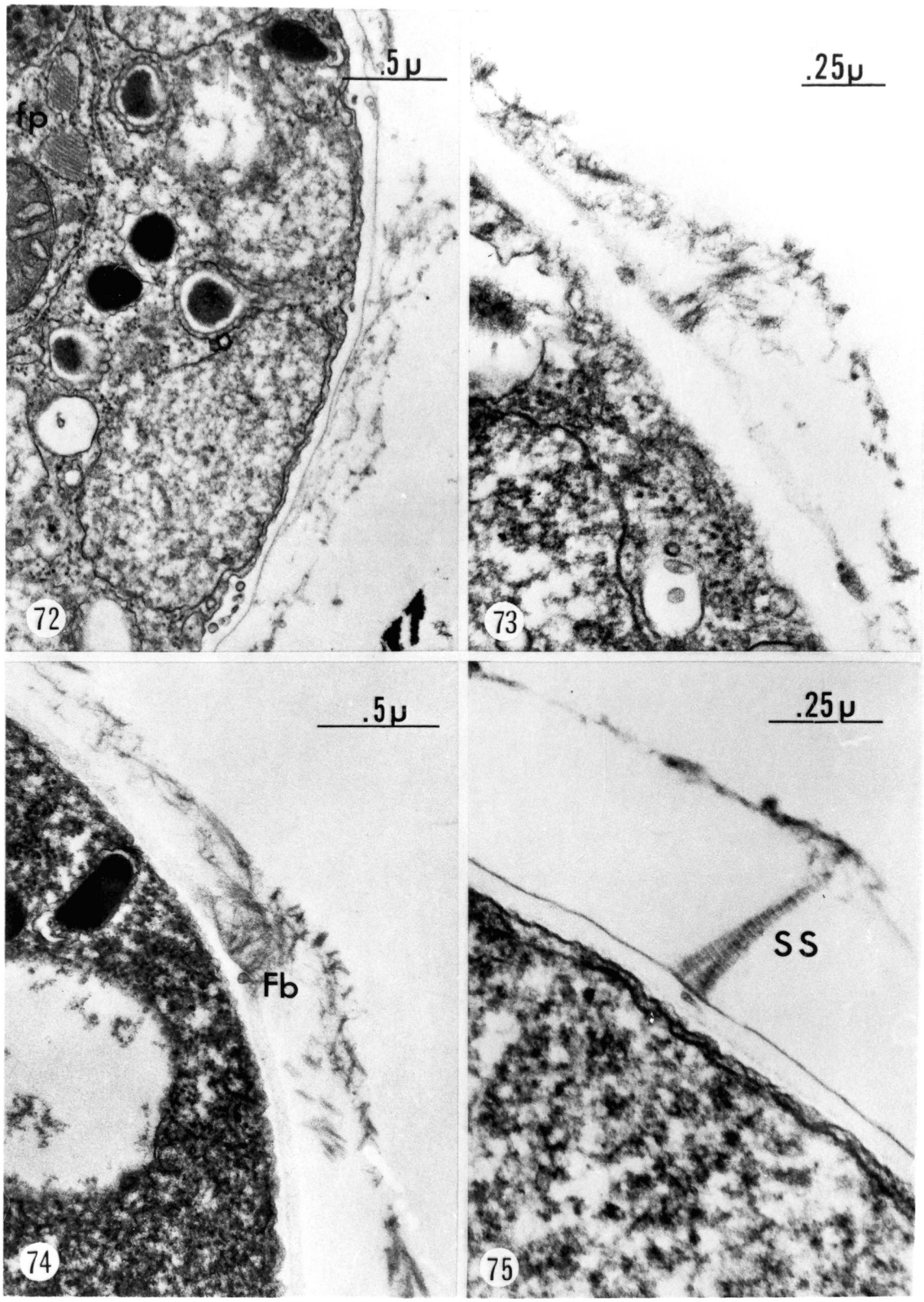

.5μ
fp
72
.25μ
73
.5μ
Fb
74
.25μ
SS
75

Figure 76-77. Formation and movement of cell wall vesicles. Fig. 76. Formation of cell wall vesicles in nuclear region. Fig. 77. Cell wall vesicles have migrated to the plasmalemma.

Figure 78. Type C vesicle formed during late encystment.

Figure 79. Encysted spore containing axoneme of retracted flagellum.

Abbreviations: cwv - cell wall vesicle, C - type C vesicle, Fr - retracted flagellum.

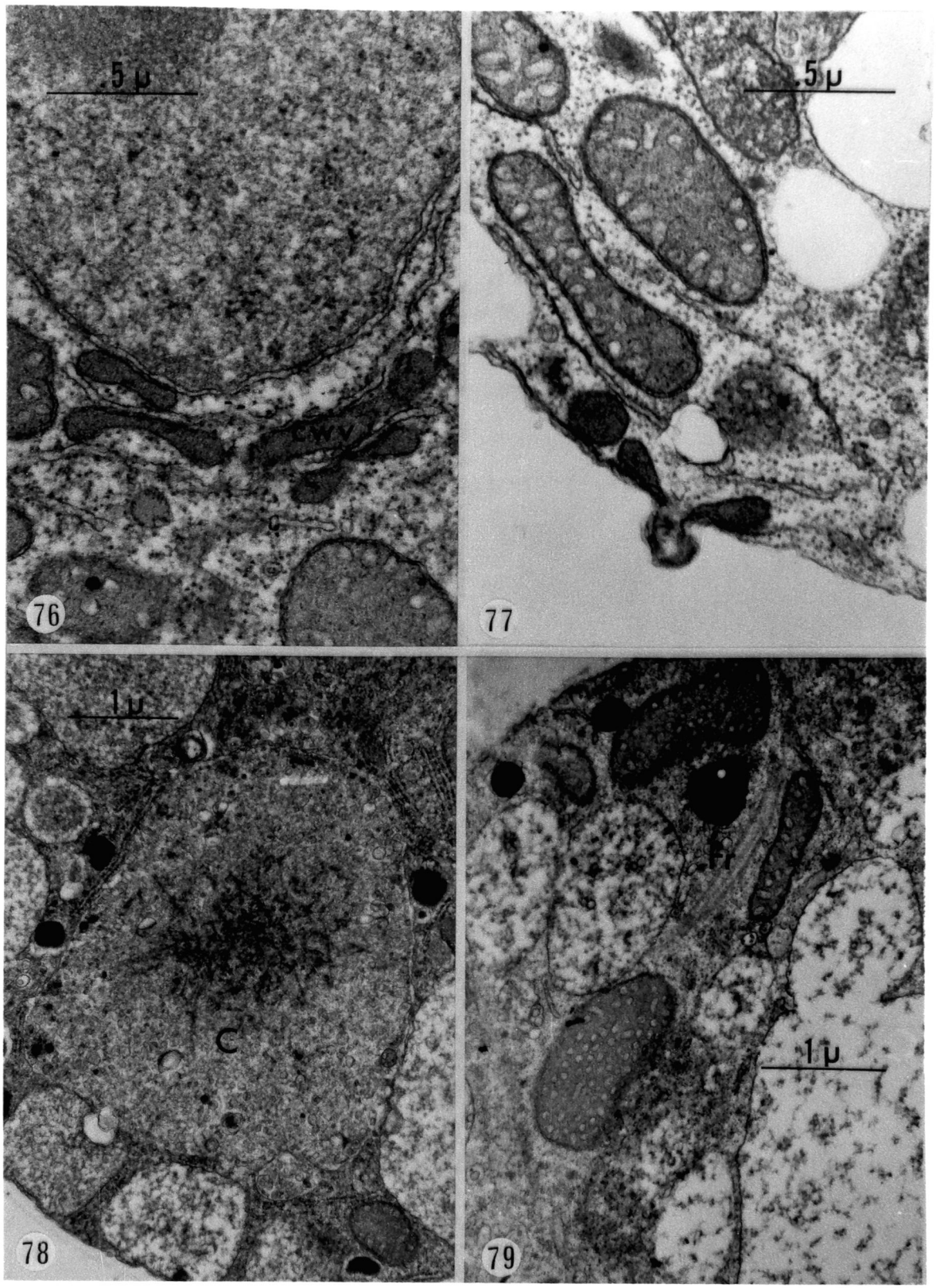

.5 μ
76
.5 μ
77
1 μ
C
78
Fr
1 μ
79

Figure 80. Release of fibrous material from type A vesicle.

Figure 81. Release of intact type B vesicle at spore surface.

Figure 82. Early stage of encystment with type B, unbound complex near the plasmalemma.

Abbreviations: A - type A vesicle, B - type B vesicle, Be - early type B vesicle, M - mitochondria, Pb - phospholipid body.

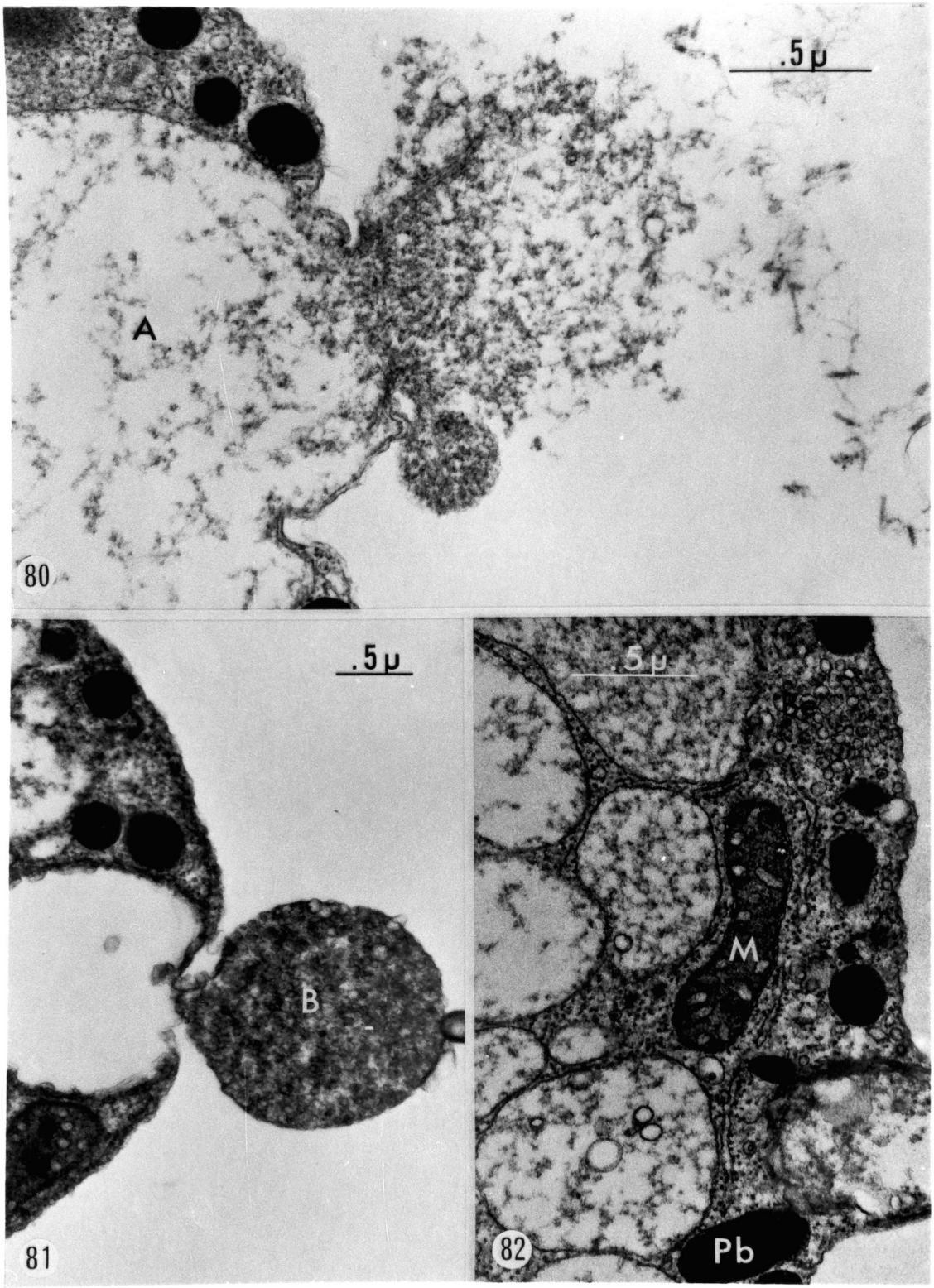

.5 µ
A
80
.5 µ
B
81
.5 µ
M
82
Pb

Figure 83-84. Portions of encysting spores showing variously modified phospholipid bodies (arrows).

Figures 85-88. Sequential stages in the modification of phospholipid bodies of an encysted spore. Note that modification ends with empty scalloped fringed vesicles (Fig. 88).

Figure 89. Encysted spore with central centriole.

Abbreviations: A - type A vesicle, Ce - centriole, Dy - dictyosome.

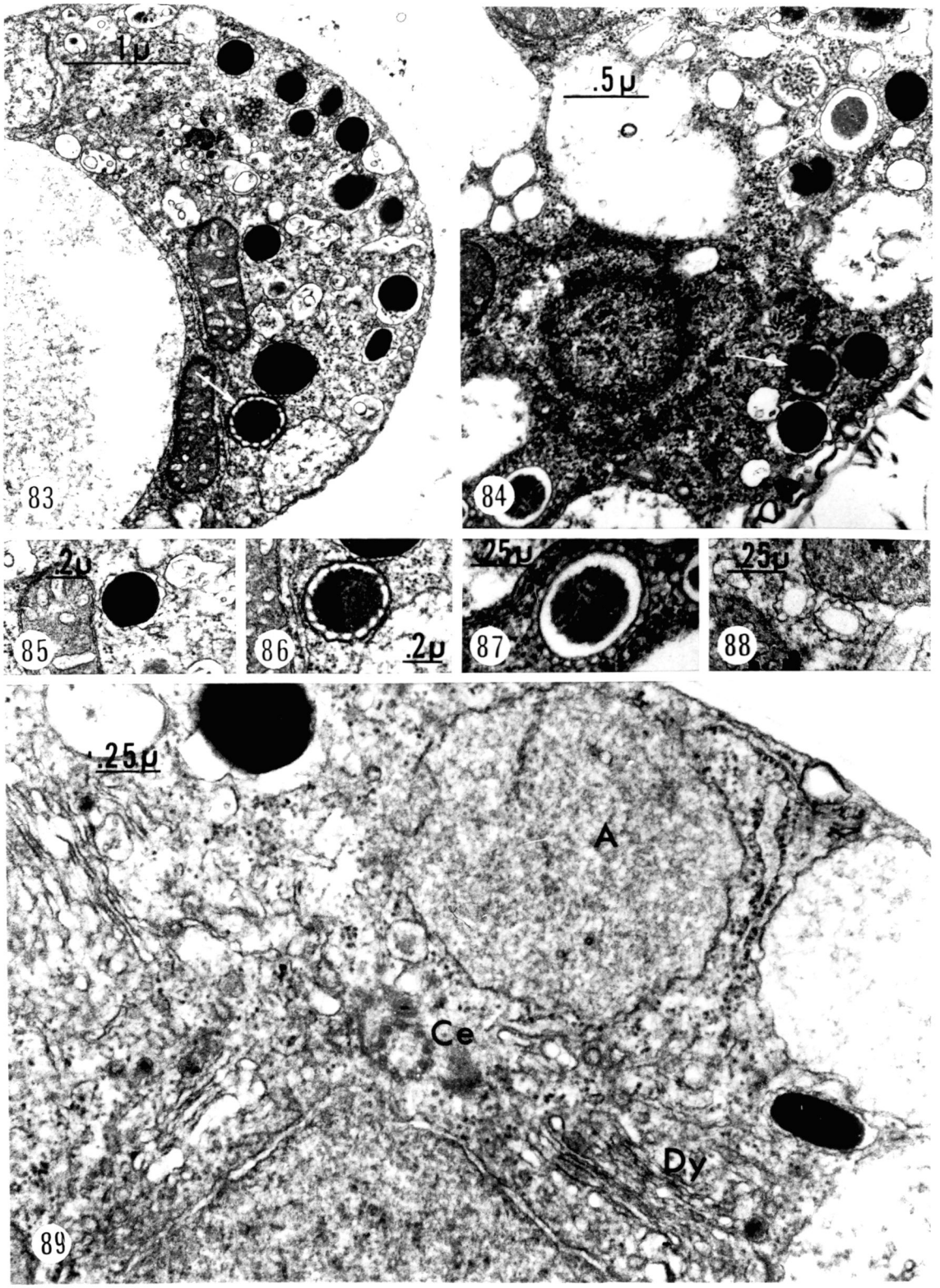
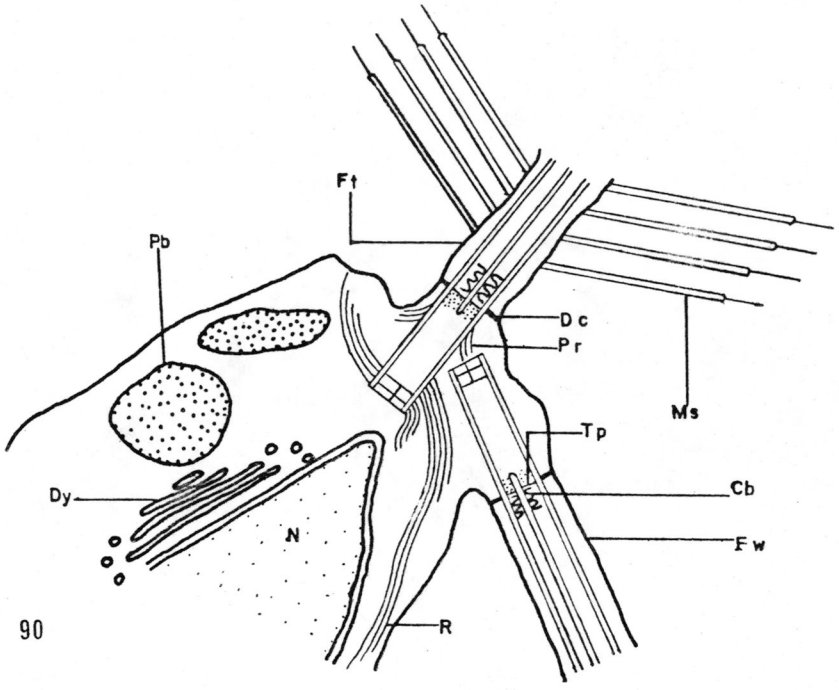

1μ
83
.5μ
84
.2μ
85
.2μ
86
.25μ
87
.25μ
88
.25μ
A
Ce
Dy
89

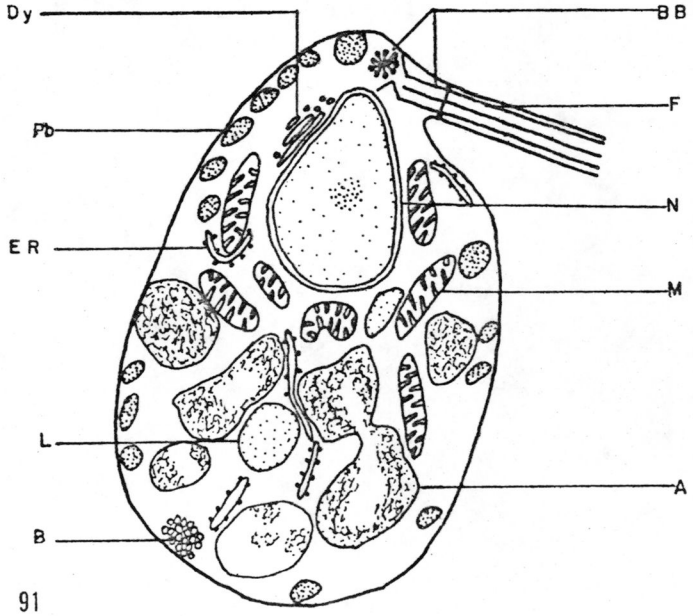
Figure 90. Line drawing showing details of flagellar apparatus.

Figure 91. Line drawing showing internal organelle arrangement in a swimming zoospore.

Abbreviations: A - type A vesicles, B - type B vesicles, BB - basal body, Cb - coiled body, Dc - dense connection, Dy - dictyosome, ER - endoplasmic reticulum, F - flagellum, Ft - tinsel flagellum, Fw - whiplash flagellum, L - lipid, M - mitochondria, N - nucleus, Pb - phospholipid body, Pr - prop, R - rootlet, Tp - terminal plate.



90

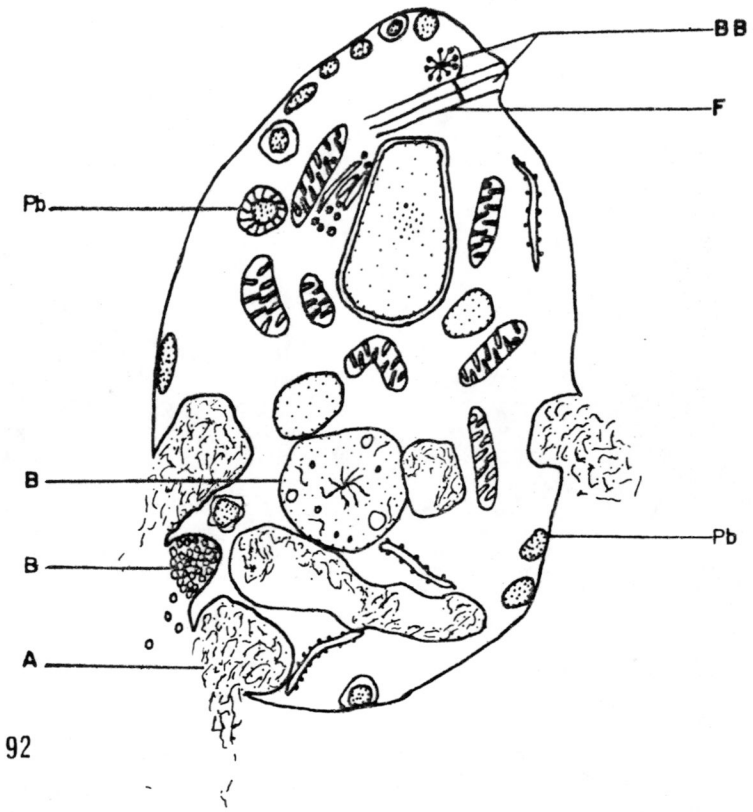


91

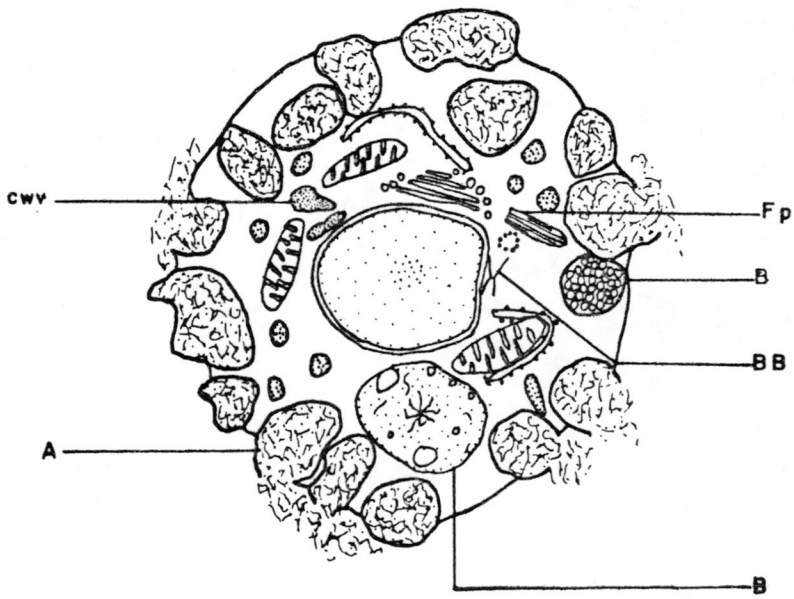
Figure 92. Initial stage of encystment. Note retraction of flagellum and release of fibrous material from type A vesicles.

Figure 93. Early stage of encystment. Note rounding up of the plasmalemma, and rearrangement of internal organelles.

Abbreviations: A - type A vesicle, B - type B vesicle, cwv - cell wall vesicles, BB - basal body, F - flagellum, Fp - flimmer packet, Pb - phospholipid body.



92

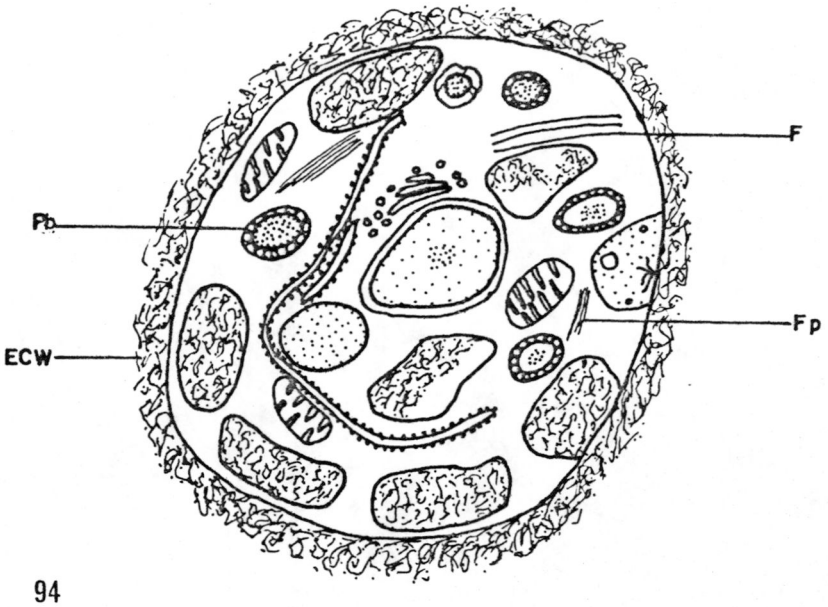


93

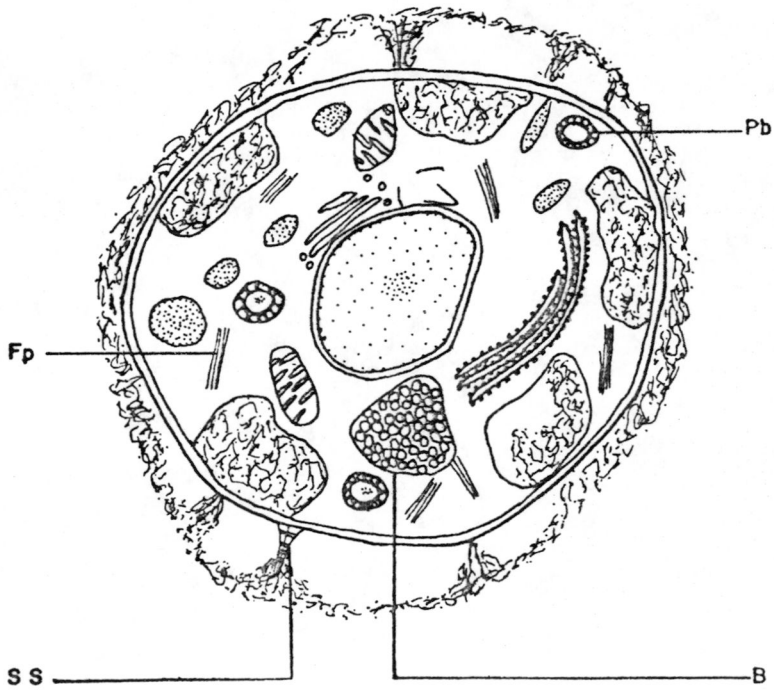
Figure 94. Middle stage of encystment showing increased production of endoplasmic reticulum, structural modification of phospholipid bodies and a thick fibrous mat surrounding periphery.

Figure 95. Middle stage of encystment showing separation of fibrous material from spore surface leaving numerous fiber bundles. Note also increased production of flimmer packets.

Abbreviations: B - type B vesicle, ECW - early cyst wall, F - flagellum, Fp - flimmer packets, Pb - phospholipid body, SS - striated spine.



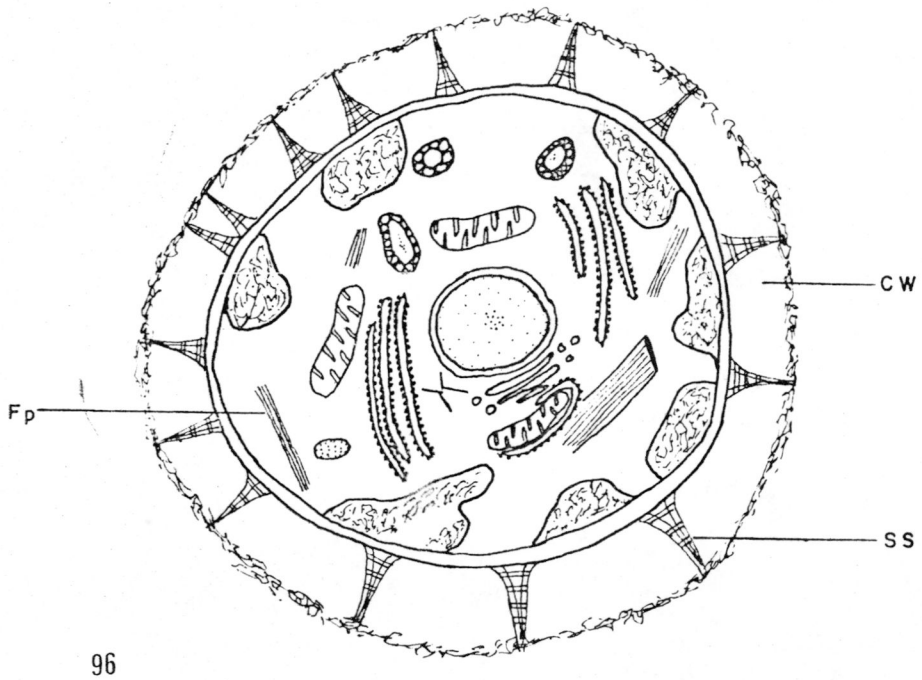
94



95

Figure 96. Line drawing showing completely encysted spore. Note final arrangement of all internal organelles, the three layered cyst wall containing striated spines, and the completely modified phospholipid bodies.

Abbreviations: CW - cyst wall, Fp - flimmer packet, SS - striated spine.


CW
Fp
SS

LITERATURE CITED

Bimpong, C. E. and C. J. Hickman. 1975. Ultrastructural and cytochemical studies of zoospores, cysts, and germinating cysts of Phytophthora palmivora. Can. J. Bot. 53: 1310-1327.

Bland, C. E. and H. V. Amerson. 1973. Electron microscopy of zoosporogenesis in the marine phycomycete, Lagenidium callinectes Couch. Archiv. Mikrobiol. 94: 47-64.

Bland, C. E. and H. V. Amerson. 1973. Observations on Lagenidium callinectes Couch: Isolation and sporangial development. Mycologia. 65: 310-320.

Bouck, G. B. 1971. The structure, origin, isolation, and composition of tubular mastigonemes of the Ochromonas flagellum. J. Cell Biol. 50: 362-384.

Desjardins, R. R., G. A. Zentmeyer and D. A. Reynolds. 1969. Electron microscopic observations of the flagellar hairs of Phytophthora palmivora zoospores. Can. J. Bot. 47: 1077-1079.

Desjardins, R. R., M. C. Wang and S. Bartnicki-Garcia. 1973. Electron microscopy of zoospores and cysts of Phytophthora palmivora: Morphology and surface texture. Arch. Mikrobiol. 88: 61-70.

Dick, M. W. 1973. Saprolegniales. Pages 113-141 in Ainsworth, Sparrow, and Sussman, eds. The Fungi. Academic Press.

Fisher, W. S., E. H. Nilson and R. S. Shleser. 1975. Effect of the fungus Haliphthoros milfordensis on the juvenile stages of the american lobster Hamarus americanus. J. Invert. Path. 26: 41-45.

Fuller, M. S., B. E. Fowles, and D. J. McLaughlin. 1964. Isolation and pure culture study of marine phycomycetes. Mycologia 56: 745-756.

Gay, J. L., A. D. Greenwood and I. B. Heath. 1971. The formation of vacuoles (vesicles) during oosphere development and zoospore germination in Saprolegnia. J. Gen. Mikrobiol. 65: 233-241.

Gotelli, D. 1974. The morphology of Lagenidium callinectes II. Zoosporogenesis. Mycologia 66: 846-858.

Grove, S. N. 1971. Protoplasmic correlates of hyphal tip initiation and development in fungi. Ph.D. dissertation. Purdue Univ.

Heath, I. B. and A. D. Greenwood. 1970. Wall formation in the Saprolegniales. Arch. Mikrobiol. 75: 67-79.

Hemmes, D. E. and H. R. Hohl. 1971. Ultrastructural aspects of encystation and cyst germination in Phytophthora parasitica. J. Cell Sci. 9:175-191.

Ho, H. H., K. Zachariah and C. J. Hickman. 1968. The ultrastructure of zoospores of Phytophthora megasperma var. sojae. Can. J. Bot. 46: 37-41.

Hoch, H. C. and J. E. Mitchell. 1972. The ultrastructure of zoospores of Aphanomyces euteiches and of their encystment and subsequent germination. Protoplasma. 75: 113-138.

Hohl, H. R. and S. T. Hamamoto. 1967. Ultrastructural changes during zoospore formation in Phytophthora parasitica. Amer. J. Bot. 54: 1131-1139.

Koch, W. J. 1968. Studies of the motile cells of chytrids. V. Flagellar retraction in posteriorly uniflagellate fungi. Amer. J. Bot. 55(7): 841-859.

Lunney, C. Z. and C. E. Bland. 1976. An ultrastructural study of zoosporogenesis in Pythium proliferum de Bary. Protoplasma. 88: 85-100.

Lunney, C. Z. and C. E. Bland. 1976. Ultrastructural observations of mature and encysting zoospores of Pythium proliferum de Bary. (in press).

Meier, H. and J. Webster. 1954. An electron microscope study of cysts in the Saprolegniaceae. J. Exp. Bot. vol. 5. 15: 401-409.

Reichle, R. E. 1969. Fine structure of Phytophthora parasitica zoospores. Mycologia (N. Y.) 61:30-50.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 17: 208-212.

Sharp, D. G., E. A. Eckert, D. Beard and J. W. Beard. 1952. Morphology of the virus of avian erythromyeloblastic leucosis and a comparison with the agent of Newcastle disease. J. Bacteriol. 63: 151-161.

Sing, V. O. and S. Bartnicki-Garcia. 1975. Adhesion of Phytophthora palmivora zoospores: Electron microscopy of cell attachment and cyst wall fibril formation. J. Cell Sce. 18: 123-132.

Snell, W. H. and E. A. Dick. 1971. A glossary of mycology. Harvard University Press. 181 pages.

Sparrow, F. K. 1960. Aquatic phycomycetes. Univ. of Michigan Press 1187 pages.

Sparrow, F. K. 1973. The peculiar marine phycomycete Atkinsiella dubia from crab eggs. Arch. Mikrobiol. 93: 137-144.

Sparrow, F. K. 1973. Lagenidiales. Pages 159-163 in Ainsworth, Sparrow, and Sussman, eds. The Fungi. Academic Press.

Sparrow, F. K. 1974. Observations on two uncommon marine fungi. Veroff. Inst. Meeresforsch. Bremerh. Suppl. 5: 9-18.

Sparrow, F. K. 1976. The present status of classification in biflagellate fungi. Pages 213-222 in Jones, ed. Recent Advances in Aquatic Mycology. Halsted Press, John Wiley & Sons, Inc. N. Y.

Vishniac, H. S. 1958. A new marine phycomycete. Mycologia (N. Y.) 50: 66-79.