OBSERVATIONS ON THE FUNGUS *SOROCHYTRIUM MILNESIOPHTHORA* SP. NOV., AN ENDOPARASITE OF THE TARDIGRADE *MILNESIUM TARDIGRADUM* DOYERE

A Thesis

by

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ABSTRACT

OBSERVATIONS ON THE FUNGUS *SOROCHYTRIUM MILNESIOPHTHORA* SP. NOV., AN ENDOPARASITE OF THE TARDIGRADE *MILNESIUM TARDIGRADUM* DOYERE (May 1984)

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This thesis describes, and documents with photomicrographs, the life cycle of *Sorochytrium milnesiophthora* sp. nov., a chytridiomycetous fungus endoparasitic upon the cosmopolitan eutardigrade *Milnesium tardigradum* Doyère. This fungus produces posteriorly uniflagellate zoospores which encyst on the cuticle of the host animal, form an appressorium, and generate a germ tube which penetrates the host cuticle to the epidermis. A thallus develops as a swelling at the tip of the penetration tube, enlarges, and moves from the epidermis into the body cavity. The thallus then cleaves into a sorus of incipient sporangia. The sorus breaks apart, and the individual sporangia produce rhizoids, enlarge, and produce a single exit papilla. The sporangial cytoplasm then cleaves into zoospores,

which are discharged from the exit papilla and infect a new host. Early host death results in sporangial formation from whatever stage of thallus is present. Evidence is provided for a polycentric rhizomycelial epibiotic growth phase resulting from the germination of encysted zoospores. *Sorochytrium milnesiophthora* does not fit into existing families within the order Chytridiales, and the erection of a new family within this order is suggested.

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I would like to thank Dr. William Dewel and Dr. Ruth Dewel for their guidance and support during my two years at Appalachian. They patiently taught me research techniques, contributed advice and ideas, and spent much of their own free time in the lab helping me with my work. I have learned much from the experience.

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I am grateful to Dr. Richard Henson, who helped me through several months of indecision concerning a thesis topic. His well-taught invertebrate class whetted my interest in those animals, and his friendly encouragement was always welcome.

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Francis Montaldi gave encouragement and advice, and loaned equipment and materials. Dr. Mary Connell delivered me from the hellish freshman labs into the comparative bliss of teaching molecular labs, for which I am most grateful.

I would especially like to thank Janice Ashley, who typed the final draft of this thesis. She is without doubt the hardest working person in the department, and one of the nicest people I know.

My mother, Blanche Joines, has generously offered encouragement and financial help when needed, which was often. Her example of patience and faith has smoothed the way through otherwise difficult situations.

Finally, I owe much to my wife Carolyn for her love and support; for tolerating my frequent absences from home; for typing the preliminary draft; and for encouraging me to quit work and return to school, which made all this possible.

DEDICATION

This work is dedicated to my father, John E. Joines, who died in April of 1982. He worked at many things in the course of his sixty-eight years: he farmed, broke horses, cut timber, hunted, distilled homemade liquor, tended an orchard, carpentered, kept bees, and read voraciously. He served as a front line medic during World War II, raised a family, and eventually embraced a vision of God full of power and wonder. He categorically rejected the values and trappings of middle class society, preferring the honesty of a way of life that was pushed aside in the headlong rush of modern times. He was irreplaceable, one of a vanishing breed, and the world will not see his like again.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The microfauna of a moss community typically comprises three main groups of metazoans: tardigrades, nematodes, and rotifers. These animals interact with each other and with the autotrophic bryophyte that forms the matrix in which they reside. The recurrent theme in any consideration of this microcommunity is the repeated wetting and drying of the habitat and its populations. The animal groups mentioned above have evolved astounding survival mechanisms by which they enter resistant states of anhydrobiosis immediately upon drying; when wetted again, they resume activity where they left off, apparently none the worse for the experience. All of their activities of maintenance and reproduction are carried out within this framework of wetting and drying, superimposed on the regular cycles of seasonality.

The fungi have an important role in this ecological interplay. Lacking autotrophic capability, they must depend on parasitic or saprophytic modes of nutrition. The parasitic or predatory fungi may invade any of the associated microinvertebrates or the bryophyte

itself. Such phenomena are well documented; nematodes, because of their economic importance as pests, have received much of the attention (Barron, 1977).

This thesis is a description of the life cycle of a particular species of endoparasitic fungus, *Sorochytrium milnesiophthora* sp. nov., which infects the eutardigrade *Milnesium tardigradum* Doyère. This fungus represents not only a new genus of predatory chytridiomycete (Dewel, et al., in preparation), but possesses a combination of characteristics that cuts across existing families and perhaps orders. These traits justify close scrutiny, as they may shed some light on the evolutionary relationships among the chytridiomycetes. Certainly the taxonomy of the chytridiomycetous fungi must be revised to accommodate this unusual species.

The work presented herein, although largely descriptive, provides some footing for the designers of more complete, statistically meaningful investigations. It should also generate additional interest in the Tardigrada, a relatively neglected invertebrate phylum.

LITERATURE REVIEW

There are few accounts of fungal parasites of tardigrades in the literature; these include four established species and several unidentified species. The paucity of information is most likely due to the incomplete state of our knowledge of the Tardigrada, a minor phylum of invertebrates which has traditionally attracted only a limited amount of study.

The four described species of such parasites belong to two genera of the order Entomophthorales. The first genus was established by Reukauf (1912) in his description of *Macrobiotophthora vimariensis* parasitizing *Macrobiotus lacustris* (= *Hypsibius dujardini*). Baumann (1961) reported the same species in *Hypsibius convergens*. Drechsler (1951) established the first species of the second genus in his description of *Ballocephala sphaerospora* parasitizing a tardigrade species tentatively identified as a *Macrobiotus*. Richardson (1970) described a second member of this genus, *Ballocephala verrucospora*, again parasitizing a tardigrade tentatively identified as a *Macrobiotus*. Hallas (1977) reported *B. verrucospora* parasitizing

Macrobiotus hufelandii. Pohlad and Bernard (1978) described the final species, *Ballocephala pedicellata*, parasitizing *Hypsibius dujardini* and *Diphascon pinguis*.

In a collection of notes on the Arctic tardigrades, Murray (1907) reported an unidentified species of fungus parasitizing a *Macrobiotus*. He gave the following account:

> In Franz Josef Land there were found several examples of a *Macrobiotus* which lays elliptical eggs in the cast skin. The eggs had been attacked by a parasite, possibly a kind of *Saprolegnia*. From each egg there proceeded a tube which penetrated the back of the skin of the parent and formed a slight expansion just outside it (Plate II. Fig. 9a). The eggs and tubes were empty, but in Scotland the living parasite has been seen infesting the body of the Tardigrade.

The figure referred to seemingly contradicts the above statement. The figure shows what appear to be elliptical sporangia with exit papillae, rather than elliptical eggs. In addition, the sketch shows the animal's pharyngeal apparatus and buccal tube, which would not be present in the shed cuticle of a tardigrade. It is likely that Murray was observing the mature sporangia of a chytridiomycete, perhaps *Catenaria*, in the body of a dead tardigrade. No connections between the

sporangia are shown, nor are any rhizoids, although these may have been overlooked depending upon the resolving power of the microscope used.

Marcus (1929), Fig. 134, showed a drawing of a specimen of *Milnesium tardigradum* infected by a fungus resembling *Catenaria*. The sporangia had exit papillae, some of which penetrated the animal's cuticle. Connections were shown between certain sporangia, and rhizoids were shown; both of these are characteristics of *Catenaria*. Marcus, in his discussion of tardigrade parasites, suggested that this undescribed fungus was the same form described by Murray (1907).

According to Cuenot (1932), parasites of tardigrades are rare. He mentioned the protozoan "Nosema", but gave no reference to fungal parasites of tardigrades other than a citation of Reukauf (1912).

Morgan (1977) reported a fungus resembling *B. verrucospora* attacking populations of *Milnesium tardigradum, Hypsibius oberhaeuseri, Macrobiotus hufelandii,* and *Echiniscus testudo;* he mentioned a second, larger filamentous fungus which entangles tardigrades rather than directly attacking them.

Nelson (1977) reported that populations of *Macrobiotus* and *Hypsibius* in New Zealand were heavily

infected by three undescribed fungal species. None of these fungi resemble *Sorochytrium milnesiophthora* (Nelson, personal communication).

MATERIALS AND METHODS

Moss samples containing populations of *Milnesium tardigradum* infected by the endoparasitic fungus *Sorochytrium milnesiophthora* were collected from two sites: Sim's Creek Bridge near Milepost 296 on the Blue Ridge Parkway, and a wall on the campus of Appalachian State University in Boone, North Carolina. Both sites are in Watauga County, North Carolina.

Samples were soaked overnight in closed containers of distilled water kept in the dark, a procedure which produces anoxic anesthesia and relaxes the tardigrades. The containers were then vigorously shaken and the contents poured into a gridded petri dish and examined at 25X magnification with a Wild binocular dissecting microscope using transmitted illumination. Anesthetized tardigrades as well as dead tardigrades containing fungal sporangia were transferred by Irwin loop to a clean drop of water on a glass slide and a coverslip was added. The coverslip was supported on each edge by an additional strip of coverslip glass; a flattened "hanging drop" preparation was thus created which allowed microscopic examination of the specimen without

smashing and destroying it. Animals were examined and photographed under 10X and 40X objectives using a Jena-Zeiss microscope equipped with differential interference optics and an automatic camera.

Live infected tardigrades were transferred to separate drops of water for observation. Dead tardigrades containing fungal sporangia were maintained in separate drops until the beginning of zoospore discharge. The soaking water from early moss samples was boiled and filtered; any animals maintained for extended periods were kept in drops of this "habitat water," to approximate natural conditions of osmolality and solute species.

Preliminary sampling of various tardigrade populations was carried out using the above procedures, and certain populations were determined to be uninfected. Live tardigrades were collected from these populations and were placed in a drop of habitat water in a plastic petri dish. A dead infected tardigrade containing actively discharging zoosporangia was placed in the drop for a given period of time and then removed. The period of exposure varied in different observation sequences depending upon the magnitude of zoospore discharge and upon the intended use of that particular group of infected animals. After such

exposure, the live tardigrades were examined at 600X as above for zoospore attachment and separated into drops of habitat water in plastic petri dishes for maintenance and observation. In initial observations, several animals were maintained per drop; but, after the value of individual observation became obvious, the animals were maintained singly. Observations of infected animals were, as nearly as possible, on a daily basis. In some groups, selected phases of fungal development were observed more closely than at other times.

Recorded data include the appearance and size of each fungal stage, the presence of rhizoids, the appearance of the host, the time of death of the host, and the time and duration of zoospore discharge. Measurements were taken with an ocular micrometer calibrated to a stage micrometer having 0.001 inch graduations. The stage micrometer was photographed for use in measurements of photographic details. Laboratory populations of infected animals were maintained by infecting successive groups of freshly hydrated tardigrades. Dried samples were obtained by placing active infected animals onto single moss shoots in plastic petri dishes and allowing these to air dry, thus inducing anhydrobiosis in the host animals.

Certain fungal stages were chosen for fixation for electron microscopy. Infected animals were kept in the dark overnight in a closed container of water along with a 50 μm-mesh bag of moss. When the anesthetized tardigrades had just started to revive, they were placed into fixative. The fixative solution was prepared by mixing one part 4% osmium tetroxide with one part 4% glutaraldehyde in 0.2M PIPES-NaCl buffer (Electron Microscopy Sciences, Fort Washington, Pa.), pH 7.2, which produced a solution of 2% osmium tetroxide - 2% glutaraldehyde - 0.1M PIPES buffer. After 30 seconds in the fixative, the animals were cut in half with a razor blade. A group of unrevived tardigrades was treated in the same fashion. Other reviving animals were cut in distilled water and immediately transferred to the fixative. Animals were fixed in the dark at 4°C for 1-2½ hours, rinsed in 0.1M PIPES buffer, post-fixed for one hour in 2% osmium tetroxide-0.1M PIPES buffer, rinsed in distilled water, and en bloc stained for 30 minutes in 2% aqueous uranyl acetate. They were then dehydrated in a graded acetone series and embedded in Spurr's Epoxy Resin (Spurr, 1969). Blocks were sectioned using glass and diamond knives on a LKB-Huxley Ultramicrotome. One-micrometer

sections were stained with methylene blue for light microscopy. Ultrathin sections were collected on 300-mesh copper grids, stained for 30 minutes in uranyl acetate and five minutes in lead citrate, and observed on a Phillips Model 201 Transmission Electron Microscope.

Zoospores were obtained for light microscopy by placing an animal containing actively discharging sporangia into a drop of water on a glass slide. After a sufficient number of zoospores had discharged, the animal was removed. A drop of fixative solution, 1.6% osmium tetroxide - 2.5% glutaraldehyde - 0.1M PIPES buffer, was then added to the water containing the zoospores. A coverslip was applied and the preparation was examined under the 100X oil immersion objective.

RESULTS

The Endobiotic Life Cycle

The endobiotic life cycle (Fig. 1) of *Sorochytrium milnesiophthora* begins with the discharge of zoospores from mature sporangia into the surrounding water. These posteriorly uniflagellate zoospores (Fig. 2A, B) locate and attach to live members of the host species *Milnesium tardigradum*. Although zoospores may attach to any part of the animal, greater numbers encyst on the dorsal posterior third of the tardigrade (Table 1).

Each zoospore retracts or loses its flagellum and forms a spherical cyst (6-7 μm diameter) on the host cuticle. This spherical cyst develops a swelling or appressorium at the point of attachment so that a smaller proximal segment and a larger distal segment are visible (Fig. 2C, D). A penetration tube originates at the proximal segment, penetrates the various cuticular layers of the host, and reaches the epidermis (Fig. 2C). As this occurs, a vacuole forms in the distal segment and replaces the cytoplasm (Fig. 2C). The tip of the penetration tube enlarges to form a spherical thallus (Fig. 2E), and a vacuole appears in the proximal

Figure 1: Endobiotic life cycle of *Sorochytrium milnesiophthora* sp. nov.

(A) Zoospore with single posterior flagellum.

(B) Zoospore encystment; production of appressorium, penetration tube, and rudimentary thallus.

(C) Sequential cleavage of thallus.

(D) Many-celled incipient sorus.

(E) Sorus with compartments rounding up.

(F) Fragmentation of sorus.

(G) Maturing sporangia.

(H) Mature sporangia with exit papillae.

(I) Host death.

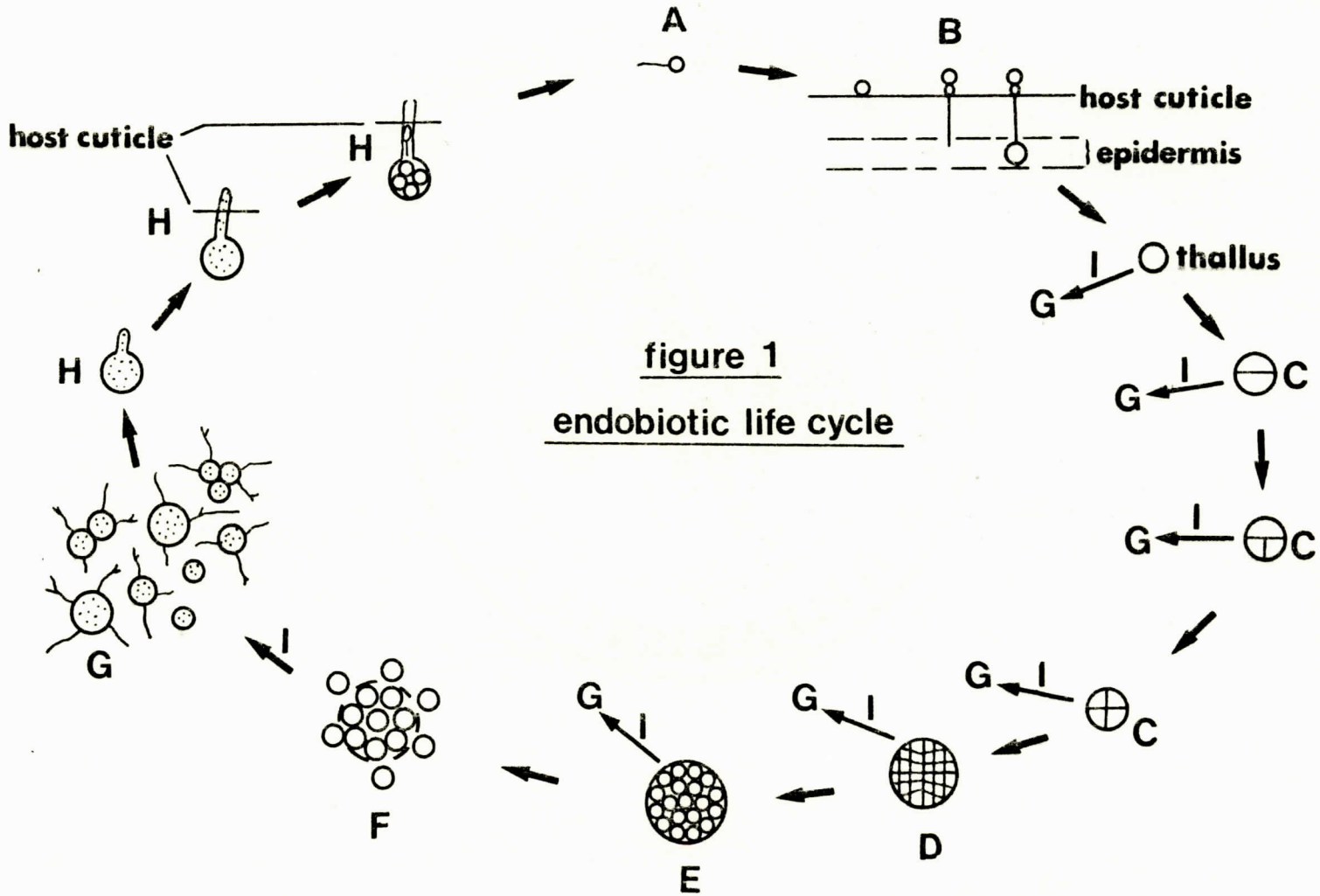


figure 1

endobiotic life cycle

Figure 2: Zoospores, zoospore attachment, and rudimentary thallus.

A. Unfixed zoospore with single whiplash flagellum (f). X1550

B. Fixed zoospore with single whiplash flagellum (f). X1550

C. Attached encysted zoospore (az); note cuticle (c), appressorium (arrow), and penetration tube extending to epidermis. X620

D. Attached encysted zoospore (az) with penetration tube (arrow); animal is dead, and body contents have receded from cuticle. X620

E. Penetration tube and rudimentary thallus (arrow); animal is dead, and body contents have receded from cuticle. X620

Bar scale (E) represents 16 μm on A-B, 40 μm on C-E.

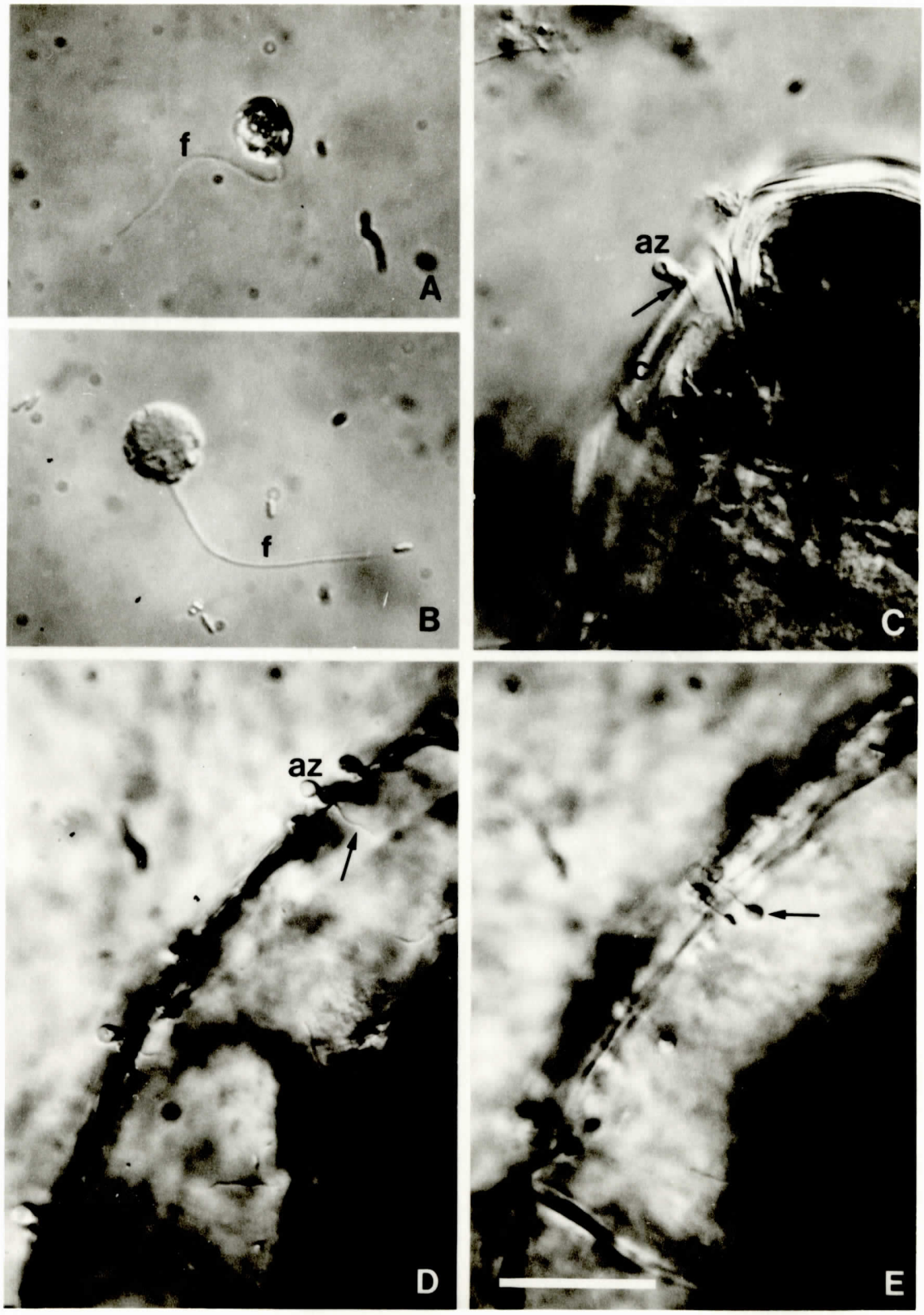

f
A
f
B
az
c
C
az
D
E

Table 1. Frequency of zoospore attachment to various regions of host cuticle.*

	Anterior	Middle	Posterior	Total
Dorsal	13	34	70	117
Ventral	10	32	41	83
Total	23	66	111	200

*Chi-square tests indicate that attachment is not uniform on anterior-posterior axis ($p<0.001$) and marginally uniform on dorsal-ventral axis ($0.10 > p > 0.05$); chi-square contingency test indicates that dorsal-ventral axis is independent of anterior-posterior axis ($0.5 > p > 0.1$).

segment, enlarges, and displaces the cytoplasm of that segment. At this point, both segments of the attached zoospore contain rapidly moving particles; this Brownian movement is an indication that they are filled with fluid (Fig. 2D).

Usually within the first two days of infection, the thallus enlarges to 5-15 μm (Fig. 3A-C). No longer situated in the epidermis, it now moves about in the body cavity of the host along with the body cavity cells. In animals collected from nature, uncleaved thalli as large as 25 μm have been observed (Fig. 3D); the age of these thalli is not known.

In a living host, the thallus cleaves sequentially into an incipient sorus of two, three, four, and then many compartments (Figs. 4A-D, 5A-D, 6A, B). The two, three, and four-celled stages, having a size range of 10-25 μm, often appear by the second or third day of infection. They are replaced by the many-celled stage by the third or fourth day. The many-celled incipient sorus persists until day five or six, increasing in size from 15-25 μm to 20-40 μm (Table 2).

At this time, the segments round up into incipient sporangia (Fig. 7A-C) and, if the animal is active the sorus breaks apart. During breakup, the sorus

Figure 3: Uncleaved thalli in epidermis.

A. Small early thallus (t). X620

B. Later thallus (t). X620

C. Thallus situated in epidermis (small arrow); note cuticle (large arrow). X620

D. Large uncleaved thallus in animal collected from nature; age of fungal stage is not known. Note epidermal ridge (arrow) around thallus. X620

Bar scale (D) represents 40 μm on A-D.

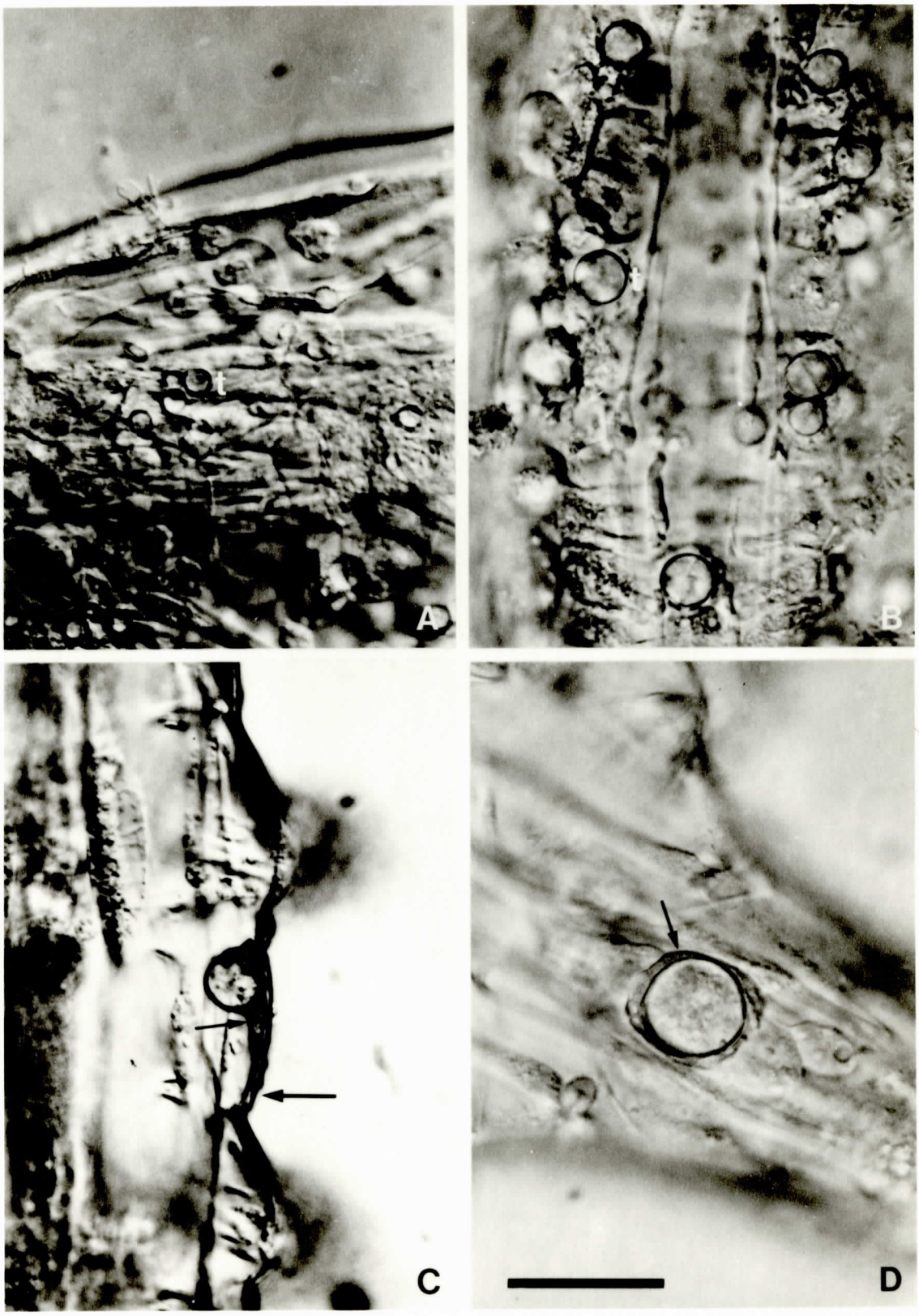

A
B
C
D

Figure 4: Sequential cleavage of thallus to form incipient sorus.

A. Two-celled stage (cl). X620

B. Two-celled stage (cl); note body cavity cells (bcc) and midgut (mg). X620

C. Three-celled stage (cl); note body cavity cell (bcc), muscle (m), and nerves (arrows). X620

D. Four-celled stage (cl). X620

Bar scale (D) represents 40 μm on A-D.

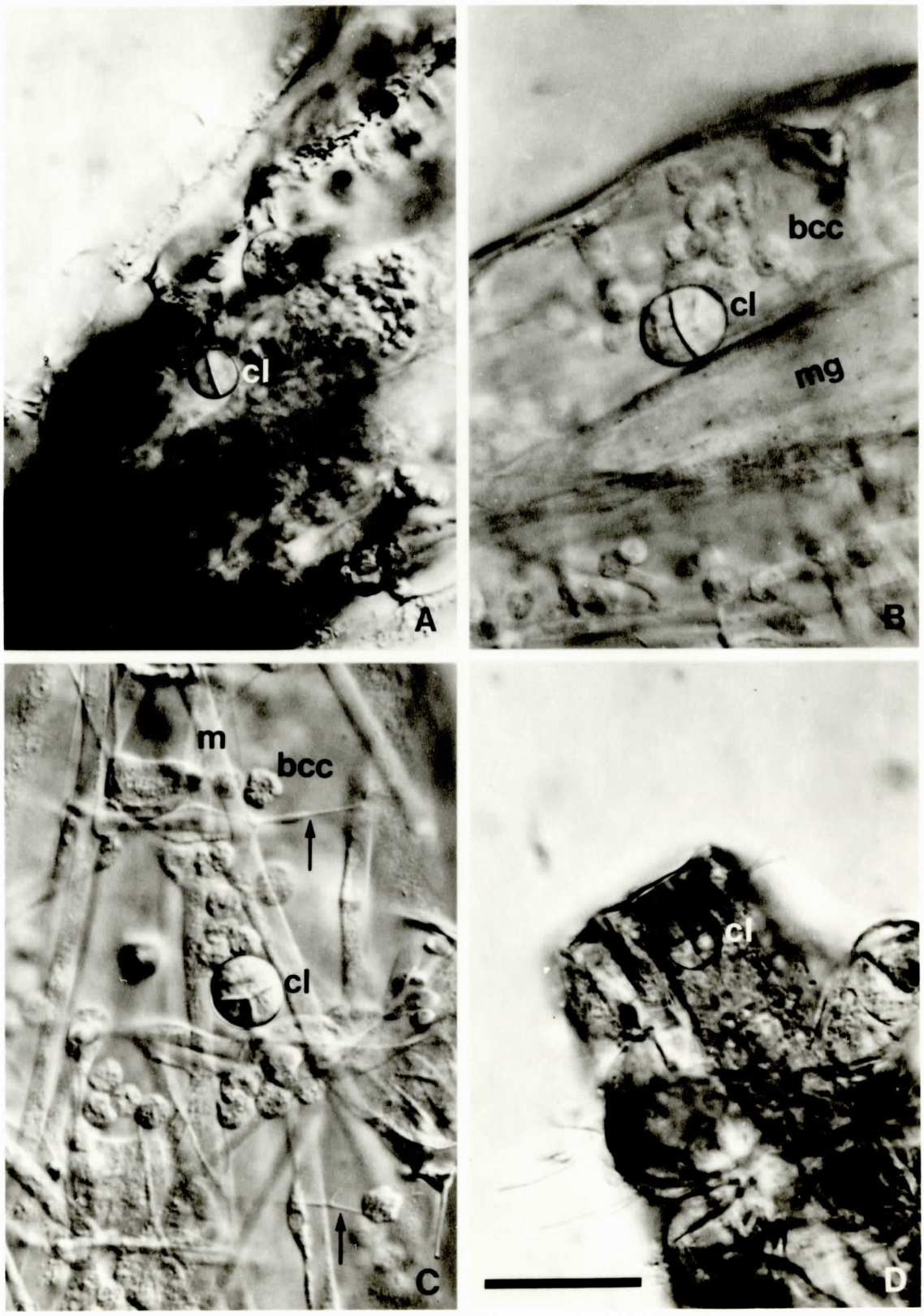

cl
A
bcc
cl
mg
B
m
bcc
cl
C
cl
D

Figure 5: Incipient sorus, many-celled stage.

A. Incipient sorus (is); note body cavity cells (bcc). X620

B. Incipient sori (is), flattened preparation. Note body cavity cells (bcc) and midgut (mg). X620

C. Incipient sorus (is) in rear of animal. X620

D. Incipient sorus (is) in rear of animal, flattened preparation. Note body cavity cells (bcc), claws (cw), and cuticle (c). X620

Bar scale (D) represents 40 μm on A-D.

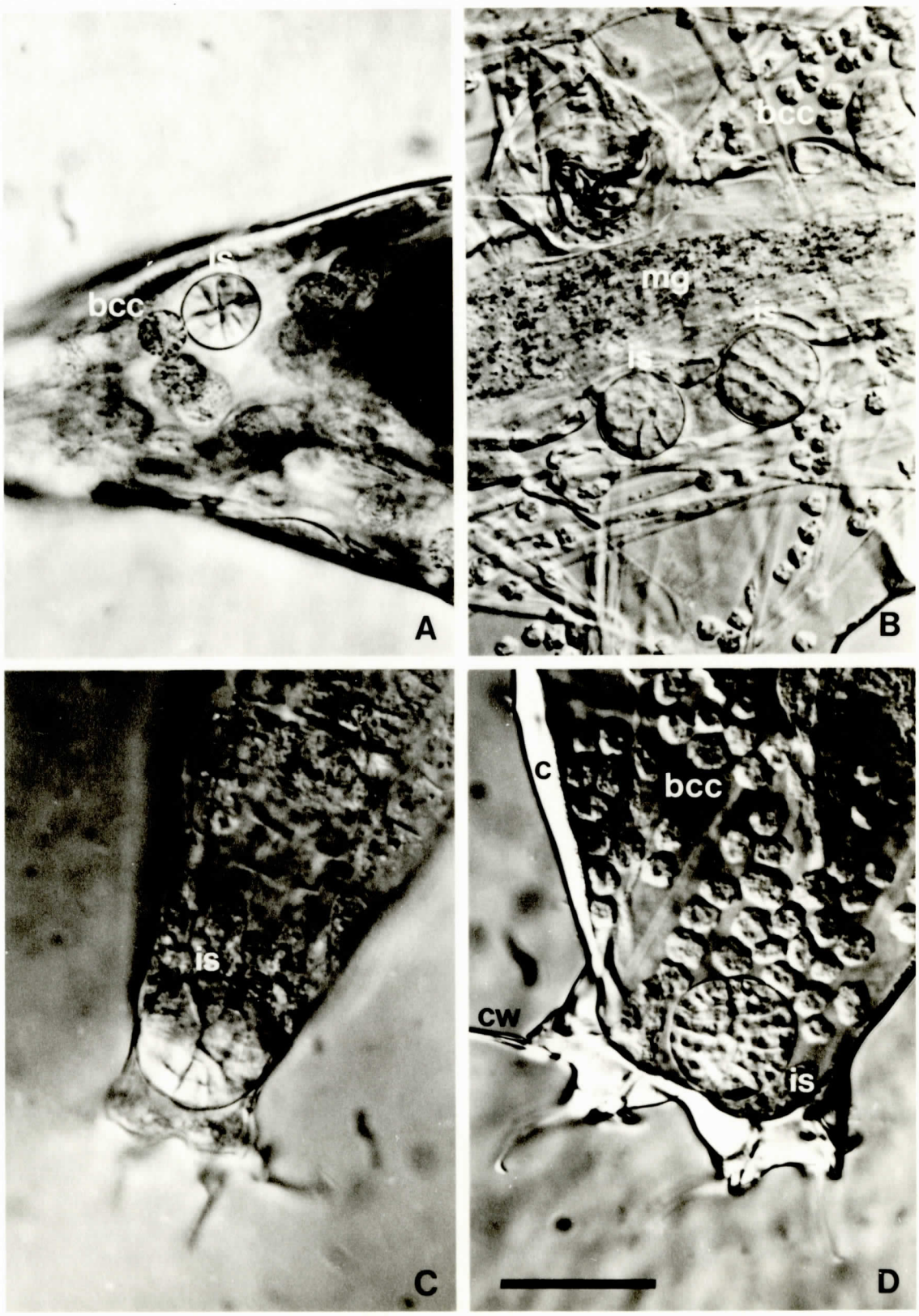
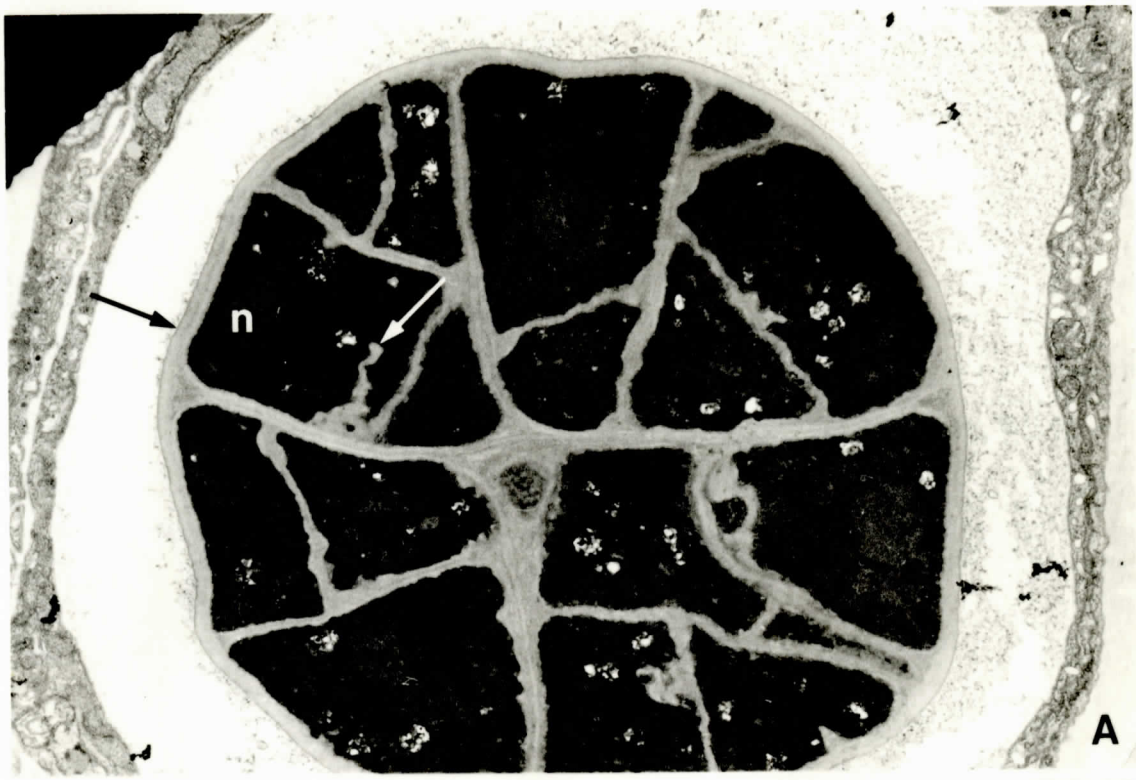

is
bcc
A
bcc
mg
is
is
B
is
C
c
bcc
cw
is
D

Figure 6. Electron micrographs of incipient sorus.

A. Section showing major cleavage planes as well as subsequent partitioning of incipient sorus. Note nuclei (n), forming partition (white arrow), and surrounding cell wall (black arrow). X7600

B. Section similar to A, but not showing major cleavage planes. Note nuclei (n) and surrounding cell wall (arrow). X7600


n
A


n
B

Table 2. Comparison of estimated sizes of incipient sori using Wilcoxon Signed Rank procedure* for paired observations.

Animal	U(1)	L(2)	D(3)	R(4)
1	23.0 µm	23.0 µm	0	
2	20.0	20.0	0	
3	23.0	29.0	+6.0	+9.5
4	18.0	25.0	+7.0	+14.5
5	22.0	13.0	-9.0	-18.5
6	25.0	33.0	+8.0	+16.5
7	19.0	32.0	+13.0	+20.0
8	19.0	27.0	+8.0	+16.0
9	18.0	24.0	+6.0	+9.5
10	19.0	13.0	-6.0	-9.5
11	23.0	25.0	+2.0	+3.5
12	25.0	32.0	+7.0	+14.5
13	25.0	40.0	+15.0	+21.0
14	19.0	35.0	+16.0	+22.0
15	20.0	22.0	+2.0	+3.5
16	19.0	25.0	+6.0	+9.5
17	25.0	31.0	+6.0	+9.5
18	19.0	19.0	0	
19	20.0	19.0	-1.0	-1.5
20	25.0	25.0	0	
21	19.0	20.0	+1.0	+1.5
22	25.0	19.0	-6.0	-9.5
23	19.0	25.0	+6.0	+9.5
24	19.0	24.0	+5.0	+5.0
25	23.0	32.0	+9.0	+18.5
26	19.0	25.0	+6.0	+9.5

(1) Column U = Upper limit of estimated size range of many-celled stage on the first day any such stage was observed in the given animal.

(2) Column L = Lower limit of estimated size range of many-celled stage on the last day any such stage was observed in the given animal.

(3) Column D = Difference in paired values of U and L.

(4) Column R = Ranked and signed value of D.

*Results: T+ = 214; T- = 39; p is less than 0.005, the two groups are significantly different.

Figure 7: Breakup of sorus.

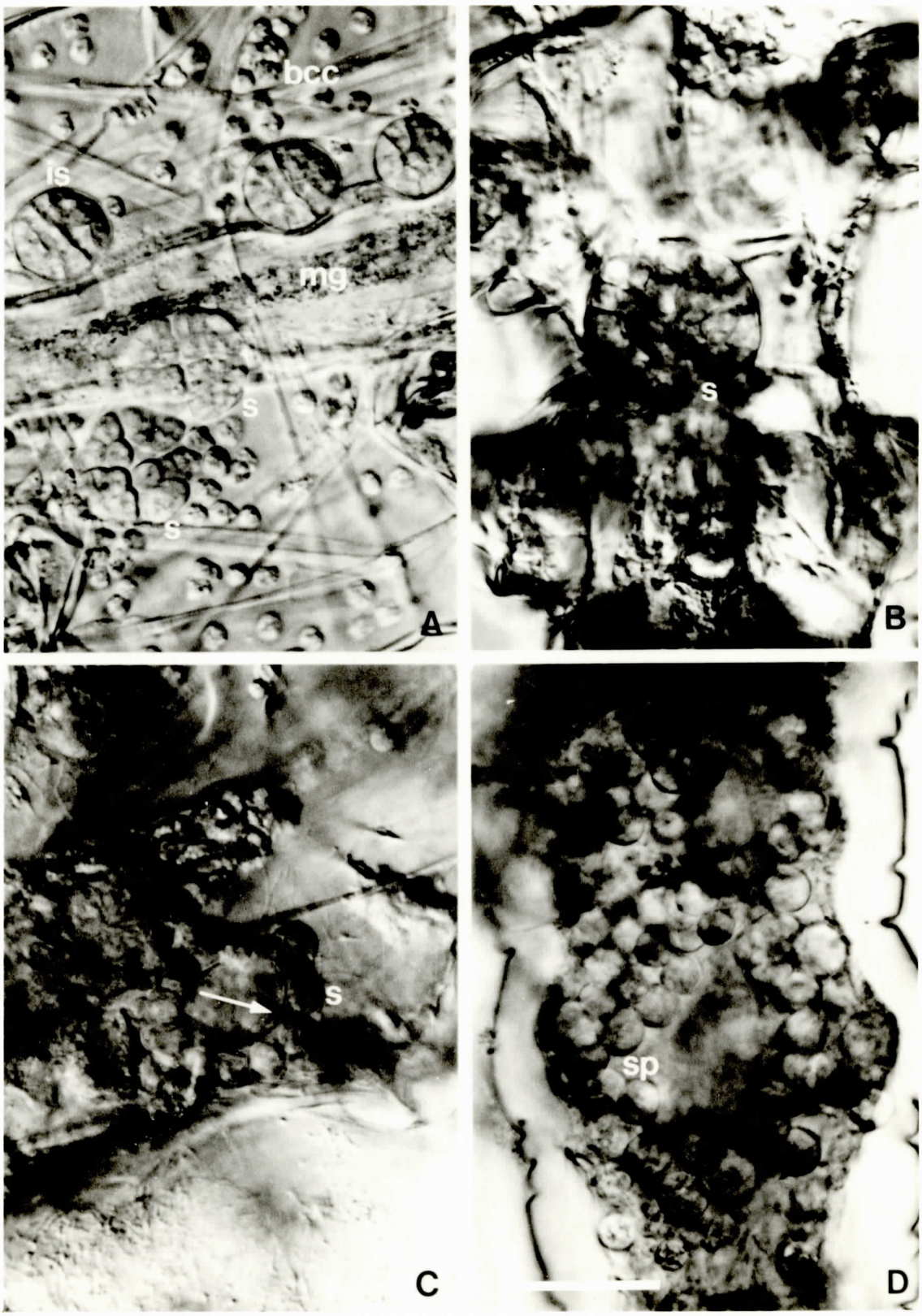
A. Sori (s) in initial stage of breakup; surrounding cell wall as well as major cleavage partitions appear to be absent. Note body cavity cells (bcc), incipient sori (is), and midgut (mg). X620

B. Sorus (s) with partially broken surrounding envelope. X620

C. Sorus (s) during breakup which appears to be a hollow ball of sporangia with "inner membrane" (arrow). Perhaps this results from mechanical eversion. X620

D. Hollow clump of sporangia (sp); no inner boundary is visible. X620

Bar scale (D) represents 40 μm on A-D.


bcc
is
mg
s
s
s
A
s
B
s
C
sp
D

sometimes appears to be a hollow ball of sporangia (Fig. 7C, D), the center of which may have a definite boundary (Fig. 7C). The sporangia released from a fragmenting sorus have a diameter of 5-10 μm, and often are incompletely divided, so that some consist of two or three compartments separated by cell walls (Fig. 7A, C). These round up into individual sporangia after a period of growth. In sluggish animals, the sorus may persist as a cluster of incipient sporangia until and beyond the time of host death (Figs. 7B, 9A, B).

When the incipient sporangia are dispersed throughout the host's body cavity following soral break-up, they are about the same size as body cavity cells (Fig. 7A); the two are difficult to distinguish in active animals.

Although the above time frame is generally followed, the time of appearance and the duration of each fungal stage varies. Occasionally, a thallus or a bipartite incipient sorus has appeared as late as day eight and then undergone cleavage and soral breakup. Also, in some animals the thallus and early cleavage stages persisted until day five or six without cleaving to the many-celled stage. These animals died on day five or six and cleavage progressed no further.

Time of host death varies depending upon degree of infection (Fig. 8). In heavy infections with many thalli produced, death often occurs within the first five days. The fungal stages present commonly develop into mature sporangia; however, such early death may occasionally stop the development of the fungus entirely. In lighter infections, the animals often live until the time of soral breakup or up to a week longer.

Following host death, the fungal stages present enlarge, produce rhizoids, exhibit increased granularity of the cytoplasm (Fig. 9A-D), and mature into sporangia. If host death precedes soral breakup, then the thalli, both cleaved and uncleaved, form sporangia. If the thallus has cleaved into two, three, four, or many compartments, then each compartment rounds up during the growth phase and forms a single sporangium. During this growth phase, the diameter may increase from 5-15 μm to 10-25 μm. If host death occurs after soral breakup, then the dispersed incipient sporangia undergo the same maturational changes, enlarging to a diameter of 8-20 μm (Table 3). Animals collected from nature seem to have more and larger sporangia than those from laboratory infection groups (Fig. 10A).

Figure 8: Survivorship curve for light and heavy infections.

FIGURE 8

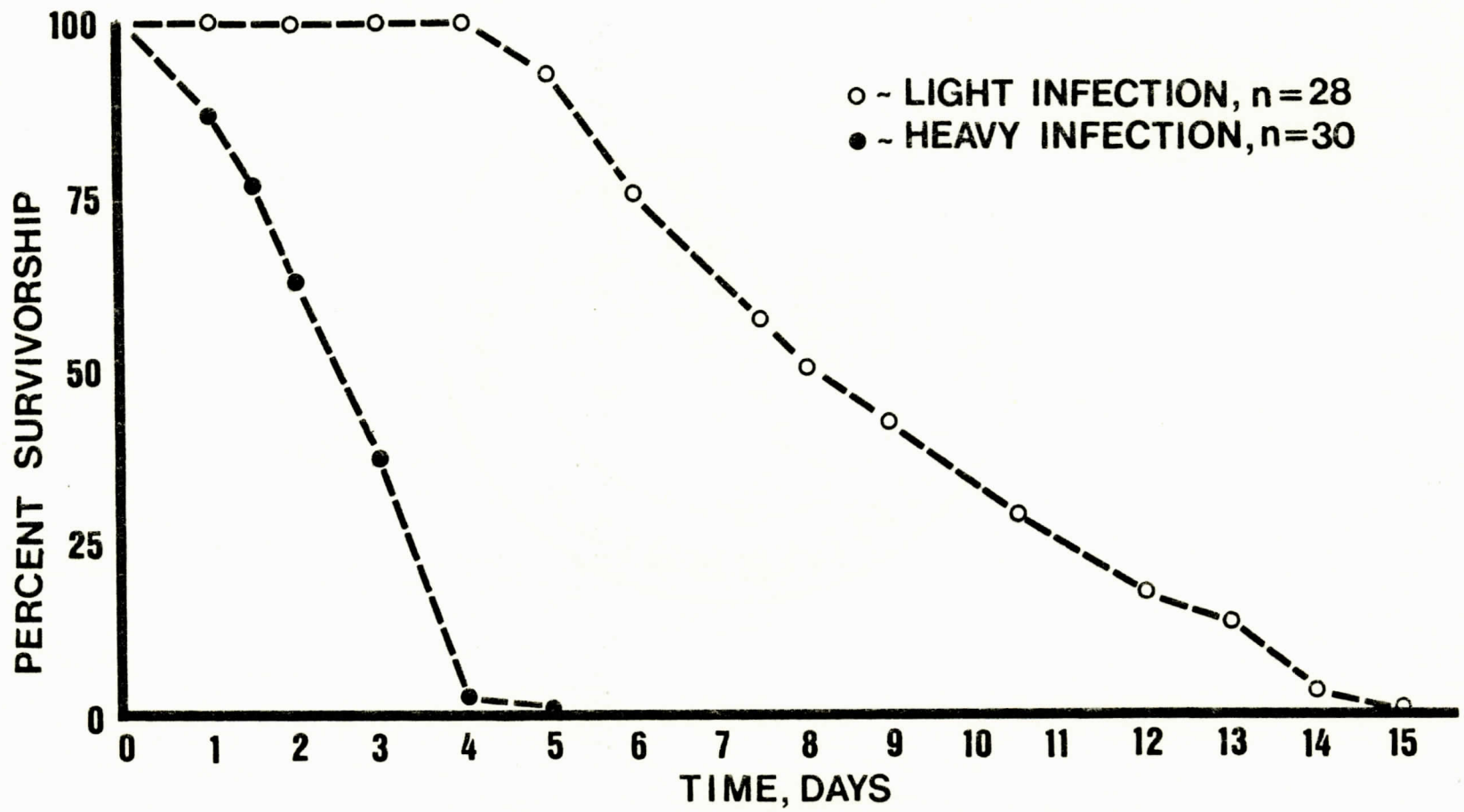

100
75
50
25
0
PERCENT SURVIVORSHIP
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
TIME, DAYS
o - LIGHT INFECTION, n=28
● - HEAVY INFECTION, n=30

Figure 9: Soral clumps and sporangia following host death.

A. Soral clump (s) with rhizoids (arrows). X620

B. Soral clump (s) with rhizoids (arrows). Note cuticle (c). X620

C. Sporangium (sp) with granular cytoplasm and rhizoids (arrows). X620

D. Sporangia (sp) with granular cytoplasm, rhizoids have disappeared. Note cuticle (c). X620

Bar scale (D) represents 40 μm on A-D.

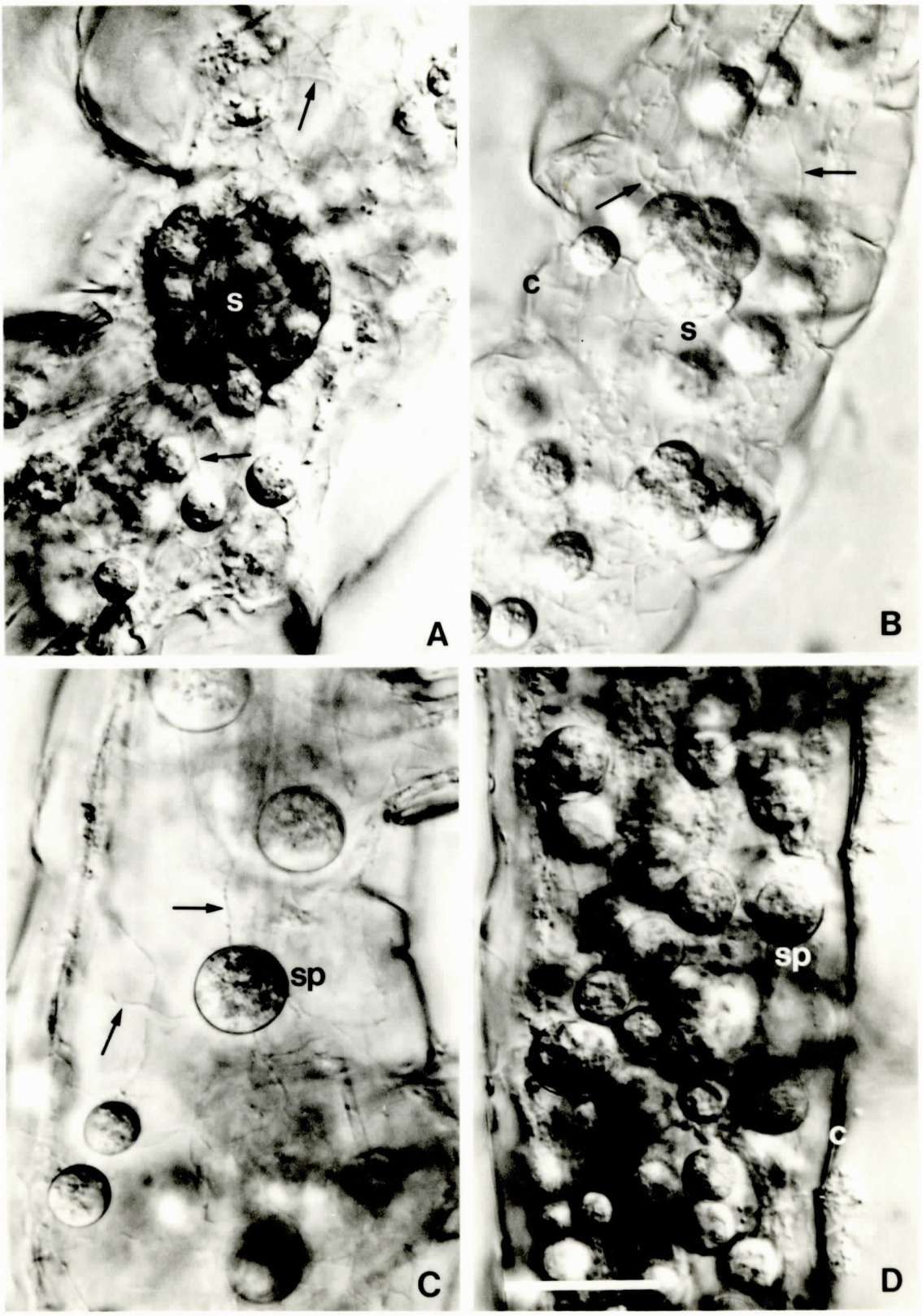

s
A
c
s
B
sp
C
sp
c
D

Table 3. Sporangial diameter following zoospore discharge, measured in 20 animals (laboratory infection group).

Animal	Number of Sporangia Meas.	Mean Diameter	SD(1)
1	101	10.8 μm	2.0
2	34	10.9	1.4
3	84	10.8	2.1
4	100	9.2	1.3
5	99	11.1	1.8
6	56	10.6	1.6
7	56	10.6	1.6
8	103	11.0	1.7
9	106	14.1	2.3
10	87	9.8	1.8
11	100	10.5	2.5
12	100	10.2	1.6
13	51	10.5	1.4
14	64	9.5	1.8
15	105	10.5	1.5
16	44	10.9	1.4
17	81	10.4	1.9
18	102	10.0	1.8
19	25	10.4	1.9
20	103	11.1	1.7

(1) Variances are significantly nonhomogeneous ($p=0.0000$) as determined by Barlett's Test for homogeneity of variance.

Figure 10: Maturing sporangia and zoospore discharge.

A. Sporangia (sp) in an animal collected from nature. X620

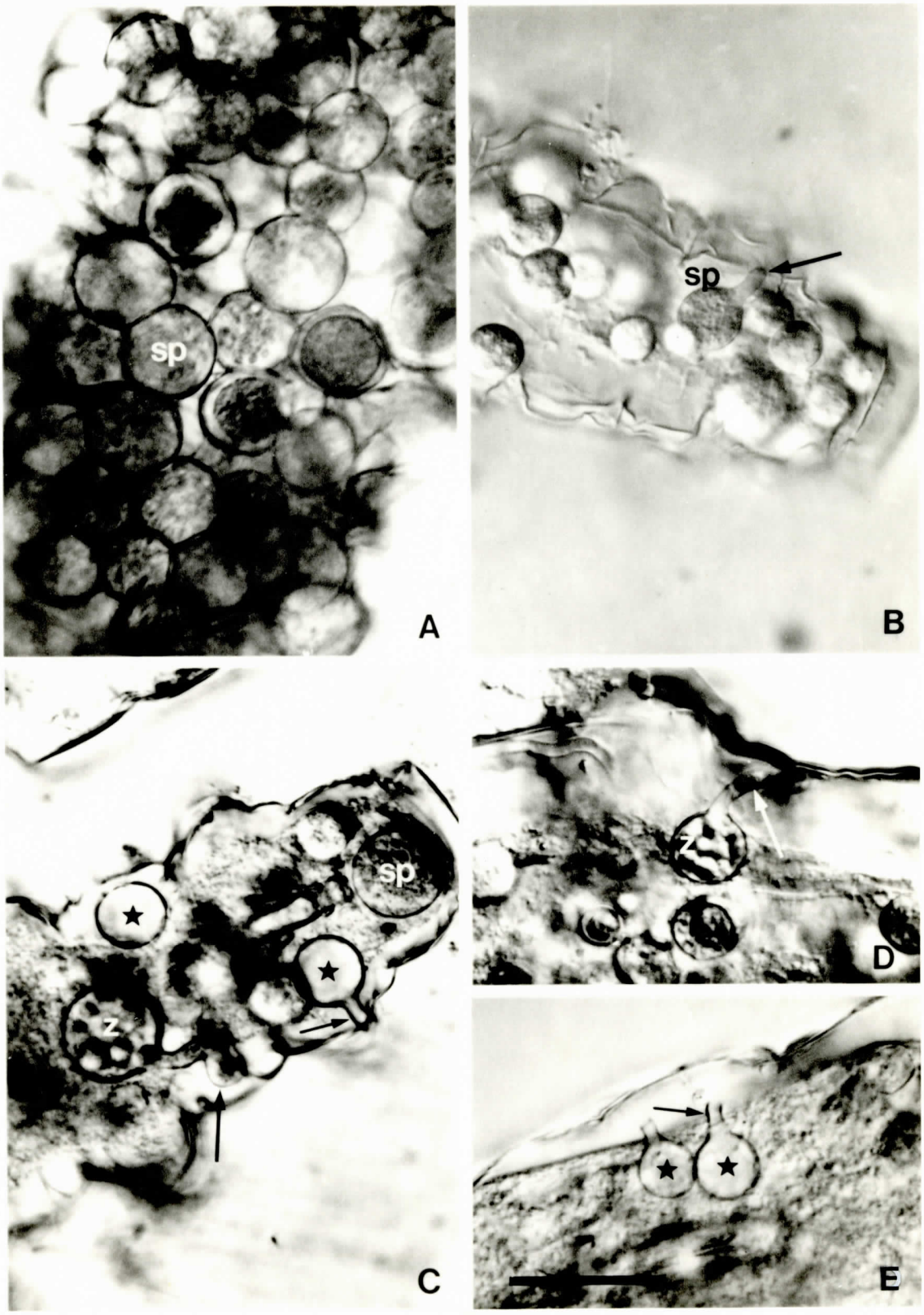
B. Sporangium (sp) with developing exit papilla (arrow). X620

C. Sporangia at various stages including granular sporangium (sp), sporangium forming zoospores (z), and empty discharged sporangia (stars). Note convoluted exit papilla (large arrow) and exit papilla extending through cuticle (small arrow). X620

D. Discharging sporangium containing zoospores (z). Note elongated zoospore (arrow) constricted within exit papilla. X620

E. Empty discharged sporangia (stars); note receded body contents and exit papilla (arrow) which did not reach to outside of animal. X620

Bar scale (E) represents 40 μm on A-E.


sp
A
sp
B
sp
z
C
z
D
E

Sporangial maturity, marked by the development of an exit papilla (Fig. 10B), is usually achieved within one to three days of host death. The exit papilla (diameter 3-4 μm) normally penetrates the host cuticle and extends 1-3 μm outside the cuticle (Figs. 10D, 11A, B). The exit papilla may twist and bend several times before exiting (Fig. 10C), especially if the body contents of the animal have receded from the cuticle as often happens in laboratory infections. Within a day of papilla formation, the sporangial cytoplasm divides into zoospores (4-6 μm diameter) (Fig. 10C, D). These begin to move about inside the sporangium, the tip of the exit papilla dehisces, and the zoospores move down the exit papilla one at a time, flagellum posterior. The zoospores are constricted by the narrow passage (Fig. 10D), and appear to move in an amoeboid fashion. After each zoospore squeezes out of the exit papilla, it rounds up into a spherical shape (Fig. 11A, B). The zoospore often hesitates for a few minutes before swimming away, so that two or more zoospores accumulate near the end of the papilla (Fig. 11B). The zoospore may then swim in a spiral path for a short distance away from the host, pause briefly, and then swim rapidly off in a straight line. Alternatively, the zoospore may swim in circles of varying diameter.

Figure 11: Zoospore discharge, encysted zoospores, and epibiotic zoosporangium.

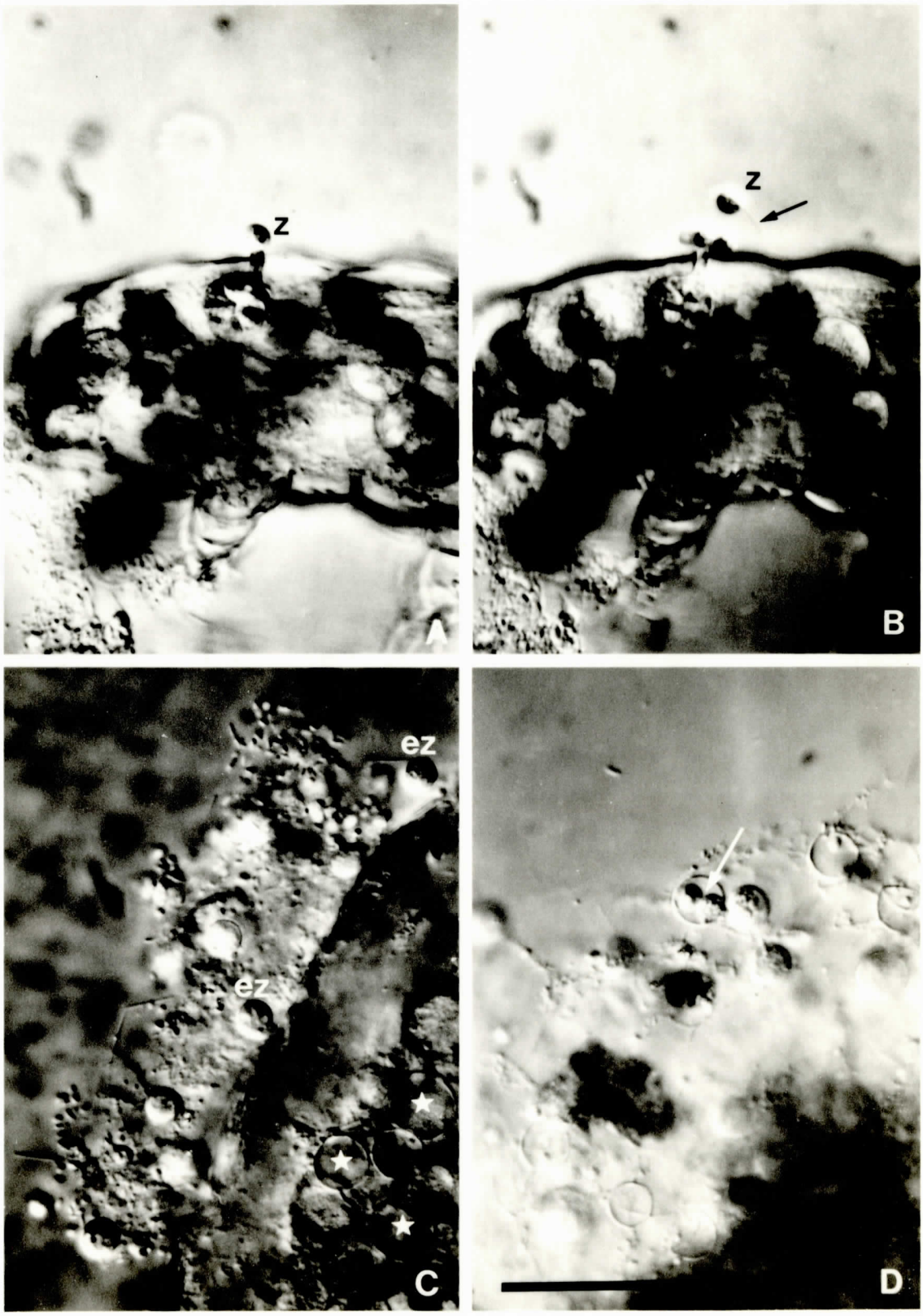
A. Exiting zoospore (z). X620

B. Zoospore (z) with visible flagellum (arrow); note cluster of zoospores near opening of exit papilla. X620

C. Encysted zoospores (ez) near dead animal. Note empty sporangia (stars). X620

D. Epibiotic fungal growth; note sporangium containing two zoospores (arrow). X620

Bar scale (D) represents 40 μm on A-D.


z
A
z
B
ez
ez
C
D

The zoospores change direction often, and sometimes pause and exhibit amoeboid movement temporarily before swimming again.

Zoospores that fail to find a new host often form spherical cysts, lose or retract their flagella, and develop no further (Fig. 11C). Sometimes, zoospores will attach to the cuticle of the original dead host; they form an appressorium, penetration tube, and rarely a rudimentary thallus, but develop no further (Fig. 2D, E). If the sporangial exit papilla fails to penetrate the host cuticle (Fig. 10E), then the zoospores become trapped inside the cuticle and form spherical cysts. Those zoospores which successfully locate a new host attach to that animal and encyst, starting the infection cycle again.

The Epibiotic Life Cycle

The first evidence to suggest an epibiotic life cycle for *Sorochytrium milnesiophthora* was observed by R. Dewel (personal communication) in dead animals from freshly hydrated Sim's Creek samples collected in May, 1983. These animals were full of orange sporangia; within one day of hydration, an epibiotic fungal growth appeared on the surface of the dead tardigrades, characterized by spherical bodies connected by a

rhizomycelium (Fig. 11D). These spheres formed sporangia, each with a single exit papilla (Fig. 11D), and discharged zoospores into the surrounding water. The zoospores resembled the typical *Sorochytrium* zoospores, with two exceptions: first, several of these zoospores were larger than normal and possessed as many as four flagella; second, limited attempts to infect live *Milnesium* by exposure to the zoospores were unsuccessful.

Continuous observations were then carried out over a 24 hour period following hydration of several Sim's Creek samples, the objective being to ascertain whether the epibiotic growth could have originated from the *Sorochytrium* sporangia inside the dead animal. Five dead infected animals were observed at approximately 15 minute intervals by J. Bond, R. Dewel, and this writer. One animal developed growth resembling fungal hyphae; however, the origin of this was not ascertainable and the growth never developed any enlargements or sporangia. Another animal, significantly free from external contamination by bacteria or other debris, produced zoospores which encysted on the glass slide or coverslip and proceeded to germinate. Many zoospores in this preparation were observed with a germ tube or branching rhizoids (Fig. 12A). In several cases, two

Figure 12: Germination of encysted zoospores, and polycentric growth on agar.

A. Germinating encysted zoospore (gz). X620

B. Germinating encysted zoospore (gz); note two connected spheres, one with contents and the other empty. X620

C. Germinating encysted zoospore (gz) with rhizoid extending back to exit papilla. X620

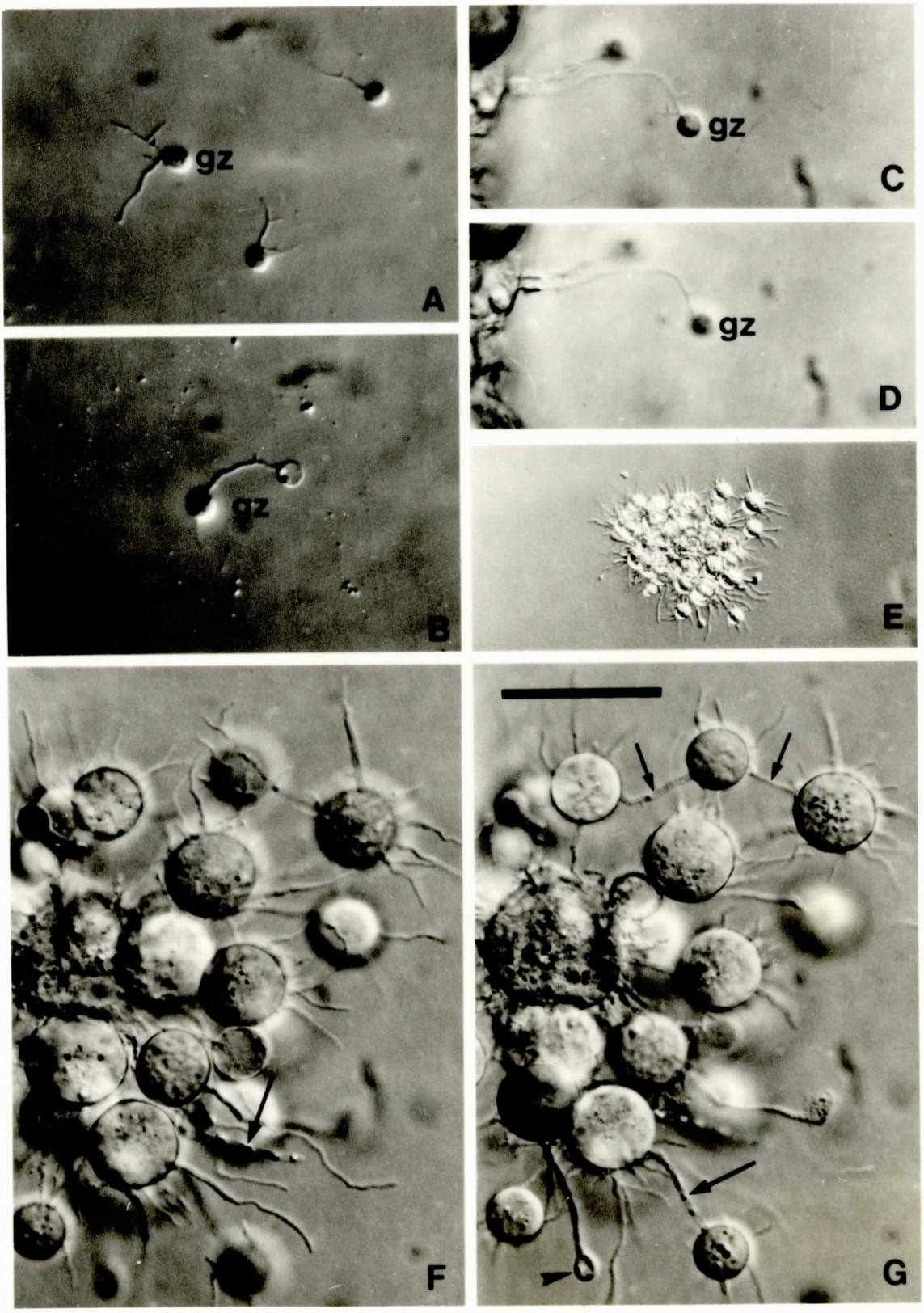
D. Same as C, different focal plane. X620

E. Low-power view of polycentric growth on agar. X155

F. Polycentric growth consisting of spherical bodies connected by a rhizomycelium. Note particles (arrow) in filament. X620

G. Same as F. Note particles in filament (large arrow), developing enlargement (arrow head) and connections (small arrows) between spheres. X620

Bar scale (G) represents 40 μm on A-D and F-G, 160 μm on E.


gz
A
gz
C
gz
D
gz
B
E
F
G

spherical structures connected by a tube or rhizoid were observed (Fig. 12B). One of these spheres had visible contents, the other looked empty except for small rapidly moving suspended particles. It was not clear whether the two connected spheres were an encysted zoospore with cytoplasm and an empty rudimentary thallus, or an empty encysted zoospore which had discharged its contents into a developing thallus.

This strong suggestion of an epibiotic growth capability was further supported by culture attempts (R. Dewel, personal communication). Several diluted drops containing zoospores from the above described specimen were applied to agar plates; several days later, a small polycentric growth was observed in the uncontaminated area of one plate (Fig. 12E-G). It is reasonable to assume that this growth originated from a single germinating zoospore of *Sorochytrium milnesiophthora*. The narrow connections between the spherical enlargements of this rhizomycelium contained small particles (Fig. 12F, G) which possibly are migrating nuclei or other cellular materials.

It is not clear what ambient conditions stimulate the germination of zoospores in the absence of a new host, since in most cases they simply form spherical

cysts and develop no further. On only one other occasion, involving an infected animal from a campus sample which died after hydration, was zoospore germination observed. In this instance, several encysted zoospores were observed with rhizoids or germ tubes (Fig. 12C, D); these zoospores were only a short distance (less than the length of the animal's legs) from the sporangial exit papillae. Three of these zoospores had a rhizoid or tube extending back to the opening of the nearest exit papilla, suggesting a possible chemotactic response to diffusing nutrients. These germinating zoospores were observed for several days and did not develop further.

The epibiotic growth on the surface of the dead infected tardigrade was observed primarily in Sim's Creek samples collected in May, 1983. It was not observed in the Appalachian State University Campus samples collected from June to December, 1983.

DISCUSSION

Sorochytrium milnesiophthora exhibits a unique combination of characters which do not fit well into present taxonomic schemes. The fungus belongs to the class Chytridiomycetes based upon the production of posteriorly uniflagellate zoospores (Alexopoulos and Mims, 1979). Within this class, *Sorochytrium* seems most closely allied with the order Chytridiales, based upon the absence of a true mycelium (Alexopoulos and Mims, 1979) and the possession of traits characteristic of certain forms within this order. Karling (1977) summarized the families within the Chytridiales as follows:

> At present, family distinctions are based primarily on thallus morphology, type of development, and organization. In these respects the families fall into broad categories of holocarpy, eucarpy, monocentricity and polycentricity. In the monocentric category some species are holocarpic without a vegetative absorbing system of rhizoids, while others are eucarpic with well-defined reproductive and vegetative portions. Among the holocarpic species, families are distinguished on the basis of whether the thallus develops into a single reproductive portion, or cleaves internally into a large number of them. Among the eucarpic monocentric species a familial distinction is usually made on whether the reproductive portion develops

Achlyogetonaceae of the order Chytridiales, whose tubular thalli are partitioned into a linear series of sporangia, and second the family Plasmodiophoraceae of the class Plasmodiophoromycetes.

According to Karling (1964), the Synchytriaceae differ from the Achlyogetonaceae in the progressive cleavage of the sorus and in the possession of a common soral membrane, which is not present in the latter. When Karling (1964) speaks of progressive cleavage in *Synchytrium*, he apparently refers to a dividing of the cytoplasm by many cleavage partitions which begins near the outside of the thallus and progresses to the center. *Sorochytrium milnesiophthora* does possess a common soral membrane, but cleaves in a different manner; the initial division occurs often by a single cleavage plane, then a second forms perpendicular to the first, and then irregular divisions occur within these major segments. This pattern is loosely reminiscent of the cleavage of a zygote into a morula.

Karling (1964) distinguishes *Synchytrium* from the Plasmodiophoraceae as follows:

> The presence of anteriorly biflagellate, heterocont zoospores, plasmodia, schizogeny, and the formation of cystosori in members of the Plasmodiophoraceae are the outstanding

> differences which separate them from *Synchytrium* and preclude chose relationship, in the author's opinion.

Sorochytrium differs from the Plasmodiophoraceae in these same characters, and a close phylogenetic position is not warranted.

The resemblance of *Sorochytrium milnesiophthora* to the chytrid family Entophlyctaceae is limited to the presence of eucarpy and the development of the thallus as an enlargement of the germ tube. Members of the Entophlyctaceae do not exhibit sorus formation or a combination of monocentric and polycentric growth phases.

Sorochytrium milnesiophthora differs from the chytrid family Physodermataceae in several aspects. Although both show monocentric as well as polycentric growth phases, the polycentric phase of the Physodermataceae is endobiotic and the monocentric phase is epibiotic (Sparrow, 1960). The reverse is true in *Sorochytrium*. The Physodermataceae are plant parasites and do not exhibit sorus formation; *Sorochytrium* is an animal parasite, possibly obligate to a single tardigrade species.

It should be emphasized that the ultrastructure of the zoospore, when elucidated, will contribute much to an understanding of the phylogenetic position of *Sorochytrium milnesiophthora*. In fact, many authorities

consider the zoospore to be the single most important taxonomic character in such fungi (Lange and Olson, 1979). Preliminary studies using transmission electron microscopy indicate that *Sorochytrium* zoospores may share important traits with members of the order Blastocladiales of the class Chytridiomycetes (R. Dewel, personal communication). Pending the outcome of such studies, this writer would suggest that a new family be erected within the order Chytridiales to accommodate this singular species of fungus.

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VITA

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