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THE ULTRASTRUCTURE OF ASCOSPOROGENESIS IN FREEZE-SUBSTITUTED EUASCOMYCETES

By

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ABSTRACT

THE ULTRASTRUCTURE OF ASCOSPOROGENESIS IN FREEZE-SUBSTITUTED EUASCOMYCETES

By

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High-pressure and propane-jet freezing in conjunction with freeze-substitution were used to examine the process of ascosporogenesis in *Thelebolus crustaceus* (Discomycete), *Sordaria humana* (Pyrenomycete), and *Emericellopsis terricola* (Plectomycete). Similarities and differences were evaluated in these three phylogenetically distinct Euascomycete groups in order to provide a coherent description of the process of ascosporogenesis with accurate terminology. The enveloping membrane system (EMS) was first observed as infoldings of the plasma membrane in all three species. The small plasma membrane invagination units were released into the ascus cytoplasm and maintained a position adjacent to and parallel with the ascus wall. Continued growth of the double-membraned EMS was either through the production of more plasma membrane invaginations or direct vesicular addition to the developing enveloping membrane cylinder. Eventually, the EMS moved inwards to encompass uninucleate portions of cytoplasm with

accompanying organelles. The process of membrane movement was facilitated by the nucleus-associated organelle with numerous microtubules. Cell wall deposition between the double membrane of each ascospore initial was variable, but appeared largely due to cisternal vesicles from within ascospore initials. During primary wall development microtubules were observed adjacent to the ascospore initial wall layer and parallel with its long axis. The secondary wall layer was derived primarily from within ascospore initials, however, some secondary wall material and/or precursors appeared to be deposited from the epiplasm.

High-pressure freezing provided overall the best preservation of the structures associated with spore formation, when compared to plunge and propane-jet freezing techniques. High-pressure frozen samples exhibited excellent preservation of microtubules, cisternae, vesicles, the enveloping membrane system and other cellular organelles. No artifacts could be specifically linked with the high pressures involved with freezing the samples or with the use of dextran (a non-membrane permeable cryo-protectant). Propane-jet frozen samples exhibited some artifacts associated with the extensive manipulation of samples prior to freezing.

Laser scanning confocal microscopy was used to examine immunofluorescently labeled microtubules of the epiplasmic microtubular network in *Thelebolus crustaceus* and to examine the fluorescently labeled FITC-conjugated lectins conconavalin A (for mannans and/or glucans) and wheat germ agglutinin (for chitin) in *Sordaria humana*.

ACKNOWLEDGEMENTS

I wish to express my appreciation to Dr. Karen Klomparens for her generous support of my numerous endeavors and for her encouragement throughout my degree. I also thank Dr. Joanne Whallon for introducing me to laser scanning confocal microscopy. I especially wish to thank my wife Monica Converse Czymmek and my son Austen Converse Czymmek for being extremely patient and supportive during my many absences while teaching or doing research.

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INTRODUCTION

Ascosporogenesis is a unique form of sexual reproduction in the group of fungi known as Ascomycetes. This process is unique because sexual spores are produced involving a distinctive form of cytokinesis, termed "free cell formation". Ascospore initials are produced when the enveloping membrane system, which is a double membrane, surrounds and delimits nucleate portions of cytoplasm with accompanying organelles. Cell wall materials are deposited between the double membranes of each ascospore initial to eventually form mature ascospores.

The vast majority of knowledge accumulated concerning the process of ascosporogenesis was obtained through ultrastructural studies using conventional chemical techniques for fixation. The limitations of conventional chemical fixation has resulted in many conflicting reports concerning the origin and development of the enveloping membrane system and many details concerning ascospore initial development. With the advent of high-pressure freezing in conjunction with freeze-substitution one is now able to observe many transient cellular events and structures which until now have been difficult or impossible. High-pressure freezing allows us to ultrarapidly immobilize cells without significant ice-crystal damage up to a

diameter of 600μm. A large number of ascomycetes have sexual structures which are smaller than 600μm making them ideal specimens for study.

Three phylogenetically distinct groups of Euascomycetes were examined; the 64-spored operculate Discomycete *Thelebolus crustaceus*, the 8-spored Pyrenomycete *Sordaria humana*, and the 8-spored winged Plectomycete *Emericellopsis terricola*. It was my goal to determine what commonalities these three Euascomycetes shared with each other during the process of ascosporogenesis. Considering the variability in size, shape, appearance, and number of spores produced within each ascus, it was expected that many differences would exist.

CHAPTER I

THE ULTRASTRUCTURE OF ASCOSPOROGENESIS IN FREEZE-SUBSTITUTED *THELEBOLUS CRUSTACEUS*: ENVELOPING MEMBRANE SYSTEM AND ASCOSPORE INITIAL DEVELOPMENT

ABSTRACT

High pressure and propane-jet freezing were essential for determining the origin and development of the enveloping membrane system during ascosporogenesis in Thelebolus crustaceus. Prior to the completion of mitotic divisions within the ascus, invaginations of the plasma membrane initiated formation of the enveloping membrane system. Fusion of individual units of the closely spaced double-membranes resulted in the formation of a cylinder around most of the ascus protoplasm. This double-membraned cylinder enveloped individual nuclei with accompanying cytoplasm and organelles to form ascospore initials. Envelopment of each ascospore initial appeared to be facilitated by a nuclear associated organelle and closely associated microtubule organizing center. Initially, cell wall materials and/or precursors were deposited between the closely spaced double membranes from within the ascospore initials. Secondary wall formation appeared to be

deposited, in part, from the epiplasm. Microtubules located adjacent to the inside cell wall of the spore initials appeared to contribute to an elliptical shape. Subsequent to epispore wall formation, numerous microtubules were found associated with the outer membranes of the ascospores and appeared to interconnect the spores into a single mass before discharge.

KEY WORDS: Thelebolus crustaceus, ascosporogenesis, high pressure freezing, propane jet freezing, ultrastructure, laser scanning confocal microscopy.

MATERIALS AND METHODS

Cultures of Thelebolus crustaceus (ATCC culture No. 76667) were grown on dialysis membrane on V-8 juice agar at 21 C in 12 hr light/12 hr dark. For freeze-substitution, samples were processed in either an RMC MF7200 propane jet ultrarapid freezer or a Balzer's HPM 010 high pressure freezer. Samples frozen using the high pressure freezer were excised and placed in gold hats with a 20% solution of dextran for 5 min prior to freezing. After storage in liquid nitrogen, samples from both methods were freeze-substituted in a RMC MS6200 holding device using a solution of 0.05% uranyl acetate and 2% osmium tetroxide in acetone for 72 hr at -85 C. Samples were then removed and slowly brought to room temperature at intervals of 2 hr each at -35 C, -25 C, -12 C, 0 C, 10 C and room temperature. The samples then were transferred to glass vials, rinsed 3X in 100% acetone, and infiltrated and embedded with Spurr's epoxy resin.

Ultrathin sections were stained with uranyl acetate and lead citrate and examined using a JEOL 100CX II transmission electron microscope. Cultures were prepared for immunofluorescence by inoculating agar plates, as described above, with coverslips attached to the petri dish lids. Within 14 days the coverslips were covered with ejected ascospores which were treated by the technique of Roberson et al (1989) with FITC anti-tubulin (Sigma Chemical Company) as the primary antibody and FITC protein A (Sigma Chemical Company) as the secondary antibody. Samples were observed using a Zeiss LSM laser scanning confocal microscope with a 488 nm argon laser. Optical serial sections (aproximately 310 nm thick) were obtained for high resolution micrographs of the three-dimensional arrangement of the microtubules.

INTRODUCTION

The fine structure of ascomycete spore development has been studied for more than three decades. The majority of these studies used conventional chemical fixation as the method of preservation. From these studies, it is generally accepted that two distinct modes of membrane development (with a few rare exceptions) occur in ascospore initial formation. These are the Hemiascomycete-type, originating "de novo" around each nucleus, and the Euascomycete-type, originating from invagination of the double-membraned cylinder termed the "ascus vesicle" (4).

Within the last decade, improved techniques in specimen preparation have been developed which avoid "wet" chemical fixation. One such alternative, freeze-substitution via plunge freezing, is a popular and relatively inexpensive procedure in which samples are rapidly frozen and fixed (23, 29, 30, 31, 40, 46). Recent cryopreservation of ascomycetes involved plunge-frozen samples of Eleutherascus (9) for ascospore wall characterization, and examination of ascosporogenesis in Ascodesmis nigricans (40).

More recently, the development of the propane jet freezer and high pressure freezer has allowed ultrastructural research to advance from preparing primarily unicellular organisms to larger and/or multicellular organisms (23). Freeze-substitution using the high pressure freezer is particularly suited to solving preparation problems associated with studying ascosporogenesis. These difficulties center around spatial and conformational preservation of the membranes and/or structures involved in ascospore initial delimitation.

The intent of this study was to define the events in the process of ascosporogenesis in Thelebolus crustaceus. Ascosporogenesis in Thelebolus crustaceus was previously examined (34) using electron microscopy, however, this study used conventional chemical fixation and lacked details concerning membrane formation and development in ascospore initial delimitation. In addition, I used this study to determine the value of high pressure freezing in conjunction with freeze-substitution for fungal ultrastructure. During this study, the discovery of an epiplasmic

microtubular network allowed us also to use the laser scanning confocal microscope to evaluate its effectiveness in studies of fungal development.

RESULTS

Origin of enveloping membrane system

Infolding of the plasma membrane at points along the ascus cell wall initiated the formation of the enveloping membrane system (EMS) (Figs. 1, 2 and 3). In the early stages, portions of the plasma membrane invaginated resulting in the exoplasmic surfaces facing each other, while the protoplasmic surfaces remained in contact with the cytoplasm (Figs. 2 and 3). Plasma membrane invaginations visualized when prepared by propane jet freezing (Fig. 3) were less electron-opaque and had a greater separation between the exoplasmic surfaces then their high pressure frozen counterparts (Figs. 1, 2, and 4). The plasmalemmal invaginations then separated from the plasma membrane and entered the cytoplasm remaining in close proximity and parallel to the ascus wall (Fig. 2). Fusion of the numerous plasmalemmal invaginations resulted in the formation of a double-membraned cylinder (EMS) which encompassed most of the ascus protoplasm (Fig. 4). Prior to the completion of the EMS, nuclei (each containing a nucleolus) (Fig. 4) could be found along the periphery of the ascus with many cisternae, mitochondria, and small vesicles/vacuoles located throughout the cytoplasm (Figs. 1 and 2).

Ascospore initial envelopment

The EMS development progressed with its inward movement and/or growth. As a result most of the cytoplasm, vesicles/vacuoles, and organelles were en masse in the center of the ascus (Fig. 5). Small portions of the EMS surrounded individual nuclei with accompanying cytoplasm and organelles with the mitochondria generally observed at the neck of each cup-shaped EMS unit (Figs. 5, 6 and 7). At this stage, the EMS was still a closely spaced, double-membraned structure (Fig. 8). Cisternae and microtubules (Figs. 6, 7 and 8) were often on the protoplasmic side of the developing ascospore initial. Invagination of the EMS around each individual nucleus appeared to be facilitated by a microtubule organizing center (MTOC) (Figs. 9 and 10) in close association with the nucleus-associated organelle (NAO) (Figs. 10 and 11). The MTOC and small disc-shaped NAO always were found oriented towards the cell wall (the location of the EMS) (Fig. 9). The MTOC consisted of an electron-transparent zone sandwiched between two narrow, parallel electron-opaque bands from which numerous microtubules emanated in various directions (Fig. 11). These microtubules, and many others which were not directly associated with the MTOC, could be found throughout the developing ascospore initial cytoplasm (Figs. 9 and 10). Many direct points of contact between microtubules and the innermost membrane of the EMS were seen (Fig. 12). No microtubules were observed in the ascus cytoplasm on the epiplasmic side of the EMS. The completion of envelopment of ascospore initials was indicated by the

presence of a maximum of sixty-four nuclei, each with accompanying cytoplasm and organelles, surrounded by two closely spaced double membranes.

Cell wall development

The electron-transparent primary wall materials and/or precursors were deposited between the closely spaced double membranes resulting in their gradual separation (Figs. 13 - 17). Prior to secondary wall deposition, many microtubules were found adjacent to the inner ascospore initial cell wall parallel to its long axis (Figs. 13 and 14). These microtubules converged at both ends of the ellipsoidal ascospore initials (Figs. 15 - 17). Concurrent with primary wall growth was the presence of many cisternae within the ascospore initial (Figs 16 and 17). Late in primary wall formation the outer ascospore initial membrane split in several places, presumedly from the expansion of the wall material and/or addition of precursors. Early development of the operculum was seen at the ascus tip as a localized thickening of the ascus wall (Fig. 18). The periplasm of the mother ascus decreased in electron-opacity but appeared to remain functional as the ascospore initials matured (Fig. 18 - 20). Few electron transparent vesicles/vacuoles remained. However, many electron dense flattened vesicle-like structures were present (Fig. 20). These flattened vesicles may have developed from the numerous electron-transparent vesicles/vacuoles (Fig. 1) formed in the earlier stages of ascosporogenesis. Formation of the

electron-opaque secondary wall layer was accompanied by the accumulation of many darkly stained cisternae from within the ascospore initial (Figs. 21, 22 and 23). Points of apparent cisternal vesicle fusion with the inner membrane of the ascospore initial were observed (Figs. 21 and 22). Microvesicles were present within the ascospore initial cytoplasm during secondary wall development (Fig. 23). No microtubules were seen along the spore initial cell walls that corresponded with those found in primary wall development. As the ascospore initials matured, the epispore wall was laid down between the electron-transparent primary wall and electron-opaque secondary wall (Fig. 24). Subsequent to epispore wall deposition, many small spherical bodies were seen in the periplasm along the spore-investing membrane, and based on location and density similarities to later stages were presumably involved in secondary wall formation (Fig. 24).

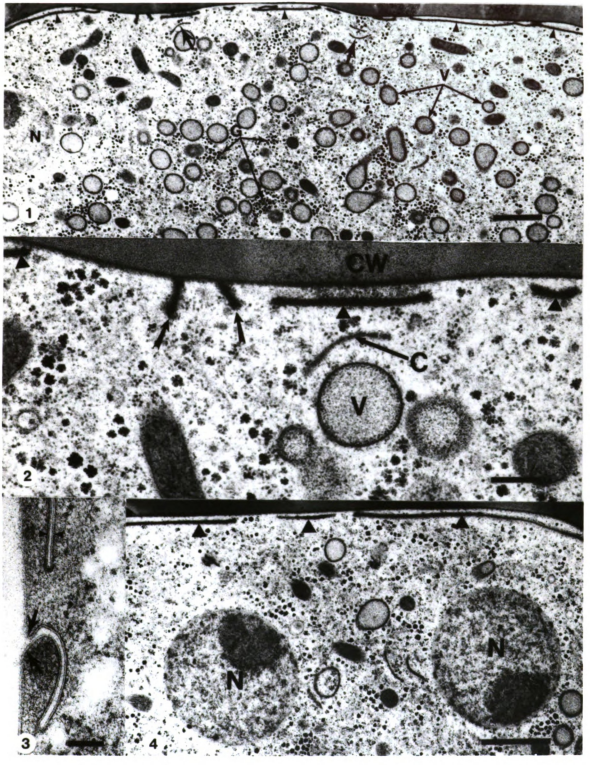
Epiplasmic microtubules

After formation of the epispore wall an accumulation of microtubules, the epiplasmic microtubular network (EMN), developed within the ascus periplasm (Fig. 25). These microtubules were attached to the outer membranes of the ascospore initials (Fig. 25 and 26). Bundles of microfilaments were also observed within the epiplasm closely associated, but not in direct contact with, the outer ascospore membranes (Fig. 27). Cross-sections of the bundles of microtubules often showed groups of one, two, or three microtubules bound by a membrane-like structure (Fig. 28).

One or more, viable-appearing, nuclei were often found excluded from the ascospore initials within the ascus periplasm and were seen well into the late stages of ascospore development (Fig.29).

Laser scanning confocal micrographs in conjunction with immunofluorescence confirmed the observations that large numbers of microtubules appeared to interconnect the individual ascospores into a single spore mass (Fig. 30). Ascospores expelled forcibly from asci usually were held into a single spore mass (Fig. 30) or occasionally separated into smaller spore clusters (Figs. 31 and 32). When individual ascospores were examined with optical sectioning, microtubules generally appeared to be attached at either end of the elliptical ascospores with a microtubule free zone around their midsections (Figs. 33, 34 and 35).

FIGS. 1-4. Thelebolus crustaceus. Fig. 1. High pressure frozen (HPF) ascus showed variable sized segments of the enveloping membrane system (EMS)(arrowheads) adjacent to the ascus wall. Note the nucleus (N), cisternae (arrows), numerous vesicles (V) and glycogen particles within the cytoplasm. Bar = 1μm. Fig. 2. Early development of the EMS as infoldings of the plasma membrane (arrows) and slightly more developed as small segments of closely spaced double membranes (arrowheads) adjacent to and parallel with the ascus cell wall (CW). Flattened, slightly concave cisternae were often found in close proximity to the EMS. Vesicle (V). Bar = 0.25μm. Fig. 3. Propane jet-frozen (PJF) sample. EMS with lower electron-opacity and wider separation between the double membranes (arrowheads) then HPF samples. Direct continuity (arrows) between the plasma membrane and the EMS was seen. Bar = 0.1μm. Fig. 4. In later stages the EMS (arrowheads) was a mostly continuous double-membraned cylinder surrounding most of the ascus cytoplasm. Nuclei (N) were lined up along the ascus periphery. Bar = 1 μm.


N
V
1
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C
V
2
N
3
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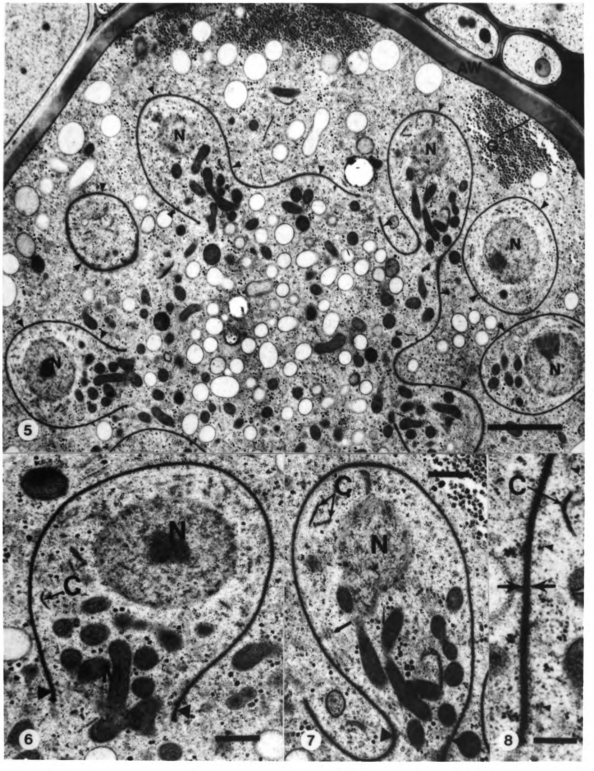
4

FIGS. 5-8. Thelebolus crustaceus. HPF. Fig. 5. Invagination of the EMS cylinder (arrowheads) around individual nuclei (N) with accompanying cytoplasm and organelles. During this process the EMS also subdivided into smaller membrane units. Ascus wall (AW), glycogen (G). Bar = 2μm. Fig. 6. The smaller units of EMS were generally cup-shaped with several mitochondria (M) located at the neck (arrowheads). Cisternae (C). Bar = .5μm. Fig. 7. As the developing ascospore initial matured, the neck (arrowheads) narrowed, presumably controlled by microtubules (arrow). Nucleus (N), cisternae (C). Bar = .5μm. Fig. 8. At this stage the EMS (arrows) was still a closely spaced double-membraned structure. Cisternae (C) and microtubules (arrowheads) were often seen in close association with the protoplasmic side of the developing ascospore initial. Bar = 2μm.


N
N
N
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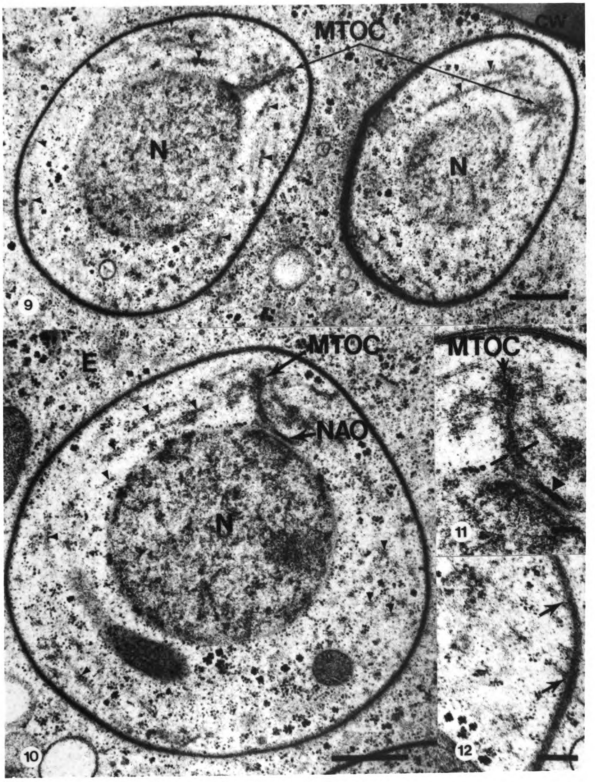
AW
5
N
C
6
C
N
7
C
8

FIGS. 9-12. Thelebolus crustaceus. HPF. Fig. 9. Two ascospore initials with oblique sections through their microtubule organizing centers (MTOC), both of which are oriented towards the cell wall (CW). Some microtubules (arrowheads) were seen radiating from the MTOC while others within the ascospore initial did not appear associated it. Nucleus (N). Bar = 0.5μm. Fig. 10. Completely delimited ascospore initial with a nucleus (N), nuclear associated organelle (NAO) and microtubule organizing center (MTOC). Microtubules (arrowheads) were seen throughout the ascospore initial protoplasm, but were absent from the epiplasm (E). Bar = 0.5μm. Fig. 11. Details from Fig. 10 showed a bridge (arrows), consisting of two electron-opaque zones separated by an electron-transparent zone interconnected the microtubule organizing center (MTOC) to the flat disc-like nucleus-associated organelle (NAO) (arrowhead). Bar = 0.1μm. Fig 12. Microtubules (arrows) were seen in direct contact with the interior membrane of the EMS. Bar = 0.2μm.


MTOC
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9
E
MTOC
NAO
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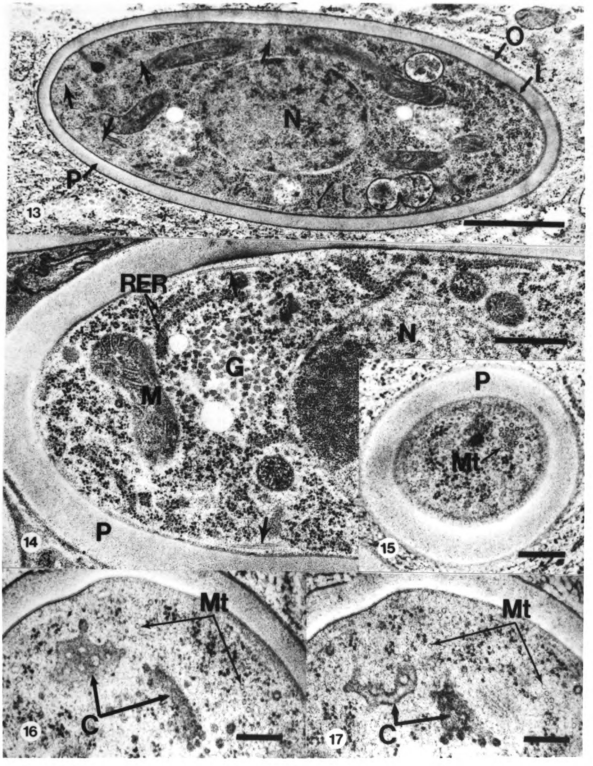
10
MTOC
11
12

FIGS. 13-17. Thelebolus crustaceus. Propane jet frozen (PJF). Fig. 13. The outer (O) and inner (I) membranes of the EMS separated as primary cell wall materials and/or precursors (P) were deposited between them. During the early stages of primary wall development microtubules (arrows) were oriented parallel with the long axis of the ascospore initial. Nucleus (N). Bar = 1μm. Fig. 14. A typical ascospore initial contained rough endoplasmic reticulum (RER), mitochondria (M), glycogen (G), microtubules (arrows) adjacent to the primary cell wall (P), and a nucleus (N). Bar = .5μm. Fig. 15. This cross-section of an ascospore initial showed a bundle of microtubules which had converged at the tip. Primary cell wall (P). Bar = 0.2μm. Fig. 16 and Fig. 17. Serial sections of an ascospore initial with waffle-like cisternae (C). Microtubules (Mt). Bar = 0.2μm.


O
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P
Mt
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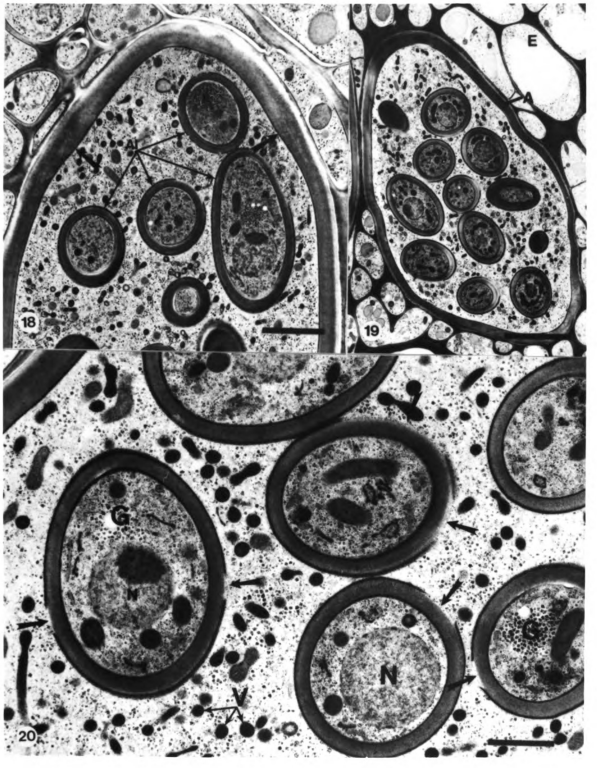
FIGS. 18-20. Thelebolus crustaceus. HPF. Fig. 18. Longitudinal section of this ascus showed the operculum consisted of a thickening (arrows) in the lateral walls below the ascus apex. Ascospore initials (AI). Bar = 2μm. Fig. 19. An oblique cross-section through an ascus (A) surrounded by enveloping hyphae (E). Bar = 4μm. Fig. 20. Late primary wall development in ascospore initials showed discontinuities (arrows) in the outer membrane suggesting primary wall materials may be largely deposited from within the spore initial resulting in expansion and rupture of the outer membrane. Electron-opaque vesicles (V) were found throughout the epiplasm. Glycogen (G), nuclei (N). Bar = 1μm.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N
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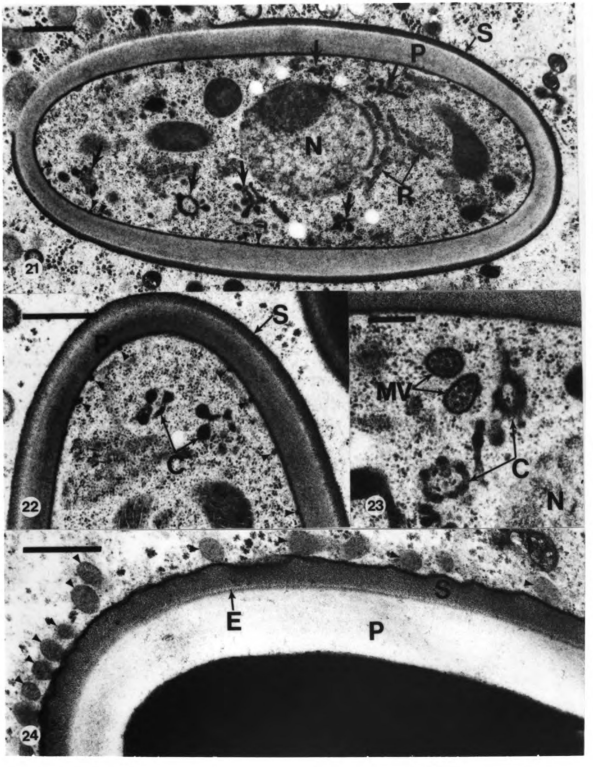
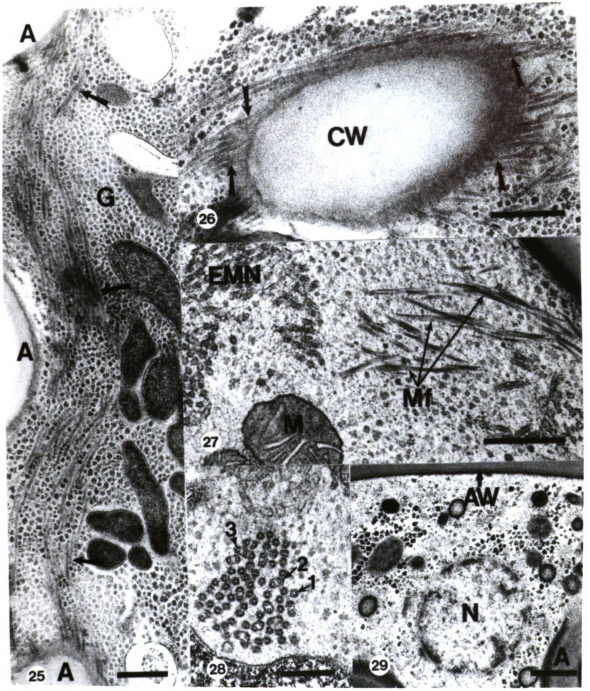
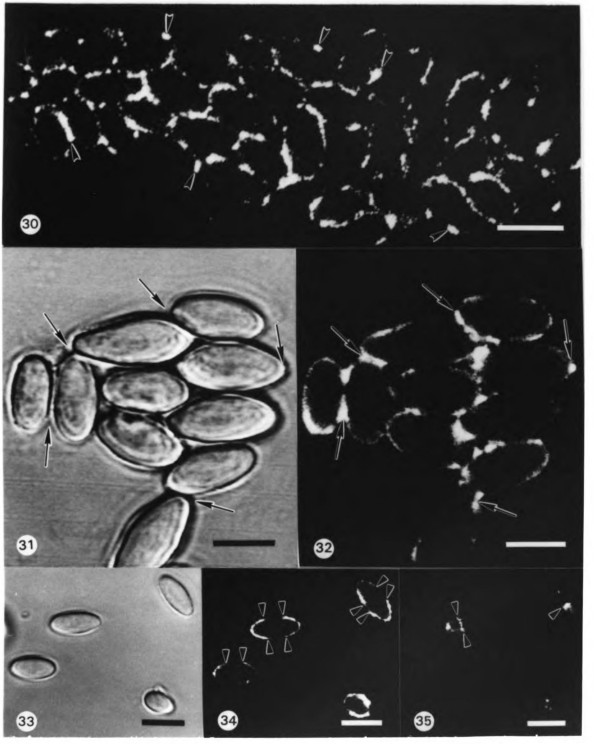
Fig. 21-24 Thelebolus crustaceus. HPF. Fig. 21. Ascospore initials during early secondary wall formation showed many cisternae (arrows) throughout the cytoplasm and many vesicles (arrowheads) fused to the inner membrane. Nucleus (N), rough endoplasmic reticulum (R), primary wall layer (P), secondary wall layer (S). Fig. 22. Unlike the cisternae during primary wall (P) development, cisternae (C) during secondary wall development were very electron-opaque and similar in appearance to the secondary wall layer (S). Bar = 0.5μm. Fig. 23. Multivesicular bodies (MV) were observed within ascospore initials. Nucleus (N), cisternae (C). Bar = 0.2μm. Fig. 24. After the primary wall (P), secondary wall (S) and epispore wall (E) layers were deposited small vesicles (arrowheads) accumulated in the epiplasm around the outer spore initial membrane, presumably to add their contents to the secondary wall. Bar = 0.5μm.


S
P
N
C
R
21
S
P
C
22
MV
C
N
23
E
S
P
24

FIGS. 25-29. Thelebolus crustaceus. Figs. 25-28 PJF . Fig. 29 HPF. Fig. 25. After the deposition of all wall layers an accumulation of microtubules (arrows) termed the epiplasmic microtubular network (EMN) interconnected ascospores (A) into a single spore mass. Glycogen (G). Bar = 0.5μm. Fig. 26. The EMN (arrows) was in direct contact with the outer membrane of the ascospores. Ascospore cell wall (C). Bar = 0.5μm. Fig. 27. Small numbers of microfibrils (Mf) were seen in the epiplasm. Epiplasmic microtubular network (EMN), mitochondrion (m). Bar = 0.5μm. Fig. 28. At late stages of development the EMN consisted of bundles of microtubules with a membranous structure surrounding individual (1), or groups of two (2) and three (3) microtubules at a time. Bar = 0.25μm. Fig. 29. One to three nuclei (N) were often seen within the epiplasm even during the late stages of ascospore (A) development. These nuclei may orchestrate the complex changes which occur in the epiplasm throughout ascospore initial development. Ascus wall (AW), ascospore (A). Bar = 0.5μm.


A
G
A
25
A
26
CW
EMN
M
27
3
2
1
28
M1
AW
N
29
A

FIGS. 30-35. Thelebolus crustaceus. Fig. 30. Laser scanning LM optical section (approximately 310 nm thick) of immunofluorescent microtubules, epiplasmic microtubular network (EMN), that interconnected these ascospores which were ejected from their ascus. Bundles of the microtubules (arrowheads) were seen at many points amongst the spore cluster. Bar = 4μm. Fig. 31. A transmitted image of a cluster of ascospores which failed to show any obvious interconnections (arrows) between ascospores. Bar = 3μm. Fig. 32. The immunofluorescent image of spores in Fig. 31 demonstrated the EMN (arrows) interconnected the ascospores to each other at many locations. Bar = 3μm. Fig. 33. Transmitted image of four individual ascospores. Bar = 3μm. Fig. 34. A median immunofluorescent optical section of the four ascospores of Fig. 33. Microtubules generally appeared to attach at the tips of the elliptical ascospores leaving a microtubule free zone around the midsection of each ascospore. Arrowheads denote the points at which the microtubules discontinue near the midsection. Bar = 3μm. Fig. 35. An optical section of the outer surface of the ascospores from Figs. 33 and 34. Arrows denote the point at which the microtubules terminate near the ascospore midsection. Bar = 3μm.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DISCUSSION

In this study, freeze-substitution using high pressure frozen fungal samples was the method of choice over plunge or propane jet-frozen samples. Plunge-frozen samples (data not shown) exhibited a gradient of minor to severe ice crystal damage within most asci, as might be expected for structures of this size (ascus approximately 40 μm in length). Propane jet-frozen samples showed significant improvement over plunge-frozen material. Some minor and occasional pockets of severe ice crystal damage were seen, however, a much greater proportion of the samples were preserved well. One major disadvantage of propane jet-frozen samples of this size was the extensive manipulation required to insert the samples between copper hats with a slotted gold spacer. Thus, the benefits of less ice crystal damage with propane jet freezing may be compromised by mechanical damage done to samples. Both ice crystal and mechanical damage were eliminated by use of the high pressure freezer. Since samples may be up to 600μm in diameter, the task of excising them from cultures grown on dialysis membrane was much less traumatic to the samples and much easier to handle for the user. With these factors in mind, ultrastructure from high pressure frozen samples would appear more accurate than any of the alternative methods, including conventional chemical fixation.

Since one of the earliest published reports of the existence of the membrane system which delimits ascospore initials (26), there have been

numerous attempts at determining its origin and nature of development. Many theories have been proposed to explain the phenomenon of ascosporogenesis (4). However, the limitations of earlier chemical techniques for membrane preservation have prevented an accurate representation of the sequence of events. Based on the data on Thelebolus crustaceus presented here, I propose a new more accurate term, the enveloping membrane system (EMS), to describe the double-membraned structure which delimits the ascospore initials.

One of the most frequently used terms in ascomycete literature to describe the EMS is the "ascus vesicle ". This term originally was introduced by Andrus and Harter (2) to describe the very thin membrane which contained all the nuclei within the ascus. The constriction of this membrane around the nuclei resulted in spore formation. Subsequent authors adopted this term to describe such membranes at the electron microscopic level for Euascomycetes. However, further ultrastructural studies revealed that the vast majority of those Hemiascomycetes examined apparently synthesized their EMS 'de novo' in association with the nuclear-associated organelles and nuclei. Hence, the term "ascus vesicle" was no longer accurate to describe the EMS for all ascomycetes.

Many terms have been suggested to more accurately describe membrane events, but only have led to more confusion as to the proper terminology. Beckett (1981) argued that the terms "prospore wall" (3, 42) and "spore wall primordium" (59) suggested that spore wall material was

laid down between the double membranes before nuclear delimitation was completed, this sequence of events has not been shown to occur. He proposed the term "spore-delimiting membrane" (SDM) as an alternative. Although the exact point at which ascospore initials become ascospores may be somewhat arbitrary, the completed deposition of all wall layers (in this case the primary, secondary, and lastly epispore) would be a reasonable prerequisite. Therefore, the term "spore-delimiting membrane" implies a membrane which delimits mature ascospores, which would not be an accurate description of membrane events.

I propose that only one term, enveloping membrane system, be used to describe the presence of a double-membraned system which delimits ascospore initials in what is called "free cell formation" (1). Such a term would apply universally to all ascomycetes, since the event is one of the few that all ascomycetes have in common. The differences between the actual origin of the EMS may be resolved by simply referring to them as the Hemiascomycete-type EMS or the Euascomycete-type EMS. The term enveloping membrane system is general enough to allow for this without leading to confusion as to its meaning. It is also a more accurate term because it may be used to describe the double-membraned system at any point during its development. As this research has shown, the EMS is dynamic and its presence as a double-membraned cylinder "ascus vesicle" surrounding most of the ascus protoplasm is transient at best.

As mentioned previously, there has been a great deal of speculation

as to the origin of the EMS. Beckett (1981) summarized the postulated origins, which included in Hemiascomycetes 'de novo' synthesis in association with the NAO (3, 5, 25, 42, 43, 59) as well as alternative modes (16, 35, 48, 58), many of which could be interpreted as 'de novo' synthesis or had inconclusive data. Taphrina deformans (51, 52) was the only exception with the plasmamembrane as its apparent origin.

In Euascomycetes, the EMS often has been found in close association with the plasma membrane (6, 17, 20, 21, 50) and in some cases appeared continuous with the plasma membrane (11, 24, 41, 50). In other cases, the EMS was reported in a position adjacent to the ascus cell wall but, several authors speculated that the origin of the EMS could have been in association with or the result of myelin figures (28, 57) mesosomes (lomasomes)(22, 56), nuclear blebs (6, 12, 13, 44, 54) or endoplasmic reticulum (37, 45, 53, 56)

Contrary to previous reports (15, 57), more recent research (7, 14, 23, 27, 29, 55) suggested that the presence of mesosomes, lomosomes and other membrane artifacts were probably the result of pretreatment of cells with chemical fixatives and/or glycerol. Many of these studies showed that membrane fusion, whorls and blebs occurred when cells were pretreated chemically but when frozen ultrarapidly, without pretreatment, by plunge, propane jet or high pressure freezing, the above membrane configurations were absent. This data supported these latter findings. Examination of members of the Basidiomycetes, Zygomycetes, and other Ascomycetes (18)

by ultrarapiid freezing have failed to reveal any such membrane artifacts.

This research showed that in the Euascomycete, T. crustaceus, the EMS was derived from the ascus plasma membrane. The exact mechanism by which the plasma membrane develops into the EMS is unknown. Flattened cisternae located within the ascus (Figs. 1 and 2), adjacent to the plasma membrane, would be a logical source of membrane. I suggest that small vesicles, with little or no cell wall material, produced by these cisternae may fuse with the plasma membrane. The excess membrane generated is eliminated in the form of plasmalemmal invaginations (EMS) which enter and accumulate in the ascus cytoplasm. Cisternae continue to add vesicles to the exposed plasma membrane and the expanding EMS. Eventually, the plasma membrane surface would be obstructed completely by EMS allowing the EMS to expand without the plasma membrane intermediate.

The EMS invagination was regulated by each individual nucleus and its affiliated NAO, MTOC and microtubules. I believe, as it has been suggested previously (6, 8, 49), that the microtubules act in a contractile manner. Beckett (1981) has suggested that contractile or barrier microtubules were not applicable to ascomycetes in general because none have been found in the Hemiascomycetes, however, this was based on chemically fixed tissues. Microtubules are poorly preserved by conventional chemical techniques, which would explain their absence in many previously studied ascomycetes, including Hemiascomycetes. Although not mentioned by the authors, Mims

et al (1990) work on freeze-substituted Ascodesmis nigricans also contained microtubules during EMS invagination around nuclei. As data accumulate using ultrarapid freezing techniques of ascomycetes, I expect the role of microtubules in EMS movement to be further resolved.

The order of ascospore cell wall layer deposition has been documented in many ascomycetes (19, 20, 21, 37, 38, 39) and, more recently, limited cell wall chemistry (17) has been performed on others. However, the source of cell wall materials and/or precursors has been a point of some controversy. Considering the diversity of ascospore morphology and physiology, it is reasonable to assume that variability exists.

My results suggested that much of the wall material and/or precursors was deposited from within the ascospore initial. This was evidenced by the numerous cisternae observed within the ascospore initials and cisternal vesicles that were fused with the innermost membrane. Also, the apparent splitting of the outer ascospore initial membrane during late primary wall deposition suggested expansion from within (cell wall growth) without outer membrane growth. It would be expected that if wall materials were being deposited from the epiplasm, then concurrent membrane growth would also occur. This was not the case, however, until late spore wall formation. This research showed that subsequent to epispore wall formation small spherical bodies accumulated in the epiplasm and added their contents to the original secondary wall deposition. The epispore wall may serve as a barrier to prevent the amalgamation of secondary wall components with the primary

wall layer.

Microtubules located adjacent to the ascospore initial wall and parallel with its long axis were commonly observed during primary wall development. These microtubules converged at each end of the elliptical ascospore initial and did not appear to be associated with a MTOC as described for Xylosphaera polymorpha (6). The microtubules were not observed during subsequent wall layer depositions and I concluded that their principal function was structural until the primary wall layer was relatively inflexible. Details within ascospore initials were often obscured or lacking when conventional chemical preparations were used. I suggest that as more data accumulate regarding ascospore initial development using freeze-substitution, microtubules will be the major factor contributing to spore shape, especially for non-spherical or unusual shaped spores.

The state of the epiplasm subsequent to ascospore initial delimitation previously has been defined by conventional chemical preparation as degenerating. In contrast, these results showed that the epiplasm was transforming rather than rapidly deteriorating. Initially, the most notable occurrences were the decrease in cytoplasmic density, accumulation of glycogen particles, presence of many electron-opaque vesicles and one or more nuclei. Later, microtubules accumulated in the epiplasm apparently interconnecting the ascospores into a single spore mass.

To date, epiplasmic microtubules have not been reported in the literature, although they have been observed in Thelebolus stercorus

(personal communication, K. O'Donnell). I observed small numbers of epiplasmic microtubules in an eight-spored Pyrenomycete, Sordaria sp. (unpublished data). The quantity, arrangement and stage during which the EMN develops may give insight as to their function. Microtubules have been implicated in playing a role in cell structure, organelle movement and/or organization, cell motility and the transport of macromolecules (33). The considerable numbers of the microtubules and the fact that they were attached to the outer membranes of the ascospores suggested that they may be involved in the movement of ascospores and/or transport of macromolecules between the ascospores. However, the density of the ascospores during EMN development suggested little, if any, metabolic activity within them, eliminating the likelihood of macromolecule transport. However, if the large numbers of microtubules which interconnect the ascospores were to depolymerize, thereby shortening their length, the resulting effect would be to draw the ascospores into a relatively tightly packed single spore mass. Rather than expelling each ascospore as an individual unit they would be expelled as one large mass.

Ingold, (1965) contended that larger spores and/or groups of spores adhering to one another as a single projectile may be shot a greater distance than smaller and/or single spores. The increased distances would have the evolutionary advantage of a greater range for the fungus, increasing the chance of finding a niche in which it could survive. Many other ascomycetes are expelled in groups, apparently held together by "mucilage",

including Dasyobolus immersus, Podospora fimicola and Podospora setosa (32). It would be reasonable to assume that if the microtubules acted in a contractile manner, large numbers of them would be needed to draw the ascospores together.

Microfilament bundles were also present in the epiplasm at the same time as EMN development. The microfilaments however, were not attached to ascospores or any other epiplasmic organelles. This might eliminate any direct role in moving ascospores or organelles. Bundles of microfilaments in the form of actin have been observed to act as stress fibers, particularly in cells which undergo high shearing forces (10, 33). Ascospores expelled from an ascus may undergo shearing forces too extreme for microtubules alone to ensure adhesion in a single spore mass. Hence, microfilaments in the form of stress fibers could function as additional reinforcement to the microtubules. Laser scanning confocal microscopy confirmed the presence of the EMN after ascospore expulsion from the ascus.

To my knowledge, this research is also the first to report the presence of nuclei in the epiplasm. Whether this is a phenomenon of multi-spored asci remains to be seen. However, the dynamic reorganization that occurred in the epiplasm and shear number of spores involved with T. crustaceus may necessitate the need for an orchestrating factor. Hence, one or more nuclei may not be encompassed by the EMS, perhaps enabling the nuclei to function efficiently within the epiplasm.

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CHAPTER II

THE ULTRASTRUCTURE OF ASCOSPOROGENESIS IN HIGH-PRESSURE FROZEN FREEZE-SUBSTITUTED *SORDARIA HUMANA*

ABSTRACT

High pressure freezing was useful for determining the origin and development of the enveloping membrane system during ascosporogenesis in *Sordaria humana*. Cisternal vesicles appeared to generate the membrane necessary for enveloping membrane system formation. Infoldings of the plasma membrane, as a result of excess membrane generated by the cisternae, appeared to be released into the cytoplasm and contribute small closely spaced double-membraned units to the enveloping membrane system. Growth and fusion of individual units resulted in the formation of a cylinder around most of the ascus protoplasm. The enveloping membrane system growth progressed with eventual delimitation of individual nuclei with accompanying cytoplasm, numerous lipid bodies and organelles to form ascospore initials. Envelopment of each ascus nucleus to form ascospore initials was facilitated by a modified nuclear-associated organelle with numerous microtubules emanating along its axis. Wall materials and/or

precursors primarily were deposited between the double membranes from within ascospore initials. The secondary wall layer appeared to be the only wall layer which may have been deposited, in part, from the epiplasm. Nuclear blebs were frequently observed during the early stages of ascospore initial wall development. Observations were also made concerning the coenocytic zone, apical apparatus, and germ pore development.

KEY WORDS: Sordaria humana, high pressure freezing, laser scanning confocal microscopy, ascosporogenesis

INTRODUCTION

Cell preservation at the ultrastructural level has been greatly enhanced by the development of cryo-techniques. The rapid immobilization of cellular structures and events allows a more accurate interpretation of cellular processes. In particular, freeze-substitution in conjunction with cryo-preparation has become a valuable method by which samples are fixed for fungal ultrastructure (1, 10, 17, 21, 34, 35, 36, 37, 47, 48, 60). Plunge-freezing and propane-jet freezing have been extremely useful for the preservation of fungal hyphae and spores. However, these techniques allow adequate freezing only to a depth of about 40μm (18, 28). A significant number of fungal structures are much larger than that, especially those involved in sexual reproduction. The development of a commercially available high-pressure freezer now allows preservation of many biological samples to a depth of about 600 μm (18, 28). High-pressure freezing

achieves this by reducing the critical freezing rate from -10,000°K/sec at ambient pressures to approximately -100 to -500°K/sec at a pressure of 2,100 bar (18, 28). To date high-pressure freezing has been reported in only a limited number of fungal samples (16, 17, 19, 21, 61).

This study was undertaken to elucidate details of enveloping membrane system (EMS) development in the Pyrenomycete S. humana and to determine whether membrane dynamics were similar to those reported for freeze-substituted samples of the Discomycete, Thelebolus crustaceus (17) or previously published data using conventional chemical fixation on Sordaria sp. In this study the term EMS has been adopted from Czymmek and Klomparens (17) and refers to the double membrane which envelopes ascospore initials. Stages during ascosporogenesis have been ultrastructurally examined in conventionally fixed Sordaria fimicola (23, 24, 42, 43, 51, 59), Sordaria brevicollis (31), and Sordaria humana (56, 57, 63, 64). In S. fimicola endoplasmic reticulum (24, 42) and golgi complex (51) were suggested as the origin of EMS. In S. brevicollis the endoplasmic reticulum (31) and in S. humana tubular elements (63) were the suggested source of membrane for the EMS.

In addition, I was interested in other features of ascosporogenesis not previously described or poorly preserved by conventional chemical techniques. Many ascomycetes form ascospore initials with multiple wall layers which tend to inhibit proper penetration by fixatives and embedment media. Freeze-substituted spores were examined by transmission electron

microscopy to determine the origin of wall materials for at least some of the wall layers.

MATERIALS AND METHODS

Cultures of Sordaria humana (isolated from horse dung at Michigan State University, East Lansing, MI) were grown on dialysis membrane on V-8 juice agar at 21 C in 12 hr light/12 hr dark for 9 days. Freeze-substituted samples were processed in a Balzer's HPM 010 high pressure freezer. Samples were excised and placed in gold specimen hats with a 20% solution of dextran (M.W. 39,100) for 5 min prior to freezing. Several perithecia ranging in size from 100μm to 450μm in diameter were placed in each gold hat in order to maximize use of space and minimize air pockets and (or) extracellular fluids. After storage in liquid nitrogen, samples were freeze-substituted in an RMC MS6200 holding device using a solution of 0.05% uranyl acetate and 2% osmium tetroxide in acetone for 72 hr at -85 C. Samples were then removed and slowly brought to room temperature at intervals of 2 hr each at -35 C, -25 C, -12 C, 0 C, 10 C and room temperature. The samples then were transferred to glass vials, rinsed 3X in 100% acetone, and infiltrated and embedded with Spurr's epoxy resin. Sections were collected on collodion substrated carbon-coated grids, stained with uranyl acetate and lead citrate and examined using a JEOL 100CX II transmission electron microscope. Thick-sections (1μm) used to monitor the location within perithecia for TEM were stained with 1% toluidine blue in

1% sodium borate, observed in a Zeiss 10 LSM with transmitted mode using the 488nm line of an Argon-ion laser, and digital filtered to improve image quality.

Samples for immunofluorescence were high-pressure frozen and freeze-substituted as described above, with the exception that samples were substituted in a 2% paraformaldehyde solution in 100% acetone. Following the 3X rinse in acetone samples were slowly rehydrated until in 100% deionized water. Samples were then placed in a 50X dilution of FITC conjugated Wheat Germ Agglutinin or FITC conjugated Concanavalin A (Sigma Chemical Co.) in 100mM KPBS (pH 6.8) for 45 minutes, rinsed 3X in deionized water, mounted in n-propyl gallate in 90% glycerol (anti-bleaching medium), and observed on the Zeiss 10 Laser Scanning Confocal Microscope using the 488 nm line of an Argon-ion laser and a FITC band-pass barrier filter.

RESULTS

Coenocytic zone

The coenocytic zone was a prominent feature of maturing perithecia (Fig. 1). It was primarily a highly vacuolate multi-nucleate region with a network of incompletely formed cross walls (Figs. 1, 2, and 3) and was located at the base of each perithecium. The cytoplasmic contents of the coenocytic zone had significantly lower electron density than did asci projecting from its upper surface (Fig. 1). Higher magnification of the

coenocytic zone revealed numerous variably stained vesicles and many multi-vesicular bodies (Fig. 2). Thick-sections of perithecia were examined by laser scanning microscopy in order to obtain a view of the overall structure of the coenocytic zone (Fig. 3), which was too large to examine with the TEM without taking numerous low magnification micrographs.

Apical apparatus

Prior to evidence of EMS formation, the apical apparatus could be observed at advanced stages in development (Fig. 4). A cross-section of the annulus at the ascus apex revealed a highly vesiculated zone, the apical vesicular zone (AVZ), which had two distinct subdivisions. The upper portion (closest to the ascus apex) was comprised primarily of numerous multi-vesicular bodies and the lower region was comprised of small vesicles which had populations of both electron dense and electron transparent vesicles (Fig. 4). Some of the small vesicles were in close association with the annulus and presumably fused to empty their contents to the ascus wall (Fig. 4). Laser scanning confocal microscopy of the apical apparatus labeled with the FITC- conjugated lectin Concanavalin A revealed that the apical apparatus contained mannans and (or) glucans (Fig 5). Immunofluorescent labelling of the ascus with the FITC-conjugated lectin Wheat Germ Agglutinin revealed the absence of chitin in the apical apparatus but heavy labelling in the remainder of the ascus wall (Fig 6).

Origin of enveloping membrane system

The enveloping membrane system (EMS) appeared as an electron-dense double membrane located parallel and adjacent to the ascus wall (Figs. 7, 8, and 9) Cisternae were frequently observed in close proximity to the EMS and had very similar staining properties. Darkly-stained microvesicles (50-60nm in diameter) appeared have been released from cisternae and to have contributed membrane to the EMS (Figs. 7 and 8). Occasionally, portions of the plasma membrane invaginated resulting in the exoplasmic surface folding in on itself, while the protoplasmic surface remained in contact with the cytoplasm (Fig. 9). The plasmalemma invaginations presumably separated from the plasma membrane and entered the cytoplasm remaining in close proximity and parallel to the ascus wall. Formation of the plasma membrane invaginations and subsequent development of the EMS appeared to be the result of numerous microvesicles generated by the many electron dense cisternae (Figs. 7 and 8) located in the ascus protoplasm. Fusion of the individual double-membraned units and continuous vesicular addition derived from cisternae resulted in the development of a relatively continuous, double-membraned cylinder (EMS) which encompassed most of the ascus protoplasm (Fig. 10).

Ascospore initial envelopment

As ascus development progressed, the large amounts of membrane

generated resulted in buckling at points around the EMS cylinder (Fig. 11 and 12). Electron dense cisternae and lightly-stained rough endoplasmic reticulum were frequently observed (Fig 11). At this stage, inward movement and/or growth appeared to occur without the assistance of a nucleus associated organelle (NAO). However, some microtubules appeared to be involved with the EMS profile, evidenced by their close association (Fig. 12). Most of the cytoplasm and organelles were accumulated in the center of the ascus while large vacuoles were observed on the presumptive epiplasmic side of the EMS (Fig. 11). Eventually, cup-shaped portions of the EMS surrounded individual nuclei with accompanying cytoplasm and organelles to form presumptive ascospore initials (Fig. 13). At this stage, the EMS was still a closely spaced, double-membraned structure. Final movement of EMS around each individual nucleus appeared to be facilitated by an elongated, modified nucleus-associated organelle (NAO) (Figs. 14,). The NAO consisted of two electron-dense bands separated by a less electron-dense band (Fig. 14). Microtubules emanated from the axis of the NAO and were coated with an electron-dense material which appeared much like the outer dense bands of the NAO (Fig. 14). The NAO involved with ascospore initial formation was modified from its mitotic (Figs. 10 and 15) and meiotic (Fig. 16) counterparts (it resembled the mitotic NAO but was approximately twice its length). The NAO was usually relatively straight (Figs. 17 and 18) but occasionally hooked at the end distal from the nucleus (Fig. 14). The modified NAO was attached to the nucleus by a narrow

electron dense band (Fig. 18).

Occasionally, mutant asci of S. humana were observed which contained four post-meiotic nuclei but no "typical" EMS cylinder. Instead, numerous variable sized double-membraned circular units were found in the ascus (Fig. 19). The NAO's of the post-meiotic nuclei (not shown) appeared to be distorted. No microtubules, NAO's or nuclei were observed within the EMS circular units. These circular units primarily contained cytoplasm but occasionally included vacuoles, mitochondria or other circular membrane units within (Fig. 19).

Direct points of contact between microtubules and the innermost membrane of the EMS were observed (Fig. 20). The microtubules emanating from the NAO were coated with the electron dense material as described previously (Figs. 20 and 21). The microtubules associated with the inner membrane were also coated, suggesting that they were NAO-associated microtubules. Epiplasmic microtubules (Fig. 21) were uncoated. However, at the opening of the presumptive ascospore initial uncoated microtubules were observed (Fig. 22) suggesting that they were not directly associated with the NAO. These microtubules appeared to be involved with inward movement of the EMS at the opening. The completion of envelopment of uninucleate ascospore initials was indicated by the presence of eight nuclei, each with accompanying cytoplasm, numerous lipid bodies and organelles, surrounded by the two closely-spaced membranes.

Ascospore initial wall development

Shortly after envelopment of the uninucleate ascospore initials, an additional mitotic event yielded binucleate ascospore initials. During early primary wall deposition, microtubules were found adjacent to the inner ascospore initial primary cell wall parallel to the long axis (Fig. 23). Concurrent with primary wall formation was the presence of rough endoplasmic reticulum, vesicles containing granular material, and microbodies within the ascospore initial (Fig. 24). Also of note, was the presence of numerous multi-vesicular bodies (Fig. 25) within the ascospore initial cytoplasm during primary wall development and microfilaments in the epiplasm. The primary wall materials and/or precursors were deposited between the closely-spaced membranes (Fig. 26) resulting in their gradual separation. The primary wall was somewhat electron dense and cisternae were frequently observed in close proximity (Fig. 26).

During secondary wall development, the binucleate ascospore initials contained many cisternae, multi-vesicular bodies, rough endoplasmic reticulum, numerous lipid bodies, microbodies, and mitochondria (Fig. 27). The epiplasm primarily contained numerous ribosomes, vacuoles, mitochondria, rough endoplasmic reticulum and many small electron-transparent vesicles (Fig. 27).

Nuclear blebs were frequently observed during secondary wall deposition (Figs 27, 28, and 29). The nuclear blebs were evaginations of

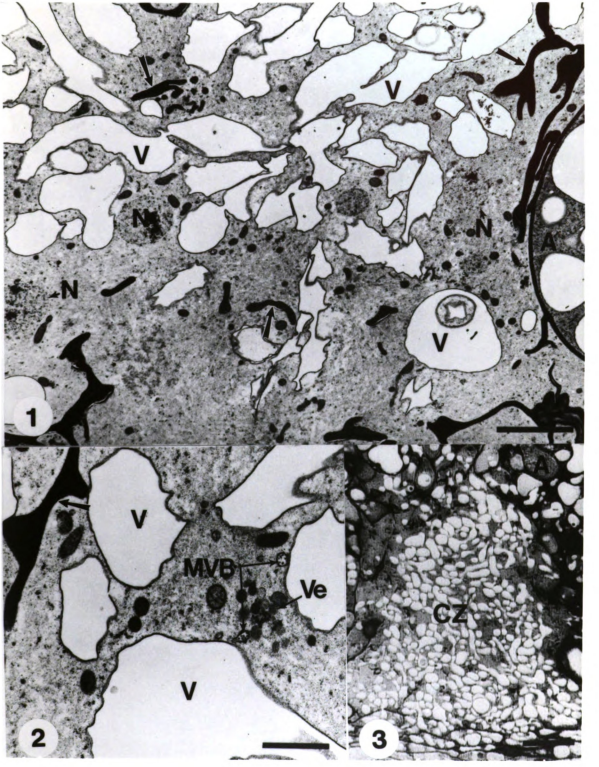
the nuclear envelope (containing nucleoplasm) with a second double-membrane closely appressed to the region of blebbing (Figs. 28 and 29). Cisternae were occasionally observed in close association with the nuclear blebs and nuclei frequently had multiple blebs (Fig. 29). The external membrane associated with the nuclear blebs had different staining properties than the nuclear envelope (Fig. 28).

Initially, the secondary wall layer was homogeneous and slightly more electron dense than the primary wall (Fig. 30). During early secondary wall deposition, the germ pore was observed as a narrow electron transparent layer between the primary and secondary wall layers (Fig. 30). Large vesicles were located within the immediate proximity of the germ pore initial (Fig. 30). The secondary wall layer became heterogeneous as small regions of electron transparent material accumulated (Figs. 31-34).

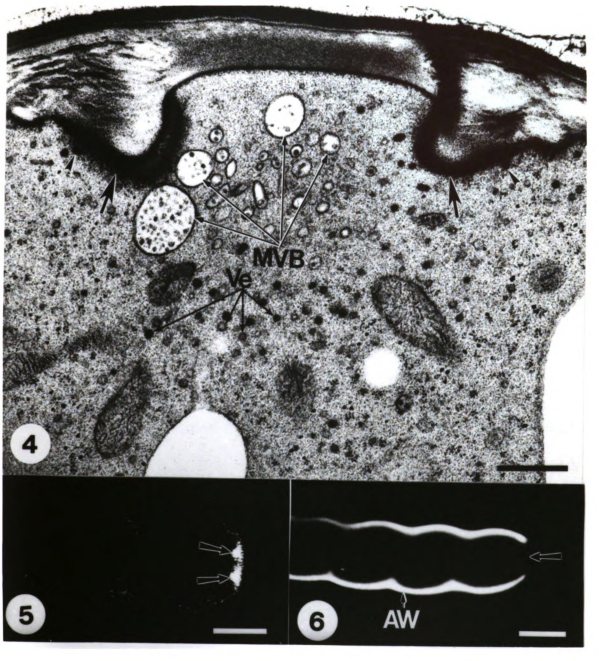
Formation of the electron-dense tertiary wall layer was accompanied by accumulation of darkly-stained cisternae (Fig. 31) from within the ascospore initial. No microtubules, corresponding to those found during primary wall development, were seen adjacent to the inside walls of ascospore initials. The germ pore at this stage consisted of a localized region with a greatly reduced primary wall layer, an expanded electron transparent secondary wall layer, and continuous tertiary wall layer (Fig. 31). Later stages showed large vesicles of unknown composition or function present (Fig. 32) but whether these were different from the vesicles during early germ pore formation, was unclear (Fig. 30). Small numbers of

epiplasmic microtubules were observed during late ascospore initial development and were associated with the outer membranes of spore initials. The binucleate ascospore initials underwent an additional mitosis to yield tetranucleate ascospores (not shown). Subsequent to epispore wall deposition, many small spherical bodies were seen in the epiplasm along the outer delimiting membrane, and based on location and density similarities were possibly involved in secondary wall formation (Fig. 34). The mature ascospore wall contained several layers. A thick quartenary wall layer was formed beneath the primary wall layer (which had become infiltrated with tertiary wall material), while the secondary wall layer remained heterogenous (Fig. 33).

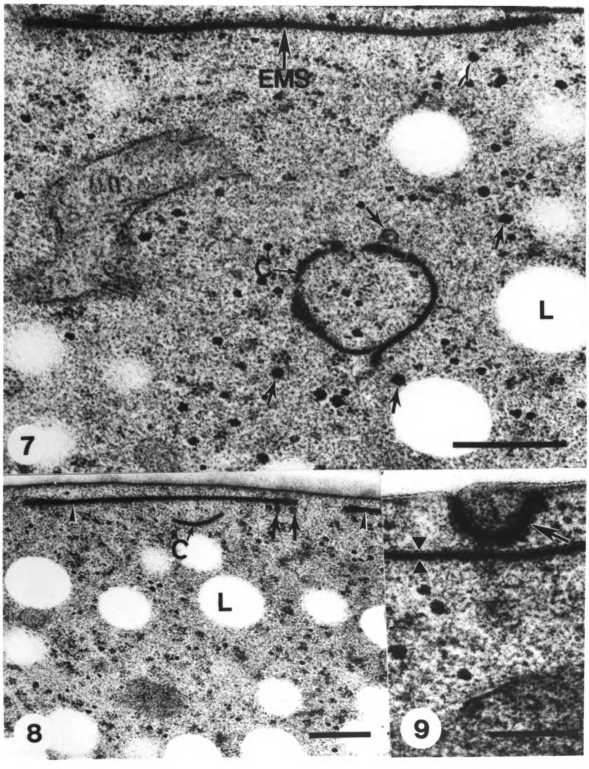
FIGS. 1-3. Sordaria humana. Fig. 1. The coenocytic zone at the base of a perithecium contained numerous vacuoles (V) and segments of darkly stained crosswalls (arrows). Nucleus (N), ascus (A), Bar = 3μm. Fig. 2. High magnification of the coenocytic zone showed many vesicles (Ve) in the cytoplasm. Crosswall (arrow), multi-vesicular body (MVB), vacuole (V). Bar = 1μm. Fig. 3. Low magnification of the coenocytic zone (CZ) using laser scanning microscopy. Ascus (A). Bar = 15μm.


V
V
V
N
N
N
V
1
V
MVB
Ve
V
2
CZ
3

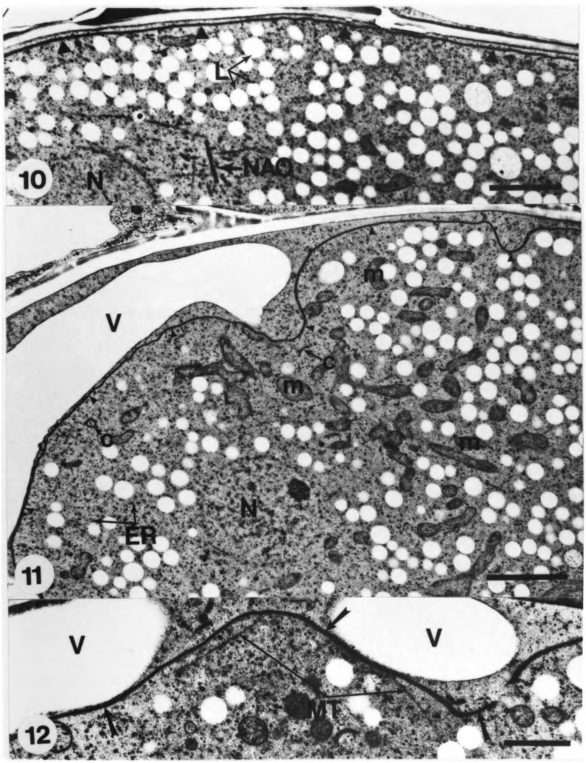
FIGS. 4-6. Sordaria humana. Fig. 4. Cross section during the development of the apical apparatus. Note the numerous multi-vesicular bodies (MVB), electron opaque and transparent vesicles (Ve) and small vesicles (arrowheads) which were presumably about to fuse with the projections of the annular ring (arrows). Bar = 0.5μm. Fig. 5. High-pressure frozen freeze-substituted laser scanning confocal image of a Concanavalin A labelled apical apparatus (arrows) indicated the presence of glucans and/or mannans. Bar = 7μm. Fig. 6. High-pressure frozen freeze-substituted laser scanning confocal image of a Wheat Germ Agglutinin labeled ascus indicated the presence of chitin in the ascus wall (AW) but was noticeably absent from the apical apparatus (arrows). Bar = 8μm.


MVB
Ve
4
5
6
AW

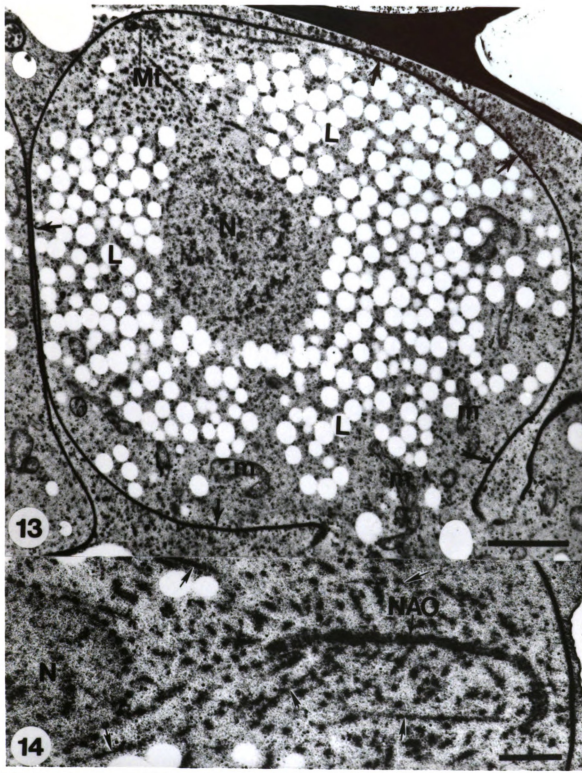
FIGS. 7-9. Sordaria humana. Fig. 7. Development of the enveloping membrane system (EMS) appeared to result from the contribution of vesicles (arrows) from darkly stained cisternae (C). Note apparent blebbing of vesicles from the cisterna and similarity in staining properties with the EMS. Bar = 0.5μm. Fig. 8. A portion of an ascus during early development of the EMS (arrowheads) which showed short segments of double membrane, a cisterna (C), and cisterna vesicles (arrows). Bar = 0.5μm. Fig.9. Infolding of the plasma membrane (arrow) appeared to contribute small double-membraned segments to the EMS (arrowheads). Lipid (L). Bar = 0.20μm.


EMS
C
L
7
C
L
8
9

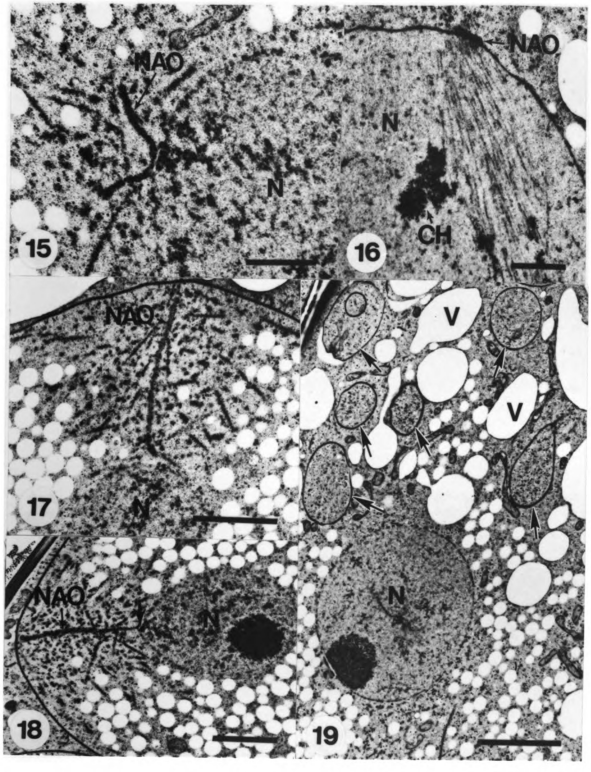
FIGS. 10-12. Sordaria humana. Fig. 10. Presumed growth and fusion of individual segments of the EMS resulted in a relatively continuous double-membraned cylinder (arrowheads) around most of the ascus cytoplasm. Note the nucleus (N) and its disassociated nucleus associated organelle (NAO). Lipids (L). Bar = 2μm. Fig. 11. The EMS (arrowheads) appeared to buckle inwards from its continuous growth. Cisternae (C), mitochondria (m), nucleus (N), endoplasmic reticulum (ER), vacuole (V). Bar = 2μm. Fig. 12. Another section of the ascus from Fig. 11 showed that microtubules (MT) appeared to be involved with the undulated appearance of the EMS (arrows) at this stage. Vacuoles (V). Bar = 1μm.


L
NAG
10
N
V
m
m
o
N
ER
11
V
V
MT
12

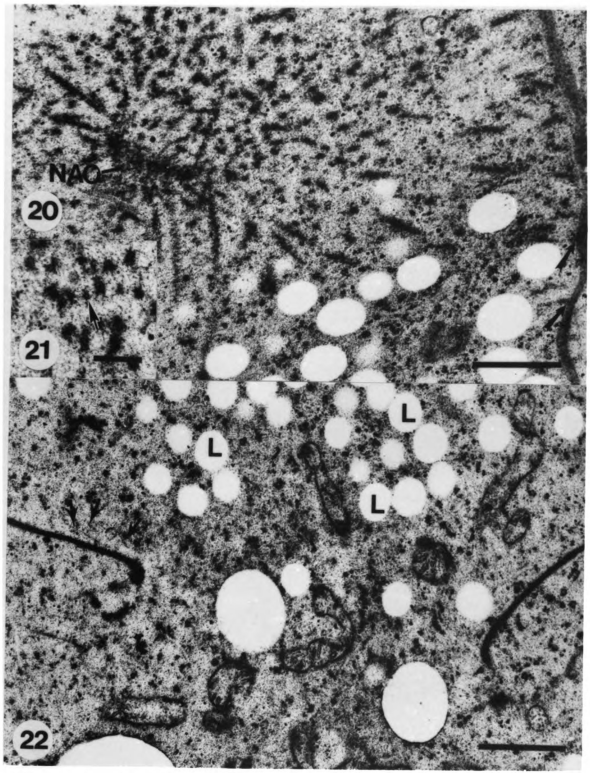
FIGS 13-14. Sordaria humana. Fig. 13. A presumptive ascospore initial with a cup-shaped EMS unit (arrowheads) encompassing a single nucleus (N), numerous lipid bodies (L), mitochondria (m), and accompanying cytoplasm. Note the microtubules (Mt) located distal to the opening of the cup-shaped EMS unit. Bar = 2μm. Fig. 14. A section from the same presumptive ascospore initial in Fig. 13 showed that a modified nucleus-associated organelle (NAO) with numerous microtubules (arrows) emanating along its axis was involved with EMS movement. Bar = 0.5μm.


Mt
L
N
L
L
m
m
13
NAG
N
14

FIGS. 15-19. Sordaria humana. Fig. 15. Post-meiotic nucleus (N) which had a V-shaped nucleus-associated organelle (NAO). Bar = 1μm. Fig. 16. A meiotic nucleus (N) with a small disk-shaped nucleus-associated organelle (NAO) closely appressed to the nuclear envelope. Chromosome (CH). Bar = 1μm. Fig. 17. The EMS-associated modified NAO appeared similar but was oriented differently and was considerably longer than its meiotic and mitotic counterparts. Nucleus (N). Bar = 2μm. Fig. 18. The point of attachment (arrow) of the modified NAO was to the nucleus (N) was very narrow. Bar = 2μm. Fig. 19. A sporulation mutant ascus which contained one of four post-meiotic nuclei (N) and numerous variable-sized circular EMS units (arrows). The circular units occasionally contained some organelles or a single vacuole but never contained a nucleus, NAO, or microtubules. Vacuole (V). Bar = 3μm.


NAO
N
15
NAO
N
CH
16
NAO
V
V
17
N
NAO
N
18
N
19

FIGS. 20-22. Sordaria humana. Fig. 20. Cross-section of a modified nucleus-associated organelle (NAO) with numerous well preserved microtubules emanating from its axis. Microtubules were observed in direct contact (arrows) with the inner membrane (arrowheads) of the EMS. Bar = 1μm. Fig. 21. High magnification of a microtubule cross-section (arrow) showed the electron dense material coating. Bar = 0.25μm. Fig. 22. The opening of the cup-shaped EMS unit showed several microtubules (arrows) which appeared to terminate near its opening. Lipid bodies (L). Bar = 1μm.


NAC
20
21
L
L
L
22

FIGS. 23-26. Sordaria humana. Fig. 23. A tangential section of the long axis of an ascospore initial during early primary wall layer (P) deposition showed the presence of microtubules (arrows) oriented parallel with the long axis. Bar = 0.5μm. Fig. 24. Granular vesicles (Ve), cisternae (C), endoplasmic reticulum (ER), and lipid bodies (L) were characteristic during early stages in ascospore initial development. Vacuoles (V). Bar = 1μm. Fig. 25. Numerous multi-vesicular bodies (arrowheads) were observed within young ascospore initials and microfilaments were observed in the epiplasm (arrow). Bar = 1μm. Fig. 26. The primary wall layer (P) appeared to be deposited by cisternae (C) within ascospore initials, and resulted in gradual separation of the double membranes. Bar = 0.25μm.

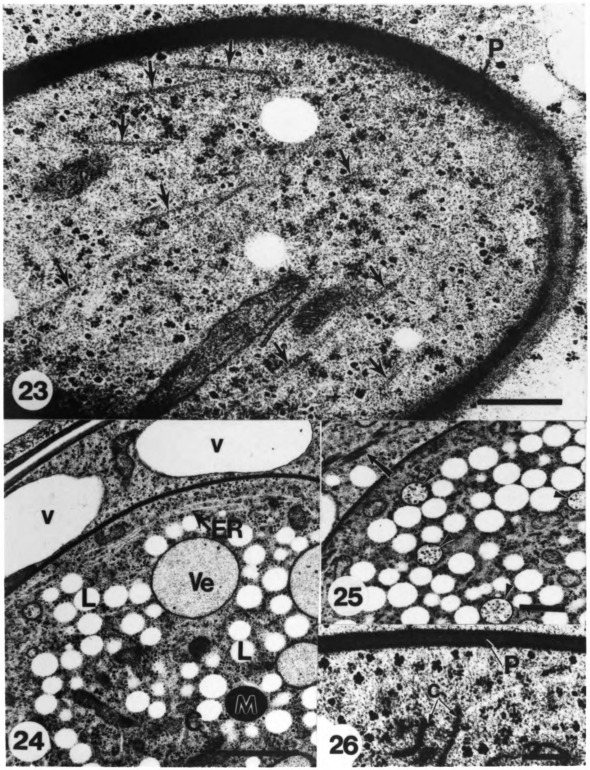
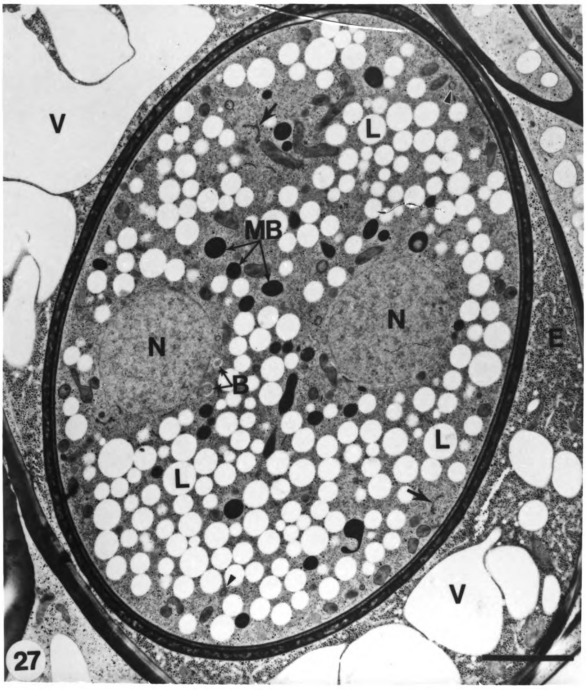
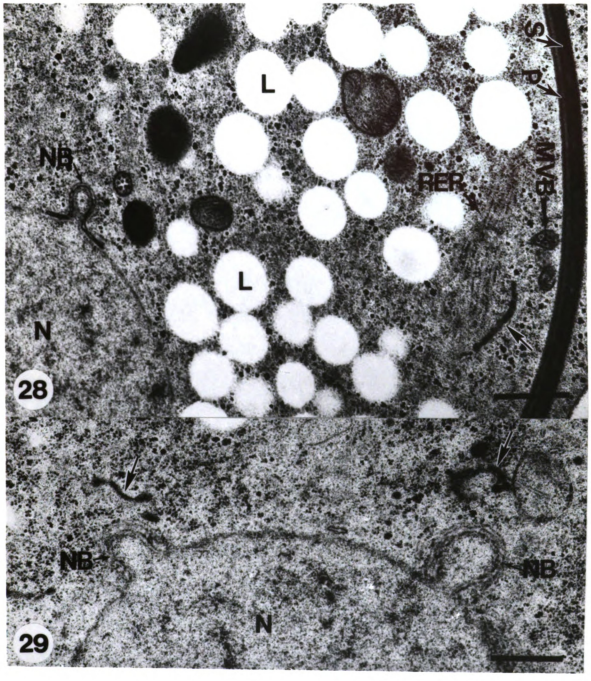

P
23
v
v
ER
Ve
L
L
M
24
25
P
26

FIG. 27. Sordaria humana. A well preserved ascospore initial during secondary wall layer deposition, contained two nuclei (N), microbodies (MB), lipid bodies (L), cisternae (arrows), multi-vesicular bodies (arrowheads) and other organelles. The epiplasm (E) contained many large vacuoles (V). Bar = 3μm.

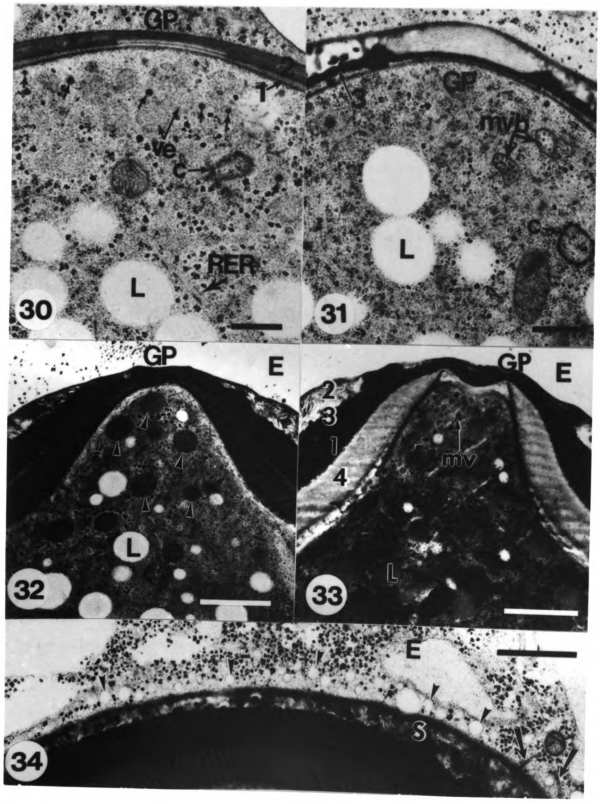


during
ies (MB),
ds) and
). Bar =

FIGS 28 & 29. Sordaria humana. Fig. 28. A serial section of Fig. 27 showed a cisterna (C) closely associated rough endoplasmic reticulum (RER) and multi-vesicular bodies (MVB) adjacent to the spore initial wall. Note the evaginated nuclear bleb (NB) with its closely appressed double membrane. Primary wall layer (P), secondary wall layer (S), lipid bodies (L), nucleus (N). Bar = 1μm. Fig. 28. Multiple nuclear blebs (NB) may exist for each nucleus (N) at this stage and frequently cisternae (arrows) were associated with the blebs. Bar = 0.5μm.


L
NB
G
RER
MVB
S
P
L
N
28
NB
NB
N
29

FIGS 30-34. Sordaria humana. Fig. 30. The germ pore was first observed during early secondary wall layer deposition as an electron transparent band (arrow) between the primary (1) and secondary (2) wall layers. Large (ve) and small (arrowheads) vesicles were located adjacent to the germ pore. Cisternae (c), rough endoplasmic reticulum (RER), lipid body (L). Bar = 0.5μm. Fig. 31. Germ pore (GP) of an ascospore initial during early tertiary wall layer (3) deposition. Note the well preserved multi-vesicular bodies (mvb), and cisterna (C). Lipid bodies (L). Bar = 0.5μm. Fig. 32. Late stage in ascospore development which showed many dark-stained spherical bodies (arrows) Fig. 33. The latest stages of germ pore development showed that primary (1), secondary (2), and quaternary (4) wall layers were greatly reduced. Numerous small vesicles (Ve) were observed near the pore. Bar = 0.5μm. Fig. 34. Numerous electron transparent bodies (arrowheads) were observed adjacent to the outer ascospore delimiting membrane and presumably add their contents to the secondary wall layer (S). Small numbers of microtubules (arrows) were associated with the outer membrane at this stage. Bar = 0.5μm. spindle plaque during meiosis in yeast. Chromosoma. **50**: 1-23.


GP
1
ve
c
RER
L
30
GP
3
mvb
c
L
31
GP
E
L
32
GP
E
2
3
1
4
mv
L
33
E
S
34

DISCUSSION

There have been numerous attempts to determine the origin and nature of the membrane system which delimits ascospore initials. Many theories have been proposed to explain the phenomenon of ascosporogenesis (4). However, the limitations of earlier chemical techniques for membrane preservation have prevented an accurate representation of the sequence of events. Based on the data on Sordaria humana presented here, I adopt the term, the enveloping membrane system (EMS) (17), to describe the double-membraned structure which delimits the ascospore initials. Czymmek and Klomparens (17) described several reasons for adopting the term EMS, and noted the inherent inaccuracies and misuse of the other terms used in the past. I present the following additional arguments in support of adopting the new term enveloping membrane system. The term spore-delimiting membrane (SDM) was introduced by Beckett (4). While some authors exclusively used this term (20, 78) a large number of publications have used alternative terms, e.g., "ascus vesicle" (47, 62, 71), "spore vesicle" (14), "ascospore-delimiting double membranes" (77), or "spore delimiting walls" (74).

In addition, confusion also existed because of the use of multiple terms to describe the EMS within the same paper. Gibson and Kimbrough (25) used "ascospore delimiting membrane", "ascus vesicle", "ascus delimiting membrane systems", and "spore delimiting membranes" in the same paper. These terms all describing what I call the EMS. Others (26,

38, 39, 79, 80,) treat the ascus vesicle separately by describing the SDM as arising from the "ascus vesicle". Still others used such terms as "peripheral membrane cylinder" and "delimiting membranes" (73), or "peripheral membrane cylinder", "ascus vesicle" and double delimiting membranous sac" (72), or "membranous sacs", "ascus vesicle", and "delimiting membranes" (70), or "ascus vesicle", "delimiting membrane cylinder" and "spore delimiting membranes" (38) within the same paper.

Although, this list of publications was not all inclusive, it represents the vast majority of literature pertaining to the ultrastructure of EMS development in ascomycetes since Beckett (4). It was clear that there was much confusion as to proper terminology amongst authors and even within individual papers.

I seek to address this problem by specifically using the term "enveloping membrane system" primarily because it accurately describes membrane events in all stages of development and is interchangeable between the Hemiascomycetes and Euascomycetes, regardless of origin.

In Euascomycetes, the EMS often has been found in close association with the plasma membrane (6, 20, 25, 26) and in some cases appeared to be continuous with the plasma membrane (11, 17, 29, 47, 62). In other cases, the EMS was reported in a position adjacent to the ascus cell wall but several authors speculated that the origin of the EMS could have been in association with or the result of myelin figures (33, 78) mesosomes (lomasomes)(27, 76), nuclear blebs (6, 12, 13, 38, 52) or endoplasmic

reticulum (42, 43, 58, 69, 75)

Beckett (4) attempted to justify the different possible origins of the EMS by interpreting and applying the endomembrane concept of Morré et. al. (50). However, Beckett's hypothesis relies on the accurate representation of membrane events using only conventional chemical fixation. Czymmek and Klomparens (17) argued that mesosomes, lomosomes and other membrane artifacts were probably the result of pretreatment of cells with chemical fixatives and/or glycerol. Myelin figures, lomasomes and mesosomes were never observed in high pressure frozen samples of Sordaria humana, however, multi-vesicular bodies corresponded with the lomasomes previously reported in S. brevicollis (31). The limitations of conventional chemical fixation in maintaining the integrity of cellular membranes casts serious doubt on the previously suggested modes of EMS formation from myelin figures, lomasomes, and mesosomes.

Multi-vesicular bodies have been noted in many studies of fungal samples using freeze-substitution (17, 35, 36, 48, 60). I note a strong correlation with their presence in the coenocytic zone, ascus apex, and during ascospore initial wall development in relatively large numbers, all regions or stages in which significant wall material was being deposited. This may be very coincidental or it may suggest a possible role (directly or indirectly) in wall formation. Similar structures have been described in animal cells (22), and as their name "secretory vacuole" implied they were believed to be involved in secretion.

This research showed that in the Pyrenomycete, S. humana, a portion of the EMS may have been derived from the ascus plasma membrane. The mechanism by which the EMS develops has not been completely elucidated. The study of high-pressure frozen samples of Thelebolus crustaceus suggested that flattened cisternae located within the ascus, adjacent to the plasma membrane, released small vesicles with little or no cell wall material, which fused with the plasma membrane (17). The excess membrane generated was eliminated in the form of plasmalemma invaginations (EMS) which entered and accumulated in the ascus cytoplasm. Cisternae continued to add vesicles to the exposed plasma membrane and the expanding EMS. Eventually, the plasma membrane surface was largely obstructed by EMS allowing the EMS to expand without the plasma membrane intermediate. The results with S. humana supported the sequence of events described in Thelebolus crustaceus.

In this research, the formation of plasma membrane invaginations, although not exclusively, was largely restricted to the earliest stages of EMS formation and that, to a great extent, EMS development was probably due to direct vesicular addition from cisternae. It appeared that the plasma membrane invaginations did contribute to the EMS. However, I did not observe the number of invaginations that I expected would be necessary for generating the large amounts of membrane required for ascospore initial delimitation. Mims et. al. (47) examined plunge-frozen freeze-substituted samples of Ascodesmis nigricans during ascosporogenesis and also observed

plasma membrane invaginations. The small segments of EMS contributed directly by the plasma membrane may serve as templates for EMS formation.

Previous reports on Sordaria sp. (24, 31, 42), using conventional chemical fixation, suggested endoplasmic reticulum (ER) as the source of EMS. It was clear from this study that during early ascus development (pre-meiotic) that an abundance of ER was observed. The amount of ER decreased significantly during meiosis and subsequent EMS development. In fact when EMS expansion was presumably at its greatest, ER was relatively scarce. In high pressure frozen samples of S. humana, the endoplasmic reticulum was clearly distinguished from the EMS and resembled it only in that they were both double-membraned structures. Although I can not rule out the fact that ER may contribute material to the numerous cisternae or directly contributes vesicles to the EMS, ER did not appear to be the major membrane contributor. Cisternae most closely resembled the staining properties of the EMS.

Formation and development of the EMS from vesicles of endoplasmic reticulum has been suggested in freeze-fractured samples of the Hemiascomycete Saccharomyces cerevisae (30). Due to the nature of freeze-fracture, a clear interpretation of the origin of the vesicles was not obvious. It would be very useful if hemiscomycetous yeasts were examined by freeze-substitution to determine if ER was the origin of the EMS. High-pressure freezing has already been successfully applied to yeast samples

(19).

Endoplasmic reticulum has been indicated in the process of membrane recycling (via plasma membrane invaginations) in high-pressure frozen plant tissue (15). However, it is known that in most plants and animals membrane flow directed toward the plasma membrane is generally from ER to golgi (golgi are presumably analogous to cisternae in fungi) (22, 53). It also has been noted that significant differences exist between the plasma membrane and ER and that golgi represent a transitional intermediate between the two (22, 50, 53). It is generally agreed upon that the EMS appears strikingly similar to the plasma membrane and has even been shown to have similar staining properties with a phosphotungstic acid-chromic acid solution in Taphrina deformans (67, 68).

Essentially, there appear to be only two logical sources of membrane for the EMS, ER and (or) cisternae. In this studies cisternae appeared to be the most likely candidate. More studies using cryo-techniques, in particular high-pressure freezing and freeze-substitution, are needed to further resolve this issue.

As mentioned previously, nuclear blebs also have been suggested as a source of membrane for the EMS. I did not observe nuclear blebs during EMS formation in this study, nor did others using freeze-substitution (17, 47). However, these results showed that nuclear blebs occurred within ascospore initials during wall development, corresponding with nuclear blebs observed in conventionally fixed samples of Sordaria brevicollis (31).

Saccabolus kerverni also showed nuclear blebs within maturing ascospore initials (13). If the generation and conversion of nuclear blebs were required for EMS formation, one could envision the nuclear material being exhausted. Nuclear blebs appeared to occur in some ascomycetes during this stage, but I suggest that they may reflect some other process within these cells, not in EMS development. I was unable to determine the fate of the nuclear blebs in S. humana.

The EMS invagination appeared to be regulated by each individual nucleus and its modified NAO and microtubules. I believe, as it has been suggested previously (6, 8, 17, 62), that the microtubules act in a contractile manner. Freeze-substituted samples of S. humana (this study), Thelebolus crustaceus (17) and Ascodesmis nigricans (47) also contained microtubules during EMS invagination around nuclei. In S. humana, an electron dense material coated the microtubules associated with the NAO. Microtubule associated proteins have been reported with some microtubule organizing centers (MTOC's) (9, 32) but the chemical nature of the electron dense coating in this samples was unknown. Mutant asci have been described previously (40, 49, 55, 65, 82). The circular EMS units described here were not considered spores or spore initials. I, therefore, would not regard them as mutant spores formed in the absence of microtubules or NAO's, which was previously described by light microscopy (65). As suggested by Beckett (4), studies of mutant asci could enhance our understanding of the role NAO's, MTOC's, or microtubules play in ascospore

initial formation. In this study of S. humana two mutant asci were observed that each contained four nuclei (presumably post-meiotic). However, rather than having a normal EMS configuration at this stage most of the EMS was observed as variable-sized, anucleate, circular units of EMS without mitochondria, microtubules, or lipid bodies. This suggested that circular units of EMS could form without the presence of nuclei, microtubules or NAO's. However it would appear that the presence of nuclei, microtubules and NAO's were necessary for the proper formation of individual ascospore initials with the normal complement of organelles. The NAO and associated microtubules appeared to be necessary in maintaining the organelles in close proximity to the nucleus and presumably pulling in the EMS to form ascospore initials.

The order of ascospore wall layer deposition has been documented in many ascomycetes (5, 17, 24, 25, 26, 41, 44, 45, 46) However, the source of ascospore wall materials and (or) precursors has been a point of some controversy. My results suggested that much of the wall materials and (or) precursors were deposited primarily from within the ascospore initial. This was evidenced by the numerous cisternae and ER observed within the ascospore initials and vesicles that were found adjacent the innermost membrane. Darkly-stained cisternae and vesicles were observed within ascospore initials during tertiary wall layer development and I suggest that the tertiary wall was derived from them. This research also showed that subsequent to tertiary wall formation, small spherical bodies

accumulated in the epiplasm adjacent to the outer delimiting membrane and presumably added their contents to the original secondary wall deposition. This research on S. humana showed similar wall deposition features as described for high-pressure frozen freeze-substituted samples of Thelebolus crustaceus (17). Namely, most wall material appeared to be derived from within ascospore initials while a portion of the secondary wall layer was derived from the epiplasm.

Laser scanning confocal microscopy of immunofluorescently- labelled asci showed the presence of mannans and (or) glucans in the apical apparatus and while chitin was detected throughout the ascus wall it was noticeably absent from the apical apparatus. Presumably there was some functional significance to the wall composition at the apical apparatus and spore discharge. It would be interesting to see if other Ascomycetes, in particular Pyrenomycetes, have similar ascus wall chemistry. Lectins have been described as useful for determining cell wall components in fungi (54). Fungal cell wall chemistry studies (2, 3) indicated that I might expect mannans in the Hemiascomycetes and glucans in the Euascomycetes. I, therefore, might speculate that the lectin Concanavalin A labelled for glucan in S. humana. The presence of glucans was also detected in young ascospore initials prior to the opaque tertiary wall formation and was localized in the gelatinous sheath (secondary wall layer) (K.J. Czymmek and K. L. Klomparens, unpublished). Recent immunogold labelling (of several polysaccharides) in high-pressure frozen plant tissue, noted the specific

location of polysaccharide components within the golgi complex (81). This type of examination could also be used to determine if cisternae from within ascospore initials are indeed involved in wall material synthesis and to which wall layers specific polysaccharides migrate.

The apical vesicular zone (AVZ) of S. humana was quite complex when compared with similar structures found at the ascus apex using conventional chemical fixation (7). Beckett (7) compared the structure of this region to the Spitzenkörper in hyphae and suggested that the structures might be homologous. I noted distinct differences between the AVZ and the Spitzenkörper previously described by freeze-substitution. I saw no evidence for a homologous origin, but due to the nature of ascus apex growth, I may reasonably suggest that the structures were analogous.

The coenocytic zone has been described previously using cryo-fractured scanning electron microscopy on samples of S. humana (57). The multinucleate region was observed at the base of each perithecium and it appeared as if a network of crosswall segments were being formed but these crosswalls were generally incomplete in the young ascocarp. Read and Beckett (57) suggested that in the perithecium this layer was crushed as the perithecium matured. I could not confirm this report but suggest here that the coenocytic zone may serve as a source of nuclei and cytoplasm for formation of asci and perhaps paraphyses. The coenocytic walls were more developed along the outer periphery of the coenocytic zone suggesting that they may be delimiting nucleate portions of cytoplasm. In the mature

perithecium, the coenocytic zone was reduced, probably due to the crosswalls eventually exhausting the supply of protoplasm.

High-pressure freezing proved very useful for examination of stages of ascosporogenesis in S. humana. Excellent preservation of many cellular organelles and structures was observed. However, in S. humana I noted one artifactual feature that I directly attribute to the technique of high-pressure freezing. It was noted that vacuoles in the coenocytic zone had a finely undulated appearance. Vacuoles found in other structures within perithecia did not exhibit this undulated appearance. I suggest that the large size of the coenocytic zone which had relatively little development of the cross walls for support made it particularly susceptible to high pressure damage. Also noted, was the presence of small tears within the cytoplasm of some perithecial structures, namely the coenocytic zone and the epiplasm of maturing asci. Although I can not rule out that high-pressure freezing caused this effect, I suggest instead that it may be due to compression and decompression of the samples during manipulations during resin infiltration and embedment. Presumably, the epiplasm and coenocytic zone were more sensitive to this manipulation due to their low density cytoplasm (therefore less crosslinking by fixatives). Similar tears were observed during propane-jet freezing which was performed at ambient pressures. Finally, it is of interest to note that in samples of Sordaria humana, approximately 75% of all perithecia were well frozen and within these perithecia I found some cell-types (paraphyses) exhibited some minor ice crystal damage while adjacent

asci were well frozen.

The technique of high-pressure freezing has proven to be very useful for examining sexual reproduction in ascomycetes. Although this technique was not without its own artifacts, it appeared that high-pressure freezing followed by freeze-substitution allowed a more accurate representation of cellular events. Due to the nature of freeze-substitution I was also able to examine ascospores much later in development which was virtually impossible using conventional chemical techniques. I expect that many larger fungal structures (as well as small ones) may be examined successfully by this technique and, thus, enhance our understanding of cellular processes in mycological research.

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CHAPTER III

THE ULTRASTRUCTURE OF ASCOSPOROGENESIS IN HIGH-PRESSURE FROZEN FREEZE-SUBSTITUTED EMERICELLOPSIS TERRICOLA

ABSTRACT

High-pressure freezing in conjunction with freeze-substitution was essential for determining many details of enveloping membrane system and ascospore initial development in Emericellopsis terricola. During meiosis I, invaginations of the plasma membrane (often very elaborate) initiated formation of the enveloping membrane system. Continued growth of individual units of the closely spaced double-membranes, due to vesicular addition by cisternae, resulted in the formation of a cylinder around most of the ascus protoplasm. This double-membraned cylinder enveloped individual nuclei with accompanying cytoplasm and organelles to form ascospore initials. Envelopment of asci nuclei to form ascospore initials was regulated by each nucleus-associated organelle and affiliated microtubules. Wall materials and/or precursors were deposited between the closely spaced double membranes primarily from within ascospore initials. However, a

close association between epiplasmic endoplasmic reticulum and the outer delimiting membrane of ascospore initials was observed. During early secondary wall formatiom, darkly-stained cisternae were observed within ascospore initials strategically located beneath the area of greatest wall growth (location of the developing winged appendages). Microtubules located adjacent to the inside wall layer of the ascospore initials appeared to contribute to an elliptical shape.

KEY WORDS: Emericellopsis terricola, ascosporogenesis, high-pressure freezing, ultrastructure, Plectomycetes

INTRODUCTION

Plectomycetes are considered a diverse group of Ascomycetes in which the asci are completely enclosed by enveloping hyphae in what is called a cleistothecium. To date all ultrastructural investigations of ascosporogenesis in the Plectomycetes (10, 11, 14, 19, 29, 31, 34, 35, 36, 37, 38, 39, 40, 41) have been done using conventional chemical techniques. In these ultrastructural studies the suggested origins and modes of development of the enveloping membrane system (EMS) were extremely variable. It was my goal to use high-pressure freezing in conjunction with freeze-substitution to find a plausible origin of the (EMS). Freeze-substitution was successfully applied to members of the Discomycetes (7, 23) and a Pyrenomycete (6, 8). In these studies, the plasma membrane was indicated as the origin of the EMS and in two of these organisms (6, 7, 8)

formation and growth of the plasma membrane invaginations and EMS was due to vesicular addition by cisternae.

In addition to EMS development, I was interested in ascospore initial formation. Due to the slow penetration of chemical fixatives through often multi-layered ascospore initial walls, conventional techniques do not adequately preserve the cytological events and structures within developing ascospore initials. In particular, multi-vesicular bodies, microtubules, and cisternae are typically absent or unrecognizable, all important components in understanding the nature of spore development.

MATERIALS AND METHODS

Cultures of Emericellopsis terricola (ATCC culture # 16431) were grown on dialysis membrane on V-8 juice agar at 21 C in 12 hr light/12 hr dark. Freeze-substituted samples were processed in a Balzer's HPM 010 high pressure freezer. Samples frozen using the high pressure freezer were excised and placed in gold hats with a 20% solution of dextran (M.W. 29,000) for 5 min prior to freezing. After storage in liquid nitrogen, samples were freeze-substituted in an RMC MS6200 holding device using a solution of 0.05% uranyl acetate and 2% osmium tetroxide in acetone for 72 hr at -85 C. Samples were then removed and slowly brought to room temperature at intervals of 2 hr each at -35 C, -25 C, -12 C, 0 C, 10 C and room temperature. The samples then were transferred to glass vials, rinsed 3X in 100% acetone, and infiltrated and embedded with Spurr's epoxy resin (30).

Ultrathin sections were stabilized on plastic substrated carbon coated grids, stained with uranyl acetate and Reynold's lead citrate (25) or Hainachi's (13), and examined using a JEOL 100CX II transmission electron microscope.

RESULTS

Origin of enveloping membrane system

Infolding of the plasma membrane at points along the ascus wall initiated the formation of the enveloping membrane system (EMS) (Fig. 1). Flattened cisternae were occasionally observed in close proximity to the plasma membrane invaginations (Fig. 1). The plasma membrane invaginations were frequently observed as U-shaped structures (Figs. 1 and 2) and often the infoldings would fuse with the plasma membrane resulting in small regions of cytoplasm being trapped (Figs. 2, 3 and 4). The plasma membrane invaginations were frequently quite elaborate (Figs. 3 and 4) and occasionally multi-vesicular bodies, small vesicles and other membranous structures were observed within the cytoplasmic inclusions. Eventually, the plasma membrane invaginations separated from the plasma membrane and entered the cytoplasm remaining in close proximity and adjacent to the ascus wall (Fig. 5). Continued growth of the small segments of the EMS resulted their fusion and the formation of a relatively continuous double-membraned cylinder around most of the ascus protoplasm (Fig. 5). The formation and growth of the EMS appeared to be the result of the flattened

cisternae which were often observed in a position approximately perpendicular (Figs 1 and 5) with the ascus wall and EMS. Eventually the EMS invaginated inwards to encompass nucleate portions of cytoplasm with accompanying organelles (Fig. 6). The nucleus-associated organelle (NAO), always oriented towards the ascus wall, appeared as a globular ribosomal zone-of-exclusion with emanating microtubules (Fig. 7). The microtubules projecting from the NAO appeared to have ribosomes and some fibrillar material closely-associated with them (Figs. 7-12). Cisternae were frequently observed in close proximity to the region of highest microtubular density (around the NAO) (Figs. 8 and 9). A typical presumptive ascospore initial contained a single nucleus, mitochondria, microtubules, cisternae, lipid bodies, endoplasmic reticulum and multi-vesicular bodies within its cytoplasm (Fig. 10). Endoplasmic reticulum was frequently observed closely associated with the EMS on the epiplasmic side of presumptive ascospore initials (Fig. 10). Microtubules emanating from the NAO appeared to extend towards the opening of the presumptive ascospore initials some of which were apparently associated with mitochondria (Figs. 11 and 12). Some microtubules appeared to terminate at or near the inner membrane of the EMS (Fig. 13). Occasionally, microtubules were observed extending beyond the boundaries of the presumptive ascospore initial into the epiplasm (Fig. 14).

Ascospore initial wall development

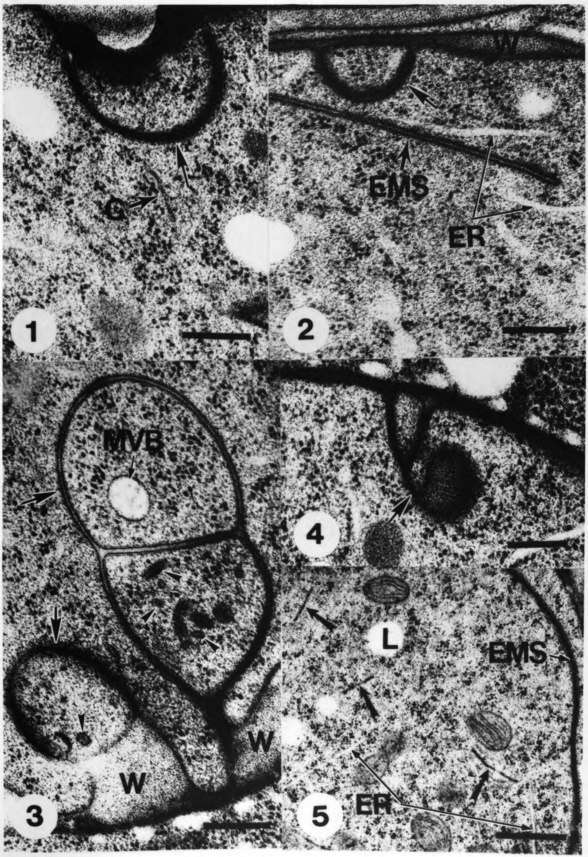
Upon delimitation, a relatively electron transparent primary wall layer developed between the double membrane of the EMS causing their eventual separation (Figs 15, 16, and 17). Endoplasmic reticulum was still closely associated with the outer delimiting membrane of ascospore initials (Fig. 15). Each uninucleate ascospore initial contained several multi-vesicular bodies (Fig. 16) and occasionally microtubules were found in a position parallel with the long axis of the spore initial (Fig. 17). A single mitotic division occurred during late primary wall deposition resulted in binucleate ascospore initials (Fig. 18).

Subsequently, secondary wall deposition appeared as an electron dense layer overlaying the well developed primary wall (Fig. 19). Localized depositions of wall materials and (or) precursors resulted in early winged-appendage formation. Generally, darkly-stained cisternae could be found within ascospore initials directly beneath the winged-appendages (Fig. 19 and 20). These darkly-stained cisternae were frequently closely associated with mitochondria (Fig. 19). During secondary wall deposition, epiplasmic endoplasmic reticulum could still be observed adjacent to the outer ascospore initial membrane (Fig. 20). Many asci with mutant ascospore initials were observed that did not have the typical winged secondary wall layer (Fig. 21). Instead, the ascospore initials contained a normal primary wall layer with a slightly granular electron transparent secondary wall layer (Fig. 21). No winged-appendages were observed in these mutants and

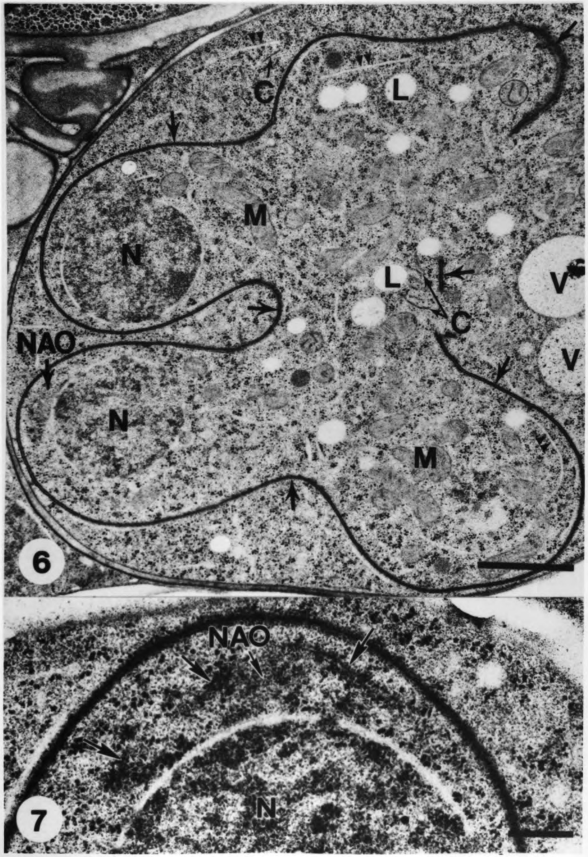
electron dense cisternae were noticeably absent within the ascospore initials.

The winged-appendages continued to lengthen accompanied by the appearance of a single lipid droplet within the center of the spore initial (Fig. 22). Electron dense cisternae were still present and numerous electron dense vesicles were also observed within the spore initial cytoplasm (Fig. 22). As the ascospore initials matured the secondary wall layer surface became irregular, an electron dense tertiary wall layer developed and electron dense cisternae and vesicles were still observed (Fig. 23). The epiplasm was filled with glycogen (Fig. 23). Following tertiary wall deposition a double-membraned structure (possibly endoplasmic reticulum) was observed closely associated with the outer ascospore membrane and frequently was seen interconnecting ascospores (Fig. 24). In addition, a different membranous structure was observed following the contour of the maturing ascospore initials secondary wall layer (Fig. 25). A few lipid bodies as well as glycogen were also observed within the epiplasm at this stage (Fig. 25). Eventually, the ascospore cytoplasm increased in electron density while the remaining epiplasm and ascus wall deteriorated to release the spores into the centrum.

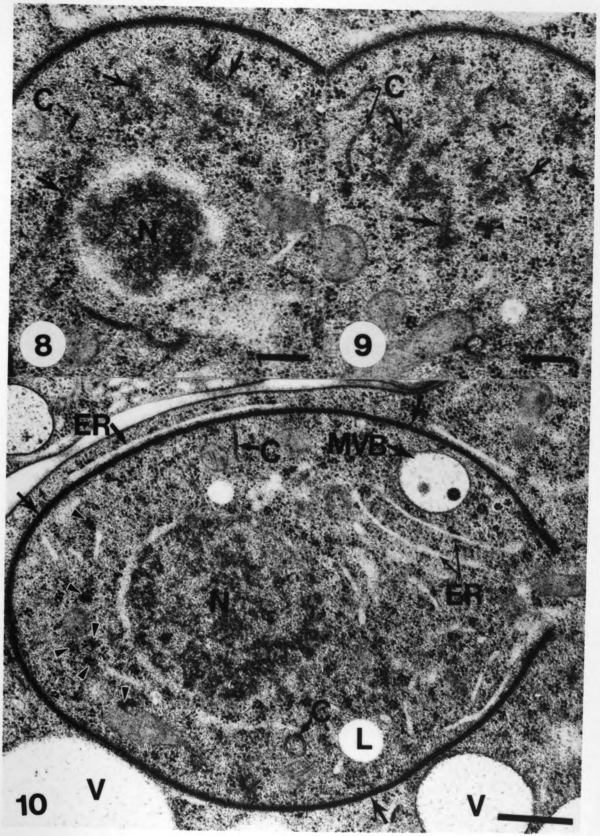
FIGS. 1-5. Emericellopsis terricola. Fig. 1. Early development of the EMS appeared as a curved infolding of the plasma membrane (arrow). Flattened, cisternae (C) were often found in close proximity to the plasma membrane invaginations. Bar = 0.25μm. Fig. 2. The curved plasma membrane infoldings (arrow) often fused with the plasma membrane delimiting small portions of cytoplasm. Ascus wall (W), enveloping membrane system (EMS), and endoplasmic reticulum (ER). Bar = 0.25μm. Fig. 3. The plasma membrane invaginations often were very elaborate (arrows) and occasionally delimited small vesicles (arrowheads) and multivesicular bodies (MVB). Bar = 0.125μm. Fig. 4. Another relatively elaborate configuration of a plasma membrane invagination (arrow). Bar = 0.5μm. Fig. 5. Release and unfolding of the plasma membrane invaginations resulted in a relatively continuous cylinder (EMS) surrounding most of the ascus protoplasm. Flattened cisternae (arrows) were located within the ascus cytoplasm oriented perpendicular to the ascus wall, presumably contributing vesicles for continued EMS growth. Endoplasmic reticulum (ER) and lipid bodies (L). Bar = 0.25μm.


1
C
2
EMS
ER
3
MVB
W
W
4
5
L
EMS
ER

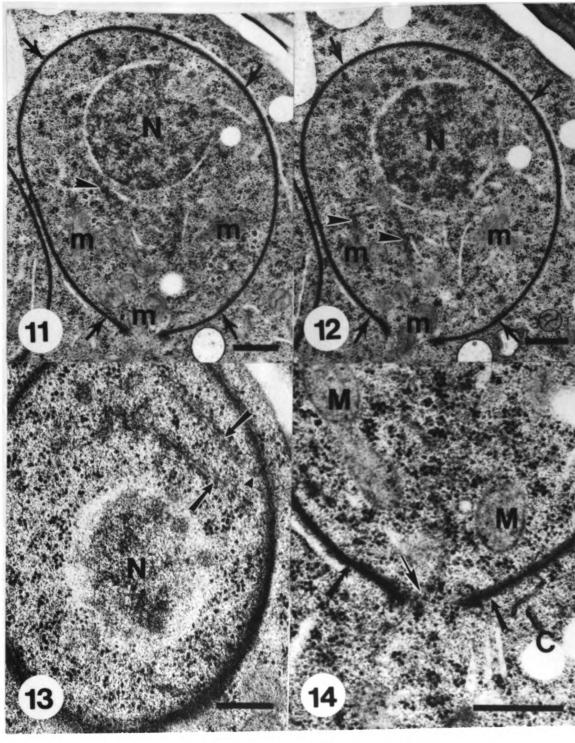
FIGS. 6-7. Emericellopsis terricola. Fig. 6. Invagination of the EMS cylinder (arrowheads) around individual nuclei (N) with accompanying cytoplasm and organelles. Nucleus-associated organelle (NAO), mitochondria (M), lipid bodies (L), vacuoles (V), cisternae (C) and endoplasmic reticulum (double arrowheads). Bar = 1μm. Fig. 7. A globular nucleus-associated organelle (NAO) with microtubules (arrows) emanating from it was present during this stage. Bar = 0.25μm.


C
L
M
N
L
V
C
V
NAO
N
M
6
NAO
N
7

FIGS. 8-10. Emericellopsis terricola. Fig. 8. A grazing section of a nucleus (N) showed an array of microtubules (arrows). These microtubules were coated with an electron dense fibrillar material and ribosomes which facilitated their identification when sectioned obliquely. Cisterna (C). Bar = 0.25μm. Fig. 9. An oblique section near an NAO which showed the numerous microtubules and also cisternae (C) were observed. Bar = 0.25μm. Fig. 10. A typical presumptive ascospore initial which showed a cup-shaped EMS (arrows), nucleus (N), cisternae (C), endoplasmic reticulum (ER), lipid body (L) and multi-vesicular body (MVB). Cross sections of microtubules (arrowheads) could also be seen. Endoplasmic reticulum (ER) was closely associated with the outer EMS membrane at this stage. Vacuoles (V). Bar = 0.5μm.


C
C
N
8
9
ER
C
MVB
N
ER
C
L
10
V
V

FIGS. 11-14. Emericellopsis terricola. Fig. 11. A section through a presumptive ascospore initial (arrows) showed a single microtubule which extended towards the narrow opening. Mitochondrion (m). Bar = 0.5μm. Fig. 12. Serial section of Fig. 12. which showed the same microtubule (arrowhead) extending towards the opening of the presumptive ascospore initial and another microtubule (arrowhead) in direct contact with a mitochondrion (m). Bar = 0.5μm. Fig. 13. Microtubules (arrows) were often observed extending towards and in close association (bordered arrow) with the inner membrane of the EMS. Nucleus (N). Bar = 0.25μm. Fig. 14. Occasionally, microtubules (arrowhead) were seen extending beyond the boundaries of the EMS (arrows) of the presumptive ascospore initial. Mitochondria (M), cisternae (C). Bar = 0.5μm.


N
m
m
m
11
N
m
m
m
12
N
13
M
M
C
14

FIGS. 15-18. Emericellopsis terricola. Fig. 15. Following complete delimitation of ascospores, electron transparent wall materials and (or) precursors were deposited between the double membranes of each ascospore initial. The elliptical ascospore initial contained a single nucleus (N), cisternae (C), endoplasmic reticulum (double arrowheads), and mitochondria (M). The epiplasm (E) contained lipid bodies (L) and endoplasmic reticulum which was closely associated with the outer membrane of the ascospore initial. Ascus wall (W), primary wall (P). Bar = 0.5μm. Fig. 16. Multi-vesicular bodies (MVB) were also frequently observed during primary wall (P) development. Bar = 0.5μm. Fig. 17. Occasionally, during the early primary wall (P) development microtubules (arrow) were observed oriented parallel with the long axis of the ascospore initial. Nucleus (N), mitochondrion (M). Bar = 0.5μm. Fig. 18. Longitudinal section of an ascospore initial during late primary wall (P) development which showed two nuclei (N) as a result of a single mitotic division. Bar = 0.5μm.

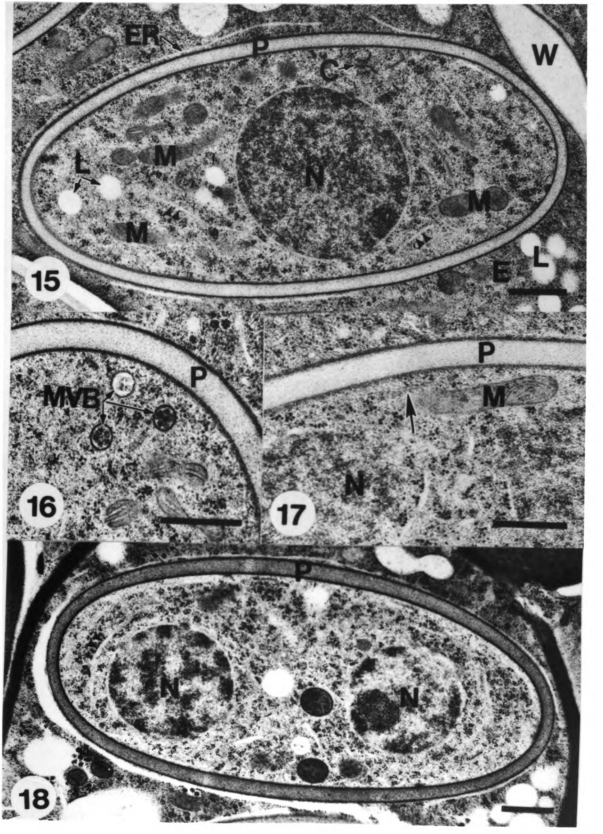
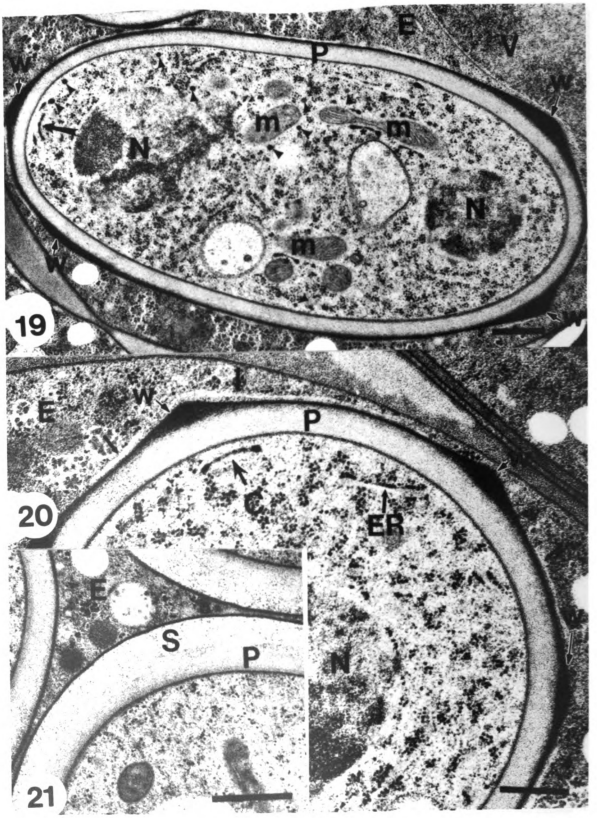
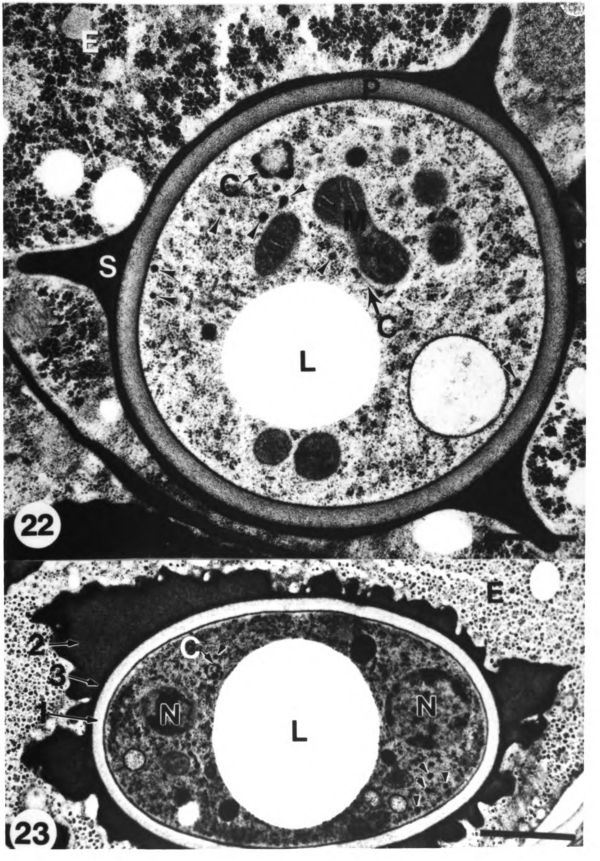

ER
P
W
L
M
N
M
M
E
L
15
P
MVB
16
P
M
N
17
P
N
N
18

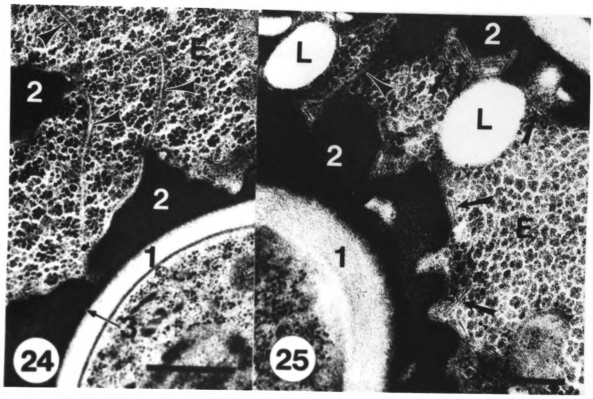
Fig. 19-21 Emericellopsis terricola. Fig. 19. Ascospore initials during early secondary wall formation showed many cisternae (arrows) throughout the cytoplasm and numerous vesicles (arrowheads). Cisternae were often located beneath the winged appendages (w) and in close association with mitochondria (m). The epiplasm (E) often contained a single large vacuole (V) with granular contents. Nucleus (N), primary wall layer (P). Bar = 0.5μm. Fig. 20. A cross section of an ascospore initial during secondary wall formation which showed the triangular winged-appendages (w) and a cisterna (C) located directly beneath one of the appendages. Epiplasmic (E) endoplasmic reticulum (arrows) was still closely associated with the outer membrane of this ascospore initial. Nucleus (N), rough endoplasmic reticulum (ER). Bar = 0.25μm. Fig. 21. Mutant ascospore initials were observed which contained an electron transparent finely granular secondary wall layer (S) without any winged appendages. Epiplasm (E), primary wall (P). Bar = 0.5μm.


E
P
V
W
m
N
19
E
W
P
20
ER
E
S
P
N
21

FIGS. 22-23. Emericellopsis terricola. Fig. 22. The secondary wall layer (S) winged-appendages were pointed at this stage and the ascospore initials contained a single lipid droplet (L), many darkly-stained cisternae (C) and vesicles (arrowheads). Mitochondrion (M). Bar = 0.5μm. Fig. 23. As the secondary wall layer (2) expanded its surface became very irregular. An electron dense tertiary wall layer (3) was also laid down between the primary (1) and secondary wall layers. The lipid body (L) enlarged within the center of the ascospore initial separating the two nuclei (N). Darkly-stained cisternae (C) and vesicles (arrowheads) were still present in the cytoplasm which had increased in electron density. The epiplasm (E) was largely filled with glycogen at this stage. Bar = 1μm.


E
C
S
C
L
22
E
2
3
C
N
N
L
23

FIGS. 24-25. Emericellopsis terricola. Fig. 24. A double membrane (presumably ER) (arrowheads) was observed in the epiplasm (E) which was closely associated with the outer membrane of the secondary wall layer (2) and frequently interconnected ascospores. Primary wall layer (1), tertiary wall layer (3). Bar = 0.5μm. Fig. 25. In addition to the ER (arrowhead) another membranous-like structure (arrows) was also observed closely associated with the outer ascospore membrane. The epiplasm (E) also contained lipid bodies (L) at this stage. Primary wall layer (1), secondary wall layer (2). Bar = 0.25μm.


2
E
2
1
24
L
2
L
2
1
E
25

DISCUSSION

The origin of the EMS has been elusive or extremely variable amongst the numerous taxonomic groupings of Plectomycetes and even within genera when conventional chemical fixation was employed for ultrastructural studies. Within the Plectomycetes the number of suggested origins was almost as numerous as the number of species examined. For example, the plasma membrane in Emericellopsis terricola (41), Thermoascus aurantiacus (10), Mycotrichum deflexum (29), and Monascus purpureus (40), in mesosomes Ajellomyces dermatitidis and Arthroderma benhamie (11) and myelin figures Nannizzia gypsea (14) associated with the plasma membrane, endoplasmic reticulum in Penicillium vermiculatum (39), electron transparent vesicles Ophiostoma davidsonii (34) Ophiostoma distortum and Ophiostoma minus (36) and Ceratocystis fimbriata (31), *de novo* in association with ribosomes in Ceratocystis moniliformis (38) or undetermined in Talaromyces striatus (19), Ophiostoma cucullatum (35), and Ophiostoma piceae (37).

All of these previous ultrastructural studies relied on conventional chemical fixation to evaluate a highly dynamic and transient process, ergo the many preposed origins. A review of these articles showed no compelling evidence that the process differed significantly from that observed for E. terricola described here. Variations probably were the result of membrane artifacts, which until the more recent development of freeze-substitution (6, 7, 15, 16, 17, 23, 24, 26, 27), were difficult to interpret. High-pressure freezing in particular, has been shown to be superior for preserving larger

samples (up to 600μm) more accurately (5, 9, 12, 42).

The plasma membrane invaginations described for E. terrricola were more elaborate than described for other high-pressure frozen freeze-substituted Euascomycetes. In Sordaria humana (6, 8, Chapter 2) and Thelebolus crustaceus (7, Chapter 1) simple plasma membrane invaginations appeared to contribute small double-membraned segments to the EMS. In E. terricola the plasma membrane invaginations often appeared labyrinthiform and were more similar to the invaginations described for Taphrina deformans (32, 33) and freeze-substituted Ascodesmis nigricans (23). Mims et. al. (23) described the more complex plasma membranes as evolving from small cytoplasmic inclusions. However, I interpret the complex appearance simply as membrane invaginations that occurred at points along the initial plasma membrane invagination. As a result, many loops occurred on a single initial plasma membrane invagination.

The initiation of the EMS and its continued growth appeared consistent with the manner described previously (7, 8), that is, generated by vesicular addition from cisternae. Interestingly, cisternae were always oriented in a position perpendicular to the ascus wall (location of the EMS). In E. terricola the cisternae were remarkably flattened and often difficult to clearly distinguish until photographically enlarged in the dark room. Combine this with the fact that cisternae are notoriously difficult to preserve or, more frequently, absent with conventional chemical techniques, and it is clear why they were not reported previously for Plectomycetes.

To date there have been no ultrastructural reports of microtubules located within asci or ascospores of Plectomycetes. High-pressure freezing in conjunction with freeze-substitution was superior to conventional chemical techniques in this respect. I determined that microtubules associated with each NAO were key in formation of ascospore initials. Movement of the EMS inwards with accompanying organelles appeared to be facilitated by the contractile nature of microtubules as suggested previously (2, 3, 7, 8, 28).

The shape of the ascospore initial also appeared to be governed by the strategic location of microtubules beneath the cell wall and parallel with its long axis. However, the microtubules were not as proliferate as described previously (2, 7, 8). This may be a function of spore size or possibly due to the fact that I missed the appropriate stage. Irrespective, I find it likely that the elliptical shape of the ascospore initials was due to microtubular reinforcement until the primary wall layer had become relatively inflexible (1, 7, 8), probably in a similar fashion as described for T. crustaceus (7).

Primary and secondary, and tertiary wall development appeared to largely occur through cisternal deposition from within ascospore initials. However, I can not rule out the possible involvement of endoplasmic reticulum directly contributing material to the cell wall layers. Particularly, since there is a close association with epiplasmic ER and the outer membrane of each ascospore initial which was present before complete

delimitation until after tertiary wall deposition. The outer ascospore initial membrane appeared to grow as the surface area of the secondary wall increased. Presumably membrane and (or) wall material must be deposited from the epiplasmic side of each ascospore initial in order for this membrane to grow. It must be noted, however, that the outer membrane was frequently ruptured during late primary wall development (not shown) in a similar fashion and stage as described for T. crustaceus (7, Chapter 1). Czymmek and Klomparens (7) suggested that at least at this stage materials may not be deposited from the epiplasm. This may be true, but the membrane rupture may also be due to an artifact of high-pressure freezing, which occurred only as a result of some characteristic of the primary wall which was not exhibited at any other stage. The only conclusive way to determine which was the case would be to examine an ascomycete amenable to good freezing at both ambient and high pressure that exhibited this characteristic at high pressure. Such membrane ruptures were absent from high-pressure frozen freeze-substituted samples of S. humana (6, 8, Chapter 2).

The formation of winged-appendages occurred associated with the localized deposition by cisternae located directly beneath a given appendage. This is the first report documenting such a phenomenon and was possible due to the nature of ultrarapid freezing and freeze-substitution being able to accurately preserve cisternae and associated vesicles. Although Wu and Kimbrough (41) documented the order of wall deposition in Emericellopsis

microspora, they were unable to determine the wall origins using conventional chemical techniques. I expect that more freeze-substitution studies will elucidate the nature of development in other appendaged ascospores. In support of localized cisternae being responsible for appendage formation was the occurrence of wingless mutants. Besides the absence of the electron dense staining property of a normal winged ascospore initial secondary wall, the ascospore initial cytoplasm was noticeably devoid of any electron dense cisternae or vesicles. The secondary wall layer was essentially electron transparent with some finely granular material interspersed. The origin of this material eluded me but may be the product of the epiplasmic ER.

Many ascomycetes have been shown to deposit material destined for the ascospore initial wall via large vesicles from the epiplasm (7, 8, 20, 21, 22). Epiplasmic vesicles were not observed associated with the outer membrane as had been described previously. However, in E. terricola the close association of ER with the outer ascospore initial membrane may eliminate the necessity to transport wall materials by way of vesicles.

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SUMMARY

The process of ascosporogenesis was variable between the three species examined during this study. However, there were several basic features that were common to all three Euascomycetes. In addition, it was clear that an accurate term was necessary to describe the double membrane system which delimited ascospore initials. The term enveloping membrane system (EMS) was introduced in this research (see Chapter 1 and 2) to describe this membrane system at any developmental stage.

1.) The enveloping membrane system (EMS) was initiated as a form of membrane recycling via plasma membrane invaginations. Cisternae added vesicles to the ascus plasma membrane and the excess membrane generated appeared as infoldings of the plasma membrane.

2.) The small plasma membrane invagination units were released into the ascus cytoplasm and maintained a position adjacent to and parallel with the ascus wall. Continued growth of the double-membraned enveloping membrane system was either through the production of more plasma membrane invaginations or direct vesicular addition to the developing EMS cylinder.

3.) Eventually the EMS enclosed most of the ascus cytoplasm and invaginated inward to surround uninucleate portions of cytoplasm with

accompanying organelles.

4.) Envelopment of ascospore initials was facilitated by a nucleus-associated organelle (NAO) (S. humana, E. terricola) or an NAO with associated microtubule organizing center (MTOC)(T. crustaceus). Whatever the means, numerous microtubules were involved and were observed emanating from the NAO or NAO-MTOC. These microtubules were observed in direct contact with the EMS and mitochondria and probably acted in a contractile manner pulling the EMS and some organelles within the boundary of each presumptive ascospore initial.

5.) Shortly after complete ascospore initial envelopment, the primary wall material and/or precursors were deposited between the double membrane delimiting each spore initial. This primary wall was relatively electron transparent and was probably deposited via cisternae, although contributions from endoplasmic reticulum could not be ruled out.

6.) During primary wall development microtubules were observed parallel with the ascospore initial wall and presumably gave each spore its charecteristic shape.

7.) Secondary wall development was variable between the three species although it was initially electron dense and always initially derived from electron dense cisternae found within the ascospore initials. The winged appendages of E. terricola were the result of localized depositions of wall materials and (or) precursors beneath the location of the developing winged-appendages.

8.) Electron transparent vesicles from the epiplasm also appeared to deposit materials to the secondary wall layer of S. humana and T. crustaceus but no evidence of epiplasmic vesicles were observed contributing material to E. terricola, however endoplasmic reticulum was observed closely associated with its outer membrane.

9.) A tertiary wall layer was deposited between the primary and secondary wall layers and was variable in appearance between species.

10.) A quarternary wall layer was deposited beneath the primary wall layer only in S. humana, but this is not atypical for Pyrenomycetes.

Many of the similarities described above were determined solely as a result of using the technique of freeze-substitution. However, this preliminary list can only be accurate if many more Ascomycetes including Hemiascomycetes are examined using a similar technique. It seems apparent that the best approach would be to use the high-pressure freezer in conjunction with freeze-substitution in order that important details of the process of ascosporogenesis not be appreciably distorted or totally lacking. I expect that other differences in detail of the process will occur but that the several basic features described above and throughout this paper were probably essential in normal ascospore formation.

Although the techniques of high-pressure freezing and freeze-substitution have proven to be invaluable for this study, they have not been without their own associated artifacts. For example, the high-pressures involved to freeze the samples may have caused the membrane ruptures

described for T. crustaceus and E. terricola. The process of freeze-substitution itself appeared responsible for extraction of lipid bodies and in some stages inadequate preservation of the nuclear envelope and endoplasmic reticulum. Infiltration by the resin may be responsible for the artificial separation of the lumen between the membranes of the nuclear envelope and endoplasmic reticulum. Due to the brittle nature of high-pressure frozen tissue I believe that the small cracks within the cytoplasm were caused by manipulation of the samples prior to embedment. Finally, the most consternating problem was the separation of the plasma membrane from the cell wall during sectioning. I attempted to remedy this problem by plastic and carbon coating the grids. This appeared to have a stabilizing effect on sections, preventing their movement under the electron beam. However, it may have been more satisfactory to use a lower viscosity resin such as Quetol or to infiltrate in smaller increments.

The advantages of high-pressure frozen freeze-substituted samples far outweighed the disadvantages, particularly concerning the process of ascosporogenesis. The transient membrane events of EMS formation and development were well preserved. Microtubules, multi-vesicular bodies, cisternae and their associated vesicles were also well preserved. All of these features were important for studying the process of ascosporogenesis and were previously lacking, unclear, or artifactually distorted using conventional chemical techniques. It is for this reason that I strongly suggest that ultrarapidly frozen freeze-substituted samples be used when

examining ascosporogenesis ultrastructurally. This would include reexamination of many previously studied conventionally fixed Ascomycetes, so that technique-associated artifacts could be determined. See appendices for exact methodology and comparisons.

APPENDIX I

Comparison of freezing methods.

Summary

Early in this study samples were frozen by plunge, propane-jet, or high-pressure freezing techniques in order to determine the most effective method with the fewest artifacts. Due to the size of the structures examined in this study (ascocarps at least 100μm in diameter) all plunge frozen samples had moderate to severe ice crystal damage, eliminating this technique as a useful alternative. The technique of propane-jet freezing lowered the overall amount and severity of ice-crystal damage. However, many significant artifacts were observed with this technique for my samples. High-pressure freezing was the method of choice for freeze-substituted samples in this study. It significantly reduced ice crystal damage and extensive manipulation of samples was avoided. The development of a commercially available high-pressure freezer allows preservation of many biological samples to a depth of about 600 μm (Chapter II, Ref. 18 and 28). High-pressure freezing achieves this by reducing the critical freezing rate from -10,000°K/sec at ambient pressures to approximately -100 to

500°K/sec at a pressure of 2,100 bar (Chapter II, Ref. 18 and 28).

Specific methods

Plunge-freezing

Samples for plunge-freezing were grown on dialysis membrane (used as a support structure) under the appropriate conditions until sporulation ocurred. The dialysis membrane (with the fungal structures adherring to it) was cut with a razor blade into ten millimeter squares and plunged into liquid propane. The dialysis membrane (with sample) was transferred to liquid nitrogen and stored until ready for substitution. Due to the size of the structures examined in this study (ascocarps at least 100μm in diameter) all plunge frozen samples had moderate to severe ice crystal damage, eliminating this technique as a useful alternative.

Propane-jet freezing

Samples for propane-jet freezing were grown as described for plunge-freezing. Ascocarps were gently removed from the culture surface and placed on a single copper hat with a 2 mm X 3 mm X 50 μm slotted gold spacer (the gold spacer provided the boundary for the samples). A second copper hat was placed on top of the slotted gold spacer and the samples were then placed in a RMC MF 7200 propane-jet freezer, frozen, and quickly transferred to liquid nitrogen for storage. The propane-jet freezing technique allowed greater depths with adequate freezing than was possible using

plunge frozen samples, however several artifacts were induced with my samples. Due to the size of the structures, much mechanical damage occurred while crushing the samples between the copper hats. In addition, some degree of desiccation (probably improving the freezing rate) occurred while samples were being placed between the copper hats and while waiting for the propane-jet freezer. Also, it was not clear what effect (if any) the copper hats may have had on samples (since copper is generally toxic to fungi). The combination of these factors lead me to believe that for my samples, propane-jet freezing would not allow accurate interpretations of the process of ascosporogenesis. Propane-jet frozen ascospores were still examined during this study due to their small size (less than 3μm in length) in hopes that they were somewhat insulated from the damage incurred during this technique.

As mentioned previously, propane-jet freezing allowed better freezing for my samples than was possible via plunge freezing, however, minor freeze damage was still frequently observed. The epiplasm (Chapter I, Figs. 13, 15, 16 and 17) showed greater freeze-damage than the ascospore initials contained within it. This may suggest that the epiplasm may have had a higher water content or fewer natural cryo-protectants. Overall appearence of the cytoplasm in propane-jet frozen ascospore initials (Chapter I, Figs. 13-17), although better than might be expected from conventional chemical fixation, was inferior to high-pressure frozen ascospore initials (Chapter I, Figs. 20 and 21; Chapter III, Figs. 13). Microtubules, cisternae,

and vesicles were preserved by both high-pressure (Chapter I, Figs. 9-12; Chapter II, Figs. 14, 20-22; Chapter III, Figs. 7-10) and propane-jet freezing (Chapter II, Figs. 13-17, 25-28) but resolvable ice crystal damage was more prevalent in the propane-jet frozen samples, probably contributing to the poorer quality appearence.

Artifacts associated with post-fixation were the separation of the lumen between the double membrane of the EMS and tears or cracks frequently observed in the cytoplasm of cells. Separation of the lumen (Chapter I, Fig. 3) appeared only in propane-jet frozen samples and may be due to the combination of propane-jet freezing and the particular resin (Spurr's epoxy resin) used. Cracks in the cytoplasm occured in both high-pressure freezing (Chapter III, Fig. 18) and propane-jet freezing (Chapter I, Fig. 25) but was much more widespread in propane-jet freezing. I suspect that this artifact was due to removal of samples from their supports. Following fixation with propane-jet frozen samples the thin cell layer often adherred to the copper hats (even after being lecithin coated) resulting in this mechanical damage. High-pressure frozen samples separated more readily and were much easier to handle, without damage, due to their size.

High-pressure freezing

High-pressure frozen samples were grown as described for plunge frozen samples. Clusters of ascocarps were gently excised from the top of the dialysis membrane and placed into a gold hat containing a drop of 15%

dextran solution (M.W. 39,000). Numerous ascocarps could easily be placed within a single gold hat and covered with the second gold hat with minimal cell disruption. Samples were then frozen in a Balzer's HPM 010 high pressure freezer and quickly transferred to liquid nitrogen for storage. Dextran was used as a non-membrane permeable cryo-protectant to fill up any exterior air spaces and to prevent ice crystal nucleation from the surface water surrounding the ascocarps. This technique provided the best overall freezing quality with minimal manipulation of samples.

APPENDIX II

Freeze-substitution

Freeze-substitution solutions

All freeze-substitution for transmission electron microscopy was done in a solution of 2% osmium tetroxide and 0.05% uranyl acetate in HPLC grade acetone. The chemicals for this solution were prepared as follows:

A solution of 4% osmium tetroxide was prepared by placing 25 ml of HPLC grade acetone into a 125 ml stoppered flask and cooled in a -80°C freezer for at least 2 hours. The HPLC acetone was then placed into an ice chest containing finely-crushed dry ice and transported to an exhaust hood where a 1 gm vial of osmium tetroxide was snapped and dropped into the solution. Care was taken to keep the acetone from being directly exposed to room atmosphere (in order to prevent condensation of water into the solution) by quickly removing and replacing the stopper when the osmium tetroxide was added. The 4% osmium tetroxide acetone solution was returned to the -80°C freezer until ready for use (at least overnight). I found fixation was the best when this solution was made one day prior to sample substitution. As a note of caution: Do not use osmium tetroxide in acetone

if solution has turned from the normal yellow to a dark brown or black.

A solution of 0.1% uranyl acetate in acetone was made by placing 0.05 gm of uranyl acetate and 50ml of HPLC grade acetone into a 100 ml opaque bottle (small acetone bottles work well), sonicated for ten minutes and placed in the -80°C freezer overnight.

Substitution

Samples stored in liquid nitrogen were transferred into a styrofoam container with at least two inches of liquid nitrogen covering the gold hats containing the samples. A styrofoam ice chest with finely crushed dry ice was used to transfer the 4% osmium tetroxide in HPLC acetone, 0.10% uranyl acetate in HPLC acetone, a flask of HPLC acetone and an RMC MS6200 holding device (both of which had been cooled to -80°C at least 2 hours prior to substitution). All transferring of samples and substitution fluids was done under an exhaust hood. The RMC holding device had three large wells to hold samples each of which held a little over 10 ml of substitution fluids. One well was partially filled with calcium sulfate and then cold HPLC grade acetone was added until the well was nearly full. This well acted as a water scavenger if any were to enter the chamber from the atmosphere. Equal amounts (approximately 5 ml) of 4% osmium tetroxide in acetone and 0.10% uranyl acetate in acetone were quickly pipetted into the two remaining wells giving a final substitution fluid of 2% osmium tetroxide and 0.05% uranyl acetate in acetone. The gold hats (with samples still

attached) were quickly transferred into the two wells. Different species could be placed within the same well because they were easily identified by light microscopy after substitution. After all samples were placed in the substitution fluid, the RMC holding device lid was replaced, and the RMC device was returned to the -80°C freezer for 72 hours. After 72 hours the RMC holding device was transferred for two hours each at the following temperatures; -35°C, -25°C, -12°C, 0°C, 10°C and room temperature. These temperatures simply reflect the temperatures in the freezers available at the time of substitution. I attempted to make the change in temperature as small as possible between freezers (in this case 10 to 12 degrees worked well). After sitting at room temperature the substitution fluid was removed and the samples were rinsed 3 times in HPLC grade acetone. Infiltration was done under desiccation (calcium sulfate and phosphorous pentoxide) under slight vacuum) at 10%, 25%, 40%, 50%, 75%, 85%, 95%, 100% and 100% Spurr's epoxy resin (with acetone as the solvent). The desiccation and slight vacuum were used to reduce the effect of the plasma membrane separating from the cell wall during sectioning (by removing any water which could prevent proper polymerization of resin). After the second change in 100% resin the gold hats and samples (which frequently separated from the gold hats during infiltration) were poured into 57mm aluminium weighing dishes (American Scientific Products) and examined under a dissecting microscope. Samples free in the resin were transferred to another weighing dish which contained a very thin layer of freshly made

100% Spurr's epoxy resin. Samples which were still attached to gold hats were gently removed with fine tipped tweezers or a sharpened wood stick and placed into the new weighing dish. The weighing dishes (with samples) were placed under the same conditions as infiltration (desiccation and slight vacuum) overnight. The weighing dishes were then transferred to a 60°C oven and polymerized for three days. After polymerization the aluminum weighing dish could be carefully peeled off and the thin layer of resin with the samples could be examined by light microscopy usually using 10X to 40X dry objectives. Samples with minor to moderate freeze-damage would appear transparent brown while minimal freeze-damaged samples were usually very transparent. Evaluation of freeze-damage in some samples was sometimes difficult due to dark pigmentation of some wall layers. However, I estimate that well over 75% of all samples examined had minimum or no resolvable ice crystal damage and severe ice crystal damage was never observed using high-pressure freezing.