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ZYGOSPOROGENESIS IN ZYGORHYNCHUS HETEROGAMUS, AND ZYGOSPOROGENESIS AND SPOROANGIOSPOROGENESIS IN MYCOTYPHA AFRICANA

presented by

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Ph.D. degree in Botany

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Major professor

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ZYGOSPOROGENESIS IN *ZYGORHYNCHUS HETEROGAMUS*, AND ZYGOSPOROGENESIS AND SPORANGIOSPOROGENESIS IN *MYCOTYPHA AFRICANA*

By

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ABSTRACT

ZYGOSPOROGENESIS IN *ZYGORHYNCHUS HETEROGAMUS*, AND ZYGOSPOROGENESIS AND SPORANGIOSPOROGENESIS IN *MYCOTYPHA AFRICANA*

By

Richard Edward Edelmann

The ultrastructure of zygosporogenesis in *Zygorhynchus heterogamus* was studied with scanning and transmission electron microscopy (SEM and TEM), using conventional chemical fixation (CF) and the cryopreservation techniques of low temperature SEM (LTSEM) and freeze-substitution (FS). Detailed observations of origin and development of the walls of the entire sexual apparatus are reported. Gametangial septa contained plasmodesmata from initial formation through full maturation of the zygospore. Wart formation was initiated with the appearance of vesiculate bodies between the zygosporangium wall and plasma membrane, and developed through deposition of a stacked series of electron-opaque disks. Rupture of the zygosporangium wall by the developing zygospore was concurrent with the formation of a contiguous warty layer. The first clear TEM observations of the multiple wall layers of the fully mature zygospore are presented. A detailed historical discussion on, and a proposal for standardized structural nomenclature are presented.

Sexual and asexual reproduction in *Mycotypha africana* was observed with

LTSEM. Sexual apparati were initiated by the contact of two zygophoric hyphae. Sub-apical contact resulted in formation of progametangia at the site of contact. Zygophoric hyphal elongation continued, forming additional sexual contacts. Progametangia enlarged, and gametangial septa were formed, isolating the gametangia which then fused, resulting in the formation of the zygosporangium. Zygospores developed internally, rupturing through the zygosporangium wall with maturation of the wart layer. Sporangiosporogenesis commenced with the formation of specialized aerial sporangiophores extending up to 5,000 μm. Apical regions of sporangiophores enlarged forming vesicles 300-400 μm long. Pedicels formed synchronously on vesicles, and ballooned outward forming sporangia. Morphologically distinct, spherical- and elongate-sporangia formed, exhibiting a highly regular spatial arrangement, alternating between the two sporangial types.

LTSEM provided excellent preservation of fragile reproductive structures free from dehydration artifacts common with CF. Nine FS fixation protocols following either propane jet freezing or high pressure freezing (HPF) of *Z. heterogamus* were compared. Both methods of freezing offered acceptable results, but HPF provided more well-frozen material. Comparison of FS with CF revealed identification of cytoplasmic ultrastructure was better with CF. FS resulted in poor membrane preservation providing few identifiable organelles, however wall ultrastructure was better than with CF.

To Dr. Terrence M. Hammill,

For introducing me to the fungi and sex in the Zygomycetes and

For allowing a sophmore to take apart his TEM.

To Dr. Karen L. Klomparens,

For being a true Mentor in every possible sense

And for allowing me to discover who and what I am.

And to

Lori A. Bridgers, BA, MA, MSEd

My Best Friend,

And all of the consequences.

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CHAPTER 1: ZYGOSPOROGENESIS IN *ZYGORHYNCHUS HETEROGAMUS* VUILLEMIN (MUCORALES), WITH A PROPOSAL FOR STANDARDIZATION OF STRUCTURAL NOMENCLATURE

ABSTRACT

The ultrastructural details of zygosporogenesis in *Zygorhynchus heterogamus* Vuillemin, 1903, were observed with scanning and transmission electron microscopy (SEM and TEM), using both conventional chemical fixation and the cryopreservation techniques of low temperature SEM (LTSEM) and freeze-substitution for TEM. Detailed observations of origin and development of the walls of the entire sexual apparatus are reported. Maturation of both gametangial septa was observed to be identical and plasmodesmata were observed to penetrate the septa from their initial formation through full maturation of the zygospore. Wart formation was initiated with the appearance of vesiculate bodies closely oppressed to the zygosporangium wall, external to the plasma membrane. Wart development progressed through the deposition of a stacked series of electron-opaque disks. Rupture of the zygosporangium wall by the developing zygospore

was concurrent with the formation of a contiguous warty layer. For the first time, clear TEM observations of the multiple wall layers of the fully mature zygospore are presented. A detailed historical discussion on, and a proposal for standardized structural nomenclature is presented. Comparisons of conventional chemical fixation and cryo-preservation is made, including the advantages of LTSEM in the preservation of delicate reproductive structures, and the benefits and failings of freeze-substitution in the preservation of mature zygospores.

Key Words: Cryo-Fixation, Freeze-substitution, High-Pressure freezing, Mucorales, Propane Jet Freezing, Ultrastructure, Zygosporangium, Zygospore.

INTRODUCTION

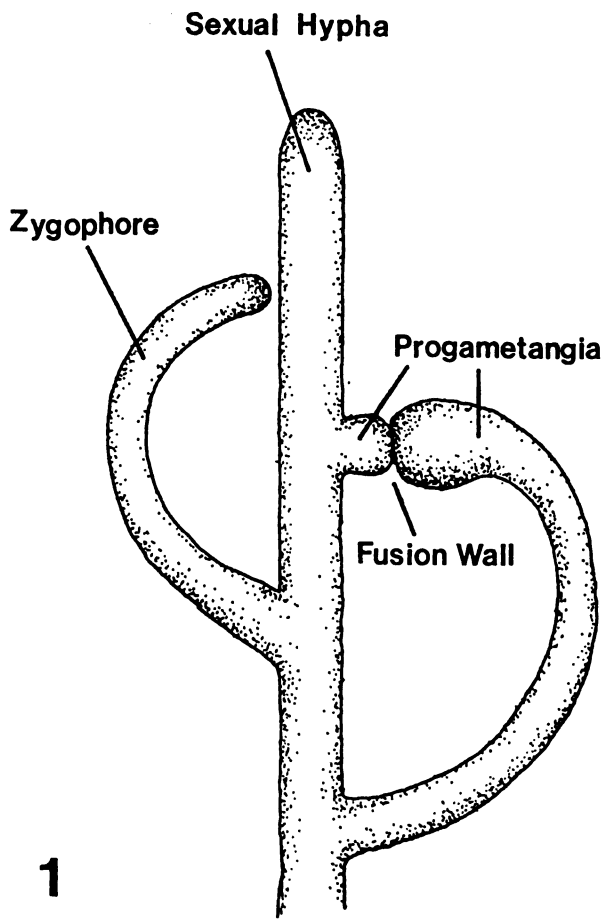
By definition the key characteristic of the class Zygomycetes is the formation of zygospores, and yet surprisingly little detail is known about this form of sexual reproduction. Numerous studies have reported on the physiology, mating type behaviors and certain limited morphological aspects such as wall structure, wart morphology, and septal structure. To date, limited studies have been published that correlate contemporary ultrastructural techniques of scanning electron microscopy, transmission electron microscopy and/or light microscopy of the developmental morphology of zygosporogenesis as a whole (Hawker and Beckett, 1971, O'Donnell, et al., 1976 & 1977,

Moss and Lichtwardt, 1977, Ansell and Young, 1983).

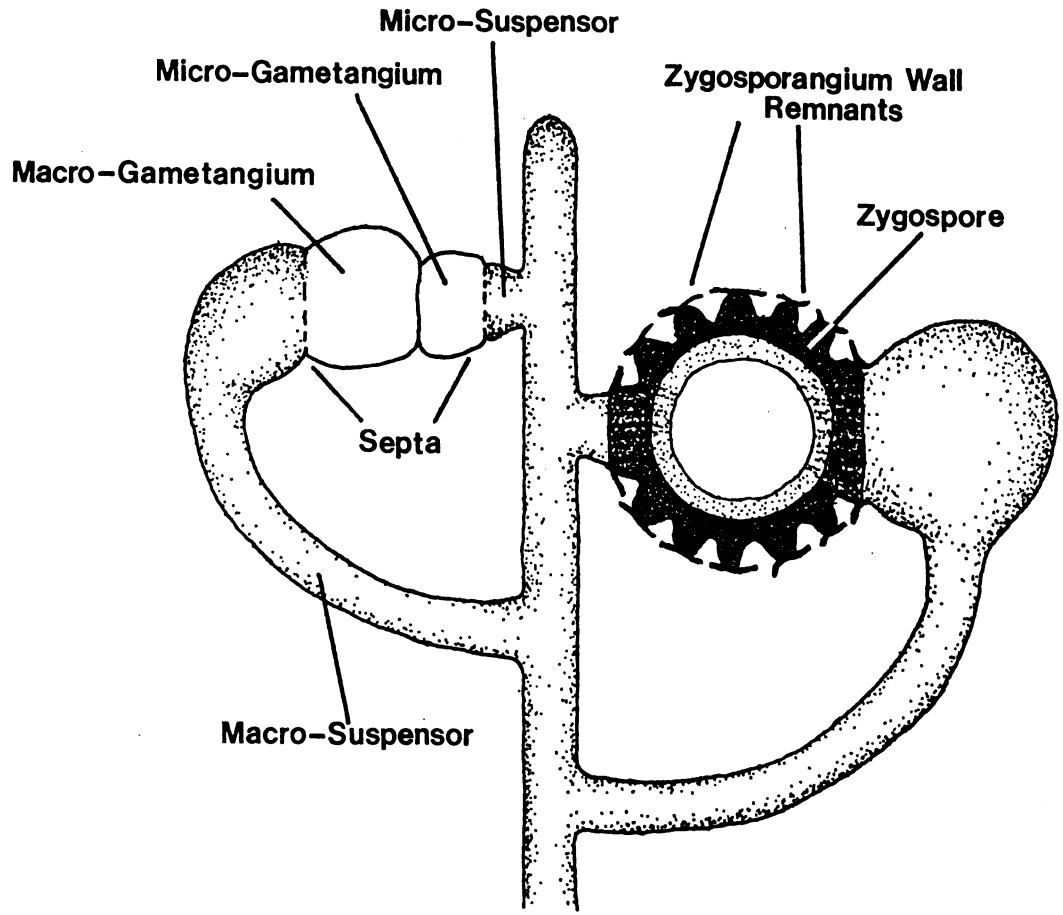
Terminology.—The terminology used throughout this paper for designating the various structures of zygosporogenesis in *Zygorhynchus heterogamus* Vuill. is detailed diagrammatically in Figure 1, and is described as follows. The term sexual apparatus was used to include all the discrete components of a single sexual reproductive complex leading to the formation of a single zygospore regardless of the developmental stage, with the exclusion of the sexual hyphae. Sexual hyphae were defined as the central, specialized aerial hyphae upon which the sexual apparati developed. Zygophores were designated as the branches arising from the sexual hypha, prior to contact and fusion, and eventual formation of the gametangia and suspensors (Blakeslee, 1904). Upon contact and adhesion of the zygophores with a sexual hypha, the zygophores were designated as progametangia (Blakeslee, 1904). After formation of the gametangial septa, the apical regions of the progametangia were defined as gametangia, and the basipetal regions as suspensors. After plasmogamy of the two gametangia the resulting structure was defined as a single zygosporangium. Zygospore was used to refer to the structure which formed de novo, within the zygosporangium. The prefixes macro- and micro- were used to distinguish the two gametangia, and their associated structures, based on size and origination point on the sexual hyphae.

The ultrastructural details of zygosporogenesis have been revealed very slowly partly because of the difficulty in preparing mature zygospores. The following study of zygosporogenesis in *Z. heterogamus* fulfills two goals: first, it is a comprehensive ultrastructural study correlating scanning and transmission electron microscopy using

Figure 1. Diagrammatic illustration of structural nomenclature used in this study of zygosporogenesis in *Z. heterogamus*.


Sexual Hypha
Zygophore
Progametangia
Fusion Wall

1


Micro-Suspensor
Micro-Gametangium
Zygosporangium Wall Remnants
Macro-Gametangium
Zygospore
Septa
Macro-Suspensor

some of the most modern techniques available. Secondly, when combined with previous research on sporangiosporogenesis (Edelmann and Klomparens, 1994), it provides an extensive study of the ultrastructural development of reproduction in *Z. heterogamus*.

Materials and Methods

Cultural Conditions.—A culture of *Zygorhynchus heterogamus* Vuillemin was obtained from E. S. Beneke and A. L. Rogers, Michigan State University. Species identification was verified using Zycha et al. (1969), Hesseltine et al. (1959), and Domsch et al. (1980). Subcultures were grown and maintained on a 1.8 % V-8 juice agar (177 ml V-8 juice, 2 g calcium carbonate, 20 g Bacto-Agar, 823 ml deionized water), incubated at 22 - 24 C, with 12 hr light/ 12 hr dark. Cultures were grown 3 - 21 days and were prepared for observation.

Electron microscopy.—Specimens for electron microscopy were prepared for each specific technique as detailed below. Scanning electron microscopy (SEM) was performed using a JEOL 35CF scanning electron microscope at 10 kV. Approximately 450 - 500 sexual apparati in the course of 12 different experimental preparations were examined with SEM. Transmission electron microscopy (TEM) was performed using a JEOL 100 CX II transmission electron microscope at 80 kV. Approximately 50 sexual apparati in the course of 27 different experimental preparations were examined with TEM.

Conventional immersion fixation. Specimens for TEM and SEM were prepared as follows: blocks of agar were cut from areas of growing colonies containing the desired stages of zygospore development. These specimens were fixed in 2.5% glutaraldehyde and 1% paraformaldehyde solution buffered with 0.05M sodium cacodylate (pH 7.2) and 1% sucrose, rinsed in buffer (6X @ 20 min), secondary fixation with buffered 1.0% osmium tetroxide, and rinsed in water (4X @ 20 min). TEM samples were en bloc stained with 0.5% uranyl acetate. All samples were dehydrated with an ethanol series (25%, 50%, 75%, 85%, 95%, 100%, 100%, 100% @ 30 min). The SEM samples were critical point dried, mounted on aluminum stubs, electrically grounded with silver paint, sputter coated with 21 nm of gold and observed. The TEM samples were infiltrated and embedded with Spurr's epoxy resin (Spurr, 1969) or Quetol/nadic methyl anhydride (Kushida, 1975), ultrathin sectioned, and stained with uranyl acetate and Reynold's (1963) lead citrate (See Edelmann and Klomparens, 1994).

Low temperature SEM (LTSEM). Samples were prepared using the procedures detailed in appendix A according to the following general protocol. Specimens were cut from the agar plates, 7 mm x 20 mm x 1 mm, mounted on to an Emscope SP 2000 cryostage, with Tissue-Tek II O.C.T. cryo-embedding medium (Lab-Tek Products, Miles Laboratories, Inc.) mixed with powdered graphite, and plunge frozen in a liquid nitrogen slush. Surface water crystals were viewed and sublimated away (10-15 min) at approximately -60 C in a JEOL 35 CF scanning electron microscope with the Emscope cryostage attachment. Specimens were then re-cooled and sputter coated with 40 nm of gold at -190 C, and observed at -80 to -90 C.

Freeze-Substitution for TEM. Specimens were selected and teased off the agar plates using a macroscope and dissecting needles. Desired samples were ultrarapidly frozen with either an RMC MF 7200 propane jet freezer or a Balzer's HPM 010 high pressure freezer. Frozen samples were freeze substituted with the following general protocol adapted from Czymmek (Czymmek and Klomparens, 1992), and specifically detailed in appendix A. Specimens were freeze-substituted with 1-2% osmium tetroxide in acetone at approx. -80 C for 3 - 4 days. Samples were then warmed to room temperature, rinsed with 100% acetone, embedded and sectioned as above.

RESULTS

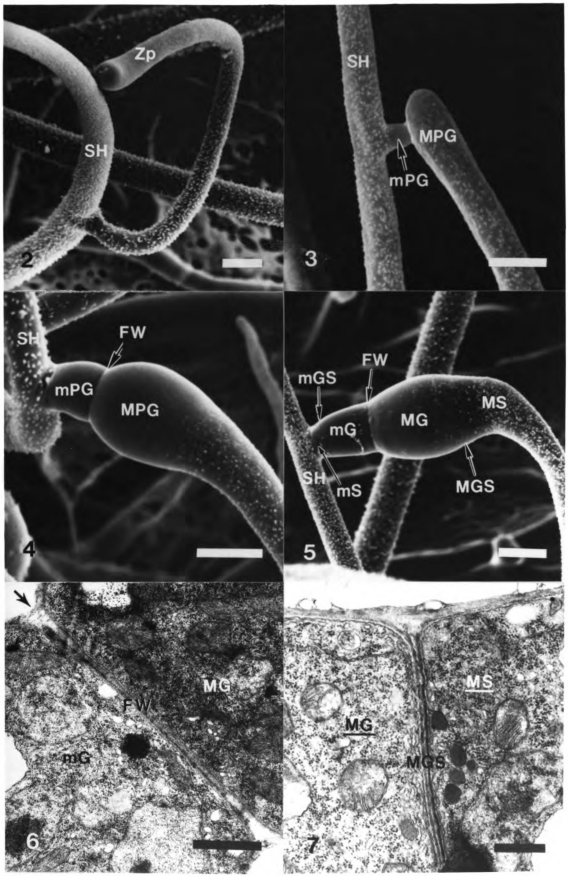
Sexual hyphae arose from below the agar surface as specialized aerial hyphae, initially indistinguishable from vegetative hyphae, 5 - 8 μm in diameter at first, enlarging up to 8 - 10 μm with maturation. Sexual apparati were never observed to develop amongst lateral hyphae growing along the agar surfaces. Upon reaching a height of 80 - 100 μm, lateral branches, the macro-zygophores, emerged subapically from the sexual hyphae. The sexual hyphae typically continued elongation ranging up to approximately 150 μm or more. Both the sexual hyphae and the macro-zygophores characteristically exhibited a greater degree of curvature than vegetative hyphae or sporangiophores which were typically straight. All hyphal surfaces, except actively growing regions, such as hyphal apices and zygosporangia, were covered with crystalline-like echinulations which enlarged with maturation of the hypha. These echinulations were observed with both conventional SEM and LTSEM, and were determined to be true features and not

preparation artifacts. It was noted that these spines appeared very sharply pointed in LTSEM but rounded or eroded after conventional fixation.

The zygophore elongated up to 80 - 100 μm until contacting either the same or a different sexual hypha from which it initially arose (Fig. 2). Contact between a specific zygophore and a sexual hypha was apparently random. Within regions of low aerial hyphal density, i.e. colony peripheries, the zygophores typically contacted the same sexual hypha from which they arose. Within denser, older growth regions, contact was often made with a sexual hypha different than that from which the zygophore arose. When contact was made with the parent sexual hypha, contact was always made further apically along the sexual hypha from the point of origination of the zygophore. The point of contact on the sexual hypha was always observed at least 10 μm from the apex and usually at a greater distance. The point of contact on the zygophore ranged from apically to 10 μm subapically, rarely further away. The number of sexual apparati per sexual hyphae typically ranged from two to five. The initial point of contact was extremely fragile and typically was dislodged during conventional fixation procedures, and was only observed with LTSEM.

Shortly after initial contact between the zygophore and the sexual hypha, the micro-progametangium arose from the sexual hypha at the point of contact (Fig. 3). Due to the rarity of observation, it was surmised that this stage was extremely short in duration. After the initial formation of the micro-progametangium, both the micro- and the macro-progametangia ballooned outward (Fig. 4). The zygophore/macro-suspensor

Figs. 2-7. Initial formation of sexual apparatus in *Z. heterogamus*. 2. LTSEM of initial contact between zygophore (Zp) and sexual hypha (SH);Bar = 10μm. 3. SEM of initial formation of micro-progametangium (mPG). At this stage the zygophore was redesignated as the macro-progametangium (MPG); bar = 10μm. 4. LTSEM of advanced development of the progametangia. The zone of contact between the two progametangia had enlarged and was firmly established as the fusion wall (FW). The apices of both progametangia were noted to be free of crystalline-like echinulations; bar = 10μm. 5. LTSEM of the transition from progametangia to discrete gametangia. With the formation of internal gametangial septa, eight distinct structures were designated: the sexual hypha, SH; the micro-suspensor, mS; the micro-gametangial septum, mGS; the micro-gametangium, mG; the fusion wall, FW; the macro-gametangium, MG; the macro-gametangial septum, MGS; and the macro-suspensor, MS. The relative size alone was the only significant difference between those structures designated micro and macro; bar = 10μm. 6. TEM of the fusion wall. The fusion wall (FW) appeared to be contiguous with the exterior walls of the micro-gametangium(mG) and the macro-gametangium (MG), with no discernable structural differences. The edges of the fusion wall where it divided to form the gametangial walls appeared to be tattered (arrow), indicative of being stressed by the expansion of the gametangia; bar = 1μm. 7. TEM of the macro-gametangial septum. The micro-gametangial septum and the macro-gametangial septum (MGS) appeared identical except for size. The gametangial septa consisted of two electron-translucent layers separated by an electron-transparent layer. With maturation an electron-opaque layer was laid down on the suspensor (MS) side of the septa; bar = 0.5μm.


Zp
SH
2
SH
MPG
mPG
3
SH
FW
mPG
MPG
4
mGS
FW
MS
mG
MG
SH
mS
MGS
5
MG
FW
mG
6
MS
MG
MGS
7

appeared to increase in diameter with maturation, ranging up to 12 - 14 μm at full maturation (as determined by SEM and LTSEM). The macro-progametangium increased in volume at a greater rate than the micro-progametangium, and maintained a volume three to four times that of the micro-progametangium throughout maturation. The surface of both progametangia remained relatively free of the crystalline-like echinulations, although a few progametangia were observed to have a few echinulations. At this stage in development, the region of contact between the two progametangia had increased in area and was noted to be firmly established and was not dislodgable by the physical forces associated with specimen preparation. With the establishment of this adhesion between the two progametangia, the region of contact was designated as the fusion wall (Fig. 4, FW).

Following the initial swelling of the two progametangia, the formation of internal gametangial septa were identifiable with SEM observation (Fig. 5, GS). With the formation of these septa, eight distinct structures were designated, as shown in Figure 5: the sexual hypha, SH; the micro-suspensor, mS; the micro-gametangial septum, mGS; the micro-gametangium, mG; the fusion wall, FW; the macro-gametangium, MG; the macro-gametangial septum, MGS; and the macro-suspensor, MS. The relative size alone was the only significant difference between those structures designated micro and macro. Though the actual sizes of individual sexual apparati differed over a wide range, particularly when correlated with colony age, the relative size ratios between specific components remained fairly consistent. It was noted, however, that the micro-suspensor was often so small as to be unobservable in the SEM.

Sectioning through this developmental stage revealed the continuous nature of the shared fusion wall (Fig. 6). No distinction was observed between the portion of the fusion wall provided by the micro-gametangium and the macro-gametangium. The fusion wall appeared to be contiguous with the exterior walls of the gametangia, with no structural differences discernable with TEM. It was noted that as the gametangia ballooned outward, the edges of the fusion wall where it divided to form the gametangial walls appeared to be tattered, indicative of being stressed by the expansion of the gametangia (Fig. 6, arrow). No significant difference between the cytoplasmic contents of the two gametangia was observable. At this stage, both gametangia contained typical zygomycetous cytoplasmic structures with the exception of an increased concentration of nuclei (relative to vegetative hyphae), very few lipid bodies, and very small amounts of endoplasmic reticulum.

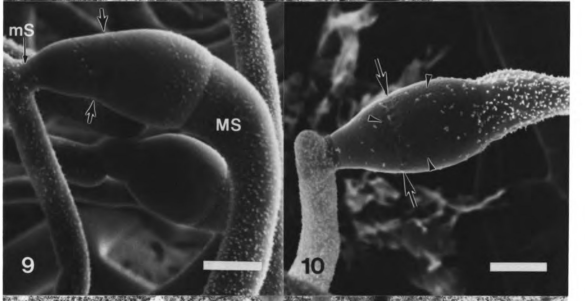
The micro- and the macro-gametangial septa were noted to be identical in structure except that the macro-gametangial septa were approximately twice the diameter of the micro-gametangial septa and therefore had greater surface area. Both gametangial septa joined to the exterior wall in a 'T' shaped junction, and marked the division between the exterior wall of the gametangia and the suspensors (Fig. 7). Shortly after the initial formation of the gametangial septa there were no structural differences between the gametangial walls and the suspensor walls. Figure 8 shows a series of micrographs revealing the development of the gametangial septa from its initial formation to just prior to complete zygospore maturation.

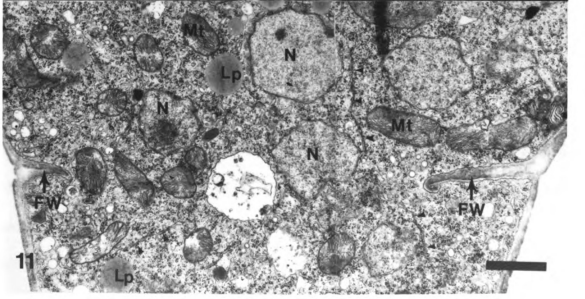
From the earliest stages of the development of the gametangial septa, numerous plasmodesmata were observed to perforate the septa (Figs. 7, 8a - e). As the gametangial septa developed these plasmodesmata were always observable, and apparently elongated as the septa thickened. The gametangial septa appeared structurally different from the exterior walls (Figs. 7, 8 a - e). Proceeding from the gametangium to the suspensor, the gametangial septa appeared to be composed of a light electron-opaque layer, an electron transparent layer, a second light electron-opaque layer, and a dark electron-opaque layer (Figs. 7, 8 c). At the earliest stages of development, only two electron-opaque layers with a central electron-transparent layer were observable (Fig. 8 a, b). At the latest stages of development, a darker electron-opaque layer was distinguishable on the interior of the gametangial side of the septa and was contiguous with zygospore wall layers in later developmental stages (Fig. 8 c, d, e). The darkest electron-opaque layer on the suspensor side extended beyond the gametangial septum itself and along the interior of the suspensor for 2 - 3 μm at later developmental stages.

Following formation of the gametangial septa, the next developmental stage was marked by the removal of the fusion wall and plasmogamy. Fusion wall removal was distinguishable in SEM observation by a transition from a distinct union of the two discrete progametangia and gametangia (Figs. 4, 5) to a slight depression indicative of the formerly separate gametangia (Fig. 9, arrows). The removal of the fusion wall was designated as a transition from the two gametangia to a single zygosporangium. Concurrent with removal of the fusion wall was a continued enlargement of the former gametangial regions, and the rounding of the zygosporangium between the two

Figs. 8-11. Gametangial septa and fusion wall. 8 a-e. TEM of developmental maturation of the gametangial septa. In all figures, a-e, the zygosporangium/zygospore is on the left and the suspensor is on the right. No differences were noted between micro- and macro-gametangial septa. a. Earliest observed stage of gametangial septum formation, with the presence of plasmodesmata (arrowheads). b. Thickening of the septa. c. Appearance of the electron opaque layer on the interior of the suspensor side of the septa (P). d. Elongation of the plasmodesmata with increased thickening of the septa (freeze-substituted). e. The gametangial septal region of a fully matured zygospore, with the presence of plasmodesmata (arrowheads) penetrating the outer most zygospore wall (*; freeze-substituted). Standard bar = 0.2μm for a-c; 1μm for d; 0.5μm for e. 9. LTSEM of zygosporangium formation: fusion wall removal and gametangial plasmogamy. Prior to completion of fusion wall removal a steady decrease in the distinguishablity of the junction of the two gametangia, the fusion wall itself (arrows), was observable; bar = 10μm. 10. SEM of initial development of the zygospore warty layer internal to the zygosporangium wall. This developing warty layer of the zygospore was observed externally by the appearance of shallow indentations (arrowheads). A thin line of scarring (arrows) marked the previous position of the fusion wall; bar = 10μm. 11. TEM of fusion wall removal and gametangial plasmogamy. No specific internal features were noted associated with fusion wall removal. At this stage the cytoplasm of the zygosporangium generally matched that of vegetative hyphae with the inclusion of numerous nuclei (N), mitochondria (Mt), few lipids (Lp), and endoplasmic reticulum (arrowheads); bar = 1μm.


8a
b
c
d
e
P

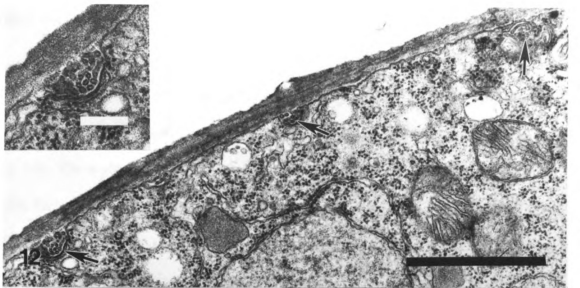

mS
MS
9
10


Mt
N
Lp
N
Mt
N
FW
FW
11
Lp

gametangial septa. At this stage it was noted that the zygosporangium was markedly larger than the suspensors. The zygosporangium continued to enlarge, forming a smooth exterior with only a fine line of scarring marking the former location of the fusion wall (Fig. 10, arrows). The surface of the zygosporangium was covered with numerous shallow depressions indicating the interior location of developing wart initials (Fig. 10, arrow heads). These shallow depressions initially appeared closest to the former fusion wall location, on the surface of the former macro-gametangial region, and advanced towards both gametangial septa with maturation.

Observations of the interior of the zygosporangium revealed a progressive removal of the fusion wall beginning from the center and proceeding towards the exterior walls (Fig. 11). From a point shortly after fusion wall removal and throughout later development, multiple observations revealed a steady increase in the number of lipid bodies present in the zygosporangium. Concurrent with the removal of the fusion wall was the appearance of regularly-spaced membrane-containing or vesiculate bodies appressed to the interior of the zygosporangium wall external to the plasma membrane (Fig. 12). These vesiculate bodies initially appeared close to the remnants of the fusion wall within the former macro-gametangium. Concurrent with the completion of fusion wall removal, these vesiculate bodies appeared generally evenly spaced throughout the entire zygosporangium (Fig. 12). Shortly after their initial appearance these vesiculate bodies were no longer observed, rather disks of electron-opaque material were noted closely appressed to the zygosporangium wall, external to the plasma membrane, exhibiting approximately the same spacing as the former vesiculate bodies (Fig. 13).

Figs. 12-16. Early development of zygospore. 12. TEM of wart initiating centers. Concurrent with the last stages of fusion wall removal, numerous regularly spaced vesiculate bodies (arrows) appeared between the zygosporangium wall and its plasma membrane; bar = 1μm, inset bar = 0.2μm. 13. TEM of wart initials. The wart initials (WI) appeared as regularly spaced electron-opaque disks appressed to the zygosporangium wall external to the plasma membrane; bar= 1μm. 14. SEM of the developing wart layer and enlargement of macro-suspensor. External observation of early wart layer development revealed the warts as hemispherical bulges of the zygosporangium wall, with some rupture of the zygosporangium wall, initially appearing on the former macro-gametangial side of the fusion wall scar (arrowhead); bar = 10μm. 15. SEM of the advancement of the rupture of the developing zygospore wall through the zygosporangium wall. The developing wart layer progressed from the location of the former fusion wall (arrow heads) towards the gametangial septa; bar = 10μm. 16. TEM of specialized stacked endoplasmic reticulum within the developing zygosporangium/zygospore. Concurrent with fusion wall removal multiple-layered, endoplasmic reticula were observed within the regions closest to both gametangial septa within the zygosporangium, and rarely within the central regions; bar = 1μm.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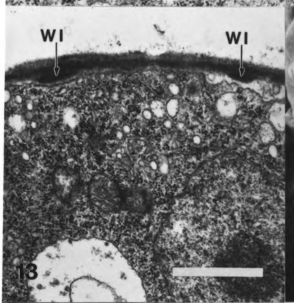
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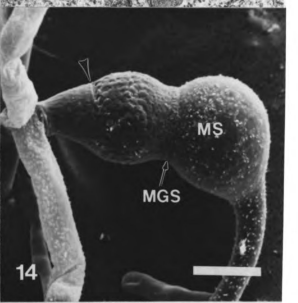
WI
13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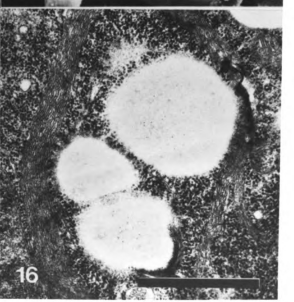
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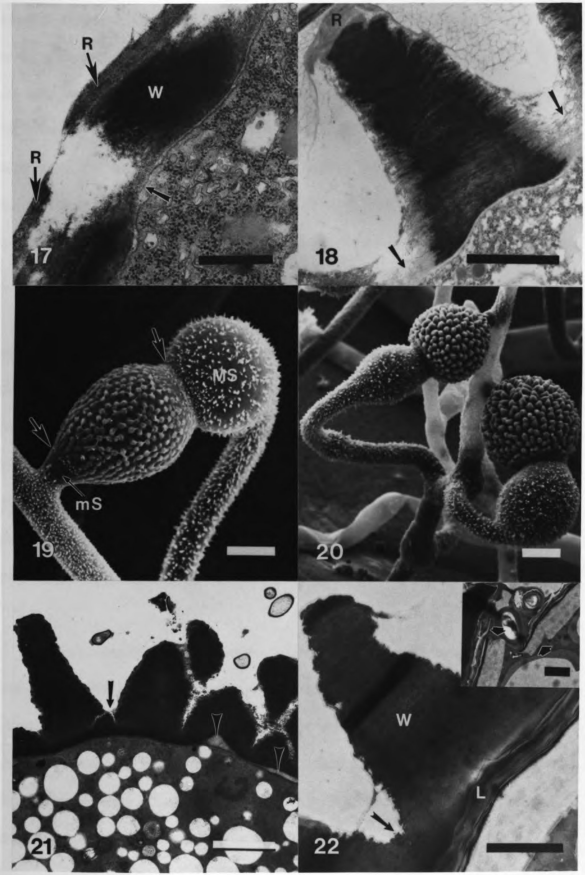
These electron-opaque disks were designated as wart initials.

External observation of the zygosporangia, shortly after internal formation of the wart initials, revealed the developing warts erumpant through the zygosporangium wall (Fig. 14). The rupture of the warts was first noted on the former macro-gametangial side of the fusion wall scar. Following their original appearance, the rupture of the warts proceeded from the fusion wall scar towards the gametangial septa (Fig. 15). Remnants of the zygosporangium wall were commonly noted adhering to the apices of the warts (Fig. 15, arrows). Concurrent with the external appearance of the warts, the macro-suspensors continued to enlarge immediately behind the macro-gametangial septa, resulting in the formation of a globose structure, with a diameter exceeding that of the zygosporangium (Fig. 14, 15). This swelling seen with the macro-suspensors was not observed to occur with the micro-suspensors. Within the developing zygosporangium in regions near to the gametangial septa, unique, multi-layered membranous structures were observed (Fig. 16). These structures appeared to be composed of closely appressed, sheet-like expanses of 6 - 20 membrane layers, similar to ER in appearance.

Sections through zygosporangia at this stage revealed that the maturing warts had formed a contiguous layer between the zygosporangium wall and the plasma membrane (Fig. 17, arrow). The formation of this wart layer was designated as marking the transition from zygosporangium to zygospore. The structure of the warts themselves appeared to be a stacked series of electron-opaque disks, increasing in diameter from the apex of the warts to their bases, resulting in individual warts having an elongated, conical

Figs. 17-22. Maturation of zygospore wall layers. 17. TEM of wart layer rupture of zygosporangium wall. The warts (W) of the outer layer of the zygospore developed as a stacked series of electron opaque disks. Enlargement of these warts apparently resulted in the rupture of the external zygosporangium wall which appeared as remnants (R) adherent to the wart apices. Coinciding with rupture of the zygosporangium wall, thin regions of wall material were observed connecting the bases of individual warts (arrows); bar = 0.5μm. 18. TEM of the advanced development of the wart layer. With maturation, the individual warts obtained a length of 2.5-3μm. The stacked electron opaque layers were still clearly observable, as well as the thin regions of wall material connecting the individual warts (arrows). Remnants (R) of the zygosporangium wall were commonly observed attached to the wart apices. This specimen was freeze-substituted; bar = 1μm. 19. LTSEM of a nearly mature zygospore. The outer wart layer of the zygospore formed a continuous wall between both gametangial septa (arrows) with the only notable remnants of the zygosporangium in the regions closest to the gametangial septa. The micro-suspensor (mS) is clearly shown to be free of the crystalline-like spines observable on the macro-suspensor (MS); bar = 10μm. 20. SEM of two fully mature sexual apparati on a single sexual hypha. At maturity the zygospores obtained a nearly spherical shape 18-25μm in diameter. Arrangement of multiple sexual apparati on a single sexual hypha was observed to be apparently random with the exception that the micro-suspensors were always noted to be acropetal to the macro-suspensors when both originated from the same sexual hypha; bar = 10μm. 21. TEM of a nearly mature zygospore wart layer. The material of the outer wart layer of the zygospore wall increased in density particularly in the regions between individual warts (arrow). At this stage initial development of the inner most electron translucent zygospore wall layer was observed (arrowheads). Freeze-substituted specimen; bar = 1μm. 22. TEM of a fully mature zygospore wall. The zygospore wall consisted of three major layers: (I) an outer electron opaque warty layer (W), approximately 0.6μm thick between the warts (arrow), (II) a middle laminate layer approximately 0.3μm thick (L), and (III) an internal electron translucent layer 0.75-0.8μm thick. The inset contains a zygospore wall fractured during specimen preparation. The arrowheads show the fracture of the inner wall layer confirming that the wall layer is not an artifact of preparation. Freeze-substituted specimen; bars = 1μm.

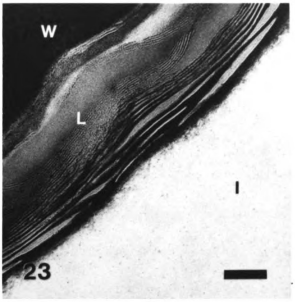

R
W
R
17
R
18
MS
mS
19
20
21
W
L
22

shape at maturity (Fig. 17, 18). The edges of the electron-opaque disks, comprising the warts, appeared extremely ragged, and without a solidly defined profile (Fig. 16, 17). The electron density differences shown within the warts in Figure 18 were believed to be artifacts of preparation and not attributable to compositional differences. The initial rupture of the warts through the zygosporangium wall occurred prior to a stable connection between individual warts. The initial contiguous wart layer was fragile and often ruptured during TEM embedment procedures, presumably due to osmotic pressures.

Coinciding with extension and maturation of the wart layer, the zygospores enlarged and rounded up, forming a globose to spherical shape at maturity (Fig. 20). The diameter of the fully mature zygospores generally exceeded that of the macro-suspensors by approximately 25 - 30%, ranging from 18 - 25 μm. Figure 20 demonstrates an example of two sexual apparati on a single sexual hypha and the diversity of sizes of sexual apparati. No pattern of arrangement of the zygospores on the sexual hypha was determined, with exception of the micro-/macro-suspensor arrangement when occurring on a single sexual hypha as mentioned previously.

Upon completion of the outer warty layer of the zygospore, inner wall layers were observed in the final developmental stages of zygospore maturation (Fig. 21). At maturation, the zygospore wall appeared to be composed of three structurally distinct regions: an electron-opaque outer warty layer, 0.6μm thick between warts (Fig. 22 & 23, W); an intermediate 0.3μm thick, laminate layer (Fig. 22 & 23, L); and an inner, 0.75-0.8μm, electron-transparent layer (Fig. 22 & 23, I). These separate wall layers appeared

Fig. 23. TEM of a fully mature zygospore wall. The individual warty (W), laminate (L), and inner (I) layers appeared to be tightly adherent to each other and no separation of the wall layers was observed. The laminate layer was composed of individual electron-transparent sheets of material embedded in a matrix of electron-opaque material. The inner layer appeared to be composed of homogenous electron-transparent material; bar = 0.1μm.


W
L
I
23

to be tightly adherent or fused to each other and no separations of the wall layers, due to specimen preparation, i.e. osmotic pressures, etc., were observed (Fig. 23). The laminate layer was composed of individual electron-transparent sheets of material embedded in a matrix of electron-opaque material. The inner layer appeared to be composed of homogenous electron-transparent material. The compositional nature of each individual zygospore wall layer was not determined in this study.

DISCUSSION

The prefixes micro- and macro- are suggested as preferable to the prejudicial terms 'male' and 'female' used by early workers (Gruber, 1912, Atkinson, 1912, and Green, 1927). Blakeslee (1904) establishes clear arguments against the use of the terms 'male' and 'female' for both heterothallic strains of zygosporic cultures (for which he suggested the use of + and -), and the "zygophoric branches" of homothallic species. Blakeslee (1904) specifically argued for the use of smaller and larger to correspondingly replace the terms 'male' and 'female' in describing gametangia of *Z. heterogamus*. I propose that the more formal prefixes micro- and macro- be substituted for 'smaller' and 'larger' due to (1) their better applicability to syntactical modification of the terms progametangium, gametangium and suspensor, and (2) their specificity in distinguishing the different developmental pathways of each.

Zygophore and Sexual Hypha.—I suggest the terminology sexual hypha in referring to the central hypha supporting the sexual apparati because it is a distinct aerial hypha upon which all the other structures associated with zygosporogenesis develop. Blakeslee (1904) defined the term zygophores as the initial hyphae which arise, are chemotactically attracted to each other, contact, fuse, and transform into the progametangia. In heterothallic species these zygophoric hypha arise separate and distinct from each other, and are justifiably called zygophores.

In some homothallic fungi, i.e. *Rhizopus sexualis* (Smith) Callen, these zygophores arise due to dichotomous branching of a single aerial hypha (Hawker and Beckett, 1971). These branches are easily distinguishable from the originating hyphal trunk, do not re-contact the originating hyphal trunk, generally appear very similar to each other, and transform into progametangia. For these reasons, the use of the term zygophores is again clear and justifiable. However, no specialized term has been used in the literature to specifically define this "originating hyphal trunk" as I have described it.

In homothallic species of *Mucor*, Blakeslee (1904) described that "the zygophores generally arise from comparatively distant parts of the mycelium, and ... yet so far as has been observed, zygospores are never formed between branches of a single aerial hypha." Blakeslee also stated that this was in "direct contrast" to *Zygorhynchus* wherein the "two zygophoric branches" usually do originate from a single aerial hypha. Furthermore Blakeslee (1904 & 1913) describes very different developmental pathways for these two heterogenous zygophoric branches, but does not suggest a specific nomenclatural

difference in terminology for these structures.

In the case of *Z. heterogamus* and other members of the genus, i.e. *Z. exponens* Burgeff, *Z. moelleri* Vuill., there is only one hyphal branch which arises from, and is distinguishable from, the original aerial hypha or hyphal trunk as described above. This zygophoric branch does eventually transform into one of the progametangia. The second "mating hypha", or zygophore, which develops or transforms into the second progametangium, and later gametangium and suspensor in other members of the zygomycetes does not form in *Z. heterogamus*. The "originating hyphal trunk" itself gives rise, de novo, to the second progametangium. This second progametangium, however, is formed only after contact by a zygophore and does not exhibit chemotactic growth. I propose that the term sexual hypha be used to describe this "originating hyphal trunk", as this distinguishes it from the zygophore. Further, I support Blakeslee's definition of zygophore as that structure which directly transforms into a progametangium.

Some researchers have designated the sexual hypha as the second zygophore in *Zygorhynchus* (Blakeslee, 1904 & 1913; Hesseltine, et al., 1959; O'Donnell, et al., 1978a), describing it as uncurving, smaller, diminutive, slender, and/or weaker. In each case their designation of the sexual hypha as the second zygophore is based on the observation of the formation of a septum either directly above, or at some distance acropetally from, the first or macro-zygophore branching, which thus distinguishes two morphologically different zygophores. My research suggests that this definition is inadequate because it only holds true for terminal sexual apparati, and does not provide a good descriptor for

the more typical case where multiple sexual apparati are found on a single aerial hypha (Fig. 20). In all other instances the zygophore is a terminal structure and does not branch to form multiple sexual apparati. The sexual hypha as I define it is analogous to an ascogenous hypha in its formation of multiple sexual apparati.

Zygosporangium and Zygospore.—The terms zygosporangium and zygospore have been used seemingly indiscriminately, even interchangeably, in the literature for over a century in referring to a variety of different structures. In fact, neither term is included in Ainsworth & Bisby's Dictionary of the Fungi (Hawksworth, et al., 1983)! Various authors have used a variety of other terms such as prozygospore (O'Donnell, et al., 1977), exospore, endospore (Benjamin, 1959, Hesseltine, et al., 1959), perispore, mesospore and fusion cell to describe either the same or differing aspects, layers and structures of the later stages of zygosporogenesis. I propose the adoption of a standardization of the terms, as originally used by Blakeslee (1904), and others (O'Donnell, et al., 1978b), in the following manner. Zygosporangium refers to the entire initial structure, between the two gametangial septa, which arises from the fusion of the two gametangia following removal of the fusion wall {Blakeslee actually used the term zygote and zygospore. Following current mycological conventions I have changed zygote to zygosporangium, similar to the changes proposed by Dangeard (1906a) of Blakeslee's (1904) progametes and gametes to progametangium and gametangium, respectively, which are now generally accepted}. The external wall of this zygosporangium is contiguous with that of the suspensors as described here with *Z. heterogamus*, and was originally the same wall which was the external wall of the progametangia. Further examples of this have been reported in *R.*

sexualis (Hawker and Gooday, 1967, 1968, and Hawker and Beckett, 1971), *Phycomyces blakesleeanus* (Kunze) Burgeff (O'Donnell et al., 1976), *P. nitens* Fries (O'Donnell et al., 1978b), and *Mucor mucedo* L.:Fr. (Edelmann and Hammill, unpublished).

The zygospore refers to the entire structure which forms de novo, internal to the zygosporangial wall. Regardless of the number of wall layers which may be distinguishable by various authors in various species, the zygospore shall describe the entire collection of layers which form internal to the zygosporangium wall. In most cases of zygosporogenesis and azygosporogenesis, the initial, outer most wall layer of the zygospore consists of a heavily pigmented, often verrucosely ornamented layer which forms de novo internal to the zygosporangium, as reported here with *Z. heterogamus*. Additionally, this de novo wall formation internal to the zygosporangium has been reported in *R. sexualis* (Hawker and Gooday, 1967, 1968, and Hawker and Beckett, 1971), *P. blakesleeanus* (O'Donnell et al., 1976), *P. nitens* (O'Donnell et al., 1978b), and in other species by Blakeslee (1904), Vuillemin (1904), Dangeard (1906b), and Ling-young (1930). The use of the terms zygosporangium and zygospore as described here are directly analogous to the asexual structures sporangium and sporangiospore, particularly as found in those members of the Zygomycetes producing unispored sporangia, *Mycotypha* Fenner and *Cunninghamella* Matruchot, as described by Khan and Talbot (1975).

The term fusion wall is preferable to the term fusion septa, as used by some authors (O'Donnell, et al.,1977, 1978a, and 1978b) due to the origin of the wall. Initially,

a fusion wall is a portion or region of the exterior wall of the zygophore, progametangia and gametangia. In contrast, septa are internal partitions formed de novo within an existing walled structure, i.e. hyphae, spore, or conidium, subdividing that structure. The fusion wall, as in *Z. heterogamus*, is an external wall which only becomes "internalized" due to the appression of the two progametangia/gametangia, thereby forming the interface between these two discrete structures. The fusion wall's role developmentally is thus directly opposite that of a septum. With development, the fusion wall is removed, allowing for the union of two separate structures, the gametangia, and the formation of a single structure, the zygosporangium.

Initiation of plasmogamy of the two gametangia in this study of *Z. heterogamus* is described as occurring with fusion wall removal, and specifically not 'fusion wall dissolution' as is generally described by most authors. Fusion wall dissolution in *R. sexualis* (Hawker and Gooday, 1969; Hawker and Beckett, 1971), *P. blakesleeanus* (Sassen, 1962), *P. nitens* (O'Donnell, et al., 1978b), and *Gilbertella persicaria* (Eddy) Hesseltine (O'Donnell, et al., 1977) is reported to have occurred through the actions of various vesicles, and microbodies associated with the fusion wall and through a putatively enzymatic degradation. In *Z. heterogamus* no observations of similar structures associated with fusion wall removal were made, and no specific proposals for the actual methods of fusion wall removal, such as an enzymatic centripetal degradation or fusion wall dissolution, can be justified at this time. Observations made of the junction between the two gametangia (Figs. 5, 6, 10 & 11) indicate that this peripheral area of the fusion wall

is under stress, presumably due to the expansion of the gametangia and zygosporangium, prior to and post plasmogamy respectively. Similar indications of physical stress were also reported in *Mycotypha africana* Novak & Backus (Chapter 2), with fully hydrated specimens (discounting them as an artifact of dehydration). O'Donnell, et al. (1978a), reported similar observations in *P. blakesleeanus*. Such observations support the notion that in addition to putative, internal enzymatic activity, the fusion wall may actually undergo an evagination, similar to that proposed for *Muc. mucedo* (Hammill, 1987).

Associated with the rupture of the zygosporangium wall by the developing warts was an apparent slime covering as observed with LTSEM in both *Z. heterogamus* (Fig. 19) and *Myc. africana* (Chapter 2). The observation of this slime covering seems to correlate with the gelatinized 'inner primary wall layer' reported in *P. nitens* (O'Donnell, 1978b), and in *R. sexualis* (Hawker and Beckett, 1971). In both *P. nitens* and *R. sexualis* this gelatinized wall layer was observed with TEM, occurring between the ruptured outer zygosporangium wall and the developing outer wart layer of the zygospore. O'Donnell, et al. (1978b), and Hawker and Beckett (1971) proposed this layer as arising from a gelatinization of the secondary wall layer of the zygosporangium. The descriptive differences between 'slime' and 'gelatinized' may be due to the nature of the methods of observation, fully hydrated and unfixed in the former, and fixed, dehydrated, and embedded in the latter, or simply a matter of semantics. I propose that the inner wall layer of the zygosporangium is softened or degraded by enzymes allowing for the expansion of the developing warts of the zygospore through the zygosporangium wall, with the end products of zygosporangial wall degradation appearing as a slimy film, or

gelatinous layer.

Blakeslee (1904) noted a distinct positioning of the micro-progametangium forming further apically on the sexual hypha from the branching point of the macro-progametangium in *Z. heterogamus*. This observation was confirmed in this current study. Since the micro-gametangium does not form until contact by the zygophore on the sexual hypha, this specific positioning of the micro-/macro-progametangia is likely due to some type of negative-geotropism of the zygophore, similar to that of the sexual hypha and the sporangiophores.

The cylindrical developmental nature of the warts as reported here in *Z. heterogamus*, was very different from the conical or "flower pot" development reported in *R. sexualis* (Hawker & Gooday, 1968, and Hawker & Beckett 1971), *Muc. mucedo* (Hammill, 1987), *Muc. hiemalis* Wehmer, and *Syzygites megalocarpus* (Ehrenberg) Fries, [= *Sporodinia grandis* (Link) Wallroth],(Ling-Young, 1931). My study of *Z. heterogamus*, reveals that the warts develop as stacks of electron-opaque disks, forming stacked columns of material. O'Donnell, et al. (1977), reports that the warts of *G. persicaria* develop as electron-opaque, stratified deposits. However his stratified layers run parallel to the long axis of the warts, and very definitely appear to be sectioning artifacts. In *P. blakesleeanus*, O'Donnell, et al. (1976), reports that the warts appear as stacks of fibrillar material, with the stacks perpendicular to the long axis of the warts. In this case it would appear that the warts of *Z. heterogamus* and *P. blakesleeanus* develop very similarly. Although, O'Donnell, et al. (1978b) report on zygosporogenesis

in *P. nitens*, it is unclear as to how wart development occurs in this species.

The structure of nearly mature and fully mature zygospore walls has been reported with mechanically broken and cryo-fractured zygospores prepared for conventional SEM in *R. sexualis* (Hawker & Beckett, 1971), *P. blakesleeanus* (O'Donnell, et al., 1976), *G. persicaria* (O'Donnell, et al. 1977), *P. nitens* (O'Donnell, et al., 1978b), and members of the genus *Zygorhynchus* (O'Donnell, et al., 1978a), although it is hard to distinguish the correct relationship of the various wall layers due to their crushed nature, poor fixation and significant dehydration artifacts. These authors and others agree that concurrent with development of the warty layer of the zygospore, penetration of fixatives and embedding resin through the wall is significantly inhibited, and very poor material is available for TEM observation following ultrathin sectioning. The use of freeze-substitution in my research has allowed the presentation of the first ultrastructurally detailed TEM observation of zygospore wall development following the formation of a contiguous wart layer.

It is clear that in *Z. heterogamus* two subsequent layers form underneath the exterior wart layer. The intermediate layer is an apparently laminate layer composed of numerous discrete sub-layers. Observations of this laminate layer indicate that the individual electron-transparent sub-layers are not continuous around the entire circumference of the zygospore but rather that these sub-layers appear individually to cover a smaller portion of the roughly spherical surface area of the zygospore and are embedded in a matrix of electron-opaque material. The structure of this laminate layer

has the appearance very similar to that of engineered laminate wooden construction material, and it is important to note that such laminate structure is extremely strong. The inner most layer is a fairly thick, electron-transparent layer. No information regarding the chemical composition of these layers can be provided at this time. Future studies will hopefully provide this information as well as show the roles of these wall layers during zygospore germination.

It is possible that *Z. heterogamus* is pseudo-homothallic. Edelmann and Klomparens (1994) reported that the sporangiospores of *Z. heterogamus* were almost exclusively binucleate. It is possible that these binucleate sporangiospores are heterokaryotic for mating type. Germination and mycelial growth from such heterokaryotic sporangiospores would yield a thallus which is composed of heterokaryotic mycelium and apparently homothallic due to the presence of both mating type nuclei. Such an occurrence would be very similar to that described in *P. blakesleeanus* by Blakeslee (1906), Burgeff (1912, 1915, and 1925) and in *Pilobolus crystallinus* (Tode) Fries as described by Krafeyzk (1935). These authors report that during zygospore germination and germsporangium development incomplete 'sex' (mating type) segregation occurs through the formation of either heterokaryotic, binucleate sporangiospores, or sporangiospores containing diploid nuclei. As a result of this, germination of these sporangiospores yield heterokaryotic or 'bisexual' mycelium, which, in *P. blakesleeanus*, produces homothallic zygospores (Blakeslee, 1906, and Burgeff, 1912). These homothallic isolates are apparently unstable and break down within a few sexual reproductive generations (Cutter, 1942). Homothallism in *Z. heterogamus* may represent

a stable occurrence of this heterokaryotic or bisexual condition.

Cryopreservation.—The use of cryopreservation techniques in this study of *Z. heterogamus* has afforded new insights into zygospore development. The use of LTSEM allowed for the preservation of the delicate initial contact of the zygophore with the sexual hypha, as well as excellent preservation of the early stages of progametangial and gametangial formation. In observations of *Z. heterogamus* it was noted that conventional immersion fixation and critical point drying of the specimens for SEM usually resulted in disruption of the initial zygophore contact, and dehydrational collapse of the early stages of the progametangia and gametangia. Due to the dehydration of the specimens in conventional SEM, it is always questionable as to what extent the specimens have shrunk (generally considered to range from 5-25% in a good preparation) in comparison to their natural, living state. Since LTSEM specimens are fully hydrated, the only reduction in size that may occur is an effect of thermal contraction, however, from +20 C to -90 C there is less than a $1x10^{-4}$% change in volume of amorphous pure water (Weast, 1984). Additionally, because of the minimal specimen treatment associated with LTSEM as compared to conventional SEM preparation, better preservation of surface material, i.e. mucoid layers, crystalline ornaments, is possible. I believe LTSEM offers the best specimen preservation currently available, and should be considered to most accurately represent the living state.

In comparison of conventional chemical immersion fixation and freeze-substitution

for TEM, numerous authors have shown the advantages of freeze-substitution for members of the Ascomycetes (Czymmek and Klomparens, 1992), and Basidiomycetes (Mims, et al., 1988). In this study of a member of the Zygomycetes, preservation of the cytoplasmic details of *Z. heterogamus* by a variety of freeze-substitution protocols was less than optimal (for a detailed discussion of these results see appendix A). In particular, poor preservation of membranes was noted, limiting organelle identification and determination of cytoplasmic events. These results were very similar to those reported by Heath and Rethoret (1982), in their observation of mitotic microtubule preservation in *Z. moelleri* via freeze-substitution. However, freeze-substitution did provide excellent preservation of the wall material in *Z. heterogamus*. In this study freeze-substitution has, for the first time, provided TEM observation of the details of the multilayered wall of fully matured zygospores.

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CHAPTER 2: LOW TEMPERATURE SCANNING ELECTRON MICROSCOPY OF THE ULTRASTRUCTURAL DEVELOPMENT OF ZYGOSPORES AND SPORANGIOSPORES IN *MYCOTYPHA AFRICANA*, AND THE EFFECTS OF CULTURAL CONDITIONS ON SEXUAL VERSUS ASEXUAL REPRODUCTION.

ABSTRACT

The ultrastructural details of sexual and asexual reproduction in *Mycotypha africana* were observed with low temperature scanning electron microscopy. Sexual reproduction was enhanced over asexual reproduction with a reduction of incubation temperature, the absence of light, and through the use of a minimal amount of inoculation material. Repeated subculturing of actively growing zygosporic cultures resulted in a loss of zygosporic competence, which was reversible following 2-3 months storage of these cultures at 14-18°C, 24 h dark. Sexual apparati were initiated by the contact of two zygophoric hyphae. Subapical contact resulted in formation of progametangia at the site of contact. Continued elongation of the zygophoric hyphae and additional, subsequent sexual contacts were observed. If sexual contact was apical, the apical region of the zygophoric hypha converted into a progametangium, stopping elongation. The progametangia enlarged, and gametangial septa were formed, isolating the gametangia,

which fused, resulting in the formation of the zygosporangium. Zygospores developed internally and were observed externally by the rupture through the zygosporangium wall of the developing wart layer of the zygospore proper. Sporangiosporogenesis commenced with the formation of specialized aerial sporangiophores extending up to 5,000 μm. The apical regions of the sporangiophores enlarged, forming vesicles 300-400 μm long. Pedicels formed synchronously on the surface of the vesicles, the tips of which ballooned outward forming the sporangia. Two morphologically distinct types of sporangia were formed: spherical- and elongate-sporangia. These sporangia were formed in a highly regular spatial arrangement, alternating between the two sporangial types.

Keywords: Unispored sporangia, Zygosporangium, Zygospores

INTRODUCTION

The fungal class Zygomycetes exhibits a wide variety of sexual and asexual reproductive structures including homo- and heterothallic zygospores, azygospores, multispored sporangia, few-spored sporangiola, unispored sporangia, and merosporangia. Within these defined developmental structures, the Zygomycetes exhibit a tremendous variety of unique and beautiful morphologies the development of which is mostly poorly understood or unknown. In her original description of the genus *Mycotypha*, E. A. Fenner (1932) stated that "the unusual nature of [this] fungus and its many variations make it an

interesting object for study from the stand point of mycology." Furthermore, the highly uniform spatial arrangement of the alternating unispored sporangia on the vesicles of *M. africana* Novak & Backus suggest an excellent model organism for the study of the fundamental aspects of subcellular structural organization.

Asexual reproduction in *M. africana* is initiated with the development of a long sporangiophore supporting an elongate vesicle covered with two distinct types of unispored sporangia. The similarity of this structure to the plant genus *Typha* L., or cattail, is the derivation of the name (i.e. *Mycotypha* = fungal cattail). *M. africana* also forms apparently homothallic zygospores. Conversion between the two forms of reproduction readily occurred with either the exposure to or the exclusion of light, respectively. The descriptive terminologies of zygosporogenesis used in this study are those which were detailed in chapter 1.

The advanced technique of low temperature scanning electron microscopy (LTSEM) was used to best preserve the extremely delicate ultrastructural details of reproduction in this fungus. Due to a minimum of specimen treatment, and the possible observation of fully hydrated specimens, LTSEM provides one of the best methods of observing fungi in their 'natural state'. LTSEM avoids the loss of aqueous or alcohol soluble surface materials, and the artifacts of specimen shrinkage due to dehydration.

This study of *M. africana* was undertaken for comparison with previous research on *Zygorhynchus heterogamus* Vuill. (Chapter 1) of homothallic zygosporogenesis in a

second genus of the order Mucorales. Additionally, the genus *Mycotypha* is in the family Thamnidiaceae, and *Zygorhynchus* is in the family Mucoraceae, allowing for a broader perspective on zygosporogenesis in general towards the future goal of establishing a general pattern for ultrastructural development of sexual reproduction in the class Zygomycetes. Thirdly, the mixture of two morphologically distinct unispored sporangia on a single vesicle is unique in the Zygomycetes. Fourthly, due to the length and delicacy of the aerial asexual reproductive structures, *M. africana* was a good test specimen for the technique of LTSEM. And finally, *M. africana* was studied simply because of its deemed beauty.

MATERIALS AND METHODS

Cultural Conditions

A culture of *Mycotypha africana* Novak & Backus was obtained from E. S. Beneke and A. L. Rogers, Michigan State University. Species identification was verified using Zycha, et al. (1969), and Hesseltine, et al. (1959), and has been submitted to the American Type-Culture Collection. Subcultures were grown and maintained on a 1.8 % V-8 juice agar (177 ml V-8 juice, 2 g calcium carbonate, 20 g Bacto-Agar, 833 ml deionized water), incubated at 14-18 °C, with 12 h light/ 12 h dark or 24 h dark. Cultures were grown 3-21 days and were prepared at the various stages of sporangiosporogenesis and

zygosporogenesis for observation.

Low Temperature Scanning Electron Microscopy (LTSEM)

Specimens were cut from the agar plates, 7 mm x 20 mm x 1 mm, mounted on to an Emscope SP 2000 cryostage, with Tissue-Tek O.C.T. cryo-adhesive (Lab-Tek Products, Miles Laboratories, inc.) mixed with powdered graphite, and plunge frozen in a liquid nitrogen slush. Surface water crystals were viewed and sublimated away (10-15 min) at approximately -60 °C in a JEOL 35CF SEM with the Emscope cryostage attachment. Specimens were then recooled and sputter coated with 40 nm of gold at -190 °C, and observed at -80 to -90 °C at 10 kV. Approximately 280 sporangial apparati and 350 zygosporic apparati were observed during the course of this research.

RESULTS

Cultural observations

Inoculation of cultures with a small agar plug, approximately 2 mm x 2 mm, from previously grown cultures, and grown at 20-22 °C, with 12 h light/ 12 h dark, resulted primarily in normal sporangial reproduction, with the colony surfaces appearing dark grey by four days. Light macro- and microscopic observations revealed that immature vesicles

and sporangial initials were hyaline, and mature sporangia were dark grey to black in color. At 10-14 days regions of zygospore formation were noted in these cultures, and generally comprised approximately 5% of the total colony. It was noted that zygospore production was not uniform throughout the colony, but rather was confined to specific regions within the colony which were almost exclusively zygosporic with few or no sporangiophores. Light microscopic observations of developing zygospores showed that the zygospores developed a dark amber coloring with maturation, becoming nearly black at maturity.

Plug-inoculated cultures never covered the entire surface of the agar plate. Colony expansion dramatically slowed after 14 days of growth, with an average colony diameter of 30-40 mm. Colony expansion appeared to stop after 8-12 weeks achieving a maximum diameter of 50-70 mm.

Cultures grown at 14-18 °C with 24 h dark, following agar plug inoculation, produced enhanced zygospore production comprising up to 25% of the total colony. Regardless of age, colonies grown in complete darkness remained hyaline to light grey in color. Apparently normal sporangial reproduction was also present, though the sporangial heads did not develop the darker pigmentation observed in cultures grown in the presence of light. Light microscopic observation of the germination of these light colored single spored sporangia indicated that they were viabile. Transfer of these dark-grown cultures to light resulted in the production of darker pigmented sporangia.

Zygospore production in 40-70% of the total colony was obtained by altering the inoculation method. Inoculation was made by wetting a flame-sterilized, stiff-wire inoculating needle in a fresh agar plate, gently passing this needle through the aerial mycelium of a 7-10 day old dark-grown culture, and then inoculating a fresh agar plate by dragging this needle in a series of parallel lines across the agar plate, approximately 1 mm below the agar surface. This inoculation method was designated few-spored inoculation. These plates were then grown at 14-18 °C in 24 h dark. This inoculation and colony transfer method became the standard procedure used throughout the rest of this study. Increased colony growth per plate was obtainable by performing a second inoculation of the plate oriented perpendicular to the first set of inoculation lines. Attempts were made to further enhance zygospore production by use of two separate colonies for each of the two series of perpendicular inoculations. No apparently significant change in zygospore production was noted in these dually inoculated plates, specifically where the lines of the two inoculation sources crossed on the agar surface.

Zygospore production was noted to decrease with repeated transfers. Zygospore production remained high, 40-70%, for 3-4 successive transfers, 10-14 days apart. By transfers 5-6, zygospore production dropped off to approximately 20% of the total colony. By transfers 7-8, zygospore production dropped to less than 1%. Allowing the cultures to remain undisturbed at 14-18 °C and 24 h dark for 2-3 months, and then re-transferring to fresh agar plates, zygosporic 'competence' was reestablished. However, following reestablishing zygosporic competence the same pattern of decreasing zygospore production with repeated transfers was observed. This procedure of loss of and

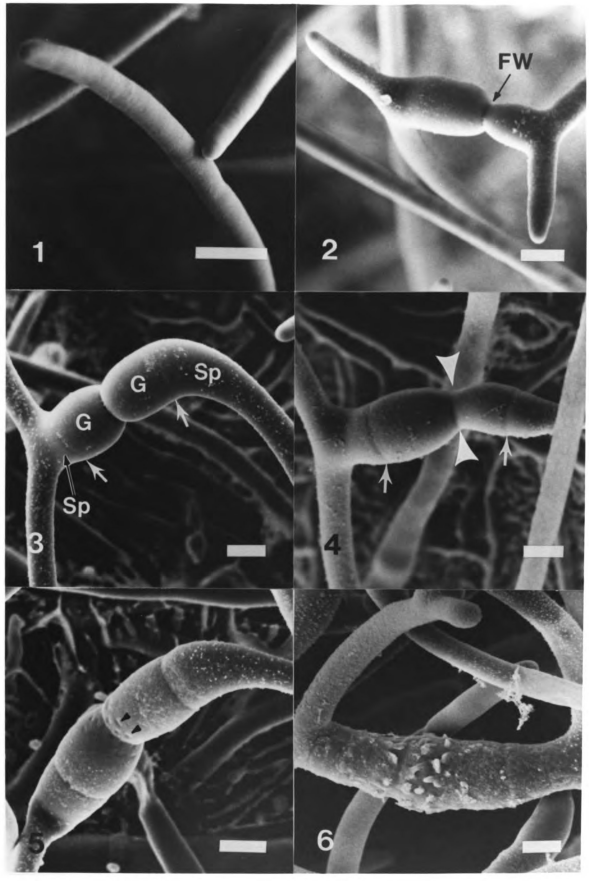
reestablishment of zygosporic competence was repeated seven times over the course of three years. No studies were undertaken to determine the specific minimum period required for reestablishment of zygosporic competence.

Low temperature scanning electron microscopy

Zygosporogenesis. Initial contact between two zygophores occurred apically to 65 μm subapically on either or both zygophores (Fig. 1). No morphologically distinguishable differences were noted between the two zygophores. Zygophores ranged 6 to 8 μm in diameter, and were never branched though any one zygophore formed up to eight sexual apparati. The zygophores extended up to approximately 500 μm in total length, and 15 to 100 μm between sexual apparati. Shortly after contact between the zygophores, the two zygophores became firmly adherent to each other and this region of contact was designated the fusion wall (Fig. 2, FW). Concurrent with the establishment of the fusion wall the regions immediately behind the fusion wall enlarged, elongating and ballooning outward (Fig. 2). The establishment of the fusion wall and this enlargement marked the initial formation of the progametangia. If contact and progametangia formation was subapical, continued elongation of the zygophore and additional sexual contacts occurred. If contact was made at the apex of a zygophore, no further elongation of that zygophore occurred.

Swelling of the progametangia continued, widening and flattening the fusion wall

Figs. 1-10. LTSEM of sexual reproduction in *M. africana*. **Fig. 1.** Initial contact between two zygophores. **Fig. 2.** Following contact, adhesion of the two zygophores resulting in the formation of the fusion wall (FW), and swelling of the progametangia occurred. When the contact point was subapical, the zygophore apices continued to elongate, forming later, additional contacts with the same or different zygophores. **Fig. 3.** After an initial period of swelling, internal gametangial septa (arrows) were formed delimiting two gametangia (G) of approximately equal size, and the suspensors (Sp). **Fig. 4.** Fusion of the two gametangia occurred with the removal of the fusion wall and was observed as a smoothing or flattening of the junction between the two gametangia (large arrowheads). Plasmogamy resulted in the formation of a single zygosporangium between the two gametangial septa (arrows). **Fig. 5.** Shortly after formation of the zygosporangium, internal wart initials (arrowheads) became externally visible in the region closest to the former fusion wall. **Fig. 6.** The development of the warts and swelling of the internal zygospore wall ruptured through the outer zygosporangium wall. Standard bar = 10 μm in figs. 1-6.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region, however, the discrete nature of the two progametangia was clearly observable (Fig. 3). No morphological differences were noted that distinguished the two progametangia. The progametangia obtained a maximum diameter of 15-18 μm, at which time internal gametangial septa formed 11-20 μm behind the fusion wall within both progametangia (Fig. 3, arrows). Formation of these gametangial septa marked the transition from progametangia to apical gametangia (Fig.3, G) and distal suspensors (Fig. 3, Sp). Apparently concurrent with completion of the gametangial septa, initiation of fusion wall removal occurred. Fusion wall removal was identified from external observation by a transition from the closely oppressed but discrete gametangia (Fig. 3) to a more continuous profile at the fusion wall (Fig. 4, arrowheads). Initiation of fusion wall removal and plasmogamy of the two gametangia marked the formation of the zygosporangium, which was designated as the region between the gametangial septa (Fig. 4).

Following formation of the zygosporangium, shallow depressions were observed in the zygosporangium wall adjacent to the site of the former fusion wall. These shallow depressions indicated the internal formation of the wart initials (Fig. 5, arrowheads). Wart development progressed from the site of the fusion wall towards both gametangial septa simultaneously. As development of the wart initials continued they enlarged, and ruptured through the zygosporangium wall (Fig. 6). Displacement of the zygosporangium wall with the internal development of a contiguous wart layer marked the development of the zygospore separate from and internal to the zygosporangium. Remnants of the zygosporangium wall were adherent to the warts at all further stages of zygospore

maturation. LTSEM observation of the ruptured zygosporangium wall and the surface of the developing external wart layer of the zygospore revealed the presence of a slimy or mucilaginous material covering the zygosporangium/zygospore complex.

Continuously from fusion wall removal, the zygosporangium and zygospore enlarged, smoothing out the depression resultant from the former fusion wall, initially forming a cylindrical zygosporangium/zygospore complex (Fig. 6) and subsequently a globose to spherical zygospore (Figs. 7-9). No major swelling was noted in the suspensors themselves. At maturity the zygospores obtained a diameter of 38-50 μm.

Development of the warts continued towards the gametangial septa until a continuous, external wart layer of the zygospore was formed between the septa (Fig. 8). Maturation of the wart layer continued with an increase in the height and diameter of the warts themselves. At maturity the warts exhibited an irregular conical shape, with a maximum height of 6-8 μm, and a diameter of approximately 8-12 μm (Fig. 9). The surfaces of the warts were crenulate with an interdigitating of the bases of adjacent warts.

No pattern of sexual apparati formation was determinable, with the exception that sexual apparati were never observed forming on sporangiophores. Figure 10 shows a typical example of the apparently random nature of sexual apparatus formation, with four zygophores forming six sexual apparati.

Fig. 7. Further swelling resulted in a spherical appearance to the developing zygosporangium/zygospore complex, with the internal zygospore warts visible through the zygosporangium wall (arrowheads). **Fig. 8.** Maturation resulted in the complete replacement of the zygosporangium by the zygospore, with the contiguous, external warty layer of the zygospore clearly visible. Remnants of the zygosporangium wall were noted adhering to the apices of the warts (arrowheads). **Fig. 9.** At maturity of the zygospore, the warts exhibited an irregular conical shape, and an interdigitation of their bases. Standard bar = 10 μm in figs.7-9. **Fig. 10a.** Convoluted sexual hyphae showing the randomness of sexual apparatus formation; bar = 50 μm. **Fig. 10b.** Diagram of fig. 10a defining the individual sexual hyphae.


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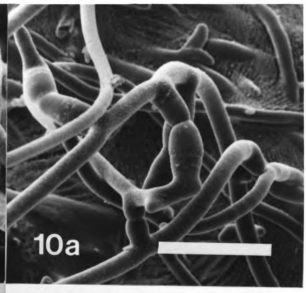
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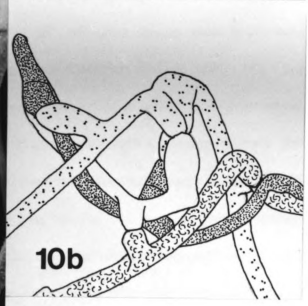
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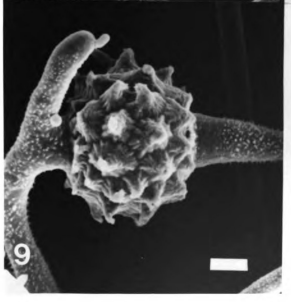
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Sporangiosporogenesis. Sporangiosporogenesis initiation was observed as the formation of a straight, unbranched, undifferentiated, aerial sporangiophore, a uniform 8-10 μm in diameter extending 800-5,000 μm from the agar surface (Fig. 11). The apical regions of the sporangiophores expanded outward forming a cylindrical vesicle (Fig. 12). Observation of intermediate stages of vesicle expansion indicate that expansion occurred uniformly within a 100-200 μm apical region. At the earliest stages of vesicle expansion, slight variations in diameter were noted (Fig. 12), but these discrepancies in diameter were not observable by the time distinct pedicels formation was seen (Fig. 13). The earliest indications of pedicel initiation were observed as a series of slightly raised, regularly spaced, latitudinal ridges on the vesicle surface (Fig. 12, arrows). Shortly after this stage, pedicel initials were clearly observable as distinct individual projections from the surface of the vesicle (Fig. 13, arrows). At this point in development the vesicles themselves exhibited a slight tapering at either end. The vesicles continued to expand in both length and diameter throughout sporangial initiation, obtaining a maximum size of 350-400 μm in length and 20-24 μm in diameter at maturity.

The pedicel initials exhibited a high degree of spacial order in their arrangement on the vesicle surface (Fig. 14). Pedicel development appeared to be generally synchronous over the entire surface of the vesicle with a slight developmental delay on the order of 1-2 h at the extreme vesicular ends. The extreme apical regions generally remained without pedicels until late in development (Fig. 15, *). Following formation of

Figs. 11-22. LTSEM of asexual reproduction in *M. africana*. **Fig. 11.** The sporangiophore, a straight, unbranched aerial hypha, extended undifferentiated for 2,000-5,000 μm above the agar. **Fig. 12.** A 100-200 μm apical region of the sporangiophore (Sph) enlarged, forming a cylindrical vesicle (V) of often irregular diameter initially. The surface of the vesicle was covered with a series of regularly spaced ridges (arrows). **Fig. 13.** Shortly after the initial enlargement, the vesicle (V) diameter became more uniform with a slight tapering at either end. Regularly arranged pedicel initials were visible on the vesicle surface (arrows). Standard bar = 50 μm in figs. 11-13.

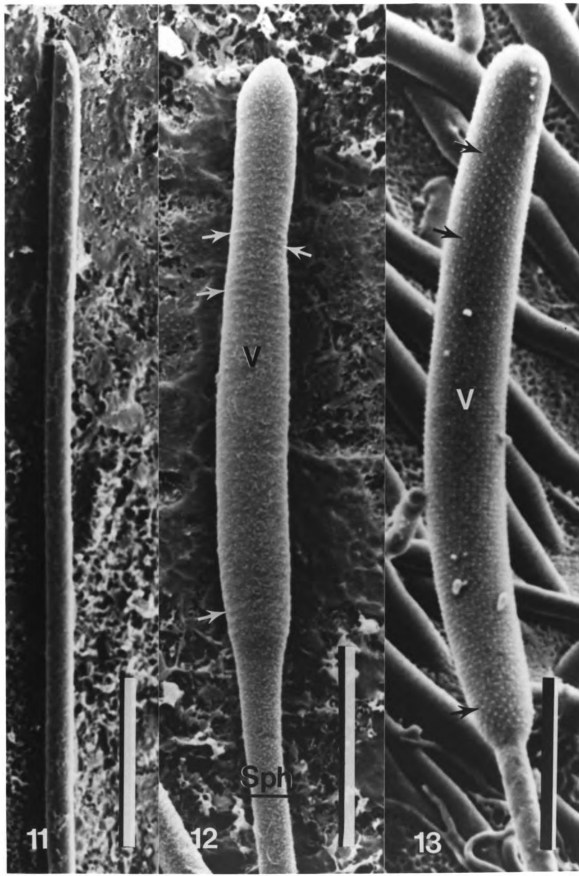
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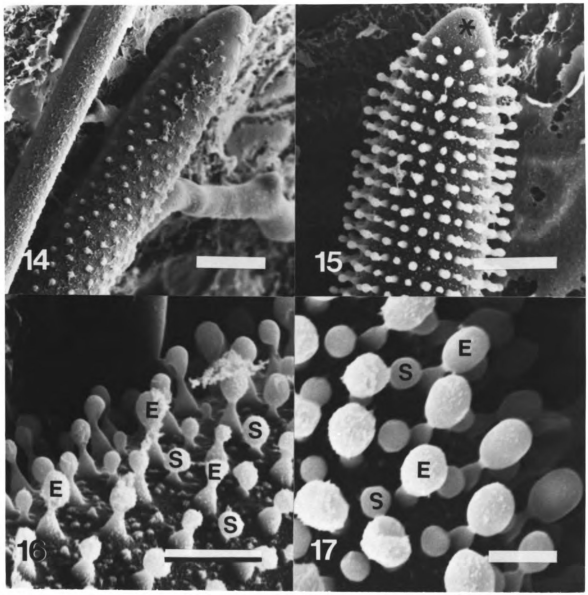
Fig. 14. Enlargement of the vesicle surface showed the uniform two-dimensional spatial arrangement of the newly-formed pedicel initials; bar = 10 μm. **Fig. 15.** The pedicels enlarged into two distinct, alternating types, and the sporangial initials formed at their apices. The spherical sporangia (S-sporangia) formed on the shorter pedicels, and the elongated sporangia (E-sporangia) formed on the large pedicels. An approximately 7 μm region of the vesicle apex remained free of sporangial initials (*); bar = 10 μm. **Fig. 16.** An enlargement of the vesicle surface shows the arrangement of the shorter pedicels supporting the S-sporangia (S) and the larger pedicels supporting the E-sporangia (E). The pedicels at this point appeared to be generally conical in shape. It was noted that there was some slight, regional asynchronous development of the pedicel-sporangia structures; bar = 5 μm. **Fig. 17.** At a slightly later stage, the S-sporangia (S) and the E-sporangia (E) were clearly visible and appeared generally uniform in their development; bar = 2.5 μm.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the pedicel initials the pedicels enlarged and elongated into two distinct sizes. The two sizes of pedicels alternated in their spacial arrangement such that no two similarly sized pedicels were adjacent to each other. Shortly after elongation of the larger pedicels above the height of the smaller pedicels, sporangial initials formed at their apices (Fig. 15).

The formation of the sporangial initials was, again, generally synchronous over the entire surface of the vesicle (Fig. 15), although slight differences in developmental progression indicated by differences in sporangial sizes were observable at the earliest stages of sporangial formation (Fig. 16). In addition to the apparent slight differences of developmental progress, two different sporangial types were observable. These sporangial types were designated elongate-sporangia (E-sporangia, Fig. 16, E) and spherical-sporangia (S-sporangia, Fig. 16, S) based on their morphologies at maturity. At this stage it was determined that the E-sporangia developed on the longer pedicels and the S-sporangia developed on the shorter pedicels. The developing sporangia continued to enlarge, becoming more uniform in size and shape (Fig. 17). The slight developmental differences within each sporangial type noted previously were no longer observable.

Maturation of the sporangia was synchronous over the entire vesicle (Fig. 18). At maturity the vesicle apical region, formerly observed to be free of sporangia, also held mature sporangia. Mature elongate sporangia were 4.5 x 1.9-2.2 μm, and mature spherical sporangia were 2.8-3.2 μm in diameter. The sporangia maintained their uniform spacing, of alternating between S- and E-sporangia, over the entire vesicle with the exception that the last 2 to 3 rows of sporangia at the base of the vesicles were generally only

Fig. 18. At maturity the entire surface of the vesicle was covered with regularly spaced unispored sporangia including the apical region; bar = 100 μm. **Fig. 19.** Enlargement of the vesicle surface shows the high degree of spatial ordering of the sporangia. The S-sporangia (S) fit neatly below and between the E-sporangia (E), which were supported further above the vesicle surface on the longer pedicels; bar = 10 μm. **Fig. 20.** Due to their orientation, perpendicular to the vesicle, the E-sporangia were found to be more susceptible to being dislodged at maturity than the S-sporangia. Upon removal of the E-sporangia, the arrangement of the S-sporangia between the longer pedicels (larger arrowheads) of the E-sporangia, was more easily observed. (smaller arrowheads show the smaller pedicels from which S-sporangia were dislodged); bar = 10 μm.

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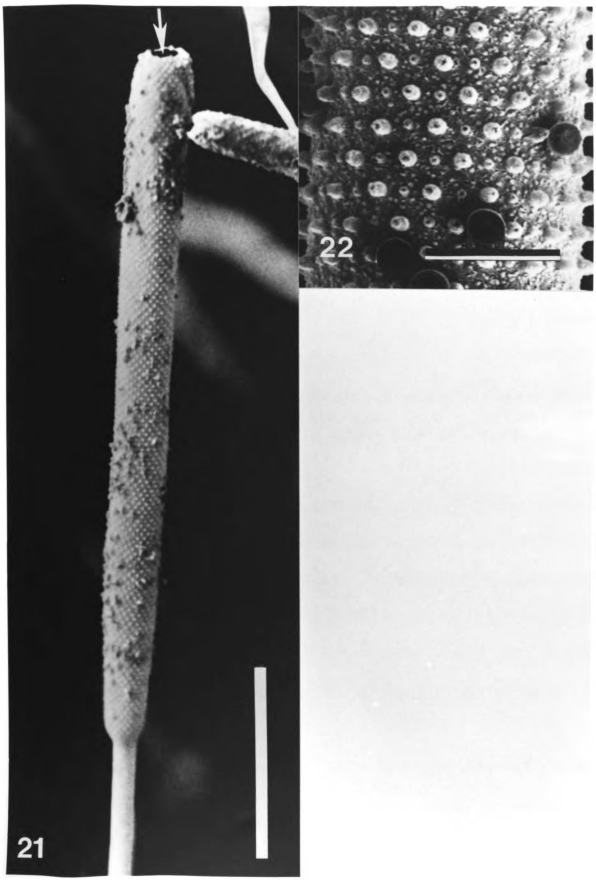
E-sporangia (Fig. 19). The longer pedicels of the E-sporangia supported these sporangia above the S-sporangia on their shorter pedicels (Fig. 19). At maturity the E-sporangia were generally noted to be dislodged from the vesicle before the S-sporangia (Fig. 20). This allowed confirmation of the arrangement of the S-sporangia on the shorter pedicels, and the spacial regularity of the pedicels and their associated sporangia (Fig. 20).

Sporangial dissemination appeared to occur due to pedicel abscision, and sporangia were easily dislodged from the vesicles by few slight disturbances of the culture plates. At sporangial dissemination it was noted that the apices of the vesicles collapsed, revealing the hollow cylindrical nature of the mature vesicles (Fig. 21). Examination of the pedicels following sporangial dissemination revealed small pores at their apices, indicative of an empty interior (Fig. 22). An apparent rigidity to the sporangiophore and vesicle walls was noted due to the fact that both structures remained upright for several months following sporangial maturity.

Fig. 21. At maturity the sporangiophore, and the vesicle remain erect for several months, with a clearly observable hollow interior (arrow); bar = 100 μm. **Fig. 22.** At maturity all the unispored sporangia had fallen from their pedicels. The apparent empty nature of the pedicels with clearly visible orifices at their apices was noted; bar = 10 μm.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DISCUSSION

Zygosporogenesis. Low temperature SEM proved to be an excellent technique for the observation of zygosporogenesis in *M. africana* for several reasons. Due to the capability of observing fully-hydrated specimens, dehydrational artifacts associated with critical point drying in conventional SEM preparation, such as a 5-25% shrinkage (Flegler et al., 1993) and specimen distortions commonly found with fungal specimens, were completely avoided. The earliest stages of sexual contact and progametangial formation are generally fragile, and easily damaged by the physical forces associated with conventional chemical immersion fixation/preparation techniques (personal observations). The limited specimen treatment of LTSEM avoids this physical damage of the specimens, and allows for the preservation of surface materials, i.e. crystalline-like ornamentations and mucoid layers, which might be washed away with conventional techniques. The only negative aspect of the observation of fully-hydrated specimens with LTSEM is in the observation of septa, which are generally more apparent with the shrinkage associated with dehydration.

The variability observed in *M. africana* in the number of sexual apparati formed by any single zygophoric hypha of from one to eight can be explained developmentally. Sexual apparati were often noted to arise generally wherever two zygophoric hyphae

randomly came into contact, without any specific arrangement, save that no single zygophoric hypha was ever observed to produce a sexual apparatus following contact with itself. No pattern of maturation, i.e. either an increase or decrease in maturation with proximity to the apex, was seen. However, observations of *M. africana* showed that if sexual contact was made at the apex of a zygophore, further elongation of that zygophore apparently did not occur. At no time during the observation of approximately 350 sexual apparati was there anything indicative of the formation of a branch from a zygophore following apical contact.

It has been established that hyphal tip elongation occurs in association with some type of apical organizing center (Bartnicki-Garcia, 1990, and Lopez-Franco & Bracker, 1993). I propose that sexual contact at the apex of a zygophore may have resulted in the disruption or replacement of its apical elongation center with the progametangial/gametangial/zygosporangial organizing architecture, and no further elongation of that zygophore was possible. When sexual contact occurred subapically, this elongation organizing center continued to function, allowing further elongation of the zygophore and additional sexual contacts. The zygophores of *M. africana* often continued to elongate and develop additional sexual apparati. It is important to keep in mind that the continued existence and development of zygophores in *M. africana* is unlike other members of the Mucorales, i.e. *Mucor mucedo* L.:Fr. (Personal observations) and *Rhizopus sexualis* (Smith) Callen (Hawker & Beckett, 1971), in which, after contact and formation of the progametangia, further development of the zygophores stops.

Initiation of plasmogamy of the two gametangia is described as occurring concurrent with fusion wall removal, and specifically not 'fusion wall dissolution' as is generally described by most authors. Fusion wall dissolution in *R. sexualis* (Hawker & Gooday, 1969; Hawker & Beckett, 1971), *Phycomyces blakesleeanus* (Kunze) Burgeff (Sassen, 1962), *P. nitens* Fries (O'Donnell, et al., 1978b), and *Gilbertella persicaria* (Eddy) Hesseltine (O'Donnell, et al., 1977) is reported to occur through the actions of various vesicles, and microbodies associated with the fusion wall and through a putatively enzymatic degradation. Since no internal ultrastructural observation of processes involved with fusion wall removal were made, no proposals for the actual methods of fusion wall removal, such as an enzymatic centripetal degradation or fusion wall dissolution, can be made. Observations made of the junction between the two gametangia (Fig. 3) indicate that this peripheral area of the fusion wall is under stress. These observations of a stretching or stressing of the external fusion wall region were repeatedly made. Since LTSEM allows for the observation of fully hydrated specimens, and extreme care was taken to ensure that significant, noticeable dehydration of the specimens did not occur, these observations are not believed to be sample preparation artifacts. Similar indications of physical stress were also reported in *Z. heterogamus* (Chapter 1), and observed in *P. blakesleeanus* (O'Donnell, 1976). Such observations support the notion that in addition to putative, internal enzymatic activity, the fusion wall may actually undergo an evagination, similar to that proposed for *Muc. mucedo* (Hammill, 1987).

Typically in zygosporogenesis, the wart initials first appear at the former site of the fusion wall, and progress toward the gametangial septa as seen here in *M. africana*,

and also many members of the Mucorales, i.e. *Z. heterogamus* (Chapter 1), *Muc. mucedo* (Edelmann & Hammill, unpublished), *G. persicaria*, (O'Donnell, et al., 1977), and *R. sexualis* (Hawker & Gooday, 1968; Hawker & Beckett, 1971). It is possible that this phenomenon may be associated with an organizing center associated with elongation and development of the progametangia and gametangia, similar to those apical organizing centers associated with hyphal elongation (Bartnicki-Garcia, 1990, and Lopez-Franco & Bracker, 1993).

Hyphal tips of the Zygomycetes do not contain specifically organized structures such as the Spitzenkörper found in members of the Basidiomycetes and Ascomycetes. The hyphal tips do have regions of high concentrations of vesicles and microbodies which have been proposed to function similarly. Similar regions of concentrations of vesicles and microbodies have been reported by Hawker & Gooday (1969), and Hawker & Beckett (1971) in the apical regions of the progametangia and gametangia of *R. sexualis*. Time-course light microscopic studies of living cultures of *Z. moelleri* Vuill. (Blakeslee, 1913, and Green, 1927), *Z. heterogamus* (Blakeslee, 1913), *Cunninghamella echinulata* Thaxter, *Muc. mucedo*, and a second species of *Mucor* [= *Mucor* v.] (Blakeslee, 1904) show that elongation/enlargement of the progametangia and gametangia specifically occurs apically. I believe it is reasonable to conclude that very similar processes and mechanisms are responsible for the apical elongation of both vegetative hyphae and progametangia and gametangia.

The apices of the progametangia and gametangia actually become the fusion wall.

Similar organizations of vesicles and microbodies have been reported in regions associated with the fusion wall of *R. sexualis* (Hawker & Gooday, 1969; Hawker & Beckett, 1971), *P. blakesleeanus* (Sasson, 1962), *P. nitens* (O'Donnell, et al., 1978b), and *G. persicaria* (O'Donnell, et al., 1977). Following removal or dissolution of the fusion wall, these organelles would still be situated in the region where initial formation of the warts are first observed to appear in these same fungi. The mechanism by which hyphal elongation occurs has been proposed to be a combination of lysis and synthesis (Bartnicki-Garcia, 1972). I propose that these same basic structures are associated in sequence with (I) adhesion of the two progametangia and formation of the fusion wall, (II) elongation of the progametangia and gametangia, (III) wall dissolution associated with fusion wall removal, and (IV) formation of the wart layer and subsequent layers of the zygosporangial wall, with a genetic regulation of the balance between lysis and synthesis. This could also explain the cessation of elongation of the zygophoric hyphae following apical sexual contact discussed above, due to genetic redirection of the apical organizing center of the zygophoric hyphae from elongation to sexual apparatus formation.

Associated with the rupture of the zygosporangium wall by the developing warts was an apparent slime covering as observed with LTSEM in both *M. africana* (Figs. 6-8) and *Z. heterogamus* (Chapter 1). The observation of this slime covering seems to correlate with the gelatinized 'inner primary wall layer' reported in *P. nitens* (O'Donnell, 1978b), and in *R. sexualis* (Hawker & Gooday, 1968; Hawker & Beckett, 1971). In both *P. nitens* and *R. sexualis* this gelatinized wall layer was observed with TEM, occurring between the ruptured outer zygosporangium wall and the developing outer wart layer of

the zygospore. O'Donnell, et al. (1978b), and Hawker & Beckett (1971), proposed this layer as arising from a gelatinization of the secondary wall layer of the zygosporangium. The descriptive differences between 'slime' and 'gelatinized' may be due to the nature of the methods of observation, fully hydrated and unfixed in the former, and fixed, dehydrated, and embedded in the latter, or simply a matter of semantics. I propose that the slime layer observed in *M. africana* is indicative of an enzymatic process occurring and associated with penetration of the zygosporangium wall by the developing warts, with the end products of a softening or degradation of the zygosporangial wall appearing as a slimy, mucoid film or a gelatinous layer external to the warts as seen in *P. nitens*, and *R. sexualis*.

In the description of the effects of various environmental conditions and observed cultural growth the terminology 'zygosporic competence' was used to describe cultures which produced zygospores. Four factors observed to affect sexual reproduction in *M. africana* were: 1) temperature, 2) light, 3) quantity of inoculum, and 4) rate of subculturing. The enhancement of sexual reproduction versus asexual reproduction by the reduction of incubation temperature is quite common in the Mucorales, although to date no specific regulatory mechanism for this effect has been reported. The conversion from sexual to asexual reproduction following exposure to light indicates the presence of a photosensitive regulatory mechanism. Both the quantity and rate of subculturing effects indicate the possible presence of some physiologically active compound also regulating sexual versus asexual reproduction. A possible mechanism that can be proposed is that actively, rapidly growing cultures of *M. africana* develop some 'anti-sexual reproduction'

compound. Repeated transfers of such actively growing cultures to fresh media every 10-14 days allows for the buildup of this putative compound. As concentration levels of this compound build it results in suppression of zygosporic competence. After 7 successive transfers this compound reaches concentration levels where almost complete suppression of sexual reproduction occurs. During continued incubation (i.e storage) of these sexually suppressed cultures, at 14-18 °C and 24 h darkness, allows for a reduction of the levels of this putative compound, possibly due to a drop in nutrient concentration in the media, and a regaining of zygosporic competence, as was observed in this study. These factors observed affecting the loss of zygosporic competence, i.e. repeated subculturing, exposure to light, or heavy inoculation, provide a fertile area for research into the physiological or genetic controlling mechanisms triggering zygosporogenesis. However, such research was beyond the scope of the present developmental study.

It is possible that sexual reproduction in *M. africana* is pseudo-homothallic. It can be conceived that each of the two types of sporangia, E- and S-sporangia, are homokaryotic for a single mating-type. Germination of a single sporangiospore would lead to the development of hyphae containing a single mating-type. Since each sporangial type matures and disperses through pedicle dehiscence simultaneously, and are inoculated, generally, simultaneously and in the same location, it is possible that following germination of each, anastomosis occurs between the single mating-type hyphae allowing for the development of hyphae heterokaryotic for both mating types, and thus a pseudo-homothallism. Determination of this could be done with single-spore culturing, and subsequent mating crosses, but such culture techniques were beyond the scope of this

current study.

Sporangiosporogenesis. Low temperature SEM was found to be an excellent technique for the preservation and observation of the asexual reproductive structures of *M. africana.* The unispored sporangia were found to be extremely fragile and very easily dislodged from the vesicles during chemical immersion fixation and preparation (unreported observations). The sporangiophores were also found to be susceptible to physical damage due to their length. This physical damage was greatly reduced with the minimal specimen preparation associated with LTSEM.

The most striking aspects of sporangiosporogenesis in *M. africana* is in its simultaneous production of two morphologically distinct types of unispored sporangia on a single vesicle and the high degree of spatial organization of these sporangia. Within the zygomycetes these reproductive features are unique to the members of this genus. Of additional note is my observations of the intermediate stages of early vesicle enlargement, which indicate that the diameter increase is simultaneous. However, due to the static nature of LTSEM observation no evidence was collected determining whether vesicle elongation with maturation occurs acropetal or basipetally or both.

As a result of these cultural and ultrastructural studies, I believe that both sexual and asexual reproduction in *Mycotypha africana* offer a number of distinctive characteristics for future study, specifically with regards to regulatory mechanisms. The

possible regulatory mechanisms associated with sexual reproduction of (1) low incubation temperature, (2) light sensitivity, and (3) the loss of zygosporic competence with repeated subculturing, would provide a choice organism for molecular and genetic developmental study. (4) The simultaneous formation of the two distinctly morphologically different unispored sporangia, spherical (S-sporangia) and elongate (E-sporangia), allow for a comparison of ultrastructural development and possible genetic control mechanisms. (5) Extremely regular structural organization in the alternation of the S- and E-sporangia, is analogous to the determinate developmental organization of members of the Laboulbeniales, although *M. africana* is easier to culture. Thus, asexual reproduction in *Mycotypha* provides a good subject for the study of subcellular developmental organization and spatial recognition.

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SUMMARY

1. The ultrastructural details of zygosporogenesis in *Zygorhynchus heterogamus* Vuillemin were observed with scanning and transmission electron microscopy (SEM and TEM), using both conventional chemical fixation and the cryopreservation techniques of low temperature SEM (LTSEM) and freeze-substitution for TEM. Detailed observations of origin and development of the walls of the entire sexual apparatus were made.

2. In *Z. heterogamus*, maturation of both gametangial septa is apparently identical and plasmodesmata were observed to penetrate the septa from their initial formation through full maturation of the zygospore.

3. In *Z. heterogamus*, wart formation was initiated with the appearance of vesiculate bodies closely appressed to the zygosporangium wall, external to the plasma membrane. Wart development progressed through the deposition of stacked series of electron-opaque disks. Rupture of the zygosporangium wall by the developing zygospore was concurrent with the formation of a contiguous warty layer.

4. For the first time, clear TEM observations of the multiple wall layers of the fully mature zygospore were observed. Three major layers were clearly distinguishable: (I) an outer, electron-opaque wart layer, (II) an intermediate, electron-translucent laminate layer, composed of closely appressed sub-layers, and (III) an internal electron-transparent layer.

5. Following a historical review of structural nomenclature used for zygosporogenesis, I propose a standardized terminology:

Zygophore — specialized sexual reproductive hyphae prior to contact and fusion, exhibiting chemotactic, pheromonal growth responses.

Progametangia — a specific hyphal structure which develops following sexual contact and fusion, and differentiates into a gametangium and suspensor.

Sexual Hypha — a central, specialized aerial hypha upon which the sexual apparati developed.

Sexual Apparatus — all the discrete components of a single sexual reproductive complex leading to the formation of a single zygospore regardless of the developmental stage, with the exclusion of the sexual hyphae.

Fusion Wall — the portion or region of the exterior wall of the zygophore which, shortly after sexual contact, fuses the progametangia, and subsequently the gametangia together, becoming a morphologically internal wall.

Zygosporangium — the single cell directly resulting from the fusion and plasmogamy of the two discrete gametangia.

Zygospore — the thick walled resting spore which forms de novo within the zygosporangium.

Macro- and *Micro-* — prefixes used to designate the larger and smaller progametangia, gametangia, gametangial septa, and suspensors of *Z. heterogamus*, based on their morphology and developmental pathways.

6. Cultural experimentation with *Mycotypha africana* revealed that reproduction was convertible from asexual to sexual by reducing the incubation temperature from 22°C to 14-18°C and switching from 12 h light/ 12 h dark to 24 h dark. The reduction in temperature for sexual reproduction is common within the zygomycetes. However, the photosensitivity is uncommon and provides a subject for future study of environmentally inducible genetic regulation of developmental pathways within the Zygomycetes.

7. Further experimentation on enhancing zygospore production in *M. africana* revealed two additional features: (I) few-spore inoculation resulted in greater zygospore production than plug-inoculation or high spore concentrations inoculation, and (II) zygosporic competence was lost following repeated subculturing of actively growing cultures, but was reversible following a minimum of 2-3 months of storage at 14-18°C and 24 h darkness. This is believed to be indicative of some type of physiologic regulator/suppressor of sexual reproduction, which builds up in actively growing cultures.

8. LTSEM of *M. africana* revealed strong similarities to homothallic zygosporogenesis in *Z. heterogamus*, particularly in the formation of multiple zygospores arising from an individual zygosporic hypha.

9. In *M. africana*, sexual apparati were initiated by the contact of two zygophoric hyphae. Subapical contact resulted in formation of progametangia at the site of contact, and continued elongation of zygophoric hypha with the formation of additional, subsequent sexual contacts. If sexual contact was apical the apical region of the zygophoric hypha converted into a progametangium, stopping elongation.

10. In *M. africana*, the progametangia enlarged, and gametangial septa were formed, isolating the gametangia, which fused, resulting in the formation of the zygosporangium. Zygospores developed internal to the zygosporangium and were observed externally by the rupture through the zygosporangium wall of the developing wart layer of the zygospore proper.

11. In *M. africana*, sporangiosporogenesis commenced with the formation of specialized aerial sporangiophores extending up to 5,000 μm. The apical regions of the sporangiophores enlarged, forming vesicles 300-400 μm long. Pedicels formed synchronously over the surface of the vesicles, the tips of which ballooned outward to form the sporangia. Two morphologically distinct types of unispored sporangia were formed and designated spherical- and elongate-sporangia (S- and

E-sporangia, respectively). These sporangia were formed in a highly regular spatial arrangement, alternating between the two sporangial types.

12. The simultaneous formation of the S-sporangia and E-sporangia, and their highly regular spatial arrangement on a single vesicle is unique in the Zygomycetes. This pattern of development allows for a comparison of ultrastructural development and possible genetic control mechanisms. The extremely regular structural organization in the alternation of the S- and E-sporangia is analogous to the determinate developmental organization of members of the Laboulbeniales - though easier to grow. Thus *Mycotypha* provides a good subject for the study of subcellular organization and spatial recognition.

13. LTSEM provided excellent preservation of fragile reproductive structures free from the dehydration artifacts common with conventional chemical fixation of fungal structures.

14. *Z. heterogamus* was preserved using nine different freeze-substitution fixation protocols for transmission electron microscopy (TEM) observation. Specimens were frozen with either propane jet freezing or high pressure freezing. Both methods of freezing offered acceptable results, but a greater quantity of well frozen material was obtained with high pressure freezing. Comparison of freeze substituted material with conventionally fixed material revealed that the observation of cytoplasmic ultrastructural details was better with conventional

fixation. In general, freeze-substitution provided poor membrane preservation providing few identifiable organelles. The only exception was found in the observation of the multiple wall layers of fully matured zygospores, which prior to this work had never been reported.

15. A bibliography of material on zygosporogenesis since the first report of zygospores by Ehrenberg, 1829, was compiled. This bibliography contains references to all known morphological and developmental studies on sexual reproduction in the Zygomycetes, *sensu lato*, though some references are more oriented towards physiology. Additionally, where known, author's full names were used in order to present an historical perspective to the development of mycology.

APPENDIX A: CRYOPRESERVATION AND FREEZE-SUBSTITUTION OF MUCORACEOUS FUNGI

ABSTRACT

Direct observation of fully hydrated specimens of *Zygorhynchus heterogamus* was made using low temperature scanning electron microscopy (LTSEM). LTSEM provided excellent preservation of fragile reproductive structures free from the dehydration artifacts common with conventional chemical fixation of fungal structures. Fungal material was preserved with freeze-substitution using nine different fixation protocols for transmission electron microscopy (TEM) observation. Specimens for freeze-substitution were frozen with either propane jet freezing or high pressure freezing. Both methods of freezing offered acceptable results, but a greater quantity of well frozen material was obtained with high pressure freezing. Comparison of freeze substituted material with conventionally fixed material revealed that the observation of cytoplasmic ultrastructural details was better with conventional fixation. In general, freeze-substitution provided poor membrane preservation and few identifiable organelles. The only exception was found in the

observation of the multiple wall layers of fully matured zygospores, which prior to this work had never been reported.

INTRODUCTION

During the past decade, the use of cryopreservation techniques for both the observation of frozen hydrated samples using low temperature scanning electron microscopy (LTSEM), and the techniques of ultrarapid freezing followed by freeze-substitution for the preparation of biological materials for transmission electron microscopy (TEM) have gained wide acceptance (Gilkey & Staelin, 1986; Menco, 1986; Dahl & Staelin, 1989). Whereas excellent results for some samples have been obtained, the techniques are still being perfected for broader applications. The variety of techniques and protocols reported to date has been limited, even though widely diverse samples have been examined. The diversity of specimen preparation protocols used for conventional chemical fixation will be required for cryopreservation as well. A "standard" technique and set of methods for all specimens will not suffice for cryopreservation, just as they have not been adequate for conventional fixation.

Since cyropreservation and freeze-substitution were introduced for fungi (Howard & Aist, 1978), a concentration of osmium tetroxide (1-2%) in acetone is the almost exclusive substitution fixative reported. For a number of tissue types this protocol produces very good results in the fungal classes Ascomycetes (Czymmek & Klomparens,

1992), and Basidiomycetes (Mims, et al., 1988). When the same protocol was followed for *Zygorhynchus heterogamus*, a member of the fungal class Zygomycetes, however, the results were far from optimal. As I began to explore a diverse set of protocols for cryopreserving and freeze-substituting these fungi, I gained some new insights into freeze-substitution and report them here to provide a basis for continuing avenues of experimentation and further development of cryopreservation techniques.

MATERIALS AND METHODS

Fungal Cultures

Zygorhynchus heterogamus Vuillemin was cultured on 1.8% V-8 juice agar at 21-24°C with 12 hr light and 12 hr dark for 3-5 days.

Low Temperature-SEM

Blocks of agar, 20 x 8 x 3mm, containing the desired stage or stages of growth were cut from actively growing fungal cultures. These blocks of agar were mounted on the Emscope SP 2000 cryo-unit specimen stage using Tissue-Tek II O.C.T. cryo-embedding (Lab-Tek Products, Miles Laboratories, inc.) medium mixed with powdered graphite. The specimens were plunge frozen in a freshly thawed liquid nitrogen slurry, and placed under vacuum. Samples were either fractured or kept whole, transferred to

a JEOL 35CF SEM with the Emscope SP 2000 cryo-stage, and sublimated at -60°C under high vacuum (10^{-6} torr) to remove surface ice. Etched specimens were transferred back to the Emscope cryo-unit, sputter coated with gold, and returned to the SEM. All observations were made with a specimen temperature of -84 to -110°C, and an accelerating voltage of 10 kV.

Freeze-substitution for TEM

Specimens were prepared according to the following general protocol. A variety of freezing and solvent/fixative combinations were used (Table 1):

(1) Specimens were ultra-rapidly frozen with either an RMC MF 7200 Propane Jet Freezing Unit or a Balzers HPM 010 High Pressure Freezing unit.

(2) Samples were incubated in a solvent/fixative solution at -85°C for approximately 72 hours.

(3) Samples were rinsed with fresh substitution solvent at -85°C prior to warming to room temperature.

(4) Samples were warmed to room temperature (-20°C, -4°C, +4°C, +22°C in 0.5 hour steps) and rinsed with the substitution solvent to remove excess fixative (1 hour). Some samples were then en bloc stained with 0.5% uranyl acetate (W:V) in the fixation solvent.

(5) Samples were infiltrated (10-25% steps, fixation solvent:resin) with either Spurr's hard (1967) or Quetol 651/NMA (Kushida, 1975) hard resin and polymerized.

(6) Polymerized specimen blocks were sectioned and post-sectioned stained with

aqueous 0.5% uranyl acetate and Reynolds' (1963) lead citrate.

Table 1. Solvent and fixative combinations used for freeze-substitution.

Freeze-substitution Solvent and Fixative(s)	RMC MF 7200 Propane Jet Freezing Unit	Balzers HPM 010 High Pressure Freezing Unit[1]
(I) 2% OsO_4 and 0.05% uranyl acetate in acetone		Figs. 6^S & 7^Q
(II) 2% OsO_4 in methanol	Figs. 8^S & 9^S	
(III) 1.0% OsO_4 and 0.25% uranyl acetate in methanol	Fig. 10^S	
(IV) 0.5% OsO_4 in acetone		Fig. 11^Q
(V) 0.1% OsO_4 in acetone	Fig. 12^Q	
(VI) 0.025% OsO_4 in acetone	Fig. 13^Q	
(VII) 1.7% glutaraldehyde in acetone	Fig. 14^Q	
(VIII) 0.5% uranyl acetate in methanol		Fig. 15^Q
(IX) 0.5% barium permanganate in acetone	Data not presented	

^{S} - Embeddment in Spurr's hard
^{Q} - Embeddment in Quetol 651/NMA

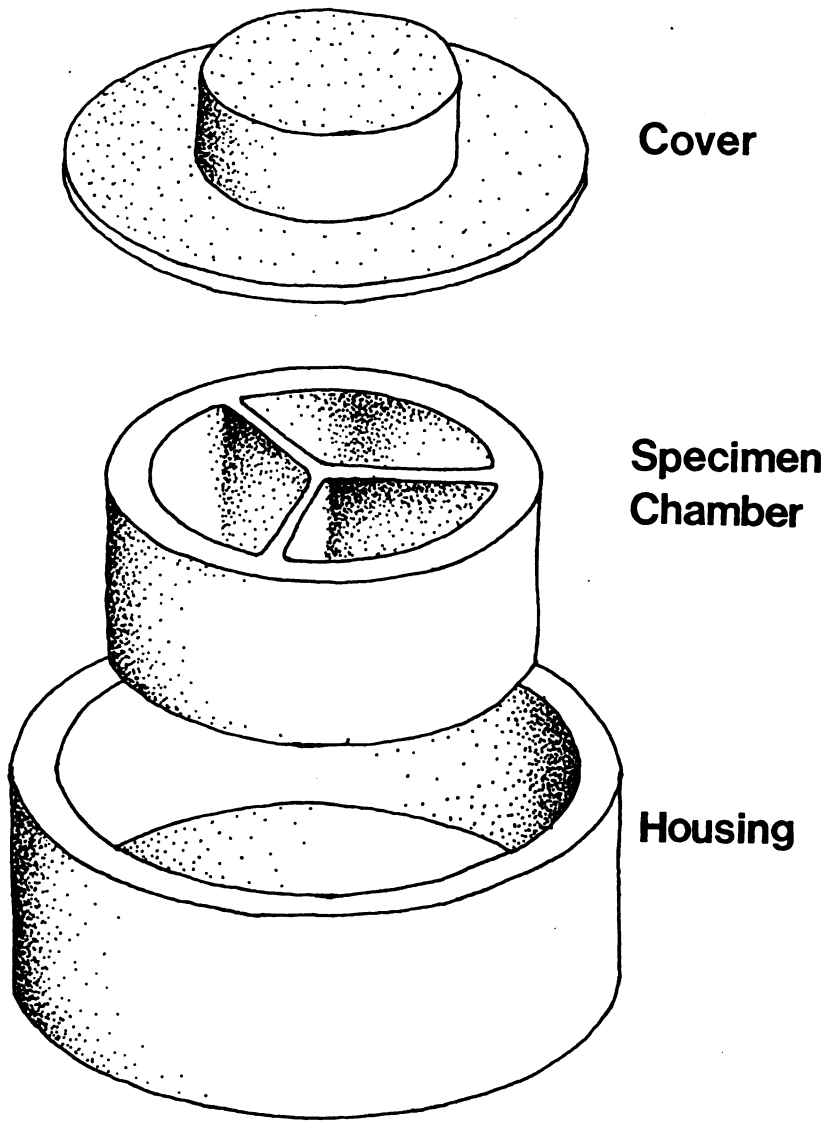
The specimen holder for the RMC MF 7200 Propane Jet Freezing Unit measured a total volume of 0.05mm deep and 1mm in diameter, and the specimen holder for the Balzers HPM 010 High Pressure Freezing Unit measured a total volume of 0.6mm deep and 1 mm in diameter.

Preparation of the solvent/fixative was conducted by adding the fixative to

[1] This was carried out at Miami University, Oxford, Ohio.

precooled (-85°C) solvent. Substitution was carried out in a precooled (-85°C) RMC three-welled aluminum substitution chamber (Figure 1). One chamber was filled with a mixture of the solvent and a desiccant (oven-dried Drierite or calcium sulfate) as a moisture scavenger (Czymmek and Klomparens, 1992). The second chamber was half filled with the solvent/fixative solution and the samples. The third chamber was either filled with additional samples for substitution or additional dehydrant mixture depending on the number of samples.

Fig. 1. Illustration of RMC three-welled aluminum freeze-substitution chamber.



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RESULTS

Low Temperature Scanning Electron Microscopy

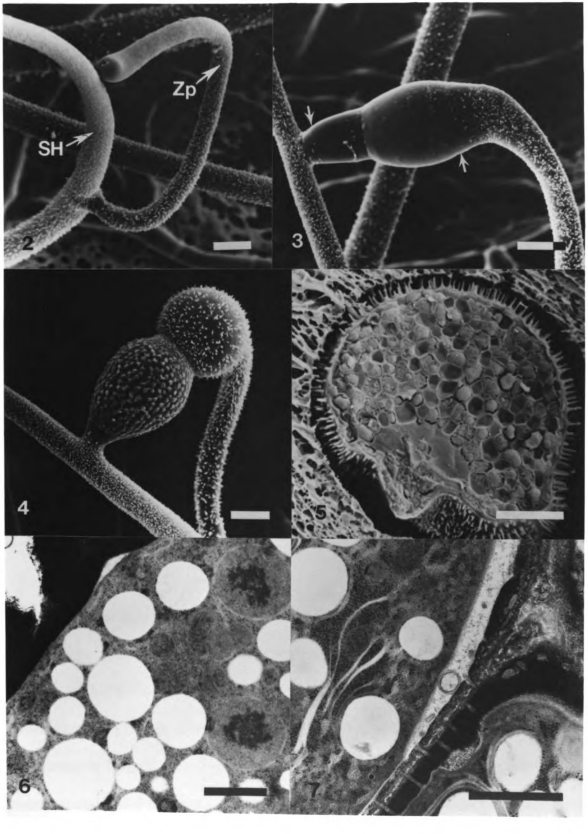
There are numerous developmental features during reproduction in *Zygorhynchus heterogamus* which are extremely fragile or ethereal and are easily lost or destroyed by the physical stresses of immersion fixation, and critical point drying. Low temperature-SEM was used to better preserve these fragile structures and provide an overview to the morphological development of sexual reproduction in the fungus *Z. heterogamus*. The general developmental scheme for sexual reproduction is highly complex and involves a number of morphological changes which are detailed in Chapter 1. Low temperature-SEM provided excellent preservation at all stages of development.

Preservation of the thin walled progametangia and the highly delicate initial contact between the two progametangia was clearly observed (Fig. 2). The enlarged gametangia, and indications of the internal gametangial walls and the fusion wall were observed with little signs of dehydration collapse, as the walls remained smooth and unwrinkled (Fig. 3). A nearly mature zygospore whose walls had ruptured through the exterior zygosporangium wall appeared to be well preserved (Fig. 4). Remnants of the zygosporangium wall were found still attached to the warty ornamentations of the

Figs. 2-16. Details of specimen preparation techniques used in the study of zygosporogenesis in *Z. heterogamus*.

Figs. 2-5. Low temperature scanning electron microscopy; bars = 10 μm. Fig. 2. Fragile initial contact of the zygophore (Zp) with the sexual hypha (SH). Fig. 3. Expansion and delimitation of the gametangia by the formation of gametangial septa. Fig. 4. Preservation of a mucoidal layer and remnants of the zygosporangial wall after rupture by the developing warty layer. Fig. 5. Fracture of an early post-cleavage sporangium showing preservation of internal matrix supporting the spore protoplasts.

Figs. 6-15. TEM of freeze-substituted preparations following either propane-jet freezing or high-pressure freezing. Figs. 6 & 7. Fixation with 2% OsO_4, and 0.5% uranyl acetate in acetone (protocol I), following high pressure freezing, resulted in little freezing damage; bars = 1 μm.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zygospore wall, as well as indications of the presence of a mucilaginous material. At all observed stages of development preservation of fine crystalline-like spines covering the fungal surfaces was noted. With deep-etching of the specimens it was determined that these spines were real structures and not ice crystal artifacts.

Fracturing of frozen aerial fungal structures was obtained (Fig. 5). These specimens, due to their aerial nature, extending up to 8 mm from the culture surface, had to be embedded in the Tissue-Tek mounting media for support and then frozen. This procedure revealed the arrangement of internal developing asexual spores within the developing asexual sporangium. Preservation of a matrix material supporting the immature spores as well as organelles within the spores themselves was observed.

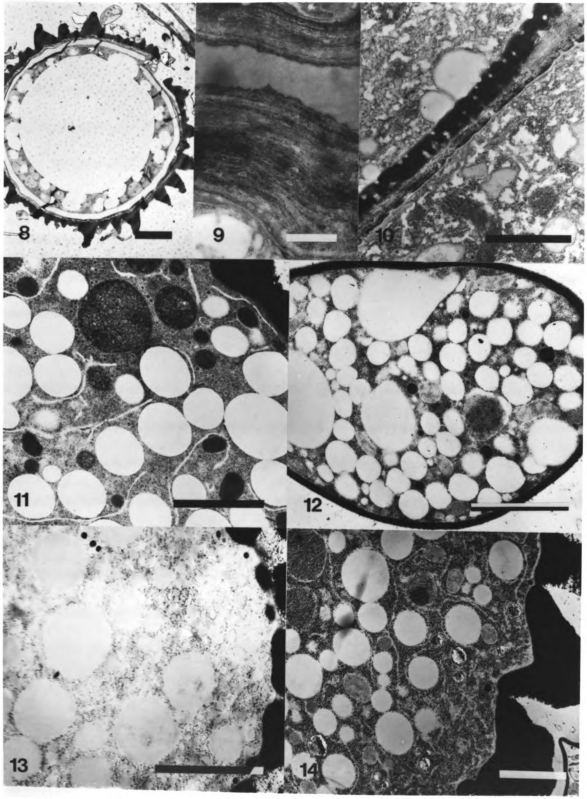
Freeze-substitution For TEM

A diverse series of freeze-substitution techniques and fixation protocols were used in an attempt to optimize ultrastructural preservation for TEM with a variety of results. Fixation with **2% OsO_4 and 0.5% uranyl acetate in acetone** (protocol I, Table 1), following high pressure freezing, resulted in little freezing damage (Figs. 6 & 7). Embedment in Spurr's resin (Fig. 6) or Quetol 661 (Fig. 7) was compared. The fixation preserved a generally finely granular matrix. Very few regions of membrane preservation were observed, rather regions of electron-transparency, where membranes were assumed

to have been located, were seen. Ribosomes were clearly identifiable, as were nuclei due to the staining of chromatin material. Mitochondria were identified by an arrangement which appeared to once have been lamella with the absence of membranes. Likewise, endoplasmic reticulum was identified as ribbon-like electron-transparent regions. Lipids were stained but appeared as electron-transparent regions due to exposure correction for the cytoplasmic density. No other organelles were identifiable. The nearly mature zygospore walls exhibited excellent preservation, and darkly stained multiple wall layers were observed. Minimal separation between the walls and the plasmalemma was noted. The Quetol resin (Fig. 7) offered improved contrast over the Spurr's resin (Fig. 6), and showed fewer separations between the walls and plasma membranes.

Fixation with **2% OsO_4 in methanol** (protocol II), following propane jet freezing, with embedment in Spurr's resin, failed to preserve the majority of the interior of fully mature zygospores and was presumed to be due to inadequate ultrarapid freezing of the large zygospores (Figs. 8 & 9). Some of the lipid material was inadequately fixed and leached out of the sections and appeared as uniformly distributed contaminations on the surface of the sections. However, some of the lipid material, closer to the periphery of the zygospores, was preserved and exhibited staining typical of conventional fixation. What appeared to be separation of the plasmalemma and the zygospore wall (Fig. 8) was determined to be a previously undescribed interior, electron-translucent wall layer, present in fully mature zygospores. Few membrane regions were preserved and few clearly identifiable organelles were noted. Excellent preservation and staining of the multi-layered walls was observed (Fig. 9).

Figs. 8 & 9. Fixation with 2% OsO_4 in methanol (protocol II), following propane jet freezing, with embedment in Spurr's resin. Fig. 8. This method failed to preserve the majority of the interior of fully mature zygospores. This lack of preservation was presumed to be due to inadequate ultrarapid freezing of the large zygospores; bar = 10 μm. Fig. 9. Excellent preservation of the wall ultrastructure; bar = 0.5 μm. Fig. 10. Fixation with 1% OsO_4, and 0.25% uranyl acetate in methanol (protocol III), following propane jet freezing, with embedment in Spurr's resin resulted in membrane preservation, but membranes appeared highly wrinkled and distorted; bar = 1μm. Fig. 11. Fixation with 0.5% OsO_4 in acetone (protocol IV), following high pressure freezing, with embedment in Quetol resulted in a finely granular cytoplasm with uniform fixation, and high contrast between organelles and the cytoplasm; bar = 2 μm. Fig. 12. Fixation with 0.1% OsO_4 in acetone (protocol V), following propane jet freezing, with embedment in Quetol resin resulted in poorer fixation than that of higher concentrations of OsO_4 as seen in lower cytoplasmic density, and a general distorted and "fuzzy" appearance of the organelles; bar = 2 μm. Fig. 13. Fixation with 0.025% OsO_4 in acetone (Protocol VI), following propane jet freezing, with embedment in Quetol again resulted in very poor fixation and low contrast within the cytoplasm, and the cytoplasm in general exhibited a "splotchy" nature; bar = 1 μm. Fig. 14. Fixation with 1.7% glutaraldehyde in acetone (protocol VII), following propane jet freezing, with embedment in Quetol produced good fixation in general, high contrast, numerous identifiable organelles, and some membrane preservation; bar = 2μm.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Fixation with **1% OsO_4, and 0.25% uranyl acetate in methanol** (protocol III), following propane jet freezing, with embedment in Spurr's resin resulted in membrane preservation, but membranes appeared highly wrinkled and distorted (Fig. 10). In general, the cytoplasm exhibited gross distortion of ultrastructural morphology with very few identifiable organelles. Lipids, in general, were identifiable by their uniform electron-translucent staining comparable to conventional fixation, but some lipids did exhibit non-uniform fixation. Wall preservation was good, with excellent preservation of the plasmodesmata perforating the gametangial septa.

Fixation with **0.5% OsO_4 in acetone** (protocol IV), following high pressure freezing, with embedment in Quetol resulted in a finely granular cytoplasm with uniform fixation, and high contrast between organelles and the cytoplasm (Fig. 11). Membranes were not observed, although regions where their presence was expected were seen as unstained, electron-transparent areas. Areas identified as endoplasmic reticulum were explicitly noted. Nuclei were presumptively identified by their size and number as were mitochondria. Lipids appeared as highly electron-transparent areas. Few separations between the plasmalemma (cytoplasm) and the walls were observed.

Fixation with **0.1% OsO_4 in acetone** (protocol V), following propane jet freezing, with embedment in Quetol resin resulted in poorer fixation than that of higher concentrations of OsO_4 (protocols I-IV) (Fig. 12). Membranes were not fixed, and the definition between organelles and the surrounding cytoplasm was indistinct. Lipids were

unstained, and nuclei were questionably identified by their size and staining patterns which appeared to distinguish chromatin material. Based on size and staining characteristics, a diverse number of additional organelles were apparent, though were not specifically identifiable. All organelles appeared to be slightly distorted in shape. Walls were darkly stained, but few details, such as multiple layers, were observed.

Fixation with **0.025% OsO_4 in acetone** (Protocol VI), following propane jet freezing, with embedment in Quetol again resulted in very poor fixation and low contrast within the cytoplasm, and the cytoplasm in general exhibited a "splotchy" nature (Fig. 13). Lipids were identifiable and had a uniform electron-translucence comparable to conventional fixation. No other organelles were identifiable.

Fixation with **1.7% glutaraldehyde in acetone** (protocol VII), following propane jet freezing, with embedment in Quetol produced good fixation in general (Fig. 14). The cytoplasm had high contrast, and numerous organelles were identifiable. Some regions of membranes were preserved, most notably within areas of endoplasmic reticulum. Nuclei were identifiable, and exhibited staining of nucleoli and chromatin material. Lipids were identifiable and showed a uniform electron-translucency comparable with conventional fixation. Few separations between the plasmalemma and the walls were noted.

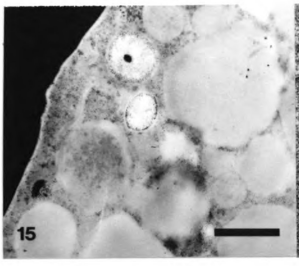
Fixation with **0.5% Uranyl acetate methanol** (protocol VIII), following high pressure freezing, with embedment in Quetol resin resulted in very low contrast in the

cytolplasm and a general fuzziness in appearance (Fig. 15). Membranes appeared to be fixed, but were indistinct. Putative endoplasmic reticulum was identifiable, but the tripartite structure of their membranes was not observable. Nuclei were identifiable by the staining of their chromatin material and size. Lipids were also identifiable and had a generally uniform electron-translucency comparable to conventional fixation. The organelles were apparently distorted in their size, shape and overall appearance, and, other than those mentioned above, could not be conclusively identified. Walls were darkly stained but revealed little structural details. Few separations between plasmalemma and the walls were noted.

Fixation with **0.5% barium permanganate in acetone** (protocol IX), following propane jet freezing, with embedment in Quetol resin was carried out, but data are not shown. No ultrastructural details were observable. Walls were identifiable, however no specific organelles were distinguishable. Gross distortions of the cytoplasm were observed, with no membranes visible. Contrast was low within the tissue. Sectioning of this material was poor, and numerous electron-opaque contaminations were noted in the sectioned material (data not shown).

In comparison, **conventional immersion fixation with aqueous 1% formaldehyde and 2% glutaraldehyde in 0.05M sodium cacodylate buffer followed by 1% OsO_4** and embedment in Spurr's resin resulted in generally good fixation, with good contrast, and clearly defined membranes which exhibited a tripartite structure (Fig. 16). Membranes did exhibit a slightly wrinkled appearance, and organelles were not as

Fig. 15. Fixation with 0.5% Uranyl acetate methanol (protocol VIII), following high pressure freezing, with embedment in Quetol resin resulted in very low contrast in the cytoplasm and a general fuzziness in appearance; bar = 1μm. Fig. 16. Conventional immersion fixation with aqueous 1% formaldehyde and 2% glutaraldehyde in 0.05M sodium cacodylate buffer followed by 1%OsO_4 and embedment in Spurr's resin resulted in generally good fixation, with good contrast, and clearly defined membranes; bar = 1μm.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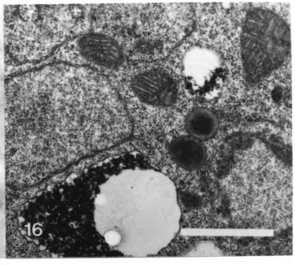
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smooth in circumference as those observed in the best freeze-substituted material. Nuclei containing chromatin material, mitochondria and their lamella, endoplasmic reticulum, numerous vesicles, and microtubules were positively identifiable. Lipids were identified and observed to frequently contain a non-uniformly distributed, darkly staining granular material in the early to mid- stages of zygospore development. Few separations between the plasmalemma and the walls were noted. Stages beyond the early zygospore wall formation exhibited poor internal fixation, and poor infiltration of the embedding resin.

Discussion

In working with the freeze-substitution of our fungal samples I recalled the statement that is taught to all beginning biological microscopists: the initial steps in sample preparation are first 'killing' and, second, fixation. These two steps are often combined both practically and intellectually when experiments use chemical immersion techniques. With the use of some combination of an aldehyde and/or osmium tetroxide in the initial preparation step both 'killing' and fixing occur somewhat simultaneously during conventional liquid chemical fixation. However, when using ultra-rapid freezing followed by freeze-substitution, this is not always the case. 'Killing' and fixation may occur separately. Consequently there are two distinct sources of artifact: ice-crystal damage and fixation artifact.

In freeze-substitution, 'killing' occurs with the cessation of all metabolic processes

and relies on thermal kinetics with the removal of enough heat energy from the system to stop all the biological functions of the cells. Under these conditions the term 'killing' is a misnomer, as can be shown in the examples of long term cryogenic storage of cell and tissue cultures. The use of the term 'immobilization' far better describes this process. Since immobilization occurs ultrarapidly (10^{-4} seconds, Gilkey and Staehlin, 1986) in relation to biological processes, even with high pressure freezing (10^{-1} seconds), ultra-rapid freezing provides one of the best methods for the preservation of biological specimens. Further, the immobilization itself results in no ultrastructural artifacts, since it involves only energies and not physical matter (i.e. changes in any atomic or molecular structures). The main artifact associated with this freeze-immobilization is the formation of ice crystals, which is related to the method and rate of freezing. These artifacts have been thoroughly covered in numerous published papers and shall not be duplicated here (for reviews see Gilkey and Staehlin, 1986, Menco, 1986, and Dahl and Staehlin, 1989).

The second major artifact associated with freeze-immobilization occurs during handling and loading of the specimens into the holders for various ultrarapid freezing devices. The samples can easily suffer physical damage and distortions prior to immobilization. It has been our observation that the best freezing of the samples occurred when the samples were tightly packed in the sample holders, eliminating all the gas pockets. Unavoidably, this tight packing may cause some physical damage and distortion to the specimens.

The primary set of artifacts of concern in this paper are those associated with the

fixation of the specimens. As with chemical immersion fixation, there is no universal fixation protocol for freeze-substitution of all samples. Each sample type and experimental condition, due to its unique physiological and biochemical composition, can require a reconfiguration of solvent, fixative(s), fixative(s) concentration and duration of -85°C incubation in order to obtain the optimal preservation.

Prior to the use of the enclosed RMC three-welled aluminum substitution chamber, individual polypropylene vials were used. Holes were made in the lids of these vials in order to accommodate pressure changes within the vials with changes in temperature. These vials were fitted into an aluminum block in which appropriate sized holes were drilled. The aluminum block was used in order to facilitate transport of the samples to and from the -85°C freezer, and to act as a heat sink during transport. Filling of the vials with fixative and samples was done in a dry-ice (solid carbon dioxide) filled styrofoam box in order to maintain temperature.

Two major problems were encountered with the use of the vials for freeze-substitution. Firstly, too much water was absorbed by the substitution fluid during freeze-substitution, resulting in poor sample dehydration. Secondly, too much carbon dioxide (from the dry-ice) was absorbed by the substitution fluid which resulted in effervescence of the fixation fluid to the point of boiling over and/or spitting through vent holes as the samples were warmed to room temperature. Both of these conditions were considered undesirable and/or dangerous. Use of the larger aluminum substitution chamber with the additional desiccant alleviated these problems, and was found to be far easier to work

with.

Comparisons of Spurr's hard resin with that of Quetol 651/NMA resin showed slightly better results with the Quetol. The Quetol resin has a lower viscosity than the Spurr's resin and thus provided improved ease of infiltration with less structural damage, i.e. fewer separations of the cytoplasm from the surrounding wall material. The Quetol resin showed very low background electron density, good staining qualities and provided excellent specimen contrast. Although the Quetol 651/NMA resin was found to be slightly more brittle than the Spurr's hard resin, no problems with trimming or sectioning were encountered. With the exception of cost, the Quetol 651 resin was believed to be superior to the Spurr's resin for the preparation of *Zygorhynchus heterogamus*.

To date only one paper has been published in which freeze-substitution of a member of the zygomycetes has been reported (Heath & Rethoret, 1982). This work dealt with the preservation of microtubules associated with mitosis in the fungus, *Z. moelleri*. Freeze-substitution in this work was done via plunge freezing in liquid-nitrogen-cooled liquid propane followed by substitution at -77°C with 2.5% osmium tetroxide and 0.1% uranyl acetate in acetone. Specimens were then rinsed with acetone and embedded in Epon. This work reports that freeze-substitution provides better preservation of microtubules when compared with conventional immersion fixation, but that little else was preserved by freeze-substitution including nucleus-associated organelles (NAOs) and nuclear envelopes. These authors conclude that for mitotic studies in "any organism" both conventional fixation and freeze-substitution should be used.

The work reported by Heath and Rethoret correlates very well with the present study of *Z. heterogamus*. The preservation of walls via freeze-substitution was believed to be superior to that of conventional fixation. With conventional fixation, during the later stages of zygosporogenesis the development of an extremely thick wall limited the penetration of fixatives and/or embedding resin yielding poor specimens for observation. The freeze-substitution of *Z. heterogamus* in this work has revealed for the first time the details of the multi-layered wall structure of fully mature zygospores (Chapter 1).

Lipids were well preserved, having an appearance and electron-opacity very similar to those found in conventionally fixed specimens. However, they appear lighter in the micrographs presented here as an artifact of photographic enlargement and contrast. Due to the overall density and low contrast of the cytoplasm preserved with freeze-substitution, the micrographs shown here have been printed lighter and with high contrast in order to show general cytoplasmic details, thus artificially lightening the lipids and giving them the appearance of being electron-transparent or unstained. It is important to note that lipids were indeed preserved, but that selective printing to highlight cytoplasmic details forfeits the obvious staining of the lipids.

In comparison of the freezing methods used, high pressure freezing yielded larger quantities of well-frozen material with less preparation damage than did propane jet freezing. Using either technique, it was found that the best preservation of material was obtained when the total volume of the sample holders was completely filled with material. Leaving air spaces within the sample matrix appeared to cause damage to the samples due

to vibration of the specimens during freezing, resulting in broken frozen tissue. Whereas high pressure freezing provided the largest quantity of frozen material, propane jet freezing did yield quite acceptable results. Due to the high equipment cost of high pressure freezing (approx. $150 k), propane jet freezing ($35 k) should be considered as a viable alternative.

A comparison of the freeze substitution techniques used with conventional immersion fixed material shows that ultrastructural details of the cytoplasmic material are best preserved with conventional fixation, with the exception of the wall layers. The question that remains is, why? In similar comparisons of specimens from the fungal classes Ascomycetes, Basidiomycetes, Chytridiomycetes, and Oomycetes freeze-substitution yields generally better preservation than conventional fixation. In general, for all tissue structures examined in *Z. heterogamus*, including asexual, sexual and vegetative hyphae and structures, conventional fixation yielded superior *usable* ultrastructural cytoplasmic details. Freeze-substitution did give smooth organelle profiles, but, poor membrane preservation with freeze-substitution limited organelle identification and the subsequent determination of cytoplasmic events. Very similar results have been found with several members of the genus *Coemansia* (K. L. Klomparens, personal communications).

The only possible explanation that can be offered for the preservational differences between the Zygomycetes and the other fungal classes is some difference in physiological makeup of members of the Mucorales from species in other fungal classes. However, no

direct evidence can be presented to support this proposal. It is necessary to keep this in mind when using freeze-substitution for any new specimens, and that various permutations on fixation protocols that have worked for similar samples may need to be tried.

Of the freeze-substitution protocols tried, the best results were obtained with 2% osmium tetroxide, 0.05% uranyl acetate in acetone with embeddment in Quetol 651/NMA resin for the preservation of *Z. heterogamus*. However, I would hesitate to say that these are the best results possible. In order to seek a better fixation protocol, I would suggest that other fixatives be sought out and tried. And I suggest that "fixatives" other than those commonly associated with electron microscopy be experimented with, even chemicals not generally considered to be fixatives. There is still debate within the literature as to how and when "fixation" takes place with freeze-substitution, and in considering a new fixative it would be absurd to accept a foregone conclusion of "well, that can not possibly work."

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APPENDIX B: BIBLIOGRAPHY OF ZYGOSPOROGENESIS LITERATURE SINCE 1820

The following is a bibliography of literature primarily covering the morphology and development of sexual reproduction in the Zygomycetes, although some citations are more oriented toward physiology. According to A. de Bary (1864), A. F. Blakeslee (1904) and M. L. Keene (1914), C. G. Ehrenburg in 1820 published the first mycological account of the observation of zygospores. Because of their better historical perspective, rather than mine nearly a century later, I have no reason to question their accuracy. Wherever possible, the full names of authors are included in order to present an historical perspective of the development of mycology.

Since the illustrations of older publications were often not interleaved with the text of the article, where known, the illustrative plates referenced by an article are included in the citation. On a number of occasions I received copied articles from other institutions in which the plates were not included because they were not specifically cited in my interlibrary requests. Although I have tried to be comprehensive, I do not claim that this list is 100% complete. This material is primarily of European and North American origins. The years 1830-1880 are particularly problematic and may very well be incomplete.

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