A FINE-STRUCTURAL STUDY OF CONIDIUM INITIATION IN STEMPHYLIIUM BOTRYOSUM WALLROTH

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SUMMARY

Conidia and conidiophores from synchronously sporulating cultures of Stemphylium botryosum were fixed and embedded at successive intervals for examination under the electron microscope.

The spore initial arises as a small bud at the tip of the conidiophore, and no discontinuity between the conidiophore wall and the spore can be detected. The single-celled bud and the tip cell of the conidiophore have very similar cytoplasmic features.

Besides the usual cellular organelles, irregularly shaped electron-transparent areas in which membrane systems seem to perform some internal secretory activity between the leaflets themselves are present. Globules of the slightly electron-dense material originating in these specialized areas can also be found next to the plasma membrane where they are released and presumably participate in wall formation. Such a secretory system has not been described before and may be of particular importance in higher fungi which are known to lack a true Golgi apparatus.

INTRODUCTION

In the past 25 years mycologists studying the Hyphomycetes have become increasingly aware of inadequacies of the Saccardoan classification (Saccardo, 1882–1931). A few examples mentioned by Simmons (1966) illustrate the artificiality and illogicality of this taxonomic system: closely related species or even strains may be put far apart in the classification on the basis of a single character such as spore colour (albino v. wild-type strain); the organisms may behave differently in nature and in culture (some Fusarium species form well defined sporodochia on their hosts but separate conidiophores in culture). Consequently, workers in the area have been searching for taxonomic criteria which better reflect the natural affinities of the Hyphomycetes.

A major step towards a more natural classification of these organisms came with S. J. Hughes's paper in 1953. Hughes suggested that 'there are only a limited number of methods whereby conidia can develop from other cells, and morphologically related imperfect states will only be brought together when the precise methods of conidium origin take first place in the delimitation of the major groupings'. In the subsequent discussion he described 8 modes of conidiation which he believed to be fundamentally different and erected a taxonomic section with illustrative specific examples for each.
Stemphylium botryosum belongs to Hughes's section VI or 'Porospore' which includes species with 'apparent development of conidia at minute single or numerous pores in the wall of the conidiophore' (Hughes, 1953).

The first ultrastructural study of porospore development was by Campbell (1969) on Alternaria brassicicola (Schw.). The spore initial was produced through a well defined pore in the primary wall (pigmented outer layer) of the conidiophore, and the outgrowth of the conidiophore secondary wall formed the primary wall of the young conidium. Additional research on an albino strain of the same organism (Campbell, 1970) showed that conidia were produced in the same manner. Finally, Campbell concluded that his results support the validity of the taxonomic concept of the porospore in both pigmented and albino strains.

Reisinger & Mangenot (1969) observed sporogenesis in Dendryphiella vinosa = Dendryphion vinosum (Berk, and Curt.) Hughes. They recognized 2 different modes of development. In the first, the conidium sprouted like a blastospore at the apex of a hyaline portion of a conidiophore formed by new growth of an old conidiophore. In the second, the spore initial broke through the differentiated pigmented outer layer of the conidiophore. As a result of their investigations the authors concluded that the differences noticed at the beginning of sporogenesis were superficial and were determined by the degree of melanization of the conidiophore; in both cases the non-melanized inner layer gave rise to the conidium. Reisinger and Mangenot's observations have led to some confusion, and thus more detailed fine-structural studies of conidium ontogeny on other organisms producing porospores are desirable to decide whether or not tretic and blastic species can be separated unequivocally.

Thus, the purpose of this investigation was to gather more information on 'porospore' ontogeny and to observe the cytological changes occurring in the conidiophore-conidium complex during conidium initiation.

Stemphylium botryosum Wallr. was chosen for the present fine-structural study for many reasons. This organism had been mentioned by Hughes (1953) as an illustrative example of his porospore section. It is the type species of the form-genus Stemphylium which has been described in great detail by Simmons (1967). Leach (1963, 1967, 1968) has carried out an extensive study of the effects of light and temperature on the sporulation of this species and determined the sequence of requirements for optimal reproduction. On the basis of Leach's work it was possible to outline a light-temperature regime which could be used routinely for the induction of synchronously developing conidiophores and conidia. An additional wounding treatment produced a denser layer of conidiophores.

MATERIALS AND METHODS

Single ascospores of Pleospora herbarum [O.S.U. isolate 132 A (1) SSI, kindly provided by Dr Charles Leach] were inoculated on 2% Difco malt extract agar and incubated in the dark at 21 °C for 5–10 days. Small plugs obtained from the periphery of these uniformly growing, dark-reared cultures were placed at the centre of 100-mm disposable Petri plates containing the same medium and were grown in complete darkness at 27 °C for 4 days. Centres of the colonies were cut out with a sterile cork borer (no. 15) and transferred to empty plates. Synchronous
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Conidiophore formation was then induced by a 12-h exposure to near ultraviolet light (Blacklight lamp BLB GE F15 T8, λ = 320–420 nm, hanging 30 cm above the plates) at the same temperature. Following return to darkness, synchronous conidiogenesis and maturation occurred in the next 30 h.

After the ultraviolet treatment, chunks of the dense conidiophore palisade were fixed at regular intervals of time (0.5 h in the early stages, 1 or 2 h in the late stages) for 2 h at room temperature in glutaraldehyde (2.5 %) in 0.1 M s-collidine buffer, pH 7.8. The addition of a drop of Tween 80 and a short vacuum treatment allowed better and quicker penetration of the fixative. The material was then washed 3 times with 0.1 M s-collidine buffer, pH 7.8, and post-fixed for 2 h at room temperature in a collidine-buffered 1 % osmium tetroxide solution at the same pH. After several rinses in water, the material was stained en bloc with 0.5 % aqueous uranyl acetate for 4 h, washed in water and dehydrated in a graded ethanol series followed by 100 % reagent grade acetone. Mollenhauer’s plastic II formulation (Mollenhauer, 1964) was used for progressive infiltration and final embedding. Polymerization of the plastic was carried out at 70 °C for 24–72 h.

Thin sections (50–75 nm) were obtained with a diamond knife (Instituto Venezolano de Investigaciones Científicas, Apartado 1827 Caracas, Venezuela) on a Porter–Blum MT-i ultramicrotome and collected on Formvar-coated 100-mesh copper grids. They were stained for 20 min at 45 °C with a fresh 5 % aqueous uranyl acetate solution (Watson, 1958) and post-stained 1–3 min with Reynolds’s lead citrate (Reynolds, 1961). The sections were stabilized with a light carbon coating and examined with a Philips 300 electron microscope working at 60 kV with a single condenser. The pictures were taken on Dupont and Kodak cut film sheets.

**RESULTS**

Throughout the following analysis the different developmental stages will be designated by the time (in hours) at which they were fixed after the beginning of the ultraviolet treatment.

**Morphology of the young conidiophore before conidium initiation**

The first conidiophore fixation was done immediately after the end of the u.v. irradiation (12 h). The conidiophores arise directly from the vegetative hyphae as lateral branches and form a dense layer. They are 50–60 μm long, 3–4 μm wide, slightly geniculate, and simple or occasionally branched. The wall of the lower part of each conidiophore appears more heavily pigmented than the wall of the tip cell. Particular attention was given to the micromorphology of the tip or sporogenous cell of the conidiophore.

In general the ground cytoplasm of young cells is electron-dense and clear recognition of organelles is sometimes hampered. The nucleus occupies a more-or-less central position and elongated mitochondria with sinuous profiles are scattered throughout the cytoplasm. Long strands of smooth endoplasmic reticulum run parallel to the main axis of the cell (Fig. 1). Irregularly shaped electron-transparent areas (approx. 0.3 × 0.6 μm), are dispersed in the cytoplasm on both sides of the nucleus. Aside from obvious cytoplasmic invaginations, they contain swirls of unit membranes interspersed with tenuous fibrillar material. Sometimes these membranes split and the space between the leaflets appears filled with some slightly electron-dense material referred to as ‘grey substance’ (Fig. 1). During the first stages examined (12–16 h), globules of this grey substance delimited by a thin electron-dense boundary can be found throughout the cytoplasm and even at the interface between the plasma membrane and the wall (Figs. 1–3).
Differentiation of the plasma membrane is pronounced. In the apical 3-4 μm of the cell, small invaginations and protrusions are visible on the plasma membrane (Figs. 1-3). In the lower part of the cell, plasmalemmasomes become abundant and reach maximal thickness near the septum (Fig. 1). Between the membranes constituting these plasmalemmasomes, microfibrils are intercalated and neatly lodged spheres of grey substance can also be noticed regularly (Figs. 1, 6, 8).

A sinuate young septum between the tip and the second cell of the conidiophore shows a narrow electron-transparent median zone (30 nm) which abuts against the septal pore. It constitutes the middle lamella of the septum and its continuity with the lateral primary wall of the cell is evident (Fig. 1). On both sides of this zone numerous electron-dense threads arise perpendicular to the convoluted plasma membrane, the first evidence of secondary wall deposition. Cytoplasmic continuity between the cells is maintained by a septal pore (0.12-0.15 μm). In the cytoplasm near the septal pore, several electron-dense membrane-bounded bodies (0.25-0.30 μm in diameter) are always found.

Primary wall material surrounds the cytoplasm of the tip cell. It is composed of a thick electron-transparent inner portion (150-300 nm) and a thin highly electron-dense outer zone (25-30 nm). Before conidium initiation, both zones reach their minimal thickness at the tip of the conidiophore where the external contour of the wall is smooth (Figs. 1, 4). Behind the tip where the transition between the 2 zones is sharp, an intermediate granular electron-dense zone appears and is considered part of the outer layer which increases in thickness as the conidiophore ages. The very first signs of secondary wall formation can be observed only in the vicinity of the first septum as described previously.

The conidiophore-conidium complex during conidium initiation

During the 16-18-h stages the conidium emerges and expands rapidly before the formation of the basal septum, the locus of the conidium secession. Although the cytoplasmic contents of the conidiophore do not show any significant difference from that of earlier stages, the plasma membrane is now sheathed with plasmalemmasomes in which fibrillar elements are interspersed (Figs. 4, 5). Spheres of grey substance (80 nm in diameter) and irregularly shaped masses (250 x 750 nm) of lesser electron-density with dark specks, here referred to as 'speckled substance', are regularly found associated with these membrane systems at the base of the cytoplasmic isthmus leading to the conidium (Fig. 5). The electron-transparent part of the wall is 180-200 nm thick and the outer electron-dense zone has acquired a verrucose aspect. The warts are filled with granular material, and fragments of membranes seem to be peeling off their outer surface (Fig. 10). The very first stage of conidium initiation is represented in Fig. 4. The conidial bud has an approximate width and height of 0.5 μm and is bounded by a very thin (5-8 nm) smooth electron-dense layer which is continuous with the verrucose layer of the conidiophore wall.

Quickly (stage 17 h) the opening at the tip of the conidiophore reaches its final width (0.8-1 μm), and simultaneously the volume of the conidium initial increases as much as 10-fold. In Fig. 5 the bud appears more or less spherical (1-1.5 μm in dia-
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meter). Within its very electron-dense cytoplasm are scattered electron-transparent areas whose contours and contents are not yet well defined. A mitochondrion can be seen passing through the cytoplasmic isthmus (Fig. 5). Very small invaginations are evident in the plasma membrane and the electron-transparent portion (100 nm) of the wall has a fibrillar aspect; the outer electron-dense zone (10–15 nm) shows the first signs of wart formation. More advanced development has been observed in samples of the same stage (Fig. 6). Within the cytoplasm of the conidiophore a nucleus, mitochondria and randomly oriented strands of endoplasmic reticulum are common features. In electron-transparent areas similar to those previously described a few peculiarities are noticed: rare globules of grey substance and completely electron-transparent polygonal areas. Just below the cytoplasmic isthmus the plasma membrane is accompanied by a few plasmalemmasomes; their number increases in the lower part of the cell and attains maximal development adjacent to the septum (Fig. 8). Besides the globules of grey substance usually observed within the plasmalemmasomes (Figs. 6, 8) irregularly shaped bodies of speckled substance are also found (Fig. 6).

Two elongate mitochondrial profiles can be discerned in the cytoplasmic channel between the conidiophore and the conidium. Small electron-transparent areas (200–400 nm in diameter) are dispersed throughout the cytoplasm of the young conidium now 4 × 4.5 μm. They contain a few membrane stacks and globules of grey and speckled substance (Figs. 7, 9). The plasma membrane is highly convoluted and in the lower half of the cell masses of speckled substance are lodged between membrane protrusions (Fig. 10). It is of particular interest to note the disappearance of the electron-dense boundary at the interface between these masses and the wall of the conidium. In the electron-transparent part of the conidium wall a subtle fibrillar substructure can be seen (Figs. 9, 10), but the outer electron-dense verrucose layer shows the same appearance as that of the conidiophore.

DISCUSSION

Conidium initiation: porospore v. blastospore

In the past decade a number of studies using the light microscope have been carried out in an attempt to verify taxonomic criteria proposed by Hughes for the Hyphomycetes in 1953. Among these studies can be mentioned Luttrell's work on Helminthosporium sp. (1963), Boerema's on Phoma sp. (1964), Kendrick & Cole's on Beauveria and Curvularia (1968), Cole & Kendrick's on Monascus ruber (1968), Kendrick, Cole & Bhatt's on Gonatobotryum apiculatum (1968) and Reisinger's on Dendryphiella and Dendryphion (1968).

Only a few of these investigations have dealt with porospore formation. Boerema (1964) has reported that conidium development in the type species of the form-genus Phoma is 'porogenous', since the conidium seems to be produced by 'an extrusion of a part of the plasma through a small pore in the apex of the parent cell'. Luttrell (1963) has emphasized the difficulty encountered in distinguishing blastogenous from porogenous conidium development in several species of Helminthosporium. Reisinger (1968) has noticed both of these types of conidiogenesis in Dendryphiella vinosa. He claims
however, that the presence of the hilum and the conidial scar allows the species to be classified in Hughes's porospore section.

Since observations with the light microscope have shown that the distinction between the blastospore and the porospore may be tenuous, it is appropriate here to compare and discuss fine-structural studies done on both types of conidial development. Boerema's early results (1965) on conidiogenesis in the form-genus Phoma are presented as diagrams traced after electron micrographs. When the first conidium is formed, an apical zone of the conidiophore about 4 μm wide protrudes like a papilla. Later this zone constricts and the young conidium assumes the shape of a bud. This study revealed that conidium development noticed with the light microscope and interpreted as porogenous (Boerema, 1964) was in fact blastogenous. Recently Hughes & Bisalputra (1970) have examined conidium ontogeny in Peziza ostracoderma. Their results show that throughout the synchronous development of blastoconidia (= blastospores) the wall of the swollen ampulla remains continuous.

To date, fine-structural investigations of conidium formation in species for which porogenous development has been alleged have been confined to Campbell's work on Alternaria brassicicola (1969, 1970) and Reisinger & Mangenot's on Dendryphiella vinosa (1969). In both strains of Alternaria brassicicola (wild type and albino) Campbell found that the conidia were produced by the extension of the inner layer of the wall of the conidiogenous cell through a well defined pore in the outer layer. Though Reisinger & Mangenot's study on Dendryphiella vinosa is not illustrated with electron micrographs of conidiogenesis itself, the authors conclude that the process does not follow the steps of blastospore formation and that it is through a break of the outer wall that the non-pigmented inner layer gives rise to the conidium. The present observations on Stemphylium botryosum do not support either Campbell's assertion of the continuity between the secondary wall of the conidiogenous cell and the primary wall of the conidium initial or Reisinger & Mangenot's statement of an actual mechanical break in the outer layer of the wall. Conidium development is simply blastogenous. If this situation is confirmed for other dark-walled species for which a tretic development has been postulated, the entire concept of the porospore may have to be discarded.

Conidiophore and conidium initial cytoplasmic features

Schemes of cytoplasmic events related to the ultrastructural aspect of apical growth and wall formation have been described by several authors. According to Bracker (1967) a vesicular component of the cytoplasm is involved in the accumulation of new wall materials. The organization and growth of fungal walls is under cytoplasmic control and wall constituents, or their precursors, must pass the plasma membrane barrier by some form of secretion. Marchant, Peat & Banbury (1967) described 2 different vesicular systems associated with wall synthesis and found only in regions of active growth or septum formation. In the apical part of the cell the endoplasmic reticulum produces vesicles which migrate through the cytoplasm, fuse with the plasma membrane, and are responsible for primary wall formation. Behind the apex, multivesicular bodies originating also from the endoplasmic reticulum are found in the cytoplasm. They move towards the plasma membrane where they give rise to lomasomes, first
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described by Moore & McAlear (1961) as 'sponge-like structures'. These structures are apparently associated with secondary wall synthesis and chitin deposition in particular. The authors could not find multivesicular bodies in *Pythium*, which is known to have non-chitinous walls. A similar system of endoplasmic reticulum and vesicles has been reported by Marchant & Smith (1967) in young buds of *Rhodotorula glutinis* and is suggested to be responsible for the transport of wall material precursors.

The irregular profile of the plasma membrane is due to the fusion of the vesicles with the plasma membrane. In *Ascodesmis sphaerospora*, Brenner & Carroll (1968) have described 3 different elements which could be involved in wall synthesis: apical vesicles fusing with the plasma membrane, electron-dense granules associated with membrane systems, and lomasomes, these 2 last elements appearing only below the apical region. Recently, Girbardt (1969) and Grove & Bracker (1970) have carried out similar studies of cytoplasmic organization of hyphal tips among fungi. The authors recognize the lack of true Golgi apparatus, replaced in higher fungi by single cisternae systems which seem to take over the secretory function.

Marchant & Robards (1968) have revised the terminology of the different structures seen in association with the plasma membrane and commonly called 'lomasomes'. They proposed a general descriptive term 'paramural bodies' and suggested that lomasomes should be used only when information about their origin or their function was available. Lomasomes originate from multivesicular bodies, occur only in zones of wall thickening and are absent from the regions of rapid elongation. For the vesicular and membranous elements apparently entirely formed by invaginations of the plasma membrane they coined the term 'plasmalemmasomes'. Marchant & Robards (1968) suggested that these structures might be involved in secondary modifications of the cell wall.

The existence of paramural bodies in live material and their role in wall formation have often been questioned. Grove, Bracker & Morré (1970) mention that in *Pythium ultimum* they cannot find any 'direct evidence to connect the lomasome-like configurations with wall deposition or tip extension'. Evidence that paramural bodies may not be artifacts has been given by Sassen, Remsen & Hess (1967) and more recently by Griffiths (1970). Freeze-etching technique revealed numerous invaginations of the plasma membrane in conidia of *Penicillium megasporum* (Sassen et al. 1967) and the occurrence of paramural bodies in hyphae of *Verticillium dahliae* (Griffiths, 1970). Griffiths observed that the paramural bodies arose as evaginations of the plasma membrane and he related their existence to original cytoplasmic vesicles which had passed through the plasma membrane and were lying between the plasma membrane and the cell wall.

In *Stemphylium botryosum* it was not possible to detect any significant apical growth of the conidiophore between the 12- and 16-h stages. True apical vesicles as described by Brenner & Carroll (1968) and Grove & Bracker (1970) could not be found. Wall formation appears to involve a unique system of internal membranous elements. Grey and speckled substances (previously called 'osmiophilic granules' and 'grey inclusions' respectively by Carroll & Carroll, 1971) are synthesized between unit membrane leaflets in electron-transparent areas; globules of these substances appear to migrate
through the cytoplasm and are found in association with the plasma membrane. These substances may represent wall material components or precursors. Through the developmental stages observed the grey substance appeared always before the speckled substance, but the available information is too scant to correlate the respective functions of these substances with primary and/or secondary wall formation.

Plasmalemmasomes, interpreted here as evaginations of the plasma membrane, associated fibrillar material and masses of grey and speckled substances have been observed only at particular stages of conidiophore and conidium development, and near forming septa. Their restricted occurrence supports Marchant & Robards' (1968) hypothesis that plasmalemmasomes may be involved in secondary transformation of wall materials.

This investigation formed part of a Ph.D. thesis. The author wishes to acknowledge and express thanks for support received from the Graduate School of the University of Oregon, the Brown-Hazen Fund and the National Science Foundation (grant no. GB 26225 to G. C. Carroll) while this work was in progress.

REFERENCES


Conidium initiation in *Stemphylium*


(Received 9 December 1971)

**ABBREVIATIONS ON PLATES**

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<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>e</td>
<td>electron-transparent area</td>
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<tr>
<td>er</td>
<td>endoplasmic reticulum</td>
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<td>gs</td>
<td>'grey substance'</td>
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<tr>
<td>m</td>
<td>mitochondrion</td>
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<td>ml</td>
<td>middle lamella</td>
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<td>n</td>
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<td>pl</td>
<td>plasmalemmosomes</td>
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<td>ss</td>
<td>'speckled substance'</td>
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<td>tw</td>
<td>Woronin Body</td>
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Fig. 1. Tip cell of conidiophore (12 h). $\times 16930$.

Fig. 2. Tip of conidiophore (14.5 h). Arrows point at globules of grey substance. $\times 16930$.

Fig. 3. Detail of Fig. 2. $\times 59850$. 

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1. ml
2. m
3. e
4. er
5. n
6. pl
7. gs
8. w
Fig. 4. Conidium initial (16 h). Note the continuity between the wall of the conidiophore and the wall of the initial. $\times 34,480$.

Fig. 5. Conidium initial (17 h). Note masses of speckled substance at the base of the cytoplasmic junction and the small invaginations in the conidium plasma membrane (arrows). $\times 34,480$. 
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Fig. 6. Conidiophore bearing young conidium (17 h). Arrows point at areas shown in detail in Figs. 7-10. × 25,050.
Figs. 7-10. Details of Fig. 6. × 77750.