

ASCOSPORE ULTRASTRUCTURE AND DEVELOPMENT IN *OPHIOSTOMA CUCULLATUM*

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ABSTRACT

Ultrastructural aspects of development of hat-shaped ascospores and centrum organization in *O. cucullatum* were studied and compared with other species in *Ceratocystis sensu lato* apparently having similar ascospores. The ascospore wall consisted of a reniform primary and ornamented secondary wall. Secondary wall ornamentation incorporated three equally spaced and parallel ridges along the length of the ascospore that gave the ascospore a triangular outline in transverse section. Brim appendages developed on the secondary wall at the rounded ends of the primary wall resulting in the hat-shaped appearance of the ascospores in side view. These appendages differed considerably from those of the symmetrically shaped and hemispherical bowler-hat ascospores previously observed in *C. moniliformis* and *C. fimbriata*. Centrum organization in *O. cucullatum* was, however, similar to that previously illustrated of *O. davidsonii*. In both of the latter species, young asci were arranged in a cluster at the ascomatal base. This was different to that in *C. moniliformis* where young asci lined the inner ascomatal wall.

Key Words: *Ceratocystis*, *Ophiostoma*, hat-shaped ascospore, centrum, ultrastructure

Ceratocystis sensu lato includes the genera *Ceratocystis* Ellis et Halsted *sensu stricto*, *Ophiostoma* H. & P. Sydow and *Ceratocystiopsis* Upadhyay et Kendrick (Upadhyay and Kendrick, 1975; Weijman and De Hoog, 1975; Upadhyay, 1981; De Hoog and Scheffer, 1984). These are insect-associated fungi and include many important plant pathogens (Boyce, 1961; Wood and French, 1963; Solheim, 1986; Clark and Moyer, 1988).

Many species of *Ceratocystis*, *Ophiostoma* and *Ceratocystiopsis* have sheathed ascospores of unusual morphology constituting an important characteristic used in their taxonomy (Hunt, 1956; Olchowecki and Reid, 1974; Upadhyay, 1981; De Hoog and Scheffer, 1984). Despite this, few ultrastructural (Garrison *et al.*, 1979; Jeng and Hubbes, 1980; Stiers, 1976; Van Wyk *et al.*, 1991), and mostly light microscopic studies, have been conducted on ascospore development and structure in *Ceratocystis s.l.* We believe that ultrastructural studies could enhance understanding

of ascospore morphology and provide us with additional tools to distinguish between genera and species (Van Wyk and Wingfield, 1990), that ultrastructural studies could lead to a better understanding of the taxonomic disposition of *Ceratocystis s.l.* within the Ascomycetes.

In a previous study the development and structure of hat-shaped sheaths of ascospores in *O. davidsonii* (Olchowecki et Reid) Solheim has been reported (Van Wyk and Wingfield, 1991). These ascospores are morphologically distinct from the hat-shaped ascospores in species of *Ceratocystis s.s.*, such as *C. fimbriata* Ellis et Halsted (Stiers, 1976) and *C. moniliformis* (Hedgcock) C. Moreau (Van Wyk *et al.*, 1991).

O. cucullatum Solheim has a *Phialographium* Upadhyay et Kendrick anamorph and is in this sense similar to *O. davidsonii* (Solheim, 1986; Wingfield *et al.*, 1989). *O. cucullatum* also resembles *O. davidsonii* in that it has reniform ascospores. Sheaths around the ascospores in *O. cucullatum* have a distinct brim, whereas those

of *O. davidsonii* are reniform without brims (Upadhyay, 1981). The aim of this investigation was therefore to study ascospore development in *O. cucullatum* and to compare it with that previously observed in *O. davidsonii*.

MATERIAL AND METHODS

Cultures of *O. cucullatum* (CBS 218.83) isolated from *Ips typographus* L. were grown at 18 C on 2% malt extract agar (20 g Difco Malt Extract, 20 g Difco Bacto Agar, 100 ml water) in Petri dishes. Cultures were illuminated by diurnal cycles of fluorescent and near-ultraviolet light. Ascospores for electron microscopic examination, attached to small (2 × 4 × 8 mm) blocks of agar were fixed in 0.1 M (pH 7.0) sodium phosphate-buffered glutaraldehyde (3%) for 3 h at 20 C, followed by 1 h fixation in similarly buffered osmium tetroxide (0.5%). The material was dehydrated in a graded ethanol series and embedded in an epoxy resin (Spurr, 1969). Ultrathin (60 nm) sections were cut with glass knives, using an LKB Ultratome III. Sections were stained for 20 min with uranyl acetate, 10 min with lead citrate (Reynolds, 1963), and examined with a Philips EM300 transmission electron microscope.

RESULTS

A vertical section through the ascoma of *O. cucullatum* showed developing asci extending upwards and outwards from the ascomatal base towards the neck (Figs. 1, 17D). The young asci occupied approximately one third of the volume of the ascoma. Mature ascospores were observed near the ascomatal neck (Figs. 1, 17D). The inner zone of the ascomatal wall at the ascomatal base, adjacent to the developing asci, consisted of 2–4 layers of large, sterile or cushion cells (Figs. 1, 17D). These cells became narrower and flattened toward the neck.

Young irregularly shaped asci, characterized by thin walls, were observed near the ascomatal base (Figs. 1, 17A). Vesicles occurred in the ascus cytoplasm (Figs. 2, 17A) and, at higher magnification, some of these vesicles appeared to be membranes arranged in circular patterns (myelin figures) (Fig. 3). These membranes appeared to coalesce to form the membrane precursors of an ascus vesicle (Figs. 4, 17A). One ascus vesicle occurred in each ascus and surrounded nuclei

and other cellular inclusions (Figs. 4, 17A). This ascus vesicle invaginated to delimit each nucleus, forming young ascospores, each surrounded by sac-like double membranes (perispore sac) and walls (Figs. 4, 17A).

At an early stage in the development of the ascospores, delimiting membranes and walls were discontinuous in certain areas (Fig. 5). It appeared that in these areas, two thin walled layers developed simultaneously with new membranes of the ascus vesicle, to enclose each young ascospore. We suggest that it is during this stage, that wall material and membranes are synthesized. Wall material is thus deposited to the inside and along the existing membranes (Fig. 5).

During further development of the ascospore wall vesicles coalesced with the delimiting membranes (Fig. 6). Additional wall substances are probably deposited between the existing thin-walled layers and membranes by these vesicles. The inner thin wall layer differentiated into a granular primary wall (endospore layer) enclosing the cytoplasm (Fig. 7). The outer wall layer differentiated into a granular to fibrillar secondary wall (mesospore) layer (Figs. 8–10, 14, 17C).

Ornamentation formed on the secondary wall through the deposition of wall material by vesicles between the outermost delimiting membrane and underlying secondary wall (Fig. 8). This deposited material differentiated into a granular layer (Figs. 9, 10), becoming more uniform in maturing ascospores (Fig. 10). Membranes of unknown origin and function were observed closely associated with the outer membrane of the ascospore (Fig. 10), during the development of secondary wall ornamentation.

Expansion of the secondary wall (Fig. 7), resulted in the enlargement of the space between the primary wall and outer membrane of the perispore sac. During this expansion, a prominent ridge developed on the long axis of each ascospore (Figs. 11, 17C). Within the ascus, the ascospores were arranged with this ridge oriented towards the middle of the ascus (Figs. 11, 17B).

Two additional ridges developed at opposite sides and simultaneously with the previously described ridge. These ridges also developed on the long axis of the ascospore at an angle of approximately 120° to each other (Figs. 12, 17B). Following further development of the ridges, the ascospores became triangular and were closely packed within the ascus as observed in transverse section through the asci (Figs. 12, 17B).

In longitudinal sections of mature ascospores, the cytoplasm of the ascospores was surrounded by a reniform primary wall (FIGS. 13, 17C). The secondary wall ridges (ornamentation) formed distinct spindle-shaped appendages at the rounded ends of the primary wall (FIGS. 13, 17C). The secondary wall ornamentation appeared dentate (FIGS. 14, 17C), whereas the secondary wall close to the primary wall had a granular to fibrillar appearance (FIG. 14). In transverse section, the primary wall was circular and the three ridges of the secondary wall gave the ascospore a triangular appearance (FIG. 15).

The asci finally disintegrated to release the mature ascospores towards the ascomatal neck. At maturity the inner zone of the ascomatal wall (cushion cells) collapsed. This resulted in the formation of a lining of membranes on the inner side of the ascomatal wall (FIG. 16).

DISCUSSION

The arrangement of asci in the centrum of *O. cucullatum* appeared to be similar to that of *O. davidsonii*. As was found in *O. davidsonii* (Van Wyk and Wingfield, 1991), young asci in *O. cu-*

cullatum were arranged in a cluster at the base of the ascoma. In both species, mature ascospores were released above the developmental area towards the ascomatal neck. In contrast, the developing asci in *C. moniliformis* formed a lining of cells around the innermost ascomatal wall. Mature ascospores were released towards the center of the ascoma (Van Wyk *et al.*, 1991).

Ascomata in both *O. davidsonii* and *O. cucullatum* are characterized by large sterile or cushion cells forming the innermost ascomatal wall layer. These cells were not observed in *C. moniliformis* (Van Wyk *et al.*, 1991). By disintegrating during ascus development, cushion cells may have the important function of providing space for the developing asci (Luttrell, 1951; Van Wyk and Wingfield, 1990). These cells were not observed in the mature ascoma. However, a lining of membranes derived from the disintegration and collapse of the cushion cells was observed adjacent to the innermost ascomatal wall. Similar membranes have also been observed in *O. stenoceras* (Robak) Melin *et Nannfeldt* (Garrison *et al.*, 1979).

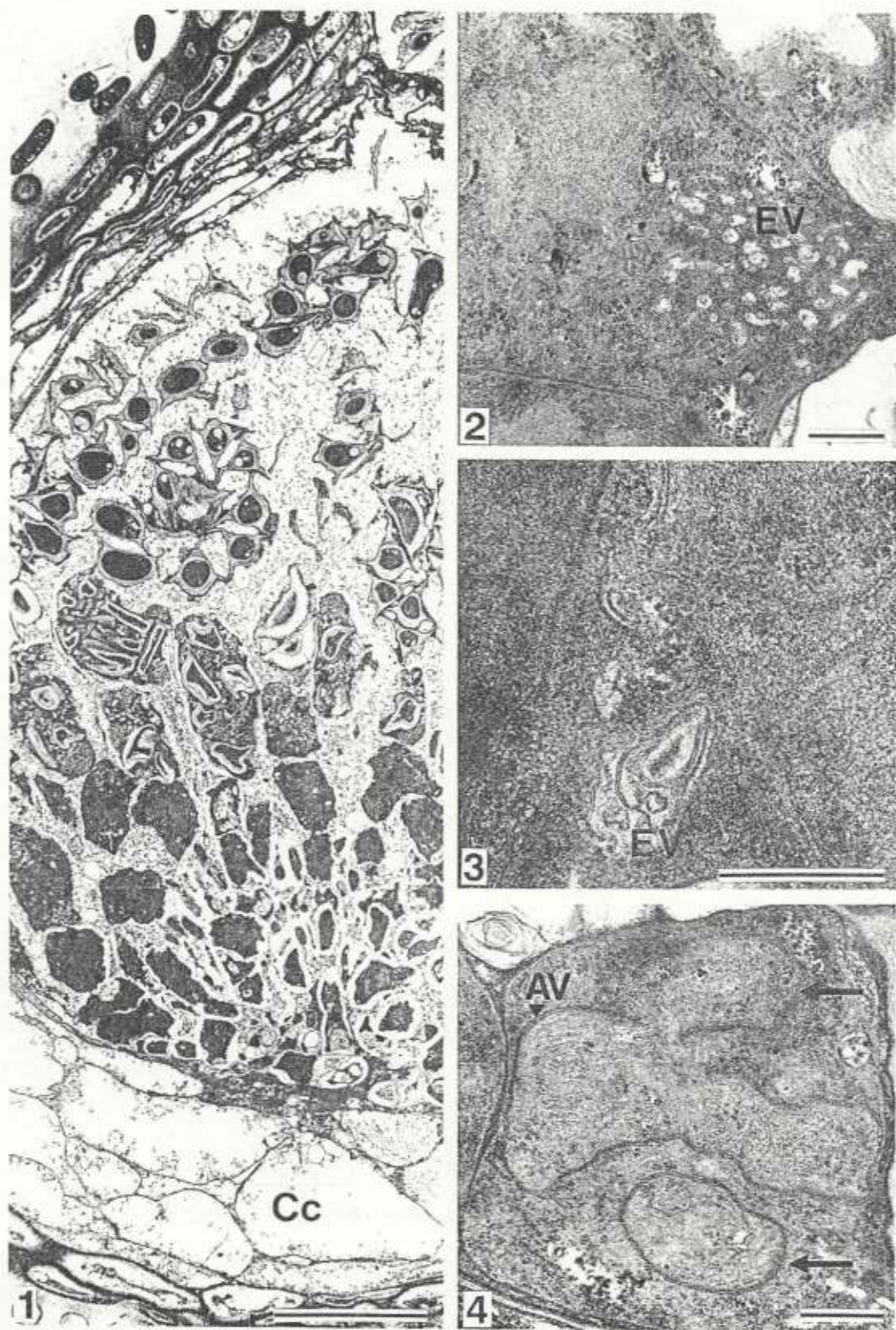
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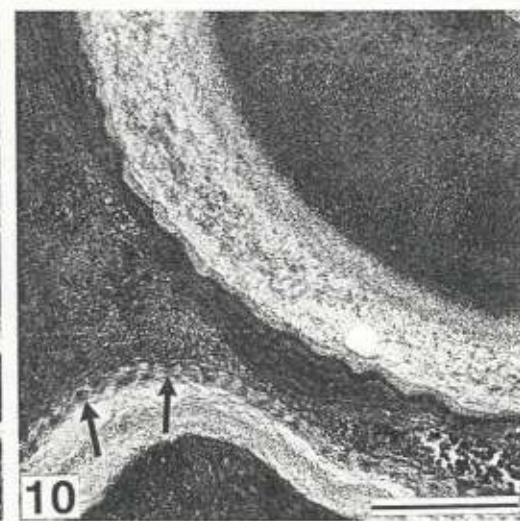
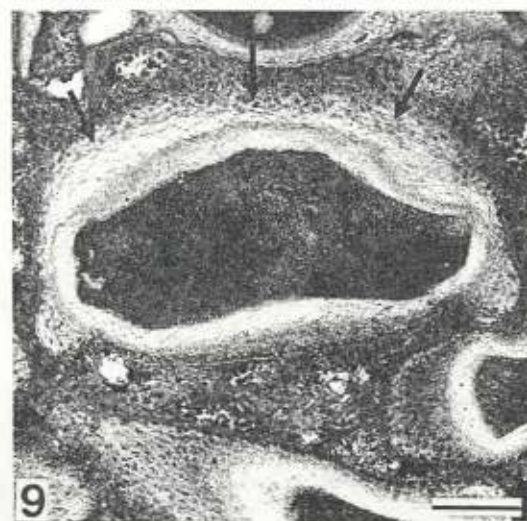
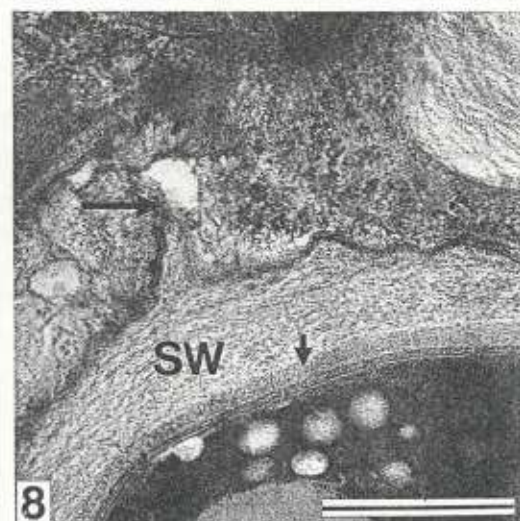
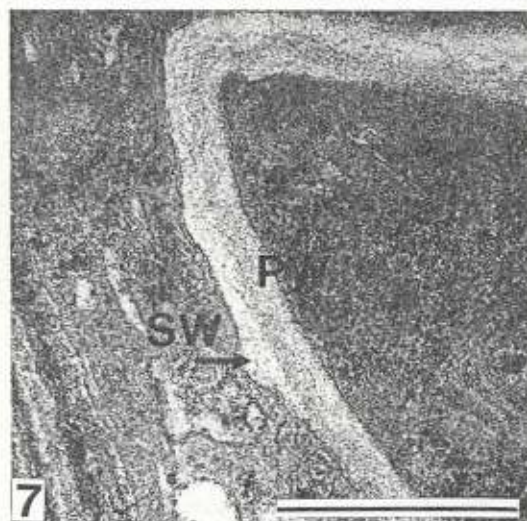
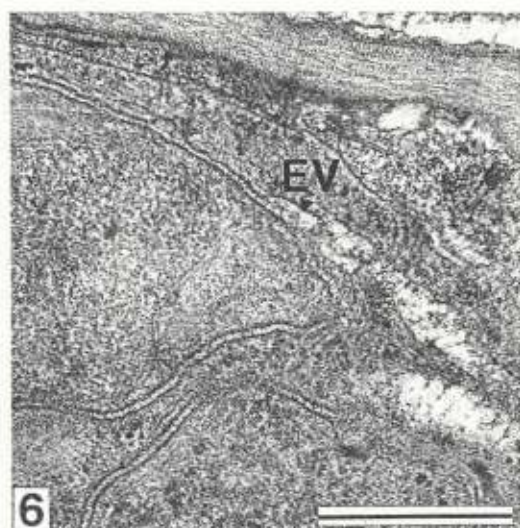
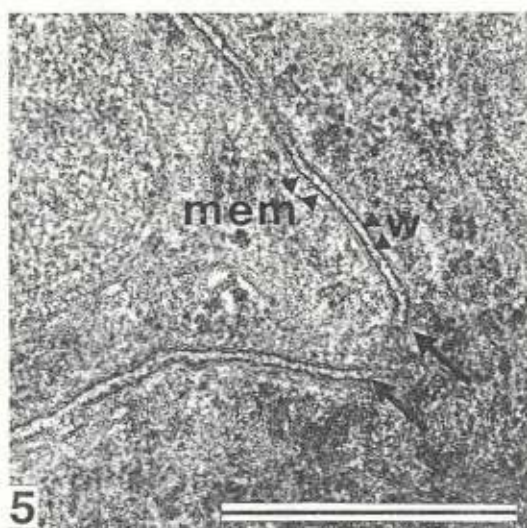
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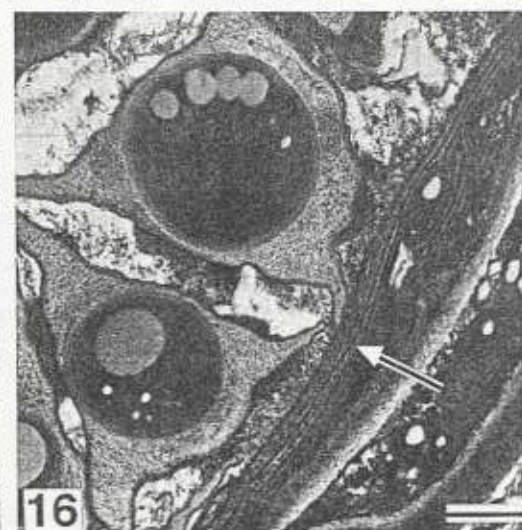
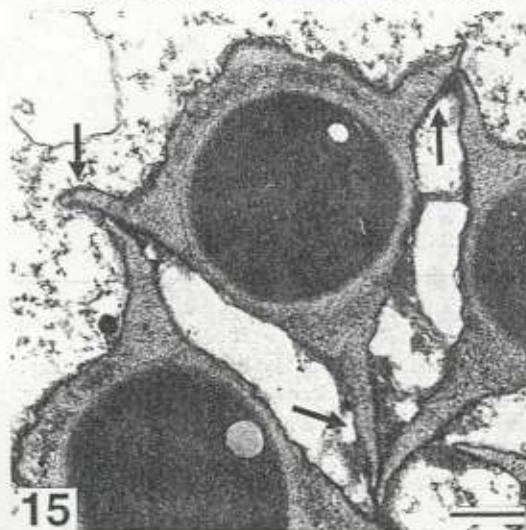
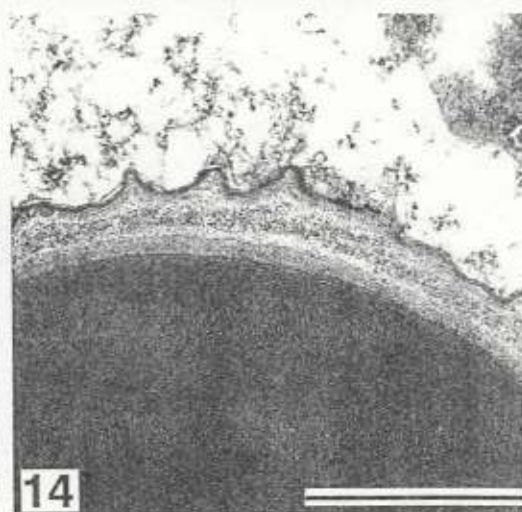
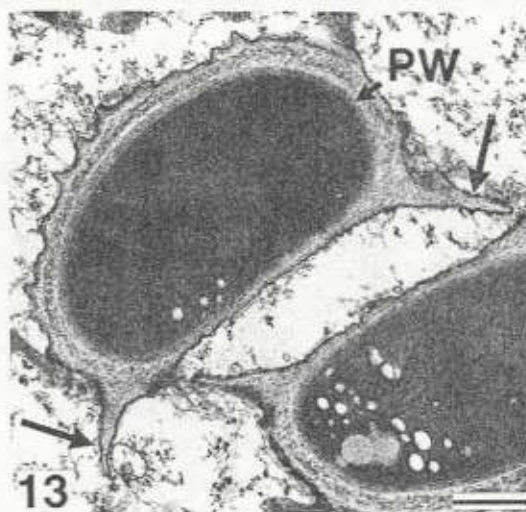
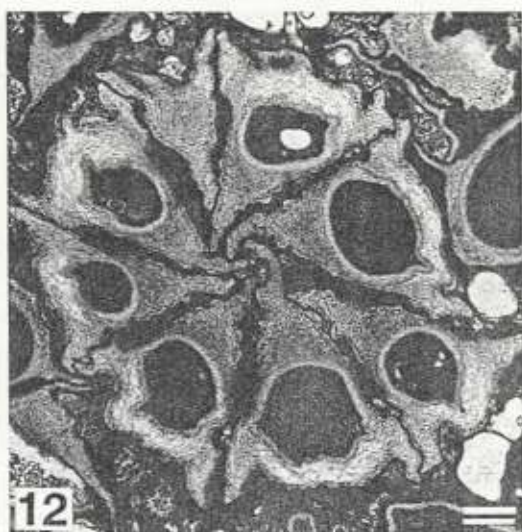
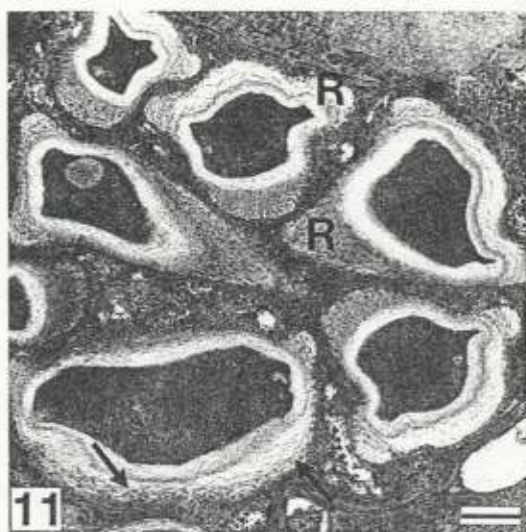
FIGS. 1-4. Transmission electron micrographs (TEM) of sections through the ascoma of *O. cucullatum* showing ascus and ascospore development. Bar = 500 nm in FIGS. 2-4. 1. Vertical section through ascoma showing developing, thin walled asci arranged in chains at the base and mature ascospores near the neck of the ascoma. Large sterile cushion cells (Cc) are arranged along the periphery of the ascomatal wall. Bar = 10 μ m. 2. Developing ascus with vesicles (EV). 3. Ascus with predecessors of an ascus vesicle formed by the coalescence of membranes and vesicles (EV). 4. Ascus containing young ascospores (arrows). Note some nuclei surrounded by developing ascus vesicle (AV).

FIGS. 5-10. TEM of sections through developing asci and ascospores. Bar = 500 nm. 5. Magnified area of young ascospore delimiting walls (w) and membranes (mem), indicating apparent synthesis of walls between and at open areas of membranes (arrows). 6. Young ascospore showing coalescence of vesicle (EV) with delimiting membrane, prior to differentiation of inner wall layer into the primary wall. 7. Ascospore showing differentiation and development of the primary wall layer (PW) between membranes. Note commencement of secondary wall development (SW). 8. Magnified area of ascospore wall showing vesicle coalesced with outer delimiting membrane (arrow) during deposition of outermost layer of the secondary wall. Note granular appearance of the primary wall (small arrow) and fibrillar appearance of the secondary wall (SW). 9. Ascospore showing initial granules of the outermost layer of the secondary wall (arrows). 10. Magnified area of mature ascospore wall showing uniformly granular appearance of the primary wall and fibrillar appearance of secondary wall. Note granules of the outermost secondary wall layer in adjacent younger ascospore (arrows) and membranes associated with the outer delimiting membrane.

FIGS. 11-16. TEM of sections through developing asci and ascospores. Bar = 500 nm. 11. Development of secondary wall ornamentation ridges (R). Note deposition of granules (arrows). 12. Ascus with eight ascospores in cross section. Note the arrangement of the ascospores and triangular appearance of secondary wall ornamentation. 13. Longitudinal section of mature ascospore. Note the reniform outline of the primary wall (PW) and brim-like appendages formed by the secondary wall (arrows). 14. Longitudinal section of part of mature ascospore showing primary wall and secondary wall with ornamentation. 15. Mature ascospores in transverse section showing ridges formed by secondary wall (arrows). 16. Innermost membranous lining of ascomatal wall (arrow) and mature ascospores in transverse section.







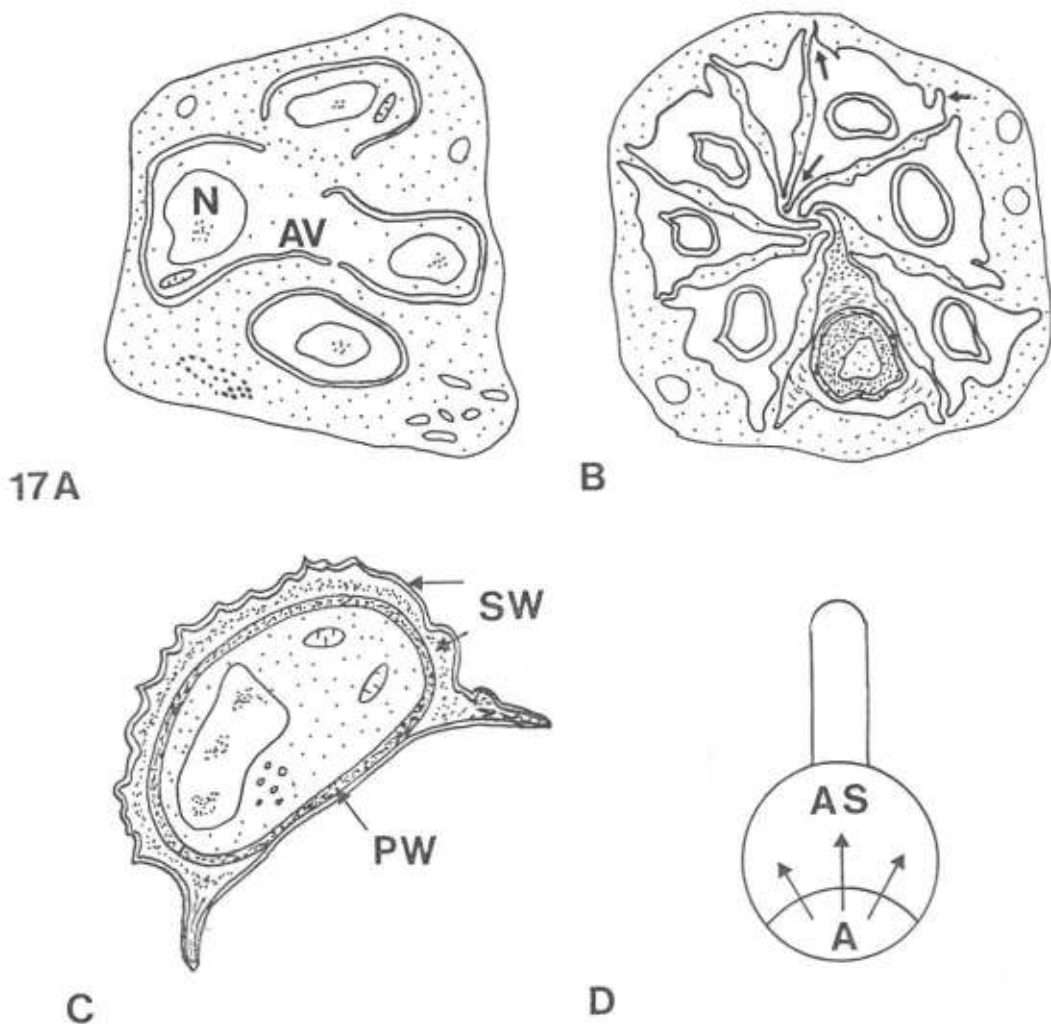


FIG. 17. Schematic representation of ascus, ascospore and centrum development in *O. cucullatum*. A. Young ascus with ascus vesicle (AV) surrounding ascospore nuclei (N). Note sac-like delimiting membranes enclosing ascospore nuclei. B. Mature ascospores in transverse section showing the sheath ridges (arrows) and triangular appearance of the wall layers. C. Mature reniform ascospores released from degenerating ascus with distinctive wall morphology (PW = primary wall, SW = secondary wall). D. Centrum of *O. cucullatum*. A, region of young developing asci; AS, region of maturing asci and mature ascospores.

terminology used for wall layers in the study of Stiers (1976) on ascospore development in *C. fimbriata*. The ascospore walls of this species were described as an innermost endospore wall and outermost episporic wall layer, with a mesospore layer separating these layers. At this stage we believe that the terminology for these layers should be standardized with that of other Ascomycetes (Parguey-Leduc *et al.*, 1987; Van Brummelen, 1989; Kimbrough *et al.*, 1990; Wu and Kimbrough, 1990). The terms endo- and

mesospore are thus replaced by the terms primary wall and secondary wall. The episporic becomes ornamentation on the secondary wall.

Ascospore formation in *O. cucullatum* commenced through the development of delimiting membranes to form a single ascus vesicle within each ascus. This ascus vesicle surrounded the nucleus in a sac-like structure. The origin of delimiting membranes in *O. cucullatum*, prior to the formation of an ascus vesicle, is unknown. Various theories, however, exist concerning the

origin of such membranes in other Ascomycetes. It has, for instance, been reported that these membranes originate from the endoplasmic reticulum, nuclear envelope (Carroll, 1967; Beckett and Crawford, 1970) or the plasmalemma (Bandoni *et al.* 1967; Kimbrough *et al.*, 1990). Delimiting membranes and walls in *C. moniliformis* appear to be synthesized by a *de novo* mechanism (Van Wyk *et al.*, 1991). In *O. cucullatum* delimiting membranes were similar to those observed in *C. fimbriata* (Stiers, 1976), *O. ulmi* (Buisman Nannfeldt (Jeng and Hubbes, 1980), *O. steno-ceras* (Garrison *et al.*, 1979) and *O. davidsonii* (Van Wyk and Wingfield, 1991), and probably have the same origin.

The synthesis of wall material observed in *O. cucullatum* appears to be different to that occurring in *C. moniliformis*. In *C. moniliformis* the ascus vesicle that surrounded all nuclei at first, consisted of membranes and wall material. The membranes and walls were synthesized simultaneously during the appearance of an ascus vesicle. The walls thus formed an integral part of the ascus vesicle at the first appearance of delimiting membranes (Van Wyk *et al.*, 1991).

In *O. cucullatum*, in contrast to *C. moniliformis*, the synthesis mechanism for wall material primarily functions during the invagination process and apparently not during the appearance of delimiting membranes. Two separate, thin wall layers are synthesized between the membranes during the invagination process of the ascus vesicle to form a sac-like structure that surrounds each nucleus. Wall formation thus appears to occur independently of the membranes associated with the formation of the initial ascus vesicle. These thin wall layers would then form the preliminary layers of the primary wall and secondary wall in *O. cucullatum*.

Ascospore wall formation in *O. cucullatum* and *O. davidsonii* was predictably similar. In both species, a primary wall developed between the delimiting membranes. The reniform primary wall surrounded the ascospore cytoplasm. The secondary wall developed between the primary wall and outermost delimiting membrane of the perispore sac. This outermost ornamentation gave the ascospores their unique shape.

Lomasomes were observed in this study of *O. cucullatum* and are believed to play a role in wall building processes (Wilsenach and Kessel, 1965; Marchant and Moore, 1973; Stiers, 1974). They have been observed in previous ultrastructural

studies of *Ceratocystis s.l.* (Stiers, 1976; Van Wyk and Wingfield, 1991), but have been absent elsewhere (Garrison *et al.*, 1979; Jeng and Hubbes, 1980). Information available on the occurrence of lomasomes in cells, their specific structure and function, appears to be contradictory (Marchant and Moore, 1973). We therefore do not consider lomasomes to be a reliable feature on which to base comparisons of species.

The most prominent difference between *O. cucullatum* and *O. davidsonii* was in the arrangement of the ascospores in the asci. In *O. davidsonii*, ascospores were randomly arranged in the ascus. In contrast, ascospores in *O. cucullatum* were arranged with one ridge on each ascospore oriented towards the center of the ascus. The differential expansion of the secondary wall and ornamentation of the ascospores to form ridges was probably a consequence of the close packaging of the ascospores in the ascus. In both *O. davidsonii* and *O. cucullatum*, the differential deposition of wall material along ridges on the ascospores within the confined space of asci probably resulted in the triangular appearance of the ascospores in transverse section.

Although ascospore walls in *O. cucullatum* and *O. davidsonii* were hat-shaped, they were distinctly different to hat-shaped walls of *C. fimbriata* (Stiers, 1976), *C. moniliformis* (Van Wyk *et al.*, 1991) and *Hansenula* spp. (Bandoni *et al.*, 1967; Black and Gorman, 1971). In the latter species the ascospores are hemispherical and the hat-shaped structure of the walls may be ascribed to the development of the ascospores in pairs, which adhere to each other at the flattened sides of the brim. In contrast, the outline of the cytoplasm of the ascospores in *O. cucullatum* and *O. davidsonii* is reniform. The ascospores form singly in the ascus with ridges of their sheaths apparently developing as a consequence of their packaging in the ascus. The ridges formed appendages that gave the sheaths a hat-shaped appearance in side view.

In this study of *O. cucullatum*, the brims on the ascospore walls were evident and consistent with light microscopic observations (Upadhyay, 1981; Solheim, 1986). This is in contrast to brims of ascospores in *O. davidsonii*, which could only be seen at the ultrastructural level (Van Wyk and Wingfield, 1991). Reference to gelatinous sheaths in descriptions (Olchowecki and Reid, 1974; Upadhyay, 1981; Solheim, 1986) is apparently in error. The "sheath" layers of ascospores ob-

served in ultrastructural studies of *O. davidsonii* and *O. cucullatum* were clearly wall layers. Ascospores are, however, released in a gloeoid or slimy matrix that should not be confused with wall layers of the ascospores.

Ultrastructural studies in *O. cucullatum*, *O. davidsonii* and in species of *Ceratocystis s.s.* have highlighted many morphological characteristics that cannot be seen reliably using light microscopy. Interpretation of the characteristics of ascospore shape based on light microscopic observations thus should be cautiously applied in the taxonomy of this group of fungi.

The taxonomy of *Ceratocystis* within the Ascomycetes (Redhead and Malloch, 1977; Benny and Kimbrough, 1980; Upadhyay, 1981) and the generic placement of this group of fungi (Upadhyay, 1981; De Hoog and Scheffer, 1984) has widely debated. In this study we have followed the taxonomic scheme of De Hoog and Scheffer (1984) where *Ceratocystis s.s.* and *Ophiostoma* are treated as distinct genera. To understand the complexity of the ascospore wall and sheath development, as well as centrum organization in *Ceratocystis s.l.*, ultrastructural studies of as many species as possible should be undertaken. We believe that such studies are required before it is possible to define new criteria that could be applied in the taxonomy of *Ceratocystis s.l.*

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