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Ascospore development in *Ceratocystis moniliformis*

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The ultrastructure of perithecium, ascus and ascospore development in *Ceratocystis moniliformis* was studied and compared with that of other Ascomycetes. Ascospore formation was preceded by the synthesis of delimiting wall structures, associated with membranes. These coalesced to form an ascus vesicle surrounding eight nuclei. The vesicle wall invaginated to form lobes or sac-like structures enclosing each nucleus. Each young ascospore was paired on one side with a second ascospore. The walls differentiated to form brim appendages at the flattened sides of the paired ascospores. Furthermore, the walls developed into two layered hat-shaped sheaths. A third allantoid wall layer was formed towards the inside of the sheath apparently by lomasomes from within the ascospore. Auxiliary cells attached to developing asci and ascospores could function in supplying additional wall material and nutrients during spore development. The ultrastructure of *C. moniliformis* is compared with that of *C. fimbriata* which also has hat-shaped ascospores.

Ceratocystis sensu lato includes *Ophiostoma* H. & P. Sydow, *Ceratocystis* Ell. & Halst. *sensu stricto* and *Ceratocystiopsis* Upadh. & Kendr. (De Hoog & Scheffer, 1984; Upadhyay, 1981; Upadhyay & Kendrick, 1975; Weijman & De Hoog, 1975). These fungi are adapted to dispersal by insects and mites (Bridges & Moser, 1986; Upadhyay, 1981; von Arx & Van der Walt, 1987; Wingfield & Marasas, 1980).

Light microscope studies of ascocarp development in species of *Ceratocystis sensu lato*, including the structure of the perithecium and ascospores, have been conducted (Andrus, 1936; Andrus & Harter, 1933; Bakshi, 1951; Dade, 1928; Elliott, 1925; Gwynne-Vaughan & Broadhead, 1936; Hutchinson, 1950; Moreau & Moreau, 1952; Rosinsky, 1961; Sartoris, 1927; Taylor-Vinje, 1940). In contrast, ultrastructural investigations have been limited to only a few species. These include *Ophiostoma stenoceras* (Robak) Melin & Nannf. (Garrison *et al.*, 1979), *O. ulmi* (Buism.) Nannf. (Jeng & Hubbes, 1980) and *C. fimbriata* Ell. & Halst. (Stiers, 1976). Additional cytological studies could thus contribute to improving the taxonomy of this group.

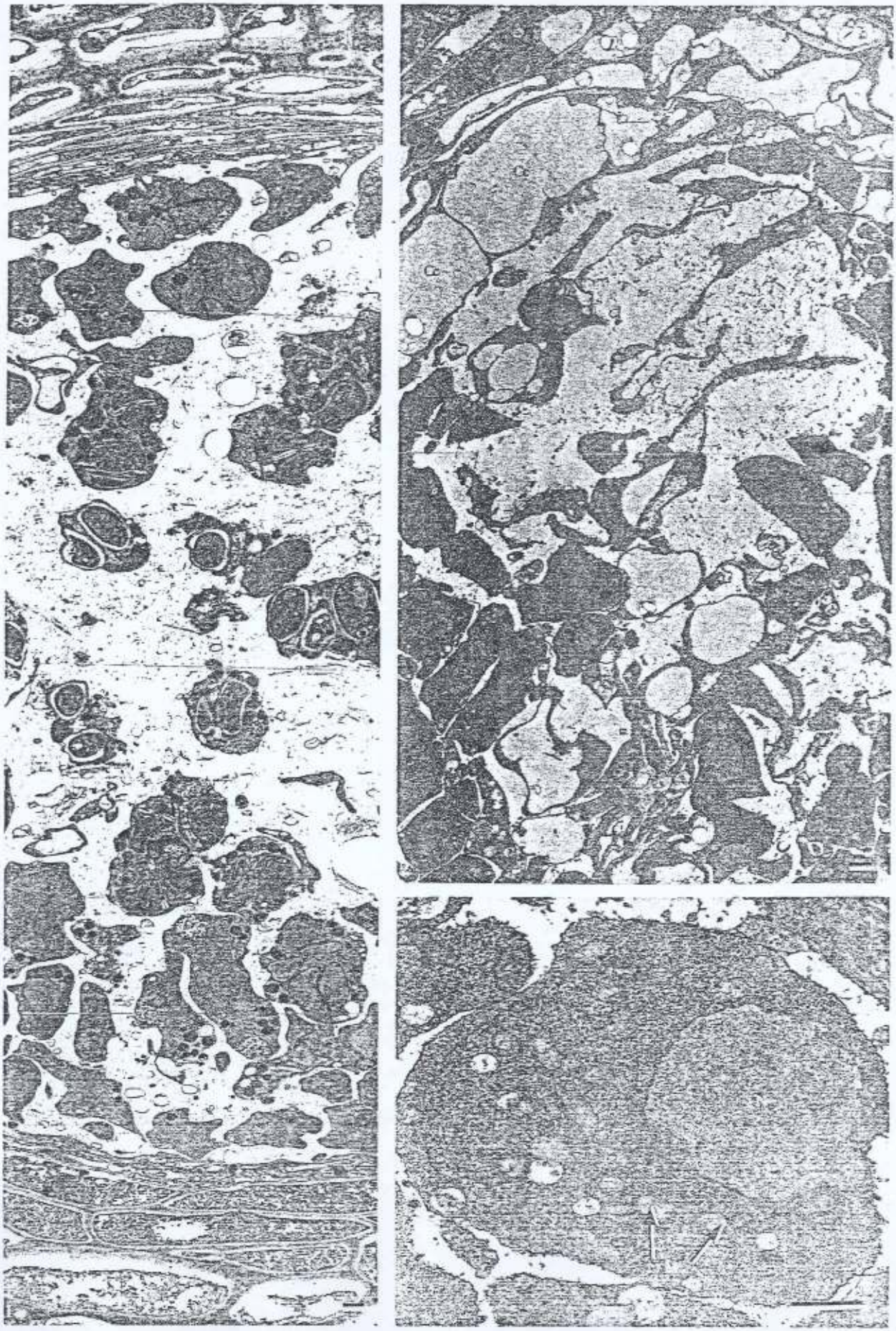
Some species of *Ceratocystis sensu lato* are characterized by having ascospores with unusual-shaped sheaths. These include spores with inequilateral, needle, hat-shaped, or pillow-shaped sheaths (Upadhyay, 1981). The only other similar ascospore forms are found in certain yeast genera (Bandoni *et al.*, 1967; Kurtzman & Ahearn, 1976). Of the five groups of ascospore forms that occur in *Ceratocystis*, only species having hat-shaped sheaths (*C. fimbriata*) and those with no sheaths (*O. ulmi* and *O. stenoceras*) have been studied ultrastructurally. This study was undertaken to compare the development and shape of ascospores with hat-shaped sheaths in *C. moniliformis* (Hedgc.) C. Moreau with those of *C. fimbriata*.

MATERIALS AND METHODS

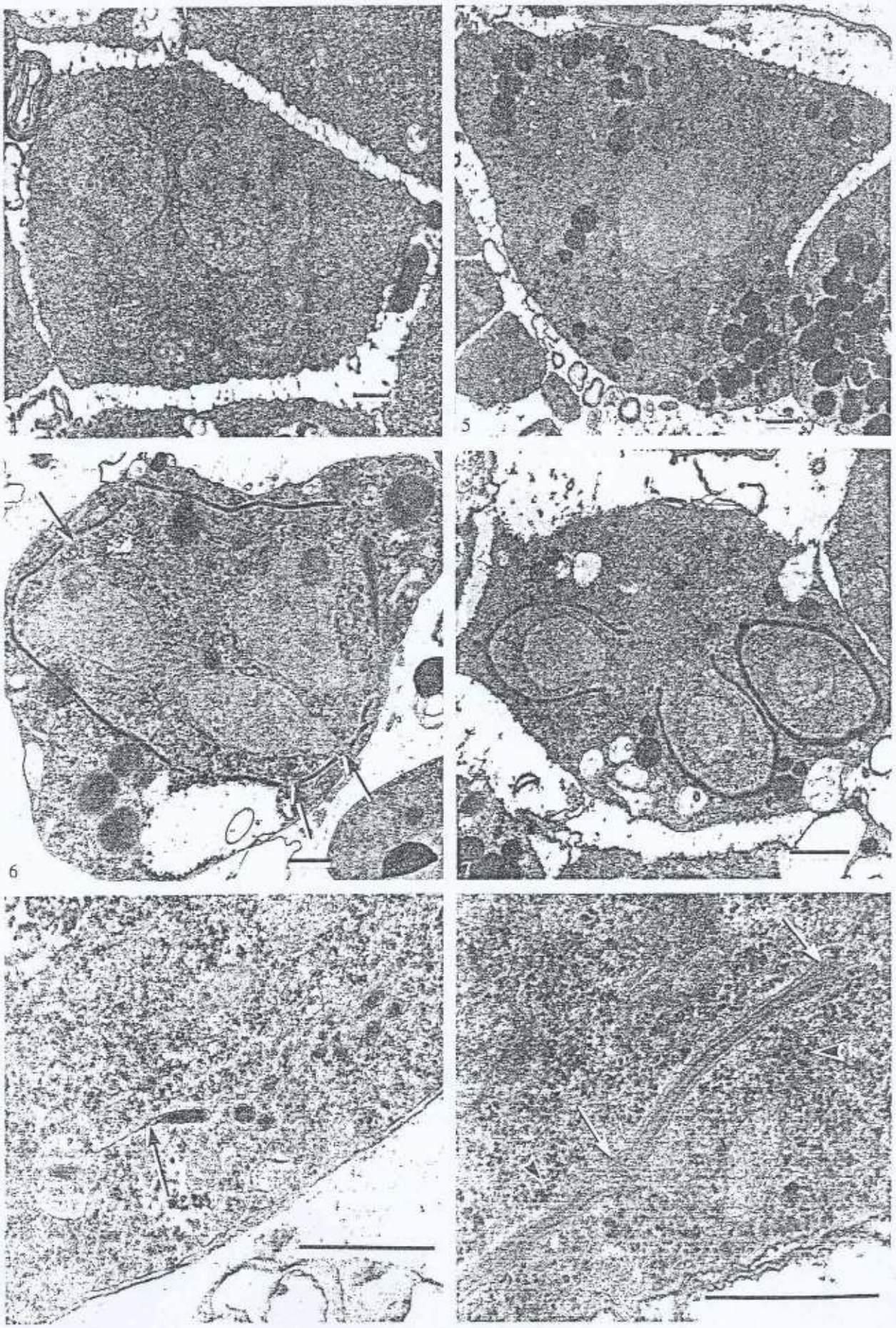
An isolate of *C. moniliformis* was obtained from the wood surface of freshly felled *Macaranga capensis* (Baill.) Benth. ex Sim, Natal, South Africa. Cultures were grown on 2% malt extract agar (20 g Difco Malt Extract, 20 g Difco Bacto Agar, 1 l H_2O), at a constant temperature of 18 °C, under uv-light to promote perithecium formation. Perithecia attached to small (2 × 4 × 8 mm) blocks of agar, were prepared for electron microscopy by fixing in 0·1 M (pH 7·0) sodium phosphate-buffered glutaraldehyde (3%) for 3 h at 20°, followed by 1 h fixation in similarly-buffered OsO_4 (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 a LKB III Ultrotome. 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

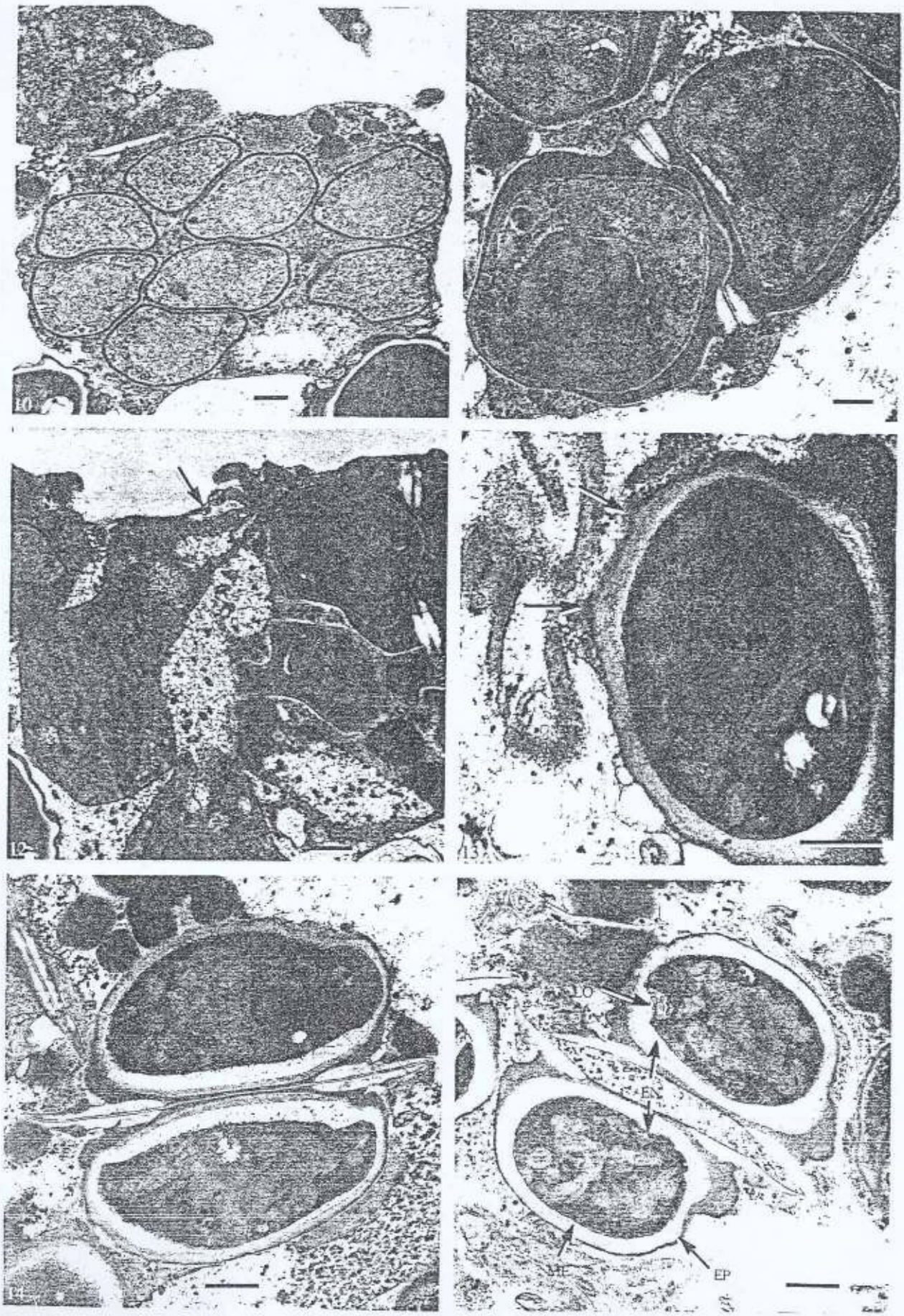
Electron micrographs comprising a cross section through a developing perithecium showed different developmental stages of asci and ascospores (Fig. 1). Asci appeared scattered throughout the perithecium, separated by large electron



Figs 1–3. For caption see p. 100.



Figs 4–9. For caption see p. 100.



Figs 10–15. For caption see p. 100.

transparent areas. These areas contained cell debris, apparently arising from degenerating mature asci. Closely packed, irregularly-shaped cells, probably ascogenous cells and young asci, were observed adjacent to the perithecium wall and mature ascospores towards the perithecium centre (Fig. 1). The asci extend from the base of the perithecium up to the neck. Asci were not observed on perithecium walls at the base of the neck (Fig. 2).

Five stages in ascospore development are described. First the young ascus contained a single nucleus, abundant mitochondria and other cellular inclusions (Figs 3, 16A). Next, nuclear divisions resulted in the formation of individual ascospore nuclei (Figs 4, 16B). The third stage was defined by the occurrence of osmiophilic bodies (Figs 5, 16C) and the fourth by the synthesis of an electron dense spore delimiting wall (Figs 6, 16D).

Delimiting walls of ascospores developed adjacent to and nearly concentric with the ascus wall surrounding most or all cellular inclusions and nuclei (Figs 6, 16D). The walls coalesced and invaginated to enclose each nucleus and other cellular inclusions forming the individual ascospores (Fig. 7). In longitudinal section, these walls appeared as sac- or pouch-like structures surrounding the nucleus of each developing ascospore. The open ends of these sac-like structures were usually turned towards one side of the ascus before final ascospore delimitation (Figs 7, 16E). Wall formation was apparently associated with membranes (Figs 8, 16D), but association with nuclear membranes or endoplasmic reticulum was not observed. Two membranes were formed, apparently through *de novo* synthesis, with an electron dense substance between them. The formation of the electron dense substance appeared to occur simultaneously with the production of the membranes themselves (Fig. 9).

In transverse section, eight ascospores could be observed in each ascus. The ascospores were formed in pairs and adhered to each other at the brim area of the developing wall (Figs 10, 16F).

The most prominent feature of the fifth stage of ascospore development, was the differentiation of the delimiting walls into a distinctive double-layered sheath, enveloping each spore (Figs 11, 14, 15). Development of the brim of the sheath was initiated by the synthesis of thickened highly electron transparent extensions formed at the edges and base of the paired ascospores (Figs 11, 16G). An inner electron transparent spore wall separated the spore from the surrounding sheath (Figs 14, 15, 16H).

During the fifth stage, unusual auxiliary cells attached to the ascus and probably the spore wall (Figs 12, 16G). Later, these auxiliary cells separated from the asci and mature ascospores. The area where the auxiliary cells were connected to the spore wall was sealed by closure of the epispore (Fig. 13). A further feature of the fifth developmental stage was the presence of lomasomes (Figs 14, 15, 16G), that appeared to contribute to the formation of the inner walls. After sheath and wall formation was completed, the asci degenerated (Figs 14, 15) and the paired ascospores separated (Fig. 15). This left individual mature ascospores amongst the debris of asci and within the perithecial cavity. The sequence of development of asci and ascospores in *C. moniliformis* is summarized diagrammatically in Fig. 16.

DISCUSSION

In *C. moniliformis* young asci appear to be formed from ascogenous cells forming a lining of developing asci adjacent to the perithecium wall. Asci do not appear to be formed in

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Figs 1–3. Development, morphology and ultrastructure of the perithecium and asci in *C. moniliformis* (bar = 1 μm). **Fig. 1.** Transverse section through middle region of the perithecium, showing developing, irregularly-shaped asci adjacent to the perithecium wall and mature asci and ascospores scattered in the centre. **Fig. 2.** Transverse section through the neck region of the perithecium. Note lack of asci near the base of the neck (NB) and sterile cushion cells (Cc). **Fig. 3.** First developmental stage represented by young ascus containing nucleus (N) and several mitochondria (M).

Figs 4–9. TEM of developing asci and ascospores in *C. moniliformis* (bar = 0·5 μm). **Fig. 4.** Second developmental stage. Ascus showing two of possible eight nuclei. **Fig. 5.** Third developmental stage of ascus with osmiophilic bodies. **Fig. 6.** Fourth developmental stage of ascus showing synthesis of preliminary ascospore delimiting wall surrounding three nuclei and cellular inclusions. Note coalescing areas of walls (arrows). **Fig. 7.** Longitudinal section of ascus and ascospores showing invaginating walls surrounding nuclei with sac-like structures forming young ascospores. **Fig. 8.** *De novo* synthesis of delimiting wall structures. Note association of the preliminary wall with membranes (arrow). **Fig. 9.** Advanced stage of delimiting wall and membrane formation. Note synthesis regions (arrows) in close association with ribosomes (R).

Figs 10–15. TEM of developing ascospores (bar = 0·5 μm). **Fig. 10.** Transverse section of ascus and ascospores showing four pairs of young ascospores during final stage wall delimitation. **Fig. 11.** Paired, young ascospores closely associated during formation of the brim of the sheath. **Fig. 12.** Sterile auxiliary cell which possibly contributes to ascospore wall formation. Note auxiliary cell appendages (arrow). **Fig. 13.** Ascospore separating from sterile auxiliary cell appendages. Note wall area (epispore) sealing off the area of appendage connexion (arrows). **Fig. 14.** Adhering paired ascospores showing development of the endospore between mesospore and plasmalemma. **Fig. 15.** Paired ascospores separating after ascus degeneration. Note the prominent two-layered sheath, epispore (EP) and mesospore (ME) and the endospore (EN) or spore wall (LO = lomasome).

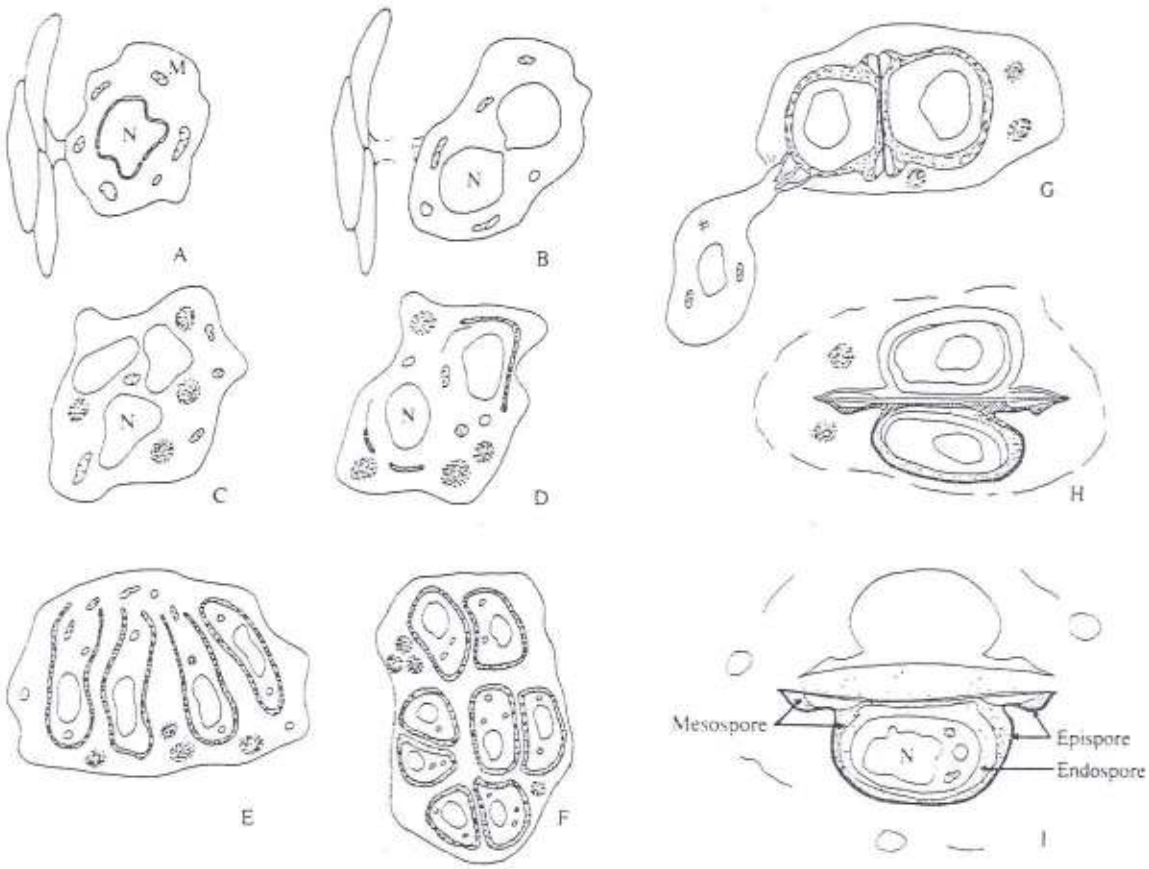

Fig. 16. Schematic representation of ascus and ascospore development in *C. moniliformis*. A. Young ascus with dividing nucleus (N); B. young ascus separating from perithecium wall with dividing nuclei; C. nuclear division completed and synthesis of osmiophilic wall material commences; D. delimiting wall formation associated with membranes; E. ascospore nuclei surrounded by sac-like delimiting walls, observed in longitudinal section; F. paired ascospores with enclosed nuclei; G. sterile auxiliary cell contributing to ascospore formation; H. degenerating ascus and ascospores adhering to each other. I. separating mature ascospore with distinctive three-layered wall.

the neck region where the ostiole will develop. Similar ascogenous cells were also reported for *O. sienoceras* (Garrison *et al.*, 1979), although their specific arrangement was not discussed.

A prominent feature of ascus and ascospore development in previous ultrastructural studies of *C. fimbriata* (Stiers, 1976), *O. sienoceras* (Garrison *et al.*, 1979) and *O. ulmi* (Jeng & Hubbes, 1980), is in the mechanism of spore delimitation. In the latter fungi, the ascospore wall is laid down between a double delimiting membrane when delimitation commences. A similar double ascospore delimiting membrane system was not observed in *C. moniliformis*. Ascospore delimitation in *C. moniliformis* is apparently achieved by wall structures and not membranes. Although these independently formed walls are associated with membranes, a separate double delimiting membrane system, was never observed.

It has been suggested that delimiting membranes originate from the endoplasmic reticulum, nuclear envelope (Beckett & Crawford, 1970; Carroll, 1967) or the plasmalemma (Bandoni *et al.*, 1967) of the ascus. The delimiting wall structures in *C. moniliformis*, however, appear to be synthesized *de novo* by a mechanism involving ribosomes (Figs 8, 9) in the ascus cytoplasm.

Stiers (1976) suggested that two possible methods of spore delimitation could occur in *C. fimbriata* which, like *C. moniliformis*, has hat-shaped ascospores. A double membrane system could form a sac around each nucleus which enclosed to form individual ascospores. Alternatively, the double membranes formed a large ascus vesicle adjacent to the ascus wall. This ascus vesicle then invaginated to form lobes, surrounding each nucleus of the young ascospores. We, however, interpret the formation of an ascus vesicle around all the nuclei adjacent to the ascus wall, as an early stage in the delimitation process. The ascus vesicle would finally invaginate to form sacs which envelop the nucleus of each young ascospore. Depending on the plane in which sections are cut, the young ascospores will either appear elongate and sac-like in longitudinal section or circular in transverse section. Two separate mechanisms of delimitation as proposed by Stiers (1976), thus seem improbable.

During the final stages of wall development the ascospores are paired side by side. This pairing is a unique feature of hat-shaped ascospores and has also been observed in yeasts such as *Hansenula anomala* (Hansen) H. & P. Syd. (Bandoni *et al.*, 1967; Black & Gorman, 1971). After spore delimitation, additional wall layers are deposited between the existing membranes and the wall layer.

Although lomasomes are apparently involved in the

deposition of wall material (Carroll, 1967; Stiers, 1974; Wilsenach & Kessel, 1965), they were not observed in *C. fimbriata* (Stiers, 1976). Structures resembling lomasomes were, however, observed in ascospores of *C. moniliformis* and it is suggested that these structures deposit wall material, contributing to inner wall (endospore) formation.

The number of wall layers that have been observed in the ascospores of other species of *Ceratocystis sensu lato* is variable. In *C. fimbriata* three layers were observed and described as an electron transparent endospore, a less electron transparent mesospore and an electron dense epispore with an extension or brim (Stiers, 1976). In *C. moniliformis* the brim of the ascospore is an electron transparent area, which is apparently not an extension of the epispore. The epispore was formed separately and enclosed the electron transparent extension of the brim. This contrasts with the uniformly electron dense brim of *C. fimbriata*.

In *O. ulmi* with slightly elongated, crescent-shaped ascospores with no sheaths, Jeng & Hubbes (1980) described the inner electron dense wall as the primary wall and the outer electron transparent wall as the secondary wall. The mesospore thus either appears to be absent or was not observed. In *C. moniliformis* the epi- and mesospore are interpreted as constituting the ascospore sheath. The endospore would thus be the spore delimiting wall. Using this terminology, it is the ascospore sheath that is hat-shaped and the spore itself thus has an allantoid shape.

A close association appears to exist between electron dense bodies (osmiophilic bodies) and wall formation in *C. moniliformis*. Similar structures have also been observed in *Saccharomyces cerevisiae* Hansen (Beckett *et al.*, 1973), *O. ulmi* (Jeng & Hubbes, 1980) and *C. fimbriata* (Stiers, 1976). Illingworth *et al.* (1973) provided evidence that the lipid content of the cells in *S. cerevisiae*, increased with an increase in lipid vesicles resembling osmiophilic bodies. Results of this study substantiate the suggestion that these bodies function in wall formation.

The final differentiation of the ascospore wall of *C. moniliformis* into three distinctive layers, was characterized by the presence of cells connected to the asci and ascospore walls. These auxiliary cells appear to be similar in shape and size to the asci. Nuclear divisions and ascospore delimiting wall structures were, however, not observed in them. They thus appear to be sterile and possibly play a role in sheath formation (epi- and mesospore). As far as we are aware, such structures have not previously been observed in studies of ascospore development. The auxiliary cells could function in providing additional wall material and nutrients during ascospore development.

Hat-shaped ascospores are found in both *C. fimbriata* and *C. moniliformis*. The two species might therefore have been expected to have similar ultrastructural characteristics. However, differences in wall-delimiting structures, ascospore walls and the presence of auxiliary cells were found between the species. This suggests that other species with similar ascospore forms should be examined before conclusions can be drawn for the group as a whole. Ultrastructural studies of ascosporogenesis in other species of *Ceratocystis* should also be undertaken. Furthermore, studies of these processes in species of *Ophiostoma* which have a more diverse range of ascospore forms requires investigation.

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