Mycol. Res. 95 (6): 725–730 (1991) *Printed in Great Britain*

Ultrastructure of ascosporogenesis in *Ophiostoma davidsonii*

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The ultrastructural development of asci, ascospores and centrum organization in *Ophiostoma davidsonii*, was examined. Mechanisms possibly involved in ascospore wall and sheath development, revealed differences between *O. davidsonii* and *Ceratocystis moniliformis*. Ascospore delimitation commenced with the formation of double delimiting membranes. The prismatic and spindle-shaped ascospores developed individually, not in pairs as in *C. moniliformis* and *C. fimbriata*. Asci of *O. davidsonii* were arranged in a cluster at the perithecium base whereas in *C. moniliformis* asci line the innermost surface, except the ostiole region.

Ceratocystis sensu lato includes the genera *Ceratocystis* Ell. & Halst. *sensu stricto*, *Ophiostoma* H. & P. Sydow and *Ceratocystiopsis* Upadh. & Kendr. (De Hoog & Scheffer, 1984; Upadhyay, 1981; Upadhyay & Kendrick, 1975; Weijman & De Hoog, 1975; Wingfield, Van Wyk & Marasas, 1988). In general, these are insect-associated fungi and also include numerous important plant pathogens (Boyce, 1961; Clark & Moyer, 1988; Marlon & French, 1967; Smith, 1967; Wingfield, Van Wyk & Marasas, 1988; Wismer, 1961; Wood & French, 1963). The taxonomic disposition of *Ceratocystis* within the Ascomycetes is not generally agreed upon (Benny & Kimbrough, 1980; Redhead & Malloch, 1977; Upadhyay, 1981). Furthermore, differentiation between genera in this group is also a matter of conjecture (De Hoog & Scheffer, 1984).

Ceratocystis sensu lato can be divided into two groups on grounds of ascospore morphology. These include species without and others with hat-, needle-, pillow-shaped and inequilateral sheaths (Upadhyay, 1981). The development of ascospores in *O. stenoceras* (Robak) Melin & Nannf. and *O. ulmi* (Buism.) Nannf. without sheaths have been examined ultrastructurally (Garrison *et al.*, 1979; Jeng & Hubbes, 1980). In a previous investigation (Van Wyk, Wingfield & Van Wyk, 1991), development of hat-shaped ascospores of *C. moniliformis* (Hedgc.) C. Moreau was studied and compared with *C. fimbriata* Ell. & Halst., previously examined by Stiers (1976). The two latter species both have *Chalara* (Corda) Rabenh. anamorphs and are best placed in *Ceratocystis sensu stricto* (De Hoog & Scheffer, 1984). *O. davidsonii* Olchowecki & Reid, which has a *Phialographium* Upadhyay & Kendrick anamorph (Olchowecki & Reid, 1974), is currently considered as a species of *Ophiostoma* (De Hoog & Scheffer, 1984).

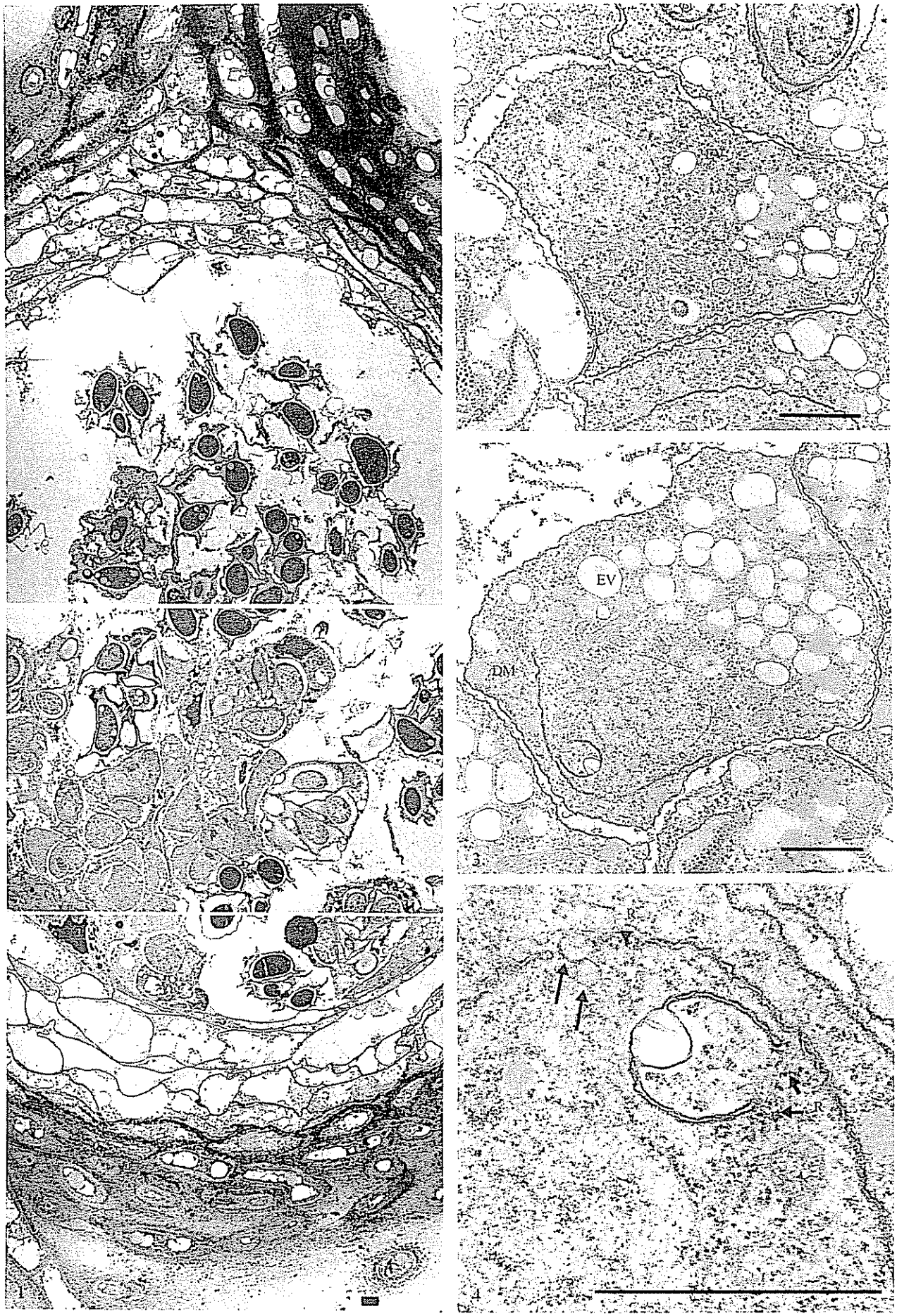
Additional information regarding ascus organization in the perithecia, as well as ascospore development and shape, could be useful in the taxonomy of this group (Van Wyk & Wingfield, 1990). Furthermore, many species of *Ceratocystis sensu lato* have ascospores of unusual shapes which are deserving of ultrastructural examination. The primary aim of this study was to examine the development of crescent shaped (reniform) ascospores with apparently hat-shaped sheaths in *O. davidsonii*. This would allow comparisons with *C. moniliformis* with similar ascospore morphology, but which is conceivably the result of convergent evolution.

MATERIALS AND METHODS

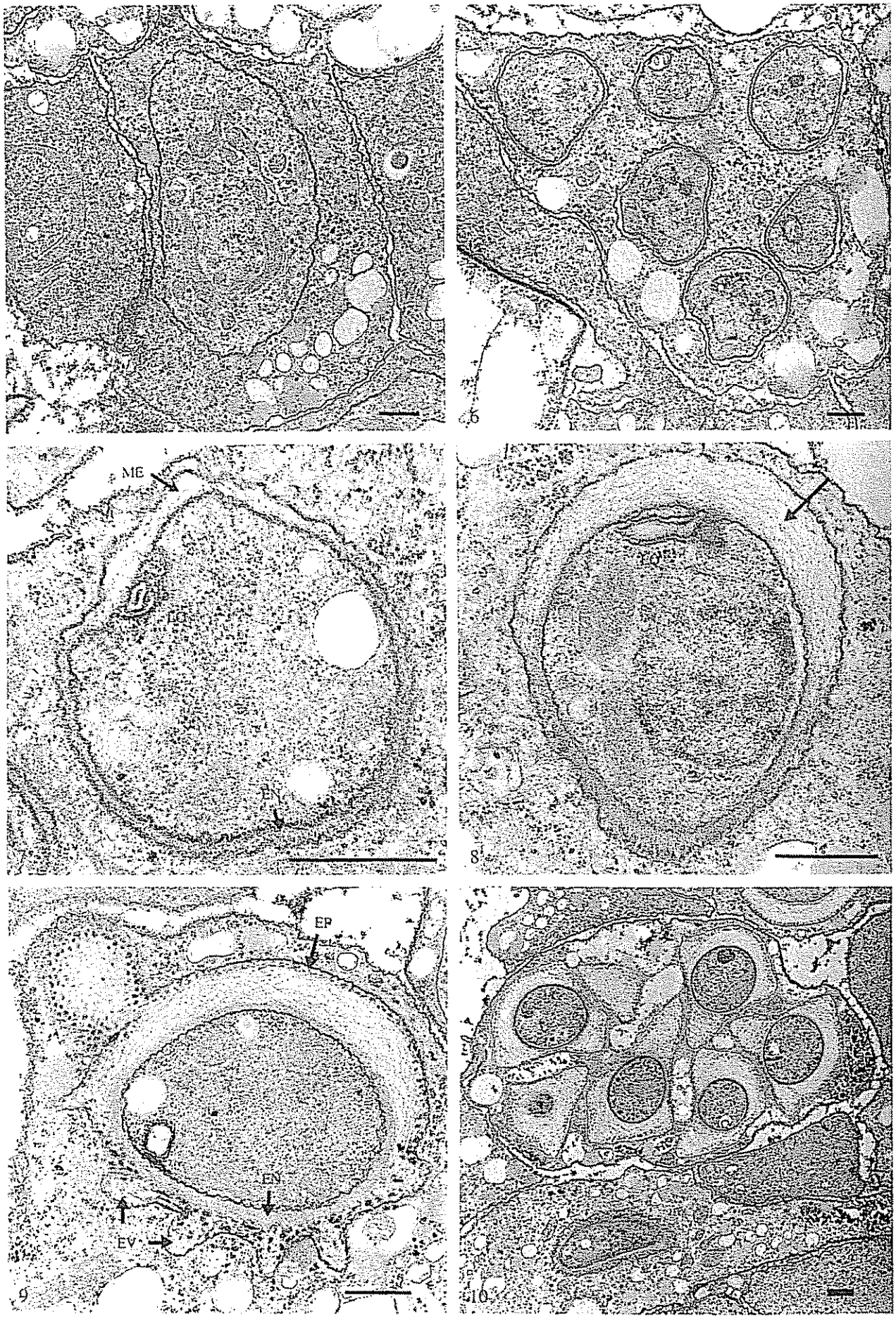
An isolate (IMI 176524) of *O. davidsonii* from the culture collection of the C.A.B. International Mycological Institute was grown at 18° on 2% malt extract agar (20 g Difco Malt Extract, 20 g Difco Bacto Agar; 1 l H_2O) in Petri dishes. Cultures were illuminated by diurnal cycles of fluorescent and near ultra-violet light. Perithecia 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°, 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 a LKB Ultrotome 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

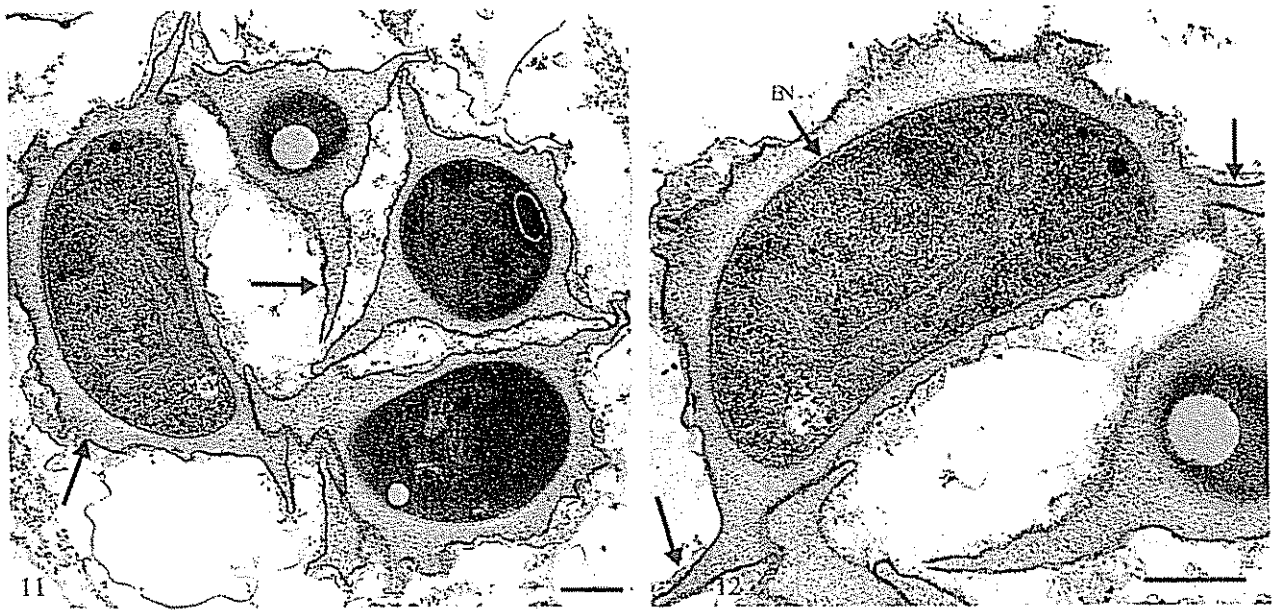
A cross section through the perithecium showed developing, irregularly shaped asci extending upwards and outwards from the perithecium base towards the neck (Fig. 1). These



Figs 1–4. For legend see p. 728.



Figs 5–10. For legend see p. 728.



Figs 11–12. TEM sections of mature ascospores (Bar = 500 nm). **Fig. 11.** Ascospores in transverse and longitudinal section showing the epi- and mesospore wall ridges (arrows). **Fig. 12.** Longitudinal section showing the reniform endospore layer (EN) and brim-like appendages of the epi- and mesospore layers, forming a spindle-shaped sheath (arrows).

structures occupied approximately one third of the volume of the perithecium. Mature ascospores were observed near the perithecium neck (Fig. 1). The perithecium wall consisted of two layers that were ultrastructurally distinct. Elements of the outermost layer were characterized by an electron-dense cytoplasm and electron-dense granules associated with the walls (Fig. 1). An innermost layer of large, electron-transparent sterile or cushion cells (Luttrell, 1951; Van Wyk & Wingfield, 1990) was observed adjacent to the developing asci (Fig. 1).

Young asci, characterized by thin walls and lobed or dividing nuclei, were observed near the perithecium base (Figs 2, 13 A). Numerous electron-transparent vesicles enclosed by single membranes were arranged near one side of the ascus (Figs 2, 13 A). An increase in the number of these vesicles was observed during later stages of ascus development (Fig. 3). Simultneously, flattened membranous vesicles or sacs, associated with ribosomes, were observed near the periphery of the ascus wall, encircling the dividing nuclei (Fig. 3). During further development, small (40 nm) electron-transparent vesicles were observed in association with the membranous sacs (Fig. 4).

Several organelles, nuclei and other cellular inclusions were enclosed by an ascus vesicle, derived from the encircling membranous sacs (Figs 5, 13 B). The ascus vesicle invaginated to enclose individual nuclei forming eight ascopsores, each surrounded by a sack-like double membrane (Figs 6, 13 C).

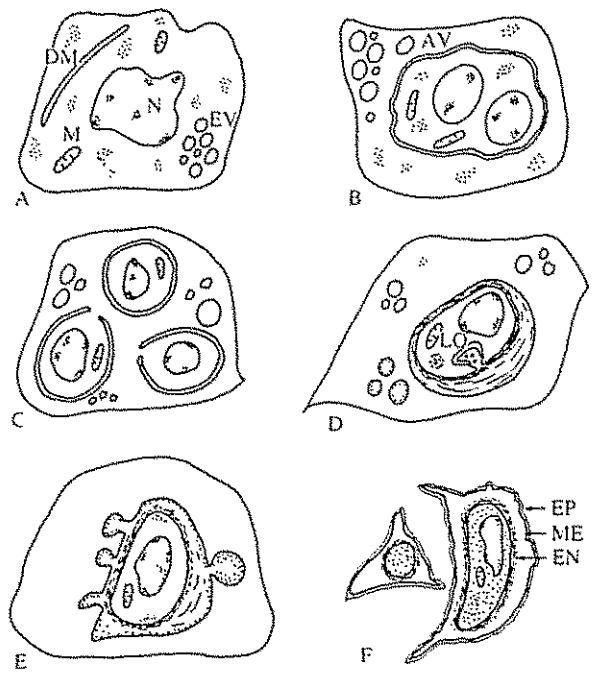
Ascospore wall development commenced with the deposition of electron-dense material between the double membranes, apparently by lomasomes (Figs 7, 13 D). The deposited material appeared to expand in the vicinity of the lomasome, resulting in enlargement of the space between the double membranes. This deposited material consisted of electron-dense fibrils which formed the sheath layer (mesospore) of the wall (Figs 8, 13 D). An electron-dense wall layer (endospore) was observed adjacent to the innermost membrane, which formed the ascospore plasmalemma (Figs 8, 13 D). The endospore and mesospore layers formed between the ascospore delimiting membranes.

Electron-transport vesicles containing electron-dense granules, were observed in the ascus cytoplasm prior to maturation of ascospores. These vesicles coalesced with the outermost delimiting membrane of each ascospore, depositing the granules between the membranes. The granules appeared to bind to the outermost membrane forming the epispore sheath layer (Figs 9, 13 E).

As the ascospore sheaths expanded, the asci became closely

Figs 1–4. TEM of sections through the perithecium of *O. davidsonii* showing ascus development (Bar = 1 µm). **Fig. 1.** Longitudinal section through perithecium showing developing, thin-walled asci at the base and mature ascospores near the neck. **Fig. 2.** Young ascus with a nucleus and single membrane bounded, electron-lucent vesicles (EV). **Fig. 3.** Developing ascus with numerous vesicles (EV) and flattened sac-like membranous vesicles (DM). **Fig. 4.** Developing membranous sacs that delimit ascospores (ascus vesicle) showing vesicles (arrows) and associated ribosomes (R).

Figs 5–10. TEM of sections through developing asci and ascospores (Bar = 500 nm). **Fig. 5.** An ascus with an ascus vesicle (AV) surrounding several organelles. **Fig. 6.** An ascus containing young ascospores after spore delimitation. **Fig. 7.** A young ascospore with a lomasome (LO), the electron-lucent rudimentary mesospore (ME) and electron-dense endospore (EN). **Fig. 8.** An ascospore with a nucleus, further development of the wall layers (arrow) and membrane or lomasome (LO) activity. **Fig. 9.** An ascus showing vesicle (EV) activity which could contribute to sheath and epispore (EP) formation, and the electron-dense endospore (EN). **Fig. 10.** An ascus with ascospores in transverse section showing the arrangement of the ascospores and triangular appearance of the wall layers.



Figs 13A–F. Schematic representation of ascus and ascospore development in *O. davidsonii*. A, Young ascus with a nucleus and preliminary delimiting membrane vesicles; B, An ascus vesicle surrounding ascospore nuclei; C, Sac-like delimiting membranes enclosing ascospore nuclei; D, A developing ascospore with a lomasome contributing to deposition of wall material; E, Vesicles contributing to epi- and mesospore wall layer formation; F, Mature reniform ascospores with distinctive wall (sheath) morphology released from a degenerating ascus.

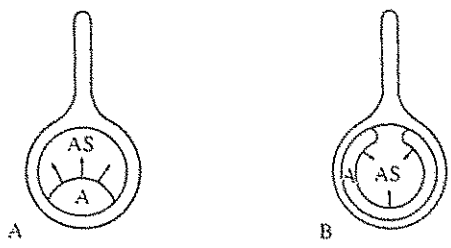


Fig. 14A–B. Schematic representation comparing centrum organization in *Ceratocystis moniliformis* and *Ophiostoma davidsonii*. A, Centrum of *O. davidsonii*; B, Centrum of *C. moniliformis*. (A = region of young developing asci, AS = region of maturing asci and ascospores).

packed with the maturing ascospores. In transverse sections of the asci, the mature ascospore sheaths appeared triangular (Fig. 10). The asci finally disintegrated to release the mature ascospores into the centrum (Figs 11, 12, 13F). In transverse and longitudinal sections mature ascospores showed a two-layered sheath (epispore and mesospore) (Fig. 11). The cytoplasm of the ascospores was surrounded by an electron-dense reniform ascospore wall (endospore) (Fig. 12). In longitudinal sections, the mature ascospore sheath showed two brim-like appendages which formed near the rounded ends of the endospore wall layer. Each appendage extended outwards and was approximately 663 nm in length and 60–80 nm diam. (Fig. 12).

DISCUSSION

Luttrell (1951) proposed that three major characteristics of the ascocarp determine relationships between species of Ascomycetes. These included centrum development and organization, the differentiation of ascocarp wall layers and the structure of asci. The development of the centrum and its mature components was considered particularly important. Despite this fact, centrum organization has received no attention in previous ultrastructural studies of *Ceratocystis sensu lato*.

There appear to be distinct patterns of centrum development in various species of *Ceratocystis sensu lato*. In *O. davidsonii*, young asci were concentrated towards the perithecium base (Figs 1, 14A). Mature ascospores were released from asci above the developmental area at the base and towards the perithecium neck (Fig. 14A). In contrast, the developing asci in *C. moniliformis* lined the perithecium except the ostiole region (Van Wyk *et al.*, 1991). Mature ascospores were then released from the asci towards the perithecium centre (Fig. 14B).

Ascospore delimitation in *O. davidsonii* commenced with the formation of double delimiting membranes during nuclear division. These membranes appear to be similar to those observed in *C. fimbriata* (Stiers, 1976), *O. ulmi* (Jeng & Hubbes, 1980) and *O. stenoceras* (Garrison *et al.*, 1979) and several yeasts (Beckett, Illingworth & Rose, 1973; Lynn & Magee, 1972). They are, however, different from delimiting wall structures in *C. moniliformis* (Van Wyk *et al.*, 1991) where formation of the ascospore wall occurred simultaneously with the delimiting membranes.

In *O. davidsonii*, small electron-transparent vesicles were apparently involved in synthesis of the double membranes. The origin of delimiting membranes in the ascus is controversial. Various mechanisms, including the involvement of nuclear membrane vesicles, endoplasmic reticulum and plasmalemma, have been proposed for certain yeast species have hat-shaped ascospores (Bandoni *et al.*, 1967; Beckett & Crawford, 1970; Carroll, 1967; Reeves, 1967).

Association of the delimiting membranes with ribosomes may indicate a *de novo* synthesis of wall material between the double membranes. Such *de novo* synthesis apparently occurred in *C. moniliformis* (Van Wyk *et al.*, 1991). In contrast, pre-existing membranes, which coalesced to form the delimiting membrane system were found in *C. fimbriata* (Stiers, 1976).

In the *de novo* synthesis of cell walls in higher plants the plasma membrane is directly involved (Giddings, Brower & Staehlin, 1980). Enzymes, incorporated into the plasma membrane, are apparently involved in the synthesis of wall microfibrils deposited against and probably between the membranes. A similar *de novo* mechanism for cell wall formation probably exists in *O. davidsonii* and *C. moniliformis* (Van Wyk *et al.*, 1991), compared with the pre-existing membrane mechanism in *C. fimbriata* (Stiers, 1976).

A unique mechanism of sheath formation appears to function in *O. davidsonii*. Here, vesicles containing electron-dense granules coalesce with the outermost delimiting membrane. The granular contents of the vesicles are then deposited between the two delimiting membranes adjacent to

the outermost membrane. This process apparently contributes to the formation of the electron-dense epispore of the sheath.

Mature ascospores in *O. davidsonii* have been described as crescent-shaped with rounded ends, surrounded by a uniform hyaline gelatinous sheath (Olchowecki & Reid, 1974; Upadhyay, 1981). The sheath, however, consists of both an epispore and mesospore layer. The electron-dense innermost layer (endospore) is reniform. However, the sheath does not appear to be uniformly shaped. Near the rounded ends of the endospore layer, extensions of the mesospore and epispore give the sheath a hat-shaped lateral appearance. Despite this hat-shaped appearance, the ascospores of *O. davidsonii* are different from those in *C. fimbriata* (Stiers, 1976), *C. moniliformis* (Van Wyk *et al.* 1991) and *Hansenula anomala* (Hansen) H. & P. Syd. (Bandoni *et al.*, 1967) where hat-shaped ascospores develop in pairs adhering at the flattened sides of the brim.

In contrast to the hat-shaped ascospores of *C. moniliformis*, ascospore sheaths in *O. davidsonii*, appear triangular in transverse section. This suggests a sheath with three longitudinal ridges converging near the rounded ends of the ascospore. These would then form a brim-like appendage. The sheath would thus be considered neither hat-shaped nor crescent-shaped, but rather prismatic and spindle-shaped. The endospore wall layer, which excludes the sheath layers, is reniform. In this way, ascospore morphology in *O. davidsonii* is different from the hat-shaped ascospores in *Ceratocystis sensu stricto*.

The triangular shape of ascospore sheaths in transverse sections, may result from the development and arrangement of the ascospores in the asci. During development, ascospores are arranged in a manner apparently resulting in the deposition of wall material selectively along three opposite ridges on the long axis of the ascospore.

Differences between ascospore development in *O. davidsonii* and other *Ceratocystis* spp. supports the contention that conclusions pertaining to the group as a whole cannot be drawn from the study of a small number of species. It appears that considerable differences could exist between ascospore development in *Ceratocystis sensu stricto* and *Ophiostoma* spp. despite their apparent similarity. In order to understand the complexity of the ascospore wall and sheath development, as well as centrum organization in this group of fungi, ultrastructural studies of as many species as possible should be undertaken. On the grounds of the few studies already available, it is expected that ultrastructural investigations of additional species will yield results of both taxonomic and evolutionary significance.

We are grateful to Dr Z. A. Pretorius and Dr B. D. Wingfield for constructive criticism of the manuscript.

(Received for publication 29 June 1990)

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