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The fine structure of ascospore shape and development in *Ceratocystis fimbriata*

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Abstract

Ascospore development in *Ceratocystis fimbriata* Ell. & Halst. commenced in an eight-nucleate ascus. A single vesicle formed along the periphery of the ascus from fragments of ascospore delimiting membranes, surrounded all eight nuclei and eventually invaginated, first forming pouches with open ends, then finally enclosing each of the eight nuclei in a separate sac, thus delimiting ascospores. Pairing of the ascospores followed and brim formation occurred at the contact area between two ascospores. Osmiophilic bodies contributed to the formation of brim-like appendages by fusing to the ascospore walls. Additional brims were observed at opposite ends of the ascospores giving them a double-brimmed appearance.

Abbreviations: AV – ascus vesicle, DM – delimiting membrane, EV – electron translucent bodies, G – granules, M – mitochondria, N – nucleus, OB – osmiophilic bodies, PMV – plasmamembrane vesicles, PW – primary wall, SW – secondary wall

Introduction

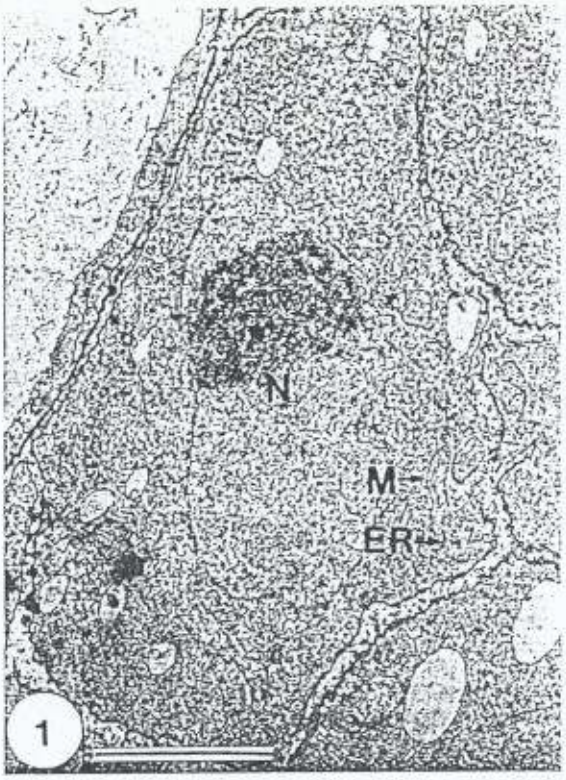
Ceratocystis sensu lato includes the genera *Ophiostoma* H. & P. Syd., *Ceratocystis sensu stricto* Ell. & Halst. and *Ceratocystiopsis* Upadhyay & Kendrick (De Hoog & Scheffer 1984). The shape of the ascospores in these genera is an important taxonomic character (Hunt 1956; Upadhyay 1981; De Hoog & Scheffer 1984), but is based on light microscopy studies and few ultrastructural studies exist. Recent ultrastructural studies have contributed to additional information on ascospore shape and development useful in the taxonomy of *Ceratocystis s. l.* (Van Wyk & Wingfield 1990; Van Wyk & Wingfield 1991a; Van Wyk & Wingfield 1991b; Van Wyk & Wingfield 1992).

The only species of *Ceratocystis s. s.* that have been examined ultrastructurally are *C. fimbriata* (Stiers 1976) and *C. moniliformis* (Hedgc.) C. Moreau (Van Wyk Wingfield & Van Wyk 1991). These two species have similar hat-shaped ascospores (Van Wyk et al. 1991). It was expected that ascospores would develop in a similar fashion in both species. However, controversy exists regarding the ultrastructure and development of these ascospores. Stiers (1976) proposed two separate mechanisms for ascospore delimitation in *C. fimbriata*. In contrast to this, Van Wyk et al. (1991) reported only one delimitation mechanism in *C. moniliformis*.

Although the ultrastructure of ascospore development in *C. fimbriata* has been examined previously, details of the appearance of the ascospores and their development remain unclear. The aim of this study was to re-examine the ultrastructure of ascospore shape and development in *C. fimbriata* in an attempt to clarify some of the aforementioned confusion.

Materials and methods

The culture of *C. fimbriata* used in this study was isolated from *Platanus* L. sp. in France and supplied by Prof. C. Grosclaude from Montfavet, France. It was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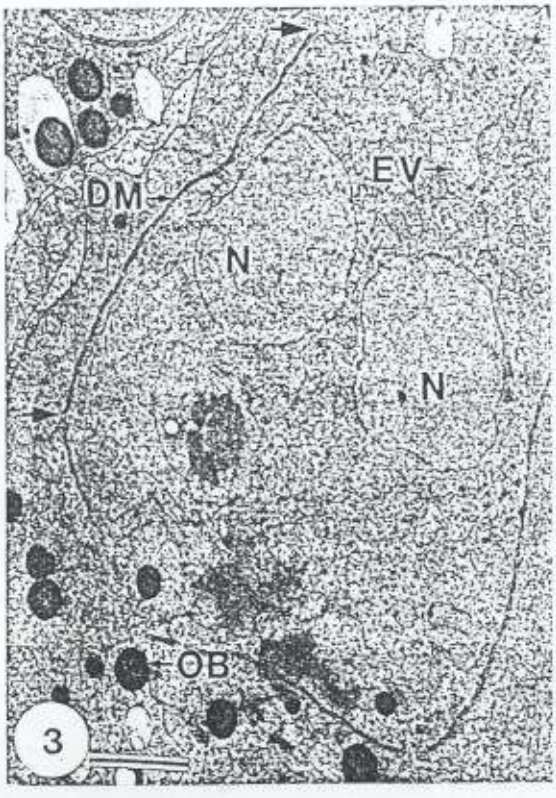
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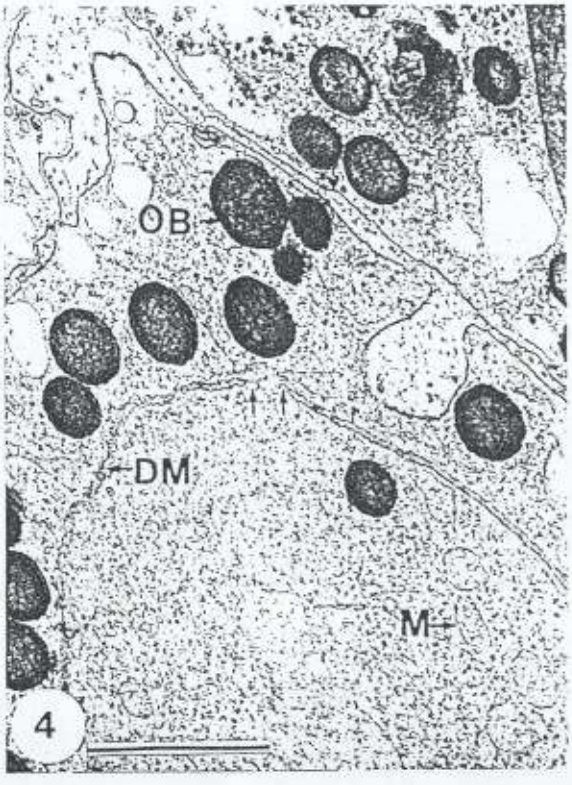
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Fig. 1–4. Transmission electron micrographs (TEM) of developing ascospores in *Ceratocystis fimbriata* (Bar = 1 μm). *Fig. 1.* Section through a young ascus containing a nucleus (N), McCartney-mitochondria (M) and endoplasmic reticulum (ER). Vesicles of variable shape and size are also present. *Fig. 2.* Young ascus with uniformly shaped plasmalemma showing nuclear division. *Fig. 3.* Ascus showing three of the eight nuclei after nuclear division. Fragments of delimiting membrane (DM) form along the ascus periphery and have light areas at their tips (arrows). Wall material exists between the delimiting membranes. Osmiophilic bodies (OB) and electron translucent bodies (EV) or vesicles occur in the asci. *Fig. 4.* Enlarged region of the delimiting membranes (DM) occurring in the ascus. The light tips (arrows) of the DM can be easily distinguished from the rest of the DM.

grown at 25° C on 2% malt extract agar (20 g Difco malt extract; 20 g Difco Bacto agar/1000 ml water). Ascomatal material was fixed in 0,1 M (pH 7) sodium phosphate buffered glutaraldehyde (3%) for 3 h and then for 1.5 h in similarly buffered OsO_4 (1%). Dehydration was in a graded acetone series and the blocks for transmission electron microscopy (TEM) were then embedded in epoxy resin (Spurr 1969) polymerised at 70° C for 8 h.

An LKB III Ultrotome was used to cut 60 nm sections with glass knives. These sections were stained with uranyl acetate for 10 min, followed by lead citrate (Reynolds 1963) for 10 min and examined with a Philips EM300 transmission electron microscope.

The acetone-dehydrated material for scanning electron microscopy (SEM) was critical-point dried, mounted and coated with gold/palladium. This was then viewed with a JEOL WINSEM (JSM 6400) scanning electron microscope.

Results

Uninucleate ascus mother cells (young asci) had mitochondria, endoplasmic reticulum and vesicles varying in shape and size (Fig. 1). Nuclear division (Fig. 2) took place in these cells early in ascospore development. Only three to four of the eight nuclei could be observed in most sections (Fig. 3). Furthermore, intensely stained bodies (OB), about 580 nm in diameter contrasted with lightly stained vesicles (EV) which were about 450 nm in diameter. These bodies and vesicles were observed at all developmental stages. Invagination of the ascus plasma membrane (Fig. 5) formed plasma membrane vesicles (PMV).

Ascospore delimiting membrane fragments formed after final nuclear division (Fig. 3) and were characterized by double membranes enclosing wall material. Electron transparent areas at the tips of the membranes (Fig. 4) functioned as synthesis areas for wall material and membranes. The membrane fragments served as precursors for large, discontinuous ascus vesicles that formed along the periphery of asci (Fig. 5) surrounding all eight nuclei. Invagination of ascus vesicles around nuclei (Fig. 6) gave rise to sac-like structures with open ends (Fig. 7). Young ascospores were delimited by fusion of the open ends (Fig. 8). Ascospores developed in four juxtaposed pairs in each ascus.

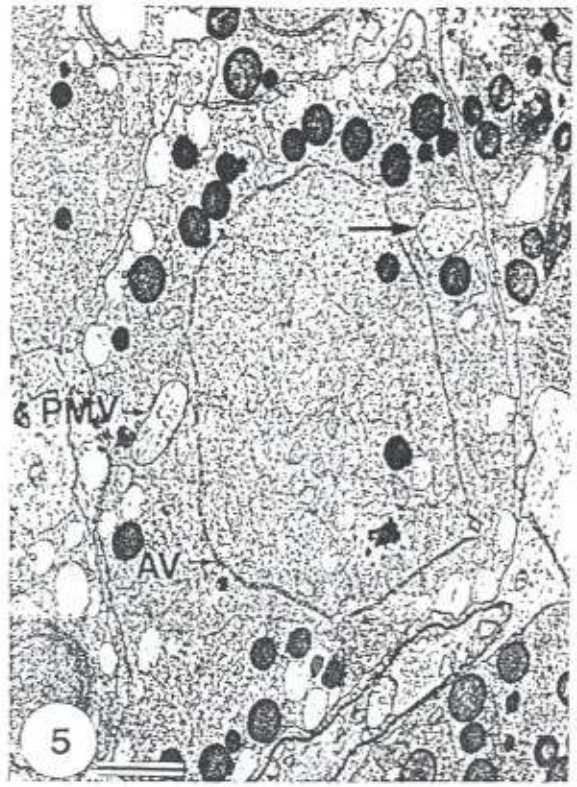
Ascospore brim formation commenced at the contact area between paired ascospores (Fig. 9). Several bodies and granules were present in the vicinity of the developing brims (Fig. 10). Osmiophilic bodies fused with the outer wall layer of the ascospores (Fig. 11) apparently contributing to wall formation. Apart from the complete brims formed between paired ascospores, additional partial brims were occasionally observed on some ascospores (Fig. 12), depending on the plane of sectioning (Fig. 13). Scanning electron microscopy revealed that these additional partial brims were present only at opposite ends of the ascospores (Fig. 14) giving the ascospores a double brimmed appearance. The brims formed part of the secondary wall (SW), which surrounded the primary wall (PW) (Figs 10, 12).

Ascospores separated before they were fully mature (Figs 10, 12). Deliquescence of the ascus preceded separation of the ascospore pairs (Fig. 10). The mature ascospores were released into the ascomatal matrix after lysis of the ascus (Fig. 12).

Discussion

Ascospore development in *C. fimbriata* observed in this study differed somewhat from that previously described by Stiers (1976). It was, however, similar to that in *C. moniliformis* (Van Wyk et al. 1991) in many aspects, such as ascospore delimitation and mechanism of wall formation. Ascospore shape was different to that previously observed for *C. fimbriata* or any other species of *Ceratocystis s. l.*

Although several mechanisms have been proposed to explain the origin of ascospore delimiting membranes in Ascomycetes, this remains uncertain. In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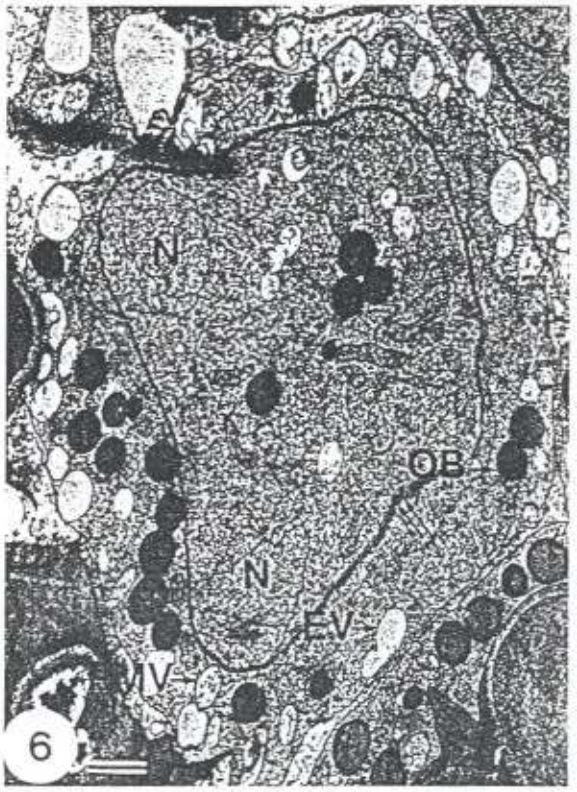
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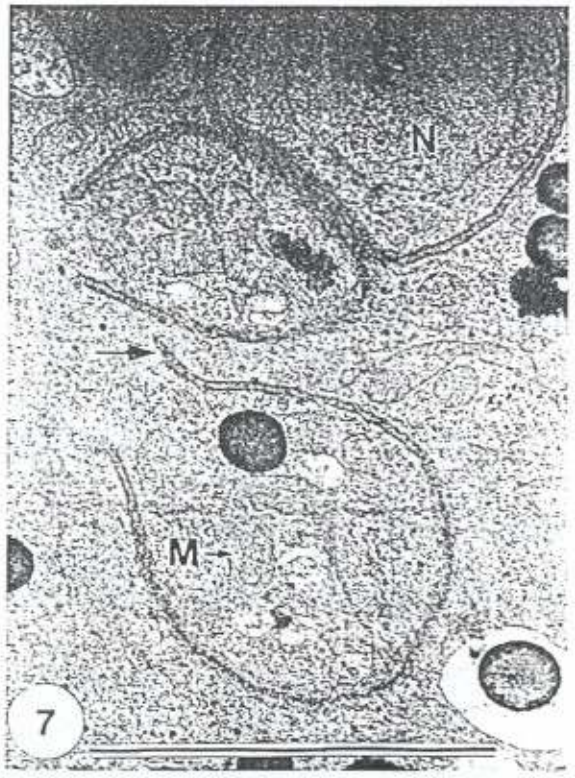
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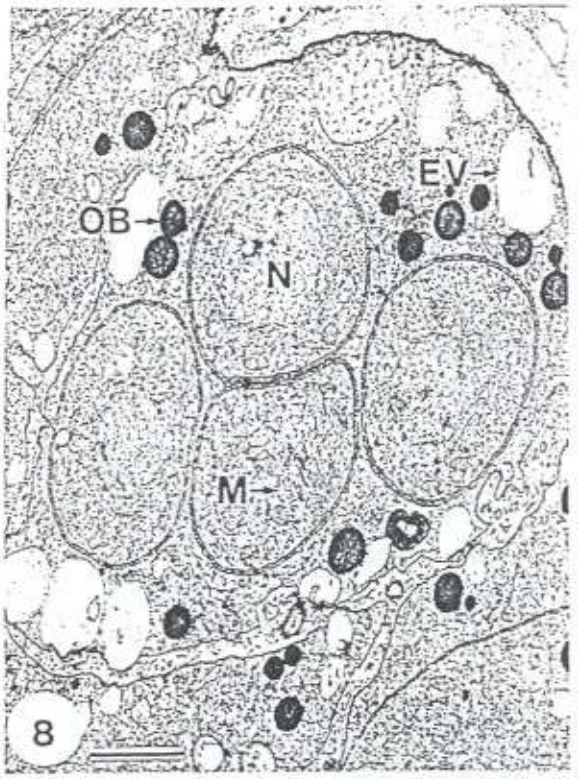
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Fig. 5-8. Transmission electron micrographs (TEM) of developing ascospores in *Ceratocystis fimbriata* (Bar = 1 μm). *Fig. 5.* Ascus with delimiting membranes, which form the ascus vesicle (AV). The ascus vesicle surrounds some cell organelles of the ascus. The ascus plasmalemma invaginates (arrow) to form plasmamembrane vesicles (PMV). *Fig. 6.* Ascus vesicle in ascus showing initial invagination at areas where nuclei occur (double arrows). Invaginations of the ascus plasmalemma also occurs. Note the plasma membrane vesicles (PMV), osmiophilic bodies (OB) and electron translucent (EV) bodies. *Fig. 7.* Enlarged region of an ascus with double delimiting membranes and walls forming pouches or sacs with open ends (arrow). Each sac engulfs a nucleus (N), cell organelles and ascus cytoplasm. *Fig. 8.* Four young ascospores in an ascus. Pairing of the ascospores had already begun with each spore apparently maintaining its integrity. Mitochondria (M), several vesicles (EV) and osmiophilic bodies (OB) occur within the ascus.

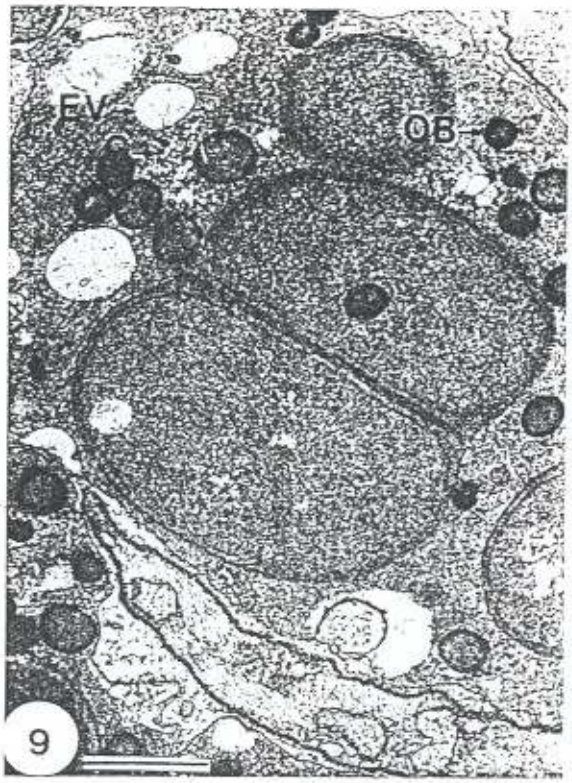
Hansenula anomala (Hansen) H. & P. Syd. and *H. wingei* Wickerham, yeasts which have hat-shaped meiospores, delimiting membranes are derived from invaginations in the meiosporangial plasmalemma and endoplasmic reticulum respectively (Bandoni et al. 1967; Gorman 1971). Van Wyk et al. (1991) also reported that wall delimiting structures were synthesized *de novo* in *C. moniliformis* by a mechanism which involves ribosomes. From observations of *C. fimbriata* in this study, it seems improbable that a single mechanism is involved in the origin of delimiting membranes. We believe that several mechanisms can occur in one species. We moreover suggest that the ER, plasmalemma and nuclei of the ascus and *de novo* synthesis of delimiting wall structures give rise to membranes that delimit the nucleus of each young ascospore.

Results of this study have shown that the formation of a single, large ascus vesicle precedes the formation of sacs around the nuclei. This is in contrast to the fact that Stiers (1976) reported two separate mechanisms for ascospore delimitation in *C. fimbriata*; in one case, double membranes appeared to form a separate sac for each of the eight nuclei in the asci, thus enclosing them individually (Table 1, 1.1–1.3). Alternatively a complete ascus vesicle surrounded the eight nuclei and eventually invaginated to enclose each nucleus (Table 1, 2.1–2.3). Van Wyk et al. (1991) proposed that in *C. moniliformis* (Table 1, 3.1–3.4), the delimitation process is a combination of the separate mechanisms suggested by Stiers (1976). The formation of a single ascus vesicle as a separate mechanism in a single fungus species seems unlikely. We thus contend that only one mechanism of ascospore delimitation exists in *C. fimbriata* and that this is the same as that in *C. moniliformis* (Table 1, 3.1–3.3).

It is uncertain whether the plasma membrane vesicles carry wall material or other cellular compounds. The osmiophilic bodies observed in this study resemble structures reported by Stiers (1976) as lysosomes. Lysosomes function in the lysis of the asci during ascospore release (Wilson et al. 1970). However, in this study, fusion of these bodies with the outer delimiting membranes suggests that they function as storage bodies of wall material and therefore cannot be considered lysosomes. The role of the granules in the vicinity of the ascospores is uncertain. It is possible, however, that they also contribute to wall formation.

Pairing after delimitation is unique to hat-shaped ascospores (Bandoni et al. 1967; Black & Gorman 1971; Stiers 1976; Van Wyk et al. 1991). This feature precedes the formation of brims which occurs through the deposition of additional wall material. In this study we have for the first time shown the presence of a second brim in ascospores of *C. fimbriata* which has also not been seen in any other species of *Ceratocystis s. l.* This second brim is discontinuous and is present only at opposite ends of the ascospores. It has probably not previously been observed because it would be visible only in sections cut in a single plane. On re-examination of micrographs of ascospore development in *C. moniliformis* (Van Wyk et al. 1991), double brims also appear to be present. These second brims are also relatively small and were not obvious to us initially. They are also not likely to be observed using light microscopy.

Ascospores in *C. fimbriata* and *C. moniliformis* develop in similar manner. This includes the origin of the delimiting membranes, which form the ascus vesicle, the mechanism of ascospore delimitation and the contribution of the osmiophilic bodies to secondary wall formation. It appears that fungi with similarly shaped ascospores have basically identical processes for ascospore development. It is, however, not known whether other fungi with hat-shaped ascospores also have double brims.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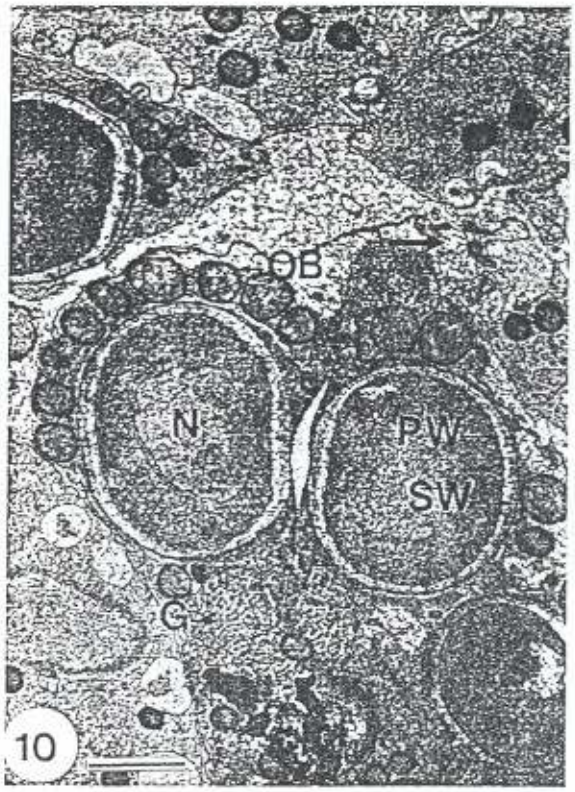
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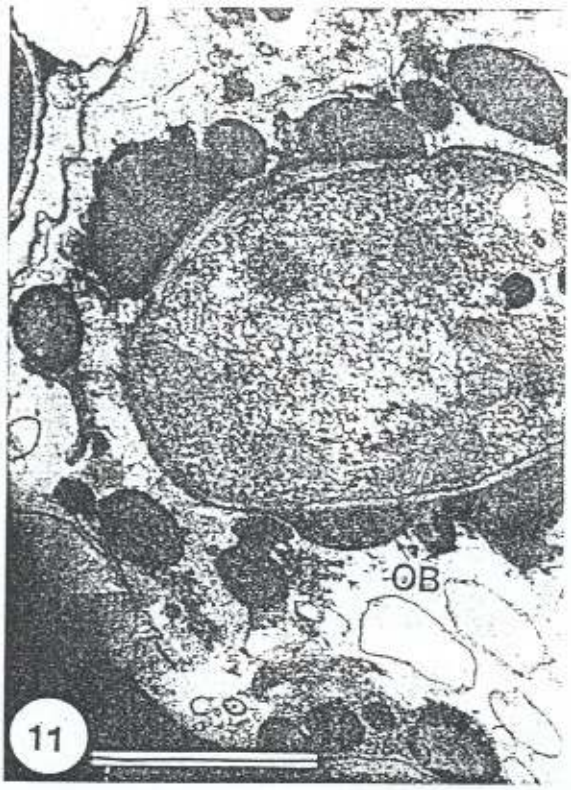
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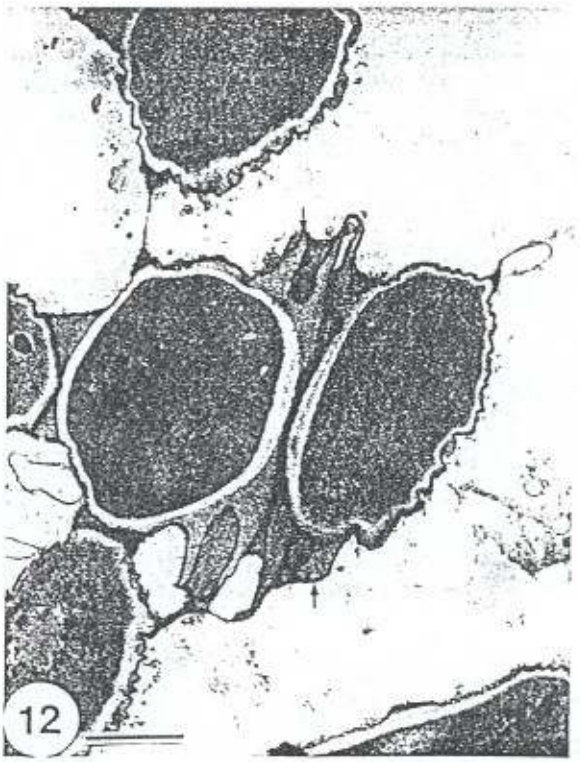
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Fig. 9-12. Transmission electron micrographs (TEM) of developing ascospores in *Ceratocystis fimbriata* (Bar = 1 μm). *Fig. 9.* Initiation of brim formation between paired ascospores at the contact area of two ascospores. Note the constant presence of osmiophilic bodies (OB) and vesicles (EV). Granules (G) are also present in the vicinity of the ascospores. *Fig. 10.* Two ascospores in an almost completely deliquescent ascus. Note lysis areas of part of the ascus (arrows). Distinct wall layers can be seen for both ascospores. The primary wall (PW) surrounds the ascospore cytoplasm, the secondary wall (SW) forming brim areas of hat-shaped ascospores. Osmiophilic bodies (OB) are in close association with the young ascospores. *Fig. 11.* Fusion of osmiophilic bodies (OB) with the outer layer of a ascospore releasing wall material. *Fig. 12.* Mature ascospores separating from each other. Note the additional brim (arrow) which gives the double brim appearance.

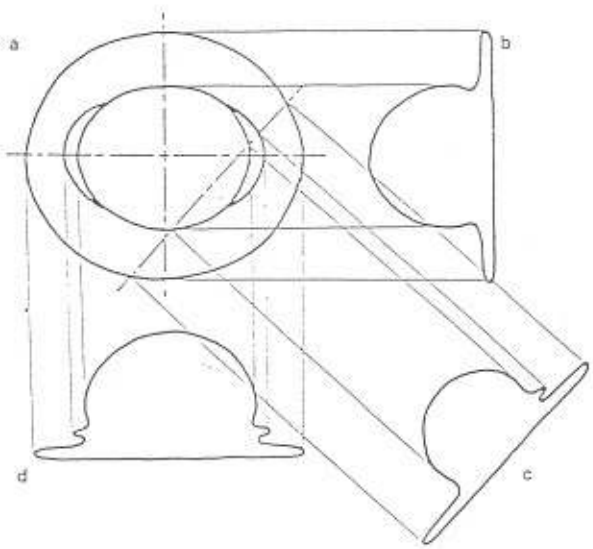

Fig. 13. Diagram showing different sections through an ascospore. a) Ascospore, as seen from the top, with additional (partial) brims at polar ends and a continuous brim at the bottom. b) Side view of a section through the ascospore. Only the continuous brim is seen. c) Side view of a section through the ascospore showing the additional brim only at one side of the ascospore. d) Side view of a section through an ascospore showing the additional brims on opposite ends of the ascospores.

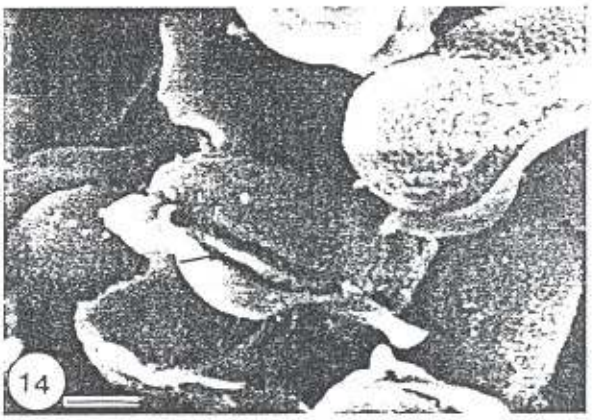

Fig. 14. Scanning electron micrograph of the hat-shaped ascospores of *Ceratocystis fimbriata* showing the presence of a second brim (arrow). Bar = 1 μm.

Acknowledgements

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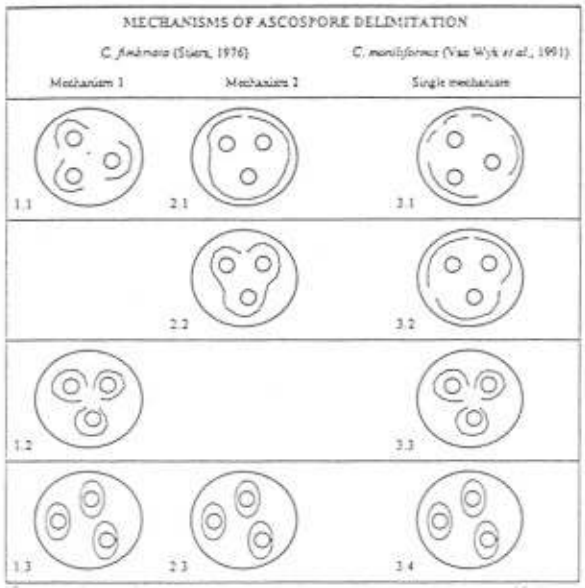
Table 1. Characteristics of ascospore delimitation in *Ceratocystis sensu stricto* based on ultrastructural observations. Two mechanisms of ascospore delimitation proposed by Stiers (1976) for *C. fimbriata* compared with a single mechanism in *C. moniliformis* determined by Van Wyk et al. (1991).



Large circles = asci; small circles = nuclei; double circles = young ascospores; thin lines = ascospore delimiting membranes.

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