Ascospore development in *Ophiostoma piceae*

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The development of ascoma, ascus, and ascospore in *Ophiostoma piceae* was studied ultrastructurally and compared with that of other Ascomycetes. Ascospore delimitation commenced with the formation of double delimiting membranes in the ascus. The ascospore wall, consisting of the primary and secondary walls, was deposited between these membranes. The elongate ascospores of *O. piceae* differed from other species having similarly shaped ascospores, with respect to the shape and arrangement of asci in the ascoma, the number of wall layers of the ascospore, and formation of the secondary wall. Asci in *O. piceae* are spindle shaped, arranged along the periphery of the ascomatal wall. The ascospores have three layered walls, whereas some other species in *Ceratocystis* s.l. also with elongated ascospores probably have only two wall layers.

Key words: *Ophiostoma*, elongated ascospores, sheaths, centrum.

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Les auteurs ont étudié le développement de l'ascoma, des asques et des ascospores de l'*Ophiostoma piceae* en microscopie électronique et ont effectué des comparaisons avec d'autres ascomycètes. La délimitation de l'ascospore commence avec la formation de membranes délimitantes doubles à l'intérieur de l'asque. La paroi de l'ascospore, constituée de composantes primaires et secondaires, se dépose entre ces membranes. Les ascospores allongées de l'*O. piceae* diffèrent de celles des autres espèces qui ont des ascospores de forme similaire, par la forme et à l'arrangement des asques dans l'ascoma, par le nombre de couches pariétales de l'ascospore, et par la formation de la paroi secondaire. Les asques den l'*O. piceae* sont en forme de fuseau et sont disposées le long de la périphérie de la paroi de l'ascoma. Les ascospores ont des parois avec trois couches, alors que d'autres espèces de *Ceratocystis* s.l. possédant également des ascospores allongées, n'ont probablement que deux couches pariétales.

Mots clés : *Ophiostoma*, ascospores allongées, enveloppes, centrum.

[Traduit par la rédaction]

Introduction

Ceratocystis Ellis et Halsted sensu lato includes the genera *Ceratocystis*, *Ophiostoma* H. et P. Sydow, and *Ceratocystiopsis* Upadhyay et Kendrick. This group of fungi includes important plant pathogens, especially of trees (Boyce 1961; Clark and Moyer 1988; Manion and French 1967). The taxonomic disposition of *Ceratocystis* s.l. within the Ascomycetes is not generally agreed upon. They have accordingly been grouped with Pyrenomycetes, Plectomycetes, and certain Endomycetous yeast genera (De Hoog and Scheffer 1984; Redhead and Malloch 1977; Upadhyay 1981; Von Arx and Van der Walt 1987). It has also been suggested that *Ceratocystis* s.l. can be divided into four groups based on different ascospore morphology as determined by light microscopy (Olchowecki and Reid 1974; Upadhyay 1981; Griffin 1968). Few ultrastructural studies, however, have been conducted on the development of the asci and ascospores of these organisms. It has been suggested elsewhere (van Wyk and Wingfield 1990, 1991*a*, 1991*b*; van Wyk *et al.* 1991) that ultrastructural studies could provide useful criteria for the taxonomy of this group. This paper forms part of an ongoing study to determine the development and morphology of ascospores amongst species of *Ceratocystis* s.l. at the ultrastructural level.

Previous ultrastructural studies on ascospore development have shown that distinct differences in the morphology of ascospore sheaths exist amongst species. For instance, species having ascospores with hat-shaped sheaths are characterized by walls consisting of three layers, with extensions of the outermost wall layers (Stiers 1976; van Wyk *et al.* 1991; van Wyk and Wingfield 1991*a*, 1991*b*). Furthermore, in species of *Ophiostoma* having ascospores without sheaths, only two wall layers have been reported by earlier authors (Garrison *et al.* 1979; Jeng and Hubbes 1980). However, in *Ophiostoma distortum* (Davidson) De Hoog et Scheffer and *Ophiostoma minus* (Hedgcock) H. et P. Sydow, both having elongated ascospores apparently without sheaths, ascospore walls consisted of three layers (van Wyk and Wingfield 1991*c*).

Controversy thus exists as to the number of wall layers of ascospores and structure of sheaths. Moreover, ascospores of all these species are released in a slimy gloeoid matrix, and terminology referring to sheaths and this matrix has in the past been confused (Olchowecki and Reid 1974; Upadhyay 1981). The question has thus arisen as to whether the matrix in which the ascospores are released forms part of the sheath or not (van Wyk *et al.* 1991; van Wyk and Wingfield 1991*a*, 1991*b*, 1991*c*). The aim of this study was to compare the development and morphology of nonsheathed elongated ascospores in *Ophiostoma piceae* (Münch) H. et P. Sydow with that in species having similarly shaped ascospores either with or without sheaths.

Materials and methods

An isolate of *O. piceae* obtained from the surface of freshly felled *Macaranga capensis* (Baill.) Benth.:Sim. was selected for this study. Cultures were grown at 18°C on 2% malt extract agar (20 g Difco malt extract, 20 g Difco bacto agar, 1 mL water) in Petri dishes and illuminated by diurnal cycles of fluorescent and near-ultraviolet light.

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Ascomata for electron microscope examination, attached to small (2 × 4 × 8 mm) blocks of agar, were fixed in 0.1 M (pH 7.0) 3% glutaraldehyde buffered with sodium phosphate 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 Ultrotome III. Sections were stained for 20 min with uranyl acetate (6%), stained for 10 min with lead citrate (Reynolds 1963), and examined with a Philips EM300 transmission electron microscope.

Results

Asci and ascospores, at different stages of development, were observed in vertical sections through developing ascomata of *O. piceae*. Spindle-shaped to ellipsoidal asci were loosely arranged in chains, separated by large electron-transparent areas (Fig. 1). Young asci, extending from the base to the neck, occurred in a distinct zone adjacent to the wall of the ascoma. The asci were thin walled and irregular in outline (Fig. 2). Mature and lysing asci, releasing the mature ascospores, occurred at the centre of the ascoma and base of the neck. Some asci, especially those near the neck, released ascospores towards the base of the neck (Fig. 13F).

In ascogenous cells adjacent to the ascomatal wall (peridium), nuclear divisions were observed. These cells resulted in the formation of multinucleate cells or asci (Figs. 2, 13A). Abundant osmiophilic bodies and several vesicles were observed in the asci (Figs. 2, 3, 4, 13B). Vesicles were often associated with the osmiophilic bodies, especially during the occurrence of saclike delimiting membrane structures (Figs. 4, 13B), the peripheral membrane cylinder (PMC). These developing membranes enclosed each nucleus to form young ascospores (Figs. 5, 13C).

The formation of the ascospore wall commenced with the deposition of a very fine granular substance between the delimiting membranes, apparently by myelin figures or more probably by lomasomes (Figs. 6, 13C). A primary wall layer was thus formed through the deposition of the wall material. A second, electron-lucent layer developed between the primary wall layer and the outermost delimiting membrane to form a rudimentary secondary wall layer (Fig. 7). No ultrastructural evidence was found for specific mechanisms involved in the formation of the secondary wall.

A layer consisting of amorphous osmiophilic granules was deposited on the outermost delimiting membrane or perisporic sac (Figs. 8, 13D). During this deposition stage, lysis of the osmiophilic bodies in the ascus was also observed (Figs. 7, 8, 13D). Disintegration of these bodies resulted in the formation of an electron-dense matrix in the ascus (Fig. 9). The secondary wall was not clearly distinguishable from this matrix in the ascus cytoplasm (Fig. 10).

The asci disintegrated through apparent enzymatic lysis (autolysis) (Figs. 9, 10, 11), releasing the mature ascospores in the centre of the ascoma (Figs. 11, 13E). During ascus and ascospore development, some of the cells of the innermost area of the ascomatal wall collapsed (Fig. 12). The remaining membranes and thin walls of these cells formed a multilayered membranous lining against the peridium (Fig. 12).

Discussion

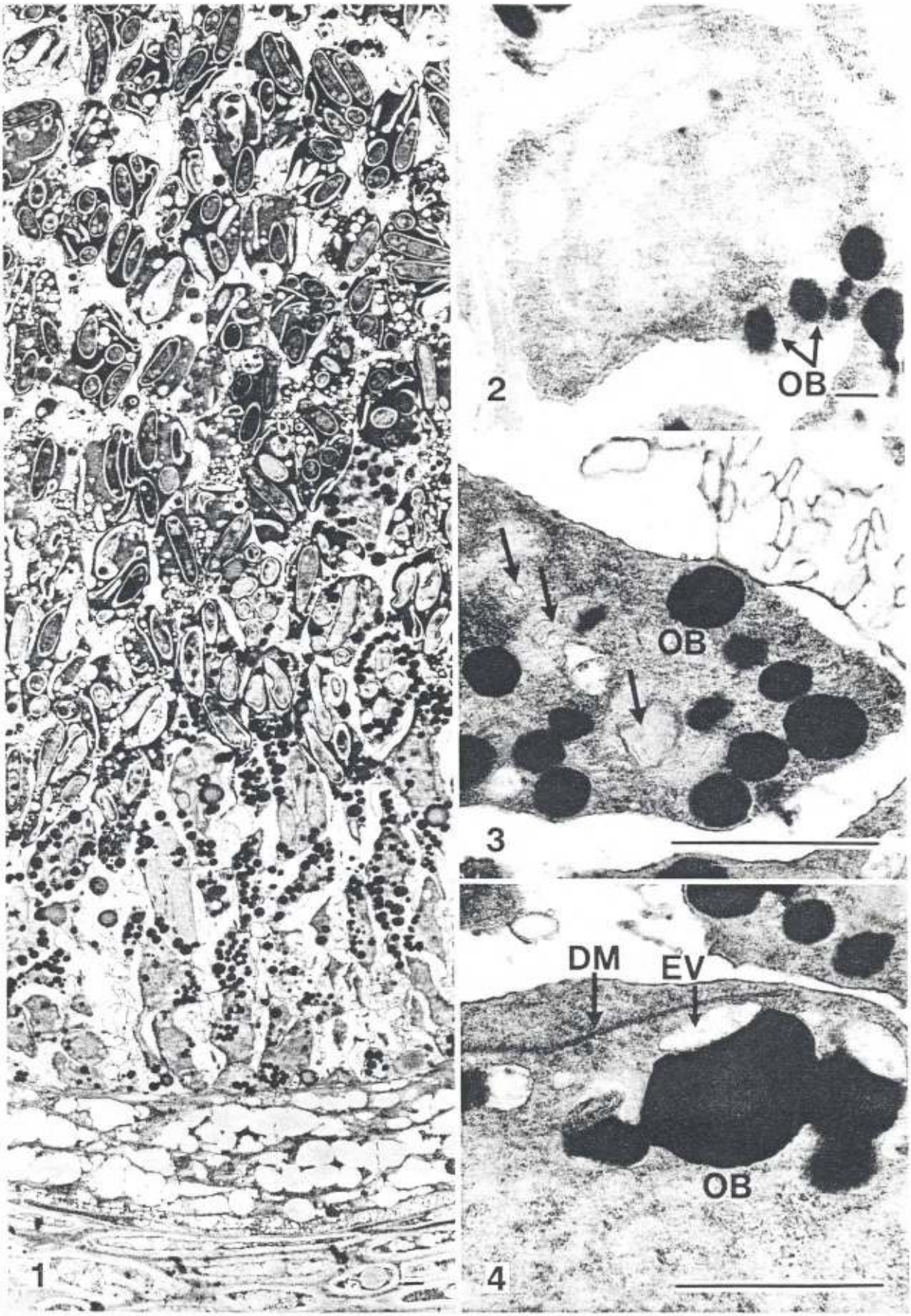
Centrum organization in *O. piceae* was different to that of *O. distortum* and *O. minus* (van Wyk and Wingfield 1991*c*). This is despite the fact that these species have similarly shaped elongate ascospores. Young asci in *O. piceae* occurred in a specific zone adjacent to the peridium. This zone of asci occurred along the periphery of the peridium and was continuous up to the base of the neck. Similarly, asci in *O. distortum* occurred adjacent to the peridium but formed a lining of cells only in the lower half of the ascoma. In *O. distortum*, mature ascospores were released towards the centre of the ascoma and towards the base of the neck. In *O. minus*, club-shaped asci were arranged in chains in the lower part of the ascoma. Tapered apices (or tips) of the asci were oriented towards a central point at the bottom of the ascomatal base, with mature ascospores released towards the upper part of the ascoma near the base of the neck. In contrast with the club-shaped asci of *O. minus* and irregularly shaped asci of *O. distortum*, asci in *O. piceae* are spindle shaped to ellipsoidal.

Ultrastructure of ascospore development has been studied in *Ophiostoma stenoceras* (Robak) Melin et Nannfeldt (Garrison *et al.* 1979) and *Ophiostoma ulmi* (Buisman) Nannfeldt (Jeng and Hubbes 1980). Both species have ascospores of a similar shape to those of *O. piceae*. Centrum development and organization of *O. stenoceras* and *O. ulmi* were not discussed in sufficient detail to make comparisons. However, in *O. stenoceras*, Garrison *et al.* (1979) reported that membranes lined the peridium. These membranes were defined as a linear membranous band. It was suggested that the membranes were probably involved in substrate transport or other synthetic activities related to ascospore formation. Similar structures were observed in *O. piceae*. We interpret these membranes as the result of the disintegration and collapse of some of the peridium cells. By disintegrating during ascus development such cells may function to provide space for the developing asci (Luttrell 1951; van Wyk and Wingfield 1990).

Ascospore formation in terms of the developing membranes of the ascus in *O. piceae* was similar to that in other species

FIGS. 1–4. Transmission electron micrographs (TEM) of sections through the ascoma of *O. piceae*, showing ascus development. Scale bars = 1 μm. Fig. 1. Vertical section through ascoma showing developing, thin-walled, and spindle-shaped to ellipsoidal asci occurring at the ascomatal base and walls, with mature asci arranged towards the neck (neck area towards the top of the figure). Fig. 2. Ascogenous cell or young ascus with dividing nucleus and some osmiophilic bodies (OB). Fig. 3. Developing ascus with several osmiophilic bodies (OB) and membranous structures or myelin figures (arrows). Fig. 4. Peripheral membrane cylinder (DM) developing, prior to the delimitation of ascospores. Electron-transparent vesicles (EV) associated with osmiophilic bodies (OB) present.

FIGS. 5–10. TEM of sections through developing asci and ascospores. Scale bars = 500 nm. Fig. 5. Ascus showing delimitation of individual nuclei by saclike delimiting membranes (arrow). Fig. 6. Young ascospore with lomasome-like structures or myelin figures (LO), contributing to primary wall (PW) formation, with wall material deposited between delimiting membranes. Fig. 7. Development of rudimentary secondary wall layer (SW). Note lysis of the osmiophilic bodies (arrows). Fig. 8. Development of electron-dense ornamentation on secondary wall (arrows) showing warty appearance of the layer and completely degenerated osmiophilic bodies (large arrow). Note lomasome or myelin figure (LO). Fig. 9. Mature ascus showing longitudinal section of elongated ascospore. Note electron-dense appearance of ascus cytoplasm forming a matrix and apparent enzymatic lysis of the matrix (E). Fig. 10. Mature ascospore showing primary wall (PW) and secondary wall (SW) with electron-dense matrix adjacent and outside to secondary wall.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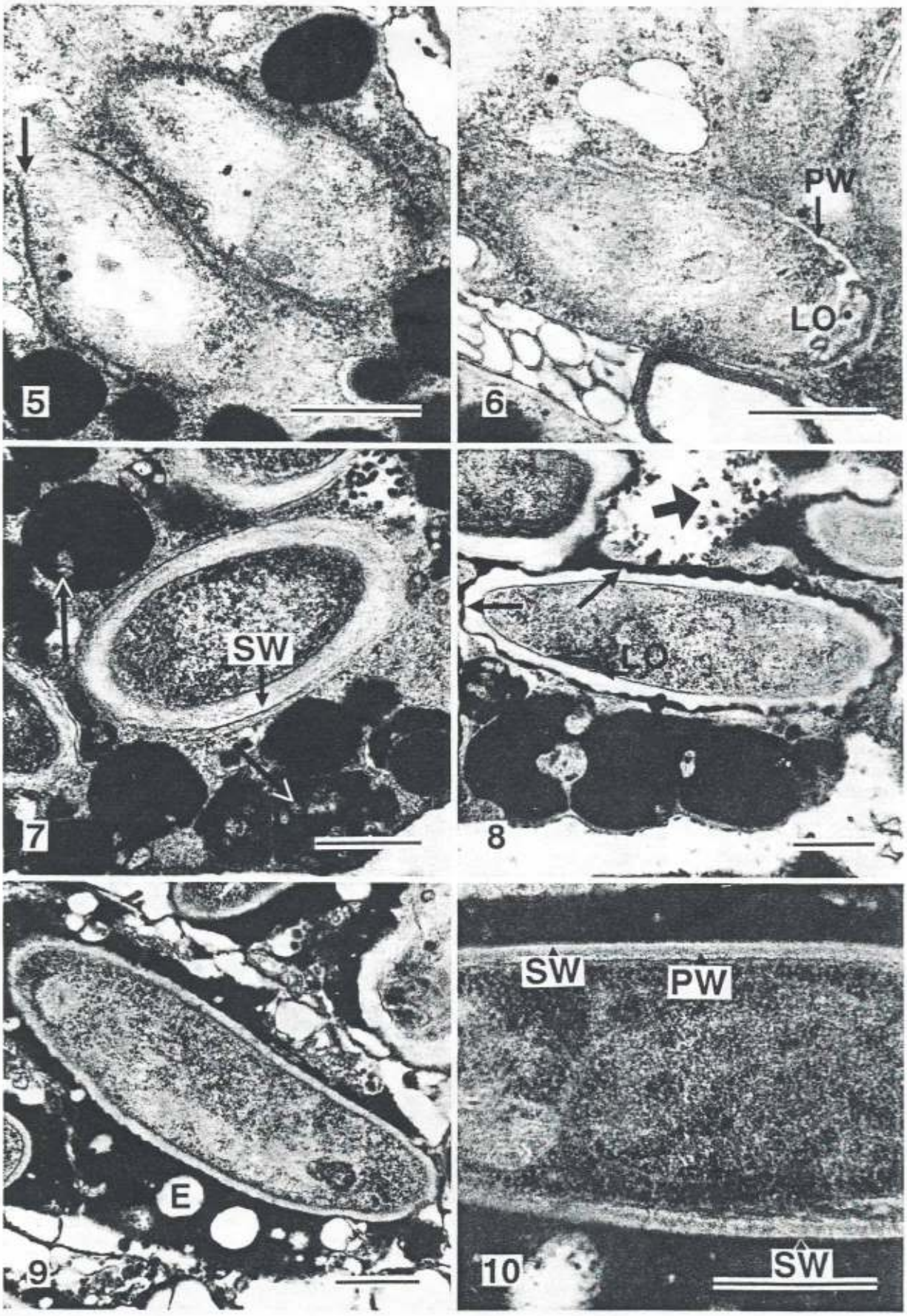
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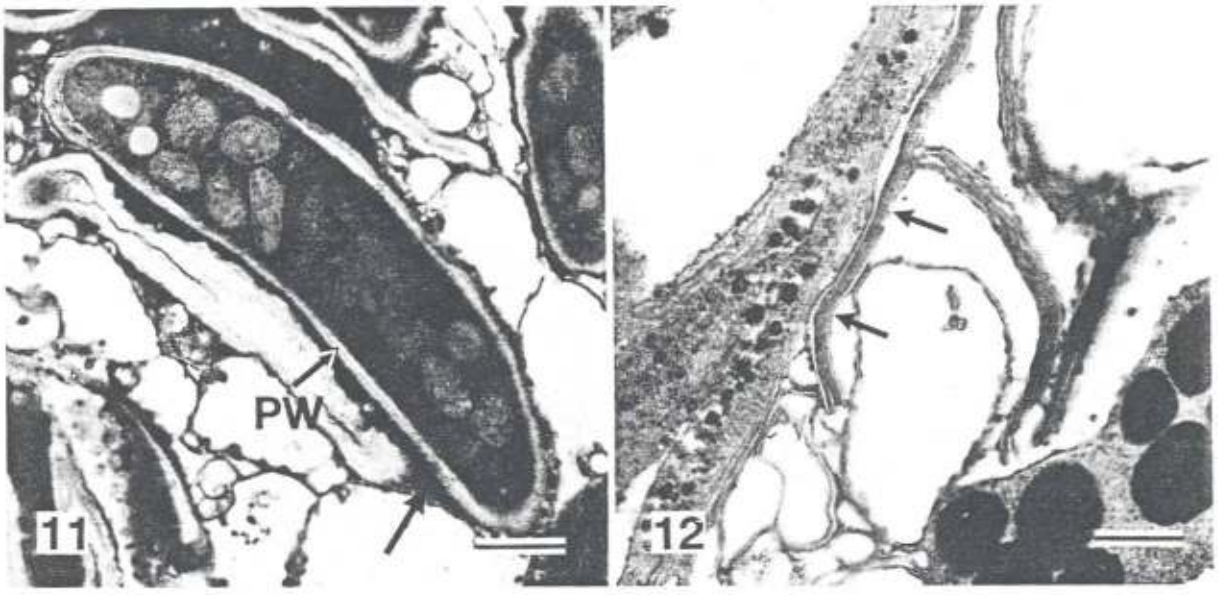

FIGS. 11 and 12. TEM of mature asci and ascospores. Scale bars = 500 nm. Fig. 11. Degenerating ascus releasing mature ascospore in the centre of the ascoma. Note primary wall (PW); secondary wall not noticeable at low magnification. Fig. 12. Collapsing sterile ascomatal wall cells membranes forming a membranous lining (arrows).

of *Ceratocystis* s.l. that have been examined ultrastructurally (Stiers 1976; Jeng and Hubbes 1980; Garrison *et al.* 1979; van Wyk and Wingfield 1991*a*, 1991*b*). The delimiting membranes develop to enclose each of the eight nuclei in the ascus within a saclike membranous structure. The ascospore walls typically develop between the delimiting membranes and consist of primary and secondary wall layers.

Controversy exists regarding the number of wall layers and the terminology to describe the different wall layers of ascospores in species of the Ascomycetes. Beckett (1981) stated that the introduction of new terms does not solve this problem and that simplification is desirable. It was suggested that simplification could be achieved by using the terminology as introduced by Carroll (1967, 1969) and Merkus (1973, 1974, 1975). According to these authors, a primary wall initially develops between the delimiting membranes. Subsequent wall layers are formed towards the outside of this primary wall, consisting of several layers collectively known as the secondary wall. These layers are designated numerically from the outside inwards (Beckett 1981). Despite the suggestions of Beckett (1981), controversy remains as new and sometimes overlapping terminology is introduced. As an example, in studies of the Pezizales (Gibson and Kimbrough 1988; Kimbrough *et al.* 1990), the term epispore was used to describe the layer between the primary and secondary walls. We and other authors have chosen to use the term epispore to describe the outermost wall layer of ascospores in species of *Ceratocystis* s.l. (Stiers 1976; van Wyk and Wingfield 1991*a*, 1991*b*; van Wyk *et al.* 1991). In this and two previous studies (van Wyk and Wingfield 1991*b*, 1991*c*) we have, however, adopted the terminology suggested by Beckett (1981).

Ascospore walls (or sheaths) in *O. piceae* were comprised of three layers, although the secondary wall could not clearly be distinguished from the primary wall and the outer osmiophilic layer adhering to the secondary wall. Other species that produced ascospores with at least three distinct wall layers include *Ceratocystis moniliformis* (Hedgcock) C. Moreau (van Wyk *et al.* 1991), *Ophiostoma davidsonii* (Olchowecki et Reid) Solheim (van Wyk and Wingfield 1991*a*), *Ophiostoma cucullatum* Solheim (van Wyk and Wingfield 1991*b*), *O. minus* and *O. distortum* (van Wyk and Wingfield 1991*c*), and *Ceratocystis fimbriata* (Stiers 1976). In ultrastructural studies of *O. ulmi* (Jeng and Hubbes 1980) and *O. stenoceras* (Garrison *et al.* 1979), only the primary and secondary walls, without any ornamentation, were described. It is uncertain why *O. piceae* has three wall layers, whereas only two layers can be distinguished in *O. ulmi* and *O. stenoceras*.

Ascospores in species of *Ceratocystis* s.l. are released in a slimy gloeoid matrix. In *O. piceae*, complete disintegration of osmiophilic bodies in the ascus resulted in the formation of an electron-dense matrix amongst the ascospores. This was also observed in an ultrastructural study of *O. ulmi* (Jeng and Hubbes 1980). Ascospores of these two species become enbedded in this matrix, and it becomes impossible to distinguish between the matrix and the secondary wall layer. Through lysis of the asci, the ascospores are released within this matrix into the ascoma. The osmiophilic bodies and ascus debris therefore probably comprise the gloeoid matrix in which ascospores are released.

In this study, ascospores of *O. piceae*, which in light-microscope studies have been described as having no sheaths (Upadhyay 1981), were shown to have walls comprised of well-developed layers. *Ophiostoma piceae* does not have an ornamented secondary wall, but the wall layers are fundamentally the same as those of species with variously shaped ascospores. The innermost primary wall surrounds the ascospore cytoplasm and thus forms the ascospore wall. Apparently, the additional wall layers that can be distinguished in the secondary wall are what have been referred to as a sheath. This has become confused with the gloeoid mass in which ascospores are released through the use of terms such as ascospores with gelatinous sheaths (Jeng and Hubbes 1980; Upadhyay 1981; Wingfield *et al.* 1988). We thus believe that the term sheath should be avoided in species of *Ceratocystis* s.l. and that

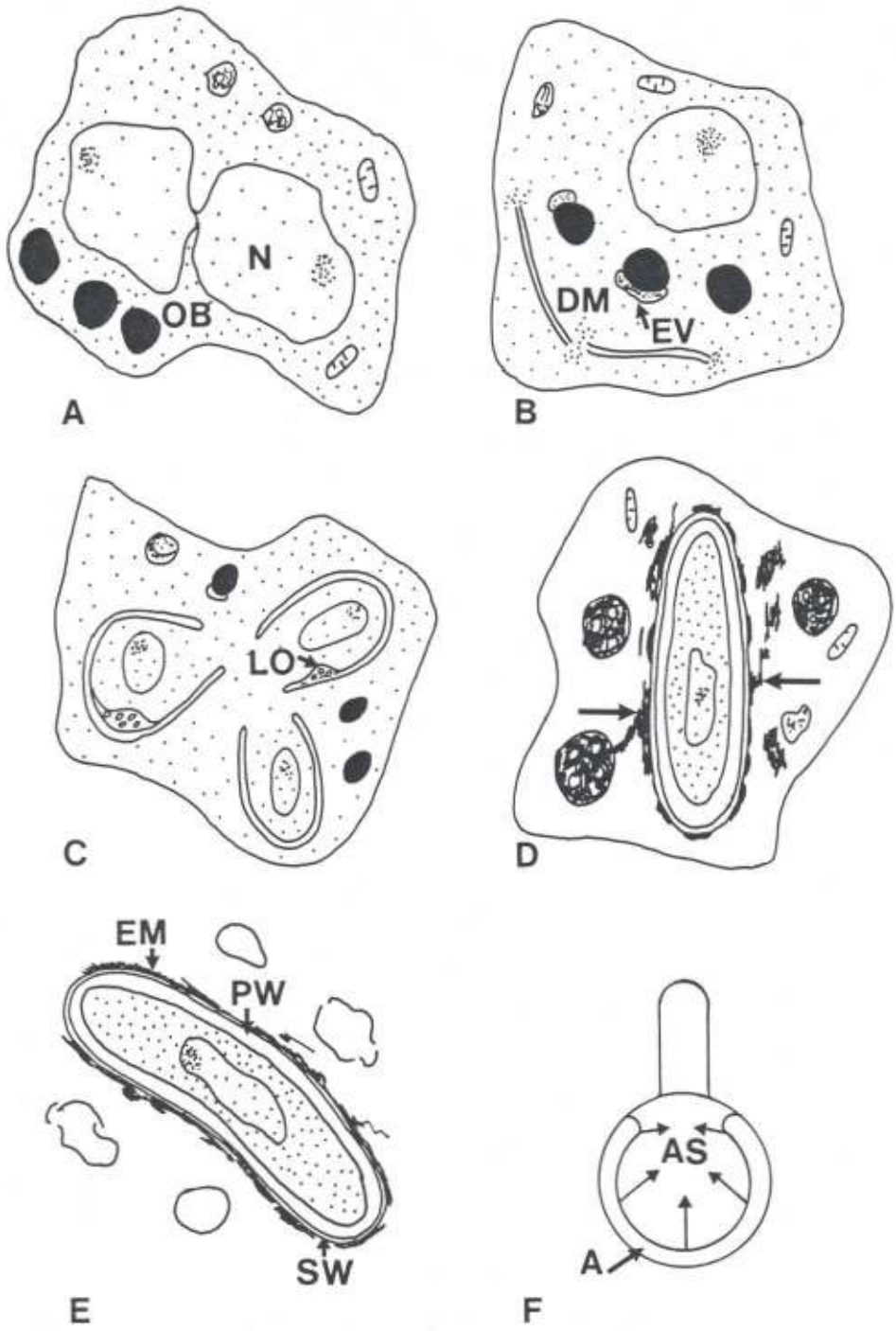

FIG. 13. Schematic representation of ascus and ascospore development in *O. piceae*. (A) Young ascus with two nuclei (N) and osmiophilic bodies (OB). (B) Delimiting membrane (DM) and vesicles (EV) associated with osmiophilic body. (C) Saclike delimiting membranes enclosing ascospore nuclei, with lomasome (LO) depositing sheath and primary wall material. (D) Mature ascus with electron-dense matrix formed by enzymatic lysis of osmiophilic bodies. Note formation of secondary wall of ascospore (arrows). (E) Mature ascospores released from lysing ascus showing the primary wall (PW), rudimentary secondary wall (SW), and electron-dense matrix (EM). (F) Centrum organization of developing asci (A) and ascospores (AS) in *O. piceae*.

the secondary wall should be described based on its characteristic shape.

Beckett, A. 1981. Ascospore formation. *In* The fungal spore: morphogenetic controls. *Edited by* G. Turian and H. R. Hohl. Academic Press, London. pp. 107–129.

Boyce, J. S. 1961. Forest pathology. McGraw-Hill Book Company, New York.

Carroll, G. C. 1967. The ultrastructure of ascospore delimitation in *Saccobolus kerverni*. J. Cell Biol. **33**: 318–224.

Carroll, G. C. 1969. A study of the fine structure of ascosporogenesis in *Saccobolus kerverni*. Arch. Mikrobiol. **66**: 321–339.

Clark, C. A., and Moyer, J. W. 1988. Compendium of sweet potato diseases. APS Press, St. Paul, Minn.

De Hoog, G. S., and Scheffer, R. J. 1984. *Ceratocystis versus Ophiostoma*: a reappraisal. Mycologia, **76**: 292–299.

Garrison, R. G., Mariat, F., Boyd, K. S., and Fromentin, H. 1979. Perithecial ultrastructure and formation of ascospores of *Ceratocystis stenoceras* (Robak) C. Moreau. Ann. Microbiol. **130**: 3–21.

Gibson, J. L., and Kimbrough, J. W. 1988. Ultrastructural observations on Helvellaceae (Pezizales). Ascosporogenesis of selected species of *Helvella*. Can. J. Bot. **66**: 771–783.

Griffin, H. D. 1968. The genus *Ceratocystis* in Ontario. Can. J. Bot. **46**: 689–718.

Jeng, R. S., and Hubbes, M. 1980. Ultrastructure of *Ceratocystis ulmi*. II. Ascogenous system and ascosporogenesis. Eur. J. For. Pathol. **10**: 104–116.

Kimbrough, J. W., and Wu, C.-G., and Gibson, J. L. 1990. Ultrastructural observations on Helvellaceae (Pezizales, Ascomycetes). IV. Ascospore ontogeny in selected species of *Gyromitra* subgenus *Discina*. Can. J. Bot. **68**: 317–328.

Luttrell, E. S. 1951. Taxonomy of the Pyrenomycetes V. The *Ophiostoma* type. Univ. Mo. Stud. **24**: 55–120.

Manion, P. D., and French, D. W. 1967. *Nectria galligena* and *Ceratocystis fimbriata* cankers of aspen in Minnesota. For. Sci. **123**: 23–28.

Merkus, E. 1973. Ultrastructure of the ascospore wall in Pezizales (Ascomycetes). I. *Ascodesmis microscopia* (Crouan) Seaver and *A. nigricans* van Tiegh. Persoonia, **7**: 351–366.

Merkus, E. 1974. Ultrastructure of the ascospore wall in Pezizales (Ascomycetes). II. Pyrenomycetaceae sensu Eckblad. Persoonia, **8**: 1–22.

Merkus, E. 1975. Ultrastructure of the ascospore wall in Pezizales (Ascomycetes). III. Otidaceae and Pezizaceae. Persoonia, **8**: 227–247.

Olchowecki, A., and Reid, J. 1974. Taxonomy of the genus *Ceratocystis* in Manitoba. Can. J. Bot. **52**: 1675–1711.

Redhead, S. A., and Malloch, D. W. 1977. The Endomycetaceae: new concepts, new taxa. Can. J. Bot. **55**: 1701–1711.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17**: 208–212.

Solheim, H. 1986. Species of Ophiostomataceae isolated from *Picea abies* infested by bark beetle *Ips typographus*. Nord. J. Bot. **6**: 199–207.

Spurr, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**: 31–43.

Stiers, D. L. 1976. The fine structure of ascospore formation in *Ceratocystis fimbriata*. Can. J. Bot. **54**: 1714–1723.

Upadhyay, H. P. 1981. A monograph of *Ceratocystis* and *Ceratocystiopsis*. University of Georgia Press, Athens.

van Wyk, P. W. J., and Wingfield, M. J. 1990. Ascospore development in *Ceratocystis sensu lato*: a review. Bothalia, **20**: 141–145.

van Wyk, P. W. J., and Wingfield, M. J. 1991*a*. Ascosporogenesis in *Ophiostoma davidsonii*. Mycol. Res. **95**: 725–730.

van Wyk, P. W. J., and Wingfield, M. J. 1991*b*. Ascospore ultrastructure and development in *Ophiostoma cucullatum*. Mycologia, **83**: 698–707.

van Wyk, P. W. J., and Wingfield, M. J. 1991*c*. Ultrastructural study of ascospore development in *Ophiostoma distortum* and *O. minus*. Can. J. Bot. **69**: 2529–2538.

van Wyk, P. W. J., Wingfield, M. J., and van Wyk, P. S. 1991. Ascospore development in *Ceratocystis moniliformis*. Mycol. Res. **95**: 96–103.

Von Arx, J. A., and Van der Walt, J. P. 1987. Ophiostomatales and Endomycetales. Stud. Mycol. **30**: 167–176.

Wingfield, M. J., van Wyk, P. S., and Marasas, W. F. O. 1988. *Ceratocystiopsis proteae* sp.nov., with a new anamorph genus. Mycologia, **80**: 23–30.