Ultrastructural study of ascoma and ascospore development in *Ophiostoma distortum* and *Ophiostoma minus*

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The development of the ascoma, ascus, and elongated ascospores in *Ophiostoma distortum* and *Ophiostoma minus* was studied at the ultrastructural level and compared with that of other related Ascomycetes. The organization of the centra in *O. distortum* and *O. minus* differed considerably. In *O. distortum*, asci were irregularly arranged in clusters that occurred in the lower half along the periphery of the ascoma. In *O. minus*, asci developed from a central cluster at the base of the ascoma. The asci extended upwards and outwards from the base. Ascospore development in both fungi commenced with the formation of double, delimiting membranes in the ascus. Between these membranes ascospore walls developed, consisting of the primary and secondary wall. In comparison with other species having similar ascospores, differences were found between the number of wall layers of the ascospores and formation of the outer wall layers. The secondary wall and perisporic sac of *O. minus* were smooth and of uniform thickness, whereas the inner layer of the secondary wall in *O. distortum* was thickened and distorted in certain areas. It is suggested that additional ultrastructural studies should be undertaken to provide a better understanding of the development and evolution of ascospore sheaths in *Ceratocystis* sensu lato.

Key words: *Ceratocystis*, elongated ascospores, centrum.

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Les auteurs ont étudié le développement des ascosmats, des asques et des ascospores en élongation chez l'*Ophiostoma distortum* et chez l'*Ophiostoma minus* au niveau des ultrastructures, et les ont comparées avec ceux de d'autres ascomycètes voisins. L'organisation des centrums chez l'*O. distortum* et l'*O. minus* diffère considérablement. Chez l'*O. distortum*, les asques sont arrangés de façon irrégulière en amas regroupés dans la moitié inférieure le long de la périphérie des ascomats. Chez l'*O. minus*, les asques se développent à partir d'amas centraux situés à la base des ascomats. Les asques s'étendent vers le haut et vers l'extérieur à partir de leur base. Chez les deux champignons, le développement de l'ascospore commence avec la formation de doubles membranes délimitant l'asque. Entre ces membranes, les parois de l'ascospore en développement comportent des parois primaires et secondaires. En comparaison avec les autres espèces montrant des ascospores similaires, les auteurs ont trouvé des différences dans le nombre de couches pariétales chez les ascospores ainsi que la formation de couches pariétales externes. La paroi secondaire et le sac périsporique sont lisses chez l'*O. minus*, alors que la couche interne de la paroi secondaire chez l'*O. distortum* est épaisse et distortionnée dans certaines de ses parties. Les auteurs suggèrent que des études ultrastructurales supplémentaires sont nécessaires à une meilleure compréhension du développement et de l'évolution des couches sporales chez le *Ceratocystis* sensu lato.

Mots clés : *Ceratocyctis*, ascospores allongées, 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. These genera represent insect-associated fungi that include many plant pathogens (Boyce 1961; Clark and Moyer 1988; Manion and French 1967; Smith 1967; Wingfield *et al.* 1988; Wismer 1961; Wood and French 1963). The taxonomic disposition of *Ceratocystis* s.l. within the Ascomycetes is not generally agreed upon and it has been grouped with Pyrenomycetes, Plectomycetes, and certain Endomycetous yeast genera (Benny and Kimbrough 1980; Luttrell 1951; Malloch 1987; Redhead and Malloch 1977). Controversy also exists on the distinct nature of *Ophiostoma* and *Ceratocystiopsis* within *Ceratocystis* sensu stricto (De Hoog and Scheffer 1984).

Few ultrastructural studies have been conducted on species of *Ceratocystis* sensu lato. This is despite the fact that many species have sheathed ascospores of unusual morphology and that the asci are evanescent and seldom seen using light microscopy. For instance, it seems unusual that some species should be without ascospore sheaths while others have sheaths of complex structure. Ultrastructural studies could provide insight into the development and evolution of these sheaths.

van Wyk and Wingfield (1990) have suggested that ultrastructural studies could lead to a better understanding of generic concepts in *Ceratocystis* s.l. Similarly we believe that such studies could improve our understanding of the placement of the group at the family and ordinal level. This paper forms part of an ongoing program to study the developmental processes in *Ceratocystis* s.l. We have previously reported on ascospore development in *Ceratocystis moniliformis* (Hedgcock) C. Moreau (van Wyk *et al.* 1991) and *Ophiostoma davidsonii* (Olchowecki et Reid) Solheim (van Wyk and

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Wingfield 1991) that have ascospores with hat-shaped sheaths. Development of the ascospores has, however, been proven to be distinct in these genera.

The aim of this study was to examine ascospore development in *Ophiostoma minus* (Hedgcock) H. et P. Sydow and *Ophiostoma distortum* (Davidson) de Hoog et Scheffer. Both species have elongated sheaths and thus belong to a group of species for which ascospore development has not been examined ultrastructurally. The sheath of *O. minus* is continuous whereas that in *O. distortum* is convoluted, suggesting that they would be interesting subjects for comparison.

Materials and methods

Isolates of *O. minus* (PREM 48205) obtained from insect-infested *Pinus resinosa* Ait. wood in Wisconsin and *O. distortum* obtained from the Centraalbureau voor Schimmelcultures, Baarn (CBS 429.82) were used in this study. These organisms were grown at 18°C on 2% malt extract agar (20 g Difco malt extract, 20 g Difco bacto agar, 1000 mL water) in Petri dishes. Cultures were illuminated by diurnal cycles of fluorescent and near-ultraviolet light. Ascomata 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 Ultrotome III. Sections were stained for 20 min with uranyl acetate and 10 min with lead citrate (Reynolds 1963), and examined with a Philips EM300 transmission electron microscope.

Results

Centrum organization

A vertical section through the ascoma of *O. minus* showed a distinct zone of developing, club-shaped asci extending upwards and outwards from the base of the ascoma toward the neck (Figs. 1, 22A). This zone of chained asci occupied approximately half the volume of the ascoma (Fig. 22A). Asci with developing ascospores occurred at the centre of the ascoma with large bodies dispersed between them (Fig. 1). Mature ascospores, with electron-dense cytoplasm, were released from disintegrating asci toward the base of the neck (Figs. 1, 22A). The diameter of the ascoma, excluding wall layers, was approximately 30–35 μm.

In *O. distortum*, ascomata were larger (approximately 150–180 μm) than those of *O. minus*. Ascomata had irregularly arranged asci occupying approximately one-third of the volume of the ascoma (Figs. 2, 22B). Clusters of asci, probably formed through crozier development, formed a lining adjacent to the inner ascomatal wall (Figs. 2, 22B). Ascospores were released toward the neck and also toward the centre of the ascoma. Mature ascospores, with electron-dense cytoplasm, were observed in the region below the neck (Fig. 22B). Ascospores were forced upwards into the neck opening (Fig. 3).

Ascus and ascospore development

Stages in the development of asci and ascospores in *O. distortum* and *O. minus* were essentially the same. We have therefore used *O. distortum* to illustrate these processes (Figs. 5 to 14). Asci in *O. distortum* were apparently formed from thin-walled cells containing single large nuclei (Figs. 4, 20A). The nuclei divided (Figs. 5, 20B), resulting in the formation of multinucleate cells (asci) (Figs. 6, 20C). The asci were irregularly shaped and contained endoplasmic reticulum, mitochondria, and other organelles dispersed between the nuclei (Figs. 6, 20C).

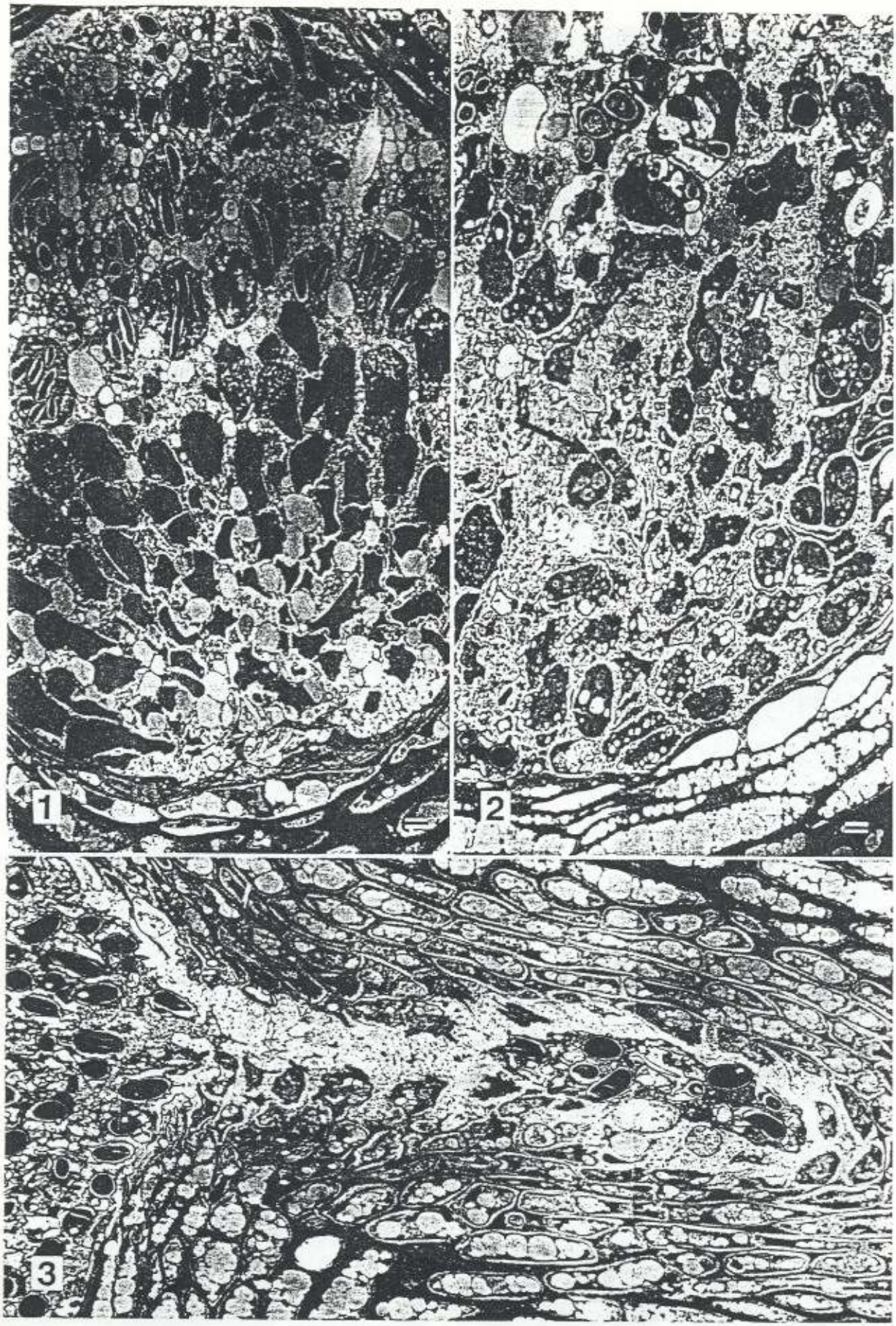
Small (approximately 65 nm) to large (between 150 and 950 nm) electron-transparent vesicles were also observed in the ascus cytoplasm (Figs. 6, 20C). The smaller vesicles, probably derived from the plasmalemma, coalesced to form elongated membranous structures, the peripheral membrane cylinder, positioned near the ascus periphery (Figs. 6, 7, 20C). During further development of the asci, the peripheral membrane cylinder developed into a double-membraned ascus vesicle, enclosing the nuclei and other organelles (Figs. 8, 20D). The ascus vesicle invaginated to enclose each nucleus by a double delimiting membranous sac, thus forming young ascospores (Figs. 9, 20E).

The larger vesicles merged with the outer delimiting membrane of the ascospore, depositing wall material between the delimiting membranes (Figs. 10, 20F). A primary wall layer was thus formed, which separated the delimiting membranes (Fig. 11). Amorphous, electron-dense granules were observed between the outer delimiting membrane and the primary wall (Figs. 11, 21A). These granules expanded to form electron-translucent areas, the perisporic sac, that completely separated

FIGS. 1–3. Transmission electron micrographs (TEM) of sections through ascomata of *O. minus* and *O. distortum* showing ascus arrangement and development. Bars = 1 μm. Fig. 1. Vertical section through the ascoma of *O. minus* showing developing thin-walled asci extending upwards and toward the neck. Degenerating asci release mature ascospores toward the neck region. Fig. 2. Vertical section through part of the ascoma of *O. distortum*; developing asci are irregularly arranged, probably derived from croziers (arrow). Fig. 3. Vertical section through ascomatal neck of *O. distortum* showing mature ascospores forced upwards into the neck opening.

FIGS. 4–9. TEM of sections through developing asci of *O. distortum*. Bars = 500 nm. Fig. 4. Section through ascogonial cell or young ascus showing single nucleus (N). Fig. 5. Dividing nucleus in ascogonial cell or ascus showing opposite nucleoli (arrows). Fig. 6. Young ascus with three nuclei and several large and small vesicles (arrows). Fig. 7. Small vesicles coalesce to form a peripheral membrane cylinder (arrows). Fig. 8. Delimiting membranous structure or ascus vesicle (AV) enclosing mitochondria (M) and other organelles. Fig. 9. Ascus showing final delimitation of individual nuclei by saclike delimiting membranes. Some saclike structures still show open regions (arrows).

FIGS. 10–15. TEM of developing ascospores. Bars = 500 nm. Fig. 10. Young ascospore with nucleus (N) and commencement of wall formation. Coalescence of vesicle with outer ascospore delimiting membrane showing deposition of wall material of the primary wall (arrow). Fig. 11. Maturing ascospores with primary wall development completed and commencement of secondary wall development. Secondary wall formed by the deposition of wall material (arrows). Fig. 12. Final stage of secondary wall development of the ascospore. Note the expansion region of deposited wall material (arrows). Fig. 13. Transverse section of mature ascospores in the intact and matured ascus showing expanded regions of the perisporic sac (arrows) and irregularly shaped secondary wall (SW). Fig. 14. Degenerating ascus releasing mature ascospores with electron-dense cytoplasm. Fig. 15. Developing ascospores in *O. minus*. Granular wall material deposited between ascospore delimiting membranes (arrows).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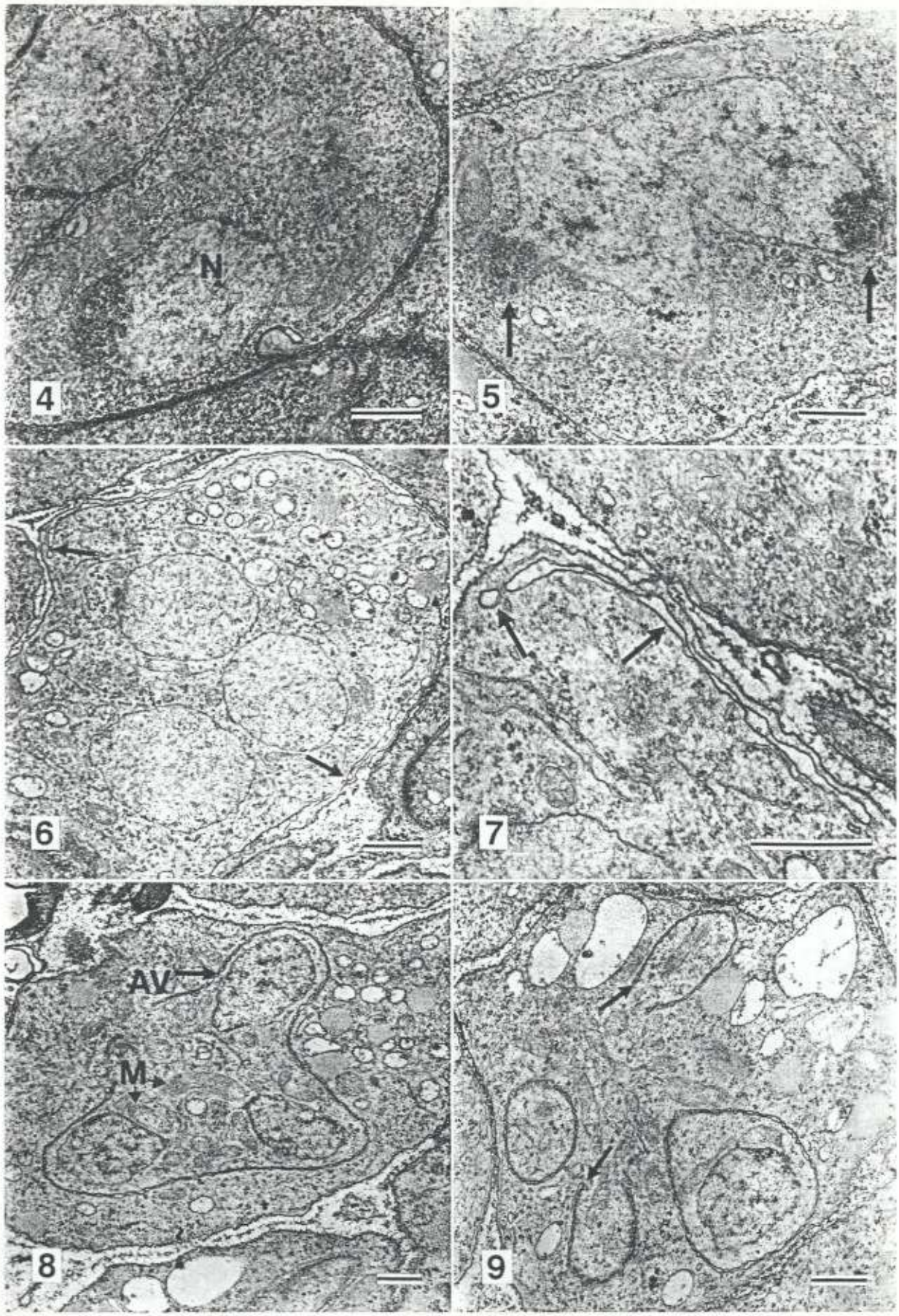
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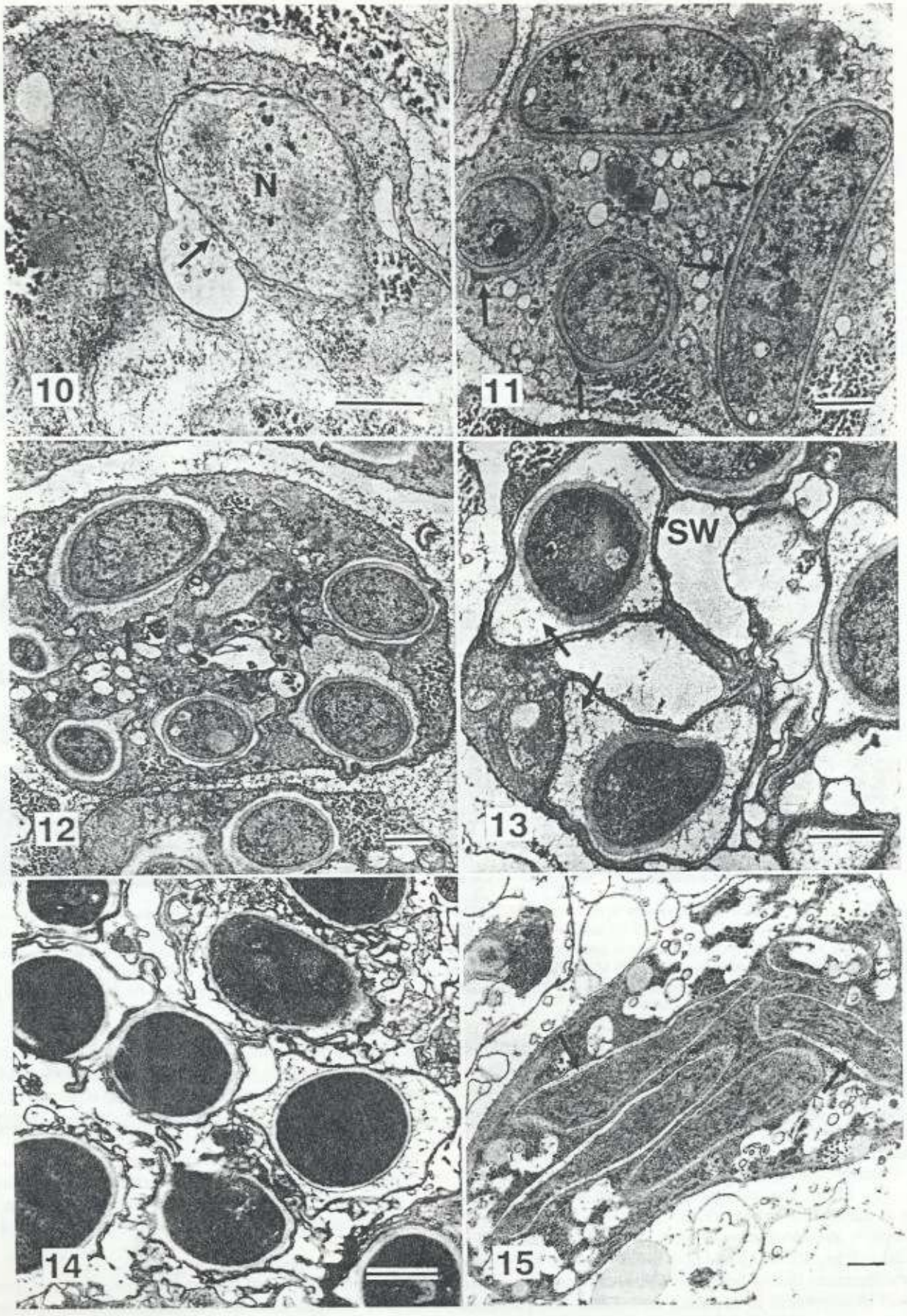
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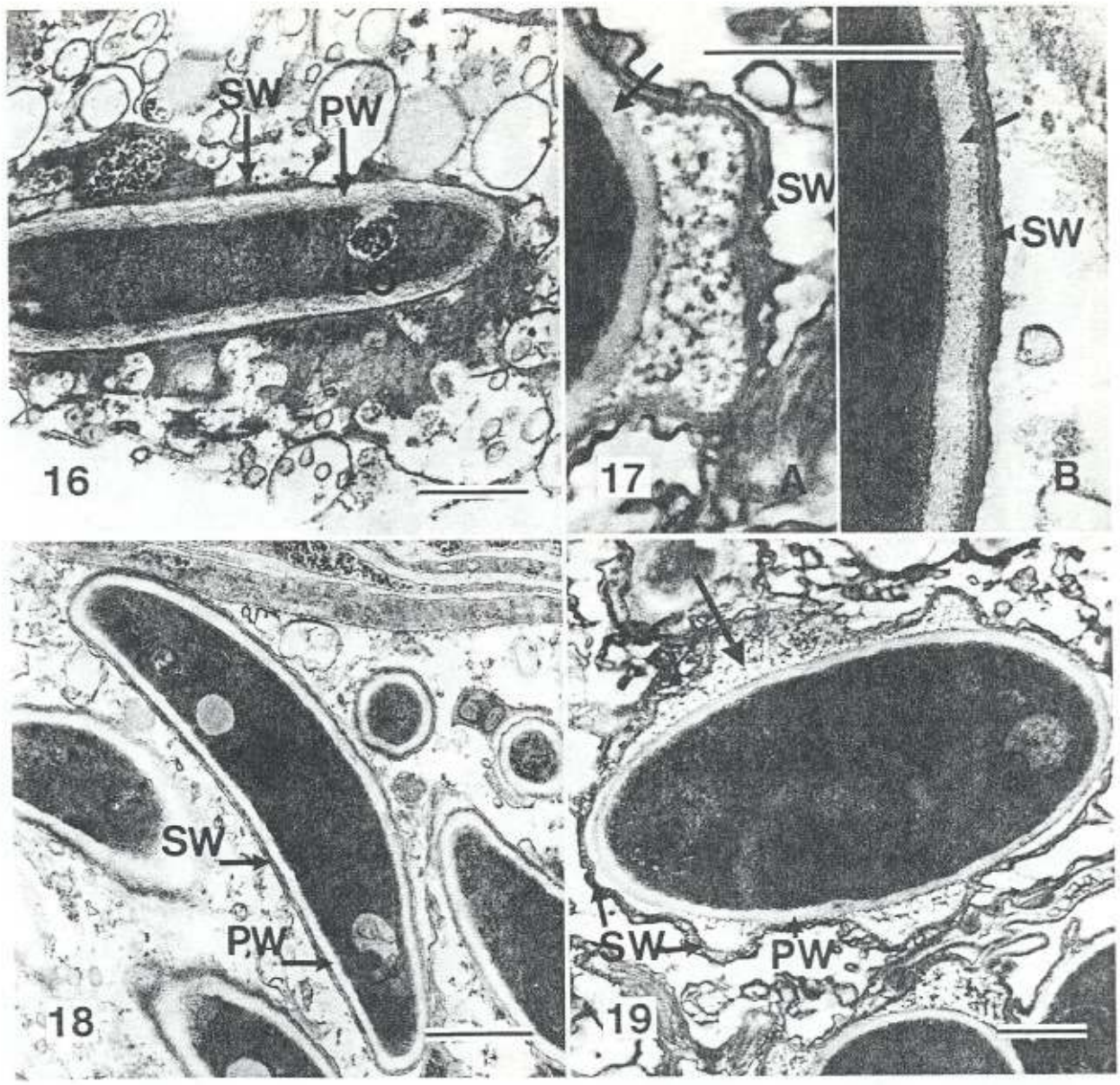

FIGS. 16–19. Development of ascospores in *O. minus*, and mature ascospores and walls of *O. distortum* and *O. minus*. Bars = 500 nm. Fig. 16. Development of the secondary (SW) and primary wall (PW) in *O. minus* with lomasome (LO). Fig. 17. Higher magnification of ascospore walls to illustrate primary (arrows) and secondary wall (SW) layers. (A) Ascospore wall of *O. distortum*. (B) Ascospore wall of *O. minus*. Fig. 18. Elongated crescent-shaped ascospore of *O. minus*, with primary (PW) and secondary wall (SW) layers. Fig. 19. Elongated ascospore of *O. distortum* showing primary wall (PW). The secondary wall (SW) is irregularly shaped ascribed to the expansion of the perisporic sac (arrow).

the primary wall from the outer delimiting membrane (Figs. 12, 21B).

After expansion, it also appeared that the granules formed the secondary wall adjacent and to the inside of the outer delimiting membrane (Fig. 12) of the ascospores. The expanded perisporic sac thus contained fibrils of secondary wall material that extended toward the inner primary wall (Figs. 13, 17A). The ascospore cytoplasm became electron dense at the time of release (Figs. 14, 19).

Although ascospore development commenced similarly in *O. minus* (Figs. 15, 21A) and *O. distortum*, there were a few minor differences during later development. For example, during wall development in maturing ascospores, the perisporic sac did not expand in *O. minus* as it did in *O. distortum*. In *O. minus*, the primary wall expanded slightly, showing a fibrillar composition (Figs. 16, 21C). Lomasomes, which contained electron-dense granules, were also observed in *O. minus* associated with the inner delimiting membrane (plasma membrane) of the ascospore during this expansion phase (Fig. 16). In contrast, lomasomes were not observed in *O. distortum*. In *O. minus* a secondary wall was formed through deposition of a uniformly thick electron-dense layer between the primary wall and outer perisporic membrane (Figs. 16, 17B, 18, 21E).

Ascospore morphology

The primary wall of mature ascospores in *O. distortum* was slightly elongate to ellipsoidal (Figs. 19, 21F). The secondary wall was irregularly shaped, ascribed to the developmental

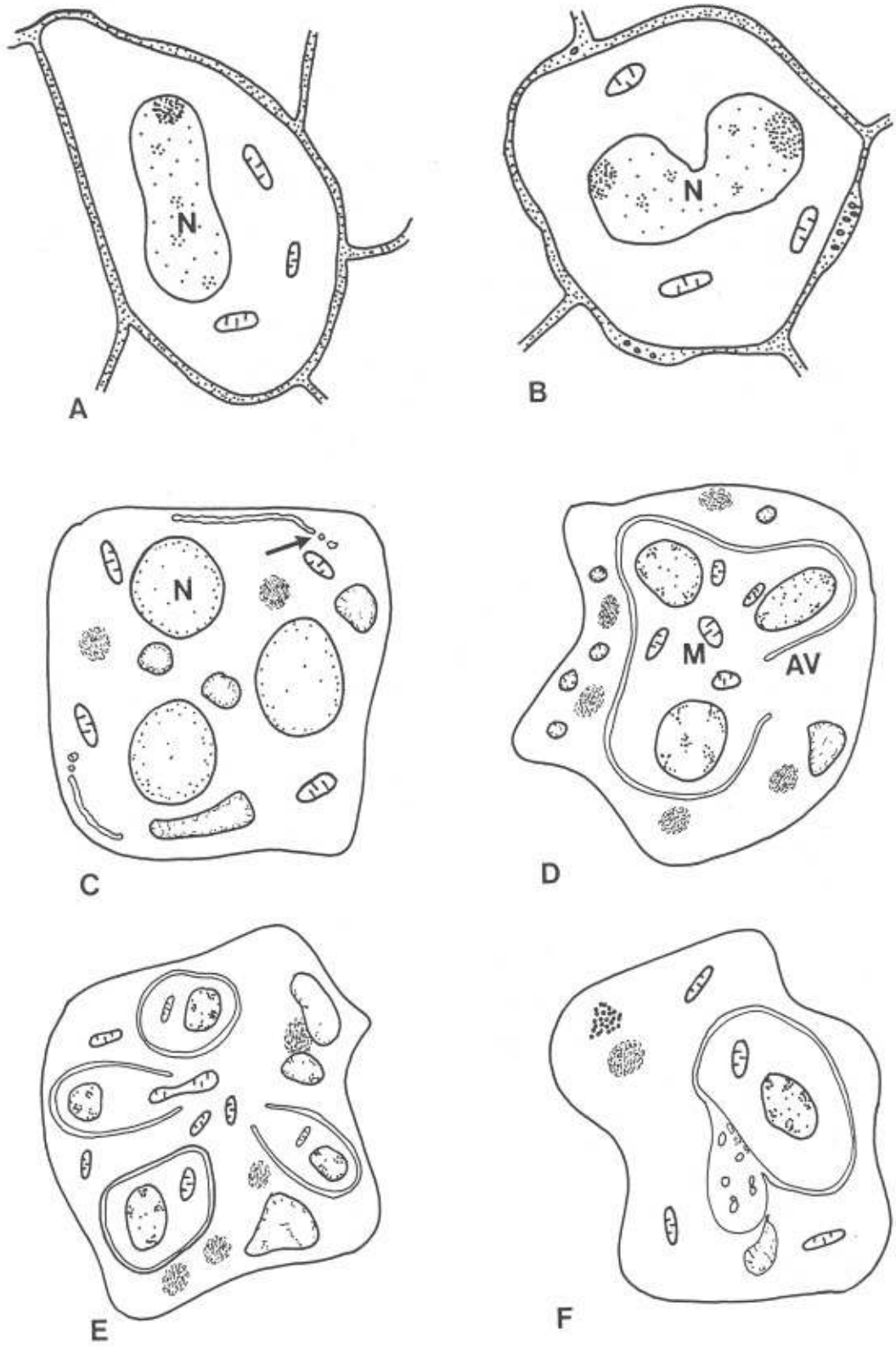

FIG. 20. Schematic representation of ascus and ascospore development in *O. distortum*. (A) Ascogonial cell prior to nuclear (N) divisions. (B) Young ascus showing nuclear (N) division. (C) Young ascus showing three of eight possible nuclei (N) and vesicles coalescing to form delimiting membranes (arrow). (D) Ascus vesicle (AV) surrounding ascospore nuclei and organelles. M, mitochondria. (E) Final stage of ascospore delimitation. (F) Commencement of primary wall formation.

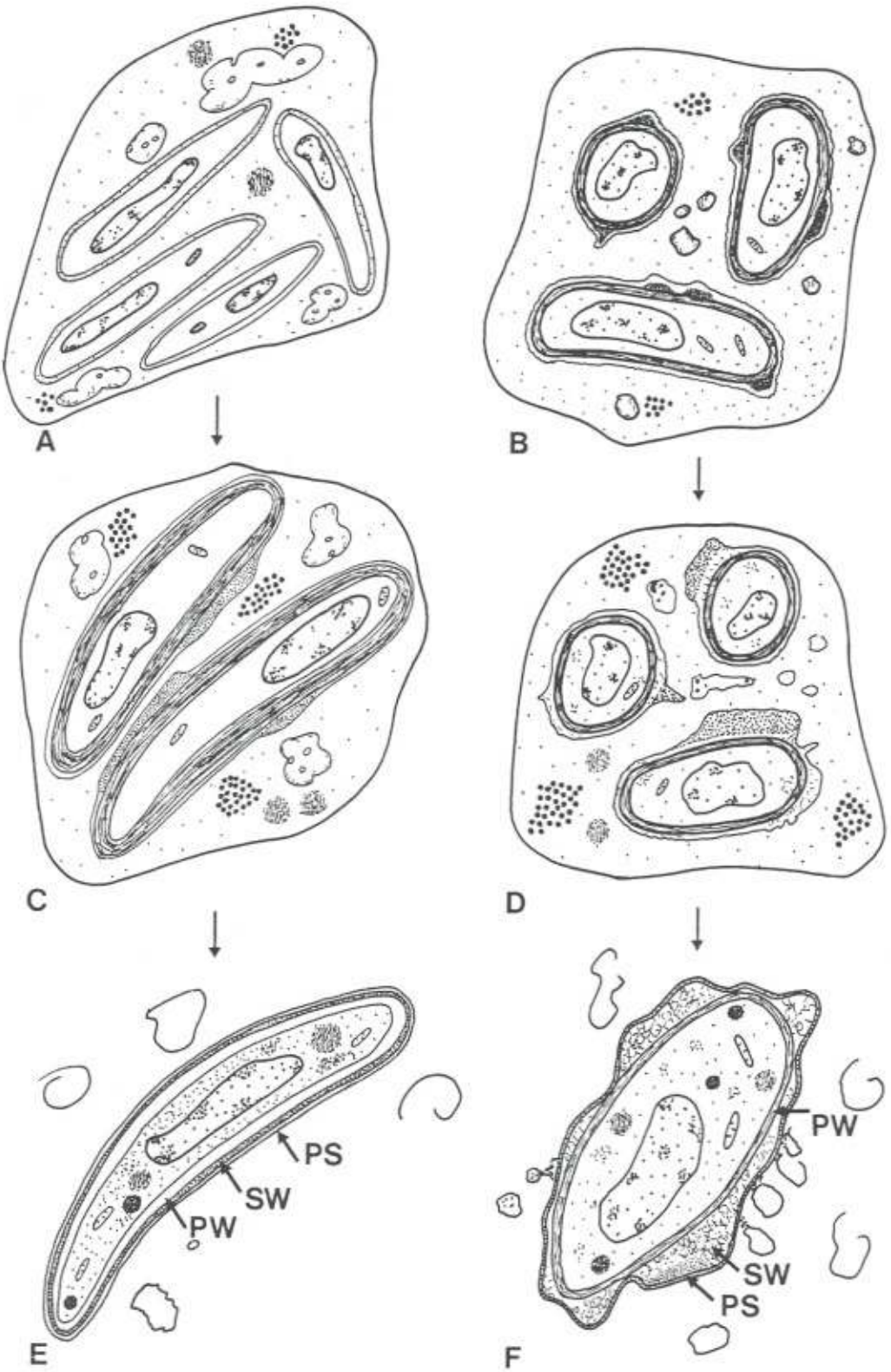

FIG. 21. Schematic representation comparing ascospore development in *O. minus* (A, C, and E) and *O. distortum* (B, D, and F). (A and B) Young ascospores during primary wall development. (C and D) Secondary wall development. (E and F) Mature ascospores. SW, secondary walls PW, primary wall; PS, perisporic sac.

phase during which the perisporic sac expanded in certain areas between the developing secondary and existing primary wall (Figs. 17, 21F). Mature ascospores in *O. minus* were elongate and crescent-shaped (Figs. 18, 21E). In contrast to *O. distortum*, the secondary wall and perisporic sac were of uniform thickness. The secondary wall and perisporic sac of *O. minus* were thus evenly shaped (Figs. 18, 21E) in contrast to the irregularly shaped secondary wall and expanded perisporic sac of *O. distortum*.

Discussion

The organization of the centra in *O. distortum* and *O. minus* differed considerably. However, there were similarities between these species and other species of *Ceratocystis* s.l. that have been examined ultrastructurally (van Wyk and Wingfield 1991; van Wyk *et al.* 1991). In *O. distortum*, irregular to club-shaped asci occurred in a distinct zone. This zone formed a lining adjacent to the ascomatal wall, arranged along the lower half of the ascoma. The tapered ends of the club-shaped asci were generally oriented toward the ascomatal base. This orientation did not appear to be uniform, some asci being irregularly arranged. It appeared that asci developed from ascogenous hyphae, which formed clusters of croziers that occurred along the periphery and lower half of the ascoma. Mature ascospores were then released toward the centre of the ascoma and neck. This pattern appeared to be similar to that in *C. moniliformis* (van Wyk *et al.* 1991). However, in the latter species the zone of young, irregularly shaped asci occurred up to the base of the neck. Mature ascospores in *C. moniliformis* were generally released toward the centre of the ascoma.

Centrum organization in *O. minus* appeared to be most similar to that in *O. davidsonii* (van Wyk and Wingfield 1991). In these species, asci originated at the base of the ascoma extending upwards and outwards from the base. However, asci of *O. minus* were uniformly club-shaped and arranged in chains with tapered ends oriented toward an area at the ascomatal base. The asci probably developed from a central cluster of ascogonial cells. In previous ultrastructural studies of *Ophiostoma ulmi* (Buisman) Nannfeldt (Jeng and Hubbes 1980), *Ophiostoma stenoceras* (Robak) Melin et Nannfeldt (Garrison *et al.* 1979), and *Ceratocystis fimbriata* Ellis et Halsted (Stiers 1976), centrum organization was not discussed.

Ascospore formation in *O. minus* and *O. distortum* commenced with the formation of double delimiting membranes. These membranes are similar to those observed in *C. fimbriata* (Stiers 1976), *O. ulmi* (Jeng and Hubbes 1980), *O. stenoceras* (Garrison *et al.* 1979), *O. davidsonii* (van Wyk and Wingfield 1991), and several yeast species (Beckett *et al.* 1973; Lynn and Magee 1970). It is suggested that the spore delimiting membranes originate from the ascus plasmalemma as they occur along the periphery of the ascus (Beckett 1981). They are, however, different from the delimiting membranes in *C. moniliformis* (van Wyk *et al.* 1991), in which the synthesis of the delimiting membranes occurred simultaneously with the development of the primary wall layer.

In *O. distortum*, vesicles in the ascus cytoplasm (epiplasm) are involved in the deposition of electron-dense material between delimiting membranes. This deposited material is apparently incorporated in the primary wall layer, adjacent to the inner membrane. In contrast to the epiplasmic vesicles, which probably contributed to the formation of the primary wall layer in *O. distortum*, lomasomes were observed in *O. minus*, apparently depositing wall material to be incorporated into the primary wall layer. Although lomasomes were not observed in *O. distortum*, lomasome activity is usually associated with ascospore wall formation (Marchant and Moore 1973; Wilsenach and Kessel 1965).

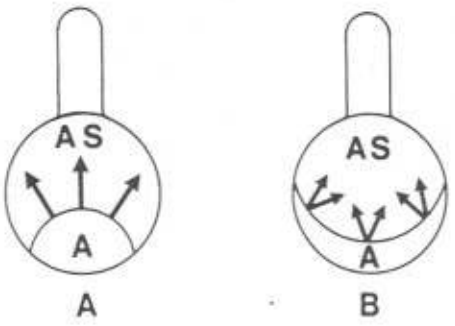

FIG. 22. Schematic comparison of centrum organization in *O. minus* and *O. distortum*. (A) Centrum of *O. minus*. (B) Centrum of *O. distortum*. A, region of young developing asci; AS, region of maturing asci and mature ascospores.

A characteristic feature of ascospore wall formation in *O. distortum* and *O. minus* was the occurrence of osmiophilic granules in the ascus during the deposition of the secondary wall. Vesicle activity and osmiophilic structures in the ascus cytoplasm (Illingworth *et al.* 1973; Parguey-Leduc *et al.* 1987) seemed involved in the formation of ascospore walls, although the physiological correlation is not clearly understood (Gibson and Kimbrough 1988). Similar structures were observed in *O. ulmi* (Jeng and Hubbes 1980), which has ascospores of the same shape as *O. minus*, although a sheath appeared to be absent (Upadhyay 1981). In *C. fimbriata*, however, Stiers (1976) interpreted these bodies as lysosomes.

Ophiostoma minus and *O. distortum* had ascospore walls composed of at least two layers. These included the outer secondary and inner primary wall. In *O. distortum* the perisporic sac was expanded and of distorted morphology, ascribed to a fibrillar matrix. In *C. fimbriata* (Stiers 1976) and *C. moniliformis* (van Wyk *et al.* 1991) the perisporic sac and secondary wall formed an appendage or brim, characteristic of hat-shaped ascospores. Although not discussed in these terms by all authors, the number and organization of wall layers appear to be similar in all species of *Ceratocystis* s.l. previously studied ultrastructurally.

The many differences in ascospore morphology within *Ceratocystis* s.l., such as the presence or absence of a sheath, appear to represent differences in the shape and structure of the perisporic sac and wall structure. From our studies and interpretation of micrographs of other authors, we believe that it is the shape and structure rather than the presence or absence of a sheath that differs between species of this genus.

Differences in the centrum organization and structure of ascospore wall layers between *O. minus* and *O. distortum* suggest that these species might not be congeneric. In *O. minus* the asci originated from a central cluster and developed in chains, while in *O. distortum* asci arose from croziers clustered at several areas along the periphery of the ascoma. The ontogeny of ascospore wall layers are strikingly different, as well as the shape and mucilaginous nature of the outer walls.

Ascus development in most species of *Ceratocystis* s.l. apparently commences in a similar fashion. However, substantial differences in ascospore delimitation, and especially wall development and structure, appear to occur between species. We also question the use of the current term sheath to describe ascospore wall layers in species of *Ceratocystis* s.l. This reaffirms our contention (van Wyk *et al.* 1991) that different ascospore forms should be examined in detail before conclusions can be drawn concerning taxonomic relationships within *Ceratocystis* s.l.

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