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Ultrastructure of ascus arrangement and ascospore development in *Ophiostoma seticolle*

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Abstract: Species in *Ceratocystis* and *Ophiostoma* have unusual ascospore morphology. Ascospores can have hat-shaped, elongate, inequilateral, needle or pillow-shaped forms. The ultrastructure and development of pillow-shaped ascospores, such as those occurring in *O. seticolle,* have never been examined critically. In *O. seticolle,* ascospore development occurs in irregularly shaped, thin-walled asci present in young ascomata. Each ascus contains eight nuclei surrounded by delimiting membranes, which lead to ascospores having at least three wall layers. The outermost wall layer is characterized by the formation of ridges that give the ascospores their pillow-shaped appearance. Ascospores of *O. seticolle* are released in a slimy matrix, and what appear to be sheaths surrounding the ascospores, reported in most light microscopic studies, are actually multilayered ascospore walls.

Key Words: ascoma, ascospores, centrum, *Ceratocystis, Ophiostoma,* pillow-shaped sheath

INTRODUCTION

Ceratocystis Ellis & Halstead, *Ophiostoma* H. & P. Sydow and *Ceratocystiopsis* Upadhyay & Kendrick are important pathogens of trees and some agricultural crops (Boyce, 1961; Clark and Moyer, 1988; Manion and French, 1967; Smith, 1967). Controversy exists regarding the taxonomy of these genera (De Hoog and Scheffer, 1984) as well as the placement of the ophiostomatoid fungi within the Ascomycetes (Benny and Kimbrough, 1980; Redhead and Malloch, 1977; Upadhyay, 1981). Recent molecular studies shed some light on the controversy defining *Ceratocystis, Ophiostoma* and *Ceratocystiopsis* as monophyletic genera (Hausner et al., 1993a, b). Ascospore shape is an important characteristic used in the taxonomy of these genera. However, there is still insufficient data on ascospore ultrastructure of the ophiostomatoid fungi (Van Wyk and Wingfield, 1990). This is a factor that had led to probably many of the taxonomic controversies.

Upadhyay (1981) divided the ophiostomatoid fungi into two genera on grounds of ascospore shape and sheath morphology. Species without sheaths and others with hat-, pillow- and inequilateral-shaped sheaths were placed in *Ceratocystis,* which included four sections to accommodate the various ascospore forms. Species with falcate sheaths, however, were placed in the genus *Ceratocystiopsis* (Upadhyay and Kendrick, 1975). The structure of sheaths and the number of wall layers that surround ascospores of species of the ophiostomatoid fungi has been the subject of debate.

In light microscopic studies, it was determined that ascospores can either have elaborate sheaths or have no sheaths (Upadhyay, 1981). Ultrastructural studies on ascospore development in the ophiostomatoid fungi, however, have shown that all species examined have ascospores with three-layered walls. Where these are elaborate or thick they have been perceived as gelatinous sheaths. These studies include species with hat-shaped ascospores (Stiers, 1976; Van Wyk et al., 1991; Van Wyk and Wingfield, 1991a, b) and species with ascospores presumed to be without sheaths, such as *O. minus* (Hedgcock) H. & P. Sydow, *O. distortum* (Davidson) de Hoog & Scheffer (Van Wyk and Wingfield, 1991c) and *O. piceae* (Münch) H. & P. Sydow (Van Wyk and Wingfield, 1992).

In ultrastructural studies on some species of *Ceratocystis* and *Ophiostoma* producing ascospores without sheaths, only two wall layers were reported (Garrison et al., 1979; Jeng and Hubbes, 1980). Ascospores of all these species were released in a slimy gloeoid matrix (Olchowecki and Reid, 1974; Upadhyay, 1981). In *O. ulmi* (Jeng and Hubbes, 1980) and *O. piceae* (Van Wyk and Wingfield, 1992) it was suggested that this matrix adhered to the outermost wall layer of the ascospores. This matrix is produced in most, if not all, ascospores in the ophiostomatoid fungi and can easily be misinterpreted as sheaths in species with well-developed outer wall layers (Van Wyk and Wingfield, 1992).

Ophiostoma seticolle (Davidson) Hoog & Scheffer was described as having ascospores with well-developed pillow-shaped hyaline gelatinous sheaths (Davidson,

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1966; Upadhyay, 1981). The ultrastructure of pillow-shaped sheaths has not previously been examined in ophiostomatoid or other fungi. The aim of this study, therefore, was to examine ascospore development in *O. seticolle* to determine the nature and ultrastructure of the prominent ascospore walls.

MATERIAL AND METHODS

A culture of *Ophiostoma seticolle* (CBS 634.66) was obtained from the Centraalbureau voor Schimmelcultures. It was grown at 18 C on 2% malt extract agar (20 g Difco malt extract, 20 g Difco Bacto agar, 1 L distilled water) in plastic 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 3%, 0.1 M, pH 7.0, sodium phosphate-buffered glutaraldehyde for 3 h at 20 C, followed by 1 h fixation in similarly buffered 0.5% osmium tetroxide. 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 collected on 50-mesh grids sustained by formvar support films. The 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

Vertical sections through ascomata showed thin-walled asci in different stages of development. The ascomata were packed with irregularly to spindle shaped asci and large unstained areas were observed between the asci (FIG. 1). Depending on the age of the ascomata, at least three distinct developmental regions were observed (FIG. 2). Young asci without any ascospores occurred in the lower region of the ascomata. An area with developing asci was observed in the middle region. In this region, early stages of wall formation of the ascospores took place. A third region, with almost mature ascospores, occurred below the neck. The ascospores that matured first were released towards the base of the neck (FIGS. 1, 2, 14A). Entirely mature ascomata contained only mature ascospores, which oozed from the ascomatal neck embedded in ascus debris.

Ascospore delimitation commenced with the formation of a single ascus vesicle involving delimiting membranes, which developed along the periphery of the ascus wall (FIGS. 3, 14B). This ascus vesicle invaginated and incompletely enclosed individual nuclei in the asci with sac-like delimiting membranes (FIGS. 4, 5, 14C). The open ends of these sac-like structures enclose nuclei and cytoplasm to form ascospore initials (FIGS. 5, 14D).

Wall formation commenced with the development of a fine granular layer between the paired delimiting membranes (FIGS. 6, 14C). Early developmental stages of ascospore wall formation were characterized by the occurrence of granules in the asci (FIGS. 6, 7, 14D). During further development two distinct wall layers were observed, an innermost primary wall and an outermost secondary wall (FIGS. 7, 14D). The outermost delimiting membrane (perisporic sac) of the ascospores appears to be in close contact with membranes, probably endoplasmic reticulum, and granules in the ascus (FIGS. 1, 7, 14D). These granules are well stained with the heavy metal salts and thus may be wall material reserves. The secondary wall layer formed characteristic protrusions (FIGS. 8, 14E). In mature ascospores three distinct wall layers were observed, i.e., the innermost primary wall, and two layers of the secondary wall (FIG. 9). The asci eventually disintegrated, releasing mature ascospores dispersed between remaining granules and ascus debris near the neck region (FIG. 10).

The protrusions that were formed in the walls of young ascospores developed into prominent ridges observed in longitudinal and transverse sections of mature ascospores (FIGS. 11, 14F). Four parallel ridges were formed opposite to each other along the length of the ascospores. This gave the ascospores a quadrangular appearance in transverse section (FIGS. 11; 14F, a). An oblique longitudinal section through the ascospores also showed these capacious parallel regions of the secondary wall and perisporic sac (FIGS. 12; 14F, b).

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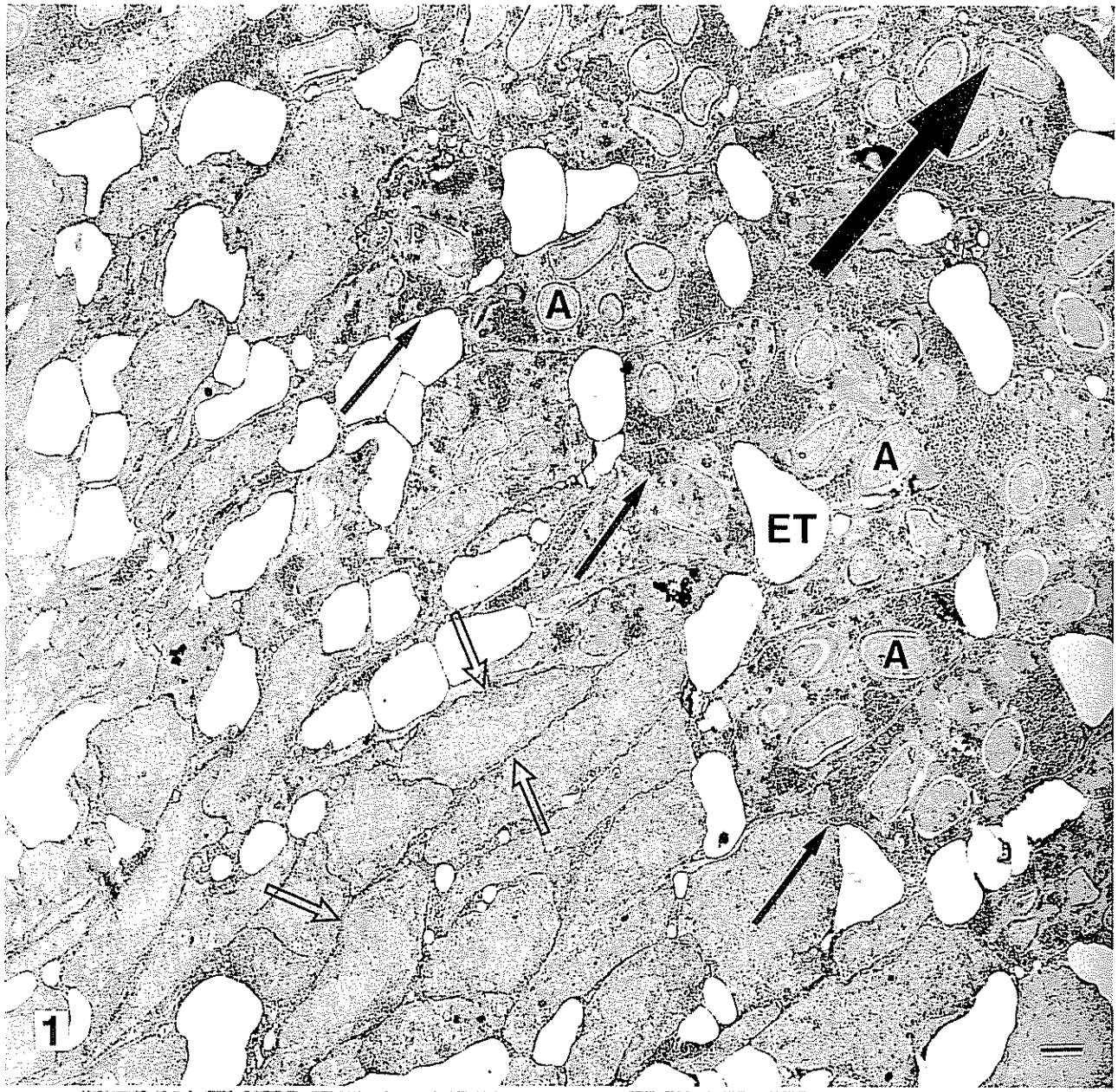
FIGS. 1–3. Transmission electron micrographs (TEM, bars = 1 μm) and light micrograph (bar = 10 μm) of sections through ascomata, asci and ascospores of *O. seticolle*. 1. TEM of vertical section through ascoma near neck region (indicated by zone A and B in FIG. 2), with mature ascospores, A, and developing asci with thin walls (outlined arrows). Note ascospores, A, embedded in electron opaque granular matrix (arrows) of asci and electron translucent areas, ET, between asci. Large arrow points towards neck opening. 2. Light micrograph of vertical section through ascoma showing three distinct zones of asci at different developmental stages: A = mature ascospores; B = maturing asci and developing ascospores; C = young asci. 3. Young ascus (TEM from zone B) indicating ascospore delimiting membranes (DM, arrows) arranged along the periphery of the ascus wall.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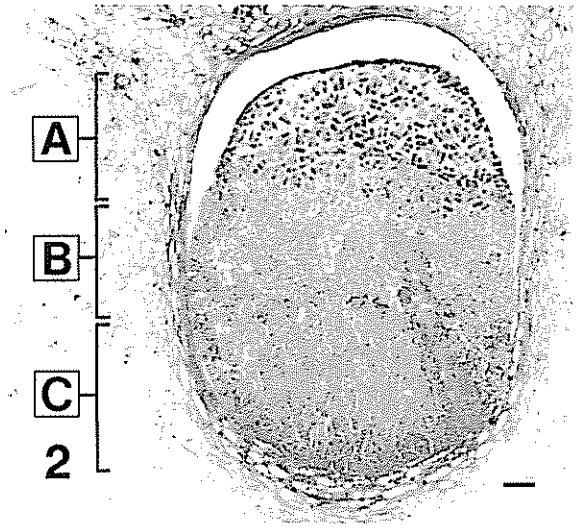
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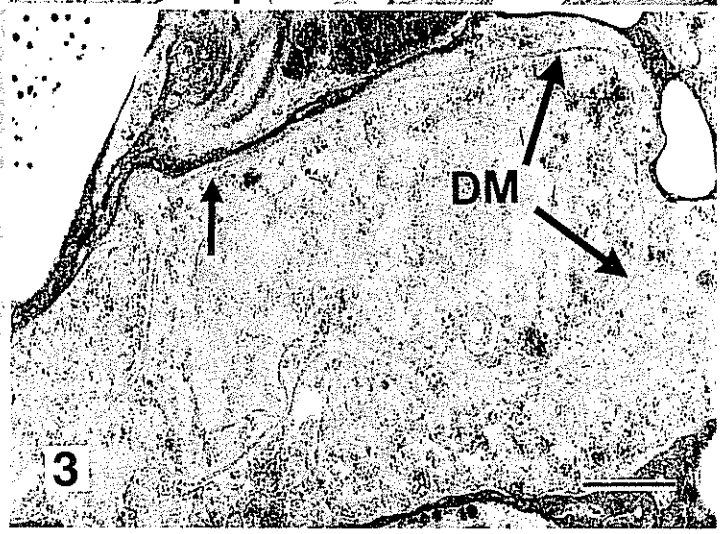
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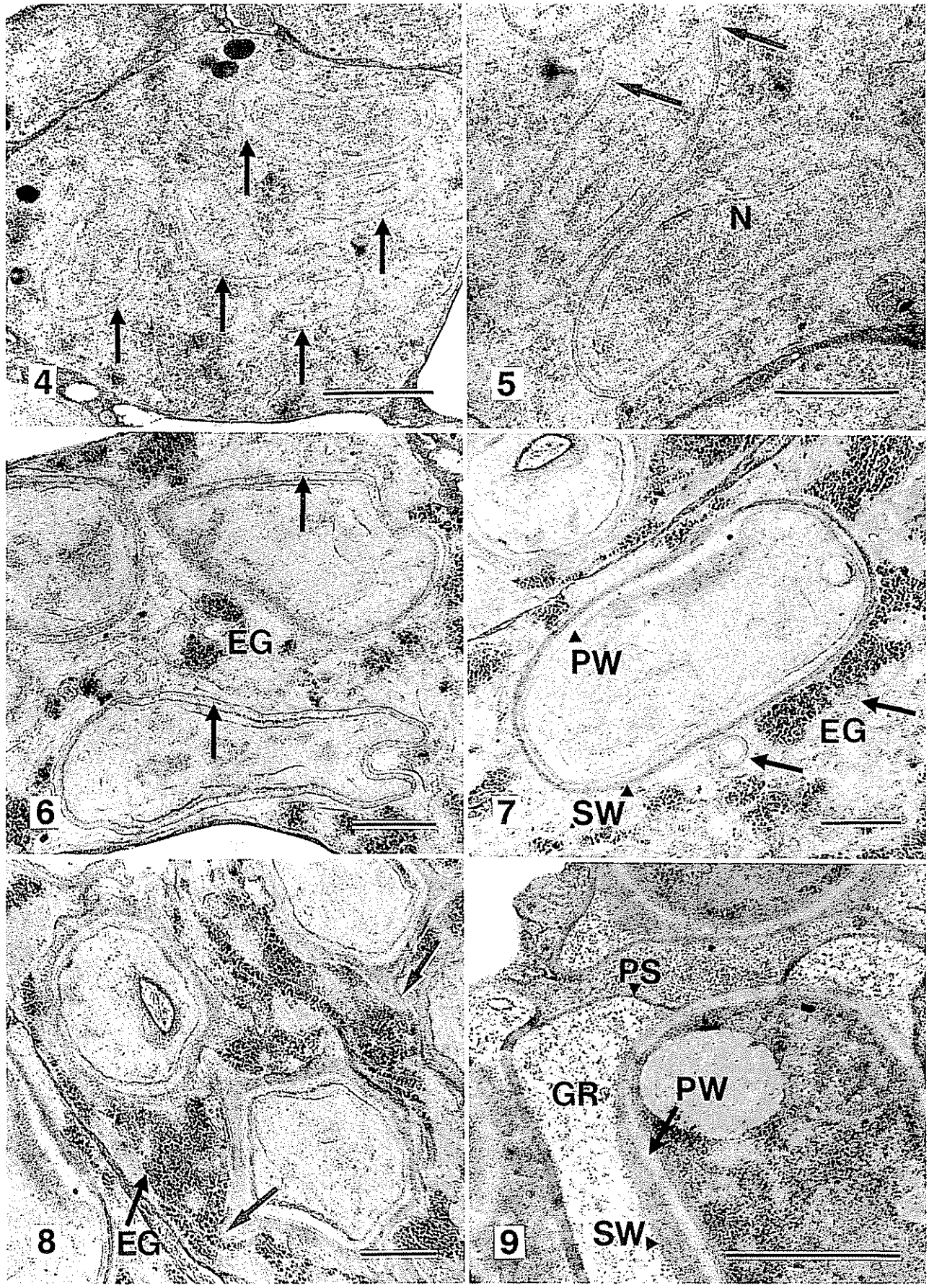
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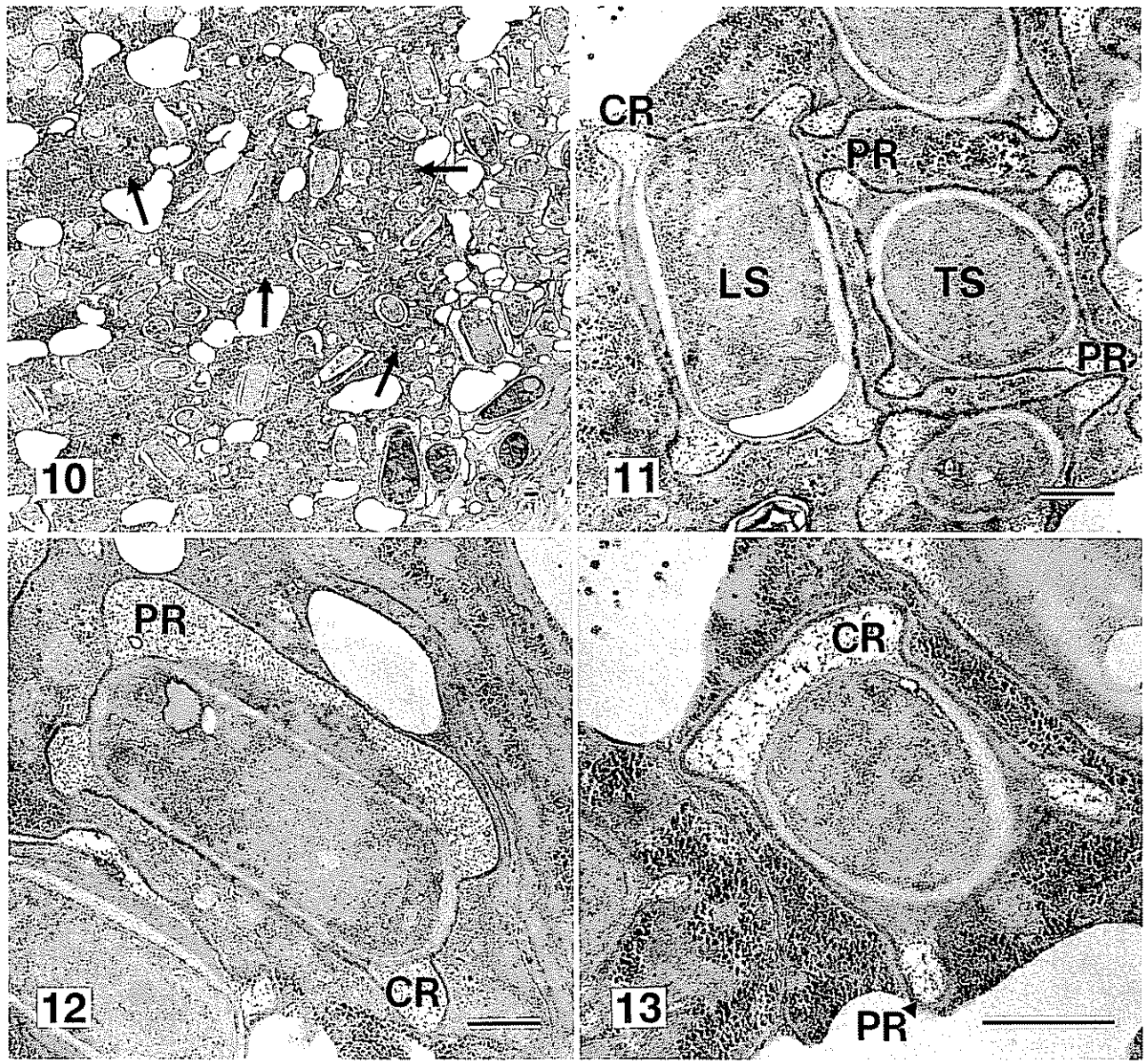

FIGS. 10–13. Sections through asci and mature ascospores of *O. seticolle*. Sectioning planes schematically illustrated in FIG. 14F. Bars = 500 nm. 10. Mature ascospores released from lysing asci within granular ascus matrix (arrows). 11. Longitudinal, LS, and transverse, TS, section through ascospores, respectively showing prominent parallel ridges, PR, and polar (circular) ridges, CR, of the secondary wall. 12. Oblique longitudinal section through ascospore. Sectioning plane is through one parallel ridge, PR, and two polar (circular) ridges, CR. 13. Oblique transverse section through rounded end of ascospore. Sectioning plane obliquely through polar (circular) ridge, CR, and parallel ridges, PR.

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FIGS. 4–9. TEM of sections through developing asci and ascospores of *O. seticolle*. Bars = 500 nm. 4. Ascus and five ascospore initials with delimiting membranes surrounding each nucleus (arrows). 5. Young ascospores delimited by sac-like membranes with open ends (arrows), surrounding each nucleus, N. 6. Commencement of wall formation stage of ascospore development. Fine granular material deposited between the delimiting membranes (arrows). Electron dense wall reserve material, EG, occurring in the ascus cytoplasm. 7. Longitudinal section through young ascospore with outermost secondary wall, SW, formed adjacent to innermost primary wall, PW. Electron dense granules, EG, in close association with membranes and perisporic sac (arrows). 8. Transverse section through ascospores showing development of secondary wall protrusion (arrows) into granular area, EG, of ascus. 9. High magnification of transverse section through ascospore wall layers. PW = primary wall, GR = gelatinous region or outside layer of secondary wall, SW = inside layer of secondary wall, PS = perisporic sac.

Circular or polar ridges at the rounded ends of the ascospores were observed in longitudinal sections (FIG. 11). Oblique sections through rounded ends of the ascospores, showed the well developed secondary wall layer which gave rise to these polar ridges (FIGS. 13; 14F, c). The primary wall gave the ascospores a cylindrical shape (FIGS. 11, 12). However, the prominent parallel and polar ridges afforded the ascospores the so-called pillow-shape.

DISCUSSION

Distinct differences were found in centrum organization between *O. seticolle* and other species of the ophiostomatoid fungi. In *O. seticolle,* the ascomata were packed with irregularly to spindle shaped asci, which occurred in three distinct layers. Asci appear to develop from a basal zone of ascogenous cells and are then pushed up into the centrum by newly developing asci. Thus, very young asci without ascospores occurred in the lower region of the ascomata, asci with developing ascospores in the middle region, and asci with mature ascospores in the upper region near the neck. The centrum is finally filled with asci that mature from the neck towards the centrum base. This arrangement of asci is a unique feature of *O. seticolle* and has not been observed in any other species of the ophiostomatoid fungi previously studied (Van Wyk and Wingfield, 1991a, b, c, 1992; Van Wyk et al., 1991).

Generally, centrum organization in the ophiostomatoid fungi can be separated into two types. In one type, young irregularly shaped asci are arranged along the periphery of the inner ascomatal wall with mature ascospores released towards the ascoma center. This was observed in *C. moniliformis* (Hedgcock) C. Moreau (Van Wyk et al., 1991), *O. distortum* (Van Wyk and Wingfield, 1991c) and *O. piceae* (Van Wyk and Wingfield, 1992). Asci in *O. piceae* were prominently spindle-shaped. In a second type, exemplified by *O. davidsonii* (Olchowecki & Reid) Solheim (Van Wyk and Wingfield, 1991a), *O. cucullatum* Solheim (Van Wyk and Wingfield, 1991b) and *O. minus* (Van Wyk and Wingfield, 1991c), young club-shaped asci were arranged in a cluster at the ascomatal base and mature ascospores were released towards the ascomatal neck. The centrum organization in *O. seticolle* appears to be reasonably similar to that of the second type.

Ascospore formation in *O. seticolle* commenced with the formation of double delimiting membranes. These membranes are similar to those observed in most species of the ophiostomatoid fungi and other Ascomycetes studied at the ultrastructural level. Similarly, the ascospore wall was formed by the deposition of wall material between the delimiting membranes (Stiers, 1976; Jeng and Hubbes, 1980; Garrison et al., 1979; Van Wyk and Wingfield, 1991c).

The occurrence of granules in the asci during the formation of the ascospore wall may indicate that wall material reserves are supplied through enzymatic processes within the ascus. These granules also appear to be associated with membranes of the ascus and delimiting membranes of the ascospores. This indicates the active involvement of the delimiting membranes in the formation and orientation of wall fibrils and fibers. Similar mechanisms were reported for higher plants (Giddings et al., 1980).

In *O. seticolle,* ascospores have been described as having pillow-shaped, hyaline, gelatinous sheaths (Olchowecki and Reid, 1974; Solheim, 1986; Upadhyay, 1981). This study has shown that the ascospores were surrounded by at least three wall layers. The outermost perisporic sac and underlying secondary wall formed characteristic ridges along the length and at the rounded ends of the ascospores. These ridges gave the ascospores their pillow-shaped appearance. Both layers, especially the secondary wall, appear to be hyaline when observed with the light microscope (Upadhyay, 1981). However, a "sheath" of hyaline and gelatinous nature was not observed.

Differences between ascospore morphologies in species of *Ceratocystis, Ophiostoma* and *Ceratocystiopsis* may be ascribed especially to the development and differentiation of their secondary walls. The hat-shaped sheaths in ascospores in *C. fimbriata* Ellis & Halstead (Stiers, 1976) and *C. moniliformis* (Van Wyk et al., 1991), are the result of the development of brim-like appendages formed by the secondary wall (epi- and mesospore layers) (Stiers, 1976; Van Wyk et al., 1991). In

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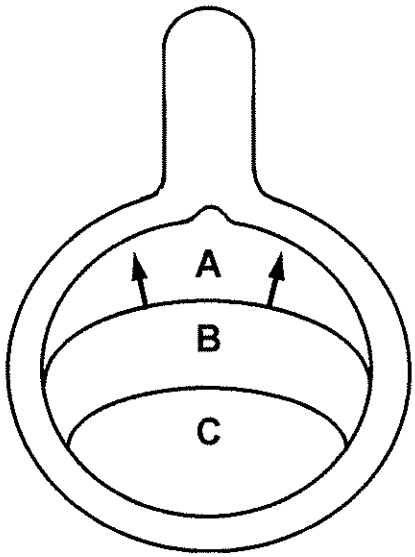
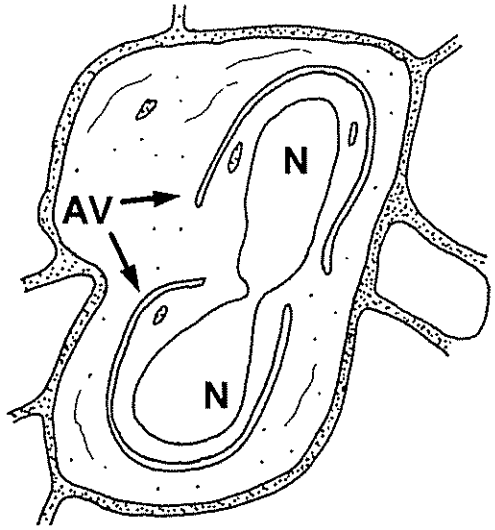
FIG. 14A–F. Schematic representation of ascus and ascospore development in *O. seticolle.* A. Schematic representation of centrum organization in *O. seticolle.* Note the distinct zones of maturing ascospores (A) developing asci and ascospores (B) and young asci (C). B. Ascospore delimiting membranes, AV, occurring along periphery of ascus wall, N = nucleus. C. Sac-like delimiting membranes, DM, enclosing ascospore nuclei. D. Ascus showing electron dense granules, EG, in close contact with membranes (arrow) and periosporic sac. Primary wall, PW, and secondary wall, SW, formed between delimiting membranes (arrow). E. Young ascospores showing secondary wall protrusions (arrows). F. Schematic representation of mature pillow-shaped ascospore in *O. seticolle* illustrating different sectioning planes through ascospore; a = transverse section (compare FIG. 11), b = oblique longitudinal section (compare FIG. 12), c = oblique transverse section (compare FIG. 13).



14 A



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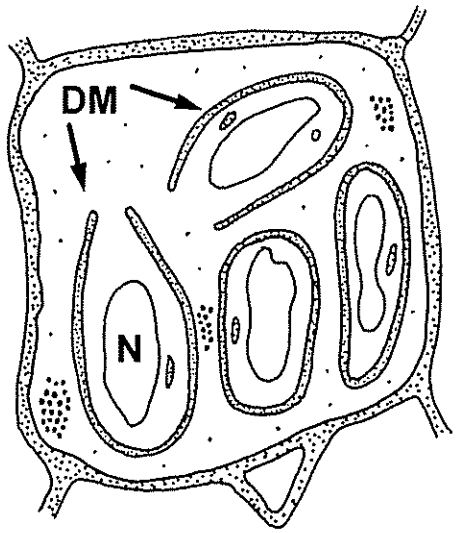

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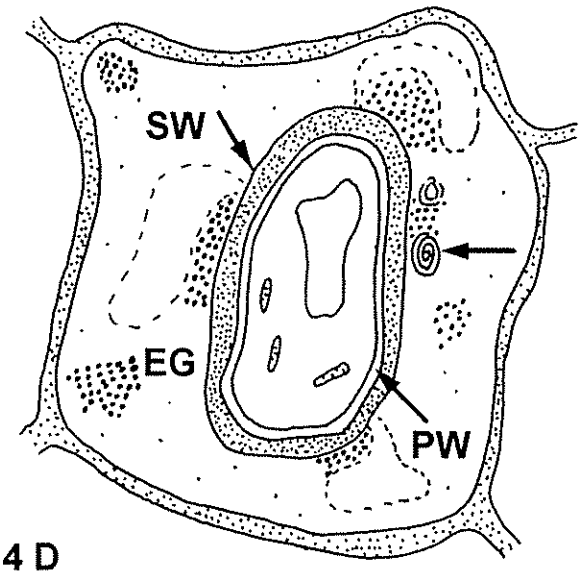

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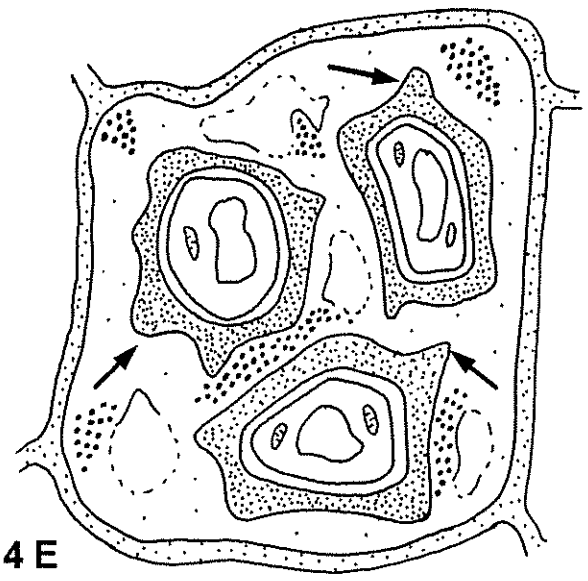

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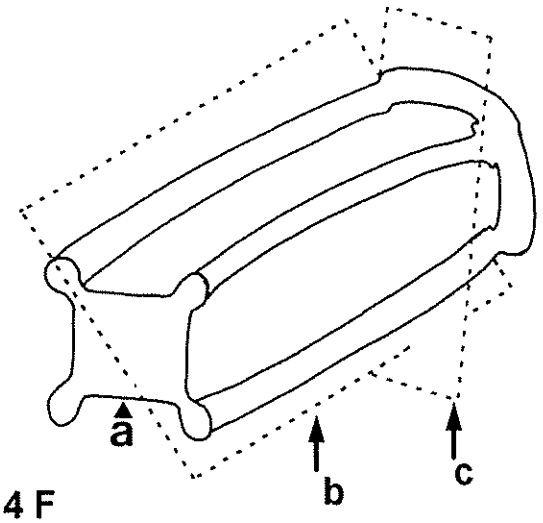

14 F

O. davidsonii (Van Wyk and Wingfield, 1991a) and *O. cucullatum* Solheim (Van Wyk and Wingfield, 1991b), which also have hat-shaped ascospores, the development of the ascospores is considerably different. In these species, the hat-shaped morphology of the ascospores results from extensions of the secondary wall at the rounded ends of the ascospores and cannot be ascribed to the development of the ascospores in pairs. Another ascospore shape arising from elaborate extensions of the secondary wall is found in *O. distortum* (Van Wyk and Wingfield, 1991c), which has elongated ascospores with convoluted secondary walls.

Ascospores of species in the ophiostomatoid fungi are released within remaining lysed ascus debris, defined as a slimy or gloeoid matrix. In a study of *O. piceae,* Van Wyk and Wingfield (1992) illustrated that this matrix adheres to the secondary wall of the ascospore. Depending on the volume of the matrix adhering to the outermost wall layer, this might be interpreted as a sheath. This matrix, however, should not be confused with the rigid wall layers that give the ascospores in many species of the ophiostomatoid fungi their characteristic shape. In this study, therefore, it has been shown that in ascospores with prominent sheaths, the sheath is actually the ascospore wall consisting of the perisporic sac, the elaborate secondary wall layers and the underlying primary wall.

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