Fine structure of ascosporogenesis in *Ceratocystiopsis proteae*

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The development and ultrastructure of the ascoma, asci, and ascospores in *Ceratocystiopsis proteae* were studied and compared with that of other genera in *Ceratocystis* sensu lato. Ascospore delimitation commenced with the formation of double delimiting membranes in the ascus as is true in other species in *Ceratocystis* s.l. The ascospore wall developed between these membranes. In contrast with three wall layers observed in other species, only two wall layers were observed in *C. proteae*. Falcate sheaths previously reported in light-microscopic studies were not observed. The falcate appearance of ascospores may be ascribed to the adherence of a matrix to the outermost wall layer during ascospores release. Differences between this species and others in *Ceratocystis* s.l. illustrate the problems that can arise from the use of single morphological characteristics in the taxonomy of this group. It is accordingly suggested that the genus *Ceratocystiopsis* requires taxonomic revision.

Key words: *Ceratocystiopsis*, falcate ascospores, sheaths, centrum.

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Les auteurs ont étudié le développement et les ultrastructures de l'ascoma, de l'asque et des ascospores chez le *Ceratocystiopsis proteae* et ils les ont comparés avec ceux de d'autres genres de *Ceratocystis* sensu lato. La délimitation de l'ascospore commence avec la formation de doubles membranes de délimitation dans l'asque, comme c'est le cas chez les autres espèces de *Ceratocystis* s.l. La paroi de l'ascospore se développe entre ces membranes. Alors qu'on observe trois couches pariétales chez les autres espèces, dans le cas du *C. proteae*, il n'y en a que deux. Les auteurs n'ont pu observer les membranes falciformes déjà rapportées par observation en microscopie photonique. L'aspect falciforme des ascospores pourrait être attribué à l'adhérence de la matrice à la couche pariétale la plus externe au cours du relâchement des ascospores. Les différences entre ces espèces et d'autres du genre *Ceratocystis* s.l. illustrent les difficultés que l'utilisation de caractéristiques morphologique uniques peuvent entrainer dans la taxonomie de ce groupe. On suggère donc qu'une revision taxonomique du genre *Ceratocystiopsis* soit effectuée.

Mots clés : *Ceratocystiopsis*, ascospores falciformes, membranes, centrum.

[Traduit par la rédaction]

Introduction

Ceratocystiopsis Upadh. and Kendr. was established as a segregate genus of *Ceratocystis* Ell. and Halst. sensu lato by Upadhyay and Kendrick (1975) to accommodate species with falcate ascospores. *Ceratocystis* s.l. also includes the genera *Ceratocystis* sensu stricto and *Ophiostoma* H. et P. Sydow. (De Hoog and Scheffer 1984; Upadhyay 1981; Weijman and De Hoog 1975). These organisms are important plant pathogens, especially of trees (Boyce 1971; Clark and Moyer 1988; Manion and French 1967), and were recently recorded with their anamorphs from South Africa, infecting pine trees (Wingfield and Knox-Davies 1980; Wingfield and Marasas 1980, 1983). *Ceratocystiopsis proteae* Wingfield, Van Wyk and Marasas, recently found within the infructescences of *Protea repens* (L.) L. (Wingfield et al. 1988), is the subject of this investigation.

Previous ultrastructural studies on ascospore development include species with hat-shaped ascospores (Stiers 1976; Van Wyk et al. 1991; Van Wyk and Wingfield 1991*a*, 1991*b*) and those with elongated ascospores with only two wall layers (Garrison et al. 1979; Jeng and Hubbes 1980). The ultrastructure and development of elongated ascospores with at least three wall layers in *Ophiostoma minus* (Hedgc.) H. and P. Syd., *Ophiostoma distortum* (Davids.) de Hoog and Scheffer (Van Wyk and Wingfield 1991*c*), and *Ophiostoma piceae* (Fries) H. and P. Syd. (Van Wyk and Wingfield 1992) were also studied. Results obtained from these investigations showed distinct differences in ascospore development and especially ultrastructural characteristics of ascospore wall layers.

The subdivision of *Ceratocystis* s.l. has been the basis of controversy (De Hoog and Scheffer 1984) because current knowledge of the shape of these ascospores is based on only light-microscope studies. This study thus forms part of an ongoing program to determine the exact nature of ascospore walls (previously defined as sheaths) in *Ceratocystis* s.l., using ultrastructural techniques. Knowledge obtained could thus improve our understanding of taxonomic relationships between genera and species of *Ceratocystis* s.l.

Ceratocystiopsis spp. are ecologically and taxonomically most closely related to *Ophiostoma* and are separated from the latter primarily on the basis of their elongate ascospores apparently with falcate sheaths (De Hoog and Scheffer 1984; Upadhyay 1981). *Ceratocystiopsis proteae* was accordingly assigned to this genus, but otherwise shares characteristics with both *Ophiostoma* and *Ceratocystis*. It is also unusual in having a *Knoxdaviesia* Wingfield, Van Wyk and Marasas anamorph opposed to the *Sporothrix* Hektoen et Perkins anamorph of other *Ceratocystiopsis* species (Mouton and Wingfield 1992). The aim of this study was to examine the ultrastructural devel-

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opment of the falcate ascospores in *C. proteae*, with the view of better understanding the generic relationships between this unusual fungus and other ophiostomatoid fungi.

Material and methods

An isolate of *C. proteae* collected from within *P. repens* (L.) L. infructescences in the Western Cape, South Africa, was 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 with 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 3% glutaraldehyde for 3 h at 20°C, followed by 1 h postfixation in similarly buffered 0.5% osmium tetroxide. The material was dehydrated in a graded ethanol series and embedded in 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

Vertical sections through the ascomata of *C. proteae* showed developing asci extending upwards and outwards from the middle of the ascoma towards the neck. The young asci occupied approximately two-thirds of the volume of the ascomata. Mature asci were observed in the vicinity of the ascomatal neck (Fig. 1).

Two types of cells were observed beneath the asci. The first cell type was characterized by a darkly stained cytoplasm and occurred just below the asci, approximately at the center of the ascomata. The cells of the other type, which had less electron-opaque cytoplasm, occurred towards the base of the ascomata. Nuclei were observed in most of these two cell types, with the exception of those adjacent to the ascomatal wall cells (peridium). The inner peridium consisted of large, sterile or cushion cells (Figs. 1 and 11F).

Young asci were thin-walled, irregularly club-shaped, and usually had more than one nucleus (Figs. 2 and 11A). Granules were arranged in clusters within the ascus cytoplasm. Vesicles containing a granular substance were also observed in the cytoplasm (Figs. 2–4). These vesicles probably contributed to the formation of saclike delimiting membranes that enclosed the nuclei and cellular inclusions to form ascospores (Figs. 3 and 11B). The open ends of the saclike structures fused to form young ascospores (Figs. 4 and 11C). Large slightly electron-opaque bodies, probably containing lipids, appeared in the ascus cytoplasm during ascospore delimitation (Figs. 4 and 11C).

Ascospore wall development commenced with the deposition of wall material between the delimiting membranes (Figs. 5 and 11C). A primary wall layer thus formed between the delimiting mebranes towards the inside of the ascospore. An additional wall layer developed through the deposition of granules at irregular intervals between the outermost delimiting membrane and primary wall. This gave the ascospore wall a characteristic warty appearance (Figs. 6 and 11D).

As the ascospores matured, the primary wall expanded, resulting in the fibrillated appearance of the primary wall (Fig. 7). The ascospores developed into elongated structures with distinctly rounded ends (Figs. 7 and 8). The primary wall and cytoplasm of mature ascospores were darker in appearance than those in younger ascospores. Granules in the ascus cytoplasm were closely associated with, or possibly incorporated into, the outermost wall layer (Fig. 8). During ascospore maturation there was also a noticeable increase in the occurrence of membrane-bound bodies, probably lysosomes, in the ascus cytoplasm (Figs. 7 and 8).

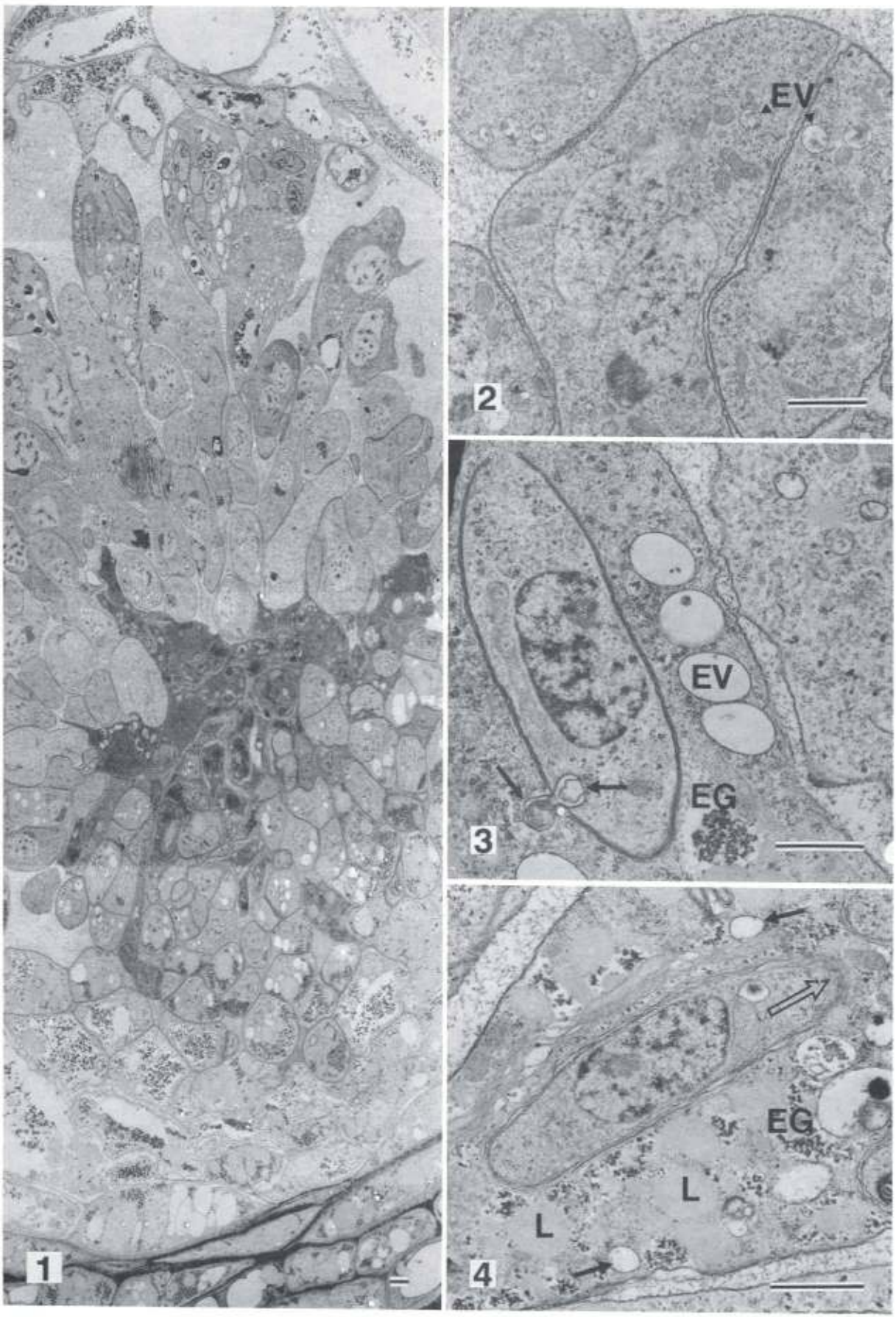
The asci finally disintegrated to release the mature ascospores into the ascoma and towards the neck. The ascospores were embedded in an osmiophilic matrix that adhered to the outermost (secondary) wall layer (Fig. 9). In transverse sections, the ascospores were circular in shape and comprised two distinct layers. An innermost, primary wall (ca. 300 nm) could be distinguished from a thinner (ca. 95 nm) secondary wall (Fig. 10).

Discussion

Young asci in *C. proteae* occurred in and above the middle of the ascomata. Mature ascospores were released towards the neck. This arrangement of asci in the centrum is different from centrum organization observed in other genera and species of *Ceratocystis* s.l. Two forms of centrum organization were recently reported to occur in *Ceratocystis* s.l. (Van Wyk and Wingfield 1991*a*). In one form, typical of *C. moniliformis* (Hedgc.) C. Moreau (Van Wyk et al. 1991), *O. distortum* (Van Wyk and Wingfield 1991*c*), and *O. piceae* (Van Wyk and Wingfield 1992), young asci occur adjacent to the ascomatal wall and represent a lining of ascus cells with mature ascospores released towards the ascoma center. In the second centrum type, typical of *O. davidsonii* Olchowecki and Reid (Van Wyk and Wingfield 1991*a*), *O. cucullatum* Solheim (Van Wyk and Wingfield 1991*b*), *O. minus* Van Wyk and Wingfield 1991*c*), and *O. seticolle* (Davids.) De Hoog and Scheffer (Van Wyk and Wingfield 1993), young asci occur at the base of the ascoma and develop towards the neck. Here, mature ascospores are released above the fertile area and towards the

FIGS. 1–4. Transmission electron micrographs of sections through the ascoma of *Ceratocystiopsis proteae*, showing ascus and ascospore development. Scale bars = 1 μm. Fig. 1. Vertical section through an ascoma showing developing, thin-walled, and mostly club-shaped asci occurring above the middle of the ascoma, extending outwards, and towards the base of the neck. Fig. 2. Young ascus showing two nuclei and small vesicles (EV) in the cytoplasm. Fig. 3. Ascus showing delimitation of an individual nucleus and cytoplasm by saclike delimiting membranes. Vesicles (arrows) contribute to the formation of the two delimiting membranes. Granules (EG) and prominent large vesicles (EV) are present. Fig. 4. Ascus showing cellular inclusions: large, slightly electron-opaque bodies (L), granules (EG), and vesicles (solid arrows). Young ascospore formed by fusion of open ends of saclike delimiting membranes (open arrow).

FIGS. 5–8. Transmission electron micrographs of sections through developing asci and ascospores. Scale bars = 500 nm. Fig. 5. Young ascospore showing deposition of primary wall (PW) between delimiting membranes. Fig. 6. Development of outermost wall layer of ascospore by deposition of granules (arrows). Fig. 7. Ascus showing longitudinal and transverse sections of elongated (falcate) ascospores. Note expanded primary wall layer (PW) and abundant electron-lucent bodies (L). Fig. 8. Ascus with mature ascospores. Ascospore walls with rounded ends, and cytoplasm darkly stained. Note granules associated with outermost delimiting membrane or perisporic sac (arrows) and fibrillated appearance of primary wall (PW). FIG. 9. Longitudinal section through mature ascospore showing primary (PW) and secondary (SW) wall layers. Note attenuated appearance of matrix adhering to rounded ends (solid arrows) and perisporic sac of the ascospore (open arrow). FIG. 10. Transverse section through mature ascospore, showing irregularly shaped secondary wall (SW) and primaray wall (PW).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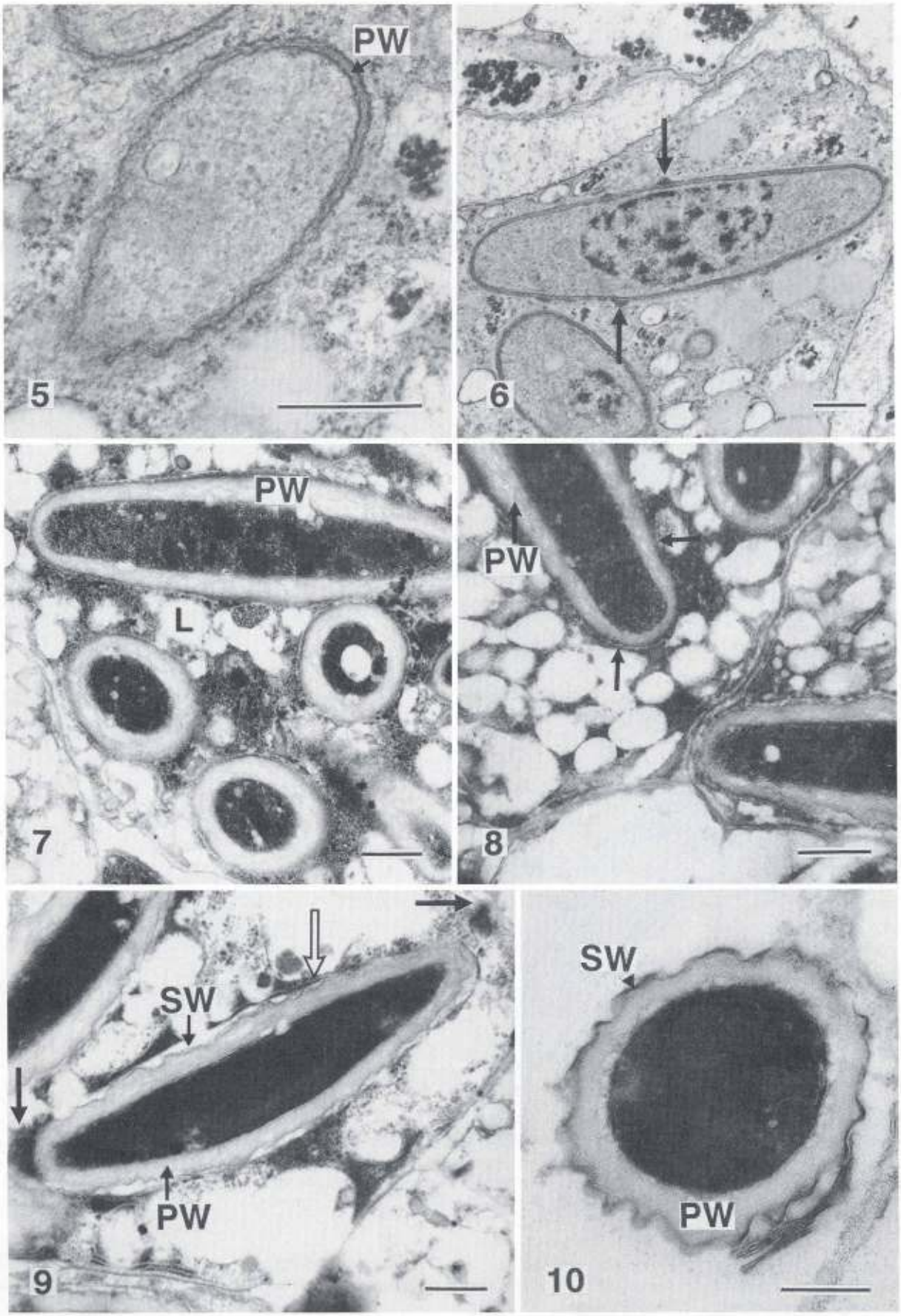
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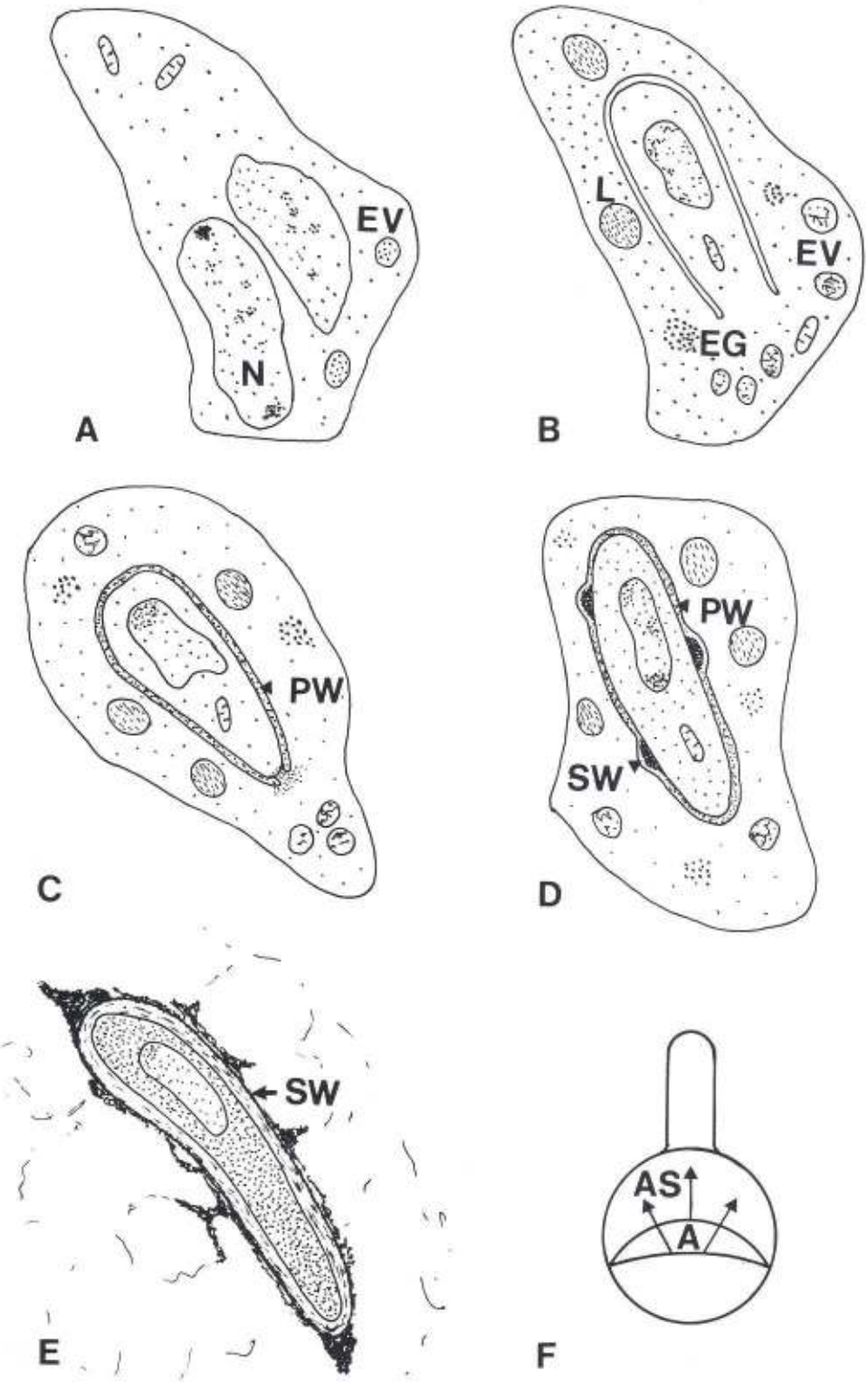

FIG. 11. Schematic representation of ascus and ascospore development in *C. proteae*. (A) Young ascus with two nuclei (N) and vesicles (EV). (B) Saclike delimiting membranes enclosing ascospore nuclei. Occurrence of bodies (L), granules (EG), and vesicles (EV). (C) Development of primary wall (PW) between delimiting membranes in young ascospores. (D) Maturation of ascospores. Secondary (SW) wall layers formed by deposition of granular substance. (E) Mature ascospore released from degenerating ascus in matrix of ascus debris adhering to secondary wall (SW). (F) Centrum organization of developing asci (A) and ascospores (AS) in *C. proteae*.

neck. The centrum organization in *C. proteae* with the development of asci at the center of the ascoma therefore appears to be a unique feature exhibited by this species.

Ascospore developemnt in *C. proteae* commenced with the formation of delimiting membranes. These membranes developed to enclose each of the eight nuclei in the ascus by a saclike membranous structure. In this matter it is thus similar to ascospore delimitation in ascomycetes in general. Charac-

teristically, the first wall layer (primary wall) develops between these delimiting membranes (Jeng and Hubbes 1980; Garrison et al. 1979; Van Wyk and Wingfield 1991*c*). However, differences were found between species in the development of additional layers of the secondary wall (Van Wyk et al. 1991; Stiers 1976).

In *C. proteae*, only a single layer of the secondary wall was observed. The development of the secondary wall was characterized by the deposition of granules between the outermost delimiting membrane (perisporic sac) and the primary wall, which gave it a warty appearance. Similar developmental processes were observed in *O. ulmi* (Buism.) Nannf. (Jeng and Hubbes 1980), *O. distortum* (Van Wyk and Wingfield 1991*c*), and *O. piceae* (Van Wyk and Wingfield 1992). In *O. distortum* and *O. piceae*, the granules deposited between the outermost delimiting membrane and primary wall function in the formation of an additional expanded layer of the secondary wall. In contrast, such a wall layer was not observed in *C. proteae*, *O. ulmi*, or *O. stenoceras* (Robak) Melin and Nannf (Garrison et al. 1979). In these species the ascospore wall consisted only of two layers, the innermost primary and an outermost secondary wall.

The genus *Ceratocystiopsis* is defined as having falcate sheaths surrounding elongate ascospores (Upadhyay and Kendrick 1975). In this study, the falcate sheaths of *C. proteae*, previously observed using light microscopy, were not observed at the ultrastructural level. *Ceratocystiopsis proteae* has elongate ascospores similar to those in *O. ulmi* (Jeng and Hubbes 1980), *O. stenoceras* (Garrison et al. 1979), and *O. piceae* (Van Wyk and Wingfield 1992). It is our opinion that these elongate ascospores are discharged from ascomata carrying material remaining from the disintegration of asci at their apices. This would thus give them a falcate appearance under the light microscope.

The falcate appearance of the ascospores in *C. proteae*, when viewed with a light microscope, is used as a constant characteristic of this fungus. However, the results of this study imply that this falcate form is not due to wall characteristics as in other ophiostomatoid fungi. The fungus would more appropriately be accommodated in *Ophiostoma*. This observation is of particular interest because Wingfield et al. (1988), in their description of *C. proteae*, made note of the fact that the fungus shared characteristics with *Ceratocystis* s.l., *Ophiostoma*, and *Ceratocystiopsis*. Clearly, in the absence of definitive falcate outer walls, *C. proteae* would be better placed in *Ophiostoma*.

Recently, a fungus very similar to *C. proteae* and also having the unusual *Knoxdaviesia* anamorph was described from *Protea* infructescences (Wingfield and Van Wyk 1993). What is particularly interesting is that this fungus has elongate ascospores that are not falcate when viewed with a light microscope. Despite its similarity to *C. proteae*, the fungus was described as *Ophiostoma capensis* Wingfield and Van Wyk (Wingfield and Van Wyk 1993). The two fungi are obviously closely related and should undoubtedly reside in the same genus. Results of the present study add further weight to this view and serve to affirm that ascospore shape, observed with the light microscope, can be a misleading characteristic in ophiostomatoid fungi.

In addition to providing further information on the ultrastructure of ophiostomatoid fungi, this study has illustrated the danger of using single morphological characteristics to segregate genera. It also lends further credence to the opinion (Wingfield et al. 1988) that *Ceratocystiopsis* requires revision. It is our contention that additional ultrastructural studies of centrum development and ascospore morphology in *Ceratocystis* s.l. will contribute significantly to our understanding of these fungi.

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