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Ultrastructural study of centrum development and ascospore morphology in *Ophiostoma europhiodes*

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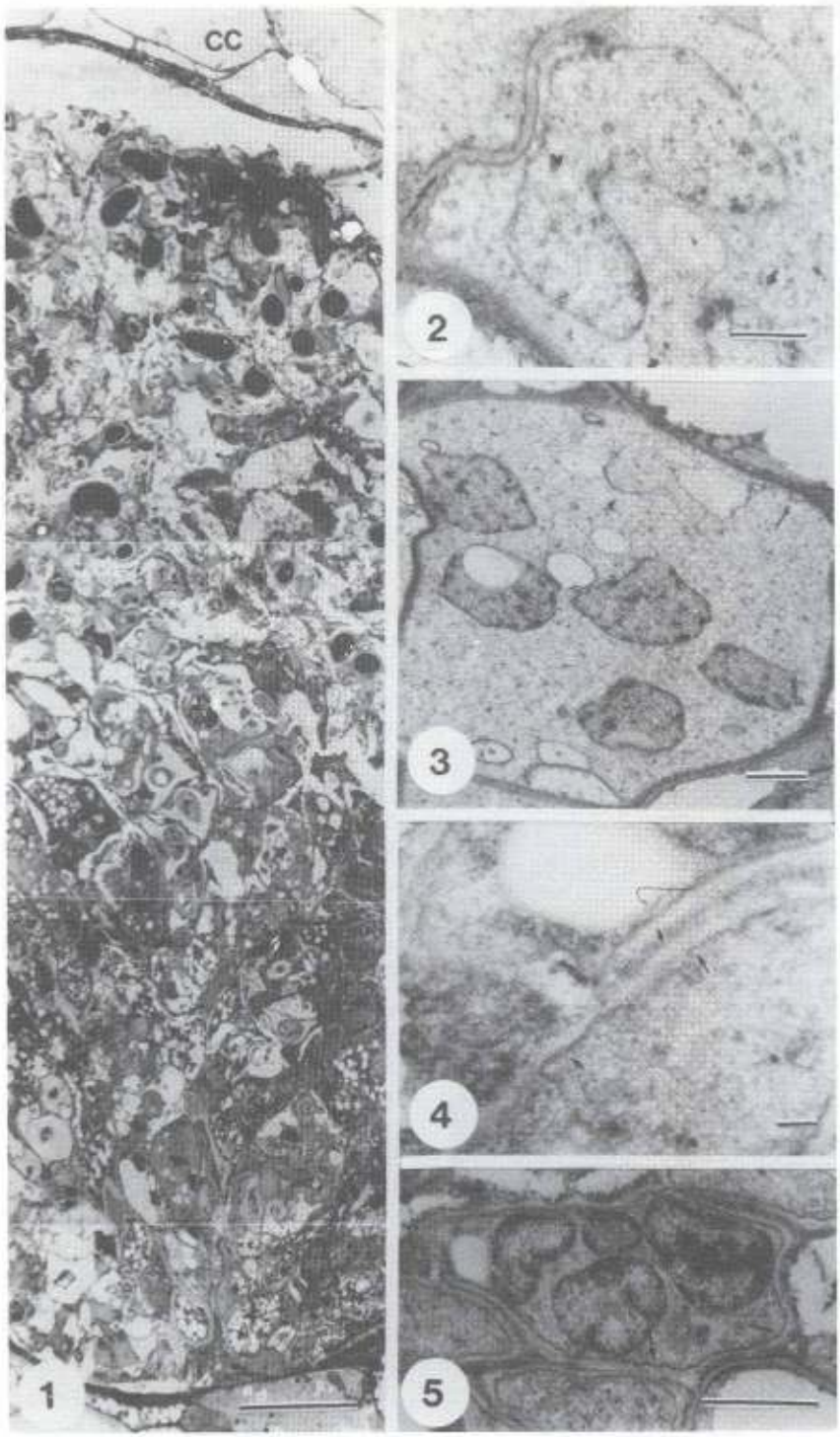
Recent ultrastructural studies have shown that species of Ophiostoma *and* Ceratocystis *differ in their centrum morphology and development. In addition, hat-shaped ascospores associated with these genera differ in their ultrastructural morphology, despite the fact that they are virtually indistinguishable, using light microscopy. The aim of this study was to establish whether the centrum morphology and ascospore development in* O. europhiodes *was similar to that found in other species of* Ophiostoma. *The arrangement of the centrum was similar to that found in other species of* Ophiostoma. *Moreover, the hat-shaped ascospores of* O. europhiodes *were typical of those found in other species of* Ophiostoma *and distinct from those in species of* Ceratocystis. *This study, therefore, confirms previous findings for* Ophiostoma *and contributes further to our understanding of centrum development and ascospore morphology in this genus.*

The ophiostomatoid fungi, which are contained in the *Ceratocystis sensu lato* complex, include the genera *Ophiostoma* Syd. & P. Syd., *Ceratocystis* Ellis & Halst. and *Ceratocystiopsis* Upadhyay & Kendr.[1–3] This group of fungi is economically important and includes numerous destructive pathogens of plants and especially of trees.[1,4–7] The taxonomy of this group has been characterized by uncertainty regarding the relationship of these genera to each other. Some authors have treated *Ophiostoma* and *Ceratocystis* as synonyms,[8–10] while others have considered them as separate genera.[2,11–15] Recent analysis of ribosomal DNA sequences has indicated that *Ophiostoma* and *Ceratocystis* are only distantly related, while *Ophiostoma* and *Ceratocystiopsis* are most likely synonyms.[16–18]

Ascospore morphology has been one of the characters used in an attempt to resolve taxonomic problems surrounding the ophiostomatoid fungi. Olchowecki and Reid[19] used this character to separate species in the ophiostomatoid fungi into four different groups. Hausner *et al.*[20] and Wingfield *et al.*,[21] however, using sequence analysis of the ribosomal RNA, showed that ascospore morphology is not conserved, and species that have similarly shaped ascospores are not necessarily phylogenetically related. Ultrastructural studies have shown that the morphology of hat-shaped ascospores differs between species of *Ophiostoma* and *Ceratocystis*,[22–26] although this observation is based on a relatively limited number of species.

It is not only the ascospore morphology that differs between species of *Ophiostoma* and *Ceratocystis*, but also the development of the ascomatal centrum. Lutrell[27] considered centrum morphology to be an important character in the taxonomy of the Ascomycetes. Few data were available regarding centrum development in the ophiostomatoid fungi until the 1990s, when Van Wyk and Wingfield[24,25,28–30] and Van Wyk, Wingfield and Van Wyk[23,26] conducted a series of studies on the centrum development and ascospore morphology in selected species of *Ophiostoma* and *Ceratocystis*, respectively. From these studies, it was found that young asci in species of *Ceratocystis* lined the walls of the ascomata and that ascospores were released towards the centre.[23] In contrast, the asci of species in *Ophiostoma* were clustered at the base of the ascomata and ascospores were released towards the necks.[24,25,28,29] They, therefore, suggested that ascospore morphology and centrum development should be important characteristics in the taxonomy of the ophiostomatoid fungi.[26,31]

The aim of this study was to investigate ascospore morphology



Figs 1–5. Transmission electron micrographs (TEM) of sections through an ascoma of *Ophiostoma europhiodes*, showing centrum morphology and ascospore development. Fig. 1. Vertical section through an ascoma showing developing asci clustered at the base of the ascoma and ascospores released towards the neck. Large cushion cells (CC) surround the centrum (bar = 10 µm). Fig. 2. Ascus mother (ascogenous) cell in mature ascoma (bar = 500 nm). Fig. 3. Three to six of the eight nuclei are visible in the young ascus (bar = 1 µm). Invagination of the ascus plasma membrane (arrows) possibly lead to the formation of membranes for the ascus vesicle. Fig. 4. The ascospore vesicle is created by the formation of double membrane fragments around the nuclei (arrows)(bar = 100 nm). Fig. 5. Nuclei surrounded by the ascus vesicle (arrows) (bar = 1 µm).

and centrum development in *Ophiostoma europhiodes* (Wright & Cain) Solheim, characterized by hat-shaped ascospores. Although other similar species have been studied previously, critical differences in ascospore form and centrum development are based on a small number of species. Our primary goal was to confirm previous findings showing that hat-shaped ascospores of *Ophiostoma* species are not fully symmetrical as in the case of *Ceratocystis* s.s. and that ascospore development began from the bases of the ascomata.

Materials and methods

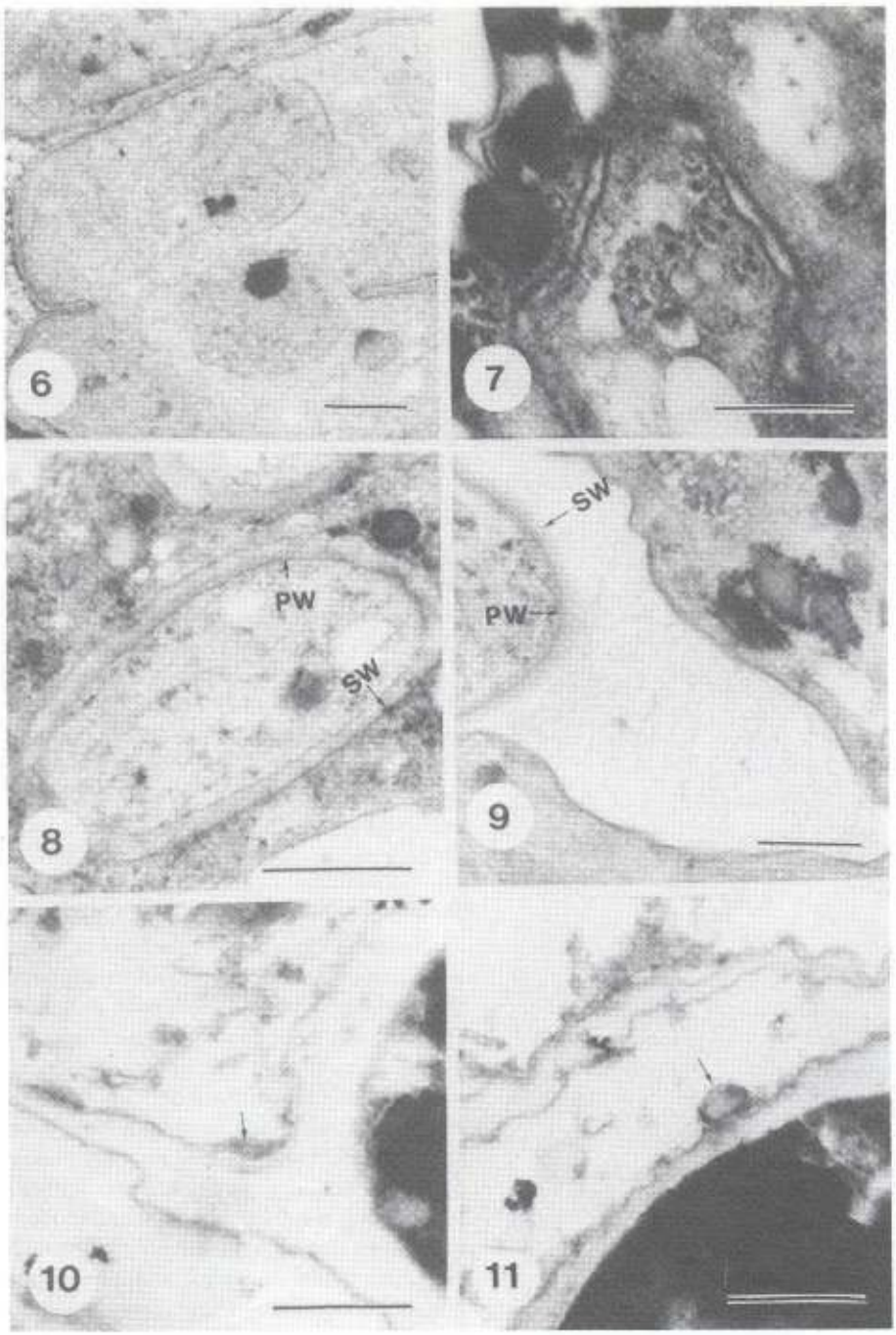
An isolate of *O. europhiodes* (CMW 483) from the USA, that produced abundant ascomata, was used in this study. The isolate was grown up on 2% malt extract agar (20 g Biolab malt extract; 20 g Biolab agar; 1000 ml distilled water) in Petri dishes at 25°C in the presence of *Pinus patula* Schlecht. & Cham. sapwood. Individual ascomata were picked from the host material and fixed in a sodium phosphate-buffered 3% glutaraldehyde solution for 12 h. This was followed by fixation with similarly buffered 0.5% osmium tetroxide for 1 h. The material was dehy-drated in a graded acetone series and embedded in epoxy resin.[32] Ultrathin sections (60 nm) were cut with glass knives, using an LKB Ultratome III. Sections were stained for 20 min with 6% uranyl acetate and 10 min in lead citrate[33] and examined with a Philips CM100 transmission electron microscope.

Results

Centrum organization. Ascospores arose late in the development of the ascomata, when the necks were already fully grown. Only sterile (cushion) cells were observed in very young ascomata with little or no cell differentiation that could lead to centrum development. Developing asci and ascospores were surrounded by three or four layers of large cushion cells. Uninucleate ascus mother cells (young asci) were clustered towards the bottom half of the centrum stretching to the middle of the centrum. Mature ascospores were released towards the necks of the ascomata and were located in the top half of the centrum (Fig. 1).

Ascospore development. Nuclear division took place early in the development of the ascospores. The nucleus in the ascus mother cell usually divided into eight separate nuclei (Fig. 2). The number of the nuclei observed varied, depending on the plane of sectioning (Fig. 3). Only three to six of the eight nuclei were observed in young asci. Vesicles varying in shape and size were observed in the young multi-nucleate asci (Fig. 3). These vesicles were formed through the invagination of the ascus plasma membrane and were apparently involved in the formation of the ascospore delimiting membranes.

Ascospore delimiting membrane fragments formed after the completion of nuclear division and were characterized by double

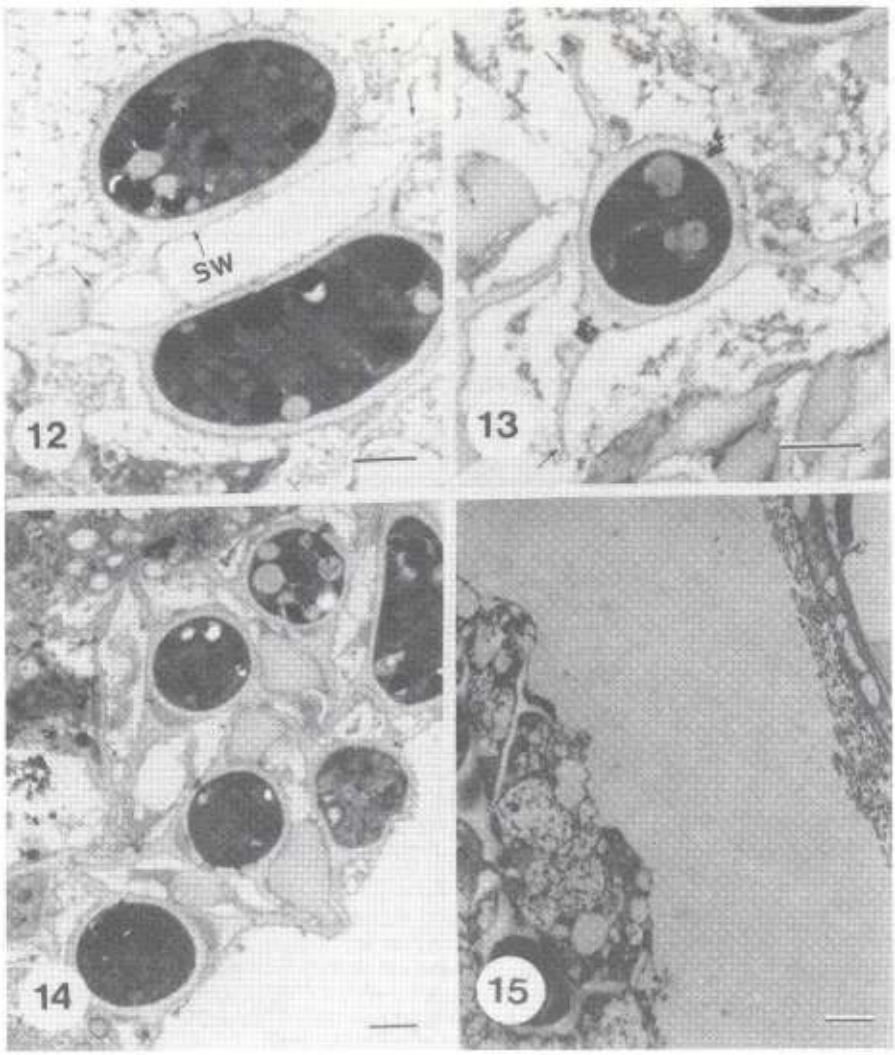


Figs 6–11. TEM of sections through developing asci and ascospores of *O. europhiodes*. Fig. 6. Invagination of the ascus vesicle (arrows) leads to the development of individual ascospores (bar = 1 μm). Fig. 7. Wall material and membranes are further synthesized at the open ends and the ends join to surround the nucleus. Fig. 8. The primary wall (PW) is formed by the expansion of the double membrane. The secondary wall (SW) is formed by the deposition of wall material under the outer plasma membrane (arrows) (bar = 1 μm). Fig. 9. Deposition of granules between the outermost delimiting membrane and the secondary wall leads to the development of the hat-shaped ornamentation of the secondary wall (SW). These ridges appear granular at first (bar = 500 nm). Fig. 10. The ornamental layer becomes more compact as the ascospores mature. Membranes of unknown origin have been seen to be associated with the formation of this ornamental layer (arrows) (bar = 500 nm). Fig. 11. Osmiophillic vesicles has been observed in close association with the development of the secondary wall (arrows) (bar = 1 μm).

delimiting membranes that surrounded the nuclei (Fig. 4). These membranes originated as small fragments of double membranes (Fig. 4). The plasma membranes (ascus vesicles) surrounding the nuclei were further synthesized to include all the nuclei (Fig. 5). Invagination of these membranes led to the formation of young ascospores, which were surrounded by double membranes, and ultimately formed the primary and secondary walls of the ascospores (Fig. 6). The double membranes separated to allow the deposition of wall material between the double membranes to form the primary walls of the ascospores (Fig. 7). The secondary walls were formed by the fusion of granules to the outer layers of the delimiting membranes (Fig. 8). This wall formation process occured simultaneously with the ascospore nucleus delimitation activity in each ascus.

During further development, the asci were characterized by the presence of electron-dense as well as lightly stained vesicles, close to the young ascospores (Fig. 8). At this stage, the ascospores were surrounded by primary as well as secondary walls. Ornamentation of the secondary walls led to formation of the brims of the hat-shaped ascospores. These brims were formed by the deposition of wall material between the outermost delimiting membranes and the secondary walls (Fig. 9). The deposited material formed a granular layer (Fig. 9) which became more compact and uniform in mature ascospores (Fig. 10). Membranes closely associated with the outer membranes of the ascospores were observed (Fig. 10) and were probably involved in the expansion of the outer membrane of the ascospore. Osmiophillic vesicles and granules were also observed in close association with the outer (primary) walls of the ascospores (Fig. 11).

Three ridges developed on the ascospores as a result of the expansion of the secondary walls and formed part of the secondary wall ornamentation. Longitudinal sections through mature ascospores showed the two walls of ascospores surrounding the cytoplasm containing vesicles of various shapes and sizes. The primary walls were uniform structures and gave the ascospores a reniform appearance. The secondary walls appeared more fibrilar and ornamentation gave the ascospores their characteristic cucullate appearance. The secondary walls had a dentate ornamentation (Fig. 12). A transverse section through mature ascospores showed that the primary walls were circular. The secondary wall ornamentation formed three ridges, giving the ascospore a triangular appearance (Fig. 13). Developing ascospores were randomly arranged through the ascus with no obvious orientation. This random dispersal of the ascospores was observed in longitudinal (Fig. 14) as well as in transverse sections through the



Figs 12–15. TEM of sections through developing asci and ascospores of *O. europhiodes*. Fig. 12. Longitudinal section through a mature ascospore. The primary wall (PW) surrounding the cytoplasm is reniform and is surrounded by an ornamented secondary wall (SW) giving the ascospore a cucullate appearance. Only two of the three ridges are visible (arrows) (bar = 1 μm). Fig. 13. Transverse section through a mature ascospore showing all three of the ridges formed by ornamentation of the secondary wall (arrows) (bar = 1 μm). Fig. 14. Longitudinal section through a mature ascus. Ascospores are randomly dispersed through the ascus (bar = 1 μm). Fig. 15. Sterile or cushion cells (CC) collapse towards the neck of the ascoma to create space for the developing ascospores.

ascomata.

Asci had started to disintegrate with the formation of the secondary wall ornamentation and the ascospores were released towards the neck of the ascoma (Fig. 14). Mature ascospores were exudated in a gelatinous mass through the neck of the ascoma. The sterile cells at the base of the neck collapsed when the ascospores were mature (Fig. 15).

Discussion

Centrum morphology in species of *Ophiostoma* and in those of *Ceratocystis* has been shown to be different. In species of *Ceratocystis*, developing asci are arranged along the periphery of the ascomatal wall and the ascospores are released towards the centre of the ascoma.[26] In species of *Ophiostoma*, the developing asci are arranged at the base of the ascoma and the ascospores are released towards the neck of the ascoma.[26] Ultrastructural observations in this study of *O. europhiodes* confirmed the findings of previous studies and add to our confidence that this is a stable and reliable taxonomic character. Until ultrastructural studies have been conducted on all species of *Ophiostoma* and *Ceratocystis*, however, it is impossible to know whether exceptions to this rule exist. The time-consuming and tedious nature of these studies, as well as the fact that most ophiostomatoid fungi do not sporulate abundantly in culture, suggests that a comprehensive view of this character remains a distant goal.

Developing asci in *O. europhiodes* were arranged in a cluster at the base of the ascomata with mature ascospores released towards the neck. This was similar to that in *O. cucullatum* Solheim, *O. davidsonii* (Olchow. & Reid) Solheim and *O. minus* (Hedge.) Syd. & P. Syd.[24,25] This arrangement was, however, found to be different from that observed in *O. distortum* (Davidson) De Hoog & Scheff., where the developing asci occurred in different zones lining the lower half of the ascomata.[28] It is curious that the latter fungus should have a unique form of centrum development, and this emphasizes the need for additional ultrastructural studies of the group.

A unique feature of the centrum of *O. europhiodes* lies in the prominent layer of sterile (cushion) cells surrounding the developing asci and ascospores. This layer is three to four cell layers thick. It has also been reported to occur in other species of *Ophiostoma*,[24,25] but in no case was it as prominent as that in *O. europhiodes*. The function of these cells is probably to provide space for the developing asci and ascospores by their collapsing during the developmental process.[31] These cells were not observed in the mature ascomata of *O. cucullatum*, whereas they were present throughout the development of the centrum in *O. europhiodes*. It is also noteworthy that ascospore development in *O. europhiodes* occurred late in the development of the ascomata.

Ascospore delimitation in *O. europhiodes* was similar to that in *O. cucullatum* and *O. davidsonii*,[23,26] both of which have hat-shaped ascospores similar to those of *O. europhiodes*, but different from the bowler hat-shaped ascospores of *Ceratocystis*.[22,23] Discontinuous membranes were observed in cells where nuclear division had already taken place and these gave rise to the ascus vesicles around the nuclei. As in the case of *O. cucullatum*, these discontinuous double membranes, making up the ascus vesicle, invaginate to enclose the nucleus of each immature ascospore. The synthesis of wall material took place during the invagination process, which was similar to that observed in *O. cucullatum*.[24] A double layer of wall material was synthesized while sac-like structures developed around each nucleus, resulting in the formation of the primary and secondary walls of the ascospore.

Ascospore wall formation was found to be similar to that of *O. cucullatum* and *O. davidsonii*.[24,26] In *O. europhiodes*, the primary walls were formed by the deposition of wall material between the double membranes. This structure resulted in the ascospores having a reniform appearance in longitudinal section. The secondary wall was formed underneath the outermost membrane by the deposition of vesicles on the outer membrane. Ornamentation of these walls through the deposition of granules and associated membranes led to the cucullate appearance of the ascospores in lateral view.

In *O. europhiodes*, the ascospores developed individually. This feature appears to be consistent with the development of ascospores in other species of *Ophiostoma*, studied at ultrastructural level.[24,25,28,29] A distinct feature of ascospore development in *C. fimbriata* and *C. moniliformis* is the development of the ascospores in pairs. This feature has not been observed in either *O. cucullatum* or *O. davidsonii*.[24–26] In contrast to *O. cucullatum*, the ascospores of *O. europhiodes* were arranged irregularly throughout the ascus, similar to those of *O. davidsonii*.[26] The ridges observed in other species of *Ophiostoma* were also observed in *O. europhiodes*. The development of these ridges gave the ascospores of *Ophiostoma* a distinct triangular appearance in transverse section. These ascospores, although similar in their hat-shaped appearance to those of species in *Ceratocystis* when viewed by optical microscopy, were distinctly different when observed at the ultrastructural level.[24–26]

This study contributes further to our knowledge of the ultrastructural arrangement and ascospore development and morphology in *Ophiostoma*. Ultrastructural studies have shown differences in ascospore morphology in *Ophiostoma* and *Ceratocystis* that cannot be seen using light microscopy. It is difficult to tell whether other species of *Ceratocystis* and *Ophiostoma*, that have not been studied ultrastructurally, will have similar ascospore morphology and centrum development. Our view is that, where suitable material can be obtained for ultrastructural study, it should be investigated.

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