Conidium development in the *Hyalorhinocladiella* anamorphs of *Ceratocystiopsis minuta-bicolor* and *Ophiostoma minus*

E. Benade, M.J. Wingfield, and P.S. Van Wyk

Abstract: The genus *Hyalorhinocladiella* was characterized by its simple conidiophores with conidiogenous cells that proliferate sympodially. However, recent studies revealed that the *Hyalorhinocladiella* anamorph of *Ophiostoma ips* has annellidic conidium development. The aim of this study was to determine whether other species in the genus share this characteristic. Conidium development was examined in the type species, *Hyalorhinocladiella minuta-bicolor*, and in the *Hyalorhinocladiella* anamorph of *Ophiostoma minus*. Light and fluorescence microscopy indicated that conidia developed by sympodial proliferation. In contrast, scanning and transmission electron microscopy revealed distinct annellations on the conidiogenous cells. Conidium development in *Hyalorhinocladiella* is therefore annellidic, and the appearance of sympodial development results from displacement of the long axis of the conidiogenous cell through percurrent proliferation. The circumscription of the genus *Hyalorhinocladiella* is therefore revised to include annellidic conidium development.

Key words: *Hyalorhinocladiella*, *Ophiostoma*, sympodial, annellidic, conidium development.

Résumé : Le genre *Hyalorhinocladiella* a été caractérisé par ses conidiophores simples, munis de cellules conidiogènes qui prolifèrent de façon sympodiale. Des études récentes ont cependant révélé que l'anamorphe de l'*Ophiostoma ips* possède un développement conidien annélidique. Le but de l'étude était de déterminer si d'autres espèces du genre partagent cette caractéristique. Les auteurs ont examiné le développement conidien chez le type de cette espèce l'*Hyalorhinocladiella minuta-bicolor* et l'anamorphe de l'*Ophiostoma minus*. La microscopie photonique et en fluorescence indique que les conidies se développent par prolifération sympodiale. Au contraire, la microscopie électronique par balayage et par transmission révèle des annellations bien visible sur les cellules conidiogènes. Le développement conidien chez les *Hyalorhinocladiella* est conséquemment annélidique et l'apparence de développement sympodial résulte d'un déplacement du long axe de la cellule conidiogène, via une prolifération percurrente. La délimitation du genre *Hyalorhinocladiella* est conséquemment revisée pour inclure le développement conidien annélidique.

Mots clés : *Hyalorhinocladiella*, *Ophiostoma*, sympodial, annélidique, développement conidien.
[Traduit par la rédaction]

Introduction

Ceratocystis Ellis & Halst. sensu lato includes the genera *Ceratocystiopsis* Upadhyay & Kendrick, *Ophiostoma* H. & P. Sydow, and *Ceratocystis* sensu stricto (Weijman and De Hoog 1975; Upadhyay 1981; De Hoog and Scheffer 1984). Species of *Ceratocystiopsis* are distinguished from the other two genera by their elongate, falcate ascospores that are attenuated at the ends and surrounded by a sheath (Upadhyay and Kendrick 1975; Upadhyay 1981). *Ophiostoma* and *Ceratocystis* species have a variety of ascospore shapes, such as cucullate, cylindrical, ossiform, hat- and pillow-shaped (Hunt 1956; Griffin 1968; Olchowecki and Reid 1974; Upadhyay 1981).

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Ceratocystis s.s. is characterized by having *Chalara* (Corda) Rabenh. anamorphs (Weijman and De Hoog 1975; Upadhyay 1981; De Hoog and Scheffer 1984), in which conidia develop through ring wall building (Minter et al. 1983; De Hoog and Scheffer 1984). In contrast, *Ophiostoma* and *Ceratocystiopsis* have anamorphs in which conidia develop through apical wall building (Minter et al. 1983). These include genera such as *Hyalorhinocladiella* Upadhyay & Kendrick, *Sporothrix* Hekt. & Perkins, *Leptographium* Lagerberg & Melin, and *Graphium* Corda (Wright and Cain 1961; Upadhyay and Kendrick 1975; Upadhyay 1981; Wingfield 1985).

Since the publication of the classic paper of Hughes (1953), anamorphic fungi have been distinguished by their mode of conidial development (Kendrick 1971). This trend has been pronounced in the anamorphs of *Ophiostoma* (Upadhyay 1981; Mouton et al. 1994). Ultimately, an excessive number of anamorph genera have been assigned to this teleomorph. Detailed examination of conidium development at the ultrastructural level in genera such as *Leptographium* and *Graphium* showed that this number of genera can be substantially reduced (Wingfield 1985; Wingfield et al. 1991). Some of the major genera that would remain include *Leptographium*, *Graphium*,

Figs. 1–6. Conidiophores and conidiogenous cells in the *Hyalorhinocladiella* anamorph of *Ceratocystiopsis minuta-bicolor*. Fig. 1. Bright-field micrograph of a conidiophore with attached hyaline, oblong conidia. Scale bar = 4 μm. Fig. 2. Fluorescence micrograph showing a sympodial arrangement of conidia on the conidiogenous cell. Scale bar = 4 μm. Fig. 3. Scanning electron micrograph of conidia revealing single attachment points. Scale bar = 2 μm. Fig. 4. Secondary conidia with narrowly spaced annellations at attachment points. Scale bar = 2 μm. Fig. 5. Scanning electron micrograph showing conidia sympodially arranged on the conidiogenous cell, although annellations (arrow) can be seen. Scale bar = 2 μm. Fig. 6. Scanning electron micrograph revealing narrowly spaced annellations at the apices of conidiogenous cells (arrows). Scale bar = 2 μm.

Sporothrix, and *Hyalorhinocladiella* (Mouton et al. 1994). Conidium development was studied in detail at the ultrastructural level in the first two genera (Wingfield 1985; Wingfield et al. 1991) but not in *Sporothrix* and *Hyalorhinocladiella*.

The anamorph of *Ceratocystiopsis minuta-bicolor* (Davids.) Upadhyay & Kendrick was described as possessing "small, rod-shaped conidia carried in groups at the tips of hyphal branches or on short side branches of hyphae" (Davidson 1966). Griffin (1968) could not determine the mode of conidium development in this fungus but described the conidia as "unicellular, hyaline and ovate." The genus *Hyalorhinocladiella* was described by Upadhyay and Kendrick (1975) to accommodate the mycelial anamorph of *Ceratocystiopsis minuta-bicolor*. This genus was characterized by having "conidiogenous cells on simple, hyaline conidiophores, that proliferate sympodially, leaving flat low-profile scars on the extending rachis" (Upadhyay 1981).

The distinction between the *Hyalorhinocladiella* and *Sporothrix* anamorphs of *Ophiostoma* is problematic (De Hoog 1993). Strictly speaking, the conidia of *Sporothrix* species have relatively narrow bases and are produced on conidiogenous cells with distinct denticles. In contrast, the conidiogenous loci in *Hyalorhinocladiella* are nondenticulate (Upadhyay and Kendrick 1975) and the conidia have more broadly truncate bases.

Ophiostoma minus (Hedgc.) H. & P. Syd. was first described as having "oval to elliptical conidia collecting in rounded masses on hyphae" (Hedgcock 1906). Rumbold (1936) confirmed the observations of Hedgcock (1906) and added that "conidia are pushed to the one side of the growing tip," suggesting sympodial development. Hunt (1956) described the anamorph of *O. minus* as having "conidiophores that sometimes arise from a brown stalk and form a brush-like mass of ramified hyphae somewhat resembling *Leptographium* sp." Upadhyay (1981) described the anamorph of *O. minus* as a *Hyalorhinocladiella* sp. in which the conidiogenous cells proliferate sympodially (Upadhyay 1981).

In a recent study of conidium development in the purportedly *Hyalorhinocladiella* anamorph of *Ophiostoma ips* (Rumb.) Nannfeldt, conidia were shown to develop through percurrent proliferation (Benade et al. 1995). This suggested that the generic circumscription of *Hyalorhinocladiella* might require revision. Alternatively, assignment of the anamorph of *O. ips* to this genus might have been inappropriate. For this reason we studied conidium development in *H. minuta-bicolor* Upadhyay & Kendrick, the type species of *Hyalorhinocladiella* and the anamorph of *Ceratocystiopsis minuta-bicolor*. The *Hyalorhinocladiella* anamorph of *O. minus* was also included for comparative purposes.

Materials and methods

An isolate of *Ceratocystiopsis minuta-bicolor* from *Dendroctonus ponderosae* Hopkins, Invermere, B.C., supplied by Y. Hiratsuka (C-1200) and an isolate of *O. minus* from *Pinus resinosa* Sol. in Wisconsin, collected by M.J. Wingfield (PREM 4820) were examined. The isolates were grown in Petri dishes on 2% malt extract agar (20 g Biolab malt extract and 20 g Biolab agar per 1000 mL water) at 25°C under cool-white fluorescent and near-ultraviolet light, for approximately 2 weeks, until the onset of sporulation.

Conidium development was examined using light and fluorescence microscopy, as well as scanning (SEM) and transmission (TEM) electron microscopy. Specimens for light microscopy were examined in lactophenol using phase- and interference-contrast microscopy. For fluorescence microscopy, conidia and conidiophores were mounted in a 0.05% (w/v) solution of cellufluor white M 2R optical brightener in 0.1 M phosphate buffer. The material was examined with a Zeiss Axioskop fluorescence microscope with dark-field and UV illumination.

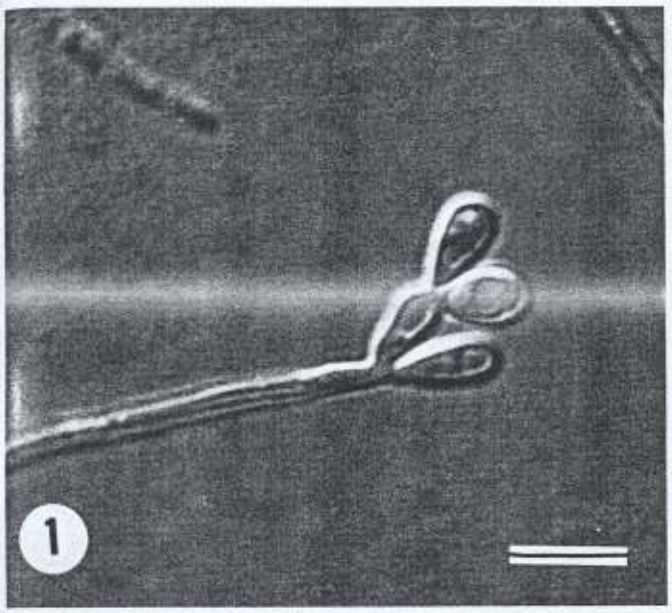
Material from cultures for SEM were cut into blocks (5–7 mm^2), fixed in 3% glutaraldehyde and 1% osmium tetroxide in 0.1 M buffer (pH 7.0), dehydrated in a graded acetone series, critical point dried, coated in gold–palladium, and viewed with a JSM 6400 scanning electron microscope. Blocks of agar (1–1.5 mm^2) for TEM were fixed and dehydrated as described above and embedded according to Spurr (1969) with the following modifications. Specimens were placed for 90 min in a mixture (1:1) of acetone and epoxy resin (at room temperature). The specimens were then placed in pure epoxy resin for 30 min at room temperature followed by 30 min at 50°C and thereafter transferred to new epoxy resin at 50°C for 60 min. Specimens were placed with the upper side facing the rounded point of preheated gelatin capsules. The epoxy resin was polymerized at 70°C overnight. Thin sections (60 nm) were cut on an ultramicrotome, mounted on copper grids, and stained with uranyl acetate (30 min) followed by lead citrate (5 min) (Reynolds 1963). Sections were viewed with a Phillips 301 transmission electron microscope.

Results

Light and fluorescence microscopy, as well as scanning electron microscopy, of *H. minuta-bicolor* showed one-celled, hyaline, oblong to ellipsoidal conidia produced in an apparently sympodial manner (Figs. 1, 2, 5). The conidia were apically rounded with truncate bases and possessed a basal, delimiting septum (Fig. 3). Secondary conidia were formed from primary conidia, leaving ringlike scars at the basal septa (Fig. 4). The presence of ringlike scars on the conidiogenous cells indicated that they proliferated percurrently (Figs. 5–7).

Transmission electron micrographs of *H. minuta-bicolor* confirmed that conidia develop through enteroblastic percurrent proliferation of the conidiogenous cells (Figs. 9, 10). This would be followed by holoblastic ontogeny. The conidiogenous cells can then also undergo angled or displaced proliferation (Figs. 7, 8), leaving denticles such as those found in *Sporothrix*.

Scanning and fluorescence microscopy of the *Hyalorhinocladiella* anamorph of *O. minus* showed simple conidiophores bearing conidiogenous cells that proliferate sympodially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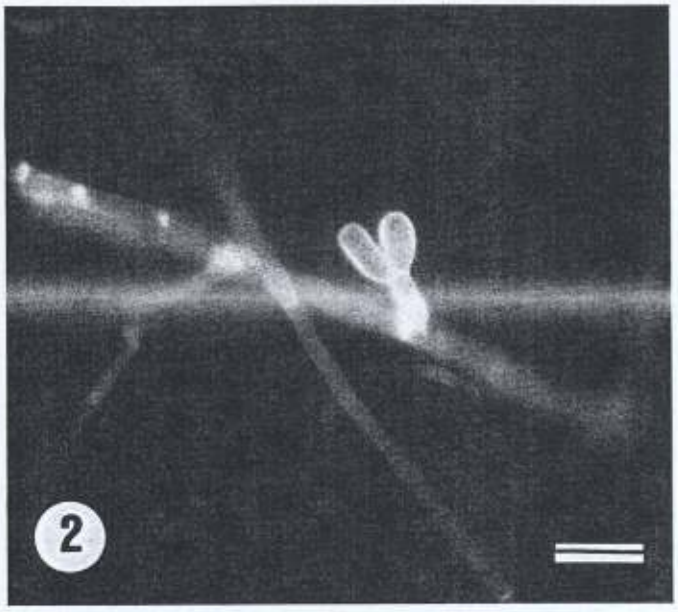
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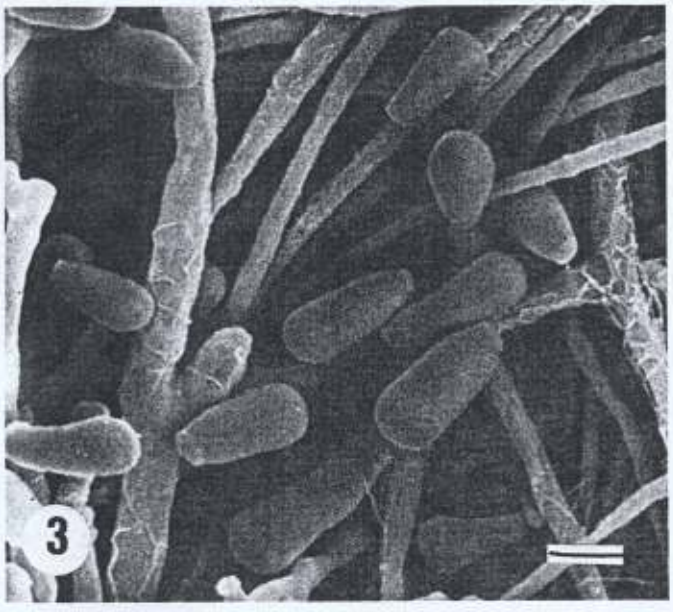
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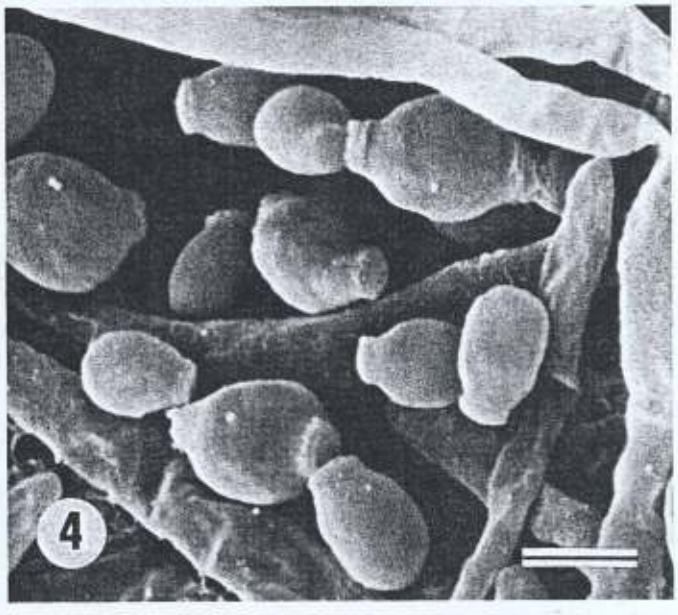
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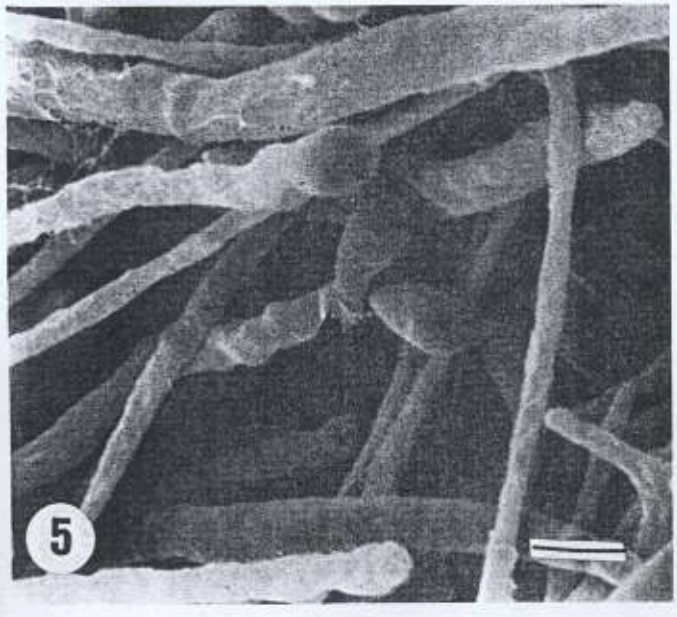
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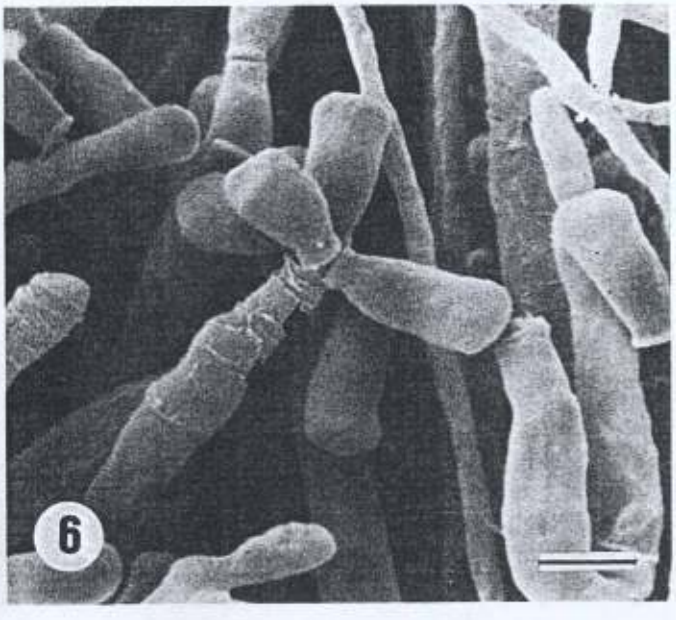
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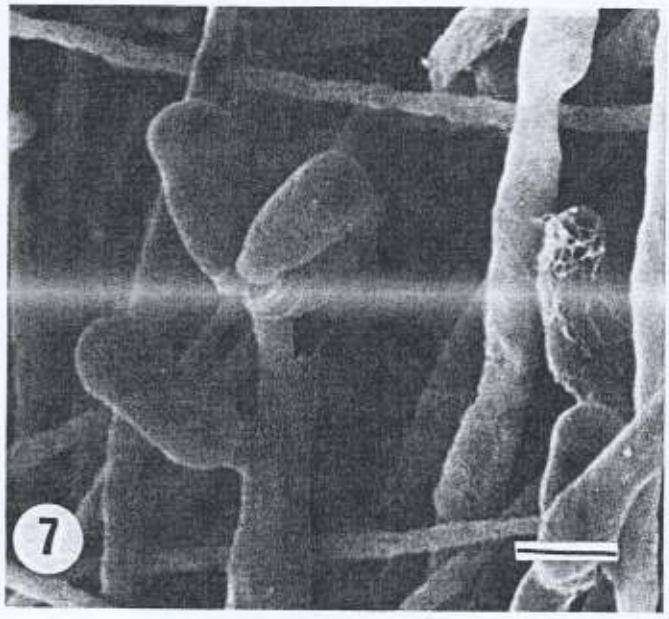
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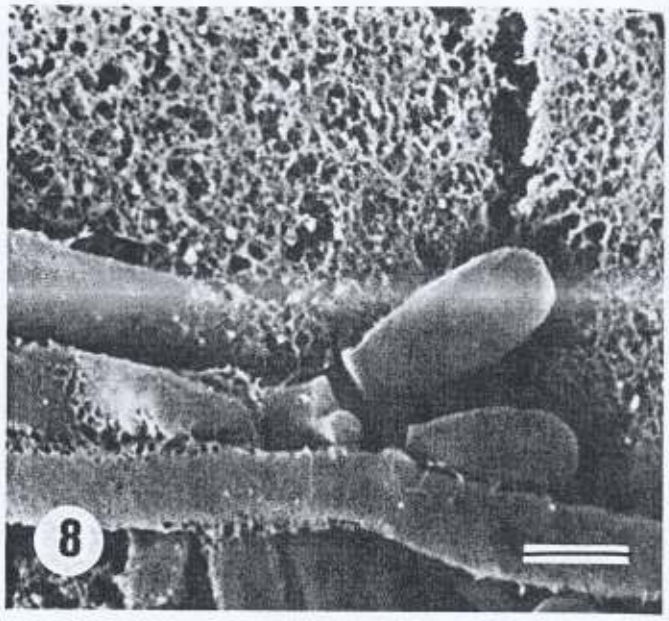
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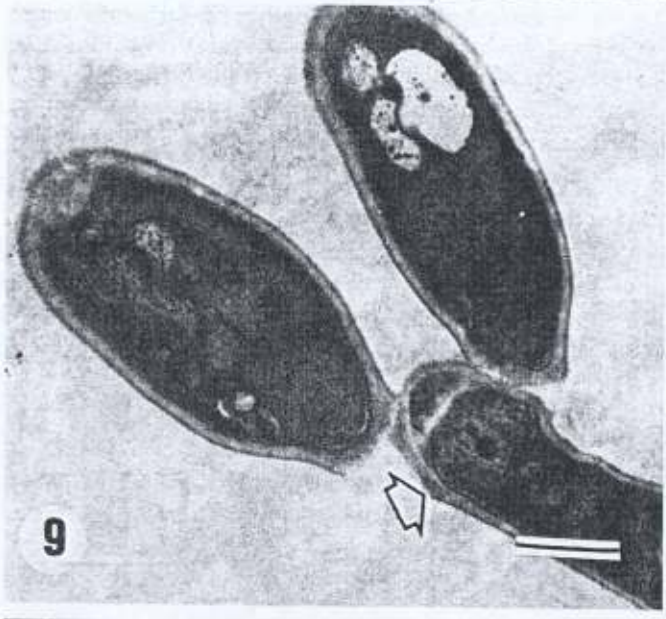
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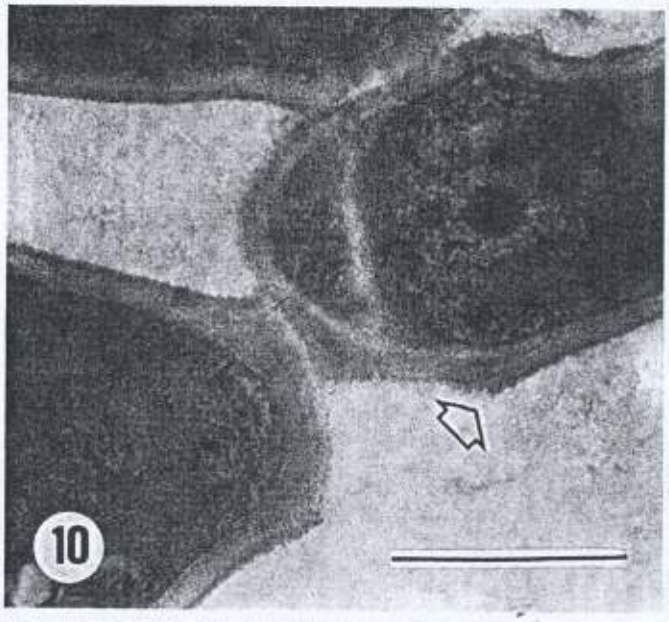
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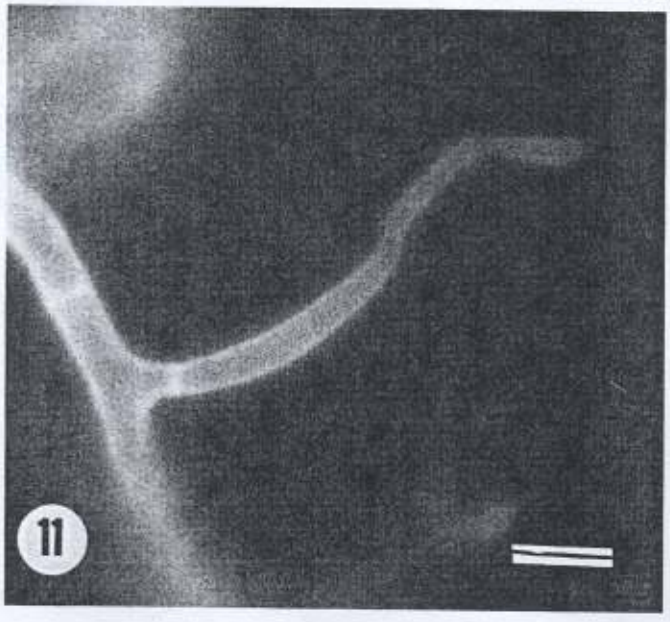
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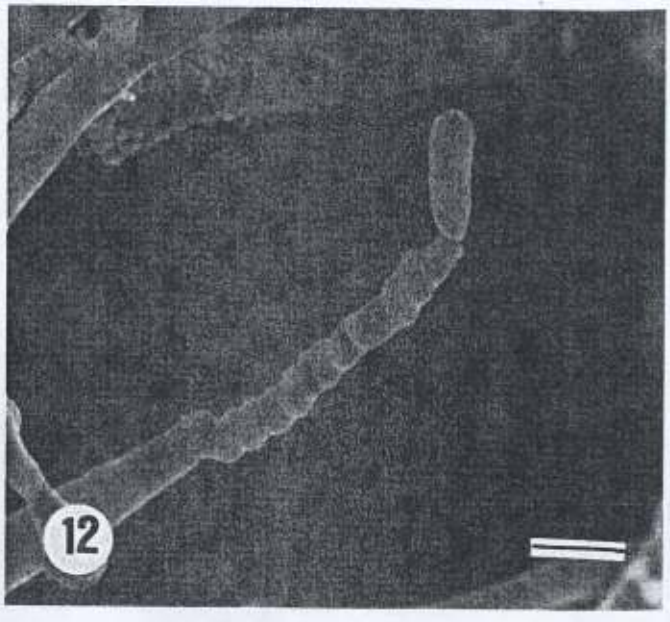
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Figs. 7–12. Conidia and conidiogenous cells of the *Hyalorhinocladiella* anamorphs of *Ceratocystiopsis minuta-bicolor* and *Ophiostoma minus*. Fig. 7. Scanning electron micrograph of *H. minuta-bicolor* revealing narrowly spaced annellations at the apices of conidiogenous cells (arrow). Scale bar = 2 μm. Fig. 8. Conidia of *H. minuta-bicolor* produced on short denticles in a *Sporothrix*-like manner. Scale bar = 2 μm. Figs. 9 and 10. Sections through conidiogenous cells of *H. minuta-bicolor* revealing small annellations at the apex of the conidiogenous cells (arrows). Scale bar = 1 μm. Fig. 11. Fluorescence micrograph of the anamorph of *O. minus* showing a conidiogenous cell with apparent sympodial proliferation. Scale bar = 4 μm. Fig. 12. SEM of small annellations on the conidiogenous cells of *O. minus*. Scale bar = 2 μm.

Figs. 13 and 14. Conidiophores, conidiogenous cells, and conidia of the *Hyalorhinocladiella* anamorph of *Ophiostoma minus*. Fig. 13. Scannning electron micrograph revealing small annellations on the conidiogenous cells. Scale bar = 2 μm. Fig. 14. Transmission electron micrograph showing annellated apices (below arrows) of the conidiogenous cells. Scale bar = 1 μm.

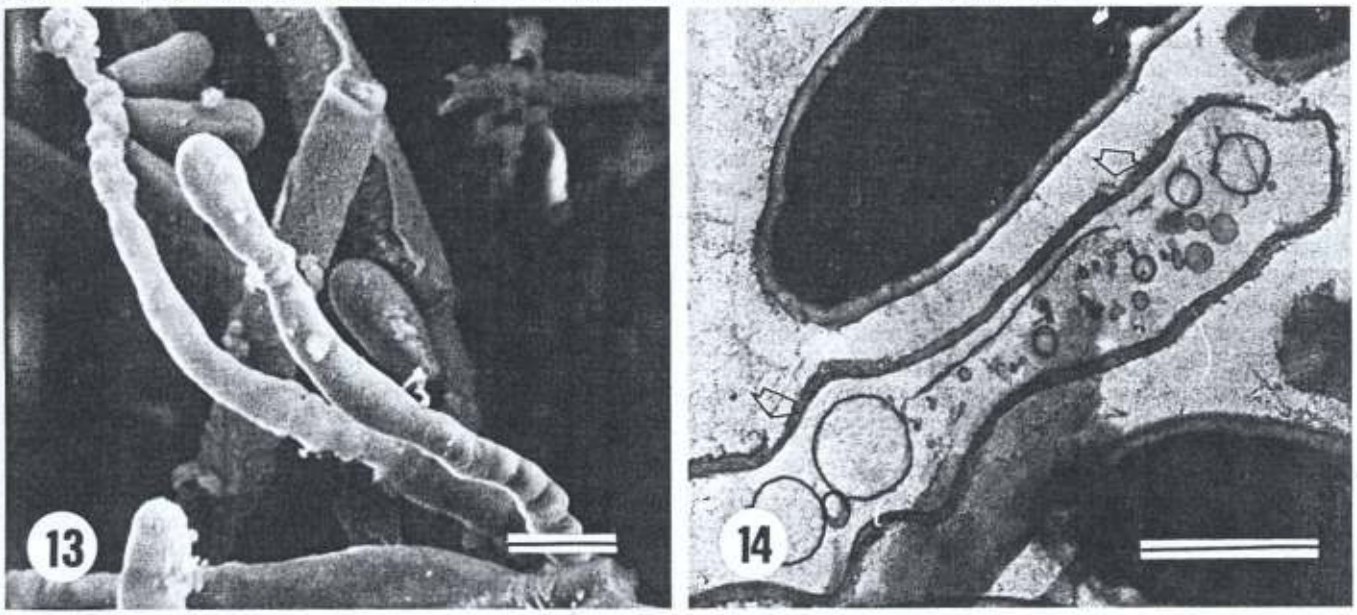
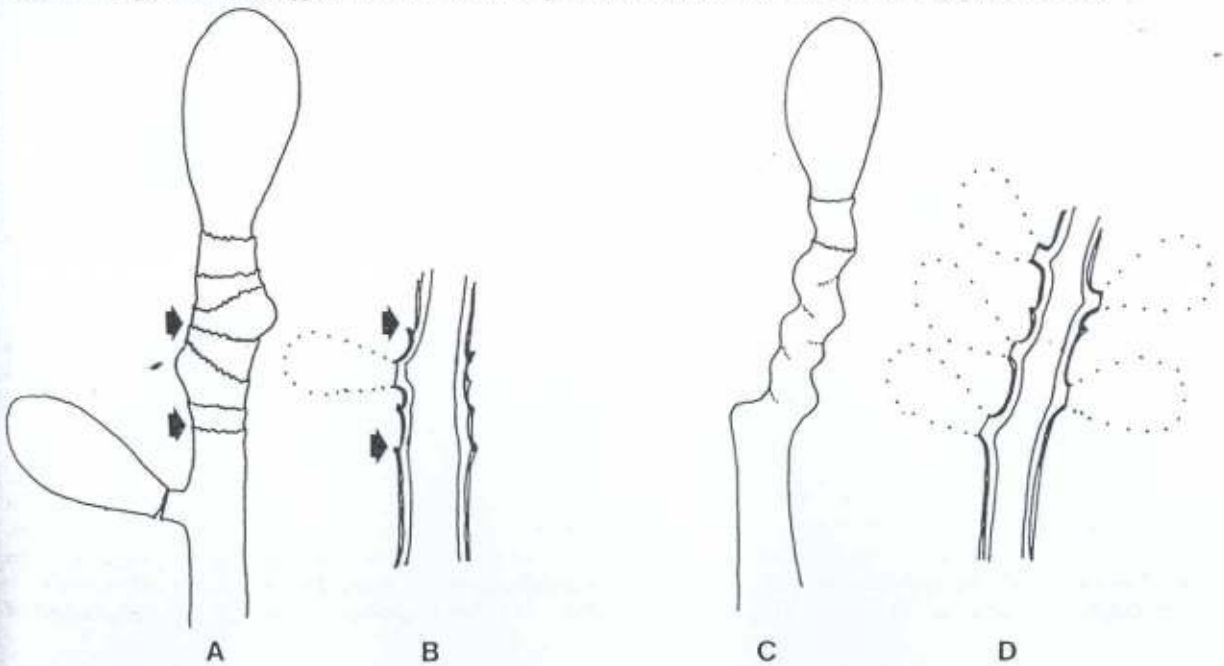


Fig. 15. Schematic illustration of conidium development in *Hyalorhinocladiella minuta-bicolor* and the *Hyalorhinocladiella* anamorph of *Ophiostoma minus*. (A and B) In *H. minuta-bicolor*, conidia are usually formed by enteroblastic percurrent proliferation of the conidiogenous cell. Distinct annellations are present on the conidiogenous cells (arrows). In some cases, however, proliferation displaces the main axis of the conidiogenous cell to form peglike denticles. (C and D) Conidiogenous cells in the *Hyalorhinocladiella* anamorph of *O. minus* also proliferate percurrently to form conidia. Here, however, an extended period of proliferation displaces the main axis of the conidiogenous cell and results in a *Sporothrix*-like appearance. The extent of proliferation determines the size of the peglike denticles.



to produce one-celled, clavate, ellipsoidal to ovoid conidia at the apices of the conidiogenous cells (Figs. 11–13). SEM of simple conidiophores also showed annellations on the conidiogenous cells (Fig. 13).

TEM of sections through the conidiogenous cells of *O. minus* revealed distinct annellations along their lengths (Fig. 14). Here, the conidiogenous cells undergo displaced or angled percurrent proliferation and extend in a geniculate fashion (Fig. 14).

Discussion

In this study of conidium development in *H. minuta-bicolor*, we demonstrated that conidia form through the percurrent proliferation of conidiogenous cells rather than through sympodial development as reported previously by Upadhyay and Kendrick (1975). Similar observations were made for the so-called *Hyalorhinocladiella* anamorph of *O. minus* and in a previous study (Benade et al. 1995) of the anamorph of *O. ips*. We conclude that the situation is similar to that in species of *Graphium* and *Leptographium*, in which delayed secession gives the appearance of sympodial development in conidiogenous cells that proliferate percurrently (Wingfield 1985, 1993; Van Wyk et al. 1988; Wingfield et al. 1991).

Basing our conclusions on the results of this study, we provide the following emendation of the generic circumscription for *Hyalorhinocladiella*.

Hyalorhinocladiella Upadhyay and Kendrick emend Benade and Wingfield

Ontogenies conidii in se habet cellae conidiogenae extensiones et percurrentes et sympodiales, non solum sympodiales ut auctores priores praesumpserunt.

Conidium ontogeny involves both percurrent and sympodial extensions of the conidiogenous cell, not solely sympodial proliferation as assumed by the original authors.

The type of percurrent proliferation of conidiogenous cells in *Hyalorhinocladiella* provides a more clear-cut distinction between this genus and *Sporothrix*. The production of conidia in *Sporothrix* through sympodial proliferation is more clearly evident than in species of *Hyalorhinocladiella*, and in contrast with the conidia of species of *Hyalorhinocladiella*, those of species of *Sporothrix* are produced on peglike denticles. However, given that both genera are anamorphs of *Ophiostoma*, it is reasonable to expect that a continuum exists between the patterns of conidial development in *Hyalorhinocladiella* and *Sporothrix*. Indeed, in some cases the anamorph of *O. minus* produced denticulate conidiogenous cells. This occurred through an extended period of proliferation that displaced the long axis of the conidiogenous cells. These differences are illustrated in a schematic representation of conidium development in *H. minuta-bicolor* and the anamorph of *O. minus* (Fig. 15).

Conidium development in *Hyalorhinocladiella* is virtually identical to that found in *Leptographium* and *Graphium*, and the difference between these genera is in the morphology of the conidiophores. *Graphium* is typified by synnematal conidiophores and *Leptographium* by macronematous and mononematous conidiophores, whereas those of *Hyalorhinocladiella* are micronematous and mycelial in nature (Upadhyay 1981).

The similarity between *Hyalorhinocladiella* and *Sporothrix* suggests that careful examination is necessary to distinguish these genera. The presence or absence of distinct denticles is perhaps the most useful character at the light microscope level. Anamorphs of *Ophiostoma* thought to belong to these genera deserve re-examination. Amongst these, many species of *Graphium* and some species of *Leptographium* apparently have *Sporothrix* synanamorphs. Given the results of this study, it would not be surprising to find that some of these would be better accommodated in *Hyalorhinocladiella*.

References

Benade, E., Wingfield, M.J., and Van Wyk, P.S. 1995. Conidium development in the *Hyalorhinocladiella* anamorph of *Ophiostoma ips*. Mycologia, **87**: 298–303.

Davidson, R.W. 1966. New species of *Ceratocystis* from conifers. Mycopathol. Mycol. Appl. **28**: 273–286.

De Hoog, G.S. 1993. *Sporothrix*-like anamorphs of *Ophiostoma* species and other fungi. *In* *Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. *Edited by* M.J. Wingfield, K.A. Seifert, and J.F. Webber. American Phytopathological Society, St. Paul, Minn. pp. 53–60.

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

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

Hedgcock, G.G. 1906. Studies upon some chromogenic fungi which discolour wood. Mo. Bot. Gard. Annu. Rep. **17**: 59–114.

Hughes, S.J. 1953. Conidiophores, conidia and classification. Can. J. Bot. **31**: 577–659.

Hunt, J. 1956. Taxonomy of the genus *Ceratocystis*. Lloydia, **19**: 1–58.

Kendrick, W.B. 1971. Taxonomy of the Fungi Imperfecti. University of Toronto Press, Toronto, Ont.

Minter, D.W., Kirk, P.M., and Sutton, B.C. 1983. Thallic phialides. Trans. Br. Mycol. Soc. **80**: 39–66.

Mouton, M., Wingfield, M.J., and Van Wyk, P.S. 1994. Conidium development in anamorphs of *Ceratocystis sensu lato*: a review. S. Afr. J. Sci. **90**: 293–298.

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

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.

Rumbold, C.T. 1936. Three blue-staining fungi, including two new species, associated with bark beetles. J. Agric. Res. **52**: 419–437.

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

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

Upadhyay, H.P., and Kendrick, W.B. 1975. Prodromus for a revision of *Ceratocystis* (Microascales, Ascomycetes) and its conidial states. Mycologia, **67**: 798–805.

Van Wyk, P.S., Wingfield, M.J., and Marasas, W.F.O. 1988. Differences in synchronization of stages of conidial development in *Leptographium* species. Trans. Br. Mycol. Soc. **90**: 451–456.

Weijman, A.C.M., and De Hoog, G.S. 1975. On the subdivision of the genus *Ceratocystis*. Antonie Leeuwenhoek, **41**: 353–360.

Wingfield, M.J. 1985. Reclassification of *Verticicladiella* on conidial development. Trans. Br. Mycol. Soc. **85**: 81–93.

Wingfield, M.J. 1993. *Leptographium* species as anamorphs of

Ophiostoma: progress in establishing acceptable generic and species concepts. *In Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. *Edited by* M.J. Wingfield, K.A. Seifert, and J.F. Webber. American Phytopathological Society, St. Paul, Minn. pp. 43–51.

Wingfield, M.J., Kendrick, B., and Van Wyk, P.S. 1991. Analysis of conidium ontogeny in anamorphs of *Ophiostoma*: *Pesotum* and *Phialographium* are synonyms of *Graphium*. Mycol. Res. **95**: 1328–1333.

Wright, E.F., and Cain, R.F. 1961. New species of the genus *Ceratocystis*. Can. J. Bot. **39**: 1215–1230.