Conidium development in *Ceratocystis autographa*

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Ceratocystis s.l. includes *Ceratocystis s.s.*, *Ophiostoma* and *Ceratocystiopsis*. These genera are distinguished by their anamorphs, ascospore morphology and sensitivity to cycloheximide. *Ceratocystis autographa* is unusual in that it reportedly has both *Ophiostoma* and *Ceratocystis* anamorphs. The aim of this study was to clarify confusion relating to the anamorphs of *C. autographa* and thus its generic placement. Light and scanning electron microscopy confirmed the presence of two distinct anamorphs in the fungus. One was *Sporothrix*-like and very rare while the other was *Chalara*-like. Light and fluorescence microscopy, as well as scanning (SEM) and transmission (TEM) electron microscopy of the *Chalara*-like anamorph revealed cylindrical conidia with only a basal delimiting septum. TEM of this state showed vesicles at the apex of the conidiogenous cell, indicating apical wall building. This differs from other species of *Chalara* where conidia have apical and basal delimiting septa and develop through ring wall building. Furthermore, *C. autographa* was tolerant to cycloheximide and is, therefore, more closely related to *Ophiostoma* than *Ceratiocystis*. Results of this study suggest that the *Chalara*-like anamorph belongs to a genus other than *Chalara* and is a probable result of convergent evolution in a fungus adapted to insect dispersal.

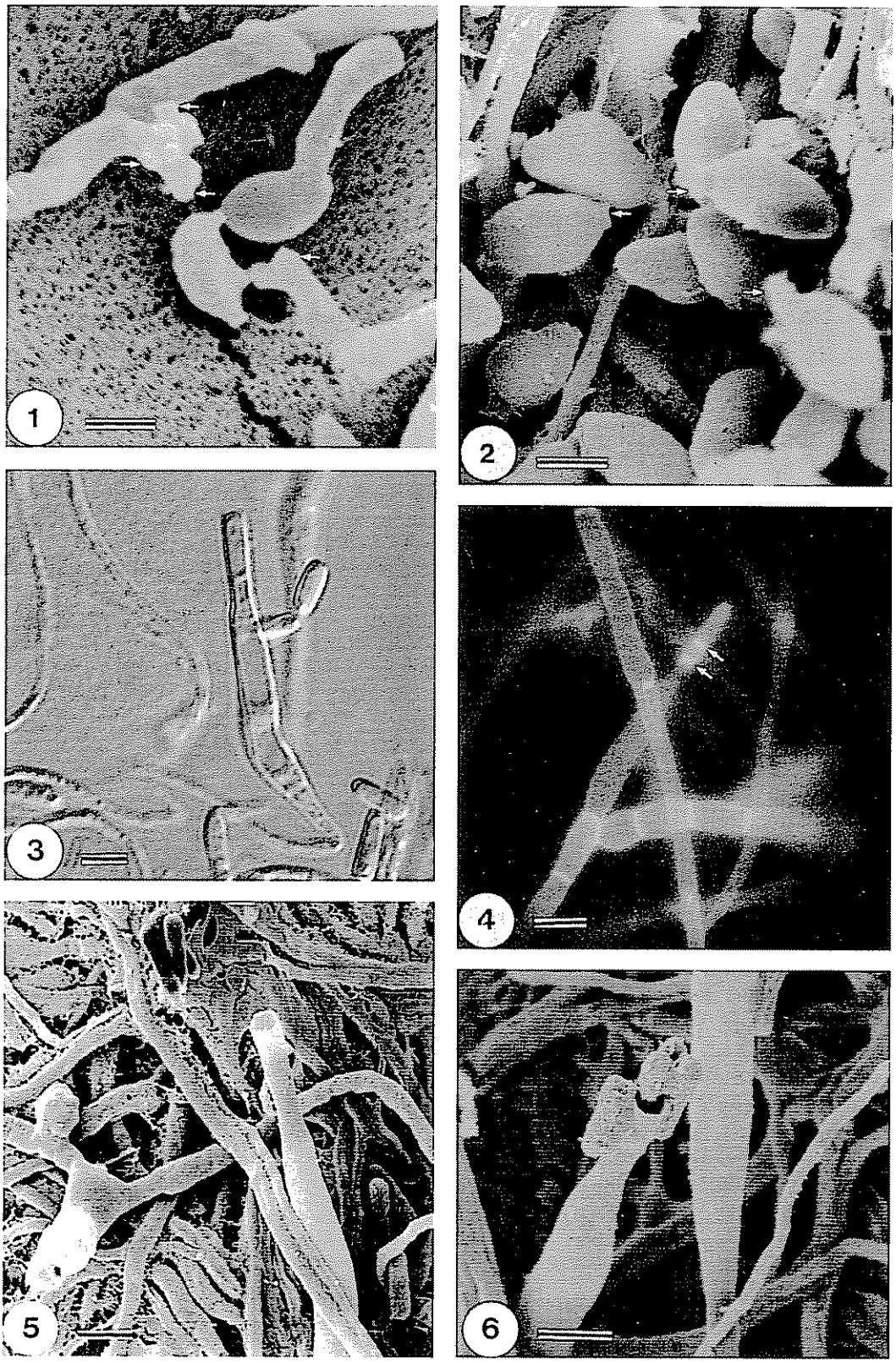
Ceratocystis Ellis & Halst. *s.s.*, *Ceratocystiopsis* H. P. Upadhyay & W. B. Kendr and *Ophiostoma* Syd. & P. Syd. form part of the *Ceratocystis s.l.* complex (Weijman & De Hoog, 1975; Upadhyay, 1981; De Hoog & Scheffer, 1984). *Ceratocystiopsis* is separated from the latter genera by the elongated, falcate, aseptate or occasionally one-septate ascospores, that are attenuated at the ends and always surrounded by a gelatinous sheath (Upadhyay & Kendrick, 1975; Upadhyay, 1981).

Species of *Ceratocystis* have short ascospores, that are not falcate, with sheaths appearing half moon-shaped, hat-shaped or cucullate. Ascospores in species of *Ophiostoma* can be surrounded by a hyaline sheath appearing ossiform, pillow, rectangular or dumbbell-shaped. Other species in *Ophiostoma*, however, can have ascospores that are sheathless, appearing lunate, cylindrical, ovate and orange section-shaped (Hunt, 1956; Griffin, 1968; Olchowecki & Reid, 1974; Upadhyay, 1981).

Species of *Ophiostoma* and *Ceratocystiopsis* are characterized by anamorphs with apical wall building (Minter, Kirk & Sutton, 1983*a*) such as *Graphium* Corda, *Sporothrix* Hektoen & C. F. Perkins, *Hyalorhinocladiella* H. P. Upadhyay & W. B. Kendr. and *Leptographium* Lagerb. & Melin (Wright & Cain, 1961; Upadhyay & Kendrick, 1975; Upadhyay, 1981; Wingfield, 1985; Harrington, 1987). In contrast, anamorphs of *Ceratocystis s.s.* are assigned to only one genus namely *Chalara* (Corda) Rabenh. (Weijman & De Hoog, 1975; De Hoog & Scheffer, 1984). This anamorph genus is characterized by phialides (Nag Raj & Kendrick, 1975, Upadhyay, 1981; De Hoog & Scheffer, 1984) from which conidia develop through ring wall building (Minter *et al.*, 1983*a*).

Species of *Ophiostoma* can be distinguished from species of *Ceratocystis s.s.* by their cell wall components. In the latter genus, cell walls contain mainly chitin with no detectable cellulose (Rosinski & Campana, 1964; Smith, Patik & Rosinski, 1967; Jewell, 1974; Weijman & De Hoog, 1975) or rhamnose (Spencer & Gorin, 1971; Weijman & De Hoog, 1975) whereas in the former genus, cellulose, chitin (Smith *et al.*, 1967; Jewell, 1974; Weijman & De Hoog, 1975) and rhamnose are present as cell components (Spencer & Gorin, 1971; Weijman & De Hoog, 1975). Furthermore, species of *Ceratocystis s.s.* are sensitive to cycloheximide while those of *Ophiostoma* can tolerate high concentrations of this antibiotic (Harrington, 1981).

Ceratocystis autographa B. K. Bakshi was first isolated from *Larix leptolepis* (Japanese larch) in England and Scotland (Bakshi, 1951). A close association was found to exist between *C. autographa* and the bark-beetles *Dryocoetes autographus* and *Hylurgops palliatus* (Bakshi, 1951). Bakshi (1951) described two distinctly different anamorphs for *C. autographa*. In the first, conidia are formed exogenously, are hyaline, round to ovoid and carried singly or in groups of three to four. In the second, endogenous form, the hyaline, barrel-shaped, one-celled conidia are produced in chains (Bakshi, 1951). Hunt (1956) confirmed Bakshi's (1951) findings but also showed that the endoconidia were cylindrical and truncate at the ends. Nag Raj & Kendrick (1975) described the barrel-shaped endoconidia from Bakshi's (1951) descriptions as short clavate, rounded at the apex with a truncate base. Furthermore, Nag Raj & Kendrik (1975) described the conidiogenous cells as being phialidic and lageniform with a highly characteristic con-



Figs 1–6. Conidia and conidiogenous cells of the endogenous and exogenous anamorphs of *Ceratocystis autographa*. **Fig. 1.** SEM of the conidiogenous cell of the exogenous *Sporothrix* anamorph with peg-like (arrows) denticles (arrows) (Bar = 2 μm). **Fig. 2.** SEM of the globose to subglobose *Sporothrix*-like conidia with single attachment points (arrows) (Bar = 2 μm). **Fig. 3.** Light micrograph of the *Chalara*-like or endogenous anamorph, showing lageniform phialidic conidiogenous cell with a cylindrical conidium inside the conidiogenous cell (Bar = 2 μm). **Fig. 4.** Fluorescence micrograph of the *Chalara*-like lageniform phialidic conidiogenous cell with two brightly fluorescing areas (arrows) at the apex of the conidiogenous cell, indicative of the single attachment points (arrows) of two succeeding conidia (Bar = 2 μm). **Fig. 5.** Scanning electron micrographs of the *Chalara*-like phialidic conidiogenous cells with a distinct collarette at the apex (Bar = 2 μm). **Fig. 6.** SEM of cylindrical conidia at the apex of a tubular collarette (Bar = 2 μm).

striction at the base of the collarette. They concluded that this anamorph of *C. autographa* is a species of *Chalara*. Upadhyay (1981) confirmed the findings of Nag Raj & Kendrick (1975) and suggested that the exogenous anamorph with its sympodial conidium development was probably a species of *Hyalorhinocladiella*.

The presence of both a *Chalara* and a *Hyalorhinocladiella* anamorph in a single species of *Ceratocystis s.l.* is enigmatic. *C. autographa* would then be the only ophiostomatoid fungus to have both ring wall building, as well as apical wall building conidial development. If a *Chalara* state were indeed present, this fungus should be retained in *Ceratocystis s.s.* and would thus be unrelated to *Ophiostoma*. In contrast, the presence of a *Hyalorhinocladiella* state would suggest that it was a species of *Ophiostoma*. The aim of this study was, therefore, to examine conidium development in *C. autographa* and to clarify the present confusion surrounding the taxonomy of this species.

MATERIALS AND METHODS

The isolate of *C. autographa* used in this study was obtained from the Centraalbureau voor Schimmelcultures Baarn (CBS 670.75). It was grown on 2% malt extract agar (MEA) in Petri dishes (20 g Biolab malt extract, 20 g Biolab agar^{-1} water) and incubated at 25 °C in the dark until sporulating. Thirty single-conidium isolates were made from this culture using the technique described by Nelson, Toussoun & Marasas (1983). The aim of this procedure was to ensure that the isolate under investigation did not represent a mixed culture. The type specimen of *C. autographa* (IMI 20162) was also examined and compared with the culture used in this study.

Material for light microscopy was mounted in lactophenol on glass slides and examined using phase- and interference contrast microscopy. Material for fluorescence microscopy was mounted on glass slides in a 0·05% w/v solution of cellufluor white M 2R optical brightener in 0·1 M phosphate buffer. A Zeiss Axioskop fluorescence microscope with dark background and uv light was used to examine the samples.

Specimens for scanning electron microscopy (SEM) were cut into blocks from the agar (approximately 5–7 mm^2) fixed in glutaraldehyde followed by 1% osmium tetroxide in a 0·1 M phosphate buffer (pH 7) and dehydrated in a graded acetone series. SEM specimens were then critical point dried, mounted, coated with gold/palladium and viewed with a JSM 6400 scanning electron microscope.

For transmission electron microscopy (TEM), material was cut into blocks from the agar (approximately 1–1·5 mm^2). Samples were fixed and dehydrated as for SEM and embedded according to Spurr (1969) with the following modifications: Specimens were placed in a mixture (1:1) of epoxy resin and acetone (100%) for 90 min at room temperature. The specimens were then placed in epoxy resin for 30 min at room temperature followed by 30 min at 50 °. The epoxy resin was replaced with new resin and placed in an oven for 60 min at 50 °. Specimens were placed in epoxy resin with the upper side facing the rounded point of the pre-heated gelatin capsule. The epoxy resin was polymerized at 70° overnight. Ultrathin sections (60 nm) were made with glass knives, mounted on copper grids and stained for 30 min in uranyl acetate followed by 5 min in lead citrate (Reynolds, 1963). Sections were examined with a Phillips 301 transmission electron microscope.

The isolate of *C. autographa* was compared with typical species of *Ophiostoma* and *Ceratocystis* for its ability to tolerate cycloheximide in culture. Isolates for comparison were one of *Ophiostoma piceae* Syd. & P. Syd. (CMW 1367; Collection of M. J. Wingfield, Department Microbiology and Biochemistry, University of the Orange Free State, P.O. Box 339, Bloemfontein, 9300) supplied by Dr J. Gibbs, Forestry Commission, Alice Holt Lodge, U.K. and an isolate of *Ceratocystis fimbriata* Ellis & Halst. (CMW 1547) originating in New Guinea. Six different concentrations (0, 0·05, 0·1, 0·5, 1·25 and 2·5 g l^{-1} MEA) of cycloheximide were used for these comparisons. The cycloheximide (Sigma Chemical Co.) was filter-sterilized using a sterile Nalgene filter holder (0·2 µm cellulose nitrate Whatman filter). The different concentrations of cycloheximide were added to the autoclaved MEA and media (15 ml/plate) was poured into 90 mm diameter Petri dishes.

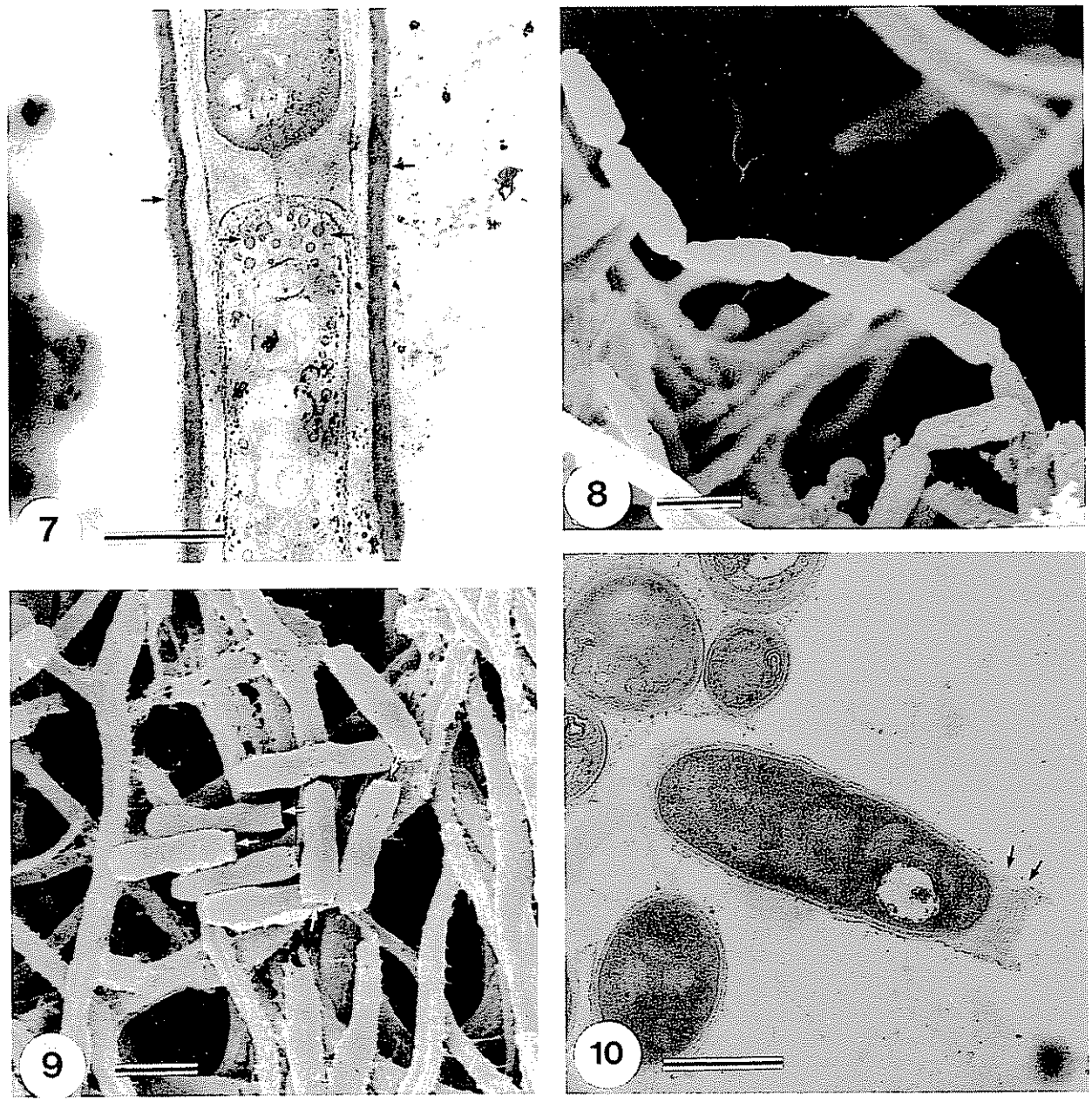
Cultures of the fungi to be tested for cycloheximide tolerance were grown on MEA for 4 weeks at 25° in the dark. A cork borer (4 mm diam.) was used to cut discs from the cultures and these were placed upside down onto the agar surface. Three discs were placed equidistant from each other on Petri dishes and each plate was replicated four times. Petri dishes were incubated at 25° under mixed light (cool-white fluorescent and nuv). Two diameter readings of each colony were made at 90° to each other and averages of the resulting 12 diameter readings were calculated. The experiment was repeated once.

RESULTS

Single-conidium cultures derived from the isolate of *C. autographa* included both apparently endoconidial and exoconidial anamorphs. There were no observable differences in growth rate or colony colour amongst single conidial cultures or between these and the original stock culture. Morphological characteristics of the *Chalara* state were also consistent with those published by Bakshi (1951) and those observed in the type specimen of the species. No teleomorph structures were found in the culture and it is impossible to be sure of the connection between the *Chalara* state and the teleomorph described by Bakshi (1951).

Scanning electron micrographs showed that the exogenous anamorph in the culture of *C. autographa* was a species of *Sporothrix* (Figs 1, 2) with prominent peg-like denticles on the conidiogenous cells, showing sympodial proliferation. Furthermore, SEM revealed that the globose to subglobose conidia produced by this anamorph, have single attachment points (Fig. 2). The *Sporothrix* anamorph of *C. autographa* occurred rarely in cultures.

Light and scanning electron micrographs revealed the presence of phialides in *C. autographa* with a gross morphology similar to *Chalara* species (Figs 3, 5, 6) with lageniform conidiogenous cells which had distinct tube-like collarettes at their apices (Figs 3, 6). Fluorescence microscopy revealed two



Figs 7–10. Conidia and conidiogenous cells of the endogenous, *Chalara*-like anamorph of *Ceratocystis autographa*. **Fig. 7.** TEM of a section through a conidiogenous locus revealing a conidium with a collar structure [arrows] and single attachment point at its base and small secretory vesicles (arrows) at apex of the conidiogenous cell indicating apical wall building (Bar = 1 µm). **Fig. 8.** SEM showing a false chain (arrows) of endoconidia (Bar = 2 µm). **Fig. 9.** Scanning electron micrograph of the cylindrical endoconidia with prominent collar structures or single attachment points (arrows) at the bases of the conidia (Bar = 2 µm). **Fig. 10.** TEM of a section through a cylindrical endoconidium revealing the distinct single attachment point (arrows) and collar structure (Bar = 1 µm).

brightly fluorescing areas at the apices of the conidiogenous cell (Fig. 4). These areas probably represent the single attachment points of two successive conidia in the tubular collarette. Scanning electron micrographs also showed cylindrical endoconidia at the apex of the conidiogenous cells (Fig. 6). Transmission electron micrographs of sections through the conidiogenous cells revealed what might be described as a wall building apex within the conidiogenous cells (Fig. 7). A wall building ring, typical of other species of *Chalara*, could not be distinguished with fluorescence or SEM.

Using SEM, chains of conidia that were rounded at the apex and narrower at the base (Fig. 8) with prominent collar structures or single attachment points (Fig. 9) could be distinguished. TEM sections through the conidia also revealed distinct single attachment points (Fig. 10). These had a basal fringe resulting in a collar structure which would presumably surround the apex of the previously formed conidium (Fig. 10).

C. autographa is a slow-growing fungus which in terms of growth rate could not be compared with *O. piceae* and *C. fimbriata*. *O. piceae* possessed a high degree of tolerance to cycloheximide, although a reduction in growth rate was observed at higher concentrations (Table 1). *C. fimbriata* could not tolerate the lowest concentration of cycloheximide tested.

Table 1. Cycloheximide sensitivity expressed as a percentage of growth in *Ophiostoma piceae*, *Ceratocystis fimbriata* and *Ceratocystis autographa*[a]

	Cycloheximide concentration (g l^{-1})					
	0	0·05	0·1	0·5	1·25	2·5
O. piceae	100	88·87	71·26	71·62	46·46	39·03
C. fimbriata	100	0·70	0	0	0	0
C. autographa	100	95·25	89·77	82·21	66·60	35·83

[a] Values represent the means of 24 readings converted to a percentage of growth of the control (0 g l^{-1} cycloheximide). Three replicate plates were used per isolate with three discs per plate, and the experiment was repeated once; colony diameters were determined after 4 weeks at 25° under mixed fluorescent and nuv light.

In contrast, *C. autographa* was tolerant to cycloheximide and significant reduction in growth was only observed at the highest concentrations of the antiobiotic (Table 1).

DISCUSSION

Results of this study have confirmed that the culture believed to represent the anamorph of *C. autographa* possesses two anamorphs with distinct modes of conidial development. These two morphological forms are retained in single-conidium cultures of the fungus and appear typical of the anamorph of the species as described by Bakshi (1951). The *Sporothrix* anamorph was rare in the culture and could not be studied in detail but the *Chalara* anamorph was indistinguishable from that associated with the type specimen and originally described by Bakshi (1951) and subsequently by other authors (Hunt, 1956; Nag Raj & Kendrick, 1975; Upadhyay, 1981).

In a comparison of DNA sequence data, Hausner, Reid & Klassen (1993, 1995) suggested that the isolate CBS 670.75 probably no longer represents the fungus originally described by Bakshi (1951). This is indeed possible although the *Chalara* state in the type specimen and that in the culture were indistinguishable. The culture in question is strange in the sense that it was collected from larch needles which is a niche rather different from galleries of larch-infesting insects. At this stage it is impossible to resolve this question but we do recognize that the culture used in this study and the *Chalara* state observed by Bakshi (1951) might not necessarily be connected to the teleomorph that he described for the fungus. For the present, we see no alternative but to treat these as connected but hope that in the future it might be possible to make further collections from the niche described by Bakshi (1951).

The presence of a *Chalara* as well as a *Sporothrix* anamorph in a species of *Ceratocystis s.l.* would be contradictory to the currently accepted generic concepts applied to the genus (Weijman & De Hoog, 1975; Upadhyay, 1981; De Hoog & Scheffer, 1984). Both light and electron microscopic examinations of the *Sporothrix* anamorph state showed it to be typical of this genus, which is a common anamorph of species of *Ophiostoma*. It was unusual only in that it occurred very rarely in cultures.

Superficial examination showed that the so-called *Chalara* anamorph, which was the predominant form present in culture, was typical of this genus. On closer examination, however, many unusual features became evident. Species of *Chalara* are characterized by ring wall building conidium development which results in chains of conidia, each with apical and basal septa (other than the first-formed conidium in each phialide) (Nag Raj & Kendrick, 1975; Hawes & Beckett, 1977; Minter *et al.*, 1983*a*; Minter, Sutton & Brady, 1983*b*). In contrast, the anamorph of *C. autographa* has conidia with single points of attachment which is typical of apical wall building development (Minter *et al.*, 1983*a*). The chains of conidia that are produced by this anamorph might best be considered as 'false chains' *sensu* Minter (Subramanian, 1972; Minter *et al.*, 1982; Minter *et al.*, 1983*a*). These chains initially establish their integrity in the long tubular collarettes and are apparently maintained by the fringe-like extension at the base of each conidium and possibly some mucus.

Although conidia in the *Chalara* state of *C. autographa* develop through apical wall building, the conidiogenous locus is distinctly different from that of most phialidic anamorphs with apical wall building conidial development. Of particular note is the absence of evidence of periclinal thickening which is a characteristic of most phialidic fungi (Minter *et al.*, 1983*a*).

Other than the presence or absence of a *Chalara* anamorph, cycloheximide tolerance provides a reliable characteristic on which to separate species of *Ophiostoma* and *Ceratocystis s.s.* (Harrington, 1981). In this study, *C. autographa* was shown to have a high degree of tolerance to this antibiotic. This suggests that the species is more closely related to *Ophiostoma* and confirms the morphological observations made here.

C. autographa has short cylindrical ascospores (Bakshi, 1951; Hunt, 1956) with sheaths appearing ossiform in side and face view (Upadhyay, 1981). This ascospore shape is unusual amongst species of *Ceratocystis s.s.* (Hunt, 1956). Cylindrical ascospores with or without sheaths are, however, common in a number of species of *Ophiostoma* (Hunt, 1956; Griffin, 1968; Olchowecki & Reid, 1974; Upadhyay, 1981). This fact further substantiates the contention that *C. autographa* is more closely related to *Ophiostoma*, than to *Ceratocystis s.s.* It is, however, possible that this fungus represents a distinct genus as suggested by the preliminary data of Hausner, Reid & Klassen (1993*a*).

Species of *Ceratocystis s.s* are well known to be adapted to insect dispersal (Upadhyay, 1981; Whitney, 1982; Perry, 1991) and it is assumed that their *Chalara* anamorphs play some part in this adaptation. The presence of a morphological form in *C. autographa*, superficially almost identical to *Chalara*, which is apparently unrelated to *Ceratocystis s.s.*, provides an excellent example of convergent evolution. Special care must, however, be taken to distinguish these products of convergence because they often, as is apparently the case with *C. autographa*, result in taxonomic confusion.

Results of this study clearly indicate that the *Chalara*-like anamorph of *C. autographa* is unlike typical species of *Chalara*, and though superficially identical to *Chalara*, would probably best be characterized by conidia having single attachment points. Although other characteristics are present in this fungus, basal delimiting septa can easily be recognized using light microscopy.

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