

## *Graphium pseudormiticum* sp. nov.: a new hyphomycete with unusual conidiogenesis

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A new synnematal hyphomycete was recently isolated from the bark beetle, *Orthotomicus erosus*, in the south-western Cape Province of South Africa. This fungus has all the features characteristic of *Graphium* species with darkly pigmented synnemata and single-celled conidia produced from annellidic conidiogenous cells. In a detailed study of conidium development using scanning and transmission electron microscopy, the conidia appear to be produced in chains and have conspicuous basal frills. The catenulate arrangement of conidia results from conidia adhering to each other because of basal frills. These prominent basal frills apparently result from advanced proliferation of the conidiogenous cells prior to conidial delimitation and secession.

The anamorph genus *Graphium* Corda was established by Corda (1837) with *G. penicillioides* Corda as the type species. Species in this genus are characterized by darkly pigmented, synnematal conidiophores that produce single-celled conidia from annellidic conidiogenous cells (Upadhyay, 1981; Wingfield, Kendrick & Van Wyk, 1991). *Graphium* species are known as anamorphs of *Ophiostoma* H. & P. Sydow and other ascomycetes in the Microascales and Sphaeriales (Seifert & Okada, 1993). The ophiostomatoid fungi are well recognized for their symbiotic relationship with insects such as bark beetles (Bakshi, 1950; Whitney, 1971; Safranyik, Shrimpton & Whitney, 1975; Goheen & Cobb, 1978), and many of them cause sap-stain in timber (Leach, Orr & Christensen, 1934; Bakshi, 1951).

Recently, Wingfield *et al.* (1991) altered the generic concept of *Graphium* to include all species formerly assigned to *Pesotum* J. L. Crane & Schokn. and *Phialographium* H. P. Upadhyay & W. B. Kendr. This study was based on mode of conidium development and *Graphium* was emended to include all darkly pigmented, synnematal species with annellidic, phialidic and apparent sympodial conidium development (Wingfield *et al.*, 1991). Mouton, Wingfield & Van Wyk (1993) then examined additional species in this group and, with further ultrastructural evidence, confirmed the synonymy of *Pesotum* and *Phialographium* with *Graphium*. Many of these *Graphium* anamorphs are also characterized by synanamorphic states, of which *Sporothrix* Hektoen & Perkins is most common (De Hoog, 1974).

An unusual and apparently new synnematal hyphomycete has been isolated in the south-western Cape Province of South Africa (Wingfield, Van Wyk & Marasas, 1988). The fungus is associated with the bark beetle, *Orthotomicus erosus* (Wollaston)

which infests *Pinus* spp. (Tribe, 1990). The insect was accidentally introduced into southern Africa from Europe.

Light microscopy showed that the conidia of the fungus were apparently produced from annellidic conidiogenous cells accumulating in gloeid masses at the apices of darkly pigmented, synnematal conidiophores, characteristic of *Graphium*. An unusual feature of this fungus was that conidia were apparently produced in chains and had conspicuous basal frills. The aim of this study was to describe this species of *Graphium*. A detailed examination of conidium development was also undertaken to characterize the unusual conidia in this fungus.

### MATERIALS AND METHODS

Cultures of the *Graphium* sp. originated from routine isolations from adult *O. erosus*. These insects were trapped in the south-western Cape Province of South Africa using bait logs as described by Tribe (1990). Insects were squashed onto the surface of 2% malt extract agar (MEA) and resulting isolates transferred to new MEA plates.

Material for scanning electron microscopy (SEM) was cut from agar into blocks (5 mm<sup>2</sup>), fixed with 3% glutaraldehyde followed by 1% osmium tetroxide, buffered by a phosphate buffer (pH = 7) and dehydrated in a graded acetone series (50, 70, 95, 100%). Specimens were then critical point dried (Cohen, 1970), mounted on stubs, coated with gold/palladium and viewed with a JSM 6400 scanning electron microscope.

Specimens for transmission electron microscopy (TEM) were fixed and dehydrated in a similar manner as those for SEM. Material was then embedded in epoxy resin (Spurr, 1969), ultrathin sections (60 nm) were made and stained in

uranyl acetate (25 min) followed by lead citrate (10 min) (Reynolds, 1963) and viewed with a Philips EM 300 transmission electron microscope.

Growth of the apparently new *Graphium* sp. was tested at different temperatures. This was done by inoculating the fungus onto 2% MEA in Petri dishes and incubating them at 10, 15, 20, 25, 30 and 35 °C, respectively. Growth of colonies was measured after 8 d by taking the average of two colony diam. measurements for each plate. Three Petri dishes were used at each temperature and the experiment was conducted in the dark and repeated once. The average colony diam. from the two experiments was then calculated.

Tolerance of species in *Ceratocystis s.l.* to cycloheximide has been an important characteristic in the taxonomy of this group of fungi (Harrington, 1981; De Hoog & Scheffer, 1984). Cycloheximide tolerance of the *Graphium* sp. was, therefore, tested. The fungus was inoculated onto MEA amended with 0, 0.05, 0.1, 0.5, 1.0, 2.5 and 5.0 g cycloheximide l<sup>-1</sup>. Growth of colonies was also calculated after 8 d and the experiment was conducted at 25° in the dark. Each concentration of the antibiotic was represented by three Petri dishes, the experiment repeated and average colony diam. calculated.

## RESULTS

*Graphium* appears to be the most appropriate disposition for the synnematal hyphomycete from *O. erosus*. This fungus also appears to be unlike any other known species of *Graphium* and we, therefore, provide the following diagnosis for it.

### *Graphium pseudormiticum* M. Mouton & M. J. Wingfield sp. nov.

Etym.: derived from Greek word meaning capable of or possessing false chains

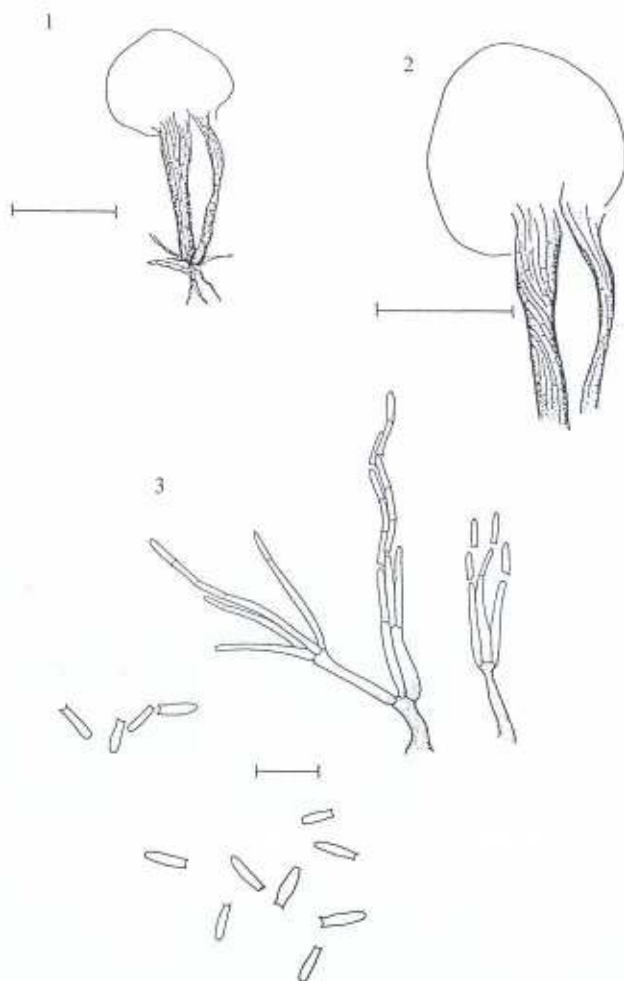
*Coloniae* in MEA ad 25° attingentes diametrum 12.5 mm post 8 dies, crescunt optime ad 30°, diametrum 18.2 mm post 8 dies, cycloheximide non tolerant, incoloratae olivaceo-viridescentes versus medietatem. *Mycelium* sparsum aerium, hyphae summersae hyalinae, regulatim septatae, parce ramosae et glabro-tunicatae. *Conidiophora* orientia singulatim vel aggregata, macronemata, synnemata, 113–263 (183) µm × 5–20 (11.3) µm ad mediam partem, septata, fusca ad basim pallescentia versus hyalinum apicem, 5–9 bene evoluta rhizoidea ad basim (Figs 1, 2, 4). *Cellulae conidiogenae* annellatae, hyalinae, 11–24 (16) µm (Figs 3, 5). *Conidia* unicellularia, hyalina, glabro-tunicata, cylindrica, 4–7.5 (6.2) µm × 1.5–2.5 (2.2) µm, subrotunda ad apicem fimbriis basilaribus prominentibus (Figs 3, 6, 7). *Conidiophora* mononemata, typi *Sporothricis* (= *Sporothrix*) conidiis unicellularibus natis sympodice in curtis denticulis aliquando praesentibus (Fig. 8).

*Colonies* on MEA at 25° attaining a diam. of 12.5 mm after 8 d, grow optimally at 30° with a diam. of 18.2 mm after 8 d, are not tolerant to cycloheximide, colourless becoming olivaceous-green towards the middle. Sparse aerial mycelium, submerged hyphae hyaline, regularly septate, sparingly branched and smooth walled. *Conidiophores* arising singly or in groups, macronematous, synnematosus, 113–263 (183) µm × 5–20 (11.3) µm in the centre, septate, dark-brown at the base becoming lighter towards the hyaline apex, 5–9 well-developed rhizoids at the base (Figs 1, 2, 4). *Conidiogenous cells*

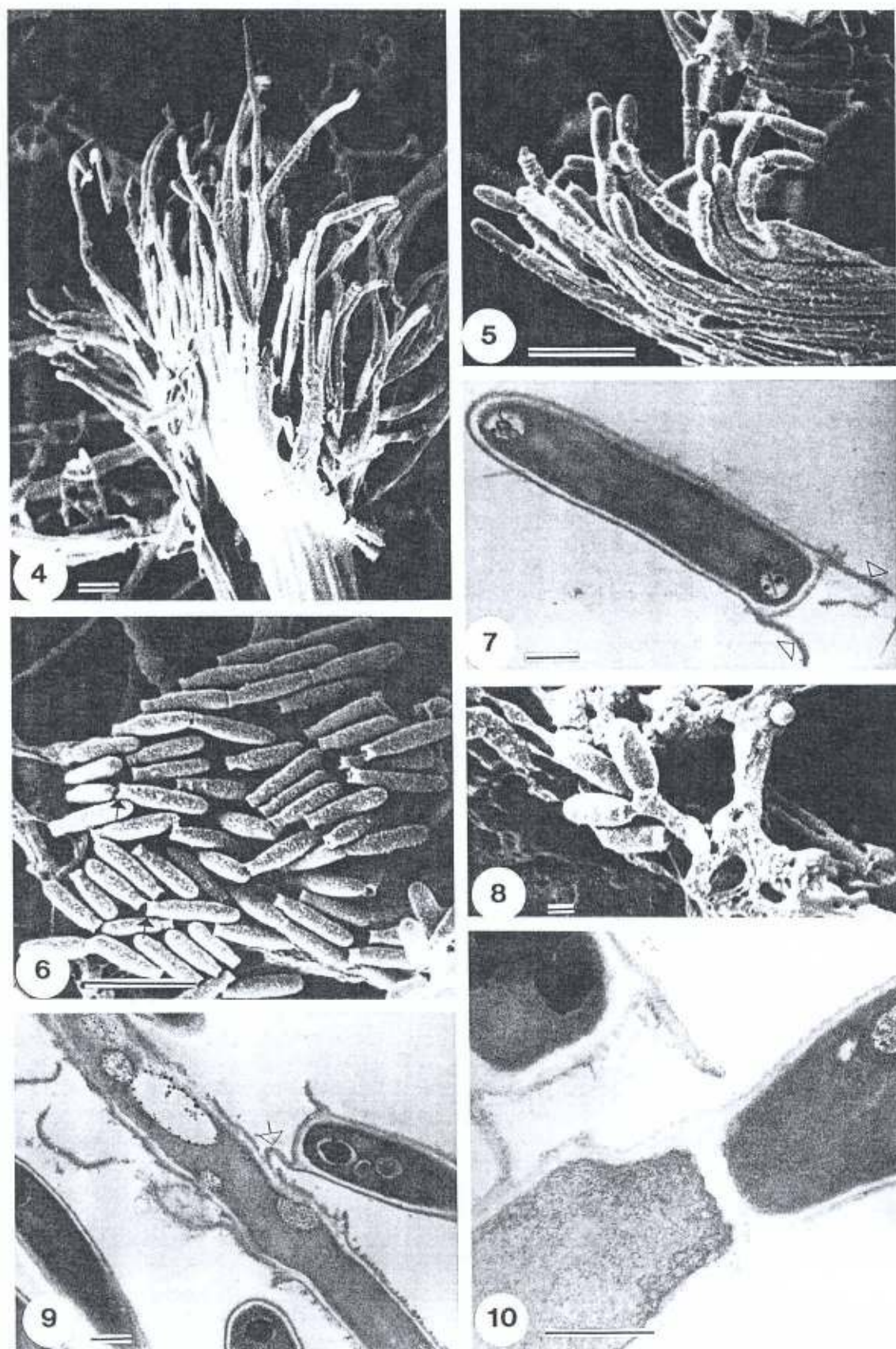
annellated, hyaline, 11–24 (16) µm (Figs 3, 5). *Conidia* one-celled, hyaline, smooth-walled, cylindrical, 4–7.5 (6.2) µm × 1.5–2.5 (2.2) µm, slightly rounded at the apex with prominent basal frills (Figs 3, 6, 7). *Conidiophores* of *Sporothrix*-type mononematous, with single-celled conidia produced sympodially on short denticles occasionally present (Fig. 8).

*Specimens examined*: Isolated from adult *Orthotomicus erosus*, which were trapped using bait logs in the south-western Cape Province, South Africa, September 1984, M. J. Wingfield, PREM 51535 HOLOTYPE.

Scanning electron micrographs showed the accumulation of annellations towards the apices of conidiogenous cells (Figs 4, 5). This fungus is, however, unusual in producing conidia in chains (Fig. 6). These conidia have conspicuous basal frills (Figs 6, 7) which result in chains where the cupulate bases adhere to apices of successive conidia (Fig. 6). Transmission electron microscopy showed sections through the annellated conidiogenous cells with the catenulate, frilled conidia still hanging along the sides of the conidiogenous cells, indicating incomplete dehiscence of the conidia (Figs 9, 10). Scanning



Figs 1–3. Conidiophores, conidiogenous cells and conidia of *G. pseudormiticum*. Figs 1 & 2. Synnematal conidiophore, capped by a slimy head of conidia (bar, 50 µm). Fig. 3. Conidiogenous cells producing chains of conidia characterized by prominent basal frills (bar, 10 µm).



**Figs 4–10.** SEM and TEM of conidia and conidiogenous cells of *G. pseudomiticum*. **Fig. 4.** SEM of the apex of the synnematosus conidiophore showing annellated conidiogenous cells (bar, 10  $\mu\text{m}$ ). **Fig. 5.** Scanning electron micrograph showing the accumulation of annellations towards apices of conidiogenous cells (bar, 10  $\mu\text{m}$ ). **Fig. 6.** Conidia characterized by conspicuous basal frills and produced in chains (arrows) (bar, 10  $\mu\text{m}$ ). **Fig. 7.** Section through a conidium revealing the prominent basal frill (bar, 1  $\mu\text{m}$ ). **Fig. 8.** SEM of the *Sporothrix* synanamorph of *G. pseudomiticum* (bar, 1  $\mu\text{m}$ ). **Fig. 9.** TEM of a section through a conidiogenous cell indicating incomplete dehiscence of conidia (bar, 1  $\mu\text{m}$ ). **Fig. 10.** Transmission electron micrograph showing an overlapping of stages of delimitation, secession and proliferation (bar, 1  $\mu\text{m}$ ).

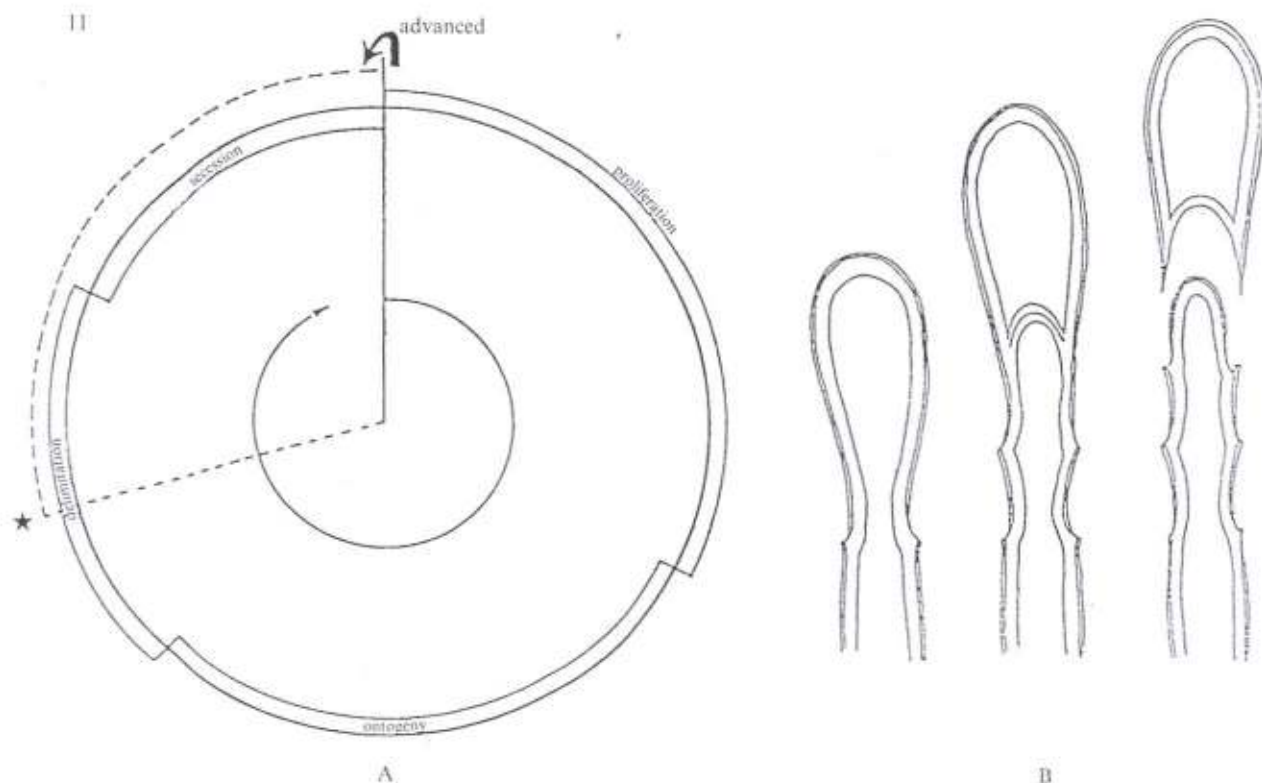


Fig. 11. Schematic representation of conidium development in *G. pseudormiticum*. Fig. 11A, B. Conidium development in *G. pseudormiticum* is characterized by advanced proliferation that overlaps the stages of conidial delimitation and secession.

electron micrographs also showed the presence of mycelial, mononematous conidiophores, producing conidia sympodially on apparent short denticles typical of *Sporothrix* (Fig. 8).

## DISCUSSION

*G. pseudormiticum* can be readily recognized as a species of *Graphium* by its darkly pigmented conidiophore, annelidic conidiogenous cells and single-celled, hyaline conidia. This fungus bears some resemblance to *G. penicillioides* although these species can easily be distinguished. Other than the absence of chains, *G. penicillioides* also has conidia that are distinctly curved (Corda, 1837). The latter species also originates from hardwood trees in Europe, a substratum substantially different from the conifer/bark beetle niche associated with *G. pseudormiticum*.

The type material of *G. penicillioides* (Herb. IMI 155518) available to us for comparison was in a poor condition and included only conidia. These conidia were curved and conformed well with those described by Corda (1837) and were very different from those of *G. pseudormiticum*. Clearly, the typification of *Graphium* requires attention but this must await collections of similar fungi from Czechoslovakia where *G. penicillioides* was originally collected (Corda, 1837; Seifert & Okada, 1993).

The presence of a *Sporothrix* synanamorph in *G. pseudormiticum* is not unusual for this group of fungi. Indeed many species of *Graphium* have a *Sporothrix* state (De Hoog, 1974; Upadhyay, 1981). The synanamorph of *G. pseudormiticum* is, however, uncommon and usually only seen using scanning electron microscopy.

*Graphium pseudormiticum* has a unique mode of conidium development as can be deduced from the conspicuous basal frills of the conidia. The mode of conidium development can be summarized as enteroblastic proliferation and holoblastic ontogeny, delimitation and secession *sensu* Minter, Kirk & Sutton (1982). The successive proliferation stage, however, overlaps the previous delimitation and secession stages (Fig. 11A, B), resulting in the prominent basal frills of the conidia. These basal frills lead to the adherence of conidia to each other giving rise to what Minter *et al.* (1982, 1983) refer to as 'false chains'.

The sensitivity of *G. pseudormiticum* to low concentrations of cycloheximide was of interest. *Ophiostoma* spp. with *Graphium* anamorphs can tolerate high concentrations of the antibiotic (Harrington, 1981; De Hoog & Scheffer, 1984). The results of this study might, therefore, imply that *G. pseudormiticum* is an anamorph of a teleomorph genus other than *Ophiostoma*.

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## REFERENCES

- Bakshi, B. K. (1950). Fungi associated with ambrosia beetles in Great Britain. *Transactions of the British Mycological Society* **33**, 111-120.  
 Bakshi, B. K. (1951). Studies on four species of *Ceratocystis*, with a discussion on fungi causing sap-stain in Britain. *Commonwealth Mycological Institute, Mycology Research Paper* **35**, 1-16.

- Cohen, A. L. (1970). Critical point drying. In *Principles and Techniques of Electron Microscopy: Biological Applications* (ed. M. A. Mayat). Van Nostrand Reinhold: New York.
- Corda, A. K. J. (1837). *Icones fungorum* 1, 1–32. Prague.
- De Hoog, G. S. (1974). The genera *Blastobotrys*, *Sporothrix*, *Calcarisporium* and *Calcarisporiella* gen. nov. *Studies in Mycology* 7, 1–88.
- De Hoog, G. S. & Scheffer, R. J. (1984). *Ceratocystis* and *Ophiostoma*: a reappraisal. *Mycologia* 76, 292–299.
- Goheen, D. J. & Cobb, F. W. Jr (1978). Occurrence of *Verticicladiella wagnerii* and its perfect state *Ceratocystis wagnerii* sp. nov. in insect galleries. *Phytopathology* 68, 1192–1195.
- Harrington, T. C. (1981). Cycloheximide sensitivity as a taxonomic character in *Ceratocystis*. *Mycologia* 73, 1123–1129.
- Leach, J. G., Orr, L. W. & Christensen, C. (1934). The interrelationships of bark beetles and blue-staining fungi in felled Norway pine timber. *Journal of Agricultural Research* 49, 315–341.
- Minter, D. W., Kirk, P. M. & Sutton, B. C. (1982). Holoblastic phialides. *Transactions of the British Mycological Society* 79, 75–93.
- Minter, D. W., Kirk, P. M. & Sutton, B. C. (1983). Thallic phialides. *Transactions of the British Mycological Society* 80, 39–66.
- Mouton, M., Wingfield, M. J. & Van Wyk, P. S. (1993). Conidium development in the synnematosus anamorphs of *Ophiostoma*. *Mycotaxon* 46, 371–379.
- Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* 17, 208–212.
- Safranyik, L., Shrimpton, D. M. & Whitney, H. S. (1975). An interpretation of the interaction between lodgepole pine, the mountain pine beetle and its associated blue stain fungi in western Canada. In *Management of Lodgepole Pine Ecosystems. Symposium Proceedings* (ed. D. M. Baumgartner), pp. 406–428. Washington State University Cooperative Service: Pullman, Washington, U.S.A.
- Seifert, K. A. & Okada, G. (1993). *Graphium* anamorphs of *Ophiostoma* species and similar anamorphs of other Ascomycetes. In *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity* (ed. M. J. Wingfield, K. A. Seifert & J. F. Webber), pp. 27–41. APS Press: St. Paul, Minnesota, U.S.A.
- Spurr, A. R. (1969). A low viscosity embedding medium for electron microscopy. *Journal of Ultrastructure Research* 26, 31–43.
- Tribe, G. D. (1990). Phenology of *Pinus radiata* log colonization and reproduction by the European bark beetle *Orthotomicus erosus* (Wollaston) (Coleoptera: Scolytidae) in the south-western Cape Province. *Journal of the Entomological Society of South Africa* 53, 117–126.
- Upadhyay, H. P. (1981). *A Monograph of Ceratocystis and Ceratocystiopsis*. The University of Georgia Press: Athens, Georgia, U.S.A.
- Whitney, H. S. (1971). Association of *Dendroctonus ponderosae* (Coleoptera: Scolytidae) with blue stain fungi and yeasts during brood development in lodgepole pine. *The Canadian Entomologist* 103, 1495–1503.
- Wingfield, M. J., Kendrick, W. B. & Van Wyk, P. S. (1991). Analysis of conidium ontogeny in anamorphs of *Ophiostoma*: *Pesotium* and *Phialographium* are synonyms of *Graphium*. *Mycological Research* 95, 1328–1333.
- Wingfield, M. J., Van Wyk, P. S. & Marasas, W. F. O. (1988). A new *Graphium* species with unusual conidial development. *Phytophylactica* 20, 103.

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