A re-examination of the vascular wilt pathogen of takamaka (*Calophyllum inophyllum*)

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The vascular wilt pathogen currently causing severe die-back and mortality of takamaka in the Seychelles has morphological and physiological characters inconsistent with its placement in *Verticillium*. Features of the pathogen warrant its transfer to *Leptographium* and *Leptographium calophylli* comb. nov. is proposed. *L. calophylli* is described in detail based on new collections.

As early as 1939, a severe vascular wilt disease of takamaka trees (*Calophyllum inophyllum* L.) was observed in Mauritius and the causal agent was identified as a new species of *Haplographium* (Wiehe, 1941). External symptoms included rapid wilting of the leaves of affected trees while internally, dark brown streaking was visible in the vascular system of the normally light coloured wood (Wiehe, 1949). Sporadic reports of wilt disease of *Calophyllum* then followed from other countries including El Salvador and Cuba (Crandall, 1949; Kudela, Hochmut & Leontovyc, 1976), apparently all caused by the same fungus.

In 1994, die-back and death of takamaka was identified on Mahé, one of 115 islands that comprise the Seychelles (Ivory & Andre, 1995). It was confirmed that the pathogen was morphologically identical to cultures of *H. calophylli* Wiehe obtained from Mauritius in the 1940s, although the species had been renamed *Verticillium calophylli* (Wiehe) W. Gams (Gams, 1971). During work on the bark beetle, *Cryphalus trypanus* Sampson, to assess its role as a vector of the takamaka wilt pathogen (Wainhouse *et al*., 1998), it became clear that some of the morphological and physiological characteristics of the pathogen were inconsistent with its placement in *Verticillium*. This study describes some of the distinctive morphological features of the pathogen which have led us to transfer it to *Leptographium* and thus to propose a new combination for the species.

MATERIALS AND METHODS

Twigs were collected from trees of *C. inophyllum* with symptoms of a vascular wilt disease located on the island of Mahé. External symptoms included wilted and withered leaves and, when the bark was peeled away from the twigs, dark brown streaking of the vascular tissue was visible. Isolations were made from the discoloured vascular tissue. In addition, some individuals of the bark beetle *Cr. trypanus* were collected as they emerged after breeding in branches of dying and dead takamaka, macerated in sterile water and the macerate spread onto agar plates (Wainhouse *et al*., 1998). The first isolations were made on 2% Oxoid MEA (33 g Oxoid malt extract agar, 10 g technical agar, 1000 ml distilled water) containing 200 μg ml^{-1} of streptomycin sulphate solution. Subsequently, 100 μg ml^{-1} of cycloheximide was also added to the agar prior to isolation. All isolates in this study, and listed in Table 1, are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute, University, University of Pretoria (CMW 4256 – CMW 4263). For comparison, a culture of *Verticillium calophylli* was obtained (IMI 28925 ex Mauritius 1945). Additional isolates, W 1648 ex Seychelles 1994 and W 1600 ex Seychelles 1996, were supplied by H. Evans.

For measurement of fruiting structures and determination of growth rate, isolates were grown on 2% MEA (20 g Difco malt extract, 20 g Difco agar, 1 l distilled water). Anamorphs were mounted in lactophenol cotton blue prior to measurement of the relevant morphological structures. Isolates were also examined using SEM. Small blocks of agar cut from sporulating colonies were fixed in 3% glutaraldehyde and 0·5% osmium tetroxide in 0·1 M phosphate buffer, dehydrated in a graded acetone series and critical-point dried. The specimens were then mounted and coated with gold palladium alloy and examined using a JSM 6400 scanning electron microscope.

The growth rate of all isolates was determined in the dark at temperatures from 5 to 40 °C at 5° intervals. Petri dishes containing 20 ml agar medium were inoculated centrally with a plug taken from the edge of an actively growing colony, with five replicate plates prepared for each isolate at each temperature. For each colony, two diameters were measured

Table 1. Isolates of the vascular wilt pathogen of takamaka (*Calophyllum inophyllum*)

Culture collection[a]	Isolate code	Origin	Source	Collection details
IMI 28925	Holotype	Mauritius	*C. inophyllum* var. *tacamaha*	P. O. Wiehe, 1945
CMW 4256	W 1600	Mahé, Seychelles	*C. inophyllum*	M. Ivory, 1994
CMW 4257	W 1648	Mahé, Seychelles	*C. inophyllum*	H. Evans, 1996
CMW 4260	B5	Mahé, Seychelles	*C. inophyllum*	J. F. Webber, 1997
CMW 4262	B7	Mahé, Seychelles	*Cryphalus trypanus*	J. F. Webber, 1997
CMW 4263	B9	Mahé, Seychelles	*Cr. trypanus*	J. F. Webber, 1997

[a] CMW refers to the culture collection of the Tree Pathology Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria, SA.

at right angles to each other and growth was calculated for each isolate from a total of 10 readings.

Cycloheximide tolerance, a characteristic used to differentiate between *Ophiostoma* and *Ceratocystis* species and their anamorphs such as *Leptographium* and *Chalara* (Harrington, 1981), was assessed using 2% MEA containing a range of different concentrations of cycloheximide (0·1, 0·5, 1, 5, 10, 25 g l^{-1}). Cycloheximide tolerance was determined by comparing the difference in growth of each isolate on cycloheximide-containing agar with growth on unamended agar. Five replicates were prepared for each isolate and cycloheximide concentration. All the listed isolates of *V. calophylli* were assessed for cycloheximide tolerance at 30°, together with isolates of *Verticillium* spp., which were included for comparison, four of *V. dahliae* Kleb. and two of *V. albo-atrum* Reinke & Berthold, which were incubated at 20°, a temperature previously been found to be much closer to their optimum for growth when compared with 30°.

RESULTS

Samples of takamaka taken from trees with typical symptoms of vascular wilt disease yielded isolates of a fungus that readily sporulated on 2% MEA. The conidiophores were branched and the conidia accumulated in slimy droplets, which is consistent with species of *Leptographium*. The same fungus was isolated from individuals of *Cr. trypanus* which had emerged from brood galleries in the bark and twigs of diseased takamaka.

All isolates from the Seychelles were similar in colony appearance (Fig. 1) and morphology (Figs 2–12), and corresponded closely to Wiehe's type specimen from Mauritius (Table 2). Isolates grew optimally at 30° and were tolerant of up to 0·5 g l^{-1} of cycloheximide with only slight growth inhibition. In contrast, growth of isolates of *V. dahliae* and *V. albo-atrum*, which were included for comparison, were completely inhibited at 0·1 g l^{-1} cycloheximide. Based on these results we have concluded that the takamaka wilt pathogen should be placed in *Leptographium*. A detailed redescription is given below based on new collections of the pathogen that have resulted from this study.

Leptographium calophylli (Wiehe) J. F. Webber, K. Jacobs and M. J. Wingf., comb. nov.

≡ *Haplographium calophylli* Wiehe, *Mycol. Pap.* **29**: 5 (1949).

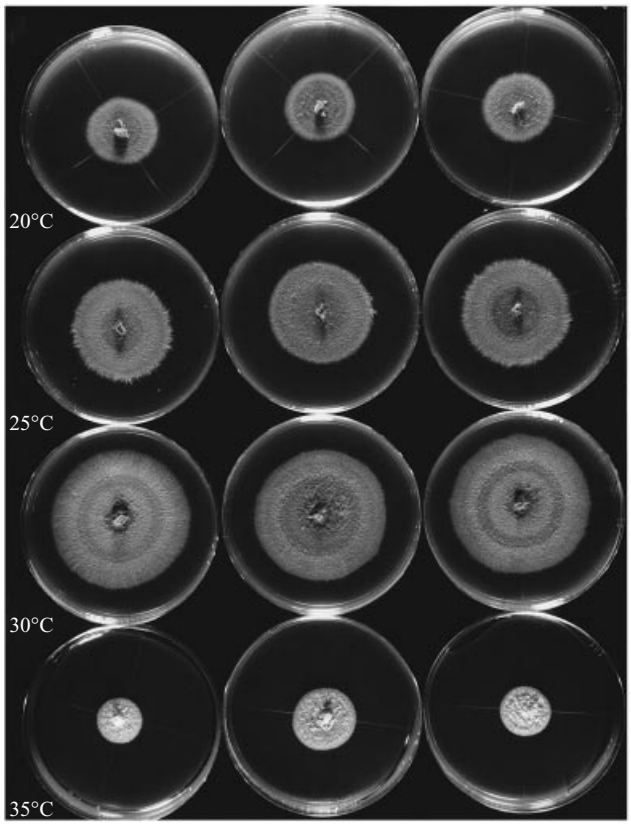


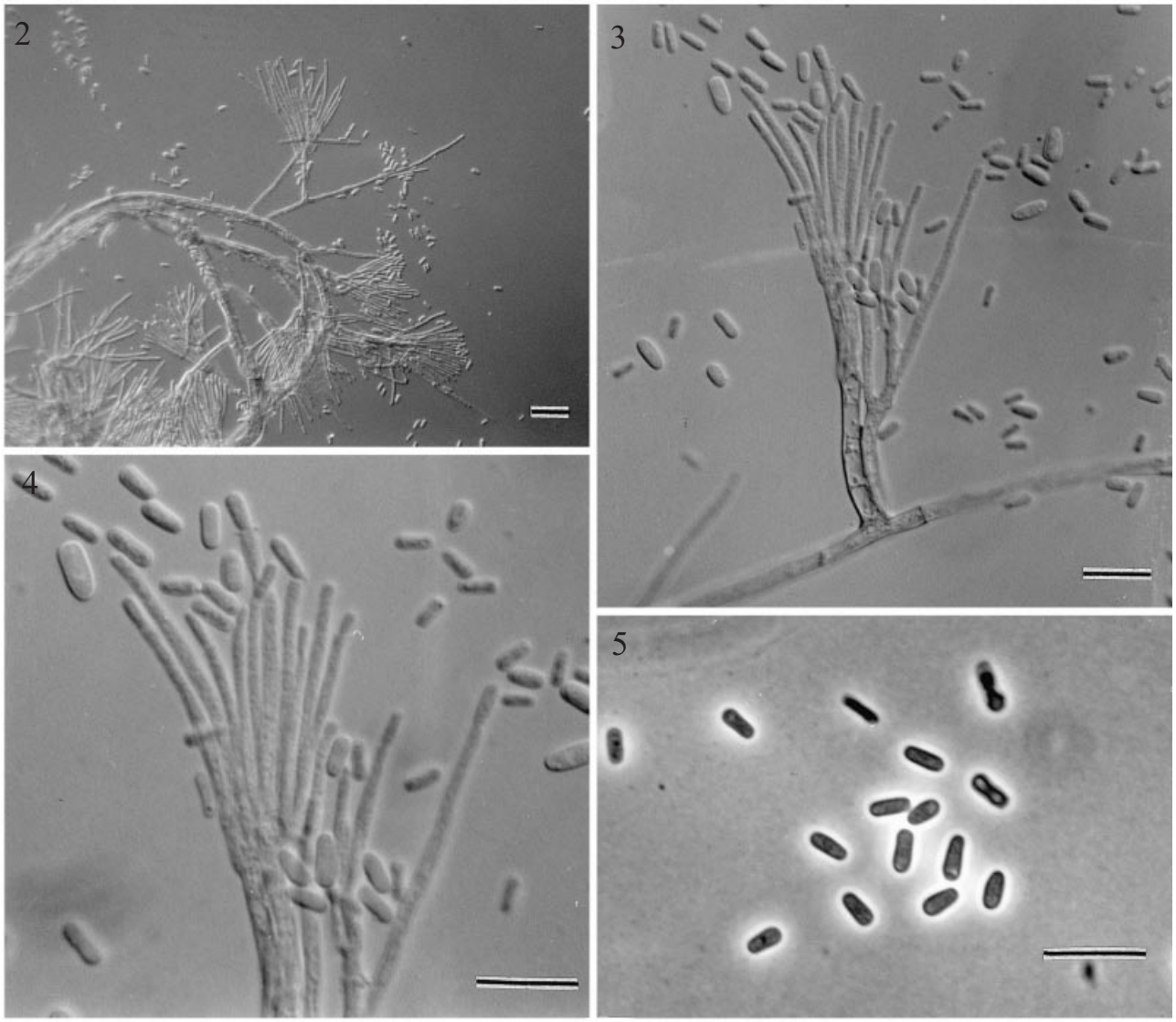
Fig. 1. Colony morphology of 7 d old cultures of *Leptographium calophylli* (left to right: isolates B5, B7 and B9) on MEA at 20, 25, 30 and 30°.

Table 2. Comparison of characters of *Haplographium calophylli* as described by Wiehe (1949) and those of more recently collected isolates

	Haplographium calophylli[1]	*Leptographium calophylli*[2]
Geographic distribution	Mauritius	Seychelles
Host	*Calophyllum inophyllum* var *tacamaha*	*Calophyllum inophyllum*
Conidiophore length	70–100 µm	41–100 mm
Stipe	Short (10 µm–?)	5–30 µm
Conidiogenous apparatus	60–80 µm	30–80 µm
Conidial shape	Oblong to cylindrical	Oblong to obovoid
Conidial size	3–5 × 1·5–5 µm	3–7 × 1–2·5 µm

[1] IMI 28925.

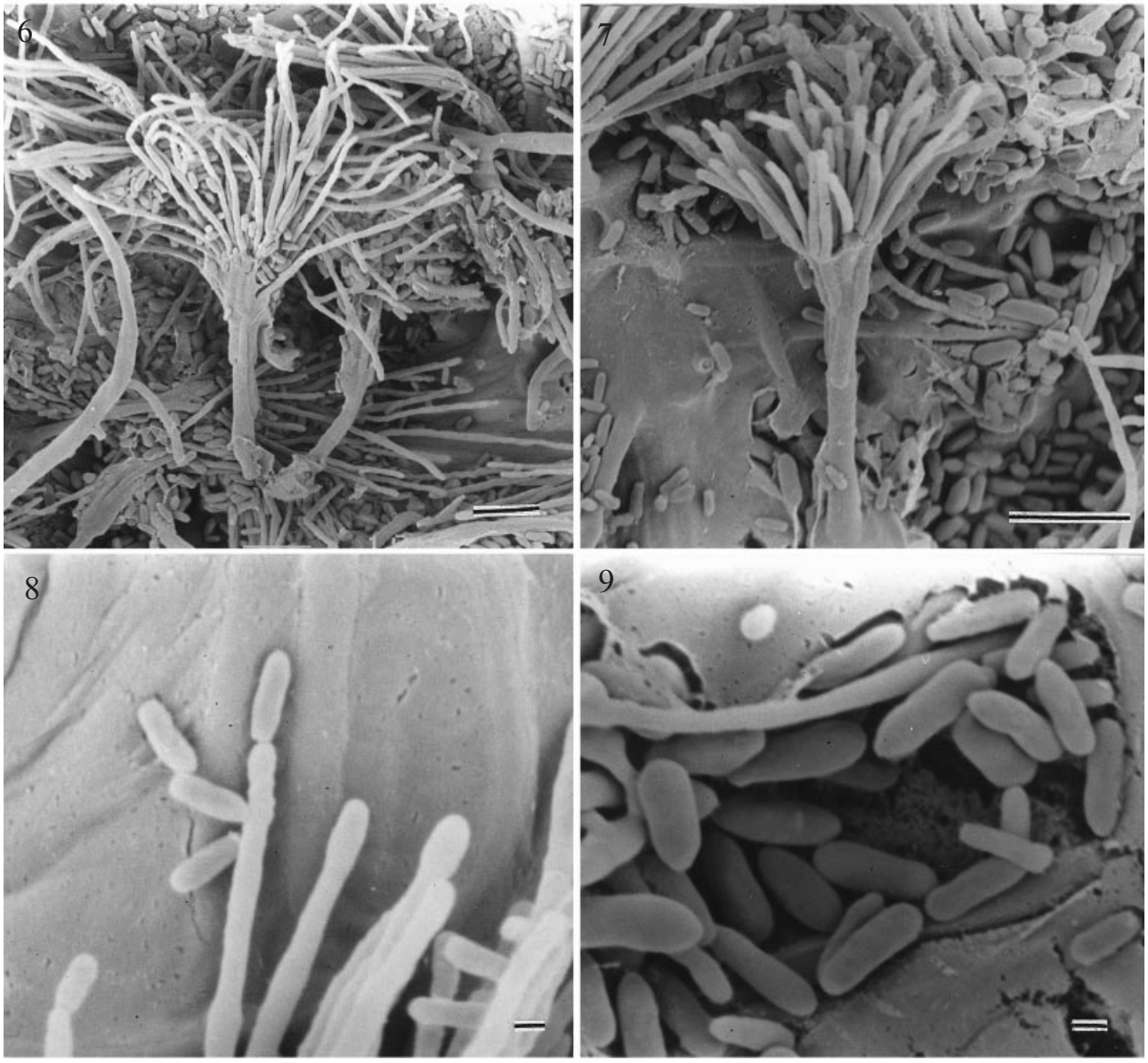
[2] Isolates CMW 4257, CMW 4256, CMW 4260, CMW 4262 and CMW 4263.



Figs 2–5. Light micrographs of *Leptographium calophylli*. Bars = 10 µm. **Fig. 2.** Conidiophores arranged on aerial mycelium. **Fig. 3.** Conidiophore; no rhizoids present. **Fig. 4.** Conidiogenous cells with annelidic conidium development. **Fig. 5.** Oblong to slightly obovoid conidia.

≡ *Verticillium calophylli* (Wiehe) W. Gams, Cephalosporium-artige Schimmilpilze, p. 206 (1971).

Colonies with optimal growth at 30° on 2% MEA, reaching 20 mm diam. in 8 d. Little growth at 15° and no growth above 40°. Able to withstand high concentrations of cycloheximide with an 80% reduction in growth on 5 g l^{-1} cycloheximide after 8 d at 30° in the dark. Colony olivaceous (21″ m) (Rayner, 1970). Colony margin smooth. Hyphae submerged on solid medium with little aerial mycelia, hyaline to light olivaceous (19″ k), smooth, straight, not constricted at the septa, 1·2–3 ($\bar{x}$ = 2) µm diam. Conidiophores occurring singly, arising directly from the mycelium, erect, macronematous, mononematous, 41–100 ($\bar{x}$ = 68) µm long, rhizoid-like structures absent (Figs 2, 10). Stipe hyaline, smooth, cylindrical, simple, 0–1 septate, 5–30 ($\bar{x}$ = 15) µm long (from first basal septum to below primary branches), 2–4 ($\bar{x}$ = 3) µm wide below primary branches, apical cell not swollen; 2–4 ($\bar{x}$ = 3) µm wide at base, basal cell swollen. Conidiogenous apparatus 30–80 ($\bar{x}$ = 50) long, excluding the conidial mass, with 2–3 series of cylindrical branches; 2–3 primary branches, hyaline, smooth, cylindrical, 0–1 septate, 7–18 ($\bar{x}$ = 12) µm long and 1·5–4 ($\bar{x}$ = 2·5) µm wide, secondary branches hyaline, aseptate, 7–15 ($\bar{x}$ = 10) µm long, 1·5–5 ($\bar{x}$ = 2) µm wide; tertiary branches hyaline, aseptate, 6–16 ($\bar{x}$ = 9) µm long, 1–2 ($\bar{x}$ = 2) µm wide (Figs 2, 6, 7, 11). Conidiogenous cells discrete, 2–4 per branch, tapering slightly from the base to the apex, 8–25 ($\bar{x}$ = 17) µm long and 1–2 ($\bar{x}$ = 1·5) µm wide (Fig. 4). Conidium development occurring through replacement wall building with holoblastic ontogeny and percurrent proliferation and delayed secession giving the false impression of sympodial proliferation (Minter, Kirk & Sutton, 1982, 1983; Van Wyk, Wingfield & Marasas, 1988) (Fig. 8). Conidia hyaline, aseptate, oblong to obovoid with truncated ends, 3–7 ($\bar{x}$ = 4·5) × 1·2–2·5 ($\bar{x}$ = 1·5) µm. Conidium frill absent (Figs 5, 9, 12). Conidia accumulating in slimy droplets at the apex of conidiogenous apparatus.



Figs 6–9. *Leptographium calophylli* (SEM). **Figs 6–7.** Conidiophores; no rhizoids present. Bars = 10 μm. **Fig. 8.** Conidiogenous cells with annelidic conidium development. Bar = 1 μm. **Fig. 9.** Oblong to slightly obovoid conidia. Bar = 1 μm.

Specimens examined: **Herbarium type** IMI 28925, *Calophyllum inophyllum* var. *tacamaha*, isolated from tracheids and medullary rays of wood, March 1945, Mauritius: Plaine Sophie, collected: P. O. Wiehe; *C. inophyllum*: **Additional Specimens** DAOM 225543 (CMW 4257), H. Evans, 1996, Mahé, Seychelles; DAOM 225547 (CMW 4256), M. Ivory, 1994, Mahé, Seychelles; CMW 4260, CMW 4262, CMW 4263, J. Webber, April 1997, Mahé, Seychelles.

DISCUSSION

Leptographium calophylli can be readily distinguished from other species in the genus. Although the sizes of the conidiogenous heads are similar to most other *Leptographium* species, the stipes are much shorter than those of any other species. A further distinguishing feature is the way the conidiophores occur mainly on aerial mycelia. This is in contrast to most of the species in the genus, which have conidiophores arising directly from the agar medium. In addition, the optimum growth temperature of 30°, is higher than is usually found in species of *Leptographium*.

The host and geographical distribution of this fungus is also unusual and must form part of the criteria used to recognize *L. calophylli*. Most species of *Leptographium* occur on coniferous hosts and only a small group occur on non-coniferous hosts (Davidson, 1976; Jooste, 1978; Harrington, 1988); *L. calophylli* now forms part of this group. Moreover, none of the species of *Leptographium* currently found on hardwoods resemble *L. calophylli*. The association of *L. calophylli* with the bark beetle *Cr. trypanus* (Wainhouse *et al.*, 1998), however, is consistent with the biology of *Leptographium*. Many species within the genus are recognized both as associates of bark beetles and as the cause of bluestain, mainly in conifer timber.

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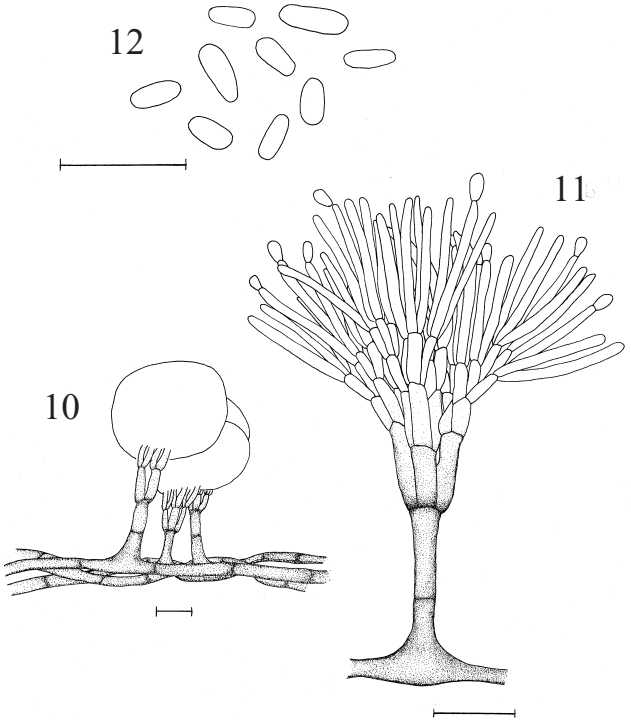

Figs 10–12. *Leptographium calophylli*. Bars = 10 μm. Conidiophores arranged on mycelium. **Fig. 11.** Conidiophore. **Fig. 12.** Oblong to slightly obovoid conidia.

supplying cultures and infected plant material. We also thank the South African Foundation for Research and Development and the Tree Pathology Co-operative Programme for financial assistance.

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