Mycoscience 42: 265–271, 2001

Morphological variation and cultural characteristics of *Coniothyrium leucospermi* associated with leaf spots of Proteaceae

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Received 9 August 2000
Accepted for publication 28 March 2001

During an examination of *Coniothyrium* collections occurring on Proteaceae one species, *C. leucospermi*, was repeatedly encountered. However, it was not always possible to identify this species from host material alone, whereas cultural characteristics were found to be instrumental in its identification. Conidium wall ornamentation, which has earlier been accepted as crucial in species delimitation is shown to be variable on host material, making cultural comparisons essential. Using standard culture and incubation conditions, *C. leucospermi* is demonstrated to have a wide host range in the Proteaceae. In addition, microcyclic conidiation involving yeast-like budding from germinating conidia and hyphae in culture is newly reported for this species.

Key Words——*Coniothyrium leucospermi*; cultural data; plant pathogen; *Protea*.

Coniothyrium Corda represents one of the anamorph genera associated with *Leptosphaeria* Ces. & De Not. (Leptosphaeriaceae) and *Paraphaeospheria* O. E. Erikss. (Phaeosphaeriaceae) in the Dothideales *sensu* Hawksworth et al. (1995) or the Pleosporales *sensu* Barr (1987). This genus has a widespread distribution and approximately 28 species are acknowledged (Hawksworth et al., 1995; Swart et al., 1998), although the Index of Fungi and the Index of Saccardo (Reed and Farr, 1993) list nearly 1100 described species. *Coniothyrium* is characterised by having unilocular, immersed, ostiolate, thin-walled and brown pycnidia. Conidia are brown, thick-walled, 0–1 euseptate and variously shaped from spheroid to ellipsoidal or cylindrical, with obtuse apices and truncate bases, and formed on annellidic conidiogenous cells (Sutton, 1971). *Coniothyrium* most closely resembles *Microsphaeropsis* Höhn. in morphology, but differs in that conidia of the latter genus are relatively thin-walled and develop from determinate conidiogenous cells with periclinal thickening (Sutton, 1980). Species of *Coniothyrium* are differentiated depending on the host, and conidium morphology (e.g. wall ornamentation, pigmentation and size) and morphology of their conidiophores, which in most cases are reduced to conidiogenous cells.

Studies of the fungi associated with necrotic leaf spots of Proteaceae (commonly known as proteas), often recover species of *Coniothyrium* (Van Wyk, 1973; Swart et al., 1998). These fungi are not considered to be serious pathogens, but may be the causal organisms of leaf spots on stressed plants. In the main, they are probably weak pathogens or opportunistic saprophytes on necrotic leaf spots.

During the course of a study investigating pathogens associated with leaf spots of Proteaceae, many collections of *Coniothyrium* were made. These collections broadly corresponded to *C. leucospermi*, but conidial morphology varied ranging from being almost hyaline with faintly verruculose walls, through to dark-brown with spinulose walls. However, when cultures made from single spores were compared they were found to be similar. Subsequent studies comparing cultural characteristics and conidium morphology *in vitro* confirmed that these collections were conspecific.

The present paper reports on the morphological variation occurring in *C. leucospermi*, and proposes that *in vitro* growth under standardised conditions is essential for the comparison of isolates.

Materials and Methods

Symptomatic leaf and stem samples of Proteaceae hosts (*Protea* (L.) L; *Leucospermum* R. Br. and *Leucadendron* R. Br.) were collected from sites throughout the Western Cape Province in South Africa, and surveyed for the presence of sporulation. This could be enhanced by incubating the specimens in damp chambers for several days. Any fertile samples were processed immediately, and the remainders were stored dry, in plastic bags, in a cold room at 4°C, for approximately 1 mo to encourage maturation of fruiting structures (Taylor and Crous, 2000). Single conidium colonies were established on 2% malt extract agar (MEA; Biolab, Midrand, South Africa), supplemented with 0.1 g/L Streptomycin sulphate. These were then transferred to divided plates containing MEA and water agar with small pieces of *Pro-*

tea repens (L.) L. leaf added to encourage sporulation. Plates were incubated at 25°C on the laboratory bench. Growth studies were then undertaken on MEA plates using 5 mm diam cores taken from the perimeter of actively growing colonies. Three replicates of five strains of each collection were grown at 5°C intervals from 5 to 40°C, and the average growth rate for each strain at each temperature interval calculated. Colony growth was measured, characteristics noted, and the colour rated (Rayner, 1970). The affect of differing light regimes on the morphology of the conidia was tested by comparing colonies grown under conditions of direct natural light (on the laboratory bench), in darkness and under near-ultraviolet light. This was carried out for each strain and conidia were checked twice over a period of a month. For microscopic examination the fungi were mounted in water as well as in lactophenol and measurements made at 1000× magnification. The 95% confidence intervals were determined from 25–50 observations and the minimum and maximum ranges given in parentheses. Herbarium specimens are lodged at PREM and reference cultures are maintained in the culture collection of the Department of Plant Pathology, University of Stellenbosch (STE-U).

Results and Discussion

Due to the variability of *C. leucospermi* on host material, it is evident that identification must be supported with cultural data. Cultural studies were therefore conducted at differing light regimes to investigate the influence that environmental factors may have on the varying conidium morphologies. The affects of the differing regimes on conidium morphology as found in this study are as follows:

In vivo: Conidia vary from hyaline and verruculose to dark red-brown and verrucose, but are most commonly medium brown and verruculose. The dimensions of the conidia are variable.

In vitro (near-ultraviolet): Either no spores were produced, or those produced were malformed (often bone-shaped), inflated and thin-walled. Colonies grew very slowly, and there was no production of conidiomata on the host material.

In vitro (dark): Shape tends to be variable, but the size, colour and the ornamentation of the conidia were consistent over the period studied (pale to medium-brown, thick-walled, verruculose). Mycelium appears 'stringy' in growth. There is some production of conidiomata on the host material at 4 wk.

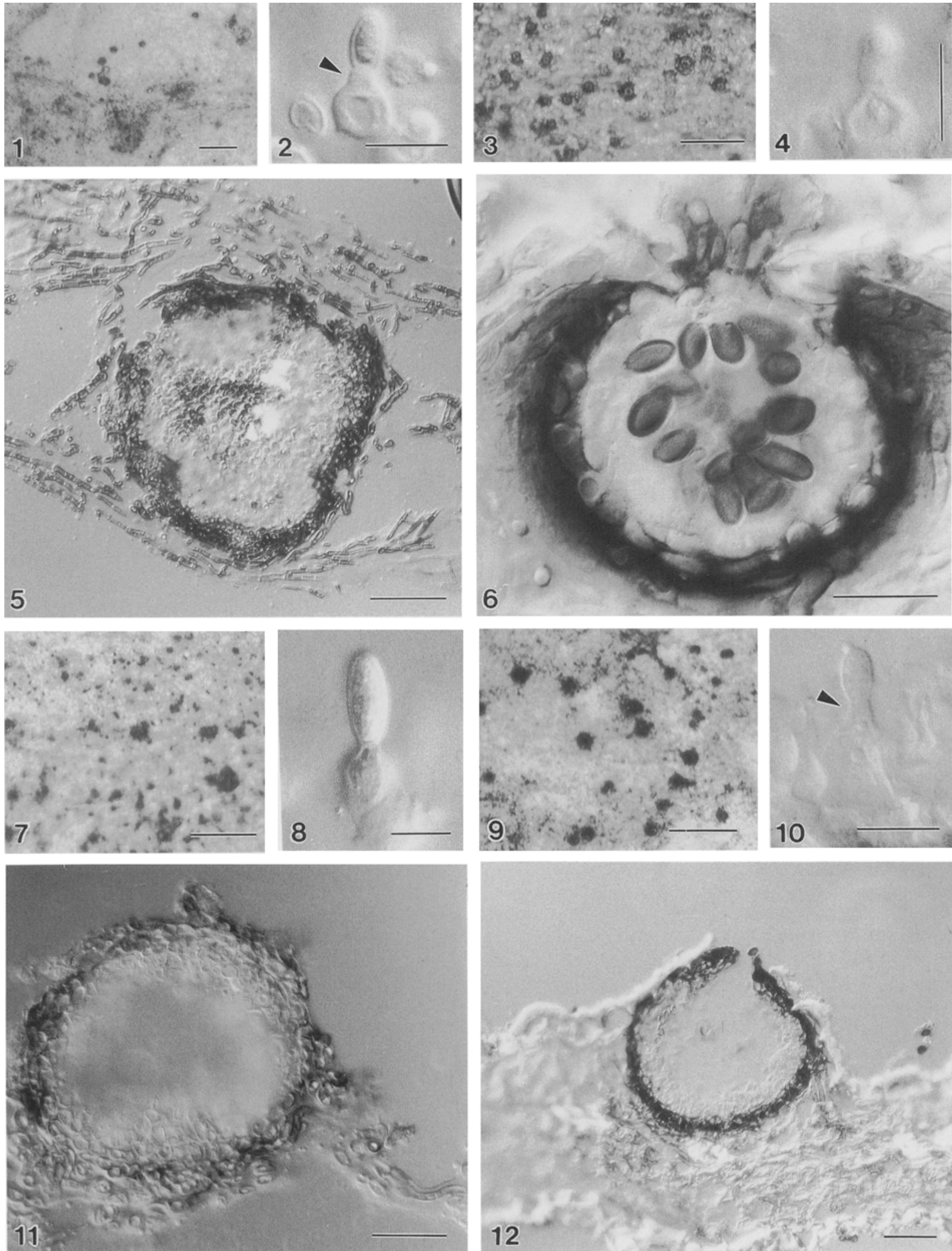
In vitro (on laboratory bench): Shape also somewhat variable. Size, colour and the ornamentation of the conidia were consistent after 2 wk of incubation (hyaline to greenish-brown, thick-walled, verruculose), but colour and ornamentation tended to vary after 4 wk. Isolates grew fastest under these conditions and conidiomata developed after 2 wk on the host material. Based to the findings of this study, the description of *Coniothyrium leucospermi* is amended and its culture characteristics are also revised.

Coniothyrium leucospermi Crous & S. Denman, S. Afr. J. Bot. **64**: 139. 1998. amend J. E. Taylor & Crous Figs. 1–32

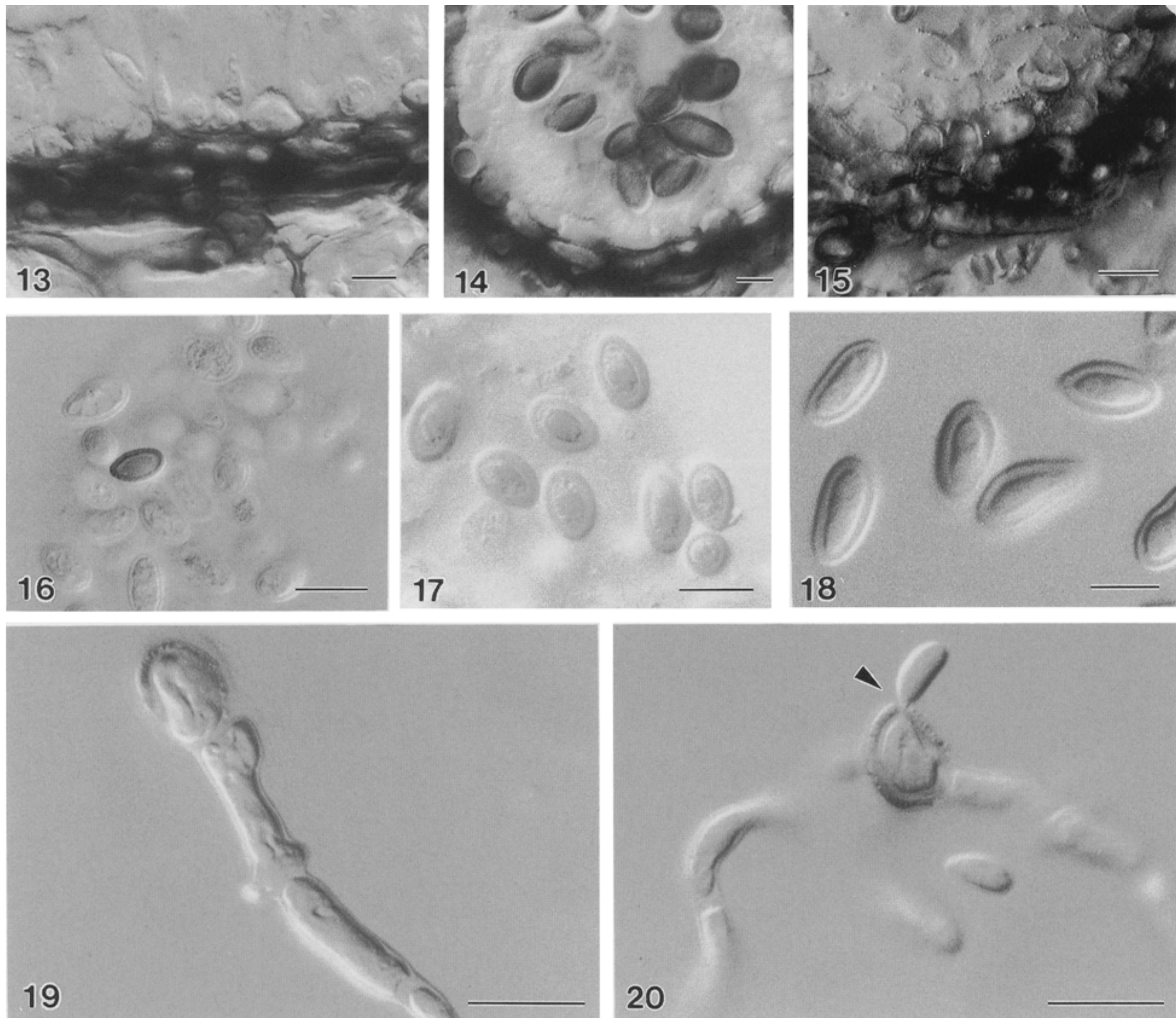
Leaf spots amphigenous, irregular, grey to light brown with a raised, dark brown border, frequently associated with tip blight or leaf margins. Conidiomata pycnidial, subepidermal, substomatal, amphigenous, separate, visible as dark brown spots (Figs. 1, 3, 7, 9); in section, globose, apapillate, sometimes surrounded by a stroma comprising host cells and dark brown fungal hyphae, 55–185 μm high, 55–205 μm wide (Figs. 6, 12); in section *in vitro*, globose sometimes becoming cupulate at maturity, 125–295 μm high, 100–300 μm wide (Figs. 5, 11). Peridium consisting of two strata of cells in a slightly compressed *textura angularis* to *globulosa*, outer stratum of thick-walled brown cells sometimes having almost occluded lumens, becoming thinner walled and hyaline inwardly, 10–42 μm (Figs. 13, 14); *in vitro* consisting of two strata of cells of *textura globulosa*, outer stratum of thick-walled brown cells, becoming thinner walled and hyaline inwardly, 8–50 μm (Fig. 15). Conidiophores reduced to conidiogenous cells, often invested in mucus. Conidiogenous cells discrete, smooth or slightly verruculose, hyaline to light brown, doliiform to ampulliform, proliferating 1–3 times enteroblastically and percurrently, 5–12 μm high, 4–9 μm wide at the base and 1.5–3 μm wide at the apex (Figs. 2, 4, 8, 10). Conidia vary in size, colouration and walled ornamentaion, but broadly similar in shape, varying from hyaline to dark red-brown, thick-walled, faintly verruculose to echinulate, aseptate, ellipsoidal to globose, apex obtuse, base bluntly rounded to truncate, 6–12 × 3–8 μm *in vivo* (Figs. 29–32); 6.5–15 × 3.5–8 μm *in vitro*, initially hyaline and thick-walled, becoming medium-brown, smooth to verruculose, ellipsoidal to globose and often irregularly shaped, apex obtuse, base bluntly rounded to truncate. Conidia show more variation in shape *in vitro*, but remain more consistent in size (Figs. 16–18, 21–28). Germinating conidia produce 1 or 2 germ tubes. Pale brown conidia with thin walls can often be seen budding from the germinating conidia, or directly from the hyphae (Figs. 19, 20).

Cultural characteristics: Colonies circular, smooth to slimy, finely radiating with regular margins, fucous black 7''''k (surface), olivaceous black 27''''m (reverse); aerial mycelium sparse, forming fine concentric zonations. Cardinal growth requirements are min 5°C, opt 20–25°C, max below 35°C. Colonies reaching 14–19(–31) mm diam on MEA after 2 wk in the dark at 25°C.

Specimens examined: South Africa, Western Cape Province. Somerset West, leaves of *Leucadendron sessile* R. Br., S. Denman & J. E. Taylor, 20 Jul. 1998, JT358, PREM 56611, STE-U 1878–1879; *ibid.*, Somerset West, leaves of *Leucadendron sessile*, S. Denman & J. E. Taylor, 20 Jul. 1998, JT337, PREM 56615, STE-U 1846–1847; *ibid.*, Stellenbosch, Simonsberg, leaves of *Protea nitida* Mill., S. Denman, 14 Feb. 1999, JT799, PREM 56612, STE-U 2340–2343; *ibid.*, Stellenbosch, Elsenberg Farm, leaves of *Protea repens*, S. Denman, 18 Jul. 1999, JT846, PREM 56614, STE-U 2828–2831;



Figs. 1–12. *Coniothyrium leucospermi.* (1, 2, 5. On *Leucadendron* sp., PREM 56613, STE-U 2566; 3, 4, 6. On *Protea neriifolia*, PREM 56610, STE-U 1824; 7, 8, 11. On *Leucadendron sessile*, PREM 56611, STE-U 1878; 9, 10, 12. On *Leucadendron sessile*, PREM 56615, STE-U 1846). 1, 3, 7, 9. Conidiomata on host substrate. 2, 4, 8, 10. Conidiogenous cells showing annellations (arrowed). 5, 11. Cross section of conidiomata (*in vitro*). 6, 12. Cross section of conidiomata (*in vivo*). Scale bars: 1, 3, 7, 9=500 μm, 5, 11=50 μm, 6, 12=30 μm, 2, 4, 8, 10=10 μm.

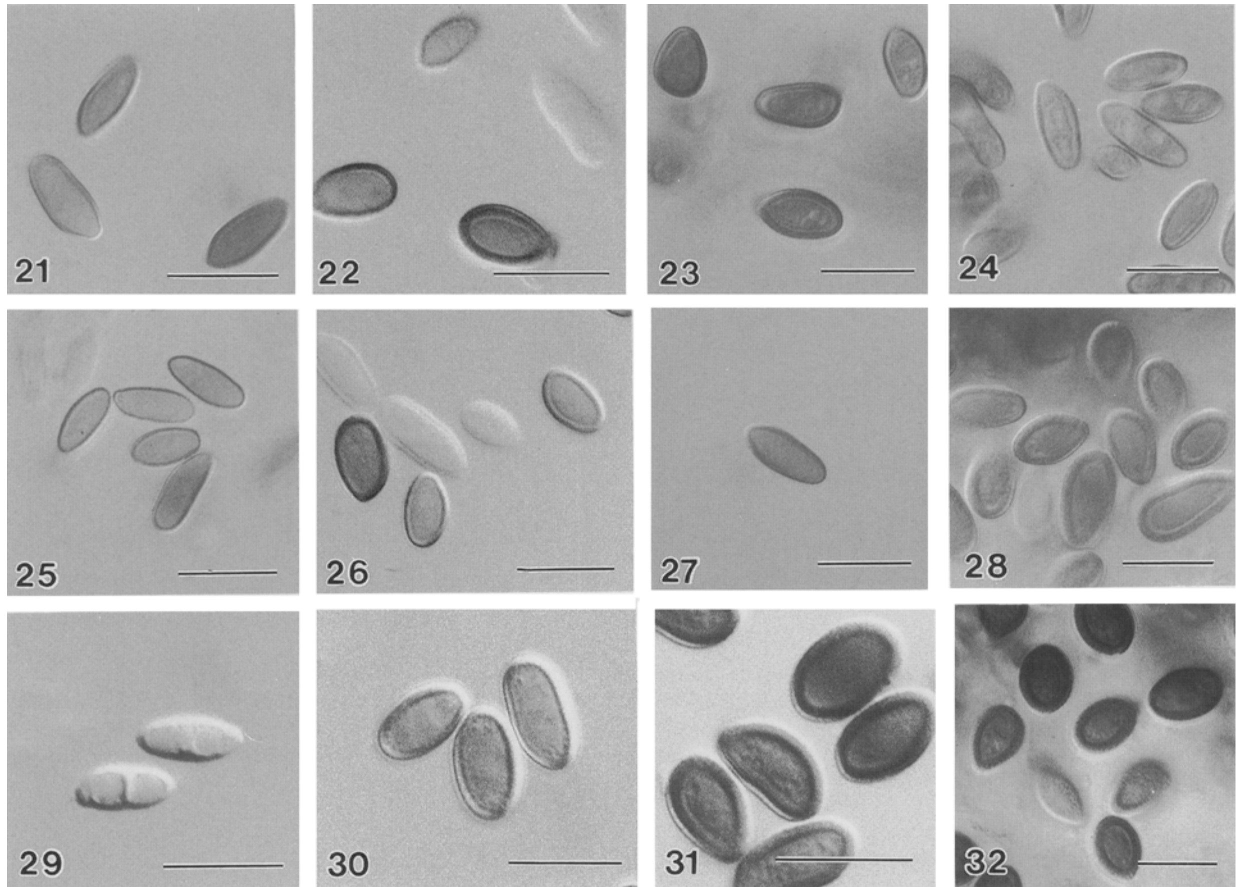


Figs. 13–20. *Coniothyrium leucospermi*. 13–15. Peridia. (13. PREM 56615 (*in vivo*); 14. PREM 56610 (*in vivo*); 15. PREM 56613, STE-U 2566 (*in vitro*)). 16–18. Immature conidia (*in vitro*). (16. PREM 56611, STE-U 1878; 17. PREM 56610, STE-U 1824; 18. PREM 56613, STE-U 2566). 19, 20. Germinating conidia (PREM 56613, STE-U 2566), showing germ tube (Fig. 19) and budding conidium (Fig. 20; arrowed). Scale bars: 13–20 = 10 μm.

Table 1. Comparison of dimensions of various characters of *Coniothyrium leucospermi* (all measurements in μm).

Collection	Conidia (*in vivo*)	Conidia (*in vitro*)	Conidiogenous cells	Conidiomata	Peridium
PREM 56611 (STE-U 1878)	(8–)8.5–9(–10.5) × (3–)3.5(–4)	(8–)9.5–11(–11.5) × (3.5–)5–6(–7.5)	(5–)6.5–8(–9) × (4.5–) 5–7(–8)	(125–)134.5–169(–200) × (100–)118–163(–200)[a]	(8–)9.5–11.5(–12)[a]
PREM 56612 (STE-U 2340)	(6–)7–8(–9) × (3–)3.5–4(–5)	(7–)–9–11(–13) × (3.5–)4.5–5(–6)	(5–)6.5–7(–8) × (4–)5–6 (–8) × (1.5–)2–2.5(–3)	(65–)73–83(–90) × (65–)68–77(–85)[b]	(12–)13–18(–20)[b]
PREM 56615 (STE-U 1846)	(7–)9–10(12) × (5–)6.5–7(–8)	(7–)9–11(–14) × (3.5–)4.5–5.5(–7)	(6–)7.5–(–11) × (4–)5.5–6.5(–9) × (1.5–)2(–3)	(110–)130–163(–185) × (130–)138–173(–205)[b]	(25–)27–35(–42)[b]
PREM 56610 (STE-U 1824)	(9–)10–11(–12) × (4–)6–6.5(–7)	(7–)9–11.5(–15) × (4–)5–6	8–9(–12) × 8–9	(55–)68–82(–85) × (55–)60–79(–90)[b]	(10–)12–16(–17)[b]
PREM 56613 (STE-U 2563)	(7–)9–10(–11.5) × (4–)5–5.5(–7)	(6.5–)8–9(–11.5) × (3.5–)5–5.5(–8)	(6–)7–8 × 6–7	(150–)198–253(–295) × (175–)204–262(–300)[a]	(22–)29–40(–50)[a]
PREM 55348 (STE-U 1426) holotype	11–13 × 5–6	(9–)10–13(–15) × 6–7	9–11 × 5–7	up to 200 μm diam.[c]	not given[c]

[a] Measurements taken *in vitro*.
[b] Measurements taken *in vivo*.
[c] Measurements from Swart et al. (1998).



Figs. 21–32. *Coniothyrium leucospermi*. Conidia *in vitro* after 28 d in the dark (Figs. 21–24), on the laboratory bench (Figs. 25–28), and *in vivo* (Figs. 29–32). (21, 25, 29. PREM 56611, STE-U 1878; 22, 26, 30. PREM 56613, STE-U 2566; 23, 27, 31. PREM 56610, STE-U 1824; 24, 28, 32. PREM 56615, STE-U 1846). Scale bars: 21–32 = 10 μm.

ibid., Cape Town, Table Mountain, leaves of *Protea neriifolia* R. Br., J. E. Taylor, 23 Jun. 1998, JT259, PREM 56610, STE-U 1824–1825; *ibid.*, Stellenbosch, leaves of *Leucadendron* sp., L. Swart, Jun. 1999, JT831, PREM 56613, STE-U 2563–2568.

Host Range: *Leucadendron* sp., *Leucadendron sessile, Leucospermum conocarpodendron* (L.) H. Beuk., *Leucospermum* sp. (Swart et al., 1998), *Protea neriifolia, P. magnifica* Link, *P. nitida*, and *P. repens*.

Geographic Distribution: Dominican Republic, South Africa (Swart et al., 1998).

Coniothyrium leucospermi was found to be very problematic to identify based on its morphology on host material. The overall morphology of the conidia was inconsistent in the coloration and ornamentation of the spore wall, and the size of the conidia also varied. It is possible that the degree of maturity at which the specimens were collected was an influential factor. The effect of conidial maturity has been shown to play a role in studies of other coelomycetous anamorphs of Pleosporales (Laundon, 1973), and also has to be taken into account when identifying anamorphs of *Botryosphaeria* spp. (Denman et al., 2000). For instance, species in certain genera, such as *Diplodia* Fr., often become pigmented only after discharge (Laundon, 1973). In contrast, conidia of *Lasiodiplodia theobromae* become pigmented and ornamented at maturity, and often mature pigmented, and immature hyaline conidia can be found in the same conidioma (Sutton, 1980).

Identification of these collections as *C. leucospermi* would have been unlikely were it not for the single conidial cultures. The culture characteristics of this species are distinctive with olivaceous-black, slimy growth, which actually resulted in many isolations of early specimens being discarded as they were assumed to be yeast contaminants. In addition, budding of cells from germ tubes and germinating conidia was noted (Figs. 19, 20) and evidence of direct conidiogenesis from hyphae was noted in culture. However, pycnidia were also formed in culture, and conidiogenous cells observed. The conidia in culture differed in dimensions (Table 1) and shape, which was more variable, than the conidia on the host material (as noted in the original description) (Swart et al., 1998). They were, however, more consistent in size and wall ornamentation (Fig. 33). Therefore, it was concluded that in order to standardise conditions for investigation of this fungus, incubation of isolates should be carried out in the dark over a period of 2 to 4 wk.

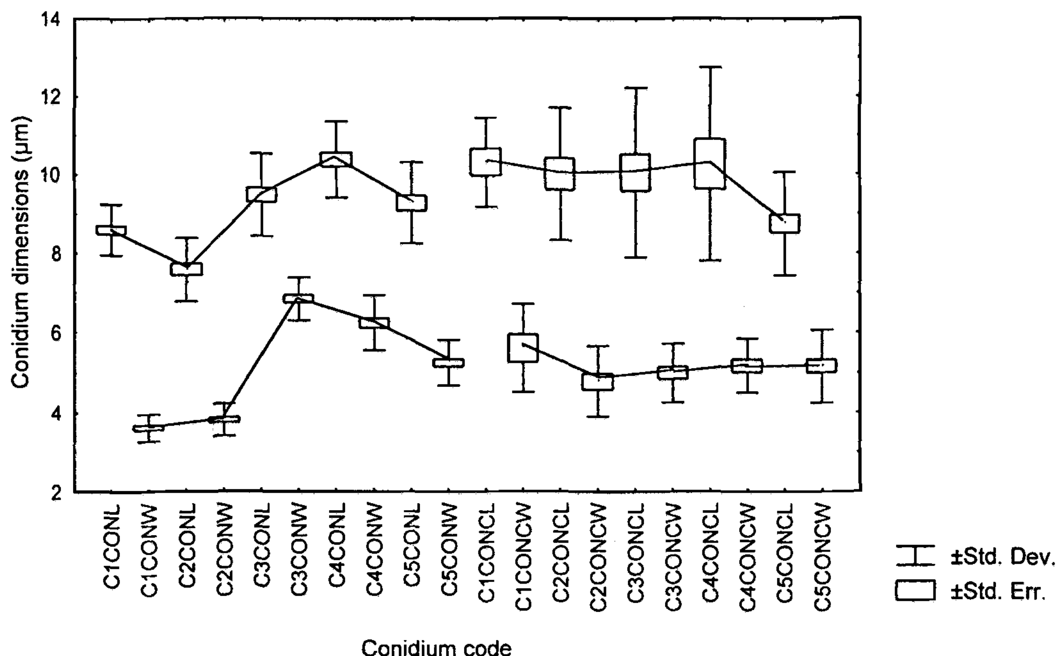


Fig. 33. Plots of conidial length and width (*in vivo* and *in vitro*). A combination of the following encode the provenance of the conidia. C1: PREM 56611, STE-U 1878; C2: PREM 56612, STE-U 2340; C3: PREM 56615, STE-U 1846; C4: PREM 56610, STE-U 1824; C5: PREM 56613, STE-U 2563.
CONL: conidial length *in vivo*; CONW: conidial width *in vivo*; CONCL: conidial length *in vitro* (4 wk at 25°C in dark); CONCW: conidial width *in vitro* (4 wk at 25°C in dark).

As mentioned, the yeast-like appearance of this species in culture did cause some confusion. Furthermore, the fungus produced other yeast-like characteristics. Conidia were produced by budding from conidia and directly from hyphae. The former can be considered as microcyclic conidiation *sensu* Hanlin (1994), whereas the latter is considered a direct from of conidiogenesis. Mangenot and Reisinger (1976) noted that in *Aureobasidium pullulans* (de Bary) G. Arnaud what they referred to as *secondary conidia* appeared from hyphal cells, and also from propagules which functioned as annellides. *Septoria* spp. have also been reported as producing conidia directly from the hyphae of germinating conidia (Jones and Lee, 1974). There have been some reports of yeast-like synanamorphs of coelomycetes in the literature (Sutton, 1980; Ramaley, 1992; Crous et al. 1995). In addition, the affinity of yeast-like states of fungi, such as the black yeasts, with members of the Dothideales *sensu* Hawksworth et al. (1995) is well documented (De Hoog, 1999). However, to our knowledge it is the first time this feature has ever been noted for a species of *Coniothyrium*.

During this study, cultural comparisons of *C. leucospermi* have been shown to be instrumental in the identification of this species. This finding may hold further repercussions for the identification and publication of other *Coniothyrium* spp. Without cultures, it appears difficult to accurately identify a *Coniothyrium* species (Taylor and Crous, unpubl. data), or distinguish whether variation is caused by environmental factors, or is due to collections representing different taxa. It is interesting to note that this species of *Coniothyrium* has been collected many times in the past, but few cultures were available (as they were probably discarded, believed to be contaminants), and variability on the host material meant that collections were often just named *Coniothyrium* sp. The regular association of *Coniothyrium* spp. with leaf spots of Proteaceae hosts can now be accounted for in many cases, and *C. leucospermi* is now considered to be a common, if not weak and opportunistic pathogen. As the culture characteristics of these fungi have been instrumental in their identification, attempts to identify *Coniothyrium* spp. that are not in culture may be erroneous. *Coniothyrium leucospermi* has been isolated as an endophyte from leaves of *Protea magnifica* which confirms its intimate relationship with Proteaceae.

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