Fungi associated with infructescences of *Protea* species in South Africa, including a new species of *Ophiostoma*

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Infructescences of different *Protea* species were collected in various parts of the western Cape Province of South Africa. Isolations were made and fungi were identified. A wide variety of Hyphomycetes and Ascomycetes including ophiostomatoid fungi were found. These included a previously undescribed *Ophiostoma* species which is described here as *O. splendens*. This fungus is remarkable in that it appears to be unrelated to previously isolated ophiostomatoid fungi from *Protea* infructescences. It will, therefore, form an important component in phylogenetic studies on this group of fungi.

The Proteaceae is one of the oldest families of flowering plants and is indigenous to South America, New Zealand, and central and southern Africa (Rousseau, 1970, 1973). Approximately 82 species of *Protea* L. occur in South Africa, with 69 species found in the western Cape Province (Rourke, 1980). There have been many studies of fungal diseases of the leaves (Marasas, Van Wyk & Knox-Davies, 1975; Van Wyk, Marasas & Knox-Davies, 1975*a*, *b*), stems (Gorter, 1976; Benic & Knox-Davies, 1983; Knox-Davies, Van Wyk & Marasas, 1987), roots (Von Broemsen & Brits, 1986) and seedlings (Knox-Davies *et al.*, 1987) of various *Protea* species. Very little attention has, however, been paid to the apparently non-pathogenic fungi associated with these plants.

Ceratocystis Ellis & Halst. *sensu lato*, which includes *Ceratocystis sensu stricto*, *Ophiostoma* H. & P. Sydow and *Ceratocystiopsis* Upadhyay & W. B. Kendr. (De Hoog & Scheffer, 1984), is a diverse aggregate that includes approximately 113 species (Wolfaardt, Wingfield & Kendrick, 1992). Anamorphs of *Ceratocystis s.s.* are accommodated in *Chalara* (Corda) Rabenh., in which conidia develop through ring-wall building (Minter, Kirk & Sutton, 1983). In contrast, *Ophiostoma* has anamorphs primarily in the genera *Sporothrix* Hektoen & C. F. Perkins, *Graphium* Corda, *Leptographium* Lagerb. & Melin and *Hyalorhinocladiella*, where conidia are produced by apical wall building (Minter, Kirk & Sutton, 1982). Species in *Ceratocystis s.s.* lack cellulose and rhamnose in their cell walls and are sensitive to cycloheximide. This is in contrast to species in *Ophiostoma* that possess rhamnose and cellulose and are tolerant to cycloheximide (Smith, Patik & Rosinski, 1967; Harrington, 1981).

Recently, two new ascomycetous species, *Ceratocystiopsis proteae* M. J. Wingf., P. S. van Wyk & Marasas (Wingfield, Van Wyk, & Marasas, 1988) from *Protea repens* (L.) L. and *Ophiostoma capense* M. J. Wingf. & P. S. van Wyk (Wingfield & Van Wyk, 1993) from various *Protea* species, have been described. The description of *C. proteae* was the first report of an ophiostomatoid fungus occurring on indigenous plants in Africa. *Knoxdaviesia* M. J. Wingf., P. S. van Wyk & Marasas was established for the anamorphs of *C. proteae* and *O. capense*. Wingfield *et al.* (1988) noted that *C. proteae* shares characteristics with *Ceratocystis s.s.*, *Ophiostoma* and *Ceratocystiopsis*, but placed it in *Ceratocystiopsis* due to its falcate ascospores. The subsequent discovery of *O. capense*, which is very similar to *C. proteae*, having a *Knoxdaviesia* anamorph but without falcate ascospores, has added confusion to the phylogenetic placement of the ophiostomatoid fungi associated with *Protea* spp.

The occurrence of ophiostomatoid fungi in infructescences of various *Protea* species is unusual, and indicates that other interesting fungi might occur in this niche. The aim of this study was to expand upon previous collections of fungi associated with the infructescences of *Protea* spp. occurring in the western Cape Province of South Africa.

MATERIALS AND METHODS

Infructescences of *Protea* spp. were collected from various parts of the Cape Peninsula (Table 1). Flowers within infructescences were incubated in moist chambers prior to isolation. Masses of conidia or ascospores were carefully removed from fruiting structures using a sterile needle bearing a small piece of agar at the tip. The spores were transferred to 2% malt extract agar (MEA) (20 g Difco malt extract + 20 g Difco Bacto Agar l^{-1} water) and incubated at 20 °C. Cultures were identified on morphology.

Perithecia of ophiostomatoid fungi were common on flower parts. Isolations from ascospore masses at the tips of these perithecia often yielded the characteristic *Knoxdaviesia* anamorphs of *C. proteae* or *O. capense*. However, a *Sporothrix* state was also often isolated. Autoclaved flowers of *Protea repens*

Table 1. Fungi isolated from infructescences of *Protea* spp. and collection sites[a]

	PPRI no.	Host	Collection site
Hyphomycetes			
Acremonium sp. 1	4739	*P. neriifolia*	Jonkershoek
		P. repens	Betty's Bay
Acremonium sp. 2	4793	*P. neriifolia*	Humansdorp, Sir Lowrey's Pass
Acremonium sp. 3	4795	*P. neriifolia*	Jonkershoek
		P. repens	Betty's Bay
Acremonium sp. 4	4780	*P. lepidocarpodendron*	Cape Point
		P. neriifolia	Humansdorp, Steenbras Dam
A. strictum W. Gams	4766	*P. neriifolia*	Steenbras Dam
Alternaria alternata (Fr.) Keissl.	4767	*P. neriifolia*	Jonkershoek
		P. repens	Du Toit Kloof, Betty's Bay
Cephalotrichum stemonitis Pers.	4771	*P. repens*	Sir Lowrey's Pass
Cladosporium sp. 1	4796	*P. repens*	Houw Hoek Pass
Cladosporium sp. 2	4797	*P. repens*	Steenbras Dam, Sir Lowrey's Pass
		P. repens	Franschoek Pass
C. cladosporioides (Fresen.) de Vries	4768	*P. neriifolia*	Sir Lowrey's Pass
C. tenuissimum Cooke	4770	*P. neriifolia*	Jonkershoek
C. sphaerospermum Penz.	4769	*P. nitida*	Jonkershoek
Fusarium anthophilum	4774	*P. burchelli*	Jonkershoek
		P. longifolia	Jonkershoek
		P. magnifica	Nuweberg
		P. neriifolia	Jonkershoek, Sir Lowrey's Pass
		P. nitida	Jonkershoek
		P. repens	Sir Lowrey's Pass
Penicillium canescens Sopp	4775	*P. longifolia*	Hermanus
		P. neriifolia	Du Toit's Kloof, Jonkershoek
		P. nitida	Jonkershoek
P. chrysogenum Thom	4776	*P. repens*	Betty's Bay
P. dendriticum Pitt	4777	*P. repens*	Du Toit's Kloof
P. funiculosum Thom	4799	*P. neriifolia*	Humansdorp
P. glabrum Westling	4778	*P. longifolia*	Hermanus
P. minioluteum Dierckx	4802	*P. neriifolia*	Du Toit's Kloof
P. novaezelandiae J. F. H. Beyma	4519	*P. neriifolia*	Jonkershoek
P. purpurescens (Sopp) Biourge	4284	*P. repens*	Du Toit's Kloof
P. rugulosum Thom	4779	*P. neriifolia*	Du Toit's Kloof
P. thomii Maire	4780	*P. repens*	Du Toit's Kloof
		P. longifolia	Hermanus
Ascomycetes			
Ceratocystiopsis proteae	4773	*P. repens*	Betty's Bay, Pringle Bay
Chaetomium indicum Corda	4772	*P. repens*	Gordon's Bay
Ophiostoma capense	4784	*P. lepidocarpodendron*	Cape Point
		P. longifolia	Franschoek Pass, Hermanus
Ophiostoma splendens	4781	*P. lepidocarpodendron*	Cape Point
		P. longifolia	Hermanus
		P. neriifolia	Jonkershoek, Sir Lowrey's Pass
		P. repens	Betty's Bay, Du Toit's Kloof, Jonkershoek, Franschoek

[a] Cultures of all species have been deposited in the culture collection of the National Collection of Fungi (PPRI).

were placed on MEA plates, which were then inoculated with this *Sporothrix* sp. and incubated at 20°. After 3 months, fully developed fertile perithecia emerged on the flowers as well as in patches on the agar surface.

Growth rate of the *Sporothrix* sp. was determined by transferring conidia from a 1-wk-old culture using a sterile needle and a small piece of agar to the surface of the agar in dishes containing 20 ml MEA. Plates were incubated at temperatures ranging from 5–35° at 5° intervals, with 3 replicate plates per temperature. Colony diameters were measured after 8 d. Cycloheximide tolerance was tested by inoculating 6 plates each amended with different concentrations (0, 0·05, 0·1, 0·5, 1·0 and 2·5 g l^{-1} MEA) of cycloheximide. Cultures were incubated at 20° for 8 d, and colony diameters were then measured.

Specimens for scanning electron microscopy were fixed in 3% glutaraldehyde and 1% osmium tetroxide (2 h) in a sodium phosphate buffer (pH 7) at room temperature. After dehydration in a graded acetone series, material was critical-point dried, coated with gold palladium and examined in a JSM 6400 scanning electron microscope.

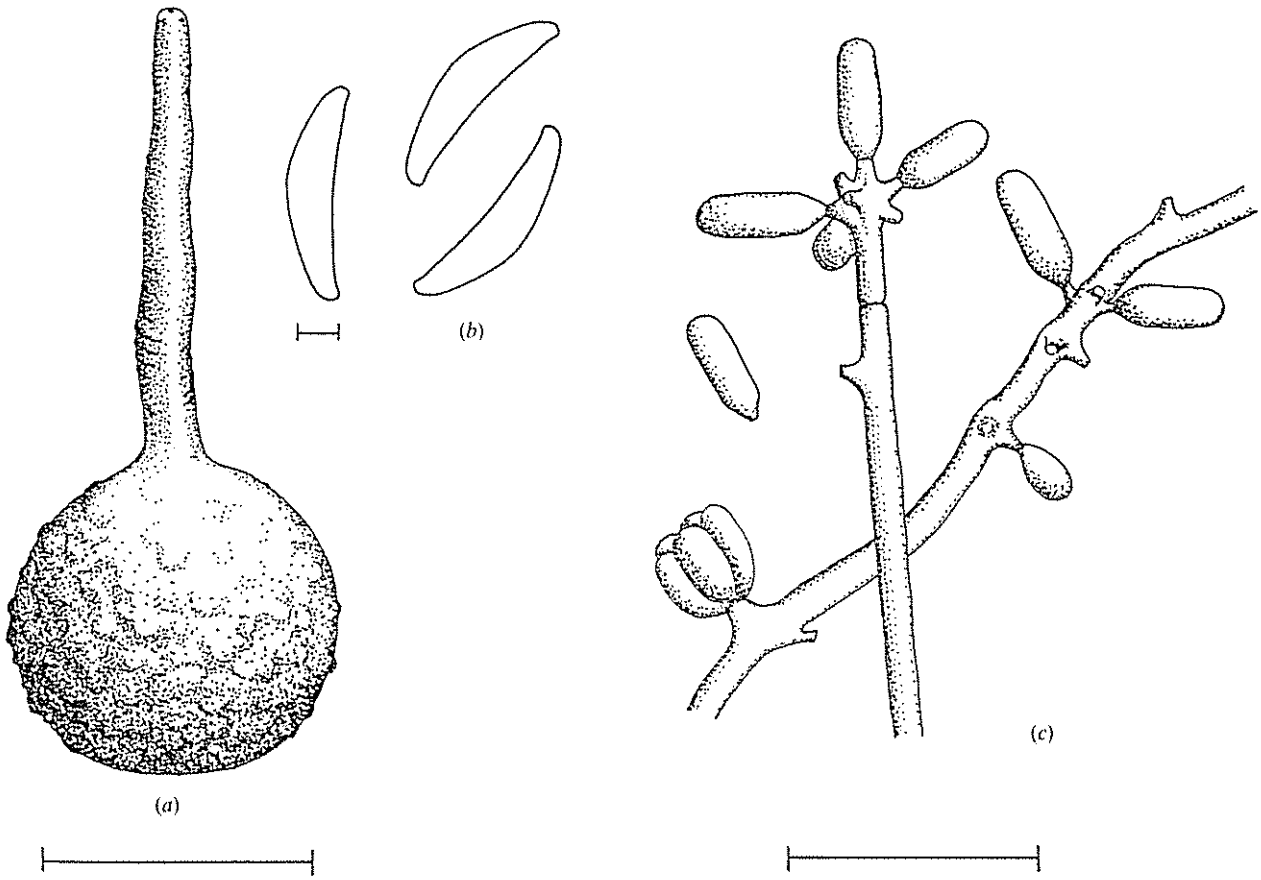


Fig. 1. *Ophiostoma splendens*. (*a*) Perithecium with a moderately long neck and unornamented base (bar, 100 μm). (*b*) Lunate ascospores without sheaths (bar, 1 μm). (*c*). *Sporothrix* anamorph with denticulate conidiogenous cells and smooth-walled clavate, hyaline conidia (bar, 10 μm).

RESULTS

A wide variety of Hyphomycetes was found in the infructescences of various *Protea* spp. (Table 1). The most dominant genera that occurred were *Acremonium* Link (four species), *Cladosporium* Link (five species) and 10 different species of *Penicillium* Link.

Fusarium anthophilum (A. Braun) Wollenw. was the only species of this genus isolated. This fungus occurred on a wide range of *Protea* species including *P. burchelli* Stapf, *P. longifolia* Andr., *P. magnifica* Link, *P. neriifolia* R. Br., *P. nitida* Mill. and *P. repens* (L.) L.

Very few teleomorphic states were encountered. The most common were those of ophiostomatoid fungi, which are apparently the dominant fungi in *Protea* infructescences. *Ceratocystiopsis proteae* occurred only in infructescences of *P. repens*, whereas *Ophiostoma capense* was found in *P. lepidocarpodendron* (L.) L., *P. longifolia* and *P. neriifolia*, but never in *P. repens*. *Chaetomium indicum* was isolated once from *P. repens*.

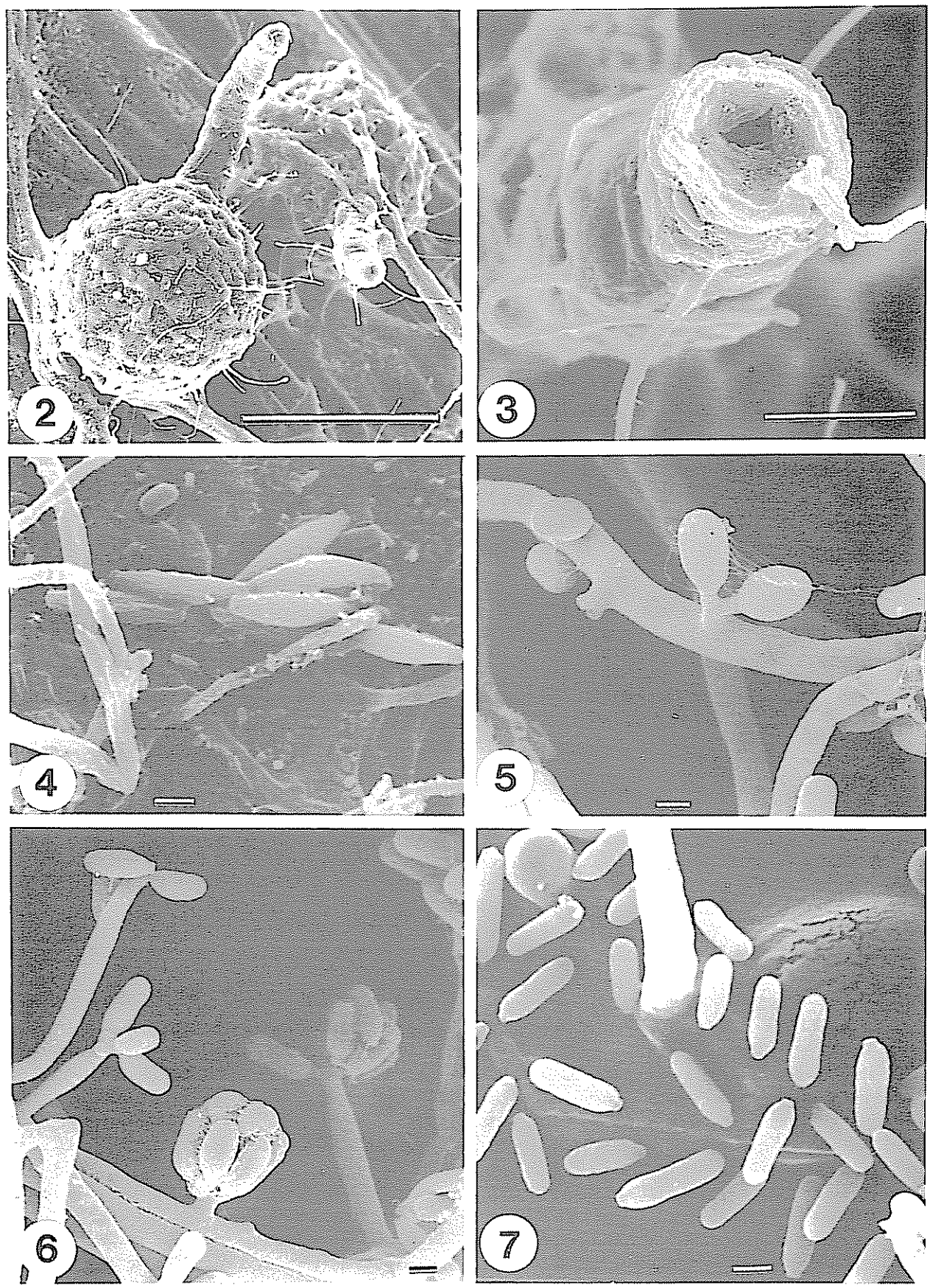
The morphological characteristics of the *Ophiostoma* sp. yielding a *Sporothrix* anamorph in culture differed from those of all previously described species in the genus. This fungus is therefore described as a new species of *Ophiostoma* with a new species of *Sporothrix* as its anamorph.

Ophiostoma splendens Marais & Wingfield, sp. nov.

Perithecia genita in textura hospitis vel in 2% MEA patellis textura hospitis praesente. Perithecia superficialia in mycelio; bases nigrae, globosae, inornatae, 100–150 (122) μm diam.; colla nigra, glabrotunicata, 143–217 (168) μm longa, 16–31 (23) μm lata ad basem, 11–19 (13) μm lata ad apicem, hyphis ostiolaribus absentibus. Asci evanescentes, non visi. Ascosporae unicellulares, hyalinae, lunatae, vaginis absentibus, 5·5–6·0 × 1·0–1·2 (5·7 × 1·1) μm.

Perithecia (Figs 1, 2) produced on host tissue or on 2% MEA plates in the presence of host tissue. Perithecia superficial on the mycelium; bases black, globose, unornamented, 100–150 (122) μm diam.; necks black, smooth-walled, 143–217 (168) μm long, 16–31 (23) μm wide at the base, 11–19 (13) μm wide at the apex, ostiolar hyphae absent (Fig. 1). Asci evanescent, not seen. Ascospores one-celled, hyaline, lunate, sheaths absent, 5·5–6·0 × 1·0–1·2 (5·7 × 1·1) μm (Figs 1, 3).

Specimens examined. On flowers within infructescences of *Protea neriifolia* infested by insects, Sir Lowrey's Pass, Cape Province, South Africa, April 1990, G. J. Marais, PREM 51054 Holotype. Paratypes, PREM 51078 on insect-infected *P. longifolia* flowers, Hermanus, April 1990, G. J. Marais; PREM 51077 on insect-infested *P. repens* flowers, Jonkershoek, April 1990, G. J. Marais.



Figs 2–7. *Ophiostoma splendens*. **Fig. 2.** Perithecium (bar, 100 µm). **Fig. 3.** Apex of perithecium neck showing absence of ostiolar hyphae (bar, 10 µm). **Fig. 4.** Lunate ascospores without sheaths (bar, 1 µm). **Fig. 5.** Conidia produced directly on hyphae (bar, 1 µm). **Fig. 6.** Conidia on typical *Sporothrix* conidiophores (bar, 1 µm). **Fig. 7.** Clavate conidia with distinct points of attachment (bar, 1 µm).

Sporothrix splendens Marais & Wingfield sp. nov.

Coloniae in MEA 3·1 cm diam. post 8 dies ad 25°, albae vel cremeae. Incrementum deminutum ad temperaturas supra vel infra 25°, paene nullo incremento ad 5° vel 35°. Sporulatio profusa et in textura hospitis et in 2% MEA. Conidiophora micronematosa, mononematosa, hyalina, septata, 1·1–1·6 (1·3) µm crassa et 8·7–21·7 (12·6) µm longa, ferentia cellas conidiogenas quae numerose gignitur sympodice dum conidia nascuntur et fiunt denticulatae; denticuli 0·5–1·1 (0·7) µm longi. Conidia quoque nascuntur ipsis in hyphis. Conidia holoblastica, hyalina, unicellularia, clavata, laevia, tenuitunicata, 5·9 × 1·7 µm, formata singulatim, aggregantur in massis mucosis.

Colonies on MEA 3·1 cm diam after 8 days at 25°, white to creamy white. Growth reduced at temperatures below and above 25° with virtually no growth at 5° and 35°. Sporulation profuse both on host tissue and 2% MEA. Conidiophores micronematous, mononematous, hyaline, septate, 1·1–1·6 (1·3) µm thick and 8·7–21·7 (12·6) µm long, bearing conidiogenous cells that proliferate sympodially during conidiation, becoming denticulate; denticles 0·5–1·1 (0·7) µm long (Figs 1, 4, 5). Conidia also produced directly on hyphae (Figs 4). Conidia holoblastic, hyaline 1-celled, clavate, smooth, thin-walled, 5·9 × 1·7 µm formed singly becoming aggregated in slimy masses (Figs 1, 5, 6).

Specimens examined. Dried cultures on 2% MEA, isolated from perithecia on flowers within inflorescences of *Protea neriifolia* infested by insects, Sir Lowrey's Pass, Cape Province, South Africa, April 1990, G. J. Marais, PREM 51079, Holotype. Paratypes, PREM 51076 on insect-infested *P. longifolia* flowers, Hermanus, April 1990, G. J. Marais; PREM 51080 on insect-infested *P. repens* flowers, Jonkershoek, April 1990, G. J. Marais.

Dried-down cultures of the holotypes of *O. splendens* and *S. splendens* as well as permanent slide preparations have been deposited in the National Collection of Fungi, Plant Protection Research Institute, Pretoria, South Africa (PREM).

O. splendens was found to be relatively tolerant to cycloheximide. Mean colony diameter declined from 2·28 cm without cycloheximide to 1·68 cm on 2·5 gl^{-1} cycloheximide.

DISCUSSION

Most species of fungi isolated from *Protea* infructescences in this study are probably saprobic, and only become obvious after the flower heads are closed. These fungi, which include species belonging to a wide range of genera, can only reach the flower by means of a vector, airborne spores or rainsplash.

Protea species are known to be rich in nectar (Cowling & Mitchell, 1981). This makes the flowers attractive for visitation by insects. They, in turn, can carry fungal spores from one infructescence to another. After a number of weeks the *Protea* infructescences close and remain intact for at least two years. During this time they are colonized by other insects (Coetzee & Giliomee, 1987). The availability of nutrients, and later, the protective and moist environment in the flower head make this environment suitable for fungal populations to flourish.

The discovery of *Ceratocystiopsis proteae* (Wingfield *et al.*, 1988), and *Ophiostoma capense* (Wingfield & Van Wyk, 1992) has placed a new perspective on the taxonomy of species in *Ceratocystis s.l.* Although the teleomorphic states of these fungi show morphological similarities to *Ophiostoma* and *Ceratocystiopsis*, the anamorph genus *Knoxdaviesia* is unusual, and new to *Ceratocystis s.l.* Although insufficient evidence is available to us at present, we believe that these fungi are unique to the group and have evolved in a close association with *Protea* spp.

The discovery of *O. splendens* in *Protea* infructescences in this study adds a further element to the taxonomy of ophiostomatoid fungi associated with Proteaceae. Until now, both species of ophiostomatoid fungi collected in this niche have been unusual in having *Knoxdaviesia* anamorphs. The *Sporothrix* anamorph of *O. splendens* is more typical of *Ophiostoma* spp. Unlike those species with *Knoxdaviesia* states, this fungus is also tolerant of cycloheximide, which is a common characteristic of *Ophiostoma* spp. (Harrington, 1981).

The most obvious characteristic distinguishing *O. splendens* from other species of *Ophiostoma* is the unusual niche in which it occurs. Morphologically it is most similar to *Ophiostoma perfectum* (R. W. Davidson) de Hoog, *O. narcissi* Limber, *Ceratocystis denticulata* R. W. Davidson and *C. tenella* R. W. Davidson. *O. perfectum* differs from *O. splendens* in having longer necks and allantoid ascospores, as well as ostiolar hyphae and ramoconidia, which are absent in *O. splendens* (de Hoog, 1974). *C. denticulata* and *O. narcissi* have perithecial neck lengths that fall in the same range of *O. splendens*. The anamorph of *O. narcissi* is also a species of *Sporothrix* and thus similar to *O. splendens*, but the conidia of these two fungi are of different sizes (Limber, 1950). Perithecia with unornamented bases and the absence of ostiolar hyphae are also common features in *O. splendens* and *O. narcissi*. The ovate to oblong ascospores of *O. narcissi*, however, separate this fungus from *O. splendens*, which has lunate ascospores. *C. denticulata* has allantoid ascospores and fine ostiolar filaments at the perithecial apices (Davidson, 1979), distinguishing *O. splendens* from this fungus. *C. tenella* has neck lengths of 105–400 µm with the ascospores orange-section-shaped (Upadhyay, 1981), which is close to *O. splendens*. However, the presence of ostiolar hyphae, crooked necks, and smaller perithecial bases separate these species.

O. splendens appears to have a relatively wide host range amongst *Protea* spp. In this respect it is similar to *O. capense* but different from *C. proteae*, which is apparently restricted to *P. repens* (Wingfield *et al.*, 1988). We have assumed that host specificity in *C. proteae* is mediated either by its having specific insect vectors which are restricted to this plant, or by some nutritional requirement of the fungus only being present in *P. repens*. The occurrence of an ophiostomatoid fungus both in infructescences of *P. repens* and other *Protea* species suggests that this specificity might be more complex than previously assumed.

Prior to the discovery of *O. splendens* in *Protea* infructescences, it was assumed that ophiostomatoid fungi occurring in this unusual niche would all have *Knoxdaviesia* anamorphs. They would thus be unrelated to other species of *Ceratocystis s.l.* Having a *Sporothrix* anamorph and being resistant to cycloheximide makes *O. splendens* much more similar to other *Ophiostoma* spp. than its co-inhabitants on Proteaceae. The hypothesis that these organisms developed separately from other ophiostomatoid fungi might thus not be correct. Further

studies concentrating on cell components and comparing the molecular evolution of these and other ophiostomatoid fungi are planned to resolve questions pertaining to their phylogeny.

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