Phomopsis saccharata sp. nov., causing a canker and die-back disease of *Protea repens* in South Africa

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Phomopsis saccharata is newly described from cankers of *Protea repens* plants collected on the Jonkershoek Mountains in Stellenbosch, South Africa. The pathogenicity of this species was established in a previous study, but the fungus has never been formally described. Morphologically this species is easily distinguished from others in the genus based on its large, chiefly 1-septate alpha conidia. Although this is an unusual feature for *Phomopsis*, ITS DNA sequence data indicate that this taxon is best accommodated in *Phomopsis*. This finding also suggests that the generic concept of *Phomopsis* should be expanded to include taxa with 1-septate alpha conidia.

Keywords: *Diaporthales*, *Phomopsis*, *Proteaceae*, stem canker.

In 1989 Orffer and Knox-Davies noted a severe canker and die-back disease on *Protea repens* (L.) L. plants in the Western Cape Province of South Africa. In their study they found a rather unusual species of *Phomopsis* associated with these disease symptoms. This fungus was found at several localities in the Western Cape Province (Orffer & Knox-Davies, 1989), and has also been observed as the most serious canker disease of *Protea repens* on commercial farms in the Eastern Cape Province (S. Denman, unpublished data). Using a variety of inoculation methods, Orffer & Knox-Davies (1989) established Koch's postulates, and confirmed that the *Phomopsis* sp. was the pathogen responsible for the stem cankers. Although the *Phomopsis* sp. was noted to have a unique morphology having large, 0–1-septate alpha conidia, it was never formally described. The aim of this study, therefore, is to name and describe the pathogen causing die-back disease of *Protea repens*. Because this species is morphologically atypical of the genus *Phomopsis* (Sacc.) Bubák, it could potentially represent a new genus in the *Diaporthales*. To determine the generic affinities of this species, phylogenetic analysis with other genera in the *Diaporthales* was carried out by analyzing the complete ITS sequence of the rDNA.

Materials and methods

Collection and morphological characterization

Shoots with canker and die-back symptoms and fruiting bodies of the fungus were collected from *Protea repens* bushes in the Jonkershoek Mountains at Stellenbosch in the Western Cape Province. Using dilution plates, single conidium isolates were established on 2% malt extract agar (MEA; Biolab, Midrand, Johannesburg, South Africa). The isolates were transferred to divided plates with potato dextrose agar (PDA, Biolab) on one side and 2% water agar with double autoclaved *Protea* L. cane pieces on the other. Plates were incubated at 25 C under continuous near-ultraviolet light to enhance sporulation. Wherever possible, thirty measurements were made of structures mounted in clear lactophenol, and the averages determined. Colony colours (top and reverse) were rated after 14 d on MEA at 25 C in the dark, using the colour charts of Rayner (1970). The holotype specimen was lodged at the National Collection of Fungi in Pretoria, South Africa (PREM), and ex-type cultures maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch, and the Centraalbureau voor Schimmelultures (CBS) in the Netherlands. Vertical sections of pycnidia were cut with a Leica CM1100 freezing microtome. Sections (10 μm) were mounted in lactic acid and structures were measured at a magnification of 1000x.

DNA sequencing and data analysis

DNA of isolate STE-U 3743 was extracted using the protocol described by Lee & Taylor (1990). The ITS1 and ITS2 internal transcribed spacers as well as the 5.8S ribosomal RNA gene were amplified with primers ITS1 and ITS4. Genomic locations and primer sequences are presented in White & al. (1990). The PCR products were purified using Wizard PCR Preps (Promega Corporation, Madison, Wisconsin). Both strands of the PCR product were sequenced using the ABI Prism 377 DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut). A Dye Terminator Cycle Sequencing Ready Reaction Kit containing AmpliTaq DNA Polymerase (Perkin-Elmer) was used for sequencing reactions. Fragments of sequencing reactions were finally purified using Centri-Sep Spin columns (Princeton Separations, Adelphia, New Jersey) and loaded onto the sequencing gel. Sequences were aligned using Sequence Navigator, from which a consensus sequence was derived. Sequences obtained from this study and GenBank retrievals were aligned with Clustal W (Thompson & al., 1994). The final alignment was optimised manually. Alignment gaps were coded as missing data in the analysis. The sequence of *Cryphonectria parasitica* (Murrill) M. E. Barr (GenBank accession:

AF172658) was used as outgroup. Phylogenetic analyses were performed with PAUP* (Phylogenetic Analysis Using Parsimony) version 4.0b2a (Swofford, 1999). Maximum parsimony analysis was performed using the branch-and-bound search option for exact solution. The unconstrained topologies of the equally parsimonious trees were compared using the Kishino-Hasegawa test. The best topology was selected as the most parsimonious tree topology and evaluated with 1000 bootstrap replications in a branch-and-bound search to test the clade stability of the tree. The decay indices were also calculated using AutoDecay (Eriksson, 1998) to further test the robustness of the branches of the tree. Other measures including tree length, consistency index, retention index, rescaled consistency index and homoplasy index (CI, RI, RC and HI) were also calculated. The DNA sequence obtained was deposited at GenBank (AF387817), and the alignment in TreeBase (S632, M478). Sequences of GenBank retrievals included in the analyses were: *Cryphonectria cubensis* (Bruner) Hodges AF046896, *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. var. *caulivora* Athow & Caldwell AF000567, *Diaporthe perjuncta* Niessl AF230744, *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. AF001028, *Diaporthe vaccinii* Shear AF191166, *Endothia eugeniae* (Nutman & F. M. Roberts) J. Reid & C. Booth AF046904, *Endothia gyrosa* (Schwein. : Fr.) Fr. AF046905, *Leucostoma niveum* (Hoffm. : Fr.) Höhn. AF191174, *Leucostoma persoonii* Höhn. AF191183, *Phomopsis amygdali* (Delacr.) J. J. Tuset & M. T. Portilla AF230755, AF230781, *Phomopsis longicolla* Hobbs AF00021, *Phomopsis* sp. U91617, *Phomopsis* sp. U94898, *Phomopsis* sp. AF230775 and *Phomopsis viticola* (Sacc.) Sacc. AF230756.

Results and discussion

Phylogenetic analysis

Nucleotide sequence of 489 bp of the 5.8S rDNA and ITS regions was determined for the *Phomopsis* isolate STE-U 3743. Sequence alignment had a total consensus length of 670 sites. Of the aligned sites, 243 ambiguous characters were excluded from the maximum parsimony analysis. Amongst 427 included characters, 279 were constant, 43 variable and 105 parsimony-informative. Ten equally most parsimonious trees (MPT, not shown) were obtained using the branch and bound option in PAUP*. In accordance with the result of the Kishino-Hasegawa likelihood test, the best tree topology (Fig. 1) of the ten MPTs was selected as the tree and evaluated with 1,000 bootstrap replications in a branch and bound search for the clade stability. In the phylogenetic tree (Fig. 1), the ingroup taxa *Phomopsis* and *Cryphonectria* formed two clades with 95% and 100% bootstrap support respectively. Isolate STE-U 3743 clustered in the *Phomopsis* clade.

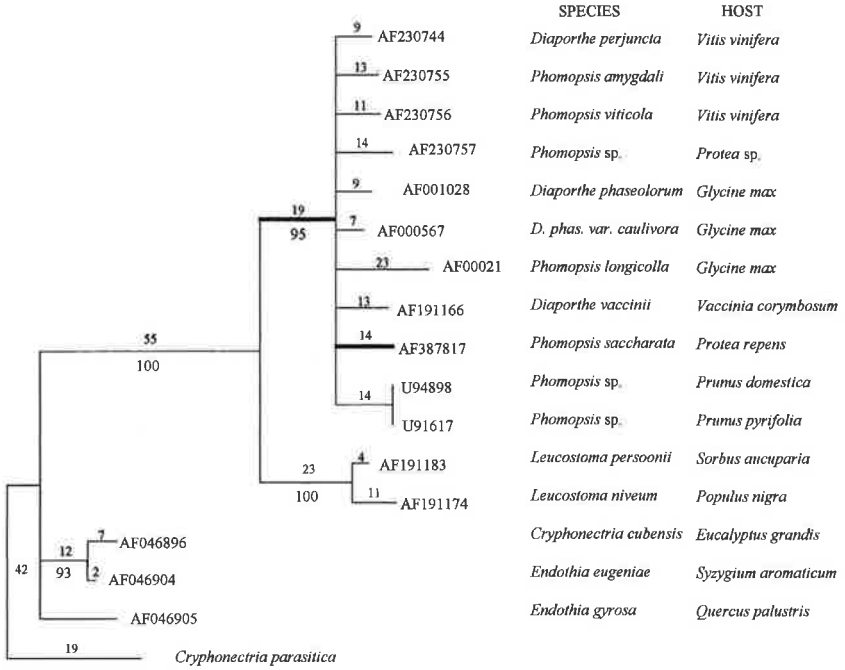


Fig. 1. – One of the ten most parsimonious trees derived from the alignment of ITS1, 5.8S and ITS2 rDNA of the *Phomopsis saccharata* (STE-U 3743) using parsimony analysis with the branch and bound option. – The tree is rooted with *Cryphonectria parasitica* (GenBank accession: AF172658) as outgroup. TL = 285 steps, CI = 0.902, RI = 0.935, RC = 0.805, HI = 0.085. Bootstrap values of 1000 replicates and decay indices are respectively indicated below and above the tree branches.

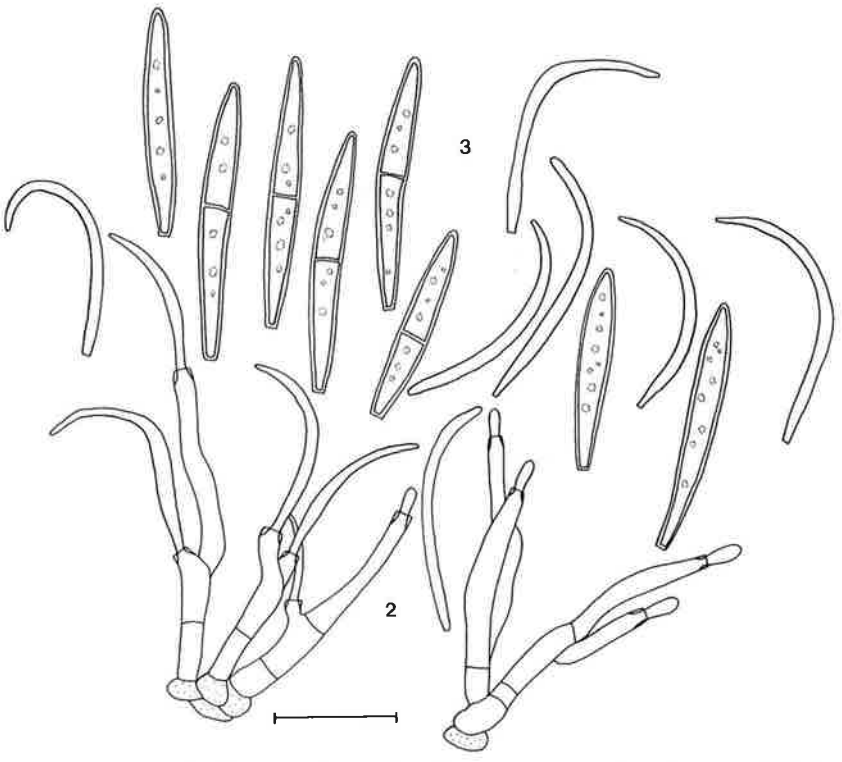
Morphological characterization

Phomopsis saccharata J.-C. Kang, L. Mostert & P. W. Crous, **sp. nov.** Figs. 2–11.

Alpha conidiophora ramosa, septata, 7–20 × 2–3 µm; beta conidiophora septata, ramosa, 4–10 × 2–3 µm (mean = 7 × 2 µm). Cellulae conidiogenae: alpha cellulae conidiogenae filiformes, 13–25 × 1–2 µm; beta cellulae conidiogenae subcylindraceae, 6–26 × 1–2 µm. Conidia: alpha conidia 0–1-septata, fusiformia ad ellipsoidea (11–)16–20(–24) × (2.5–)3(–4) µm; beta conidia aseptata, curvata ad hamata, (15–)18–20(–27) × (1–)1.5(–2) µm.

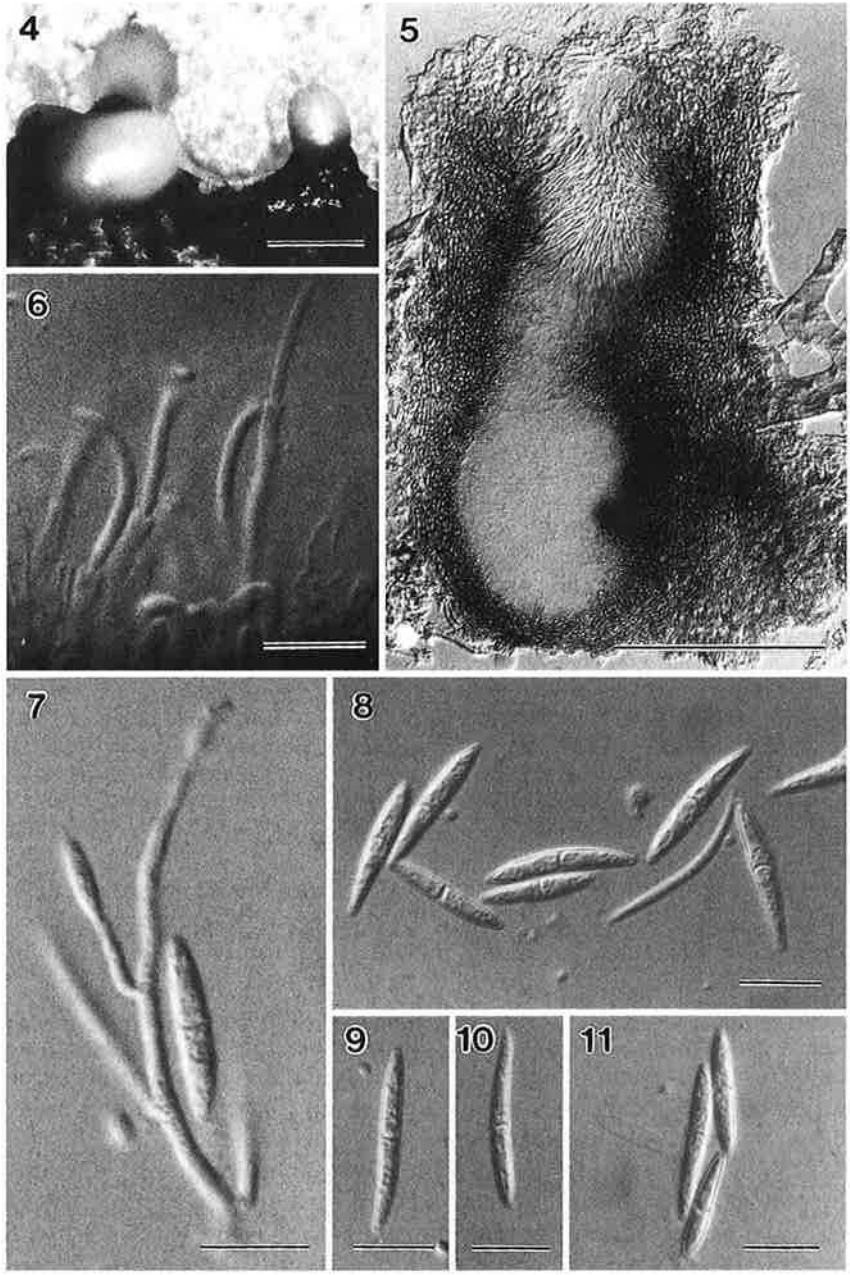
Etymology. – Named after the common name of *Protea repens*, which is sugar bush (saccharum = sugar).

Pycnidia formed after 33 d on autoclaved *Protea* stems on WA, eustromatic, dark brown to black, almost superficial, covered with hyphae, separate, sparse, ampulliform to barrel shaped, convoluted, up to 155 (mean = 115) µm wide and 170 (mean = 122) µm tall, appearing more flattened on the host, up to 290 µm wide and



Figs. 2–3. – Conidiophores and conidia of *Phomopsis saccharata*. – 2. Conidiophores giving rise to beta and alpha conidia, on the left and right, respectively. – 3. Alpha and beta conidia. – Bar = 10 µm.

410 µm tall; pycnidial wall consisting of two regions: an outer thick-walled region, 10–30 µm wide, with 3–4 layers of dark brown cells of *textura angularis*, and an inner, pale brown, thinner walled region of *textura intricata*, 3–10 µm wide. – Conidial mass hyaline to yellow. – Conidiophores: alpha conidiophores branched, septate, 7–20 × 2–3 µm (mean = 13 × 2 µm); beta conidiophores septate, branched, 4–10 × 2–3 µm (mean = 7 × 2 µm). – Conidiogenous cells: alpha conidiogenous cells filiform, tapering towards the apex, periclinal thickening present, collarette inconspicuous to absent, 13–25 × 1–2 µm (mean = 20 × 1.5 µm); beta conidiogenous cells subcylindrical to reniform, tapering towards the apex, periclinal thickening present, collarette inconspicuous to absent, 6–26 × 1–2 µm (mean = 17 × 1.5 µm). – Conidia: alpha conidia 0–1-septate, fusoid to ellipsoidal with a truncate base and acutely rounded apex, mostly multiguttulate, but also aguttulate, (11–)16–20(–24) × (2.5–)3(–4) µm (mean = 18 × 3 µm); beta conidia aseptate, rarely straight, mostly curved to hamate, (15–)18–20(–27) × (1–)1.5(–2) µm (mean = 19 × 1.5 µm). –



Figs. 4–11. – *Phomopsis saccharata* on *Protea* canes. – 4. Pycnidia. – 5. Longitudinal section through pycnidium (*in vitro*). – 6. Beta conidiophores and conidiogenous cells. – 7. Alpha conidiophores and conidiogenous cells. 8. Alpha and beta conidia. – 9–11. Alpha conidia with septa. – Bars = 200, 50, 10 μm, respectively in Figs. 4, 5, 6–11.

Pycnidia can give rise to either alpha or beta conidia, or a combination of both conidial types.

Cultural characteristics. – Colony colour predominantly buff (19″d) with white patches. Reverse, buff (19″d) with greyish sepia (15″″i) and some fuscous black (7″″k) patches. Colonies flat with smooth margins, zone lines with moderate, fluffy, aerial mycelium after 14 d at 25 °C in the dark. Orffer & Knox-Davies (1989) reported that colonies grew from 4–32 C, achieving optimal growth at 28 C, growing at a rate of 5 mm/d. The fungus could not be isolated or cultured on media containing streptomycin (0.02%).

Hosts. – *Protea repens* (L.) L., *P. obtusifolia* H. Buek ex Meisn. (Proteaceae).

Distribution. – Cape Province, South Africa.

Specimen examined. – SOUTH AFRICA. Western Cape Province, Jonkershoek Mountains, Stellenbosch, stem cankers on *P. repens*, S. Denman, 1999, PREM 57213 (holotype), STE-U 3743 (culture ex-type).

Contrary to our hypothesis, the ITS phylogeny presented in Fig. 1 clearly indicates that *P. saccharata* is a true species of *Phomopsis*, not clustering with any of the outgroups included in the Diaporthales, but falling within the *Diaporthe* Nitschke clade. Within the genus *Phomopsis, P. saccharata* is unusual in that most of its alpha conidia are 1-septate. This feature initially led us to consider that it could be a species of *Diplodina* Westend. The latter genus is also known from Proteaceae, and is associated with similar disease symptoms, namely *Diplodina melanocraspeda* Bathgate, M. E. Barr & B. L. Shearer (teleomorph: *Cryptodiaporthe melanocraspeda* Bathgate, M. E. Barr & B. L. Shearer), which causes a severe canker of *Banksia coccinea* R. Br. (Proteaceae) in Australia (Bathgate & al., 1996). However, the presence of some aseptate alpha conidia, as well as its ITS phylogeny excluded this genus. As our data indicate, *P. saccharata* is a species of *Phomopsis s. str.*, hereby expanding the generic concept of *Phomopsis* to also include taxa with septate alpha conidia.

Other species of *Phomopsis* reported in the family *Proteaceae* include *Phomopsis banksiae* Novos. on *Banksia integrifolia* L. (alpha conidia 6–12 × 2.5–3 µm) (Gutner & Novoselova, 1936; Petrak, 1950), *Phomopsis hakeae* Lebedeva (alpha conidia 10–12 × 4 µm) on *Hakea sericaea* Schrad. (Petrak, 1953), and *Phomopsis briosii* Mutto on a *Roupala* sp. (Trotter, 1931). A presumed isotype of the latter (BPI 358308) was examined and found to be *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. None of these species has conidia that agree to those of *P. saccharata* in dimensions or morphology.

There are several other reports of *Phomopsis* spp. on Proteaceae. Forsberg (1983) reported *Phomopsis* spp. from several *Protea* hosts in Australia, namely *Protea cynaroides* (L.) L., *Protea repens*, *Protea neriifolia* R. Br. as well as a *Leucadendron* sp. Mostert & al. (2001) reported a *Phomopsis* sp. from stems of an unidentified species of *Protea* in South Africa, while Greenhalgh (1981) reported a Phomopsis canker on *Protea* from Queensland, Australia.

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