# Characterisation of *Phomopsis* spp. associated with die-back of rooibos (*Aspalathus linearis*) in South Africa

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**Abstract:** Die-back of rooibos (*Aspalathus linearis*) causes substantial losses in commercial *Aspalathus* plantations in South Africa. In the past, the disease has been attributed to *Phomopsis phaseoli* (teleomorph: *Diaporthe phaseolorum*). Isolates obtained from diseased plants, however, were highly variable with regard to morphology and pathogenicity. The aim of the present study was thus to identify the *Phomopsis* species associated with die-back of rooibos. Isolates were subjected to DNA sequence comparisons of the internal transcribed spacer region (ITS1, 5.8S, ITS2) and partial sequences of the translation elongation factor-1 alpha gene. Furthermore, isolates were also compared in glasshouse inoculation trials on 8-mo-old potted plants to evaluate their pathogenicity. Five species were identified, of which *D. aspalathi* (formerly identified as *D. phaseolorum* or *D. phaseolorum* var. *meridionalis*) proved to be the most virulent, followed by *D. ambigua*, *Phomopsis theicola*, one species of *Libertella* and *Phomopsis*, respectively, and a newly described species, *P. cuppatea*. A description is also provided for *D. ambigua* based on a newly designated epitype specimen.

**Taxonomic novelties:** *Diaporthe aspalathi* Janse van Rensburg, Castlebury & Crous stat. et nom. nov., *Phomopsis cuppatea* Janse van Rensburg, Lamprecht & Crous sp. nov.
**Key words:** Elongation factor 1-alpha gene, *Diaporthe*, endophytes, ITS, pathogenicity, Phomopsis die-back, systematics.

## INTRODUCTION

Rooibos (*Aspalathus linearis*) is a leguminous shrub that is indigenous to the Western Cape Province of South Africa, and used for the production of rooibos tea. A serious die-back disease of plants in the Clanwilliam area was first observed in 1977 and officially reported in the scientific literature by Smit & Knox-Davies (1989a, b), who identified the causal organism as *Phomopsis phaseoli* (Desm.) Sacc. [teleomorph: *Diaporthe phaseolorum* (Cooke & Ellis) Sacc.]. Since die-back of rooibos was originally reported, it has developed into a disease of considerable economic importance, affecting up to 89 % of plants in 3-yr-old plantations (Lamprecht *et al*., unpubl. data).

The genus *Phomopsis* (Sacc.) Bubák contains a large number of cosmopolitan plant pathogens, many of which incite blights, cankers, die-backs, rots, spots, and wilts in a wide assortment of plants of economic importance (Kulik 1984, Uecker 1988). *Phomopsis* diseases usually manifest themselves in the production of characteristic symptoms, some of which can culminate in the death of the host plant (Kulik 1984). In rooibos, symptoms manifest themselves as a die-back of harvested branches, with pycnidia forming on dead tissue, and a characteristic internal discoloration of infected branches. Eventually this leads to death of the host plant, after which perithecia form just below the soil surface (Fig. 1).

Contrary to earlier reports (Smit & Knox-Davies 1989a, b), preliminary surveys and pathogenicity studies revealed the *Phomopsis* isolates associated with the disease to be highly variable with regards to morphology and virulence, indicating the possible existence of more than one species. To develop a sustainable die-back management programme for the rooibos industry, it was necessary to determine which species were involved in this disease complex and which of these were the most important pathogens. The aim of the present study was to characterise the *Phomopsis/Diaporthe* spp. associated with die-back symptoms of rooibos bushes. This was done by generating DNA sequence data of the ITS region and partial translation elongation factor-1 alpha (TEF1 or EF1-α) gene and analysing these data with morphological and cultural observations. A further aim was to conduct pathogenicity studies with the various species identified and to determine which of these were the most virulent pathogens involved with the die-back disease of *Aspalathus*.

## MATERIALS AND METHODS

### Isolates

Symptomatic plants were collected throughout the rooibos-producing area ranging from Citrusdal in the south to Nieuwoudtville in the north. Isolations were made from surface-disinfected host tissue onto Petri dishes containing 2 % potato-dextrose agar (PDA; Difco, Becton Dickinson, Sparks, MD, U.S.A.). A total of 28 *Phomopsis* isolates representing the different morphological groups recognised on PDA were selected for further molecular characterisation. The origin of isolates, as well as plant parts from which they were isolated, are listed in Table 1. Reference strains were deposited in the Centraalbureau voor Schimmelcultures in Utrecht, the Netherlands (CBS).

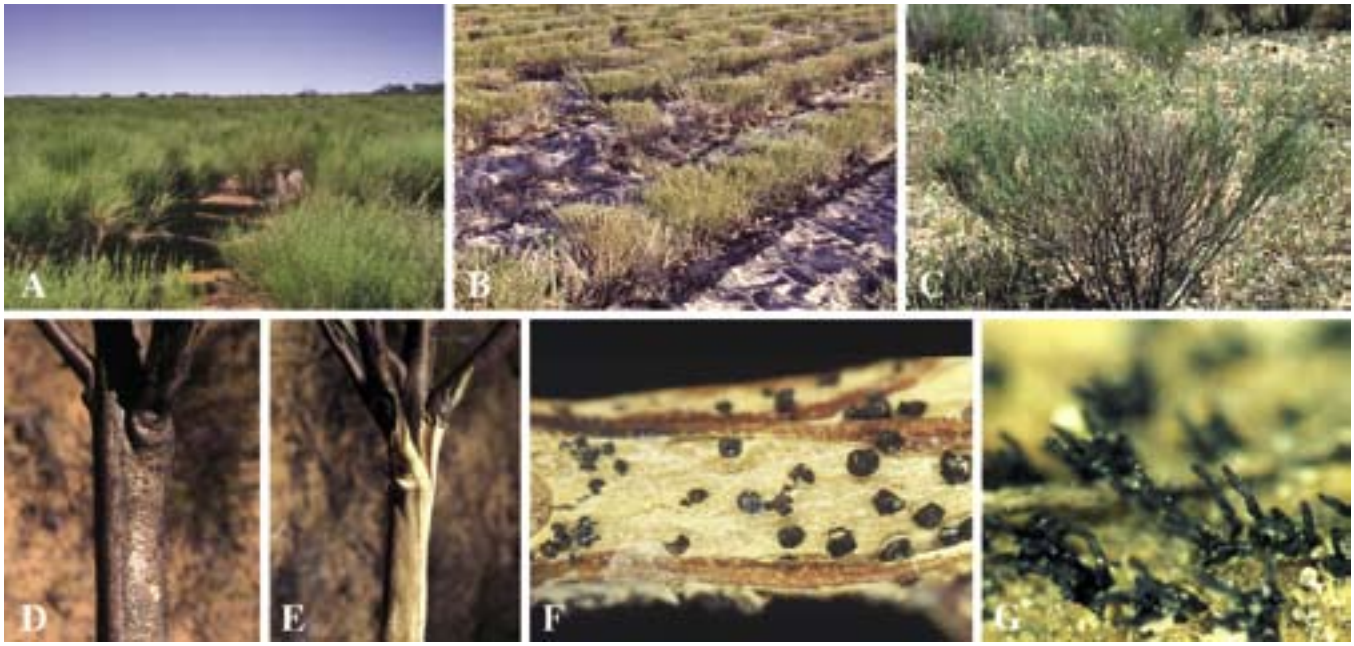


**Fig. 1.** *Aspalathus linearis* plants with Phomopsis die-back. A. Healthy plants under cultivation. B. Plants after harvest. C. *Aspalathus* bush with die-back symptoms. D–E. Stem cankers. F. Pycnidial formation on dead stem tissue. G. Formation of perithecia on stems just below the soil surface.

**Table 1.** *Phomopsis* and *Diaporthe* isolates from South Africa used in this study.

| Species | Strain no.[1] | Farm, area | Rainfall[2] | Plant part | Lesion length (cm)[3] | Collector | GenBank numbers (EF, ITS)[4] |
|---|---|---|---|---|---|---|---|
| *D. ambigua* | CBS 117167; CPC 5414; R86AM | Taaibosdam, Gifberg | High | Crown | 3.44d–g | J.C. Janse van Rensburg | DQ286237, DQ286263 |
| | CBS 117170; R59O | Vaalkrans, Nardouwsberg | Low | Branch | 5.63b | J.C. Janse van Rensburg | DQ286238, DQ286264 |
| | CBS 117371; CPC 5421; R350U | Uitsig, Agter-Pakhuis | Low | Branch | 4.83b–d | J.C. Janse van Rensburg | DQ286239, DQ286265 |
| | CBS 117372; CPC 5411; 10040K | Clanwilliam | High | Root | 2.17g–k | S.C. Lamprecht | DQ286240, DQ286266 |
| | CBS 117373; CPC 5427; R408AP | Langebergpunt, Clanwilliam | High | Root | 0.69j–l | J.C. Janse van Rensburg | DQ286241, DQ286267 |
| | CBS 117374; CPC 5418; R165AI | Karnemelksvlei, Citrusdal | High | Crown | 5.36b–c | J.C. Janse van Rensburg | DQ286242, DQ286268 |
| | CPC 5409; 9963K | Clanwilliam | High | Crown | 4.00b–e | S.C. Lamprecht | DQ286243, DQ286269 |
| | CPC 5412; R66I | Taaiboskraal, Agter-Pakhuis | Low | Root | 0.61j–l | J.C. Janse van Rensburg | DQ286244, DQ286270 |
| | CPC 5413; R78U | Nardouw, Nardouwsberg | Low | Crown | 0.94i–l | J.C. Janse van Rensburg | DQ286245, DQ286271 |
| | CPC 5419; R337AR | Vaalkrans, Nardouwsberg | Low | Branch | 3.06d–h | J.C. Janse van Rensburg | DQ286246, DQ286272 |
| | CPC 5423; R366A | Taaibosdam, Gifberg | High | Branch | 2.25e–j | J.C. Janse van Rensburg | DQ286247, DQ286273 |
| | CPC 5425; R379S | Vondeling, Nardouwsberg | Low | Branch | 2.25e–j | J.C. Janse van Rensburg | DQ286248, DQ286274 |
| *D. aspalathi* | CBS 117168; CPC 5420; R338E | Vaalkrans, Nardouwsberg | Low | Crown | 10.00a | J.C. Janse van Rensburg | AY339353, AY339321 |
| | CBS 117169; CPC 5428; R412AY | Langebergpunt, Clanwilliam | High | Branch | 10.00a | J.C. Janse van Rensburg | DQ286249, DQ286275 |
| | CBS 117500; CPC 5408; 9940AF | Clanwilliam | High | Crown | 9.67a | S.C. Lamprecht | DQ286250, DQ286276 |
| | CPC 5410; 9996D | Clanwilliam | High | Crown | 10.00a | S.C. Lamprecht | DQ286251, DQ286277 |

**Table 1.** (Continued).

| Species | Strain no.[1] | Farm, area | Rainfall[2] | Plant part | Lesion length (cm)[3] | Collector | GenBank numbers (EF, ITS)[4] |
|---|---|---|---|---|---|---|---|
| | CPC 5430; R425B | Koelfontein, Clanwilliam | High | Branch | 10.00a | J.C. Janse van Rensburg | DQ286252, DQ286278 |
| *Libertella* sp. | CBS 117163; CPC 5426; R380Z | Vondeling, Nardouwsberg | Low | Branch | 1.81g–k | J.C. Janse van Rensburg | DQ286253, DQ286279 |
| | CBS 117164; CPC 5429; R424T | Koelfontein, Clanwilliam | High | Crown | 2.64e–i | J.C. Janse van Rensburg | DQ286254, DQ286280 |
| | R686I | Snorkfontein, Gifberg | High | Root | 0.72j–l | J.C. Janse van Rensburg | DQ286255, DQ286281 |
| | R699H | Pendoringkraal, VanRhynsdorp | Low | Root | 0.78j–l | J.C. Janse van Rensburg | DQ286256, DQ286282 |
| *P. cuppatea* | CBS 117499; CPC 5431; R433R | Kossak se werf, Clanwilliam | High | Branch | 1.19i–l | J.C. Janse van Rensburg | AY339354, AY339322 |
| | CPC 5416; R162AO | Berg-en-dal, Citrusdal | High | Crown | 3.94b–f | J.C. Janse van Rensburg | DQ286257, DQ286283 |
| | R164AN | Karnemelksvlei, Citrusdal | High | Branch | 1.61h–l | J.C. Janse van Rensburg | DQ286258, DQ286284 |
| *Phomopsis* sp. 9 | CBS 117165; CPC 5417; R162L | Berg-en-dal, Citrusdal | High | Crown | 0.42k–l | J.C. Janse van Rensburg | DQ286259, DQ286285 |
| *P. theicola* | CBS 117166; CPC 5415; R120AB | Boskloof, Niewoudtville | High | Branch | 4.75b–d | J.C. Janse van Rensburg | DQ286260, DQ286286 |
| | CBS 117501; CPC 5422; R353AH | Uitsig, Agter-Pakhuis | Low | Branch | 5.39b–c | J.C. Janse van Rensburg | DQ286261, DQ286287 |
| | CPC 5424; R374AP | Snorkfontein, Gifberg | High | Branch | 3.72c–f | J.C. Janse van Rensburg | DQ286262, DQ286288 |
| Control | | | | | 0.00l | | |

[1]CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC & R: Culture collection of Pedro Crous, housed at CBS.

[2]Low = 180–200 mm/yr; High = 250–400 mm/yr.

[3]Values in a column followed by the same letter do not differ significantly (P = 0.05).

[4]EF: partial elongation factor 1-alpha gene; ITS: internal transcribed spacer region.

**Sequence Analysis**

Mycelium was grown on PDA plates and isolated using the protocol of Lee & Taylor (1990). PCR amplification and sequencing of the ITS rDNA, as well as partial EF1-α gene introns and exons, were performed as described by Van Niekerk *et al*. (2004). Newly generated sequences have been deposited in GenBank (Table 1) and the alignment in TreeBASE (S1506, M2708).

Sequences were manually aligned using Sequence Alignment Editor v. 2.0a11 (Rambaut 2002). Additional sequences were obtained from GenBank and added to the alignment. In the phylogenetic trees, downloaded sequences are labelled with GenBank accession numbers; newly generated sequences are indicated with strain numbers. Two datasets were created and analysed using PAUP v. 4.0b10 (Swofford 2002) as described by Van Niekerk *et al*. (2004).

**Taxonomy**

Strains were grown under continuous near-ultraviolet light (400–315 nm) (Sylvania Blacklight-Blue, Osram Nederland B.V., Alphen aan den Rijn, The Netherlands) at 25 °C. Media used were PDA, and 2 % water agar containing pieces of autoclaved *Aspalathus* twigs using 9 cm diam Petri dishes. Growth rates and colony diameters of cultures incubated in darkness were measured on PDA. Structures were mounted in lactic acid, and 30 measurements at × 1000 magnification were made of each structure. The 95 % confidence levels were determined, and the extremes of spore measurements given in parentheses. Images were taken from slides mounted in lactic acid. Macroscopic characters of colonies were described after 14 d using the colour charts of Rayner (1970).

**Pathogenicity**

The 28 isolates used in the molecular studies were also used to conduct the pathogenicity trials. Rooibos plants were cultivated for 8 mo in 18 cm diam pots in a pasteurised sand : soil : perlite medium (1 : 1 : 1) (3 plants per pot). Plants were maintained in a glasshouse at 25 ºC (night) and 30 ºC (day) temperature, and watered three times a week. Nitrosol (Fleuron) (Universal selected services, Braamfontein, S.A.) fertiliser was applied every second week at 200 mL/pot. Colonised PDA agar plugs (5 mm diam) of the respective isolates were used to inoculate plants (three pots per isolate, with three plants per pot). Plant stems were trimmed to a uniform length of 20 cm. A cut was made 10 cm from the top of the main stem of a rooibos seedling, and an

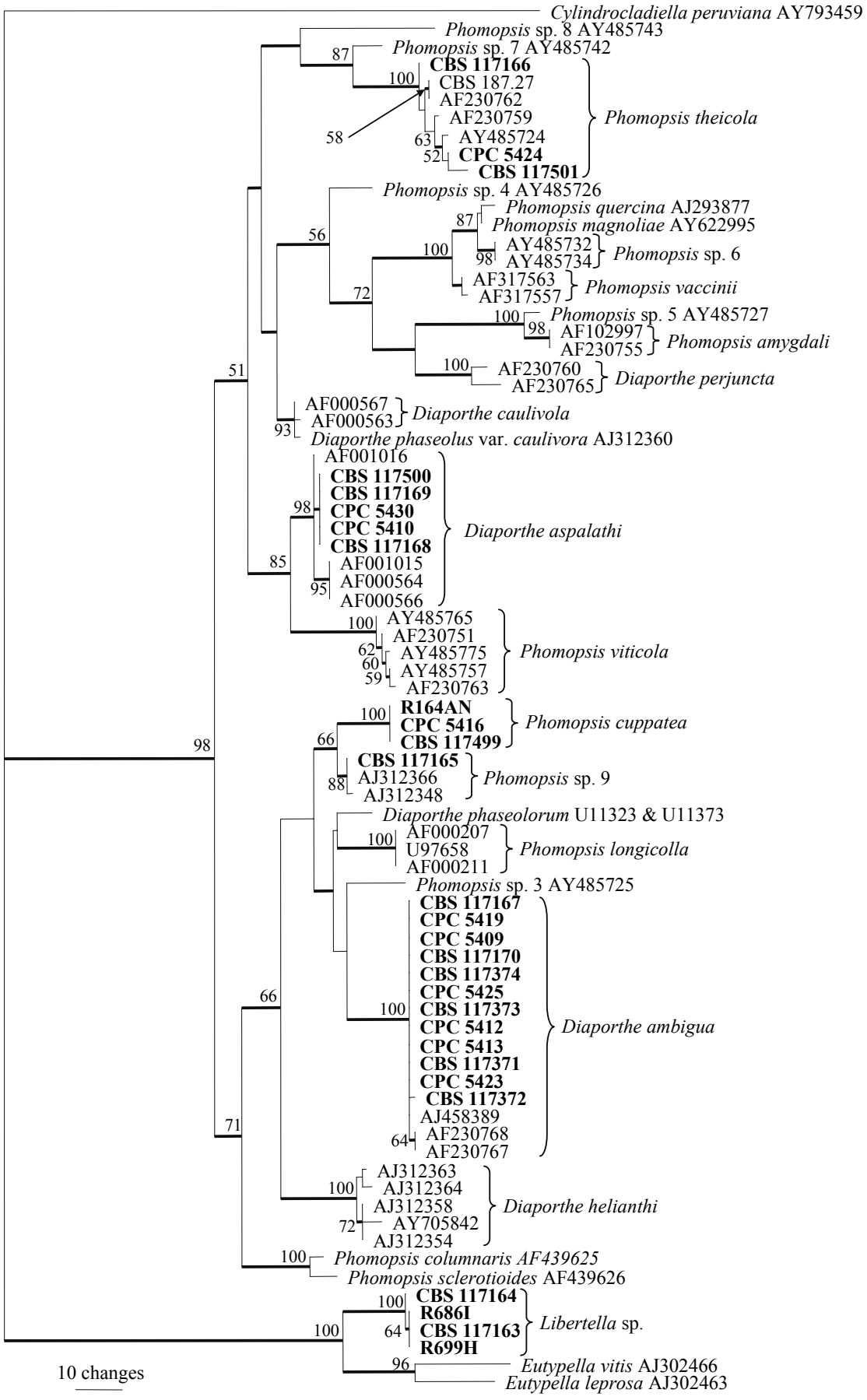


**Fig. 2.** One of 176 equally parsimonious trees obtained from a heuristic search with 10 random addition replicates of the ITS sequence alignment. The scale bar shows ten changes and bootstrap support values from 1000 replicates are shown at the nodes. Thickened lines indicate branches present in the strict consensus tree and isolates obtained from *Aspalathus linearis* are shown in bold print. The tree was rooted to *Cylindrocladiella peruviana*.

agar plug inserted into the cut, and sealed with Parafilm. Three months after inoculation, plants were evaluated for disease symptoms, and lesion length measured. Survival of plants was also recorded. Re-isolations were made from plant material with disease symptoms onto PDA amended with 0.02 % novostreptomycin. A standard one-way analysis of variance was performed on these data using SAS statistical software v. 6.08 (SAS Institute, Cary, NC). The Shapiro-Wilk test was performed to test for normality (Shapiro & Wilk 1965). There was no evidence against normality and the original data were analysed. Student's t-least significant differences were calculated at the 5 % level to compare ranked means.

## RESULTS

### Sequence Analysis

Approximately 510 and 340 bases were sequenced for ITS and EF1-α, respectively. As EF1-α sequences were not available for the taxa for which ITS sequences could be downloaded from GenBank, a tree was generated for all of them using only ITS sequences (Fig. 2). A partition homogeneity test did not indicate significant incongruence between the two genes (P = 0.7630) and the two genes were combined into a single alignment for isolates with both gene sequences (Fig. 3). Using different outgroups did not change the clades presented in Figs 2–3; nor did analyses using 1000

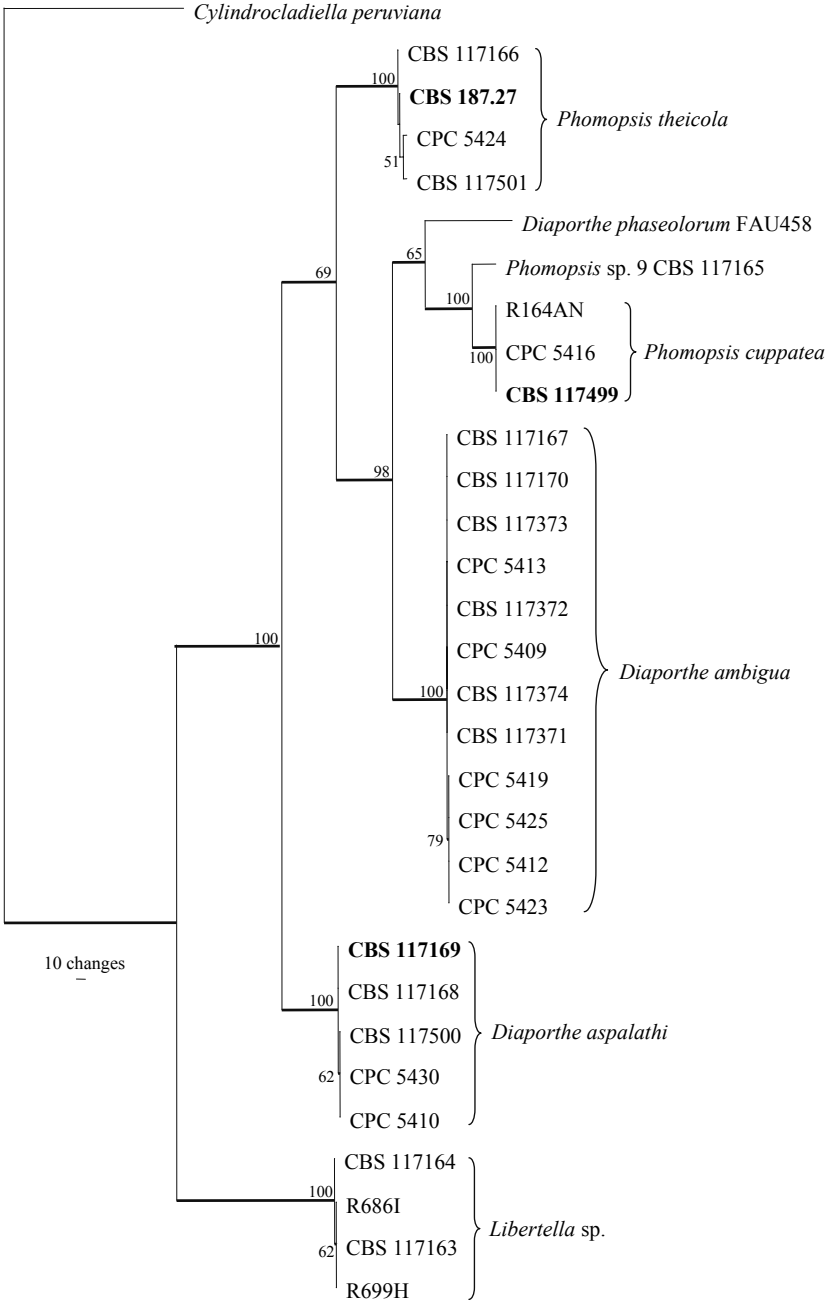


**Fig. 3.** One of eight most parsimonious trees obtained from a heuristic search with 10 random addition replicates of the combined ITS and EF1-α alignment. The scale bar shows ten changes; bootstrap support values from 1000 replicates are shown at the nodes. Thickened lines indicate the strict consensus branches and type strains are shown in bold print. The tree was rooted to *Cylindrocladiella peruviana* (ITS: AY793459; EF: AY725736).

random taxon additions change the number of trees found or the scores calculated (data not shown).

The ITS data matrix contains 78 taxa (including the outgroup) and 353 positions including alignment gaps (the sequence of the 5.8S rDNA gene of *Diaporthe phaseolorum* strain FAU458 was not available on GenBank and this region was therefore excluded from the analysis). Only the sequences of the *Eutypella* and *Libertella* species were variable in this excluded region. Of these characters, 191 are parsimony-informative, 49 are variable and parsimony-uninformative, and 113 are constant. Neighbour-joining analysis using substitution models representative of three different assumptions (uncorrected "p", Kimura-2-parameter and HKY85) on the sequence data yielded trees with similar topology and bootstrap values. Parsimony analysis of the alignment yielded 176 equally parsimonious trees (Fig. 2; TL = 698 steps; CI = 0.626; RI = 0.896; RC = 0.561), most of which differed only in the order of taxa within terminal clades. The same terminal clades were found in both the parsimony and neighbour-joining trees, although the order of branching was not always congruent (data not shown). Isolates from *Aspalathus linearis* are present in six clades (Fig. 2). The first clade (100 % bootstrap support) is identified as *P. theicola* Curzi, containing sequences from the ex-type strain CBS 187.27. The second clade (98 % bootstrap support) is identified as *D. aspalathi* sp. nov. and contains isolates from *A. linearis*, as well as isolates from *Glycine max*. The third and fourth clade containing *Aspalathus linearis* isolates are described as *P. cuppatea* sp. nov., and *Phomopsis* sp. 9, with bootstrap support values of 100 % and 88 %, respectively. Two sequences obtained from GenBank from *Helianthus annuus* isolates group together with the *A. linearis* isolate in the *Phomopsis* sp. 9 clade. *Aspalathus linearis* isolates in the final two clades belong to *Diaporthe ambigua* (containing GenBank sequence AF230767 of the ex-epitype; 100 % bootstrap support) and a *Libertella* sp. (100 % bootstrap support). The *Libertella* Desm. species groups with 100 % bootstrap support with sequences of *Eutypella* (Nitschke) Sacc. species obtained from GenBank.

The combined data matrix contains 31 taxa (including the outgroup) with 755 positions including alignment gaps. Of these characters, 438 are parsimony-informative, 142 are variable and parsimony-uninformative, and 175 are constant. Parsimony analysis of the alignment yielded eight most parsimonious trees (TL = 1196 steps; CI = 0.823; RI = 0.931; RC = 0.766), one of which is shown in Fig. 3. The same six species that were identified in the ITS tree (Fig. 2) were found in the analysis of the combined dataset. In all cases where multiple isolates are available, the bootstrap support for the species is 100 %. Neighbour-joining analysis using three substitution models (uncorrected "p", Kimura 2-parameter and HKY85) on the sequence data yielded trees with similar topology and bootstrap values, except for the position of the *Libertella* Desm. sp. clade which was placed as a sister clade to *D. aspalathi* sp. nov. in the Kimura 2-parameter (57 % bootstrap support) and HKY85 (59 % bootstrap support) analyses (data not shown). The trees obtained using neighbour-joining differed from those obtained using parsimony only with respect to the branching of the deeper nodes, which are not supported by bootstrap analysis (data not shown).

**Taxonomy**

***Diaporthe ambigua*** Nitschke, in Nitschke, *Pyrenomycetum Germanicum*: 311. 1867. Fig. 4.

*Anamorph*: *Phomopsis ambigua* (Sacc.) Traverso, Fl. Ital. Cryptog., Pars 1: Fungi. *Pyrenomycetae. Xylariaceae, Valsaceae, Ceratostomataceae* 2(1): 266. 1906.

≡ *Phoma ambigua* Sacc. Grevillea 2: 91. 1880.

*Perithecia* globose, solitary to aggregated, up to 500 µm diam. *Perithecial neck* dark brown to black, subcylindrical, smooth, tapering towards the apex, up to 1000 µm long, 250 µm wide at the base, 70 µm wide at the apex; ostiole red-brown, obtusely rounded. *Asci* unitunicate, cylindrical–clavate with a refractive apical ring, 8-spored, biseriate, 50–60(–65) × 7–8(–9) µm. *Paraphyses* constricted at the septa, unbranched, tapering towards the apex with a rounded tip, extending above the asci, up to 150 µm long, and up to 7 µm wide at the base, and 3–4 µm wide at the apex. *Ascospores* hyaline, smooth, fusoid–ellipsoidal, widest just above the septum, tapering towards both ends, medianly septate, constricted at the septum at maturity, with 1–2 guttules per cell, (12–)13–15 × (3–)3.5–4 µm. *Pycnidia* formed on PDA and on *Aspalathus* twigs. *Conidiophores* subcylindrical, branched below or unbranched, 0–1-septate, 15–45 × 2–3 µm. *Alpha-conidia* ellipsoidal, biguttulate, with an obtuse apex, tapering to an obtuse or bluntly rounded base with a visible scar, 6–7(–8) × 2(–3) µm, corresponding to the dimensions reported for the anamorph (Uecker 1988). *Beta-conidia* not seen. Description based on CBS 114015 = CPC 2657; cultures homothallic.

*Cultural characteristics*: Colonies on OA olivaceous-black, spreading with patches of white, with sparse aerial mycelium and cream conidial masses; colonies on PDA flat, spreading, with sparse, white, dense aerial mycelium; surface with solid patches of olivaceous-black in the central part; outer region dirty-white to cream; aerial mycelium sparse, consisting of a dense layer of dirty white to cream mycelium; reverse with solid, iron-grey patches in the central part, also with isolated patches in the outer region, surrounded by cream areas.

*Specimens examined*: **Germany**, Nordrhein-Westfalen, Landkreis Unna, on *Pyrus communis*, Th. Nitschke, Aug. 1866, **holotype** in B. **South Africa**, on *Pyrus communis*, S. Denman, CBS H-19685, **epi-type designated here,** culture ex-epitype CBS 114015 (= AF230767).

*Notes*: As shown in the present study, the host range of *D. ambigua* is wider than originally suspected by Nitschke (1867), but not as extreme as stated by Wehmeyer (1933). The type specimen is depauperate, containing perithecia of a *Pleospora* sp. and *Togninia minima*, and some remnants of *Diaporthe ambigua*. A few ascospores were observed, 12–15 × 3.5–4 µm, that were constricted at the median septum, and guttulate. *Diaporthe ambigua* was originally described

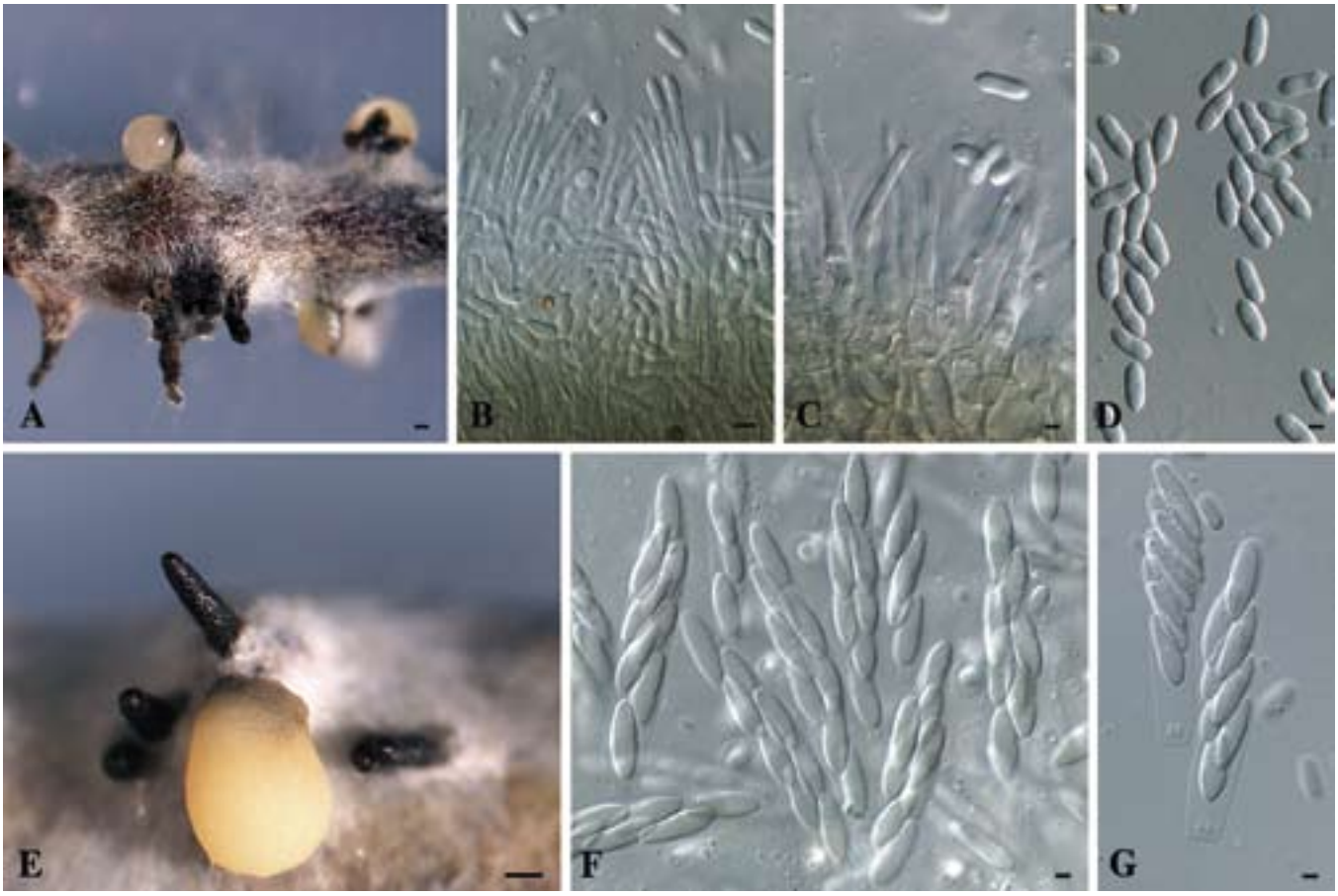


**Fig. 4.** *Diaporthe ambigua* (epitype). A. Pycnidia forming on *Aspalathus* stems in culture. B–C. Conidiophores. D. Alpha-conidia. E. Perithecia. F–G. Asci with ascospores. Scale bars: A, E = 70 µm, B = 4 µm, C–D, F–G = 2 µm.

from cankers on pear in Germany (Nitschke 1867), and the name was subsequently used for the organism causing cankers on apples, pears and plums in South Africa (Smit *et al*. 1996).

***Diaporthe aspalathi*** Janse van Rensburg, Castlebury & Crous, **nom. et. stat. nov.** MycoBank MB500803. Fig. 5.

≡ *Diaporthe phaseolorum* var. *meridionalis* F.A. Fernández, Mycologia 88: 438. 1996. [non *D. meridionalis* Sacc., Syll. Fung. I: 638. 1878].

*Etymology*: Named after *Aspalathus*, on which it causes a prominent die-back disease.

*Perithecia* globose, solitary, scattered to aggregated, up to 500 µm wide. *Perithecial neck* black, cylindrical, mostly smooth, but tapering near the apex, up to 800–1000(–2000) µm long, 150 µm wide at the base, 90 µm wide at the apex; ostiole widening once spores discharge, 90–130 µm wide. *Asci* unitunicate, cylindrical with a refractive apical ring, 8-spored, biseriate, 52–55(–60) × 7–8(–10) µm. *Paraphyses* septate, unbranched, tapering towards the apex with a rounded tip, extending above the asci, up to 110 µm long, and up to 8 µm wide. *Ascospores* hyaline, smooth, fusoid, widest at the septum, tapering towards both ends, medianly septate, not constricted at the septum, with 1–2 guttules per cell, (12–)13–15(–16) × 3(–3.5) µm. *Pycnidia* formed on PDA and on *Aspalathus* twigs. *Alpha-conidia* biguttulate, fusoid with obtuse ends, (6–)7–8(–9) × (2–)2.5(–3) µm. *Beta-* and *gamma-conidia* absent. Description based on CBS 117169; cultures homothallic.

*Cultural characteristics*: On OA flat, spreading with sparse to no aerial mycelium; surface with irregular patches of pale white to cream and olivaceous-grey, with sparse strands of pale white aerial mycelium; on PDA flat, spreading, with sparse to no aerial mycelium; surface smoke-grey; reverse smoke-grey to olivaceous-grey.

*Specimen examined*: **South Africa**, Western Cape Province, Clanwilliam, Langebergpunt, on *Aspalathus linearus*, J. Janse Van Rensburg, CBS H-19686, culture CBS 117169.

*Notes*: Isolates (see Table 1) readily produce perithecia on PDA and on *Aspalathus* twigs. *Diaporthe phaseolorum* var. *meridionalis*, which was described as causing soybean stem canker in the South-eastern U.S.A. (Fernández & Hanlin 1996), is not closely related to *D. phaseolorum* as earlier expected. Although morphologically similar, this species clusters apart from the reference strain of *D. phaseolorum* (Figs 2–3). *Diaporthe phaseolorum* var. *meridionalis* is also the main causal organism of canker and die-back of rooibos, and not *D. phaseolorum* as reported earlier (Smit & Knox-Davies 1989a, b). The name *D. meridionalis* Sacc. (1878) is preoccupied, and represents a species similar to *D. eres* Nitschke (Wehmeyer 1933), and hence a new name, *D. aspalathi* is proposed here for the species pathogenic to *Aspalathus* and soybean.

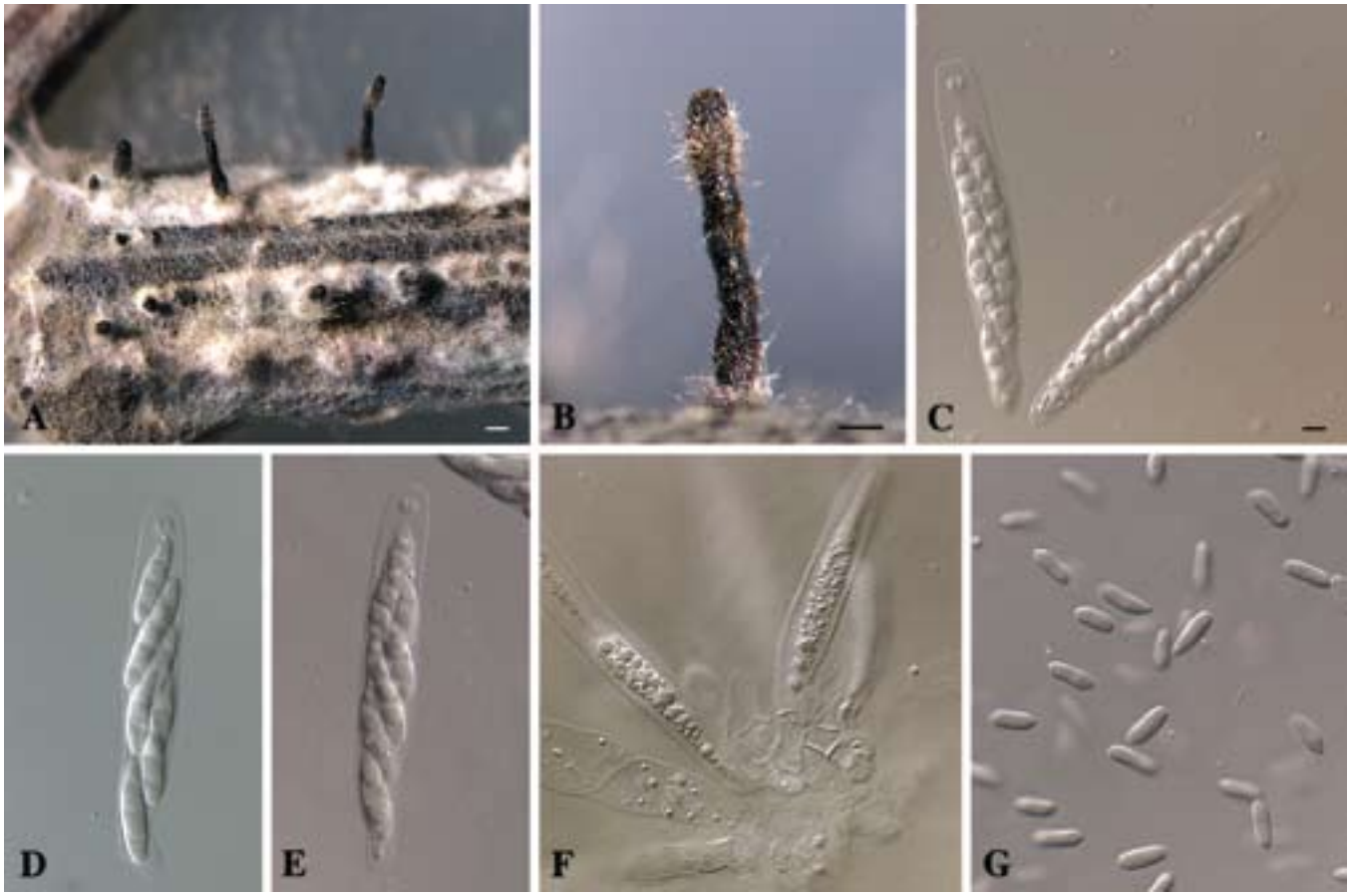


**Fig. 5.** *Diaporthe aspalathi* (CBS 117169). A–B. Perithecia. C–F. Asci and ascospores. G. Conidia. Scale bars: A = 150 µm, B = 70 µm, C = 3 µm.

***Phomopsis cuppatea*** Janse van Rensburg, Lamprecht & Crous, **sp. nov.** MycoBank MB500804. Fig. 6.

*Etymology*: Named after the primary use of the host substrate, which is to make "rooibos" tea.

Conidiophora cylindrica, 1–3-septata, 30–80 × 3–5 µm. Cellulae conidiogenae rectae vel curvatae, in collare modice distensum ad 3 µm longum exeuntes, exigue periclinaliter inspissatae, 10–35 x 1.5–2 µm. Alpha-conidia fusoidea–ellipsoidea, sursum hebeter rotundata, ad basim obtusa vel subtruncata, bi- vel multiguttulata, (10–)12–13(–14) × (3–)4(–5) µm.

*Pycnidia* eustromatic, black, scattered or aggregated, globose to conical, convulated to unilocular, singly ostiolate, up to 400 µm wide; pycnidial wall consisting of brown, thick-walled cells of *textura angularis*; conidial mass globose, pale-luteous to cream. *Conidiophores* cylindrical, noticeably flexuous and tall, well-developed, branched above or below, 1–3-septate, 30–80 × 3–5 µm. *Conidiogenous cells* straight to curved, tapering slightly towards the apex, collarettes slightly flaring, up to 3 µm long, with minute periclinal thickening, 10–35 × 1.5–2 µm. *Alpha-conidia* fusoid–ellipsoidal, apex bluntly rounded, base obtuse to subtruncate, bi- to multiguttulate, (10–)12–13(–14) × (3–)4(–5) µm; *beta-* and *gamma-conidia* not observed. Description based on CBS 117499.

*Cultural characteristics*: Colonies on OA flat, spreading, with sparse, dirty white aerial mycelium; surface with irregular patches of olivaceous-black and pale olivaceous-grey; on PDA flat, spreading, with sparse aerial mycelium; surface smoke-grey to pale olivaceous-grey; reverse smoke-grey.

*Specimen examined*: **South Africa**, Western Cape Province, Clanwilliam, Kossakse werf, on *Aspalathus linearis*, J. Janse van Rensburg, CBS H-19687, culture CBS 117499.

***Phomopsis* sp. 9**

*Cultural characteristics*: Colonies on OA flat, spreading with sparse dirty-white aerial mycelium; surface and reverse with diffuse patches of fuscous-black and dirty-white; colonies on PDA flat, spreading, with sparse, dirty-white aerial mycelium at the edge of the dish; surface and reverse having a translucent to ochreous central part; outer region umber. Description based on CBS 117165.

*Notes*: When CPC 5417 was deposited in the CBS collection as 117165, it was sterile, and thus could not be named in the present study. Connecting to the numbering system used by Van Niekerk *et al.* (2005), it is thus referred to as *Phomopsis* sp. 9. Two ITS sequences obtained from GenBank represent strains isolated from *Helianthus annuus*, and grouped together with this isolate, proving that this species may have a wider host range than just *Aspalathus*.

**Pathogenicity**

In inoculation experiments, the longest lesions were observed for isolates of *D. aspalathi*, which proved to be the most virulent species. Significantly shorter

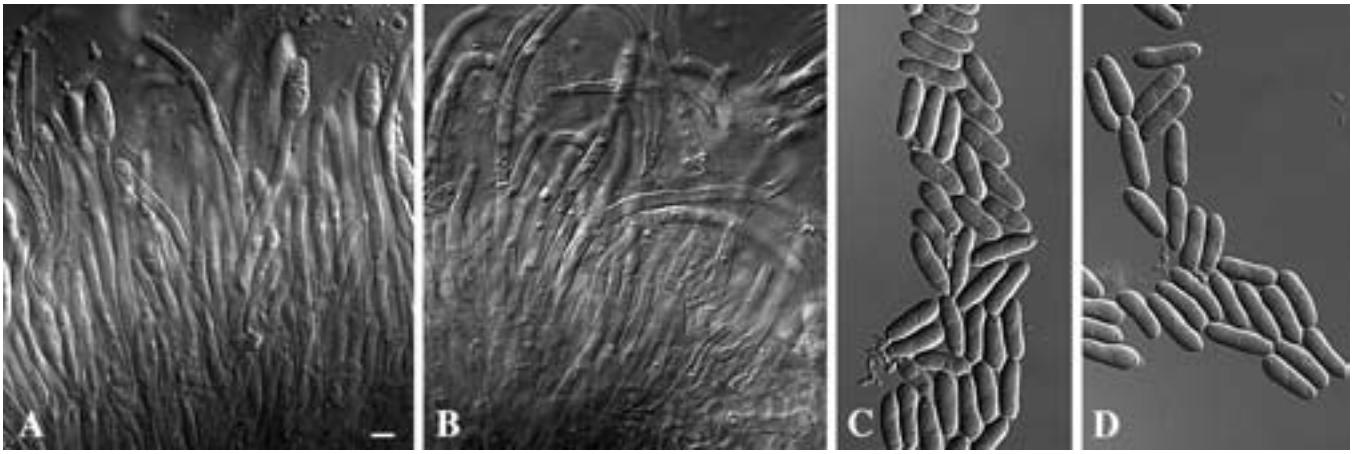


**Fig. 6.** *Phomopsis cuppatea* (holotype). A–B. Conidiophores. C–D. Conidia. Scale bar = 4 µm.

lesions were observed for isolates of the other taxa tested (P = 0.05) (Table 1).

Three months after inoculation, 95.56 % of plants inoculated with isolates belonging to *D. aspalathi* were dead, followed by 25.71 % of plants inoculated with *P. theicola*, and 16.67 % of plants inoculated with the *Libertella* sp. Only 14.14 % of plants inoculated with isolates of *D. ambigua* died. The new species, *P. cuppatea* and *Phomopsis* sp. 9, proved to be the least virulent, and in both cases only 8.33 % of the inoculated plants died. All inoculated taxa could be successfully re-isolated from inoculated plants, which in many cases ended up with dead tissue being covered in pycnidia and perithecia. None of the controls died, or showed any disease symptoms.

## DISCUSSION

Contrary to earlier reports which were based on morphological observations alone (Smit & Knox-Davies 1989a, b), the current study has revealed that up to five *Phomopsis* spp. are involved with die-back of rooibos bushes, while a sixth *Phomopsis*-like taxon proved to be better accommodated in *Libertella*, which clustered among *Eutypella* teleomorphs.

Mostert *et al*. (2001) isolated a species of *Phomopsis* from grapevines, which also proved to be present on *Protea* and *Pyrus* in countries such as Australia, Portugal and South Africa. As no name could be attributed to this species it was eventually referred to as Taxon 3. The same species was again encountered in the *Phomopsis* study on grapevines by Van Niekerk *et al*. (2005), where 15 species were distinguished, and taxon 3 was referred to as *Phomopsis* sp. 1. In the current study we finally managed to identify this species, as its ITS DNA sequence is identical to that of the ex-type strain of *P. theicola* Curzi (CBS 187.27), which was originally described from *Camellia sinensis* in Italy (Uecker 1988). This species obviously has a wide host range and distribution, which once again underlines the difficulties mycologists encounter when trying to identify species of *Phomopsis*.

Several isolates which also formed a *Diaporthe* state in culture, proved to be identical to *D. phaseolorum* var. *meridionalis* based on morphology and sequence data. Although this pathogen was originally identified as *D. phaseolorum* by Smit & Knox-Davies (1989a, b), the reference strain available to us of *D. phaseolorum* (Figs 3–4) (treated as authentic by F.A. Uecker and preserved at BPI), clustered apart from the *Aspalathus* pathogen. Furthermore, as *D. phaseolorum* var. *meridionalis* is clearly not a variety of *D. phaseolorum*, and as the name *D. meridionalis* is already preoccupied, a new name is proposed for this pathogen as *D. aspalathi*.

Another species which proved to be very common on *Aspalathus* matched GenBank sequences for *Diaporthe ambigua*. *Diaporthe ambigua* was originally described from branches of *Pyrus communis* from Germany, and was later associated with a *Diaporthe* canker of apple, pear and plum rootstocks in South Africa (Smit *et al.* 1996). To reduce any further confusion surrounding this name, we have thus chosen to designate an epitype specimen and ex-epitype culture in the present study, from which DNA sequence data are derived.

Two new *Phomopsis* spp. were also encountered during the current study, namely *P. cuppatea*, and *Phomopsis* sp. 9. As the culture of the latter proved to be sterile, further collections are required before its taxonomy can be resolved.

Several isolates of a *Libertella* sp. were also isolated from *Aspalathus* during the present study. Although *Libertella* is an anamorph of *Eutypella* and *Eutypa* Tul. & C. Tul. and this taxon grouped with *Eutypella* spp. known from GenBank sequence data, no teleomorph was ever observed on host material or induced in culture.

Lesions and pycnidia formed faster downwards than upwards on stems of inoculated plants. Previous reports stated that after infection of blueberry twigs, hyphae of *Phomopsis* spp. move to the stem cortex (Daykin & Milholland 1990). Movement through the stem cortex takes place through the intercellular spaces, and intracellularly through the parenchyma of the outer cortex. Only after the cortex has been completely colonised, does the fungus invade the vascular tissue and pith (Daykin & Milholland, 1990). *Phomopsis* infection of sunflower follows the same pattern where, after penetration in the host, infection hyphae invade the intercellular spaces in the cortex (Muntañola-Cvetković

*et al*. 1981). Xylem elements are invaded, but affected less than the phloem and parenchyma tissues which disintegrate completely (Muntañola-Cvetković *et al*. 1981). Daykin & Milholland (1990) suggested that the formation of vast numbers of tyloses inside the xylem in advance of infection together with phloem plugging due to gums and hyphae, causes the lesions associated with die-back. From these masses of hyphae pycnidia are initiated (Muntañola-Cvetković *et al*. 1981). Tyloses form in xylem vessels of most plants under various conditions of stress and during invasion by a pathogen (Agrios 2004). Formation of these structures is an attempt by the plant to close off invaded cells to limit fungal movement in the plant (Sinclair *et al.* 1987). This shutting down of infected vascular tissues reduces the flow of water from the roots upward. At this point, reduced water flow and toxins often result in external symptoms (Sinclair *et al.* 1987). In rooibos these external symptoms are twig die-back, reduction in biomass and eventually plant death.

Although the pathogenicity data obtained in the present study are still preliminary, and need to be confirmed in the field, they clearly show that *D. aspalathi* was the most virulent taxon, producing the longest stem lesions and also causing the most plant death. In contrast, the *Libertella* sp. hardly caused any tissue discoloration. The latter could be due to the fact that symptoms were rated after 3 mo, and that species from the *Eutypa/Eutypella* complex, as observed in grapevines, generally take much longer for symptom expression. Further studies will be necessary, however, to fully resolve the phylogenetic status of the various other *Phomopsis* spp. associated with die-back of *Aspalathus*, which in this study appeared to be of less importance than *D. aspalathi*.

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