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.

**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* (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.

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

| Species     | Strain no. | Farm, area                  | Rainfall | Plant part | Lesion length (cm) | Collector                  | GenBank numbers | EF, ITS |
|-------------|------------|-----------------------------|----------|------------|--------------------|----------------------------|-----------------|---------|
| *D. ambigua*| CBS 117167; CPC 5414; R86AM | Taibosdam, Gifberg          | High     | Crown     | 3.44d–g           | J.C. Janse van Rensburg    | DQ286237, DQ286263 |         |
|             | CBS 117170; R59O        | Vaalkrans, Nardousberg      | 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  | Karmemelksvlei, Citrusdal   | 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         | Taibosskraal, Agter-Pakhuis | Low      | Root      | 0.61j–l           | J.C. Janse van Rensburg    | DQ286244, DQ286270 |         |
|             | CPC 5413; R78U         | Nardouw, Nardousberg       | Low      | Crown     | 0.94i–l           | J.C. Janse van Rensburg    | DQ286245, DQ286271 |         |
|             | CPC 5419; R337AR       | Vaalkrans, Nardousberg     | Low      | Branch    | 3.06d–h           | J.C. Janse van Rensburg    | DQ286246, DQ286272 |         |
|             | CPC 5423; R366A        | Taibosdam, Gifberg         | High     | Branch    | 2.25e–j           | J.C. Janse van Rensburg    | DQ286247, DQ286273 |         |
|             | CPC 5425; R379S        | Vondeling, Nardousberg     | Low      | Branch    | 2.25e–j           | J.C. Janse van Rensburg    | DQ286248, DQ286274 |         |
| *D. aspalathi*| CBS 117168; CPC 5420; R338E | Vaalkrans, Nardousberg    | 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 |         |
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).

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

Table 1. (Continued).

| Species                  | Strain no.¹ | Farm, area       | Rainfall² | Plant part | Lesion length (cm)³ | Collector                      | GenBank numbers (EF, ITS)⁴ |
|--------------------------|-------------|------------------|-----------|------------|---------------------|-------------------------------|-----------------------------|
| **Libertella sp.**       | CBS 117163; CPC 5426; R380Z | Vondeling, Nardouswberg | Low       | Branch     | 1.81g–k            | J.C. Janse van Rensburg       | DQ286253, DQ286279         |
| **CBS 117164; CPC 5429; R424T** | Koelfontein, Clanwilliam | High       | Branch     | 2.64e–i    | J.C. Janse van Rensburg       | DQ286254, DQ286280         |
| **R586I**                | 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       | DQ286257, DQ286274         |
| **CPC 5416; R162AO**     | Berg-en-dal, Citrusdal | High       | Crown      | 3.94b–f    | J.C. Janse van Rensburg       | DQ286258, DQ286283         |
| **R164AN**               | Karmemelkslei, Citrusdal | High       | Branch     | 1.61h–l    | J.C. Janse van Rensburg       | DQ286259, 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         |
| **CBS 117166; CPC 5415; R120AO** | Boskloof, Niewoudtville | High       | Branch     | 4.75b–d    | J.C. Janse van Rensburg       | DQ286260, DQ286296         |
| **CBS 117501; CPC 5422; R353AH** | Uitsig, Agter-Pakhuys | Low       | Branch     | 5.39b–c    | J.C. Janse van Rensburg       | DQ286261, DQ286297         |
| **CPC 5424; R374AP**     | Snorkfontein, Gifberg | High       | Branch     | 3.72c–f    | J.C. Janse van Rensburg       | DQ286262, DQ286288         |
| **Control**              |              |                  |           |            | 0.00l               |                               |                             |

¹CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC & R: Culture collection of Pedro Crous, housed at CBS.
²Low = 180–200 mm/yr; High = 250–400 mm/yr.
³Values in a column followed by the same letter do not differ significantly (P = 0.05).
⁴EF: partial elongation factor 1-alpha gene; ITS: internal transcribed spacer region.
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*.

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**Phomopsis theicola**

**Phomopsis sp. 7** AY485742

CBS 117166

AF187272

AF230759

AY485724

CPC 5424

CBS 117501

**Phomopsis sp. 4** AY485726

**Phomopsis quercina** AJ293877

**Phomopsis magnoliae** AY622995

AY485732

AY485734

**Phomopsis vaccinii**

AF317563

AF317557

**Phomopsis sp. 5** AY485727

AF106997

AF230755

**Phomopsis amygdali**

**Diaporthe caulivola**

**Diaporthe phaseolus var. caulivora** AJ312360

AF0001016

**Diaporthe aspalathi**

**Diaporthe phaseolus var. caulivora**

CPC 5410

CPC 5416

CBS 117499

AF001015

AF000564

AF000566

**Phomopsis viticola**

AF104765

AF230751

AF230755

**Phomopsis cuppatea**

**Diaporthe phaseolorum** U11323 & U11373

**Phomopsis longicolla**

**Diaporthe ambigua**

**Diaporthe helianthi**

**Phomopsis columnaris** AF439625

**Phomopsis sclerotioides** AF439626

**Eutypella vitis** AJ302466

**Eutypella leprosa** AJ312358
agiar 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).](image-url)
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 described as representative of three different assumptions. Two sequences obtained from GenBank and this region was therefore excluded from the analysis). Only the sequences of the Anamorph Phomopsis ambigua (Sacc.) Traverso, Fl. Ital. Cryptog., Pars 1: Fungi. Pyrenomycetaceae. Xylariaeaceae, Valsaceae, Ceratostomataceae 2(1): 266. 1906.

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 unilunulate, 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 creamy 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-19865, epithe type designated here, culture ex-epitype CBS 114015 (= AF230767).

Notes: As shown in the present study, the host range of D. ambigu a 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 ambigu a. A few ascospores were observed, 12–15 × 3.5–4 µm, that were constricted at the median septum, and guttulate. Diaporthe ambigu a was originally described
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. erez* Nitschke (Wehmeyer 1933), and hence a new name, *D. aspalathi* is proposed here for the species pathogenic to *Aspalathus* and soybean.
Phomopsis cuppatea Janse van Rensburg, Lamprecht & Crous, sp. nov. MycoBank MB 500804. 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 exeuntis, exigue periclinaliter inspissatae, 10–35 × 1.5–2 µm. Alpha-conidia fusoida–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, convoluted 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 multiguttulata, (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 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
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 ambiguа. Diaporthe ambiguа* 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. cuppateа*, 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 (Muntañolа-Cvetković...
Xylem elements are invaded, but affected less than the phloem and parenchyma tissues which disintegrate completely (Muntañola-Cvetković et al. 1981). Daykin & Miholland (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 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. aspalathii.

REFERENCES

Agrios GN (2004). Plant Pathology. 3rd ed. Elsevier Academic Press, Netherlands.

Daykin ME, Miholland RD (1990). Histopathology of blueberry twig blight caused by Phomopsis vaccini. Phytopathology 80: 736–740.

Kulik MM (1984). Symptomless infection, persistence, and production of pycnidia in host and non-host plants by Phomopsis batatae, Phomopsis phaseoli, and Phomopsis sojae, and the taxonomic implications. Mycologia 76: 274–291.

Lee SB, Taylor JW (1990). Isolation of DNA from fungal mycelia and single spores. In: PCR protocols: a guide to methods and applications. (Innis MA, Gelfand DA, Sninsky JJ, White TJ, eds). Academic Press, San Diego, CA, U.S.A.: 282–287.

Mostert L, Crous PW, Kang J-C, Phillips AJL (2001). Species of Phomopsis and a Libertella sp. occurring on grapevines with specific reference to South Africa: morphological, cultural, molecular and pathological characterization. Mycologia 93: 146–167.

Muntañola-Cvetković M, Mihaličević M, Petrov M (1981). On the identity of the causative agent of a serious Phomopsis-Diaporthe disease in sunflower plants. Nova Hedwigia 34: 417–435.

Niekerk JM van, Groenewald JZ, Farr DF, Fourie PH, Halleen F, Crous PW (2005). Reassessment of Phomopsis species on grapevines. Australasian Plant Pathology 34: 27–39.

Niekerk JM van, Groenewald JZ, Verkley GJ, Fourie PH, Wingfield MJ, Crous PW (2004). Systematic reappraisal of Coniella and Pilidiella, with specific reference to species occurring on Eucalyptus and Vitis in South Africa. Mycological Research 108: 283–303.

Rambaut A (2002). Sequence Alignment Editor. Version 2.0. Department of Zoology, University of Oxford, Oxford.

Rayner RW (1970). A mycological colour chart. CMI and British Mycological Society. Kew, Surrey, England.

Shapiro SS, Wilk MB (1965). An analysis of variance test for normality (complete samples). Biometrika 52: 591–611.

Sinclair WA, Lyon HH, Johnson WJ (1987). Diseases of Trees and Shrubs. Cornell University Press. U.S.A.

Smit WA, Knox-Davies PS (1989a). Die-back of rooibos tea caused by Diaporthe phaseolorum. Phytophylactica 21: 183–188.

Smit WA, Knox-Davies PS (1989b). Comparison of Diaporthe phaseolorum isolates from rooibos tea, Aspalathus linearis. Phytophylactica 21: 301–306.

Smit WA, Viljoen CD, Wingfield BD, Wingfield MJ, Calitz FJ (1996). A new canker disease of apple, pear, and plum rootstocks caused by Diaporthe ambigua in South Africa. Plant Disease 80: 1331–1335.

Swoford DL (2002). PAUP 4.0b10: Phylogenetic analysis using parsimony. Sinauer Associates, Sunderland, MA, U.S.A.

Niekerk JM van, Groenewald JZ, Farr DF, Fourie PH, Halleen F, Crous PW (2005). Reassessment of Phomopsis species on grapevines. Australasian Plant Pathology 34: 27–39.

Niekerk JM van, Groenewald JZ, Verkley GJ, Fourie PH, Wingfield MJ, Crous PW (2004). Systematic reappraisal of Coniella and Pilidiella, with specific reference to species occurring on Eucalyptus and Vitis in South Africa. Mycological Research 108: 283–303.

Rambaut A (2002). Sequence Alignment Editor. Version 2.0. Department of Zoology, University of Oxford, Oxford.

Rayner RW (1970). A mycological colour chart. CMI and British Mycological Society. Kew, Surrey, England.

Shapiro SS, Wilk MB (1965). An analysis of variance test for normality (complete samples). Biometrika 52: 591–611.

Sinclair WA, Lyon HH, Johnson WJ (1987). Diseases of Trees and Shrubs. Cornell University Press. U.S.A.