A novel species of *Microsphaeropsis* causing cankers on *Rafnia amplexicaulis* in South Africa

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**Key words:** multigene phylogeny, new taxon, pathogenicity, stem canker

**Abstract:** Cankers leading to branch, stem and plant death were observed on the South African endemic *Rafnia amplexicaulis* (*Fabaceae*) in the Cederberg Wilderness Area, South Africa, during September 2021. Conidiomatal pycnidia were found developing on the cankers, and isolations consistently yielded a *Microsphaeropsis* species. Phylogenetic analysis based on partial nucleotide sequences of the internal transcribed spacers (ITS), the nuclear large subunit (LSU) and RNA polymerase II second largest subunit (*Rpb2*) regions showed that the fungus represented an undescribed species. Based on the multigene phylogeny and morphological characteristics, we describe the species here as *M. rafiae* sp. nov. Pathogenicity tests and the fulfilment of Koch’s postulates confirmed that *M. rafiae* sp. nov. is the cause of the cankers of *R. amplexicaulis*. Presently, this disease is known from a single location in South Africa, and further surveys are required to determine its distribution and relative importance.

**INTRODUCTION**

*Rafnia amplexicaulis* (*Fabaceae*) is a perennial, woody shrub endemic to the Northern and Western Cape Provinces of South Africa. As a resprouter, coppice shoots are produced from an underground lignotuber following fire, resulting in plants being multi-stemmed at ground level (Campbell & van Wyk 2001). The leaves and roots of *R. amplexicaulis* have been utilised as traditional remedies by the local Cape indigenous communities (Kinfe et al. 2015).

During a field visit to the Cederberg mountains in September 2021, yellowing and dying *R. amplexicaulis* shrubs were observed in a single location within the Cederberg Wilderness Area, Western Cape Province, South Africa. Closer inspection revealed girdling cankers present on the symptomatic stems. Fungal structures (pycnidia) characteristic of a *Microsphaeropsis* species were visible on the surface of the cankers.

*Microsphaeropsis* (*Didymellaceae*) was introduced by Von Höhnel (1917) to accommodate pycnidial fungi with small dark asceptate conidia produced from phialides. The genus has a cosmopolitan distribution, with species commonly described as plant endophytes or saprophytes. The genus also contains a number of plant pathogens described from necrotic spots and/ or lesions on leaves and twigs (Swart et al. 1998, Hou et al. 2020).

The aim of this study was to describe the disease occurring on *R. amplexicaulis* and identify its causal agent.

**METHODS AND METHODS**

Disease description and isolations

The diseased *R. amplexicaulis* plants were restricted to an area of approximately 1 000 m² on a south-east facing slope within the Cederberg Wilderness Area, Western Cape Province, South Africa (-32.412743, 19.174894). Shrubs were visibly yellowing, and on closer examination cankers were commonly found on symptomatic stems and branches. In instances where cankers were girdling, these led to stem and branch death (Fig. 1). Removal of the outer bark showed distinct necrosis of the cambium at the leading edges of the lesions. Sections of symptomatic stems were removed from plants, placed in brown paper bags and transported to the laboratory for further examination.

Conidiomatal pycnidia that oozed conidial masses typical of *Microsphaeropsis* species were observed on the surfaces of the cankers. Conidia were lifted from the pycnidia using a sterile hypodermic needle and transferred to the surface of 2 % malt
extract agar (MEA: 20 g Biolab malt extract, 20 g Difco agar, 1 L deionised water) amended with 1 % streptomycin sulphate (Sigma-Aldrich). Cultures were purified by transferring single hyphal tips to fresh MEA plates and maintained at 25 °C.

The resulting cultures were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Lunnion Road, Pretoria, South Africa. The holotype and ex-holotype were deposited in the H.G.W.J. Schweickerdt Herbarium (PRU) and the culture collection of Innovation Africa (CMW-IA), respectively, at the University of Pretoria, Lunnion Road, Pretoria, South Africa.

Morphology

The top part of pycnidial structures were cut with a scalpel. The exposed insides of the structures were moistened with a piece of agar, and conidiogenous cells and conidia were extracted and mounted on slides in water. For measurements, the water was replaced with 85 % lactic acid. Images were captured using Nikon microscopes (Eclipse Ni and SMZ18, Japan) mounted with a DS-Ri2 camera. The image program NIS-Elements BR was used for measurements and taking photos. Bark samples containing pycnidia were cut into small pieces. The pieces were boiled for a few seconds to soften the structures and mounted on a disc with freezing medium. Vertical sections were prepared in 10–12 µm thickness using a cryomicrotome (Leica CM1520, Germany). The sections were mounted in 85 % lactic acid for observation. Fifty conidia were measured and presented as min–max (average ± standard deviation), whereas less than ten structures were measured for conidiogenous cells and conidiomata and presented as min–max, due to the shortage of samples.

Isolate CMW 57792 was used for the growth study and colony morphology. Culture characteristics and growth rates were determined on MEA, potato dextrose agar (PDA; BD Difco) and oatmeal agar (OA; liquid extract of 30 g of oats cooked in 800 mL water for an hour used to make 1 L, 20 g Difco agar). Colours were described using the colour chart of Rayner (1970).

Cultures were grown at seven temperatures, ranging from 5 to 35 °C in 5 °C intervals. At each temperature, five replicates of the isolate were incubated in the dark. Diameters perpendicular to each other were measured after 10 d, when the colony margins reached the edges of the Petri-dishes at optimum temperature. After measuring the diameters, the plates were returned to the incubators for an additional few weeks to observe possible changes with age. An NaOH spot test was performed on a culture grown on OA (Boerema et al. 2004).

DNA isolation, PCR amplification and sequencing

DNA was extracted from 7-d-old isolates grown on 2 % MEA at 25 °C using Prepmen Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s protocols. The nuclear internal transcribed spacer regions 1 and 2 (ITS), including the 5.8S rRNA gene region, were amplified using primers ITS1F and ITS4 (White et al. 1990, Gardes & Bruns 1993); part of the nuclear large subunit (LSU) of ribosomal RNA gene with primers LR0R and LR5 (Vilgalys & Hester 1990, Rehner & Samuels 1994) and a fragment of the DNA-directed RNA polymerase II second largest subunit gene (RPB2) with primer pair RPB2-5F2 and fRPB2-7cR (Liu et al. 1999; Sung et al. 2007). PCR amplifications were prepared following the protocols described by Pham et al. (2019). For ITS and LSU regions, the thermal cycling included an initial denaturation at 95 °C for 5 min followed by 10 primary amplification cycles of 30 s at 95 °C, 30 s at 56 °C, and 60 s at 72 °C, then 30 additional cycles of the same reaction sequence, with a 5 s increase in the annealing step per cycle, and the reactions were completed with a final extension at 72 °C for 10 min. The amplification for RPB2 was performed following the method of Liu et al. (2020). Amplified fragments of all loci were purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Amplicons were sequenced in both directions using an ABI PRISM™ 3100 DNA sequencer (Applied Biosystems, USA) at the Sequencing Facility.
Table 1. Collection details and GenBank accessions of isolates included in the phylogenetic analyses.

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CBS = culture collection of Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CMW = culture collection of the FABI, University of Pretoria, Pretoria, South Africa; CMW-IA = culture collection of Innovation Africa, University of Pretoria, Pretoria, South Africa; CPC = culture collection of Pedro Crous, housed at Westerdijk Fungal Biodiversity Institute; MFLUCC = Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; UPSC: Uppsala University Culture Collection, Sweden.

ITS = internal transcribed spacer regions 1 & 2 including the 5.8S region of the nrRNA; LSU = 28S large subunit of the nrRNA; RPB2 = DNA-directed RNA polymerase II second largest subunit gene.

1Denotes ex-type strain.

Isolates and sequences obtained in this study are indicated in bold.
of the Faculty of Natural and Agricultural Sciences, University of Pretoria, South Africa. Geneious Prime v. 2022.1.1 was used for assembling and editing raw sequences (https://www.geneious.com). All sequences generated in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov) (Table 1).

Phylogenetic analyses

Reference sequences for species closely related to those emerging from this study were downloaded from the GenBank nucleotide database (Table 1). All sequences were aligned using MAFFT v. 7 (http://mafft.cbcr.jp/alignment/server/) (Katoh & Standley 2013), then confirmed manually in MEGA v. 7 (Kumar et al. 2016) where necessary. Maximum likelihood (ML) and Bayesian inference (BI) analyses were performed on the combined dataset of three regions. The most appropriate model was obtained using the software jModeltest v. 1.2.5 (Posada 2008). For ML, analyses were conducted using RAxML v. 8.2.4 on the CIPRES Science Gateway v. 3.3 (Stamatakis 2014) with default GTR substitution matrix and 1 000 rapid bootstraps. For BI, analyses were performed using MrBayes v. 3.2.6 (Ronquist et al. 2012) on the CIPRES Science Gateway v. 3.3. Four Markov chain Monte Carlo (MCMC) chains were run from a random starting tree for five million generations and trees were sampled every 100th generation. The first 25 % of trees sampled were eliminated as burn-in and the remaining trees were used to determine the posterior probabilities. Calophoma parvula (CBS 620.68) was used as the outgroup taxon. Resulting trees were viewed using MEGA v. 7 (Kumar et al. 2016) and FigTree v. 1.4.3 (Rambaut 2010).

Pathogenicity tests

The pathogenicity of the isolated Microsphaeropsis species towards R. amplexicaulis was determined in a natural population of the host plant in the Cedarberg Wilderness Area, Western Cape Province, South Africa (-32.42956; 19.15981). Inoculations were initiated during spring (October) of 2021 using two isolates (CMW 57792 and CMW 57793).

Thirty plants were randomly chosen for inoculation, distributed in an area of approximately 1 ha. A 7-mm-diam cork borer was used to remove the bark and expose the cambium on a single branch (2–3 cm diam) per plant. Similar sized discs, taken from the actively growing margins of 2-wk-old cultures on PDA, were inserted into these wounds with the mycelial growth facing the xylem (n = 10 branches per isolate). Each branch received only one inoculation. An additional 10 branches were inoculated with an agar-only control. Wounds were covered with Parafilm™ (Amcor, Zürich, Switzerland) to prevent desiccation and contamination by other organisms.

After 7 wk, inoculated branches were harvested by removal at the base of the main stem, and transferred in brown paper bags to the laboratory for assessment. Leaves were removed from the branches, and the length of the lesions (mm) was determined by removing the bark around each inoculation point with a sterile scalpel and measuring the length of the longest distance of the stained portion of the vascular tissues using digital calipers. Isolations were made from stained portions of vascular tissues of all inoculated branches including control treatments, to determine whether Microsphaeropsis was the causal agent for lesion development. Branch sections containing lesions were surface sterilised with 70 % ethanol for 1 min, after which approximately 5 mm³ sections from the leading edges of lesions were plated onto ½ PDA. These were incubated in the dark at 25 °C for approximately 7–10 d and resulting fungal cultures were morphologically evaluated to confirm identity.

Lesion length data were normally distributed after implementing a Shapiro-Wilks test (W = 0.931, p = 0.053) using R Software v. 3.6.3 (https://www.rstudio.com). The influence of treatment (different isolates and controls) on lesion length data was thereafter tested using a linear model (lm) using base R. Significant main effects were separated using a conservative Tukey post-hoc test in the multcomp package in R (Hothorn et al. 2008). A probability level of 5 % was considered significant.

Permitting

Permission to collect samples was provided by the Western Cape Nature Conservation Board. Collections were made under permit number CN44-87-16977.

RESULTS

Disease description and pathogen identification

A Microsphaeropsis species was consistently found sporulating on cankered R. amplexicaulis stems. Four isolates (CMW 57792, CMW 57793, CMW 57794 and CMW 57795), originating from separate shrubs, were purified and used for further morphological study and molecular identification. Two isolates (CMW 57792 and CMW 57793) were used in the pathogenicity trials.

Phylogenetic analyses

Amplicons of approximately 520 bp were generated for the ITS region, 900 bp for the LSU, and 880 bp for the RPB2. The concatenated aligned dataset consisted of 31 ingroup taxa and 1 835 characters, including alignment gaps. Based on the results of JModeltest, a TrNef+G model was selected for ITS, the TPM1uf for LSU and the TIM3+I for RPB2, and these models were applied to individual loci in the concatenated dataset for the BI analyses. ML and BI analyses resulted in phylogenetic trees with concordant topologies and showed similar phylogenetic relationships between taxa. The ML tree with bootstrap support values, and the posterior probabilities obtained from BI, is presented in Fig. 2. The four isolates considered in this study were identical and clustered in a well-supported clade (ML/BI = 100/1.00), clearly distinct from the most closely related species, Microsphaeropsis proteae, and thus represent a novel taxon.

Taxonomy

Microsphaeropsis rafniae M.J. Wingf., N.Q. Pham & Marinc., sp. nov. MycoBank MB 849562. Fig. 3.

Etymology: Name refers to Rafnia, the host genus on which it occurs.

Diagnosis: Similar to M. proteae (conidia 5–8 × 3.5–4 µm in vivo) but differs in its smaller conidial dimensions (3–5 × 2–3 µm in vivo).
Fig. 2. Phylogenetic tree based on a Maximum Likelihood (ML) analysis of a combined DNA data set of ITS, LSU and RPB2 sequences representing Microsphaeropsis spp. and closely related groups in Didymellaceae. Isolates sequenced in this study are presented in bold face. Bootstrap values ≥ 70 % for ML analyses and posterior probabilities values ≥ 0.90 obtained from Bayesian inference (BI) are indicated at the nodes as ML/BI. Isolates representing ex-type cultures are marked with a “T”. Calophoma parvula (CBS 620.68) represents the outgroup taxon.
**Typus:** South Africa, Western Cape Province, West Coast District, Cederberg Mountains, Rafnia amplexicaulis, 4 Sep. 2021, M.J. Wingfield (holotype PRU(M) 4552; ex-holotype culture CMW-IA 52, CMW 57792), [GenBank: OR209698 (ITS), OR209716 (LSU), OR211858 (RPB2)].

**Description:** Sexual morph not observed. Conidiomata in substrate pycnidial, solitary, scattered, immersed becoming erumpent, subglobose, 159 × 149 μm; ostiole inconspicuous; pycnidial wall pseudoparenchymatous, consisting of 3–4 layers of compressed cells, outer layers pale brown. Conidiophores reduced to conidiogenous cells, formed along pycnidial cavity. Conidiogenous cells phialidic, hyaline, ampulliform to lageniform, 4–8 × 3–4 μm, showing periclinal thickening or percurrent proliferation. Conidia oozing out in yellow mass, becoming brown droplets at tip of ostiole, ellipsoidal, ovoid, rarely pyriform, hyaline, thin-walled at beginning, becoming sub-

**Fig. 3.** Micrographs and culture characteristics of *Microsphaeropsis rafniae* sp. nov. (holotype: PRU(M) 4551, ex-holotype CMW-IA 52, CMW 57792). A. Conidiomata immersed in the substrate. B, C. Conidioma with protruding ostiole and conidial mass on the tip. D. Vertical section of conidioma in the substrate. E. Conidiogenous cells showing periclinal thickening (left) or percurrent proliferation (middle). F. Conidiomatal wall. G. Conidia. H–J. Colonies of the ex-holotype grown at six temperatures after 10 d (left) /30 d (right) in the dark on PDA (H), MEA (I) and OA (J).
A novel species of Microsphaeropsis from Rafnia amplexicaulis

**Culture characters:** Colonies on PDA in the dark for 10 d, optimum growth temperature at 20 °C reaching 68.6 mm, followed by 25 °C (61.3 mm), 15 °C (52.1 mm), 5 °C (17.1 mm), 30 °C (10.5 mm) and 35 °C (no growth); on MEA optimum growth temperature at 20 °C reaching 71.5 mm, followed by 25 °C (65.3 mm), 15 °C (52.6 mm), 5 °C (18.6 mm), 30 °C (12 mm) and 35 °C (no growth). Colonies on PDA, MEA and OA showing circular growth with smooth margins, with superficial, flat and medium dense mycelia, fertile in 30 d, morphology homogeneous in 10 d, becoming diverse with age, having streaks or patches of white aerial mycelia. Colony colours on PDA and MEA in 10 d above peach (7d) to sienna (13i), fading towards edges, in 30 d sienna, umber (13m) to olivaceous grey (21i”) at higher temperatures, covered with white aerial mycelia partly or in patches. Colony colours on OA in 10 d colourless with tint of citrin (21k) near centre, in 30 d greenish olivaceous (23”) streaks to olivaceous grey with white aerial mycelial patches or streaks and black fruiting structure.

**Distribution:** South Africa, Western Cape Province, West Coast District, Cederberg Wilderness Area.

**Additional specimens examined:** South Africa, Western Cape Province, West Coast District, Cederberg Wilderness Area, -32.412743, 19.174894, on stems and branches of Rafnia amplexicaulis, 4 Sep. 2021, M.J. Wingfield, PRU(M) 4552, culture CMW-IA 53, CMW 57793 [GenBank: OR209699 (ITS), OR209717 (LSU), OR211859 (RPB2)]; PRU(M) 4553, culture CMW-IA 54, CMW 57794 [GenBank: OR209700 (ITS), OR209718 (LSU), OR211861 (RPB2)]; PRU(M) 4554, culture CMW 57795 [GenBank: OR209701 (ITS), OR209719 (LSU), OR211862 (RPB2)].

**Notes:** Microsphaeropsis rafniae is phylogenetically close to M. proteae and M. fusca. However, it can be distinguished from those species by its smaller conidia (3–5 × 2–3 μm in vivo); M. fusca (5–10.5 × 3.5–6.5 μm in vitro) (Hou et al. 2020) and M. proteae (5–8 × 3.5–4 μm in vivo) (Swart et al. 1998, Crous et al. 2011). Microsphaeropsis fusca was originally isolated from twig lesions of Sarothamnus scoparius (Fabaceae) in the Netherlands, and M. proteae from leaves of Protea nitida (Proteaceae) in Hermanus, Western Cape Province, South Africa (Swart et al. 1998, Hou et al. 2020). The NaOH spot test was positive on OA, turning from pale luteous to sienna colour, whereas M. fusca was reported as negative (Hou et al. 2020). There is no record of a spot test for M. proteae. The species showed strong growth at lower temperatures (5–25 °C). Cultures at 35 °C failed to grow when plates were returned to an optimum temperature (20 °C) and incubated for another 10 d, indicating this temperature results in death.

**Pathogenicity tests:** After 7 wk of incubation, lesions were evident around the inoculation points under the bark of the hosts. These consisted of dark brown to reddish-brown vascular staining in the form of streaks. Controls showed similar staining, but this did not extend far from the inoculation points. Microsphaeropsis rafniae was consistently isolated from the stained areas of the treatments but was never recovered from the controls. Treatment had a significant effect on lesion length (F = 35.83, DF = 2, 27, p < 0.001). Post-hoc analyses revealed that lesions caused by the two isolates were significantly longer than those caused by the controls, but lesion length did not differ between the two test isolates (Fig. 4).

**DISCUSSION**

A previously unreported canker disease was observed on stems and branches of Rafnia amplexicaulis in the Cederberg Wilderness Area, Western Cape, South Africa. Microsphaeropsis isolates were recovered from these cankers and identified based on multi-locus (ITS, LSU and RPB2) phylogenetic analyses and

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**Fig. 4.** Lesion length of two isolates of Microsphaeropsis rafniae sp. nov. (R1: CMW 57792, R2: CMW 57793) inoculated into Rafnia amplexicaulis branches after 7 w of incubation. Boxes indicating a 25–75 % data range, whiskers indicating a 1.5 × interquartile range.
morphological characters. These analyses revealed the isolates represented a novel species, described here as *M. rafniae*. Pathogenicity tests confirmed that *M. rafniae* is a canker pathogen of *R. amplificaualis*.

The genus *Microsphaeropsis* was originally placed in *Montagnulaceae*, a family established to accommodate species with pigmented, phoma-like conidia. De Gruyter et al. (2009) proposed the family *Didymellaceae* to accommodate species in *Phoma* s. l. and related genera, including *Microsphaeropsis*. A new family, *Microsphaeropsidaceae*, was introduced to accommodate the genus by Chen et al. (2015). However, Hou et al. (2020) found that *Microsphaeropsis* spp. clearly reside in the *Didymellaceae* and reduced *Microsphaeropsidaceae* to synonymy, with *Microsphaeropsis* again placed in *Didymellaceae*.

*Microsphaeropsis* spp. have been reported from a wide variety of plant hosts, including as pathogens causing twig lesions (Hou et al. 2020) and leaf spots (Swart et al. 1998). This study characterised a novel pathogenic *Microsphaeropsis* species, *M. rafniae*, causing cankers on *R. amplificaualis*, an endemic South African shrub. *Microsphaeropsis rafniae* is phylogenetically closest to *M. proteae*, also described from an endemic South African plant, *Protea nitida* (Swart et al. 1998). The two species are, however, phylogenetically distinct, with all isolates of *M. proteae* clustered in a well-supported clade. While morphologically similar, the two species can be distinguished based on their conidial size. Currently, neither the distribution range nor epidemiology of *M. rafniae* are known, with further studies required to better understand the role of this fungus as a disease-causing agent of *R. amplificaualis*.

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