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Microfungi associated with dying *Euphorbia mauritanica* in South Africa and their relative pathogenicity

S. Marincowitz[1*], N.Q. Pham[2], B.D. Wingfield[1], F. Roets[3], M.J. Wingfield[1]

[1]Department of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Private Bag X20, Hatfield, 0028, South Africa
[2]Department of Plant and Soil Sciences, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Private Bag X20, Hatfield, 0028, South Africa
[3]Department of Conservation Ecology and Entomology, Stellenbosch University, Private Bag X1, Matieland, 7602, South Africa

*Corresponding author: seonju.marincowitz@fabi.up.ac.za

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Abstract: *Euphorbia mauritanica* is a succulent shrub that is indigenous to South Africa and widely distributed throughout the country. Dying plants have been observed in their natural habitat in the Northern and Western Cape Provinces of South Africa in recent years. Stems displaying lesions were collected and the emerging cultures were identified based on ITS, LSU, *ACT*, *RPB2*, *TEF1* and/or *TUB2* sequence data. Four filamentous fungi were consistently observed and isolated. One was identified as *Alanphillipsia* (*Ala.*) *aloes*, and the other three were new to science and are described here as *Cytospora euphorbiicola sp. nov.*, *Nothomicrosphaeropsis namakwaensis sp. nov*. and *Austrophoma* (*Aus.*) *euphorbiae gen. et sp. nov.* These new species and *Ala. aloes* were the most commonly encountered, and their pathogenicity was tested on *E. mauritanica* plants in a greenhouse trial. All four species gave rise to lesions that were significantly larger than those associated with the controls, but they were not significantly different to each other. Although the lesions associated with the inoculations were well-developed, they did not give rise to plant death, suggesting that they are not responsible for the large-scale die-off of *E. mauritanica* in the field. The primary cause of the death of *E. mauritanica* in the studied area remains unknown and could be due to environmental factors such as has been found with the die-off of *Euphorbia ingens* in South Africa.

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INTRODUCTION

During the course of the last few decades, the die-off of *Euphorbia* species, particularly the woody *Euphorbia*, *E. ingens*, has occurred in South Africa. Intensive studies have been conducted on these die-offs considering both possible biotic and abiotic factors that might explain the problem (Roux *et al*. 2008, Van der Linde *et al.* 2011, 2012a, b, 2016, 2017a, b, 2018). These studies led to the identification of a relatively large number of insect pests and pathogens associated with the observed symptoms. Among them were *Lasiodiplodia theobromae* and *L. mahajangana*, and weevil-associated *Gondwanamyces serotectus* and *G. ubusi*, which showed significant disease development in pathogenicity trials (Van der Linde *et al*. 2011, 2012b). However, the overall conclusions were that environmental factors predisposed trees to damage by opportunistic fungal pathogens and aggressive insects, eventually leading to tree mortality.

During routine plant disease surveys, large numbers of *E. mauritanica* plants have been observed dying in the Northern and Western Cape Provinces of South Africa. Typically, the plants had symptomatic branches with discolouration that extended down to the base. The widespread nature of the die-off and the disease symptoms raised concerns that fungal pathogens might be involved. Unlike *E. ingens* which grows as a tree, *E. mauritanica* is a shrub growing up to 1.5 m high with many succulent stems branching at the base and with small leaves. It is native to Africa with a wide distribution in South Africa and grows in dry climates, mainly in coastal and inland areas with colder winters (Bruyns 2022). A fungus, *Endoconidioma euphorbiae* (*Dothioraceae*, *Dothideales*), was reported from the leaves of *E. mauritanica* (Crous *et al.* 2020). However, no extensive studies have been conducted regarding fungi associated with *E. mauritanica* or the die-offs of the plant.

The aim of this study was to identify the fungi commonly associated with the die-offs of *E. mauritanica* plants, primarily utilising DNA sequence analyses. Furthermore, their possible contribution to plant death was assessed using artificial inoculations under greenhouse conditions.

MATERIALS AND METHODS

Field observations and isolations

Dying *E. mauritanica* plants (Figs 1, 2), common in areas in the vicinity of Clanwilliam (Western Cape Province) and Nieuwoudtville (Northern Cape Province), were visited in August 2020. Initial symptoms were distinct lesions on the stems that appeared to extend rapidly downwards. Eleven stem pieces from different plants with incipient lesions were transferred to the laboratory, and isolations were made from fungal structures present on the lesions. Spores taken from sporocarps were plated on malt extract agar (MEA: 20 g Biolab malt extract, 20 g Difco agar, 1 L deionised water) supplemented with 100 mg streptomycin. Pure cultures were established from the primary isolations on MEA and on water agar (WA: 20 g Difco agar, 1 L deionised water), where autoclaved bamboo toothpicks had been placed on the agar surface to induce sporulation.

Fungal structures produced on WA, MEA or toothpicks were studied under a compound microscope (Nikon Eclipse Ni, Japan). The structures were initially mounted in water and later replaced with 85 % lactic acid, in which all micrographs and measurements were done. Vertical sections of sporocarps formed on media or toothpicks were prepared using a Leica Cryomicrotome (Leica Biosystem, Germany). The sporocarps were mounted in a freezing medium, cut in 10–12 µm thickness, and mounted in 85 % lactic acid for observation. Up to 50 measurements were made for key morphological structures, if available, and presented in minimum–maximum (average ± standard deviation).

For growth characteristics, 5 mm diam mycelial plugs taken from the leading edges of 7 d-old cultures were placed at the centres of 90 mm Petri dishes containing 2 % MEA. The cultures were incubated in the dark at temperatures ranging from 10–35 °C at 5 °C intervals. Colonies were observed daily, and when the fastest growing cultures reached the edges of the Petri dishes, two measurements of colony diameter, perpendicular to each other were taken, and the averages, as well as the daily growth rates, were computed. Culture characteristics were described after growth for 28 d in the dark, and colony colours were described using the colour charts of Rayner (1970).

All cultures were stored in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI),

Fig. 1. Locations where dying *Euphorbia mauritanica* were observed and the samples collected: Clanwilliam (Western Cape Province) and Nieuwoudtville (Northern Cape Province), South Africa.

Fig. 2. Dying *Euphorbia mauritanica* in the fields close to Nieuwoudtville, Northern Cape Province, South Africa. **A, B.** Dead and dying shrubs. **C–E.** Lesions observed in the stems of dying plants.

University of Pretoria, Pretoria, South Africa. The type specimens and ex-type cultures were lodged in the H.G.W.L. Schweickerdt herbarium (PRU) and the culture collection of the Innovation-Africa (CMW-IA), University of Pretoria, South Africa.

DNA extraction, PCR amplification and sequencing

Isolates grown on 2 % MEA for 7 d at 25 °C were used for DNA extraction. Mycelium was scraped from the surface of the media using sterile needles and transferred to 1.5 mL Eppendorf tubes. DNA was extracted using Prepman® Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the protocols suggested by the manufacturer. The regions targeted for PCR amplification and subsequent sequencing are listed in Table 1. PCR reactions were conducted using the Applied Biosystems ProFlex PCR System (Thermo Fisher Scientific, Waltham, MA, USA) following the preparation described by Pham *et al.* (2019). Amplified fragments were cleaned using an 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 of the Faculty of Natural and Agricultural Sciences, University of Pretoria, South Africa. Geneious Prime v. 2022.1.1 (https://www.geneious.com) was used to assemble and edit the raw sequences. All sequences generated in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov) (Supplementary Tables S1–S3).

Phylogenetic analyses

The ITS and LSU regions were sequenced for all isolates obtained in this study. Preliminary identification was made by performing a nucleotide BLAST search using the ITS and LSU sequences against the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) to identify the isolates to genus and closest species level. This information was then used to generate the datasets for further phylogenetic analyses. Sequences for species closely related to those emerging from this study were sourced from GenBank (Supplementary Tables S1–S3). All sequences were aligned with MAFFT v. 7 (Katoh & Standley 2013) and inspected manually using

Table 1. Primers used in this study, with sequences and sources.

Region	Primer	Direction	Sequence	Annealing temperature	References	*Alanphilipsia*	*Didymelaceae*	*Cytospora*
Internal transcribed spacer regions 1 and 2 and the 5.8S gene of the ribosomal RNA (ITS)	ITS-1F	Forward	CTTGGTCATTTAGAGGAAGTAA	56 °C	Gardes & Bruns (1993)	v	v	v
	ITS-4	Reverse	TCCTCCGCTTATTGATATGC		White *et al*. (1990)			
Nuclear large subunit of the ribosomal RNA (LSU)	LR0R	Forward	ACCCGCTGAACTTAAGC	55 °C	Rehner & Samuels (1994)	v	v	v
	LR5	Reverse	TCCTGAGGGAAACTTCG		Vilgalys and Hester (1990)			
DNA-directed RNA polymerase II second largest subunit (*RPB2*)	fRPB2-5F2	Forward	GGGGWGAYCAGAAGAAGGC	58 °C	Sung *et al*. (2007)	n/a	v	v
	fRPB2-7cR	Reverse	CCCATRGCTTGYTTRCCCAT		Liu *et al*. (1999)			
β-Tubulin 2 (*TUB2*)	BT2a	Forward	GGTAACCAAATCGGTGCTGCTTTC	52 °C	Glass & Donaldson (1995)	n/a	v	v
	BT2b	Reverse	ACCCTCAGTGTAGTGACCCTTGGC		Glass & Donaldson (1995)			
Actin (*ACT*)	ACT-512F	Forward	ATGTGCAAGGCCGGTTTCGC	55 °C	Carbone & Kohn (1999)	n/a	n/a	v
	ACT-783R	Reverse	TACGAGTCCTTCTGGCCCAT		Carbone & Kohn (1999)			
Translation elongation factor 1-α (*TEF1*)	EF1-728F	Forward	CATCGAGAAGTTCGAGAAGG	52 °C	Carbone & Kohn (1999)	n/a	n/a	v
	EF1-986R	Reverse	TACTTGAAGGAACCCTTACC		Carbone & Kohn (1999)			

MEGA v. 7 (Kumar *et al.* 2016). The most appropriate model for analyses was obtained using the software jModeltest v. 1.2.5 (Posada 2008). Bayesian inference (BI) analyses were performed using MrBayes v. 3.2.6 (Ronquist *et al.* 2012) on the CIPRES Science Gateway v. 3.3 (Miller *et al.* 2010). 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 the trees sampled were eliminated as burn-in, and the remaining trees were used to determine the posterior probabilities. Maximum-likelihood (ML) analysis was conducted using RAxML v. 8.2.4 (Stamatakis 2014) on the CIPRES Science Gateway v. 3.3, with default GTR substitution matrix and 1 000 rapid bootstraps. Final consensus trees were viewed using MEGA v. 7 (Kumar *et al.* 2016).

Pathogenicity tests

Five representative isolates of four species that were obtained in this study were selected for an inoculation trial to determine the pathogenicity of the fungi. Three isolates, CMW 56355, CMW 56351 and CMW 56352, each represented a species, and two isolates, CMW 56344 and CMW 56346, represented a single species. Plants of *E. mauritanica* were grown from branch cuttings taken from a single plant and thus representing a single clone. The rooted cuttings were maintained in individual pots in a greenhouse where the temperature was adjusted to approximately 25 °C.

Plants with main stems ranging in 5–8 mm diam were selected for inoculation. The inoculum was prepared by first soaking toothpicks in malt extract broth that had been cut in half and thus 3 cm in length. The toothpicks were autoclaved twice and placed onto the surface of potato dextrose agar in 90 mm Petri dishes (PDA: Difco potato dextrose agar 24 g, 1 L deionised water) under sterile conditions. A plug of agar taken from the actively growing margins of the test fungi was then placed at the centre of the plates containing two half-length toothpicks, which were placed approximately 10 mm apart from each other and from the inoculum source. These plates were incubated at 25 °C in the dark for 7 wk until the toothpicks were completely overgrown by fungal hyphae. Toothpicks on non-inoculated plates were maintained under the same conditions to be used as controls.

Fifteen plants per isolate and an equal number of controls were inoculated. The inoculated plants were kept in a greenhouse where the temperature was adjusted to approximately 25 °C and exposed to a natural light cycle. The plants were watered twice a week. Toothpicks overgrown by mycelium and sterile toothpicks for the controls were inserted into the main stem to a depth of 2–3 mm. After 8 wk, the toothpicks were removed from the inoculated stems that were then dissected lengthwise. The pathogenicity test was not repeated. A transparent mica sheet (210 × 297 mm) was placed directly on the freshly cut branches, and the area of infection was traced on the sheet using a marker. The sheet with the traced outlines and a ruler were transferred to a flatbed scanner (Epson Perfection V700 photo, China) for image processing. The image files created were loaded into Adobe Photoshop 2021 (Adobe Systems Incorporated), and the lesion area was measured. The experimental data were analysed statistically using R software v. 4.2.1 (R Core Team 2021) by performing the Kruskal-Wallis Rank Sum test (Kruskal & Wallis 1952) and the Dwass-Steele-Critchlow-Fligner all-pairs test for pairwise comparisons (Dwass 1960, Steel 1960, Critchlow & Fligner 1991).

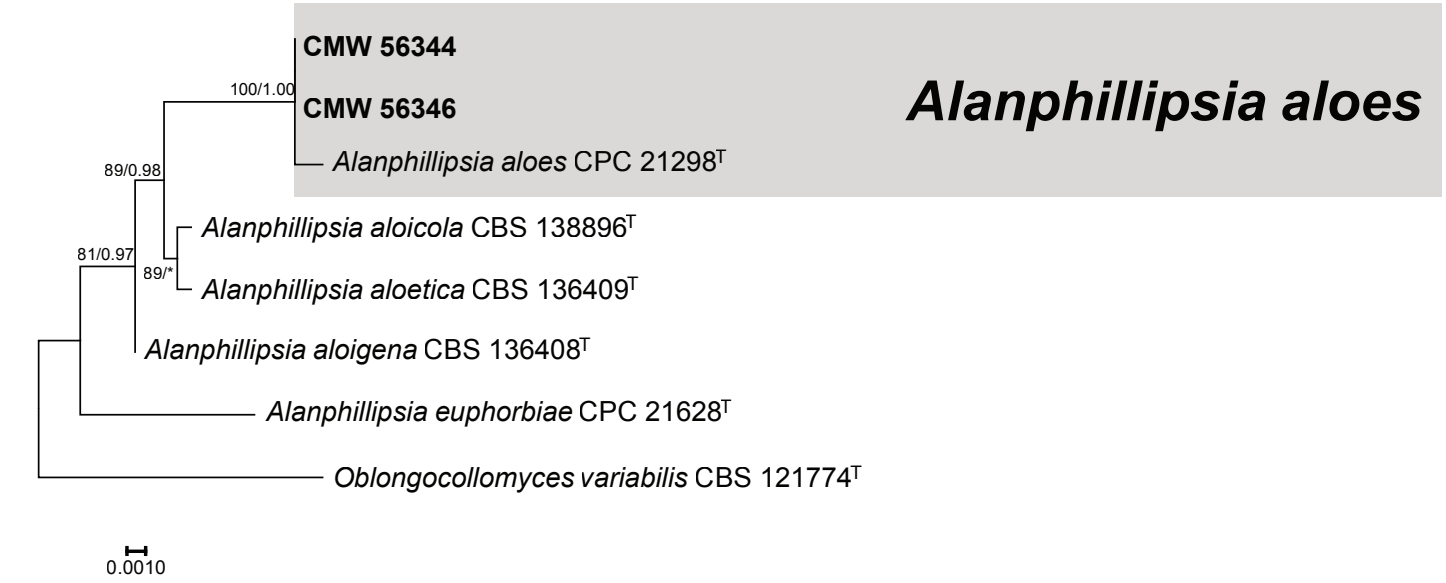

Fig. 3. Phylogenetic tree based on maximum likelihood (ML) analysis of a combined DNA data set of the ITS and LSU sequences for *Alanphillipsia* spp. Isolates sequenced in this study are presented in boldface. Bootstrap values ≥ 60 % for ML analyses and posterior probabilities values ≥ 0.9 obtained from Bayesian inference (BI) are indicated at the nodes as ML/BI. Bootstrap values < 60 % or probabilities values < 0.9 are marked with "*". Isolates representing the ex-type material are marked with "T". *Oblongocollomyces variabilis* (isolate CBS 121774) represent the outgroup.

Re-isolations were made from the tissues close to the point of inoculation onto MEA. The resulting isolates were identified based on their colony morphology, and a representative sample of these was sequenced for the ITS region.

RESULTS

Identification of isolates

Four fungal taxa were consistently found present on the infected tissues, and pure cultures were produced for them. An initial BLAST search using generated ITS and LSU sequences against the NCBI GenBank database identified these isolates as members of *Alanphillipsia* (*Botryospheriaceae*, *Botryosphaeriales*, *Dothideomycetes*), *Cytospora* (*Cytosporaceae*, *Diaporthales*, *Sordariomycetes*), *Nothomicrosphaeropsis* (*Didymellaceae*, *Pleosporales*, *Dothideomycetes*) and a didymellaceous fungus.

Two isolates (CMW 56344, CMW 56346) were identified as species of *Alanphillipsia* based on the BLAST search using the ITS and LSU sequences. Five previously described *Alanphillipsia* spp. and *Oblongocollomyces variabilis* (CBS 121774) as the outgroup were included in the phylogenetic analyses. Maximum likelyhood and BI analyses were performed based on the combined sequences of the ITS and LSU region. The concatenated aligned dataset consisted of seven ingroup taxa and 1 401 characters, including alignment gaps. Based on the result of jModeltest, the TrNef+I model was selected for ITS and the Tim2+G for LSU, and these models were applied to individual loci in the combined dataset for the BI analysis. The ML tree for *Alanphillipsia* with bootstrap support values of the ML and the posterior probabilities obtained from the BI analysis is presented in Fig. 3. The two isolates obtained in this study had identical sequences and clustered in a well-supported clade (ML/BI = 100) that includes the ex-type isolate of *Alanphillipsia aloes* (CPC 21298). The two isolates were thus identified as *Alanphillipsia aloes*.

The phylogenetic placement of the *Cytospora* isolate (CMW 56355) obtained was confirmed based on the analyses of the concatenated dataset of six regions (ITS+LSU+*ACT*+*RPB2*+*TEF1*+*TUB2*). This combined dataset consisted of 36 ingroup taxa and 3 558 characters with alignment gaps. Sequences for *Diaporthe eres* (AR5193) were used as the outgroup. Based on the results of jModeltest, BI analysis was performed applying the TIMef+I+G substitution model for ITS region, the TrN+I+G for LSU, the TPMIuf+G for *ACT*, the TIM3+I+G for *RPB2*, the TIM2+G for *TEF1,* and the TPM2uf+G for *TUB2*. The isolate CMW 56355 collected in this study represented a new lineage, clearly distinct from the most closely related species, *Cytospora tibouchinae* (CPC 26333) and *Cytospora myrtagena* (CBS 116843) (Fig. 4).

For the two taxa in *Didymellaceae* (CMW 56351, CMW 56352), the concatenated aligned dataset including sequences of four gene regions (ITS+LSU+*RPB2*+*TUB2*) consisted of 88 ingroup taxa and 2 291 characters, including alignment gaps. Two isolates of *Pleiochaeta setosa* (CBS 118.25, CBS 496.63) were used as the outgroup taxon. Based on the results of jModeltest, the TIM2+I+G model was selected for *RPB2* and LSU, the TVM+I+G for *TUB2*, and the GTR+I+G for ITS. These models were applied to individual loci in the concatenated dataset for the BI analysis. The 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 of the ML and the posterior probabilities obtained from the BI analysis is presented in Fig. 5. The isolate CMW 56351 formed a lineage, which was separated and distinct from all other genera in the *Didymellaceae*, representing a novel genus (Fig. 5). The isolate CMW 56352 clustered within *Nothomicrosphaeropsis*, was closely related but clearly distinct from the ex-type isolate

Cytospora - ITS+LSU+*ACT*+*RPB2*+*TEF1*+*TUB2*

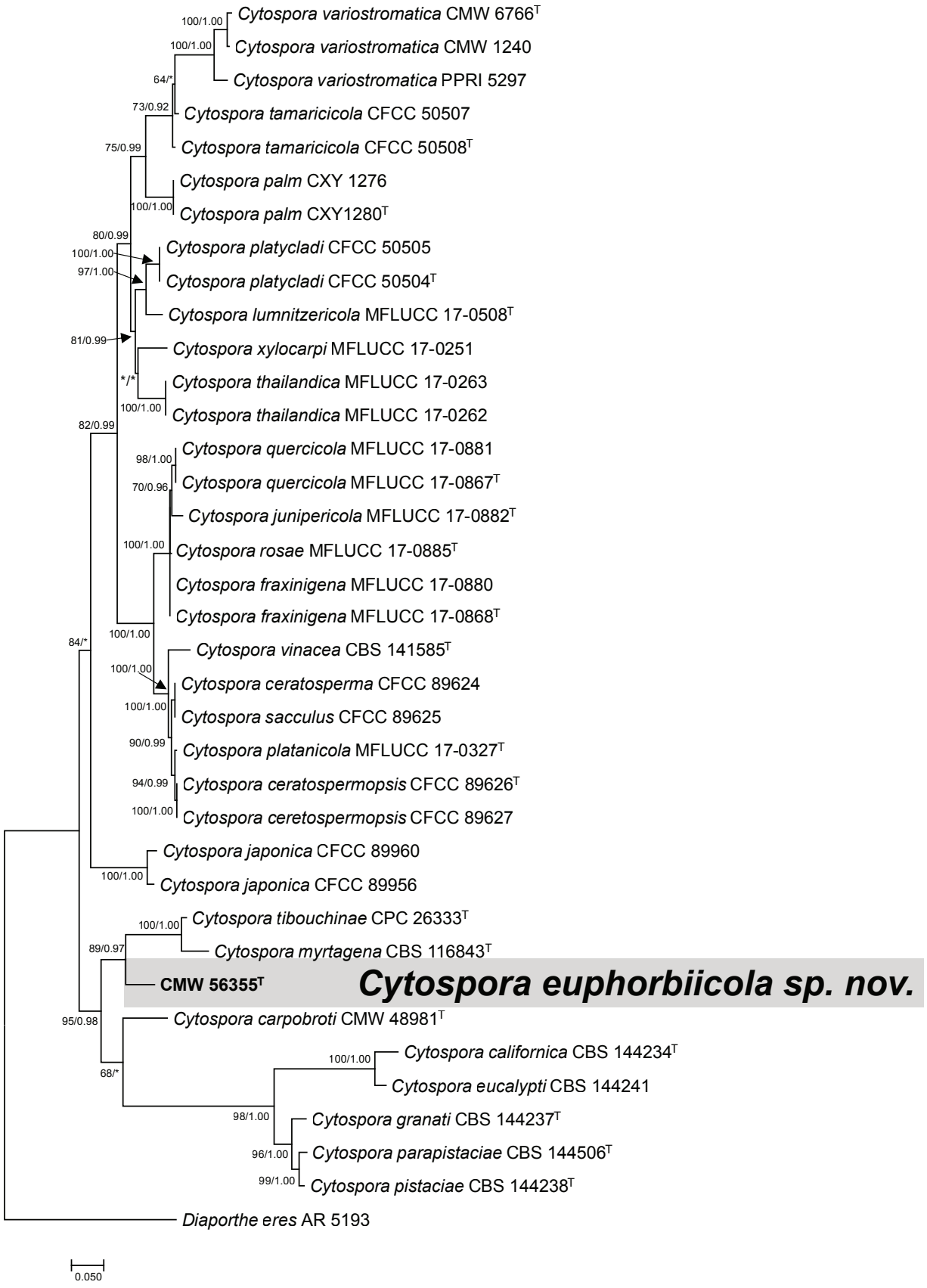

Fig. 4. Phylogenetic tree based on maximum likelihood (ML) analysis of a combined DNA data set of the ITS, LSU, *ACT*, *RPB2*, *TEF1* and *TUB2* sequences for *Cytospora* spp. Isolates sequenced in this study are presented in bold face. Bootstrap values ≥ 60 % for ML analyses and posterior probabilities values ≥ 0.9 obtained from Bayesian inference (BI) are indicated at the nodes as ML/BI. Bootstrap values < 60 % or probabilities values < 0.9 are marked with "*", and nodes lacking the support values are marked with "–". Isolates representing the ex-type material are marked with "T". *Diaporthe eres* (isolate AR5193) represents the outgroup.

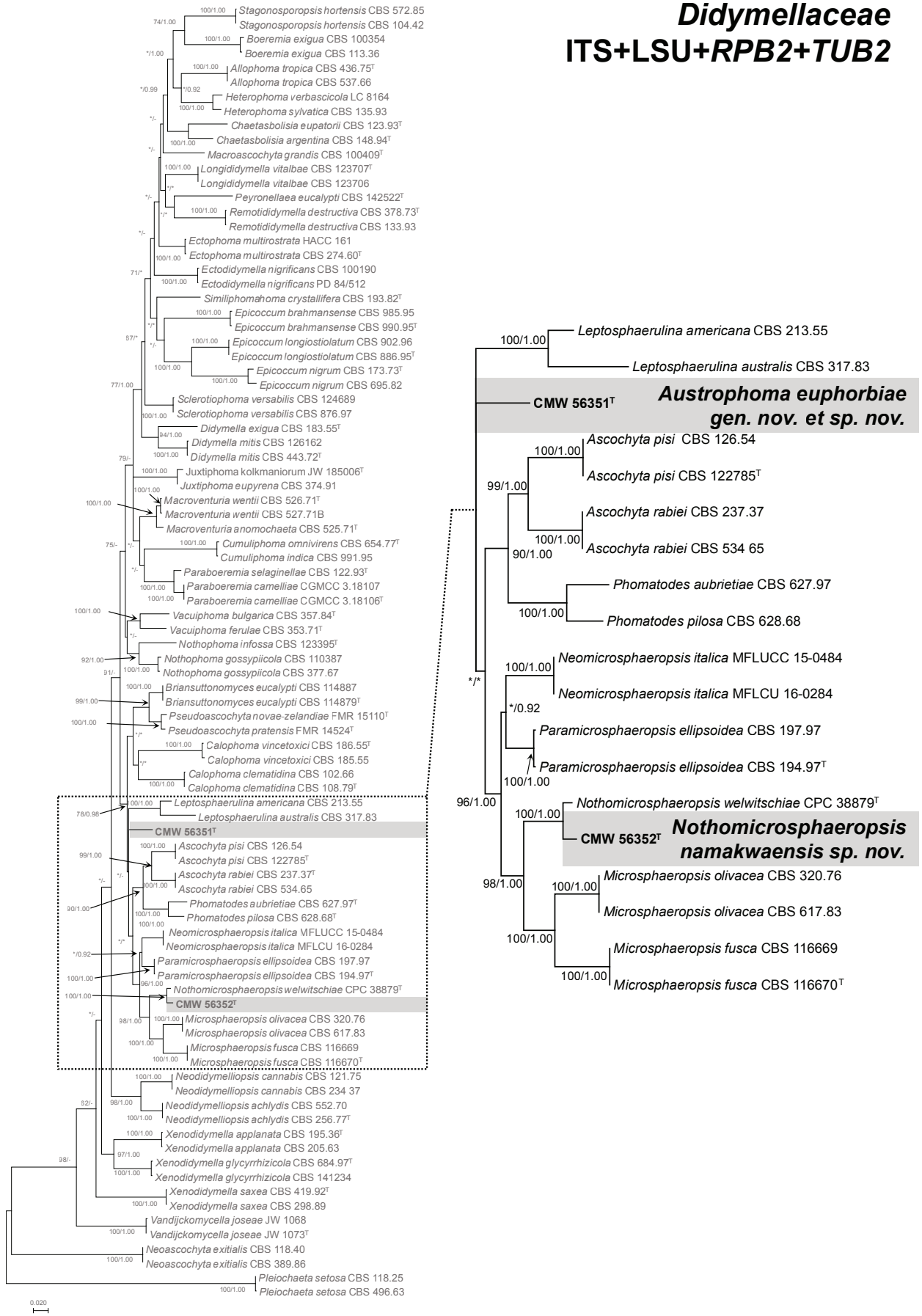

Fig. 5. Phylogenetic tree based on maximum likelihood (ML) analysis of a combined DNA data set of the ITS, LSU, *RPB2*, and *TUB2* sequences representing species of the *Didymellaceae*. Isolates sequenced in this study are presented in bold face. Bootstrap values ≥ 60 % for ML analyses and posterior probabilities values ≥ 0.9 obtained from Bayesian inference (BI) are indicated at the nodes as ML/BI. Bootstrap values < 60 % or probabilities values < 0.9 are marked with "*", and nodes lacking the support values are marked with "–". Isolates representing the ex-type material are marked with "T". Two isolates of *Pleiochaeta setosa* (CBS 118.25 and CBS 496.63) represent the outgroup.

of its sister species, *N. welwitschiae*. This isolate was thus recognised as a novel species of *Nothomicrosphaeropsis*.

Three fungi which are found to be a new species of *Nothomicrosphaeropsis, Cytospora* and a new genus of the *Didymellaceae*, are described and introduced here.

Taxonomy

Austrophoma N.Q. Pham, Marinc. & M.J. Wingf.***, gen. nov.*** MycoBank MB 849172.

Type: *Austrophoma euphorbiae* N.Q. Pham, Marinc. & M.J. Wingf.

Etymology: The name refers to a similarity to *Phoma* and its distribution in the Southern Hemisphere.

Diagnosis: Similar to phoma-like didymellaceous genera, but differs in its hyaline, cylindrical (rectangular in side view) conidia and dark colony colour at 25 °C on 2 % MEA.

Description: *Conidiomata* on MEA pycnidial, papillate or with short neck. *Conidiophores* reduced to conidiogenous cells lining the hymenial layer inside conidioma. *Conidiogenous cells* hyaline, holoblastic, doliiform to lageniform. *Conidia* hyaline, cylindrical, with round apex and flat base, straight or slightly curved, aseptate, rarely 1-septate.

Austrophoma euphorbiae N.Q. Pham, Marinc. & M.J. Wingf. ***sp. nov*.** MycoBank MB 849173. Fig. 6.

Etymology: The name refers to the host genus *Euphorbia*.

Diagnosis: Similar to other phoma-like species but differs in having hyaline and rectangular-shaped (in side view) conidia.

Typus: **South Africa**, Northern Cape Province, Namakwa District Municipality, Nieuwoudtville Falls, from dying *Euphorbia mauritanica*, Aug. 2020, *M.J. Wingfield* (**holotype** PRU(M) 4548, culture ex-type CMW-IA 49, CMW 56351). GenBank: OR198840 (ITS); OR198846 (LSU); OR211854 (*TUB2*); OR211851 (*RPB2*).

Fig. 6. Micrographs of *Austrophoma euphorbiae sp. nov.* (ex-type: CMW-IA 49, CMW 56351). **A.** Colony with abundant pycnidia on 2 % MEA after 60 d in the dark at room temperature. **B, C.** Young and mature pycnidia with ostiolar protruding, single or multiple necks. **D, E.** Vertical sections through pycnidia. **F.** Pycnidial wall. **G.** Conidiogenous cells. **H.** Conidia. **I.** Colony morphology on 2 % MEA incubated in the dark for 28 d at different temperatures. Scale bars: A = 1 mm; B, C = 100 µm; D–F = 50 µm; G, H = 10 µm.

Description: *Sexual morph* unknown. *Asexual morph* on 2 % MEA. *Conidiomata* pycnidial, immersed to superficial in medium, globose to ellipsoidal, ovoid; base pale brown when young becoming dark brown to black with age, 122–396 × 91–490 (214 ± 64.1 × 197 ± 100.5) µm; protruding ostioles, or necks absent or present, single or multiple when present, 72–533 µm long. *Conidiomatal walls* pseudoparenchymatous, *textura angularis*, in two tiers, 16–34 (25 ± 4.3) µm thick, outer tier composed of 3–5 layers of thick-walled, moderately compressed, brown cells, inner tier composed of thin-walled, compressed, hyaline cells. *Conidiophores* on entire layer of inner cavity, reduced to conidiogenous cells. *Conidiogenous cells* holoblastic, cylindrical. *Conidia* hyaline, cylindrical, with round apex and flat base, mostly straight, occasionally curved, aseptate, rarely 1-septate, 8–14 × 3–4 (10.4 ± 1.37 × 3.2 ± 0.32) µm, oozing out in milky slimy mass.

Culture characteristics: Colonies on MEA showing optimum growth at 25 °C (5.3 mm/d), followed by 20 °C (5.2 mm/d), 15 °C (4 mm/d), 10 °C (2.5 mm/d), 30 °C (0.5 mm/d) and 35 °C (0.4 mm/d). Colonies on MEA at 25 °C in the dark for 28 d fuscous black (3''''k) above and reverse, sparse aerial hyphae, with even edge. Colonies at lower temperatures colour becoming paler.

Host: *Euphorbia mauritanica*.

Distribution: South Africa (Northern Cape Province).

Notes: *Austrophoma* had a close affinity to phoma-like genera in the *Didymellaceae* in multi-gene analyses. The most closely related genera were *Ascochyta*, *Leptosphaerulina*, *Microsphaeropsis*, *Neomicrosphaeropsis*, *Nothomicrosphaeropsis*, *Paramicrosphaeropsis* and *Phomatodes*. In addition to its differences in molecular characteristics, *Austrophoma* can be distinguished from *Ascochyta* by producing pycnidia (on MEA) with papilla or distinguishing necks. *Ascochyta* produces ostiolate or poroid pycnidia, without a distinct form of neck (Chen *et al*. 2015). *Austrophoma* is different from *Microsphaeropsis* and its derivative genera containing the name 'microsphaeropsis' by having hyaline conidia: *Microsphaeropsis*, *Paramicrosphaeropsis*, (Hou *et al*. 2020), *Nothomicrosphaeropsis* (Crous *et al*. 2021) and *Neomicrosphaeropsis* (Thambugala *et al*. 2016) produce pigmented conidia. *Austrophoma* and *Phomatodes* (Chen *et al*. 2015) are morphologically similar to each other in having hyaline conidia in allantoid to cylindrical conidia, but combined sequence data separate them into distinct lineages. No sexual morph was found with *Austrophoma*, whereas *Leptosphaerulina* and *Neomicrosphaeropsis* have sexual morphs producing muriform ascospores.

Nothomicrosphaeropsis namakwaensis N.Q. Pham, Marinc. & M.J. Wingf., ***sp. nov.*** MycoBank MB 849174. Fig. 7.

Etymology: The name refers to the District Municipality Namakwa, where the host plant grows naturally.

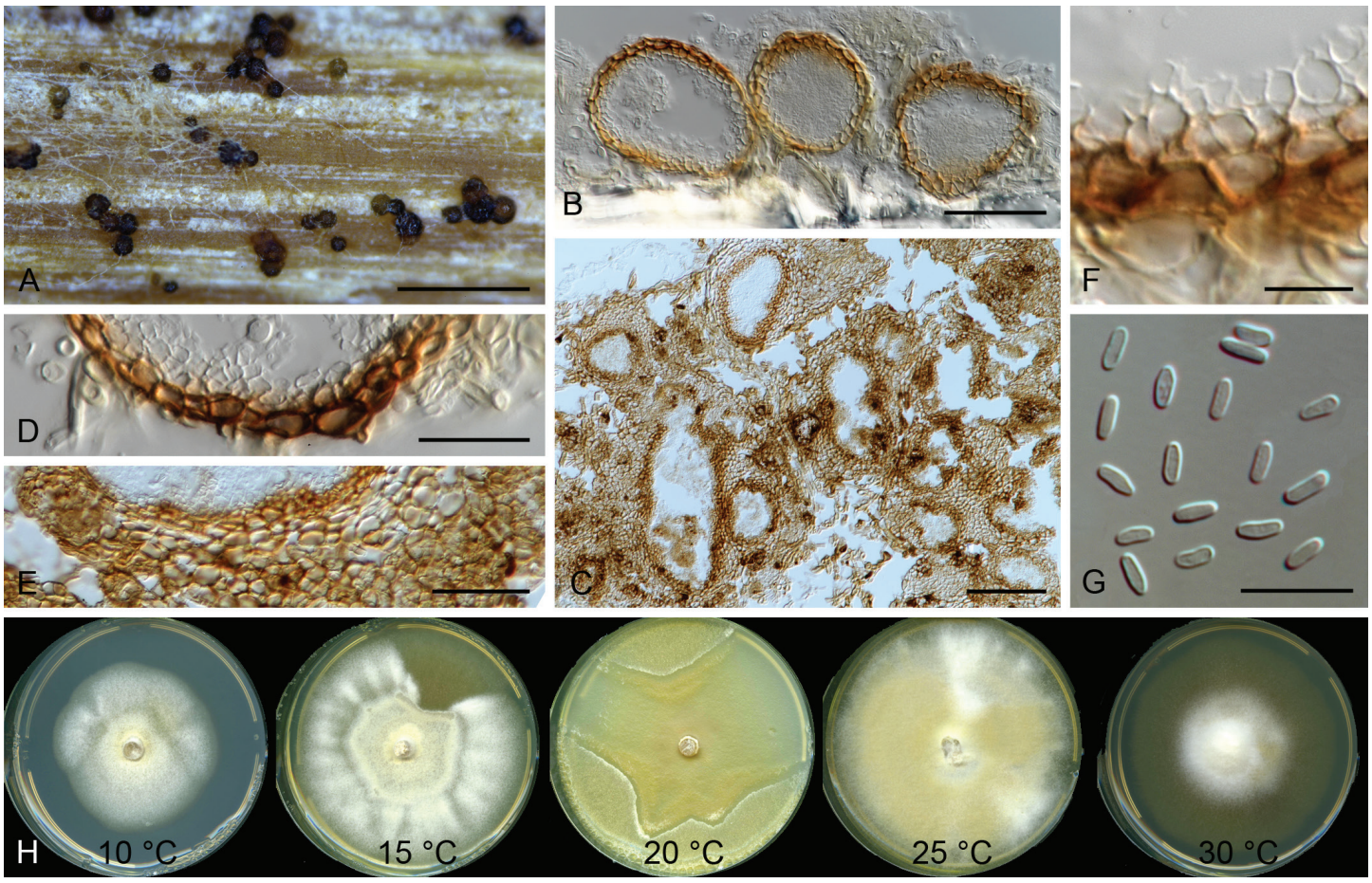

Fig. 7. Micrographs of *Nothomicrosphaeropsis namakwaensis sp. nov.* (ex-type: CMW-IA 50, CMW 56352). **A.** Pycnidia formed on toothpick. **B.** Vertical section through pycnidia. **C.** Vertical section through eustroma formed on MEA with pycinidal locules connected by *textura angularis* tissue. **D, E.** Conidiomatal wall (D) and pycnidial locule (E). **F.** Conidiogenous cells. **G.** Conidia. **H.** Colony morphology on 2 % MEA in the dark for 28 d at different temperatures. Scale bars: A = 500 µm; B, E = 50 µm; C = 100 µm; D = 25 µm; F, G = 10 µm.

Diagnosis: Similar to *N. welwitschiae* but differs in conidial dimensions (3–5 × 1.5–2 µm, oblong to ellipsoidal) from *N. welwitschiae* (4–7 × 2–2.5 µm, sub-cylindrical). It can be differentiated from *N. welwitschiae* by the LSU (1 bp), *RPB2* (9 bp) and *TUB2* (5 bp) sequences.

Typus: **South Africa**, Northern Cape Province, Namakwa District Municipality, Nieuwoudtville Falls, from dying *Euphorbia mauritanica*, Aug. 2020, *M.J. Wingfield* (**holotype** PRU(M) 4549, culture ex-type CMW 56352, CMW-IA 50). GenBank: OR198841 (ITS); OR198847 (LSU); OR211855 (*TUB2*); OR211852 (*RPB2*).

Description: *Sexual morph* unknown. *Asexual morph* on toothpicks in WA. *Conidiomata* pycnidial or eustromatic, 50–238 × 46.5–172 (89.3 ± 33.82 × 83.1 ± 26.49) µm; when pycnidial, separate, immersed to superficial on media or superficial on toothpicks with base attached, globose to ellipsoidal, single or gregarious, base often missing when immersed in media; when eustromatic, stromata consisting of brown, thick-walled *textura angularis* tissues connecting pycnidial locules. *Ostioles* inconspicuous. *Conidiomatal walls* pseudoparenchymatous, composed of two tiers, 7–17 (11.3 ± 2.79) µm thick, inner tiers consisting of a few layers of sub-hyaline, thin-walled cells, outer tiers consisting of 1–3 layers of thick-walled, brown, compressed cells. *Conidiophores* borne along hymenial walls of inner cavity, reduced to conidiogenous cells, 4–6 × 3–5 µm. *Conidiogenous cells* blastic, doliiform. *Conidia* hyaline, oblong to ellipsoidal, aseptate, 3–5 × 1.5–2 (3.8 ± 0.35 × 1.5 ± 0.11) µm.

Culture characteristics: Colonies on MEA showing optimal growth at 25 °C showing 7.5 mm/d, followed by 20 °C (6.4 mm/d), 15 °C (4.2 mm/d), 10 °C (2.8 mm/d), 30 °C (2.4 mm/d) and 35 °C (0.5 mm/d). Cultures on MEA at 25 °C for 28 d in the dark, sterile, showing circular growth with even margin, aerial mycelia medium dense, cottony, colour above saffron (13d) to pale luteous (17d), reverse luteous (19b).

Host: *Euphorbia mauritanica*.

Distribution: South Africa (Northern Cape Province).

Notes: *Nothomicrosphaeropsis namakwaensis* grouped with *N. welwitschiae* in a well-supported clade (Fig. 5). The genus accommodates only one other species, *N. welwitschiae* isolated from dead leaves of *Welwitschia mirabilis* in Namibia (Crous *et al*. 2021). *Nothomicrosphaeropsis namakwaensis* can be distinguished from *N. welwitschiae* by its hyaline conidia, which do not darken with age and larger conidial dimensions.

Cytospora euphorbiicola N.Q. Pham, Marinc. & M.J. Wingf., ***sp. nov*.** MycoBank MB 849175. Fig. 8.

Etymology: The name refers to the host plant, *Euphorbia.*

Diagnosis: Similar to *C. tibouchinae* and *C. myrtagena* but differs in larger conidial dimensions (*C. euphorbiicola* 4–7 × 1–2 µm; *C. tibouchinae* 3–4 × 1–1.5 µm; *C. myrtagena* 3–4 × 1 µm). It can be differentiated from *C. tibouchinae* by the ITS (90 bp) and LSU (10 bp) sequences and *C. myrtagena* by the ITS (99 bp) sequence.

Typus: **South Africa**, Northern Cape Province, Namakwa District Municipality, Nieuwoudtville Falls, from dying *Euphorbia mauritanica*, Aug. 2020, *M.J. Wingfield* (**holotype** PRU(M) 4550, culture ex-type CMW 56355, CMW-IA 51). GenBank: OR198839 (ITS); OR198845 (LSU); OR211853 (*TUB2*); OR211850 (*RPB2*); OR211857 (*TEF1*); OR211856 (*ACT*).

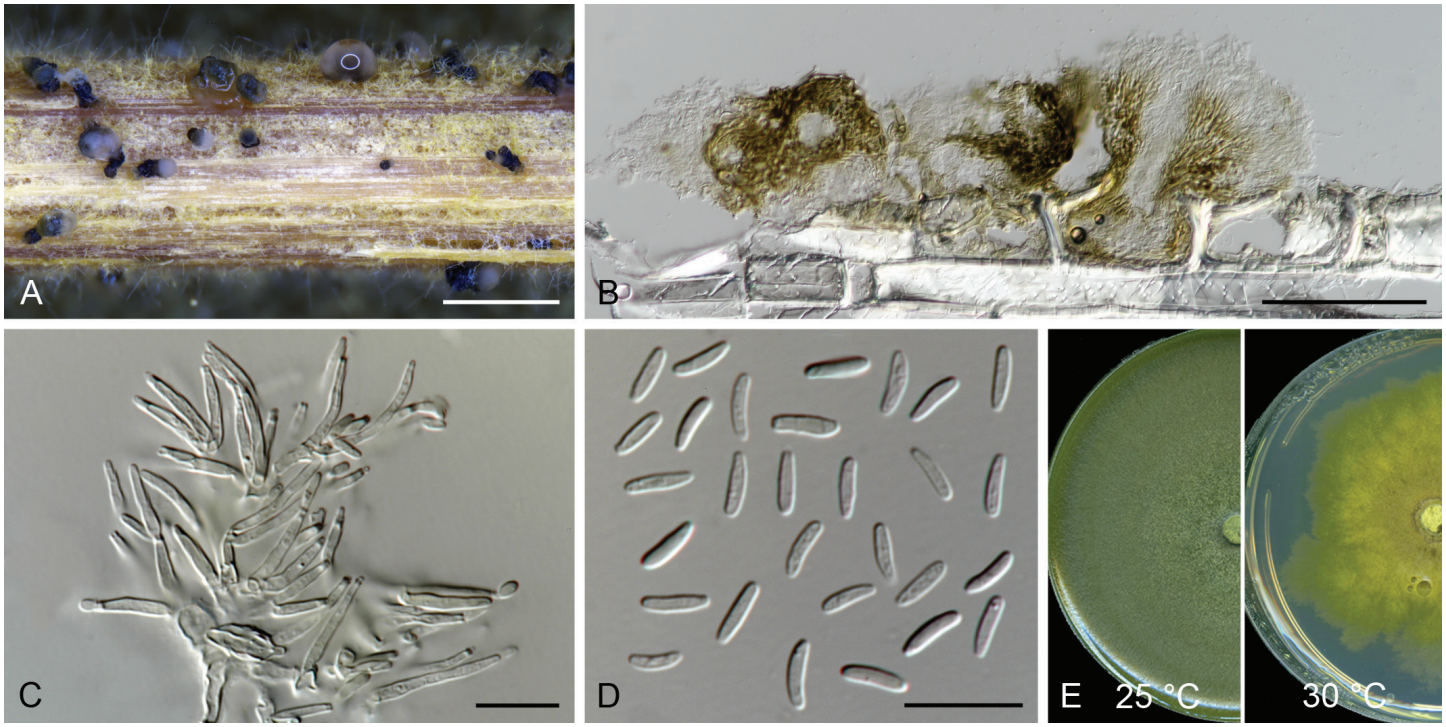

Fig. 8. Micrographs of *Cytospora euphorbiicola sp. nov*. (ex-type: CMW-IA 51, CMW 56355). **A.** Sporodochia formed on toothpick. **B.** Vertical section through sporodochia. **C.** Conidiophores and conidiogenous cells. **D.** Conidia. **E.** Colony morphology on 2 % MEA incubated in the dark for 28 d at 25 °C and 30 °C. Scale bars: A = 1 mm; B = 100 µm; C, D = 10 µm.

Description: *Sexual morph* unknown. *Asexual morph* on toothpicks placed on WA medium. *Conidiomata* sporodochial. *Conidiophores* borne on hymenial layer inner cavity, branched irregularly at the base and above. *Conidiogenous cells* phialidic, cylindrical tapering towards apex, integrated, acropleurogenous, collarette minute, 7.5–16 × 1–2 (10.6 ± 1.59 × 1.5 ± 1.82) µm. *Conidia* hyaline, aseptate, fusiform to allantoid, straight or curved, 4–7 × 1–2 (5.3 ± 0.52 1.3 ± 0.15) µm, in slimy milky mass or droplets.

Culture characteristics: Colonies on MEA showing optimal growth at 25 °C showing 13.7 mm growth per day, followed by 20 °C (13.2 mm/d), 15 °C (9 mm/d), 10 °C (3.9 mm/d), 30 °C (3.3 mm/d) and 35 °C (growth only on the plug). Cultures on MEA for 28 d in the dark at 25 °C sterile, showing circular growth with even edge, aerial mycelia flat, medium dense, colour above and reverse citrine (21k); at 30 °C circular growth with uneven margin, colour above amber (21'b), at 10 °C above pale luteous (19d).

Host: *Euphorbia mauritanica*.

Distribution: South Africa (Northern Cape Province).

Notes: *Cytospora euphorbiicola* shows close phylogenetic affinity to *C. tibouchinae* and *C. myrtagena*, both isolated from members of the *Myrtales*. *Cytospora tibouchinae* was reported from *Tibouchina semidecandra* in La Réunion (Crous *et al*. 2016), and *C. myrtagena* from *Eucalyptus grandis* and *Tibouchina urvilleana* in Sumatra (Indonesia) and Hilo (Hawaii, USA) (Adams *et al*. 2005, Rossman *et al*. 2015). *Cytospora euphorbiicola* (4–7 × 1–2 µm) can be differentiated from both species by its larger conidial dimensions (*C. tibouchinae* 3–4 × 1–1.5 µm; *C. myrtagena* 3–4 × 1 µm). No sexual morph is known for *Cytospora euphorbiicola* and *C. tibouchinae* (Crous *et al*. 2016), but one is known for *C. myrtagena* (Adams *et al.* 2005)

Pathogenicity

Eight weeks after inoculation, all inoculated *Euphorbia* stems remained living, but those inoculated with fungi had distinct lesions around the points of inoculation. In contrast, there were only small areas of wound-associated discolouration for the control inoculations. Mean lesion area associated with five fungal isolates did not differ significantly from each other, but they were all significantly larger than the lesions associated with the controls ($p < 0.05$) (Fig. 9). Re-isolations from the lesion margins resulted in 100 % recovery of the inoculated fungi, while isolations made from the control inoculations yielded either nothing or only secondary contaminant fungi.

DISCUSSION

Dying *E. mauritanica* shrubs such as those examined in this study have been observed in parts of the Western and Northern Cape Provinces of South Africa for at least five years. The results of this study showed that a number of fungi are present on the primary lesions associated with the die-off of these plants. These included some known fungi as well as the various new taxa described here. The greenhouse pathogenicity test confirmed that these fungi were opportunists and most likely contributed to the die-off of the shrubs, possibly predisposed by environmental factors.

Alanphillipsia aloes (*Botryosphaeriaceae*) was originally reported from dark lesions associated with dying *Aloidendron* (*Aloe) dichotoma* in South Africa (Crous *et al*. 2013). Five species are known in the genus, including *Ala. aloes*, *Ala. aloetica*, *Ala. aloicola*, *Ala. aloigena* and *Ala. euphorbiae*. They have been reported exclusively from indigenous *Aloe* and *Euphorbia* trees in arid regions of South Africa (Crous *et al*. 2013, 2014). Members of the *Botryosphaeriaceae* (Slippers & Wingfield 2007) are well-known opportunistic fungi that tend to become active under conditions of host plant stress. It was thus not surprising that *Ala. aloes* did not emerge as a primary pathogen in our inoculation studies.

Two pycnidia-producing isolates represented two new taxa in the *Didymellaceae*, *Austrophoma eugeniae* and *Nothomicrosphaeropsis namakwaensis*. The phylogenetic placement of these fungi with closely-related genera within the family was well supported in multi-gene analyses. The only species in the genus *Nothomicrosphaeropsis*, *N. welwitschiae*, was isolated from dead leaves of *Welwitschia mirabilis*, which is found in the Namib desert (Crous *et al*. 2021). *Nothomicrosphaeropsis namakwaensis* described here is the second species to be described in the genus and can be clearly differentiated from *N. welwitschiae* based on the sequences of *RPB2* and *TUB2* regions. It showed some degree of pathogenicity to the *E. mauritanica* plants, but it did not result in death.

Austrophoma is introduced here as a new genus in the *Didymellaceae*, with *Aus. euphorbiae* as the type species. Although only a single isolate of this fungus was collected, its unique habitat justified our decision to describe it. As in the case of *Nothomicrosphaeropsis,* in which we discovered a second species in this study, it is reasonable to anticipate that additional

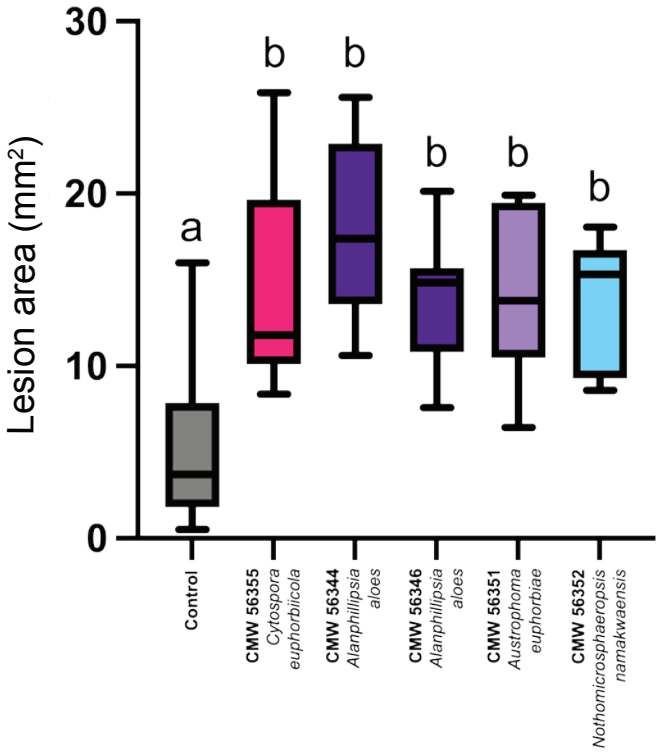

Fig. 9. Boxplots indicating lesion area resulting from inoculations with five fungal isolates and the control on *Euphorbia mauritanica*. Vertical bars represent standard error of the means. Bars with different letters indicate statistically significance at $P \leq 0.05$.

species of *Austrophoma* will emerge as studies in this unusual and arid environment are undertaken. *Austrophoma euphorbiae* showed significant lesion development in the pathogenicity tests but is unlikely to have contributed to disease development.

Cytospora euphorbiicola (*Cytosporaceae*) grouped with *C. tibouchinae* (La Réunion) and *C. myrtagena* (North Sumatra, Indonesia; Hawaii, USA) (Adams *et al*. 2005, Crous *et al*. 2016, Rossman *et al.* 2015). *Cytospora* spp. are well-known opportunistic fungi, having a cosmopolitan distribution and commonly occurring in healthy woody plants as asymptomatic endophytes. However, they can also cause diseases in various woody plants, usually under conditions of stress (Adams *et al*. 2005, Mehrabi *et al*. 2011, Zhang *et al*. 2012, Du *et al*. 2013, Palavouzis *et al*. 2015). An extensive study by Adams *et al*. (2006) identified 14 *Cytospora* spp. from South Africa, and later Jami *et al*. (2018) described *C. carpobroti* causing disease on sour fig, *Carpobrotus edulis*. *Cytospora euphorbiicola* is the 16th species of *Cytospora* to be described from South Africa. It was able to cause lesions on the stems of *Euphorbia* plants but, like all other fungi considered in this study, did not result in plant death.

Greenhouse inoculations with the fungi most commonly encountered in this study showed that they are all able to cause lesions, but none were found to be primary pathogens and able to kill *E. mauritanica*. We thus conclude that the fungi most commonly associated with the die-off of *E. mauritanica* are not the primary cause of the observed disease. This situation could be similar to the case of *E. ingens* plants that are dying in large numbers where they occur naturally in South Africa (Van der Linde *et al*. 2012a). The die-off of *E. ingens* plants is associated with a suite of complex stress factors, including biotic and abiotic agents and anthropogenic activities in their habitats (Van der Linde *et al.* 2011, 2012a, b, 2016, 2017a, b, 2018). A particular interest was in *Lasiodiplodia theobromae*, *Gondwanamyces serotectus* and *G. ubusi*, which in pathogenicity tests, gave rise to extensive internal rot and lesions in the succulent branches (Van der Linde *et al.* 2011, 2012b). Apart from biotic agents, moisture deficiency resulting from increasing temperatures and decreasing precipitation was noted as a major abiotic contribution to the die-off of *E. ingens* (Van der Linde *et al.* 2012a).

It is reasonable to assume that the death of *E. mauritanica* plants considered in this study has been subjected to stress since the sampling took place after a prolonged drought. The fungi encountered are likely endophytes that have proliferated under these stressful conditions resulting in large-scale die-offs.

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Conflict of interest: The authors declare that there is no conflict of interest.

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Supplementary information

Table S1. Collection details and GenBank accessions of *Alanphillipsia* isolates included in the phylogenetic analyses.

Table S2. Collection details and GenBank accessions of *Cytospora* isolates included in the phylogenetic analyses.

Table S3. Collection details and GenBank accessions of *Didymellaceae* isolates included in the phylogenetic analyses.