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Greater Botryosphaeriaceae diversity in healthy than associated diseased *Acacia karroo* tree tissues

Fahimeh Jami · Bernard Slippers · Michael J. Wingfield · Marieka Gryzenhout

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Abstract Botryosphaeriaceae are common endophytes of trees. Some species are also known to be pathogens. It is, therefore, assumed that endophytic Botryosphaeriaceae are often involved in general die-back diseases. Here we test this assumption in severe branch die-back observed on *Acacia karroo* trees in the Pretoria area of South Africa. The presence of the Botryosphaeriaceae was compared between healthy and diseased tissue on the same trees. Eight Botryosphaeriaceae species were isolated from die-back and healthy branches. Of these, six species, namely *Tiarosporella urbis-rosarum, Diplodia allocellula, Phaeobotryosphaeria variabilis, Dothiorella brevicollis* and *Neofusicoccum vitifusiforme* were obtained from healthy tissues, and only two species, *Dothiorella dulcispinae* and *Spencermartinsia pretoriensis*, were exclusively found in die-back branches. *Spencermartinsia viticola* was found in both tissue types and this fungus was also the most commonly isolated species from both healthy and die-back samples. Results of pathogenicity trials showed highly variable results for the isolated species and that the two species associated only with die-back symptoms, were weakly pathogenic. These results suggest that the Botryosphaeriaceae found in these trees were not directly associated with the die-back symptoms, despite their diversity and common occurrence in these tissues. The situation is different in other tree systems where dominant species, often with wide host ranges, have been shown to be involved in die-back diseases. This indicates the importance of characterizing the unique aspects of each tree disease system.

Keywords Botryosphaeriaceae · *Acacia karroo* · Die-back · Diversity · South Africa

F. Jami · M. J. Wingfield
Department of Microbiology and Plant Pathology, Forestry & Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa

B. Slippers
Department of Genetics, Forestry & Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa

M. Gryzenhout (✉)
Department of Plant Sciences, University of the Free State, Bloemfontein, South Africa
e-mail: Gryzenhoutm@ufs.ac.za

Introduction

Species residing in the fungal family Botryosphaeriaceae include latent pathogens that occur asymptomatically as endophytes for extended periods, but cause disease under stress conditions (Slippers and Wingfield 2007; Smith et al. 1996; Van Niekerk et al. 2011; Denman et al. 2000). Symptoms of these diseases include die-back followed by resin exudation, blackish discoloration of the heartwood and pith, fruit rot, leaf blight, premature leaf drop, gummosis and in severe cases tree death (Slippers et al. 2007; Slippers and Wingfield 2007). Some species of the Botryosphaeriaceae have wide host ranges and they also occur on all continents other than Antarctica (Slippers and Wingfield 2007; Taylor et al. 2009).

The pathogenicity of some Botryosphaeriaceae species has been well established, but the true role of most described species in disease is poorly studied. Many Botryosphaeriaceae have been isolated from die-back symptoms, others only from asymptomatic tissues and some have been found in both tissue types (Slippers and Wingfield 2007). When tested, many species have been shown to be pathogenic (Slippers and Wingfield 2007). For instance, species from die-back

symptoms that have been shown to be aggressive pathogens in artificial inoculation trials include *Diplodia africana* on *Juniperus phoenicea* (Linaldeddu et al. 2012), *Neofusicoccum parvum* on *Eucalyptus globulus* (Iturritxa et al. 2011) and *Syzygium paniculatum* (Ploetz et al. 2009), *Lasiodiplodia theobromae, L. pseudotheobromae* and *L. egyptiacae* on *Mangifera indica* (Ismail et al. 2012) and *Botryosphaeria dothidea, N. luteum, N. mediterraneum* and *N. parvum* on *Ficus microcarpa* (Mayorquin et al. 2012). However, *L. gonubiensis* has been isolated from asymptomatic tissues of the native tree *S. cordatum* (Pavlic et al. 2004), but was also shown to cause lesions in pathogenicity trials (Pavlic et al. 2007). Several species have been isolated from both healthy and die-back. *Lasiodiplodia theobromae* were isolated from necrotic branches of *Vaccinium* species (Wright and Harmon 2009), vine die-back (Taylor et al. 2005; Van Niekerk et al. 2004) and healthy tissues of *Terminalia catappa* (Begoude et al. 2010) and *Eucalyptus* spp. (Pérez et al. 2010), while *L. margaritacea* were isolated from both healthy and die-back symptoms on native *Adansonia gregorii* (Pavlic et al. 2008; Sakalidis et al. 2011). All of these species were shown to be pathogenic in pathogenicity tests. The pathogenicity of some other Botryosphaeriaceae isolated from die-back symptoms, such as *N. protearum* from die-back of native *Protea* spp. (Denman et al. 2003), *Spencermartinsia viticola* and *Dothiorella iberica* on grapevine in New South Wales and South Australia (Luque et al. 2005; Pitt et al. 2010) still remain unknown in plant pathogenicity tests. This matter is further complicated because many studies fail to clearly indicate whether isolates have been obtained as endophytes or from diseased tissue.

Acacia karroo or sweet thorn (Fabales: Mimosoideae) is the most widespread native *Acacia* in southern Africa (Timberlake et al. 1999) and plays an important role in increasing soil fertility through nitrogen fixation with rhizobia nodules (Barnes et al. 1996). Die-back symptoms on branches of *A. karroo* are common in South Africa but they have become quite severe in the Pretoria area (Gauteng Province). Larval tunnels of an unidentified cerambycid beetle were sometimes observed in these dieback symptoms, and especially in the necrotic parts of the branch samples (Fig. 1). Cerambycid beetles have been reported from *Acacia* species in various parts of the world (Eisa and Roth 2009; Elliott and De Little 1985; Watt 1983). The larvae of cerambycid beetles (Coleoptera: Cerambycidae) are xylophagous and create a network of tunnels while feeding in different tissues of healthy, dead or decaying woody tissues of plants (Haack and Slansky 1987). Some cerambycid beetles can directly kill the trees or branches because their feeding in the cambium layers destroys the vascular tissues (Rad 2006; Hawkeswood 2011). These larvae could thus be involved in causing some of these symptoms or cause stress to the tree, but were not associated with them frequently enough to be the main causal agent.

Botryosphaeriaceae species are known to be associated with die-back symptoms on *A. karroo* and other *Acacia* trees in South Africa (Jami et al. 2012; Van der Walt 2008), and could thus be associated with the increased die-back of *A. karroo* in the Pretoria area. The aim of this study was to determine whether these species of Botryosphaeriaceae are also present and associated with the striking branch die-back symptoms in Pretoria, which have not been sampled previously. Due to the endophytic nature of the Botryosphaeriaceae, species of these fungi would most likely be associated with branches. Species occurring in die-back branches were thus compared with those found in asymptomatic tissues to establish a better understanding of the diversity of species existing as endophytes on these trees, and their potential relationship with those involved in the die-back symptoms.

Materials and methods

Collection of samples

Samples were collected from 40 *A. karroo* trees having branches with die-back at different locations in the greater Pretoria area, Gauteng Province. The die-back begins where leaves on branch tips begin to wilt, turn brown and die, but they remain attached to the plant resulting in "flagging" symptoms (Fig. 1). Other than wilting, no symptoms were observed on the leaves. Lesions were formed in the woody tissue, with dead tissue extending internally within branches and often associated with gum production on the outside of the branches. These lesions were clearly the cause of the die-back symptoms as the wilting only occurred to the terminal ends of these lesions and wilting due to problems at the roots were unlikely.

A single branch showing die-back with internal lesions and an asymptomatic branch were collected from each tree. The branch samples were placed in paper bags and transported to the laboratory. For endophyte isolations, a selected portion of each branch was cut into 0.5 cm and twelve pieces were randomly selected from each branch. From the die-back branches, the portions selected were taken from the border zone between healthy and discolored wood. All of these amounted to 480 pieces in total from die-back and 480 from healthy branches, which were surface-disinfested in 10 % hydrogen peroxide for 2 min, and rinsed three times in sterile water. Representative samples from all branches were placed onto 2 % malt extract agar (four pieces per plate) (Biolab, Midrand, South Africa). Pieces

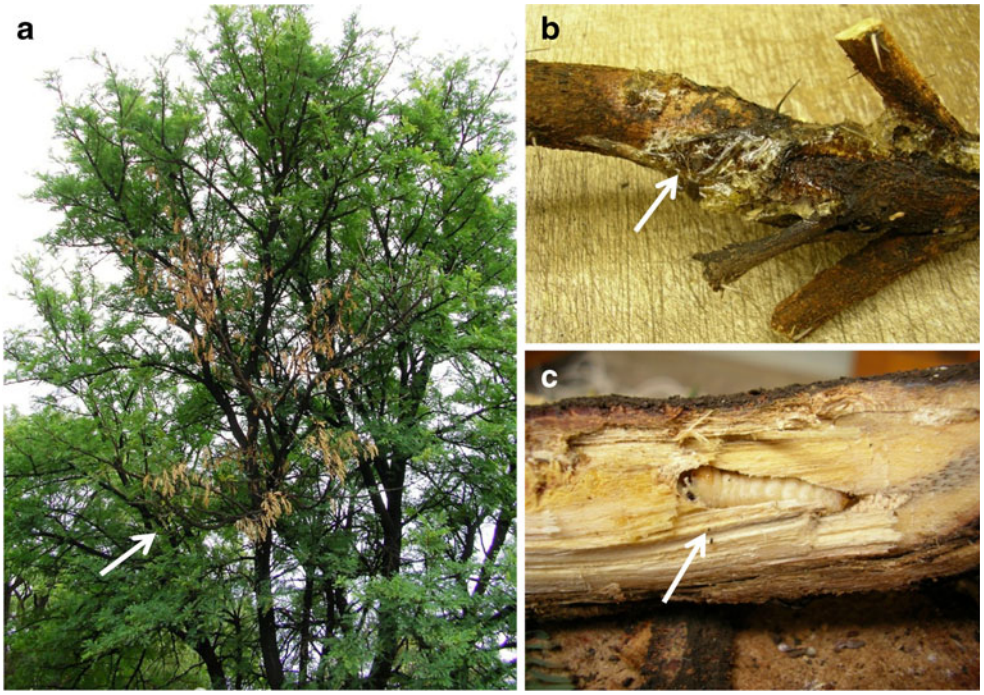

Fig. 1 **a** *Acacia karroo* tree with die-back branches (*arrow*). **b** Gummosis (*arrow*) on die-back branch. **c** Cerambycid larva (*arrow*) that are occasionally found within a die-back branch

from diseased tissue were thus also placed onto agar selective for *Phytophthora* (NARPH) (Shearer and Dillon 1995) and between two slices of carrot for the isolation of *Ceratocystis* species (Moller and Devay 1968).

The plates and carrot pieces were incubated at 24 °C for 7 days and the fungal growth from each wood sample showing morphology characteristic of the Botryosphaeriaceae was transferred from the primary isolations to new MEA plates. After 4–5 days, all those isolates showing typical fast growing, white to black cultures with fluffy aerial hyphae were transferred to 15 % WA (water agar) in order to make single hyphal tip sub-cultures. These isolates are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

DNA sequence analyses

Isolates (Table 1) were initially grouped based on culture morphology (fast growing, white to black cultures with aerial hyphae). DNA was extracted from the mycelium of 5-day-old single hyphal-tip cultures (Lee and Taylor 1990) representing three cultures for each morphological group. Sequence data were obtained for the internal transcribed spacer region of the ribosomal RNA (rRNA) operon using primers ITS-1 (Gardes and Bruns 1993) and ITS-4 (White et al. 1990), the β-tubulin gene using primers Bt2a and Bt2b (Glass and Donaldson 1995), the translation elongation factor 1-α (TEF-1α) gene using primers EF1-728F and EF1-986R (Carbone and Kohn 1999) and the large subunit rDNA (LSU) gene region using primers LR0 and LR5 (Vilgalys and Hester 1990).

The PCR reaction mixture, PCR conditions and visualization were as described by Jami et al. (2012). The amplified PCR fragments were purified with Sephadex (Sigma, Steinheim, Germany) and sequenced with the BigDye terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, California, USA) in both directions, with the same primers used for the PCR reactions. PCR products were sequenced using an ABI 3730 48 capillary sequencer (Perkin-Elmer Applied Biosystems).

Sequences of the isolates were edited using Vector NTI 11 (Lu and Moriyama 2004). DNA sequences for relevant Botryosphaeriaceae species previously published were retrieved from GenBank (http://www.ncbi.nlm.gov). The resulting data matrices for each gene region were rooted with *Pseudofusicoccum stromaticum* following the example of Phillips et al. (2008). The data matrices were aligned online using MAFFT (http://align.bmr.kyushuu.ac.jp/mafft/online/server/) version 6 (Katoh et al. 2005) and checked manually for alignment errors.

Phylogenetic analyses of sequence data for Maximum Parsimony (MP) and Maximum Likelihood (ML) were made using PAUP* v.4.0b10 (Swofford 2001). Maximum parsimony (MP) genealogies for single genes were constructed with the heuristic search option (100 random taxa additions, tree bisection and reconstruction or TBR in PAUP). The uninformative aligned regions within each dataset were removed from the analyses, gaps were treated as fifth character and all characters were unordered and of equal weight. Branches of zero length

Table 1 Representative isolates from *Acacia karroo* used in the phylogenetic analyses

Isolate No.	Identity	Host	Location	Symptoms	Collector	GenBank			
						ITS	EF1-α	LSU	β-tubulin
CMW37928	*Spencermartinsia viticola*	Tree no.6	Pretoria, South Africa	Healthy	F. Jami & M. Gryzenhout	JX283730	JX283741	JX456000	JX283717
CMW37929	*S. viticola*	Tree no.22	Pretoria, South Africa	Healthy	F. Jami & M. Gryzenhout	JX283731	JX283744	JX456001	JX283720
CMW37930	*S. viticola*	Tree no.19	Pretoria, South Africa	Healthy	F. Jami & M. Gryzenhout	JX283732	JX283745	JX456002	JX283721
CMW37931	*S. viticola*	Tree no.34	Pretoria, South Africa	Die-back	F. Jami & M. Gryzenhout	JX283733	JX283742	JX456003	JX283716
CMW37932	*S. viticola*	Tree no.33	Pretoria, South Africa	Die-back	F. Jami & M. Gryzenhout	JX283734	JX283743	JX456004	JX283718
CMW37933	*S. viticola*	Tree no.1	Pretoria, South Africa	Die-back	F. Jami & M. Gryzenhout	JX283735	JX283745	JX45599	JX283719
CMW36463	*Dothiorella brevicollis*	Tree no.6	Pretoria, South Africa	Healthy	F. Jami & M. Gryzenhout	JQ239403	JQ239390	JQ239416	JQ239371
CMW36464	*Do. brevicollis*	Tree no.38	Pretoria, South Africa	Die-back	F. Jami & M. Gryzenhout	JQ239404	JQ239391	JQ239417	JQ239372
CMW36460	*Dothiorella dulcispinae*	Tree no.39	Pretoria, South Africa	Die-back	F. Jami & M. Gryzenhout	JQ239400	JQ239387	JQ239413	JQ239373
CMW36461	*Do. dulcispinae*	Tree no.12	Pretoria, South Africa	Die-back	F. Jami & M. Gryzenhout	JQ239401	JQ239388	JQ239414	JQ239374
CMW36462	*Do. dulcispinae*	Tree no.14	Pretoria, South Africa	Healthy	F. Jami & M. Gryzenhout	JQ239402	JQ239389	JQ239415	JQ239375
CMW36480	*Spencermartinsia pretoriensis*	Tree no.34	Pretoria, South Africa	Die-back	F. Jami & M. Gryzenhout	JQ239405	JQ239392	JQ239418	JQ239376
CMW36481	*S. pretoriensis*	Tree no.36	Pretoria, South Africa	Die-back	F. Jami & M. Gryzenhout	JQ239406	JQ239393	JQ239419	JQ239377
CMW37934	*Neofusicoccum vitifusiforme*	Tree no.35	Pretoria, South Africa	Healthy	F. Jami & M. Gryzenhout	JX283728	JX283746	JX456005	JX283722
CMW37935	*N. vitifusiforme*	Tree no.14	Pretoria, South Africa	Healthy	F. Jami & M. Gryzenhout	JX283729	JX283747	JX456006	JX283723
CMW36482	*Phaeobotryosphaeria variabilis*	Tree no.21	Pretoria, South Africa	Healthy	F. Jami & M. Gryzenhout	JX283726	JX283738	JX456007	JX283714
CMW36483	*P. variabilis*	Tree no.23	Pretoria, South Africa	Healthy	F. Jami & M. Gryzenhout	JX283727	JX283739	JX456008	JX283715
CMW36468	*Diplodia allocellula*	Tree no.7	Pretoria, South Africa	Healthy	F. Jami & M. Gryzenhout	JQ239397	JQ239384	JQ239410	JQ239378
CMW36469	*D. allocellula*	Tree no.8	Pretoria, South Africa	Healthy	F. Jami & M. Gryzenhout	JQ239398	JQ239385	JQ239411	JQ239379
CMW36470	*D. allocellula*	Tree no.9	Pretoria, South Africa	Healthy	F. Jami & M. Gryzenhout	JQ239399	JQ239386	JQ239412	JQ239380
CMW36465	*Tiarosporella urbis-rosarum*	Tree no.4	Pretoria, South Africa	Healthy	F. Jami & M. Gryzenhout	JX283736	JX283748	JX45597	JX283724
CMW36466	*T. urbis-rosarum*	Tree no.5	Pretoria, South Africa	Healthy	F. Jami & M. Gryzenhout	JX283737	JX283749	JX45598	JX283725
CMW36479	*T. urbis-rosarum*	Tree no.3	Pretoria, South Africa	Healthy	F. Jami & M. Gryzenhout	JQ239409	JQ239396	JQ239422	JQ239383

were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the tree(s) obtained was evaluated by 1,000 bootstrap replications. Congruence between the different datasets was tested using the Partition Homogeneity Test (PHT) in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Farris et al. 1995; Huelsenbeck et al. 1996), with the uninformative characters removed before analysis. Other measures such as tree length (TL), consistency index (CI), rescaled consistency index (RC), and the retention index (RI) (Hillis and Huelsenbeck 1992) were recorded.

For ML analyses, the best nucleotide substitution models for each dataset were found separately with Modeltest 3.7 (Posada and Buckley 2004). All three datasets best fitted the GTR model with the ITS, TEF-1α, β-tubulin and combined datasets having the following parameters: for ITS G=0.337, I=0.468; for TEF-1α G=0.819, I=0.759;

for β-tubulin G=0.246, I=0; for LSU G=0.310, I=0 and for combined datasets G=0.318, I=0.436. The analyses were performed in PAUP 4.0b10 and confidence levels were determined with 1,000 bootstrap replications.

Pathogenicity tests

One-year-old *A. karroo* seedlings with stems ranging in height from 70–110 cm and 0.7–1.5 cm in diameter, and growing in a 1:2:4 mixture of river sand, red top soil and pine bark potting soil in 5-L plastic bags, were maintained in a greenhouse at 26 °C and watered once each day. Two to three isolates of each Botryosphaeriaceae species were randomly selected and each isolate was inoculated into 10 trees with one additional tree in each replication inoculated as a control. In total, 23 isolates were inoculated into 230 plants, totaling to 253 plants including the plants inoculated as negative controls.

For inoculation, a section of bark was removed from the main stems of the seedlings 15 cm above the soil level with a 6 mm sterilized cork-borer to expose the cambium. A 6 mm plug of agar, covered with mycelium of the test fungus, was placed with the mycelium surface facing inwards onto the wound, while clean agar discs were used in control inoculations. The inoculated wounds were sealed with Parafilm to minimize contamination and to prevent desiccation of the inoculum. Lesion lengths were measured 6 weeks after inoculation. Variation in the extent of the lesions was analyzed through a one-way analysis of variance (Karadzic 2003) with the general linear model procedure from SAS (version 9.3).

To re-isolate the inoculated fungi in order to confirm Koch's postulates, one plant was chosen from those inoculated for each isolate (in total 23 plants) and from the controls of each species (in total 8 plants). These were identified based on the conidial morphology. A small sample of tissue was cut from the lesions, including the inoculation points, surface disinfested with 10 % hydrogen peroxide for 2 min, and rinsed three times in sterile water. The tissue samples were plated onto 2 % malt extract agar and incubated at 24 °C for 7 days.

Results

Collection of samples

In total 164 Botryosphaeriaceae isolates were isolated from *A. karroo* branches. Of these 84 isolates were from the 480 healthy wood pieces and 80 isolates were from the equivalent number of wood pieces displaying die-back symptoms. At least 50 % of sampled trees yielded isolates and these were from across the geographical range sampled. No isolates of *Ceratocystis* or *Phytophthora* were obtained in this study.

DNA sequence analyses

The datasets for the ITS, TEF-1α and β-tubulin sequences were analyzed individually and in combination. The ITS sequence dataset contained 546 characters (4 parsimony-uninformative, 148 parsimony-informative, 370 constant characters) with CI=0.602, RI=0.902, RC=0.543, HI=0.398 and TL=321. The TEF-1α dataset contained 362 characters (3 parsimony-uninformative, 224 parsimony-informative, 135 constant characters) with CI=0.651, RI=0. 919, RC=0.550, HI=0.383 and TL=986. The β-tubulin dataset contained 471 characters (0 parsimony-uninformative, 142 parsimony-informative, 329 constant characters) with CI=0.698, RI=0.901, RC=0.629, HI=0.302, and TL=304. The LSU dataset contained 845 characters (7 parsimony-uninformative, 72 parsimony-informative and 766 constant characters) with CI=0.642, RI=0.875, RC=0.587, HI=0.328 and TL=138. The tree statistics for the combined dataset (TreeBase Accession No. S12358) were CI=0.487, RI=0.854, RC=0.416, HI=0.513, TL=2,148, and the partition homogeneity test (PHT) on the datasets produced a P-value of 0.01.

The topology of the trees obtained using the ML and MP analyses were similar for the individual gene regions, as well as in the combined analysis with regards to the clades representing species isolated in this study. However, clades representing genera occasionally collapsed in individual analyses. Isolates resided in five genera and eight species that were identified as follows: *Spencermartinsia pretoriensis*, *S. viticola, Dothiorella dulcispinae, Do. brevicollis*, *Diplodia allocellula, Phaeobotryosphaeria variabilis*, *Tiarosporella urbis-rosarum* and *Neofusicoccum vitifusiforme* (Fig. 2).

Of the eight Botryosphaeriaceae species, *S. viticola* was most common and dominant in both healthy and die-back tissue. Of total isolates, 77.3 % represented *S. viticola*, 2.3 % *Do. brevicollis*, 9.5 % *D. allocellula,* 3.5 % *P. variabilis,* 3.5 % *T. urbis-rosarum* and 3.5 % *N. vitifusiforme* (Table 1). The isolates of *Spencermartinsia pretoriensis* (CMW36480 and CMW36481), *Dothiorella dulcispinae* (CMW36460, CMW36461 and CMW36462) and *S. viticola* (CMW37931, CMW37932 and CMW37933) were representative of isolates from branches with die-back (2.5 % *S. pretoriensis*, 10 % *Do. dulcispinae* and 87.5 % *S. viticola*) (Table 1). Representative isolates from healthy branches included *S. viticola* (CMW37928, CMW37929 and CMW37930), *Do. brevicollis* (CMW36464 and CMW 36463), *Diplodia allocellula* (CMW36468, CMW36469 and CMW36470), *Phaeobotryosphaeria variabilis*

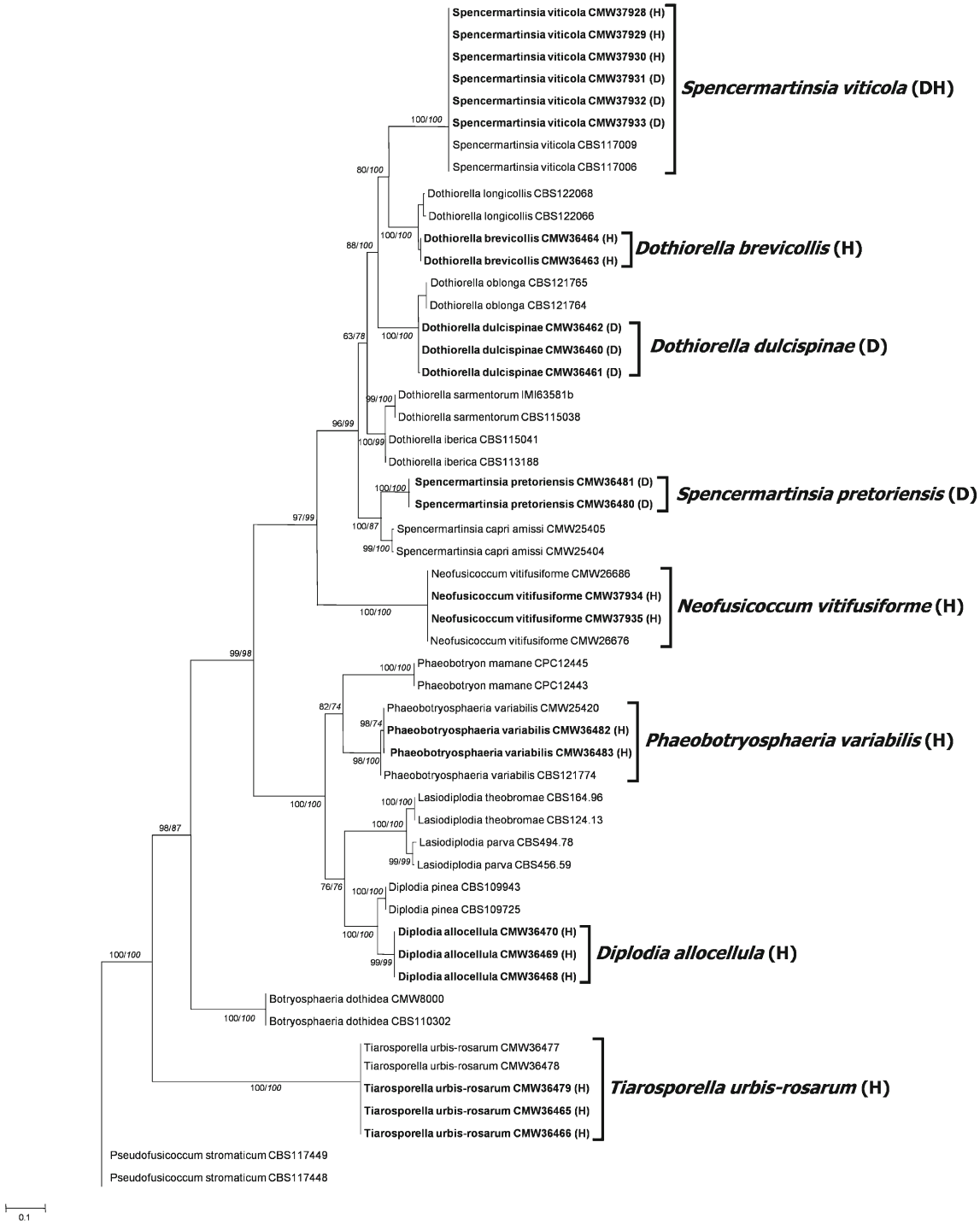

Fig. 2 Maximum Likelihood (ML) tree of the combined data set of ITS ribosomal DNA, TEF-1α, β-tubulin and LSU gene region sequences. Bootstrap values for ML (Piano et al. 2005) and Maximum Parsimony (*italic*) above 60 % are given at the nodes. The tree was rooted to *Pseudofusicoccum stromaticum* (CBS117448 and CBS117449). H = Isolates from healthy branches, D = Isolates from die-back branches

(CMW36482, CMW36483), *Tiarosporella urbis-rosarum* (CMW36479, CMW36465 and CMW36466) and *Neofusicoccum vitifusiforme* (CMW37934, CMW37935). Some isolates were obtained from the same tree such as those of *Do. brevicollis* (CMW 36463) and *S. viticola* (CMW37928), *S. viticola* (CMW37931) and *S. pretoriensis* (CMW36480), and *Do. dulcispinae* (CMW36462) and *N. vitifusiforme* (CMW37935), respectively (Table 1). The rest of the isolates were obtained from different trees.

Fig. 3 **(a)** Stem of seedling with a distinct lesion resulting from inoculation with *Phaeobotryosphaeria variabilis*. **(b)** Seedling inoculated as a control with no evidence of lesion development

Pathogenicity tests

All 23 isolates of the eight species produced lesions in the cambium of inoculated branches within 6 weeks (Fig. 3), and the average lesions length were significantly ($P<0.05$) larger than those observed for the controls. Statistical analyses showed that lesion sizes varied little between the ten inoculated trees for each isolate, but did vary considerably between the isolates used for some species, namely *Do. brevicollis*, *T. urbis-rosarum* and *S. viticola*. The longest lesions were produced by isolates of *P. variabilis* (average lesion length 30.5 mm), one isolate of *Do. brevicollis* (average lesion length 17 mm), one isolate of *S. viticola* (average lesion length 12 mm) and one isolate of *T. urbis-rosarum* (average lesion length 11 mm). All these isolates were obtained from healthy tissues, even though the pathogenicity test showed that they were the most pathogenic isolates. Isolates obtained from die-back tissues represented some of the isolates producing the shortest lesions, namely isolates of *S. pretoriensis* (average lesion length 5 mm) and *Do. dulcispinae* (average lesion length 8.5 mm). Two isolates of *S. viticola* (average lesion length 5 mm), of which one originated from die-back and another from healthy tissues, and two isolates of *T. urbis-rosarum* (average lesion length 4.5 mm) that were both from healthy tissues (Fig. 4)

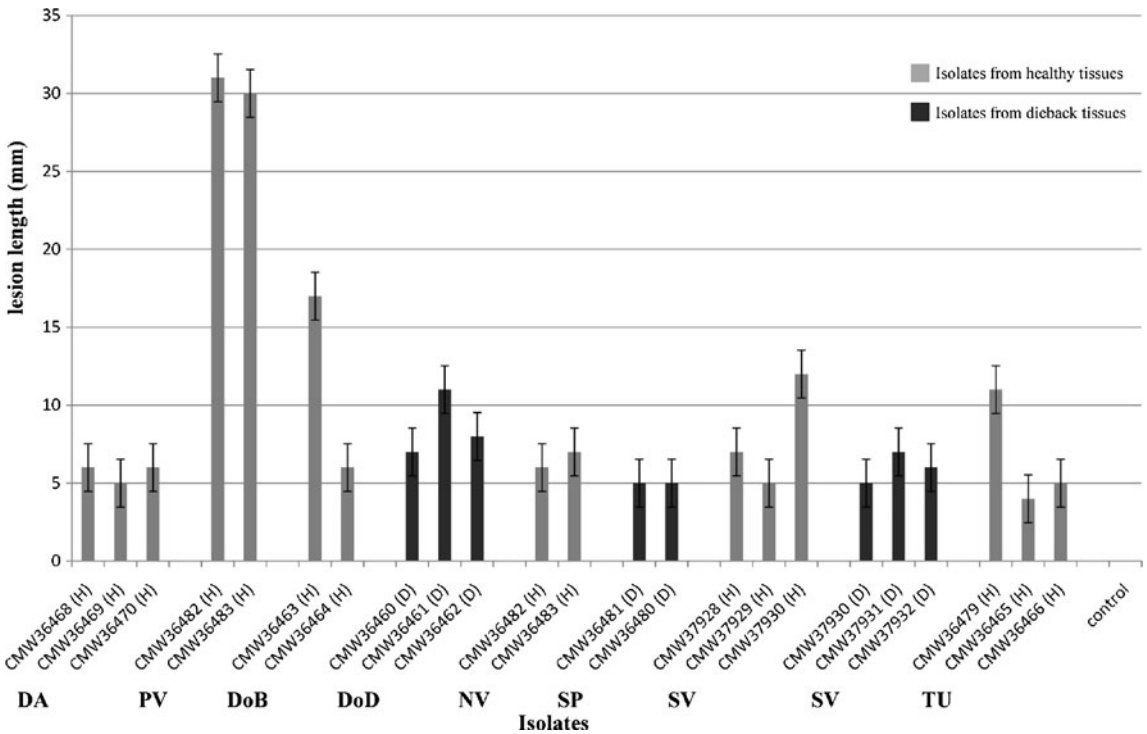

Fig. 4 Average length (mm) of lesions produced on *Acacia karroo* seedlings inoculated with 19 isolates of eight Botryosphaeriaceae species (isolates were obtained from healthy and diseased tissue) and a negative control after 5 weeks in a greenhouse. All isolates produced lesions significantly greater ($P<0.05$) than those of the controls. H = Isolates from healthy branches, D = Isolates from die-back branches. *DA Diplodia allocellula*, *PV Phaeobotryosphaeria variabilis*, *DoB Dothiorella brevicollis*, *DoD Do. dulcispinae*, *NV Neofusicoccum vitifusiforme*, *SP Spencermartinsia pretoriensis*, *SV S. viticola*, *TU Tiarosporella urbis-rosarum*

were among those isolates that produced the smallest lesions. All eight Botryosphaeriaceae species were re-isolated from lesions and no Botryosphaeriaceae were isolated from the controls.

Discussion

Eight species of Botryosphaeriaceae were isolated from branches of 40 *A. karroo* trees, either healthy or displaying symptoms of die-back. Most of these species, including *Tiarosporella urbis-rosarum, Diplodia allocellula, P. variabilis, Dothiorella brevicollis, Neofusicoccum vitifusiforme* and *Spencermartinsia viticola*, were obtained from healthy tissues. In contrast, only three species, namely *Dothiorella dulcispinae, S. pretoriensis* and *S. viticola*, were isolated from die-back tissue. Thus only *S. viticola* occurred on both healthy and diseased tissue types and only two species, *Do. dulcispinae* and *S. pretoriensis,* were found exclusively in die-back tissue. A maximum of two species co-occurred in a single tree, including *Do. brevicollis*, *S. viticola, S. pretoriensis*, *Do. dulcispinae* and *N. vitifusiforme*.

All the isolated species of Botryosphaeriaceae produced lesions on inoculated *A. karroo* branches. Surprisingly, the two species found only in branches with die-back, namely *S. pretoriensis* and *Do. dulcispinae,* were among those producing the smallest lesions. These two species also represented only 12.5 % of the total number of isolates from diseased tissue while 87.5 % of isolates were those of *S. viticola*. These results suggest that the Botryosphaeriaceae isolated in this study play little if any role in the development of die-back symptoms on *A. karroo*.

Spencermartinsia viticola was isolated from both die-back and healthy tissue, and this species was also the most dominant species isolated. *S. viticola* was described for the first time from decline of *Vitis vinifera* in Spain as a saprophyte (Luque et al. 2005). However, since then it has been reported from diseases on many other plants such as warts on trunks of *Populus cathayana* in China (Zhang et al. 2009), canker of citrus in California (Adesemoye and Eskalen 2011) and from healthy tissues of *Acacia mellifera* in South Africa (Van der Walt 2008). The results of our study confirm that this species can produce lesions on *A. karroo* in pathogenicity tests, but it was amongst those producing fairly short lesions and this species thus does not appear to be a particularly aggressive pathogen on *A. karroo*.

In a previous study, *Phaeobotryosphaeria variabilis* was isolated from die-back branches on *A. karroo*, but its pathogenicity was not considered (Van der Walt 2008). In the present study, we found *P. variabilis* only in healthy tissues, but it produced the longest lesions of all the species in the pathogenicity trial. This species thus appears to have the capacity to cause disease on *A. karroo*, although it was apparently not involved with the die-back symptoms studied here.

Isolates of some species differed significantly in their aggressiveness in pathogenicity tests. For example, some isolates of *T. urbis-rosarum*, *S. viticola* and *Do. brevicollis* produced long lesions in the inoculation tests while the other isolates of these species were amongst those that produced small lesions. This could be due to genetic differences in the isolates themselves or differences in susceptibility of genotypes of seedlings, which is not unusual as it is known that isolates of species can differ considerably in their pathogenicity (Mayer 2006; Müller et al. 2001). Little variation was observed in our study between the trees inoculated with the particular isolates, and the variation observed thus appears linked to differences in virulence of the various isolates. This implies that due to these differences in virulence the most virulent isolates may not have been used for the pathogenicity tests, and that more virulent isolates for each species could occur in branches, contributing to die-back.

It is possible that the damage caused by the feeding cerambycid larvae or other biological agents places the branches under stress allowing endophytic, opportunistic Botryosphaeriaceae to have the opportunity to cause disease symptoms (Slippers and Wingfield 2007). Further studies will be necessary to determine if there is any direct association between the Botryosphaeriaceae species occurring naturally as endophytes in *A. karroo* and such other biological agents, or whether the presence of the Botryosphaeriaceae and possible die-back symptoms are only co-incidental and opportunistic. Such further studies would, for example, include determining whether these fungi are associated with the larvae, targeting development of lesions linked to earlier stages of beetle larval infestation, and considering possible transmission mechanisms by the beetles.

The results of this study emphasize the extensive diversity of the Botryosphaeriaceae community that can exist on trees in a limited area. Furthermore, these are all potential pathogens. Our study also elucidates the necessity to study each tree system separately to determine the ecological roles of the Botryosphaeriaceae found in the tree species, which may differ those in other tree systems. Expanding in depth sampling in particular areas, such as on more than one host, together with extensive pathogenicity studies will be very helpful to further characterize the potential role these fungi can play in disease of such native communities.

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