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Quambalaria leaf and shoot blight on *Eucalyptus nitens* in South Africa

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Abstract. *Quambalaria* spp. cause leaf and shoot dieback diseases on young *Eucalyptus* trees in Australia, Thailand, South America and South Africa. The disease was first recorded in South Africa in the early 1990s but has been restricted to nurseries in the subtropical north-east coastal area of the country, without resulting in great effect. Recent disease surveys in the Mpumalanga Province of South Africa have revealed extensive shoot and leaf dieback, as well as stem cankers on 1-year-old *E. nitens* trees. Some symptoms of the disease resembled Quambalaria leaf and shoot blight. However, this was the first time it had occurred on the stems of larger trees, on *E. nitens* or in the cold temperate region of the country. The aim of this study was to identify the causal agent of the disease and to test different *Eucalyptus* spp. and clones of relevance to the South African forestry industry for their susceptibility to the pathogen. Comparisons of DNA sequence data for the ITS and 5.8S regions were used to identify the fungus. Results showed that the pathogen represented *Q. eucalypti*. Inoculation trials showed that all the material tested was susceptible to infection by *Q. eucalypti*. This study shows that *Q. eucalypti* has become elevated from a relatively minor nursery pathogen to one that can cause significant damage on a wide range of *Eucalyptus* spp. in both temperate and sub-tropical areas of South Africa.

Additional keywords: plantation forestry, selection.

Introduction

Quambalaria leaf and shoot blight of *Corymbia* and *Eucalyptus* spp. is caused by three species of *Quambalaria* (Simpson 2000). These are *Q. eucalypti* (M.J. Wingf., Crous & W.J. Swart) J.A. Simpson, *Q. pitereka* (J. Walker & Bertus) J.A. Simpson and *Q. pusilla* (U. Braun & Crous) J.A. Simpson (Simpson 2000). Infection by *Quambalaria* spp. results in leaf lesions and shoot blight and is characterised by the occurrence of powdery white fungal spore masses on the lesions (Wingfield *et al*. 1993; Simpson 2000). In Australia, the disease caused by *Q. pitereka* results in the death of young shoots in *Corymbia* plantations in Queensland and New South Wales (Simpson 2000), while in South Africa and Brazil, *Q. eucalypti* is associated with leaf lesions and death of young shoots on rammets in clonal nurseries (Wingfield *et al*. 1993; Alfenas *et al*. 2001). Although *Q. eucalypti* has not been considered a serious pathogen in South Africa, it is reported as destructive in Brazil (Zauza *et al*. 2003). Likewise, *Q. pitereka* causes serious damage in the establishment of new *Corymbia* plantations in Queensland and New South Wales (Simpson 2000; Pegg *et al*. 2005).

The three species of *Quambalaria* occurring on *Eucalyptus* and *Corymbia* spp. appear to be geographically restricted. *Quambalaria pitereka* is known only from Australia, where it is an economically important pathogen in eastern Australia (Simpson 2000; Pegg *et al*. 2005). It has, however, also been recorded from Western Australia as *Sporotrichum destructor* H.A. Pittman (Cass Smith 1970; Simpson 2000). *Quambalaria eucalypti* occurs in South Africa (Wingfield *et al*. 1993) and Brazil (Alfenas *et al*. 2001; Zauza *et al*. 2003), while *Q. pusilla* has been reported only from Thailand (Simpson 2000).

Quambalaria pitereka was first described from Australia as *Ramularia pitereka* J. Walker & Bertus (Walker and Bertus 1971). Braun (1998) transferred *R. pitereka* to the genus *Sporothrix*. Simpson (2000) established the new genus *Quambalaria*, establishing the names *Q. pitereka* and *Q. eucalypti* and suggested that they might be Basidiomycetes within the Exobasidiales or Ustilaginales. Recent research has shown experimentally that *Quambalaria* is a basidiomycete representing a unique family, the Quambalariaceae in the Microstromatales (De Beer *et al*. 2006).

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Quambalaria leaf and shoot blight was first recorded in South Africa in 1987 from two *E. grandis* clones in a nursery in the KwaZulu/Natal Province (Wingfield *et al*. 1993). The susceptible clones were removed from production, and the disease seemed to disappear. Approximately 10 years later, Quambalaria leaf and shoot blight reappeared in the area, in a second nursery, causing damage to *E. grandis* × *E. urophylla* hybrid clones.

In January 2005, a disease resembling Quambalaria leaf and shoot blight was observed on *E. nitens* trees in a provenance trial in the Mpumalanga Province of South Africa. The area of occurrence, symptoms and species affected were unusual for this fungus. The aim of this study was to identify the causal agent of the disease and to consider the susceptibility of various *Eucalyptus* spp. and some *E. grandis* hybrid clones planted in South Africa.

Methods

Diagnosis and isolation

Disease was observed on 1-year-old *E. nitens* trees in a single plantation (Rooihoogte) (S26°03.840, E050°19.335) near Carolina in the Mpumalanga Province. The surveyed compartment consisted of 100 different *E. nitens* genotypes, planted as single rows, in eight tree plots with three replications. The compartment was evaluated for diseases in January 2005 and in June 2005.

Leaf and shoot samples were collected, placed in paper bags and transported to the laboratory for isolation and identification of the disease agent. Isolations were made by scraping white powdery spore masses from the lesions on the leaf and stem surfaces and transferring them to 2% Malt Extract Agar containing streptomycin sulphate (MEAS) (20 g Biolab Malt Extract, 15 g Biolab Agar, 100 mg Sigma-Aldrich Chemie Gmbh streptomycin sulphate). Plates were incubated at a room temperature of 22–24°C and cultures purified by repeated transfer to clean Petri plates containing MEAS. All isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria (Table 1). Representative isolates have also been deposited in the culture collection of the Centralbureau voor Schimmelcultures, Utrecht, The Netherlands. Dried cultures have been deposited with the National Collection of Fungi (PREM), Pretoria, South Africa.

DNA sequencing

Four isolates of the *Quambalaria* sp. from the Rooihoogte plantation and two previously collected from Kwambonambi were grown on MEA plates for 14 days. Mycelium was scraped from the surfaces of the Petri dishes using a scalpel and placed in 2-mL microcentrifuge tubes. Mycelium was frozen in liquid nitrogen and ground into a fine powder using plastic rods that had been sterilised with 70% ethanol. DNA was extracted from the ground mycelium using a modification of the method described by Möller *et al*. (1992). Using a pipette and sterile lab tips, 500 µL of TES buffer (100 µL Tris; 10 µL EDTA; 2% SDS) and 200 µL of proteinase K were added to each sample. Tubes were incubated for 1 h at 60°C. The concentration of salt was adjusted by adding 140 µL NaCl. 65 µL 10% CTAB were added to each centrifuge tube and incubated at 60°C for 10 min. 905 µL of 24 : 1 chloroform : isoamylalcohol were added to the tubes and incubated for 10 min at 4°C. Tubes were then centrifuged for 10 min at 12 000 rpm using an Eppendorf benchtop centrifuge. The aqueous phases were transferred to fresh, sterile 1.5 mL microcentrifuge tubes. Isopropanol (440 µL) was added and tubes were incubated for 30 min on ice to precipitate the nucleic acids. After centrifugation (12 000 rpm for 5 min) the aqueous phases were discarded and the DNA pellets dried by inverting the tubes onto a paper towel for 5 min. DNA pellets were washed with 1000 µL ice-cold 70% ethanol and dried at 60°C in a SpeedyVac rotator. DNA pellets were resuspended in 50 µL sterile water and 6 µL RNAseA was added.

The internal transcribed spacer (ITS) region and the 5.8S gene of the rDNA (rDNA) operon were amplified using the primers ITS1 (5′TCCGTAGGTGAACCTGCGG3′) and ITS4 (5′TCCTCCGCTTATTGATATGC3′) (White *et al*. 1990). In each microcentrifuge tube, 1 µL $MgCl_2$, 2 µL dNTP's, 2.5 µL buffer with $MgCl_2$, 0.5 µL primer, 17 µL water, 2 µL DNA and 0.5 µL Taq was included. The PCR programme consisted of an initial denaturation

Table 1. List of *Quambalaria* isolates used in sequence and inoculation studies

Isolate number[A]	CBS numbers	GenBank accession	Species	Host	Origin	Collector
CMW1101[B]	CBS118844	DQ317625	*Q. eucalypti*	*E. grandis*	Kwambonambi, South Africa	M. J. Wingfield
CMW11678[C]	CBS119680	DQ317626	*Q. eucalypti*	*E. grandis*	Kwambonambi, South Africa	L. Lombard
CMW14329		DQ317614	*Q. eucalypti*	*E. grandis* × *E. camaldulensis* clone	Kwambonambi, South Africa	J. Roux
CMW17252	CBS118615	DQ317609	*Q. eucalypti*	*E. nitens*	Rooihoogte, South Africa	Z. L. Mthalane and J. Roux
CMW17253		DQ317610	*Q. eucalypti*	*E. nitens*	Rooihoogte, South Africa	Z. L. Mthalane and J. Roux
CMW17254		DQ317611	*Q. eucalypti*	*E. nitens*	Rooihoogte, South Africa	Z. L. Mthalane and J. Roux
CMW17255		DQ317612	*Q. eucalypti*	*E. nitens*	Rooihoogte, South Africa	Z. L. Mthalane and J. Roux
CMW17771	CBS118616	DQ317613	*Q. eucalypti*	*E. grandis* clone	Kwambonambi, South Africa	J. Roux
CMW5318	CBS118828	DQ317628	*Q. pitereka*	*Corymbia citriodora* subsp. *variegata*	Queensland, Australia	M. Ivory
CMW6707		DQ317627	*Q. pitereka*	*Corymbia maculata*	New South Wales, Australia	M. J. Wingfield
JCM3928[B]	CBS6526	AB038129	*Rhodotorula bacarum*	*Ribes nigrum*	United Kingdom	R. W. M. Buhagiar
JCM9030[B]	CBS8079	AB038130	*R. hinnulea*	*Banksia collina*	Australia	R. G. Shivas
JCM9035[B]	CBS8073	AB038131	*R. phylloplana*	*B. collina*	Australia	R. G. Shivas

[A]CMW represents cultures in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), Pretoria, South Africa; JCM: Japan Collection of Microorganisms, RIKEN BioResource Center, Saitama, Japan; CBS: the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

[B]Ex-type cultures.

[C]Ex-epitype cultures.

step at 96°C for 2 min, followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 45 s and elongation at 72°C for 1 min and ended with a final elongation step at 72°C for 7 min. All PCR products were purified using centri-sep columns with Sephadex G-50 (Sigma-Aldrich, Amersham Biosciences Limited, Sweden). The purified PCR products were sequenced following the methods of Nakabonge *et al*. (2006).

DNA sequence alignments were conducted online using MAFFT ver.5.667 (http://timpani.genome.ad.jp/~mafft/server/, verified 14 June 2006). Sequences were analysed using Molecular Evolutionary Genetic Analyses (MEGA) Version 3.1 (Kumar *et al*. 2004). Neighbour joining analyses were calculated with the Kimura-2 parameter and bootstrap values were calculated for 1000 replicates (Felsenstein 1988). In addition, a heuristic search based on parsimony with random stepwise addition and tree bisection reconnection was conducted. For comparisons, we included ITS sequences of two South African isolates of *Q. eucalypti* from *E. grandis* as well as two isolates representing *Q. pitereka* from *Corymbia* spp. in Queensland, Australia (Table 1). Other species previously shown to belong to the Microstomatales (Begerow *et al*. 2001) were also included in the analyses. ITS sequences of these species (Table 1) were obtained from the GenBank, National Centre for Biotechnology Information nucleotide database (http://www.ncbi.nlm.nih.gov/, verified 14 June 2006).

Inoculation of leaves and shoots

Ten different clones and five species of *Eucalyptus* were included in pathogenicity tests using two isolates (CMW14329, CMW17253) of *Q. eucalypti*. These clones and species were obtained from South African forestry companies and are used in commercial plantings. The clones are hybrids between *E. grandis* × *E. urophylla* (GU). For each isolate, a suspension of 10^6 mitospores/mL was prepared using a haemocytometer and a dilution series in sterile water. A drop of sterile Tween 20 was added to each spore suspension to facilitate adhesion of spores to plant surfaces. Spore suspensions were transferred to sterilised 500 mL spray bottles. Spores were sprayed onto the leaves of each tree until run-off. Trees were then covered with plastic freezer bags for 48 h to ensure a high relative humidity and to facilitate fungal infection. For each *Quambalaria* isolate and each *Eucalyptus* genotype, 20 trees were sprayed with spores and 10 were treated as controls. The controls were not sprayed with spores and covered only with plastic bags.

Trees were inspected daily for symptom development. After 14 days, each tree was scored for infection by counting the number of leaves with spots from the bottom to the top, and assigning it a disease rating using a scale of between 0 and 5 (0 = no leaf spots; 1 = 1–3 leaves with spots; 2 = 4–6 leaves with spots; 3 = 7–10 leaves with spots; 4 = >10 leaves with spots; 5 = spots on leaves and stem). Results were analysed using SAS (SAS Institute Inc., Cary, NC) and data were tested for adherence to the normal distribution. Data were analysed using General Linear Model (GLM) and least square means were calculated at 95% confidence limits to distinguish between different clones and species.

Stem inoculations with Quambalaria

Eucalyptus dunnii and an *E. grandis* clone (TAG5) were used to determine whether a stem inoculation technique, used successfully for other tree pathogens (Roux *et al*. 2004; Nakabonge *et al*. 2006), could be useful for *Quambalaria* spp. A total of 40 trees were used for the inoculations. Of these, 20 were *E. dunnii* and 20 TAG5. For each genotype, nine trees were inoculated with isolate CMW17253 and nine were inoculated with isolate CMW14329. Two trees for each genotype served as controls. Using a cork borer that was sterilised with 70% alcohol, wounds were made on the stems ~1 m above soil level. Only the bark was removed in such a way that the cambium was exposed without being damaged. A sterile scalpel was used to place discs of agar bearing 2-week-old mycelium into the wounds, with the fungus facing the cambium. The wounds were then covered with a strip of Parafilm (Pechiny Plastic Packaging, Chicago) to prevent desiccation or contamination of the inoculation sites. Results were collected after 6 weeks by measuring the lengths of bark and cambial lesions that had developed.

Results

Disease description and pathogen isolation

Quambalaria leaf and shoot blight on *E. nitens* near Carolina was characterised by the formation of leaf spots (Fig. 1*a*, *b*) and stem cankers (Fig. 1*d*). Under the moist conditions experienced during the first evaluation of the trial in January 2005, white spore masses were present on both the leaf lesions and stem cankers. Infection of some trees resulted in shoot and leaf death (Fig. 1*c*). Infection of the main stems was also observed, resulting in sunken, black cankers which, under moist conditions, contained white powdery spore masses (Fig. 1*d*). During the second evaluation in the dry winter month of June, stem cankers remained visible, but spores masses could not be seen. A few protected leaves were found bearing some old dry spores in the lesions.

DNA sequencing

DNA was isolated from all isolates (CMW14329, CMW17252, CMW17253, CMW17254, CMW17255, CMW17771) selected. PCR products of between 608–625 bp in size were obtained for these isolates. DNA for all of these isolates was sequenced to confirm their identities. After alignment, the dataset consisted of 642 bp. The six isolates sequenced in this study were 100% identical to the two *Q. eucalypti* isolates from South Africa and, in both analyses, grouped separately from the cluster containing the Australian isolates of *Q. pitereka*. Parsimony analyses resulted in 130 most parsimonious trees (CI = 0.923; RI = 0.918; length 182 teps). A 50% majority rule consensus tree showed that the two clusters containing *Quambalaria* spp. each had 100% bootstrap support, which was the same as the bootstrap values obtained in the neighbour-joining analyses (Fig. 2).

Inoculation of leaves and shoots

Eucalyptus trees sprayed with *Q. eucalypti* isolates developed symptoms after 2 weeks. Characteristic white spore masses were observed in leaf spots and, in some cases, lesions were also observed on growth tips and stems. *Quambalaria eucalypti* was successfully re-isolated from symptomatic leaves. All genotypes tested in this study showed some level of susceptibility to infection by *Q. eucalypti*. Significant differences ($R^2 = 0.44$; CV = 64.19) were found between the controls and the inoculated plants (95% confidence level) and between different genotypes of trees (Fig. 3). The *E. smithii* trees, from both companies, were the most susceptible to infection by *Q. eucalypti*, while two of the GU clones (E, F) were the least susceptible.

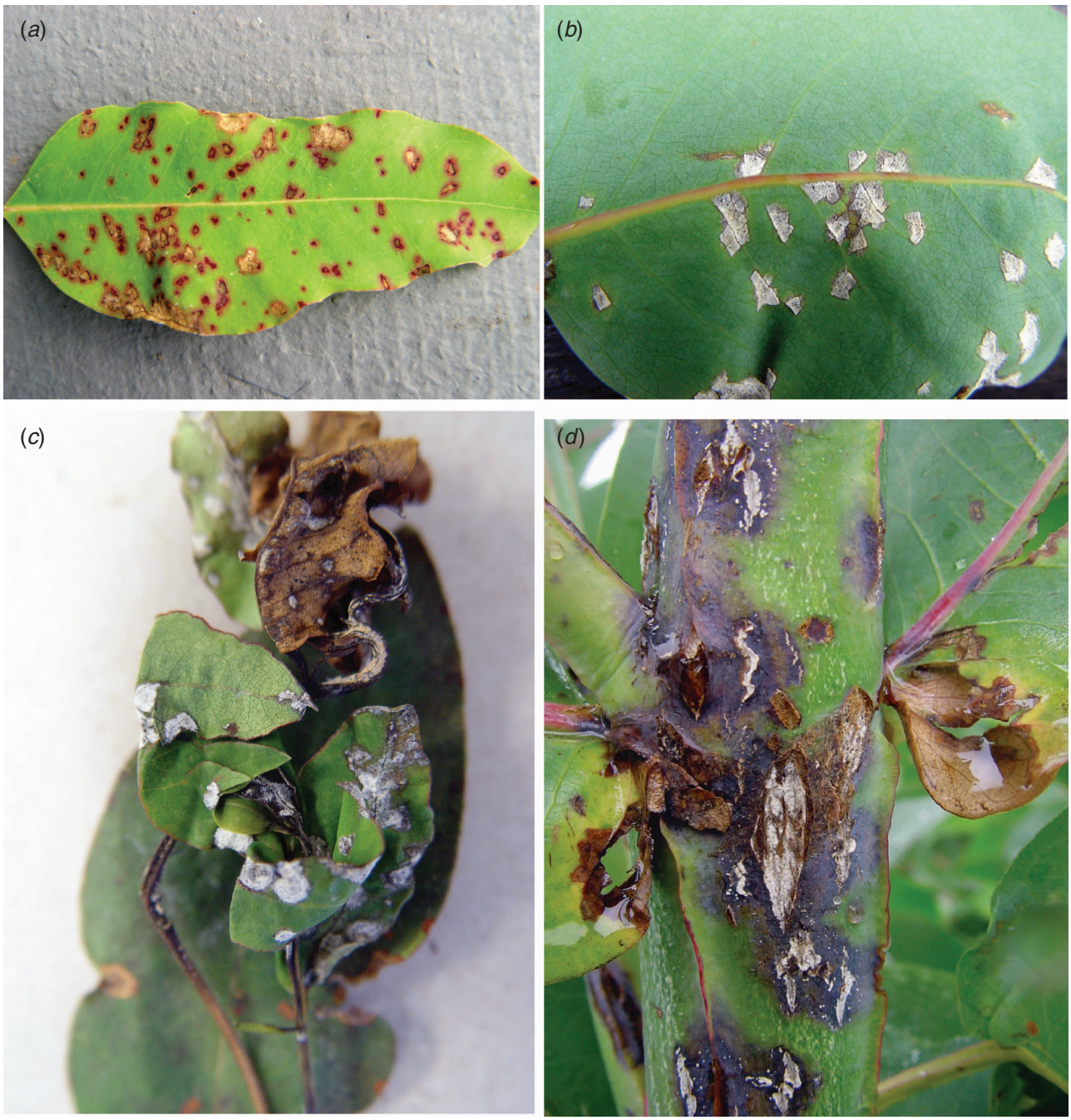

Fig. 1. Symptoms of *Quambalaria eucalypti* infection on *Eucalyptus* spp. (*a*) developing leaf spots on a *E. grandis* × *E. camaldulensis* clone, (*b*) leaf spots with white fungal spore masses, (*c*) leaf spot, blight and shoot die-back, (*d*) stem lesions and white spore masses on *E. nitens*.

Stem inoculations

No lesions developed on the 18 trees inoculated in this part of the study. Treatments differed from the controls only in the fact that the control inoculation wounds had begun to heal through callus production. Wounds on inoculated trees had not started healing after 6 weeks. Some white spores of the inoculated fungi could be found in the wounds.

Discussion

Results of this study provide the first observation of *Q. eucalypti* and the associated Quambalaria leaf and shoot blight disease outside the nursery environment in South Africa. This is also the first report of this disease in the Mpumalanga Province and from *E. nitens*. Both field evaluations and greenhouse inoculations show that *Q. eucalypti* should be considered a potential restraint to the

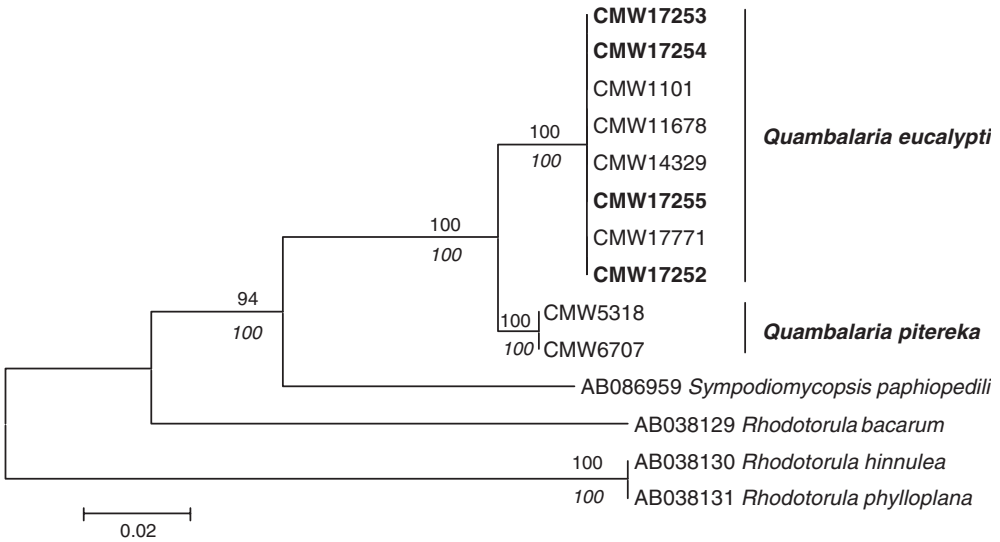

Fig. 2. NJ tree obtained from ITS sequence data of *Quambalaria* isolates obtained from South Africa and Australia. Bootstrap values are for 1000 replicates. Bar = 0.02 expected changes per site. Bootstrap values obtained for maximum parsimony are indicated in italics below the branches. Isolates of *Q. eucalypti* from *E. nitens* appear in bold. Others are those from Kwambonambi from *E. grandis* clones.

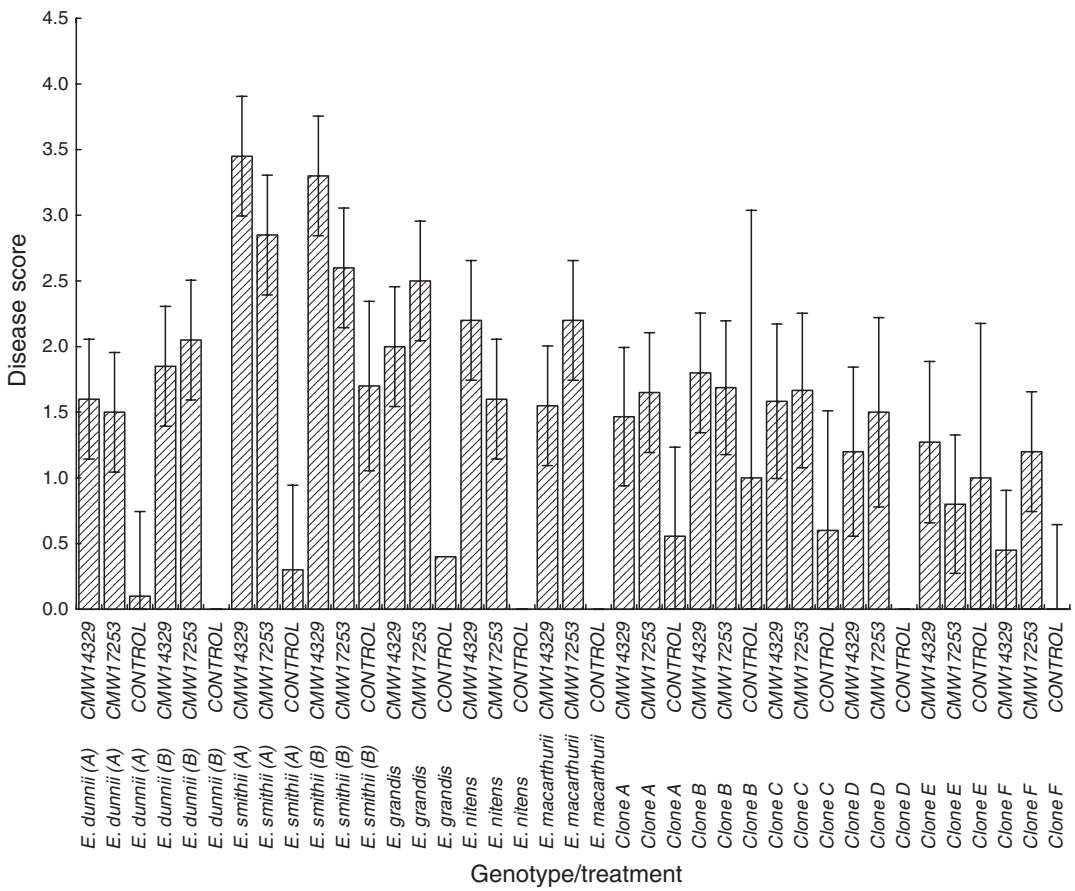

Fig. 3. Histogram showing variance in susceptibility to *Quambalaria eucalypti* between different commercial South African *Eucalyptus* spp. (A and B indicate different companies) and hybrid GU clone selections (vertical bars indicate 95% confidence limits; $R^2 = 0.44$; CV = 64.19).

growth and production of *Eucalyptus* in South Africa. This would be similar to the situation with *Q. pitereka* in Australia (Simpson 2000; Pegg *et al*. 2005).

Quambalaria leaf and shoot blight in South Africa has previously been restricted to the subtropical KwaZulu/Natal north coast. The incidence of the disease in that case was limited to two nurseries, with the result that it was considered a relatively minor problem. The occurrence of the disease in a sub-tropical environment was also consistent with reports of the disease elsewhere in the world. For example, *Q. pitereka* causes considerable damage in young plantations in subtropical Queensland and New South Wales (Pegg *et al*. 2005). Likewise, *Q. eucalypti* can be an important pathogen in Brazilian nurseries and plantations (Alfenas *et al*. 2001; Zauza *et al*. 2003). The high level of incidence of *Q. eucalypti* infections on 1-year-old *E. nitens* in the considerably colder and higher altitude area of Carolina was thus unusual.

Comparison of DNA sequence data showed clearly that the fungus responsible for the new disease outbreaks in an atypical environment and on a new host in South Africa is *Q. eucalypti*. The fungus grouped separately from *Q. pitereka* and other closely related species. The origin of *Q. eucalypti* remains uncertain although it is believed to occur in Australia (G. Pegg, pers. comm.). Both South Africa and Brazil have commonly received seed from Australia and they have exchanged seed between themselves. This could have facilitated movement of the pathogen from Australia into these countries.

Foliar inoculation trials under greenhouse conditions showed that *Q. eucalypti* has a wide host range in the genus *Eucalyptus*. All species and hybrid clones tested in this study were susceptible to infection by the fungus. *Eucalyptus smithii* seedlings were the most susceptible to *Q. eucalypti* infection. On average, the hybrid *E. grandis* clones were less susceptible to infection than the pure species. Follow-up surveys to the Rooihoogte trial site showed that the most severely affected trees were malformed as a result of *Q. eucalypti* infection. This, together with the greenhouse trials strongly suggest that this disease has increased in importance and could hinder establishment of new *Eucalyptus* plantations in some areas.

Successful disease management relies on a number of factors. These include quarantine to keep new pests and diseases out of the country, or out of a specific area in the country, chemical and biological control, site species/clone matching, good silvicultural practices and the selection and breeding of disease tolerant planting stock. Although screening trees based on natural infection provides useful information, this is not always the most rapid or reliable way in which to obtain information pertaining to the susceptibility of a particular genotype to fungal infection. Artificial inoculation trials under field or nursery conditions provide valuable information and have been used successfully in a number of breeding programmes, such as that used against *Fusarium circinatum* on *Pinus* spp. (Hodge and Dvorak 2000) and for *Chrysoporthe cubensis* on *Eucalyptus* spp. (Alfenas *et al*. 1983). Results of the current study confirm that foliar spray inoculations provide reliable information regarding disease susceptibility to *Q. eucalypti*. However, stem inoculations, similar to those used for other pathogens such as *Ceratocystis* spp. (Roux *et al*. 2004) and *Chrysoporthe* spp. (Van Zyl and Wingfield 1999; Nakabonge *et al*. 2006) were not successful for *Q. eucalypti*. This suggests that *Q. eucalypti* typically infects fresh, unwounded tissue. The selection of disease tolerant material against *Q. eucalypti* in the future will most likely rely on greenhouse foliar inoculation studies.

It seems likely that *Q. eucalypti* has entered South Africa accidentally. The fact that it was first found in a nursery suggests that it was introduced on seed as seedlings and cuttings are prohibited from importation. Whether this was from Brazil, Australia or a country where the disease is present, but not yet recognised, is unknown. The fact that the pathogen has been shown in this study to infect a wide range of *Eucalyptus* spp. and under very different climatic conditions suggests that it will increase in importance in the future. The presence of *Q. eucalypti* in South Africa also emphasises the dynamic nature of tree diseases and the threat that they hold to the sustainability of an industry that has typically been relatively free of disease and pest problems.

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