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The *Eucalyptus* stem canker pathogen *Teratosphaeria zuluensis* detected in seed samples

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The global spread of *Eucalyptus* pathogens is thought to be facilitated by the trade in seed for the establishment of new plantations and breeding programmes. In this study, we used high-throughput amplicon sequencing to elucidate whether this might be true for the *Eucalyptus* stem pathogen *Teratosphaeria zuluensis* in South Africa. DNA libraries were obtained for seed-capsules, clean seed, 1-month-old seedlings grown in a phytotron, plants grown *in vitro* in a tissue culture facility as well as leaves and stems of 3-month-old seedlings exposed to a *Eucalyptus* plantation environment. The DNA libraries were sequenced using high-throughput Illumina MiSeq and 454 technologies. Sequencing revealed the presence of several OTUs in the Botryosphaeriaceae, Mycosphaerellaceae and Teratosphaeriaceae in seed and seed-capsules and amongst these, OTUs that cluster close to *T. zuluensis*. OTUs from the Mycosphaerellaceae were also recovered from seedlings grown in a phytotron, *in vitro* propagated seedlings and those exposed to the plantation environment. The results support the view that important *Eucalyptus* pathogens, including *T. zuluensis*, can be spread via the trade in seed.

Introduction

The growing international trade in plants and plant products is contributing substantially to the spread of invasive alien plant pests and pathogens (Palm and Rossman, 2003; Brasier, 2008; Aukema *et al*., 2010; Liebhold *et al*., 2012). Several recent studies have shown that the global movement of germplasm, particularly of living plants, as well as plant products such as untreated timber, is responsible for ~50 per cent of new invasions by plant pathogens and pests (e.g. Aukema *et al*., 2010; Haack *et al*., 2010; Liebhold *et al*., 2012; Garbelotto *et al*., 2013; Santini *et al*., 2013). These have resulted in significant economic and ecological losses to agriculture and forestry (Pimentel *et al*., 2005; Rossman, 2009).

The global domestication of Australian *Eucalyptus* species for plantation establishment has resulted in a substantial movement of seed and timber products across borders (Wingfield *et al*., 2008, 2010). It is currently estimated that >20 million hectares of *Eucalyptus* species are cultivated globally (Iglesias-Trabado and Wilstermann, 2008), with significant expansion continuing to occur in developing countries. Because of the need for rapid plantation development, many of these plantations are initially established using germplasm, such as seed and cuttings, obtained from already established operations elsewhere in the world. Substantial quantities of *Eucalyptus* seed are being collected in native stands in Australia and disseminated worldwide (Ciesla *et al*., 1996). In areas where these trees are non-native, there are often intensive breeding programmes involving the import of superior seed from other countries, the establishment of seed orchards and often subsequent export of improved seed globally (Wingfield *et al*., 2001, 2013). The movement of *Eucalyptus* seed is assumed to be a major source for the global spread of pathogens (Ciesla *et al*., 1996; Burgess *et al*., 2007; Wingfield *et al*., 2008).

Important diseases of *Eucalyptus*, particularly those caused by species in the Botryosphaeriaceae, Mycosphaerellaceae and Teratosphaeriaceae, are thought to have been spread via the trade in seed (Ciesla *et al*., 1996; Slippers and Wingfield, 2007; Wingfield *et al*., 2008; Hunter *et al*., 2011). However, this has not been proven experimentally. Studies conducted so far detected these fungi in leaves, twigs and trunk increment cores of *Eucalyptus* species, where they occur as endophytes (Pillay *et al*., 2012; Kemler *et al*., 2013; Marsberg *et al*., 2014). Of particular interest was the recent detection of *Teratosphaeria zuluensis* (M.J. Wingf., Crous and T.A. Cout) M.J. Wingfield and Crous (Crous *et al*., 2009a,b; Cortinas *et al*., 2006b), an important *Eucalyptus* stem canker pathogen, in leaves of *Eucalyptus grandis* (Marsberg *et al*., 2014). This discovery has renewed discussions regarding the possible spread of this important stem pathogen in seed.

Teratosphaeria stem canker is an important disease of *Eucalyptus* species grown outside Australia. It is caused by two closely related pathogens, *T. zuluensis* and *T. gauchensis* (M.-N. Cortinas,

Crous and M.J. Wingf.) M.J. Wingfield and Crous (Cortinas *et al.*, 2006b) in the class Dothideomycetes, family Teratosphaeriaceae. *Teratosphaeria zuluensis* was first described in South Africa (Wingfield *et al.*, 1997) and is known to cause disease of *Eucalyptus* species in Africa (Roux *et al.*, 2005; Chungu *et al.*, 2010; Jimu *et al.*, 2014), Asia (van Zyl *et al.*, 2002; Old *et al.*, 2003; Cortinas *et al.*, 2006a) and Central America (Roux *et al.*, 2002). *Teratosphaeria gauchensis* was first described from Uruguay and Argentina (Cortinas *et al.*, 2006b) and has been reported in Africa (Gezahgne *et al.*, 2005; Roux *et al.*, 2005; Jimu *et al.*, 2015), Hawaii (Cortinas *et al.*, 2004) and recently Europe (Silva *et al.*, 2014).

The aim of this study was to consider the hypothesis that the *Eucalyptus* stem canker pathogens, *T. gauchensis* and *T. zuluensis*, have been spread globally with seed of these trees. We used Illumina MiSeq sequencing to identify, at genus level, the fungal communities in seed-capsules, clean seed and seedlings. This was then followed by 454 pyrosequencing to retrieve longer sequences required for species-specific identification of fungi.

Materials and methods

In this study, we focused on recovering important fungal pathogens on *Eucalyptus* seed as well as seed-capsules, which form the bulk of impurities in seed batches. Furthermore, we tested *Eucalyptus* seedlings grown in protected environments, i.e. phytotron and tissue culture for vertical transmission of pathogens. Some of the seedlings raised in a phytotron were exposed to the plantation environment and then tested for horizontal transmission of pathogens.

Sample collection

Samples of seed-capsules were collected from six mature *E. grandis* trees in a commercial plantation in the KwaMbonambi (28° 37′ S, 32° 09′ E, 65 m above sea level) area of South Africa. This site was specifically chosen because it is one of the first plantations where Teratosphaeria stem canker was detected and the chosen trees were in a heavily diseased compartment. The seed-capsule samples were packed in separate paper bags and transported to the laboratory for further study.

Seed germination and seedling tending

In the laboratory, the collected *Eucalyptus* seed was released from air-dried capsules by vigorous shaking. The seed was then cleaned by removing branch, leaf and capsule residue, followed by sieving using a 1.2-mm sieve, similar to the process followed during commercial seed collection operations. Sub-samples of the seed and seed-capsules were stored in a −20°C freezer for DNA extraction. The remaining seed was pre-treated by chilling at 4°C overnight and then germinated in two controlled environments to test for vertical transmission of fungi.

One batch was propagated on in a Unigro 98 tray, filled with autoclaved ground pine bark in a phytotron. The seed was watered once every 2 days until germination. The seed germinated after 5–9 days after planting. The temperature was maintained at 27°C. Two weeks after germination, the watering regime was reduced to once every 3 days. The seedlings were grown for 30 days after germination. DNA was extracted from 31 seedlings. The other 28 seedlings were moved to a commercial nursery near KwaMbonambi where they were exposed to the natural forestry environment for 2 months. The seedlings were then retrieved for DNA extraction and to test for horizontal transfer of pathogens from the surrounding *Eucalyptus* plantations.

To test whether potential endophytes could be transmitted vertically in the seed, a second batch of seed was surface-disinfested in three steps. The seed was placed in a muslin bag and washed with running water for 5 min and then washed with deionised and autoclaved water. The seed was then immersed in 70 per cent alcohol for 30 s, washed again with deionised and autoclaved water and immersed in 2.5 per cent NaOCl present in a commercial bleach solution for 20 min to degrade DNA on the seed coat. The seed was finally washed twice with deionised and autoclaved water and then germinated on 40 mL Murashige and Skoog (MS) growth medium (Murashige and Skoog, 1962), which had been adjusted to 5.8 pH with HCl (1 M) and autoclaved at 121°C for 25 min. Twelve successfully germinated seedlings (of a total of 20 sown) were grown in the tissue culture facility at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. DNA was extracted 30 days after *in vitro* germination of the seed.

DNA extraction

For DNA extraction from seedlings, a sterile surgical blade was used to remove the roots. Seedlings were then surface-disinfested with 2.5 per cent NaOCl present in commercial bleach solution for 20 min and rinsed in deionised and autoclaved water. For 1-month-old seedlings, DNA was extracted from leaves and stems combined. For 3-month-old seedlings, leaves were cut from stems for separate DNA extraction. Genomic DNA was extracted from seed, seed-capsules, seedlings, leaves and stems using the Zymo plant/seed extraction Kit™ (Zymo Research, Irvine, USA). DNA concentrations were measured using a Thermo Scientific NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Illumina MiSeq sequencing

Six individual libraries were generated for the *E. grandis* germplasm in this study. These were for ground seed-capsules (18 g), seeds (18 g), seedlings grown in a phytotron (31), seedlings grown *in vitro* in a tissue culture facility (12) as well as leaves (20) and stems (20) of seedlings exposed to the plantation environment. The nuclear rDNA ITS region of the samples was amplified using the fungal-specific primer pair ITS 1F (Gardes and Bruns, 1993) and ITS 2 (White *et al.*, 1990). Illumina MiSeq sequencing of the amplicons was performed by Inqaba Biotech (Pretoria, Gauteng, South Africa, www.inqababiotec.co.za).

The ITS reads generated from Illumina MiSeq sequencing were processed using the QIIME pipeline (Caporaso *et al.*, 2010). The sequences were subjected to quality trimming and chimera removal. Sequences with a Phred quality score of <30 and a length of <200 bp were removed. The sequences were then clustered into Operational Taxonomic Units (OTUs) with a sequence similarity level of 95 per cent. The longest sequence from each OTU was selected as the representative sequence. Representative sequences for each OTU were queried against the UNITE database (released 13.04.2012, including fungal ITS sequences from the International Nucleotide Sequence Databases (Abarenkov *et al.*, 2010)) for taxonomic assignment.

454 GS-FLX amplicon pyrosequencing

Three individual libraries of *E. grandis* ground seed-capsules (18 g), seed (18 g) and seedlings grown in a phytotron (31) were generated for 454 pyrosequencing. Pyrosequencing was performed on a Genome Sequencer FLX 454 Titanium (454 Life Sciences/Roche Applied Biosystems, Bradford, USA) by Inqaba Biotech. The nuclear rDNA ITS region of the samples was amplified using the fungal-specific primer pair ITS 1F (Gardes and Bruns, 1993) and ITS 4 (White *et al.*, 1990).

Raw 454 reads were processed using the SEED pipeline (version 1.1.35) (Větrovský and Baldrian, 2013). Denoising and chimera removal (Phred quality score of 20) were performed using MOTHUR version 1.31.2 (Schloss *et al.*, 2009) and UCHIME (Edgar *et al.*, 2011), respectively. Reads

of <400 bp after denoising and chimera removal were discarded. Reads were aligned using MAFFT Version 7 (Katoh *et al*., 2005), and clustering was performed using USEARCH Version 5.2.32 (Edgar, 2010). Clustering was performed at 97 per cent similarity level (O'Brien *et al*., 2005). Consensus sequences of each OTU were assigned to taxons using BLASTn with an e-value of $<10^{-80}$ and minimum query coverage of >80 per cent. Sequences highly similar to important *Eucalyptus* pathogens were extracted, and Maximum Parsimony analyses were conducted to identify the fungi to species level, using PAUP v. 4.0b10 (Swofford, 2002).

Results

Illumina MiSeq sequencing

A total of 3805 reads were recovered from seed-capsules (996), seed (654), seedlings grown in a phytotron (1039) and those grown *in vitro* in a tissue culture facility (8) as well as leaves (709) and stems (399) of seedlings exposed to the plantation environment, after quality trimming. Taxonomic composition assessment of *E. grandis* seed-capsules, seed and all seedlings revealed a predominance of fungi belonging to the Ascomycota (71.3–99.3 per cent) followed by Basidiomycota (0.1–15.6 per cent) (Table 1, Figure 1). A total of seven fungal classes were identified, dominated by the Dothideomycetes and Sordariomycetes. At least 29 families were identified from the samples. The Dothideomycetes were dominated by Mycosphaerellaceae (29.3 per cent), Pleosporaceae (11.2 per cent), Dothioraceae (6.4 per cent), Botryosphaeriaceae (3.1 per cent) and Cucurbitariaceae (0.3 per cent). Sordariomycetes included Nectriaceae (10.2 per cent), Amphisphaeriaceae (4.9 per cent), Valsaceae (1 per cent), Diaporthaceae (0.1 per cent) and Glomerellaceae (0.1 per cent). Families belonging to the other five classes included Trichocomaceae (10 per cent), Polyporaceae (2.2 per cent), Ustilaginaceae (0.3 per cent), Pezizaceae (0.2 per cent), Herpotrichiellaceae (0.1 per cent) and Trichosporonaceae (0.1 per cent). A total of 38 genera were identified with 11 of these predominant and accounting for 76.5 per cent of the total sequences. Predominant genera were *Mycosphaerella* (24 per cent), *Alternaria* (10.8 per cent), *Aspergillus* (9.8 per cent), *Fusarium* (9.5 per cent), *Hormonema* (6.3 per cent), *Pestalotiopsis* (4.9 per cent), *Cladosporium* (4.6 per cent), *Trametes* (2.1 per cent), *Neofusicoccum* (1.7 per cent), *Nigrospora* (1.5 per cent) and *Botryosphaeria* (1.3 per cent) (Table 1).

Seed-capsules were dominated by Eurotiomycetes (58.9 per cent), Dothideomycetes (29.5 per cent) and Sordariomycetes (10.8 per cent). All reads belonging to the Eurotiomycetes represented Trichocomaceae. Dothideomycetes were composed of Dothioraceae (13.6 per cent), Mycosphaerellaceae (10.2 per cent) and Pleosporaceae (5.7 per cent). Sordariomycetes included the Amphisphaeriaceae (3.4 per cent), Nectriaceae (3.3 per cent), Valsaceae (0.3 per cent) and unidentified Trichosphaeriales (3.5 per cent) and Xylariales (0.3 per cent). No Botryosphaeriaceae or Teratosphaeriaceae were recorded from seed-capsules. Predominant genera were *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Hormonema*, *Nigrospora* and *Pestalotiopsis*, representing 98.6 per cent of the total reads (Table 1).

Seed samples were dominated by Dothideomycetes (54.9 per cent) and Sordariomycetes (23 per cent). Other classes included the Eurotiomycetes (0.7 per cent), Tremellomycetes (0.5 per cent), Microbotryomycetes (0.2 per cent) and Pezizomycetes (0.2 per cent). Dothideomycetes consisted of the families Dothioraceae (23.5 per cent), Mycosphaerellaceae (15.1 per cent), Pleosporaceae (10.6 per cent), Teratosphaeriaceae (2.9 per cent), Botryosphaeriaceae (1.9 per cent), Cucurbitariaceae (0.3 per cent) and two unidentified Pleosporales (0.6 per cent). Sordariomycetes were composed of fungi belonging to the Amphisphaeriaceae (11.9 per cent), Nectriaceae (7.1 per cent), Valsaceae (1.9 per cent), Glomerellaceae (0.6 per cent), Diaporthaceae (0.2 per cent), unidentified Xylariales (0.9 per cent), Diaporthales (0.2 per cent) and Trichosphaeriales (0.2 per cent). Eurotiomycetes were represented by Trichocomaceae (0.5 per cent) and Herpotrichiellaceae (0.2 per cent) whereas Tremellomycetes were represented by Trichosporonaceae (0.2 per cent) and an unidentified family (0.3 per cent). Microbotryomycetes were composed of unidentified Sporidiobolales whereas Pezizomycetes were composed of the Pezizaceae family. At the genus level, the seed was dominated by *Alternaria*, *Cladosporium*, *Cytospora*, *Fusarium*, *Hormonema*, *Neofusicoccum*, *Pestalotiopsis* and *Teratosphaeria* (Table 1).

Seedlings grown in a phytotron were dominated by fungi belonging to the Sordariomycetes (54.9 per cent) and Dothideomycetes (34.5 per cent), with a very low representation of Eurotiomycetes (0.2 per cent). Sordariomycetes were dominated by fungi belonging to the family Nectriaceae (50.8 per cent), with lower representation of fungi belonging to the Amphisphaeriaceae (3.8 per cent), Valsaceae (0.1 per cent) and unidentified Trichosphaeriales (0.2 per cent). Dothideomycetes were composed of fungi belonging to the Pleosporaceae (29.7 per cent), Cucurbitariaceae (1.3 per cent), Dothioraceae (1.3 per cent), Teratosphaeriaceae (1 per cent), Mycosphaerellaceae (0.2 per cent) and unidentified Pleosporales (1 per cent). The predominant genera were *Alternaria*, *Curreya*, *Fusarium*, *Hormonema*, *Phoma*, *Pestalotiopsis*, *Peziza* and *Teratosphaeria*, accounting for ~88 per cent of the reads (Table 1).

Seedlings grown *in vitro* in a tissue culture facility were dominated by Mycosphaerellaceae (62.5 per cent) and Polyporaceae (12.5 per cent). The fungi belonged to the genera *Mycosphaerella* and *Trametes*, respectively (Table 1).

Eucalyptus grandis stems were dominated by fungi belonging to the Dothideomycetes (40.6 per cent) and Sordariomycetes (34.5 per cent), with lower representation of Agaricomycetes (11.8 per cent), Microbotryomycetes (0.3 per cent) and Ustilaginomycetes (0.3 per cent). Dominant families were Pleosporaceae (19.5 per cent), Botryosphaeriaceae (15.8 per cent), an unidentified Agaricomycete family (11.8 per cent), Amphisphaeriaceae (10 per cent) and Valsaceae (3.3 per cent). Predominant genera were *Alternaria*, *Botryosphaeria, Cladosporium, Mycosphaerella, Neofusicoccum, Nigrospora, Pestalotiopsis, Pseudozyma* and *Valsa* representing >59 per cent of the total reads. Unlike the stems, leaves of the seedlings exposed to the plantation environment were predominated by fungi belonging to the Dothideomycetes (79.4 per cent), followed by Sordariomycetes (2.5 per cent) and then Agaricomycetes (0.1 per cent). They were dominated by fungi belonging to *Mycosphaerella*, representing 78.1 per cent of the reads.

454 GS-FLX amplicon pyrosequencing

A total of 2417, 1842 and 2023 sequences of ≥400 bp were respectively recovered from seed-capsules, seed and seedlings after quality trimming. Identification of fungal organisms in seed-capsules and seed revealed the presence of at least 102 fungal taxa. The most common taxa across the two samples (Table 2) were *Hormonema* sp. (45.2 per cent), uncultured endophytic

Table 1 Percentages of fungal communities identified in *E. grandis* seed-capsules, seed and seedling libraries as revealed by Illumina MySeq sequencing

Family	Genus	Total reads	Seed-capsules	Seed	Phytotron seedlings	*In vitro* seedlings	Stems of seedlings exposed to the forest plantation	Leaves of seedlings exposed to the forest plantation
Mycosphaerellaceae	*Mycosphaerella*	24	–	0.3	0.1	62.5	3.3	78.1
Pleosporaceae	*Alternaria*	10.8	5.7	9.8	29.2	–	19.5	0.7
Trichocomaceae	*Aspergillus*	9.8	58.9	–	–	–	–	–
Nectriaceae	*Fusarium*	10.2	3.3	7.1	50.8	–	–	–
Dothioraceae	*Hormonema*	6.3	13.6	22.9	1.3	–	–	–
Amphisphaeriaceae	*Pestalotiopsis*	4.9	3.4	11.9	3.8	–	10	0.1
Mycosphaerellaceae	*Cladosporium*	4.6	10.2	14.8	0.1	–	2	0.1
Polyporaceae	*Trametes*	2.1	–	–	–	12.5	–	–
Botryosphaeriaceae	*Neofusicoccum*	1.7	–	0.9	–	–	9	0.1
Incertae sedis	*Nigrospora*	1.5	3.5	0.2	0.1	–	3	2.1
Botryosphaeriaceae	*Botryosphaeria*	1.3	–	0.5	–	–	6.8	0.3
Valsaceae	*Valsa*	0.9	–	1.7	0.1	–	3.3	0.1
Teratosphaeriaceae	*Teratosphaeria*	0.6	–	2.9	1	–	–	–
Cucurbitariaceae	*Curreya*	0.3	–	0.3	1.3	–	–	–
Ustilaginaceae	*Pseudozyma*	0.3	–	–	–	–	2	–
Incertae sedis	*Phoma*	0.2	–	0.5	0.9	–	–	–
Pezizaceae	*Peziza*	0.2	–	–	1.4	–	–	–
Dothioraceae	*Sydowia*	0.1	–	0.6	–	–	–	–
Botryosphaeriaceae	*Lasiodiplodia*	0.1	–	0.5	–	–	–	–
Pleosporaceae	*Curvularia*	0.1	–	0.2	0.4	–	–	–
Trichocomaceae	*Penicillium*	0.1	–	0.5	–	–	–	–
Valsaceae	*Phomopsis*	0.1	0.3	0.2	–	–	–	–
Glomerellaceae	*Glomerella*	0.1	–	0.6	–	–	–	–
Mycosphaerellaceae	*Guignardia*	<0.1	–	–	–	–	–	0.1
Pleosporaceae	*Bipolaris*	<0.1	–	0.2	–	–	–	–
	Cochliobolus	<0.1	–	0.2	–	–	–	–
Herpotrichiellaceae	*Exophiala*	<0.1	–	0.2	0.1	–	–	–
Trichocomaceae	*Eupenicillium*	<0.1	–	–	0.1	–	–	–
Incertae sedis	*Scolecobasidium*	<0.1	–	–	–	–	–	0.1
	Myrothecium	<0.1	–	–	–	–	–	0.1
	Khuskia	<0.1	–	–	0.1	–	–	0.1
Diaporthaceae	*Diaporthe*	<0.1	–	0.2	–	–	–	–
Polyporaceae	*Laetiporus*	<0.1	–	–	–	–	–	0.1
Trichosporonaceae	*Trichosporon*	<0.1	–	0.2	–	–	–	–
Incertae sedis	*Rhodotorula*	<0.1	–	–	–	–	0.3	–
	Sporobolomyces	<0.1	–	0.2	–	–	–	–
Other Auriculariales	Other	2	–	–	–	–	11.8	–
Other Diaporthales	Other	<0.1	–	0.2	–	–	–	–
Other Pleosporales	Other	0.1	–	0.3	0.1	–	–	–
Other Tremellales	Other	0.1	–	0.3	–	–	–	–
Other Xylariales	Other	0.2	0.3	0.9	–	–	–	–
Other Ascomycete	Other	9.5	–	0.2	1.5	25	14.5	15.8
Other Basidiomycete	Other	1.9	0.1	8	1.8	–	1.5	–
Unclassified Fungi		4.2	0.5	12.4	4.8	–	12.8	0.8
No blast hit		0.5	0.2	0.6	1.1	–	0.3	1.3

fungus (6.9 per cent), uncultured Basidiomycete (6.5 per cent), *Alternaria alternata* (Fr.) Keissl (4.6 per cent), *Davidiella tassiana* (De Not.) Crous and U. Braun (4.6 per cent) and *T. zuluensis* (4.5 per cent). Besides *T. zuluensis*, other *Eucalyptus*-associated fungi were *Teratosphaeria* sp. (0.6 per cent), *Neofusicoccum eucalyptorum* Crous, H. Smith and M.J. Wingf (0.3 per cent), *Davidiella* sp. (0.2 per cent) and a *Mycosphaerellaceae* sp. (0.2 per cent).

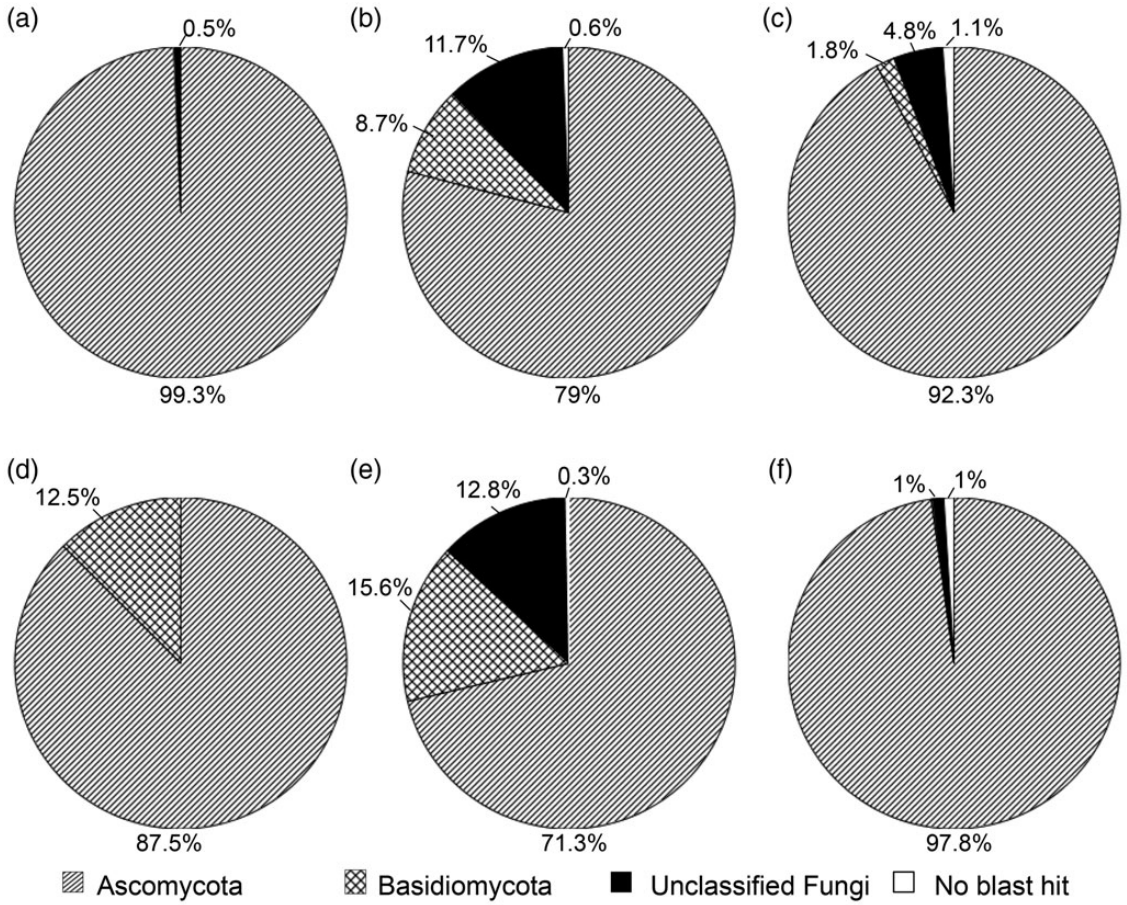

Figure 1 Illumina sequence read abundance for fungi belonging to phylum Ascomycota, Basidiomycota, unclassified fungi and no blast hit associated with *E. grandis*. (a) Seed-capsules, (b) seed, (c) seedlings grown in a phytotron, (d) seedlings grown *in vitro* and (e) stems and (f) leaves of seedlings exposed to forest plantation environment.

A total of 38 fungal taxa were identified for 1-month-old-seedlings grown in a phytotron. The most dominant taxa were *Cladosporium* sp. (47.7 per cent), *Pestalotiopsis microspora* (Speg.) Bat. and Peres (16.5 per cent), *Phoma glomerata* (Corda) Wr. and Hochapf (6.9 per cent), *Erythrobasidium* sp. (4.3 per cent), *Pleosporales* sp. (2.4 per cent) and *Pseudozyma* sp. (1.6 per cent). Other fungi included *Pestalotiopsis aquatica* (Ellis and Everh.) Steyaert (0.7 per cent), *Hyalodendron* sp. (0.7 per cent), *Alternaria* sp. (0.6 per cent) and *Pestalotiopsis disseminata* (Thüm.) Steyaert (0.6 per cent).

454 *Pyrosequencing-based identification of* T. zuluensis *and* N. eucalyptorum

A total of 64 sequences recovered from *E. grandis* seed-capsules ($n = 46$) and seed ($n = 18$) showed high similarity to *T. zuluensis* sequences found on GenBank. These sequences met the requirement that their query coverage was >99 per cent and that their identities to the closest hit are >90 per cent. Sequences recovered from seed-capsules ranged from 501 to 533 bp with identities ranging from 97 to 99 per cent. Sequences recovered from seed ranged from 504 to 559 bp with identities ranging from 98 to 99 per cent.

When a more stringent requirement of ≥99 per cent query coverage and ≥97 per cent identity was applied, only 12 sequences remained from the original 64, 7 from seed-capsules and 5 from seed (Table 3). Identities to the closest *T. zuluensis* match ranged from 97 to 99 per cent for seed-capsules and 98 to 99 per cent for seed. These sequences displayed high query coverage (88–90 per cent) and identities (98–99 per cent) to the ex-type sequences DQ240207 and DQ240214 (Cortinas *et al.*, 2006b). These highly matching sequences were used for phylogenetic reconstructions.

Phylogenetic analyses of the ITS gene region of DNA sequences of fungi tentatively identified through BLASTn searches showed that they were identical to *T. zuluensis* (Figure 2). Besides, *T. zuluensis*, another important *Eucalyptus* pathogen tentatively identified to species level, was *N. eucalyptorum*. Two sequences recovered from *E. grandis* seed were identified as *N. eucalyptorum*, with 98–99 per cent query coverage and a common identity of 89 per cent (Table 3). Both sequences were 533 bp in length.

Discussion

The results of this study provide unequivocal evidence that the stem canker pathogen *T. zuluensis* occurs in seed and seed-capsules of *E. grandis* when this material is collected from a site

where the disease is common. The fungus was never identified from seedlings grown from cleaned seed and grown in an isolated phytotron or under sterile conditions on agar. This suggests that the fungus is not vertically transferred from seed to plants, which is similar to the situation for fungi such as the pine pathogen *Diplodia sapinea* (Bihon *et al*., 2010). The results suggest that *T. zuluensis* was probably introduced into South Africa in seed batches, as has been previously proposed (Cortinas *et al*., 2006b; Hunter *et al*., 2011), but not tested.

Table 2 Percentages of fungal communities identified in *E. grandis* seed-capsules, seed and seedling libraries as revealed by 454 sequencing

Genus	Seed-capsules	Seed	Phytotron seedlings
Hormonema	62.1	29.7	–
Uncultured fungi	12.7	40.0	15.8
Teratosphaeria	8.6	1.7	–
Fungal spp.	8.2	0.4	–
Davidiella	6.1	3.7	–
Cladosporium	0.9	7.0	48.2
Mycosphaerellaceae	0.3	–	–
Ascomycete	0.2	–	–
Comminutispora	0.2	–	–
Alternaria	0.2	11.1	0.6
Neofusicoccum	0.2	0.4	–
Cercospora	0.2	0.3	–
Curvularia	–	0.9	–
Cylindrocarpon	–	0.8	–
Macrophomina	–	0.3	–
Fungal endophyte	–	0.3	0.6
Pestalosphaeria	–	0.3	–
Pestalotiopsis	–	–	18.3
Phoma	–	–	6.9
Erythrobasidium	–	–	4.4
Pleosporales sp.	–	–	2.4
Pseudozyma	–	–	1.6
Herpotrichiellaceae sp.	–	–	0.2
Rhodosporidium	–	–	0.2
Exophiala	–	–	0.2
Other organisms	0.2	3.3	0.7

High-throughput sequencing of *E. grandis* seed-capsules, seed and seedlings revealed a dominance of Dothideomycetes and Sordariomycetes. The Dothideomycetes included fungi in the Botryosphaeriaceae, Mycosphaerellaceae and Teratosphaeriaceae, which include major *Eucalyptus* stem and leaf pathogens (e.g. Keane *et al*., 2000; Old *et al*., 2003; Wingfield *et al*., 2008). The Sordariomycetes also constitute important stem canker pathogens of *Eucalyptus* in the Diaporthaceae, Nectriaceae and Valsaceae (e.g. Keane *et al*., 2000; Old *et al*., 2003). Saprophytic fungi, dominated by the Dothioraceae, Pleosporaceae and Trichocomaceae, were previously isolated from *Eucalyptus* seed using culture-based techniques (Mittal and Sharma, 1982; Mwanza and Kellas, 1987; Yuan *et al*., 1997; Mehrotra and Singh, 1998; Keane *et al*., 2000). A study on *E. grandis* leaves, petioles, twigs and trunk increment core samples similarly reported the occurrence and high sequence abundance of Botryosphaeriaceae, Mycosphaerellaceae and Teratosphaeriaceae (Kemler *et al*., 2013).

Our experiment, investigating vertical transmission of pathogens from seed to seedlings, yielded inconsistent results with the two chosen high-throughput sequencing techniques. Illumina MiSeq sequencing showed the occurrence of *Mycosphaerella* species in both seed and seedlings grown *in vitro* in tissue culture and those grown in a phytotron, both controlled environments. This raises the possibility that this group of fungi might be vertically transmitted from seed into seedlings. This is strongly supported by the low number of reads recovered from seedlings grown *in vitro*, which can be attributed to the high sterility in the tissue culture facility, increasing the likelihood that *Mycosphaerella* species were vertically transmitted from seed into seedlings. However, these findings were not confirmed by 454 pyrosequencing that showed only the presence of these fungi in seed-capsules. The failure of 454 sequencing to detect these fungi could be a result of primer bias (Bellemain *et al*., 2010) because different primers were used for 454 and Illumina sequencing technologies. The two sequencing technologies are expected to give the same fungal diversity results when the same primers are used (Luo *et al*., 2012). We failed to find the evidence of vertical

Table 3 Sequences recovered from *E. grandis* seed and seed-capsules matching *T. zuluensis* and *N. eucalyptorum* sequences in NCBI GenBank

Seed-capsule						Seed					
Sample name	GB[1]	CA[2]	QC[3]	E[4]	ID[5]	Sample name	GB[1]	CA[2]	QC[3]	E[4]	ID[5]
HXOG0ZS01B2W9T	*T. zuluensis*	JQ732913	99	0	98	HXOG0ZS01BJH5A	*T. zuluensis*	JQ732913	99	0	98
HXOG0ZS01A7Z1S	*T. zuluensis*	KF454696	99	0	99	HXOG0ZS01BEK0B	*T. zuluensis*	KF454696	100	0	99
HXOG0ZS01ALYPR	*T. zuluensis*	JQ732913	99	0	99	HXOG0ZS01BEFW2	*T. zuluensis*	KF454696	100	0	99
HXOG0ZS01BOVQ5	*T. zuluensis*	KF454696	100	0	98	HXOG0ZS01A8JYI	*T. zuluensis*	KF454696	100	0	99
HXOG0ZS01A9HQ3	*T. zuluensis*	KF454696	99	0	98	HXOG0ZS01AJ8WY	*T. zuluensis*	JQ732913	99	0	98
HXOG0ZS01AWRDT	*T. zuluensis*	JQ732913	100	0	99	HXOG0ZS01BGLOD	*N. eucalyptorum*	KC479188	99	0	89
HXOG0ZS01AA89V	*T. zuluensis*	JQ732913	99	0	97	HXOG0ZS01AGALJ	*N. eucalyptorum*	KC479188	98	0	89

[1]Closest GenBank match.
[2]Three Closest Accessions.
[3]Query Cover (per cent).
[4]E-value.
[5]Identity.

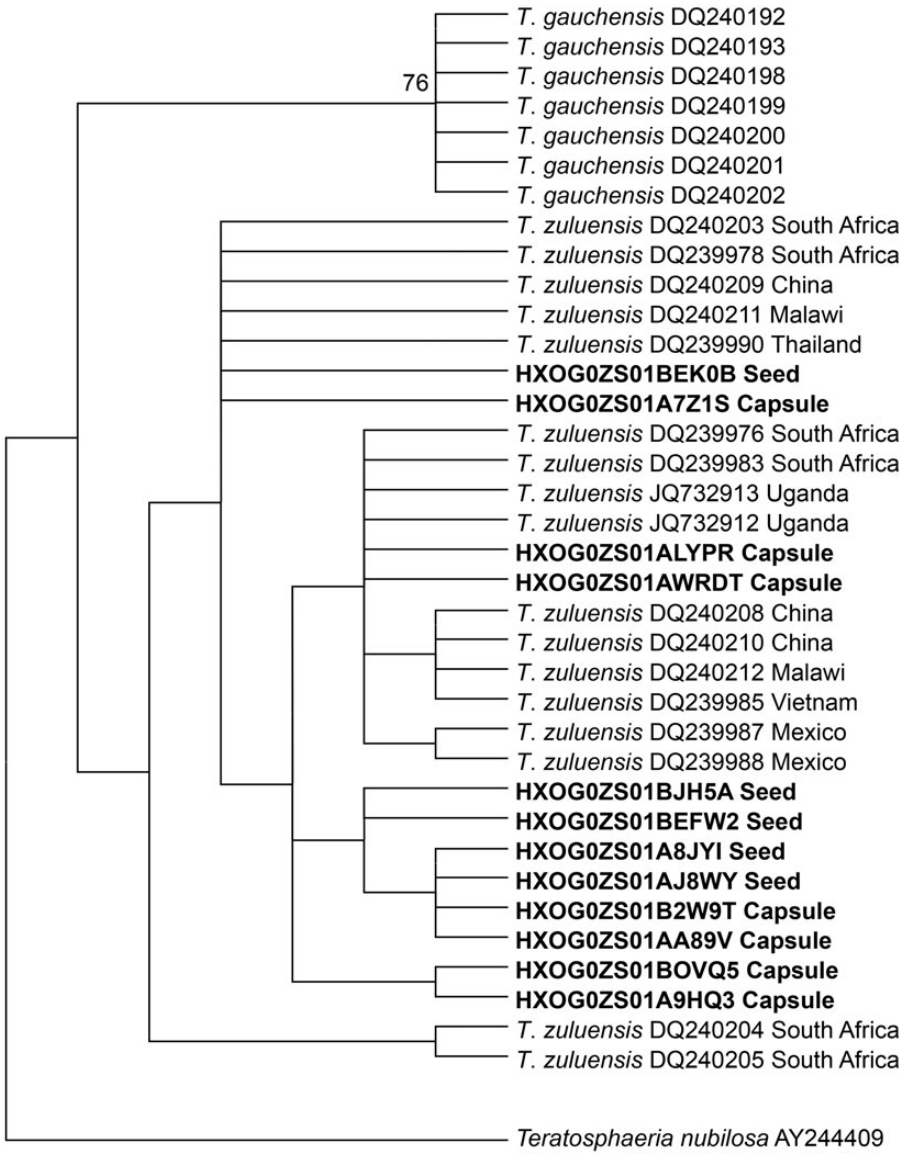

Figure 2 Majority-rule consensus of the 18 most parsimonious trees obtained from a heuristic search with 37 random taxon additions of ITS sequences using PAUP v4.0b10. Bootstrap support values (after 1000 replicates) of >75% are shown at the nodes. 454 reads are shown in bold font. *Teratosphaeria nubilosa* was used as an out-group.

transmission of *N. eucalyptorum* and *T. zuluensis* from seed to seedlings, increasing the likelihood that these pathogens could be horizontally transmitted. The occurrence of Botryosphaeriaceae in seedlings exposed to the plantation environment suggests horizontal transmission of some of these species and genera, as proposed previously by Slippers and Wingfield (2007) and for *D. sapinea* shown by Bihon *et al*. (2010).

The occurrence of Botryosphaeriaceae, Mycosphaerellaceae, Teratosphaeriaceae and particularly *T. zuluensis* in *E. grandis* seed batches requires further study. This is especially important to determine how these pathogens in seed end up infecting growing *Eucalyptus* trees. There is little evidence to demonstrate that pathogens identified in seed result in infections in *Eucalyptus* trees (Slippers and Wingfield, 2007). Yet, it is of great importance to fully understand the role of seed movement in spreading these important *Eucalyptus* pathogens. This is especially true given that these pathogens survive as endophytes in healthy plant material (Pillay *et al*., 2012; Marsberg *et al*., 2014), making it difficult or even impossible for commonly applied quarantine programmes to detect them. This could be leading to these important pathogens spreading unnoticed. Once introduced into new areas, the pathogens would likely infect eucalypt species in plantations, leading to the spread of diseases.

Sequencing techniques used in this study have their limitations despite their high resolution in detecting microbes on environmental samples such as seed. A major drawback is their inability to distinguish viable from non-viable DNA, leading to overestimation of viable fungal species (e.g. Amend *et al*. 2010; Vaishampayan *et al*., 2013). It is also difficult to tell whether the nucleic acids amplified during PCR were from filamentous hyphae or spores. Different

proportions of hyphae and spores lead to biases during DNA extraction and sequence abundances (Amend *et al*., 2010). Despite these limitations, we are confident of our findings as similar studies dealing with viable fungal cultures from leaf tissue similarly identified the endophytic fungi identified in this study, including *T. zuluensis*, in *E. grandis* plant material (Pillay *et al*., 2012; Marsberg *et al*., 2014). However, future work will be required to test the viability of *T. zuluensis* and other pathogenic fungi detected on *E. grandis* seed.

Conclusions

This study has provided evidence that the stem canker pathogen *T. zuluensis* occurs on seed and seed-capsules of *E. grandis* when this material is collected from a site where the disease is common. This also applies to some of the most important *Eucalyptus* pathogens, particularly those belonging to the Botryosphaeriaceae, Mycosphaerellaceae and Teratosphaeriaceae. These fungi were detected in seed and seed-capsules, which form part of the chaff in seed batches moved around the world for *Eucalyptus* plantation establishment.

Endophytic fungi or fungi on the surface of seed are difficult to detect, allowing them to escape quarantine. It is, therefore, important to ensure that seed batches are free of chaff and that surface disinfestation of seed is applied to reduce the load of pathogens. Additional studies to consider the vertical and horizontal transmission of these pathogens should be conducted to gain a better understanding of the spread of important *Eucalyptus* diseases.

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Conflict of interest statement

None declared.

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