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Seed health status and germination of *Eucalyptus* spp.

E. Mangwende · P. W. Chirwa · T. A. S. Aveling

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Abstract The presence of disease causing microorganisms on seeds raises serious quarantine and economic concerns to nurserymen, foresters and seed traders. The agar plate method was used to examine seed-borne mycoflora associated with *Eucalyptus* seed lots and their effect on seed germination was determined. A total of 35 fungal species from 29 genera were identified from 12 different *Eucalyptus* species. The *Eucalyptus nitens* seed lot was the most infested, whereas the lowest incidence of fungi was from the *E. dorrigoensis* seed lot. *Penicillium* was the most abundant fungus. *Colletotrichum*, *Aureobasidium* and *Disculoides* were recorded for the first time associated with *Eucalyptus* seeds. There was a significant reduction in seed germination of seed lots inoculated with selected seed-borne fungi compared to non-inoculated controls. *Fusarium oxysporum* and *F. solani* reduced seed germination the most on *E. badjensis*, *E. dorrigoensis*, *E. nitens*, *E. pellita*, *E. teritecomis* and *E. urophylla* seed lots with percentage germination of 31.3 and 33.5; 30.5 and 30.0; 38.8 and 37.0; 30.5 and 32.3; 25.0 and 26.8; 33.3 and 31.8; 31.3 and 33.5%, respectively. Similarly, seed germination was lowest on the *E. benthamii* seed lot (29.8%) inoculated with *C. gloeosporioides,* whilst germination of *E. grandis*, *E. smithii* and *E. viminalis* seed lots inoculated with *Botrytis* sp. and *F. solani* were 37.0 and 37.5%; 35.8 and 36.3%; 28.3 and 30.0%, respectively. This study has shown that commercial *Eucalyptus* seed lots carry a wide diversity of fungi and suggests that infested seeds may be a primary reason for poor seed germination.

Keywords Seed-borne · Seed germination · *Colletotrichum* · *Disculoides* · *Fusarium*

E. Mangwende · P. W. Chirwa · T. A. S. Aveling
Department of Plant and Soil Sciences, University of Pretoria, Pretoria 0002, South Africa

E. Mangwende · T. A. S. Aveling (✉)
Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria 0002, South Africa
e-mail: terry.aveling@fabi.up.ac.za

Introduction

The ideal for foresters is to obtain high *Eucalyptus* seedling survival rates above 85% (Stape et al. 2001), but delay of seedling emergence and poor survival of seedlings remain a common nursery challenge. Several factors can reduce seedling emergence, among them is seed health status (Brown and Ferreira 2000; Lilja et al. 2010). In almost every harvested seed lot, chaff and other debris together with a variety of microorganisms are naturally present at least in small quantities (Boland et al. 1980). Seed-borne fungi can cause seed rot, delay seed germination or threaten establishment of plant stands due to pre- and/or post-emergence damping-off (Cram and Fraedrich 2010; Evira-Recuenco et al. 2015; Tobias et al. 2017). During processing or storage, infested seed batches may contaminate other clean seed lots (Agarwal and Sinclaire 1997).

Apart from seeds acting as primary sources of inoculum of diseases in nurseries, there is increased risk of

spread of diseases across geographical borders through the seed trade (Elmer 2001; Santini et al. 2013). The rise in the seed trade in the last decades has increased the risk of spread of forestry pathogens such as *Botryosphaeria dothidea* (Moug.) Ces. & De Not., *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *Mycosphaerella nubilosa* (Cooke) Hansf. and *Teratosphaeria zuluensis* (M.J. Wingf., Crous & T.A. Cout.) M.J. Wingf. & Crous (Slippers et al. 2009; Hunter et al. 2011; Jimu et al. 2015; Maciel et al. 2015). In the last decade, different governments have passed tougher quarantine laws in trade of agricultural goods and services, but new pests and diseases continue to appear in *Eucalyptus* plantations (Graziosi et al. 2019). Hence, regular seed health tests are a prerequisite as decision-making tools for detecting and quantifying inoculum loads on seeds.

Although reports on seed-borne mycoflora associated with *Eucalyptus* have appeared from time to time (Mittal 1986; Farr et al. 1989; Mittal et al. 1990; Pongpanich 1990; Mehrotra and Singh 1998), most of these studies merely listed seed-borne mycoflora on a few *Eucalyptus* spp. without examining the effects of specific fungi on seed germination and seedling development. Jimu et al. (2015) investigated the mycoflora associated with *Eucalyptus grandis* W. Hill ex Maiden seed samples produced in South Africa, however the diversity of seed-borne mycoflora associated with various *Eucalyptus* species largely remains unknown. Therefore, the aim of this study was to investigate seed-borne mycoflora associated with commercial seeds of 12 different *Eucalyptus* spp., evaluate their effect on seed germination and use a detached leaf assay to determine their pathogenicity.

Materials and methods

Source of seed

One sample of each *Eucalyptus* spp. (Table 1), supplied by commercial forestry seed companies in South Africa, were used in this study. Seed lots were tightly sealed in plastic bags and stored at 4 °C until use.

Seed health tests

Seed-borne mycoflora associated with *Eucalyptus* spp. seeds were investigated using the agar plate method. A weighed replicate (ISTA (International Seed Testing Association) 2018) of 0.1 g of each *Eucalyptus* spp. was wrapped in sterile cheesecloth and surface disinfected by soaking in 1% sodium hypochlorite solution for 5 min. After rinsing in sterile distilled water, seeds were spread out and air dried on sterile paper towels in a laminar flow. Ten seeds were plated in each 90 mm diameter Petri dish containing potato dextrose agar (PDA, Biolabs, Midrand, South Africa). Petri dishes were sealed with Parafilm® and transferred to a 25 °C incubator (Labcon growth chamber, Krugersdorp, South Africa). For each *Eucalyptus* species, four replicates of 10 Petri dishes were arranged in a completely randomised design. After 5 days of incubation, fungi growing from seeds were isolated, sub-cultured on PDA and incubated at 25 °C for 7 days under alternating cycles of 12 h near ultra violet (UV) (365 nm) light and darkness. Fungal genera and species were identified with the aid of various references of Ellis and Ellis (1997), Mathur and Kongsdal (2003) and Leslie and Summerell (2006). Incidences of seed-borne fungal species were determined by counting the number of times each fungal species appeared, and expressed as a percentage of seeds tested in each seed lot. Relative incidences of isolation of each fungal species were expressed as a percentage of the total number of fungal species observed in all four replicates. Fungal isolates were stored on PDA slants at 4 °C for further experiments.

Molecular identification

The molecular technique based on the Polymerase Chain Reaction (PCR) was used to confirm identity of selected seed-borne fungal isolates. From 7-day-old cultures, 100 mg of mycelium was scraped and DNA was isolated using Zymo DNA extraction kits (Zymo Research, USA) following the manufacturer's protocol. Primer pairs ITS 1F and ITS 4R were used to amplify the Internal Transcribed Spacer (ITS1 and 2) conserved regions (White et al. 1990). Each 50-µl reaction mixture included 21 µL of PCR-grade water, 1 µL of DNA template, 1.5 µM of each primer, and 1 µL of PCR Master Mix (2X) (0.25 µL Taq DNA polymerase, reaction buffer, 4 mM MgCl2 and 0.4 mM of each dNTP; Thermo Scientific, Waltham, USA). The PCR conditions consisted of a denaturation step at 94 °C for 2 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min and a final elongation step at 72 °C for 10 min. The amplified DNA was purified using a Zymo

Table 1 Incidences of fungi (%) associated with commercial *Eucalyptus* spp. seed lots produced in South Africa

Fungi	*Eucalyptus* species from which fungi were isolated												Total (%)
	E. badjensis	*E. benthamii*	*E. dorrigoensis*	*E. dunnii*	*E. grandis*	*E. macarthurii*	*E. nitens*	*E. pellita*	*E. smithii*	*E. tereticornis*	*E. urophylla*	*E. viminalis*	
Alternaria alternata	1.1	1.9	–	1.9	4.0	6.7	10.3	1.7	3.7	5.6	4.4	5.0	7.4
Aspergillus niger	–	4.1	3.4	8.4	4.2	6.2	3.0	4.8	2.5	3.3	–	3.6	6.9
Aspergillus fumigatus	–	–	1.0	0.6	–	–	0.6	–	1.3	–	–	–	0.5
Aspergillus flavus	0.1	0.1	–	–	0.5	1.4	1.0	–	0.5	–	0.5	0.5	0.7
Aureobasidium pullulans	–	–	–	–	0.2	–	–	–	–	–	–	–	0.0
Bipolaris peregianensis	–	–	0.5	–	–	–	–	0.1	–	–	–	–	0.1
Botryobasidium aureum	–	–	–	0.1	–	0.3	0.2	–	–	–	–	0.2	0.1
Botryosphaeria dothidea	0.2	0.7	1.0	0.3	2.3	–	1.8	–	0.4	–	–	–	1.0
Botrytis cinerea	–	0.5	0.6	0.2	0.2	–	0.7	–	0.6	–	–	0.5	0.5
Chaetomium globosum	7.4	6.4	–	2.6	4.4	–	8.9	4.3	2.2	–	–	8.4	7.1
Cladosporium sphaerospermum	–	–	–	–	4.2	3.2	2.8	–	–	–	–	–	1.6
Colletotrichum gloeosporioides	–	–	–	4.0	–	0.8	3.8	–	–	–	–	–	1.4
Curvularia brachyspora	–	–	–	0.4	2.0	–	–	–	1.8	–	–	–	0.7
Curvularia lunata	–	–	–	0.7	0.5	0.5	–	–	0.5	1.2	–	1.2	0.7
Curvularia spicifera	–	–	–	–	1.0	1.8	–	1.5	1.4	2.8	1.0	1.2	1.7
Disculoides eucalypti	–	–	0.1	–	–	–	0.1	–	–	–	–	–	0.0
Epicoccum nigrum	0.7	0.4	0.5	–	1.2	–	1.2	–	0.5	–	–	0.8	0.8
Epicoccum purpurascens	–	0.1	–	0.5	1.4	–	1.9	1.8	1.1	1.8	2.5	2.2	2.1
Fusarium oxysporum	–	–	–	–	–	–	1.8	–	–	–	–	1.5	0.5
Fusarium solani	–	0.1	–	–	0.0	–	–	–	–	–	0.1	1.5	0.3
Gliocladium penicillioides	2.1	2.2	–	–	2.8	–	3.6	3.1	–	–	2.5	2.5	3.0
Gliocladium roseum	–	2.5	–	3.4	5.1	3.9	2.5	–	3.2	4.0	–	–	3.9
Lasiodiplodia theobromae	0.6	–	–	–	–	–	0.3	–	–	–	0.1	–	0.2
Mycosphaerella marksii	–	–	–	0.3	–	–	0.4	0.5	–	0.2	0.5	0.4	0.4
Neofusicoccum ribis	–	–	–	0.3	–	–	–	0.3	–	–	–	–	0.1
Nigrospora sphaerica	–	–	–	0.5	–	1.1	–	–	–	2.2	–	–	0.6
Paecilomyces marquandii	–	–	0.3	–	0.5	–	–	–	–	–	0.3	–	0.2

Table 1 (continued)

Fungi	*Eucalyptus* species from which fungi were isolated											Total (%)
	E. badjensis	*E. benthamii*	*E. dorrigoensis*	*E. dunnii*	*E. grandis*	*E. macarthurii*	*E. nitens*	*E. pellita*	*E. smithii*	*E. tereticornis*	*E. urophylla*	*E. viminalis*	
Penicillium spp.	24.0	22.5	21.5	7.4	43.2	22.7	39.7	32.8	28.2	26.4	17.7	27.0	49.9
Pestalotiopsis funerea	–	2.5	–	2.2	2.5	2.5	1.6	1.8	–	–	–	1.5	2.3
Phoma glomerata	–	–	–	–	0.5	–	0.2	–	–	–	–	–	0.1
Preussia africana	0.5	0.4	–	0.6	–	0.3	–	0.1	2.0	–	–	–	0.6
Stachybotrys chartarum	–	–	–	–	–	–	0.3	–	0.1	–	–	–	0.1
Sydowia polyspora	–	–	–	–	0.1	–	–	–	–	0.3	0.1	–	0.1
Talaromyces purpurogenum	–	–	–	–	3.9	4.0	3.5	–	3.1	–	–	3.5	2.9
Trichoderma viride	4.2	2.8	–	–	–	–	2.5	–	–	–	–	–	1.5
Ulocladium atrum	–	–	–	–	–	–	–	–	–	0.3	–	–	0.0

purification kit (Inqaba Biotech, South Africa), concentration was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and adjusted to 50 ng/μL.

The purified PCR product was sequenced with PCR primers ITS 1F and ITS 4R and the BigDye terminator sequencing kit v.3.1 (Applied Biosystems, USA) with AmpliTaq® DNA Polymerase (Applied Biosystems, Warrington, UK). From forward and reverse sequences obtained, consensus sequences were compiled using BioEdit (www.mbio.ncsu.edu/BioEdit/BioEdit.html), and subjected to Blast searches in in GenBank [National Centre for Biotechnology Information (NCBI), (www.ncbi.nlm.nih.gov/BLAST)]. Fungal cultures were deposited in the National Collection of Fungi, ARC-Plant Health and Protection, Roodeplaat, Pretoria, South Africa and the respective sequences were deposited in GenBank at NCBI, (www.ncbi.nlm.nih.gov/genbank) (Table 2).

Seed germination tests

The effect of 16 molecularly identified fungi isolated from *Eucalyptus* seeds (one isolate for each fungal species) on seed germination were evaluated for their effect on seed germination *in -vitro*. From 7-day-old cultures of each fungus, mycelia was scrapped and spores suspended in sterile distilled water amended with two drops of Tween 20 (Merck Ltd., Johannesburg, South Africa). The concentration of inoculum was adjusted to 1×10^5 spores/mL. Twelve *Eucalyptus* spp. seed lots, surface sterilised as described above, were inoculated with each of the sixteen fungi by soaking in 10 mL inoculum contained in a 150 mm glass Petri dish for 5 h at room temperature. Inoculated seeds were air dried on sterile paper towels in the laminar. Surface sterilised *Eucalyptus* seed lots soaked in sterile distilled water served as controls. Subsequently, seed germination was tested on four replicates of 50 inoculated and control seeds using the on-top paper method (ISTA (International Seed Testing Association) 2018). In each 150 mm glass Petri dish, 25 seeds were evenly spread out on top of two layers of moistened sterile filter papers (Whatman No. 1). Petri dishes containing plated seeds were incubated in a walk-in growth chamber (Seed Science Laboratory, University of Pretoria, South Africa). The plates received an alternating cycle of 10/14 h cool white light and darkness and temperature was maintained at 25 ±

1 °C. After 21 days, assessment of seed germination was done according to ISTA (International Seed Testing Association) (2018). Results of the experiment were scores of either healthy germinated seedlings without symptoms or diseased seedlings. Healthy germinated seedlings have intact primary roots and fully developed hypocotyls, whereas diseased seedlings were identified as those with necrotic spots or discolouration on the hypocotyl or seminal roots.

Seed-borne mycoflora pathogenicity assays

Pathogenicity assays were performed on detached leaves collected from 3-year old *Eucalyptus* plants grown in a nursery of the Forestry and Agricultural Biotechnology Institute (FABI, University of Pretoria, South Africa). Freshly collected, healthy looking leaves of *E. benthamii*, *E. camaldulensis*, *E. dorrigoensis, E. dunnii*, *E. grandis*, *E. macarthurii*, *E. nitens*, *E. tereticomis*, and *E. viminalis* were surface sterilized with 70% ethanol and rinsed thoroughly with sterile distilled water. Sixteen fungi isolated from *Eucalyptus* seed lots, listed in Table 2, were used. For each fungus, a 5 mm diameter mycelial plug of a 5-day-old culture was placed with the top side facing down on a sterilised leaf surface. Thereafter, inoculated leaves (three for each *Eucalyptus* sp.) were aligned on two layers of sterile moistened Whatman No.1 filter papers in glass Petri dishes. Inoculated *Eucalyptus* leaves were maintained in a walk-in growth chamber at 25 ± 1 °C. Control leaves were inoculated with 5 mm diameter agar plugs without fungi. Visual assessments of symptom development were recorded after five days of incubation based on relative size and colour of spots on inoculated leaves compared with non-inoculated controls. The experiment was repeated.

Data analysis

Results of germination tests were combined and subjected to analysis of variation (ANOVA) using SAS Version 9.4 statistical software (SAS Institute 2016), with the Fisher's Least Significance Difference test (LSD, $p = 0.05$) separating significant differences between means. For pathogenicity tests, observations of infection of detached leaves were recorded in contrast with untreated controls.

Results

Seed health status

In this study, a total of 35 fungal species from 29 genera in addition to *Penicillium* species that was not identified to species level were found naturally associated with *Eucalyptus* seed lots. A total of 220 fungal isolates were obtained from *Eucalyptus* seed lots, among which 106 could be identified morphologically to the species level. The remaining 114 fungal isolates were left unidentified as fungi did not sporulate or produce other reproductive structures. The *Eucalyptus nitens* seed lot was the most infested, whereas the lowest incidence of fungi occurred on the *E. dorrigoensis* seed lot (Table 1). Taxonomic composition assessments showed a predominance by three genera: *Penicillium*, followed by *Aspergillus* and *Alternaria*. Genera rarely isolated in order of frequency included *Stachybotrys*, *Ulocladium*, *Aureobasidium* and *Disculoides*. Of the isolated fungi, confirmation of 16 seed-borne isolates exhibited high similarities with ITS sequences of reference isolates from GenBank (Table 2).

Seed germination tests

Percentage germinated seeds of the 12 *Eucalyptus* spp. inoculated with the 16 selected fungi are given in Table 3. Highest seed germination percentages were from non-inoculated seed lots, where *E. dunnii*, *E. teritecomis* and *E. urophylla* seed lots had percentages germination above 90%. However, seed germination was significantly reduced when seeds were inoculated with seed-borne fungi ($p < 0.05$). The lowest seed germination was recorded on *E. badjensis* (30.5%), *E. benthamii* (29.8%), *E. dorrigoensis* (37.0%), *E.* dunii (32.2%), *E. grandis* (37.0%), *E. macathurii* (28.3%), *E. nitens* (25.0%), *E. pellita* (30.5%), *E. smithii* (33.5%), *E. tereticomis* (31.8%), *E. urophylla* (31.3%) and *E. viminalis* (28.3%). On the contrary, inoculating *Eucalyptus* seed lots with *S. polyspora* and *Chaetomium* sp. had the least effect on seed germination. Germination was reduced the most by *Botrytis sp.* in *E. benthamii* and *E. viminalis* seed lots and by *Colletotrichum* in *E. benthamii*. Germination was most affected by *Botrytis* sp. in *E. benthamii*, *E. dorrigoensis* and *E. grandis*, *F. oxysporum* in *E. nitens* and *F. solani* in *E. macathurii* and *E. nitens* (Table 3).

Table 2 Sequences recovered from fungi isolated from seed lots of *Eucalyptus* spp. matching sequences in NCBI GenBank

Sample Name	Closest GenBank match	GenBank accession	Closest accession	Query Cover (%)	E-value	Identity (%)
PPRI 26850	*Aureobasidium pullulans*	MN200199	KT693733	97.0	0.0	99.2
PPRI 26848	*Botryosphaeria dothidea*	MN200200	KF766151	99.0	0.0	98.5
PPRI 26854	*Botrytis cinerea*	MN200201	KX858922	99.0	0.0	96.6
PPRI 26859	*Chaetomium globosum*	MN200202	MH858130	98.0	0.0	97.1
PPRI 24314	*Colletotrichum gloeosporioides*	MG641892	JX010155	100.0	0.0	99.0
PPRI 23538	*Disculoides eucalypti*	MN200203	NR120089	100.0	0.0	97.5
PPRI 26851	*Fusarium oxysporum*	MN200204	U28160	98.0	0.0	97.1
PPRI 26857	*F. solani*	MN200205	NR163531	99.0	0.0	98.1
PPRI 26855	*Gliocladium roseum*	MN200206	AJ309334	98.0	0.0	95.8
PPRI 26858	*Lasiodiplodia theobromae*	MN200207	NR111174	98.0	0.0	96.1
PPRI 26847	*Mycosphaerella marksii*	MN200208	AY152600	97.0	0.0	98.2
PPRI 26852	*Nigrospora sphaerica*	MN200209	MF467244	98.0	0.0	99.5
PPRI 26856	*Phoma glomerata*	MN200210	AF126819	99.0	0.0	98.7
PPRI 26860	*Preussia africana*	MN200211	JQ031265	98.0	0.0	97.6
PPRI 26849	*Sydowia polyspora*	MN200212	MH198272	97.0	0.0	99.0
PPRI 26853	*Ulocladium atrum*	MN200213	JF417684	98.0	0.0	94.8

Seeds inoculated with seed-borne fungi yielded significantly higher numbers of diseased seedlings ($p < 0.05$) compared with non-inoculated controls which were naturally infested. The most diseased seedlings occurred in *E. badjensis*, *E. benthamii*, *E. dorrigoensis*, *E. dunnii*, *E. pellita*, *E. smithii*, *E. tereticornis* seed lots inoculated with either *F. oxysporum* (61.8, 55.8, 51.5, 57.8, 60.0, 55.0 and 57.5%, respectively) or *F. solani*. (60.8, 59.3, 53.0, 55.0, 57.5, 57.3 and 54.3%, respectively) when compared to their respective controls (Table 4). Similarly, inoculating *E. benthamii*, *E. dorrigoensis*, *E. grandis*, *E. smithii* and *E. urophylla* seed lots with *Botrytis* sp. yielded the most diseased seedlings (59.8, 52.3, 49. 0, 54.5 and 55.3%, respectively) when compared to their respective controls. Seedlings of *E. benthamii* were most susceptible to infection with either *Botrytis* sp. or *Colletotrichum* sp. *E. nitens* had highest disease susceptibility to *F. oxysporum* whilst *E. macarthurii, E. nitens* and *E. urophylla* were most susceptible to *F. solani* (Table 4).

Seed-borne mycoflora pathogenicity assays

There were dark brown-black leaf spots on *E. benthamii*, *E. camaldulensis*, *E. dorrigoensis*, *E. dunnii*, *E. grandis*, *E. macarthurii*, *E. nitens*, *E. tereticornis*, and *E. viminalis* leaves inoculated with *Disculoides* sp., *F. oxysporum*, *Lasiodiplodia* sp. and *Mycosphaerella* sp. Inoculation with *Botrytis* sp., *Botryosphaeria* sp., *F. solani*, *Phoma* sp., *Preussia* sp., *Nigrospora* sp. or *Ulocladium* sp. produced light brown leaf spots on leaves of *E. benthamii*, *E. dunnii* and *E. nitens*. However, no leaf symptoms appeared on non-treated controls and *Eucalyptus* leaves inoculated with any of *Aureobasidium*, *Chaetomium*, *Gliocladium* and *Sydowia* species.

Discussion

Testing health status of seeds is essential for monitoring presence or absence of disease causing microorganisms that may affect seed germination and seedling development. Despite several countries implementing stricter phytosanitary regulations in the trade of agricultural products including live plants and seed (Cleary et al. 2019), phytosanitary requirements for most tree species, even the dominant tree species in commercial forest plantations, are minimal (Cleary et al. 2019).

Tree seeds are often infested with large numbers of fungi (Mittal 1986; Yuan et al. 1990; Mamatha et al. 2000; Sutherland et al. 2002; Cleary et al. 2019). This study showed that *Eucalyptus* seed lots were naturally

Table 3 Percentage healthy seedlings from 12 *Eucalyptus* spp. seed lots inoculated with 16 selected fungi isolated from *Eucalyptus* spp.

Treatment	*Eucalyptus* species											
	E. badjensis	*E. benthamii*	*E. dorrigoensis*	*E. dunii*	*E. grandis*	*E. macathurii*	*E. nitens*	*E. pellita*	*E. smithii*	*E. tereticomis*	*E. urophylla*	*E. viminalis*
Aureobasidium sp.	39.8*f**wx	40.0defwx	46.0dev	41.5gw	43.5efw	40.5fgw	30.0ghz	39.3ghwx	33.5jy	42.3fgw	40.3fgwx	37.0gx
Botryosphaeria sp.	55.3bcv	43.5cdex	39.0fyz	37.8hz	37.5ijz	41.0fgyz	36.5efz	42.0efxy	43.3ghxy	48.5dew	50.5cdvw	36.5gz
Botrytis sp.	34.8gxy	33.0fyz	37.3fwxy	35.0ixy	37.0jwx	38.8gwx	30.0ghz	35.0ixy	35.8jxy	39.0ghw	37.3ghwx	30.0hz
Chaetomium sp.	56.3bctu	42.8cdez	54.3buv	46.0efy	47.3dxy	59.5brst	63.5br	52.8buvw	51.3cdvwx	50.0cdwx	60.3brs	59.0bst
Colletotrichum sp.	42.0fxy	29.8fz	38.0fy	50.3cw	56.8bv	46.0ewx	39.5ey	38.5hy	48.3dewx	53.0cvw	45.8ex	47.3cdwx
Disculoides sp.	34.5gz	44.8cdv	45.3euv	35.0iyz	40.5ghwx	43.8efvw	32.8fgz	38.5hxy	40.5hiwx	39.0ghx	48.5deu	40.0efgx
Fusarium oxysporum	30.5hy	34.5efxy	38.8fwx	32.3jy	40.0hiw	39.5gw	25.0iz	30.5jy	39.3iw	33.3iy	31.3jy	41.3efw
F. solani	33.0ghvwx	35.0efvwx	37.0fv	37.0hiv	37.5ijv	28.3hyz	26.8hiz	32.3jwxy	36.3jwxy	31.8ixy	33.5ijvwx	28.3hyz
Gliocladium roseum	52.5cdvwx	56.8bvwx	56.3bvwx	41.3gv	54.0cv	57.0bcyz	60.5bz	55.3bwxy	51.0cdvw	53.3cxy	53.8cvwx	49.0cyz
Lasiodiplodia sp.	53.5cuv	55.3bu	48.8cdwx	47.0defwx	45.8dexy	50.5dvw	55.0cu	49.0cwx	48.5dewx	37.8hz	40.0fgz	43.8dey
Mycosphaerella sp.	49.5dexy	35.0efz	50.8cxy	49.0cdxy	52.8cx	46.8ey	45.3dy	48.8cxy	51.5cx	48.0dexy	49.3dexy	47.5cdxy
Nigrospora sp.	42.8fz	56.3bw	56.8bw	49.0cdxy	51.3cx	55.0cw	39.5ez	47.5cy	49.3cdexy	49.0dxy	46.5ey	41.8efz
Phoma sp.	40.5fxy	49.0bcv	46.3devw	49.8cv	41.5fghx	39.3gxy	31.3ghz	41.3fgxy	45.3fgw	48.0devw	39.0fghxy	38.5fgy
Preussia sp.	40.5fyz	50.5bcv	47.0dew	44.8fvw	43.3efgvwx	40.3fgyz	39.8ez	44.5devw	45.3fgvw	45.3efvw	42.5fxyz	42.3efxyz
Sydowia sp.	58.8bwx	55.8bx	56.0bx	58.5bwx	57.3bwx	56.5bcwx	47.3dz	55.3bx	57.8bwx	60.0bw	51.0cdy	58.3bwx
Ulocladium sp.	48.3ev	48.0bcdvw	48.0cdevwx	47.8cdewxy	45.0dewxy	42.3fgy	37.8ez	44.8dxy	46.8efvwx	48.3dev	36.3hiz	48.0cvw
Control	80.3ay	88.3awx	89.8awx	91.3aw	89.5awx	87.8ax	75.8az	88.0ax	89.3awx	90.3awx	90.5awx	88.8awx

*In each column, means with the same letters do not differ significantly according to Fisher's LSD test at $p < 0.05$

**Means within a row not followed by the same letter are significantly different from each other ($p < 0.05$)

Full names of fungi are given in Table 2

infested with several fungi, where the highest incidence was recorded on *E. nitens* seed lot and the least on *E. dorrigoensis*. Variation of incidences of fungi on seed lots can be attributed to the influence of external environments of seed orchards but also different sources of possible contamination sites from harvesting to processing and storage (Cram and Fraedrich 2010). The season seeds are harvested and the level of maturity of capsules can influence the pattern of fungal richness isolated from seeds. Such variations are expected to be more pronounced due to morphological differences of seeds of species examined (Boland et al. 1980). Seed size, surface texture and shape are important characteristics that may influence the amount of fungi harboured in seed lots. Wrinkled seeds are more likely to harbour more pathogens than smooth surfaced seeds (Charkowski et al. 2001). This is particularly true for findings of this study, where fewer fungi were isolated from seeds of *E. dorrigoensis* and *E. grandis* as they have a uniform, more or less smooth, surface compared with more wrinkled and rough surfaced seeds of *E. nitens* (Boland et al. 1980).

The majority of fungi associated with seeds tend to have saprotrophic lifestyles with minimal negative effect on seed germination and seedling growth. A total of 29 fungal genera were found naturally associated with *Eucalyptus* seed lots. Taxonomic composition assessments showed that *Eucalyptus* seeds were predominantly infested with saprotrophs, *Penicillium* (49.9%), *Aspergillus* (8.1%) and *Alternaria* (7.4%), which have been previously reported to cause significant reduction of *Eucalyptus* seed germination and seedling emergence (Doshi et al. 1993; Yuan et al. 1997). Moreover, due to their fast growing saprotrophic characteristic, slow growing fungi were inhibited and obscured. In general, many pathogenic fungi are characterised by slow growth on media, such as *Teratosphaeria*, taking more than 4 weeks to reach a diameter of 40–50 mm (Cortinas et al. 2006). Since isolations of fungi in this study were done using the culture based approach, estimates of fungal incidence in this study were conservative as several isolates were left unidentified due to lack of sporulation. Although isolations on media is cheap, it is limited and often fails to detect certain fungal groups such as basidiomycetes that seldom produce asexual or sexual spores in culture upon which identification is based.

The trade of seed carries with it risks of inadvertent introduction of pests and pathogens to previously unaffected regions. The majority of seed-borne fungi such as *Lasiodiplodia*, *Neofusicoccum* and *Mycosphaerella* found on commercial seed lots are already widely distributed geographically and do not pose a significant quarantine threat. However, there is a quarantine concern as this study reports first occurrences of *Aureobasidium pullulans* and *Disculoides eucalypti* on *Eucalyptus* seeds. The genus *Disculoides* was described in 2012 with *D. eucalypti* and *Disculoides eucalyptorum* Crous, Pascoe, I.J. Porter & Jacq. Edwards, being isolated from diseased *E. viminalis* leaves in Australia (Crous et al. 2016). In New Zealand, *Disculoides eucalypti* Crous, Pascoe, I.J. Porter & J. Edwards, intercepted on imported *Eucalyptus leucoxylon* F. Muell., was short-listed as a quarantine threat to the country's biodiversity (Surveillance 2016; Crous et al. 2016). Detection of *Botryosphaeria dothidea* (Moug. ex Fr) Ces. & De Not on commercial *Eucalyptus* seeds is of quarantine significance as it appears on the European and Mediterranean Plant Protection Organization (EPPO) database of quarantine pests (https://gd.eppo.int/taxon/BOTSDO).

Seeds infected or contaminated with fungi may be damaged and fail to germinate, produce weak seedlings or may develop diseases on seedlings. Findings of this study showed that germination of *Eucalyptus* seed lots inoculated with seed-borne fungi resulted in a wide range of symptoms that included rotting of seeds, formation of lesions on newly developed hypocotyls and seminal roots or abnormal twisting of germinated seedlings. After inoculation, seed germination was less than 62% and as low as 25%, which potentially translates to low chances of seedling survival in nurseries. However, occurrence of diseased seedlings from non-inoculated controls suggest the presence of natural infection as confirmed by seed health tests. *Botrytis* and *Fusarium* spp. inoculated seed consistently yielded the lowest percentage of healthy seedlings on all *Eucalyptus* species. The notoriety of *Fusarium* as a serious threat to seedling emergence in numerous forest nurseries is well documented (Omokhua et al. 2009; Gordon et al. 2015; Won et al. 2019). The pathogen is a persistent problem in nurseries as it can cause severe pre- and post-emergence damping-off, and mortality of mature trees in forest plantations. In the seed lot samples examined in this study, soil-borne pathogens such as *Fusarium oxysporum* and *F. solani* might have been introduced

Table 4 Percentage diseased seedlings from 12 *Eucalyptus* spp. seed lots inoculated with 16 selected fungi isolated from *Eucalyptus* spp.

Treatment	*Eucalyptus* species											
	E. badjensis	*E. benthamii*	*E. dorrigoensis*	*E. dunii*	*E. grandis*	*E. macathurii*	*E. nitens*	*E. pellita*	*E. smithii*	*E. tereticomis*	*E. urophylla*	*E. viminalis*
Aureobasidium sp.	54.0*c**wx	52.3cdxy	44.0cdz	46.5dyz	44.8cdz	53.3cdwx	57.8cdew	49.8cdexyz	53.8bwx	47.5dyz	51.8cdwx	51.8dexy
Botryosphaeria sp.	39.0ghz	54.8bcvw	50.8axy	52.3bcwxy	41.8defz	53.0cdvwx	57.5cdeu	49.3dexy	48.5cdy	37.8fz	41.3hz	57.3abuv
Botrytis sp.	55.8bcwx	59.8av	52.3ay	53.5bcxy	49.0abz	57.0bvw	54.8efwxy	54.0bwxy	54.5abwxy	52.3bcy	55.3abcwxy	54.0bcdwxy
Chaetomium sp.	36.5hv	32.5iwx	31.8fwx	31.5gwx	33.8gw	36.3ghv	29.3hxyz	31.5hwx	30.0hxy	27.0gyz	26.8iz	30.0hxy
Colletotrichum sp.	53.8cw	59.0abv	44.3cdx	39.5fz	39.5efyz	44.8fx	46.3fx	53.0bw	47.5cdex	43.3exy	44.0fghx	45.8fx
Disculoides sp.	58.8abtu	48.3dexyz	46.3bcyz	46.5dyz	46.8bcyz	56.0bcuv	61.0bcdt	52.0bcwx	49.8cwxy	48.5cdxy	44.5fghz	53.3cdevw
Fusarium oxysporum	61.8au	55.8abcvwx	51.5axy	57.8auvw	47.3bcz	55.8bcvw	71.8at	60.0auv	55.0abwxy	57.5auvw	58.8auvw	50.3defvw
F. solani	60.8axyz	59.3abxyz	53.0az	55.0abyz	51.5az	67.0ax	65.0bxy	57.5axyz	57.3axyz	54.3abz	54.5bcxy	59.0axyz
Gliocladium roseum	35.0hvw	30.8ixy	31.0fxy	30.3gyz	31.5gxy	34.3iwx	31.0hxy	29.8hyz	30.0hyz	37.8fv	26.8iz	30.0hyz
Lasiodiplodia sp.	39.3ghwxy	35.5hiz	39.0exyz	52.0ct	40.5efwx	46.5fu	37.0gyz	44.8fguv	42.5fgvw	54.0abt	53.0bct	51.5det
Mycosphaerella sp.	42.0fgyz	45.8efvwx	47.5bvw	40.8fyz	38.8fz	49.0efvw	50.3fvw	44.8fgwxy	41.3gyz	41.5efxyz	45.5efgvwx	49.5efv
Nigrospora sp.	45.8dewx	40.3ghz	41.8dexyz	42.0efxyz	41.5defyz	40.5gz	57.3cdev	44.3gwxyz	45.0efwxy	43.3ewxyz	46.8efw	53.8bcdv
Phoma sp.	45.0efyz	42.3fgz	52.3avwx	46.3dy	46.8bcyz	55.3bcvwx	62.3bcu	50.5cdx	44.0efgyz	51.8bcwx	55.0abcvw	56.0abcv
Preussia sp.	49.0dwx	44.8efgyz	43.8cdyz	44.3deyz	42.8dez	54.0bcuv	56.0deu	47.3efxy	47.0cdexy	55.3abu	48.8dewx	51.3devw
Sydowia sp.	38.0hwx	33.0iy	32.5fy	31.5gy	33.8gxy	39.3ghw	32.5hy	25.3iz	31.5hy	25.5gz	41.8ghw	34.8gxy
Ulocladium sp.	47.8devwx	42.8fgy	47.0bwx	45.3dxy	39.0fz	50.5dev	54.8efu	46.3fgwx	46.0dewxy	48.8cdvw	56.5abu	47.3fvwx
Control	5.8ixy	4.5jyz	3.5gyz	2.3hz	3.0hz	7.5jx	6.3ixy	4.8jyz	5.3iy	4.5hyz	6.0jxy	3.8iyz

*In each column, means with the same letters do not differ significantly according to Fisher's LSD test at $p < 0.05$

**Means within a row not followed by the same letter are significantly different from each other ($p < 0.05$)

Full names of fungi are given in Table 2

on seeds at harvesting as capsules often fall on the ground of seed orchards. Thus, the impact of superficial contamination on seed germination and subsequent seedling damage in nurseries at a later stage is not to be underestimated.

In -vitro assays showed that inoculum of seed-borne *A. alternata*, *B. dothidea*, *C. globosum*, *C. brachyspora*, *P. curvatum*, *D. eucalypti*, *L. theobromae*, *N. sphaerica* and *P. africana* did not only reduce seed germination percentages but were also pathogenic on detached leaves of *Eucalyptus.* Although the leaf detached assay is a fast means of evaluating pathogenicity and severity of fungi, *in -vitro* detached leaves and plantlets are more susceptible than intact leaves of plants in the greenhouse or field (Townley et al. 2001; Liu et al. 2007).

In conclusion, findings of this study showed a large diversity of fungi associated with commercial *Eucalyptus* seed lots, some of which were pathogenic in a detached *Eucalyptus* leaf assay, and many reduced seed germination of *Eucalyptus* seed lots. The importance of the seed health and testing of *Eucalyptus* seed lots has been highlighted.

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Compliance with ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of interest Authors declare that they do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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