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Identification and control of *Colletotrichum* species associated with *Eucalyptus* seeds

by

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DECLARATION

I, the undersigned, declare that the thesis, which I hereby submit for the degree Doctor of Philosophy in Plant Pathology at the University of Pretoria is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Edgar Mangwende

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DEDICATION

To my parents, Peter and Christinah Mangwende.

EPIGRAPH

He who sells seeds of an inferior quality for sowing in the farms (telling that it is of good quality) deserves to be punished through physical torture i.e chopping off hand, foot, ear, nose, etc. Manu (2nd Century BC).

Identification and control of *Colletotrichum* species associated with *Eucalyptus* seeds

SUMMARY

The South African forestry industry largely relies on seed for its replanting programs following clear-felling. The high cost of *Eucalyptus* seed and planting targets makes it imperative to start with pathogen-free seeds to avoid inconsistences of emergence and development of seedlings. Together with 34 other fungal species, two *Colletotrichum* species in the *Colletotrichum gloeosporioides* species complex were found to be naturally associated with 14 commercial *Eucalyptus* seed lots. *Eucalyptus nitens* seed lot had the highest incidence of fungi (92.4%), whereas the lowest incidence occurred on *E. dorrigoensis* seed lot (28.9%). Seed germination of seed lots inoculated with seed-borne fungi was less than 62% and as low as 25%. Multiloci phylogenetic analyses of ITS, *β*-tubulin, actin, and glyceraldehyde-3-phosphate dehydrogenase gene regions identified *Colletotrichum* isolates PPRI 24314 as *C. fructicola* Prihastuti. and PPRI 24315 as *C. kahawae* Waller and Bridge. Biochemical tests based on utilisation of ammonium tartrate confirmed the latter isolate as *C. kahawae* subsp. *cigarro*. *Colletotrichum fructicola* and *C. kahawae* subsp. *cigarro* were naturally associated with seed lots of *E. dunnii* (3.5 and 0.5%, respectively), *E. nitens* (2.6 and 1.2%, respectively) and *E. macarthurii* (0.8% for only *C. fructicola*). Greenhouse pathogenicity tests showed that both *C. fructicola* and *C. kahawae* subsp. *cigarro* caused the highest incidence of anthracnose leaf spot on *E. nitens* (65 and 55%, respectively), with a severity of 67 and 63%, respectively. Sowing seeds inoculated with *C. fructicola* demonstrated the pathogen to be vertically transmitted into *E. camaldulensis*, *E. dunnii* and *E. nitens* seedlings (25.5, 38.3 and 64.0%, respectively), whereas *C. kahawae* subsp. *cigarro* inoculated seeds transmitted the pathogen in 27.3, 30.5 and 56.3% of the seedlings, respectively. Since both pathogens were seed-borne and seed-transmitted, non-chemical seed treatments that included *Bacillus* and *Trichoderma*, hot-water, microwave radiation and hydrogen peroxide were evaluated for their efficacy at sanitising seed lots of *E.*

nitens and *E. viminalis* artificially inoculated with either *C. fructicola* or *C. kahawae* subsp. *cigarro*. Soaking *Eucalyptus* spp. seed lots in 15% H_2O_2 for 10 min effectively reduced incidences of *Colletotrichum* spp. but negatively reduced seed germination. Instead, soaking *Eucalyptus* spp. seeds in 10% H_2O_2 for 5 min and 10 min were optimal treatment/time combinations with an acceptable reduction of *Colletotrichum* spp. and significantly high seed germination percentages. Hot water optimum treatment/time parameters were 50 °C for 30 min against *C. kahawae* subsp. *cigarro*, and 55 °C for 15 min against *C. fructicola*. Exposure of moist *Eucalyptus* spp. seeds to microwave radiation at powers levels of 1 400 w for 30 s significantly improved seed germination similar to that of the Celest® XL treatment. However, sowing seeds treated with H_2O_2, hot water and microwave radiation failed to control anthracnose leaf spot developing on seedlings grown under greenhouse conditions. Seeds treated with a *Bacillus* biocontrol agent consistently reduced incidences of *Colletotrichum* spp. on *Eucalyptus* spp. and effectively suppressed appearance of anthracnose leaf spot on seedlings grown under greenhouse conditions. Due to the high efficacy of Celest® XL and *Bacillus in-vitro* and *in-vivo*, they can be recommended as sanitisers of commercial *Eucalyptus* seed lots against anthracnose leaf spot.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

BCA= biological control agent

cm= centimetre

CV= Coefficient of Variation

DAS= days after sowing

^{o}C = degree Celsius

DNA= Deoxyribonucleic acid

h= hour

ha= hectare

ISTA= International Seed Testing Association

ISR= induced systemic resistance

ITS= Internal transcribed spacer

L= litre

LSD= Least Significant Difference

m= metre

µm= micrometre

mg= milligram

min= minute

mm = millimetre

mM= millimolar

%= percentage

PCR= Polymerase chain reaction

PDA= Potato dextrose agar

rDNA= ribosomal deoxyribonucleic acid

®= Registered

RFLP= Restriction fragment length polymorphism

s= second

SAS= Statistical Analysis Software

™= Trademark

w= watt

CHAPTER 1

1.1 Introduction

Eucalyptus L'He´r, family Myrtaceae, is one of the most planted hardwood tree genera in the world (Nicolle and Jones 2013). In South Africa, *Eucalyptus* is extensively planted as an exotic over approximately 450 000 ha, which is 42% of the total forestry plantation area (Forestry South Africa (FSA) 2018). The most widely grown species include *Eucalyptus dunnii* Maiden, *E. grandis* W. Hill, *E. nitens* (H. Deane & Maiden) Maiden, *E. saligna* Sm. and *E. urophylla* S.T. Blake, and several hybrids of these species particularly of *grandis* x *urophylla* (FSA 2018). In the 2016/17 production period, exports of forest products amounted to US$1.7bn, of which 75% of pulpwood sales were derived from *Eucalyptus* (FSA 2018).

Commercial *Eucalyptus* plantations are intensively managed under short rotation cycles of six to ten years (FSA 2018). Hence, it is essential to regenerate clear-felled plantation areas for sustainable forestry productivity. Despite advances in technologies of clonal vegetative propagation, seeds remain the most commonly used reproductive material for plantation forest regeneration, not only in South Africa but internationally too (Griffin 2014; Cleary et al. 2019). Due to scarcities of water and arable land, forestry plantations are expanding to marginal areas characterised with semi-arid and/or frost conditions, where trees grown from seeds are better adaptable than clones (Dye 2013; Steane et al. 2017; Whyte et al. 2016; Barradas et al. 2018).

Seeds for restocking large-scale commercial forests are collected from seed orchards across the country or even imported from other forestry regions of the world (Seedling Growers Association of South Africa (SGASA) 2018). However, the trade of forestry propagative material is associated with an inevitable risk of spread of pathogens (Hurley et al. 2016). Forestry pathogens such as *Botryosphaeria*, *Mycosphaerella*, and *Teratosphaeria* spp. have been found associated with seed lots of *Eucalyptus* and other tree species (U'ren et al. 2009;

Jimu et al. 2016; Tobias et al. 2017). Viljoen et al. (1992) reported *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. as a serious pathogen causing seedling blight on *Eucalyptus* cuttings and seedlings in nurseries.

The genus *Colletotrichum* is known to cause diseases on a wide range of hosts, where *Eucalyptus* plants grown both in nurseries and plantation fields are reported to be susceptible (Zakaria and Bailey 2000; Zwolinski and Bayley 2001). The genus of *C. gloeosporioides* has been shown to be a cryptic species of one of the 22 closely similar members of the *C. gloeosporioides* species complex. *Colletotrichum theobromicola* Delacr. is an example of a closely related species, and has been reported causing stem girdling and death of rooted *Eucalyptus* mini-cuttings in nurseries (Rodrigues et al. 2014). The accurate identification of species in this genus is not only important for understanding epidemiology but also to improve biosecurity for effective disease management (Cannon et al. 2012; De Silva et al. 2017).

Although several governments have taken concerted actions of implementing strict quarantine restrictions on traded plants and agricultural products, new pests and diseases continue being reported in forest plantations (Ikegami et al. 2018; Sikes et al. 2018; Meurisse et al. 2019). Consequently, testing health status of traded propagative material has become a prerequisite (Liebhold and Wingfield 2014; Wingfield et al. 2015). As seeds are often contaminated by seed-borne pathogens, application of seed treatments warrantees the supply of disease-free forestry genetic material (Evira-Recuenco et al. 2015; Nelson 2018).

From the late 1960s, synthetic chemicals such as Carboxin, Chlorothalonil, Captan, Thiram and Triforne were main means of controlling seed-borne pathogens (Brown and Ferreira 2000). Nonetheless, the Forestry Stewardship Commission (FSC), an international non-governmental organisation promoting responsible management of the world's forests, discourages further use

of synthetic chemicals due to their hazardous effects to non-target pests and the environment (Lemes et al. 2017; Skrzecz and Perlińska 2018).

Planting resistant genotypes would be the most effective alternative strategy to manage anthracnose leaf spot (Arentz 1991). However, resistance observed on *Eucalyptus deglupta* Blume is not yet incorporated in most commercially grown species and hybrids. Hence, the interest in non-chemical seed treatments. Although the subject of non-chemical disease management is now like a cliché in vegetable and field crops, most of the methods have not been explored sufficiently in sanitising forestry seeds. Hydrogen peroxide (H_2O_2), a natural chemical compound with low mammal toxicity that slowly decomposes in the presence of light (Liao et al. 2003), is a potential alternative. Few studies have reported effectiveness of H_2O_2 in sanitising seeds against a wide range of seed-borne mycoflora (Dashtban et al. 2010; Veal and Day 2011; Nandi et al. 2017). However, seed germination is reduced at higher concentrations of H_2O_2 (Szopińska 2014). Donald and Lundquist (1988) found that seed-borne fungi were effectively controlled when *Eucalyptus* seeds were soaked in a 33% H_2O_2 solution, but this study did not take its effect on seed germination into consideration.

Soaking seeds in hot water baths is a classical method that was mainly used to sanitise barley and wheat seeds infected with the loose smut pathogen, *Ustilago nuda* (C.N. Jensen) Kellerm. & Swingle (Sharma et al. 2017). Due to its effectiveness, usage of hot water seed treatment now includes several pathogens associated with seed lots of many hosts other than cereals (Alam et al. 2014; Berbegal et al. 2015; Koudela and Novotný 2016; Mandiriza et al. 2018). Even though hot water seed treatments at 50 ^{o}C for 5, 10 or 20 min was previously mentioned to be effective against seed-borne fungi associated with *Eucalyptus* seed lots (Brown and Ferreira 2000), this is a generalisation for locally produced landraces and provenances. Seeds of different plants exhibit varying thermal tolerances due to differences in biochemical

compositions, level of maturity of seeds, and various physical conditions of external layers of the seed (Forsberg 2004). Hence, the need for research to investigate the treatment time/temperature regimes suitable for an effective control of *Colletotrichum* spp. without affecting *Eucalyptus* seed germination.

The challenge with hot water seed treatment is that its efficacy is limited to a few internal layers of seed coat (Forsberg 2004). This drawback makes microwave radiation an attractive option due to its mode of action, which can effectively control fungi in deeply embedded tissues of seeds (Friesen 2014). However, excessive irradiation negatively affects physiology of seed (Grondeau et al. 1994; Han 2010). Hence, further studies are required to establish a range of microwave radiation treatments required to control *Colletotrichum* spp. associated with different *Eucalyptus* spp., without overheating and killing the seed.

The use of biocontrol microorganisms as seed dressing agents have been widely accepted as a viable alternative or complementary method to synthetic chemical treatment, with the relative ease of registration with Environment Protection Agencies and Organisations (Sharma et al. 2015). *Bacillus* and *Trichoderma* species are among the most used biological control agents (BCA) globally (Velivelli et al. 2014; Waghunde et al. 2016; Gupta and Vakhlu 2018; De Silva et al. 2019). Although several studies have reported efficacy of these BCAs against various seed-borne fungi in a wide variety of crops, their performance has not been evaluated as seed treatments of *Eucalyptus* seed lots.

In conclusion, the poor emergence in most forest nurseries supports investigations on seed quality and health of *Eucalyptus* seeds. In this study, seed-borne mycoflora associated with locally produced *Eucalyptus* seed lots were identified and seeds were sanitised with non-chemical control measures that include microwave irradiation, biocontrol agents, soaking seeds in hot water and hydrogen peroxide to control *Colletotrichum* spp.

1.2 Aims of the study

The use of inferior and diseased seed is costly both to the seed trader and nursery manager as this translates to considerable reduction of seedling emergence which in turn results in replanting. Hence, the aim of this study was to investigate the health status of locally produced *Eucalyptus* spp. seed lots and evaluate sustainable non-chemical means of sanitising infected and/or infested seed.

1.3 Hypothesis tested

- There are no seed-borne pathogens associated with locally produced *Eucalyptus* spp. seed lots.
- Seed-borne mycoflora associated with *Eucalyptus* spp. seed lots will not have any effect on seed germination.
- Seed treatments with biocontrol agents, or soaking in hot water and hydrogen peroxide, and exposure in microwave irradiation, will not control *Colletotrichum fructicola* or *C. kahawae* subsp. *cigarro*. associated with *Eucalyptus* spp. seed lots.

1.4 Thesis structure

Chapter 2 of the thesis reviews literature of seed-borne mycoflora associated with *Eucalyptus* seed lots. It describes how *Eucalyptus* seed serve as primary source of inoculum of anthracnose leaf spot in nurseries and twig die-back in forest plantations. Since previous surveys reported *Colletotrichum gloeosporiodes* as a common pathogen, limitations of morphological characters in identifying these previously reported species are highlighted. The challenges of traditional identification of pathogens are highlighted and means to resolve the *Colletotrichum gloeosporiodes* species complex using molecular methods are incorporated in the discussion. Limitations of current biosecurity measures in detecting seed-borne *Colletotrichum fructicola*

and *C. kahawae* subsp. *cigarro* and ways of reducing inoculum loads on infected and/or infested seedlots are discussed. As forest operations are now complying with environmentally friendly regulations of the FSC, potential non-chemical methods, including hot water, microwave radiation, hydrogen peroxide and biological agents, *Bacillus subtilis* (Ehrenberg) Cohn and *Trichoderma harzianum* Rifai to be used against seed-borne and seed-transmitted *Colletotrichum* spp. on *Eucalyptus* seed lots, are reviewed.

Chapter 3 presents outcomes of seed health and germination tests performed on commercial *Eucalyptus* spp. seed lots produced in South Africa. The objective of this Chapter was to evaluate the effects of various seed-borne mycoflora on seed germination and assess their pathogenicity on *Eucalyptus* spp. This work has been submitted to the journal, New Forests.

Chapter 4 elucidates the identity of different *Colletotrichum* spp. associated with *Eucalyptus* spp. seed lots. Although anthracnose leaf spot and twig dieback are commonly recurring diseases in *Eucalyptus* plantations, there has been a lot of taxonomic revision that necessitated investigations to identify and confirm currently assigned names of the causal agents. *Eucalyptus* seed was screened for the presence of *C. fructicola* and *C. kahawae* subsp. *cigarro*, and their effects on seed germination and pathogenicity investigated. This work has been submitted to the journal, Forestry.

Chapter 5 describes assessments of efficacy of non-chemical methods that included the BCAs *Bacillus* and *Trichoderma*, hot-water, microwave radiation and hydrogen peroxide at sanitising artificially inoculated *E. nitens* and *E. viminalis* seed lots against *C. kahawae* subsp. *cigarro*. This work has been submitted to the journal, Crop Protection.

Chapter 6 details *in-vitro* and greenhouse evaluations of non-chemical seed treatments *viz*. hot water, microwave radiation, *Bacillus* and *Trichoderma* BCAs and hydrogen peroxide against

anthracnose leaf spot caused by *C. fructicola* on *E. nitens* and *E. viminalis*. This work has been submitted to the journal, Forestry.

Chapter 7 highlights overall findings of this thesis on *Eucalyptus* seed pathology research. Non-chemical control methods that effectively sanitised artificially inoculated seeds are presented and recommendations are made to the forestry seed and nursery industry based on performance of the seed treatments against *C. fructicola* and/or *C. kahawae* subsp. *cigarro*.

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CHAPTER 2

Literature review

2.1 Introduction

Early *Eucalyptus* plantings in Africa were introduced as a source of fuel wood, windbreaks and sawlogs (Turnbull 2000). Over the years, *Eucalyptus* was commercialised and extensively planted to meet the global demand of pulp and paper (Pallett and Sale 2004; Raj et al. 2014). In South Africa, the forestry industry largely relies on *Eucalyptus* together with pine and wattle, which all have a significant contribution to the country's economy (Forestry South Africa (FSA) 2018). In the 2010/2016 production period, South African forest industry contributed about 4% to GDP, where close to 70% of pulpwood sales were derived from *Eucalyptus* with an estimated total of 9 587 262 tons (FSA 2018). Since *Eucalyptus* plantations are intensively managed under shorter rotation cycles of six to ten years (Derikvand et al. 2019), vast tree stands are clear-felled every year and must be replanted to ensure a continued supply of forest products. Forest plantations may be regenerated from cuttings or allowed to coppice, but majority of the trees grown from seedlings (Southern African Tree Seed Working Group 2012).

Due to the relative ease and affordability of regenerating trees from seed, there is a high demand of quality seeds by many foresters across the globe (Griffin 2014). Naturally, *Eucalyptus* seeds have high germination capacity, but poor seedling emergence, and losses of up to 40% continue to be experienced in nurseries (Boland et al. 1980; Mangwende et al. 2018). Apart from physiological abnormalities influenced by genetics, research has shown that *Eucalyptus* seed often comes with a wide variety of contaminants such as seed-borne mycoflora that may threaten seedling health and productivity in forest plantations (Yuan et al. 1997; Mehrotra and Singh 1998; Wingfield et al. 2001; Jimu et al. 2015). Apart from reducing seedling stands in nurseries, infected *Eucalyptus* seed risks spread of diseases to previously non-diseased areas

(Elmer 2001; Brasier 2008; Aukema et al. 2010). Together with several other fungi, *Eucalyptus* seeds have been shown to be infected with *Botryosphaeria*, *Mycosphaerella*, and *Teratosphaeria*, which are often reported to cause diseases in forest plantations (Slippers and Wingfield 2007; Andjic et al. 2011; Liebhold et al. 2012).

Together with cuttings and seedlings, *Eucalyptus* seed risks movement of invasive alien pathogens around the world. However, the trade of forestry seed is likely to increase as commercial forest companies and various research institutes strengthen partnerships in tree improvement research. Hence, the importance of routine seed health tests prior to trading infected and/or infested seeds as different governments passed strict phytosanitary controls in trade of agricultural goods and services (Wingfield et al. 2015; Burgess and Wingfield 2016; McTaggart et al. 2016). Thus, the next section highlights *Eucalyptus* seed anatomy to elucidate how pathogens might be carried.

2.2 *Eucalyptus* seed as a source of inoculum

Seeds carry a wide diversity of seed-borne mycoflora, which may survive throughout different seasons on seed surfaces or inside seeds (Agarwal and Sinclair 1987). Few of the microorganisms may help improve plant performance yet others are a threat to seed health (Barret et al. 2015). Details of the location of seed-borne fungi and the respective physiochemical properties of the seed are crucial as they determine the difficultness of detecting the pathogen and influence the choice of seed treatment selected to effectively sanitise infected and/or infested seed lots.

Depending on the course of penetration and the availability of soft tissue, infection may either be limited to the external surfaces of the seed coat or transverse deep to affect seed components lying below the epidermal layer (Agarwal and Sinclair 1987). During early to mid-stages of seed development, the seed coat acts as a maternal conduit to convey nutrients for the

developing embryo (Debeaujon et al. 2007). Vascular infecting fungi such as *Fusarium*, *Mycosphaerella* and *Alternaria* often invade through the funiculus into the integument of developing seed coat or raphe, if present (Singh and Mathur 2004). At later stages of seed primordium, morphological barriers established within the seed may hinder penetration of hyphae into deeper layers. Hence, if invasion happens when the seed is nearing full maturity, infection will only be limited to external surfaces of the seed, thus localised around the seedcoat and few of the epidermal layers (Agarwal and Sinclair 1987).

2.2.1 Seed coat

A mature, healthy, seed coat forms a protective covering enveloping the embryo (Figure 2.1). Histological studies suggest that most fungi penetrate epidermal layers of seed coats by mechanical means or with the aid of secreted of enzymes, or both (Agarwal and Sinclair 1987; Singh and Mathur 2004). For example, *Colletotrichum gloeosporiodes* (Penz.) Penz. and Sacc, a fungal pathogen reported to cause capsule disease on *Eucalyptus camaldulensis* Dehnh., may directly penetrate the thin epidermal layer by enzymatic hydrolysis. Secretion of cutinases, amylases, cellulases, lipases, nucleases and proteases by the fungus during surface penetration will hydrolyse and weaken epidermal layers of the seed coat. Morphologically, large epidermal cells are more likely to be heavily colonised by sporulating mycelium. Penetration may occur through any part of the epidermis, sometimes between the vertical walls of the epidermal cells, often through the center of the cell wall (Agarwal and Sinclair 1987). At point of entry, an appresorium is formed from which vigorous pointed hyphae penetrate and infect the host (Kunwar et al. 1985).

In the last stages of seed maturation, the lumina of the epidermal cells, the parenchyma and chalaza cork tissues are increasingly impregnated with pigments such as cutin, lignin and suberin that are filled as amorphous deposits (Moïse et al. 2005). Apart from giving mature

seeds more mechanical resistance, low-molecular weight fragments of lignin such as p-hydroxycinnamyl alcohols, phenol and ether derivatives, possessing varying antimicrobial properties, are increasingly secreted preventing biological degradation (Telysheva et al. 1968; Zemek et al. 1979). The excessively centripetally thickened walls are lignified in *Eucalyptus triantha* Link, *Eucalyptus laevopinea* R.T. Baker, Proc. Linn. Soc, *Eucalyptus* F. Muell. ex Benth (Gauba and Pryor 1958). However, no phloroglucin reaction was obtained in *Eucalyptus haemastoma* Sm., indicating the absence of lignin and a comparatively weaker barrier, where fungi may display increased activity and penetrate the seed coat without much difficulty (Moïse et al. 2005).

Below the epidermal layer is the outer and inner integuments of the seed coat. The outer integument is composed of dead and hard tissues that thicken to form inner tangential and radial walls composed of sclereids (Vaughan and Whitehouse 1971; Bouman 1975). Because of presence of a thicker wall of sclereids cells, the outer integument that acts as a mechanical barrier making it difficult for fungi to penetrate (Agarwal and Sinclair 1987). However, the integrity of closely packed epidermal sclereids may be broken by mechanical damage, usually incurred during processing operations such as artificial drying before sorting and storage of seed. This may cut open the thin epidermal layer, exposing few cells of the subepidermal layer, with the parenchyma layer usually remaining intact. *Colletotrichum gloeosporiodes* was shown to penetrate seed coat of flax seed, comprised of palisade and sclerenchyma layers, within 24 to 30 h (Makowski and Mortensen 1998), and is expected to be more pronounced in *Eucalyptus planchoniana* F. Muell. Fragm., *E. laevopinea* R.T. Baker and others with a relatively small outer epidermal layer.

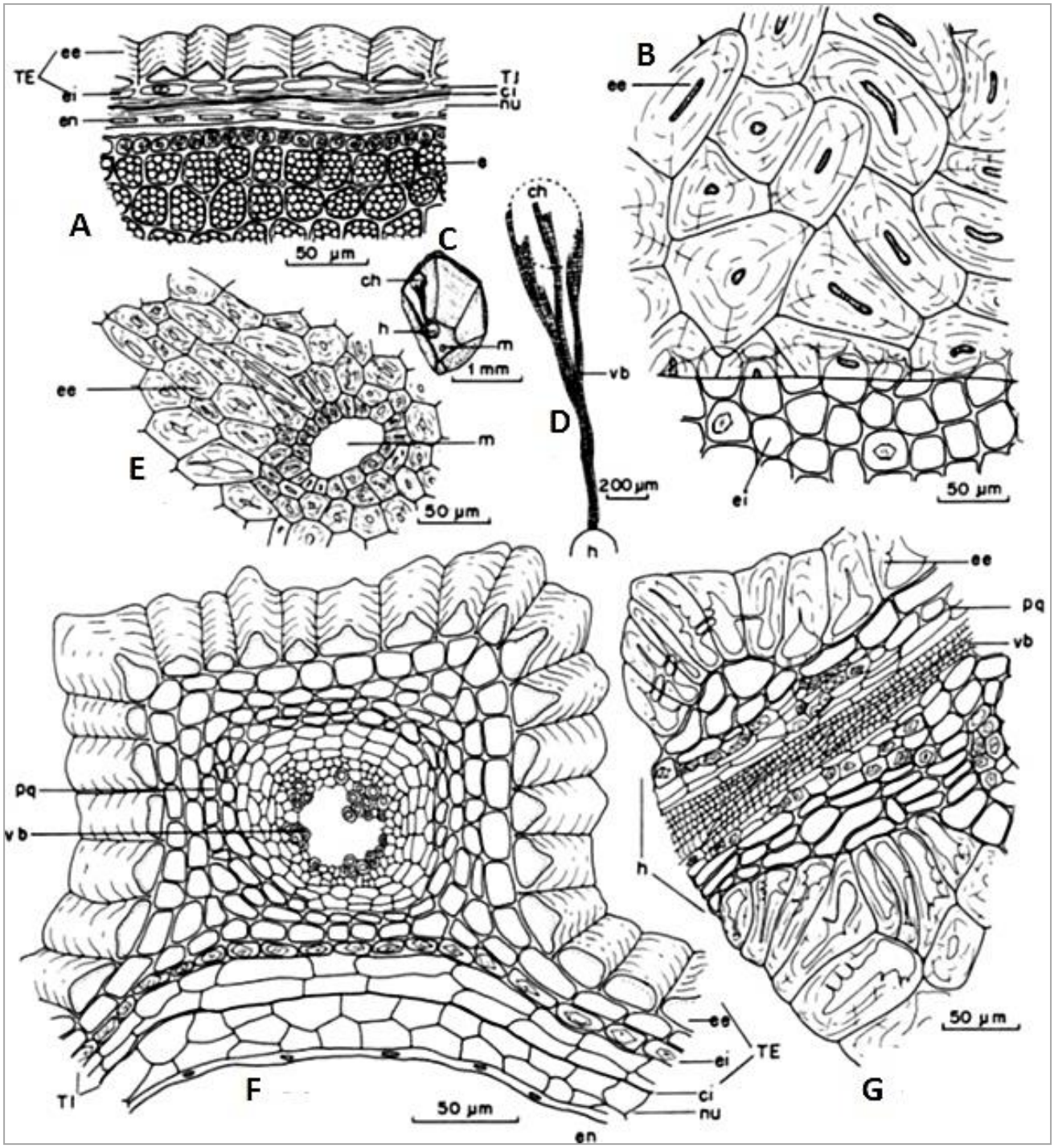

Figure 2.1: Generalised morphology and anatomy of the *Eucalyptus* seed coat. Section A and B- are cross and paradermal sections of the fertile seed coat, respectively; C-Seed diagram showing hilum, chalaza and micropyle positions; D-Vascularisation pattern; E- paradermal section at the micropylar region. F-raphe transversal section; G-longitudinal section through the hilum region. (Abbreviations: vb, vascular bundles; TI, inner integument; en, endosperm; m, micropylar gap; ch, chalaza; nu, nucellar tissue, h, hilum; gm, ground meristem; tp, parenchyma; TE, testa; ee, exotesta; ei, inner epidermis; rc, root cap; hy, hypocotyl; rm, root meristem; co, cupuliform organ; cot, cotyledons; r, long raphe; nu, nucellar tissue; pr, procambial cells; gl, oil glands; sc, seed coat; p, epicotyl) (Beltrati 1981).

Fungi may also gain easy entry through the edges and ribs of seeds. These constitutes of relatively isodiametrical epidermal cells elongated parallel to the surface making them easy to break such as observed on seeds of *Eucalyptus oreades* F. Muell. ex R.T. Baker, *Eucalyptus kybeanensis* Maiden & Cambage, *Eucalyptus stellulata* Sieber ex D.C. and *Eucalyptus piperita* Sm. (Boland et al. 1980). Some of the breaks in seed coat are genetically controlled (Boland et al. 1980). For example, cracking of the seed coat is a known natural phenomenon in the series Eximiae (*Eucalyptus eximia* Schauer, *Eucalyptus watsoniana* F.Muell.), Torellianae (*Eucalyptus torelliana* F. Muell.) and Maculatae (*Eucalyptus citriodora* Hook.) (Boland et al. 1980; Carr and Carr 1962).

The inner integument is immediately below the outer integument. The suberized inner integument is a dead tissue due to excretion of oxalate crystals and phlobaphenes into the outer integument that excludes its cells from metabolic activities (Gauba and Pryor 1958). Normally after 48 hr, fungal hyphae that would have colonised the palisade layer, transverses to the integuments due to chemotactic action originating from the young embryo (Schopfer et al. 2001; Nelson 1991). The fact that the inner integument is under programmed cell death (Wan et al. 2002; Lijuan et al. 2003), as evidenced by accumulation of peptidases, carbohydrates and nucleases in the proximal region, may enhance the easiness of hyphae to invade dismantled components of this organelle (Gauba and Pryor 1958; Rudall 2007).

The integuments do not enclose the nucellus completely but retain an opening at the apex referred to as the micropyle (Singh and Mathur 2004). Fungal inoculum may penetrate through an open micropyle, invades and colonise readily in spaces between the components of the ovule and developing seed (Agarwal and Sinclair 1987). For example, Kulik and Yaklich (1991) found that soybean [*Glycine max* (L.) Merr.] seeds with open micropyle had higher incidence of infection by *Phomopsis* sp. compared with closed micropyle seeds.

2.2.2 Hilum

The vascular connection between mother plant and seed is severed in the last stages of seed maturation. Abscission of the seed from the funiculus makes it autonomous, with a well distinct scar left on the surface where a seed was previously attached to the mother plant (Agarwal and Sinclair 1987). The area around the abscission scar is normally characterised by an uneven distribution of parenchyma cells unlike the rest of the seedcoat. Although raised rims of sclereids surrounds the hilum, the broad surface lacks the usual protective cuticle layer, which is neither cutinised nor suberized leaving the cavity below the scar defenceless for fungal invasion (Agarwal and Sinclair 1987; Gauba and Pryor 1958). Sometimes pycnida may develop profusely on the seed coat around the hilum. Further colonisation by fungal mycelia beyond the air-filled hollow of the hilum is often interrupted as the immediate vascular system is aligned with helically thickened tracheids that extends in the expanded raphe parenchyma right up to the chalaza.

2.2.3 The embryo

Eucalyptus produces exalbuminous seed, i.e. without an endosperm, and food reserves for germination are stored in the large embryo (Boland et al. 2006). The embryo is boarded by a thin cuticle, semisolid envelope, which envelops the cotyledons and hypocotyl (Figure 2.2). In addition, the remaining cavity is filled with food storage substances consisting of fat, in the form of oil droplets and aleurone protein.

Studies have shown that, the relative size of the embryo in relation to the seed coat is an essential feature that may affect location, infection and transmission of a given race or strain of a pathogen within an embryo (Agarwal and Sinclair 1987). Due to its small embryo size, *Eucalyptus* seed infected during the early stages of capsule development often results in seed abortion. For example, *Ramularia pitereka* J. Walker & Bertus. [Current name: *Quambalaria*

pitereka (J. Walker & Bertus) J.A. Simpson] a common pathogen transmitted by wasps [*Megastigmus judikingae* Doganlar & Hassan, *Megastigmus zwimendeli* Doganlar & Hassan (Hymenoptera: Torymidae)] is known to enter systemically through the vascular system (Drake 1974). In such cases of infection of the embryo, physiological activities of the developing seed are interrupted with an eventual pre-mature death and significant reduction of potential yield of seed orchards.

Ramularia sp. has been reported to cause seed losses on different *Eucalyptus* species, where loses of 2.3% was recorded on *Eucalyptus crebra* F. Muell., 5.3% on *Eucalyptus populnea* F. Muell., 30% on *Eucalyptus melanophloia* F. Muell. and 34% on a hybrid between *E. populnea* x *E. crebra* in Australia (Drake 1974; Drake 1981). Infection of embryos at the sub-cellular level includes the broadening of plasmodesmata and depletion of plasma membrane, the degradation of cytoplasm and cell organelles, and the deformation of protein bodies, lipid bodies, and nuclear membrane (Agarwal and Sinclair 1987; Singh and Mathur 2004).

2.3 Significance of seed-borne mycoflora associated with *Eucalyptus* seed

The quality of planted seeds may influence the ability of seedlings to be established to realize their full potential of yield (Eldridge et al. 1994). Generally, forestry seeds are naturally infected or infested with a small proportion of seed-borne mycoflora (reference). Due to its tiny seed size, it is difficult to separate infected and/or infested seed lots by visual means (Boland et al. 1980). Furthermore, the fact that previous studies mainly isolated saprophytes or storage fungi such as *Aspergillus*, *Curvularia*, *Gliocladium*, *Penicillium* and *Pestalotiopsis* has led to a general neglect of the aspect of seed health by foresters (Yuan et al. 1990; Ennos 2014).

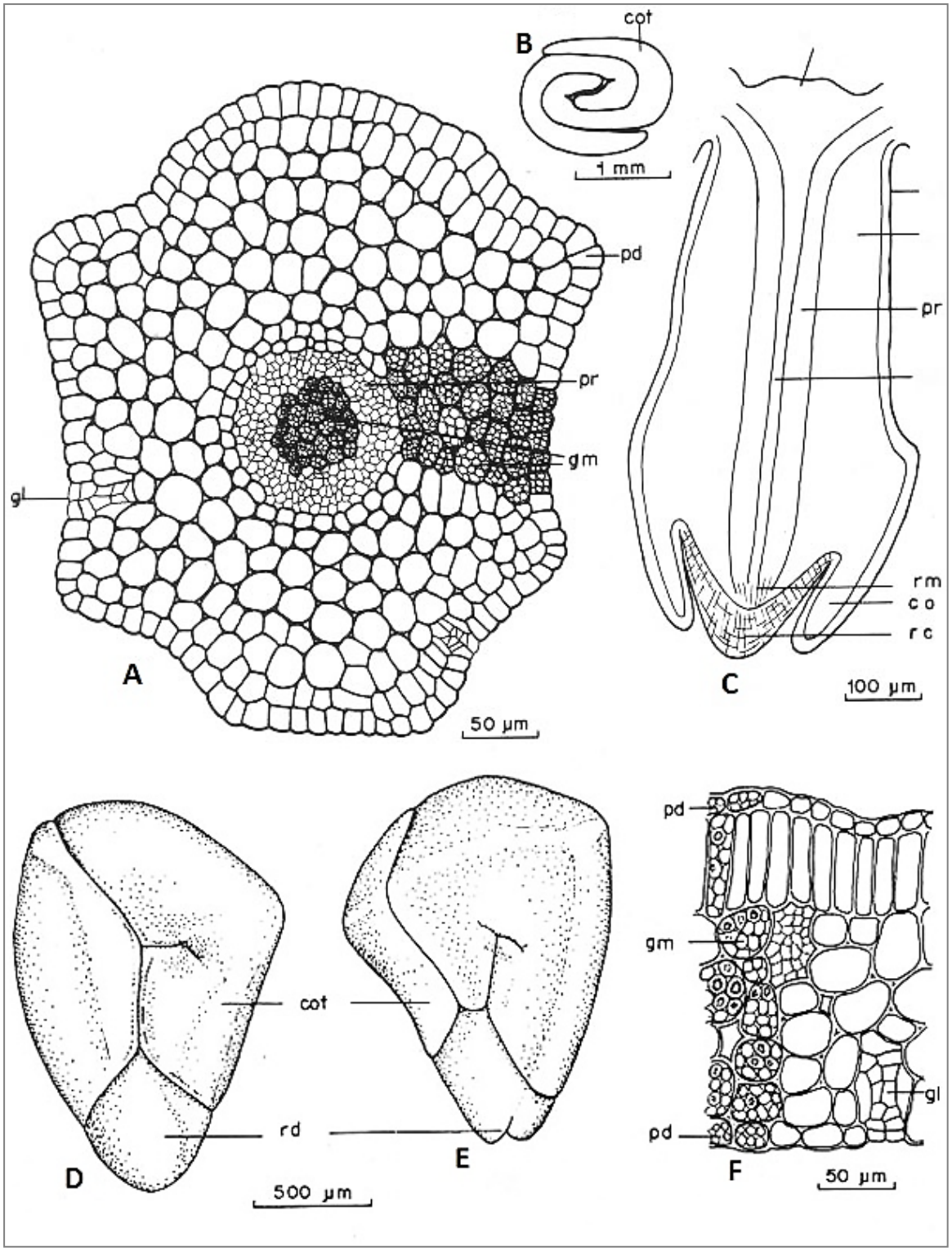

Figure 2.2: Middle cross section through the embryo of *Eucalyptus* seed (A), a cross section through the cotyledons (B), longitudinal section through the embryo axis (C), the surface view of the embryo (D), surface view of the embryo (E), cotyledon cross section (F). [Abbreviations: cot, cotyledons; co, cupuliform organ; gm, ground meristem; gl, oil glands; pr, procambial cells; pd, protoderm; rd, radicular primordium; rc, root cap; rm, root meristem] (Beltrati 1981).

Thus, seed health testing of forest seeds is an overlooked subject where potential effects of seed-borne fungi on seedling development are often underestimated. Consequently, infected and/or infested seeds are sown in nurseries. However, some of the saprophyte fungi infect and cause plant diseases under certain circumstances. Saprophytic seed-borne mycoflora associated with seed lots were previously shown to cause seeds to rot, or develop some discolorations and some form of necrotic infections that may reduce germination capacity (Mwanza and Kellas 1987; Mittal et al. 1990; Sutherland et al. 2002).

Seed-borne mycoflora may simply remain dormant on and/or in seed (Agarwal and Sinclair 1997). However, as seed is graded or passed to other processing operations, saprophytes and other fungi loosely attached on the surfaces of the seed may be spread further contaminating non-infested seed batches (Brown and Ferreira 2000; Sutherland et al. 2002). This may promote 'false' seed-transmission of diseases that are not of seed-borne nature such as *Phytophthora* and *Pythium* and *Verticillium* (Otten and Gilligan, 2006).

The close association of fungi with seeds facilitates widespread dissemination of such pathogens across vast distances and natural barriers (Wingfield et al. 2001; Jimu et al. 2015). For example, the pathogen that causes *Teratosphaeria* leaf disease, *Teratosphaeria nubilosa* (Cooke) Crous, first reported in Victoria, Australia, but is now widespread in Australia and several countries, including South Africa (Hunter et al. 2008; Hunter et al. 2009; Pérez et al. 2009; Andjic et al. 2016). Infected and/or infested propagative materials were reported to spread the pathogen to forest plantation areas (Old et al. 2003; Wingfield et al. 2001; Wingfield et al. 2008, Hunter et al. 2008; Andjic et al. 2011; Jimu et al. 2015).

Apart from introducing new diseases in forest plantations, infected and/or infested propagative material increases the likelihood of uniting already existing but previously geographically separated, mating types of pathogens (Neergaard 1969; Brasier 2008). At the population level,

introduction of different genotypes in any given forest plantation alters the genetic diversity of already existing fungal population (Desprez-Loustau et al. 2007). This increases chances of recombination and hybridisation between introduced and resident species resulting in interspecific gene flow that may either affect levels of pathogenicity or contribute to evolution of new hybrid species with novel host ranges (Groenewald et al. 2007; Brasier 2008; Wilken et al. 2012).

Seed health testing of forestry seed is a relatively new subject, and the number of pathogens being shortlisted and categorised as of quarantine nature are increasing yearly (Wingfield et al. 2015; Maier et al. 2016; Crous and Wingfield 2018). Since trade of forestry seed for research and commercial purposes is likely to continue in future, it is important that research efforts are concentrated on improving and optimising early and accurate pathogen detection (McTaggart et al. 2016; Guarnaccia et al. 2017). Although reports on seed-borne mycoflora associated with *Eucalyptus* appeared from time to time (Saxena 1985; Mittal et al. 1990; Yuan et al. 1990, 1997), little is known about seed-borne mycoflora associated with local provenances of *Eucalyptus* seed lots (Jimu et al. 2015). In this regard, information is scarce on the general distribution and extent of damage caused by seed-borne pathogens, and the level of infestation and/or infection that can be tolerated are not yet established making it difficult for a regulatory technical officer to advice whether to tolerate or reject an infected or infested seed batch.

2.4 *Colletotrichum* species associated with *Eucalyptus* seed

Together with many other fungi, the genus *Colletotrichum* has been reported associated with *Eucalyptus* seed (Table 2). The occurrence of mixed fungal taxa presents a complex challenge in elucidating collective effects and overall implication on *Eucalyptus* seed lots. Such studies on forest seeds are scarce, their interaction effects with respect to their pathogenicity, and transmission to seedlings remains unknown for most plants. Few studies of this nature were

conducted on vegetable seeds and cereals, where *Colletotrichum* co-occurring with a few other fungal species exhibited varying levels of aggressiveness when infecting and colonising seed tissues. For example, Kunwar et al. (1985) observed varying interaction effects of *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore, *Phomopsis* spp. and *Cercospora sojina* Hara naturally co-occurring on soybean seeds. In the presence of *Colletotrichum* sp., hyphae of *Phomopsis* spp. were shown to be restricted to the upper layers of the seed coat. Similarly, *C. sojina* hyphae co-occurring with *C. truncatum* restricts *C. sojina* in the seed coat layers. Given the limit on availability of literature, the discussion will exclusively narrow focus on seed-borne *Colletotrichum* species with respect to identification and management.

2.5 Systematics of *Colletotrichum*

2.5.1 Morphological taxonomy

Taxonomy of *Colletotrichum* has undergone several changes over the years, starting from the host-specific independence, through the morphology-based system to the currently used molecular system (Baxter et al. 1985; Cannon 2002). Initially described as *Vermicularia* by Tode in 1790, the genus was renamed to its current name *Colletotrichum* by Corda in 1831 (Sutton 1992; Hyde et al. 2009). As an anamorphic genus, taxonomy mainly relied on hyaline, straight or falcate conidia and setose acervuli isolated from susceptible hosts, where around 50 species had been described by 1900 (Damm et al. 2012). Due to ambiguity of taxonomic notes, taxons were described each time susceptible hosts were discovered (Mills 2001). Consequently, von Arx counted close to 750 *Colletotrichum* species by 1956 (Cannon et al. 2012). Several mycologists observed that many of the species described based on host preference were in fact the same, rejecting apparent differences in host preference as a basis for taxonomic segregation (Simmonds 1966; Sutton 1966, 1968; Smith and Black 1990; Walker et al. 1991).

Although few *Colletotrichum* species are known to produce a *Glomerella* teleomorph, existence of strictly homothallic or heterothallic strains due to compactible gene mutations within same *Colletotrichum* species makes production of ascospores unreliable (Perfect et al. 1999; Curry and Baird 2004). Moreover, optimum conditions for ascospores formation are extremely unpredictable (Vaillancourt et al. 2000). Hence, morphological classification of the genus is based on the asexual *Colletotrichum* state (Perfect et al. 1999). In 1957, von Arx carefully studied the genus sorely on morphological features with little or no emphasis on pathology reducing the number of described taxa to 11 accepted species (Bailey 1992). However, due to high similarity of morphological and cultural features, *Colletotrichum* species were broadly identified as fungi producing cylindrical conidia. This presented a huge risk of misidentification between closely related *Colletotrichum* species, most of which were inaccurately identified as *C. gloeosporioides* (Figure 2.3). For example, it is difficult to distinguish between any of *Colletotrichum fructicola* Prihast., L. Cai & K.D. Hyde (Figure 2.6), *Colletotrichum kahawae* Waller and Bridge (Figure 2.4 and 2.5), and *Colletotrichum theobromicola* Delacr. (Figure 2.7). For example, Sutton (1992) reported that average c*onidia* size *of C. gloeosporiodes* measured from 10.4 to 16.7 μm × 4.2 to 5.8 μm (n=100), and is likely to be confused with conidia of *C. fructicola* also measuring 15.2 to 15.6 × 4.7 to 4.9 μm (n = 45) (Prihastuti et al. 2009). In this regard, closely related species from potentially distinct taxa were lumped together.

These subtle differences in size and shape of conidial and appressoria and other overlapping features contributed to a slow increase in the number of newly described taxons (Hindorf 1970; Baxter et al. 1983; Gubler and Gunnell 1991; Gunnell and Gubler 1992; Johnston 2000; Gautam 2005). However, in 1990 a comprehensive taxonomic revision officially accepted 39 *Colletotrichum* species by integrating both morphological and cultural characteristics (Sutton 1992).

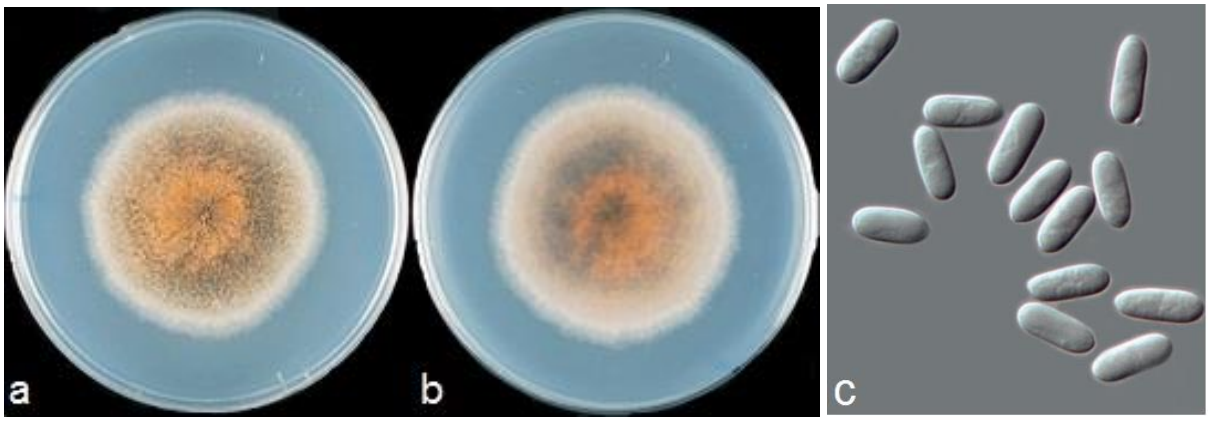

Figure 2.3: Ten day old cultures of *Colletotrichum gloeosporioides* (ICMP 17821) on PDA, from above (a) and below, and ascospores (c) (Weir et al. 2012).

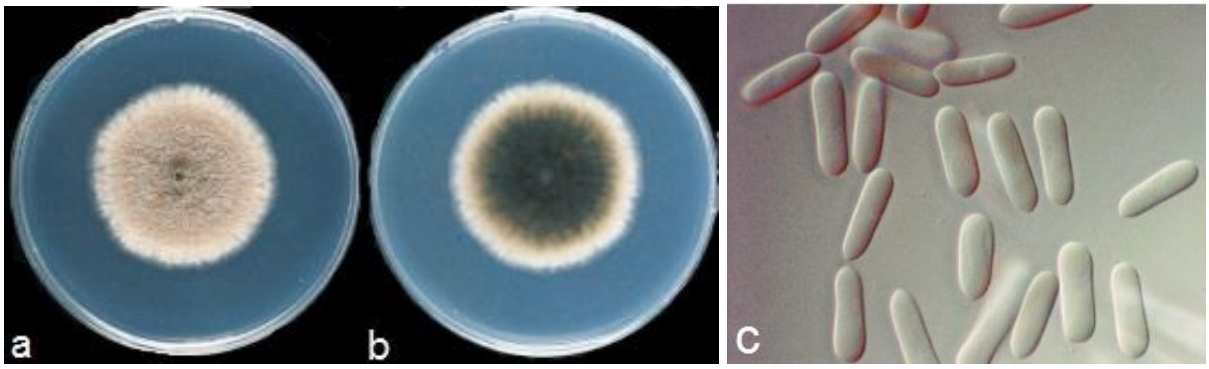

Figure 2.4: Ten day old cultures of *Colletotrichum* kahawae subsp. kahawae (ICMP 17905) on PDA, from above (a) and below, and ascospores (c) (Weir et al. 2012).

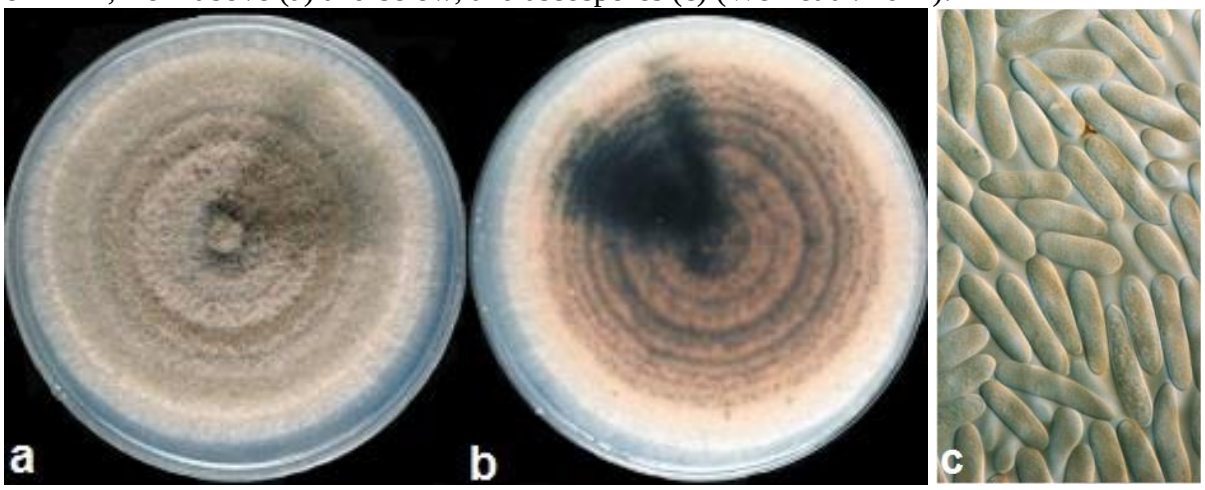

Figure 2.5: Ten day old cultures of *Colletotrichum kahawae subsp. cigarro* on PDA, from above (a) and below, and ascospores (c) (Weir et al. 2012).

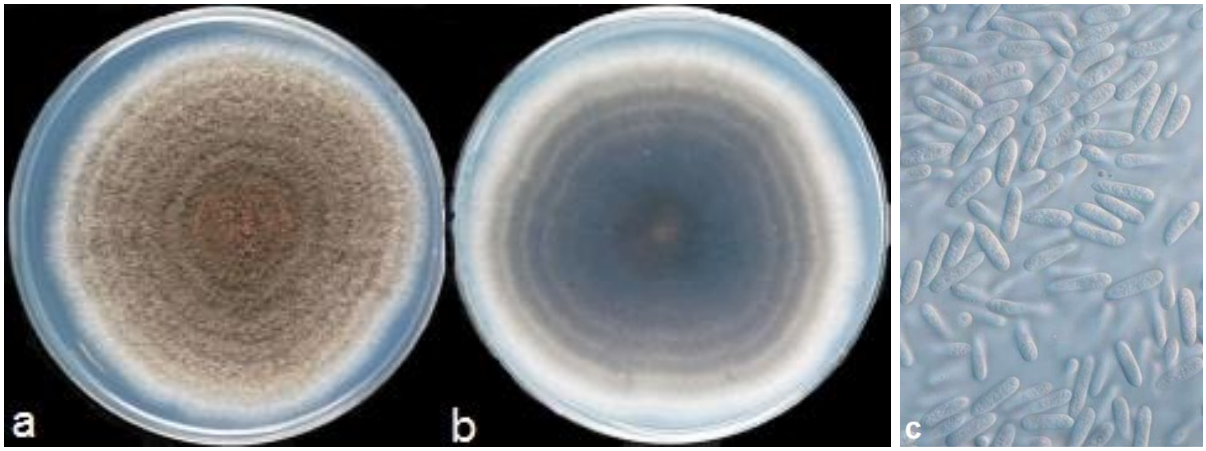

Figure 2.6: Ten day old cultures of *Colletotrichum fructicola* (ICMP 18615) on PDA, from above (a) and below, and ascospores (c) (Weir et al. 2012).

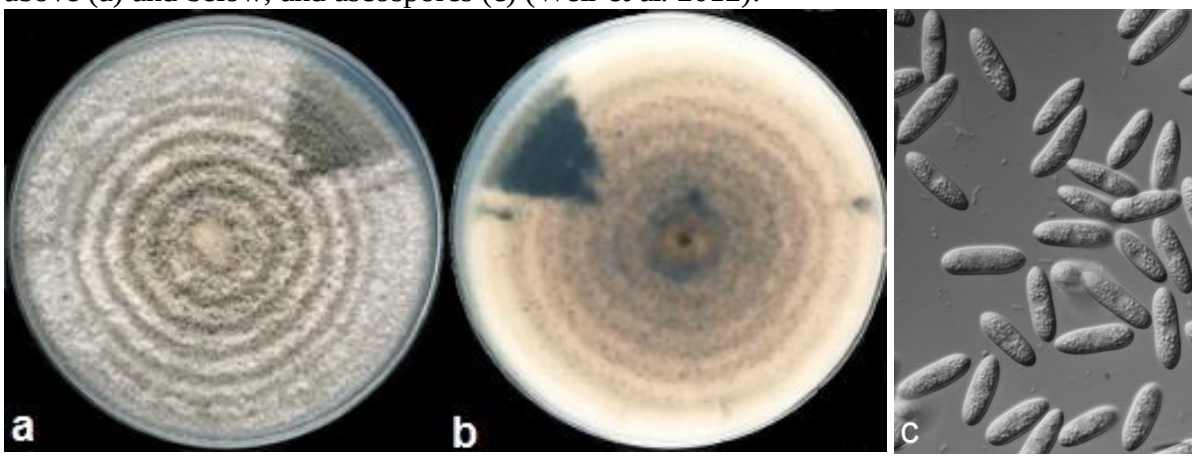

Figure 2.7: Ten day old cultures of *Colletotrichum theobromicola* (ICMP 17957) on PDA, from above (a) and below, and ascospores (c) (Weir et al. 2012).

Unfortunately, this did not resolve the accurate identification of *Colletotrichum* species. The lack of standardised protocols and plasticity of *Colletotrichum* species when grown under different media with varying temperature, light regime makes morphological features not a suitable method to delineate species boundaries of *Colletotrichum* spp. (Johnston 2000; Hyde et al. 2009).

2.5.2 Molecular systematics

The genus *Colletotrichum* consists of groups of species that are morphologically similar, but genetically different (Noireung et al. 2012). Prior to the molecular era, diagnosis based on morphological methods were limited on availability of type material that resulted in a lot of incorrect synonyms for each species examined (Johnston et al. 2005). For example, more than

600 synonyms can be named for *C. gloeosporiodes*. The same is true for other *Colletotrichum* spp. viz. *Colletotrichum acutatum* J.H. Simmonds, *Colletotrichum dematium* (Pers.) Grove and *Colletotrichum graminicola* (Ces.) G.W. Wilson etc., which have been broadly accepted to be occurring as species complexes (Johnston et al. 2005; Phoulivong et al. 2010).

Cryptic species may be mistaken for a single species compromising on the species' integrity and understanding (Sharma and Shenoy 2016). Due to previous confusion in systematics of the genus *Colletotrichum*, the reliance on morphological criteria in diagnosing diseases led to many closely related species to be wrongly assigned names with majority of them misidentified as *C. gloeosporiodes*. This has contributed to the notion that *C. gloeosporiodes* is the prominent cause of anthracnose throughout the world (Phoulivong et al. 2010).

The widespread adoption of molecular techniques in the 1990's has greatly improved precision in the systematics and identification of the fungi (Crouch et al. 2009). Initially, sequence variation in the internal transcribed spacer (ITS1) flanking the 5.8 S ribosomal DNA (rDNA) was accepted as the universal barcode for identifying a broad range of fungi. Mills et al. (1992) and Sreenivasaprasad et al. (1992) were among the first workers to distinguish between *Colletotrichum* species using DNA sequence data. As time lapsed, studies increasingly showed that ITS sequences have limited resolving ability of delineating closely related species within *Colletotrichum* species complexes (Cai et al. 2009; Crouch et al. 2009; Damm et al. 2009; Roy et al. 2010). It has been reported that the commonly used genes such as actin, beta-tubulin and calmodulin are equally not comprehensive enough in delineating cryptic species in the genus *Colletotrichum* (García-Serrano et al. 2008; Silva et al. 2012). To date more informative gene regions such as glyceraldehyde-3-phosphate dehydrogenase and glutamine synthetase were shown to be more objective at resolving the genetic differences within *Colletotrichum* species complexes (Silva et al. 2012). To yield improved delineation of cryptic species, single-locus

phylogenies were adopted (Johnston and Jones 1997; Johnston 2000). Sherriff et al. (1994) were among the first workers to construct bootstrapped Neighbour Joining (NJ) trees for *Colletotrichum* spp. using ITS2 and LSU sequences. Ever since, single-locus phylogenies dominated molecular systematics of *Colletotrichum*, but species boundaries remain vague and relationships within some of these species complexes were poorly resolved (Sreenivasaprasad et al. 1993, 1996; Johnston and Jones, 1997; Polashock et al. 2009; Nguyen et al. 2010).

Majority of early molecular studies were not reliable due to unavailability of type or authentic living cultures preserved in culture collections, which is a limitation when reconstructing a natural classification system of *Colletotrichum* (Cai et al. 2009; Hyde et al. 2009). Thus, introduction of ex-type strains revolutionised systematics of the genus *Colletotrichum* as they provided a solid reference of comparing sequence data (Cannon et al. 2008). As a standard, the systematic International Code of Nomenclature (ICN) for algae, fungi and plants accentuate application of epi-or neotypes in all taxonomic work on *Colletotrichum* (Hawksworth 2011).

Today, a polyphasic approach is recommended, where both epitypification and multilocus phylogenetic analysis are integrated for robust and comprehensive analyses to unravel the species richness in various *Colletotrichum* species complexes (Weir et al. 2012). Prihastuti et al. (2009) has shown that the use of both DNA sequences and ex-epitype strains is a more robust method, where they successfully detected six new species that represented phylogenetically distinct lineages of the *C. gloeosporioides* complex. Hyde et al. (2009) accepted 66 *Colletotrichum* species based on epitypification and multiple sequence analysis. It has been shown that *C. gloeosporiodes* occur within the *C. gloeosporiodes* complex, which consists of 22 closely related species viz. *Colletotrichum asianum* Prihast., L. Cai & K.D. Hyde, *Colletotrichum cordylinicola* Phouliv., L. Cai & K.D. Hyde, *C. fructicola, C. gloeosporiodes, Colletotrichum horii* B.S. Weir & P.R. Johns, *Colletotrichum kahawae* subsp.

kahawae J.M. Waller & Bridge, *C. musae*, *Colletotrichum nupharicola* D.A. Johnson, Carris & J.D. Rogers, *Colletotrichum psidii* Curzi, *C. siamense*, *C. theobromicola*, *Colletotrichum tropicale* E.I. Rojas, S.A. Rehner & Samuels, and *Colletotrichum xanthorrhoeae* R.G. Shivas, Bathgate & Podger, along with the taxa described here as new, *Colletotrichum aenigma* B.S. Weir & P.R. Johnst., *Colletotrichum aeschynomenes* B.S. Weir & P.R. Johnst., *Colletotrichum alatae* B.S. Weir & P.R. Johnst., *Colletotrichum alienum* B.S. Weir & P.R. Johnst., *Colletotrichum aotearoa* B.S. Weir & P.R. Johnst., *Colletotrichum clidemiae* B.S. Weir & P.R. Johnst., *Colletotrichum kahawae* subsp. *cigarro* B.S. Weir & P.R. Johnst., *Colletotrichum salsolae* B.S. Weir & P.R. Johnst. and *Colletotrichum ti* B.S. Weir & P.R. Johnst. (Weir et al. 2012). After this major taxonomic revision, other *Colletotrichum* species not *C. gloeosporiodes* have been reported as causal agents of anthracnose. For example, *C. theobromicola* was reported as a serious pathogen causing severe seedling blights on *Eucalyptus* cuttings grown in commercial nurseries in Brazil (Rodrigues et al. 2014).

2.6 Implication of *Colletotrichum* spp. associated with *Eucalyptus*

The genus *Colletotrichum* contains anamorphic fungi that can cause disease on a wide range of hosts including *Eucalyptus*. Early in their life, some *Colletotrichum* species in the *boninense*, *gloeosporioides* and *graminicola* species complexes were reported as endophytes, exhibiting a latent or quiescent and endophytic phase, which is usually short-lived as the fungi switch to assume its necrotrophic life style (Lu et al. 2000; Guozhong et al. 2004; Wang et al. 2008; Vieira et al. 2014). This is the reason why most *Colletotrichum* species are referred as the hemibiotrophs. Literature reports *C. gloeosporiodes* to be commonly associated with healthy leaves of *Eucalyptus dives* Schauer, *E. grandis* and *E. globulus* seedlings (Viljoen et al. 1992). The period an endophyte takes to switch to its pathogenic state depends on host genotype, physiological condition, and the environmental conditions (Mendgen and Hahn 2002; Kogel et al. 2006; Delaye et al. 2013). However, some endophytic *Colletotrichum* spp. have been

reported to switch to its destructive form within a period of about 48 to 72 h after inoculation (Brown 1975; Wharton and Diéguez-Uribeondo 2004; O'Connell et al. 2012).

All plant parts of a susceptible host are infected with pathogenic *Colletotrichum* spp. at all stages of growth and development. Sowing infected seeds lots drastically reduces germination capacity, yet few of seeds that germinate often results in deformed and poor stand seedlings (Brown and Ferreira 2000). Infection of leaves initially produces discrete round light brown to blackish spots known to as anthracnose, which may be surrounded by a red-purple border. As the disease spread further, the lesions coalesce turning to a dark brown to black colour (Sharma et al. 1984; Rodrigues et al. 2014). Eventually, diseased vegetative parts develop a chlorotic yellowing accompanied with curling, and maybe covered with concentric rings of acervuli and yellowish to pinkish conidial masses (Baxter et al. 1983). Similarly, young stems are often seen with subcircular or angular depressed black lesions, which subsequently coalesce causing seedling blights (Dodd et al. 1992).

Anthracnose on *Eucalyptus* was first reported in India (Sharma 1984), but the disease is generally distributed throughout the rest of the world (Viljoen et al. 1992; Rodrigues et al. 2014). Since *Colletotrichum* abundantly releases highly dispersible conidia, the densely populated seedlings growing in nutrient rich and moist nursery conditions are at greater risk of rapid spread with a possibility of a disease outbreak (Figure 2.8). Dispersed conidia may remain on a plant surface and retain its potential to cause disease for periods of over 7 days (Estrada et al. 1993). The disease usually appears in the beginning of the rainy season and causes damage particularly when the host's immune system is not yet fully developed or might remain latent and manifest disease symptoms only when the host is exerted under some form of stress (Smith et al. 1998).

Although seed infected with seed-borne *Colletotrichum* spp. presents a high chance of disease transmission into seedlings, the disease may remain quiescent and manifest symptoms at later growth stages of the plant (Viljoen et al. 1992). For example, extreme weather events such as drought, frost and hot winds that are occurring more frequently in South African forest plantation areas are known to stress and increase susceptibility of *Eucalyptus* to *C. gloeosporiodes* resulting in outbreaks of tree diebacks (Smith et al. 1998). Despite recurring occurrences of *Colletotrichum* spp. in *Eucalyptus* nurseries and forest plantations (Viljoen et al. 1992), anthracnose leaf spot of *Eucalyptus* are generally neglected, and their overall impact underestimated. Consequently, this has attracted little research interest and epidemiology of the disease remain undetermined.

Knowledge of specific *Colletotrichum* species associated with trees in forest plantations is important particularly for estimating risks involved in trade, which will help develop biosecurity policies and disease management (Johnston and Jones 1997; Freeman et al. 1998; Cai et al. 2009). Recently, *C. fructicola* and *C. kahawae* subsp. *cigarro* were found associated with *Eucalyptus* seed produced in South Africa (Mangwende et al. 2018). Since both fungi are both seed-borne and seed-transmitted (Mangwende et al. 2018), seed trade risks spreading them to places they are not known to occur. *Colletotrichum kahawae*, commonly known to cause anthracnose of coffee, is only restricted to African regions where coffee (*Coffea arabica* L.) is grown.

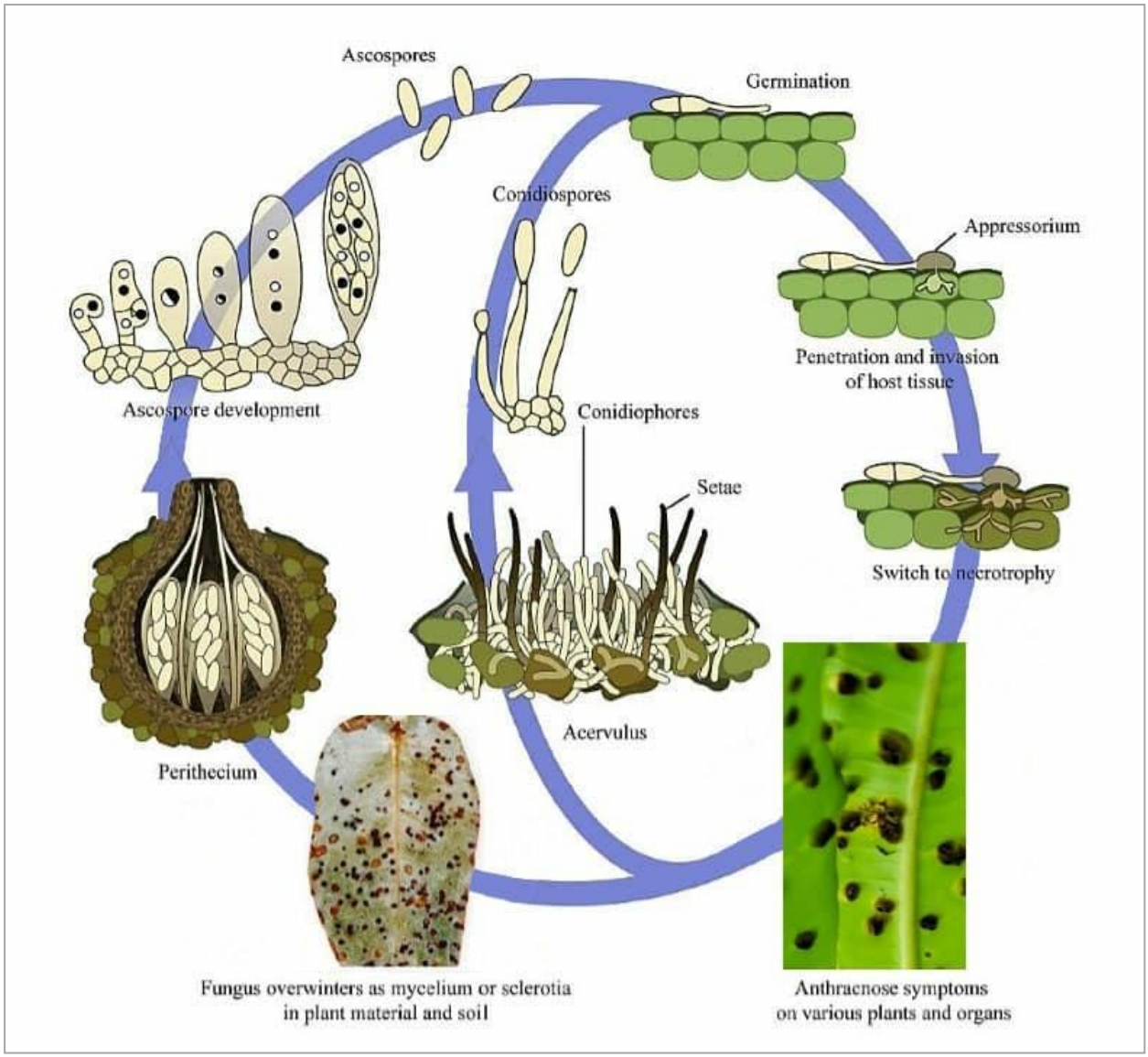

Figure 2.8: Disease cycle of *Colletotrichum* spp. on *Eucalyptus* (Agrios 2005)

Coffee berry disease has been reported to be most severe in high altitude areas that lie above 1400 m (Bedimo et al. 2007), where it can cause up to 80% yield loss if control measures are not applied (Van der Vossen and Walyaro 2009; Hindorf and Omondi 2011). Thus, the disease has a potential of severely affecting the economy of countries strongly relying on coffee production. For this reason, *C. kahawae* is listed as a quarantine pathogen in many coffee producing countries outside Africa such as Australia and China (Jayawardena et al. 2016). On that note, occurrence of *C. kahawae* on forestry seed produced in South Africa is an issue that must be handled with caution considering the ongoing exchange of genetic material between

South America and some Asian countries. However, there has been an increase of reports of *C. kahawae* on several hosts outside Africa including on Andean raspberry (*Rubus glaucus* Benth.) (Afanador-Kafuri et al. 2014), olives (*Olea europaea* L.) (Schena et al. 2014), rocket (*Eruca sativa* Mill.) (Garibaldi et al. 2016a) and American sweetgum (*Liquidambar stryraciflua* L.) (Garibaldi et al. 2016b). Although these studies employed phylogenetic studies to identify and confirm reported *Colletotrichum* spp., none of these studies seem be showing consistency on the use of primer sets that would delineate the pathogens up to the subspecies level (Batista et al. 2017). This has already caused a technical distress within the teams closely monitoring biosecurity implications of this pathogen.

2.7 Management of seed-borne *Colletotrichum* spp.

Once seed health status of a given seed lot has been determined, if it is above the accepted threshold level, the seed lot is either subjected to quarantine regulations, where the seed lot may be rejected from import or export (Neergaard 1969). However, in the event that a pathogen is detected above an accepted threshold level, surface disinfectants or seed treatment may be applied to eradicate seed-borne inoculum protecting the seed lot from reaching economic injury levels.

There is little information on tolerance levels of commonly occurring seed-borne pathogens on forestry seed. Together with notions and perceptions that regard tree seeds as free from pests, seeds are hardly treated risking the global spread of forest pathogens. Furthermore, lack of availability of registered seed treatments for forestry seeds have forced local foresters to use unregistered chemicals. Foresters are reported to apply chemicals registered on other crops such as benomyl, carboxin, triforine, Thiram and Captain as prophylactic seed treatments (Chalermpongse 1987). A study by Harsh et al. (1992) showed that dressing *Eucalyptus* seed lots with 0.2% thiophanate-methyl, carbendazim and mancozeb significantly reduces seedling

losses caused by seed-borne fungi. Currently, about 82% of commercial plantation areas in South Africa have achieved the global Forest Stewardship Council certification (FSC) (Molnar 2003; Rametsteiner and Simula 2003; FSC 2004). As a regulating body to promote responsible management of the world's forests, the FSC has set standard production practices that stipulates registered forest plantations to avoid further use of synthetic chemicals and fertilisers (Worrell 2000; Fortier and Messier 2006). Hence, much of the discussion will focus on different non-chemical seed treatments to be used for producing healthy, disease free, seed in compliance with FSC principles and criteria. In this discussion, literature on alternative, non-chemical, treatments such as physical methods and biological control agents as seed treatments was reviewed.

2.7.1 Hydrogen peroxide

Hydrogen peroxide (H_2O_2), a strong oxidizer, is a chemical compound with low mammal toxicity, and slowly decomposes in the presence of light. The superficial contamination of seeds by fungal pathogens can be eradicated by seed disinfectants such as hydrogen peroxide (H_2O_2). Hydrogen peroxide can be used in sanitising seeds and has been shown to be highly effective against a wide range of seed-borne mycoflora (Dashtban et al. 2010; Veal and Day 2011). Depending on the concentration, H_2O_2 is ideal for inactivating or eradicating pathogens already established on the seed coat or present in the solution containing the seed to prevent cross contamination of seed. Apart from reducing seed-borne infection, H_2O_2 also oxidises various germination inhibitors present in the pericarp that improves seed germination (Barnett and McGilvray 2002). Hydrogen peroxide has been reported to improve the germination of eastern gamagrass [*Tripsacum dactyloides* (L.) L.] as well as rice (*Oryza sativa* L.), maize (*Zea mays* L.), watermelon [*Citrullus lunatus* (Thunb.) Matsum. & Nakai] and muscadine (*Vitis rotundifolia* Michx.). Although Donald and Lundquist (1988) proved soaking *Eucalyptus* seeds

in a 33% H_2O_2 solution for 1, 2 or 4 min to restrict fungal germination and development, this was broadly generalised as the study did not take into consideration effects of H_2O_2 on seed germination. Several studies have shown reduction of seed germination at higher concentrations of H_2O_2. For example, Szopińska (2014) reported a significant reduction in the occurrence of seed-bone *Alternaria zinniae* M.B. Ellis associated with zinnia (*Zinnia elegans* Jacq.) seed lots if seeds were soaked in 6 and 9.1% H_2O_2 solutions for 60 and 20 min, respectively. Increasing concentrations of H_2O_2 above 9% significantly resulted in drastic reductions both seed germination and seedling vigour.

Nandi et al. (2017) conducted a study to improve seedling emergence of chilli (*Capsicum annum* L.). Seeds treated with 1, 2 and 3% H_2O_2 were tested for the presence of seed-borne fungi together with their germination capacity. Together with other seed-borne fungi, *Colletotrichum capsici* (Syd.) E.J. Butler & Bisby was isolated with the highest incidence from untreated chilli seed lots. The highest inhibition of colony growth of *C. capsici* was observed by the treatment of 3% H_2O_2 followed by 2 and 1% H_2O_2. Treatment of seeds with H_2O_2 regardless of concentration, positively improved seed health with a significant increase in number of seeds free from fungi. Throughout the different assessment intervals that were done at 7, 10 and 15 Days after Sowing (DASO) and germination was highest on seeds treated with 1% H_2O_2 (55.5, 60.5 and 84.8%, respectively). However, germination percentages decreased with an increase of H_2O_2 concentration. Germination percentages were lowest for seeds treated with 3% H_2O_2 (4.3, 5.6 and 5.9%, respectively). Similarly, seedling vigour was influenced by different concentrations of H_2O_2. The highest seedling vigour index of 64.7% was recorded on seeds soaked in 1% H_2O_2, and the lowest seedling vigour index of 23.2% was obtained from seeds treated with 3% H_2O_2. Hence, investigations to determine the concentrations and durations of exposure of various *Eucalyptus* spp. seeds to H_2O_2 are critical for optimizing its use in eradicating see-borne *Colletotrichum* spp. without reducing seed germination.

2.7.2 Thermotherapy

The use of heat to terminate growth of mycoflora associated with seed, popularly known as thermotherapy, is based on the elevation of ambient temperature through water or air. The principle and mechanism of action works by direct lethal action of heat, thermal coagulation of proteins and lipids, which disrupts the ultrastructure of the pathogen (Groot et al. 2006). Seeds of different host species, because of variation in their biochemical compositions, have different temperature tolerances (Baker 1962). Even seed lots of the same species may show variation to thermal tolerances due to their differences physiological maturity and moisture content (Forsberg 2004). In this regard, it is important to determine temperature-time combinations that will effectively control seed-borne mycoflora associated with seed, without injuring seed components.

2.7.2.1 Hot water

Hot water seed treatments was a classical method used to disinfect barley and wheat seed lots infected with the loose smut pathogen *Ustilago nuda* (C.N. Jensen) Kellerm. & Swingle (Doling 1965). Today, there is a wide body of literature showing efficacy of both hot water and microwave radiation as seed treatments on a wide range of pathosystems (Nega et al. 2003; Du Toit and Hernandez-Perez 2005; Tylkowska et al. 2010; Agustí-Brisach et al. 2012; Koch et al. 2014; Mandiriza et al. 2018). Since it is an economical, practically easy to use method without need for registrations and licenses, hot water treatments are now widely adopted in most modern-day seed sanitation operations. Although Donald and Lundquist (1988) mentioned a general recommendation of sanitising *Eucalyptus* seed using hot water baths set at 50 ^{o}C for 5, 10 or 20 minutes, further investigations are required to confirm efficacy against seed-borne *Colletotrichum* spp. associated with various *Eucalyptus* spp. seed lots.

Seed health tests occasionally detect *Colletotrichum spinaciae* Ellis & Halst., *Fusarium* spp. *and Phoma betae* A.B. Frank on commercial red beet and sugar beet seed lots (Maude et al. 1969). Severe damping off of seedlings are often recorded when crops are grown from seeds infected with both *P. betae* and *C. spinaceae.* Complete disease eradication was achieved by Thiram soaking, whereas hot water seed treatment at 50 °C for 25 min failed to eliminate the seed-borne pathogens resulting in 10.5 and 42.5% infection of the crops, respectively (Maude et al. 1969).

A total of 27 seed-borne fungi were isolated from 16 soybean seed lots (Nik 1980), where *Colletotrichum dematium* (Pers.) Grove. was isolated on 75.0 and 56.2% of the samples on blotter and PDA, respectively. Treating soybean seeds with Benomyl, Captan, Dithane M45, Thiram and a 10 min soak in a 55 °C hot water bath significantly reduced the incidence of fungi recovered on blotters and PDA compared to the control. Benomyl yielded the highest reduction of seed-borne fungi. Although soaking soybean seeds in a hot water bath effectively lowered the incidence of seed-borne fungi, seed germination was impaired.

Seedling damping-off and diebacks are common diseases limiting Protea (*Protea compacta* R.Br.) productivity. The disease is caused by *C. gloeosporioides*, a fungus that has been shown to be both seed-borne and seed-transmitted (Benić 1987; Botha and Le Maitre 1992; Bayman et al. 1998). In a study to improve seedling emergency of infected *P. compacta* seed lots, Benic and Knox-Davies (1983) observed that untreated seeds initially had higher germination capacity (71.0%), but the number of surviving seedlings was reduced to 52.5% by damping-off caused by *Colletotrichum* and a few other seed-borne fungi. Soaking seed in a 30 °C Thiram seed soak for 24 h and in a 50 °C hot water bath for 30 min, followed by Thiram dusting effectively reduced the incidence of seed-borne *C. gloeosporioides*. In addition, soaking infected seed lots in a 50 °C hot water bath for 15 min significantly improved seed germination

and seedling emergency. Although complete elimination of seed-borne inoculum was observed at an extended soaking period, this drastically reduced both germination capacity and seedling survival.

From 16% annual crop losses recorded on sorghum due to plant diseases in Bangladesh, seed-borne diseases cause at least 10% of the losses (Fakir 1983). Islam et al. (2009) treated sorghum seed lots with hot water treatment at 55 °C for 10 mins, garlic tablet, neem leaf extract, BAU-Biofungicide and Vitavax-200 to reduce presence of important seed-borne pathogens including anthracnose stalk rot pathogen (*C. graminicola),* seed rot causing pathogen (*Fusarium moniliforme* J. Sheld.), and seedling blight or charcoal rot producing pathogen [*Macrophomina phaseolina* (Tassi) Goid.]. Comparing with the untreated control, all seed treatments significantly reduced all seed-borne pathogens. Treating sorghum seeds with the synthetic seed treatment, Vitavax 200, eradicated seed-borne pathogens resulting in the highest increase in germination. However, alternative non-chemical seed treatments showed good performance of hot water seed treatment in reducing incidence of pathogenic *Colletotrichum* sp., and the least efficacy against the pathogen recorded on seeds treated with BAU-Biofungicide. Despite its efficacy against seed-borne *Colletotrichum* sp., seeds treated with hot water recorded the lowest seed germination percentages of 82.0 and 86.1% for sorghum seeds collected from Mymensingh and Sherpur (Bangladesh), respectively.

Ripe fruit rot of Chilli is an important seed-borne and seed-transmitted disease caused by *C. capsici* (Hadden and Black 1989; Surekha et al. 1990; Than et al. 2008). Vivekanand et al. (2018) conducted an efficacy evaluation of two chemicals, Captan and Mancozeb (2.5g/kg), two bioagents *Trichoderma harzianum* Rifai and *Pseudomonas fluorescens* (Flügge) Migula (5g/kg) and hot water treatment (55 °C for 30 min) as seed treatment against anthracnose fruits rots under in vivo conditions. Chilli seed lots treated with Mancozeb resulted in the highest

suppression of disease at 75 and 105 days after transplanting (DAT). Similarly, seeds treated with Captan had the lowest incidence of anthracnose fruit rots at 90 DAT. Performance of biocontrol agents in supressing anthracnose fruit rots was satisfactory throughout the test periods, but seeds soaked in hot water bath recorded were least effective as it resulted in the most severe and highest incidence of disease.

2.2.2.2 Microwave radiation

Microwave radiation energy is a particularly attractive method of controlling seed-borne pathogens deeply seated inside tissues of the seed (Bloomberg 1966; Graham et al. 1983). Although microwave radiation also uses heat as the lethal mode of action (Grondeau et al. 1994; Reddy et al. 1998), short exposure time is required for high frequency alternating electromagnetic radiation, 300 MHz-300 GHz (Pozar 1993), to be transmitted to the various components of seeds where pathogens are imbedded.

Depending on the dielectric properties of atoms and molecules of seed and seed-borne mycoflora associated with it, bipolar components may become polarized allowing them to store electric energy when exposed under an electromagnetic radiation (Yadav et al. 2014). As electrical charges flow through, bipolar molecules are forced to rearrange and align with the rapidly changing electric field (Kappe et al. 2008). The resistance of bipolar molecules to change with the rapid movement creates friction, which generate heat after exposure to microwave radiation (Lozano et al. 1986). The amount of heat generated within the seed tissues and seed-borne pathogen is influenced by moisture content, bulk density, and the frequency of electric field applied (Knox et al. 2013; Friesen et al. 2014). For example, Najah et al (2016) showed that chilli seeds with a moisture content of 4.3% recorded the most effective reduction of seed-borne *C. acutatum* when exposed to microwave irradiation for 40 seconds. However,

microwave thermotherapy at low water content level of around 0.08g g^{-1} for less than 30 s, has been found to cause bursting of soybean seeds (Reddy et al. 2000).

Thus, research is needed to assess specific time-temperature regimes, required to sufficiently kill the pathogen without overheating the seed itself (Grondeau et al. 1994). A few studies done on seeds of different crops have successfully established optimum microwave radiation time-temperature regimes required to effectively reduce inoculum loads whilst improving seed germination and vigour of different. For example, Lozano et al. (1986) showed that true seed of cassava (Manihot esculenta Crantz) infected with *Colletotrichum* spp., *Fusarium* spp. and *Xanthomonas campestris* pv. *manihotis* (Bondar) Dye were effectively controlled when temperatures reached 77 ^{o}C after 120 seconds of exposure to microwave radiation at 1400 W, 2450 MHz.

An extended exposure to high levels of irradiation can negatively affect the physiology of seedlings (Berbert et al. 2002; Han 2010). A linear decrease in growth of *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara was observed when dry bean (*Phaseolus vulgaris* L.) seeds were exposed to microwave radiations ranging from 0-90 s applied at 10 s intervals. High seed germination percentages were recorded for seeds exposed to microwave radiation for 0-40 s, but seed germination drastically reduced as the exposure periods exceeded 60 seconds (Friesen et al. 2014). Reddy et al. 2000 demonstrated that soybean seeds exposed for 30 s in microwave irradiation effectively controlled all internal fungi without any deleterious effects on viability, vigour, moisture content or ultrastructure of the seeds. However, extended exposure to microwave radiation resulted in a significant increase in abnormal seedlings. The ultra-structural properties showed that neither axis nor cotyledon of 30 s microwave seed treatment cells differed from control seeds. Exposure of seeds for an extended time period of 45 or 60 s subsequently lowered seed viability and vigour and resulted

in some significant ultra-structural changes (Reddy et al. 2000). In addition, changes in the nuclei and mitochondria of axis cells were evident after 45 s irradiation, while changes in cell walls, membranes, nuclei and other organelles of axis tissue, with tissues of cotyledons showing the greatest degree of disruption when exposed for 60 s (Reddy et al. 2000).

The type of seed, location and morphology of inoculum affects efficacy of microwave radiation on seed-borne pathogens (Cavalcante and Muchovej 1993; Tylkowska et al. 2010). In general, the capacity to tolerate long exposure to microwave radiation decreases as seed size increases (Hankin and Sands 1977). More time will be required for the interior of a seed to be sufficiently affected by microwave energy to eliminate deep-seated inoculum, and this will compromise the integrity peripheral tissues. Morphology of inoculum plays a large role in determining efficacy of microwave seed treatments (Cavalcante and Muchovej 1993). Fungal spores react differently to microwave radiation based on cell composition. Dry bean seeds were exposed to microwaves of 650 w, 2450 MHz for 15, 30, 45, 60, 90 and 120 s (Tylkowska et al. 2010). Microwaves were not effective in controlling *Alternaria alternata* (Fr.) Keissl. and *Fusarium* spp., whereas diminished the presence of *Penicillium* spp., both on the seed surface and in the inner seed tissues. Fungi that produce hyaline single-celled spores, like *C. lindemuthianum*, are more sensitive to microwave radiation than fungi that produce multi-celled or dark pigmented spores such as *Alternaria* spp. and *Fusarium* spp. (Tylkowska et al. 2010). However, other studies have shown contrary results where microwave radiation failed to control *A. alternata* and *Fusarium* spp. (Lozano et al. 1986; Reddy et al. 1998).

2.7.3 Biological control

Biological control makes use of living organisms to suppress growth or reproduction of a pathogen or disease (Burges and Jones 2012). Due to pressure of adopting environmentally friendly means of controlling pests and disease, use of biological agents in seed sanitation is

gaining momentum with their ease to register with Environment Protection Agencies (Velivelli et al. 2014). Because of the commercial success of *Bacillus* and *Trichoderma* particularly for controlling a wide range of pests and diseases, this review was narrowed to consider potential use of these biocontrol agents for sanitising *Eucalyptus* seed lots infected and/or infested with seed-borne Colletotrichum.

2.7.3.1 *Bacillus subtilis*

Bacillus subtilis (Ehrenberg) Cohn is a motile, rod-shaped Gram-positive bacterium, traditionally considered as a soil-dwelling microorganism mutually associating with plant roots in the upper rhizosphere (Barea et al. 2005; Pérez-Garcíaet al. 2011). Under stressful conditions, *B. subtilis* sporulate releasing dormant spores called endospores that enable them to adapt to high temperatures, extreme pH, drought and various other forms of stress (Higgins and Dworkin 2012). This is advantageous for stability giving *B. subtilis* formulations to have an extended shelf life (Pérez-Garcíaet al. 2011; Vejan et al. 2016). Strains of *Bacillus subtilis* including QST713, GBO3, MB1600 and many others are active ingredients of commercial plant protection products such as Kodiak®, Companion®, HiStick N/T® and Serenade® (Wang et al. 2018).

The direct beneficial effects of treating seeds with *B. subtilis* include promotion of tissue growth that facilitate fast development of seedlings through an increase in nitrogen uptake, phosphate solubilisation, and siderophore and phytohormone production (Raupach and Kloepper 1998; Nagórska et al. 2007). When applied directly to seeds, *B. subtilis* out-competes various plant pathogens colonizing the developing root system, where the bacteria feed off plant root exudates depriving the pathogens of a food source (Adebanjo and Bankole 2004). *Bacillus* sp. may also suppress diseases by induced systemic resistance with the release of phytohormones such as indole acetic acid (IAA) (Ongena et al. 2005; 2007). Several strains of

Bacillus sp. have been shown to produce iturin, a secondary antifungal metabolite that activates induced defence responses in plants (Hiradate et al. 2002). Other studies have confirmed that *Bacillus* sp. supress fungal pathogens by producing inhibitory metabolites (Leelasuphakul et al. 2008; Mondol et al. 2013). Chitin and β-1,3-glucans are major constituents of many fungal cell walls, and various strains of *B. subtilis produce* extracellular enzymes such as microbial chitinase or β-1,3-glucanase which significantly reduced mycelial growth of fungal pathogens (Kim and Chung 2004).

Several studies have reported formulations containing *B. subtilis* to be effective against various *Colletotrichum* spp. causing anthracnose diseases in a wide variety of crops including avocado (*Persea americana* Mill.), beans (*Phaseolus vulgaris)*, cucumber (*Cucumis sativus* L.), tobacco (*Nicotiana tabacum* L.), watermelon etc. (Ongena et al. 2004). However, few studies have investigated the efficacy of formulations containing *B. subtilis* applied as a seed treatment to control seed-borne *Colletotrichum* spp.

Raupach and Kloepper (2000) conducted field trials to evaluate effect of plant growth-promoting rhizobacteria on cucumber growth and supressing naturally occurring anthracnose disease caused by *Colletotrichum orbiculare* Damm, P.F. Cannon & Crous. Cucumber seeds were treated with *Bacillus pumilus* strain INR7, *Curtobacterium flaccumfaciens* strain ME1, and *Bacillus subtilis* strain GB03 at 108 to 109 CFU/seed. Treated seeds were sown in 1996 and 1997 and growth promotion monitored comparing to controls that consisted of seed beds fumigated with methyl bromide and non-fumigated seedbeds. In both years, there was a significant reduction of severity of foliar diseases on plants grown from seeds treated with PGPRs compared to plants grown in fumigated and non-fumigated seedbeds. In addition, plants grown from seeds treated with PGPR recorded significantly higher plant growth compared to the non-bacterized controls in fumigated and non-fumigated seedbeds.

Chill seeds suspended in 10^8 cfu/ml of *Pseudomonas* sp. and *Bacillus* sp. strains and evaluated against *C. capsici* causing chilli fruit rot and dieback (Bharathi et al. 2004). In addition, seeds were also treated with different mixtures of bacteria containing chitin and/or neem, where a total of 13 treatments were evaluated and their effects on seed germination and seedling vigour recorded. Among the antagonistic strains evaluated, seeds treated with *B. subtilis* alone revealed the highest effect on seedlings vigour (1405.0) and germination percentage (96.1%). However, seeds that were not treated with the PGPRs had the highest susceptibility to anthracnose infection with a poor plant growth. Treating chilli seeds with a mixture of PGPR + neem + chitin had a significant reduction on the incidence of *C. capsici* that positively increased the number of flowers, fruits, average fruit length and total yield of chilli plants compared to the controls.

Greenhouse trials were conducted to test the efficacy of commercially formulated microorganisms that consisted of BA2552 [*Pseudomonas chlororaphis* (Guignard and Sauvageau) Bergey], MBI600 (*B. subtilis*), FZB24 (*B. subtilis*), Serenade (*B. subtilis*), Mycostop Mix (*Streptomyces griseoviridis* Anderson), F251/2 (*Fusarium oxysporum* strain 251/2) compared to a chemical Pomarsol (Thiram) as seed treatments against anthracnose leaf spot of beans caused by *C. lindemuthianum* (Tinivella et al. 2009). It was observed that seedling emergence rates were not affected for seeds treated with commercial microbial products, and *B. subtilis* based formulations provided the best protection from anthracnose. Although disease was suppressed a little lower compared to *B. subtilis* formulation, seeds treated with *Pseudomonas putida* Trevisan and *Fusarium oxysporum* Schltdl. were also effective against bean anthracnose.

Tinivella et al. (2009) investigated the efficacy of coating bean and pea seeds with resistance inducers, *B. subtilis* based commercial formulations, and non-formulated selected bacterial and

yeast strains in controlling anthracnose disease caused by *C. lindemuthianum.* Formulations containing *B. subtilis* (MBI600, FZB24 and Serenade) provided the best protection from anthracnose on bean and pea. Satisfactory disease control was observed on plants grown from seed treated with thyme (*Thymus vulgaris* L.) oil, *Clonostachys rosea f. rosea* (Link) Schroers, resistance inducers, a saprophytic strain of *F. oxysporum* and the mustard powder-based product Tillecur.

Ashwini and Srividya (2014) characterised *B. subtilis* obtained from the rhizosphere of chilli, and they observed it to be effective at controlling *C. gloeosporioides* strain OGC1. Treatment of chilli seeds with *B. subtilis* showed 100% germination index similar to the untreated seeds. However, sowing treated seeds treated with *Bacillus* sp. resulted in 65.0% reduction in disease incidence compared to untreated controls that recorded 77.5% disease incidence.

Tumpa et al. (2016) treated vegetable seeds with an aqueous formulation of *B. subtilis* to suppressing seed-borne fungal pathogens. For summer vegetable crops, the following results were obtained: Treating sweet gourd seed with *Bacillus* sp. completely suppressed the growth of *Aspergillus niger* van Tieghem, *Rhizopus* sp., *Macrophomina phaseolina* (Tassi) Goid. and *Phoma exigua* Sacc. A satisfactory level of suppression of seed-borne fungi viz. *F. moniliforme*, *Botrytis cinerea* Pers., *M. phaseolina*, *Aspergillus flavus* Link, *Colletotrichum* sp., *Cercospora* sp., *Penicillium* sp., and *P. exigua* was observed on cucumber seeds treated with *Bacillus* sp. Snake gourd seeds treated with *B. subtilis* resulted in a partial suppression of *B. cinerea* (20.0%), *F. moniliforme* (40.0%), *A. flavus* (30.1%), *F. oxysporum* (20.0%), while complete suppression was also recorded for *M. phaseolina*, *A. niger* and *Rhizopus* sp. and *Penicillium* sp. For winter vegetable crops, bottle gourd and *P. vulgaris* seeds treated with *Bacillus* sp. completely suppressed incidence of *M. phaseolina*, *Cercospora* sp., *P. exigua*, and *A. niger*.

2.7.3.2 *Trichoderma harzianum*

Among biocontrol agents reported to be effective in plant disease management, the ascomycetous fungus *T. harzianum* Rifai is widely accepted as an effective alternative to synthetic fungicides. Some internationally registered commercial biopesticides containing *T. harzianum* as the principle ingredient include Supresivit®, Tri002 and Trichodex® TrichoFlow™, ArborGuard™, GreenMax and Trichoplus (Woo et al. 2014; Fraceto et al. 2018).

Several studies show that biological control agents often utilise one or more mechanisms in combating seed-borne and soil-borne pathogens (Howell 2003; Benítez et al. 2004; Harman et al. 2004). *Trichoderma harzianum* may mycoparasitise through competition for nutrients or antagonise pathogens through secondary metabolites such as phytohormones (Sivan and Chet 1989; Haran et al. 1996; Elad 2000). Alternatively, *T. harzianum* may secrete cell-wall degrading enzymes such as chitinases (endochitinases, exochitinases, and *β*-N-acetilhexosaminidases), cellulases (exoglucanases, endoglucanases, and *β*-1-3-glucanases) and proteases that allows it to penetrate obtain nutrients from the pathogen (Harman et al. 2004; Harman 2006).

Extensive research has been done where the efficacy of *Trichoderma* applied as a seed treatment was evaluated against pathogenic seed-borne *Colletotrichum* spp. associated with chilli, soybean and bean seed lots (Suthin-Raj and Christopher 2009; Padder et al. 2010; Padder and Sharma 2011). Begum et al. (2008) investigated potential of biocontrol agents to replace synthetic fungicides in controlling soybean crop losses caused by *Colletotrichum truncatum* [(Schw.) Andrus and W. D. Moore]. Seven isolates of *Trichoderma* and three isolates of bacteria were tested, where two isolates of *Trichoderma* namely *Trichoderma virens* (J.H. Mill., Giddens & A.A. Foster) Arx isolate UPM23, *T. harzianum* isolate UPM40 and a

bacterium namely *P. aeruginosa* isolate UPM13B8 effectively inhibited mycelial growth of *C. truncatum* under in vitro conditions, *and* were selected for further tests under greenhouse conditions. Seeds treated with 2.2 x10^5 conidia for *T. virens,* and 2.4x 10^5 conidia $seed^{-1}$ for *T. harzianum* and 2.4x 10^5 conidia seed-1 for *P. aeruginosa* resulted in a significant increase in seed germination, seedling emergency and length. The highest germination was recorded for seeds treated with UPM23 (98.0%) and UPM40 (97.4%), and seedling establishment was significantly higher in UPM40 (98.0%) treated seeds followed by UPM23 (96.0%), UPM13B8 (92.0%) and the control (93.0%). Although treating seeds with *P. aeruginosa* resulted in a healthy seedling stand with higher fresh and dry weights, both *T. virens and T. harzianum* did not result in significant increase of fresh and dry weights of seedlings.

Colletotrichum capsici causes serious dieback and anthracnose fruit rot of chilli. Sowing infected and infested seed lots may result in a significant reduction of seedling emergence. Seed treatment with *T. harzianum* + thiram gave maximum seedling emergence and reduced seed rot (Khilendra et al. 2009). Similarly, Suthin-Raj and Christopher (2009) observed that treating chilli seed lots with talcum based formulations of 2.8×10^7 cfu g^{-1} *P. fluorescens* (5g/kg seed) and 1.9×10^7 cfu g^{-1} *T. harzianum* (10g/kg seed) reduced the incidence of *C. capsici* (25.0 and 24.1%, respectively) and increased seedling vigour of chilli by 13.7 and 12.1%, respectively.

Padder et al. (2010) evaluated efficacy of biocontrol agents, *T. viride*, *T. harzianum* and *Gliocladium virens* J.H. Mill., Giddens & A.A. Foster, and five biopesticides, Achook, Neemgold, Wannis, Spictaf and Neemazal, in controlling seed-borne *C. lindemuthianum* associated with common bean seed. Although biocontrol agents significantly reduced mycelial growth under in vitro conditions, sowing seed treated with biocontrol agents under greenhouse conditions failed to reduce the incidence and severity of anthracnose disease compared to the

biopesticides. In this investigation, evaluations might have been conducted too early before biocontrol agents could have colonized beyond 2 cm depth of the rhizosphere from the seed to observe significant effects on disease development (Howell 2003).

Performance of different bioagents, viz. *T. viride*, *T. harzianum*, *Trichoderma hamatum* (Bonord.) Bainier and *G. virens* were compared for their effectiveness in controlling anthracnose disease of beans under in vivo conditions (Padder and Sharma 2011)**.** Coating seed lots with biocontrol agents significantly reduced anthracnose disease compared to when biocontrol agents were mixed in the soil. All seeds treated with bioagents recorded a significant increase in seed germination of more than 73.0% compared to the control (65.5%), where the maximum increase was recorded on seeds treated with *T. viride* (85.8%) and the lowest on seeds treated with *T. hamatum* (79.3%). *T. viride* had the most disease suppressing effect (86.4% reduction), which was comparable to that of *T. harzianum*.

Farmers in Ethiopia rely on the use of last season's saved seed for cultivation of haricot beans. As seeds act as a source of inoculum of *C. lindemuthianum*. Mohammed et al. (2014) investigated the effect of coating naturally infected seed lots with Talc based formulations (2.8 x 10^{-6} cfu/g product) of *T. viride* and *T. harzianum applied at* 40 g/Kg of seeds and *P. fluorescence applied at* 10 g/Kg of seeds to reduce losses from anthracnose disease. Naturally infected seed lots of the Mexican 142 variety were treated. Assessments of disease showed that incidence and severity of anthracnose disease was more plants grown from untreated seed lots than on seed lots treated with biocontrol agents. Thus, at 81 day after planting the incidence and severity level of the disease on untreated plot were 93.3 and 86.3%, respectively. In addition, lower hundred seed weight with the most infected pods per plant were recorded on plants grown from untreated seeds. Plants grown from seeds treated with *P. fluorescence*, *T.*

harzianum, and *T. viride* recorded a disease incidence of 76.7, 70.0 and 66.7%, respectively. The severity of anthracnose disease was 45.7, 65.9 and 57.9%, respectively.

2.8 Conclusion

In trying to understand seedling losses experienced in nurseries, the health status of *Eucalyptus* seed produced in local seed orchards is questioned. This review covers aspects of seed morphology to understand how seeds become infected and/or infested and serve as sources of primary inoculum of diseases. This review connected the different seed components with possible location of seed-borne mycoflora for optimising detection an insight that may help in selecting effective seed treatments. Currently, majority of foresters harvest and store their seed without applying any seed treatment. Implications of seed-borne *Colletotrichum* spp., *Colletotrichum fructicola* and *C. kahawae* subsp. cigarro associated with *Eucalyptus* seed lots in forestry productivity and seed trade indicated the potential danger of introduction of diseases into nurseries; thereby motivating the need for effective management. Since effective disease management relies on accurate pathogen detection, challenges of using morphological techniques to identify *Colletotrichum* spp. were highlighted, but molecular techniques have also their limitations particularly in distinguishing *Colletotrichum kahawae* up to the subspecies level. As one of the loophole in biosecurity regulation, the implications associated with this complex subject were highlighted. Furthermore, the increase in registration of commercial forests to attain the global Forest Stewardship Council certification calls for restriction of use of synthetic chemicals in plant disease management in forestry production. This also implies the need for strict environmentally benign ways of forestry production from seedlings throughout plantation fields until harvesting. Today, few seeds and seedlings are produced complying with these regulations. Hence, the focus on non-chemical means of seed sanitation. Studies have shown various non-chemical methods such as hot water seed

treatments, microwave radiation, biocontrol agents and hydrogen peroxide to be effective against seed-borne *Colletotrichum* associated with seeds of vegetables and field crops. In this regard, these methods are covered in this review as alternative replacements of chemical seed sanitation of *Eucalyptus* seeds infected and/or infested with *C. fructicola* and *C. kahawae* subsp. *cigarro*.

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Table 2: Seed-borne fungi associated with *Eucalyptus* spp. seed

Fungal species	*Eucalyptus* species	References
Acremonium rutilum W. Gams	*E. pellita*	Yuan et al. (1997)
Acremonium strictum W. Gams	*Eucalyptus* spp.	Reddy et al. (1982); Mittal et al. (1990)
Acrostaphylus lignicola Subram.	*E. tereticornis*	Reddy et al. (1982); Mittal et al. (1990)
Alternaria alternata (Fr.) Keissl.	*E. camaldulensis, E. grandis, E. nitens, E. pellita*	Yuan et al. (1990); Harsh et al. (1992); Yuan et al. (1997)
Aspergillus alutaceus Berk. & M.A. Curtis	*Eucalyptus* spp.	Mehrotra and Singh (1998)
Aspergillus candidus Link	*Eucalyptus* spp.	Reddy et al. (1982); Mittal (1985); Mittal et al. (1990)
Aspergillus candidus Link	*Eucalyptus* spp.	Reddy et al. (1982); Mittal et al. (1990)
Aspergillus flavipes (Bainier & R. Sartory) Thom & Church	*Eucalyptus* spp.	Reddy et al. (1982); Mittal (1985); Saxena (1985); Pongpanich (1990)
Aspergillus flavus Link	*E. alba, E. deglupta, Eucalyptus* hybrid	Mittal (1986); Yuan et al. (1997); Mehrotra and Singh (1998)
Aspergillus fumigatus Fresen.	*E. alba, E. deglupta, Eucalyptus* hybrid	Mittal (1985); Mittal (1986); Mittal et al. (1990); Pongpanich (1990)
Aspergillus nidulans (Eidam) G. Winter	*E. deglupta*	Mittal et al. (1990); Pongpanich (1990)
Aspergillus niger Tiegh.	*E. alba, E. camaldulensis, E. deglupta, E. globulus, E. grandis, E. nitens, E. pellita*	Yuan et al. (1990); Harsh et al. (1992); Mehrotra and Singh (1998)
Aspergillus sulphurous (Fresen,) Thom & church	*Eucalyptus* spp.	Mittal (1985); Mittal et al. (1990); Mehrotra and Singh (1998)
Aspergillus sydowii (Bainier & Sartory) Thom & Church	*Eucalyptus spp., Eucalyptus hybrid*	Reddy et al. (1982); Mittal (1985); Mittal (1986); Mittal et al. (1990)
Aspergillus terreus Thom	*Eucalyptus* spp.	Reddy et al. (1982); Mwanza and Kellas (1987); Mittal et al. (1990)
Aspergillus unguis (Émile-Weill & L. Gaudin) Thom & Raper	*Eucalyptus* spp.	Reddy et al. (1982); Mittal et al. (1990)
Bipolaris tetramera (McKinney) Shoemaker	*Eucalyptus* spp.	Saxena (1985); Mittal et al. (1990)
Botryodiplodia sp.	*E. grandis*	Mittal et al. (1990)
Botrytis cinerea Pers.	*E. camaldulensis*	Saxena (1985); Mittal et al. (1990); Yuan et al. (1990)
Cephalosporium sp.	*E. deglupta, E. globulus*	Mittal et al. (1990)

Chaetomium cochliodes Palliser	*E. camaldulensis, E. globulus, E. grandis, E. pellita*	Yuan et al. (1990); Yuan et al. (1997)
Chaetomium funicola Cooke	*E. pellita*	Yuan et al. (1997)
Chaetomium globosum Kunze	*E. pellita*	Saxena (1985); Mittal et al. (1990); Yuan et al. (1990)
Choanephora cf. *cucurbitarum* (Berk. & Ravenel) Thaxt.	*E. pellita*	Yuan et al. (1997)
Cladosporium cladosporioides (Fresen.) G.A. de Vries	*E. camaldulensis, E. pellita*	Harsh et al. (1992); Yuan et al. (1997); Mehrotra and Singh (1998)
Cladosporium herbarum (Pers.) Link	*Eucalyptus* spp.	Reddy et al. (1982); Saxena (1985); Mittal et al. (1990)
Cladosporium orchidis E.A. Ellis & M.B. Ellis	*E. pellita*	Yuan et al. (1997)
Cladosporium tenuissimum Cooke	*Eucalyptus* spp.	Reddy et al. (1982); Mittal et al. (1990)
Coniella australiensis Petr.	*E. pellita*	Yuan et al. (1997)
Coniochaeta ligniaria (Grev.) Cooke	*E. pellita*	Yuan et al. (1997)
Curvularia eragrostidis (Henn.) J.A. Mey.	*E. alba, E. pelita*	Pongpanich (1990); Yuan et al. (1997)
Curvularia fallax Boedijn	*E. pelita*	Yuan et al. (1997)
Curvularia geniculata (Tracy & Earle) Boedijn	*Eucalyptus* spp.	Reddy et al. (1982); Mittal et al. (1990)
Curvularia lunata (Wakker) Boedijn	*E. camaldulensis, E. deglupta, E. grandis, E. pellita, E. robusta, E. tereticornis*	Pongpanich (1990); Yuan et al. (1997); Mehrotra and Singh (1998)
Curvularia pallescens Boedijn	*E. alba, E. camaldulensis, E. deglupta, E. robusta*	Reddy et al. (1982); Saxena (1985); Mittal et al. (1990); Pongpanich (1990)
Curvularia senegalensis (Speg.) Subram.	*E. camaldulensis, E. laevopinea, E. nitens*	Yuan et al. (1990); Yuan et al. (1997)
Curvularia verruculosa Tandon & Bilgrami ex M.B. Ellis	*Eucalyptus* spp.	Saxena (1985); Mittal et al. (1990); Yuan et al. (1997)
Cylindrocladium scoparium Morgan	*Eucalyptus* spp.	Mittal et al. (1990)
Dothiorella eucalypti (Berk. & Broome) Sacc.	*E. camaldulensis*	Farr et al. (1989)
Drechslera australiensis Bugnic. ex M.B. Ellis	*E. pelita*	Saxena (1985); Mittal et al. (1990); Yuan et al. (1997)
Drechslera spicifera (Bainier) Arx	*E. camaldulensis*	Yuan et al. (1990)
Emericella nidulans (Eidam) Vuill.	*Eucalyptus* spp.	Reddy et al. (1982)

Epicoccum nigrum Link	*E. camaldulensis, E. grandis, E. nitens*	Mwanza and Kellas (1987); Yuan et al. (1990)
Exserohilum rostratum (Drechsler) K.J. Leonard & Suggs	*E. saligna, E. tereticornis*	Reddy et al. (1982); Saxena (1985); Mittal et al. (1990)
Fairmaniella leprosa (Fairm.) Petr. & Syd.	*E. camaldulensis*	Farr et al. (1989)
Fusarium equiseti (Corda) Sacc.	*E. deglupta*	Saxena (1985); Mittal et al. (1990)
Fusarium graminearum Schwabe	*Eucalyptus* spp.	Mehrotra and Singh (1998)
Fusarium moniliforme J. Sheld.	*E. camaldulensis, E. grandis, E. tereticornis*	Reddy et al. (1982); Saxena (1985); Mittal et al. (1990)
Fusarium oxysporum Schltdl. Emen. W.C. Snyder & H.N. Hansen	*E. deglupta*	Saxena (1985); Mittal et al. (1990)
Fusarium poae (Peck) Wollenw	*Eucalyptus* spp.	Saxena (1985); Mittal et al. (1990)
Fusarium semitectum Berk. & Ravenel	*E. camaldulensis, E. globulus*	Saxena (1985); Mittal et al. (1990); Mehrotra and Singh (1998)
Fusarium solani (Mart.) Sacc.	*E. camaldulensis*	Mittal (1985); Mittal et al. (1990); Yuan et al. (1990)
Gliocladium roseum Bainer	*E. camaldulensis, E. pelita*	Yuan et al. (1990); Yuan et al. (1997)
Gloeosporium capsularum Cooke & Harkn.	*Eucalyptus* spp.	Farr et al. (1989)
Harknessia fumaginea B. Sutton & Alcorn	*E. pelita*	Yuan et al. (1997)
Harknessia hawaiiensis F. Stevens & P.A. Young	*E. pelita*	Yuan et al. (1997)
Harknessia uromycoides (Speg.) Speg.	*E. globulus, E. odorata*	Farr et al. (1989)
Humicola cf. *uscoatra* Traaen	*E. pelita*	Yuan et al. (1997)
Lewia infectoria (Fuckel) M.E. Barr & E.G. Simmons	*Eucalyptus* spp.	Saxena (1985); Mittal et al. (1990)
Macrophomina phaseolina (Tassi) Goid.	*Eucalyptus* spp.	Saxena (1985); Mittal et al. (1990)
Memnoniella echinata (rivolta) L.D. Galloway	*E. camaldulensis, E. tereticornis*	Reddy et al. (1982); Mittal (1986); Mittal et al. (1990); Yuan et al. (1990)
Monocillium sp.	*E. globulus, E. grandis, E. tereticornis*	Mittal et al. (1990)
Mucor hiemalis Wehmer	*Eucalyptus* spp.	Saxena (1985); Mittal et al. (1990)
Mucor plumbeus Bonord.	*Eucalyptus* spp.	Mwanza and Kellas (1987)
Myrothecium roridum Tode: Fr.	*E. grandis*	Saxena (1985); Mittal et al. (1990)
Nectria sp.	*E. pelita*	Yuan et al. (1997)
Paecilomyces spp.	*E. alba, E. deglupta, E. globulus*	Mittal et al. (1990); Pongpanich (1990)

Penicillium albicans Biner	*Eucalyptus* hybrid	Mittal (1985); Mittal (1986); Mittal et al. (1990)
Penicillium brevicompactum Dierckx	*Eucalyptus* spp.	Mwanza and Kellas (1987)
Penicillium *chrysogenum* Thom	*Eucalyptus* hybrid; *Eucalyptus* spp.	Saxena (1985); Mittal et al. (1990); Harsh et al. (1992)
Penicillium citrinum Thom	*Eucalyptus* spp.	Reddy et al. (1982); Mittal et al. (1990); Mehrotra and Singh (1998)
Penicillium dodgei Pitt	*Eucalyptus* spp.	Mittal (1985); Mittal et al. (1990); Mehrotra and Singh (1998)
Penicillium expansum Link	*E. pelita*	Mittal (1985); Mittal et al. (1990); Yuan et al. (1997)
Penicillium glabrum (Wehmer) Westling	*E. camaldulensis, E. grandis*	Saxena (1985); Mittal et al. (1990); Yuan et al. (1990)
Penicillium kloeckeri Pitt	*Eucalyptus* spp.	Saxena (1985); Mittal et al. (1990)
Penicillium olsonii Bainier & Sartory	*Eucalyptus* spp.	Mwanza and Kellas (1987)
Penicillium spinulosum Thom	*Eucalyptus* spp.	Mwanza and Kellas (1987)
Periconia spp.	*Eucalyptus* spp.	Mittal et al. (1990)
Pestalotia sp.	*E. deglupta*	Quiniones and Zamora (1987); Mittal et al. (1990)
Pestalotiopsis disseminata (Thüm.) Steyaert	*E. pellita*	Yuan et al. (1997)
Pestalotiopsis funerea (Desm.) Steyaert	*E. alba, E. grandis*	Mittal et al. (1990); Yuan et al. (1997)
Pestalotiopsis mangiferae (Henn.) Steyaert	*E. tereticornis*	Reddy et al. (1982); Mittal et al. (1990)
Pestalotiopsis neglecta (Thüm.) Steyaert	*E. pellita*	Yuan et al. (1997)
Phoma eucalyptica Sacc.	*E. amplifolia, E. angulosa, E. coccifera, E. dives, E. fastagata, E. kybeanensis, E. laevopinea, E. microcerys, E. nidularis, E. pauciflora*	Girard (1973)
Pithomyces maydicus (Sacc.) M.B. Ellis	*E. tereticornis*	Reddy et al. (1982); Mittal et al. (1990)
Preussia sp.	*E. pellita*	Yuan et al. (1997)
Ramularia sp.	*E. cebra, E. drepanophylla, E. melanoploia, E. populnea*	Drake (1974); Drake (1981); Mittal et al. (1990)
Rhizopus oryzae Went & Prins. Geerl.	*Eucalyptus* spp.	Mittal (1985); Saxena (1985); Mittal (1986); Mittal et al. (1990)
Rhizopus stolonifer (Ehrenb.) Vuill.	*E. camaldulensis, E. globulus, E. grandis, E. pellita*	Mwanza and Kellas (1987); Yuan et al. (1990); Yuan et al. (1997)
Spicaria sp.	*Eucalyptus* spp.	Mehrotra and Singh (1998)
Stachybotrys atra Corda	*E. nitens, E. tereticornis*	Reddy et al. (1982); Saxena (1985); Mittal et al. (1990); Yuan et al. (1990)
Stachybotrys sp.	*E. globulus*	Mittal et al. (1990)

Syncephalastrum racemosum Cohn ex J. Schröt.	*E. alba, E. pellita*	Mittal et al. (1990); Pongpanich (1990); Yuan et al. (1997)
Teratosphaeria	*E. grandis*	Jimu et al. (2015)
Thamnostylum lucknowense (J.N. Rai, J.P. Tewari & Mukerji) Arx & H.P. *Upadhyay*	*Eucalyptus* hybrid	Mittal (1986); Mittal et al. (1990)
Torula sp.	*Eucalyptus* hybrid	Harsh et al. (1992)
Trichoderma viride Pers.	*E. globulus, E. pellita, Eucalyptus* hybrid	Mittal (1985); Mittal (1986); Mittal et al. (1990); Yuan et al (1990)
Trichothecium roseum (Pers.) Link	*E. camaldulensis, Eucalyptus hybrid, E. pellita*	Saxena (1985); Mittal et al. (1990); Yuan et al. (1990)
Ulocladium atrum Preuss	*Eucalyptus* spp.	Mwanza and Kellas (1987)
Verticillium albo-atrum Reinke & Berthold	*Eucalyptus* spp.	Saxena (1985); Mittal et al. (1990)
Xylaria sp.	*Eucalyptus* spp.	Mehrotra and Singh (1998)

CHAPTER 3

Health status and seed germination of *Eucalyptus* spp. seed lots produced in South Africa

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 produced in South Africa. A total of 35 fungal species from 29 genera were identified from 14 different *Eucalyptus* species. *Eucalyptus nitens* seed lot was the most infested (88.6%), whereas the lowest incidence of fungi was from *E. dorrigoensis* seed lot (28.9%). *Penicillium* was the most abundant fungus (49.9%). *Colletotrichum*, *Aureobasidium* and *Disculoides* are recorded for the first time associated with *Eucalyptus* seeds. There was a significant reduction in seed germination of *Eucalyptus* seed lots inoculated with seed-borne fungi. Seeds inoculated with either *Fusarium oxysporum* or *F. solani* reduced seed germination the most on *E. badjensis*, *E. dorrigoensis*, *E. nitens*, *E. pellita*, *E. teritecomis* and *E. urophylla* seed lots (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 *E. benthamii* seed lots (29.8%) inoculated with *C. gloeosporioides,* whilst germination of *Botrytis* sp. or *F. solani* inoculated seed of *E. grandis*, *E. smithii* and *E. viminalis* was reduced to 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, which suggests that infested seeds may be a primary reason for poor seed germination.

Key words: Seed-borne; seed germination, *Colletotrichum*, *Disculoides; Fusarium*

3.1 Introduction

Eucalyptus is second to *Pinus* in global importance as plantation trees (Chen et al. 2013; Phiri 2013). In South Africa, 80 % of the total pulpwood sales with a gross value of more than ZAR 8 billion are derived from eucalypts (Clarke 2018). In a bid to boost productivity, eucalypt plantations are intensively managed under short rotation cycles of six to ten years (Gabrielle et al. 2013). In this regard, thousands of hectares of tree stands are clear-felled every year, and approximately 100 million seedlings grown in forest nurseries are required for plantation regeneration (FSA 2012; SGASA 2018).

The ideal for foresters is to obtain high seedling survival rates above 85% (Stape et al. 2001), but delay of seedling emergence and poor survival of seedlings remain as 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 *Eucalyptus* seed lot, chaff and other debris are found on naturally harvested seed lots together with a variety of microorganisms present at least in small quantities (Boland et al. 1980). This facilitates infestation of clean seed batches through cross contamination during processing and storage (Agarwal and Sinclaire 1997). 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).

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 seed trade (Elmer 2001; Santini et al. 2013). The rise in seed trade in the last decades has increased the risk of spread of forestry pathogens such as *Botryosphaeria*, *Lasiodiplodia*, *Mycosphaerella* and *Teratosphaeria* (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 (Bulman et al. 2018; Cleary et al. 2019; Graziosi et al. 2019). Hence, regular seed health tests are a prerequisite as decision-making tools for reducing inoculum loads on seeds.

Although reports on seed-borne mycoflora associated with *Eucalyptus* appeared from time to time (Mittal 1986; Farr et al. 1989; Mittal et al. 1990; Pongpanich 1990; Mehrotra and Singh 1998), results of 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. According to literature, Jimu et al. (2015) was the only study that investigated the mycoflora associated with *Eucalyptus* seed samples produced in South Africa. Since this study narrowly focused on communities associated with *Eucalyptus grandis* W. Hill ex Maiden seed samples, the diversity of seed-borne mycoflora associated with various local provenances of *Eucalyptus* seed lots largely remains unknown. Therefore, the aim of this study was to investigate seed-borne mycoflora associated with commercial seeds of 14 different *Eucalyptus* spp. and evaluate their effect on seed germination.

3.2 Materials and methods

3.2.1 Source of seed

A total of 12 different *Eucalyptus* spp. (Table 3.1), 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.

3.2.2 Seed health tests

Seed-borne mycoflora associated with *Eucalyptus* spp. seed lots were detected using the agar plate method. A weighed replicate of 0.1 g of *Eucalyptus* spp. seeds 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 wrapped 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 ultra violet (UV) (365 nm) light and darkness. Fungal genera and species were identified with the aid of various morphological references of Ellis and Ellis (1997), Mathur and Kongsdal (2003) and Leslie and Summerell (2006). Incidences of seed-borne fungal species were determined and recorded. Fungal isolates were stored on PDA slants at 4 °C for further experiments.

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 pair 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 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 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 3.2).

3.2.3 Seed germination tests

The effect of molecularly identified seed-borne fungi on seed germination were evaluated *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). The concentration of inoculum was adjusted to $1x10^5$ spores/mL. Surface sterilised seed lots of each *Eucalyptus* sp. were inoculated with an inoculum of each fungus by soaking in 10 mL inoculum contained in a 150 mm glass Petri dish for 5 h. 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 (International Seed Testing Association (ISTA) 2019). 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 temperature was maintained at 25±1 °C. After 21 days, assessments of seed germination was done

according to ISTA (2019). Results of the experiment were scores of either germinated seeds or diseased seedlings, where diseased seedlings were identified as those with discolorations on hypocotyl or seminal roots.

3.2.4 Seed-borne mycoflora pathogenicity assays

Pathogenicity tests 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 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. From 5-day-old cultures, 5 mm diameter mycelial plugs from the edges were placed, with the top side facing down, on a sterilised leaf surface and leaves were aligned on two layers of moistened Whatman No.1 filter papers in glass Petri dishes. Inoculated *Eucalyptus* leaves were maintained in a walk-in growth chamber at 25±1 ℃. Control leaves were inoculated with 5 mm diameter agar plugs without fungi. Visual assessments of symptom development were recorded with descriptions of leaf spots after five days of incubation, and the experiment was repeated.

3.2.5 Data analysis

Results of germination tests from experiment one and two 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.

3.3 Results

3.3.1 Seed health status

In this study, a total of 29 fungal genera 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. *Eucalyptus nitens* seed lot was the most infested (92.4%), whereas the lowest incidence of fungi occurred on *E. dorrigoensis* seed lot (28.9%) (Table 3.1). Taxonomic composition assessments showed a predominance by three genera: *Penicillium* (49.9%), followed by *Aspergillus* (8.1%) and *Alternaria* (7.4%). Genera rarely isolated in order of frequency included *Stachybotrys*, *Ulocladium*, *Aureobasidium* and *Disculoides*. Confirmation of 16 randomly selected seed-borne fungal isolates exhibited high similarities with ITS sequences of reference isolates from GenBank (Table 3.2).

3.3.2 Seed germination tests

Results of seeds that germinated from *Eucalyptus* seed lots inoculated with seed-borne fungi are given in Table 3.3. Highest seed germination percentages were from non-inoculated seed lots, where *E. dunnii*, *E. teritecomis* and *E. urophylla* seed lots had seedling germination above 90%. However, seed germination was significantly reduced when seeds were inoculated with seed-borne fungi ($p<0.05$).

There were no significant differences ($p>0.05$) in percentages of seed germination of *E. badjensis*, *E. dorrigoensis*, *E. nitens*, *E. pellita*, *E. teritecomis* and *E. urophylla* seed lots, inoculated with either *Fusarium oxysporum* (31.3, 30.5, 38.8, 30.5, 25.0, 33.3 and 31.3 %, respectively) or *F. solani* (33.5, 30.0, 37.0, 32.3, 26.8, 31.8 and 33.5 %, respectively) when compared to their respective controls, from which the lowest seed germination percentages were recorded (Table 3.3). A statistically similar trend was observed for *E. badjensis* (42.0%), *E. benthamii* (29.8%), and *E. dorrigoensis* (38.0%) seeds inoculated with *C. gloeosporioides*

when compared to their controls. In addition, seed germination was significantly reduced on *E. grandis, E. smithii* and *E. viminalis* seed lots inoculated with either *Botrytis* sp. or *F. solani* (37.0, 37.5%; 35.8, 36.3%; 28.3, 30.0%, respectively) when compared to their respective controls. On the contrary, inoculating *Eucalyptus* seed lots inoculated with *S. polyspora* and *Chaetomium* sp. had the least effect on seed germination. Germination was affected most by *Botrytis* sp. in *E. benthamii* and *E. viminalis* seedlots and by *Colletotrichum* in .*E. benthamii*. Germination was the most affected by *F. oxysporum* in *E. nitens* and *F. solani* in *E. macathurii* and *E. nitens* (Table 3.3).

Seeds inoculated with seed-borne fungi yielded significantly higher numbers of diseased seedlings ($p<0.05$) compared with controls. 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, 51.5, 51.5, 57.8, 60.0, 55.0 and 57.5%, respectively) or *F. solani*. (60.8, 53.0, 53.0, 55.0, 57.5, 57.3 and 54.3%, respectively) when compared to their respective controls (Table 3.4). 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 3.4).

3.3.3 Effects of seed-borne fungi on detached leaves

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. or *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 *Eucalyptus* leaves inoculated with any of *Aureobasidium*, *Chaetomium*, *Gliocladium* and *Sydowia* species.

3.4 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. Over the years, *Eucalyptus* seed health testing was done occasionally but is becoming common practice as several countries implement stricter phytosanitary regulations in the trade of agricultural products including live plants and seed (Cleary et al. 2019). Apart from a study on *E. grandis* by Jimu et al. (2015), this is the first comprehensive study profiling seed-borne mycoflora associated with several species of *Eucalyptus* seeds produced in South Africa.

This study showed that *Eucalyptus* seed lots were naturally infested with several fungi, where the highest incidence was recorded on *E. nitens* seed lot (92.4%) and the least on *E. dorrigoensis* (28.9%). Variation of incidences of fungi on *Eucalyptus* seed samples can be attributed to the influence of external environment where commercial seed is cultivated, collected or processed (Cram and Fraedrich 2010). Moreover, the season seeds were 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, where 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).

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). In this study, a total of 29 fungal genera were found naturally associated with *Eucalyptus* seed lots, and Jimu et al. (2015) detected 31 fungal genera on a *E. grandis* seed lot. This difference could be due to the type of method used to isolate and quantify seed-borne fungi. Although the media culture based approach is cheap, it is limited in detecting certain fungal groups, such as basidiomycetes, that seldom produce sexual structures in culture upon which identification is based. Similarly, estimates of fungal incidence in this study were conservative as several isolates were left unidentified as fungi did not sporulate. In addition, it is challenging to isolate slow growing fungi using cultivation based techniques. For example, *Teratosphaeria* was previously detected by Illumina MySeq sequencing on *E. grandis* seeds (Jimu et al. 2015), but could not be isolated from any of the commercial seed lots tested in their study. It is likely that seed health assays were terminated too early before visual growth of *Teratosphaeria*, a slow growing fungus taking about six weeks at 25 °C to reach a diameter of 40–50 mm (Cortinas et al. 2006). Furthermore, it is difficult to isolate slow growing fungi with the presence of saprotrophs and other fungi that rapidly grow on media inhibiting and obscuring slow growing fungi.

It is also likely that important soil-borne fungi such as *Fusarium* spp. might have been introduced on seeds at harvesting as capsules often fall on the floor of seed orchards, and may contaminate other seed batches during grading and storage prior their entry in nurseries. 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. To our knowledge, this is the first report of *Aureobasidium* and

Disculoides associated with *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 was intercepted on imported *Eucalyptus leucoxylon* F. Muell. and 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).

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 germinants. Seed germination was less than 62% and as low as 25%, which potentially translates to low chances of seedling survival in nurseries. Taxonomic composition assessments showed that *Eucalyptus* seeds were predominantly infested with saprotrophs, *Penicillium* (51%), *Aspergillus* (8.3%) and *Alternaria* (7.5%), and such fungi are often neglected as they are regarded as common storage fungi with little or no effect on seed germination. However, *in - vitro* seed germination assays showed that seeds inoculated with storage fungi such as *Nigrospora* and *Gliocladium* had significantly lower seed germination percentages than controls ($p<0.05$). Similarly, saprotrophs such as *Penicillium*, *Aspergillus* and *Mucor* were previously reported to cause significant reduction of *Eucalyptus* seed germination and seedling emergence (Yuan et al. 1997; Doshi et al. 1993).

All commercial seed lots examined in this study were susceptible to fungal infection following inoculation of seeds. Although seed-borne fungi significantly reduced seed germination, it is

important to note that concentrations of inoculum ($1x10^5$ spores/mL) used in this study were excessively high as opposed to what occurs in in nature as demonstrated by results of seed health tests of this study. *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).

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, expression of disease symptoms vary depending on environmental conditions of greenhouse, growth chamber or lab bioassays. *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). Spore load per unit leaf surface area translating to tissue infection and disease development differ depending on the overall integrity of the plant tissues and environmental or weather conditions (Hayden et al. 2011).

In conclusion, findings of this study showed a large diversity of fungi associated with commercial *Eucalyptus* seed lots. Furthermore, many of these fungi result in 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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Table 3.1: Incidences of fungi (%) associated with commercial *Eucalyptus* spp. seed lots produced in South Africa

Fungi	*Eucalyptus* species												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

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
Total	40.8	46.9	28.9	34.0	84.3	55.2	92.4	52.7	53.0	48.1	29.7	61.3	

Table 3.2: 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

Table 3.3: Effects of inoculation of seed with fungi isolated from *Eucalyptus* seed lots on percentage seed germination

	***Eucalyptus* species**											
Treatment	*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)

Table 3.4: Effects of inoculation of seed with fungi isolated from *Eucalyptus* seed lots on percentage diseased seedlings

	***Eucalyptus* species**											
Treatment	*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)

CHAPTER 4

Anthracnose leaf spot pathogens, *Colletotrichum fructicola* and *Colletotrichum kahawae* subsp. *cigarro*, associated with *Eucalyptus* seed produced in South Africa

Abstract

The high cost of *Eucalyptus* seed, together with adoption of strict phytosanitary regulation on seed trade makes it imperative to monitor the quality and ensure supply of pathogen-free seeds for both forestry regeneration and research. From seed health tests, two species in the *Colletotrichum gloeosporioides* species complex were found to be naturally associated with *Eucalyptus dunnii*, *E. nitens* and *E. macarthurii* seed lots produced in South Africa. Multiloci phylogenetic analyses based on the concatenated sequences of the ITS regions, *β*-tubulin, actin, and glyceraldehyde-3-phosphate dehydrogenase genes, identified representative isolates PPRI 24314 as *Colletotrichum fructicola* and PPRI 24315 as *C. kahawae*. In addition, biochemical tests confirmed identity of PPRI 24315 to be *C. kahawae* subsp. *cigarro* as it was able to utilise either ammonium tartrate or citric acid as a sole carbon source. Pathogenicity tests showed that both *C. fructicola* and *C. kahawae* subsp. *cigarro* caused anthracnose leaf spot on *E. camaldulensis*, *E. dunnii*, *E. nitens* and *E. viminalis* seedlings. Disease symptoms included irregular dark-brown leaf spots on seedlings six days after inoculation. The two fungi were exclusively re-isolated from disease spots, thereby fulfilling Koch's postulates. Sowing *Eucalyptus* spp. seed artificially inoculated with either of the two pathogens showed the seed-transmissibility of *C. fructicola* and *C. kahawae* in *E. camaldulensis, E. dunnii* and *E. nitens* seedlings. To our knowledge, this is the first report of *C. fructicola* and *C. kahawae* subsp. *cigarro* associated with *Eucalyptus*.

Key words: Anthracnose, *Colletotrichum* spp., *Eucalyptus* seed, seed-borne, seed-transmitted

4.1 Introduction

Anthracnose caused by *Colletotrichum* spp. is one of the most devastating plant diseases affecting commercial nursery seedlings and tree plantations globally (Shivanna 2005; Peres et al. 2008; Rodrigues et al. 2014). In South Africa, anthracnose leaf spot disease is commonly reported in *Eucalyptus* plantations, where above ground plant parts of susceptible hybrids and clones may be infected, resulting in partial or complete defoliation, or is later manifested as twig dieback of terminal leader shoots of trees (Viljoen et al. 1992; Smith et al. 1998).

Colletotrichum infections are most severe in nurseries with closely spaced seedlings under high temperature conditions and with prolonged exposure to excess moisture (Brown and Ferreira 2000). The disease symptoms appear as necrotic lesions on leaves and stems, as well as seedling blights (Viljoen et al. 1992). Necrotic lesions on leaves are light brown circular spots that are surrounded by a red-purple border in the early stages of symptom development. As the disease progress, the spots become more pronounced, with increased visibility of acervuli containing yellowish to pinkish conidial masses (Viljoen et al. 1992). As lesions coalesce, the photosynthetic leaf area is severely reduced.

Since the genus *Colletotrichum* consists of several cryptic species complexes (Damm et al. 2009; Weir et al. 2012), accurate identification is important not only to understand the epidemiology, but to improve biosecurity and for effective management of plant diseases (Cannon et al. 2012; De Silva et al. 2017b). Although previous surveys have reported *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. as an important pathogen in *Eucalyptus* plantations (Sharma et al. 1984; Smith et al. 1998), with the longstanding taxonomic confusion of *Colletotrichum* species (Hyde et al. 2009), there is need for confirming accuracy of names assigned to these pathogens. Prior to the molecular era, identification of *Colletotrichum* spp. was primarily based on highly variable methods that employed morphological and physiological characters such as conidial size and shape of appressoria, presence or absence of

setae, growth rate, host range etc. (Than et al. 2008). This is the reason several other workers prior to 2009 tended to support the hypothesis that *C. gloeosporioides* was the sole pathogen associated with anthracnose disease occurring on a wide range of hosts around the world (Martínez-Culebras et al. 2000; Benyahia et al. 2003; Lubbe et al. 2004; Phoulivong et al. 2010).

Due to paucity of useful morphological and physiological characters, DNA-sequence based identification methods were widely adopted to resolve taxonomy of *Colletotrichum* species complexes. Initially, multi-locus sequence datasets were not prevalent so single gene regions mainly ITS or *β*-tubulin were used (Cai et al. 2009). Thus, accuracy of species names in many of these previous studies remain doubtful, and authenticity of previously reported species are yet to be confirmed. Multigene phylogenetic analysis has successfully delineated cryptic species within species complexes such as *Colletotrichum acutatum* J.H. Simmonds, *C. gloeosporioides*, etc. (Weir et al. 2012). Following this taxonomic correction, there has been an increase in reports of several *Colletotrichum* species apart from *C. gloeosporioides* causing anthracnose on *Eucalyptus* and other hosts (Rodrigues et al. 2014; Diao et al. 2017; De Silva et al. 2017a; Guarnaccia et al. 2017; Rojas et al. 2018; Shi et al. 2018; Nodel et al. 2019).

In light with reassessments on the systematics of *Colletotrichum* species complexes, this study was initiated to advance knowledge on *Colletotrichum* species associated with *Eucalyptus* seed produced in South Africa. A polyphasic approach that used both morphological characters and multi-gene sequence analysis was employed to confirm identify of *Colletotrichum* species. Furthermore, effects of seed-borne *Colletotrichum* species on seed germination were determined, and their rates of transmission and pathogenicity were evaluated in greenhouse trials.

4.2 Materials and Methods

4.2.1 Screening *Eucalyptus* seed for the presence of *Colletotrichum* species

The presence of *Colletotrichum* species on commercially produced *Eucalyptus* seed lots was determined using the agar plate method. Screening tests were performed on a total of 27 locally produced seed lots belonging to 15 species, *viz. Eucalyptus badjensis* Beuzev. & Welch, *Eucalyptus benthamii* Maiden & Cambage, *Eucalyptus camaldulensis* Dehnh., *Eucalyptus dorrigoensis* (Blakely) L.A.S. Johnson & K.D. Hill, *Eucalyptus dunnii* Maiden, *Eucalyptus globulus* Labill., *Eucalyptus grandis* W. Hill, *Eucalyptus macarthurii* H. Deane & Maiden, *Eucalyptus nitens* (H. Deane & Maiden) Maiden, *Eucalyptus pellita* F. Muell., *Eucalyptus saligna* Sm., *Eucalyptus smithii* F. Muell. ex R.T. Baker, *Eucalyptus tereticornis* Sm., *Eucalyptus urophylla* S.T. Blake, and *Eucalyptus viminalis* Labill. Seed lots were surface disinfected in 1% sodium hypochlorite (NaOCl) solution for five minutes and rinsed three times in sterile distilled water. Seeds were dried under sterile conditions inside a laminar flow cabinet and plated on potato dextrose agar (PDA) (Biolabs, Midrand, South Africa), and incubated for 7 days at 25 ±1 °C under alternating cycles of 12 h ultra violet (UV) (365 nm) light and 12 h darkness. Fungi growing from plated seeds were identified on the basis of their macroscopic and microscopic features (Cai et al. 2009), and incidence of *Colletotrichum* species was recorded. Selected fungal isolates were purified on PDA, stored at 4 °C on PDA slants, and selected isolates were deposited in the National Collection of Fungi, ARC-Plant Health and Protection, Roodeplaat, Pretoria, South Africa.

4.2.2 Molecular identification

Genomic DNA was extracted from 7-day-old *Colletotrichum* cultures using a Zymo Fungal DNA MiniPrep extraction Kit™ (Zymo Research Corp., Irvine, USA) following the manufacturer's instructions. The DNA extracts were used as templates in polymerase chain

reactions (PCR) with amplification of the partial glyceraldehyde-3-phosphate dehydrogenase (GPDH) gene region using primers GDF1 and GDR1 (Templeton et al. 1992); chitin synthase (CHS-1) using primers CHS I-79F and CHS I-354R (Carbone and Kohn 1999); actin (ACT) using primers ACT-512F and ACT7-83R (Carbone and Kohn 1999; Glass and Donaldson 1995); *β*-tubulin (TUB2) using primers T1/Bt2b (Glass and Donaldson 1995; O'Donnell and Cigelnik 1997) and the ribosomal internal transcribed spacer (ITS) region using primers ITS 1 and ITS 4 (White et al. 1990). PCR amplicons were submitted to Inqaba Biotechnical Industries (Pty) Ltd (Inqaba Biotec, Pretoria, South Africa) for Sanger sequencing. From forward and reverse sequences obtained, consensus sequences were compiled using BioEdit (www.mbio.ncsu.edu/BioEdit/BioEdit.html), and subjected to nBLAST searches in GenBank [National Centre for Biotechnology Information (NCBI, http:/www.ncbi.nlm.nih.gov/BLAST)]. Sequences derived in this study were deposited in GenBank at National Centre for Biotechnology Information GenBank (NCBI, www.ncbi.nlm.nih.gov/genbank).

Colletotrichum species sequences from our study, together with reference sequences selected from Weir et al. (2012) were aligned for phylogenetic analyses using MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/index.html). Maximum Likelihood analyses were performed using PhyML 3.0 (Guindon and Gascual 2003). Statistical selection of best-fit models of nucleotide substitution using the Akaike information criteria (AIC), was determined with jModeltest 2.1.7 (Darriba et al. 2012). Initial analyses showed that individual genes were broadly congruent, thus nucleotide alignments of all the genes were concatenated. A TIM3+I+G model was used for the concatenated dataset, and 1000 bootstrap replicates were conducted. *Colletotrichum boninense* Moriwaki, Toy. Sato & Tsukib. (ICMP 17904) and *Colletotrichum hippeastri* Yan L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai (ICMP 17920) were used as outgroups in the analysis (Weir et al. 2012).

4.2.3 Substrate utilisation tests

To determine the subspecies of *Colletotrichum kahawae* isolate, a biochemical assay was conducted following a protocol used by Waller et al. (1993). In this test, agar plugs of 7-day-old cultures were inoculated on to a basal medium (Lynch et al. 1981), supplemented with either ammonium tartrate or citric acid (1% *w/v*). Visual assessments of growth of fungi were done by comparing with positive and negative controls that contained glucose or no additional carbon source, respectively.

4.2.4 Pathogenicity and host range tests

To evaluate the effects of the two *Colletotrichum* species on seed germination, *Eucalyptus* seed lots were surface disinfected by immersion in 1% NaOCl solution for 5 min, rinsed in sterile distilled water and soaked for 10 min in a 1 x 10^5 spores mL^{-1} *Colletotrichum* inoculum suspension. Inoculated seeds were air dried in the laminar flow cabinet for 4 h. Surface disinfected seeds soaked in sterile distilled water served as controls. Thereafter, 25 seeds were plated on top of three layers of sterile moistened blotters in a Glass Petri dish (110 x 17 mm), where a total of 200 seeds were plated out for each seed lot. Plated seeds were incubated in a growth chamber set at 25 ±1 ºC with alternating cycle of 16 h cool white light (58 w Osram fluorescent tubes; Russia) and 8 h dark period. Experimental units were laid in a completely randomised design (CRD), and repeated. Seedlings were evaluated according to International Seed Testing Association (ISTA) rules, where final counts of seed germination and diseased seedlings were recorded after 21 days. Seedlings with discolorations on hypocotyls or seminal roots were scored as “diseased”.

Seed-transmission tests were performed in a greenhouse located at the Hatfield Experimental farm (University of Pretoria, Pretoria, South Africa, latitude: 25° 45' 6.94" S; longitude: 28°15’ 34.69’’ E; 1 380 m above sea level). Inoculated seeds of *E. camaldulensis*, *E. dunnii*, *E. grandis*, *E. macarthurii*, *E. nitens*, and *E. viminalis* were sown singly in pasteurised loamy soil

filled 5 cm diameter pots. Greenhouse conditions were adjusted and maintained at 25 °C /17°C for day-night temperatures, respectively, and plants were watered every second day. Each experiment consisted of fifteen pots in a randomised complete block design and the experiment was repeated. Assessments of transmission of anthracnose disease from seeds into seedlings were done after six months, where number of seedlings that developed anthracnose disease symptoms from inoculated seeds were counted in comparison with seedlings grown from non-inoculated seeds. To confirm if *Colletotrichum* spp. were the cause of leaf spot, pathogens were re-isolated from symptomatic plants. Leaf sections of 5 mm x 5 mm were surface sterilised in 1% NaOCl solution for 5 min, rinsed in sterile distilled water and plated on PDA. After incubation at 25 °C for 5 d fungi were identified.

Pathogenicity of the two *Colletotrichum* species was determined by detached leaf assays. Healthy leaves from six-month-old seedlings of *E. benthamii*, *E. camaldulensis*, *E. dorrigoensis*, *E. dunnii*, *E. grandis*, *E. macarthurii*, *E. nitens*, *E. tereticomis*, and *E. viminalis* were surface sterilized in 70% ethanol, rinsed thrice in sterile distilled water, and transferred to moistened sterilised blotters in Glass Petri dishes. Inoculum of each of the two *Colletotrichum* species was sprayed on leaf surfaces at a concentration of 1x 10^5 spores mL^{-1} until surface runoff. Leaves sprayed with sterilized distilled water served as controls. For each *Eucalyptus* species, two leaves were placed inside a Glass Petri dish, and this was replicated five times. Experimental units were arranged in a CRD in a growth chamber that was maintained at 25 °C with light /darkness cycles of 16 h and 8 h, respectively. Inoculated and non-inoculated leaves were monitored daily until appearance of disease symptoms, where results were recorded either as “symptom” or “symptom-free”.

To investigate susceptibility of different *Eucalyptus* species seedlings to the two *Colletotrichum* species, greenhouse pot assays were performed on six-month-old seedlings of *E. camaldulensis*, *E. dunnii*, *E. grandis*, *E. macarthurii*, *E. nitens*, and *E. viminalis*. Leaf

surfaces of 10 healthy seedlings of each species were surface disinfected by spraying with 70% ethanol and washing with a continuous flow of sterile distilled water. Thereafter, leaf surfaces were sprayed with a 10^5 spores mL^{-1} inoculum of each of the two *Colletotrichum* species until surface run-off, and seedlings were covered with polythene bags for 72 h to maintain a high humidity. Controls also consisted of ten *Eucalyptus* species seedlings that were surface disinfected as above, but were sprayed with sterile distilled water until run-off. To fulfil Koch's postulates, isolations were made from leaves of diseased and healthy plants and the isolated fungi were identified as described above.

Assessments of disease development were done 14 days after inoculation and the experiment was repeated twice. Data of lesions that developed on the leaves were recorded, where percentage disease incidence (DI) and disease severity (DS) were calculated using a formulae described by Awa et al. (2012). Severity of leaf spot disease was scored using a 1 to 5 point scale, where 1 = no visible symptoms, 2 = 1 to 3 lesions, 3 = 4 to 6 lesions, 4 = lesions coalesce to cover <30% of leaf surface area, and 5 = >30% leaf area covered with lesions with severe to complete necrosis of the leaf.

$$\% DI = x/N * 100$$

$$\% DS = \sum(a+b)/(N*Z) * 100$$

Where a = number of infected leaves

b = corresponding score of leaf infection

N= Total number of sampled leaves

Z= Highest score scale

x= Number of infected leaves

The data were arcsine transformed and analysed using SAS v. 9.0 (Statistical Package, Cary, NC, USA), and comparisons between means used the Fisher's least significant difference (LSD) test ($P<0.05$). However, results are presented as untransformed data.

4.3 Results

4.3.1 Morphological Identification

Two morphologically different *C. gloeosporioides*-like strains were isolated from the *Eucalyptus* seed lots. Mycelia in pure cultures of all isolates were light to dark gray. Cultures that produced a white to gray cottony upper surface and an orange to gray appearance on the reverse side, produced cylindrical, aseptate, hyaline conidia that had obtuse to slightly rounded ends, and were 10.5 to 22.5 µm long and 3 to 6.5 µm wide, consistently matched descriptions of morphological characteristics described for *Colletotrichum fructicola* Prihast., L. Cai & K.D. Hyde within the *C. gloeosporioides* species complex (Weir et al. 2012). However, some dark gray cultures produced aerial mycelium with stromatic acervuli, and abundantly formed sclerotia after two weeks. Conidia were hyaline, cylindrical tapering slightly from only one side measuring 16 - 20 × 3.5 - 5.5 µm, and these morphological descriptions matched those of *C. kahawae* J.M. Waller & Bridge (Weir et al. 2012). Purified *C. fructicola* and *C. kahawae* subsp. *cigarro* isolates were assigned South African National Collection of Fungi voucher numbers PPRI 24314 and PPRI 24315, respectively.

Screening *Eucalyptus* seed lots showed natural infection with *C. fructicola* and C. *kahawae* subsp. *cigarro* on *E. dunnii* (3.5 and 0.5%, respectively), *Eucalyptus nitens* (2.6 and 1.2%, respectively), and *E. macarthurii* (0.8% for only *C. fructicola*). However, *Colletotrichum* species were not present on the other *Eucalyptus* species tested.

4.3.2 Molecular identification

To confirm the identity of *C. fructicola* and *C. kahawae* subsp. *cigarro* isolates, the ACT, CHS, GAPDH, ITS and TUB2 gene regions were sequenced (GenBank Accession Nos for PPRI 24314: MK512735, MK512737, MK512733, MG641892 and MK512739; GenBank

Accession Nos for PPRI 24315: MK512736, MK512738, MK512734, MG641893 and MK512740) and subjected to BLAST and Maximum likelihood analyses. The BLAST searches showed that both isolates belonged to the *C. gloeosporioides* species complex. Maximum likelihood analyses of the concatenated dataset of 2260 bp and 50 taxa, with representative reference isolated of all the accepted species in this species complex, gave strong support for PPRI 24314 belonging to *C. fructicola* and PPRI 24315 belonging to *C. kahawae* subsp. *cigarro* (Figure 4.1).

4.3.3 Substrate utilisation test

Biochemical tests showed that the *C. kahawae* isolate PPRI 24315 grew on basal medium containing either glucose or citric acid or ammonium titrate as a sole carbon source, confirming the identity of the isolate as *C. kahawae* subsp. *cigarro*.

4.3.4 Pathogenicity and host range tests

Germination capacity was significantly reduced on all *Eucalyptus* species seed lots inoculated with either *C. fructicola* or *C. kahawae* subsp. *cigarro* (Table 4.1). For *Eucalyptus* species seed lots inoculated with *C. fructicola*, the highest percentage of seed germination was recorded from *E. grandis* (56.8%), and lowest seed germination observed on *E. benthamii* (29.8%). However, seed germination of *Eucalyptus* species seed lots inoculated with *C. kahawae* subsp. *cigarro* ranged from 32.3 to 54.8%, with the highest and lowest percentage seed germination recorded from *E. urophylla* (54.8%) and *E. benthamii* (32.3%), respectively. Seeds of *E. badjensis*, *E. globulus* and *E. tereticornis* were most susceptible to *C. kahawae* subsp. *cigarro*, whereas seeds germination was reduced the most on seeds of *E. camaldulensis*, *E. dorrigoensis*, *E. urophylla* inoculated with *C. fructicola*. There were statistically similar responses to seed germination on *E. benthamii*, *E. dunnii*, *E. grandis*, *E. macarthurii*, *E. nitens*, *E. pellita*, *E. saligna*, *E. smithii* and *E. viminalis* seeds inoculated with either *C. fructicola* or *C. kahawae* subsp. *cigarro*.

Inoculating *Eucalyptus* species seed lots with *Colletotrichum* species significantly increased the incidence of seedlings that developed disease symptoms ($P<0.05$) (Table 2). Disease symptoms initially appeared as slight discolorations with pale brown markings developing on green plant parts of seedlings, which decayed with spread of secondary infection. The number of diseased seedlings grown from *Eucalyptus* species seed lots inoculated with *C. fructicola* ranged from 39.0 to 59.0%. The highest and lowest number of diseased seedlings were grown from *E. benthamii* (59.0%) and *E. camaldulensis* (39.0%), respectively. For *Eucalyptus* species seed lots inoculated with *C. kahawae* subsp. *cigarro*, the number of seedlings that developed disease ranged from 35.5% to 53.8%, where highest and lowest number of diseased seedlings were grown from *E. pellita* (53.8%) and *E. camaldulensis* (35.5%), respectively. Seedlings grown from *E. camaldulensis*, *E. dorrigoensis*, *E. saligna*, *E. smithii* and *E. urophylla* seed lots inoculated with *C. fructicola* were most susceptible to diseases.

Leaf detached assays showed that both *C. fructicola* and *C. kahawae* subsp. *cigarro* were pathogenic on *Eucalyptus* species leaves (Figure 4.2). Six days after inoculation, all leaves showed irregular dark-brown leaf spot, whereas no symptoms were observed on controls. However, in greenhouse pot assays, anthracnose leaf spot appeared on all inoculated *Eucalyptus* seedlings after 11 days of inoculation, except for *E. grandis*, *E. macarthurii* and the non-inoculated control seedlings (Figure 4.3). Anthracnose leaf spot caused by *C. fructicola* and *C. kahawae* subsp. *cigarro* occurred mainly on *E. dunnii* and *E. nitens* (65.0% and 55.0%, respectively), and were most severe on *E. nitens* (67.0% and 63.0%, respectively). Both *C. fructicola* and *C. kahawae* subsp. *cigarro* were re-isolated from diseased seedlings, thereby fulfilling Koch's postulates and confirming them as causal agents of anthracnose leaf spot on *Eucalyptus*.

Sowing *E. camaldulensis*, *E. dunnii* and *E. nitens* seed lots artificially inoculated with *C. fructicola* resulted in transmission of the pathogen into the seedlings (25.5%, 38.3% and 64.0%,

respectively), whereas the same seed lots inoculated with *C. kahawae* transmitted the pathogen in 27.3%, 30.5% and 56.3 of the seedlings, respectively. Contrary, *Eucalyptus grandis* seed lots inoculated with either *C. fructicola* or *C. kahawae* subsp. *cigarro* did not result in transmission of either pathogen into the seedlings.

4.4 Discussion

It is imperative to regularly monitor the health status of seed lots intended for seedling production. Apart from a study by Jimu et al. (2015), no work was previously done to explore seed-borne mycoflora associated with *Eucalyptus* seed lots produced in South Africa. In this study, *C. fructicola* and *C. kahawae* subsp. *cigarro* are reported for the first time associated with *Eucalyptus* species seed lots. Investigations were done to determine the pathogenicity of the two seed-borne fungi, their effects on seed germination and rates of transmission from seed to seedlings.

Screening tests showed that *Colletotrichum* species were naturally associated with *E. dunnii*, *E. nitens* and *E. macarthurii* seed lots. The isolate PPRI 24314 was initially identified morphologically as *C. gloeosporioides* species complex. Despite being less expensive than molecular analysis, morphological assessments are not always able to delineate species boundaries of cryptic species and reliance on these methods has resulted in taxonomic confusion of *Colletotrichum* species. For instance, conidia *of C. gloeosporioides* are described as aseptate, cylindrical with blunt ends, measuring from an average size of 10.4 to 16.7 µm × 4.2 to 5.8 µm (n=100) (Sutton 1992) and may easily be confused with similar closely relating species in the *C. gloeosporiodes* species complex such as *C. fructicola* that produces one celled conidia with obtuse to slightly rounded ends measuring 15.2 to 15.6 × 4.7 to 4.9 µm (n = 45) (Prihastuti et al. 2009).

Today, molecular methods have proven to be more objective in unravelling species richness of *Colletotrichum* species complexes (Chen et al. 2017; Gan et al. 2017; Hassan et al. 2018; Moreira et al. 2019). Based on multi-gene analyses of concatenated ATC, ITS, CHS, GAPDH and *β*-TUB2 sequences, PPRI 24314 isolate was identified as *C. fructicola* and PPRI 24315 as *C. kahawae* subsp. *cigarro.* Although *Colletotrichum* spp. have been identified on *Eucalyptus* spp. in South Africa (Viljoen et al. 1992; Smith et al. 1998), these isolates were no longer available for inclusion in this study. Few studies have successfully distinguished between the subspecies *C. kahawae* subsp. *kahawae* and *C. kahawae* subsp. *cigarro* on the basis of gene analyses of concatenated ATC, ITS, CHS, GAPDH, *β*-TUB2, calmodulin (CAL), glutamine synthetase (GS) and manganese-superoxide dismutase (SOD2) genes (Batista et al. 2017). Failure to distinguish *C. kahawae* to the subspecies level has serious biosecurity implications considering that *C. kahawae* subsp. *kahawae* is listed as a quarantine pathogen outside the African continent (Batista et al. 2017). Furthermore, complementary biochemical assays confirmed the identity of isolate PPRI 24315 to be *C. kahawae* subsp. *cigarro* as it utilised either ammonium tartrate or citric acid as a sole carbon source. This is a distinct characteristic separating it from *C. kahawae* subsp. *kahawae* that cannot metabolise any of the aforementioned substances as sole carbon sources (Waller et al. 1993).

Colletotrichum kahawae subsp. *cigarro* has been reported on various hosts including coffee (*Coffea arabica* L.), *Proteaceae* cut-flowers, tree tomato (*Solanum betaceum* Cav.), blackberry (*Rubus glaucus* Benth.), olives (*Olea europaea* L.), mango (*Mangifera indica* L.) and mandarin (*Citrus reticulate* Blanco) (Liu et al. 2013; Afanador-Kafuri et al. 2014; Mosca et al. 2014; Ismail et al. 2015; Perrone et al. 2016; Rojas et al. 2018). Previous studies have reported *C. fructicola* causing anthracnose lesions on other hosts including spotted laurel (*Aucuba japonica* Thunb.); chilli (*Capsicum* spp.), pear (*Pyrus bretschneideri* Rehder), apple (*Malus pumila* Miller), tea (*Camellia sinensis* (L.) Kuntze), cassava (*Manihot esculenta* Crantz), and fatsia

(*Fatsia japonica* (Thunb.) Decne. & Planch.) (Sharma and Shenoy 2014; Li et al. 2016; Bragança et al. 2016; Shi et al. 2017, 2018; Nodet et al. 2019).

This study demonstrated a significant interaction effect of the two fungi, *C. fructicola* and *C. kahawae*, on seed germination, where they reduced seed germination of *E. benthamii*, *E. dunnii*, *E. grandis*, *E. macarthurii*, *E. nitens*, *E. pellita*, *E. saligna*, *E. smithii* and *E. viminalis* seed lots with a statistically similar effect. Similarly, significantly high percentages of diseased seedlings were comparable for the two pathogens when inoculated on *E. badjensis*, *E. benthamii*, *E. dunnii*, *E. globulus*, *E. grandis*, *E. macarthurii*, *E. nitens*, *E. pellita*, *E. tereticomis* and *E. viminalis* seed lots.

Leaf detached assays and greenhouse pot assays proved the potential of both *C. fructicola* and *C. kahawae* subsp. *cigarro* to cause anthracnose leaf spot on *E. dunnii*, *E. nitens* and *E. viminalis* seedlings. Similarly, several *Eucalyptus* species and hybrids grown in plantations in Mpumalanga and KwaZulu-Natal provinces of South Africa are susceptible to anthracnose leaf spot (Smith et al. 1998). Although previously reported to be caused by *C. gloeosporioides*, anthracnose leaf spot were prominently reported on *Eucalyptus* cuttings and on *E. dives*, *E. grandis* and *E. globulus* seedlings (Viljoen et al. 1992). However, greenhouse pathogenicity investigations in this study showed that *E. grandis* and *E. macarthurii* were not susceptible to anthracnose disease, caused by both *C. fructicola* and *C. kahawae* subsp. *cigarro*. Though causes of these differences are not clear, it is possible that *Colletotrichum* species entered an extended quiescent phase or were only endophytes as previously reported on other hosts (Cannon et al. 2012; O'Connell et al. 2012; Ranathunge et al. 2012; Manamgoda et al. 2013), which might have caused asymptomatic infection of *E. grandis* and *E. camaldulensis*. Smith et al. (1998) reported *C. gloeosporioides* causing leaf spot and dieback on trees exposed to environmental stress such as drought, frost and hot winds. Hence, it is possible that growth

conditions in our study were equally stressful enough to allow similar development of disease symptoms.

In conclusion, this study demonstrated that *C. fructicola* and *C. kahawae* subsp. *cigarro* are both seed-borne and seed-transmitted but their overall impact of the two *Colletotrichum* species on *Eucalyptus* seedling production in nurseries is not clear at this stage. Although pathogenicity tests showed high infection and more severe anthracnose leaf spot on *Eucalyptus* leaves, this was derived with artificially inoculated seed lots. Effects of seed-borne pathogens on plant growth and development depends on its transmissibility, but seed health tests showed that natural incidences of *Colletotrichum* spp. on *Eucalyptus* seed lots were low. Thus, local transmission via the seed pathway may not be as important as airborne dispersal; however, long distance transport through pathogen infected seed is very important as illustrated by seed import regulations.

4.5 References

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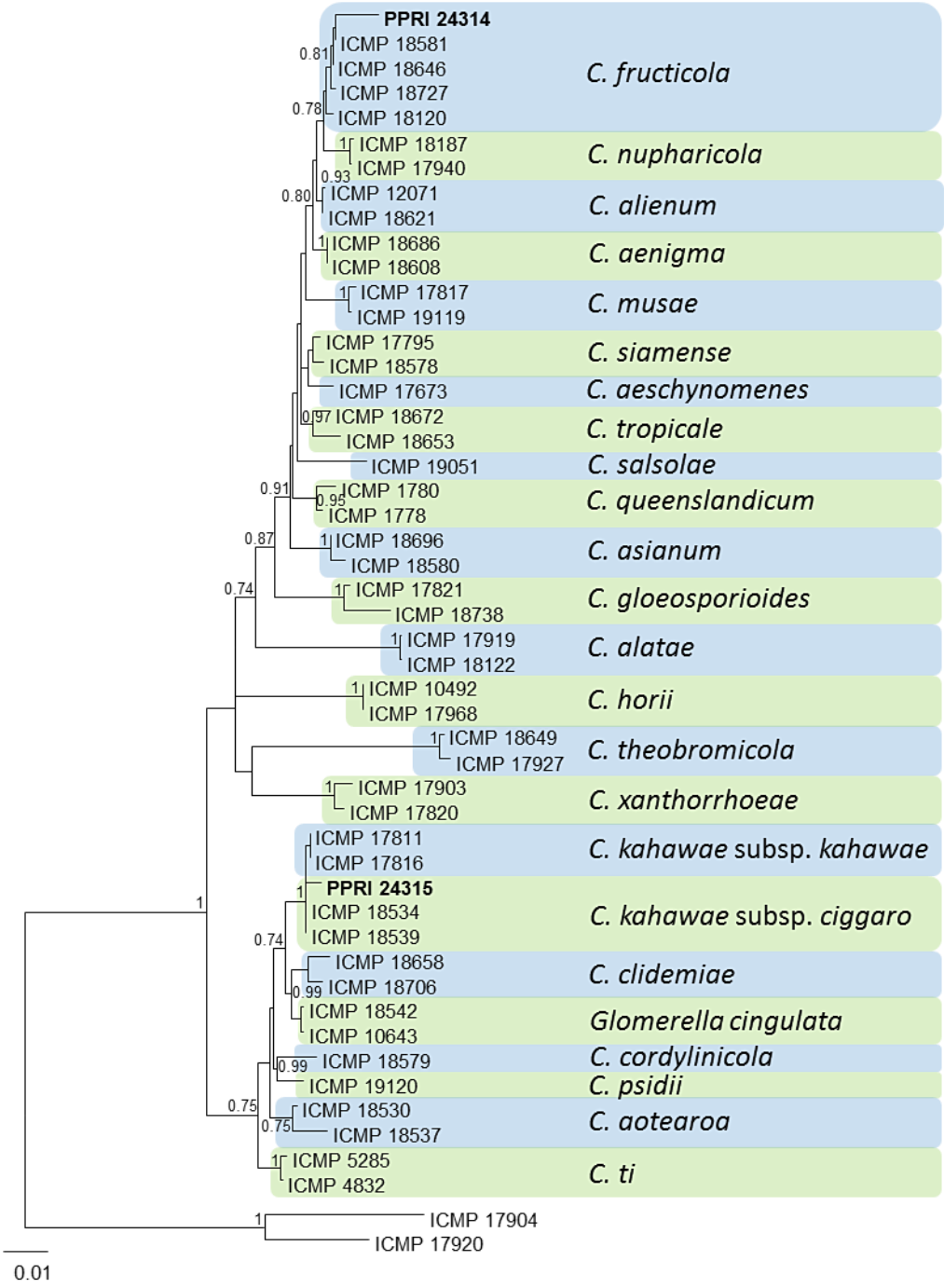

Figure 4.1: A maximum likelihood phylogenetic tree of 50 isolates in the *Colletotrichum gloeosporioides* species complex. The tree was build using concatenated sequences of ACT, GAPDH, CHS, ITS and *β*-tubulin genes. Maximum Likelihood vales ≥0.7 are shown above nodes. The tree is rooted with *C. boninense* ICMP 17904 and *C. hippeastri* ICMP 17920.

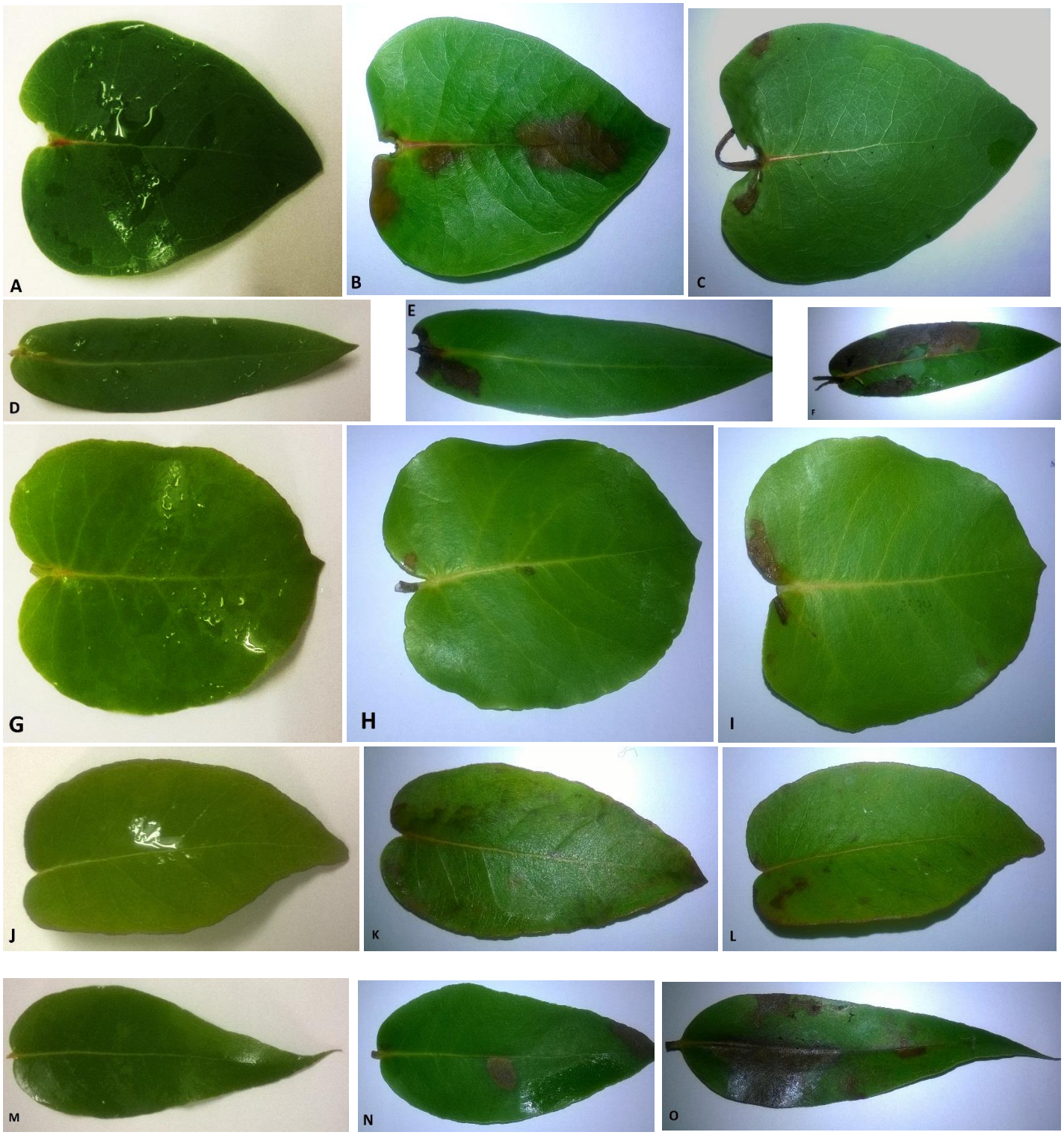

Figure 4.2: Symptoms of anthracnose leaf spot on: (A) *E. benthamii* control, (B) *E. benthamii* inoculated with *C. fructicola*, (C) *E. benthamii* inoculated with *C. kahawae* subsp. *cigarro*, (D) *E. camaldulensis* control, (E) *E. camaldulensis* inoculated with *C. fructicola*, (F) *E. camaldulensis* inoculated with *C. kahawae* subsp. *cigarro*, (G) *E. dorrigoensis* control, (H) *E. dorrigoensis* inoculated with *C. fructicola*, (I) *E. dorrigoensis* inoculated with *C. kahawae* subsp. *cigarro*, (J) *E. dunnii* control, (K) *E. dunnii* inoculated with *C. fructicola*, (L) *E. dunnii* inoculated with *C. kahawae* subsp. *cigarro*, (M) *E. grandis* control, (N) *E. grandis* inoculated with *C. fructicola*, (O) *E. grandis* inoculated with *C. kahawae* subsp. *cigarro*.

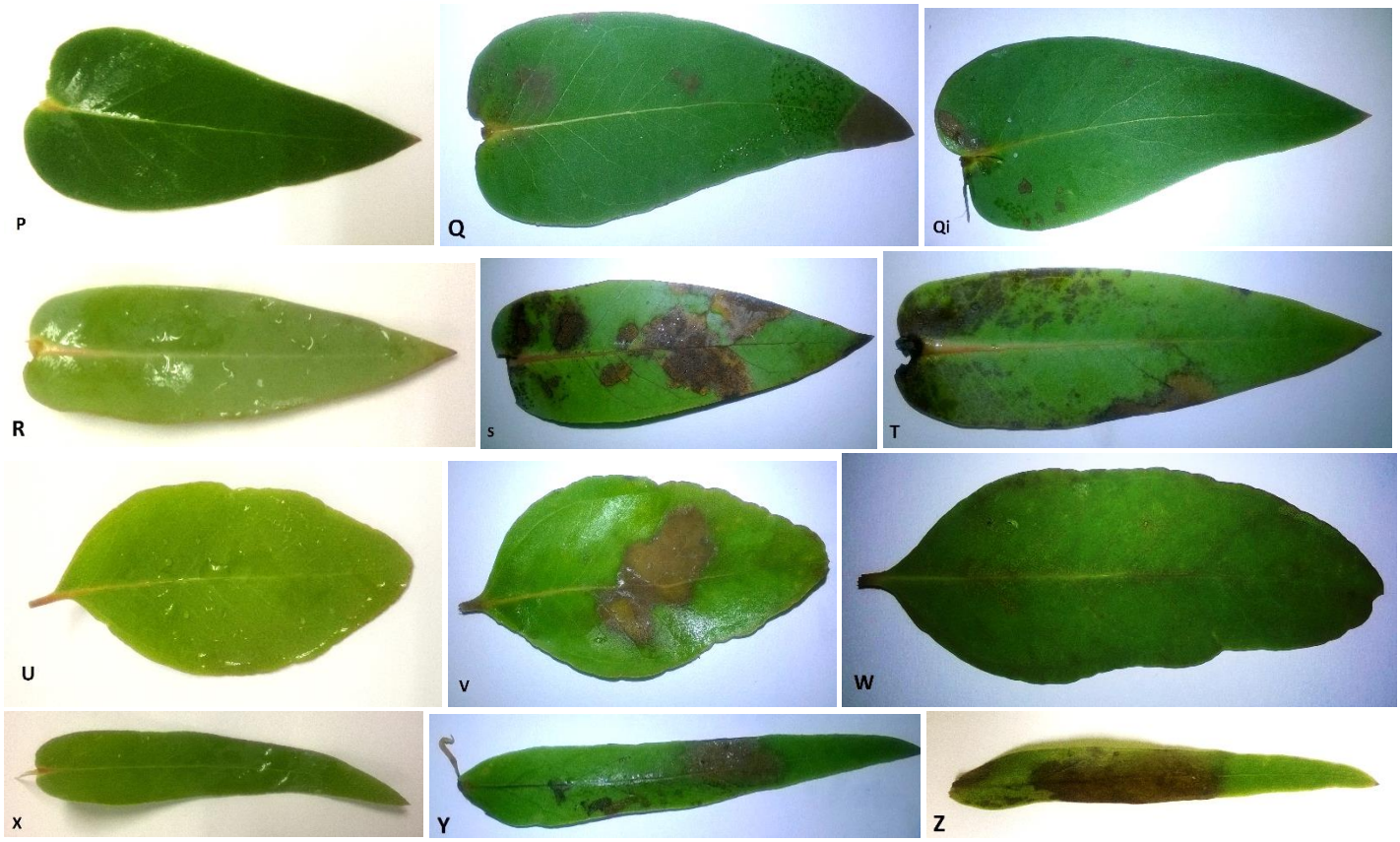

Figure 4.2: Symptoms of anthracnose leaf spot on: (P) *E. macarthurii* control, (Q) *E. macarthurii* inoculated with *C. fructicola*, (Qi) *E. macarthurii* inoculated with *C. kahawae* subsp. *cigarro*, (R) *E. nitens* control, (S) *E. nitens* inoculated with *C. fructicola*, (T) *E. nitens* inoculated with *C. kahawae* subsp. *cigarro*, (U) *E. teritecomis* control, (V) *E. teritecomis* inoculated with *C. fructicola*, (W) *E. teritecomis* inoculated with *C. kahawae* subsp. *cigarro*, (X) *E. viminalis* control, (Y) *E. viminalis* inoculated with *C. fructicola*, (Z) *E. viminalis* inoculated with *C. kahawae* subsp. *cigarro*.

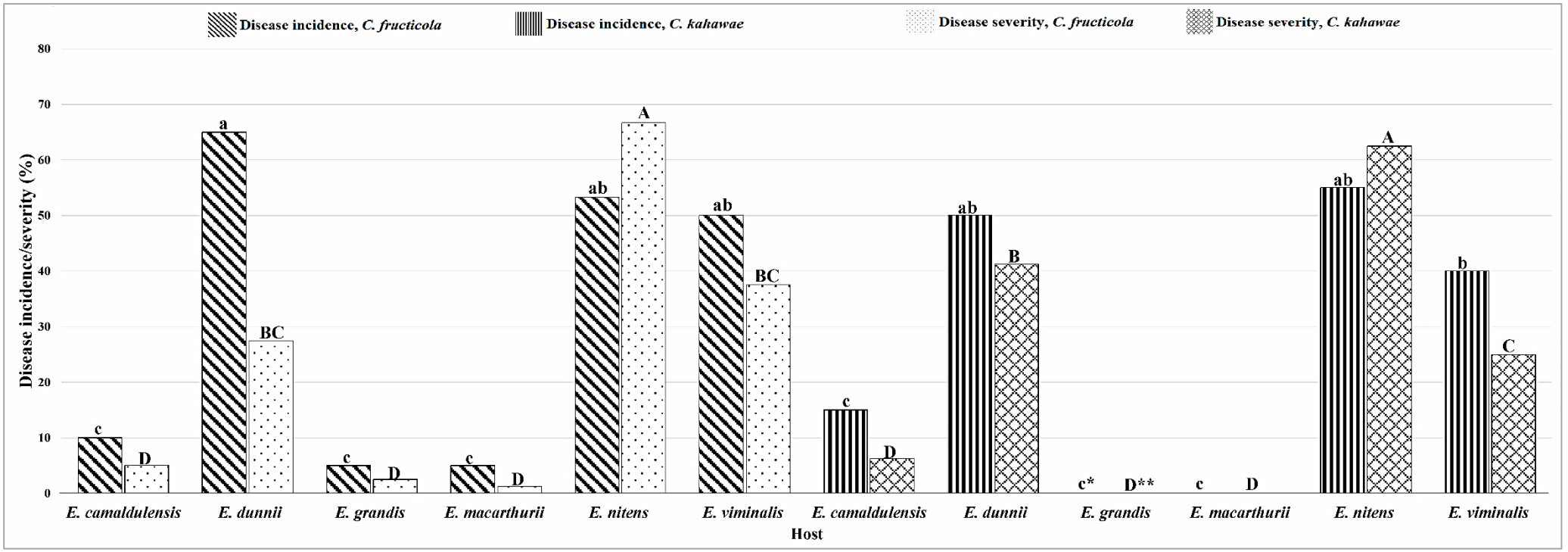

Figure 4.3: Mean disease incidence and severity of anthracnose leaf spot disease on *Eucalyptus* seedlings. Bars with the same letter (s) are not significantly different according to Fischer's LSD test ($p<0.05$).

Table 4.1: Effects of *Colletotrichum* species on percentage seed germination of *Eucalyptus* seed lots

Species	Non-inoculated control	Inoculated with *C. fructicola*	Inoculated with *C. kahawae*
E. badjensis	78.3*gh** x	42.0ey	36.0ez
E. benthamii	74.3hx	29.8gy	32.3fy
E. camaldulensis	85.3defx	46.3dz	52.0ay
E. dorrigoensis	88.3bcdex	33.5fz	41.8dy
E. dunnii	94.3ax	51.5bcy	50.8by
E. globulus	93.5ax	47.5dy	43.5cdz
E. grandis	89.0bcdx	56.8ay	54.5ay
E. macarthurii	88.8bcdx	46.0dy	45.8cy
E. nitens	85.8cdefx	39.5ey	40.8dy
E. pellita	90.5abx	33.5fy	33.3efy
E. saligna	90.0abcx	48.8cdy	50.5by
E. smithii	84.0efx	47.0dy	44.3cdy
E. tereticornis	90.5abx	53.0by	45.5cz
E. urophylla	91.3abx	45.8dz	54.8ay
E. viminalis	81.5fgx	47.0dy	43.5cdy
LSD	4.4	3.1	3.7
Cv%	2.0	2.0	2.0

*Means in the same column followed by the same letter do not differ significantly according to Fisher's LSD test at $p < 0.05$.

**Means in the same row for percentage germination not followed by the same letter are significantly different from each other ($p>0.05$).

Table 4.2: Effects of *Colletotrichum* species on percentage diseased *Eucalyptus* seedlings

Species	Non-inoculated control	Inoculated with *C. fructicola*	Inoculated with *C. kahawae*
E. badjensis	3.0aby	53.8bx	55.3ax
E. benthamii	4.3ay	59.0ax	58.3ax
E. camaldulensis	2.0bcdez	39.0ex	35.5gy
E. dorrigoensis	1.0defz	44.3cx	38.0efgy
E. dunnii	0.8efy	39.5dex	38.0efgx
E. globulus	0.8efy	43.8cdx	47.3bx
E. grandis	1.5cdefy	39.5dex	38.3efgx
E. macarthurii	2.3bcdy	44.8cx	41.8cdex
E. nitens	2.0bcdey	46.3cx	46.3bcx
E. pellita	1.0defy	53.0bx	53.8ax
E. saligna	1.0defz	44.0cx	36.5fgy
E. smithii	2.0bcdez	47.5cx	43.5bcdy
E. tereticornis	0.5fy	43.3cdex	42.8bcdex
E. urophylla	1.0defz	44.0cx	39.8defgy
E. viminalis	2.8bcy	45.8cx	41.5cdefx
LSD	1.5	4.5	5.2
Cv%	2.0	2.0	2.0

Explanations: see Table 1

CHAPTER 5

Evaluation of seed sanitation methods against *Colletotrichum kahawae* subsp. *cigarro* on *Eucalyptus* spp.

Abstract

To promote the supply of healthy or disease-free planting stock, non-chemical seed treatments that included *Bacillus*, *Trichoderma*, hot water, microwave radiation, and hydrogen peroxide were evaluated for their efficacy at sanitising seeds inoculated with the anthracnose leaf spot pathogen, *Colletotrichum kahawae* subsp. *cigarro*. Efficacy of seed treatments were assessed on *Eucalyptus nitens* and *E. viminalis* seed lots. When both reduction in incidence of *Colletotrichum* sp. and of seed germination are considered, hot water seed treatments at 50 °C for 30 mins and 60 °C for 1 min were optimum treatment/time parameters for *Eucalyptus*. Most improvements on seed germination were observed on *Eucalyptus* spp. seeds soaked in 10% H_2O_2 for 10 min having similar efficacy as the chemical seed treatment (Celest® XL). Exposure of moist *Eucalyptus* seeds to microwave radiation of 1400 w for 30 s was the only microwave power-time combination that significantly improved seed germination similar to that of the Celest® XL treatment. *In-vitro* assays showed no disease on seedlings grown from seeds soaked in hot water baths set at 55 and 60 °C for 15 min and above, and seeds soaked in 10 and 15 % H_2O_2 for 5 min and above. Moreover, no disease symptoms were observed on seedlings grown from moist seeds exposed to microwave radiation at 1400 w for 90 s and above or dry seeds exposed to microwave radiation at 1400 w for 120 s and above. However, the *Bacillus* treatment was the only non-chemical seed treatment that demonstrated effectiveness against anthracnose leaf spot under greenhouse conditions.

Key words: anthracnose, seed treatment, biocontrol, hot water, hydrogen peroxide, microwave radiation

5.1 Introduction

Seeds represent a long-term investment for plant regeneration (De Frenne et al. 2016). Despite advances in technologies of clonal vegetative propagation, foresters continue using seeds as a means of regenerating *Eucalyptus* plantations as they are economical and simple in practice (Griffin 2014). *Eucalyptus* seed germination percentages are often high under laboratory conditions, but seedling emergence is inconsistent in nurseries compelling foresters to sow more than one seed per container cavity (Luna et al. 2009).

Consistent seedling emergence in nurseries ensure production of sufficient quantities of reforestation planting stock (Thomas 2009). Apart from physiological abnormalities influenced by genetics, seed contaminants particularly mycoflora accrued from the field, during processing or in storage are important determinants to the success or failure of seedling establishment (Yuan et al. 1997; Rodrigues et al. 2014; Jimu et al. 2015). Together with several other fungi associated with *Eucalyptus* seeds, *Colletotrichum* found on and/or inside the seed may delay or impair seed germination and cause seedling death (Reglinski et al. 2015; Mangwende et al. 2018).

Despite presence of multiple pathogens, infected seeds often appear healthy and retain viability under laboratory seed germination tests (Facelli et al. 1999; Close et al. 2002). This is particularly alarming as such seeds indisputably pass through visual phytosanitary inspections, risking introduction and spread of forest pathogens to previously non-diseased areas (Cleary et al. 2019). The recent detection of polyphagous fungi such as *Botryosphaeria*, *Colletotrichum* and *Mycosphaerella* on commercial seeds is strong evidence that seed trade risks introduction and spread of pathogens (Mangwende et al. 2018).

The fungus *Colletotrichum kahawae* subsp. *cigarro* B.S. Weir & P.R. Johnst. is commonly misidentified as *C. kahawae* subsp. *kahawae* J.M. Waller & Bridge a specialized hemi-

biotrophic pathogen of coffee (*Coffea arabica* L.) (Jayawardena et al. 2016; Batista et al. 2017). On *Eucalyptus*, the pathogen causes anthracnose leaf spot and twig die-back (Viljoen et al. 1992; Smith et al. 1998; Mangwende et al. 2018). Furthermore, *C. kahawae* subsp. *cigarro* is both seed-borne and seed-transmitted (Mangwende et al. 2018).

The management of seed-borne diseases is not easy as there are limited number of registered seed treatments in South Africa. Although foresters occasionally use synthetic fungicides registered for other crops (Prahodsky et al. 2018; Garrett et al. 2018), there are concerns about their negative impacts on the environment and development of fungicide resistance in some pathogens (Tremolada et al. 2010; Mendell et al. 2015; Lemes et al. 2017). Therefore, the search for non-chemical methods to prevent spread of seed-borne pathogens is of great practical significance particularly in fulfilling phytosanitary requirements.

As alternatives to synthetic chemicals, seeds can be treated biologically or physically. Seed treatments with hot water or microwave radiation have successfully been applied against a range of pathogens and are in commercial use mainly on vegetable seeds (Tylkowska et al. 2010; Koch and Roberts 2014; Sharma et al. 2015). However, seeds of different plant species have unique biochemical compositions, which grant them different thermal tolerances (Forsberg 2004). Thus, the need to optimise temperature-time combinations that will effectively control target pathogens without negatively affecting seed viability. There is also potential in the use of natural chemicals such as hydrogen peroxide and biocontrol agents, but their application as seed treatments has been limited to a few agronomic and vegetable crops (Tinivella et al. 2009; Woo et al. 2010; Szopińska 2014; van Lenteren et al. 2018).

Due to the lack of registered seed treatments for use in seed trade and FSC certified nurseries, non-chemical methods that included biocontrol agents, viz. *Bacillus* and *Trichoderma*, physical methods, hot-water and microwave radiation, and a natural chemical, hydrogen peroxide, were

evaluated for their efficacy at sanitising seed lots of *Eucalyptus nitens* (H. Deane and Maiden) Maiden and *Eucalyptus viminalis* Labill. artificially inoculated with *C. kahawae* subsp. *cigarro*. Efficacy was also determined against transmission of the pathogen from seed to seedlings in the greenhouse.

5.2 Materials and Methods

5.2.1 Source of materials

Seeds of *E. nitens* and *E. viminalis* were supplied by commercial forestry seed companies. They were selected based on the levels of susceptibility to anthracnose leaf spot disease i.e. highly and moderately susceptible, respectively (Chapter 4). Pathogenic *C. kahawae* subsp. *cigarro* (PPRI 24314, ITS GenBank accession number: MG641893) isolated from *Eucalyptus* seeds (Chapter 4 section 4.3.1) was used in this study. Commercial biocontrol agents, *Trichoderma harzianum* Rifai ($2x10^9$ spores/g) (Plant Health Products (Pvt.) Ltd., Kwazulu-Natal, South Africa) and *Bacillus subtilis* (Ehrenberg) Cohn strain MBI 600 ($2x10^{11}$ spores/ml) (Becker Underwood (Pvt) Ltd., Kwazulu-Natal, South Africa), and a fungicide Celest® XL (25 ai/L fludioxonil and 10 g ai/L mefenoxam) (Syngenta (Pvt.) Ltd., Midrand, South Africa) were used for the study. Ensure® ISO (30% hydrogen peroxide) was sourced from Merck (Pvt.) Ltd. (Midrand, South Africa).

5.2.2 Seed inoculation

Seeds of both seed lots were surface disinfected in 1% sodium hypochlorite solution for 5 min and artificially inoculated by soaking in 20 mL of a 1×10^5 conidia ml^{-1} inoculum of *C. kahawae* subsp. *cigarro* amended with 2 drops of Tween-20 for 4 h, with occasional hand shaking. Inoculated seeds were air-dried overnight on sterile paper towels in a laminar flow cabinet, and plated (50 per sample) on potato dextrose agar (PDA, Biolabs, South Africa).

Plated seeds were incubated at 25 ℃ for 7 days under alternating cycles of 12 h ultra violet (UV) (365 nm) light and darkness. To confirm that inoculation was successful, fungi was re-isolated on PDA and identity confirmed in comparison with positive reference plates of *C. kahawae* subsp. *cigarro* grown alongside incubated seeds from which more than 90% was re-isolated from inoculated seeds.

5.2.3 Hot water seed treatment

Artificially inoculated *Eucalyptus* seed lots were enclosed in double cheesecloth to form aliquots of 200 seeds per cheesecloth bag. Initially, aliquots were soaked in sterile distilled water at room temperature for 2 h prior treatment in a hot water bath (Model: 132A; Labotec, South Africa). The temperatures of sterile distilled water in glass beakers was equilibrated to the target temperatures of 35, 40, 45, 50, 55 and 60 ±1 °C before the start of the experiment, and were constantly monitored. Aliquots containing seeds were soaked at the different hot water temperatures for different periods namely, 1, 15, 30, 45 and 60 min. Seeds left soaked in sterile distilled water at standard room temperature at equivalent time points served as positive controls, whereas seeds soaked in Celest® XL at the recommended rate of 1 mL/kg seed at above mentioned times served as negative controls. Immediately after hot water treatment, aliquots were submerged in sterile distilled water at room temperature for 5 min. Subsequently, aliquots were spread onto sterile paper towels and left to air dry on a laminar flow bench.

5.2.4 Seed treatments with microwave radiation

Dry and moist *Eucalyptus* seed lots were exposed to microwave radiation. To moisten seeds, inoculated seeds were wrapped in double cheesecloth and soaked in sterile distilled water at room temperature for 2 h prior to treatment. Seeds were evenly spaced on top of two layers of dry Whatman filter papers aligned in a glass Petri dish. A 1400 w and 2450 MHz consumer grade microwave oven (Samsung microwave model: ME9114W1, Malaysia) with digital

adjustable power levels was used. A total of 200 seeds for each seed lot were exposed to microwave radiation with three levels of power, 250, 600 and 1400 w. For each power level, exposure times ranged from 0 to 180 s with 30 s increments. The glass Petri dish containing seeds was placed in the centre of the rotating plate of the microwave oven. Soon after treatment, seeds were cooled by submerging in sterile distilled water at standard room conditions for 5 min and then air dried on a laminar flow bench. Efficacy of microwave radiation was measured against non-treated inoculated seeds and inoculated seeds treated with Celest® XL.

5.2.5 Seed treatment with hydrogen peroxide

Cheesecloths containing 200 inoculated seed per bag per seed lot were soaked in sterile distilled water at room temperature for 2 h before transferring the individual aliquots to beakers containing aqueous solutions of 1, 5, 10 or 15% (*v/v*) hydrogen peroxide (H_2O_2) at standard room temperature. For each concentration of H_2O_2, seeds were soaked for 1, 5, 10, 30 and 45 min. Inoculated seeds soaked in sterile distilled water at room temperature at these same time points served as positive controls, whilst seeds soaked in Celest® XL at aforementioned times were negative controls. After treatment, cheesecloths containing seeds were rinsed in sterile distilled water and seeds were left to dry on a laminar flow bench.

5.2.6 Effects of seed treatments on incidence of *C. kahawae* subsp. *cigarro*

The agar plate method was used to determine the incidence of *C. kahawae* subsp. *cigarro* on treated and non-treated (controls) seeds. Four replicates of 50 seeds per seed lot were plated on PDA media (10 seeds per Petri dish) and randomly arranged in a 25 °C incubator (Labcon, Gauteng, South Africa) with alternating 12 h white fluorescent light/12 h dark regime. The experiment was repeated. A Petri dish inoculated with *C. kahawae* subsp. *cigarro* was also included, from which fungi growing from the seeds was compared with. After 5 days of

incubation, fungi growing from seeds were examined and percentages of seeds infected with *C. kahawae* subsp. *cigarro* was determined.

5.2.7 Effects of seed treatments on seed germination

Seed germination of treated and non-treated seed lots was done using the on-top of paper method (ISTA, 2019). Four replicates of 50 seeds were maintained, with sub-replicates of 25 seeds evenly spaced on three layers of moist Whatman No. 1 filter paper aligned in a glass Petri dish. Plates were incubated in a germination growth cabinet maintained at 25 ^{o}C with alternating cycles of 12 h white light (58 w Osram fluorescent tubes; Russia)/12 h dark cycle. Final germination counts were conducted after 21 days of plating. Numbers of germinated seeds and seedlings that developed diseases were recorded. Diseased seedlings were identified by lesions developing on hypocotyls and/or primary roots.

5.2.8 Greenhouse trials

Greenhouse trials were conducted in a greenhouse located at the Experimental Farm of the University of Pretoria, South Africa (25° 45′ S, 28°15’ E). Trials were repeated, where the first trial was sown on 24 August (spring) and the second on 5 October (summer). Following treatment with the best performing seed treatments from aforementioned *in-vitro* tests, *Eucalyptus* seeds of both seed lots were sown singly in 15 cm diameter pots filled with pasteurised sandy loam soil. Pots were randomly arranged in blocks in the greenhouse, each treatment with ten individually seeded pots replicated three times. Greenhouse conditions were maintained at 25/20 ±1 °C day and night, respectively, and plants watered every second day. At 21 days after sowing (DAS), the number of emerged seedlings was recorded and assessments for anthracnose leaf spot disease done before harvest at 180 DAS. Evaluation of disease severity was done using a scale of 1–5 described in Chapter 4 section 4.2.4 and average

diameters of anthracnose leaf spot. Plants were harvested 180 DAS and seedling length (cm) and total dry mass (g) recorded.

5.2.9 Statistical analysis

Statistical analyses was conducted using the General Linear Model procedure of Statistical Analysis System (SAS, version 9.4) (SAS Institute, 2016). Analysis of variance (ANOVA) was performed on data and means compared with the Fischer's least significant differences (LSD, $p<0.05$).

5.3 Results

5.3.1 Effects of seed treatments on the incidence of *C. kahawae* subsp. *cigarro*

Hot water seed treatments significantly reduced incidences of *C. kahawae* subsp. *cigarro* on *Eucalyptus* spp. seed lots compared with controls ($p<0.05$), except for seeds soaked in hot water baths set at 40 °C for 1 min (Table 5.1 and Appendix 5.1). The incidence of *C. kahawae* subsp. *cigarro* persisted on *E. nitens* seed lots soaked in hot water baths for 1 min regardless of the temperature increment.

At the same soaking period, hot water seed treatment at 60 °C effectively reduced the incidence of *C. kahawae* subsp. *cigarro* on *E. viminalis* seeds and was comparable with the biocontrol agents and Celest® XL treatment (Appendix 5.1). At soaking periods of 15 min and above, setting hot water baths at 60 °C effectively eliminated incidences of *C. kahawae* subsp. *cigarro* on both *Eucalyptus* spp. seed lots.

Effects of soaking *E. nitens* (Table 5.2) and *E. viminalis* (Appendix 5.1) seed lots in H_2O_2 significantly reduced the incidence of *C. kahawae* subsp. *cigarro* on *Eucalyptus* spp. seed lots compared to untreated controls ($p<0.05$), except for seeds soaked in 1% H_2O_2 for 1 min. There was a significant increase in efficacy of H_2O_2 against *C. kahawae* subsp. *cigarro* as the soaking

period was increased, where seeds soaked in 15% H_2O_2 for 10 min and above effectively eradicated incidences of *C. kahawae* subsp. *cigarro*.

Both *Eucalyptus* spp. seed lots exposed to microwave radiation had significantly lower incidences of *C. kahawae* subsp. *cigarro* compared with inoculated controls ($p<0.05$), except for dry seeds exposed at 250 w microwave radiation for 30 s (Table 5.3 and Appendix 5.3). At exposure periods of 60 s and below, all power-time parameters of microwave radiation were significantly less effective at reducing incidences of *C. kahawae* subsp. *cigarro* than seed treatments with biocontrol agents and Celest® XL ($p<0.05$). Microwave radiation of moist seeds at 1400 w for 90 s and above, together with microwave radiation of dry seeds at 1400 w for 120 s and above, eliminated incidences of *C. kahawae* subsp. *cigarro* on both *Eucalyptus* spp. seed lots.

5.3.2 Effects of seed treatments on seed germination

Seed treatments significantly increased seed germination of both *Eucalyptus* seed lots compared to non-treated controls ($p<0.05$) (Tables 5.1-3 and Appendices 5.1-3). Soaking *Eucalyptus* seeds in hot water baths set at 50 for 30 and 60 °C for 1 min were the most effective temperature-time combinations that resulted in the most improvement of seed germination (Table 5.1 and Appendix 5.1). Further increase of hot water bath temperature beyond these limits greatly reduced seed germination.

Results of effects of H_2O_2 on *Eucalyptus* seed germination are presented in Table 5.2 and Appendix 5.2. At a soaking period of 10 min and below, there were significant increments to seed germination as concentration of H_2O_2 was increased from 1 to 10%. However, increasing the concentration of H_2O_2 beyond 10% resulted in reduction of seed germination. Most improvements on seed germination were observed on *E. viminalis* seeds soaked in 10% H_2O_2 for 10 min, which had similar efficacy as the Celest® XL treatment (Appendix 5.2) but not in

the case of *E. nitens* seeds (Table 5.2). Regardless of concentration of H_2O_2, seed germination of *E. nitens* was significantly lower than seed treatments with biocontrol agents and Celest® XL ($p<0.05$).

The effects of microwave seed treatments on germination of both *Eucalyptus* seed lots are displayed in Table 5.3 and Appendix 5.3. Microwave radiation of moist seeds significantly increased seed germination better than dry seeds ($p<0.05$). In fact, exposure of moist *Eucalyptus* seeds to microwave adjusted to 1400 w for 30 s was the only microwave power-time combination that significantly improved seed germination with a similar level of efficacy as the Celest® XL treatment. However, prolonged exposure to microwave radiation at 1400 w above 60 s significantly reduced seed germination ($p<0.05$). Microwave radiation of dry seeds at 1400 w for 120 s and above completely reduced seed germination.

5.3.3 Effects of seed treatments on diseased seedlings

Hot water seed treatments and soaking both *Eucalyptus* spp. seed lots in H_2O_2 significantly reduced the proportion of diseased seedlings in the greenhouse trials compared with inoculated controls ($p<0.05$) (Table 5.4). There were no diseased seedlings from *Eucalyptus* spp. seeds soaked in hot water baths set at 55 and 60 °C for 30 min and above (Table 5.1 and Appendix 5.1). Similarly, there were no diseased seedlings from seeds soaked in 10 and 15 % H_2O_2 for 5 min and above (Table 5.2 and Appendix 5.2).

Microwave radiated seeds had significantly lower numbers of diseased seedlings than non-treated controls ($p<0.05$), except for dry *Eucalyptus* seeds exposed at 250 w microwave radiation (Table 5.3 and Appendix 5.3). At each exposure period, the number of seedlings developing diseases were significantly lowered with an increase of microwave power level. At the same power level, moist *Eucalyptus* seeds had greater sensitivity to microwave radiation than dry seeds with significantly less diseased seedlings. In fact, there were no diseased

seedlings from moist seeds exposed to microwave radiation at 1400 w for 60 s and above, which had the same effect as non-inoculated controls. Similarly, no diseased seedlings were grown from dry seeds exposed to microwave radiation at 1400 w for 120 s and above.

5.3.4 Effects of seed treatments on disease development

5.3.4.1 Incidence of anthracnose leaf spot

Seed treatments in the greenhouse trials significantly suppressed appearance of anthracnose leaf spot on both *Eucalyptus* spp. seedlings compared with seedlings grown from non-treated seeds inoculated with *C. kahawae* subsp. *cigarro* (Table 5.4). Despite treating seeds with seed treatments, significantly higher incidences of anthracnose leaf spot were observed on *Eucalyptus* seedlings ($p<0.05$), even on *E. nitens* seedlings grown from Celest® XL treated seeds. Biocontrol agents, *Bacillus* and *Trichoderma,* significantly suppressed appearance of anthracnose leaf spot on *Eucalyptus* spp. seedlings better than the other non-chemical seed treatments.

5.3.4.2 Severity of anthracnose leaf spot

5.3.4.2.1 Disease scores

Anthracnose leaf spot disease was more pronounced and most severe on seedlings grown from inoculated controls in the greenhouse trials (Table 5.4). Non chemical seed treatments did not significantly suppress ($p>0.05$) severity of anthracnose leaf spot on *Eucalyptus* seedlings compared with Celest® XL, except for seedlings grown from *Bacillus* treated seeds.

5.3.4.2.2 Diameter of leaf spot

Seedlings grown from inoculated controls had the largest leaf spot (Table 5.4), and statistically similar diameters of anthracnose leaf spot were recorded on seedlings grown from *E. nitens* seed lots treated with hot water at 60 °C for 1 min and dry seeds exposed to microwave radiation

at 1 400 w for 60 s. Although a different response was observed on seedlings grown from *E. viminalis*, biocontrol agents were the only non-chemical seed treatments that consistently suppressed appearance of anthracnose leaf spot on seedlings of both *E. nitens* and *E. viminalis* ($p<0.05$) and were as effective as the Celest® XL treatment.

5.3.5 Effect of seed treatments on *Eucalyptus* seedling growth

5.3.5.1 Emergence

Seed treatments significantly improved *Eucalyptus* seedling emergence in the greenhouse trials compared with inoculated controls ($p<0.05$) (Table 5.5). The *Bacillus* treatment was the only non-chemical seed treatment that consistently increased seedling emergence of both *E. nitens* and *E. viminalis* as effective as Celest® XL.

5.3.5.2 Seedling length

Sowing non-treated seeds inoculated with *Colletotrichum* sp. yielded the smallest seedlings in all trials. The average length of seedlings grown from *E. nitens* seed lots ranged from 17.3 to 32.7 cm, and 23.0 to 41.6 cm from *E. viminalis* seed lots (Table 5.5). Seedlings from treated seeds were significantly longer compared to seedlings grown from inoculated controls ($p<0.05$), except for *E. nitens* seedlings grown from microwave treated seeds at 1400 w for 60 s. In all trials, biocontrol agents consistently improved length of seedlings and were comparable to the Celest® XL treatment, which had the longest seedling length. A similar response was observed on seedlings from *E. nitens* seed lots treated with hot water at 60 °C for 1 min.

5.3.5.3 Seedling dry mass

Greenhouse trials showed that microwave radiation of moist seeds at 1400 w for 30 s and seed treatment with *Bacillus* and *Trichoderma* significantly increased dry seedling mass compared with dried seedling mass from non-inoculated controls $p<0.05$ (Table 5.5). However, the

Bacillus treatment was the only non-chemical seed treatment that yielded seedlings with dry seedling mass statistically similar to that of Celest® XL treated seeds.

5.4 Discussion

Delays in emergence and poor survival of seedlings remains a common challenge in most forest nurseries (Lilja et al. 2010; Fendrihan 2015; Mattsson 2016). Seed sanitation is not only appealing to nursery managers but also to forest seed traders where healthy seeds warrantee compliance with strict regional and international plant quarantine regulations (Cleary et al. 2019). Although synthetic chemicals are widely accepted as reliable means of managing pests and diseases, further use of synthetic chemicals in forestry operations are being discouraged as forestry production is becoming progressively compliant with the guidelines of the Forestry Stewardship Commission (Mendell et al. 2015; Lemes et al. 2017).

This study showed that hot water seed treatments of *Eucalyptus* seed lots significantly reduced ($p<0.05$) incidences of *C. kahawae* subsp. *cigarro* and improved seed germination. Hot water seed treatments have been used to sanitise *Colletotrichum* infected seeds of different plant species including lupins (*Lupinus angustifolius* L.) and corms (*Anemone coronaria* L.) (Zinnen and Sinclair 1982; Doornik 1992; Thomas and Adcock 2004). Hot water seed treatment acts by thermal disruption of proteins, lipids and other structural components of cells (Abu-Shakra and Ching 1967). Similarly, hot water seed treatment temperatures of 50 °C were previously shown to be effective at sanitising *Eucalyptus* seeds against a broad range of fungi (Donald and Lundquist 1988). In addition, this study showed high efficacy against *C. kahawae* subsp. *cigarro* for short exposure periods of seeds at higher water bath temperature (60 °C for 1 min).

The main challenge with hot water is that it is limited to a few internal layers of the seed coat. Anthracnose leaf spots were observed on seedlings grown from the optimal hot water temperature-time combinations (60 ºC for 1 min and 50 ºC for 30 min) under greenhouse

conditions. It is possible that incidence of *C. kahawae* subsp. *cigarro* was retained on *E. nitens* seeds soaked in hot water bath set at 60 ^{o}C for 1 min as heat was not effectively conducted to reach some of the spores that were embedded deeper inside seed coat crevices. Similarly, studies on cabbage seed infested with *Leptosphaeria maculans* Ces. & De Not. showed a 2% retention of infestation after hot water seed treatments (Williams 1967). Since there were no diseased seedlings under *in-vitro* conditions, it is possible that the concentration of pathogen inoculum was significantly reduced to an extent that it did not cause pronounced disease symptoms particularly considering the latent and biotrophic nature of *Colletotrichum* species. Moreover, *C. kahawae* subsp. *cigarro* might have been poorly transmitted from seed into seedlings.

Soaking *Eucalyptus* seed lots in H_2O_2 significantly improved seed germination of *Eucalyptus* spp. Similarly, seed germination was increased when seeds of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco), zinnia (*Zinnia elegans* Jacq.), switchgrass (*Panicum virgatum* L.), big bluestem (*Andropogon gerardii* Vitman) and Indian grass (*Sorghastrum nutans* (L.) Nash) were soaked in H_2O_2 (Ogawa and Iwabuchi 2001; Lee et al. 2004; Sarath et al. 2008). Soaking both *Eucalyptus* seed lots in 10 % H_2O_2 for 5 min and 10 min were the optimal treatment/time combinations that gave highest improvements on seed germination and were equally effective as seed treatments with *Bacillus* and Celest® XL. Regardless of concentration of H_2O_2, seed germination of *E. nitens* seed lots was significantly lower than seed treatments with the biocontrol agents and Celest® XL ($p<0.05$).

Incidences of *C. kahawae* subsp. *cigarro* were significantly reduced on *Eucalyptus* seed lots soaked in H_2O_2, which might have contributed to increments in seed germination. Hydrogen peroxide has antimicrobial properties against *Colletotrichum* spp. (Peng and Kuc 1992; Nandi et al. 2017). Although there were positive increments of seed germination with gradual increase

of concentration of H_2O_2 from 1 to 10%, presence of *C. kahawae* subsp. *cigarro* persisted on treated seeds. Use of higher concentrations is discouraged as high concentrations of H_2O_2, a strong oxidant, can cause skin and eye injuries (Barnett and McGilvray 1997). Furthermore, seed treatment with H_2O_2 is non-systemic and was not effective at controlling anthracnose leaf spot developing on seedlings grown under greenhouse conditions.

Although microwave radiation also makes use of heat as the lethal mode of action against pathogens (Grondeau et al. 1994; Reddy et al. 1998), it differs from hot water treatments in that heat generated by high-frequency alternating electromagnetic radiation (EMR) of 300 MHz-300 GHz act directly on atomic level of cellular structures through dipole rotation and ionic polarization (Bouraoui et al. 1993). Thus, microwave radiation can rapidly penetrate seeds at the cellular level killing seed-borne pathogens deeply imbedded in seed tissues (Grondeau et al. 1994). Due to its ability to rapidly generate heat, it is crucial to optimise the power-time combinations for effective control of pathogens without overheating seeds (Berbert et al. 2002; Han 2010). In this study, moist *Eucalyptus* spp. seed lots irradiated in a microwave oven at 1400 w for 30 s was the only microwave power-time combination that significantly improved seed germination with a similar level of efficacy as the Celest® XL treatment. Prolonged exposure of seeds to microwave radiation above 60 s significantly reduced germination ($p<0.05$).

Microwave radiation of moist seeds significantly increased seed germination above that of dry seeds ($p<0.05$). Efficacy of seed treatments with microwave radiation is dependent on the dielectric permittivity of the materials involved (Nelson 1996; Jiao et al. 2011). The overall moisture content, temperature, bulk density and frequency of applied electric fields affects the extent to which heat is produced and transferred between molecules, warming the material thoroughly (Bouraoui et al. 1993). Hence, moistening seeds elevates permittivity of microwave radiated seeds that generates an elevated amount of heat compared with dry seeds. Furthermore,

moist *Eucalyptus* seeds had significantly lower percentage of diseased seedlings than dry seeds. This confirms studies that showed that higher seed moisture content translates to an increase in efficacy of microwave radiation against seed-borne fungi (Bouraoui et al. 1993; Berbert et al. 2002; Jiao et al. 2011; Knox et al. 2013).

In conclusion, investigations of this study were very rigorous considering that seeds used were artificially inoculated with high concentrations of *C. kahawae* subsp. *cigarro* ($1x10^5$ spores/mL), which is a rare scenario under natural circumstances. When both seed disinfection and seed germination are considered, non-chemical seed treatments *viz*. soaking seeds in hot water baths set at 50 °C for 30 min, 60 °C for 1 min, soaking seeds in 5% H_2O_2 for 10 min, 10% H_2O_2 for 10 min, microwave radiation of moist seeds at 1400 w for 30 s and 600 w for 60 s proved to be effective under laboratory conditions. However, these same seed treatments were not consistent in greenhouse studies except for the *Bacillus* treatment. Since there are limited chemicals registered as seed treatments of *Eucalyptus* seeds, the effectiveness of Celest® XL and the *Bacillus* treatment against the pathogen *in-vitro* and under greenhouse conditions suggests recommendation as sanitisers of commercial *Eucalyptus* seed lots.

5.5 References

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Table 5.1: Effects of hot water seed treatments of artificially inoculated *E. nitens* seed lots on seed germination, diseased seedlings and incidence of *C. kahawae subsp. cigarro*

	Soaking period														
	1 min			15 min			30 min			45 min			60 min		
Treatment	**Inc[a]**	**Germ[b]**	**Dis[c]**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**
40 °C	92.0[*]a[**]w	42.3f[***]D	50.3b[****]X	82.3bx	53.3eC	47.5bX	81.0bx	59.8dB	23.8bY	67.0by	77.3bA	20.3bZ	45.3bz	78.3bA	19.5bZ
45 °C	84.5bv	57.3eC	24.5cX	59.3cw	70.8dB	17.8cY	43.8cx	73.5cAB	7.8dZ	21.3cy	78.0bA	10.0cZ	14.8cz	77.0bA	8.3cZ
50 °C	42.8cv	69.8dD	12.0dX	22.5dw	79.5cB	6.8dY	14.0dx	90.5aA	6.8cY	8.0dy	74.8bC	0.0dZ	0.0dz	70.3cD	0.0dZ
55 °C	42.0cw	77.0cB	8.5dX	12.0dx	85.0bA	0.0eY	3.0ey	73.0cC	0.0dY	0.0ez	62.3cD	0.0dY	0.0dz	41.5dE	0.0dY
60 °C	5.5ex	87.0aA	6.5eX	0.0ey	22.3fB	0.0eY	0.0ey	13.0fC	0.0dY	0.0ey	0.0eD	0.0dY	0.0dy	0.0fD	0.0dY
Bacillus	8.5dy	84.0bB	5.0eX	0.0ez	89.3abA	0.0eY	0.0ez	90.8aA	0.0dY	0.0ez	90.0aA	0.0dY	0.0dz	90.0aA	0.0dY
Trichoderma	10.5dy	83.5bB	9.3dX	0.0ez	86.3bAB	0.0eY	0.0ez	87.3bAB	0.0dY	0.0ez	89.5aA	0.0dY	0.0dz	89.0aA	0.0dY
Celest® XL	0.5fz	88.0aB	0.0fZ	0.0ez	90.3aAB	0.0eZ	0.0ez	91.3aAB	0.0dZ	0.0ez	92.5aA	0.0dZ	0.0dz	91.8aA	0.0dZ
Control	93.0ayz	21.8gA	67.3aZ	91.0az	22.3fA	70.0aZ	95.5ay	19.3eB	70.3aZ	94.8ay	18.5dB	68.5aZ	93.3ayz	17.8eB	71.0aZ

Inc[a]: Percentage incidence of *C. kahawae* subsp. *cigarro*, Germ[b]: seed germination, Dis[c]: diseased seedlings. [*]Means sharing a common letter in a column differ significantly according to the Fisher's LSD test at $p<0.05$. [**]In each row, means with the same lowercase letters do not significantly differ from each other at $p = 0.05$. [***]Means within a row not followed by the same uppercase letter are significantly different from each other ($p = 0.05$). [*****] Means within a row followed by same uppercase letter are not significantly different from each other.

Table 5.2: Effects of hydrogen peroxide seed treatments of artificially inoculated *E. nitens* seed lots on seed germination, diseased seedlings and incidence of *C. kahawae subsp. cigarro*

	Period seeds soaked in H_2O_2														
	1 min			5 min			10 min			30 min			45 min		
Treatment	**Inc[a]**	**Germ[b]**	**Dis[c]**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**
1% H_2O_2	95.3[*]a[**]x	43.5e[***]B	53.3b[****]Y	86.5by	49.8eA	49.3bZ	84.8by	50.5eA	47.0bZ	84.3by	43.5fB	53.3bY	76.0bz	42.8fB	53.8bY
5% H_2O_2	68.3bw	58.0dC	40.0cX	53.5cx	82.3bcAB	10.5cY	44.5cy	84.5bA	10.0cY	40.0cy	84.3cA	8.5cZ	33.8cz	80.5cB	9.0cYZ
10% H_2O_2	43.8cw	80.0bB	14.5dY	41.3dw	80.8cB	0.0dZ	35.5dx	83.0cA	0.0dZ	23.3dy	81.0dAB	0.0dZ	15.5dz	77.8dC	0.0dZ
15% H_2O_2	34.8dx	77.0cA	0.0gZ	5.3ey	77.5dA	0.0dZ	0.0gz	77.8dA	0.0dZ	0.0ez	70.0eB	0.0dZ	0.0ez	70.0eB	0.0dZ
Bacillus	9.3ex	81.0aC	6.3fY	4.3ey	84.3bB	0.0eZ	1.8fz	86.3bB	0.0dZ	0.0ez	88.8aA	0.0dZ	0.0ez	89.5aA	0.0dZ
Trichoderma	11.0ex	79.5bC	9.3eY	5.5ey	81.5cB	0.0eZ	3.5ey	82.8cB	0.0dZ	0.0ez	86.5bA	0.0dZ	0.0ez	87.8bA	0.0dZ
Celest® XL	0.0fz	82.5aC	0.0gZ	0.0fz	87.5aB	0.0eZ	0.0gz	88.3aAB	0.0dZ	0.0ez	90.5aA	0.0dZ	0.0ez	90.0aA	0.0dZ
Control	96.3az	35.8fA	59.8aZ	96.0az	35.5fA	60.5aZ	95.5az	35.5fA	60.5aZ	95.8az	33.3gAB	60.0aZ	98.0az	31.0gB	61.5aZ

Inc[a]: Percentage incidence of *C. kahawae* subsp. *cigarro*, Germ[b]: seed germination, Dis[c]: diseased seedlings. [*]Means sharing a common letter in a column differ significantly according to the Fisher's LSD test at $p<0.05$. [**]In each row, means with the same lowercase letters do not significantly differ from each other at $p = 0.05$. [***]Means within a row not followed by the same uppercase letter are significantly different from each other ($p = 0.05$). [*****] Means within a row followed by same uppercase letter are not significantly different from each other.

Table 5.3: Effects of microwave irradiation seed treatments of artificially inoculated *E. nitens* seed lots on seed germination, diseased seedlings and incidence of *C. kahawae subsp. cigarro*

	Microwave exposure Time																	
	30 sec			60 sec			90 sec			120 sec			150 sec			180 sec		
Treatment	**Inc[a]**	**Germ[b]**	**Dis[c]**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**
dry 250 w	85.5*ab**x	36.0g***BC	60.3a****X	84.8bxy	35.8dC	56.5bY	84.0by	38.3gB	58.0bXY	82.3bz	37.3eB	52.3bZ	83.3byz	42.0dA	51.8bZ	82.0bz	42.5dA	50.3bZ
dry 600 w	83.3bv	41.3fAB	54.0bX	75.8cw	39.5dB	59.3aW	66.8cx	44.8fA	57.8bW	45.8cy	43.5dAB	54.0bX	31.3cz	44.0dA	51.3bXYZ	33.0cz	42.8dAB	49.0cZ
dry 1400 w	60.5ew	68.0dA	28.0dV	52.5ex	72.5bA	21.5dW	28.3fy	37.8gB	17.0eXY	0.0fz	0.0fC	13.8deY	0.0fz	0.0gC	0.0fZ	0.0fz	0.0gC	0.0gZ
wet 250 w	74.5cw	54.3eC	48.8cW	68.3dx	60.5cB	50.3cW	51.0dy	54.0eCD	43.5cX	30.3dz	49.5cE	38.0cY	28.5cz	51.3cDE	37.3cY	31.0cz	69.3cA	22.0dZ
wet 600 w	71.0dv	70.0cdA	28.8dW	46.0fw	74.0bA	20.0dX	32.0ex	61.8dB	21.8dX	26.0dy	35.3eC	13.5eY	20.8dy	27.0fD	10.8dY	14.5dz	21.5fE	5.5fZ
wet 1400 w	47.0fx	81.8aA	9.5gY	30.5gy	72.3bB	0.0fZ	0.0iz	42.5fC	0.0gZ	0.0fz	0.0fD	0.0fZ	0.0fz	0.0gD	0.0fZ	0.0fz	0.0gD	0.0gZ
Bacillus	13.0hx	74.5bB	20.5fX	10.0iyz	74.8bB	17.8eX	10.3hxy	76.0bAB	13.0fY	11.5exy	75.5bB	12.0eY	9.8eyz	77.5abA	8.5eZ	8.5ez	78.0bA	7.0efZ
Trichoderma	19.5gx	71.5cB	23.8eX	17.3hx	72.0bB	21.0dX	14.0gy	72.8cB	18.5eY	12.5eyz	74.0bB	15.5dY	11.8ez	76.0bAB	10.0dZ	10.0ez	77.5bA	9.3eZ
Celest® XL	3.8iy	79.8aA	5.5hY	0.0jz	79.5aA	0.0fZ	0.0iz	79.8aA	0.0gZ	0.0fz	79.5aA	0.0fZ	0.0fz	80.0aA	0.0fZ	0.0fz	81.5aA	0.0gZ
Control	87.0az	32.0hA	59.0aZ	91.0ay	32.5dA	58.8aZ	90.0ay	33.0hA	60.3aZ	90.0ay	34.3eA	60.0aZ	90.0ay	32.8eA	59.3aZ	90.0ay	31.5eA	60.0aZ

Inc[a]: Percentage incidence of *C. kahawae* subsp. *cigarro*, Germ[b]: seed germination, Dis[c]: diseased seedlings. *Means sharing a common letter in a column differ significantly according to the Fisher's LSD test at $p<0.05$. **In each row, means with the same lowercase letters do not significantly differ from each other at $p = 0.05$. ***Means within a row not followed by the same uppercase letter are significantly different from each other ($p = 0.05$). ***** Means within a row followed by same uppercase letter are not significantly different from each other.

Table 5.4: Assessment of anthracnose leaf spot disease on seedlings grown in a greenhouse from *Eucalyptus* spp. seeds inoculated with *Colletotrichum* spp.

	Incidence (%)				Severity (%)				Ø leaf spots (mm)			
	E. nitens		*E. viminalis*		*E. nitens*		*E. viminalis*		*E. nitens*		*E. viminalis*	
Treatment	**Trial I**	**Trial II**	**Trial I**	**Trial II**	**Trial I**	**Trial II**	**Trial I**	**Trial II**	**Trial I**	**Trial II**	**Trial I**	**Trial II**
HWT 50 °C for 30 min	30.5f	27.5d	14.3ef	20.8ef	48.4f	50.1d	40.5c	62.4b	3.7b	3.8bc	2.7b	5.5b
HWT 60 °C for 1 min	44.9b	39.9b	28.1b	44.0b	64.5b	75.8b	36.1d	48.1cd	5.6a	6.3a	2.0c	3.7cd
5% H_2O_2 for 10 min	29.2g	26.6d	20.0c	27.0c	60.6cd	49.9d	33.7d	40.3e	5.3ab	3.8bc	1.8c	2.8f
10% H_2O_2 for 10 min	29.0g	26.6d	25.1b	18.1fg	56.7e	48.1d	34.7d	45.0d	4.9ab	3.6c	2.0c	3.0ef
Wet 1400 w for 30 s	31.6e	26.6d	11.1f	17.2g	57.4de	47. 9d	44.2b	46.0d	5.3ab	3.5c	2.9b	3.4de
Wet 600 w for 60 s	33.8d	31.5c	15.5de	25.4cd	61.3bc	55.7c	54.3a	51.3c	5.2ab	4.8b	4.8a	4.1c
Dry 1400 w for 60 s	39.1c	34.2c	18.6cd	23.0de	73.9a	79.2ab	44.7b	65.4b	6.4a	6.7a	3.0b	5.8b
Bacillus	4.2i	3.8f	0.7g	3.0h	1.1h	1.1f	3.9f	6.5g	0.2c	0.2d	0.7d	0.8g
Trichoderma	13.1h	10.6e	0.0g	5.5h	11.1g	13.7e	8.2e	27.2f	0.8c	0.9d	0.9d	1.2g
Celest® XL	1.8j	1.3f	0.0g	0.0i	0.9h	0.5f	0.0g	4.0g	0.1c	0.1d	0.0e	0.7g
Inoc control	65.9a	62.0a	46.9a	80.3a	76.0a	81.1a	55.3a	78.3a	6.2a	6.7a	4.8a	6.6a
Non-Inoc control	0.0k	0.0f	0.0g	0.0i	0.0h	0.0f	0.0g	0.0h	0.0c	0.0d	0.0e	0.0h
LSD	0.2	3.9	3.5	3.1	3.8	3.8	3.6	3.6	1.6	1.1	0.4	0.5
CV%	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0

Means sharing a common letter in a column differ significantly according to the Fisher's LSD test at $p<0.05$.

Table 5.5: Effects of seed treatments on the growth and development of seedlings grown in a greenhouse from *Eucalyptus* spp. seeds inoculated with *Colletotrichum* spp.

	Emergence (%)				Seedling length (cm)				Total dry mass (g)			
	E. nitens		*E. viminalis*		*E. nitens*		*E. viminalis*		*E. nitens*		*E. viminalis*	
Treatment	**Trial I**	**Trial II**	**Trial I**	**Trial II**	**Trial I**	**Trial II**	**Trial I**	**Trial II**	**Trial I**	**Trial II**	**Trial I**	**Trial II**
HWT 50 °C for 30 min	78.3cde	80.2bcd	80.0de	85.4c	24.6bcd	26.0bcd	32.3e	30.3def	3.0de	3.2d	3.0de	2.6d
HWT 60 °C for 1 min	76.1de	72.8e	73.2f	75.2f	27.0ab	30.6a	34.6cde	32.3bcde	3.5cd	3.9bc	3.1cd	2.9bcd
5% H2O2 for 10 min	82.1bc	79.6bcd	82.3cd	84.7cd	23.8bcd	29.8abc	34.3cde	31.6cdef	2.4fg	3.2d	2.6e	2.7cd
10% H2O2 for 10 min	80.3cd	77.5cd	85.1bc	84.0cde	24.8bc	25.8cd	31.0e	35.5abc	2.7efg	3.3d	2.9de	2.6d
Wet 1400 w for 30 s	82.5bc	81.9b	88.0ab	87.3bc	25.7bc	30.4a	37.2bcd	33.6abcd	3.7bc	4.3ab	3.7ab	3.1bc
Wet 600 w for 60 s	79.5cde	80.8bc	80.0de	86.4c	23.5bcd	23.7de	33.0de	31.1def	2.4fg	3.4cd	3.6ab	3.0bcd
Dry 1400 w for 60 s	75.5e	76.6cde	77.3ef	81.0de	20.6de	21.5e	32.0e	28.7ef	1.9h	2.4e	2.8de	2.7cd
Bacillus	86.5ab	87.5a	87.0ab	91.2ab	30.6a	31.8a	39.1ab	36.0ab	4.1ab	4.6a	3.6ab	3.3ab
Trichoderma	79.0cde	83.0b	85.0bc	80.9de	26.7abc	30.2ab	37.4abc	34.4abcd	3.7bc	4.1ab	3.4bc	3.1bc
Celest® XL	88.4a	90.0a	91.0a	92.2a	30.7a	32.7a	41.6a	36.9a	4.5a	4.6a	3.9a	3.6a
Inoc control	44.9f	46.0f	50.4g	52.1g	17.3e	19.8e	26.4f	23.0g	2.3gh	2.4e	1.9f	1.8e
Non-Inoc control	78.6cde	76.5de	82.3cd	80.0e	22.6cd	23.7de	31.7e	27.8f	2.8ef	3.0d	3.0de	2.6d
LSD	4.5	4.3	4.2	4.2	4.3	4.3	4.3	4.4	0.5	0.5	0.5	0.4
CV%	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0

Means sharing a common letter in a column differ significantly according to the Fisher's LSD test at $p<0.05$.

Supplementary material:

Appendix 5.1: Effects of hot water seed treatments of artificially inoculated *E. viminalis* seed lots on seed germination, diseased seedlings and incidence of *C. kahawae subsp. cigarro*

	Soaking period														
	1 min			15 min			30 min			45 min			60 min		
Treatment	**Inc[a]**	**Germ[b]**	**Dis[c]**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**
40 °C	91.0[*]ab[**]v	48.5e[***]B	49.3b[****]Y	80.5bw	50.0eB	48.5bY	75.0bx	63.3dA	46.5bY	52.0by	66.5dA	30.8bZ	24.8bz	69.5cA	27.8bZ
45 °C	89.0bw	61.3dB	27.5cX	32.0dx	66.8dB	24.0cX	21.0cy	79.0cA	9.5cZ	17.5cy	81.0cA	16.5cY	8.0cz	77.5bA	15.0cY
50 °C	58.0cw	77.5cC	10.8eXY	17.8ex	74.8cC	11.0dX	7.3dy	92.0aA	8.0cXY	0.0dz	85.5bB	7.3dY	0.0dz	69.8cD	0.0dZ
55 °C	39.8dx	80.8bB	15.0dX	12.0fy	87.3bA	6.8dY	0.0ez	76.5cB	0.0dZ	0.0dz	55.0eC	0.0eZ	0.0dz	40.0dD	0.0dZ
60 °C	0.0fz	88.3aA	17.0dY	0.0gz	25.8hB	0.0eZ	0.0ez	9.3fC	0.0dZ	0.0dz	0.0gD	0.0eZ	0.0dz	0.0fD	0.0dZ
Bacillus	6.5ey	82.5bB	3.5fY	0.0gz	90.0abA	0.0eZ	0.0ez	91.8aA	0.0dZ	0.0dz	91.5aA	0.0eZ	0.0dz	91.0aA	0.0dZ
Trichoderma	8.5ey	80.5bB	7.8efY	0.0gz	88.5bA	0.0eZ	0.0ez	89.0bA	0.0dZ	0.0dz	90.0aA	0.0eZ	0.0dz	90.0aA	0.0dZ
Celest® XL	0.0fz	90.5aA	0.0gZ	0.0gz	92.5aA	0.0eZ	0.0ez	93.3aA	0.0dZ	0.0dz	92.8aA	0.0eZ	0.0dz	93.0aA	0.0dZ
Control	92.3az	31.0fB	62.0aZ	91.0az	36.0fA	59.8aZ	89.8az	27.5eB	61.3aZ	90.8az	28.0fB	62.3aZ	92.3az	27.3eB	62.0aZ

Inc[a]: Percentage incidence of *C. kahawae* subsp. *cigarro*, Germ[b]: seed germination, Dis[c]: diseased seedlings. [*]Means sharing a common letter in a column differ significantly according to the Fisher's LSD test at $p<0.05$. [**]In each row, means with the same lowercase letters do not significantly differ from each other at $p = 0.05$. [***]Means within a row not followed by the same uppercase letter are significantly different from each other ($p = 0.05$). [*****]Means within a row followed by same uppercase letter are not significantly different from each other.

Appendix 5.2: Effects of hydrogen peroxide seed treatments of artificially inoculated *E. viminalis* seed lots on seed germination, diseased seedlings and incidence of *C. kahawae subsp. cigarro*

	Period seeds soaked in H_2O_2														
	1 min			5 min			10 min			30 min			45 min		
Treatment	**Inc[a]**	**Germ[b]**	**Dis[c]**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**
1% H_2O_2	83.3[*]a[**]x	45.8d[***]B	47.3b[****]Y	80.5bx	53.5eA	42.8bZ	68.0by	51.8dA	42.5bZ	66.8byz	50.5fA	48.0bY	63.8bz	50.0fA	49.3bY
5% H_2O_2	41.8by	60.5cC	39.5cX	39.8cy	85.0bAB	8.0cZ	39.5cy	87.3bA	9.3cYZ	26.3cz	84.8dAB	12.0cY	21.8cz	82.5cB	10.8cY
10% H_2O_2	29.0cx	83.3aB	9.8dY	27.0dx	87.3abAB	0.0fZ	18.3dy	89.5abA	0.0dZ	7.0dz	83.5dB	0.0dZ	4.5dz	80.3dB	0.0dZ
15% H_2O_2	23.5dy	78.5bA	0.0gZ	0.0gz	78.8dA	0.0fZ	0.0ez	79.0cA	0.0dZ	0.0ez	72.3eB	0.0dZ	0.0ez	71.3eB	0.0dZ
Bacillus	7.0ey	80.0bB	5.5fY	3.5fy	87.5abA	3.3eY	0.0ez	90.0aA	0.0dZ	0.0ez	90.8bA	0.0dZ	0.0ez	91.5aA	0.0dZ
Trichoderma	9.8ey	78.3bB	7.8eY	6.8ey	82.5cB	5.0dY	0.0ez	87.8bA	0.0dZ	0.0ez	88.5cA	0.0dZ	0.0ez	89.0bA	0.0dZ
Celest® XL	0.0fz	84.5aB	0.0gZ	0.0gz	89.3aA	0.0fZ	0.0ez	91.3aA	0.0dZ	0.0ez	93.0aA	0.0dZ	0.0ez	93.5aA	0.0dZ
Control	86.3az	39.3eA	55.3aZ	86.8az	40.8fA	53.8aZ	90.0az	39.5eA	56.3aZ	88.3az	37.8gA	57.0aZ	88.5az	39.0gA	58.5aZ

Inc[a]: Percentage incidence of *C. kahawae* subsp. *cigarro*, Germ[b]: seed germination, Dis[c]: diseased seedlings. [*]Means sharing a common letter in a column differ significantly according to the Fisher's LSD test at $p<0.05$. [**]In each row, means with the same lowercase letters do not significantly differ from each other at $p = 0.05$. [***]Means within a row not followed by the same uppercase letter are significantly different from each other ($p = 0.05$). [*****]Means within a row followed by same uppercase letter are not significantly different from each other.

Appendix 5.3: Effects of microwave radiation seed treatments of artificially inoculated *E. viminalis* seed lots on seed germination, diseased seedlings and incidence of *C. kahawae subsp. cigarro*

	Microwave exposure Time																	
	30 sec			60 sec			90 sec			120 sec			150 sec			180 sec		
Treatment	**Inc[a]**	**Germ[b]**	**Dis[c]**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**
dry 250 w	83.5[*]a[**]y	39.5e[***]A	51.8ab[****]Z	78.5bz	39.8fA	56.5aY	79.8ayz	39.3fA	54.3aYZ	79.3byz	39.3fA	54.8aY	81.5ayz	40.3fA	53.5aYZ	80.3ayz	40.8fA	52.8aYZ
dry 600 w	80.8bv	37.3efC	50.0bX	74.3cw	40.3fBC	51.8bX	65.8bx	40.0efBC	51.5bX	41.8cy	42.8eAB	42.0bY	29.0bz	44.0eA	40.0bYZ	30.8bz	45.8eA	37.3bZ
dry 1400 w	65.0ex	68.0cB	34.0dX	36.5ey	74.8bcA	25.5cY	32.3dy	68.3cB	23.0dY	0.0gz	8.3gC	0.0fZ	0.0ez	0.0gD	0.0gZ	0.0ez	0.0gD	0.0gZ
wet 250 w	78.0cw	48.3dD	49.5cX	76.0bcw	55.5eB	51.0bX	58.8cx	47.3dD	38.8cY	34.5dy	51.0dC	35.3cY	25.8bz	65.5cA	28.0dZ	21.5cz	68.8cA	29.3cZ
wet 600 w	74.8du	69.8cA	29.0eY	59.8dv	72.0cdA	21.8cZ	31.3dw	47.5dD	40.0cW	18.5ex	51.8dCD	32.3dX	13.0cy	55.8dBC	33.0cX	8.5dz	58.5dB	23.0dZ
wet 1400 w	55.8fx	82.3aA	8.5hY	22.8fy	69.0dB	0.0gZ	0.0gz	41.5eC	0.0gZ	0.0gz	0.0hD	0.0fZ	0.0ez	0.0gD	0.0gZ	0.0ez	0.0gD	0.0gZ
Bacillus	11.0hy	80.5abB	12.5gX	10.3hyz	81.5aB	10.5fXY	9.8fyz	82.0bB	8.8fYZ	8.0fyz	82.5bB	8.3eYZ	7.5dz	85.0aA	7.0fYZ	7.0dz	87.3aA	5.3fZ
Trichoderma	15.3gx	77.1bB	19.5fW	13.5gxy	77.8bB	15.0eX	12.5exy	78.5cB	11.5eXY	10.8fyz	79.0cAB	10.0eYZ	9.5dz	80.3bAB	8.8eYZ	8.3dz	82.0bA	7.5eZ
Celest® XL	1.5iz	82.8aC	4.5iY	0.0iz	83.0aC	0.0gZ	0.0gz	85.8aBC	0.0gZ	0.0gz	86.3aAB	0.0fZ	0.0ez	87.8aA	0.0gZ	0.0ez	89.0aA	0.0gZ
Control	82.8abz	35.0fA	52.0aZ	83.3az	38.3fA	52.5bZ	82.0az	38.0fA	53.0aZ	82.8az	38.5fA	52.3aZ	83.0az	38.0fA	53.5aZ	83.8az	38.0fA	53.0aZ

Inc[a]: Percentage incidence of *C. kahawae* subsp. *cigarro*, Germ[b]: seed germination, Dis[c]: diseased seedlings. [*]Means sharing a common letter in a column differ significantly according to the Fisher's LSD test at $p<0.05$. [**]In each row, means with the same lowercase letters do not significantly differ from each other at $p = 0.05$. [***]Means within a row not followed by the same uppercase letter are significantly different from each other ($p = 0.05$). [*****]Means within a row followed by same uppercase letter are not significantly different from each other.

CHAPTER 6

Seed treatments against anthracnose leaf spot of *Eucalyptus* spp. caused by *Colletotrichum fructicola*

Abstract

Starting with quality, disease free, propagative material is essential for successful seedling establishment. Disease management is a challenge particularly in forests certified by the Forestry Stewardship Commission (FSC) due to lack of alternatives to synthetic chemicals. Non-chemical seed treatments that included hot water, microwave irradiation, hydrogen peroxide were evaluated for their efficacy against anthracnose leaf spot on *Eucalyptus nitens* and *E. viminalis* seedlings. *In -vitro* tests showed that soaking seeds in hot water baths set at 55 °C for 15 min and 60 °C for 1 min, soaking seeds in 5% H_2O_2 for 10 min and 10% H_2O_2 for 10 min, microwave radiation of dry or moist seeds at 1400 w for 30 s and 60 s, were optimal individual treatments that significantly reduced incidences of *Colletotrichum fructicola* with significant increments in seed germination. Greenhouse pot assays showed that a biocontrol agent (BCA) *Bacillus* application was the only non-chemical seed treatment that consistently suppressed incidence of anthracnose leaf spot on both *Eucalyptus* spp. seedlings, and was as effective as the fungicide Celest® XL. Sowing seeds treated with *Bacillus* or *Trichoderma*, or seeds soaked in hot water at 60 °C for 1 min consistently yielded significantly longer *E. nitens* seedlings than those from inoculated controls, and were comparable with seedling lengths from Celest® XL treated seeds. Similarly, seedlings grown from *E. viminalis* seeds treated with *Bacillus* BCA or microwave radiation of moist seed lots at 1 400 w for 30 s were significantly longer and comparable with seedlings from Celest® XL treated seeds. Therefore, the *Bacillus* BCA application showed potential as an effective alternative treatment to promote growth of seedlings free from anthracnose leaf spot.

Key words: Anthracnose, Biocontrol agent, Hydrogen peroxide, Hot water, Microwave radiation

6.1 Introduction

In South Africa, approximately 100 million tree seedlings are produced annually in more than 15 certified nurseries for afforestation and reforestation programmes [Forestry South Africa (FSA) 2012; Seedling Growers Association of South Africa (SGASA) 2018]. Due to the economies of scale, production practices in most nurseries are characterised by high seedling densities, application of excessive fertilisers and frequent irrigation routines (Haase et al. 2016). These growing conditions are favourable for development of diseases particularly during summer periods (Brown and Ferreira 2000). In general, among other diseases affecting seedlings grown in nurseries, anthracnose leaf spot caused by *Colletotrichum* species are frequently reported (Lundquist and Roux 1984; Viljoen et al. 1992; Rodrigues et al. 2014).

Anthracnose disease often occur at phenological stages A and B causing interconnected ellipsoidal lesions and stem cankers on *Eucalyptus* seedlings (Rodrigues et al. 2014). Leaf spots initially appear as circular, light brown to red lesions, and as the lesion enlarge, they may coalesce and cause leaf curling (Baxter et al. 1983; Smith et al. 1998). During severe cases, infection can spread to lower vegetative parts to cause stem girdling and eventual seedling mortality (Dodd et al. 1991).

Since *C. fructicola* and *C. kahawae* subsp. *cigarro*, causal pathogens of anthracnose leaf spot, are both seed-borne and seed-transmitted (Mangwende et al. 2018), precautions should be taken to ensure that seeds used for propagation are free from the pathogens (McGee 1997). Despite persistent diseases and culling percentages in nurseries, forestry seeds are seldom treated. As there are no registered chemicals available to treat *Eucalyptus* seeds in South Africa, foresters intermittently dress seeds with synthetic fungicides such as Captan, Dithane M-45, Thiram and Carbendazim registered on other crops to supress seed-borne and soilborne pathogens (Taylor and Harman 1990; Sharma et al. 2015; Croplife 2019). Moreover, it has

become more challenging to sanitise tree seeds as forestry operations are increasingly complying with principles and regulations of the Forest Stewardship Council (FSC), which restrict use of synthetic chemicals due to concerns on human health and the environment (Tomkins 2004; Mendell et al. 2015; Lemes et al. 2017). A conservative solution is to breed for resistance, but variable resistance observed on provenances of *E. deglupta* is not yet successfully incorporated in commercially grown *Eucalyptus* species (Arentz 1991). Hence, the interest in exploring alternative non-chemical disease management strategies.

Physical methods such as hot water and microwave radiation can be used. Despite a wide body of literature showing efficacy of hot water and microwave radiation on controlling *Colletotrichum*, their application as seed treatments have been limited to a few agronomic and vegetable crops (Lozano et al. 1986; Benic and Knox-Davies 1983; Islam et al. 2009; Han 2010; Tylkowska et al. 2010; Vivekanand et al. 2018). Another environmentally benign approach is to use biocontrol agents (BCAs) (Raupach and Kloepper 2000; Begum et al. 2008; Padder and Sharma 2011; Ashwini and Srividya 2014; Mohammed et al. 2014; Tumpa et al. 2016). However, few studies have investigated BCAs in controlling seed-borne mycoflora associated with forest seed lots (Iturritxa et al. 2017; Silva et al. 2019).

The aim of this study was to evaluate the efficacy of seed treatments *viz*. physical methods, hot water and microwave; BCAs *Bacillus subtilis* (Ehrenberg) Cohn and *Trichoderma harzianum* Rifai; and a natural chemical, hydrogen peroxide, at sanitising seed lots of *Eucalyptus nitens* (H. Deane and Maiden) Maiden and *Eucalyptus viminalis* Labill. artificially inoculated with *Colletotrichum fructicola* Prihastuti L. Cai & K.D. Hyde. Effectiveness of seed treatments was also confirmed by reduction in transmission of the pathogen from seeds into seedlings as observed by development of anthracnose leaf spot on seedlings.

6.2 Materials and methods

6.2.1 Source of materials

Eucalyptus viminalis and *E. nitens* seeds were supplied by South African forest seed companies. *Eucalyptus* species were selected based on susceptibility to anthracnose leaf spot (Chapter 4). The anthracnose leaf spot pathogen, *C. fructicola* (PPRI 24314), isolated from *Eucalyptus* seeds (Chapter 4 section 4.3.1) was used in this study. Commercial BCAs used in this study included EcoT™, a powdered formulation of *T. harzianum* at a concentration of $2x10^9$ spores/g (Plant Health Products (Pvt.) Ltd. (Kwazulu-Natal, South Africa), and Integral®, a liquid formulation of *B. subtilis* strain MBI 600 at a concentration of $2x10^{11}$ spores/ml (Becker Underwood (Pvt) Ltd. (Kwazulu-Natal, South Africa). In addition, the chemical Celest® XL consisting of 25 ai/L fludioxonil and 10 g ai/L mefenoxam (Syngenta (Pvt.) Ltd., Midrand, South Africa) and Ensure® ISO (30% hydrogen peroxide) (Merck (Pvt.) Ltd. Midrand, South Africa) were used in this study.

6.2.2 Seed inoculation

Pathogen inoculum was prepared from 14-day-old *C. fructicola* cultures. Mycelia was gently scrapped with a scalpel and plates flooded with sterile distilled water. Mycelia suspension was filtered through a cheesecloth to obtain spore suspensions. Two drops of Tween-20 were added and the concentration of inoculum adjusted to 1×10^5 conidia/mL.

Seeds of the two *Eucalyptus* spp. were immersed in 1% sodium hypochlorite solution for 5 min, rinsed thoroughly in sterile distilled water and air dried on sterile paper towels. Surface sterilised seeds were artificially inoculated by soaking in the inoculum for 4 h with occasional hand shaking. Thereafter, seeds were left to air dry on the laminar flow. The success of seed inoculation was confirmed by plating inoculated seeds, 50 per sample on potato dextrose agar (PDA, Biolabs, South Africa), where incidences of *C. fructicola* of more than 90% were observed.

6.2.3 Seed treatments

6.2.3.1 Hot water

A hot water bath (Model: 132A; Labotec, South Africa) was used in this study. The two inoculated *Eucalyptus* spp. seed lots were treated with hot water seed treatments following the protocol of Masum et al. (2009). Prior to treatment, hot water baths were adjusted until desired consistent temperatures of 35, 40, 45, 50, 55 and 60 ±1 °C were achieved. Aliquots comprising of 200 inoculated seeds wrapped in each cheesecloth were individually immersed in hot water baths set at each temperature point for different time periods of 1, 15, 30, 45 and 60 min. At each temperature-time combination, a negative control was included comprising of seeds soaked in sterile distilled water at standard room temperature and a positive control of seeds soaked in Celest® XL at the recommended rate of 1 mL/kg. After treatment, seeds were cooled in sterile distilled water, spread on sterile paper towels and left to air dry in the laminar flow.

6.2.3.2 Microwave radiation

Following seed inoculation, some seeds were either left to dry in the laminar flow or directly treated wet with microwave radiation (Samsung microwave model: ME9114W1, Malaysia). *Eucalyptus* seeds of each species, 200 for each microwave power-time combination, were spread on two Whatman filter papers aligned inside a glass Petri dish. Microwave radiation was evaluated at three microwave power levels of 250, 600 or 1400 w with exposure periods ranging from 0 to 180 s with increments of 30 s. Inoculated seeds that were left untreated for the different times served as positive controls, whereas positive controls consisted of inoculated seeds treated with Celest® XL. Treated seeds were immersed in sterile distilled water for 5 min, and air dried in the laminar flow.

6.2.3.3 Hydrogen peroxide

Inoculated seeds wrapped in cheesecloths forming aliquots of 200 in each bag, were soaked in aqueous solutions of 1, 5, 10 or 15% (*v/v*) hydrogen peroxide for 1, 5, 10, 30 or 45 min. Inoculated seeds immersed in sterile distilled water at the aforementioned time points served as positive controls, whilst seeds soaked in Celest® XL served as negative controls. Thereafter, seeds were rinsed in sterile distilled water and left to dry in the laminar flow.

6.2.4 Effects of seed treatments on incidence of *C. fructicola*

The efficacy of the above-mentioned seed treatments at reducing incidences of *C. fructicola* on artificially inoculated seed lots was determined by the agar plate method. Recovery of *C. fructicola* was examined from treated *Eucalyptus* spp. seed lots, four replicates of 50 seeds, plated on PDA media (10 seeds per Petri dish). After 5 days of incubation in a in a 25 °C incubator (Labcon, Gauteng, South Africa) with alternating 12 h white fluorescent light/12 h dark regime, counts of seeds infected with *C. fructicola* were recorded. The experiment was repeated.

6.2.5 Effects of seed treatments on seed germination

Germination assays were conducted following the on-top of paper method (ISTA 2019) with minor modifications. Seed were germinated on moist Whatman No. 1 filter papers, where 25 seeds were plated in each 11 cm glass Petri dish. Each experiment included four replicates of 50 seeds and was repeated. The Petri dishes were incubated in a growth cabinet maintained at 25 ^{o}C with alternating cycles of 12 h white light (58 w Osram fluorescent tubes; Russia)/12 h dark cycle for 21 days. Counts of seeds that germinated or seedlings that developed diseases were recorded. Seedlings scored as diseased were decayed or had lesions on hypocotyls and/or seminal roots.

6.2.6 Greenhouse trials

Two trials were conducted at the start of spring and summer to investigate efficacy of selected non-chemical seed treatments against anthracnose leaf spot in a greenhouse located at the Hillcrest campus of the University of Pretoria, South Africa (25° 45′ S, 28°15’ E). Treated. Seeds of both *Eucalyptus* spp. were sown singly in 15 cm diameter pots filled with pasteurised sandy loam soil (ten pots for each treatment). Each trial followed a completely randomised block experimental design with inoculated and non-inoculated controls. The greenhouse received a natural photoperiod of approximately 12 h and temperature was maintained at 25 ±1 °C and a night time temperature of 20 ±1 °C. Plants were watered every second day.

Seedling emergence was checked at 21 days after sowing (DAS). Plants were assessed for incidence and severity of anthracnose leaf spot at 60, 120 and 180 DAS. Disease severity was determined visually following a five point disease severity rating scale described described in Chapter 4 section 4.2.4. At the end of each trial, surviving plants were harvested, sizes of anthracnose lesions measured and plant length recorded. Harvested plants were dried for 72 h at 70 °C and weighed separately for each treatment to determine the total biomass.

6.2.7 Statistical analysis

Experimental data was computated for analysis of variance using the General Linear Model procedure of Statistical Analysis System (SAS, version 9.4) (SAS Institute, 2016) and means were compared through the Fisher’s Least Significant Differences test (LSD, $p<0.05$).

6.3 Results

6.3.1 Effects of seed treatments on the incidence of *C. fructicola*

Hot water seed treatments significantly reduced incidences of *C. fructicola* on both *Eucalyptus* spp. seed lots compared with controls ($p<0.05$), except for seed lots soaked in hot water bath

set 40 °C for 1 min (Table 6.1 and Appendix 6.1). Although incidences of *C. fructicola* persisted on *E. nitens* seed lots soaked in hot water baths for 1 min, adjusting the hot water bath to 60 °C effectively reduced incidences of *C. fructicola* on *E. viminalis* seeds and was comparable with the *Bacillus* BCA and Celest® XL seed treatments. Soaking *Eucalyptus* spp. seed lots in a hot water bath set at 55 °C for 30 min and above, and 60 °C for 15 min and above effectively eliminated incidences of *C. fructicola*.

Soaking *Eucalyptus* spp. seed lots in H_2O_2 significantly reduced incidences of *C. fructicola* compared with untreated controls ($p<0.05$), except for seeds soaked in 1% H_2O_2 for 1 min (Table 6.2 and Appendix 6.2). At a soaking period of 5 min and above, 15% H_2O_2 was the only concentration that effectively eliminated incidences of *C. fructicola* on *Eucalyptus* spp. seeds as effectively as Celest® XL.

Microwave irradiated seeds had significantly lower incidences of *C. fructicola* compared with controls ($p<0.05$), except for dry *E. nitens* seed lots exposed at 250 w microwave radiation for 30 s and microwave radiation of dry *E. viminalis* seed lots at 250 w for 30, 60 or 90 s (Table 6.3 and Appendix 6.3). Exposure of both dry and moist *Eucalyptus* spp. seed lots to microwave radiation of 1400 w for 90 s and above effectively eliminated incidences of *C. fructicola*.

6.3.2 Effects of seed treatments on seed germination and seedling anthracnose leaf spot

The effects of hot water seed treatments on *Eucalyptus* spp. seed germination are shown in Table 6.1 and Appendix 6.1. Soaking *Eucalyptus* spp. seed lots in hot water baths set at 55 °C for 15 min and 60 °C for 1 min were the only temperature-time combination that significantly improved seed germination as effective as seed treatment with *Bacillus* BCA and Celest® XL ($p<0.05$).

Despite significant increase of seed germination for *Eucalyptus* spp. seeds soaked in H_2O_2 compared with untreated controls, none of the concentration-time combinations improved seed germination of *E. nitens* as effectively as Celest® XL (Table 6.2). Significantly high seed germination were observed from *E. viminalis* seeds soaked in 5% H_2O_2 for 5 or 10 min, and 10% H_2O_2 for 1 or 5 min, which were equally as effective as seeds treated with Celest® XL (Appendix 6.2).

Seed germination percentages of moist *Eucalyptus* spp. seed lots microwave irradiated at 1 400 w for 30 s were significantly increased comparable to Celest® XL treated seed lots ($p<0.05$) (Table 6.3 and Appendix 6.3). Seed germination was also significantly high for moist *E. nitens* seed lots exposed to microwave radiation of 600 w for 60 s (Appendix 6.3).

The percentage of diseased seedlings was significantly reduced when seeds were soaked in hot water baths and H_2O_2 compared with the untreated controls ($p<0.05$) (Tables 6.1 and 6.2 and Appendices 6.1 and 6.2). Diseases were effectively suppressed from appearing on seedlings of both *E. nitens* and *E. viminalis* seed lots soaked in hot water baths set at 60 °C for 15 min and longer. However, longer soaking periods were required to yield the same level of disease suppression on *E. viminalis* seedlings. Thus, whilst hot water treatment parameters of 50 °C for 45 min and 55 °C for 15 min and longer effectively suppressed appearance of diseases on *E. nitens* seed lots, *E. viminalis* seed lots had to be soaked for 60 min and 30 min, respectively to yield disease free seedlings. Similarly, there were no diseases on seedlings grown from both *Eucalyptus* spp. seed lots soaked in 15% H_2O_2 for 5 min or longer, and 10% H_2O_2 for 10 min or longer.

There was a significant reduction in percentage of diseased seedlings grown from seed lots exposed to microwave radiation compared with untreated controls ($p<0.05$), except for dry seed lots exposed to microwave radiation of 250 w (Table 6.3 and Appendix 6.3). Microwave

radiation of moist *Eucalyptus* spp. seed lots at 1400 w for all exposure periods effectively suppressed appearance of diseases on seedlings. Dry *Eucalyptus* spp. seed lots had to be exposed to microwave radiation for 150 s and longer to achieve similar levels of disease suppression.

6.3.3 Effects of seed treatments on development of anthracnose leaf spot in the greenhouse

Seedlings from treated seed lots had significantly lower incidences of anthracnose leaf spot than inoculated controls (Table 6.4). Highest incidences of anthracnose leaf spot were observed on seedlings grown from inoculated controls, whereas no anthracnose leaf spot appeared on seedlings from non-inoculated controls. *Bacillus* BCA was the only non-chemical seed treatment that consistently suppressed incidence of anthracnose leaf spot on *Eucalyptus* spp. seedlings, and was as effective as Celest® XL.

Anthracnose leaf spot was less severe on seedlings grown from treated seeds, which was significantly lower than inoculated controls ($p<0.05$) (Table 6.4). Severity of anthracnose leaf spot was least severe on seedlings grown from seeds treated with *Bacillus* BCA or Celest® XL, and appeared to be as healthy as those of the non-inoculated controls.

The average diameter of anthracnose leaf spot measured on *Eucalyptus* spp. seedlings are presented in Table 6.4. Seedlings from inoculated controls had the largest leaf spot, and were not significantly larger than those of seedlings grown from microwave irradiated *E. nitens* seed lots or seed lots soaked in a 60 °C hot water bath ($p>0.05$). However, seedlings grown from treated *E. viminalis* seed lots had significantly smaller lesion sizes than those from inoculated seeds ($p<0.05$). The smallest anthracnose leaf spot was observed on seedlings grown from *Eucalyptus* spp. seed lots treated with Celest® XL, which were not significantly different from seed lots treated with the BCAs ($p>0.05$).

6.3.4 Effect of seed treatments on emergence and seedling growth

Seed treatments significantly improved *Eucalyptus* seedling emergence in the greenhouse trials compared with the inoculated controls ($p<0.05$) (Table 6.5). Trial I results showed that sowing *E. nitens* seeds treated with BCAs and seeds soaked in 5% H_2O_2 for 10 min had significantly higher seedling emergence than non-inoculated controls ($p<0.05$), and compared well with Celest® XL. However, *Bacillus* BCA seed treatment was the only non-chemical treatment that consistently increased seedling emergence significantly more than non-inoculated controls in both trials I and II ($p<0.05$) (Table 6.5). There was a significant increase in seedling emergence of *E. viminalis* treated seeds compared with non-inoculated controls ($p<0.05$), except for dry seeds exposed to microwave radiation at 1400 w for 60 s and seeds soaked in a hot water bath at 60 °C for 1 min.

Seedlings grown from treated seed lots were significantly longer than seedlings of the inoculated controls ($p<0.05$), except those from moist *E. nitens* seed lots exposed to microwave radiation of 1400 w for 60 s (Table 6.5). The average lengths of *E. nitens* seedlings ranged from 21.3 to 36.8 cm, and from 22.3 to 43.4 cm for *E. viminalis* seedlings, where longest seedlings were recorded from seeds treated with Celest® XL and shortest seedling lengths from inoculated controls. For *E. nitens* seed lots, treatments with the BCAs *Bacillus* and *Trichoderma*, or soaking seeds in hot water bath set at 60 °C for 1 min consistently yielded significantly longer seedlings than those of the inoculated controls, and were comparable with the Celest® XL treatment. However, effects of non-chemical seed treatments on *E. viminalis* seedling growth were inconsistent. Trial II results showed that *Bacillus* BCA and microwave radiation of moist seed lots were the only non-chemical seed treatments that yielded significantly longer *E. viminalis* seedlings comparable with the Celest® XL seed treatment.

Seed treatments in both greenhouse trials significantly increased seedling dry mass compared with inoculated controls ($p<0.05$), except for seedlings grown from *E. nitens* seed lots soaked in 5% H_2O_2 for 10 min in Trial I (Table 6.5). *Bacillus* BCA treatment was the only non-chemical seed treatment from which dry seedling masses of both *E. nitens* and *E. viminalis* were comparable to those grown from Celest® XL treated seeds.

6.4 Discussion

Anthracnose leaf spot caused by *Colletotrichum* species are frequently reported in *Eucalyptus* nurseries (Viljoen et al. 1992; Rodrigues et al. 2014). Disease management in most forest nurseries primarily relies on synthetic pesticides, but their use is being discouraged as they may pose a health hazard to humans and the environment (Reynolds et al. 2015; Ye et al. 2015). Due to limited availability of alternatives to synthetic chemicals, only small percentages of forest seed and nursery seedlings are produced following the FSC's rules and regulations (Klooster 2010).

This study showed that *C. fructicola* may significantly reduce *Eucalyptus* seed germination and seedling emergence. This fungus was described in 2009 and has been reported as an important fungal pathogen in several plantations in China, Japan, Thailand and Uruguay (Prihastuti et al. 2009; Alaniz et al. 2015; Zhang et al. 2015; Gan et al. 2017). Recently, *C. fructicola* was detected on *Eucalyptus* spp. seed lots (Mangwende et al. 2018). These infected and/or infested seed lots were destined to restock large-scale commercial forests in South Africa. Moreover, with active participation of South African forest companies in tree improvement programmes, seeds and other propagative material are distributed across geographical regions increasing the risk of introduction of insects and pathogens in areas they are not known to occur. In order to attempt to reduce the spread of *C. fructicola*, efficacy of non-chemical seed treatments including microwave radiation, BCAs, hot water baths and hydrogen peroxide were evaluated.

Although incidences of *C. fructicola* were effectively eliminated on both *Eucalyptus* spp. seed lots soaked in hot water baths set at 55 °C for 30 min and above, and 60 °C for 15 min and 15% H_2O_2 for 5 min and above, these seed treatment conditions significantly reduced seed germination. Similarly, microwave irradiation of dry or moist *Eucalyptus* spp. seed lots at 1400 w for 90 s and above seems unsuitable regardless of their success at controlling *C. fructicola*. In considering both seed disinfection and seed germination, non-chemical seed treatments *viz*. soaking seeds in hot water baths set at 55 °C for 15 min, 60 °C for 1 min, soaking seeds in 5% H_2O_2 for 10 min, 10% H_2O_2 for 10 min, microwave radiation of dry or moist seeds at 1400 w for 30 s and 60 s, respectively, and 600 w for 60 s were optimal treatment conditions under laboratory conditions.

Greenhouse trials demonstrated higher seedling emergence from treated seed lots than possible losses observed in untreated controls. However, viable fungal inoculum retained on both treated *Eucalyptus* spp. seed lots was sufficient enough to cause anthracnose leaf spot. Several C*olletotrichum* species are seed-borne and seed-transmitted pathogens causing anthracnose blight on young germinants without major decline in stand establishment (Latunde-Dada et al. 1999; Yoshida and Tsukiboshi 2002; Holzmueller et al. 2006; Sergeeva et al. 2008; Zhang 2008). Thus, ineffectively of non-chemical seed treatments at eliminating incidences of *C. fructicola* permitted retention and transmission of low levels of the pathogen from seeds into seedlings. Furthermore, the non-systemic nature of non-chemical seed treatments translated to poor efficacy at suppressing growth of the fungus and resulted in appearance of high incidences of anthracnose leaf spot under greenhouse conditions.

Sowing seeds treated with *Bacillus* BCA was the only non-chemical seed treatment that was comparable with Celest® XL at suppressing development of anthracnose leaf spot on both *Eucalyptus* spp. seedlings. Several studies have shown that *Bacillus* sp. residing in seeds are

often vertically transmitted throughout various developmental stages including seed germination and root and stem growth (Wulff et al. 2003; Truyens et al. 2015; Bodhankar et al. 2017; Glassner et al. 2018; Marag and Suman 2018; Shahzad et al. 2018). *Bacillus* secretes compounds such as hydrogen cyanide and mycelial cell wall degrading enzymes such as cellulase, chitosanase and glucanase that directly inhibit growth of fungi or induce the host's systemic resistance (Reetha et al. 2014; Thadathil and Velappan 2014; Gajera et al. 2016; Kilani-Feki et al. 2016).

Seedling size is an essential growth attribute critical for post-planting success (Close et al. 2010; FSA 2012). Large seedlings perform better than small seedlings after planting, particularly under stressful plantation conditions where there is constant competition for resources with weeds (Close et al. 2003). Sowing untreated seeds inoculated with *Colletotrichum* spp. yielded the shortest seedlings and the lowest average dry masses. The quality of seedlings were poor and severely diseased with low prospects of surviving after transplanting in forest plantations. Seed treatments with commercial bio-control agents and a hot water bath set at 60 °C for 1 min consistently produced the longest seedlings in all trials, which compared well with seedlings grown from Celest® XL treated seeds. Dried seedling mass from seed treatments with BCAs were comparable with those from Celest® XL treated seeds.

In conclusion, in view of the restrictions on further use of synthetic pesticides in FSC registered forest plantations, nursery managers can consider treating *Eucalyptus* seeds with Integral® (*Bacillus* BCA) as an effective alternative to synthetic fungicides against anthracnose leaf spot. Although it was not demonstrated in this study, it is possible to achieve better disease control and significantly higher increments of plant growth from seeds treated with a combination of seed treatments. Soaking seeds in hydrogen peroxide or use of physical methods such as hot

water and microwave before seeds are treated with *Bacillus* or *Trichoderma* BCAs can reduce initial inoculum of *C. fructicola*. Greater disease control can be expected after application of BCAs as they have a systemic mode of action that can effectively minimise disease caused by pathogen inoculum retained from the initial treatment. Similarly, combination of biocontrol formulations should reinforce each other at supressing anthracnose leaf spot and needed to be tested in future over a variety of environmental conditions.

6.5 References

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Table 6.1: Effects of hot water seed treatments of artificially inoculated *E. nitens* seed lots on seed germination, diseased seedlings and incidence of *C. fructicola*

	Soaking period														
	1 min			15 min			30 min			45 min			60 min		
Treatment	**Inc[a]**	**Germ[b]**	**Dis[c]**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**
40 °C	96.3[*]a[**]v	45.0e[***]C	46.0b[****]X	81.8bw	49.5eB	42.5bX	72.0bx	65.8eA	18.5bY	53.0by	68.0dA	15.0bZ	35.0bz	68.8cA	14.5bZ
45 °C	85.0bv	69.0dC	19.5cW	54.0cw	74.0dB	12.8cX	40.0cx	78.0cAB	7.0cY	31.0cy	80.3bA	5.8cZ	9.0cz	79.0bA	5.0cZ
50 °C	37.0cv	78.5cB	8.3deY	20.0dw	80.8cB	3.5dZ	14.0dx	89.3bA	3.5dZ	8.0dy	71.3cC	0.0dZ	0.0dz	69.8cC	0.0dZ
55 °C	25.5dx	81.5bB	6.5eY	11.0ey	88.0aA	0.0eZ	0.0ez	76.5dC	0.0eZ	0.0ez	65.8dD	0.0dZ	0.0dz	41.3dE	0.0dZ
60 °C	7.8ey	83.0abA	3.5fZ	0.0fz	22.3fB	0.0eZ	0.0ez	9.8gC	0.0eZ	0.0ez	0.0fD	0.0dZ	0.0dz	0.0fD	0.0dZ
Bacillus	6.3ey	81.0bB	5.0efY	0.0fz	86.5abA	0.0eZ	0.0ez	90.0bA	0.0eZ	0.0ez	89.8aA	0.0dZ	0.0dz	90.0aA	0.0dZ
Trichoderma	7.0ey	82.3abB	9.5dY	0.0fz	84.0bB	0.0eZ	0.0ez	89.5bA	0.0eZ	0.0ez	89.0aA	0.0dZ	0.0dz	89.8aA	0.0dZ
Celest® XL	0.0fz	84.8aB	0.0gZ	0.0fz	88.5aA	0.0eZ	0.0ez	91.5aA	0.0eZ	0.0ez	91.0aA	0.0dZ	0.0dz	91.5aA	0.0dZ
Control	95.8az	24.3fAB	62.8aZ	95.0az	23.3fB	65.3aYZ	95.0az	21.8fB	66.3aYZ	96.0az	25.3eAB	65.7aYZ	97.8az	27.5eA	67.3aY

Inc[a]: Percentage incidence of *C. fructicola*, Germ[b]: seed germination, Dis[c]: diseased seedlings. [*]Means sharing a common letter in a column differ significantly according to the Fisher's LSD test at $p<0.05$. [**]In each row, means with the same lowercase letters do not significantly differ from each other at $p = 0.05$. [***]Means within a row not followed by the same uppercase letter are significantly different from each other ($p = 0.05$). [*****] Means within a row followed by same uppercase letter are not significantly different from each other.

Table 6.2: Effects of hydrogen peroxide seed treatments of artificially inoculated *E. nitens* seed lots on seed germination, diseased seedlings and incidence of *C. fructicola*

	Period seeds soaked in H_2O_2														
	1 min			5 min			10 min			30 min			45 min		
Treatment	**Inc[a]**	**Germ[b]**	**Dis[c]**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**
1% H_2O_2	92.0[*]a[**]x	45.0e[***]BC	44.5b[****]Y	82.5by	54.0eA	39.5bZ	81.3by	52.3dA	42.8bYZ	80.5by	47.3eB	50.3bX	72.3bz	42.3eC	54.3bX
5% H_2O_2	69.3bw	51.0dD	28.8cW	49.3cx	72.5dC	9.3cZ	41.0cy	82.8bA	16.8cY	39.0cy	79.5cAB	20.0cX	32.8cz	78.0cB	19.5cXY
10% H_2O_2	40.5cw	77.3bAB	14.3dX	37.8dwx	80.5bA	4.8dY	35.0dx	80.8bA	0.0dZ	31.0dy	79.3cA	0.0dZ	21.3dz	75.8cB	0.0dZ
15% H_2O_2	31.3dx	73.3cA	10.5edY	2.3fy	72.5cAB	0.0eZ	0.0ez	73.0cAB	0.0dZ	0.0ez	69.5dBC	0.0dZ	0.0ez	67.8dC	0.0dZ
Bacillus	7.0ey	80.5aB	6.5eY	3.8eyz	82.8aB	0.0eZ	0.0ez	83.8aB	0.0dZ	0.0ez	90.3abA	0.0dZ	0.0ez	89.0abA	0.0dZ
Trichoderma	9.0ey	78.3aC	9.3eY	6.3ey	82.0aBC	0.0eZ	0.0ez	82.5abB	0.0dZ	0.0ez	88.0bA	0.0dZ	0.0ez	88.3bA	0.0dZ
Celest® XL	0.0fz	81.5aB	2.0fZ	0.0fz	83.5aB	0.0eZ	0.0ez	84.5aB	0.0dZ	0.0ez	91.8aA	0.0dZ	0.0ez	91.0aA	0.0dZ
Control	94.0az	34.3fA	56.5aZ	93.5az	35.3fA	53.5aZ	94.0az	36.0eA	53.5aZ	94.0az	36.0fA	53.5aZ	94.3az	29.0fB	61.0aY

Inc[a]: Percentage incidence of *C. fructicola*, Germ[b]: seed germination, Dis[c]: diseased seedlings. [*]Means sharing a common letter in a column differ significantly according to the Fisher's LSD test at $p<0.05$. [**]In each row, means with the same lowercase letters do not significantly differ from each other at $p = 0.05$. [***]Means within a row not followed by the same uppercase letter are significantly different from each other ($p = 0.05$). [*****] Means within a row followed by same uppercase letter are not significantly different from each other.

Table 6.3: Effects of microwave radiation seed treatments of artificially inoculated *E. nitens* seed lots on seed germination, diseased seedlings and incidence of *C. fructicola*

	Microwave exposure Time																	
	30 sec			60 sec			90 sec			120 sec			150 sec			180 sec		
Treatment	**Inc[a]**	**Germ[b]**	**Dis[c]**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**
250 w dry	87.5*a**x	34.5f***B	57.5a****Z	82.5by	35.0eAB	57.5aZ	80.8by	35.8gAB	56.3aZ	80.5by	38.5fA	54.8aZ	78.0bz	37.8fAB	56.0aZ	79.8byz	37.0fAB	55.8aZ
600 w dry	79.0bv	37.0efB	55.3bY	69.3cw	38.8eB	53.5bY	48.8cx	43.5fA	51.8bY	40.8dy	44.8eA	44.5bZ	38.0cy	44.5eA	40.3bZ	29.5cz	42.5eAB	41.0bZ
1400 w dry	54.8dx	65.3dB	39.0dV	33.5fy	71.0bA	30.8dW	0.0fz	65.3cB	25.0dX	0.0gz	0.0gC	17.0dY	0.0fz	0.0gC	0.0eZ	0.0fz	0.0gC	0.0eZ
250 w wet	75.3cu	39.8eD	48.8cW	66.3dv	51.5dB	42.8cX	49.8cw	45.0fC	40.0cX	43.8cx	53.3dB	32.5cY	35.5cy	63.5cA	27.0cYZ	28.8cz	67.8cA	26.3cZ
600 w wet	71.5cv	68.0dB	30.3eW	39.8ew	79.5aA	25.0eX	30.5dx	60.0dC	24.0dX	24.5ey	57.3cC	16.8dY	20.0dy	52.0dD	10.5dZ	16.0dz	48.5dD	7.5dZ
1400 w wet	42.0ex	86.0aA	5.5hY	19.3gy	67.0cB	0.0hZ	0.0fz	54.8eC	0.0gZ	0.0gz	0.0gD	0.0fZ	0.0fz	0.0gD	0.0eZ	0.0fz	0.0gD	0.0eZ
Bacillus	11.5gy	75.3bB	19.0gX	9.5iyz	76.5aB	14.3gXY	8.5eyz	77.3bAB	11.5fY	8.5fyz	79.5aA	9.8eY	7.8ez	80.0aAB	8.0dYZ	8.0eyz	80.5aA	6.5dZ
Trichoderma	16.0fy	71.0cB	21.3fX	12.3hyz	71.5bB	17.5fXY	10.5ez	74.0bB	15.0eY	10.5fz	75.8bAB	11.5eYZ	9.0ez	77.3bAB	10.8dZ	11.5dyz	79.0bA	8.3dZ
Celest® XL	0.0hz	77.5bB	2.5hZ	0.0jz	79.0aAB	0.3hZ	0.0fz	81.5aAB	0.0gZ	0.0gz	81.8aAB	0.0fZ	0.0fz	82.5aAB	0.0eZ	0.0fz	83.0aA	0.0eZ
Control	89.5az	37.5eAB	56.8aZ	88.8az	37.0eAB	53.8bZ	88.0az	38.5gA	56.8aZ	90.5az	35.8fAB	56.0aZ	89.5az	35.5fAB	56.5aZ	89.0az	34.5fB	56.0aZ

Inc[a]: Percentage incidence of *C. fructicola*, Germ[b]: seed germination, Dis[c]: diseased seedlings. *Means sharing a common letter in a column differ significantly according to the Fisher's LSD test at $p<0.05$. **In each row, means with the same lowercase letters do not significantly differ from each other at $p = 0.05$. ***Means within a row not followed by the same uppercase letter are significantly different from each other ($p = 0.05$). ***** Means within a row followed by same uppercase letter are not significantly different from each other.

Table 6.4: Disease assessment on seedlings grown from *Eucalyptus* spp. seeds inoculated with *C. fructicola.*

	Incidence (%)				Severity (%)				Ø leaf spots (mm)			
	E. nitens		*E. viminalis*		*E. nitens*		*E. viminalis*		*E. nitens*		*E. viminalis*	
Treatment	**Trial I**	**Trial II**	**Trial I**	**Trial II**	**Trial I**	**Trial II**	**Trial I**	**Trial II**	**Trial I**	**Trial II**	**Trial I**	**Trial II**
HWT 55 °C for 15 min	11.8de	18.3c	19.5bcd	12.0ef	44.2e	44.7d	30.3e	41.4e	3.1bc	4.1bcd	1.7e	2.8de
HWT 60 °C for 1 min	21.3b	21.3bc	22.0bc	17.4bc	49.8d	62.2b	58.0b	38.4e	4.2ab	5.3abc	5.0b	2.5ef
5% H_2O_2 for 10 min	12.9d	14.8de	16.7de	10.5fg	39.8f	45.1d	32.6e	45.6d	2.3cd	3.6cd	1.9e	3.2d
10% H_2O_2 for 10 min	10.3e	11.0f	11.2fg	14.0de	36.8g	42.3d	40.4d	40.6e	2.3cd	2.8de	2.6d	2.6e
Wet 1400 w for 30 s	12.5d	13.0ef	14.0ef	11.2ef	50.0d	51.1c	30.4e	38.1e	4.1ab	4.6abcd	1.8e	2.1f
Wet 600 w for 60 s	16.1c	18.0cd	18.6cd	15.4cd	55.2c	49.1c	41.5d	54.0c	4.6ab	3.9cd	2.8d	4.5c
Dry 1400 w for 60 s	22.8b	23.8b	23.0b	20.0b	60.0b	66.2b	52.5c	65.0b	5.2a	6.1ab	4.4c	5.7b
Bacillus	4.6f	2.3g	0.0h	3.2h	3.0i	5.1f	2.3g	0.0g	0.8e	0.7ef	0.5fg	0.0h
Trichoderma	11.2de	10.0f	9.5g	7.4g	14.9h	15.8e	8.0f	7.4f	0.9de	1.1ef	0.8f	0.8g
Celest® XL	2.1g	2.2g	0.0h	2.6h	1.31ij	1.7fg	0.8g	0.0g	0.3e	0.2f	0.1g	0.0h
Inoc control	42.8a	51.3a	52.0a	47.1a	72.5a	77.6a	68.2a	75.9a	5.4a	6.2a	6.1a	6.4a
Non-Inoc control	0.0h	0.0g	0.0h	0.0h	0.0j	0.0g	0.0g	0.0g	0.0e	0.0f	0.0g	0.0h
LSD	2.0	3.3	3.7	3.4	2.8	3.6	3.4	3.7	1.5	2.1	0.6	0.5
CV%	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0

Means sharing a common letter in a column differ significantly according to the Fisher's LSD test at $p<0.05$.

Table 6.5: Effects of seed treatments on the growth of *Eucalyptus* spp. seedlings grown from seeds inoculated with *C. fructicola*

	Emergence (%)				Seedling length (cm)				Total dry mass (g)			
	E. nitens		*E. viminalis*		*E. nitens*		*E. viminalis*		*E. nitens*		*E. viminalis*	
Treatment	**Trial I**	**Trial II**	**Trial I**	**Trial II**	**Trial I**	**Trial II**	**Trial I**	**Trial II**	**Trial I**	**Trial II**	**Trial I**	**Trial II**
HWT 55 °C for 15 min	80.9cd	80.4c	87.0def	79.5de	28.7cd	30.9bcd	30.1cd	29.0d	3.3def	3.5ef	3.1c	2.9d
HWT 60 °C for 1 min	80.9cd	73.7d	85.4efg	75.3e	31.2abc	34.4ab	36.4bc	34.0bc	3.6cde	4.6bc	3.3c	3.2cd
5% H_2O_2 for 10 min	86.5ab	77.1cd	92.3ab	86.0bc	28.0cde	33.0abc	30.0cd	32.0bcd	2.6ghi	4.2cd	2.7d	3.1cd
10% H_2O_2 for 10 min	79.8cd	75.5d	88.2cde	82.6cd	28.1cde	30.4bcd	34.9bcd	33.4bc	3.1efg	4.0d	3.3c	3.0cd
Wet 1400 w for 30 s	81.3bcd	80.3c	90.8abcd	88.1b	29.1bcd	32.6abc	35.4bcd	35.5ab	3.9bc	5.0ab	3.8b	3.3bcd
Wet 600 w for 60 s	83.7bc	80.7c	89.4bcde	83.0cd	26.5de	28.4cde	35.0bcd	34.5abc	2.8fgh	4.9b	3.8b	3.2cd
Dry 1400 w for 60 s	77.3d	75.9d	83.3fg	80.0d	24.4ef	25.0ef	33.1cd	31.3cd	2.1i	3.2f	3.2c	2.9d
Bacillus	89.5a	88.0b	91.6abc	89.7b	32.9ab	36.2a	39.0b	36.0ab	4.4ab	5.5a	4.1ab	3.7ab
Trichoderma	86.4ab	81.0c	89.0bcde	81.3d	30.9abc	34.3ab	37.9b	33.9bc	3.8bcd	4.6bc	3.7b	3.5abc
Celest® XL	90.1a	93.0a	94.7a	95.0a	34.6a	36.8a	43.4a	38.3a	4.6a	4.7b	4.4a	4.0a
Inoc control	46.6e	51.5e	54.8h	58.1f	21.3f	23.6f	25.3e	22.3e	2.4hi	2.3g	2.2e	2.2e
Non-Inoc control	79.2cd	76.7cd	82.3g	80.0d	24.7ef	27.6def	31.3d	28.0d	2.8fgh	3.7de	3.3c	3.0cd
LSD	5.4	4.4	3.9	4.2	3.9	4.6	4.2	4.2	0.6	0.5	0.4	0.5
CV%	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0

Means sharing a common letter in a column differ significantly according to the Fisher's LSD test at $p<0.05$.

Supplementary material:

Appendix 6.1: Effects of hot water seed treatments of artificially inoculated *E. viminalis* seed lots on seed germination, diseased seedlings and incidence of *C. fructicola*

	Soaking period														
	1 min			15 min			30 min			45 min			60 min		
Treatment	**Inc[a]**	**Germ[b]**	**Dis[c]**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**
40 °C	91.5[*]a[**]v	51.0e[***]D	45.3b[****]X	77.0bw	55.5eC	45.8bX	69.0bx	61.3eB	31.0bY	46.0by	63.5eB	29.5bY	20.0bz	70.0dA	24.5bZ
45 °C	77.0bv	62.3dC	25.8cW	62.5cw	67.5dB	21.3cX	44.0cx	70.5dB	7.3cZ	33.3cy	78.0dA	14.3cY	6.0cz	79.8cA	11.5cY
50 °C	39.5cx	70.5cC	10.0eX	10.0dy	75.0cC	8.3dXY	11.0dy	89.3bA	5.8cY	0.0dz	82.3cB	4.5dY	0.0dz	81.5cB	0.0dZ
55 °C	20.3dx	84.0bB	12.0deY	5.0ey	90.5aA	4.0eZ	0.0ez	78.0cB	0.0dZ	0.0dz	60.8eC	0.0eZ	0.0dz	37.3eD	0.0dZ
60 °C	0.0fz	88.0aA	14.3dY	0.0fz	24.0gB	0.0fZ	0.0ez	0.0hC	0.0dZ	0.0dz	0.0gC	0.0eZ	0.0dz	0.0fC	0.0dZ
Bacillus	2.8efz	87.8abA	4.0gY	0.0fz	89.5aA	0.0fZ	0.0ez	91.3aA	0.0dZ	0.0dz	91.0aA	0.0eZ	0.0dz	90.5aA	0.0dZ
Trichoderma	4.3ey	83.5bB	7.3fY	0.0fz	88.0bA	0.0fZ	0.0ez	89.5bA	0.0dZ	0.0dz	88.8bA	0.0eZ	0.0dz	88.0bA	0.0dZ
Celest® XL	0.0fz	89.0aB	0.0hZ	0.0fz	91.0aAB	0.0fZ	0.0ez	94.0aA	0.0dZ	0.0dz	94.0aA	0.0eZ	0.0dz	92.8aAB	0.0dZ
Control	93.8az	29.8fB	59.5aZ	92.0az	31.0fB	58.5aZ	98.0ay	33.0fAB	60.0aZ	92.0a	36.5fA	59.0aZ	95.0ayz	36.5eA	61.3aZ

Inc[a]: Percentage incidence of *C. fructicola*, Germ[b]: seed germination, Dis[c]: diseased seedlings. [*]Means sharing a common letter in a column differ significantly according to the Fisher's LSD test at $p<0.05$. [**]In each row, means with the same lowercase letters do not significantly differ from each other at $p = 0.05$. [***]Means within a row not followed by the same uppercase letter are significantly different from each other ($p = 0.05$). [*****]Means within a row followed by same uppercase letter are not significantly different from each other.

Appendix 6.2: Effects of hydrogen peroxide seed treatments of artificially inoculated *E. viminalis* seed lots on seed germination, diseased seedlings and incidence of *C. fructicola*

	Period seeds soaked in H_2O_2														
	1 min			5 min			10 min			30 min			45 min		
Treatment	**Inc[a]**	**Germ[b]**	**Dis[c]**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**
1% H_2O_2	86.3[*]a[**]x	47.8d[***]BC	48.3b[****]XY	83.0bx	52.8cA	41.5bZ	71.0by	50.0dAB	46.5bY	69.8byz	47.8dBC	48.0bXY	66.8bz	45.8dC	50.5bX
5% H_2O_2	44.8by	58.3cC	25.0cX	42.8cyz	87.3aA	9.0cZ	42.0cyz	91.8aA	26.0cX	39.3cz	82.0bB	13.3cYZ	38.8cz	80.0bB	14.5cY
10% H_2O_2	32.0cx	81.8aB	15.0dX	30.0dx	86.8aA	6.8dY	27.3dy	85.3bAB	0.0dZ	26.0dyz	81.8bB	0.0dZ	23.0dz	79.3bB	0.0dZ
15% H_2O_2	18.8dy	75.8bA	7.3eY	0.0fz	76.8bA	0.0fZ	0.0ez	77.3cA	0.0dZ	0.0ez	71.3cB	0.0dZ	0.0ez	70.0cB	0.0dZ
Bacillus	6.3ey	82.0aC	4.5efY	4.0eyz	88.5aB	0.0fZ	0.0ez	91.0aAB	0.0dZ	0.0ez	92.5aA	0.0dZ	0.0ez	91.8aAB	0.0dZ
Trichoderma	7.0ey	77.3bB	6.0eY	5.5ey	79.0bB	2.8eYZ	0.0ez	90.5aA	0.0dZ	0.0ez	91.8aA	0.0dZ	0.0ez	92.5aA	0.0dZ
Celest® XL	0.0fz	83.5aC	1.8fZ	0.0fz	89.0aB	0.0fZ	0.0ez	92.3aAB	0.0dZ	0.0ez	94.0aA	0.0dZ	0.0ez	93.3aA	0.0dZ
Control	89.0az	37.5eB	56.8aY	90.0ayz	42.8dA	52.3aZ	93.3ay	39.5eAB	57.0aY	93.3ay	39.5eAB	57.0aY	92.3ayz	42.0eA	52.3aZ

Inc[a]: Percentage incidence of *C. fructicola*, Germ[b]: seed germination, Dis[c]: diseased seedlings. [*]Means sharing a common letter in a column differ significantly according to the Fisher's LSD test at $p<0.05$. [**]In each row, means with the same lowercase letters do not significantly differ from each other at $p = 0.05$. [***]Means within a row not followed by the same uppercase letter are significantly different from each other ($p = 0.05$). [*****]Means within a row followed by same uppercase letter are not significantly different from each other.

Appendix 6.3: Effects of microwave radiation seed treatments of artificially inoculated *E. viminalis* seed lots on seed germination, diseased seedlings and incidence of *C. fructicola*

	Microwave exposure Time																	
	30 sec			60 sec			90 sec			120 sec			150 sec			180 sec		
Treatment	**Inc[a]**	**Germ[b]**	**Dis[c]**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**	**Inc**	**Germ**	**Dis**
250 w dry	85.0*a**x	38.8d***A	51.8a****Z	83.5axy	39.8eA	55.8aY	81.8axy	40.3efA	55.0aYZ	81.0by	38.3fA	55.0aYZ	77.3bz	38.5eA	55.5aYZ	78.5byz	38.3fA	56.0aY
600 w dry	74.5bu	41.5dB	49.5bX	70.0bv	41.5eB	46.3bXY	58.5cw	43.8eB	45.8bXY	44.8cx	43.8efB	45.0bYZ	28.8dy	50.8dA	43.3bYZ	23.3cz	52.8dA	42.5bZ
1400 w dry	70.8cx	75.8bA	28.0dV	43.0dy	78.3bA	23.5dW	0.0fz	62.8cB	19.8eX	0.0gz	5.0gC	9.5eY	0.0gz	0.0fD	0.0fZ	0.0fz	0.0gD	0.0fZ
250 w wet	73.3bcv	51.3cB	52.5aW	68.5bw	49.8dB	47.0bX	63.5bx	53.0dB	40.3cY	40.3dy	58.3cA	29.3cZ	36.0cy	61.8cA	28.5cZ	25.3cz	60.5cA	28.0cZ
600 w wet	69.3cv	73.0bB	37.5cW	61.0cw	80.0bA	31.0cX	42.8dx	41.5efC	26.3dX	37.0ey	52.0dB	18.8dY	20.8ez	55.3dB	11.0dZ	17.8dz	44.8eC	8.0dZ
1400 w wet	47.8dx	84.5aA	35.8cY	37.0ey	70.8cB	0.0gZ	0.0fz	52.3dC	0.0hZ	0.0gz	0.0gD	0.0gZ	0.0gz	0.0fD	0.0fZ	0.0fz	0.0gD	0.0fZ
Bacillus	10.5ey	82.0abB	9.3fY	9.5fyz	84.8aB	8.0fYZ	9.0eyz	85.8aAB	6.3gYZ	7.2fyz	87.0aA	5.8fYZ	6.8fyz	87.5abA	5.0eZ	6.3ez	88.8abA	4.5eZ
Trichoderma	13.0ey	77.3bC	13.0eX	11.3fy	78.0bC	10.5eXY	11.0eyz	79.3bBC	9.8fY	9.5fyz	81.5bAB	8.5eYZ	8.3fz	83.0bAB	6.8eYZ	7.0ez	84.5bA	6.0eZ
Celest® XL	0.8fz	83.5aC	0.0gZ	0.0gz	85.5aBC	0.0gZ	0.0fz	87.0aAB	0.0hZ	0.0gz	88.3aA	0.0gZ	0.0gz	89.5aA	0.0fZ	0.0fz	90.5aA	0.0fZ
Control	87.5az	38.5dA	53.5aZ	86.0az	38.5eA	53.5aZ	85.3az	38.5fA	53.5aZ	87.5az	38.5fA	53.5aZ	87.0az	39.0eA	53.5aZ	86.3az	38.5fA	53.5aZ

Inc[a]: Percentage incidence of *C. fructicola*, Germ[b]: seed germination, Dis[c]: diseased seedlings. *Means sharing a common letter in a column differ significantly according to the Fisher's LSD test at $p<0.05$. **In each row, means with the same lowercase letters do not significantly differ from each other at $p = 0.05$. ***Means within a row not followed by the same uppercase letter are significantly different from each other ($p = 0.05$). *****Means within a row followed by same uppercase letter are not significantly different from each other.

Chapter 7

7.1 Conclusion

Commercial *Eucalyptus* plantations are managed on a short to medium rotation cycle of six to ten years, where it is imperative to regenerate the clear-felled tree stands every year (Forestry South Africa (FSA) 2018). Planting targets as high as 100 million seedlings are expected each year, but the challenge of poor and inconsistent seedling emergence with losses up to 40% continue being reported in nurseries (Seedling Growers Association of South Africa (SGASA) 2018). Although *Eucalyptus* seed germination is generally high under laboratory conditions, seedling emergence is inconsistent in commercial nurseries (Brown and Ferreira 2000). Studies done in the past suggested changes in planting media and types of seedling containers (Humara et al. 2002; Close et al. 2006; Fernández et al. 2007), but challenges of low initial survival and delay of seedlings to emerge still persist in nurseries. With this background, it was hypothesised that commercial *Eucalyptus* seeds infected and/or infested with seed-borne mycoflora are the cause of poor seedling emergence and growth in nurseries.

This study showed that locally produced *Eucalyptus* spp. seed lots may be an important source of seed-borne mycoflora. *Eucalyptus nitens* seed lot had the highest incidence of fungi (92.4%) and *E. dorrigoensis* seed lot had the least (28.9%). Despite a popular notion that recognises tree seeds to be resilient to pathogen attack (Ennos 2014), *Eucalyptus* spp. seed lots examined in this study were all susceptible to infection following inoculation with seed-borne fungi. Seed-borne fungi were shown to impair seed germination and also resulted in significantly high percentages of diseased *Eucalyptus* spp. seedlings, which potentially translates to low chances of seedling emergence in nurseries or poor survival in the field under favourable conditions at sowing.

Findings of this study are also concerning to seed traders particularly with South Africa's active involvemet in forest tree improvement programmes. Over the years, South Africa has grown to be a global supply of forestry genetic material including seed (Southern African Tree Seed Working Group (2012). However, the increase in reports of new pests and diseases in forest plantations makes it difficult to exchange infected and/or infested seeds with introduction of stricter border inspection on traded agricultural goods and products (Bulman et al. 2018; Sikes et al. 2018). As *Aureobasidium, Colletotrichum* and *Disculoides* species are reported for the first time associated with *Eucalyptus* seeds, this has significant implications in seed trade. There is a gradual increase in studies showing exchange of infected and/or infested seeds as an important pathway for global and regional distribution of important plant pathogens (Evira-Recuenco et al. 2015; Jimu et al. 2016; Cleary et al. 2019). In fact interception of *Disculoides* sp. was previously implicated with a quarantine consequence on *E. leucoxylon* imported in New Zealand (Crous et al. 2016; Surveillance 2016), and this can serve as a lesson demonstrating the need for regular health tests on traded seed. In the current study, the fungus failed to cause disease symptoms on seedlings and repeated repeated efforts to fulfil the Kotch postulates were unsuccessful. Furthermore, this study showed that *D. eucalypti* was not transmitted from inoculated seeds into seedlings. More studies are needed to establish the etiology for better understanding of the infection mechanism of *D. eucalypti.* Since the first report of *D. eucalypti* was from mature diseased *E. viminalis* (Crous et al. 2012), it is possible that it has a long latent phase or might only be pathogenic on host tissues undergoing senescence.

This study has revealed the need to be more vigilant in phytosanitary inspections especially with the current taxonomic revisions being done on most fungal genera. *Colletotrichum* species associated with locally produced *Eucalyptus* seed may have serious biosecurity implications and

correct identification and control of member species of this genus is important. Previous surveys based on morphological characters identified *Colletotrichum gloeosporiodes* as the sole causal pathogen of anthracnose leaf spot on *Eucalyptus* (Sharma et al. 1984; Smith et al. 1998). However, taxonomic revisions have shown *C. gloeosporiodes* to be one of 22 cryptic species of the *C. gloeosporiodes* species complex (Weir 2012). Among these closely related species is the quarantine coffee berry pathogen *C. kahawae*, which is believed to be confined to Africa (Batista et al. 2017). In the past decade, there has been an increase of reports of this pathogen from different regions of the world (Liu et al. 2013; Afanador-Kafuri et al. 2014; Mosca et al. 2014; Schena et al. 2014; Ismail et al. 2015; Perrone et al. 2016). Studies in molecular genetics segregate *C. kahawae* to a subspecific level, *C. kahawae* subsp. *kahawae*, and it clusters together with *C. kahawae* subsp. *ciggaro*, a generalist and cosmopolitan, which does not cause disease on coffee berries (Weir etal. 2012). Although these reports refer to *C. kahawae* subsp. *cigarro*, some of them did not distinguish the pathogen at the subspecific level leaving a wave of confusion of whether the pathogen has been introduced outside Africa. In this study, concatenated sequences of ACT, CHS, GAPDH, ITS and TUB2 gene regions initially failed to delineate the *C. kahawae* to subspecies level, but complementary biochemical tests revealed the identitity of isolate PPRI 24315 to be *C. kahawae* subsp. *cigarro*.

Implications of seed-borne *Colletotrichum* spp. must not be underestimated considering that this study showed them to be seed-tramissible and pathogenic on *Eucalyptus* spp. Although anthracnose leaf spot is a common disease in *Eucalyptus* nurseries (Smith et al. 1998), it might be difficult to fully relay the message to to foresters as tolerance thresholds have not been established for most forestry seed-borne pathogens. Nonetheless, caution is required in trading such seeds as this may facilitate introduction of different strains or physiologic races of already existing

pathogens giving rise to more severe disease outbreaks. Isolation of up to about six isolates of *Colletotrichum* sp. from 400 seeds plated (1.4%) may seem trivial, but a kilogram of pure *Eucalyptus* seed contains above 500 000 individual seeds. This translates to approximately 7 500 naturally infected seeds per kilogram. There is risk that nursery managers are blindly sowing seeds infected and/or infested not only with *Colletotrichum* spp. but several other pathogens. Caution is also to be taken on seed-borne fungi such as *Botryosphaeria, Lasiodiplodia* and *Sydowia* that may not necessarily cause immediate stand reductions but may be spread latently in forest plantation areas (Münch et al. 2008; De Silva et al. 2017). This is particularly true for *Colletotrichum* as it is known to be a hemibiotroph with a latent infectious stage and switches to be pathogenic, causing visible disease symptoms, when the host is stressed (Viljoen et al. 1992; Smith et al. 1998).

Since there are no registered chemical seed treatments on *Eucalyptus*, disease management in most forest nurseries is achieved with fungicides registered on other crops such as Benomyl, Captan, Carboxin and Thiram (Brown and Ferreira 2000). However, further use of synthetic pesticides is being discouraged in FSC certified plantation operations. Therefore, alternative non-chemical measures that included BCAs, hot water, microwave radiation, and H_2O_2, and also a synthetic fungicide Celest XL® as a control, were evaluated at sanitising infected and/or infested *Eucalyptus* seed lots. The practical relevance of non-chemical seed treatments was assessed by whether they significantly reduced incidences of *Colletotrichum* spp. without impairing seed germination and seedling emergence. Despite high germination and emergence rates observed when seeds were treated at optimum treatment conditions, viz. 10 % H_2O_2 for 5 min and 10 min, soaking in hot water baths set at 60 °C for 1 min and 50 °C for 30 min against *C. kahawae* subsp. *cigarro*, and 55 °C for 15 min against *C. fructicola* and microwave irradiation of moistened *Eucalyptus* spp. seeds at 1 400 w for 30 s, there is further research required on seeds sown under less favourable

conditions. Assays such as conductivity test of seeds and cold soil tests are some of the assays that might be included to give better estimations of field emergence (ISTA, 2019).

In-vitro assays indicated significant reduction of anthracnose leaf spot on seedlings grown from seeds treated with non-chemical seed treatments. Nevertheless, none of the non-chemical seed treatments effectively supressed appearance of anthracnose leaf spot on seedlings grown under greenhouse condition, except for treatments with the BCAs or Celest® XL. The fact that no chemicals are listed as seed treatments for *Eucalyptus* seeds makes Celest® XL a suitable candidate against anthracnose leaf spot, but also soil-borne pathogens such as *Pythium*, *Fusarium* and *Rhizoctonia* (Croplife 2019). However, registration of Celest® XL will be challenging particularly as management of diseases in FSC certified forest plantations is stipulated to be done using non-chemical methods. From an ecological and biological point of view, BCA's should be easier to be approved by Envrionmental Protection Agencies as they generally have minimum negative impact on fauna and flora (Harman et al. 2010; Velivelli et al. 2014). Despite widespread availability of information and proven efficacy of BCA's in this study, it might be a challenge for foresters to adopt them in their crop protection operations particularly due to previous lack of reliability and failure when applied under field conditions for most vegetable and agronomic crops (Nicot and Bardin 2012; Nicot et al. 2012; Velivelli et al. 2014). Consisteny of seed treatments results both *in-vitro* and *in-vivo* is a positive indication and seed and nursery managers can consider control of anthracnose leaf spot using Integral® on *Eucalyptus* spp. grown in semi-controlled microclimatic conditions. There is need to make use of existing partnerships to collaborate with key forestry industry stakeholders to disseminate outcomes of this study to safeguard seed trade and quality seedling production outputs from nurseries. Through these channels, further large scale tests should be conducted to confirm this. Above all, the overall decision on registration of seed

treatments has to pass through local stakeholders and key forestry consultans that operate under the standards principles and regulations of the FSC.

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