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

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ABSTRACT

Anthracnose leaf spot is a common disease caused by *Colletotrichum* species. Non-chemical seed treatments that included *Bacillus*, *Trichoderma*, hot water, microwave radiation, and hydrogen peroxide were evaluated at disinfecting *Eucalyptus* seeds infected with *Colletotrichum kahawae* subsp. *cigarro*. The seed treatments were assessed on *Eucalyptus grandis* and *E. nitens* seed lots. When both reduction in the incidence of *Colletotrichum* and increased seed germination are considered, hot water seed treatments at 55 °C for 15 min and 60 °C for 1 min were optimum treatment/time parameters for *Eucalyptus*. Seed germination improved when *Eucalyptus* seeds were soaked in 10% H_2O_2 for 10 min to the equivalent of that of 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 diseases on seedlings raised 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 raised 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. *Bacillus*, however, was the only non-chemical seed treatment that demonstrated effectiveness against anthracnose leaf spot under greenhouse conditions.

1. Introduction

Seeds represent a long-term investment for plant regeneration (De Frenne et al., 2012). 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; Mangwende, 2020).

Despite presence of multiple pathogens, infected seeds often appear healthy and retain viability under laboratory seed germination tests (Facelli et al., 1999; Close and Wilson, 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, 2020).

Although anthracnose leaf spot disease is reported in *Eucalyptus* nurseries, advances in molecular techniques has shown possible misidentifications of previously identified pathogens in the genus *Colletotrichum*. Over the years, *C. gloeosporiodes* has been identified as the sole causal of anthracnose leaf spot (Sharma et al., 1984; Smith et al., 1998) but taxonomic revisions have shown several cryptic species in the *Colletotrichum gloeosporiodes* species complex (Damm et al., 2009; Weir

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et al., 2012). Contrary to previous studies that isolated *C. gloeosporiodes* from *Eucalyptus* (Viljoen et al., 1992; Smith et al., 1998), Mangwende (2020) found that *Eucalyptus* seed lots were infected with *Colletotrichum. kahawae* subsp. *cigarro* B.S. Weir & P.R. Johnst. The fungus *C. kahawae* subsp. *cigarro* 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, 2020). Furthermore, *C. kahawae* subsp. *cigarro* is both seed-borne and seed-transmitted (Mangwende, 2020).

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., 2014; 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 grandis* W. Hill and *Eucalyptus nitens* (H. Deane and Maiden) Maiden artificially inoculated with *C. kahawae* subsp. *cigarro*. Efficacy of non-chemical seed treatments to limit transmission of the pathogen from seed to seedlings in the greenhouse was compared with Celest® (a synthetic pesticide registered as a seed treatment on several crops in South Africa).

2. Materials and methods

2.1. Source of materials

Seeds of *E. grandis* and *E. nitens* were supplied by commercial forestry seed companies. They were selected based on the levels of susceptibility to anthracnose leaf spot disease i.e. not susceptible and highly susceptible, respectively (data not shown). Pathogenic *C. kahawae* subsp. *cigarro* (PPRI 24314, GenBank accession numbers for ACT, CHS, GAPDH, ITS and TUB2 gene regions: MK512735, MK512737, MK512733, MG641892 and MK512739, respectively) isolated from *Eucalyptus* seeds (Mangwende, 2020) was used in this study. The identity of *C. kahawae* subsp. *cigarro* isolate was confirmed using a biochemical assay, where the pathogen was able to grow on basal medium containing either glucose or citric acid or ammonium titrate as a sole carbon source (Waller et al., 1993). Commercial biocontrol agents, *Trichoderma harzianum* Rifai (2×10^9 spores g^{-1}) (Plant Health Products (Pty.) Ltd., Kwazulu-Natal, South Africa) and *Bacillus subtilis* (Ehrenberg) Cohn strain MBI 600 (2×10^{11} spores mL^{-1}) (Becker Underwood (Pty) Ltd., Kwazulu-Natal, South Africa), and a fungicide Celest® XL (25 ai L^{-1} fludioxonil and 10 g ai L^{-1} mefenoxam) (Syngenta (Pty.) Ltd., Midrand, South Africa) were used for the study. Ensure® ISO 103 (30% hydrogen peroxide) was sourced from Merck (Pty.) Ltd. (Midrand, South Africa).

2.2. Seed inoculation

Seeds 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 °C for 7 days under alternating cycles of 12 h ultra violet (UV) (365 nm) light and darkness. To confirm that inoculation was successful, fungi were re-isolated from inoculated seeds on PDA and identity confirmed in comparison with positive reference plates of *C. kahawae* subsp. *cigarro*.

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 to 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 °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 room temperature at equivalent time points served as negative controls, whereas seeds soaked in Celest® XL at the recommended rate of 1 mL kg^{-1} seed at equivalent times served as positive 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.

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.

2.5. Seed treatment with hydrogen peroxide

Cheesecloths containing 200 inoculated seed per bag 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 negative controls, whilst seeds soaked in Celest® XL at aforementioned times were positive controls. After treatment, cheesecloths containing seeds were rinsed in sterile distilled water and seeds were left to dry on a laminar flow bench.

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 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 percentage of seeds infected with *C. kahawae* subsp. *cigarro* was determined.

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 spaced evenly 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 °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 scores of seedlings that developed diseases were recorded. Diseased seedlings were identified by lesions developing on hypocotyls and/or seminal roots.

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 (winter) and the second on 5 October (spring). Following treatment with the best performing seed treatments from *in -vivo* tests, *Eucalyptus* seeds 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 assessment of plant health was done at 60, 120 and 180 DAS. Evaluation of disease severity was done using a scale of 1–5 according to Mangwende (2020) and average diameters of anthracnose leaf spots. Plants were harvested 180 DAS and seedling length (cm) and total dry mass (g) recorded.

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). A two-way analysis of variance (ANOVA) was performed on data and means compared with the Fischer's least significant differences (LSD, $p \leq 0.05$).

3. Results

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

Seed treatments significantly reduced incidences of *C. kahawae* subsp. *cigarro* on *Eucalyptus* spp. seeds compared with controls ($p \leq 0.05$), except for seeds soaked in hot water baths set at 40 °C for 1 min (Table 1 and Appendix A). The incidence of *C. kahawae* subsp. *cigarro* persisted on *E. grandis* seed lots soaked in hot water baths for 1 min regardless of the temperature increment (Appendix A). At the same soaking period, hot water seed treatment at 60 °C effectively reduced the

Table 1
Effects of hot water seed treatments on seed germination, diseased seedlings and incidence of *C. kahawae* subsp. *cigarro* from artificially inoculated *E. nitens* seed lots.

Treatment	Soaking period													
	1 min			15 min			30 min			45 min			60 min		
	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	50.3bX	82.3bx	53.3eC	47.5bX	81.0bx	59.8 dB	23.8bY	67.0by	77.3bA	20.3bZ	45.3bz	78.3bA	19.5bZ
45 °C	84.5bv	57.3eC	24.5cX	59.3cw	70.8 dB	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	79.5 cB	6.8dY	14.0dx	90.5 aA	6.8cY	8.0dy	74.8bC	0.0dZ	0.0dz	70.3cD	0.0dZ
55 °C	42.0cw	77.0 cB	8.5dX	12.0dx	85.0bA	0.0eY	3.0ey	73.0 cC	0.0dY	0.0ez	62.3cD	0.0dY	0.0dz	41.5 dE	0.0dY
60 °C	5.5ex	87.0 aA	6.5eX	0.0ey	22.3 fB	0.0eY	0.0ey	13.0 fC	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	90.8 aA	0.0dY	0.0ez	90.0 aA	0.0dY	0.0dz	90.0 aA	0.0dY
Trichoderma	10.5dy	83.5bB	9.3dX	0.0ez	86.3bAB	0.0eY	0.0ez	87.3bAB	0.0dY	0.0ez	89.5 aA	0.0dY	0.0dz	89.0 aA	0.0dY
Celest® XL	0.5fz	88.0 aB	0.0fZ	0.0ez	90.3aAB	0.0eZ	0.0ez	91.3aAB	0.0dZ	0.0ez	92.5 aA	0.0dZ	0.0dz	91.8 aA	0.0dZ
control	93.0ayz	21.8gA	67.3aZ	91.0az	22.3 fA	70.0aZ	95.5ay	19.3eB	70.3aZ	94.8ay	18.5 dB	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 do not differ significantly according to the Fisher's LSD test at $p \leq 0.05$. **In each row, means with the same lowercase letters do not significantly differ from each other at $p \leq 0.05$. ***Means within a row not followed by the same uppercase letter are significantly different from each other ($p \leq 0.05$).

incidence of *C. kahawae* subsp. *cigarro* on *E. nitens* seeds and was comparable with the biocontrol agents and Celest® XL treatment. 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* seed lots.

Effects of soaking *Eucalyptus* seed lots in H_2O_2 on the incidence of *C. kahawae* subsp. *cigarro* are presented in Table 2 and Appendix B. Seed treatments significantly reduced the incidence of *C. kahawae* subsp. *cigarro* on *Eucalyptus* seed lots compared to untreated controls ($p \leq 0.05$), except for seeds soaked in 1% H_2O_2 for 1 min. At a soaking period of 1 min, H_2O_2 was the least effective seed treatment. However, there was a significant increase in efficacy of H_2O_2 at reducing incidences of *C. kahawae* subsp. *cigarro* as the soaking period was increased. Soaking *E. grandis* seeds in 15% H_2O_2 for 5 min significantly reduced incidences of *C. kahawae* subsp. *cigarro* even better than biocontrol agents ($p \leq 0.05$) (Appendix B). Soaking *Eucalyptus* seeds in 15% H_2O_2 for more than 5 min effectively eradicated incidences of *C. kahawae* subsp. *cigarro*.

Eucalyptus seeds exposed to microwave radiation had significantly lower incidences of *C. kahawae* subsp. *cigarro* compared with inoculated controls ($p \leq 0.05$), except for dry seeds exposed at 250 W microwave radiation for 30 s (Table 3 and Appendix C). 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 ($p \leq 0.05$). Exposure of moistened seeds to microwave radiation of 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 *Eucalyptus* seeds.

3.2. Effects of seed treatments on seed germination

Seed treatments significantly increased seed germination of *Eucalyptus* seed lots compared to non-treated controls ($p \leq 0.05$) (Tables 1–3 and Appendix A-C). Soaking *Eucalyptus* seeds in hot water baths set at 55 and 60 °C for 30 and 1 min, respectively, were the most effective temperature-time combinations that resulted in the most improvement of seed germination (Table 1 and Appendix A). Further increase of hot water bath temperature beyond these limits greatly reduced seed germination.

There was a positive response to seed germination with gradual increments of concentration of H_2O_2 from 1 to 10% (Table 2 and Appendix B). However, increasing the concentration of H_2O_2 beyond 10% resulted in reduction of seed germination. Most improvements on seed germination were observed on seeds soaked in 10% H_2O_2 for 10 min, which had similar efficacy as the Celest® XL treatment, except for *E. nitens* seed lots. Regardless of concentration of H_2O_2, germination of *E. nitens* was significantly lower than seed treatments with biocontrol agents and Celest® XL ($p \leq 0.05$).

The effects of microwave seed treatments on germination of *Eucalyptus* seeds are displayed in Table 3 and Appendix C. Microwave radiation of moist seeds significantly increased seed germination better than dry seeds ($p \leq 0.05$). 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 ($p \leq 0.05$). Microwave radiation of dry seeds at 1400 W for 120 s and above severely affected seed germination.

3.3. Diseased seedlings

Seed treatments including soaking seeds in hot water baths and H_2O_2 significantly reduced the proportion of diseased seedlings compared with controls ($p \leq 0.05$) (Tables 1 and 2 and Appendices A-B). There were no diseased seedlings from seeds soaked in hot water baths set at 55 and 60 °C for 15 min and above (Table 1 and Appendix A). Similarly,

Table 2
Effects of hydrogen peroxide on seed germination, diseased seedlings and incidence of *C. kahawae* subsp. *cigarro* from artificially inoculated *E. nitens* seed lots.

Treatment	Period seeds soaked in H_2O_2														
	1 min			5 min			10 min			30 min			45 min		
	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	53.3bY	86.5by	49.8eA	49.3bZ	84.8by	50.5eA	47.0bZ	84.3by	43.5 fB	53.3bY	76.0bz	42.8 fB	53.8bY
5% H_2O_2	68.3bw	58.0 dC	40.0cX	53.5cx	82.3bcAB	10.5cY	44.5cy	84.5bA	10.0cY	40.0cy	84.3 cA	8.5cZ	33.8cz	80.5 cB	9.0cYZ
10% H_2O_2	43.8cw	80.0bB	14.5dY	41.3dw	80.8 cB	0.0dZ	35.5dx	83.0 cA	0.0dZ	23.3dy	81.0dAB	0.0dZ	15.5dz	77.8 dC	0.0dZ
15% H_2O_2	34.8dx	77.0 cA	0.0gZ	5.3ey	77.5 dA	0.0dZ	0.0gz	77.8 dA	0.0dZ	0.0ez	70.0eB	0.0dZ	0.0ez	70.0eB	0.0dZ
Bacillus	9.3ex	81.0 aC	6.3fY	4.3ey	84.3bB	0.0eZ	1.8fz	86.3bB	0.0dZ	0.0ez	88.8 aA	0.0dZ	0.0ez	89.5 aA	0.0dZ
Trichoderma	11.0ex	79.5bC	9.3eY	5.5ey	81.5 cB	0.0eZ	3.5ey	82.8 cB	0.0dZ	0.0ez	86.5bA	0.0dZ	0.0ez	87.8bA	0.0dZ
Celest® XL	0.0fz	82.5 aC	0.0gZ	0.0fz	87.5 aB	0.0eZ	0.0gz	88.3aAB	0.0dZ	0.0ez	90.5 aA	0.0dZ	0.0ez	90.0 aA	0.0dZ
Control	96.3az	35.8 fA	59.8aZ	96.0az	35.5 fA	60.5aZ	95.5az	35.5 fA	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 do not differ significantly according to the Fisher's LSD test at $p \leq 0.05$. **In each row, means with the same lowercase letters do not significantly differ from each other at $p \leq 0.05$. ***Means within a row not followed by the same uppercase letter are significantly different from each other ($p \leq 0.05$).

Table 3
Effects of microwave radiation on seed germination, diseased seedlings and incidence of *C. kahawae* subsp. *cigarro* from artificially inoculated *E. nitens* seed lots.

Treatment	Microwave exposure Time																	
	30 s			60 s			90 s			120 s			150 s			180 s		
	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	35.8 dC	56.5bY	84.0by	38.3gB	58.0bXY	82.3bz	37.3eB	52.3bZ	83.3byz	42.0 dA	51.8bZ	82.0bz	42.5 dA	50.3bZ
dry 600 W	83.3bv	41.3fAB	54.0bX	75.8cw	39.5 dB	59.3 aW	66.8cx	44.8 fA	57.8bW	45.8cy	43.5dAB	54.0bX	31.3cz	44.0 dA	51.3bXYZ	33.0cz	42.8dAB	49.0cZ
dry 1400 W	60.5ew	68.0 dA	28.0 dV	52.5ex	72.5bA	21.5 dW	28.3fy	37.8gB	17.0eXY	0.0fz	0.0 fC	13.8deY	0.0fz	0.0gC	0.0fZ	0.0fz	0.0gC	0.0gZ
wet 250 W	74.5cw	54.3eC	48.8 cW	68.3dx	60.5 cB	50.3 cW	51.0dy	54.0eCD	43.5cX	30.3dz	49.5 cE	38.0cY	28.5cz	51.3cDE	37.3cY	31.0cz	69.3 cA	22.0dZ
wet 600 W	71.0dv	70.0cdA	28.8 dW	46.0fw	74.0bA	20.0dX	32.0ex	61.8 dB	21.8dX	26.0dy	35.3eC	13.5eY	20.8dy	27.0fD	10.8dY	14.5dz	21.5 fE	5.5fZ
wet 1400 W	47.0fx	81.8 aA	9.5gY	30.5gy	72.3bB	0.0fZ	0.0iz	42.5 fC	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	71.5 cB	23.8eX	17.3hx	72.0bB	21.0dX	14.0gy	72.8 cB	18.5eY	12.5eyz	74.0bB	15.5dY	11.8ez	76.0bAB	10.0dZ	10.0ez	77.5bA	9.3eZ
Celest® XL	3.8iy	79.8 aA	5.5hY	0.0jz	79.5 aA	0.0fZ	0.0iz	79.8 aA	0.0gZ	0.0fz	79.5 aA	0.0fZ	0.0fz	80.0 aA	0.0fZ	0.0fz	81.5 aA	0.0gZ
Control	87.0az	32.0 hA	59.0aZ	91.0ay	32.5 dA	58.8aZ	90.0ay	33.0 hA	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 do not differ significantly according to the Fisher's LSD test at $p \leq 0.05$. **In each row, means with the same lowercase letters do not significantly differ from each other at $p \leq 0.05$. ***Means within a row not followed by the same uppercase letter are significantly different from each other ($p \leq 0.05$).

there were no diseased seedlings from seeds soaked in 10 and 15% H_2O_2 for 5 min and above (Table 2 and Appendix B).

Microwave radiated seeds had significantly lower numbers of diseased seedlings than non-treated controls ($p \leq 0.05$), except for dry *Eucalyptus* seeds exposed at 250 W microwave radiation (Table 3 and Appendix C). At the same exposure period, the number of seedlings developing diseases were significantly lowered with each 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, efficacy of microwave radiation of moist seeds at 1400 W was similar to non-inoculated controls without any diseased seedlings. In addition, there were no diseased seedlings raised from moist seeds exposed to microwave radiation at 1400 W for 90 s and above. Similarly, dry seeds exposed to microwave radiation at 1400 W for 120 s and above had no diseased seedlings.

3.4. Effects of seed treatments on disease development

3.4.1. Incidence of anthracnose leaf spot

Seed treatments significantly suppressed appearance of anthracnose leaf spot on *Eucalyptus* seedlings compared with seedlings from non-treated seeds inoculated with *C. kahawae* subsp. *cigarro* (Table 4). The highest incidences of leaf spot were recorded at 180 DAS. Despite treating seeds with seed treatments, significantly higher ($p \leq 0.05$) incidence of anthracnose leaf spot was observed on *Eucalyptus* seedlings, even on *E. nitens* seedlings raised from Celest® XL treated seeds, compared with non-inoculated controls.

3.4.2. Severity of anthracnose leaf spot

3.4.2.1. Disease scores. Anthracnose leaf spot was more pronounced at 180 DAS and were most severe on seedlings raised from inoculated and untreated seeds (Table 4). Seed treatments did not significantly suppress ($p > 0.05$) severity of anthracnose leaf spot on *Eucalyptus* seedlings compared with Celest® XL, except for seedlings raised from *Bacillus* treated seeds.

3.4.2.2. Diameter of leaf spots. Seedlings raised from non-treated seeds inoculated with *C. kahawae* subsp. *cigarro* had the biggest leaf spots and were statistically similar to those of seedlings raised from seeds treated with hot water at 60 °C for 1 min and microwave radiation of dry seeds at 1400 W for 60 s (Table 4). *Bacillus* was the only non-chemical seed treatment that significantly suppressed ($p \leq 0.05$) appearance of anthracnose leaf spots on *E. nitens* seedlings equally as the Celest® XL treatment.

3.5. Effect of seed treatments on Eucalyptus seedling growth

3.5.1. Emergence

Seed treatments significantly improved *Eucalyptus* seedling emergence compared with inoculated controls ($p \leq 0.05$) (Table 5). Trial I results showed that *Bacillus* was the only non-chemical seed treatment that had similar effect as Celest® XL on increasing *Eucalyptus* seedling emergence. Trial II results showed that sowing *Eucalyptus* seeds treated with *Bacillus* and moist seeds exposed to microwave radiation had significantly higher seedling emergence than non-treated seeds, and compared well with the Celest® XL treatment ($p \leq 0.05$).

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 raised from *E. nitens* seed lots ranged from 17.3 to 32.7 cm (Table 5). The longest seedlings were recorded at 180 DAS, where seedlings from treated seeds were significantly longer compared to seedlings grown from inoculated controls ($p \leq 0.05$), except for *E. nitens* seedlings raised

Table 4
Assessment of anthracnose leaf spot disease on seedlings raised from *E. nitens* seeds inoculated with *C. kahawae* subsp. *cigarro*.

Treatment	Incidence (%)						Severity (%)							
	Trial I			Trial II			Trial I			Trial II			Ø leaf spots (mm)	
	60 DAS	120 DAS	180 DAS	60 DAS	120 DAS	180 DAS	60 DAS	120 DAS	180 DAS	60 DAS	120 DAS	180 DAS	Trial I	Trial II
HWT 55 °C for 15 min	1.2f	5.0h	30.5f	1.1ef	6.0f	27.5d	2.0d	22.0de	48.4f	3.2c	16.6ef	50.1d	3.7b	3.8bc
HWT 60 °C for 1 min	0.9g	11.9c	44.9b	1.0ef	9.7c	39.9b	1.8d	28.0c	64.5b	2.8c	22.4cd	75.8b	5.6a	6.3a
5% H_2O_2 for 10 min	1.3e	7.8e	29.2g	1.1de	8.0e	26.6d	5.8b	25.5cd	60.6cd	3.3bc	19.5de	49.9d	5.3 ab	3.8bc
10% H_2O_2 for 10 min	0.8h	5.3g	29.0g	1.6cd	4.8g	26.6d	3.1c	17.9f	56.7e	2.4c	13.3f	48.1d	4.9 ab	3.6c
Wet 1400 W for 30 s	2.1c	5.8f	31.6e	1.9c	4. 8g	26.6d	1.9d	23.0de	57.4de	2.5c	17.6e	47. 9d	5.3 ab	3.5c
Wet 600 W for 60 s	1.5d	9.3d	33.8d	1.6cd	8.5d	31.5c	2.6cd	20.5ef	61.3bc	2.7c	24.8c	55.7c	5.2 ab	4.8b
Dry 1400 W for 60 s	3.3b	14.0b	39.1c	3.4b	14.5b	34.2c	3.5c	34.7b	73.9a	5.0b	31.4b	79.2 ab	6.4a	6.7a
Bacillus	0.5i	2.0j	4.2i	0.4gh	3.2i	3.8f	0.1e	0.2h	1.1h	0.0d	0.2h	1.1f	0.2c	0.2d
Trichoderma	0.9g	3.4i	13.1h	0.6 fg	4.2h	10.6e	0.7e	7.3g	11.1g	0.3d	6.3g	13.7e	0.8c	0.9d
Celest® XL	0.0j	1.3k	1.8j	0.0h	1.1j	1.3f	0.0e	0.1h	0.9h	0.0d	0.1h	0.5f	0.1c	0.1d
Inoc control	13.7a	41.2a	65.9a	11.0a	45.4a	62.0a	52.4a	68.7a	76.0a	58.5a	69.3a	81.1a	6.2a	6.7a
Non-Inoc control	0.0j	0.0l	0.0k	0.0h	0.0k	0.0f	0.0e	0.0h	0.0h	0.0d	0.0h	0.0f	0.0c	0.0d
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	2.0	2.0
LSD	0.1	0.1	0.2	0.5	0.2	3.9	1.0	3.8	3.8	1.7	3.6	3.8	1.6	1.1

Means sharing a common letter in a column do not differ significantly according to the Fisher's LSD test at $p \leq 0.05$.

Table 5
Effects of seed treatments on the growth and development of seedlings raised from *E. nitens* seeds inoculated with *C. kahawae* subsp. *cigarro*.

Treatment	Emergence (%)		Seedling length (cm)						Total dry mass (g)	
			Trial I			Trial II			Trial I	Trial II
	Trial I	Trial II	60 DAS	120 DAS	180 DAS	60 DAS	120 DAS	180 DAS	180 DAS	180 DAS
HWT 55 °C for 15 min	78.3cde	80.2bcd	13.7abcd	20.4cd	24.6bcd	12.6abcd	20.0bcd	26.0bcd	3.0de	3.2d
HWT 60 °C for 1 min	76.1de	72.8e	14.6abc	22.3abc	27.0 ab	15.8 ab	24.3a	30.6a	3.5cd	3.9bc
5% H2O2 for 10 min	82.1bc	79.6bcd	10.0de	16.9def	23.8bcd	11.5bcde	22.8abc	29.8abc	2.4 fg	3.2d
10% H2O2 for 10 min	80.3cd	77.5cd	12.0bcde	19.4cde	24.8bc	10.7cde	18.9cde	25.8cd	2.7efg	3.3d
Wet 1400 W for 30 s	82.5bc	81.9b	12.0bcde	19.8cde	25.7bc	13.8abc	23.9 ab	30.4a	3.7bc	4.3 ab
Wet 600 W for 60 s	79.5cde	80.8bc	11.2cde	18.7cdef	23.5bcd	10.5cde	17.3de	23.7de	2.4 fg	3.4cd
Dry 1400 W for 60 s	75.5e	76.6cde	8.6e	15.7ef	20.6de	8.2e	15.3e	21.5e	1.9h	2.4e
Bacillus	86.5 ab	87.5a	15.6 ab	25.6 ab	30.6a	15.4 ab	24.7a	31.8a	4.1 ab	4.6a
Trichoderma	79.0cde	83.0b	13.7abcd	21.4bc	26.7abc	13.6abc	22.6abc	30.2 ab	3.7bc	4.1 ab
Celest® XL	88.4a	90.0a	16.4a	25.8a	30.7a	16.4a	26.4a	32.7a	4.5a	4.6a
Inoc control	44.9f	46.0f	8.6e	14.6f	17.3e	8.4de	15.1e	19.8e	2.3gh	2.4e
Non-Inoc control	78.6cde	76.5de	10.7cde	16.5def	22.6cd	12.2abcde	17.5de	23.7de	2.8ef	3.0d
CV%	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
LSD	4.5	4.3	4.1	4.3	4.3	4.3	4.3	4.3	0.5	0.5

Means sharing a common letter in a column do not differ significantly according to the Fisher's LSD test at $p \leq 0.05$.

from microwave treated seeds at 1400 W for 60 s. In both trials, there was consistency on seedling lengths from seeds treated with biocontrol agents and hot water at 60 °C for 1 min comparable to the Celest® XL treatment, which had longest seedlings.

3.5.3. Seedling dry mass

Greenhouse trials showed that microwave radiation of moist seeds at 1400 W for 30 s and seed treatments with *Bacillus* and *Trichoderma* significantly increased the dry seedling masses compared with dried seedling masses from controls ($p \leq 0.05$)(Table 5). However, dried mass of seedlings raised from *Bacillus* treated seeds was the only non-chemical seed treatment that was statistically similar to seedling mass from Celest® XL treated seeds.

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 disinfection 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 ($p \leq 0.05$) incidences of *C. kahawae* subsp. *cigarro* and improved seed germination. Hot water seed treatments have

been used to disinfect *Colletotrichum* infected seeds of different plant species including lupins (*Lupinus angustifolius* L.) and *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). Hot water seed treatment temperatures of 50 °C between 5 and 20 min were previously shown to be effective at disinfecting *Eucalyptus* seeds against a broad range of fungi (Donald and Lundquist, 1988). However, incidences of *C. kahawae* subsp. *cigarro* were effectively reduced at higher temperatures of 55 °C and above. This variation may be attributed to differences in levels of physiological maturity of seeds in the studies or differences in agro-ecological zones of seed orchards influencing variations in bio-chemical compositions (Forsberg, 2004).

The main challenge with hot water seed treatments is that it is limited to a few internal layers of seedcoat. Although soaking *E. nitens* seeds in a hot water bath set at 60 °C for 15 min and above effectively reduced incidences of *C. kahawae* subsp. *cigarro*, anthracnose leaf spot was still observed on seedlings raised from these seeds 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 °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 concentrations of pathogen inoculum was significantly reduced to the extent that it was not sufficient to cause well 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 as reported by Mangwende (2020).

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 and Mitchell, 2008). Soaking *Eucalyptus* seeds in 10% H_2O_2 for 5 min and 10 min were the most effective combinations to give the highest improvement on seed germination and were equally effective as that of seeds treated with *Bacillus* and Celest® XL. Regardless of concentration of H_2O_2, seed germination of *E. nitens* seed lots was significantly lower than in seed treatments with biocontrol agents and Celest® XL ($p < 0.05$).

There was a significant reduction of incidences of *C. kahawae* subsp. *cigarro* on *Eucalyptus* seeds soaked in H_2O_2, which resulted to direct increments of 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. Seeds of *E. nitens* have rough outer surfaces and deep crevices that may harbour spores of the pathogen thereby lowering efficacy of H_2O_2 at disinfecting seeds with a direct reduction in seed germination. Desperate attempts to disinfect infected seeds might lure usage of higher concentrations, but this must be discouraged as a high concentration of H_2O_2 is a strong oxidant that 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 with 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* seeds 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 ($p \leq 0.05$).

Microwave radiation of moist seeds significantly increased seed germination better than dry seeds ($p \leq 0.05$). Efficacy of seed treatments with microwave radiation is depended on the dielectric permittivity of the materials involved (Nelson, 1991; Jiao et al., 2011). As seeds are exposed to high-frequency electromagnetic radiation (EMR) (300 MHz-300 GHz), heat energy is generated within the molecules and structural compounds of seeds and pathogens. 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. In fact, microwave radiation of moistened *Eucalyptus* seeds at powers levels of 1400 W for 30 s was the best power-time treatment combination, from which the highest seed germination percentage was recorded. In this same way, spores on moistened seeds were easily killed. Contrasts at each power level showed greater sensitivity to microwave radiation response where moist *Eucalyptus* seeds had significantly lower percentages 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* (1×10^5 spores mL^{-1}), 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 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 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 *Bacillus*. Since there are limited chemicals registered as seed treatments of *Eucalyptus* seeds, high effectiveness of Celest® XL and *Bacillus* against the pathogen *in -vitro* and anthracnose leaf spot under greenhouse conditions gives high confidence in recommending them for disinfecting commercial *Eucalyptus* seed lots.

Contribution of authors

All authors played a significant role in the work presented. Conceptualization: Edgar Mangwende and Theresa Aveling; Material preparation, data collection and analysis: Edgar Mangwende; Writing – preparation of the original draft: Edgar Mangwende; Writing - review and editing: Theresa Aveling and Paxie Chirwa; Funding and resources acquisition: Theresa Aveling and Paxie Chirwa; Supervision: Theresa Aveling and Paxie Chirwa. All authors read and approved the final manuscript.

Declaration of competing interest

None.

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Appendix A-C. Supplementary data

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