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

2   ***Eucalyptus* spp.**

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12 **Abstract**

13 Anthracnose leaf spot is a common disease caused by *Colletotrichum* species. Non-chemical

14 seed treatments that included *Bacillus*, *Trichoderma*, hot water, microwave radiation, and

15 hydrogen peroxide were evaluated at disinfecting *Eucalyptus* seeds infected with

16 *Colletotrichum kahawae* subsp. *cigarro*. The seed treatments were assessed on *Eucalyptus*

17 *grandis* and *E. nitens* seed lots. When both reduction in the incidence of *Colletotrichum* and

18 increased seed germination are considered, hot water seed treatments at 55 °C for 15 min and

19 60 °C for 1 min were optimum treatment/time parameters for *Eucalyptus*. Seed germination

20 improved when *Eucalyptus* seeds were soaked in 10% H_2O_2 for 10 min to the equivalent of

21 that of the chemical seed treatment (Celest® XL). Exposure of moist *Eucalyptus* seeds to

22 microwave radiation of 1400 W for 30 s was the only microwave power-time combination

23 that significantly improved seed germination similar to that of the Celest® XL treatment. *In -*

24 *vitro* assays showed no diseases on seedlings raised from seeds soaked in hot water baths set

25 at 55 and 60 °C for 15 min and above, and seeds soaked in 10 and 15 % H_2O_2 for 5 min and

26 above. Moreover, no disease symptoms were observed on seedlings raised from moist seeds

27 exposed to microwave radiation at 1400 W for 90 s and above or dry seeds exposed to

28 microwave radiation at 1400 W for 120 s and above. *Bacillus*, however, was the only non-

29 chemical seed treatment that demonstrated effectiveness against anthracnose leaf spot under

30 greenhouse conditions.

31 **Key words:** anthracnose, seed treatment*,* biocontrol, hot water, hydrogen peroxide,

32 microwave radiation

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 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, *Eucalyptus viminalis* Labill. 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

104   Seeds of *E. grandis* and *E. nitens* were supplied by commercial forestry seed companies.

105   They were selected based on the levels of susceptibility to anthracnose leaf spot disease i.e.

106   not susceptible and highly susceptible, respectively (data not shown). Pathogenic *C. kahawae*

107   subsp. *cigarro* (PPRI 24314, GenBank accession numbers for ACT, CHS, GAPDH, ITS and

108   TUB2 gene regions: MK512735, MK512737, MK512733, MG641892 and MK512739,

109   respectively) isolated from *Eucalyptus* seeds (Mangwende, 2020) was used in this study. The

110   identity of *C. kahawae* subsp. *cigarro* isolate was confirmed using a biochemical assay,

111   where the pathogen was able to grow on basal medium containing either glucose or citric acid

112   or ammonium titrate as a sole carbon source (Waller et al. 1993). Commercial biocontrol

113   agents, *Trichoderma harzianum* Rifai ($2x10^9$ sporesg^{-1}) (Plant Health Products (Pvt.) Ltd.,

114   Kwazulu-Natal, South Africa) and *Bacillus subtilis* (Ehrenberg) Cohn strain MBI 600

115   ($2x10^{11}$sporesmL^{-1}) (Becker Underwood (Pvt) Ltd., Kwazulu-Natal, South Africa), and a

116   fungicide Celest® XL (25 aiL^{-1} fludioxoniland 10 g aiL^{-1}mefenoxam) (Syngenta (Pty.) Ltd.,

117   Midrand, South Africa) were used for the study. Ensure® ISO 103 (30% hydrogen peroxide)

118   was sourced from Merck (Pvt.) Ltd. (Midrand, South Africa).

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2.2. Seed inoculation

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122   Seeds were surface disinfected in 1% sodium hypochlorite solution for 5 min and artificially

123   inoculated by soaking in 20 mL of a 1×10^5 conidia mL^{-1} inoculum of *C. kahawae* subsp.

124   *cigarro* amended with 2 drops of Tween-20 for 4 h, with occasional hand shaking. Inoculated

125   seeds were air-dried overnight on sterile paper towels in a laminar flow cabinet, and plated

126   (50 per sample) on potato dextrose agar (PDA, Biolabs, South Africa). Plated seeds were

127   incubated at 25 °C for 7 days under alternating cycles of 12 h ultra violet (UV) (365 nm) light

128   and darkness. To confirm that inoculation was successful, fungi was re-isolated from

129   inoculated seeds on PDA and identity confirmed in comparison with positive reference plates

130   of *C. kahawae* subsp. *cigarro.*

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2.3. Hot water seed treatment
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134   Artificially inoculated *Eucalyptus* seed lots were enclosed in double cheesecloth to form

135   aliquots of 200 seeds per cheesecloth bag. Initially, aliquots were soaked in sterile distilled

136   water at room temperature for 2 h prior to treatment in a hot water bath (Model: 132A;

137   Labotec, South Africa). The temperatures of sterile distilled water in glass beakers was

138   equilibrated to the target temperatures of 35, 40, 45, 50, 55 and 60 °C before the start of the

139   experiment, and were constantly monitored. Aliquots containing seeds were soaked at the

140   different hot water temperatures for different periods namely, 1, 15, 30, 45 and 60 min. Seeds

141   left soaked in sterile distilled water at standard room temperature at equivalent time points

142   served as negative controls, whereas seeds soaked in Celest$^{®}$ XL at the recommended rate of

143   1 mLkg^{-1} seed at equivalent times served as positive controls. Immediately after hot water

144   treatment, aliquots were submerged in sterile distilled water at room temperature for 5 min.

145   Subsequently, aliquots were spread onto sterile paper towels and left to air dry on a laminar

146   flow bench.

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2.4. Seed treatments with microwave radiation
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150   Dry and moist *Eucalyptus* seed lots were exposed to microwave radiation. To moisten seeds,

151   inoculated seeds were wrapped in double cheesecloth and soaked in sterile distilled water at

152 room temperature for 2 h prior to treatment. Seeds were evenly spaced on top of two layers of

153 dry Whatman filter papers aligned in a glass Petri dish. A 1400 W and 2450 MHz consumer

154 grade microwave oven (Samsung microwave model: ME9114W1, Malaysia) with digital

155 adjustable power levels was used. A total of 200 seeds for each seed lot were exposed to

156 microwave radiation with three levels of power, 250, 600 and 1400 W. For each power level,

157 exposure times ranged from 0 to 180s with 30 s increments. The glass Petri dish containing

158 seeds was placed in the centre of the rotating plate of the microwave oven. Soon after

159 treatment, seeds were cooled by submerging in sterile distilled water at standard room

160 conditions for 5 min and then air dried on a laminar flow bench. Efficacy of microwave

161 radiation was measured against non-treated inoculated seeds and inoculated seeds treated

162 with Celest® XL.

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164 **2.5. Seed treatment with hydrogen peroxide**

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166 Cheesecloths containing 200 inoculated seed per bag were soaked in sterile distilled water at

167 room temperature for 2 h before transferring the individual aliquots to beakers containing

168 aqueous solutions of 1, 5, 10 or15% (*v/v*) hydrogen peroxide (H_2O_2) at standard room

169 temperature. For each concentration of H_2O_2, seeds were soaked for 1, 5, 10, 30 and 45 min.

170 Inoculated seeds soaked in sterile distilled water at room temperature at these same time

171 points served as negative controls, whilst seeds soaked in Celest® XL at aforementioned

172 times were positive controls. After treatment, cheesecloths containing seeds were rinsed in

173 sterile distilled water and seeds were left to dry on a laminar flow bench.

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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 percentages 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 were 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 21days 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

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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.

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2.9. Statistical analysis

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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≤0.05).

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219

220   **3. Results**

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222   **3.1. Effects of seed treatments on the incidence of *C. kahawae* subsp*. cigarro***

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224   Seed treatments significantly reduced incidences of *C. kahawae* subsp. *cigarro* on *Eucalyptus*

225   spp. seeds compared with controls (p≤0.05), except for seeds soaked in hot water baths set at

226   40 °C for 1 min (Tables 1 and Appendix A). The incidence of *C. kahawae* subsp. *cigarro*

227   persisted on *E. grandis* seed lots soaked in hot water baths for 1 min regardless of the

228   temperature increment (Appendix A). At the same soaking period, hot water seed treatment at

229   60 °C effectively reduced the incidence of *C. kahawae* subsp. *cigarro* on *E. nitens* seeds and

230   was comparable with the biocontrol agents and Celest® XL treatment. At soaking periods of

231   15 min and above, setting hot water baths at 60 °C effectively eliminated incidences of *C.*

232   *kahawae* subsp. *cigarro* on all *Eucalyptus* seed lots.

233

234 **Table 1:** Effects of hot water seed treatments on seed germination, diseased seedlings and incidence of *C. kahawae* subsp. *cigarro* from

235 artificially inoculated *E. nitens* seed lots

	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	50.3bX	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

236 Inc[a]: Percentage incidence of *C. kahawae* subsp. *cigarro*, Germ[b]: seed germination, Dis[c]: diseased seedlings. *Means sharing a common letter in

237 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

238 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

239 each other ($p \leq 0.05$)

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242   Effects of soaking *Eucalyptus* seed lots in H_2O_2 on the incidence of *C. kahawae*

243   subsp. *cigarro* are presented in Table 2 and Appendix B. Seed treatments significantly

244   reduced the incidence of *C. kahawae* subsp. *cigarro* on *Eucalyptus* seed lots compared to

245   untreated controls ($p \leq 0.05$), except for seeds soaked in 1% H_2O_2 for 1 min. At a soaking

246   period of 1 min, H_2O_2 was the least effective seed treatment. However, there was a significant

247   increase in efficacy of H_2O_2 at reducing incidences of *C. kahawae* subsp. *cigarro* as the

248   soaking period was increased. Soaking *E. grandis* seeds in 15% H_2O_2 for 5 min significantly

249   reduced incidences of *C. kahawae* subsp. *cigarro* even better than biocontrol agents ($p \leq 0.05$)

250   (Appendix B). Soaking *Eucalyptus* seeds in 15% H_2O_2 for more than 5 min effectively

251   eradicated incidences of *C. kahawae* subsp. *cigarro*.

252   *Eucalyptus* seeds exposed to microwave radiation had significantly lower incidences

253   of *C. kahawae* subsp. *cigarro* compared with inoculated controls ($p \leq 0.05$), except for dry

254   seeds exposed at 250 w microwave radiation for 30 s (Table 3 and Appendix C). At exposure

255   periods of 60 s and below, all power-time parameters of microwave radiation were

256   significantly less effective at reducing incidences of *C. kahawae* subsp. *cigarro* than seed

257   treatments with biocontrol agents and Celest[®] XL ($p \leq 0.05$).Exposure of moistened seeds to

258   microwave radiation of 1400 W for 90 s and above, together with microwave radiation of dry

259   seeds at 1400 W for 120 s and above, eliminated incidences of *C. kahawae* subsp. *cigarro* on

260   *Eucalyptus* seeds.

261

262   **3.2. Effects of seed treatments on seed germination**

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264   Seed treatments significantly increased seed germination of *Eucalyptus* seed lots compared to

265   non-treated controls ($p \leq 0.05$) (Tables 1-3 and Appendix A-C). Soaking *Eucalyptus* seeds

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266   **Table 2:** Effects of hydrogen peroxide on seed germination, diseased seedlings and incidence of *C. kahawae* subsp. *cigarro* from artificially

267   inoculated *E. nitens* seed lots

| | **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	53.3bY	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

268   Inc[a]: Percentage incidence of *C. kahawae* subsp. *cigarro*, Germ[b]: seed germination, Dis[c]: diseased seedlings. *Means sharing a common letter in

269   a column do not differ significantly according to the Fisher's LSD test at p≤0.05. **In each row, means with the same lowercase letters do not

270   significantly differ from each other at p≤0.05. ***Means within a row not followed by the same uppercase letter are significantly different from

271   each other (p ≤0.05)

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273   in hot water baths set at 55 and 60 °C for 30 and 1 min, respectively, were the most effective

274   temperature-time combinations that resulted in the most improvement of seed germination

275   (Table 1 and Appendix A). Further increase of hot water bath temperature beyond these

276   limits greatly reduced seed germination.

277   There was a positive response to seed germination with gradual increments of

278   concentration of H_2O_2 from 1 to 10% (Table 2 and Appendix B). However, increasing the

279   concentration of H_2O_2 beyond 10% resulted in reduction of seed germination. Most

280   improvements on seed germination were observed on seeds soaked in 10% H_2O_2 for 10 min,

281   which had similar efficacy as the Celest® XL treatment, except for *E. nitens* seed lots.

282   Regardless of concentration of H_2O_2, germination of *E. nitens* was significantly lower than

283   seed treatments with biocontrol agents and Celest® XL ($p \leq 0.05$).

284   The effects of microwave seed treatments on germination of *Eucalyptus* seeds are

285   displayed in Table 3 and Appendix C. Microwave radiation of moist seeds significantly

286   increased seed germination better than dry seeds ($p \leq 0.05$). In fact, exposure of moist

287   *Eucalyptus* seeds to microwave adjusted to 1400 W for 30 s was the only microwave power-

288   time combination that significantly improved seed germination with a similar level of

289   efficacy as the Celest® XL treatment. However, prolonged exposure to microwave radiation

290   at 1400 W above 60 s significantly reduced seed germination ($p \leq 0.05$). Microwave radiation

291   of dry seeds at 1400 W for 120 s and above completely reduced seed germination.

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 sec | | | 60 sec | | | 90 sec | | | 120 sec | | | 150 sec | | | 180 sec | | |
	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 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 ($p \leq 0.05$)

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 1-2 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 (Tables 1 and Appendix A). Similarly, 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

17

323   leaf spot was observed on *Eucalyptus* seedlings, even on *E. nitens* seedlings raised from

324   Celest® XL treated seeds, compared with non-inoculated controls.

325

3.4.2. Severity of anthracnose leaf spot

3.4.2.1. Disease scores

328   Anthracnose leaf spot was more pronounced at 180 DAS and were most severe on seedlings

329   raised from inoculated and untreated seeds (Table 4). Seed treatments did not significantly

330   suppress ($p > 0.05$) severity of anthracnose leaf spot on *Eucalyptus* seedlings compared with

331   Celest® XL, except for seedlings raised from *Bacillus* treated seeds.

3.4.2.2. Diameter of leaf spots

333   Seedlings raised from non-treated seeds inoculated with *C. kahawae* subsp. *cigarro* had the

334   biggest leaf spots and were statistically similar to those of seedlings raised from seeds treated

335   with hot water at 60°C for 1 min and microwave radiation of dry seeds at 1 500 W for 60 s

336   (Table 4). *Bacillus* was the only non-chemical seed treatment that significantly suppressed

337   ($p \leq 0.05$) appearance of anthracnose leaf spots on *E. nitens* seedlings equally as the Celest®

338   XL treatment.

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

Treatment	Incidence (%)						Severity (%)						Ø leaf spots (mm)	
	Trial I			Trial II			Trial I			Trial II			Trial I	Trial II
	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	5.3ab	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	4.9ab	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	5.3ab	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	5.2ab	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	79.2ab	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	0.6fg	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

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

341

342 **Table 5:** Effects of seed treatments on the growth and development of seedlings raised from *E. nitens* seeds inoculated with *C. kahawae* subsp.
343 *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	27.0ab	15.8ab	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	2.4fg	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	23.9ab	30.4a	3.7bc	4.3ab
Wet 600 W for 60 s	79.5cde	80.8bc	11.2cde	18.7cdef	23.5bcd	10.5cde	17.3de	23.7de	2.4fg	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	86.5ab	87.5a	15.6ab	25.6ab	30.6a	15.4ab	24.7a	31.8a	4.1ab	4.6a
Trichoderma	79.0cde	83.0b	13.7abcd	21.4bc	26.7abc	13.6abc	22.6abc	30.2ab	3.7bc	4.1ab
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

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

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 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 (Table5). 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 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 treatment 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 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). 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).

392   The main challenge with hot water seed treatments is that it is limited to a few internal

393   layers of seedcoat. Although soaking *E. nitens* seeds in a hot water bath set at 60 °C for 15

394   min and above effectively reduced incidences of *C. kahawae* subsp. *cigarro*, anthracnose leaf

395   spot was still observed on seedlings raised from these seeds under greenhouse conditions. It

396   is possible that incidence of *C. kahawae* subsp. *cigarro* was retained on *E. nitens* seeds

397   soaked in hot water bath set at 60 ^{o}C for 1 min as heat was not effectively conducted to reach

398   some of the spores that were embedded deeper inside seed coat crevices. Similarly, studies on

399   cabbage seed infested with *Leptosphaeria maculans* Ces. & De Not. showed a 2% retention

400   of infestation after hot water seed treatments (Williams 1967). Since there were no diseased

401   seedlings under *in -vitro* conditions, it is possible that concentrations of pathogen inoculum

402   was significantly reduced to the extent that it was not sufficient to cause well pronounced

403   disease symptoms particularly considering the latent and biotrophic nature of *Colletotrichum*

404   species. Moreover, *C. kahawae* subsp. *cigarro* might have been poorly transmitted from seed

405   into seedlings as reported by Mangwende (2020).

406   Soaking *Eucalyptus* seed lots in H_2O_2 significantly improved seed germination of

407   *Eucalyptus* spp. Similarly, seed germination was increased when seeds of Douglas fir

408   (*Pseudotsuga menziesii* (Mirb.) Franco), zinnia (*Zinnia elegans* Jacq.), switch grass (*Panicum*

409   *virgatum* L.), big bluestem (*Andropogon gerardii* Vitman) and Indian grass (*Sorghastrum*

410   *nutans* (L.) Nash) were soaked in H_2O_2 (Ogawa and Iwabuchi, 2001; Lee et al., 2004; Sarath

411   et al., 2008). Soaking *Eucalyptus* seeds in 10 % H_2O_2 for 5 min and 10 min were the most

412   effective combinations to give the highest improvement on seed germination and were

413   equally effective as seeds treated with *Bacillus* and Celest® XL. Regardless of concentration

414   of H_2O_2, seed germination of *E. nitens* seed lots was significantly lower than seed treatments

415   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 deeper crevices that may harbour spores of the pathogen thereby lowering efficacy of H_2O_2 at disinfecting seeds with a direct reduction on seed germination. Desperate attempts to disinfect infected seeds might lure usage of higher concentrations, but this must be discouraged as high concentrations 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

440 treatment. Prolonged exposure of seeds to microwave radiation above 60 s significantly

441 reduced germination (p≤0.05).

442   Microwave radiation of moist seeds significantly increased seed germination better

443 than dry seeds (p≤0.05). Efficacy of seed treatments with microwave radiation is depended

444 on the dielectric permittivity of the materials involved (Nelson, 1996; Jiao et al., 2011). As

445 seeds are exposed to high-frequency electromagnetic radiation (EMR) (300 MHz-300 GHz),

446 heat energy is generated within the molecules and structural compounds of seeds and

447 pathogens. The overall moisture content, temperature, bulk density and frequency of applied

448 electric fields affects the extent to which heat is produced and transferred between molecules,

449 warming the material thoroughly (Bouraoui et al., 1993). Hence, moistening seeds elevates

450 permittivity of microwave radiated seeds that generates an elevated amount of heat compared

451 with dry seeds. In fact, microwave radiation of moistened *Eucalyptus* seeds at powers levels

452 of 1400 W for 30 s was the best power-time treatment combination, from which the highest

453 seed germination percentage was recorded. In this same way, spores on moistened seeds were

454 easily killed. Contrasts at each power level showed greater sensitivity to microwave radiation

455 response where moist *Eucalyptus* seeds had significantly lower percentages of diseased

456 seedlings than dry seeds. This confirms studies that showed that higher seed moisture content

457 translates to an increase in efficacy of microwave radiation against seed-borne fungi

458 (Bouraoui et al., 1993; Berbert et al., 2002; Jiao et al., 2011; Knox et al., 2013).

459   In conclusion, investigations of this study were very rigorous considering that seeds

460 used were artificially inoculated with high concentrations of *C. kahawae* subsp. *cigarro*

461 ($1x10^5$ sporesmL^{-1}), which is a rare scenario under natural circumstances. When both seed

462 disinfection and seed germination are considered, non-chemical seed treatments *viz.* soaking

463 seeds in hot water baths set at 55 °C for 15 min, 60 °C for 1 min, soaking seeds in 5% H_2O_2

464 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.

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