Susceptibility of *Eucalyptus grandis* and *Acacia mearnsii* seedlings to five *Phytophthora* species common in South African plantations

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ABSTRACT

Eucalyptus grandis and its hybrids, as well as *Acacia mearnsii*, are important non-native trees commonly propagated for forestry purposes in South Africa. In this study, we conducted pathogenicity trials to assess the relative importance of five commonly isolated *Phytophthora* spp. (*Phytophthora alticola*, *P. cinnamomi*, *P. frigida, P. multivora* and *P. nicotianae*) from the plantation environment on *E. grandis* and *A. mearnsii* seedlings. Overall *E. grandis* was more susceptible to the tested *Phytophthora* spp. than *A. mearnsii*. *Phytophthora cinnamomi* was the only pathogen that had a significant negative effect on both the host tree species, leading to a reduction in root and shoot weight as well as to death in the case of *E. grandis*. *Phytophthora alticola* and *P. nicotianae* exclusively affected *E. grandis* and *A. mearnsii,* respectively. This study updated the current knowledge on the pathogenicity of *Phytophthora* spp. on two important non-native commercially propagated tree species from South Africa.

Keywords Black-butt disease, plantation forestry, pathogenicity, sand-infestation pot trial, tree health

1 | INTRODUCTION

Commercial forestry in South Africa depends on plantations of non-native tree species. Two commonly planted non-native trees are *Acacia mearnsii* and various species and hybrids of *Eucalyptus*. Among these two non-native tree species, *Eucalyptus* species are the most widely planted, encompassing around 42% of the total commercial plantation area in the country, while *Acacia* plantations account for approximately 10% (Forestry South Africa 2018), Various native and introduced pests and pathogens, including *Phytophthora* spp., result in diseases of these non-native trees (Roux & Wingfield 1997; Roux et al., 2012; Wingfield & Swart 1994; Wingfield et al., 2001).

Phytophthora cinnamomi has been reported to cause root and collar-rot of *Eucalyptus* spp. (Linde et al., 1994b). More recently *Phytophthora alticola* and *Phytophthora frigida* were found to cause collar-rot of cold-tolerant *Eucalyptus* spp. in the KwaZulu-Natal Province (Maseko et al., 2007). The most common *Phytophthora*

disease of *A. mearnsii* is 'black-butt' caused by *P. nicotianae* (Zeijlemaker 1971; Zeijlemaker & Margot 1970). This disease is characterized by black discoloration of the bark around the bases of trees followed by cracking of the bark and gummosis (Roux & Wingfield 1997; Roux et al., 1995; Zeijlemaker 1971; Zeiljemaker 1967). Black-butt does not kill older trees but reduces bark yield. Other *Phytophthora* spp. reported to infect *A. mearnsii* are *P. boehmeriae* and *P. meadii* (Roux & Wingfield 1997).

In a recent study considering the diversity of *Phytophthora* spp. in plantations of *E. grandis* and *A. mearnsii* in South Africa, *P. alticola*, *P. cinnamomi*, *P. frigida* and *P. multivora* were commonly isolated species (Bose et al., 2018). The pathogenicity of some of these species has not been tested on these trees. In this study, we evaluated the pathogenicity of *P. alticola*, *P. cinnamomi, P. frigida, P. multivora* and *P. nicotianae* on *E. grandis* and *A. mearnsii* under greenhouse conditions using a sand-infestation technique.

2 | MATERIALS AND METHODS

2.1 | Biological materials

Seeds of *E. grandis* (EG66839) and *A. mearnsii* (AM69218) were sourced from commercial forestry companies in South Africa. Isolates of *Phytophthora alticola*

(CMW48711), *P. cinnamomi* (CMW48774), *P. frigida* (CMW48733), *P. multivora* (CMW48804) and *P. nicotianae* (CMW50379) were retrieved from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

Phytophthora spp. were selected based on previous reports of their pathogenicity to either of the host genera. However, *P. multivora* was selected as it was commonly isolated from soil in plantation environments, but its pathogenicity has never been tested in South Africa. Isolates for each of the *Phytophthora* spp. were selected arbitrarily. Isolates of *P. alticola, P. cinnamomi* and *P. frigida* originated from *Eucalyptus* soil, *P. multivora* from *A. mearnsii* soil and *P. nicotianae* from bark tissue of *A. mearnsii.*

2.2 | Seed germination and transplanting of seedlings

Prior to sowing, *A. mearnsii* seeds were heat treated with boiling water for five minutes at a ratio of 1:20 (seed: boiling water); the treated seeds were allowed to dry for two weeks at room temperature. No pre-treatment was necessary for the *E. grandis* seeds. All seeds were germinated on autoclaved vermiculite (Culterra, South Africa) in plant trays and the plants were irrigated regularly.

Sand-infestation pot trials (Simamora 2016) were prepared using sterilized, washed river sand as the growth medium. The sand was sterilized thrice by autoclaving on three consecutive days. Free-draining polyurethane pots were also

sterilized with 2% (v/v) sodium hypochlorite solution followed by rinsing twice with sterilized deionized water.

Three weeks post-germination, the seedlings were transferred to pots containing the autoclaved sand. In each pot, two 10 ml sterile plastic tubes (10 × 1.5 cm) were inserted during transplanting such that each could later receive ~2.5 g inoculum (Simamora 2016). All the seedlings were maintained in a phytotron at a temperature ranging between 18-24°C with a relative humidity of 60-70%. Seedlings were irrigated every day and were fertilized using Nitrosol® (Fleuron Pty Ltd, South Africa) once every two weeks following the manufacturer's instructions.

2.3 | Inoculum preparation

For each *Phytophthora* isolate, the inoculum was prepared in a 1 L Erlenmeyer flask containing 500 ml of vermiculite, 5 g millet seeds and 300 ml 10% clarified V8 juice (Campbell Soup Company USA). All the flasks were plugged with non-absorbent cotton wool and autoclaved over three consecutive days and inoculated upon cooling. Agar blocks from six-day-old *Phytophthora* cultures growing on 10% clarified V8 Agar served as the inoculum (Simamora 2016). After inoculation, the flasks were incubated at 20°C in the dark. All the flasks were gently shaken every four days to distribute the inoculum evenly. After six weeks of incubation, the inocula were rinsed with sterilized deionised water to remove excess nutrients (Jung

et al., 1996; Matheron & Mircetich 1985), immediately before inoculating the pots in which plants had been propagated.

2.4 | Experimental design

Three months after the seedlings had been planted, the pots were inoculated by removing the plastic tubes and filling each hole with ~ 2.5 g vermiculite inoculum. The holes were covered with sterilized sand. For each *Phytophthora* spp., ten pots each of *E. grandis* and *A. mearnsii* were inoculated. Ten seedlings of each species (ten pots) were mock inoculated with sterilized vermiculite to serve as controls. In order to stimulate the production of sporangia and the release of zoospores from the inoculum source, the pots were flooded overnight in polyurethane trays filled with sterile distilled water on three occasions: after inoculation, at 14 d and 28 d. Pots were arranged randomly on benches in the phytotron. The arrangement of the pots was changed once per week. The entire pathogenicity trial was repeated once.

2.5 | Measurements of pathogenicity

Three months after inoculation, the inoculated seedlings were harvested. The shoots were separated from the root systems and assessed separately. The fresh weight of the shoots was recorded. Dry weight was determined by desiccating the shoots at 40°C for 15 days in paper bags and weighed subsequently. Roots were rinsed with deionized water and blotted dry. The roots were visually rated for root rot on a scale of 0 to 4 (0 = no visible root damage, 1 = ~20% of the roots with lesions

and with loss of fine roots, 3 = >20 - <50% of the roots with lesions and loss of fine roots, 3 = >50% of the roots with lesions and loss of fine roots, 4 = dead). The fresh weight of the roots was measured, followed by the root volume using a water displacement method. For dry weight, the roots were dried at 40°C for 15 days in paper bags.

2.6 | Re-isolation and identification of *Phytophthora* spp.

Isolations to fulfill Koch's postulates were done from five arbitrarily selected symptomatic plants for each treatment per trial, including the controls. For each plant, five root tips were plated onto NARPH medium (Masago et al., 1977) selective for *Phytophthora* species (modified from Hüberli et al., 2000). The re-isolated *Phytophthora* spp. were identified using DNA sequencing of the region spanning the internal transcribed spacer (ITS1-5.8S-ITS2) region followed by sequence similarity searches using the BLAST algorithm (Altschul et al., 1990) available on GenBank.

2.7 | Statistical analyses

Using the statistical analyses mentioned below, we individually analyzed three datasets, one from each replicate of the trial together with a concatenated dataset. In the 'root health rating,' analysis the concatenated dataset was excluded.

A One-way Analysis of Variance (ANOVA) was used to determine statistically significant differences between the *Phytophthora* treatments and controls for both *E. grandis* and *A. mearnsii*. An individual analysis was performed for each dependent

variable and each plant species, on the original, repeated and combined trial datasets. Assumptions of normality and homogeneity of variance were assessed prior to the analysis. Additionally, plants that had died before the end of the experiment and outliers were removed from the datasets.

Normality was assessed with Q-Q plots, observation frequency histograms and the Shapiro-Wilk test. Homogeneity of variance was tested using Levene's and Bartlett's tests of homogenous variance. The majority of dependent variables violated the assumption of homogenous variance, and a Welch's ANOVA (Welch 1951) was used to analyze the data. A Kruskal-Wallis test (Kruskal & Wallis 1952) was performed on a dependent variable when data were non-normally distributed. Tukey HSD, Games-Howell, and Nemenyi *post hoc* tests were performed after One-way ANOVA, Welch's ANOVA, and Kruskal-Wallis tests, respectively.

A Fisher's exact test was used to analyze the health rating dependent variable for each plant species due to small sample sizes in several categories. A Two-way ANOVA compared the original and the repeated trials to examine if there were statistically significant differences between the *Phytophthora* treatments. Assumptions for the Two-way ANOVA were tested as above. If the variance was heteroscedastic, trial and treatment were unified into a single factor, and a Welch ANOVA with a Games-Howell *post hoc* was used to determine if the treatments were significantly different between trials.

All analyses were conducted using the statistics program R (R Core Team, 2018), the "car" (Fox & Weisberg 2017), "graphics", "PMCMR" (Pohlert 2014), "rcompanion" (Mangiafico 2018), "stats" and "userfriendlyscience" (Peters 2015) packages.

3 | RESULTS

Successful infection of both *A. mearnsii* and *E. grandis* was achieved using the root inoculation technique described in this study (Fig. 1, 2 and 3). Except for disease rating and re-isolation of pathogens from infected plants, this section is based on the outcome of statistical analyses using concatenated datasets.

3.1 | Pathogenicity trials on *Acacia mearnsii*

3.1.1 | Comparison between the trials

Only two measurements were significantly different between the trails; *P. multivora* [fresh root weight; $F(11, 108) = 38.1$, $p < 0.001$, Games-Howell *post hoc* p-value = 0.001] and *P. frigida* [root volume; $F(11, 108) = 67.3$, $p < 0.001$, Games-Howell p-value = 0.006]. Overall, the difference between the trials was negligible, and for all further analyses, the the trials were combined.

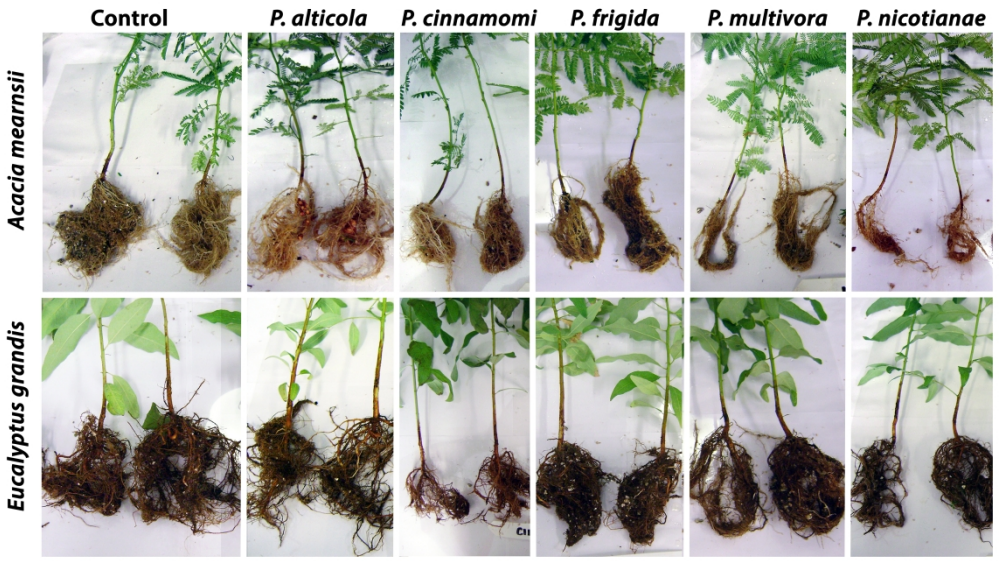

FIGURE 1 The difference in root morphology of *Eucalyptus grandis* and *Acacia mearnsii*, three months after inoculating with five *Phytophthora* spp. Compared to the controls, both the host plants infected with *P. cinnamomi* showed a significant reduction in root parameters measured in this study.*Acacia mearnsii* infected with *P. nicotianae* showed a significant reduction in root weight and volume.

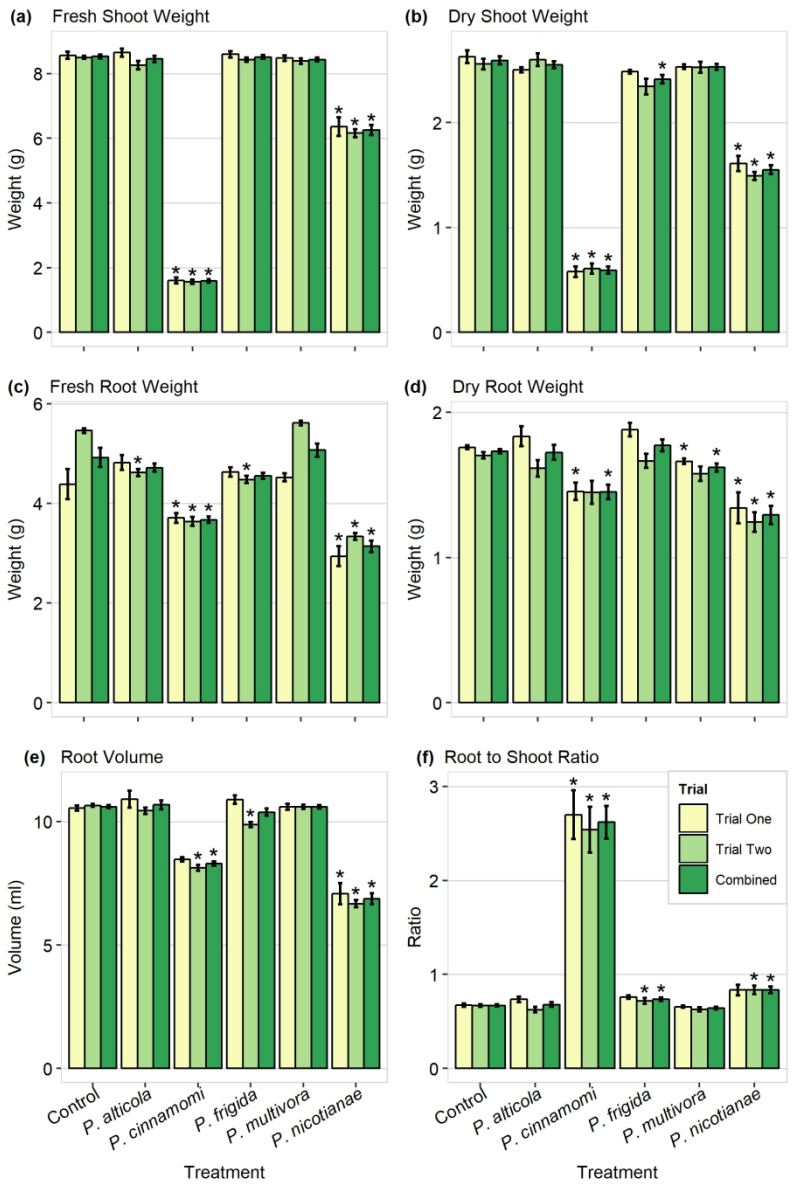

FIGURE 2 Graphical representations of *Acacia mearnsii* pathogenicity trials using five *Phytophthora* species. Each bar plot shows a different post-harvest measurement. The bars indicate standard errors. Statistically significant ($p \leq 0.05$) measurements for each *Phytophthora* sp. is indicated by an asterisk on the bar plot.

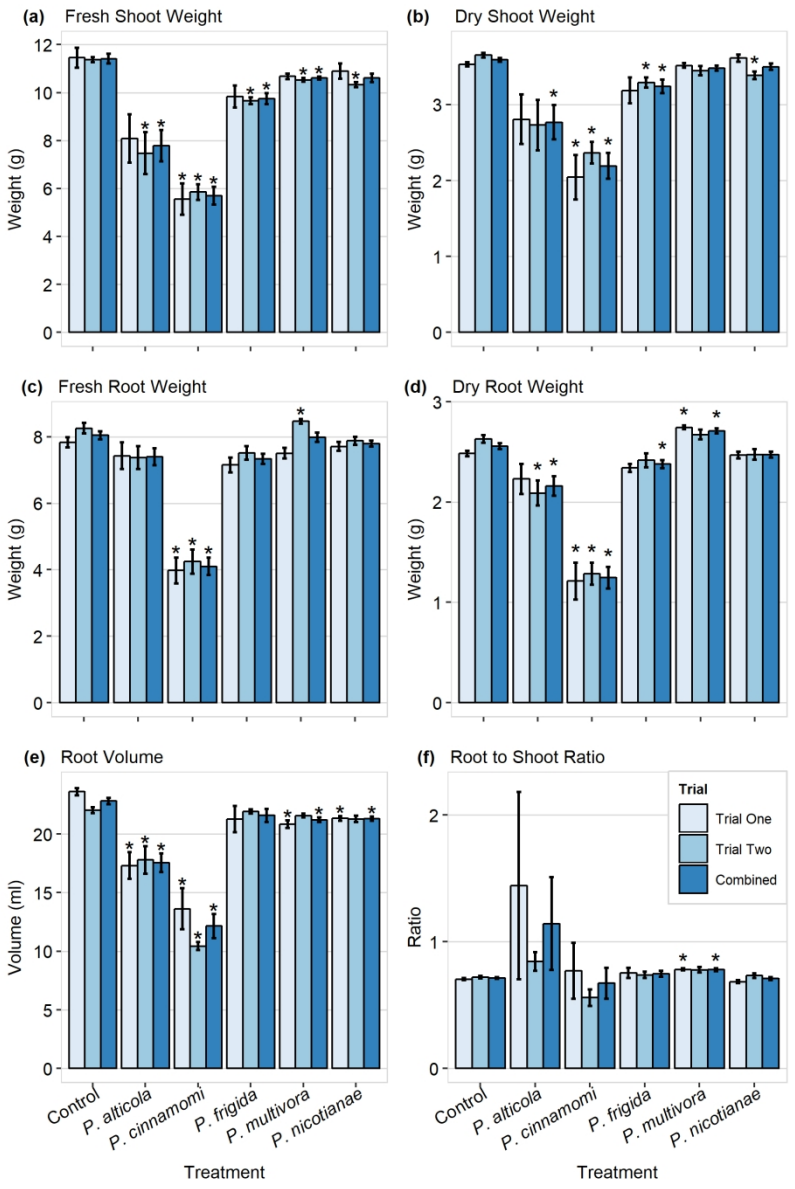

FIGURE 3 Graphical representations of *Eucalyptus grandis* pathogenicity trials using five *Phytophthora* species. Each bar plot shows a different post-harvest measurement. The bars indicate standard errors. Statistically significant ($p \leq 0.05$) measurements for each *Phytophthora* sp. is indicated by an asterisk on the bar plot.

3.1.2 | Phytophthora treatments

Phytophthora cinnamomi and *P. nicotianae* significantly reduced the weight of fresh shoot, dry shoot, fresh root, dry root and root volume (Fig. 2 a-e; $p < 0.05$). *Phytophthora frigida* and *P. multivora* significantly reduced only the dry shoot and dry root weights, respectively (Fig. 2 b, d; $p < 0.05$). *Phytophthora cinnamomi*, *P. frigida* and *P. nicotianae* significantly affected the root to shoot ratio (Fig. 2f; $p < 0.05$). *Acacia mearnsii* infected with *P. cinnamomi* showed an increased root to shoot ratio compared to the controls (Fig. 2f; $p < 0.05$).

3.1.3 | Disease rating

For both the trials, the health rating was significant only for seedlings infected with *P. nicotianae* (Fig. 4, $p < 0.05$). Almost all *A. mearnsii* seedlings, from both the trials, infected with *P. nicotianae* showed mild to severe signs of root-rot (Fig. 4). There was no plant mortality in any of the trials. In the case of the health rating, apart from *P. nicotianae,* none of the other *Phytophthora* spp. had any significant effect *A. mearnsii*.

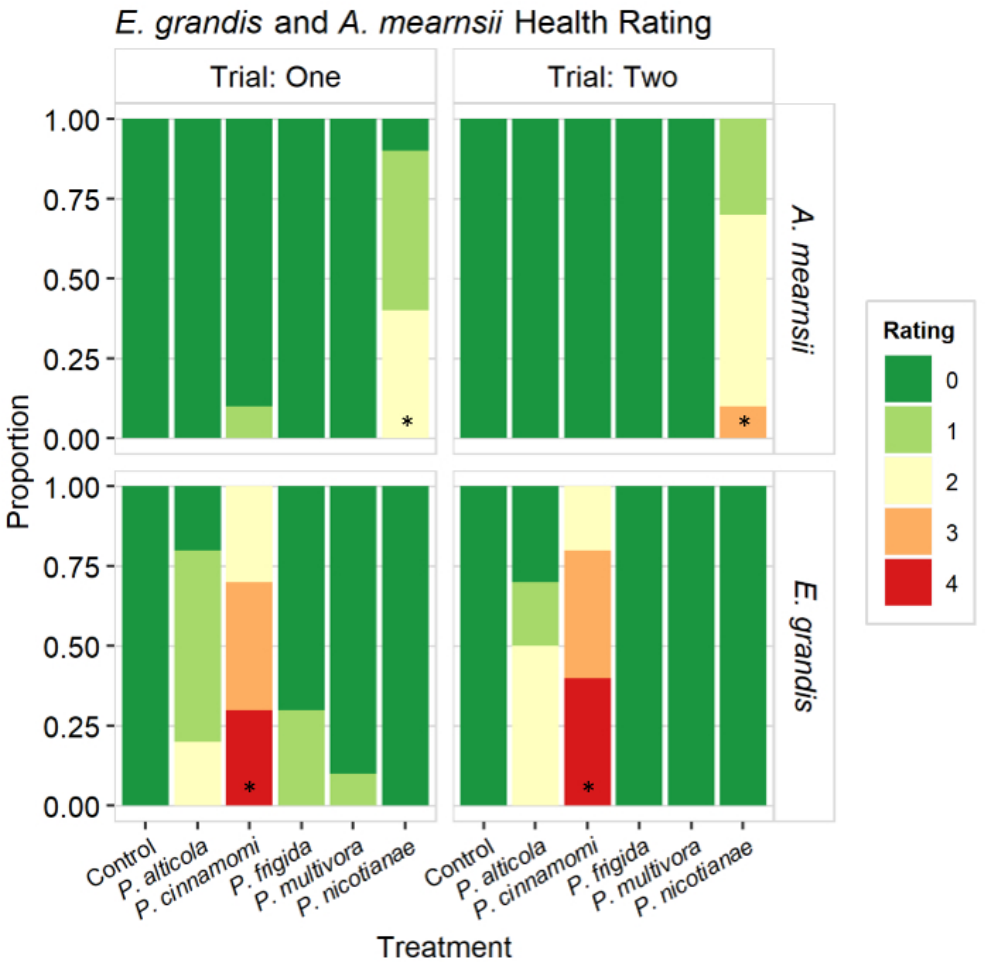

FIGURE 4 Stacked bar graphs representing the root health of *Eucalyptus grandis* and *Acacia mearnsii* on a scale 0-4 (0 = no damage, 4 = dead). An asterisk represents statistical significance ($p \leq 0.05$).

3.2 | Pathogenicity trials on Eucalyptus grandis

3.2.1 | Comparison between the trials

Only two measurements were significantly different between the trails; *P. multivora* [fresh root weight; $F(11, 101) = 28.3$, $p < 0.001$, Games-Howell *post hoc* p-value < 0.003] and control [root volume, $F(11, 101) = 21.9$, $p < 0.001$, Games-Howell *post hoc* p-value = 0.039]. Overall, the difference between the trials was negligible and for all further analyses, the trials were combined.

3.2.2 | *Phytophthora* treatments

The weights of fresh shoots and dry roots were significantly reduced by *P. alticola*, *P. cinnamomi* and *P. frigida* (Fig. 3 a, d; $p < 0.05$). *Phytophthora alticola*, *P. cinnamomi* and *P. frigida* significantly reduced the dry shoot weight (Fig. 3 b; $p < 0.05$). *Phytophthora cinnamomi* was the only pathogen that significantly affected the weight of fresh roots (Fig. 3 c, $p < 0.05$). Root volume of *E. grandis* plants was significantly reduced by *P. alticola*, *P. cinnamomi* and *P. nicotianae* (Fig. 3 f; $p < 0.05$).

Phytophthora multivora had a contrasting effect on *E. grandis* plants. It was found to significantly reduce the fresh shoot weight and root volume (Fig. 3 a, e; $p < 0.05$), while significantly increasing the dry root weight and fresh shoot weight (only in the second trial). This anomaly resulted in a significant change in root to shoot ratio among the *E. grandis* plants affected by *P. multivora* (Fig. 3 f; $p < 0.05$).

3.2.3 | Disease rating

For both the trials, the health rating was significant only for E. grandis seedlings infected with P. cinnamomi ($p < 0.05$), all the infected seedlings had medium to severe

TABLE 1 The number of *Phytophthora* species re-isolated from the infected host plants after each pathogenicity trials. *Phytophthora* species were re-isolated from five random plants per treatment for both the trials.

	Eucalyptus grandis		*Acacia mearnsii*		Re-isolation %
	Trial 1	Trial 2	Trial 1	Trial 2	(total number of positive isolations/20×100)
Phytophthora alticola	3	2	1	0	30
Phytophthora cinnamomi	5	3	3	4	75
Phytophthora frigida	3	2	1	0	30
Phytophthora multivora	1	0	0	1	10
Phytophthora nicotianae	1	0	3	4	40
Control	0	0	0	0	00

signs of root-rot Fig. 4). Mortality was recorded among the *E. grandis* seedlings infected with *P. cinnamomi,* where three seedlings died in the first and four in the second trial (Fig. 4). Although statistically insignificant, seedlings infected with *P. alticola* also showed mild to medium signs of root-rot (Fig. 4).

3.3 | Re-isolation and identification of *Phytophthora* isolates

While *Phytophthora* could always be isolated from the soil, it could not always be recovered from the inoculated plants. Based on consolidated results from both the trials, *Phytophthora cinnamomi* (75%) was most commonly re-isolated from the inoculated plants (Table 1). This was followed by *P. nicotianae* (40%), *P. alticola* (30%) and *P. frigida* (30%) (Table 1). *Phytophthora multivora* was re-isolated only twice; once from an *E. grandis* from the first trial and once from an *A. mearnsii* plant in the second trial (Table 1).

4 | DISCUSSION

This study evaluated the pathogenicity of five commonly occurring *Phytophthora* spp. isolated from commercially managed plantations of *E. grandis* and *A. mearnsii* in South Africa. Results showed that *P. cinnamomi* was the most pathogenic species and that it affected both *E. grandis* and *A. mearnsii*. The other tested species differed in both pathogenicity and host specificity.

Phytophthora cinnamomi was the only pathogen that had a significant effect on both *E. grandis* and *A. mearnsii*. This confirms observations from previous pathogenicity studies conducted in South Africa where various *Eucalyptus* spp. have shown susceptibility to *P. cinnamomi* (Maseko 2010; Maseko et al., 2007; Wingfield & Kemp 1994). Maseko (2010), compared the pathogenicity of *P. cinnamomi, P. alticola* and *P. frigida* on *E. dunnii* and found that *P. cinnamomi* produced significantly longer under-bark lesions compared to *P. frigida* and *P. alticola*.

Phytophthora cinnamomi has an extensive host range (Burgess et al., 2017), yet it has never been reported to infect *A. mearnsii*. In this study, compared to the control, *A. mearnsii* infected with *P. cinnamomi* showed a significant reduction in some of the post-harvest measurements. This may have been due to: (i) higher inoculum threshold in the sand-infestation pot trials in contrast to the field situation, (ii) abiotic conditions such as temperature, humidity, along with regular flooding, and (iii) inoculated plants were three-months-old, potentially making them more vulnerable to infection.

Phytophthora alticola and *P. frigida* were first recovered from several cold-tolerant *Eucalyptus* species in South Africa (Maseko et al., 2007). However, prior to the present study, the pathogenicity of these species had not been tested on *E. grandis*, which is one of the important *Eucalyptus* species in South African forestry. In this study, *P. alticola* and *P. frigida* displayed similar pathogenicity on *E. grandis*. This is in contrast to a previous pathogenicity study (Maseko 2010), which showed that *P.*

frigida was a more aggressive pathogen than *P. alticola* on *E. dunnii.* This difference could be due to: (i) variation in host response, (ii) dissimilar trial designs, and (iii) different inoculation techniques (under-bark inoculations versus sand-infestation pot trial).

Phytophthora nicotianae is considered as the most serious root and collar pathogen of *A. mearnsii*, causing a disease known as 'black-butt' in South Africa. Since the first report of this disease (Zeiljemaker 1967), several pathogenicity trials have been conducted (Roux & Wingfield 1997; Zeijlemaker & Margot 1970). However, the effect of *P. nicotianae* on the overall health of *A. mearnsii* has not previously been evaluated. Apart from above ground symptoms such as cankers and gummosis that are often observed in plantation environments, through this study, we were also able to measure the effect of *P. nicotianae* on the root system of *A. mearnsii*. *Acacia mearnsii* infected with *P. nicotianae* had significantly reduced root system that could not have been documented using under-bark inoculation trials.

Phytophthora multivora has commonly been isolated from soil in South Africa (Oh et al., 2013), but has never been found associated with declining native or non-native vegetation. In the present study, *P. multivora* significantly reduced the fresh shoot weight and root volume for *E. grandis* and dry root weight for *A. mearnsii*. Compared to the control, *E. grandis* plants infected with *P. multivora* showed a significant increase in the fresh root weight (only in the second trial), dry root weight and root to shoot ratio. *Phytophthora multivora* has been implicated in the decline of

Eucalyptus spp. and other native shrubs in Western Australia (Scott et al., 2009). Although *P. multivora* infected both *E. grandis* and *A. mearnsii* in this study, there was no sign of visible root-rot. Based on our observations, it is possible that *P. multivora* infects both the host tree species in plantations, but at low levels and without producing obvious damage.

5 | CONCLUSIONS

In the present study, a pathogenicity trial was conducted using five *Phytophthora* spp. commonly isolated from the plantation forestry environment in South Africa. These trials were repeated once, and they yielded the same results. The trials were conducted under greenhouse conditions and although the results were broadly consistent with the field situation, they may not fully reflect the natural situation. A single isolate of each of the *Phytophthora* spp. was used in the pathogenicity tests, allowing for increased replication and the inclusion of a relatively large number of *Phytophthora* spp. However, the trial design from this study did not account for the variation in the pathogenicity among different isolates of *Phytophthora spp.* considered in this study. This drawback can be best exemplified using *P. multivora*. In various trials conducted globally, a substantial variation in the pathogenicity towards an assortment of hosts has been reported among the isolates of *P. multivora* (Belhaj et al., 2018; Croeser et al., 2018; Rodriguez-Padron et al., 2018).

Therefore, larger trails, including greater numbers of isolates, should be considered in the future.

All previous studies to evaluate the pathogenicity and aggressiveness of *Phytophthora* spp. on *Eucalyptus* spp. and *A. mearnsii* plants in South Africa were conducted using an under-bark inoculation technique (Linde et al., 1994a; Maseko et al., 2007; Roux & Wingfield 1997; Zeijlemaker 1971). Although this is a commonly used technique, it has two main limitations: (i) use of mycelium as the inoculum rather than allowing zoospores to infect naturally, and (ii) the extent of pathogenicity is exclusively measured based on lesion length. In this regard, using a sand-infestation technique such as the one utilized in this study more accurately reflects the natural mode of infection. However, there are several factors that predispose plants to infection by *Phytophthora* and these include factors that influence the production of zoospores. Thus, under-bark inoculations in concert with non-wounding techniques could provide deeper insights into host susceptibility and pathogen aggressiveness on the host.

ACKNOWLEDGEMENTS

We thank Sappi Forests (Pty) Ltd. and Mondi South Africa for providing seeds of *E. grandis* and *A. mearnsii*. Dr. Julian Chan from ICFR, Pietermaritzburg, South Africa is thanked for kindly sharing the protocol for geminating *Acacia mearnsii*

seeds, as is the DNA Sequencing Facility of the University of Pretoria that provided assistance in sequencing our amplicons. Ms. Izette Greyling (FABI) collected black-butt samples for isolation of *P. nicotianae* for which we are most grateful. We acknowledge funding from the University of Pretoria, members of the Tree Protection Co-operative Programme (TPCP), and the DST-NRF Centre of Excellence in Tree Health Biotechnology (CTHB), South Africa.

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