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ORIGINAL ARTICLE

Hybrid vigor in *Eucalyptus* increases resistance against *Phytophthora* root rot

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Abstract

Eucalyptus nitens is a cold-tolerant eucalypt that is native to Eastern Australia. Pure *E. nitens* as well as its hybrids, such as *Eucalyptus grandis* × *Eucalyptus nitens*, is propagated commercially in various regions of the southern hemisphere, including South Africa. In a plantation environment, *E. nitens* is susceptible to a variety of native and invasive pathogens, including *Phytophthora alticola* and *P. cinnamomi*. Recently, there have been increasing reports of root and collar rot in *E. nitens* in South Africa. The severity of this disease was substantially lower among interspecific hybrids of *E. grandis* × *E. nitens* compared to purebred *E. nitens*. In South Africa, the susceptibility of commercially propagated provenances of pure *E. nitens* and varieties of hybrid *E. grandis* × *E. nitens* to *Phytophthora* species is unknown. Therefore, we conducted greenhouse trials to evaluate the pathogenicity of *P. alticola* and *P. cinnamomi* to two families of pure *E. nitens*, one self-fertilized and the other outcrossed, as well as a single clonal variety of the most widely planted interspecific hybrid, *E. grandis* × *E. nitens*. The outcomes from these trials revealed that both self-fertilized and outcrossed families of *E. nitens* were highly susceptible to the tested *Phytophthora* species. The severity of root rot was greatest among plants inoculated with *P. cinnamomi.* The tested interspecific hybrid was tolerant to both *Phytophthora* species and developed new lateral and fine roots to offset the effects of root rot.

Keywords *Eucalyptus nitens* · *Eucalyptus grandis* × *E. nitens* · Commercial forestry · Pathogenicity · *Phytophthora alticola* · *Phytophthora cinnamomi*

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Introduction

Eucalyptus nitens is native to Eastern Australia. This tree is frequently planted in temperate regions of the southern hemisphere, such as in parts of Australia, Chile, New Zealand, and South Africa (Parsons et al. 2006; INFOR 2004; Lausberg et al. 1995; Purnell 1988). *Eucalyptus nitens* is often preferred over other cold-tolerant eucalypts because of its rapid growth (Clarke 2000; Pérez et al. 2006) and its frost hardiness (Pérez et al. 2006; Nakhooda and Jain 2016). This tree species is vulnerable to a wide range of pests and diseases (Roux et al. 2006; Carnegie et al. 2005; Adam et al. 2013), including various *Phytophthora* species, especially *Phytophthora cinnamomi* (de Andrade Lourenço et al. 2020; Studholme et al. 2019; Dick et al. 2006; Cahill et al. 2008).

Phytophthora species are important pathogens of trees in both natural and planted forests worldwide (Nagel et al. 2013; Burgess et al. 2017; Grünwald et al. 2012; Hansen 2008). *Phytophthora cinnamomi*, for example, affects the

productivity of *Eucalyptus* plantations globally (Sena et al. 2018; Nagel et al. 2013; Burgess et al. 2021) while also causing serious damage to trees in native *Eucalyptus* woodlands (Dell and Malajczuk 1989; McDougall et al. 2002). *Phytophthora* species, including *P. cinnamomi*, *P. alticola*, and *P. frigida*, infect various *Eucalyptus* species in South Africa (Linde et al. 1994b; Maseko et al. 2007; Nagel et al. 2013; Bose et al. 2017). Due to the susceptibility of *Eucalyptus fastigata* and *E. fraxinoides* to *P. cinnamomi*, commercial deployment of these cold-tolerant species was substantially reduced in South Africa (Linde et al. 1994a; Wingfield and Kemp 1994).

One of the principal cold-tolerant tree species commercially propagated in South Africa is *Eucalyptus nitens* (Jones et al. 2004). Between 2010 and 2020, several incidences of root- and collar-rot-related mortality of *E. nitens* were observed. Several surveys and long-term research projects were launched to determine the factor(s) causing this previously unknown post-planting mortality among provenances of pure *E. nitens* (Jones 2019). Data from these studies indicated that the mortality rate of *E. nitens* was highest in the first year after planting, and it was significantly higher in lower altitude sites (Jones 2019). Both *P. cinnamomi* and *P. alticola* were isolated from the roots of some symptomatic trees (Plant Diagnostic Clinic, Forestry and Agricultural Biotechnology Institute). This disease outbreak, however, did not affect trees of the most commonly planted interspecific hybrid *E. nitens* × *E. grandis* (GN).

Field and greenhouse studies were conducted to evaluate the role of *P. alticola* and *P. cinnamomi* in the mortality of purebred *E. nitens*. In our greenhouse trials, a GN hybrid was included as a negative control because this variety was rarely affected by *Phytophthora* root and collar rot. We selected these two *Phytophthora* species because both were isolated from pure *E. nitens* trees affected by post-planting mortality. Based on field observations, we hypothesized that (1) the progeny from self-fertilized *E. nitens* would be more susceptible to *P. alticola* and *P. cinnamomi* than the outcrossed provenance, and (2) the interspecific hybrid would show tolerance to the tested *Phytophthora* species. In addition, based on published data (Bose et al. 2019; Maseko 2010), we hypothesized that (3) the severity of root rot would be highest among the plants inoculated with *P. cinnamomi*.

Materials and methods

Monitoring of *E. nitens* plantations

Two *E. nitens* compartments (F15 and D38A) in Lothair, Mpumalanga Province (26° 24′ 18″ S, 30° 26′ 29″ E) were monitored to better comprehend the causes of the post-planting mortality. These two compartments were specifically chosen because the mortality rate was more than 30%. The seed lot numbers of the trees were used to identify their parents. Following that, microsatellite-based DNA fingerprinting was used to reconstruct the pedigree of 20 randomly selected trees, including 10 healthy and 10 symptomatic. The DNA fingerprinting of the sampled trees was outsourced to the Precision Tree Breeding Platform, Forest Molecular Genetics Programme, University of Pretoria (https://www.fabinet.up.ac.za/index.php/research-groups/forest-molecular-genetics).

Greenhouse pathogenicity trials

Greenhouse trials were conducted to investigate the relative susceptibility of two different *E. nitens* families and an interspecific GN hybrid to two *Phytophthora* species. The pure *E. nitens* were from a self-fertilized family (selection 104; family 37656) and an outcrossed family (selection 131; family 37254). These two families were selected based on the outcomes of the field study. Seeds for both these pure *E. nitens* families were collected from Sappi's *Eucalyptus* seed orchard located in Lebanon, KwaZulu-Natal Province. DNA fingerprinting was performed to validate their genetic background. The interspecific hybrid (PP2107) was developed by the Institute for Commercial Forestry Research (ICFR), Pietermaritzburg, KwaZulu-Natal Province, and is the most widely planted GN hybrid in South Africa.

Selection of *Phytophthora* isolates

Isolates of *P. alticola* (CMW48711) and *P. cinnamomi* (CMW48774) were retrieved from the microbial culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. Both isolates were previously used in a pathogenicity study of various *Eucalyptus* species (Bose et al. 2019) and originated from soil samples collected from a *Eucalyptus grandis* plantation located in Commondale, Mpumalanga Province (S27° 17′ 39″ E30° 53′ 56″).

Assessment of virulence

After retrieving both *Phytophthora* isolates from the culture collection, the virulence of each species was reassessed with an under-bark inoculation method using *E. nitens* as the host. Twenty-one days after inoculation the pathogens were re-isolated from infected plant tissue onto a *Phytophthora*-selective medium, NARPH (50 mg nystatin, 200 mg ampicillin, 10 mg rifampicin, 25 mg pentachloronitrobenzene, and 50 mg hymexazol per 1 L of deionized water and 15 g cornmeal agar) as described by Hüberli et al. (2000). The identity of the isolated pathogens was determined by

amplifying the complete internal transcribed spacer region using primers ITS6 and ITS4 (Cooke et al. 2000; White et al. 1990). These reisolated cultures were used for subsequent inoculation studies.

Seed germination and transplanting of seedlings

Seeds of the self-fertilized and outcrossed families of *E. nitens* were germinated under natural light in steam-sterilized polystyrene trays with autoclaved composted pine bark medium. Thereafter, the trays were incubated in covered tunnels (mean temperature 25 °C; mean relative humidity 65%) at the Sappi Shaw Research Centre, Howick, KwaZulu-Natal Province. All trays were irrigated once every day. The plants were fertilized with Osmocote® controlled release fertilizer pellets that were sprinkled on the surface of the potting medium.

Plants of the interspecific GN hybrid were propagated from shoot cuttings. Selected shoot segments were allowed to root in a sterile mixture of coir and perlite (9:1) in an enclosed rooting tunnel where the relative humidity was set between 60 and 70% and the mean temperature ranged between 18 and 24 °C with underfloor heating. All the cuttings were irrigated with overhead misting at regular intervals throughout the day. Before initiation of the trial, the rooted cuttings were hardened in a separate covered tunnel for a period of 5 to 6 weeks under ambient temperature.

For each pure family, one *E. nitens* seedling measuring 5–8 cm in height was transplanted into sixty 0.5 L pots containing sterile sand. For the interspecific GN hybrid, a single-rooted cutting was transferred into a pot with sterile sand. Two plastic tubes measuring 10 × 1.5 cm were inserted into each pot such that each can receive approximately 2.5 g of *Phytophthora* inoculum.

Irrespective of family, all pots had one seedling of approximately the same height. All the plants were allowed to grow in a covered tunnel with temperatures ranging between 23 and 28 °C and relative humidity of 50–60%.

Preparation of inoculum

For both *Phytophthora* species, the inocula were prepared in vermiculite as described by Bose et al. (2019). Each flask contained 600 mL of sterile growth medium (600 mL coarse vermiculite from Culterra, South Africa; 10 g millet seeds; 170 mL of 10% V8 juice, Campbell Soup Company, USA; 150 mL of deionized water; and 2 g calcium carbonate, Sigma-Aldrich, USA). The flasks were inoculated with *Phytophthora* cultures grown on 10% V8-agar (100 mL clarified V8 juice; 900 mL deionized water; 15 g Difco Agar, Becton, Dickinson and Company, Sparks, USA) for 8 days in darkness. The inoculated Erlenmeyer flasks were incubated in darkness at 20 °C for 6 weeks. During this time, the flasks were gently rotated at regular intervals for even distribution of the mycelia.

Inoculation of plants

Three months after transplanting into pots, seedlings of the *E. nitens families* and clonal variety of the GN hybrid were inoculated with the *Phytophthora* species. For this, both plastic tubes were removed from the pot, and approximately 2.5 g of the *Phytophthora* inoculum was dispensed into each cavity. For negative controls, the cavity was filled with an equal amount of sterile vermiculite medium. The cavities were then sealed with sterile sand.

Experimental design

The first replicate of the trial was conducted in 2019. Seedlings and cuttings were transplanted in June, inoculated in September, and harvested in December. In 2020, the second replicate of the trial was completed. The seedlings and cuttings were transplanted in April, inoculated in July, and harvested in October. Regardless of the trials, the first week of each month was dedicated to transplanting, inoculating, and harvesting.

In both repeats of the trial, there were 20 biological replicates per treatment (control, *P. alticola*, and *P. cinnamomi*) for each host genotype (self-fertilized, outcrossed, and interspecific hybrid). After inoculation, pots were arranged randomly in plastic trays on a bench in the covered tunnel. The negative controls were arranged in separate trays from trees that received *Phytophthora* inoculum. The arrangement of the pots within trays was changed regularly to reduce any microclimate effect. The plants were flooded three times: immediately after inoculation, then after 14- and 28-day post-inoculation, by filling the trays with water. All the plants were irrigated once every day until they were harvested.

Measurement of symptoms

Seedlings and cuttings were carefully harvested 3 months after inoculation. This was done by rinsing off all the growth media from the roots, under running tap water. The severity of root rot was evaluated on a scale of 0–4 (0 = no visible root damage, 1 = ~ 20% roots with lesions, 2 = > 20% roots with lesions, 3 = > 50% roots with lesions, 4 = dead). During harvesting, the fresh shoot and root masses and root volumes were measured using a volume displacement method (Harrington et al. 1994). For the dry weights of shoots and roots, plants were dried at room temperature (21–24 °C) for a period of 30 days and then weighed using an analytical scale.

Re-isolation and identification of *Phytophthora* species

To fulfil Koch's Postulates, root sections with lesions from all plants were plated onto the *Phytophthora* selective medium NARPH. Mycelia emerging from plated root tissues were subcultured onto half-strength potato dextrose agar medium (19.5 g PDA powder, Merck, South Africa; 7 g Difco agar; 1 L of deionized water). Molecular identification of a selection of isolates was done by amplifying the complete internal transcribed spacer (ITS) region of the rDNA using the primer pair ITS6 and ITS4. The sequences were identified using the BLAST algorithm (Altschul et al. 1990) available through the NCBI GenBank.

Statistical analyses of datasets

All datasets were statistically analyzed using R v4.2.1 (R Core Team 2021). A Wilcoxon signed rank test with continuity correction was used to determine whether the genetic background of pure *E. nitens* affected post-planting mortality. Growth parameter data from the two replicate trials could not be transformed towards normality and was analyzed using the Kruskal–Wallis chi-squared test. Differences between treatments were consistent between the two trials. For analysis of the combined dataset, data from trial 2 was normalized towards the mean values of trial 1 using a factor of 5 for fresh shoot weight, dry shoot weight, and root volume, and a factor of 9 for fresh and dry root weight. The normalized dataset was analyzed using the Kruskal–Wallis chi-squared test, and differences between individual treatments were determined using Dunn's test. Disease severity data were analyzed using the Kruskal–Wallis chi-squared test, and differences between individual treatments were determined using model simplification.

Results

Monitoring of *E. nitens* in plantations

Significant differences were found in the number of *E. nitens* trees affected by the post-planting root rot disease in the two compartments (F15 and D38A) monitored for the disease. The results of DNA fingerprinting revealed a significant relationship between the survival of *E. nitens* in the field and their genetic background. Post-planting mortality affected self-fertilized families more than outcrossed families ($p = 0.001904$; Fig. 1).

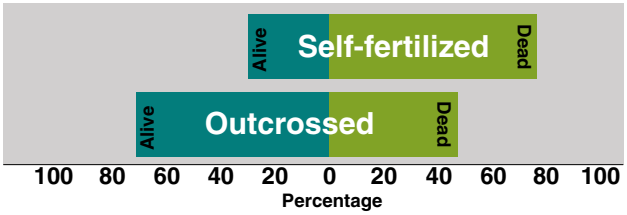

Fig. 1 *Eucalyptus nitens* survival after planting in the field is influenced by their genetic background. DNA-fingerprinting data revealed that self-fertilized *E. nitens* provenances were more susceptible to post-planting mortality compared to outcrossed provenances

Greenhouse pathogenicity trials

DNA fingerprinting data of purebred *E. nitens* showed that selection 131 was completely outcrossed, whereas the self-pollination percentage in selection 104 ranged from 40 to 100%.

In both replicates of the greenhouse trial, compared to the GN hybrid, the severity of root rot was higher for the purebred *E. nitens* seedlings, with no statistical differences between the self and outcrossed seedlings ($p = 2.637e-7$ (trial 1); $p = 1.198e-2$ (trial 2); Fig. 2A, B). The severity of root rot was the highest among plants infected with *P. cinnamomi* compared to *P. alticola* ($p \leq 2.2e-16$ (trial 1); $p \leq 2.2e-16$ (trial 2); Fig. 2A, B).

In both outcrossed and self-fertilized *E. nitens* seedlings, compared to the controls, both pathogens significantly reduced fresh and dry shoot weights, fresh root weights, and root volumes (Table 1; Fig. 3). However, the dry root weight of outcrossed and self-fertilized seedlings produced variable results with only one of the treatments resulting in significant differences for each (Table 1; Fig. 3). For the interspecific hybrid, only dry root weight and root volumes were significantly reduced compared to the controls. Although fresh and dry shoot weights and fresh root weights were reduced, the differences with the controls were not significant (Table 1; Fig. 3). While *P. cinnamomi*-infected GN clones had decreased dry root weight and volume, clones that were infected with *P. alticola* had significantly reduced dry shoot and root weights, and root volume (Table 1; Fig. 3). In all instances *E. nitens* plants were more severely affected by the pathogens than the GN hybrid.

When the parameters of pathogenicity were compared between the three genotypes, they were significantly different across all the treatments (Table S1). However, when these same parameters were compared between outcrossed and self-fertilized *E. nitens*, differences were negligible (Table S1). This showed that disease severity was nearly uniform between the pure families of *E. nitens*.

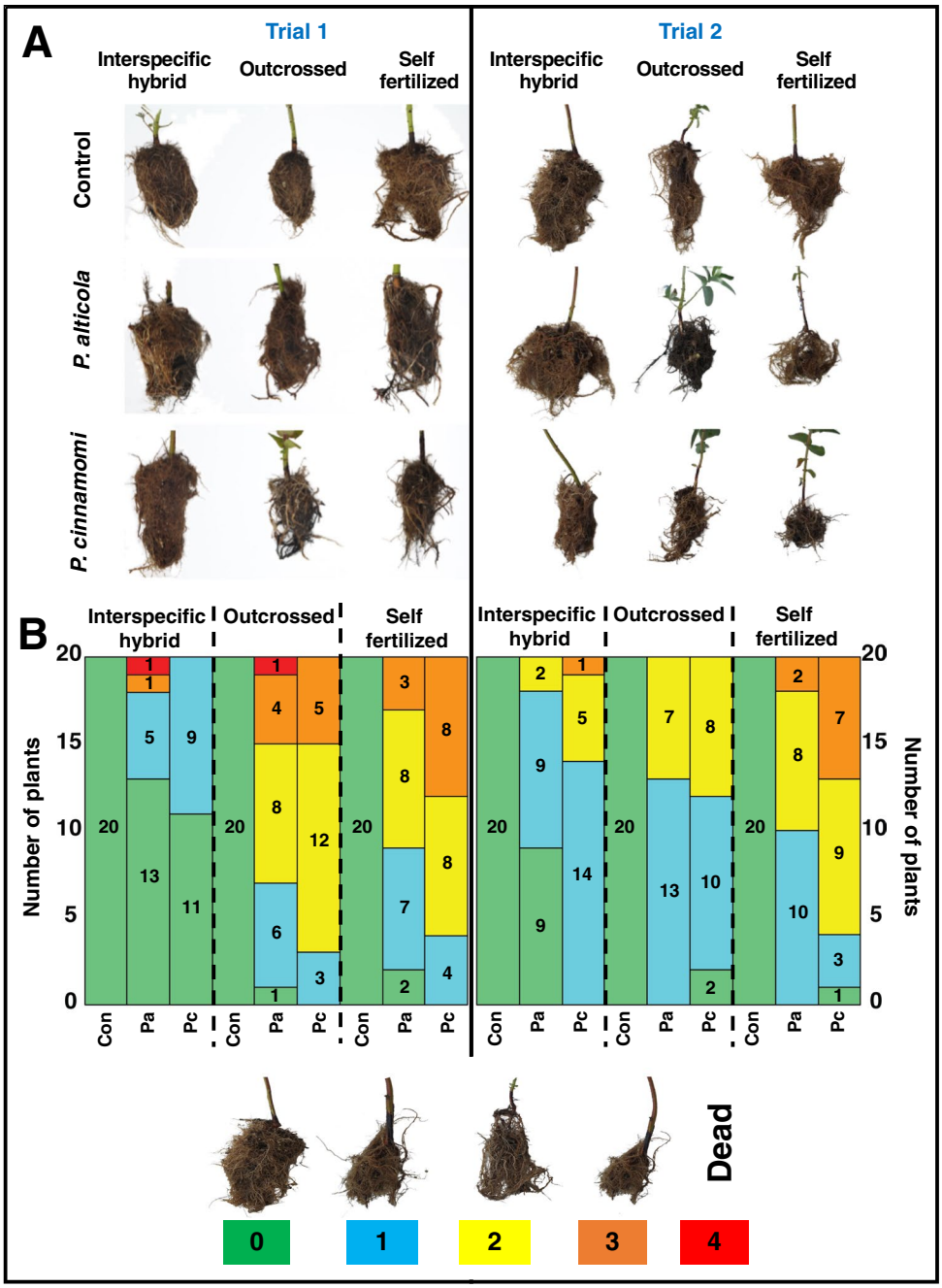

Fig. 2 (**A**) The difference in root morphology of self-fertilized and outcrossed provenances of *E. nitens* and clonal progeny from an *E. grandis* × *E. nitens* hybrid infected with *P. alticola* or *P. cinnamomi* from two replicates of the trial. (**B**) Bar plots were constructed using data from root rot severity assessments on a scale of 0–4 (0 = no damage, 4 = dead)

Re-isolation and identification of *Phytophthora* isolates

In both trials, the greatest number of *Phytophthora* isolates were recovered from self *E. nitens* seedlings followed by the outcrossed family and then the GN hybrid (Fig. 4). Based on consolidated results from both trials, *P. cinnamomi* was most consistently recovered from the inoculated plants (Fig. 4).

Discussion

Pathogenicity trials were conducted to assess the susceptibility of a self-fertilized and outcrossed provenance of *E. nitens* and an interspecific GN hybrid to infection by *P. alticola* and *P. cinnamomi*. Statistical analyses of the data emerging from these pathogenicity trials showed that both self-fertilized and outcrossed *E. nitens* seedlings were equally susceptible to both pathogens. In contrast, the interspecific GN hybrid

Table 1 Medians and statistical significance (*p* value) of quantitative measurements for disease severity in inoculated and control genotypes of *Eucalyptus nitens* seedlings and an interspecific hybrid (GN). The data was analyzed using the Kruskal–Wallis chi-squared test, and differences between different treatments were determined using Dunn's test. Letters next to the numbers denote statistical differences ($n = 20$)

Families	Parameters of pathogenicity	Control median	*P. alticola* median	*P. cinnamomi* median	*p* values
Interspecific hybrid	Fresh shoot weight (g)	36.85[a]	33.00[a]	23.66[a]	1.18e − 1
	Dry shoot weight (g)	5.513[a]	3.813[b]	4.548[a]	2.0e − 3
	Fresh root weight (g)	45.675[a]	34.200[b]	35.650[b]	2.0e − 5
	Dry root weight (g)	15.975[a]	10.960[b]	11.690[b]	4.0e − 4
	Root volume (mL)	70.0[a]	45.0[b]	45.0[b]	9.32e − 10
Outcrossed	Fresh shoot weight (g)	19.915[a]	17.500[b]	16.500[b]	1.78e − 2
	Dry shoot weight (g)	4.269[a]	2.108[b]	2.453[b]	1.11e − 8
	Fresh root weight (g)	20.70[a]	16.98[b]	17.74[b]	9.28e − 3
	Dry root weight (g)	8.160[a]	6.660[b]	7.065[ab]	1.3e − 2
	Root volume (mL)	40.0[a]	25.0[b]	25.0[b]	9.50e − 9
Self-fertilized	Fresh shoot weight (g)	17.700[ab]	18.515[a]	16.500[b]	3.19e − 2
	Dry shoot weight (g)	3.908[a]	2.260[b]	2.077[b]	1.97e − 6
	Fresh root weight (g)	23.400[a]	19.225[b]	16.230[c]	3.50e − 6
	Dry root weight (g)	9.70[a]	7.47[ab]	5.76[b]	3.89e − 8
	Root volume (mL)	37.25[a]	25.20[b]	22.40[b]	7.03e − 9

showed higher levels of tolerance to infection by both of the tested *Phytophthora* species.

Under plantation conditions, we observed that the self-fertilized *E. nitens* family was more susceptible to post-planting mortality compared to the outcrossed selection. Self-fertilization is expected to increase homozygosity, which can result in inbreeding depression (Cheptou 2018; Pupin et al. 2019). Hence, it was not surprising that the self-fertilized *E. nitens* seedlings were more severely affected by post-planting mortality under field conditions. In contrast, outcrossing promotes the emergence of new traits. Consequently, tree breeders frequently utilize outcrossing for plant improvement (Chen et al. 2021). Yet, in our greenhouse inoculation trials, there were no statistically significant differences in susceptibility to inoculation with *Phytophthora* species between self and outcrossed seedlings. This could be due to a higher or more consistent inoculum load in the greenhouse trials compared to the plantation situation.

The interspecific GN hybrid displayed tolerance towards infection by *P. alticola* and *P. cinnamomi*. Among eucalypts, interspecific hybridization is promoted by their remarkably conserved genomic structure (Butler et al. 2017). These hybrids with novel gene combinations can include desirable traits from both parents, including rapid growth and disease resistance (Teixeira et al. 2009; Guimarães et al. 2010; Bradshaw and Grattapaglia 1994). It remains unknown whether interspecific hybridization among *Eucalyptus* species can confer resistance to *Phytophthora* diseases. However, in other trees, QTL-mediated resistance to *P. cinnamomi* has been achieved by backcrossing American and Chinese chestnuts (*Castanea* species), where the latter was resistant to the pathogen (Zhebentyayeva et al. 2019). This might be partially true for GN because, in a previous trial, *E. grandis* showed tolerance towards *P. alticola* but not to *P. cinnamomi* (Bose et al. 2019; Maseko 2010).

Tolerance of the GN hybrid to infection by the two tested *Phytophthora* species in the study could also be due to unique phenotypic characteristics in the hybrid and not in the parent plants. For example, this tolerance could stem from the rapid growth traits inherited from *E. grandis*, allowing the hybrid variety to endure the impact of the root rot by producing new lateral and fine roots to offset the effect of the pathogens. A similar situation was seen in an earlier study, where *P. multivora*-infected *E. grandis* also displayed a defense response by producing a considerably larger root mass and root-to-shoot ratio than the control (Bose et al. 2019).

In this trial, the severity of root rot was greater in plants infected with *P. cinnamomi* than in those infected with *P. alticola*. Both of these *Phytophthora* species, however, are known to induce root rot in *E. nitens* (Maseko 2010). The observed differences might be attributed to the *E. nitens* provenances used in this study, which may have been less susceptible to *P. alticola* than to *P. cinnamomi*. The lower level of susceptibility might also account for the fact that we re-isolated *P. alticola* from the roots of the GN hybrid

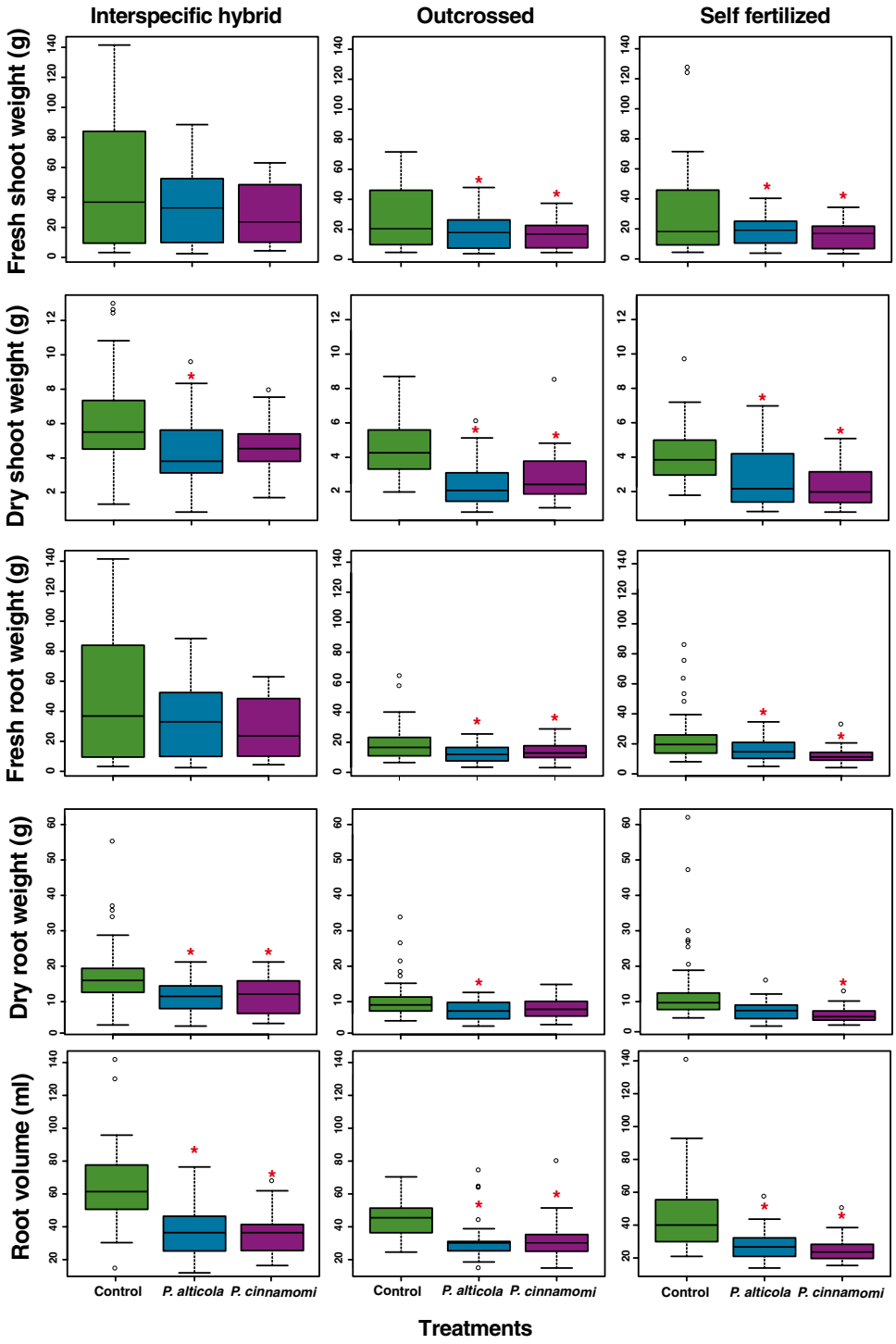

Fig. 3 Comparison of the shoot and root weights and root volumes between provenances of self-fertilized and outcrossed *E. nitens* and an interspecific hybrid (*E. grandis* × *E. nitens*) inoculated with *Phytophthora alticola* or with *P. cinnamomi*. Controls were mock-inoculated with sterile vermiculite inoculum. Box plots were constructed using combined, normalized datasets from two trials. Asterisks in red indicate statistical significance ($p \leq 0.05$; Kruskal–Wallis chi-squared test; $n = 20$)

that did not exhibit root rot symptoms. This could be attributed to the fact that some *Phytophthora* species can induce asymptomatic infections (Migliorini et al. 2019; Belhaj et al. 2018). Similarly, in our previous trials involving *E. grandis* and *A. mearnsii*, *P. alticola* was also re-isolated from the asymptomatic roots of both hosts (Bose et al. 2019). This suggests that isolating *P. alticola* from the roots of trees and soils does not necessarily equate to its role in tree decline.

Phytophthora root rot can be a serious constraint to *Eucalyptus* propagation in South Africa. Results of this study have revealed opportunities to reduce this problem through the utilization of *Eucalyptus* hybrids such as those between *E. grandis* and *E. nitens*. It is also relevant that we observed

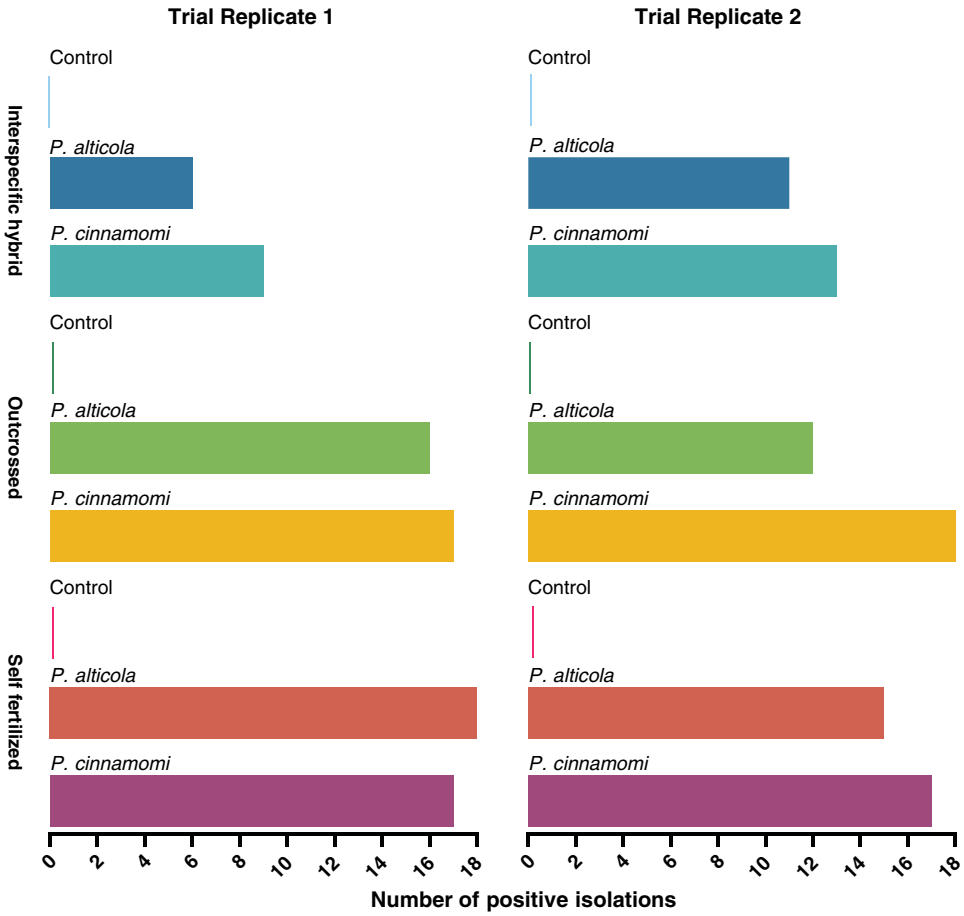

Fig. 4 Bar plots illustrating the number of positive isolations of *Phytophthora alticola* and *P. cinnamomi* from control and infected *Eucalyptus* plants. This trial was conducted using progeny from self-fertilized and outcrossed *E. nitens* and an interspecific hybrid (*E. grandis* × *E. nitens*)

serious root rot among the pure families of *E. nitens* in our trials, but no mortality. This is likely due to our trials being conducted in greenhouses where the plants were regularly irrigated and fertilized. In the plantation situation, however, loss in root structure combined with drought substantially increases mortality. This would also be consistent with the fact that the most severe post-planting mortality of *E. nitens* occurred in 2016 when severe drought was experienced, similar to the decline of oak and beech in Italy (Colangelo et al. 2018; Seddaiu et al. 2020) and Central Europe (Jung 2009), respectively, as well as other parts of the Mediterranean region (Peñuelas and Sardans 2021) where *Phytophthora* is causing severe losses during extreme climatic events.

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Author contribution W. Jones conducted the field trial and collected data from the field trial. The greenhouse trial was conducted by T. Bose, W. Jones, and J. Roux. Statistical analyses of the data were done by A. Hammerbacher. The first draft of the manuscript was written by T. Bose, and all authors commented on previous versions of the manuscript. The funding was procured by M. J. Wingfield, B. Slippers, and J. Roux. This study was supervised by M. J. Wingfield. All authors read and approved the final manuscript.

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Data availability Not applicable.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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