

***In vitro* assays of *Phytophthora agathidicida* on kauri leaves suggest variability in pathogen virulence and host response**

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Abstract *Phytophthora agathidicida* is an aggressive soil-borne oomycete pathogen that kills New Zealand kauri trees (*Agathis australis*). When artificially inoculated, *P. agathidicida* causes lesions on leaves as well as roots, providing a non-invasive method for virulence screening. However, little is known about the extents to which the pathogen varies in virulence and kauri trees vary in disease susceptibility. Three isolates of *P. agathidicida* grown in culture were inoculated onto detached leaves from six kauri trees. Visible disease lesions were measured and the extent of asymptomatic leaf colonisation determined by culturing. All six trees were susceptible to *P. agathidicida*, but one showed higher susceptibility than the others. The pathogen also showed variability in virulence among isolates. Asymptomatic colonisation of leaf tissue was also found, suggesting a latent or biotrophic phase for the pathogen. Although further work is needed, the variability of both pathogen virulence and host susceptibility have important implications for management of kauri dieback. Furthermore, asymptomatic colonisation of kauri tissues suggests that *P. agathidicida* could be present outside of regions with visible disease symptoms.

Keywords *Phytophthora*, kauri, asymptomatic infection, kauri dieback.

INTRODUCTION

The iconic New Zealand kauri is under serious threat from the soil-borne kauri dieback pathogen, *Phytophthora agathidicida* (Weir et al. 2015). This destructive pathogen can kill all life stages of kauri and has been identified throughout the upper North Island of New Zealand, where kauri naturally occur (Waipara et al. 2013). Symptoms of infection include aggressive root and collar rot, canopy thinning, yellowing of foliage, bleeding resins at the base of the trunk and tree mortality (Beever et al. 2009).

A screening assay that can rapidly identify resistant kauri trees would provide an effective management tool for this disease. *Phytophthora*

agathidicida naturally infects roots but detached leaves may be a good alternative source of material to test that is non-destructive and could accelerate the screening process. Leaves are also commonly used as bait material for these pathogens, providing a scalable and rapid means of initial compatibility testing (Tahi et al. 2006). This approach has been used in screening with other species of *Phytophthora* (Hüberli et al. 2008; Ireland et al. 2012) and successfully integrated in breeding and screening for *Phytophthora* spp. resistance (Tahi et al. 2006).

Kauri live for hundreds of years with the oldest living specimens estimated to be more than 1500 years old. With direct inoculation of these iconic

trees not being an option, a non-invasive means of determining the susceptibility of established trees would be advantageous. Foliar inoculations with two *P. agathidicida* isolates were previously conducted on glasshouse-grown two-year-old kauri (Horner & Hough 2014) but have yet to be conducted on older forest-grown kauri that may differ in disease tolerance. The current study focused on whether three different isolates of *P. agathidicida* vary in virulence and whether kauri trees aged 7–8 years vary in disease susceptibility. Using a wounded detached foliar assay, we also investigated whether this pathogen is present in asymptomatic tissue in advance of visible disease lesions.

MATERIALS AND METHODS

Phytophthora agathidicida isolates and kauri trees

The *P. agathidicida* isolates used were from the Scion collection and had been baited from kauri rhizosphere soil in the Coromandel (NZFS 3813 and NZFS 3814), or the Waitākere ranges (NZFS 3118). They were grown in carrot agar (CA): 200 g minced frozen carrots, mixed with 15 g agar (BBLTM Grade A, BD Life Sciences) per litre. Six kauri saplings (T1–T6), 7–8 years in age, from the Scion Long Mile Reserve (Long Mile Rd., Rotorua) were used. These were second-generation saplings propagated in 2007/2008 from seed from Holt's Forest (Hawke's Bay) kauri that were originally planted in the 1980s from seed from the Waipoua grafted nursery. All lines were open pollinated seed from bulk collections, with details of the maternal parents not recorded. The trees were selected to represent a diverse range of foliar and structural phenotypes.

Leaf assays and statistical analysis

Forty leaves were collected from each kauri tree and placed abaxial side up on wet paper towels in a plastic tray. In a randomised split-plot design, ten leaves were inoculated with each *P. agathidicida* isolate by placing a colonised CA plug over a small surface needle wound at the base of the leaf, or with an uncolonised CA plug as a control. The trays were sealed with plastic film and incubated for 6 days in a controlled atmosphere at 20–23°C and

with 12 h light/12 h dark. The distance of lesion spread from the inoculation point was measured. To determine the extent of pathogen growth beyond the disease lesion, four 5-mm segments of leaf (see Fig. 1) were plated onto CRNH medium (Jeffers & Martin 1986) and incubated for 6 days at 20°C. Colony growth was checked every 2 days and the identity of *P. agathidicida* colonies confirmed microscopically (Weir et al. 2015). The data shown in Table 1 were log-transformed to satisfy assumptions of normal distribution and homogeneity of variance for ANOVA, followed by Tukey's multiple comparisons test, using GraphPad Prism 7 software.

RESULTS

Mean disease lesion lengths (Table 1) were calculated for each tree and isolate to determine differences in pathogen virulence and host response to infection by *P. agathidicida*. None of the uninoculated controls showed lesions.

A two-way ANOVA showed significant differences between trees ($P < 0.0001$) and between pathogen isolates ($P < 0.0001$) but no significant interaction. Tukey's multiple comparisons test showed Tree T4 had significantly larger lesions than all other trees ($P < 0.0002$ in all pairwise comparisons) and significant differences between pathogens (3118 significantly different to both 3813 and 3814 with $P < 0.0001$).

Phytophthora agathidicida was recovered from asymptomatic leaf tissue beyond the visible disease lesion (Fig. 1). On average, the pathogen was found 7.42 mm ahead of the visible lesion. Given the 6-day observation period and lesion growth rate of 3.03 mm/day, this indicates a direct latency between infection and visible lesion development of 2.5 days, but this varied with tree and pathogen isolate. The mean extent of growth beyond the lesion margin was significantly different among trees ($P < 0.0001$), with T4 hosting more asymptomatic growth than the others. However, there were only marginal differences among pathogen isolates ($P = 0.038$); 3118 had shorter mean asymptomatic lesion length (6.3 ± 2.0 mm) than 3813 (8.0 ± 2.4 mm) and 3814 (8.0 ± 1.7 mm).

Table 1 Mean visible disease lesion length by isolate and tree for leaves collected from six kauri trees (T1–T6) inoculated with three *P. agathidicida* isolates (NZFS).

Tree (T)	Disease lesion length (mm) Mean \pm SD (n=10)			
	NZFS 3813	NZFS 3814	NZFS 3118	Tree Means ¹
T1	19.5 \pm 6.9	14.9 \pm 5.0	10.7 \pm 3.4	15.0 \pm 4.4 ^a
T2	19.8 \pm 3.7	18.3 \pm 2.9	9.9 \pm 3.0	16.0 \pm 5.3 ^a
T3	22.5 \pm 3.0	17.3 \pm 6.5	10.1 \pm 2.9	16.6 \pm 6.3 ^a
T4	33.2 \pm 4.1	28.4 \pm 4.0	24.5 \pm 10.5	28.7 \pm 4.3 ^b
T5	20.6 \pm 3.5	19.1 \pm 2.1	10.4 \pm 4.2	16.7 \pm 5.5 ^a
T6	20.3 \pm 3.4	18.8 \pm 8.2	9.5 \pm 4.3	16.1 \pm 5.9 ^a
Path. Means ¹	22.6 \pm 5.3 ^a	19.4 \pm 4.6 ^a	12.5 \pm 5.9 ^b	

¹Different letters between means within row or column indicate significant differences.

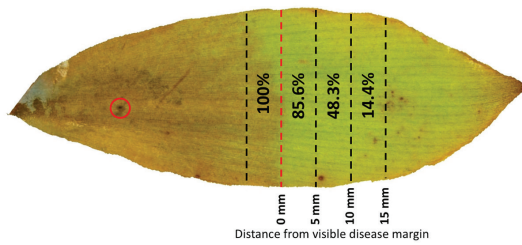


Figure 1 Recovery of *Phytophthora agathidicida* from asymptomatic leaf tissue. The inoculation point (red circle) and visible disease front (red line) are indicated. Percentages of all inoculated leaf samples (n=180) are shown from which *P. agathidicida* was recovered by plating leaf tissue segments from up to 5, 10 and 15 mm ahead of the lesion front.

DISCUSSION

This study provided evidence for differences in pathogen virulence and host response to infection by *P. agathidicida*. Asymptomatic growth ahead of the lesion front further suggests a biotrophic or latent stage in leaf tissue which is common for *Phytophthora* pathogens on compatible hosts. This phase provides an opportunity for the pathogen to develop persistent structures ahead of necrotrophic responses by the host (Crone 2012). It is likely that a similar latent period would be observed during root infection, although this was not investigated here. Our work does, however,

highlight the importance of considering latency when assessing host susceptibility. Complete resistance has not yet been observed in kauri inoculated with *P. agathidicida* so the ability for the host to mount a rapid response that minimises the extent of unimpeded biotrophic infection may be a key factor in host resistance.

This study confirmed earlier findings (Horner & Hough 2014) that leaves can be used in screening kauri for susceptibility to kauri dieback, although those authors did not report differences in lesion advance between the two isolates they tested. However, variation among isolates was observed in the current study, which suggests possible differences in virulence. Variation was also observed among trees, which may relate to the susceptibility of the tree as a whole. However, because this was done as a screening assay for a disease that impacts the root and collar of the tree, it can only be taken as an initial screen for true susceptibility. Future studies should apply this approach across replicate clonal trees and/or families of kauri with parallel comparisons to root-inoculation studies, to determine if results from leaf and root inoculations are comparable. A kauri-leaf assay could also be used to test the efficacy of products such as elicitors, which could be applied to the trees and then leaves bio-assayed to determine if host defences were induced that help to protect against infection. Our work also suggests that detached kauri leaves can be infected

by *P. agathidicida* and may present an alternate source of host material in which the pathogen can persist in the forest.

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