

Visualizing the early infection of *Agathis australis* by *Phytophthora agathidicida*, using microscopy and fluorescent *in situ* hybridization

By S. E. Bellgard^{1,4}, M. Padamsee¹, C. M. Probst¹, T. Lebel² and S. E. Williams³

¹Landcare Research Private Bag 92170, Auckland 1142, New Zealand; ²Melbourne Botanic Gardens, South Yarra, VIC, Australia; ³University of Wyoming, Laramie, WY, USA; ⁴E-mail: bellgards@landcareresearch.co.nz (for correspondence)

Summary

Phytophthora agathidicida (PTA) causes a root rot and collar rot of New Zealand kauri (*Agathis australis*). This study developed techniques to visualize early infection of kauri by PTA in deliberately inoculated seedlings. Conventional light microscopy was carried out on cleared and stained roots using trypan blue to observe PTA structures. Additionally, scanning electron microscopy (SEM) was used to study the PTA root structures at a higher resolution. A fluorescent *in situ* hybridization assay (FISH) was developed using a PTA-specific probe to label PTA structures *in planta*. Infection progression in roots of 2-year-old kauri inoculated with PTA at 5, 10, 16 and 20 days post-inoculation (d.p.i.) was compared using these three approaches. Light microscopy identified no *Phytophthora*-like structures in the control treatments. In PTA-inoculated plants, lignitubers were produced 5 d.p.i. in cortical cells. Infection was localized after 5 days, but as the infection progressed (up to 20 d.p.i.), the 'degree' of root infection increased, as did the number of replicates in which structures were observed. SEM provided higher resolution images; again, no PTA structures were observed in the negative control material examined. The slide-based FISH-specificity assay successfully hybridized with PTA hyphae. Fluorescence was observed using 330–380 nm excitation and an emission filter at 420 nm (DAPI), with PTA nuclei fluorescing a bright greenish-yellow. Cross-reactivity was not observed when the assay was applied to six other non-target *Phytophthora* species. Successful hybridization reactions occurred between the primer and PTA structures *in planta*. Applying this FISH assay has allowed clear differentiation of the intracellular and intercellular structures of PTA. The technique can be applied to longer term studies or analysis of *ex situ* inoculation studies aiming to elucidate differential host-responses to the pathogen. Additionally, the technique could be applied to study the interactions with other fungal endophytes (e.g. mycorrhizal fungi), which could be assessed for biocontrol potential as part of the integrated management of the disease.

1 Introduction

Phytophthora agathidicida, the organism formerly known as *Phytophthora* 'taxon Agathis' 'PTA', is the causal agent of a root rot and collar rot of kauri (*Agathis australis* (D. Don) Loudon) in the northern forests of New Zealand (Weir et al. 2015). The distribution of the disease has now been confirmed throughout the geographic range of the remnant forest (Waipara et al. 2013). The pathogen was first mis-identified as *P. heveae*, impacting upon kauri in 1971 on Great Barrier Island (Gadgil 1974). Beever et al. (2009) were the first to make the connection between the disorder observed on Great Barrier Island in the 1970s and the 'dieback' observed on the mainland in 2006 and that a novel *Phytophthora* pathogen was involved in the disease. The disorder has been demonstrated to be transferred in soil (Beever et al. 2010) and also via root pieces colonized by the pathogen (Bellgard et al. 2013). Early infection is facilitated although the fine roots, with the disease progressing along larger woody roots, parasitizing the cambium but rarely invading the xylem. When it reaches the collar, it causes a canker, which is characterized by the production of a resinous exudate associated with parasitism of the cork cambium (Beever et al. 2010). The lesion can extend around the trunk, effectively girdling the tree. A crown decline follows, as a result of the vascular dysfunction, but this takes years to develop, with symptoms including chlorosis, and crown decline (Bellgard et al. 2013).

Jung et al. (2013) consider that soilborne *Phytophthora* species have developed a specific life history that is characterized by relatively short, active phases of spread via zoospores during periods of wet soil conditions and subsequent primary invasion of healthy tissue. The time course between the primary infection and onset of disease symptoms is not known in the PTA-kauri pathosystem. It is hypothesized that primary infection of healthy kauri roots occurs during periods of favourable environmental conditions, but the nature of the early infection process is unknown as well as the morphology of the organism as it infects through the root cortex. Widmer et al. (1998) utilized light and transmission electron microscopy to study the early infection of the fibrous roots of disease tolerant and susceptible citrus hosts by *P. nicotianae* and *P. palmivora*. They were able to describe differences in hyphal colonization in the cortex of resistant cultivars. This study builds on this earlier work, through the development of a species-specific, fluorescent assay that will preferentially bind to our target pathogen. In addition, the survival strategies and long-term resting structures produced by PTA over time after primary infection are not well understood. This study aimed to compare and contrast three microscopic visualization techniques using the homothallic *Phytophthora agathidicida* and New Zealand kauri as the test system. The specific aims were to:

1. provide a visual description of the infection of 2-year-old kauri plants over a time course of 20 days;
2. develop and test the specificity of a PTA fluorescent *in situ* hybridization (FISH) assay;
3. describe the infection process using microscopy and the FISH assay.

2 Materials and methods

2.1 Inoculum production and procedure

Inoculation experiments were conducted in a quarantine (Physical Containment [PC], Level 1), naturally lit glasshouse at Landcare Research, St Johns, Auckland. All research at the PC-1 laboratories were carried out under a CTO permit (Biosecurity Act 1993) approving propagation and communication of PTA. PTA inoculum was prepared by growing isolate ICMP 18403 (provided by the International Collection of Microorganisms from Plants, [ICMP]) for 4–6 weeks at 20°C on sterilized millet seeds thoroughly moistened with V8-juice broth (Vettraino et al. 2001; Jeffers 2006). The inoculum was repeatedly rinsed (three times) with sterile reverse osmosis (RO) water to remove unassimilated nutrients before being added to sterile potting mix at a rate of 25 ml per litre of the potting mix. Kauri seed was provided by Dr B. Burns (University of Auckland) from a residence in the West Auckland suburb of Titirangi. The seed was sourced from an open-pollinated tree. Seeds were germinated and grown in sterile potting mix for 2 years in the naturally lit PC-1 glasshouse. The temperature in the glasshouse over the 3-week period of the experiment ranged between 18° and 24°C. Mean relative humidity was 70–80%.

The same 2-year-old kauri seedlings were transplanted into the inoculated potting mix and then flooded to induce sporulation of PTA (Vettraino et al. 2001). There were 20 replicate black, polythene, planter bags (size 1, 600 ml) treated with PTA. Another set of four bags were established as a control using sterilized millet seeds thoroughly moistened with V8-juice broth and incorporated into sterile potting mix at a rate of 25 ml per litre. Bags were arranged in a randomized block design, with PTA-treated plants segregated from negative controls. Plants were watered to field capacity with tap water every other day. The experiment ran from 1 to 20 December 2012. All liquid run off from pot watering was captured and sterilized in an autoclave prior to disposal.

2.2 Progressive harvesting and root segregation

Five, 10, 16 and 20 days post-inoculation (d.p.i.), four randomly selected plants were extracted from the potting mix. The used potting mix was disposed of into a quarantine waste bin, which was collected by a certified biological hazard waste disposal company. The shoot height and mass and root length and mass were recorded, and any signs of disease – root necrosis, shoot chlorosis, shoot desiccation, leaf loss/necrosis – were assessed qualitatively (Vettraino et al. 2001). At each of the sampling dates, and at the end of the 20 days, root and collar pieces were surface disinfested in 70% ethanol (EtOH) for 30 seconds and then rinsed in sterile RO water. The root pieces were blotted dry on clean paper towels and plated to *Phytophthora*-selective agar (Jeffers 2006). The plates were wrapped in foil and incubated at 18°C. Characteristic oospores of PTA were observed in 7 days, and representative cultures were subcultured to fresh Potato Dextrose Agar (PDA) plates for DNA analysis and ITS-sequence confirmation of PTA.

Following on from this, the entire root system was fixed in FAA (ethyl alcohol 50 ml, glacial acetic acid 5 ml, formaldehyde (37–40%) 10 ml, RO water 35 ml; Talbot and White 2013). A similar recovery exercise was carried out on the control kauri plants. The individual kauri roots from each sample were carefully segregated into four root classes: root collar, primary roots, secondary roots and tertiary roots. Root metrics were estimated prior to partitioning of the root systems. For a given kauri plant, replicate subsamples of the root system were distributed to four different groups of cryotubes for light microscopy, SEM, FISH analysis and a set kept for future RT-PCR analysis. Each of the tube had been supplied with fresh FAA.

2.3 Light and SE microscopy

Clearing of root cytoplasm to remove dark pigments followed the method described in Vierheilig et al. (2005), with the following modifications. Roots were cleared with 10% potassium hydroxide by heating at 90°C for 15–45 min in a water bath. Roots were washed in tap water and treated with 30% hydrogen peroxide for a maximum of 1 h. The peroxide was removed, and the roots rinsed in tap water to remove peroxide. Roots were acidified in 0.1 N HCl. The acid was poured off, but the roots were not rinsed. Samples were stained in 0.5% trypan blue (in lactic acid–glycerol–water) for 20 min at 90°C. Samples were de-stained with fresh lactic acid glycerol solution. Roots were mounted in clear lactoglycerol (Dickson and Smith 1998). Roots were viewed under a Nikon ECLIPSE 80i compound light microscope (Nikon Corporation, Tokyo, Japan). Photomicrographs were obtained using a Nikon DS-Fi1 camera. Images were processed using NIS-Elements Basic Research (version 2.35).

The SEM preparation followed the protocol of Brundrett et al. (1996) with the following amendments. The dehydration process was commenced by placing the samples in 50% EtOH ensuring the samples were covered. The EtOH was removed and followed with 70% EtOH for 24–48 h. This reagent was pipetted off and followed with 90% EtOH for 24 h. This was finally pipetted off and followed with 95% EtOH for 24 h. The root pieces were carefully removed and placed onto a clean Petri dish and air-dried for 30–60 min. Once dried, specimens were kept in a desiccator supplied with fresh desiccant. SEM stubs were prepared by writing a unique stub number on the underside of each stub, which was recorded in a spreadsheet with the sample. Gold sputter coating was undertaken in a vacuum chamber in the presence of the inert gas argon. The fine tertiary roots needed a maximum of 2 min to achieve suitable imaging. The larger root/stem samples needed at least 2 min (better with 3 min) coating to achieve the optimal imaging. Images were taken using a JCM-5000 NeoScope™ Table Top SEM (JEOL Products, Akishima, Tokyo, Japan). Parameters for imaging were 10 KV, high VAC, high resolution (=spot size of 4–5).

2.4 FISH assay

A slide-based FISH assay was developed for the visualization of pure cultures of PTA based upon the protocol described by Li et al. (2014), and to assess any potential cross-reactivity with six other *Phytophthora* species: *P. cinnamomi*, *P. citrophthora*, *P. gonapodyides*, *P. kernoviae*, *P. multivora* and *P. pluvialis*. Pure cultures were produced on 5% clarified V8-juice agar at 20°C in the dark (Jeffers 2006). Mycelium was harvested from the advancing edge of 10-day-old cultures of each *Phytophthora* species using a fresh pipette tip. The *Phytophthora* samples were transferred to 1.5 ml tubes to which 500 μ l of 4°C fixative buffer (44 ml of 95% EtOH, 10 ml RO water and 6 ml of 25 \times SET buffer [3.75 M NaCl, 25 mM EDTA, 0.5 M Tris-HCl pH 7.8]) was added and allowed to incubate at 4°C for 1 h. The tubes were centrifuged, and the fixative buffer was removed. The samples were rinsed carefully with 500 μ l of PBS (800 ml RO water, 8 g NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄ and 0.24 g of KH₂PO₄) (pH 7.4). Tubes were placed on a heating block at 50°C for 5 min. The samples were then gradually dehydrated with EtOH by first washing with 50% EtOH solution for 90 seconds followed by 80 and 96% EtOH solutions. Tubes were centrifuged for 30 seconds and ethanol was pipetted off. The hybridization procedure was performed in the tubes in a darkened room. A hybridization mix was prepared by adding 2 μ l of the PTA-specific probe (see Appendix 1), to 125 μ l of preheated 50°C hybridization buffer [5 \times SET buffer, 0.1% (v/v) Igepal-CA630 (Sigma, Auckland, New Zealand), and 25 μ l/ml polyA potassium salt (Sigma)]. The hybridization mix was added to the tubes and incubated at 50°C for 1.5 h in the dark. The hybridization mix was removed, and 120 μ l of 50°C preheated 5 \times SET buffer was added and incubated at 50°C for 15 min in the dark. SET buffer was syphoned off and the incubation treatment with 5 \times SET buffer repeated. To reduce autofluorescence, 500 μ l of 1% toluidine blue was added. Samples were washed in PBS buffer until clear. The samples were transferred to wetted slides (the well was made with multiple layers of clear nail polish), and a drop of Prolong Gold (Thermo Fisher Scientific, Inc., Albany, New Zealand) was added to the samples. A 32-mm cover slip was placed onto the slide. Slides were stored in the dark at room temperature until viewed. Slides were viewed under a Nikon Ni compound microscope. Fluorescence was observed using 330–380 nm excitation and an emission filter at 420 nm (DAPI: 4',6-diamidino-2-phenylindole, dihydrochloride) using a Nikon Intensilight C-HGFI (mercury light source). Photomicrographs were obtained using a Nikon DS Ri1 camera. Images were processed using a Nikon Digital Sight DS-U3 processor and NIS-Elements Basic Research (version 2.35) software.

For the root-based FISH assay, kauri roots were transferred into 1.5-ml Eppendorf tubes containing 50% EtOH and stored at 4°C. The roots were cut into 5 mm pieces. One piece of root was mounted on a stub with a drop of tissue freezing medium onto frozen deionized water. The root samples were tangentially cut into 22.5 μ m thick sections using a Leica CM1850 cryostat (Leica Biosystems, Albany, New Zealand), following the manufacturer's protocol. The sections were transferred onto a microscope slide with a nail polish rim (24 \times 40 mm) with 50% EtOH. The root sections were kept at 4°C overnight. The sections were successively washed on the slides with 70, 80 and 96% EtOH for 90 seconds. Ethanol was removed by pipetting it off the slides. The following steps were similar to those used for pure cultures: the sections were incubated once with 200 μ l of hybridization mix at 60°C for 1.5 h in the dark and twice with 5 \times SET at 60°C for 15 min. Two drops of 1% toluidine blue were added to the slides and root sections were washed in PBS buffer until clear. Two drops of Prolong Gold were added to the samples which were then covered with a cover slip. The slides were stored and visualized as stated previously for the pure cultures.

3 Results

3.1 Onset of disease symptoms and *post hoc* recovery of PTA

The onset of root infection by 5 days was not reflected with above-ground symptoms until day 16, when leaf desiccation became visible on the lower leaves (Table 1). By day 20, there was a constriction in the collar and the shoots were visibly chlorotic (Table 1). At day 20, PTA was recovered from the tertiary, secondary and primary roots and collar region of the seedlings inoculated with PTA (Table 1). Mean shoot mass and root mass of the PTA-treated seedlings were less than those of the control treatment (Table 2).

3.2 Ontogeny of infection

No *Phytophthora*-like structures were observed in the control-inoculated treatments (Table 1). No hyphal structures were observed within or on the outside of the control-inoculated roots.

In PTA-inoculated plants, zoospore encystment (Fig. 1a) occurred by day 5. Extramatrix colonization of the outer root surface was initially limited (Fig. 1b), but by 20 d.p.i., the surface of the roots were completely covered with PTA hyphae (Fig. 1c). Penetration of the epidermal layer led to colonization of the cortical cells. Types of intercellular hyphae included simple hyphae and also hyphal aggregations (Fig. 1d).

Lignitubers were produced by 5 days in cortical cells (Table 1, Fig. 2a). Infection rates were localized after 5 days and only occurred in the tertiary roots (Table 1). As the infection progressed through to 16 d.p.i., lignitubers were also produced in cortical cells of secondary roots. At 20 d.p.i., the degree of root infection increased, as did the number of replicates in which structures were observed. Intracellular hyphae appeared to be encased in lignitubers as digitate protuberances within the cortical cells (Fig. 2b). Papillae formed as either hemispheric pads (Fig. 2c) or as elongated 'palisade-like' layers along the inner cell wall (Fig. 2d).

Table 1. Summary table derived from observation of light microscopy.

	5 d.p.i.				10 d.p.i.				16 d.p.i.				20 d.p.i.			
	Co	K1	K7	K12	Co	K14	K10	K3	Co	K4	K5	K6	Co	K2	K9	K8
Lignitubers	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
Primary root	n	n	n		n	n		n	n		KP	KP	n	n	KP	KP
Secondary root	n	n	n	KP	n	n	n	KP		KP	KP	n	n	KP	n	KP
Tertiary root	n	n	n	KP	n	KP	KP	KP	n	KP	KP	KP	n	KP	KP	KP
Hyphal network	n	n	n		n	n			n			n	n	n	n	n
Internal hyphae	n	n	n		n				n				n			
Chlamydospores	n	n	n	n	n	n		n	n	n		n	n	n	n	n
Oospores	n	n	n	n	n	n	n		n	n	n		n	n	n	n
Stromata-like structures	n	n			n	n	n	n	n	n	n	n	n			
Root necrosis																
Leaf desiccation										LD	LD	LD		LD	LD	LD
Shoot chlorosis												SC		SC	SC	SC

'K'-labelled plant replicates are those inoculated with PTA. No *Phytophthora*-like structures were recorded in the control treatments (Columns labelled: 'Co' in light grey shading): 'n' = no structures observed. The size of the dark grey rectangles indicates the relative 'degree' of infection, with 'KP' indicating where PTA was recovered *post hoc*, and 'LD' and 'SC' indicating the onset of disease symptoms.

Table 2. Mean (SEM) shoot mass and root mass (g) after 20 days.

Inoculation tt	Shoot mass	Primary root mass	Secondary root mass	Tertiary root mass
PTA-treated	12.4 (2.8)	150.89 (22.2)	192.22 (28.1)	1263.21 (18.6)
Negative control	15.2 (0.0)	347.39 (0.0)	233.41 (0.0)	2870.85 (0.0)

Stromata-like survival propagules were observed in the plants deliberately inoculated with PTA (Fig. 3). The root cells were reddish brown to dark brown due to the accumulation of phenolic substances. Oospores of PTA were not definitively observed in this study at 20 d.p.i.

3.3 Slide-based FISH sensitivity assay

The PTA-specific probe successfully hybridized to PTA hyphae. PTA nuclei fluoresced a 'bright blue', in comparison with *P. multivora* under DAPI excitation. Viable nuclei in PTA hyphae appear as 'yellow' in the captured image (Fig. 4). In contrast, hyphae of *P. cinnamomi* did not fluoresce under 330–380 nm excitation and an emission filter at 420 nm (DAPI) (Fig. 5). Comparative studies of images under 330–380 nm excitation (Fig. 6a) vs. conventional bright field (Fig. 6b) also demonstrated the utility of the PTA probe to enable the differentiation of PTA hyphae.

Specificity of the PTA-specific probe was confirmed against six other *Phytophthora* species: *P. cinnamomi*, *P. citrophthora*, *P. gonapodyides*, *P. kernoviae*, *P. multivora* and *P. pluvialis*. (Hybridization temperature for *P. pluvialis* was 65°C cf. 50°C for the others.)

3.4 Application of FISH assay to tangential root sections

Kauri roots from the control treatment that were processed through the FISH reaction showed autofluorescence (Fig. 7). PTA-inoculated roots were compared with the control treatment for the presence of PTA structures in roots. When the PTA-inoculated plant material was assayed, PTA mycelium present in the root tissue hybridized with the probe and formed bright green fluorescence under UV excitation (Fig. 8). The green mycelium present in plant tissue conformed to the shape and morphology of the lignitubers identified from the light microscopy (Fig. 8a). Hyphal aggregates and stromata-like structures were also identified at 20 d.p.i. (Fig. 8b). Hyphae linking lignitubers were clearly differentiated and encircled the entire cortical cell (Fig. 8c).

4 Discussion

The assays described, using three visualization techniques, facilitated the detection of different PTA life stages within root material. Studies into the root invasion of *Phytophthora* species have been undertaken using light and transmission electron

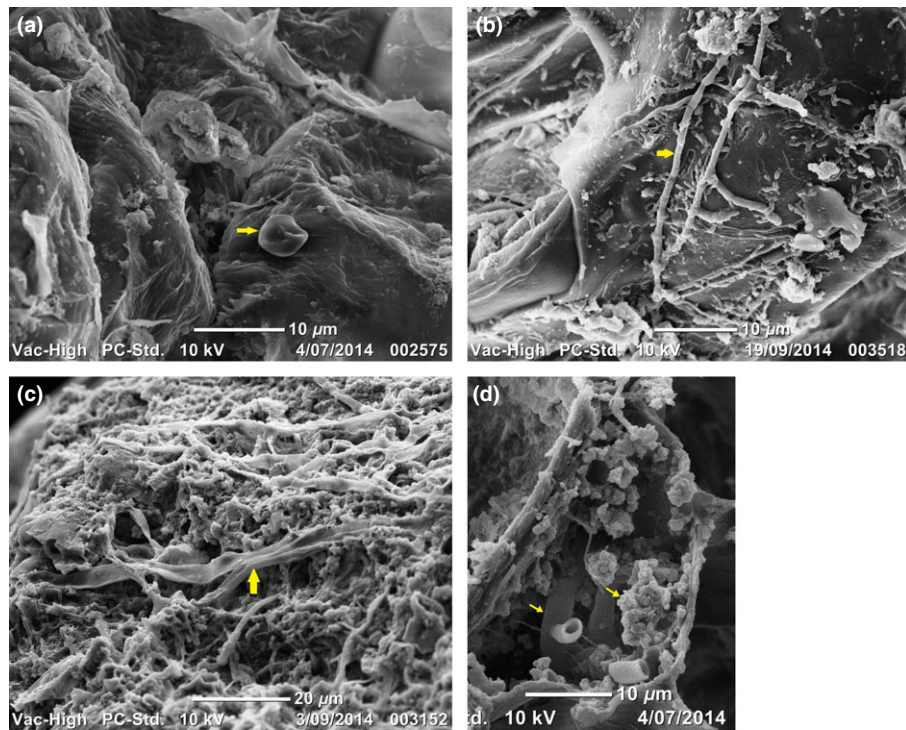


Fig. 1. (a) Zoospore encystment 5 days after inoculation (d.p.i.) ('cyst'). (b) Hyphal ramification across fine root surface 5 d.p.i. (arrow) (c) Hyphal ramification across root surface 20 d.p.i. (arrow) (d) Internal 'trunk' hyphae with hyphal aggregations.

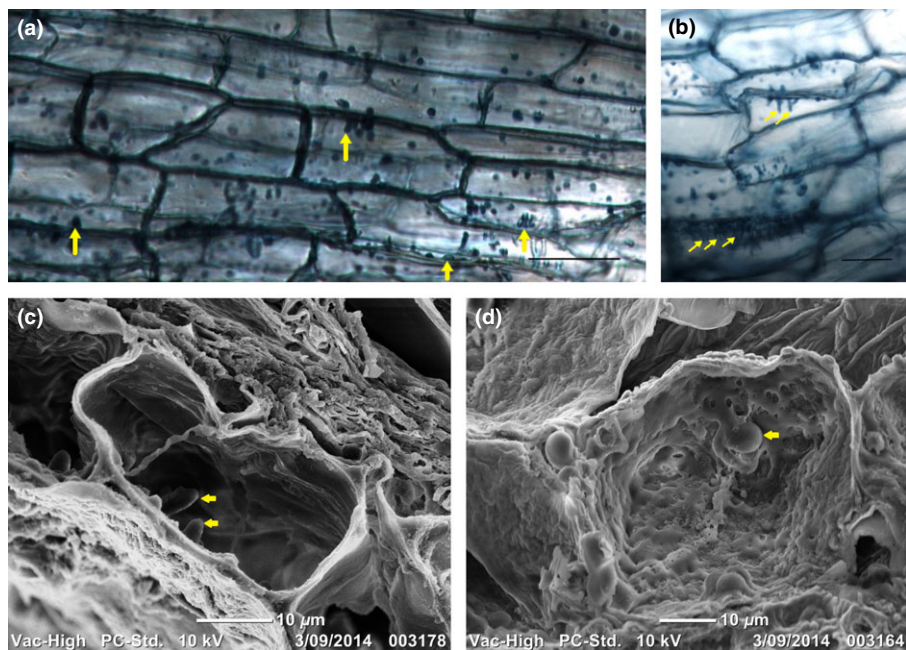


Fig. 2. (a) Lignitubers development in cortical cells 5 days after inoculation. In PTA-inoculated plant roots. (Scale bar equals 40 μm) (b) Digitate lignitubers (arrows). (Scale bar equals 40 μm) (c) Digitate lignitubers invaginating cortical cells (arrows). (d) Lignituber with callose deposition (arrow).

microscopy (e.g. Widmer et al. 1998). The successful development of a species-specific probe allowed PTA to be readily distinguished from plant cells and allowed visualization of PTA within plant tissues. As demonstrated here, PCR-specific primers can be adapted for FISH applications, by centralizing polymorphic regions within the probe as opposed to having them located at the 3' end of the primer, which is favoured for PCR reaction protocols (Appendix 1).

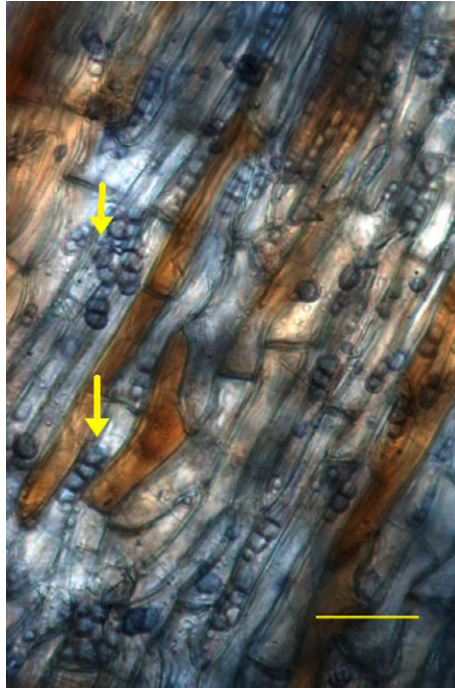


Fig. 3. Stromata-like structures of PTA developed between the cortical cells highlighted (arrow). (Scale bar equals 40 μm).

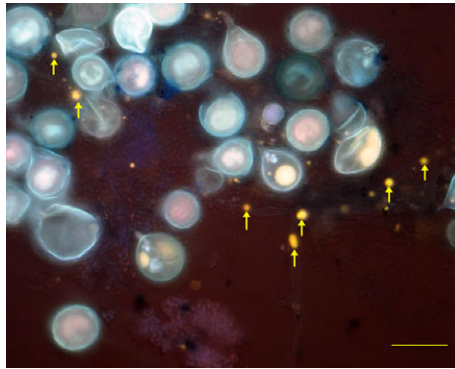


Fig. 4. Hyphae of PTA (arrow), noting yellow fluorescence of the probe, vs. the light blue autofluorescence of the oospores (some empty) (Scale bar equals 60 μm).

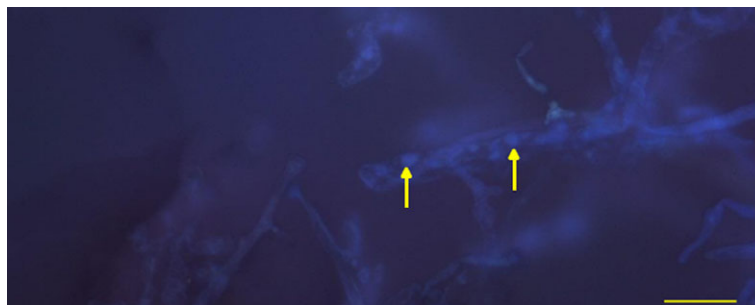


Fig. 5. Hyphae of *P. cinnamomi* (arrow), noting no fluorescence of the probe (Scale bar equals 60 μm).

This comparative inoculation study (described here) identified different structures of the PTA pathogen that have not been seen in axenic culture. Deliberate root inoculation of 2-year-old kauri seedlings was associated with a decrease in both shoot and root mass in response to PTA infection, and Koch's postulates were satisfied with the *post hoc* recovery of

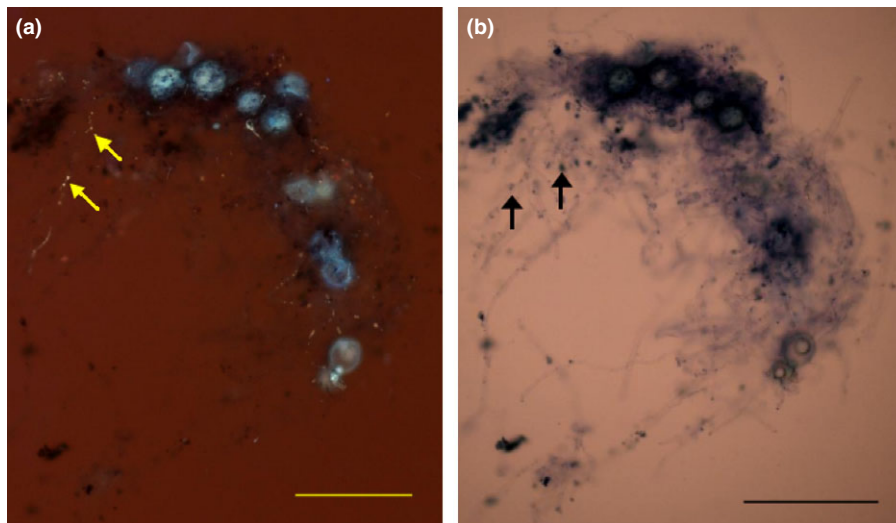


Fig. 6. Nuclei in hyphae of PTA: (a) fluorescing yellow under 330–380 nm excitation (arrows) (Scale bar equals 100 μm), (b) as viewed using conventional bright field (black arrows) (Scale bar equals 100 μm).

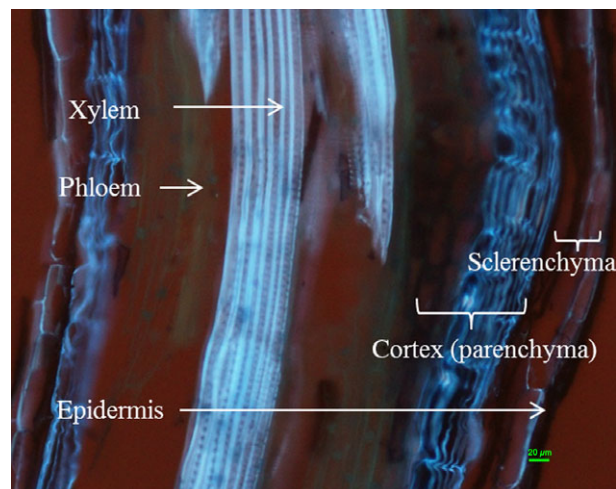


Fig. 7. Tangential section of tertiary root of uninoculated kauri seedling.

PTA from PTA-inoculated plants (and not from the negative control). Light microscopy incorporating clearing and staining was an essential process for viewing the *Phytophthora* structures within the roots/collar material of 2-year-old kauri seedlings. The clearing (and in particular bleaching) made the roots semi-transparent. The amount of time used in the clearing and bleaching steps was determined in part by the amount of pigments present in the root material.

The ability of the pathogen to grow as a hemibiotroph is indicated by the presence of lignitubers. During the very early stage of infection (5 d.p.i.), the pathogen proliferated asymptotically, both within and on the outside of the plant root. The internal hyphae continued to infiltrate through the fine roots to the secondary roots and reached the primary roots by day 16. By this time, above-ground symptoms were expressed as desiccation of the lower leaves. The constriction of the collar region observed by day 20 reflected the vascular dysfunction, which also was associated with shoot decline.

Stromata-like structures have not been observed in PTA before in pure axenic culture on either Potato Dextrose Agar, Malt Extract Agar, V8-juice agar, Sabouraud Dextrose Agar or Corn Meal Agar. Stromata-like structures (produced during the putative, necrotrophic stage) were first described from natural ecosystems of Western Australia by Jung et al. (2013), who studied the roots of herbaceous understorey plants infected by *P. cinnamomi*. They hypothesized that these structures could play a part in the persistence of the pathogen in the soil around an infected host. Stromata-like structures housed in decaying root material are more or less protected, and if they are able to germinate and produce hyphae, which in turn, sporulate under flooded-soil conditions, they can re-commence the fine root infection process. PTA also recovered 5 d.p.i. from asymptomatic fine roots. So possibly, the stromata-like structures and hyphal aggregations germinated and were able to produce hyphae. Hyphal aggregations formed within cortical cells, 5 d.p.i., and there was evidence that the hyphae remained obvious as the structure matured (20 d.p.i.). Both stromata-like structures and hyphal aggregations may be

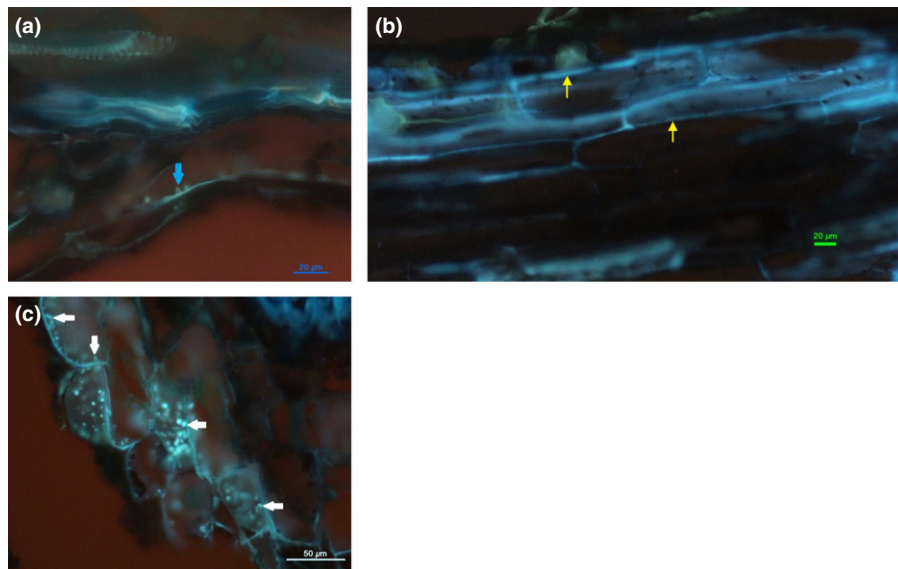


Fig. 8. PTA hyphae under fluorescence: (a) connecting papillate lignitubers; (b) surrounding and connecting infection with contiguous cortical cells; (c) interconnecting series of stromata-like structures and hyphal aggregations within and between cortical cells (20 d.p.i.).

curiosities for *Phytophthora*, but are regular components of higher fungal biological entities. The fact that hyphal organization approaching tissue-like structures occur in roots infected by *Phytophthora*, may be a phenomenon that has been largely overlooked in crop pathosystems, as the hosts' roots of crops are less durable than tree roots. The persistence of the pathogen in root pieces has implications for land management decisions around infected trees adjacent to tracks and road ways. Potentially infested sediment must be segregated from any material to be re-used post-clearance and properly contained and disposed of by a certified disposal contractor (Fichtner et al. 2011). Temporary and/or permanent modifications to drainage courses around or adjacent to infected trees must also consider the destination of potentially contaminated run-off to ensure that it is diverted from healthy roots and uninfested forest.

The slide-based FISH assay successfully hybridized with hyphae of PTA. The probe was demonstrated to be specific for PTA. Further confirmation of the specificity of the probe was made through failed hybridization with non-target *Phytophthora* species that have been recovered from the kauri forest, that is *P. cinnamomi*, *P. citrophthora*, *P. multivora* (Bellgard et al. 2013) and *P. gonapodyides*, and *P. kernoviae*. Application of this assay to naturally infected field samples from a range of plant species (other than kauri) could see the assay used to rapidly identify PTA within environmental samples mounted onto microscope slides for direct observation. Further, information with regard to infection, colonization and survival in the presence of other co-infected *Phytophthora* species will provide insights into the apparent host specificity of PTA for kauri and potential interactions with other known pathogens of kauri, for example *P. cinnamomi* (Johnston et al. 2003).

Obtaining freeze-microtome, tangential sections helped the probe penetrate the layers of plant cell walls and hybridize with PTA embedded within, between and upon plant cells. The application of the assay to woody roots is limited by the thickness of the sections. An extension to the development of the FISH assay for woody root tissue would be some form of semi-permanent embedding (e.g. paraffin wax) to assist with attaining root sections of <math><15\ \mu\text{m}</math>. The presence of a small quantity of paraffin in the root section may not be an issue, as the repeated number of ethanol rinses as part of the FISH assay protocol will dissolve the wax component.

FISH hybridization was used to help guide the interpretation of results from both light and SEM microscopy by corroborating the morphology of the structures and confirming that they are formed by PTA. SEM preparation of root tissues preserved in FAA was possible using a standard progressive dehydration process. Suitable resolution was achieved by gold coating for 2 min; however, larger specimens needed a thicker layer of gold prior to visualization. The contrasting, relative benefits of the three methods are determined by the objectives of the study and the amount of funding available to support the research effort. Clearing and staining of roots is cheap and quick, and very 'low-tech', and enabled visualization of the intercellular and extracellular colonization features. The technique has been modified to eliminate the need to use phenol; however, the FAA fixative and stain are toxic substances which require appropriate training and disposal of wastes. Light microscopy requires a compound microscope and camera to take images of suitable resolution – which is challenging due to the thickness of the stained material. SEM is of course a step-up in resolution, but also in cost and time investment. Specialist, and dedicated equipment and training are necessary; however, existing protocols can be adapted for use with kauri roots. The development and optimization of the FISH assay required a substantial investment of time and resources. The synthesis of the probe required a species-specific primer, before the specificity testing could be commenced against other *Phytophthora* species. The sectioning of roots and laborious and tedious assay protocol requires at least 1 day to prepare the samples. A specialist epifluorescent microscope is required, equipped with a series of filters which selects a specific segment of the light spectrum to excite the stained preparation and then allows only excited (fluoresced) light to pass to the ocular lenses.

Widmer et al. (1998), through the use of light and transmission EM, were able to identify differences in the hyphal colonization (in 6-week-old seedlings) between resistant and susceptible orange cultivars. Part of the long-term management strategy for kauri is the identification of resistance in the remaining population. The use of the FISH assay could assist to screen seedling lines for differences in hyphal colonization when exposed to a set amount of inoculum. By integrating digital image analysis, the extent of hyphal colonization in the cortical cells could be quantified and compared between replicates of the different seedling lines assessed. Another application of the assay would be for the study of interactions between PTA and other endophytic fungi present in the roots of kauri. Beneficial arbuscular mycorrhizal fungi have been observed in the short, lateral root protuberances (nodules) and the fine roots of kauri (Morrison and English 1967; Padamsee et al. 2016). Mycorrhizal associations have been implicated in the biocontrol of plant diseases (Hooker et al. 1994), and the FISH assay could be integrated into dual inoculation studies. In a potential experimental scenario, a host root can be deliberately pre-infected with a symbiotic partner and then the plant exposed to the pathogen. The assay can also be used to study sublethal infections in non-target hosts, to get some insights into resistance reactions. The assay can also be applied to the study of mycoparasite relationships of non-associated fungi, as modifications in hyphal morphology of PTA and/or growth habit in relation to natural or synthesized extracellular substances can be assessed as part of screening studies for potential biocontrol agents.

The development of Kauri Dieback forest hygiene management guidelines relies on a comprehensive understanding of the survival and transmission components of the disease and life cycle of *P. agathidicida*. This glasshouse research identified that (i) *P. agathidicida* infects fine roots of kauri and colonizes root cells and produces lignitubers within 5 d.p.i., (ii) inoculum of *P. agathidicida* resides in the infected roots of kauri seedlings as stomata-like structures and/or hyphal aggregations, and (iii) these structures were able to germinate and produce oospores (*in vitro*) when the infected root pieces were plated to selective agar. The results of this study suggest that roots infected with *P. agathidicida* may be a source of inoculum that remains dormant in the soil of dieback infested sites. This underscores the need for ongoing, containment-management practices, to prevent the unintentional spread of soil and root material from dieback infested sites into healthy forest.

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APPENDIX 1

PTA-SPECIFIC PROBE DESIGN

The probe used was first published in Than et al. (2013, table 3). The sequence 5'–3' has a length of 20 oligonucleotides: GGCGGCTGCTGGCTTTGGCT. The probe was commercially synthesized and labelled with AlexaFluor350 dye at the 5' end, which excites at 350 nm and emits at 442 nm, and a black hole quencher at the 3' end (IDT Integrated DNA Technologies, Indiana, USA <http://sg.idtdna.com/site>).