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Manatū Ahu Matua

Specialist Phytophthora Research: Biology, Pathology, Ecology and Detection of PTA

Final Report

MPI Contract 11927

Prepared for Chris Green on behalf of the Planning & Intelligence team, Kauri Dieback Joint Agency Response

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Frontispiece

“Me he kauri whakaruruhau, ka toro ngā peka, hei awhi i te wao”

Like a kauri I stretch my branches to embrace the forest

Hirini Melbourne

Contents

Tihe mauriora

1 Executive summary

In 1970 a collar-rot caused by a *Phytophthora* species (originally identified as *P. heveae*) was first recognised as impacting upon kauri on Great Barrier Island. Re-examination of the original isolates, with the added help of molecular identification methods, has shown this original identification was incorrect and the informal name *Phytophthora* "taxon Agathis" (PTA) was ascribed to the causative agent (Beever et al. 2009). Recent surveys have found that root- and collar-rot symptoms and crown decline are distributed in the Waitakere Ranges (e.g., Huia and Piha), north of Auckland (Rodney, Pakiri and Albany), Whangarei, (Glenbervie Forest), Great Barrier Island, and the northern kauri forest (e.g., Raetea, Waipoua, Trounson, Punaruku). Surveys instigated by the Kauri Dieback Response have failed to detect PTA in the Coromandel Peninsula, and surveys by Auckland Council have failed to detect PTA disease symptoms in the Hunua Ranges Regional Park. Knowing where PTA occurs at the regional level is critical for understanding the full scope of the problem.

This report documents a 3-year project aimed at obtaining urgently needed information to better understand the threats posed by PTA to kauri trees and kauri-forest ecosystems. The expectations of the contract were to supply specialist *Phytophthora* research on pathology, biology and ecology. To achieve these expectations the following were investigated:

- Identify potential non-*Agathis* hosts of PTA
- Demonstrate pathways by which PTA can be spread
- Study the plot-scale impacts of PTA
- Study the vertical distribution of PTA in the root system under diseased trees
- Map the small-scale distribution of PTA around an infected tree
- Develop a RT-PCR, species-specific assay for PTA

The aims of the concurrent Landcare Research-funded complementary research project were to:

- Resolve the taxonomic uncertainty around PTA and provide a formal binomial name
- Investigate relationship of PTA to other members of *Phytophthora* ITS Clade 5
- Provide evidence on the putative origins of PTA.

Emphasis was placed on the development of tools and providing the scientific data to allow evidence-based management decisions to replace previous approaches based largely on the precautionary principle.

1.1 Unified species concept for PTA and closely allied taxa

On the basis of rigorous multi-gene genetic analysis, morphological segregation and host-differentiation, we find that PTA is a well-supported, discrete taxonomic entity, separate from *P. heveae* (which is associated with rubber *Hevea brasiliensis* [Euphorbiaceae], and avocado *Persea americana* [Lauraceae]) and *P. castaneae* (associated with sweet chestnut *Castanea* spp. [Fagaceae]). In a forthcoming publication we propose to formally name PTA as *Phytophthora "agathidicida"* (ined.; the "*Agathis*-killing" *Phytophthora*), a new species within ITS Clade 5. Additionally, we have identified another new morphospecies within Clade 5, *P. "cocois"* (ined.; the "coconut" *Phytophthora*), previously misidentified as *P. castaneae*. We have thus doubled the number of species in *Phytophthora* ITS Clade 5.

Agathis [Araucariaceae] is distributed from peninsular Malaysia to New Zealand, including Malesia, the Philippines, New Guinea, Melanesia and Australia. The highest concentration of species diversity of Araucariaceae in New Caledonia, with five endemic *Agathis* species, all of which are well diversified in their morphological characteristics. The known distribution of *Phytophthora* species closely allied to PTA, namely *P. heveae* and *P. castaneae,* are present in indigenous soils of Papua New Guinea and tropical rainforests of northern Queensland and Taiwan. If isolates matching PTA can be found in these places it would add support to the proposal that PTA is exotic.

1.2 Host range of PTA

Nineteen native plant species of New Zealand, and Queensland kauri (*Agathis robusta*) were screened for susceptibility as hosts of PTA. For the purpose of this study, a host is defined as a plant on or in which PTA can live under glasshouse conditions. Inoculation of the following woody plant species indicated susceptibility as hosts for PTA: kauri *Agathis australis*, rimu *Dacrydium cupressinum*, māmāngi *Coprosma arborea*, pōhutukawa *Metrosideros excelsa*, rewarewa *Knightia excelsa*, mānuka *Leptospermum scoparium*, kānuka *Kunzea ericoides*, pigeonwood *Hedycarya arborea*, tawa *Beilschmiedia tawa*, taraire *B. tarairi*, and korokio *Corokia buddleioides*. Of these, only tawa and taraire did not display any significant decrease in shoot or root weight as a consequence of PTA infection.

The results presented in this study are based upon the responses of young plants to soilborne inoculation with PTA in a glasshouse context. To date no non-*Agathis* species have been observed with collar rot symptoms, little-leaf syndrome or canopy thinning. Symptomology and evidence of root infections of non-*Agathis* hosts in kauri forests needs to be confirmed before PTA is considered to pose a risk to any other NZ native plants.

1.3 Pathways of infection

Plant tissues infected with PTA from the root and collar could transmit infection to the roots of 18-month old kauri seedlings. The type of roots that could convey infection were; fine, secondary, and primary roots. Koch's postulates were satisfied for all symptomatic kauri plants through the recovery of PTA from PTA-inoculated plants. Thick-walled, globose to sub-globose oospores were observed in dead, secondary roots of kauri.

1.4 Spatial extent of PTA in a forest stand

Our study area was the Twin Peaks Track, near the Huia Dam Reservoir in the Waitakere Ranges, west of Auckland City. The kauri stand represents a mixed age, ricker stand. Disease was first recorded at this site 6 years ago, and tree-deaths are now present across the entire site. Collar and lower-trunk symptoms were characterised by an advancing lesion (canker) with profuse production of resin at the base of the trunk. Seventy five percent of PTA recovery was associated with symptomatic trees (i.e., stem bleeding and a crown health rank >3). PTA was not readily recovered from soil collected beneath trees with "stag heads" (PTA detected from 15% of samples) and rarely from below healthy trees (PTA from 1% of samples). In addition to PTA, *P. cinnamomi, P. multivora* and *P. nicotianae* were recovered from soil samples taken from below kauri trees at very low frequencies (2.5%). The extent of disease spread is difficult to interpret as not all dead trees could be confirmed as having been killed by PTA, and other *Phytophthora* species were present along the transect. The derived extent of the infections after 6 years was between 1 and 19 m. Calculated on the basis of circular spread, the "linear" spread over 6 years is 3.41 m.

1.5 Vertical distribution of PTA in roots below infected trees

The soil profiles under two intensively sampled diseased trees are similar in that they are highly leached, have similar parent materials, and podsolisation is the overall soil process. The soils both have mostly substantial accumulations of organic materials at the surface. The subsoil, which comprises 0.5–1.0 m of the soil profile, is dominated by clays derived from volcanic ash. For the two intensively-sampled diseased trees, the surface lateral roots were found to possess few or no fine roots. The recovery of PTA by soil bioassay was restricted to the upper 15–20 cm of mineral soil under both trees. There were healthy fine roots present below the zone of diseased roots (i.e., below 20 cm). Healthy rootlets, present at a depth of approximately 70 cm under both trees, were infected by *P. cinnamomi* as detected by root plating to *Phytophthora*-selective media. No *Phytophthora* species were recovered by soil bioassay beyond a depth of 20 cm. The role of *P. cinnamomi* at depth in the predisposition of kauri trees to PTA collar-rot is unknown.

1.6 PTA distribution around a diseased tree

The spatial distribution of soil inocula around an infected focus was studied using a non-specific, indirect, soil bioassay. PTA was recovered 6.5 m from the base of a PTA-positive tree. Soil baiting also recovered *P. cinnamomi* and *P. multivora* from around this PTA-positive tree.

1.7 Developing a species-specific PTA assay

A real time PCR assay was developed for the detection of PTA from field-collected soil. A species-specific primer for PTA was designed from the internal transcribed spacer (ITS) region of 18S ribosomal DNA (18S rDNA) of PTA. Specific PCR amplification of PTA using the primer was demonstrated against three species of *Pythium* and 31 species of *Phytophthora.* The sensitivity of the primer (on the basis of TaqMan chemistry), was 2 fg (femtograms) from pure mycelial cultures of PTA. This primer detected PTA at levels as few as four oospores per 1 g of soil. This approach has been shown to be faster than the soil bioassay used for detection of PTA in naturally infested soil. Compared to the soil bioassay, this assay had diagnostic sensitivity of 75% and a diagnostic specificity of 68.8%.

1.8 Recommendations for Future Research

Biology and origin of PTA

- Continue population evaluation, and determine genetic diversity within PTA.
- Ascertain whether the presence/absence of genetic structure within the NZ population of PTA reflects a "founder population".

Clarifying PTA disease aetiology and host range

Develop an agreed priority list of *at risk* plant species, for inoculation trials using a root-based inoculation system. Relate this to observations of mature plant specimens in the forest. Potential species could include, Kirk's daisy *Brachyglottis kirkii*, neinei *Dracophyllum latifolium*, and tāwari *Ixerba brexioides*. Additionally, important monocotyledons associated with kauri should also be screened for their susceptibility to PTA. This selection of plants species was in no way exhaustive and further consultation is needed to identify a priority list of *at risk* taxa.

- Elucidate factors governing fine-root infection of kauri, and progression of disease from fine roots to collar.

- Study environmental conditions controlling zoospore release (e.g., temperature, electrical conductivity, extracellular root exudates), and density-dependent zoosporic thresholds necessary for fine root infection of kauri.
- Study environmental factors controlling oospore dormancy and germination.

Temporal and spatial soil inoculum dynamics of PTA in a forest stand

- Add georeference data for all trees in the Twin Peaks Track transects.
- Establish further long-term study plots in other kauri forest systems – Waipoua, Trounson, Albany, Coromandel, and Hunua. Ideally, these will be placed in healthy and infested kauri forests, and monitored regularly.
- Establish soil test pits to better characterise soils in healthy and diseased kauri forests throughout the geographic range of kauri (see above).
- Undertake plant health analysis of any non-*Agathis* species displaying collar-rot, or crown decline in PTA-infected forests to confirm susceptibility to PTA.
- Review by-catch data from Surveillance 2, to identify the frequency and map distribution of other post-border *Phytophthora* species present in kauri forest.

PTA detection

- Develop a duplex assay, integrating real time polymerase chain reaction (RT-PCR) technology with conventional soil bioassay, to increase the speed and accuracy of the PTA detection process.

2 Introduction, aims and report structure

2.1 Introduction

Phytophthora "taxon Agathis" (PTA) was identified as the causal agent of the collar-rot of kauri (*Agathis australis*) by the late Dr Ross Beever (Beever et al. 2009). PTA-mediated root- and collar-rot of kauri was initially recorded as *P. heveae* in 1970 (Gadgil 1974), but this species identification was incorrect as PTA does not have caducous sporangia, has larger amphigynous oogonia, and has an ITS sequence distinct from *P. heveae* (Weir et al. 2012). Pathogenicity tests have confirmed that PTA is highly pathogenic to kauri (Beever et al. 2010 unpubl. report). Recent surveys have found that collar-rot symptoms and crown decline are distributed throughout the kauri forest north of Auckland (e.g., Pakiri, Albany, Rodney) and the Waitakere Ranges (e.g., Huia and Piha). Results of the two rounds of PTA surveillance also confirmed the presence of PTA in Waipoua Forest, Trounson Kauri Park, Raetea Plantation, and Punaruku. PTA was also confimed at Franklin and Awhitu. Soil detection failed to recover PTA from any of the sites sampled in the Coromandel Peninsula (Bellgard et al. 2011; Bellgard 2013). Surveys by Auckland Council have failed to detect PTA disease symptoms in the Hunua Ranges Regional Park.

Conjecture remains about the origins of PTA, and whether PTA is an exotic incursion. The speed of disease spread within infested stands and the distribution of PTA at the "tree-scale" (both subsoil and lateral spread of PTA inoculum) around diseased foci are not well understood. How the pathogen is spread in soil also remains largely unknown, with oospores suspected to be the long-term survival structure of other soilborne *Phytophthora* species (Sneh & McIntosh 1974; Stack & Millar 1985). The stem-inoculations of seedlings of New Zealand plant other than kauri, carried out by Beever et al. (2007), found only kauri to be susceptible to PTA. Little is known about the soil-based susceptibility of other New Zealand plants to PTA.

The taxonomic identity of PTA has been problematic since the 1970s (Gadgil 1974), and its relationship to other closely allied, morphologically similar taxa, *P. heveae, P. castaneae* (erroneously renamed as *P. katsurae* by Ko & Chang 1979 – see Pennycook 2012), and a *castaneae*-like species recovered from *Cocos* in Hawai'i and the Ivory Coast was also unknown. Resolution of the taxonomic identity of PTA and its relationship with other *Phytophthora* ITS Clade 5 taxa is essential for the development of PTA-specific primers for application in real-time PCR detection technologies.

Four other species of *Phytophthora* have been recorded from kauri or soil in kauri forests: *P. cinnamomi, P. cryptogea, P. kernoviae,* and *P. nicotianae* (Beever et al. 2006, unpubl. report). In New Zealand, *P. cinnamomi* has been isolated from soil beneath a number of native communities. Newhook (1960) recovered *P. cinnamomi* from mature kauri forest in the Waitakere Ranges and from soil beneath 9 out of 10 kauri trees sampled in undisturbed virgin forest on Little Barrier Island. Hepting & Newhook (1962) reported isolation of *P. cinnamomi* in 40% of soil samples in old growth forest at Waipoua. Plantings of kauri at Waipoua also experienced heavy losses of nursery seedlings attributed to the root-rot *P. cinnamomi* as reported by Morrison & Lloyd in 1972, with the earliest records of "damping off" being recorded in the early 1950's associated with unseasonally heavy rainfall (Morrison 1955).

2.2 Generic life cycle of *Phytophthora*

The complete life cycle of PTA has not as yet been articulated, but we have observed oospores, sporangia and zoospores (Fig. 1). Assuming that PTA accords to the generic life cycle of soilborne *Phytophthora* species presented in Fig. 1, it has the potential to survive adverse environmental conditions over several years as dormant resting spores (oospores). When and if environmental conditions become suitable (high soil moisture, soil temperature > 10°C) *Phytophthora* resting spores may germinate and form sporangia, releasing motile biflagellate zoospores into the soil water (Agrawal 2009; Widmer 2010). These zoospores are chemotactically attracted by young fine-root tips. After encysting and penetrating the exoderm (or the periderm in suberised fine roots), *Phytophthora* exhibit an early, asymptomatic *biotrophic* phase and a later *necrotrophic* stage that is characterised by tissue degradation and disease symptoms (Lee & Rose 2010[1]). In the early stages of infection, they establish haustoria and commence feeding. Meanwhile, they continue to grow between and within cells of the fine roots, with typical coralloid to irregular, non-septate hyphae. In response to nutrient depletion, competition by secondary antagonistic fungi or strong defence reactions by the root, the *Phytophthora* hyphae have the capacity to form resting spores. After decomposition of the root by saprophytic fungi, breakdown by soil mesofauna or chewing and disturbance (e.g., by pigs), or other perturbation under the canopy and close to the trunk of infected trees, the resting spores have the opportunity to be liberated into the soil environment, and the zoosporic cycle can commence, once again.

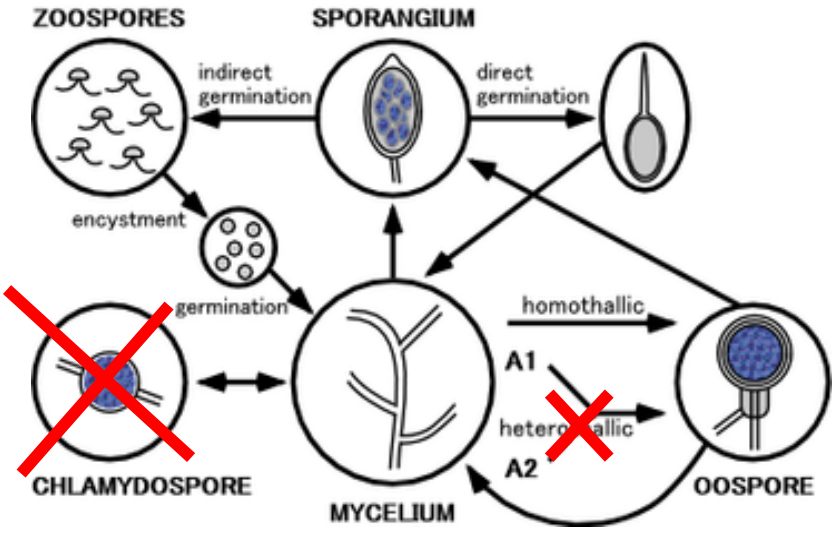

Figure 1. Generic *Phytophthora* life cycle[2]. N.B. Chlamydospore phase not as yet observed in PTA; and "heterothallic" deleted because PTA is homothallic.

Phytophthoras can increase and disseminate their inoculum from low, nearly undetectable levels, during a relatively short period of favourable environmental conditions (Jung et al. 2013). On the other hand, for some host–pathogen combinations there may need to be decades of inoculum build-up and progressive fine-root destruction before a mature tree begins to show visible crown symptoms (Tsao 1990). Therefore, the epidemiology of *Phytophthora*-induced fine-root diseases is considered to be multi-cyclic (Erwin & Ribeiro

[1] *Phytophthora* species are considered *hemibiotrophic.*

[2] http://commons.wikimedia.org/wiki/File:Phytophthora_life_cycle_English_text.png

1996). Predisposing factors such as waterlogging, extreme droughts, or defoliation by insects can reduce the vitality of the tree. Conditions such as excess soil moisture following heavy rain, flooding or irrigation can favour the pathogen and accelerate or facilitate the onset of the disease process.

2.3 Project aims

This report documents a 3-year project aimed at obtaining urgently needed information to better understand the threats posed by PTA to kauri trees and kauri-forest ecosystems. The expectations of the contract were to supply specialist *Phytophthora* research on pathology, biology and ecology:

- Identify the host range of PTA
- Demonstrate pathways by which PTA can be spread
- Study the plot-scale impacts of PTA
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2.4 Report structure

This report is structured with seven main chapters covering:

1 The basis for the species description of PTA (Chapter 3)

2 The testing of NZ plants by soil-based inoculation with PTA (Chapter 4)

3 The transfer of PTA infection from infected plant material (Chapter 5)

4 The plot-scale progression of PTA (Chapter 6)

5 The vertical distribution of PTA in roots below infected trees (Chapter 7)

6 The spatial distribution of soil inoculum of PTA around an infected tree (Chapter 8)

7 The development and testing of a species-specific assay for PTA (Chapter 9)

Each of these chapters is divided into four components: introductory statements, materials and methods, results and discussion. A concluding discussion and concise list of PTA research recommendations are provided at the end of the report (Chapters 10 and 11).

3 A unified species concept for PTA

3.1 Introductory statements

The *Phytophthora* genus currently recognises 117 "species": with a number of distinct taxa and others in the process of formal description (Martin et al. 2012). The genus is divided into 10 major clades (Cooke et al. 2000), with PTA proposed to sit within Clade 5 together with *P. castaneae* [≡ *P. katsurae*] and *P. heveae* (Weir et al. 2012) (Fig. 2). Arentz (1986) reported both those species are present in soils in Papua New Guinea, including under rainforests containing *Agathis robusta*, *Araucaria cunninghamii* and *Araucaria hunsteinii*, although without obvious linkage to disease. Brown (1999), in a study focused on the association between *P. cinnamomi* and patch death in tropical rainforests of northern Queensland (containing both *Agathis* and *Araucaria*), also reported both *P. castaneae* and *P. heveae*, with *P. heveae* more frequent at higher elevations under dead patches of rainforest.

The original identification of the kauri pathogen was *P. heveae* based upon the morphological classification system that existed in the 1970's (i.e., Waterhouse 1963; 1970). As part of the earlier MAF contract to sequence the collection of *Phytophthora* species held in ICMP, the late Ross Beever identified that the kauri *Phytophthora* was not *P. heveae*, based upon analysis of the ITS-region (Beever et al. 2006). Since that time, we have progressed our understanding of the taxonomic position of 'PTA' in ITS Clade 5 through the use of multi-gene analysis, morphology of oospores and growth rate. This chapter describes the process behind the determination of the species concept and proposed formal nomenclature of PTA and resolution of novel species relationships in ITS Clade 5. Results from multi-gene analysis, morphological analysis and host-range differentiation are used as the basis for the species concept of PTA.

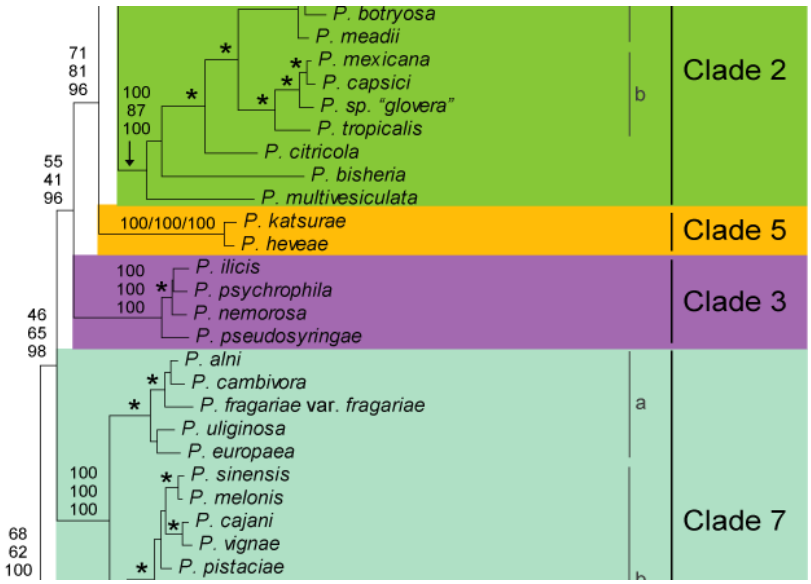


Figure 2. Extract from ITS-based *Phytophthora* phylogeny highlighting the location of Clade 5 (Cooke et al. 2000). N.B. The correct name for *P. katsurae* is *P. castaneae.*

3.2 Materials and Methods

Isolation methods

Isolates from New Zealand were recovered from either cambium samples taken from bleeding trunk lesions or from soil samples taken from the base of kauri trees. PTA recovered from soil was isolated using soil baiting where 200 g samples of soil were flooded with Reverse Osmosis (RO) water and baited with either carnation petals or freshly picked azalea leaves and floated on the surface of water (adapted from Vettraino et al. 2001; and following Vannini pers. comm.). After 2–7 days' incubation at 18–20°C, discoloured baits were blotted on clean paper-towels to remove excess moisture, cut into small pieces and sterilised in 70% ethanol solution for 30 seconds. The pieces were then rinsed in sterilised RO water, blotted dry and plated to *Phytophthora*-selective agar (Jeffers 2006). Colonies growing from the baits were transferred to fresh PDA for DNA sequence analysis. The 11 PTA isolates upon which the species description is based and the geographic location of their recovery are provided in Table 1. The origin of the PTA isolates used in this study are provided in Appendix 1A.

Table 1. Isolates of PTA examined

ICMP	Host	Location	Year of isolation	Substrate	Other references
16471	**Kauri**	**Gt Barrier**	**1971**	**Soil**	
17027	**Kauri**	**Gt Barrier**	**2006**	**Cambium**	**REB 316-14(K2)**
18244	Kauri	Pakiri	2008	Cambium	REB 326-1
18358	Kauri	Waitakere	2009	Cambium	REB 326 221
18360	Kauri	Gr Barrier	2009	Cambium	REB 326 155
18401	Kauri	Raetea	2010	Cambium	REB 327-34
18404	Kauri	Trounson	2010	Cambium	REB 327-41
18406	Kauri	Huia	2010	Soil	REB 327-46
18407	Kauri	Waipoua	2010	Cambium	REB 327-47
18408	Kauri	Waipoua	2010	Cambium	REB 327-53
18410	Kauri	Trounson	2010	Soil	REB 327-60

N.B. The isolate in **red bold** is the original "Gadgil" isolate from Great Barrier Island; and the Isolate in **black bold** is the proposed "type" isolate for PTA. We wish to acknowledge the issue of the high impact DoC permit to sample Northland kauri in 2010 negotiated by Tony Beauchamp.

BLAST search

A BLAST search of GenBank – using the ITS sequence of PTA – was used to select a genetically diverse set of isolates. Voucher cultures representing this diversity and that of named *P. heveae* (6 isolates) and *P. castaneae* (8 isolates), as well as 3 isolates of *P. "cocois"* (the un-named species ex-coconut) were obtained from international culture collections (Appendix 1B lists the non-PTA isolates studied). All cultures are held in the ICMP culture collection, Landcare Research, Auckland, where isolates are stored in a metabolically inactive state in liquid nitrogen at −196°C; additional details on each culture are available on the ICMP[3] website.

[3] http://scd.landcareresearch.co.nz/Search/Search/ICMP

DNA isolation, amplification and sequencing

Mycelium was collected from isolates grown on PDA agar, and manually comminuted with a micropestle in 420 µl of Quiagen DXT tissue digest buffer; 4.2 µl of proteinase K was added before incubation at 55°C for 1 h. After a brief centrifugation 220 µl of the supernatant was placed in a Corbett X-tractorGene automated nucleic acid extraction robot. The resulting 100 µl of pure DNA in TE buffer was stored at −30°C in 1.5 ml tubes until use. Gene sequences were obtained from six nuclear and two mitochrondrial gene regions: actin (ACT) [316 bp], calmodulin (CAL) [756 bp], chitin synthase (CHS-1) [229 bp], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [308 bp], the ribosomal internal transcribed spacer[4] (ITS) [615 bp], glutamine synthetase (GS) [907 bp], manganese-superoxide dismutase (SOD2) [376 bp], and β-tubulin 2 (TUB2) [716 bp].

DNA sequences in both directions (forward and reverse) were obtained on an Applied Biosystems 3130xl Avant Genetic analyzer using BigDye v. 3.1 chemistry. Electropherograms were analysed and assembled in Sequencher v. 4.10.1 (Gene Codes Corp.).

Phylogenetic statistical analysis

Multiple sequence alignments of each gene were made with Geneious Pro v. 6.1.6 (Drummond et al. 2011). Bayesian inference (BI) was used to reconstruct the phylogenies using MrBayes v. 3.2.1 (Ronquist et al. 2012). jModelTest v. 2.1.4 (Posada 2008) was used to carry out statistical selection of best-fit models of nucleotide substitution using the corrected Akaike information criteria (AICc). Initial analyses showed that individual genes were broadly congruent, thus nucleotide alignments of all genes were concatenated using Geneious, and separate partitions created for each gene with their own model of nucleotide substitution. Analyses were run twice for 5×10^7 generations. Samples were taken from the posterior every 1000 generations. Convergence of all parameters was checked using the internal diagnostics of the standard deviation of split frequencies and performance scale reduction factors (PSRF), and then externally with Tracer v. 1.5 (Rambaut & Drummond 2007). On this basis the first 25 % of generations were discarded as burn-in. *Phytophthora multivora* was used as the outgroup.

Morphology and physiology of isolates

Isolates were grown at 20°C in darkness on clarified V8-juice agar (V8A), malt extract agar (MEA), corn-meal agar (CMA) and potato-dextrose agar (PDA) (all from BBL, Becton, Dickinson & Co., Sparks, MD 21152, USA). Colony morphologies were photographed and described according to Brasier & Griffin (1979), Erwin & Ribeiro (1996) and Jung et al. (2003) after 10 days.

Growth rates on V8A, MEA, CMA and PDA were measured on replicate plates in an incubator set at 20°C. To establish the cultures for the growth studies, 6 mm plugs of mycelium were taken from the edge of actively growing of cultures on PDA, and placed in the centre of fresh plates. Radial growth rate was recorded at two perpendicular diameters after 5, 7, 9, 12, and 14-days incubation (Jung et al. 1999). Daily growth (mm) was determined by the equation: (colony diam. at 7^{th} day – diam. of inoculum plug)/7(d) (Englander et al. 2006).

[4] This is the target locus routinely sequenced for *Phytophthora* species (see Cooke et al. 2000).

To determine the cardinal temperatures for optimal PTA growth (a necessary part of the species description process), the 28 isolates (i.e., 11 PTA, 8 *P. castaneae*, 3 *P. "cocois"*, 6 *P. heveae*) were subcultured onto PDA plates and incubated for 24 h at 20°C to initiate growth. Five replicate plates for each isolate and temperature were then transferred to incubators set at 0, 5, 10, 15, 17.5, 20, 22.5, 25, 30 and 32.5°C, and radial colony growth was measured as above after 5–7 days.

Sporangia were produced by incubating 10-day-old cultures of the 28 isolates, grown on 10% clarified V8 juice agar in non-sterile soil extract overnight, at 20°C, under continuous light. Non-sterile soil extract is produced by mixing 200 g of soil with RO water, mixing and then coarsely filtering through paper towels (Jeffers 2006). The following day, the cultures were incubated at 4°C for 15 min prior to fixing with Aniline Blue and/or Acid Fuchsin in lacto-glycerol solution and microscopic examination of sporangial length × breadth, shape, assessment of caducity and size of exit pore. Fifty sporangia of each isolate were assessed.

Oospores were produced by growing the 28 isolates on 10% clarified V8-juice agar, for 10 days, in the dark, at 20°C. After this time, samples were fixed with Aniline Blue and/or Acid Fuchsin in lacto-glycerol solution for microscopic examination of oogonia/oospore diameter and length, gametangial morphology, and wall morphology. Fifty oogonia/oospores were assessed for each isolate.

Statistical analysis of morphology and physiology data

Statistical analyses and data visualisation were conducted with R 3.0.2 (R Development Core Team 2013), using the ggplot2 package.

For the radial growth analyses (Fig. 5), box plots show the median, upper and lower quartiles, and the 'whisker' extends to the outlying data up to a maximum of 1.5× the interquartile range. Individual outliers outside this range are shown as dots.

For comparative oogonial widths (Fig. 9), tests of significance were conducted using the Welch Two Sample t-test, with p values reported.

3.3 Results

Phylogenetics

Based upon the multi-gene analysis, and concatenation of eight loci, PTA is a well-supported, discrete taxonomic entity (Fig. 2) separate from *P. heveae* (associated with rubber (*Hevea brasiliensis* Euphorbiaceae) and avocado (*Persea americana* Lauraceae)) and *P. castaneae* (associated with sweet chestnut species *Castanea* spp. and *Castanopsis* spp. Fagaceae). PTA is most closely related to *P. "cocois"*, a new species isolated from coconut (*Cocos* Arecaceae), previously misidentified as *P. castaneae* (Fig. 3). We have thus contributed two new species to ITS Clade 5.

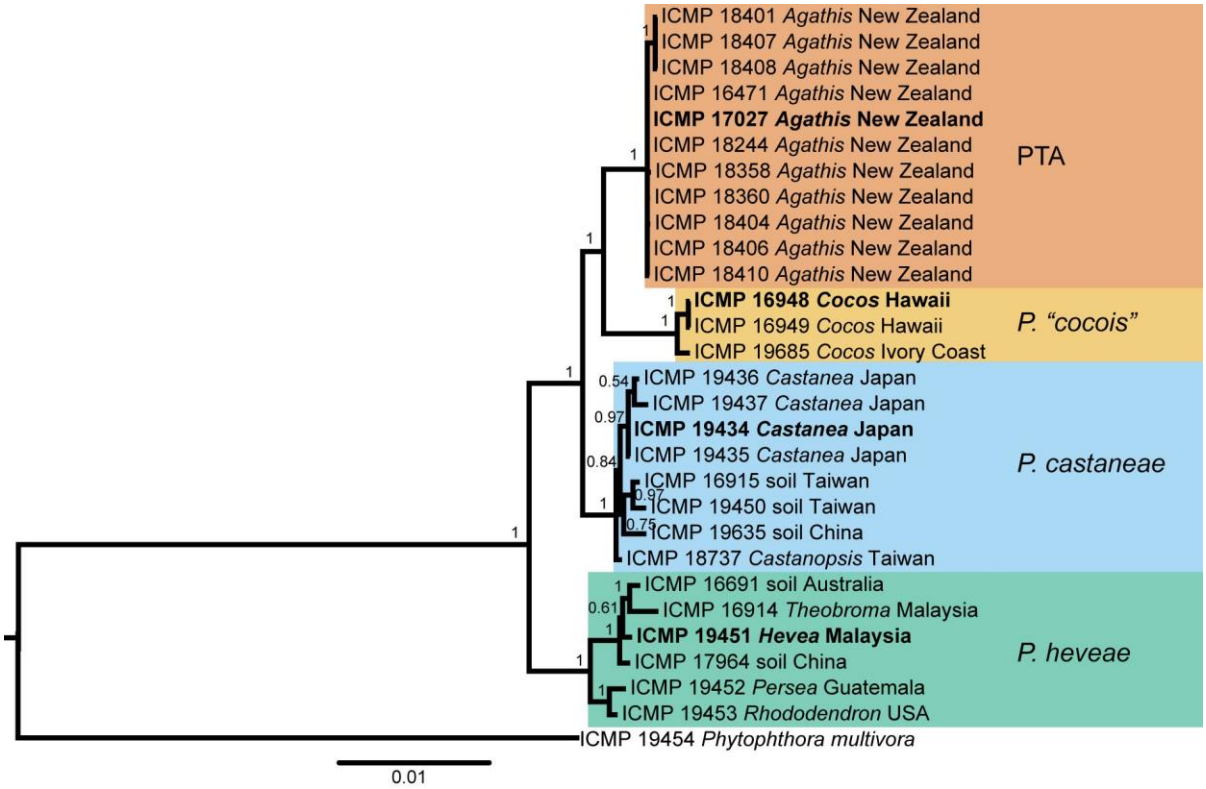


Figure 3. Resolved phylogenetic tree of Phytophthora ITS Clade 5 using eight concatenated loci. Isolates in bold type are types or proposed "types" of the respective subclades.

Morphology and physiology

PTA colony morphology after 7 days was very uniform across the three isolates of PTA tested on most of the media examined (Fig. 4). Colonies are loosely aerial. On 5% clarified V8-juice agar, there is a "radial" pattern ("weakly stellate") (Fig. 4).

There were different growth-rate responses from each of the four species studied (Fig. 5). Overall, *P. castaneae* was the fastest growing on three of the four media tested (Fig. 5).

PTA had a cardinal temperature growth optimum of 22.5°C, similar to those of the other three Clade 5 *Phytopthora* species (Fig. 6). However, the cardinal temperature plot for PTA appeared to be skewed compared to those of the other three species, with PTA growing relatively faster than the other species at 5–22.5°C, but slightly slower at >25°C. Unlike the other three species, PTA did not grow at 30°C.

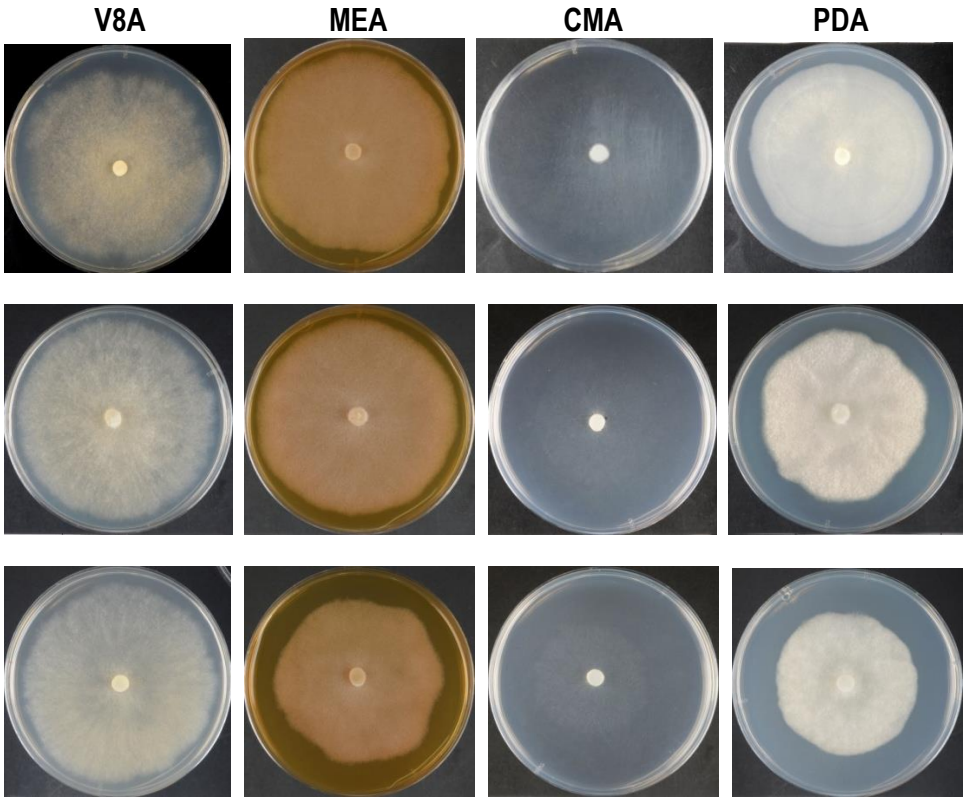


Figure 4. Comparative colony morphology of three PTA isolates: (Top) ICMP 18404 ex Trounson; (Centre) ICMP 18406 ex Huia; I(Bottom) CMP 18407 ex Waipoua, on V8-juice agar, malt-extract agar, corn-meal agar, and potato-dextrose agar after 7-days at 25°C.

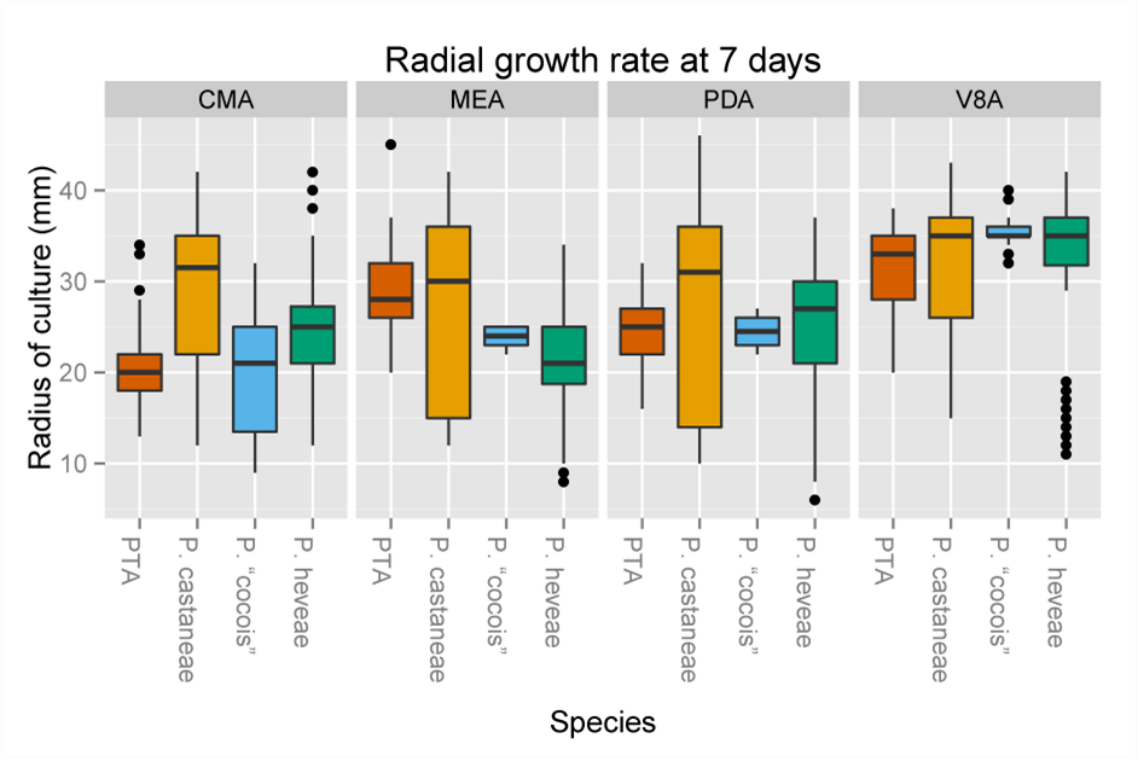


Figure 5. Comparative growth of PTA, *P. castaneae*, *P. "cocois"*, and *P. heveae* on four selected agar media: (L–R) corn-meal, malt extract, potato-dextrose, V8-juice.

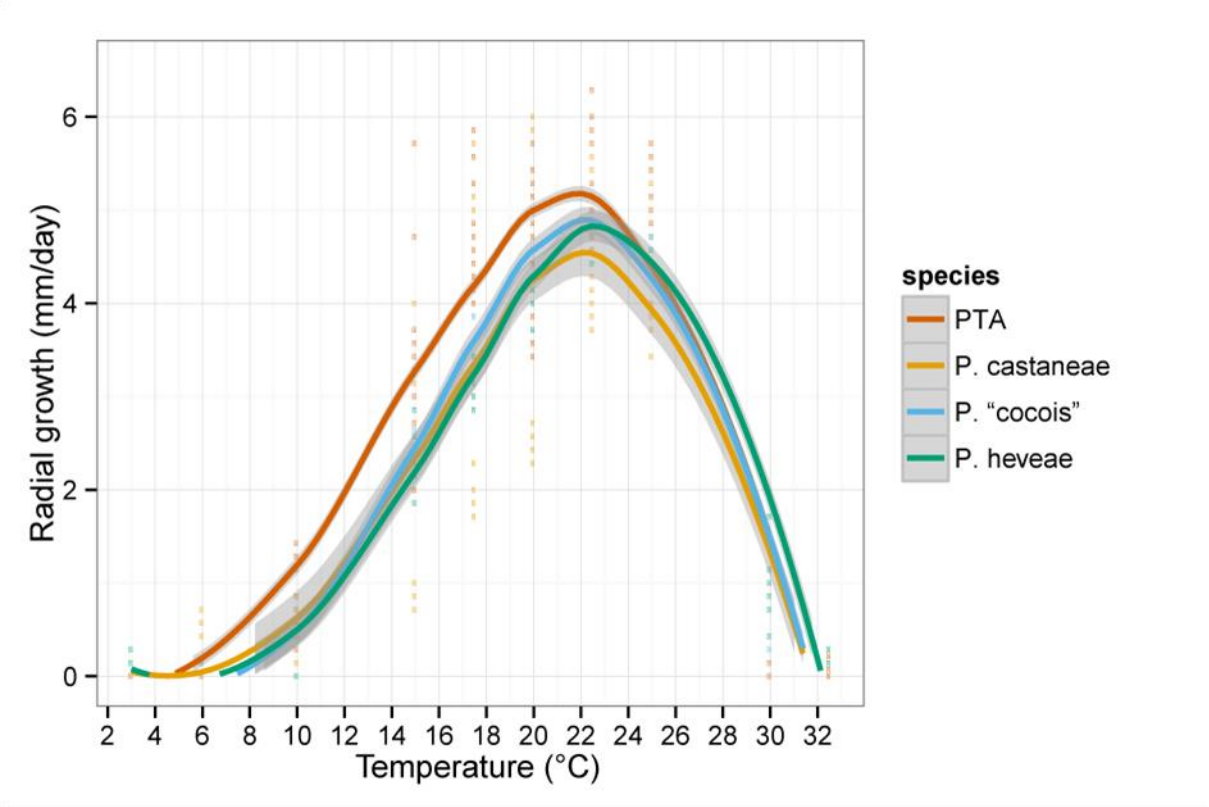


Figure 6. Cardinal temperature plot (on 5% V8-juice agar) of 28 isolates in *Phytophthora* Clade 5. Data variation smoothed using loess curves, with 95% confidence band (grey shading).

Sporangia were globose to ovoid-ellipsoid, and could be formed via internal proliferation. Sporangia were non-caducous (although some isolates had a somewhat defined septum at the base of the sporangium), semi-papillate. The range of length × breadth dimensions of sporangia from 11 PTA isolates was 30–45 µm × 18–25 µm (Fig. 7). Vegetative hyphae were simple, with slight swellings, and lacking chlamydospores in culture.

Figure 7. Photomicrograph of sporangium of PTA (ICMP 18403). Scale bar equals 25 µm.

PTA is homothallic, with isolates forming oogonia quickly (3–4 days) and abundantly on V8A. Oogonia from 11 PTA isolates had a median width of 32.2 µm. Antheridia are amphigynous (Fig. 8). Oospores nearly fill the oogonia, and the median oospore width of 11 PTA isolates was 27.5 µm.

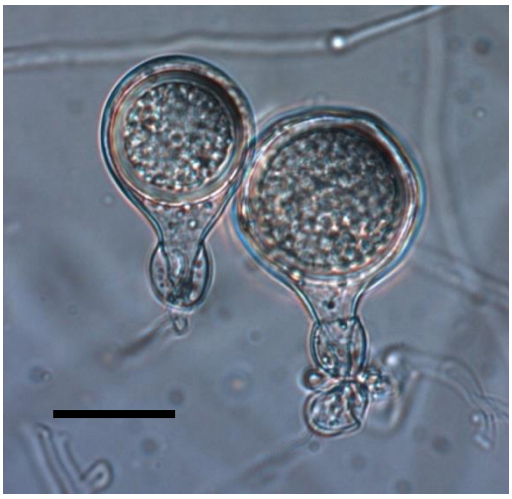


Figure 8. Oogonia of PTA (image courtesy of Margaret Dick). Scale bar equals 30 µm.

Mean oospore and oogonia widths were significantly differentiated between the four species (Fig. 9). PTA gametangia were significantly larger (p-value < 2.2×10^{-16}) than those of *P. heveae*, *P. castaneae* and *P. "cocois"*. Mean PTA oospore width was 27.7 µm (range 19.8–35 µm; n = 440); and mean oogonium diameter was 31.9 µm (range 22.2–45 µm; n = 440).

PTA oogonium wall morphology can be described as mildly stipulate, and the antheridia are globose (Table 2). PTA gametangia can be differentiated from those of *P. castaneae* which have coarsely bullate, rugose oogonium walls and narrow antheridia; and from those of *P. "cocois"* which have mildly bullate oogonium walls and strongly reflexed antheridia (Table 2). *P. heveae* oogonia are distinguished from those of any of the other three species by having very smooth walls and narrow, funnel-shaped antheridia (Table 2).

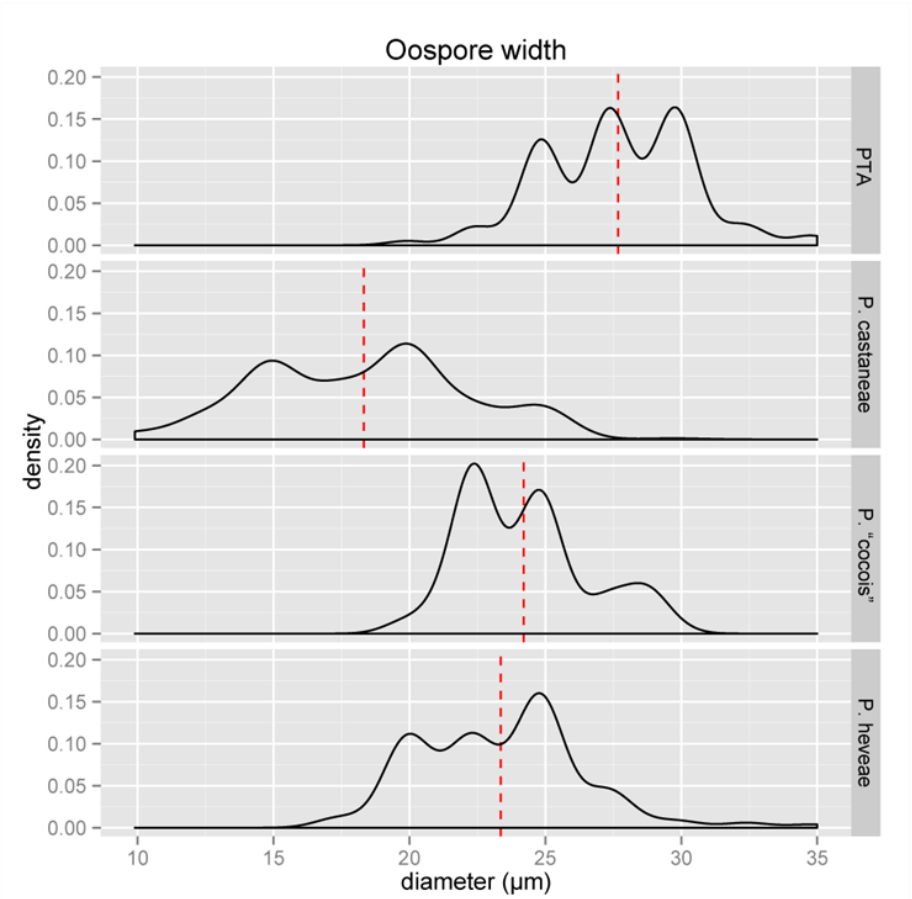
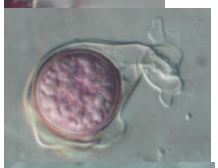


Figure 9. Frequency distribution of oospore width variation of PTA, *P. castaneae*, *P. "cocois"* and *P. heveae*. Red dashed lines indicate mean values.

Table 2. Comparative gametangial morphology between members of Clade 5

Phytophthora spp.	Gametangia	Morphology
P. castaneae		Coarsely bullate oogonium with rugose protuberances Narrow amphigynous antheridia
P. "cocois"		Mildly bullate oognium Reflexed antheridia
PTA		Mildly stipulate oogonium Globose amphigynous antherida
P. heveae		Smooth walled oogonium Funnel-shaped, amphigynous antheridia

3.4 Discussion

Fungal taxonomy has evolved since the times of Gadgil in the 1970s, with morphology now only one component in the identification of unknown organisms. Since publication of the first ITS-based molecular phylogeny of the genus *Phytophthora* by Cooke et al. (2000), DNA sequence analysis has become routinely applied to assist with the resolution of species boundaries. Moreover, the limitations of the early ITS-based systems have been realised, and now there is a prerequisite number of other gene regions that need to be sequenced before species-based validations can be proposed. This is the case with PTA, as when the organism is studied by ITS alone it is a 100% match to *P. castaneae* (Beever et al. 2009). Our multi-gene analysis has assisted in the differentiation of the specific entities within ITS Clade 5. Not only is there strong genetic evidence, there is also morphological segregation, both in oospore diameter and sporangial papillation, caducity (i.e., persistence of sporangia) and cardinal temperature for growth. Further there is host differentiation, with *P. castaneae* being associated with sweet chestnut *Castanea* and *Castanopsis* from Taiwan and Japan (Fagaceae), and *P. heveae* having a wider host range including rubber *Hevea brasiliensis* (Euphorbiaceae) and cacao *Theobroma cacao* (Malvaceae). On the basis of genetic data, comparative morphology and host specificity, we propose in a forthcoming publication to formally name PTA as *Phytophthora "agathidicida"* (ined.; the "*Agathis*-killing" *Phytophthora*), a new species within ITS Clade 5. Additionally, we have identified another new morphospecies within Clade 5, *P. "cocois"* (ined.; the "coconut" *Phytophthora*), previously misidentified as *P. castaneae*. We have thus doubled the number of species in *Phytophthora* ITS Clade 5.

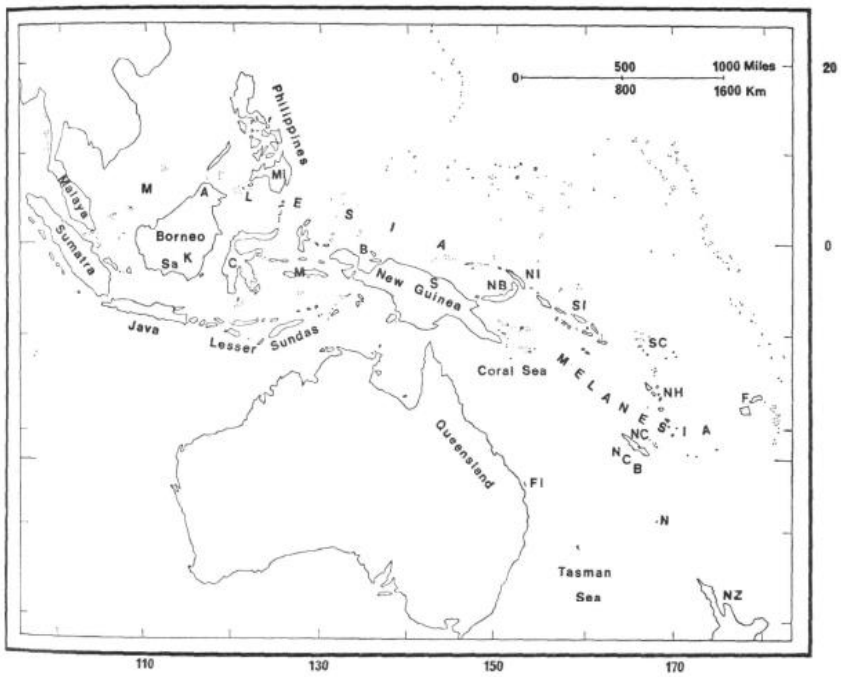


Fig. 1. Malesia, Melanesia and Australasia showing places mentioned in the text. B, Biak Island; C, Celebes; F, Fiji; K, Kalimantan; FI, Fraser Island; M, Moluccas; Mi, Mindanao; N, Norfolk Island; NB, New Britain; NC, New Caledonia; NCB, New Caledonia Basin; NH, New Hebrides; NI, New Ireland; NZ, New Zealand; S, Sepik; Sa, Sampit; SC, Santa Cruz island; SI, Solomon islands.

Figure 10. Distribution of *Agathis* species (extracted from Whitmore 1980).

In considering the potential origins of PTA, it is useful to consider the distribution of *Agathis* species (Fig. 10). *Agathis* is distributed from peninsular Malaysia to New Zealand, including

Malesia, the Philippines, New Guinea, Melanesia and Australia (Whitmore 1977). The classification of *Agathis* remains somewhat controversial. In his 1980 revision of the taxa, Whitmore recognised 13 species (Whitmore 1980). De Laubenfels (1988) recognised 21 species, but his treatment has not gained acceptance (Setoguchi et al. 1998). The highest concentration of species diversity of Araucariaceae is in New Caledonia, with five endemic species of *Agathis*, all well diversified in their morphological characteristics. Vavilov proposed that the centre of diversity of a species might well be the centre of origin of the species (Zakharov 2005). As such, New Caledonia may well represent the centre of diversity of *Agathis.* Understanding the spectrum of soilborne *Phytophthora* species that have co-evolved in New Caledonia may give further clues as to where PTA was translocated from to New Zealand.

It may be speculated that PTA was introduced from a region where *Agathis* species are present (Figure 10) or from where *P. castaneae* and *P. heveae* co-occur (e.g., Papua New Guinea, Australia, Taiwan) into the northern parts of New Zealand back in the early 1900s. One possible pathway could be via soil ballast of timber ships. For example, Cumberland (1966) describes the introductory pathway of Manchurian rice grass (*Zizania latifolia*) into Northland via the soil ballast of ships. Ironically, timber ships using clay-brick ballast translocated seeds of *Zizania* from its home in East Asia to the ports of Northland, New Zealand, and in return loaded kauri logs on board (Arnold 1959). What soil pathogens were also deposited together with the clay-brick ballast? It is apparent that further study of morphological and molecular variation of isolates from around the world, particularly from areas where *Agathis* species occur, is warranted to better clarify the number and relationships of the ITS Clade 5 species, and the discovery of PTA overseas would help confirm its exotic status.

4 Host range of PTA

4.1 Introduction

Phytophthora spp. are particularly devastating when the invaded system possesses multiple unrelated susceptible host species (Goss et al. 2009). Previous tests using stem-wounding inoculation gave no indication that PTA could establish on such wounded tissue of any species other than kauri (Beever et al. 2007). However, these studies did not preclude the ability of PTA to infect root tissue and establish disease in species other than kauri. This chapter aimed to assess the effect of a root-based inoculation of PTA on young kauri and non-kauri plant species, without deliberate wounding. This was achieved by placing the plants into a potting medium in which PTA has been added using colonized millet seed. In this manner, PTA infection was provided via soil; however, one of the limitations of this approach is the use of young plants as test-hosts, which does not reflect the biology of the non-kauri plants in their native forest context.

4.2 Materials and Methods

4.2.1 Preliminary pathogenicity studies

Trial design

A series of preliminary trials were commenced in September 2010 to assess the suitability of the soil-based root inoculation methodology of Vettraino et al. (2001). This study also aimed to assess the pathogencity of PTA isolates and the duration of the test-period for symptoms to be displayed in kauri. In this pilot-study, three isolates of PTA: ICMP; 18403 (ex Raetea), 18404 (ex Trounson), 18405 (ex Trounson) were tested (Appendix 1A). The preliminary host comparisons included; 3- and 5-year old kauri, 3-year old Queensland kauri and 2-year old rimu in the combinations and timings articulated in Table 3.

Table 3. Plants tested for susceptibility to PTA as part of preliminary studies. Date of inoculation indicated

	18403	18404	18405	Sequential harvests
Kauri 3-year old	21/09/10	21/09/10	21/09/10	Yes
Kauri 5-year old	27/01/11	27/01/11	Not tested	Yes
Qld kauri 3-year old	24/09/10	24/09/10	24/09/10	Yes
Rimu 2-year old	28/01/11	28/01/11	Not tested	Yes

N.B. ICMP 18404 and 18405 were isolated from separate cambium samples from the same tree.

These trial plants were grouped in three comparative prelininary studies:

- 1: (September 2010) 3-year old kauri v. Queensland kauri (using three isolates of PTA
- 2: (January 2011) 5-year old kauri (using two isolates of PTA)
- 3: (January 2011) 2-year old rimu (using two isolates of PTA).

Inoculation procedure

Inoculation experiments/tests were conducted in a PC-1, naturally lit growth house at Landcare Research, St Johns, Auckland, conditions for which are provided in Appendix 2A. All research at LCR was carried out under a CTO permit approving propagation and communication of PTA. PTA inoculum was prepared by growing isolate ICMP 18403 (selected as the PTA test isolate at the end of the preliminary pathogenicity studies) for 4–6 weeks at 20°C on sterilised millet seeds thoroughly moistened with V8-juice broth (Vettraino

et al. 2001; Jeffers 2006). The inoculum was repeatedly rinsed with sterile RO water to remove unassimilated nutrients before being added to sterile potting mix at a rate of 25 ml per litre of potting mix. The inoculum and potting mix were mixed in a sterilised cement mixer and then put into No. 4 or No. 6 planter bags. Plants were removed from their original potting bags and the potting medium shaken off, before the plants were placed in the inoculum mix and the roots were covered. There were nine replicate bags treated with PTA. Another set of nine pots were established as a control, using sterilised millet seeds thoroughly moistened with V8-juice broth, and incorporated into sterile potting mix at a rate of 25 ml per litre. Plants were immediately watered with tap-water to field capacity, and then watered every second day The PTA-treated plants were placed on one side of the glasshouse, and the controls on the other side of the glasshouse, in separate metal trays to catch all run-off. The plants were blocked according to test-plant species, and these blocks randomised on the bench.

Preliminary trial inoculations

For Comparisons 1 and 2, six test plants were inoculated with each PTA isolate, and 6 plants with control inoculum. For Comparison 3, 12 rimu plants were inoculated with each PTA isolate and 12 rimu plants with control inoculum. Sequential harvests were made of PTA-treated plants (and paired controls) to study the development of disease progression. In Comparison 1, the first harvest was carried out after 4 days (Table 4), with subsequent harvests after 21, 30, and 35 days.

Table 4. Comparison 1: timing of sequential harvests after inoculation (21/09/10) for 3-year old kauri v. 3-year old Queensland kauri

	18403	18404	18405	Controls
Kauri 3-year old	35 days	4, 21 days	30 days	4, 21, 30, 35 days
Qld kauri 3-year old	35 days	4, 21 days	30 days	4, 21, 30, 35 days
Sample size	n = 6	n = 3	n = 6	n = 1, 1, 1, 3

For the study on 5-year old kauri (Comparison 2), the experiment commenced on 27/01/11, with the first sample occurring 10 days after inoculation (Table 5).

Table 5. Comparison 2: timing of sequential harvests after inoculation (27/01/11) for 5-year old kauri

	18403	18404	Controls
Kauri 5-year old	10, 20, 30 days	10, 20, 30 days	10, 20, 30 days
Sample size	n = 2	n = 2	n = 2

The periodicity of the timing for the sampling of the rimu study (Comparison 3) was 35, 60, and 90 days after inoculation (Table 6).

Table 6. Comparison 3: timing of sequential harvests after inoculation (28/01/11) for 2-year old rimu

	18403	18404	Controls
Rimu 2-year old	35, 60, 90 days	35, 60, 90 days	35, 60, 90 days
Sample size	n = 4	n = 4	n = 3, 3, 6

Plant dissection and post-harvest treatment

Whole plants were removed from the glasshouse to the Ecology Laboratory where the plants were removed from their pots and the adhering soils washed off. All liquid waste was captured and later steam sterilised. The plants roots were divided into three size classes: 1) primary root (or tap root), secondary roots (originating from primary root), distal roots (originating from secondary roots). The collar material was from the junction between primary root and stem (Fig. 11)

Figure 11. 3-year old kauri control (L) and PTA-treated (R) plants four days after inoculation

Plant material was surface sterilised in 70% ethanol solution for thirty seconds, and then rinsed twice in sterile reverse osmosis (RO) water. The plant materials were blotted dry on clean paper towels and plated to *Phytophthora*-selective agar (Jeffers 2006). Plates were wrapped in foil and incubated at 18°C. Characteristic oospores of PTA were observed in 4–7 days.

4.2.2 Host range investigation

Species tested as potential hosts

Twenty plant species were screened as hosts of PTA. The majority of these plants are either species in the forest succession to kauri or species that are part of the terminal forest understory (Tony Beauchamp pers. comm.). Pōhutukawa and Queensland kauri were also included to test their susceptibility to PTA using the soil-based inoculation method (Table 7). The kauri plants (3-year old) were sourced from Scion Nursery in 2010. These plants originated from six trees from Waipoua Nursery, Northland, planted in Scion, Rotorua, 1953–54 (Margaret Dick pers. comm.). Scion also supplied the Queensland kauri, and rimu seedlings[5]. All of the other plants were sourced as nursery stock from Oratia Nursery.

[5] No further information available on the provenance of these seedlings (personal communication Margaret Dick).

Approximate ages of the plants from the commercial nursery ranged between 6–24 months (Table 7).

Inoculation procedure

Inoculations were conducted as detailed under 4.2.1 (p. 19).

Table 7. Plants tested for susceptibility to PTA

Common name	Binomial	Approx. age	Date of inoculation
Gymnosperm trees			
Kauri	*Agathis australis*	*4-years*	*21/09/2010/*
Rimu	*Dacrydium cupressinum*	*24-months*	*28/01/2011*
Tānekaha	*Phyllocladus trichomanoides*	*18-months*	*30/01/2012*
Kahikatea	*Dacrycarpus dacrydioides*	*18-months*	*20/03/2012*
Tōtara	*Podocarpus totara*	*6-months*	*11/03/2011*
Dicotyledonous trees and shrubs			
Māmāngi	*Coprosma arborea*	*6-months*	*01/12/2012*
Karamū	*Coprosma robusta*	*18-months*	*10/02/2013*
Kānono	*Coprosma grandifolia*	*18-months*	*10/02/2013*
Pōhutukawa	*Metrosideros excelsa*	*18-months*	*17/08/2012*
Rewarewa	*Knightia excelsa*	*12-months*	*17/08/2012*
Mānuka	*Leptospermum scoparium*	*6-months*	*01/12/2012*
Kānuka	*Kunzea ericoides*	*6-months*	*01/12/2012*
Māpou	*Myrsine australis*	*18-months*	*10/02/2013*
Pigeonwood	*Hedycarya arborea*	*24-months*	*01/12/2012*
Karaka	*Corynocarpus laevigatus*	*24-months*	*20/10/2011*
Tawa	*Beilschmiedia tawa*	*12-months*	*20/10/2011*
Taraire	*Beilschmiedia tarairi*	*12-months*	*20/10/2011*
Korokio	*Corokia buddleioides*	*12-months*	*01/12/2012*
Tanguru	*Olearia albida*	*18-months*	*20/11/2011*
White maire	*Nestegis lanceolata*	*24-months*	*10/02/2013*
Exotic gymnosperm tree			
Queensland kauri	*Agathis robusta*	*3 years*	*21/09/2010*

Harvesting and processing of plant material

Three-months after inoculation, the seedlings were extracted from the potting mix. The used potting mix was disposed of in an Interwaste Quarantine Refuse Bin (Interwaste bin). The roots were segregated into three size classes: primary roots, secondary roots and the distal roots (as defined earlier). The primary root of the seedlings represented the original tap root, which joins the main stem at the collar. The collar material (i.e., the junction between primary root and stem) was also recovered (Fig. 12). A similar recovery exercise was carried out on the control plants. The experiments began in January 2011, and were concluded in May 2013.

The shoot height and mass, and root mass were recorded (Appendix 2B), as well as any signs of disease: root necrosis, shoot chlorosis, shoot desiccation, leaf-loss, leaf necrosis, and/or shoot death were assessed qualitatively (Vettraino et al. 2001). For each host species, 20 root pieces and five collar pieces were surface-sterilised in 70% ethanol for 30 seconds and then rinsed in sterile RO water. The plant 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 4–7 days, and representative cultures were sub-cultured to fresh PDA plates for DNA analysis.

Figure 12. Image depicting white-coloured, collar region of tawa (*Beilschmiedia tawa*) (above profusion of roots) inoculated with ICMP 18403.

All scalpels and sampling implements were decontaminated by flame sterilisation between the sampling of plant root systems. All benches were decontaminated with 70% ethanol. All solid waste was disposed of in an Interwaste bin. All liquid wastes were captured and autoclaved at 121°C for 20 min prior to disposal.

In order to visualise PTA *in planta*, root samples taken during the host-studies carried out in 2013 were fixed, cleared and stained by the methodology of Jung et al. (2013). Representative samples (n = 5) of all infected root and collar material and negative controls were fixed in the histology preservative, FAA (Formalin–Acetic acid–Alcohol solution; 50:10:5% w/v). This material was cleared with 10% KOH for 30 min and then bleached in 5% alkaline H_2O_2. The plant material was then acidified in 5% HCl, and stained with Aniline Blue (Brundrett et al. 1996). The material was then destained overnight in a lactic acid glycerol solution (Koske & Gemma 1989). The material was examined under a compound light microscope, and photo-micrographs captured using a Nikon DSi-1 camera.

Statistical analysis

A randomised block design was used for this experiment with PTA-treated plants segregated from negative controls, and plants "blocked" according to species. There were nine replicate plants for each species, treated with and without PTA. Data sets for shoot height and biomass, and root mass for PTA-treated and negative control plants were all analysed statistically using a two-tailed Student t-test.

4.3 Results

4.3.1 Preliminary pathogenicity studies

Onset of infection

PTA infection had commenced after 4 days in 3-year old kauri (Table 8). After four days, PTA was recovered from distal roots, with the disease progressing to secondary and primary

roots after 10 days (Table 8). PTA was recovered from the primary root after 21 days (Fig. 13), and had reached the collar by 21 days, by which stage, the plants were dead.

Table 8. Onset of infection for 3-year old and 5-year old kauri, 3-year old Queensland kauri and 2-year old rimu (D.a.i. = days after inoculation).

	3-yr old kauri	5-yr old kauri	Qld kauri	rimu
D.a.i.	**PTA recovery**	**PTA recovery**	**PTA recovery**	**PTA recovery**
4-days	Distal	n/t	Nil	n/t
10-days	Distal, 2°	Distal	Nil	n/t
21-days	Distal, 2°, 1°, Collar	Distal, 2°	Nil	n/t
30-days	n/t	Distal, 2°, 1°	Nil	Nil
35-days	n/t	Distal, 2°, 1°, Collar	n/t	n/t
60-days	n/t	n/t	Nil	Distal
90-days	n/t	n/t	Nil	Distal, 2°
Sample size	n = 6	n = 6	n = 6	n = 12

Figure 13. PTA infection causing dicolouration of primary root of 3-year old kauri (21 days after inoculation).

For 5-year old kauri, PTA was recovered from distal roots after 10 days, with disease progressing to the collar, resulting in death by 35 days after inoculation (Table 8). In sharp contrast, Queensland kauri displayed no root dysfunction and foliar symptoms (Fig. 14) after 90 days.

Figure 14. Queensland kauri control (left) and PTA-treated (right) plants showing healthy distal root growth.

After two months the lower leaves of rimu started to display symptoms of desiccation (Fig. 15). At this time, PTA was only recovered from the distal roots of rimu (Table 8). After 90

days, PTA-treated rimu were harvested, and PTA was recovered from both secondary and distal roots of rimu. At the end of these preliminary experiments, the trial test period for hosts other than kauri was set at 3 months.

Figure 15. 2-year old rimu PTA-treated plant showing signs of lower leaf desiccation

Controls and variation between PTA isolates

No *Phytophthora* species were recovered from any of the control test plants. There was no difference observed in the onset of infection between the different isolates of PTA tested on 3- and 5-year old kauri and rimu; ICMP 18403 was selected as the PTA test isolate for the subsequent soil-based inoculation studies.

4.3.2 Host range investigation

Glasshouse conditions

Mean monthly temperatures varied according to seasonal variation in the naturally-lit glasshouse used for the pathogenicity studies (Fig. 16). January–April and October–December mean temperatures were above 18°C; temperatures were coolest in the winter months of June–September, with mean temperatures below 15°C (Fig. 16). Mean relative humidity (RH) January–April and October–December was 70–80% (Fig. 17). Relative humidity peaked during June–July (80–90%). Because of the seasonal variation in growth conditions, there was a temperature and RH differential between the months: 1) Jan–Apr and Oct–Dec; and 2) May–Sep. In order to minimise the potential temperature and RH differences, no root inoculation studies were carried out during May–July.

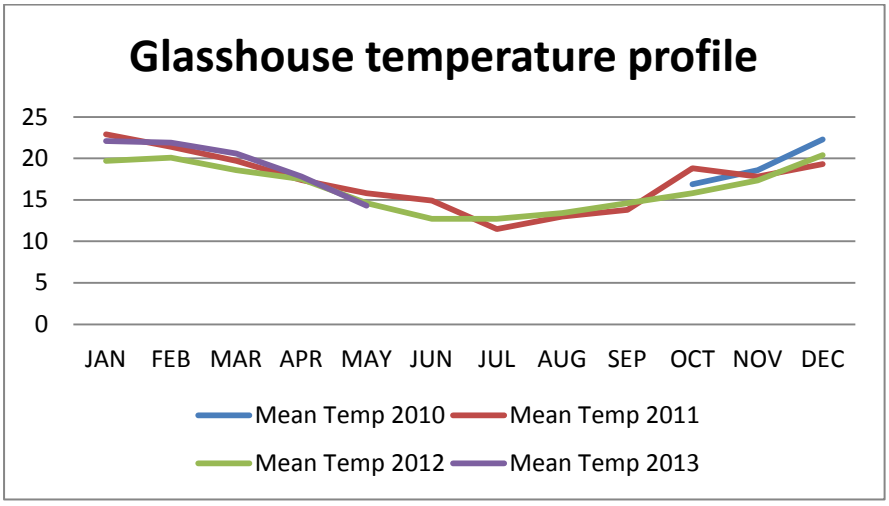


Figure 16. Glasshouse temperature profile Oct 2010–May 2013

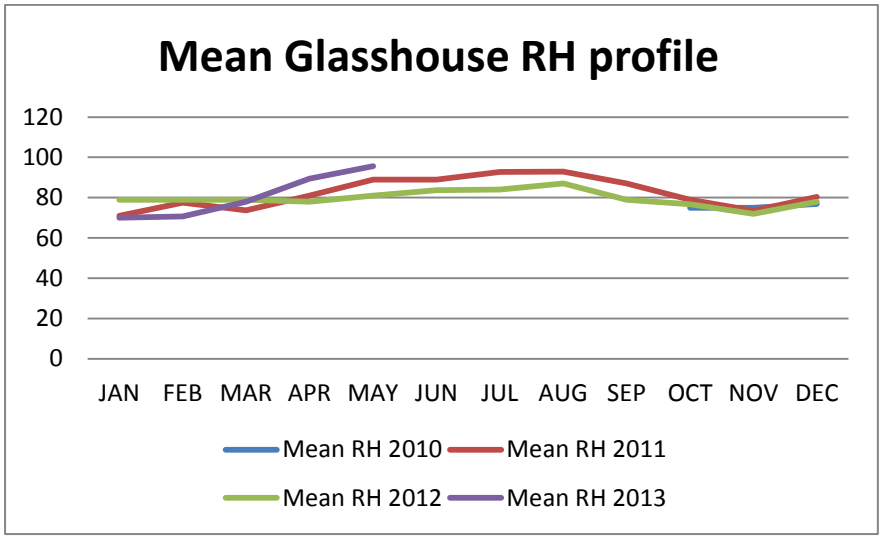


Figure 17. Glasshouse relative humidity (RH) profile Oct 2010–May 2013.

Condition of control plants after three months

PTA was not recovered from any of the plants inoculated with "control" millet. The general condition of the plants inoculated as the negative control, was a profusion of healthy "distal" roots (e.g., pōhutukawa; Fig. 18). Additionally, above-ground responses of the seedlings inoculated as "control" plants was a "healthy", turgid, green foliage (Fig. 19).

Figure 18. Healthy profusion of roots of pōhutukawa inoculated with negative control.

Figure 19. Healthy foliage and roots of *Coprosma arborea* 3 months after inoculation with "control" millet

Responses of plant species to PTA

Significant treatment effects of PTA on plants species were observed. Five types of foliar and root responses were observed following soil inoculation with PTA.

(1) The most severe disease impact was observed on four-year-old kauri plants, which were dead within 21 days of inoculation (Fig. 20; Table 9; Appendix 2B). Also dead at the end of the 3 month inoculation period, were kānuka and mānuka. For these three plant species, PTA had a significant negative impact upon root biomass (Table 9), and PTA was recovered from primary, secondary and distal roots, and collar material (Table 9). *P. cinnamomi* was also recovered from a single individual each of kānuka and mānuka treated with PTA (Table 9).

The other member of the Myrtaceae tested, pōhutukawa, also showed root decline symptoms and significant reduction in root biomass (Table 9; Fig. 21). PTA was recovered from primary, secondary and distal roots, but not from the collar of PTA-treated pōhutukawa after three months.

Rimu (Podocarpaceae) displayed lower leaf death and desiccation of foliage and root death, and a significant reduction in root biomass (Table 9), with PTA being recovered from primary, secondary and distal roots. *P. europaea* was recovered from one individual of rimu inoculated with PTA (Table 9).

Root biomass of rewarewa (the only representative of the Proteaceae) was significantly reduced by PTA (Table 9). PTA was recovered from diseased primary, secondary and distal roots of rewarewa inoculated with PTA.

Table 9. Plant responses to deliberate inoculation with PTA. (Each recovery of a non-PTA *Phytophthora* sp. represents a single isolation.)

Plant binomial	Root health status	Mean root mass Control (g)	Mean root mass PTA (g)	PTA recovery	Site of PTA recovery
Gymnosperm trees					
Agathis australis	Dead	86.9a	69.5b	Yes	1°, 2°, D, C
Dacrydium cupressinum	Unhealthy (foliage desiccated)	899a	505b	Yes; (+ *P. europaea*)	1°, 2°, D
Phyllocladus trichomanoides	Healthy	77.3a	118b	No	—
Dacrycarpus dacrydioides	Healthy	23.8a	42.6b	No	—
Podocarpus totara	Growth promotion	89.5a	117.1b	No	—
Other woody spp.					
Coprosma arborea	Unhealthy	5.2a	4.6a	Yes	1°, 2°, D
Coprosma robusta	Growth promotion	103.5a	134.4b	No	—
Coprosma grandifolia	Healthy	53.9a	49.9a	No	—
**Metrosideros excelsa*	Unhealthy	187.4a	157.7b	Yes	1°, 2°, D
Knightia excelsa	Unhealthy	67.9a	52.2b	Yes	1°, 2°, D
Leptospermum scoparium	Dead	21.2a	11.9b	Yes; (+ *P. cinnamomi*)	1°, 2°, D, C
Kunzea ericoides	Dead	45.3a	18.1b	Yes; (+ *P. cinnamomi*)	1°, 2°, D, C
Myrsine australis	Healthy	21.1a	24.2a	No	—
Hedycarya arborea	Unhealthy	68.7a	52.7b	Yes	1°, 2°, D
Corynocarpus laevigatus	Healthy	13.1a	12.4a	No	—
Beilschmiedia tawa	Healthy	69.2a	72.5a	Yes	1°, 2°, D, C
Beilschmiedia tarairi	Healthy	75.9a	80.9a	Yes	1°, 2°, D, C
Corokia buddleioides	Root decline	25.3a	18.4b	Yes	1°, 2°, D
Olearia albida	Unhealthy	273.5a	228.4b	No	—
Nestegis lanceolata	Healthy	128.5a	144a	No; (+ *P. cinnamomi*)	—
Exotic tree					
Agathis robusta	Growth promotion	40.3a	46.6b	No	—

N.B. Paired PTA/Control root mass values followed by different letters are significantly different (P<0.05). 1°= primary roots; 2° = secondary roots; D = distal roots; C = collar

Figure 20. Kauri seedlings, negative control (L) and PTA-treated (R) 3 months after soil inoculation with PTA ICMP 18403.

Figure 21. Roots of pōhutukawa (*Metrosideros excelsa*) control plant (L), PTA-treated (R) 3 months after soil inoculation with PTA ICMP 18403.

(2) The second type of response was displayed as a dysfunction either above or below ground, but rarely both together. These symptoms in general were less pronounced, and did not result in mortality after 3 months. For example, the roots of pigeonwood were unchanged by inoculation, but the young leaves of inoculated plants showed marginal necrosis and tip death (Table 9). In contrast, korokio remained healthy above ground, but the root systems were stunted (Table 9). Māmāngi (Fig. 22) and tanguru also showed some shoot and root decline, with tanguru treated with PTA having a significantly less root biomass than the control plants (Table 9).

(3) The third type of response was characterised by very slight disease symptoms, with PTA recovered from healthy root systems. Both species of *Beilschmiedia* (Lauraceae) were hosts of PTA (Table 9). Tawa showed very slight constriction and discoloration at the collar (Fig. 12). PTA was recovered from both of their primary, secondary and distal roots, and from the collar of these two species (Table 9).

The fourth and fifth types of responses were: (4) that the plants were unchanged after 3 months; and (5) that there was evidence of synergistic growth promotion of either above- or below-ground parts. For example, the other gymnosperm tree species, tōtara (Fig. 23a, b), kahikatea and tānekaha, were healthy and even more vigorous after inoculation (Table 9). Other species that remained unchanged by inoculation were māpou, karaka, white maire and kānono (Table 9).

The three species of *Coprosma* displayed markedly different host reactions: (i) growth promotion (karamū); (ii) severe symptoms and death after 4 months (māmāngi; Fig. 22); (iii) no effect (kānono) (Table 9).

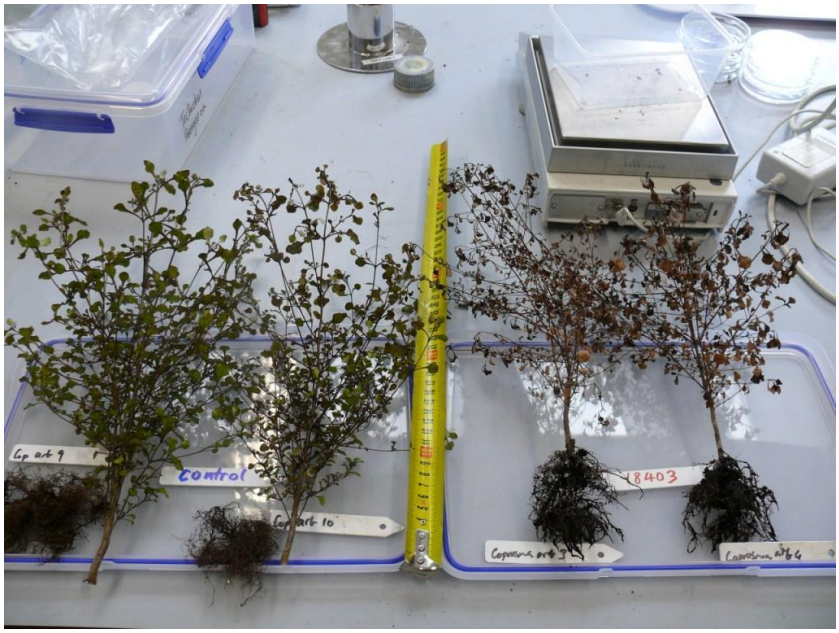


Figure 22. Māmāngi (*Coprosma arborea*) Control plants (L), and PTA-treated plants (R) 3 months after soil inoculation with PTA ICMP 18403. Note dried shoots of PTA-treated plants.

(a)

(b)

Figure 23. Tōtara (*Podocarpus totara*) growth increase: (a) roots and (b) shoot in relation to control 3-months after soil inoculation with PTA ICMP 18403 (inoculated treatment on left, doubled-over).

Kauri, kānuka, mānuka, pigeonwood, korokio and māpou roots and collar material from 2013 PTA inoculations were fixed in FAA, then cleared and stained. Typical arbuscular colonisation (by unknown species) was observed in most roots. Oospores of PTA were observed in the secondary roots of kauri at a density of 79.9 ± 12.6 oospores/mm^2 (Fig. 24). Of the other hosts, only the roots of korokio (Argophyllaceae) were found to contain oospores of PTA – these occurred at much lower density 6.5 ± 1.3 oospores/mm^2 (Fig. 25). PTA was recovered directly from the roots of this species (see Appendix 2).

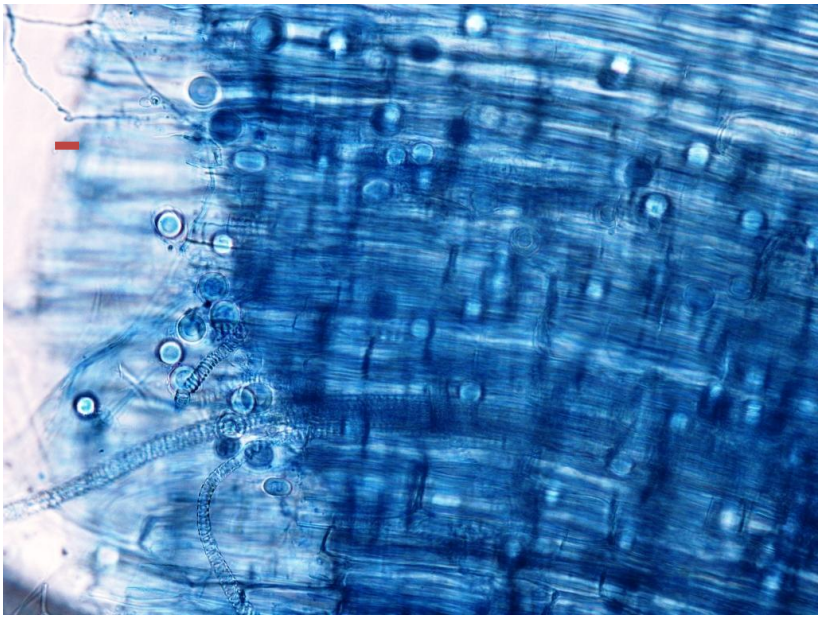


Figure 24. Cleared roots of kauri (*Agathis australis*) with thick-walled, globose to sub-globose oospores stained blue. Scale bar equals 35 µm.

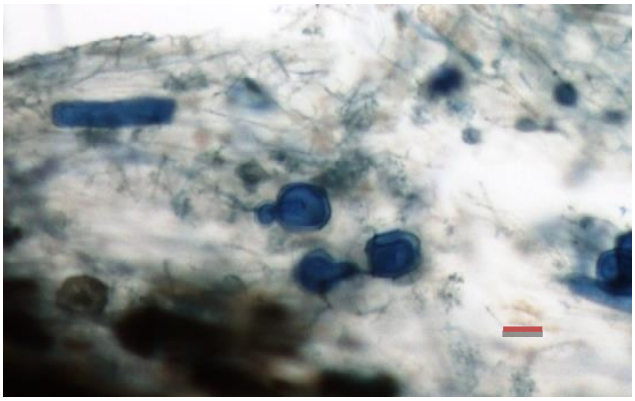


Figure 25. Cleared roots of korokio (*Corokia buddleioides*) with thick-walled, globose to sub-globose oospores stained blue. Scale bar equals 35 µm.

4.4 Discussion

The work of Beever et al. (2009) found that, apart from kauri, no other woody plant species showed any response when deliberately stem-inoculated with PTA. We have defined a host as any plant that PTA can live on, or in, and this was assayed by the recovery of PTA by plating plant tissues to selective media. From our study, the woody plant species that could become infected by PTA were kauri, rimu, māmāngi, pōhutukawa, rewarewa, kānuka,

mānuka, pigeonwood, korokia, tawa and taraire. All of the above species, except tawa and taraire showed an adverse reaction of seedlings to deliberate soil-based inoculation with PTA under glasshouse conditions.

Three-year old kauri were killed by PTA in 21 days, and 5-year old kauri in 35 days. This confirms the primary pathogenicity of PTA to kauri. Susceptible reactions resulting in death after 3 months were observed in kānuka and mānuka – both in the Myrtaceae. However these seedlings were the youngest of the plants tested, i.e., 6 months old; the observed death after 3-months may have been due to a seedling sensitivity, associated with the young plant's inability to ward off infection. Future screening studies comparing hosts susceptibility should aim to standardise the age of the ramets, and possibly also to develop an inoculation technique that more closely reflects the field situation (i.e., using inoculum levels more comparable with field-soil levels).

Severe root necrosis was observed in rewarewa (Proteacae) and rimu (Podocarpaceae). Root infections of non-*Agathis* hosts by PTA have not been studied in the field situation. If there are any other hosts of PTA identified from the kauri forest community, they may play a key role in the epidemiology of PTA at the local scale by serving as a source of inoculum. A survey of the roots for the presence of oospores in other hosts from field samples would be one method to confirm the existence of other PTA hosts. It has been hypothesised for *P. ramorum* that the pathogen may need to build inoculum levels on a range of "associated hosts", to serve as a springboard to the terminal host (e.g., Rizzo et al. 2003). As such, even hosts with little or no above- or below-ground symptoms may be important in the transmission biology.

The "hosting response" of the two members of Lauraceae, tawa and taraire, to deliberate root inoculation with PTA was not associated with any disease symptoms, or significant change in root mass. Robertson (1970) identified tawa and taraire as susceptible to *P. cinnamomi.* In-field validation of the presence of PTA associated with these two laurels remains to be confirmed, especially in fully grown trees, which were not assessed as part of this study.

Up-regulation in plants in response to deliberate infection by pathogens can occur (e.g., Dellagi et al. 2005). The growth promotion above and below ground observed in tōtara in response to deliberate root inoculation with PTA again shows the difficulty in generalising across a plant family, as rimu was observed to be susceptible in the glasshouse to PTA, while tānekaha and kahikatea remained healthy. Avrova et al. (2004) identified that potato oxysterol-binding protein and cysteine protease cathepsin B were up-regulated in response to infection with *P. infestans.* These were specifically up-regulated in the early, *biotrophic* phase of the infection. Tōtara provides a fascinating model for comparative infection and resistance studies with kauri, because of the relatively close phylogenetic relationship of Araucariaceae and Podocarpaceae (Quinn et al. 2002). Potentially, a lot could be learned about resistance responses and control of programmed cell death through comparative transcriptomic studies of tōtara and kauri inoculated and challenged by PTA.

Oospores have been suspected to be part of the way that *Phytophthora* species can perpetuate in the rhizosphere soil of infected host plant roots (e.g., Horner & Wilcox 1995). Homothallic species of *Phytophthora* like PTA are self-compatible and can produce oospores in single spore culture. Oospores are formed after an antheridium fertilises an oogonium. When the oospore germinates, the germ tube can produce either a sporangium or a mycelium (Schmitthenner 2000). Thick-walled, globose to sub-globose oospores were observed in deliberately inoculated, dead secondary roots of kauri (Fig. 24). The early stage of infection

of some other pathogenic *Phytophthora* species, involves asymptomatic proliferation in the host tissue by suppressing programmed cell death or by thwarting host defence responses (Lee & Rose 2010). During the later stages of the infection process, these species undergo a physiological transition from asymptomatic biotroph growth to a highly destructive necrotrophic phase (Münch et al. 2008).

Jung et al. (2013) identified that survival structures in fine roots, secondary roots and root-collar debris are likely to be responsible for long-term survival of *P. cinnamomi* in jarrah forests. PTA oospores were found in the diseased roots of inoculated kauri and korokio, but in the field PTA has been recovered only from the roots of kauri. Dispersal of PTA could also be facilitated through the physical transfer of infected root material contained in soil adhering to footwear, tools, machinery and logs (see Colquhoun & Kerp 2007). Of the other species screened as potential hosts of PTA, no members of the Ericaceae were studied (e.g., neinei *Dracophyllum latifolium*). This particular family is known to be susceptible to *P. cinnamomi* in south-west Australia (Shearer et al. 2010), and is worthy of some form of preliminary risk assessment to susceptibility to PTA. Another common kauri associate, Kirk's daisy *Brachyglottis kirkii* (Asteraceae), should be considered for susceptibility testing. It would be desirable also to test the large lilies/grasses/sedges that are associated with kauri and include important successional species such as sedges (*Gahnia xanthocarpa* and *G. pauciflora*) and kauri grass (*Astelia trinervia*). Weste (2001), in her survey of the Grampian Ranges (Victoria, Australia) for plant species susceptible to *P. cinnamomi*, found that very few species in the monocot families Cyperaceae, Liliaceae, Juncaceae, Poaceae, and Restionaceae showed disease symptoms. Prioritisation for all future comparative host screening studies should consider a methodology with a larger screening capacity, and with relevance to adult trees and shrubs of the natural forest. In this way, knowledge of the possible presence of other hosts in the kauri forest can be progressed.

From the nursery-sourced plants used in host range studies, *P. cinnamomi* was recovered from single individuals of kānuka and mānuka, and *P. europaea* from a single individual of rimu. *P. cinnamomi* is widely spread throughout the kauri forest, and has been been linked with root death and decline of young kauri on flooded sites in the Cascades, Auckland (Podger & Newhook 1971). *P. europaea* has not been found in any forest; its recovery from the present study of *P. europaea* (ICMP 19116) is only the second record of the pathogen in New Zealand (previously recorded in South Canterebury from *Iris sibirica* – ICMP 16258). How did *P. europaea* arrive in New Zealand from Europe? The international movement of living plants has been recognised as a pathway for the movement of pathogens (Brasier 2008).

The combined impact of PTA with the presence of *P. cinnamomi* and/or *P. europaea* has not been reported or studied before. To-date we have no evidence for dual-infections in kauri, caused by more than PTA. Pot-trial pathogenicity studies confirmed the susceptibility of "young"[6] kauri to *P. cinnamomi* (Podger & Newhook 1971). Interactions between root-rot basidiomycetes and *Phytophthora* spp. through artificial inoculation of oak seedlings have shown that application of pathogens in combinations resulted in significantly greater damage to the oak host than the sum of the damages induced by individual pathogen-challenges (Marçais et al. 2011).

[6] No mention of age of kauri test plants in Podger & Newhook 1971.

5 Transfer of PTA infection

5.1 Introduction

Spread of PTA to new localities will likely occur through vectors such as pigs, capable of moving large amounts of infected soil (Weste & Marks 1987), as well as human-mediated soil movements (Shearer & Tippett 1989). The complete life cycle of PTA has not been described, but from the chapter characterising the morphology of PTA, sporangia, zoospores, oogonia and oospores have been observed in PTA *in vitro*. Additionally, from the previous chapter, oospores of PTA were observed *in planta* . It has been hypothesised that when environmental conditions are made suitable (i.e., high soil moisture), PTA infection may be initiated through fine-root infection. Which life-stage of PTA (e.g., oospores) in the soil or present in infected root tissues, germinates to produce sporangia? This chapter describes an investigation into the ability of PTA-infected plant material (i.e., roots and collar tissue of diseased kauri) to transmit PTA infection to healthy, fine roots of 18-month old kauri seedlings. Five-year old kauri plants were infected with PTA via the millet methodology described in the previous chapter. The infected kauri plant material was harvested and partitioned into various inoculum fractions and then this diseased material used to inoculate 18-month old kauri seedlings.

5.2 Materials and Methods

Generation of PTA-infected root and collar inoculum

Infected 5-year old kauri plants were generated using the millet method described in the previous chapter. PTA inoculum was prepared by growing isolate ICMP 18403 for 4–6 weeks at 20°C on sterilised millet seeds thoroughly moistened with V8-juice broth (Jeffers 2006). The inoculum was repeatedly rinsed with sterile RO water to remove unassimilated nutrients then added to sterile potting mix at a rate of 25 ml per litre of potting mix. The inoculum and potting mix were mixed in a cement mixer and then put into No. 4 planter bags. Five-year old kauri were transplanted into the inoculated potting mix, and then flooded to induce sporulation of PTA (Vettraino et al. 2001). There were 15 replicate bags treated with PTA. Another set of 15 bags were established as a control, using sterilised 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 randomised block design, with PTA treated plants segregated from negative controls. Plants were watered to field capacity with tap water every other day. Plants were grown in a PC-1 naturally lit growth house at Landcare Research, St Johns (temperature and RH conditions provided in Appendix 2A; experiment commenced August 2011). The experiments were carried out under a CTO permit approving usage of PTA.

Twenty-one days after inoculation, the plants were extracted from the potting mix. The used potting mix was disposed of in an Interwaste bin. The roots were segregated into the three size classes described from the previous chapter: 1) primary roots, 2) secondary roots, and 3) distal roots. The collar material (i.e., the junction between primary root and stem) was also recovered (Fig. 26). A similar recovery exercise was carried out on the control kauri plants.

Figure 26. Infected root and collar material used for root inoculation (anti-clockwise from bottom left: distal, secondary, collar and primary root material).

Inoculation of kauri seedlings with PTA-inoculum

The root and collar material salvaged from the plant harvest was used as the inoculum to study the transmission of PTA infection. Root or collar material was placed along the roots of 18-month-old kauri seedlings which had been grown in "root trainers" (Fig. 27). The root-trainers were opened, and the pieces of root and collar material inserted adjacent to the fine-roots without any disturbance to the roots.

Figure 27. Placement of infected root material along roots of 18-month old kauri seedlings grown in root trainders.

The root trainer "books" were then tied closed with flagging tape. The negative controls were segregated from the PTA-inoculated plants. A replicated set of positive controls was also established using 6 mm plugs of PTA mycelial culture which were laid against the seedling roots (5 plugs per seedling). Positive control treatments were segregated from the other treatments and watered every 2–3 days with potable tap-water (Fig. 28). Plants were grown in a PC-1 naturally lit growth house at Landcare Research, St Johns (temperature and RH conditions provided in Appendix 2A; experiment commenced late-August 2011). The experiments were carried out under a CTO permit approving usage of PTA. All run-off from PTA treatments were captured and heat sterilised 121°C for 20 mins prior to disposal. All benches were decontaminated with 70% ethanol, and all solid waste materials placed in an Interwaste bin for certified disposal.

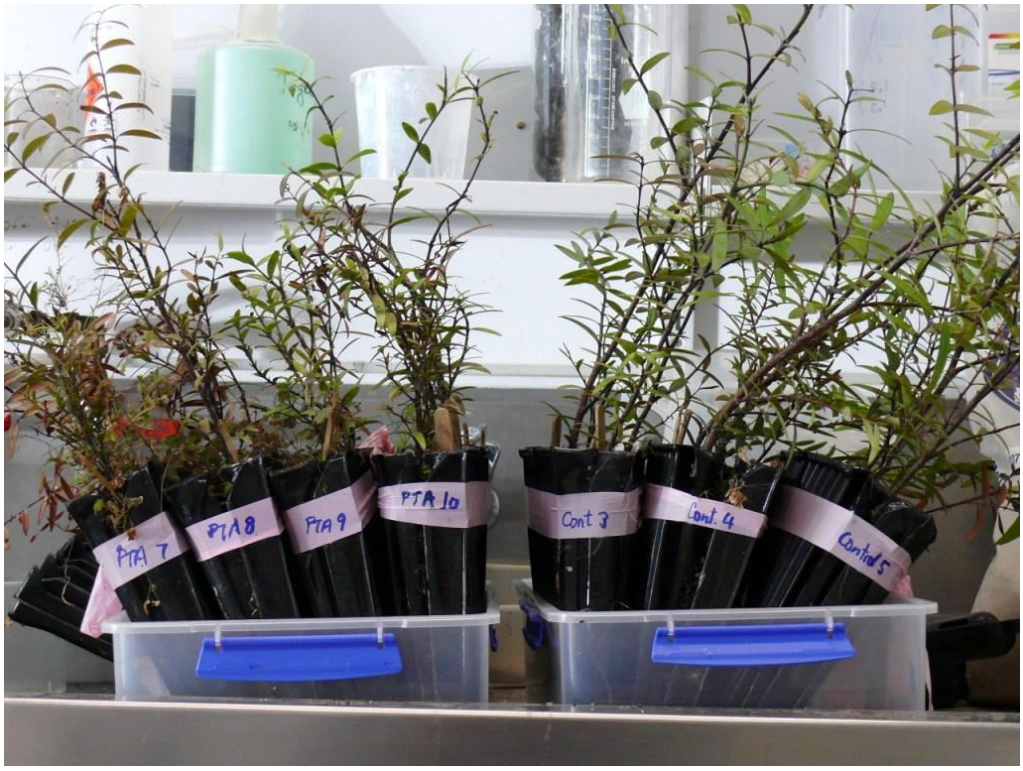


Figure 28. Experimental set-up of the PTA transfer experiment.

Processing of plant materials

Plant health was monitored over a period of 6 months to account for seedling deaths. At the end of the experiment, shoot height, and mean shoot and root fresh mass were measured. Roots were segregated into three size classes described previously, and placed in clean labelled Petri dishes. Each of the root pieces and collar material was surface sterilised in 70% ethanol for 30 seconds and then rinsed in sterile RO water. The plant pieces were blotted dry on clean paper towels and plated to *Phytophthora*-selective agar. Plates were wrapped in foil and incubated at 18°C in the dark to allow PTA to grow-out from the infected plant material (Jeffers 2006). Characteristic oospores of PTA formed profusely after 4–7 days. All scalpels and sampling implements were decontaminated by flame sterilisation between the sampling of plant root systems. All benches were decontaminated with 70% ethanol. All solid waste was disposed of in an Interwaste bin. All liquid wastes were captured and autoclaved at 121°C for 20 min prior to disposal.

Representative samples (n = 5) of all infected root and collar material and negative controls were fixed in the histology fixative, FAA (Formalin–Acetic Acid–Alcohol solution; 50:10:5% w/v). This material was cleared with 10% KOH for 30 min and then bleached in 5% alkaline H_2O_2. The plant material was then acidified in 5% HCl, stained with Aniline Blue (Brundrett et al. 1996), destained overnight in a lactic acid glycerol solution (Koske & Gemma 1989), and examined under a compound light microscope to search for characteristic oospores of PTA. Photo-micrographs captured using a Nikon DSi-1 camera.

Experimental design and statistical analysis

There were two replicate sets of 5-seedling root trainers inoculated with each of the four types of root/collar material (i.e., ten seedlings / treatment). Two root trainers were established as negative controls, and two root trainers were inoculated as positive controls.

PTA treatments were segregated from control treatments by being placed on the opposite bench. The experiment was a randomised block design, with plants blocked according to the type of root inoculum (i.e., 1° roots, 2° roots, distal roots, and collar material). Data sets for shoot and root biomass of PTA-inoculum and negative control plants were all analysed statistically using a two-tailed Student t-test.

5.3 Results

Glasshouse conditions over trial period

Mean monthly temperatures in the naturally lit glasshouse used for the root inoculum study varied from August 2011 to February 2012. Mean temperature was 13°C for August–September and 18–20°C for October–February. Mean relative humidity (RH) was 87–93% for August–September, and c. 80% for October–February.

Recovery of PTA from seedlings

No PTA was recovered from any of plants inoculated with negative control root- and collar-inoculum. All positive controls died within 6 weeks of the commencement of the experiment.

After 6 months, significant disease impacts were observed in the kauri seedlings inoculated with PTA-infected plant material. PTA infection was effectively transmitted from the following types of PTA-infected material: a) primary roots, b) collar material and c) secondary roots (Fig. 29).

Figure 29. Seedlings of kauri 6 months after being inoculated with infected primary roots (left), infected collar material (centre), and infected secondary roots (right).

The disease symptoms observed in the inoculated kauri seedlings associated with PTA-infection were reduction in fine roots, discoloration of the primary root system, constriction at the collar, leaf loss and shoot death. The disease symptoms were also associated with a significant reduction in root and shoot biomass after 6 months (Table 10). Koch's postulates were satisfied for all symptomatic plants through the post hoc recovery of PTA from the diseased plants.

Inoculation with PTA-infected primary (1°) roots and PTA-infected collar material caused the most significant decrease in mean root biomass (Table 10; Appendix 3) – and in some cases death of the inoculated plants. PTA-infected primary (1°) root material was the only plant material tested (other than the positive control) that resulted in plant death (Table 10).

Table 10. Mean root and shoot mass (g) of kauri seedlings 6 months after inoculation with either PTA-infected or Control (uninfected) plant inoculum

Type of inoculum	Distal roots		2° roots		1° roots		Collar-material	
	PTA	Control	PTA	Control	PTA	Control	PTA	Control
Root mass	2.4	5.8	3.0	13.9	0.5 †	13.9	7.9	21.0
Shoot mass	5.2	12.4	6.1	22.6	0.9 †	22.9	2.9	14.8

N.B. All differences between paired PTA/Control root mass values and between paired PTA/Control shoot mass values are significantly different ($P < 0.05$). † Eight out of ten replicates of the 1° root PTA treatment were dead at the end of the trial.

Visualisation studies

Cortical cells of the secondary (2°) roots of kauri infected with PTA were sparsely colonised by unidentified arbuscular mycorrhizal fungi (Fig. 30). These fungi formed typical hyphal structures that could be differentiated from oospores of PTA. Oospores of PTA were observed in the cortical cells of the PTA infected secondary roots. Fungal structures (i.e., pelotons) were also observed in the cortical cells of the root nodules (Figs 31 and 32).

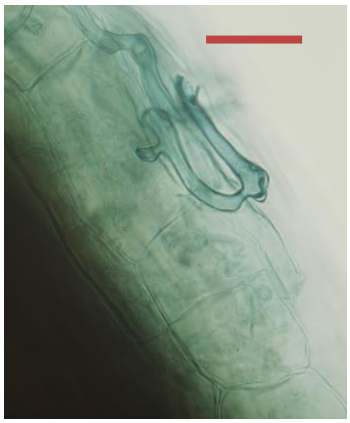


Figure 30. Cortical cell of secondary root of kauri showing typical branching pattern of arbuscular mycorrhizal fungus. Scale bar = 1 µm.

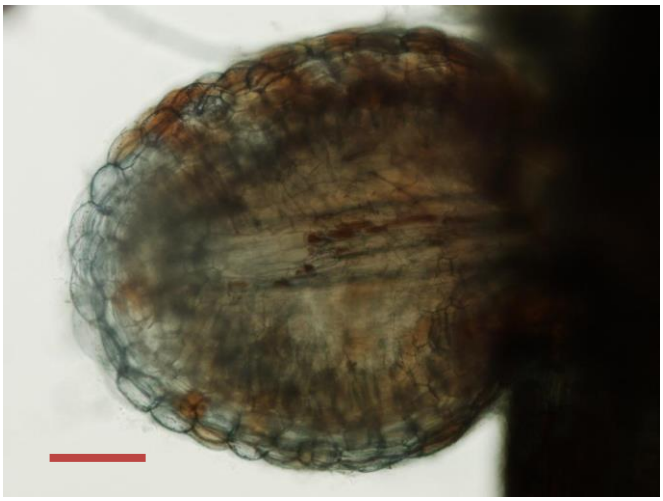


Figure 31. Light micrograph of kauri root nodule. Scale bar = 25 µm.

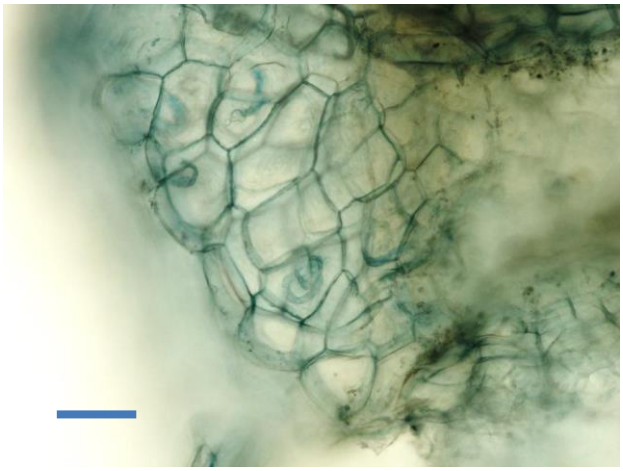


Figure 32. Pelotons in the root cortical cells of kauri root nodule. Scale bar = 5 µm.

5.4 Discussion

This study provides evidence for the transmission of PTA from infected plant material to healthy roots of 18-month old kauri seedlings, from which PTA was subsequently recovered from secondary roots, primary roots, and the junction of the root and stem (collar region). Oospores of PTA were observed in the secondary root material of the dead seedlings.

It is hypothesised that in the field oospores contained in the roots of infected kauri plant material are one source of PTA transmission. The kauri root root system is composed of shallow lateral roots and vertical (sinker) roots that arise along major laterals and penetrate the soil profile. The infection of surface lateral roots by PTA and the progression of infection to the collar of trees has not been demonstrated in the field. It is hypothesised that extensive infection of lateral roots is necessary before severe crown symptoms are expressed. Quantitative studies of fine root biomass of live kauri trees on infested and uninfested sites need to be carried out to correlate canopy decline symptoms with root system dysfunction.

Symptoms in kauri growing on dieback sites include severe root necrosis and death, gummosis at the collar, gradual dying back of the crown, through to wilting and death. Large, lateral roots of diseased kauri need to be dissected to understand the lateral and tangential spread of PTA along and around bark lesions in large roots. The research of Tippett et al. (1983) demonstrated that the roots of jarrah (*Eucalyptus marginata*) were not girdled by the invasion of the inner phloem in the first 12 months after deliberate inoculation with *P. cinnamomi*. The root phenology of kauri needs to be studied with respect to seasonal changes, especially the relationship between the activation of cambium, soil moisture, and timing of main leaf-flush, to see if there is a correlation between the extent of root or collar infection and effects on tree growth (Davison & Tay 1995). Histological investigations of kauri roots, especially focusing on the inner and outer phloem of inoculated roots and collar regions of trees of all age classes (e.g., seeding, saplings, rickers, mature, senescent), need to be undertaken to understand the nature of host responses to deliberate infection. In this way, positive host responses to phosphite interventions can be recognised and interpreted in infected kauri trees treated with phosphite (see Fernandez-Escobar et al. 1999; Horner & Hough 2013).

6 Spatial extent of PTA in a forest stand

6.1 Introductory statements

As well as understanding the regional occurrence of PTA, it is also important to understand its occurrence at the landscape level. Beever et al. (2009) proposed a rate of spread of approximately 3 m per year on the assumption of a circular area of infection at a Great Barrier Island site. This corresponds to the rate of spread associated with *P. cinnamomi* in south-western Australia estimated by Strelein et al. (2006). Dawson et al. (1985) estimated rates of spread of 400 m per year in post-fire vegetation communities in the Brisbane Ranges, 80 km west of Melbourne. This chapter describes the results of analysis of soil under two PTA-diseased trees and a plot-scale study of kauri infested with PTA. The establishment of the study plot was carried out by Auckland Council. Survey work was undertaken in 2006, 2009 and 2012.

6.2 Study area

The study area was the Twin Peaks Track near the Huia Dam Reservoir (GPS: 2649586E; 6466012S) in the Waitakere Ranges, west of Auckland City (Fig. 33). The kauri stand at this site represents a mixed-age ricker stand. The study area is approximately 1 ha in area (Fig. 34) and is described as "stratified kauri–broadleaf evergreen forest" (Clunie 1970). The tree species associated with and under kauri are māpou, māmāngi, rewarewa, lancewood, and more occasionally tānekaha, miro and rimu emerging over mingimingi, kānono, and ground-covering kauri grass and cutty grass. The scientific names of the most common species associated with kauri are provided in Table 11.

Figure 33. Location map of Twin Peaks Track study area and the Huia Dam.

The climate, physiography, vegetation and soils of the area have been described previously (Gibbs et al. 1968; Clunie 1970; Jongkind & Buurman 2006). The climate is temperate oceanic with warm, humid summers and mild winters. The mean annual rainfall is 1820 mm and rainfall is distributed throughout the year, but is highly variable between years, especially

during the summer months. The average monthly maximum temperature is highest in February (c. 21°C) and lowest in July (c. 10°C).

Table 11. The most common plant species in the Twin Peaks Track study area

Common name	Binomial
Gymnosperm trees	
Kauri	*Agathis australis*
Rimu	*Dacrydium cupressinum*
Tānekaha	*Phyllocladus trichomanoides*
Miro	*Prumnopitys ferruginea*
Sedges	
Cutty grass	*Gahnia xanthocarpa*
Monocotyledonous trees, lianes and herbs	
Nīkau	*Rhopalostylis sapida*
Kiekie	*Freycinetia banksii*
Supplejack	*Ripogonum scandens*
Kauri grass	*Astelia trinervia*
Dicotyledonous trees and shrubs	
Māmāngi	*Coprosma arborea*
Kānono	*Coprosma grandifolia*
Mingimingi	*Leucopogon fasciculatus*
Rewarewa	*Knightia excelsa*
Mānuka	*Leptospermum scoparium*
Māpou	*Myrsine australis*
Lancewood	*Pseudopanax crassifolius*
Karaka	*Corynocarpus laevigatus*
Tawa	*Beilschmiedia tawa*
Taraire	*Beilschmiedia tarairi*
Ferns	
Ponga	*Cyathea dealbata*
Whekī	*Dicksonia squarrosa*

Figure 34. Aerial view of Twin Peaks Track study area (2012).

6.3 Material and Methods

Establishment of soil pits

Adjacent to Monique's Tree[7] (Fig. 35(L), DBH = 88 cm) and to Joan's Tree[8] (Fig. 35(R), DBH = 27 cm), a vertical soil pit 1 × 1 m was dug to a depth of 1.2 m next to the trunk. Both trees displayed gummy lesions, and had been confirmed as PTA-positive from previous studies (Beever et al. 2010). The soil vertical profiles were described, including the colour, texture and mottling of soil, and abundance of roots. Soil samples (total approx. mass of 500 g) were taken within each horizon and stored in a chilly-bin. An aliquot of the soil sample (200 g) was sent to the Landcare Research soil testing laboratory for a range of chemical analyses (Appendix 4), and the remainder stored for soil bioassay (see Chapter 7, Section 7.2). All tools and sampling implements were decontaminated with 70% ethanol between the sampling of each soil horizon, and between each soil pit. All solid waste (i.e., used waste paper towels) was collected in Autoclave bags and returned to Landcare Research, St Johns, for disposal in Interwaste bins.

Plant health studies

A National Vegetation System (NVS[9]) plot was established by Auckland Council together with Landcare Research staff in 2006, approx. 400 m south of Monique's Tree. The NVS vegetation mapping system uses a standardised survey sampling protocol and pro forma monitoring sheets, upon which tree and vegetation data are captured. The central sampling quadrat is 20 × 20 m, in which each tree is tagged and numbered. Four, 20 m wide sampling corridors were also established in 2006.

All kauri within the central grid were assessed for tree health status by in the summer of: 2006 (Nick Waipara, Ross Beeever† and two summer students); 2009 (Bec Stanley, Nick Waipara and Karl Crosby); and 2012 (Monique Wheat, Lee Hill, Bec Stanley and Nick Waipara). Tree health status in the adjacent four transects was also assessed in 2009 and 2012. The tree diameter at breast height (DBH) was determined, and any signs of pig-rooting or other disturbance to the root bole area were recorded. To measure tree health status, the condition of the canopy was scored (crown index rating) on the basis of the following criteria: healthy (1), presence of foliar thinning (2), some branch dieback (3), severe shoot dieback (4) and dead (5). In addition, the circumference of old and new resin-bleeds and the height and extent of collar lesions were documented for each tree. The presence of borers and/or wood-decay fungi (e.g., *Ganoderma* sp.) was also recorded in the 2012 survey.

All tapes and sampling implements were decontaminated with 2% TriGene II Advance between the surveying of each corridor.

Confirmation of presence of PTA

From 48 kauri trees along the 20 m wide transect corridors, soil samples were taken 0.5 m from the base of the tree in March 2011 (Appendix 5B). The "duff" of a small area of ground was cleared away either opposite an active lesion in a symptomatic tree or downslope from a

[7] Confirmed as PTA-positive via soil bioassay in 2009 [2649474E, 6465892S]

[8] Confirmed as PTA-positive via cambium sample in 2009 [2649586E, 6466012S]

[9] http://nvs.landcareresearch.co.nz

non-symptomatic tree. Using a trowel, a sample of c. 500 g of soil to a depth of approximately 15 cm was removed from each of four cardinal points around the tree. The soil was held in zip-lock bags that were clearly labelled with the location, tree number and sampling date. A duplicate, second label, written on waterproof note paper, was inserted into the bag, and the samples placed in chilly-bins. All tools and sampling implements were cleaned of soil and decontaminated with 70% ethanol after each soil sample was recovered from each tree. These soil samples were retained at 10°C for soil bioassay, to identify the presence of PTA.

Figure 35. (L) Monique's Tree (2011) with advancing gummy lesion extending up to 1.5 m. (R) Joan's Tree (2009) showing characteristic gummy bleeding in the collar region of the lower trunk.

Two hundred grams of each soil sample was analysed using the extended soil-baiting bioassay protocol (Beever et al. 2010 unpubl. report). This involved a period of air-drying, and then moist incubation prior to flooding and baiting with cedar needles *Cedrus deodara* and lupin *Lupinus angustifolius* cotyledons. The bait tissues were extracted after two days incubation in a Contherm Incubator (20°C, 64% RH, light intensity of 164 μE[10] on 12 hour light/dark diurnal cycle). The bait tissues were surface-sterilised in 70% ethanol for 30 seconds and then rinsed in sterile RO water. The bait pieces were blotted dry on clean paper towels and plated to *Phytophthora*-selective agar (Jeffers 2006) and wrapped in foil and incubated at 18°C. After 4–7 days characteristic oospores of PTA were observed. Representative subcultures were transferred to fresh plates of PDA for DNA sequence confirmation. Ten mls of Trigene Advance (II) was added to each of the used bait boxes at the end of the experiment and disposed of in an Interwaste bin.

Estimation of disease spread

Disease haloes around dead tree foci identified in 2006 and from dead/symptomatic trees (either as individuals or as clumps) identified in 2012 were plotted over the 2012 transect plot (Fig. 40; Appendix 6). The diameters of the haloes were calculated and the mean diameter estimated (Fig. 40; Appendix 6). The average linear rate of disease spread over 6 years was calculated by halving the mean diameter of the haloes (i.e., by calculating the radius).

[10] 164 μE (μm-^{2}s^{-1}) = 8856 Lux (Photometric units)

Statistical analysis

A chi-squared contingency statistic was used to analyse the change in proportion of asymptomatic, symptomatic and dead trees using: 1) data derived directly from the transect plots (i.e., Figs 38 and 39), and 2) using the plant health data[11] from the three surveys carried out in 2006, 2009, 2012 (i.e., Appendix 5A).

6.4 Results

Soil description

The topography of the site is gently sloping (25°), with a north-eastern aspect. The soils at the two trees are similar in that they are highly leached, have similar parent materials, and podsolisation is the overall soil process (Appendix 4). The soils all have accumulations, and mostly substantial accumulations, of organic materials at the surface (Fig. 36). The mineral soil at the surface has much organic material incorporated such that the change from the surface "O-horizon" materials or "duff" to the mineral soil is gradual.

Figure 36. Soil profile under Monique's Tree showing organic litter surface horizon ("O-horizon").

Ultimately the subsoil is dominated by clays derived from volcanic ash, which comprises 0.5–1.0 m of the surface and sub-surface soil layers. Under this are concretions of iron having red and green colours, many of which harden irreversibly on drying. Soil pHs are acidic, some as low as 3.2, and nutrients are limiting in most horizons. Carbon (C) : nitrogen (N) ratios are 20:1 and even higher, indicating that carbon in the system is unlikely to decompose readily. Extractable phosphorus (P) is also very low – up to 5 ppm. The very low pH strongly suggests that nutrient cations (e.g., calcium, magnesium, potassium) are low. Total organic C averaged 41.8% (standard deviation (SD) = 15.8), total N 1.4% (SD = 0.399) and total P 0.0522% (522 mg/kg, SD = 59). C:N:P ratios average approximately 25,000:60:1 on a molar basis (range from 5000:60:1 to 32,000:60:1). The C:N ratio averages 417:1 (range from 92:1 to 525:1). Given that optimum C:N:P ratios and C:N ratios are around 100:10:1 and 10:1 respectively, these values suggest that the kauri system is severely limited in N and P (Russell 1973; Troeh & Thompson 1993). Indeed, available N and P are very low in these soils.

[11] Symptomatic tress were defined by possessing a crown index rating ≥3

Nitrate N ranges from 1.2–7.1 mg/kg and ammonium N from 7–66 mg/kg. Available phosphate P ranges from <1–6 mg/kg.

Tree health

Based upon an interpretation of the most recent survey data (2012), across the site tree disease symptoms increased in frequency comperd with 2006, as did the number of dead trees (Figs 38 and 39). Crown and foliar symptoms ranged from chlorosis, thin crown and markedly reduced growth, to dead trees with bleached "stag-heads" (Fig. 37). Collar and lower-trunk symptoms were characterised by an advancing lesion (canker) with a profuse production of resin at the base of the trunk (Fig. 35). Trees which exhibited a combination of collar rot and foliar dieback (crown index >3) were considered to be "symptomatic". The progression of the lesion around the base of the tree can result in "girdling", which is the chronic phase of the disease. Decay of the larger roots of kauri was uncommon but there was extensive decay of distal feeder and secondary roots, their cortex stripping readily from the stele. PTA was recovered from both suberised secondary roots and dead root nodules.

(a)

(b)

(c)

Figure 37. (a) Little-leaf syndrome. (b) Advanced crown decline. (c) Dead, "stag-head".

Transect trends

The size of the NVS plot is 20 × 20 m, with four radiating transects (Fig. 38): 1) southwestern transect of 115 m; 2) northwestern transect of 75 m; 3) northeastern transect of 40 m; and 4) the southeastern transect being 30 m in length (Fig. 38).The NVS plot is gently sloping (≈12–15°) with a southeasterly aspect[12].

[12] Auckland Council have confirmed that the GPS coordinates for the NVS plots and transects are not available (Nick Waipara pers. comm.).

PTA presence was confirmed across the axes of the NVS transects, by the recovery of PTA from the base of 15 out of 48 trees sampled (Appendix 5B). Sixty eight percent of the PTA positive recoveries came from trees displaying a collar bleeds and/or crown index >3 (Appenidix 5A). PTA was not readily recovered from soil collected from beneath trees that had been dead for a long time (Table 12). Out of 31 asymptomatic trees, PTA was only recovered 19% of samples (Table 12).

Table 12. Tree health status and PTA recovery from 100 trees along NVS axes (March 2011)

Health category	Asymptomatic	Symptomatic*	Dead	Σ
PTA positive	6	9	0	15
PTA negative	25	6	2	33
Σ	31	15	2	48

N.B. $\chi^2 = 8.72$, df = 2 ($p < 0.01$). * Symptomatic trees exhibited lower stem resinosis and/or foliar decline (i.e. Crown index rating ≥3).

Tree health significantly decreased over the 6-year monitoring period. In 2006, 16 trees were recorded as being dead (Fig. 38), and by by 2012, 37 trees had perished (Fig. 39, Table 13). A pictorial representation of the distribution of trees and their health status for 2006, and 2012 is presented in Figs 38 and 39. It is clear from these figures that tree deaths occur in all directions – upslope, downslope, and across the NVS central-plot area. There also appears to be a clustering in the distribution of symptomatic and dead trees (Fig. 40) – around either a symptomatic or dead tree focus present in 2006 (Fig. 38).

In addition, the data presented in Appendix 5A also show that tree health significantly decreased over the 6-year monitoring period (Table 13).

Table 13. Tree health status between 2006–2012 along NVS axes

Health status	2006	2009	2012	Σ
Asymptomatic	110	107	89	273
Symptomatic	30	37	28	99
Dead	16	18	37	71
Σ	156	156	154	

NB. $\chi^2 = 34.4$, df = 4, ($P < 0.001$).

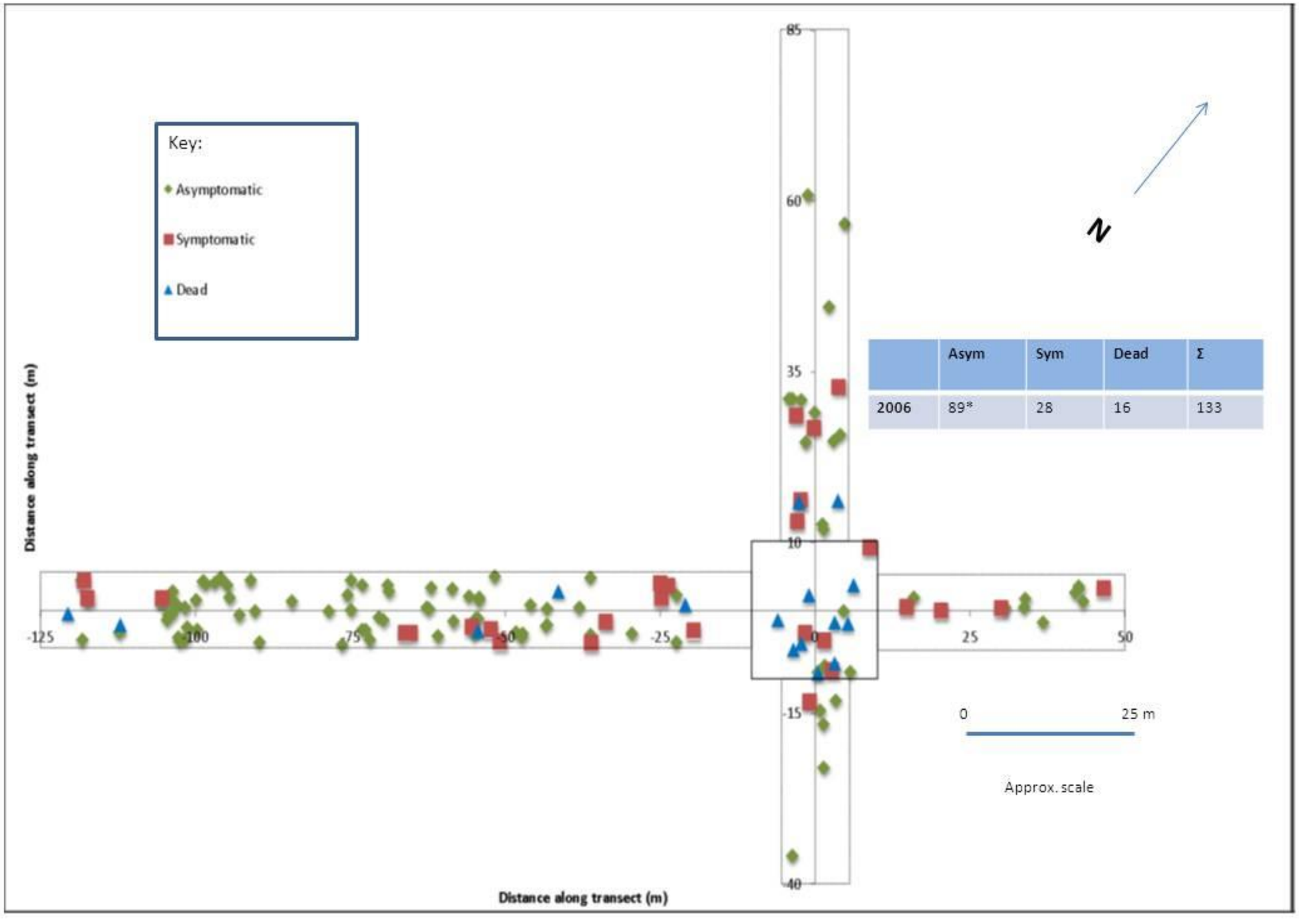
Other *Phytophthora* species, *P. cinnamomi*, *P. multivora* and *P. nicotianae* were also recovered from soil samples taken from below kauri trees in the transects, but at much lower frequency than PTA (Appendix 5A).

Expansion of infection foci

A depiction of the disease haloes interpolated from the 2006 and 2012 tree health maps is presented in Fig. 40. The range in the diameter of the haloes of infection around the dead foci was 1–19 m (Appendix 6; Table 14). The halo of a single infected tree was assumed to be 1 m in diameter. The average diameter of the infection haloes was 6.825 m (Table 14). Assuming a circular infection pattern, the inferred linear extent is considered to be half the average diameter, i.e., 3.41 m (Table 14).

Table 14. Size of infection haloes in 2012, extrapolated from Fig. 40

Derived factors	2012
Range in diameter of haloes	1–19 m
Total diameter of haloes	136.5 m
Average diameter of haloes	6.825 m (± 1.03 SEM)
Linear distance of spread over 6 years	3.41 ± 0.52 m
Number of dead trees	37
Number of symptomatic trees	28
Number of haloes assessed	20



	Asym	Sym	Dead	Σ
2006	89*	28	16	133

Figure 38. Twin Peaks Track transect plant health status 2006

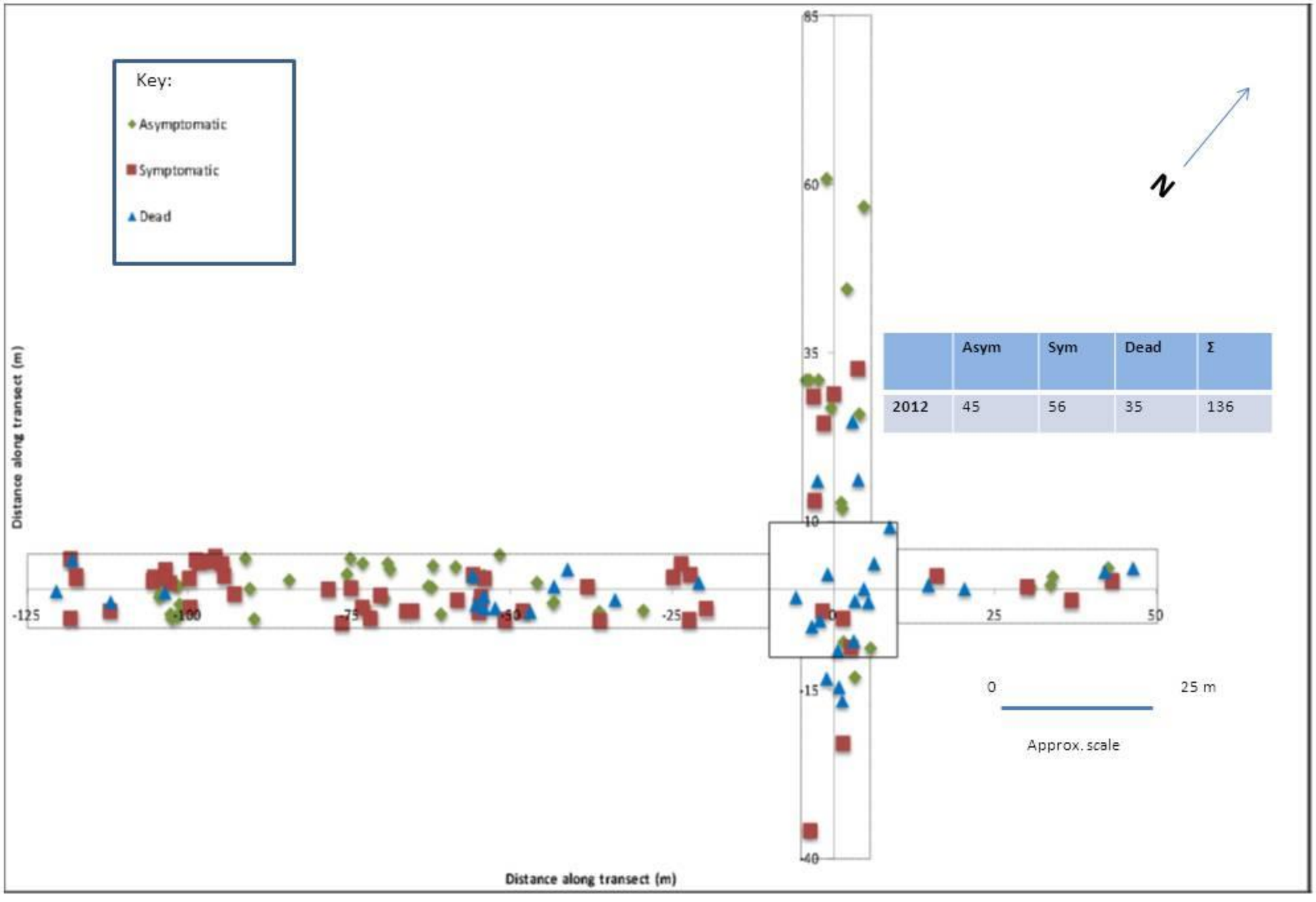


Figure 39. Twin Peaks Track transect plant health status 2012.

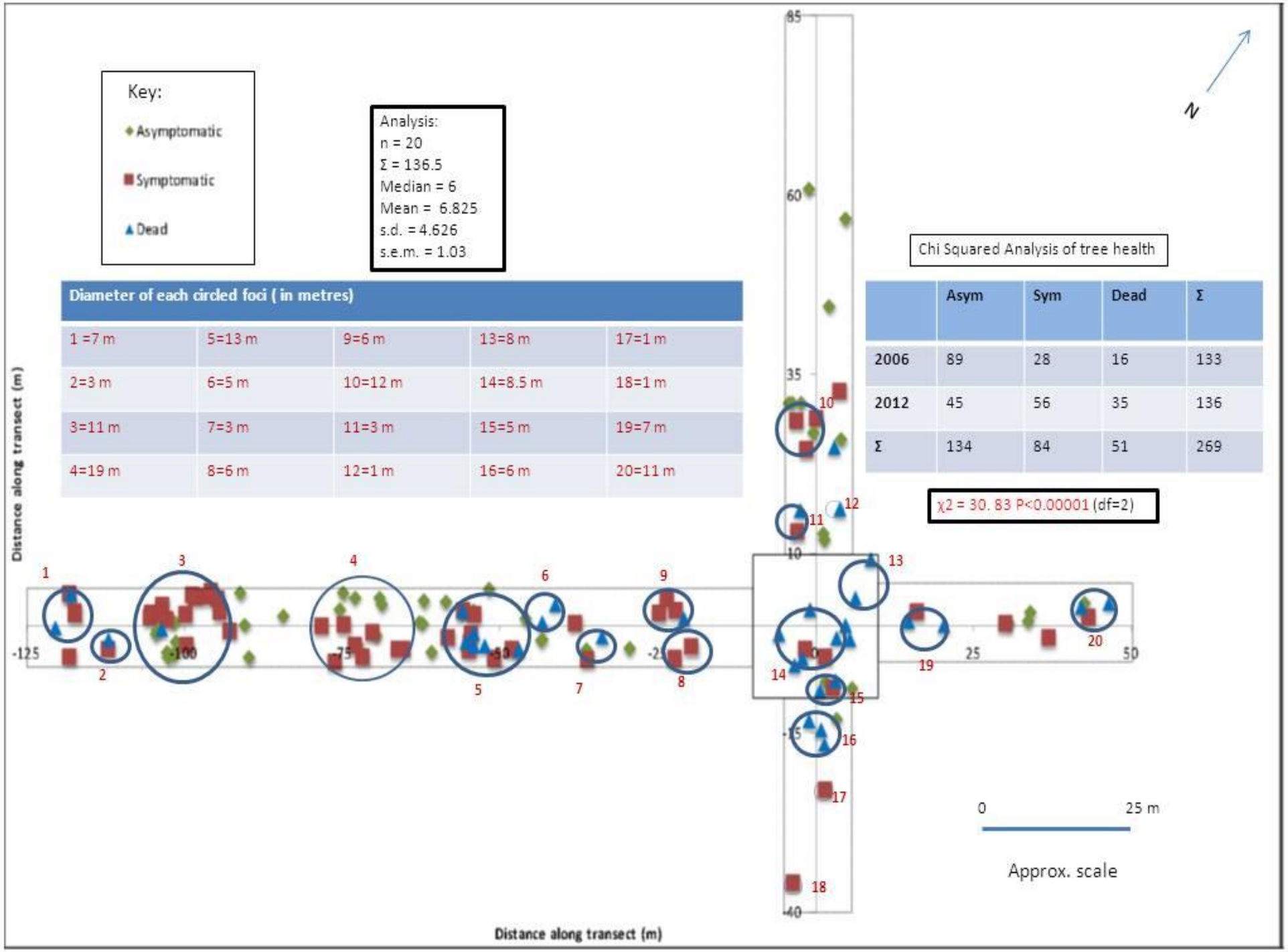


Figure 40. Twin Peaks Track transect with diseased haloes plotted on the 2012 plant health data. The halo of a single infected tree was assumed to be 1 m.

6.5 Discussion

The soil profiles under two diseased trees at the PTA-infested Twin Peaks Track site were characterised by having a shallow organic-rich topsoil overlying a deep clayey subsoil. Soil pHs were acidic, some as low as 3.2, and nutrients are limiting (esp. N and P) in most horizons. Kauri has been previously associated with intense podsolisation in the Waitakere Ranges (Jongkind & Buurman 2006). The clayey nature of the subsoil profile suggests impeded vertical drainage. Depressed plant growth and root weights due to *Phytophthora cinnamomi* root rots of avocado have been associated with low soil pH, and especially when low soil pH is coupled with high soil moisture contents (Fernandez Falcon et al. 1984). It is proposed that the subsoil profile of the Twin Peaks Track site possesses characteristics that are conducive to PTA disease development and expression, as there is potential for prolonged periods of wet soil conditions including during warmer summer months. It is hypothesised that when soil environmental conditions are favourable (high soil moisture, soil temperature >10°C), *Phytophthora* species' resting spores germinate by forming sporangia that release motile, biflagellate zoospores into the soil water. Serious fine-root infection by *P. kernoviae* and *P. ramorum* has been correlated with high soil moisture, low nutrients and low pH, which all make for appropriate conditions for sporangium development and zoospore release (Kong et al. 2012).

It is also important to consider how associated environmental factors affect the physiological limitations of both host and pathogen because this may provide insights into how to predict when and where deaths may occur, and potentially, how to reduce their incidence (Davison 1994). The extent and progression of *Phytophthora* diseases in European oak forests and in south-west Australia forests have been strongly linked with edaphic and weather conditions (Jung et al. 2002; Jung et al. 2013). Tree health along the transect deteriorated over time; however, significantly lower frequency of PTA isolation was made from dead trees (Table 12). To assist with the interpretation of diseased symptoms, soil samples need to be taken periodically (e.g., monthly) over a full year to determine seasonal fluctuations in soil inoculum of PTA – especially in relation to rainfall, temperature and soil water content (e.g., Shearer et al. 2010). This is not a minor undertaking as in order to account for tree and site variation along the Twin Peaks Track transect, the potential study would need to have 132 trees sampled every month (i.e., >1500 soil bioassays). Application of a high-throughput screening process using a molecular assay to determine DNA content would be necessary. Determining the spatial distribution of viable PTA inoculum beneath and around diseased trees will inform management at the local level, for example in the design and routing of tracks in diseased/healthy forests.

Beever et al. (2009) proposed a rate of spread of approximately 3 m per year on the assumption of a circular area of infection at a Great Barrier Island site. This corresponds to the rate of spread associated with *P. cinnamomi* in south-western Australia (Strelein et al. 2006). After 6 years, the approximate size of infection haloes around twenty dead or symptomatic tree foci identified in 2006 ranged from 1–19 m. No knowledge of the tree health at this transect was available prior to 2006. Consequently, it is very difficult to extrapolate a linear rate of spread around the diseased foci identified in 2006 because not all the dead trees were surveyed in 2012. In addition to PTA, *P. cinnamomi*, *P. multivora* and *P. nicotianae* were also recovered (at much lower frequencies) from soil samples taken from below kauri trees along this transect. PTA is the primary pathogen; however, no information is available about the combined impact of multiple *Phytophthora* species. Because of the co-occurrence of PTA and *P. cinnamomi* in soil near the two trees we studied here, it would

seem appropriate to further investigate the interrelationship between PTA and *P. cinnamomi* in kauri decline.

Five species of *Phytophthora* are presently known from kauri (Beever et al. 2009), and all may impact upon kauri health, both individually and synergistically. *P. cinnamomi* is already confirmed as a primary pathogen of juvenile kauri (Podger & Newhook 1971; Horner 1984; Johnston et al. 2003). Other species include *P. kernoviae*, first described in the UK (Brasier et al. 2005), and recovered in Trounson Park in soil under a large dead kauri along with PTA and *P. cinnamomi* in the same 2 m^2 plot (Tony Beauchamp pers. comm.). Co-occurrence of *P. cinnamomi* with PTA implies that the interaction between dual infections by PTA and *P. cinnamomi* could represent a very important research component for future management of kauri, as *P. cinnamomi* is known to have a very wide distribution in the kauri estate (Johnston et al. 2003).

7 Vertical distribution of PTA in roots below infected trees

7.1 Introductory statements

We propose that PTA is primarily soilborne. On the basis of other *Phytophthora* species that cause collar-rot symptoms, it is likely that initial infection is through part of the root system and that the infection spreads along the large surface roots (via pathogenic lesions), eventually reaching the lower trunk (Davison & Tay 1995). From Chapter 6, describing the deliberate inoculation of 18-month old kauri seedlings with PTA-infected plant material, it was identified that fine roots were one point of entry for PTA into seedlings of kauri. It is hypothesised that damage to surface roots by PTA infection undermines the longevity of infected hosts. This chapter describes the excavation of the root systems from two PTA-diseased kauri at the Twin Peaks Track site. The soil pits adjacent to these trees were excavated as part of the study of the soil profile described in the previous chapter. The distribution of roots under two PTA-infected trees was compared using the extended soil-baiting bioassay, and direct root-plating of root tissue to *Phytophthora*-selective media.

7.2 Methods

Soil samples

The soil samples obtained in this study were recovered from the vertical soil pits established at Monique's Tree and Joan's Tree, described in the previous chapter. Soil samples were recovered from the walls of the pits at eight depths for Monique's Tree and seven depths for Joan's tree. Bulk soil samples were taken, and 200 g used for soil analyses by the Landcare Research soil analysis laboratory (Palmerston North). The remainder of the soil was analysed detailed below.

Soil bioassay

A 200 g sample was taken from each of the 8 depths under Monique's Tree and the 7 depths under Joan's Tree. The samples were air-dried for two days, then moist incubated for 4 days before being flooded and baited. Bait tissues were surface sterilised and plated to *Phytophthora*-selective media (Jeffers 2006). The plates were wrapped in foil and incubated at 18°C. In 4–7 days characteristic oogonia of PTA were observed.

Direct root assay plates

A 50 g sample from each of the 8 depths under Monique's Tree and the 7 depths under Joan's Tree was wet-sieved through a 1 mm brass sieve. The roots from each sample were collected and placed in a clean petri dish. The roots were surface-sterilised in 70% ethanol 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) and incubated in the dark for up to 10 days. (This incubation time allowed the development of characteristic oogonia of PTA, that could be visually identified; and it is also long enough for the development of the coralloid hyphae characteristic of *P. cinnamomi* (Stamps et al. 1990).) Representative pure cultures were isolated and maintained on potato dextrose agar (PDA) and identity confirmed using PCR.

DNA confirmation

Confirmation of the presence of PTA involved PCR sequencing of the isolates. This process commenced with collecting mycelium from isolates grown on PDA agar, and manual comminuting with a micropestle in 420 µl of Quiagen DXT tissue digest buffer; 4.2 µl of

proteinase K was added before incubation at 55°C for 1 h. After a brief centrifugation 220 µl of the supernatant was placed in a Corbett X-tractorGene automated nucleic acid extraction robot. The resulting 100 µl of pure DNA in TE buffer was stored at −30°C in 1.5 ml tubes until use. Gene sequences were obtained from the 18S rDNA of the ITS region. Sequences were obtained in both directions (forward and reverse primers) on an Applied Biosystems 3130xl Avant Genetic analyser using BigDye v. 3.1 chemistry. Electropherograms were analysed and assembled in Sequencher v. 4.10.1 (Gene Codes Corp.).

7.3 Results

Soil PTA recoveries

For both of the PTA-positive trees, PTA was readily recovered from the surface-soil associated with the collar infection. PTA recovery by soil bioassay was restricted to the upper 15 cm of mineral soil under Monique's Tree, and up to 20 cm below Joan's Tree (Tables 15 and 16). No *Phytophthora* species were recovered by soil bioassay below a depth of 20 cm, suggesting that the concentration of PTA inoculum is in the surface soil. The distribution of PTA, restricted to the surface soil, coincides with the concentration of roots at the soil surface.

Description of root distributions

The diseased lateral surface roots possessed few or no fine roots (Fig. 41). The upper soil horizon under both trees contained diseased, suberised, secondary roots (Tables 15 and 16). Determination and/or delimitation of lesions on the secondary roots were very hard to visualise, due to the deeply pigmented nature of the root material. PTA was recovered from roots in the surface soil from depths of 0–14 cm for Monique's Tree and 0–20 cm for Joan's Tree.

For both trees, there were healthy fine roots present below the zone of diseased roots throughout the full vertical extent of the soil pits, i.e., at 14–74 cm for Monique's Tree and at 20–70 cm below Joan's Tree. Via the direct root plating of healthy rootlets, *P. cinnamomi* was identified as being present at a depth of 64–74 cm under Monique'sTree and at a depth 61–70 cm below Joan's Tree (Tables 15 and 16).

Figure 41. Diseased lateral-surface-root system – lack of fine feeder roots.

Table 15. Vertical distribution of root health status and PTA under Monique's Tree

Depth (cm)	Soil description	Root health	Soil bioassay	Root plates
0–4	Clay minerals & organics	Diseased secondary roots	PTA	PTA
5–14	Clay, mixed, massive	Diseased secondary roots	PTA	PTA
15–24	Clay, mixed, massive	Healthy fine roots	Nil	Nil
25–34	Clay, mixed, massive	Healthy fine roots	Nil	Nil
35–44	Clay, mixed, massive	Healthy fine roots	Nil	Nil
45–54	Clay, mottles 2 mm	Healthy fine roots	Nil	Nil
55–64	Clay, mottles 0.5–1.5 mm	Healthy fine roots	Nil	Nil
65–74	Clay, mottles 5 mm	Healthy fine roots	Nil	*P. cinnamomi*

Table 16. Vertical distribution of root health status and PTA under Joan's Tree

Depth	Soil descripiton	Root health	Soil bioassay	Root plates
0–10	Organic & clay; kauri gum	Diseased secondary roots	PTA	PTA
11–20	Clay, some mottles	Diseased secondary roots	PTA	PTA
21–30	Clay, mottles	Healthy fine roots	Nil	Nil
31–40	Clay, mottles	Healthy fine roots	Nil	Nil
41–50	Clay, mottles	Healthy fine roots	Nil	Nil
51–60	Mottles 50% by vol.	Healthy fine roots	Nil	Nil
61–70	Large concretions	Healthy fine roots	Nil	*P. cinnamomi*

7.4 Discussion

The recovery of PTA was restricted to the upper 14 cm of mineral soil under Monique's Tree, and to the upper 20 cm under Joan's Tree. The validity of these results was confirmed by both the extended soil bioassay and the direct plating of diseased roots of kauri to *Phytophthora*-selective media. Soil baiting is a useful technique to study the viability of PTA inoculum in bulk soil samples, from both symptomatic and healthy kauri. The steps of the extended bioassay aims to stimulate living (or dormant) inoculum of PTA into activity. So the extended soil bioassay can be used to study temporal and spatial soil inoculum dynamics of PTA in and around disease foci, and also to help identify sites where PTA is present but the above-ground symptomology is not as yet evident. In the jarrah forest of south-west Australia inoculum of *P. cinnamomi* could be recovered more frequently from soil sampled between 10 and 80 cm below the soil surface than from the top 10 cm of the soil (Shearer & Shea 1987). The top soil profile, by containing the majority of PTA inoculum, also has the potential to transfer PTA infection to other trees and/or forests if translocated. This also applies to vertebrate vectors such as feral pigs, whose "rooting" activity involves the surface soil around tree bases as they forage for invertebrates and fungi below ground (Krull et al. 2013). Moreover, their bioturbation could potentially physically damage surface roots and expose healthy roots lower in the profile to PTA-laden topsoil.

Data relating the extent of root or collar infection/dysfunction to subsequent whole-tree health is unavailable. PTA was readily recovered from the soil around kauri showing collar infection. The absence of healthy fine feeder roots from the upper soil layers and recovery of PTA from the remaining secondary, suberised roots suggests that PTA from the remaining secondary, suberised roots suggest that PTA has killed the fine roots and now resides in the secondary, suberised roots. Direct plating of surface-sterilised roots/lesion-material to *Phytophthora*-selective agar is useful for the study of lesions caused by PTA in the secondary phloem of invaded roots or stems of kauri. The interpretation of lesions on the secondary,

suberised roots of kauri was made difficult by the degree of discoloration of the necrotic root tissue. Our studies are based upon only two trees, and further examination of larger specimens (3–4 m DBH) is required before we can extrapolate from our preliminary data on the vertical distribution of PTA under diseased kauri trees.

The recovery of *P. cinnamomi* from deeper in the soil profile was also observed by Newhook (1958); *P. cinnamomi* was recovered from depths of up to 60 cm under diseased *Pinus radiata* and other conifers in diseased shelterbelts. *P. cinnamomi* is already known to be pathogenic to kauri and has been documented as being responsible for the deaths of young rickers (Podger & Newhook 1971; Horner 1984). From the previous chapter, *P. cinnamomi,* together with *P. multivora* and *P. nicotianae,* were recovered at low frequencies from the Twin Peaks Track transects. Multiple alien *Phytophthora* taxa have been discovered on diseased ornamentals and in natural forest ecosystems in Europe and North America (Vettraino et al. 2001; Reeser et al. 2007; Moralejo et al. 2009). No knowledge exists of the combined threat of PTA and *P. cinnamomi* to kauri, but recent research of Kong et al. (2010) suggests that interspecific signalling between zoospores of different *Phytophthora* species can promote plant infection by one or more *Phytophthora*. No *Phytophthora* species were recovered by soil bioassay below a depth of 20 cm, yet *P. cinnamomi* was detected (by root plating) in the apparently undiseased roots of kauri at depth. In contrast, PTA was clearly associated with root necrosis at shallower depths under two PTA-infected trees. While this confirms PTA as the primary collar-rot pathogen of kauri, these first recoveries of *P. cinnamomi* and PTA from two PTA-infected trees, suggest that the interaction between single and dual inoculations of PTA with *P. cinnamomi* against kauri warrants future investigations. (This suggestion is reinforced by the subsequent recovery of both PTA and *P. cinnamomi* from the same surface-soil samples around one of these PTA-infected trees; see Chapter 8.)

8 Spatial distribution of soil inoculum of PTA around an infected tree

8.1 Introductory statements

We propose that PTA is primarily soilborne. On the evidence from other species that cause collar-rot symptoms, it is likely that dormant resting structures (e.g., oospores) in the soil or in infected root tissue will be the primary source of inoculum. Preceding chapters have provided evidence of the potential for PTA to form oospores in kauri roots as part of the necrotrophic phase of the disease. The temporal and spatial soil–inoculum dynamics of PTA in infested sites are not known. In this chapter, soil baiting was used to study the spatial distribution of PTA inoculum in the surface soil around an infected tree, using a systematic sampling grid superimposed over the infective focus. The tree chosen for this study is Monique's Tree, for which the vertical root and PTA distributions were mapped out in the previous chapter.

8.2 Material and Methods

Tree health status

Monique's Tree (initially labelled Hw003) was the focus of a dendochronology study by Monique P. Wheat (Wheat 2011). Initial photographs were obtained in 2008, and the collar lesion has progressively increased its vertical and horizontal extent up and around the lower trunk (Fig. 42).

Figure 42. Progressive advancement of collar-lesion up and around Monique's Tree

Soil sampling protocol

Around Monique's Tree, a 9 × 9 m grid was set up using tape measures with the tree as the central focus (Figs 43 and 44). From each of the 80 surrounding 1 m squares, an area of the soil surface was cleared away and, using a trowel, a 200–300 g sample of soil was extracted

from the top 15 cm of the soil profile. Sampling was undertaken in March 2011. The samples were extracted sequentially in a spiral-fashion beginning at the periphery, to ensure minimal disturbance to neighbouring, as yet unsampled quadrats. The zip-lock bags containing the soil samples were clearly labelled with the location and quadrat number (i.e., 1–80). A duplicate label, written on waterproof note paper, was inserted into the bag, and the samples placed in chilly-bins. All tools and sampling implements were decontaminated with 70% ethanol between the sampling of each quadrat. All solid waste (e.g., contaminated paper towels) was collected in autoclave bags which were returned to Landcare Research, St Johns for certified disposal in an Interwaste bin.

Soil bioassay

A 200 g soil sample from each of the 80 squares was analysed using the extended soil-baiting bioassay protocol. Each sample was air-dried for two days, then moist incubated for 4 days before being flooded with RO water (approx. 500 ml). Two forms of bait tissues were floated on the water surface: cedar needles (*Cedrus deodara*) and cotyledons of lupin (*Lupinus angustifolius*). The bait boxes were incubated in a Contherm Incubator at 20°C, 64% RH, and 12-hour diurnal cycle of light and dark for two days. Bait tissues were removed to clean petri dishes and surface sterilised in 70% ethanol for 30 seconds. The baits were rinsed twice in sterilised RO water, blotted dry and plated to *Phytophthora*-selective media (Jeffers 2006). The plates were wrapped in foil and incubated at 18°C in the dark. After 4–7 days, characteristic oogonia of PTA were observed. At the end of the bioassay, each bait box was dosed with 1 ml Trigene II Advance, the lids replaced, before being disposed of in an Interwaste bin for certified disposal.

DNA confirmation

PTA identifications were confirmed by PCR sequencing of the isolates. Mycelium was collected from isolates growing on PDA agar, and manually comminuted with a micropestle in 420 µl of Quiagen DXT tissue digest buffer; 4.2 µl of proteinase K was added before incubation at 55°C for 1 h. After a brief centrifugation, 220 µl of the supernatant was placed in a Corbett X-tractorGene automated nucleic acid extraction robot. The resulting 100 µl of pure DNA in TE buffer was stored at −30°C in 1.5 ml tubes until required for sequencing. Gene sequences were obtained from the 18S rDNA of the ITS region. Sequences were obtained in both directions (forward and reverse primers) on an Applied Biosystems 3130xl Avant Genetic analyser using BigDye v. 3.1 chemistry. Electropherograms were analysed and assembled in Sequencher v. 4.10.1 (Gene Codes Corp.).

8.3 Results

For this study, carried out in late summer/early autumn of 2011, PTA was recovered by conventional soil baiting from 26 of the 80 samples (32.5%); PTA was recovered by both bait tissues from 16 samples, only by lupin cotyledons from 5 samples, and only by cedar needles from 5 samples (Appendix 7). The 16 samples from which PTA was detected by both baits were all grouped in the area immediately adjacent to the collar lesion and extending level with, or downslope from, the lesion (Figs 42 and 43). No PTA was detected in the three samples (Samples 73–75) immediately adjacent to the tree on the side opposite the collar lesion; and PTA was recovered from only 5 upslope samples (each by only one of the bait tissues).

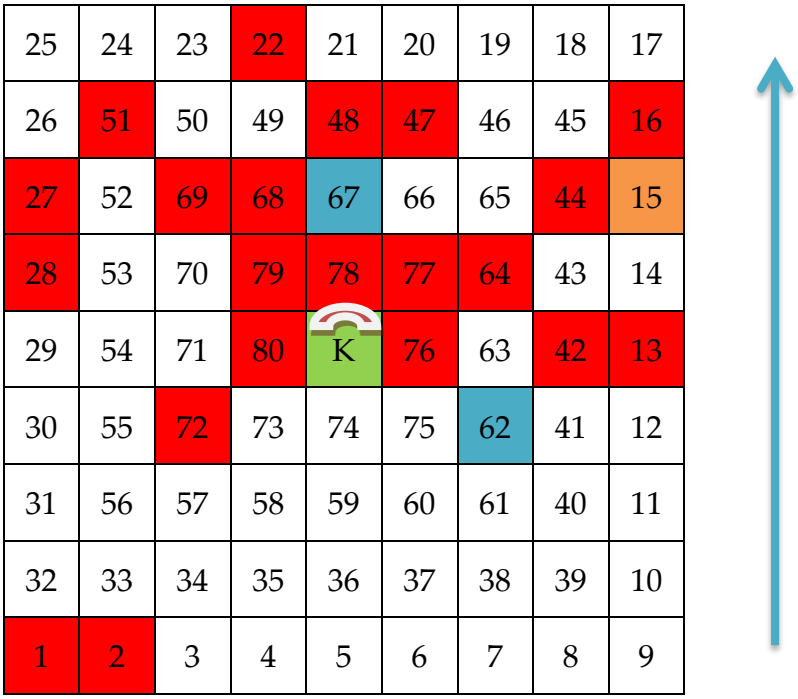


Figure 43. Lupin cotyledon recovery of PTA (red squares), *P. cinnamomi* (orange square; also Sample 2) and *P. multivora* (blue squares) from around Monique's Tree (K). Each square is 1 m², with light-blue arrow showing downward direction of slope. N.B. Location of lesion identified adjacent to sample 78.

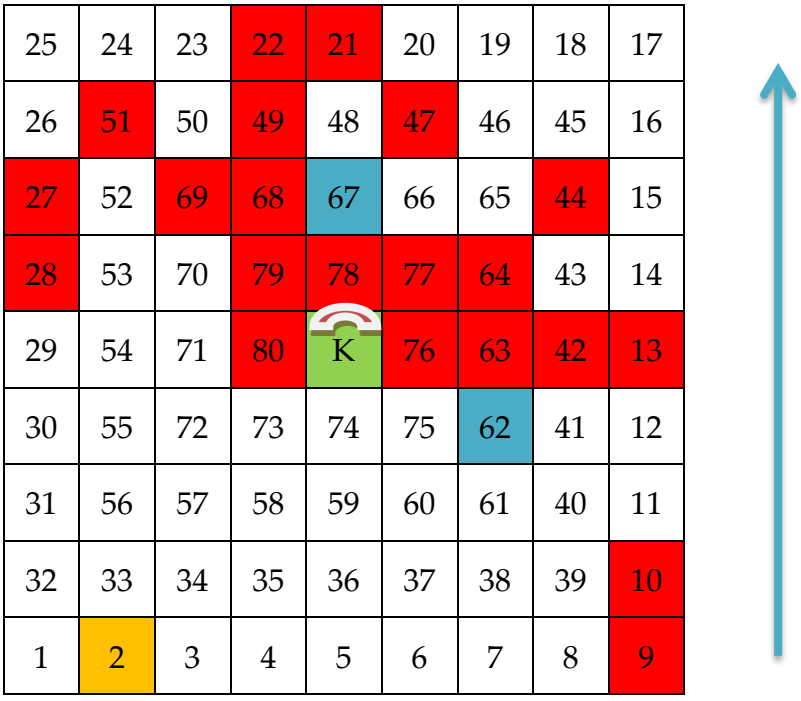


Figure 44. Cedar needle recovery of PTA (red squares), *P. cinnamomi* (orange square; also Sample 77) and *P. multivora* (blue squares) from around Monique's Tree (K). Each square is 1 m², with light-blue arrow showing downward direction of slope. N.B. Location of lesion identified adjacent to sample 78.

P. cinnamomi and *P. multivora* were recovered by both bait tissues, but the overall frequency was low (*P. cinnamomi* 3.75%; *P. multivora* 2.5%). From two samples, both PTA and *P. cinnamomi* were recovered (Sample 2, Fig. 43; Sample 77, Fig. 44), mostly from a distance of 5–5.5 m from the base of the PTA-positive tree.

8.4 Discussion

Recovery of PTA inoculum extends beyond the base of the infected kauri to a distance of at least 5.5 m (for an 88 cm DBH tree). It has already been established from the preceding chapter that the depth from which PTA was most readily recovered from soil and root assays was 0–14(–20) cm. However, from the results of this intensive sampling programme, there was a patchy distribution of PTA recoveries around the central focus, with PTA being readily recovered adjacent to the lesion. Around the other side of the tree, within 1 m of the trunk of the PTA-positive tree were soil samples that were negative for PTA. This suggests that in order to to get a comprehensive sample of soil around a symptomatic or asymptomatic kauri tree, all cardinal points around the target tree need to be sampled. Additionally, if possible, soil samples should be taken from distances stepped out from the centrum to the edge of the canopy drip-line. Further, high density samples need to be taken around trees of a range of DBH size classes to establish if there is a consistent spatial pattern in the distribution of PTA inoculum in the upper soil layers around infected trees. This of course will be regulated by the length of time since infection, the nature and distribution of the initial point(s) of infection, and a series of co-factors governing disease development and display of host symptoms.

The contiguity of "red" squares identified from the intense soil sampling effort could reflect the movement of PTA infection in the shallow, lateral roots, as it progresses towards to the lower trunk / collar region. The relationship between the soil-based detection needs to be correlated with development of lesions caused by PTA in the secondary phloem of roots and collar of kauri, across a range of sites, and age classes. This could be achieved by excavation of the surface roots and dissection out of samples. Consideration must be given to the impact of such an investigation on the surrounding trees. Further, high-density samples need to be taken around trees of a range of DBH size classes to establish if there is a consistent spatial pattern in the distribution of PTA inoculum in the upper soil layers around infected trees. These types of studies will also help resolve difficulties in standardising the soil-based recovery of PTA and other *Phytophthora* species. There are a range of biotic and abiotic factors which make the diagnosis and management of *Phytophthora* collar- and root-rots challenging (Tsao 1990). These include the ability of *Phytophthora* species to lay down long-lived, resistant and/or dormant resting structures. PTA is homothallic and has the ability to produce oospores from a single culture. PTA has been reported to have approximately 80% dormancy of oospores produced on artificial media (Bellgard et al. 2010 unpubl. data). Over 80%, and up to 95%, of all oospores produced by the three different *Phytophthora* species studied by Sutherland & Cohen (1983) were in a dormant phase. One of the challenges to the conventional extended soil bioassay is the risk that dormant structures will fail to be detected. If dormant oospores fail to be stimulated to germinate and produce a sporangium, PTA will not be detected.

Three other *Phytophthora* species were recovered at a very low frequencies from around Monique's tree: *P. cinnamomi* and *P. multivora*. We have already discussed the historical relationships of *P. cinnamomi* and kauri decline in the Waitakere Ranges as first reported by Podger and Newhook (1971). Over the past 4 years, ICMP has curated a number of *P. multivora* isolates from a diverse range of hosts, most recently from the soil around

apparently healthy planted kauri from Otari-Wilton's Bush, Wellington (Wynn Udall pers. comm., Victoria University, Wellington) (Table 17).

Table 17. Host and locality data for New Zealand records of *Phytophthora multivora*

ICMP #	Host	Locality, contributor, date
18451	*Griselinia littoralis*	Auckland, R. Thangavel, 2010
19002	*Sophora microphylla*	Pt. Chevalier, C. Inglis, 2010
19030	*Dacrydium cupressinum*	Warkworth, D. Poulter, 2011
19049	*Acer palmatum*	Otahuhu, C. Inglis, 2010
19175	*Metrosideros kermadecensis*	Wiri, R. Thangavel, 2011
19508	*Hebe stricta*	Swanson Stream, N. Waipara, 2012
20019	*Agathis australis*	Otari, Wellington, S. Bellgard, 2013

Multiple *Phytophthora* species were found to be associated with stem cankers on tanoak in south-western Oregon (Reeser et al. 2007). From 263 cankered tanoak trees sampled, 97 were culture negative for *Phytophthora* species, and 123 were culture positive for *P. ramorum*; additional *Phytophthora* species isolated from tanoak stem cankers were *P. cambivora* (4×), *P. cinnamomi* (1×), *P. gonapodyides* (4×), *P. nemarosa* (31×), and an undescribed *Phytophthora* species (1×). While the focus of the Sudden Oak Death response centres on *P. ramorum*, other *Phytophthora* species, although recovered less frequently, are also considered to be playing a role in the syndrome.

The previous chapter reported the recovery of *P. cinnamomi* from healthy roots at depth under two PTA-infected trees. In this study, around a single tree we recovered both *P. cinnamomi* and *P. multivora* from the upper soil layer at low frequencies. This represents the first record of this nature, and further intensive sampling around PTA-infected trees is necessary to corroborate this preliminary finding, which may necessitate an investigation into the single and dual inoculation of kauri with *P. multivora* and PTA to see if there is any additive effect of two *Phytophthora* species on kauri decline. While evidence in the preceding chapters clearly shows PTA is the primary collar-rot pathogen of kauri, the role of co-factors such as other pathogens (e.g., *P. cinnamomi* and *P. multivora*), climate change, pig-rooting and other localised disturbances cannot be discounted from exacerbating kauri dieback. Any future research should include at a minimum, a risk-based assessment of the low-level presence of *P. multivora* in kauri forests.

9 Development of a species-specific assay for PTA

9.1 Introductory statements

Identification of *Phytophthora* spp. has been assisted through advances in molecular-based systems for identification. Species specific polymerase chain reaction (PCR) technologies are routinely used for the rapid and accurate identification of *Phytophthora* species (Minerdi et al. 2008). PCR can exponentially amplify specific DNA sequences of *Phytophthora* by *in vitro* DNA synthesis. Nuclear molecular loci used to discriminate *Phytophthora* spp. include: ITS (Cooke et al. 2000), β-tubulin (Kroon et al. 2004a; Blair et al. 2008; Bilodeau et al. 2007a), translation elongation factor 1-α (Kroon et al. 2004a; Blair et al. 2008), elicitin (Bilodeau et al. 2007 a, b), 60 Ribosomal protein L10, enolase, heat shock protein, TigA gene fusion protein (Blair et al. 2008) and *Ypt* 1 (e.g., Cooke et al. 2000; Schena et al. 2008). Mitochondrial multiple-copy gene regions that have been successfully used for phylogeny and molecular diagnostics include: cox 1 (Kroon et al. 2004a, b), cox 2 (Martin & Tooley 2003), cox 1 and cox 2 spacer (Martin & Tooley 2003), nad 1 (Kroon et al. 2004a), nad 5 (Ivors et al. 2004).

Conventional PCR-based techniques have alleviated some of the problems associated with traditional mycological approaches. However, technical limitations related to post-amplification procedures and cross contamination have seen real-time PCR (RT-PCR) techniques replace conventional approaches for high-throughput diagnostic applications (Schena et al. 2004). RT-PCR enables automation of techniques and is suitable for large-scale sample processing. Several RT-PCR assays have been described for detection of the important quarantine pathogen *P. ramorum.* Bilodeau et al. (2005) described an assay based upon ITS, β-tubulin, and elicitin regions using TaqMan and SYBR Green assays. Hughes et al. (2006) described an ITS-based RT-PCR assay that uses TaqMan chemistry and has been adapted for field use with a SmartCyber unit. The aim of this study was to develop a species-specific PCR assay for PTA that could be used to detect PTA in plant tissue and soil.

9.2 Materials and Methods

Conventional PCR and sequencing

Sequences of four gene regions were determined for the isolates listed in Tables 18 and 19. These represent *Phytophthora* and *Pythium* species that have been isolated from soil in kauri forests. The gene regions selected were NADH dehydrogenase subunit 1 (NADH1), translation elongation factor 1-α (TEF), β-tubulin (β-Tub) and 18S rRNA. NADH1, TEF and β-Tub genes were amplified using the primers and method described in Kroon et al. (2004a). The 18S rRNA SSU ITS region was amplified using primers ITS6 and ITS4 (White et al. 1990). Successful amplifications were then confirmed by visualisation of the PCR by electrophoresis. The PCR products were then sequenced using the ABI Genetic Analyser 3130xl sequencing machine (Applied Biosystems).

Table 18. *Phytophthora* and *Pythium* species recovered from kauri forests used for cross reactivity and specificity testing.

Species	Isolate reference	Culture collection/ sample identifier	Host	Method of recovery	Location
Phytophthora cinnamomi	REB326-68	LCR	Fuschia leaf	Stream bait	Waitakere
P. citricola	REB326-61	LCR	Fuschia leaf	Stream bait	Waitakere
P. citricola	REB326-67	LCR	Fuschia leaf	Stream bait	Waitakere
P. cryptogea	REB326-60	LCR	Fuschia leaf	Stream bait	Waitakere
Phytophthora sp.	REB326-69	LCR	Fuschia leaf	Stream bait	Waitakere
Pythium sp.	REB326-70	LCR	Fuschia leaf	Stream bait	Waitakere
Pythium sp.	REB326-73	LCR	Fuschia leaf	Stream bait	Waitakere
Pythium sp.	REB326-74	LCR	Fuschia leaf	Stream bait	Waitakere
Pythium sp.	REB326-75	LCR	Rhododendron	Stream bait	Waitakere
Pythium sp.	REB326-77	LCR	Rhododendron	Stream bait	Waitakere
PTA	REB327-68	LCR	Kauri	Soil bioassay	Twin Peak Track
PTA	REB327-73	Phy 155	Kauri	Soil bioassay	Waitakere
PTA	REB327-80	Phy 131	Kauri (Trounson Tr 1-1)	Soil bioassay	Trounson Kauri Park[13]

Table 19. *Phytophthora* species used for cross-reactivity and specificity testing (carried out at DEFRA[14] Laboratories)

Species	Culture collection
Phytophthora boehmeriae	DEFRA
Phytophthora botryosa	DEFRA
Phytophthora cactorum	DEFRA
Phytophthora cambivora	DEFRA
Phytophthora cinnamomi	DEFRA
Phytophthora citricola	DEFRA
Phytophthora citrophthora	DEFRA
Phytophthora cryptogea	DEFRA
Phytophthora europaea	DEFRA
Phytophthora fragariae var. *rubi*	DEFRA
Phytophthora gonapodyides	DEFRA
Phytophthora heveae	DEFRA
Phytophthora hibernalis	DEFRA
Phytophthora ilicis	DEFRA
Phytophthora insolita	DEFRA

[13] We acknowledge access to sample Trounson Kauri Park under DoC High Impact Permit.

[14] DEFRA: Department of Environment, Food and Rural Affairs, London, UK.

Species	Culture collection
Phytophthora kernoviae	DEFRA
Phytophthora lateralis	DEFRA
Phytophthora megasperma	DEFRA
Phytophthora nemorosa	DEFRA
Phytophthora palmivora	DEFRA
Phytophthora parasitica	DEFRA
Phytophthora pseudosyringae	DEFRA
Phytophthora ramorum	DEFRA
Phytophthora richardiae	DEFRA
Phytophthora syringae	DEFRA
Phytophthora uliginosa	DEFRA

TaqMan primers and probe for RT-PCR

Alignments of sequences of PTA (target sequence) and non-target *Phytophthora* and *Pythium* isolated from kauri forest were constructed using Geneious Pro software (Biomatters) and used as the basis for primer and probe design. Initial examination of alignments of NADH, TEF, β-Tub and ITS indicated that the ITS region displayed the highest variability between target and non-target species, so this region was selected for the design of primers and probe specific to PTA. The sequences used for the alignment on which primer design was based are shown in Table 20. Primer Express 3 software (Applied Biosystems) was used to design the primers and probe for the TaqMan assay.

Table 20. Sequences of real-time PCR primers and TaqMan probe targeting the ITS-region for PTA detection

Primer/Probe	Sequence (5'–3')	5' base position in sequence accession EF067922.1 for PTA
PTA_ITS_F2	AACCAATAGTTGGGGGCGA	36
PTA_ITS_R3	GACGAGCTCTATCATGGCGAG	97
PTA_ITS_Probe[1]	GGCGGCTGCTGGCTTTGGCT	62

[1] Labelled with 5' reporter FAM (6-carboxyfluorescein) and 3' quencher BHQ1 (Black Hole Quencher 1).

TaqMan RT-PCR and assay specificity

The assay was tested against all the isolates collected from kauri forests and an additional 26 *Phytophthora* isolates from DEFRA. A TaqMan RT-PCR was carried out on a Rotorgene 6000 instrument (Qiagen) using TaqMan Environmental Master Mix (Applied Biosystems). Each 15 µl reaction contained primers at a final concentration of 350 nM and probe at 80 nM and DNA at 1 µl DNA. The reaction conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 61°C for 60 s. Potential false-negatives due to PCR inhibition were monitored using an internal positive control from Applied Biosystems (TaqMan Exogenous Internal Positive Control Reagent using VIC probe). Negative controls containing water instead of DNA and positive controls consisting of PTA genomic DNA (REB327-68) were included in all TaqMan RT-PCR runs. Reactions were carried out in triplicate except where otherwise stated.

Assay sensitivity

Assay sensitivity was determined by testing a 10-fold dilution series of PTA DNA (REB327-68) ranging from 2 ng/µl to 0.2 fg/µl. DNA concentrations were determined using a fluorimeter. The average amount of DNA in 100 g of soil sample is about 180 ng, averaged from isolations of five soil samples. To investigate the effects of inhibitors carried over in DNA extraction from soil, the same dilution series was tested with the addition of 180 ng of DNA extracted from PTA-free soil to each reaction (a brown sandy loam soil used for native amenity planting at Landcare Research, St Johns). The top 0–10 cm of soil was used. Four replicate reactions were used to test each dilution.

Oospore detection in artificially inoculated soil

Soil was collected from the Landcare Research gardens and prepared for DNA extraction as described above. A 10-fold dilution series of PTA oospores (isolate ICMP 18407) was prepared. Mean oospore density was estimated by 10 counts using a haemocytometer. Known numbers of oospores were added to 100 g samples of dry soil, ranging from 40,000 to 400 oospores per sample. Samples were subjected to DNA extraction; unspiked soil from the same source was used as the extraction negative control. DNA was extracted from five replicate samples at each oospore dilution, and each extract was tested by TaqMan RT-PCR in triplicate.

Application of assay to field samples

The TaqMan RT-PCR assay was validated using field-soil samples collected as part of the study described in section 6.3. DNA was extracted from 40 soil samples and tested for PTA using the TaqMan RT-PCR assay. Eight were previously known to be positive for PTA from the conventional lupin baiting technique (also, one had *P. cinnamomi,* and one had *P. multivora*). Two soil samples, spiked with PTA oospores (40,000), were used as positive controls, and four soil samples were collected from PTA-free sites and used as negative controls.

Statistical analysis

Regression analysis

The diagnostic sensitivity and specificity were calculated from the 2×2 contingency table following the method described by Lane et al. (2006) and Hughes et al. (2006).

9.3 Results

TaqMan real-time PCR assay development

The ITS gene region was selected for having the most variability in the target sequence compared with the non-target *Phytophthora* and *Pythium* sequences. The other gene regions were too similar to achieve discrimination between PTA and the non-target species. It was also selected due to its presence in high copy numbers, providing more sensitivity. Forward and reverse primers, and a TaqManprobe were designed in the ITS1 region of PTA (Table 20). When tested against all non-target cultures, this primer combination amplified only the target PTA cultures (Table 21).

Table 21. Results of specificity testing using RT-PCR assay.

Species	Isolate reference	Culture collection	CT value	Result
Phytophthora cinnamomi	REB326-68	LCR	>40	–
Phytophthora citricola	REB326-61	LCR	>40	–
Phytophthora citricola	REB326-67	LCR	>40	–
Phytophthora cryptogea	REB326-60	LCR	>40	–
Phytophthora sp.	REB326-69	LCR	>40	–
Pythium sp.	REB326-70	LCR	>40	–
Pythium sp.	REB326-73	LCR	>40	–
Pythium sp.	REB326-74	LCR	>40	–
Pythium sp.	REB326-75	LCR	>40	–
Pythium sp.	REB326-77	LCR	>40	–
PTA	REB327-68	LCR	16.3	+
PTA	REB327-73	LCR	16.2	+
PTA	REB327-80	LCR	19.1	+
Phytophthora boehmeriae	—	DEFRA	>40	–
Phytophthora botryosa	—	DEFRA	>40	–
Phytophthora cactorum	—	DEFRA	>40	–
Phytophthora cinnamomi	—	DEFRA	>40	–
Phytophthora citricola	—	DEFRA	>40	–
Phytophthora citrophthora	—	DEFRA	>40	–
Phytophthora cryptogea	—	DEFRA	>40	–
Phytophthora europaea	—	DEFRA	>40	–
Phytophthora fragariae var. *rubi*	—	DEFRA	>40	–
Phytophthora gonapodyides	—	DEFRA	>40	–
Phytophthora heveae	—	DEFRA	>40	–
Phytophthora hibernalis	—	DEFRA	>40	–
Phytophthora ilicis	—	DEFRA	>40	–
Phytophthora insolita	—	DEFRA	>40	–
Phytophthora kernoviae	—	DEFRA	>40	–
Phytophthora lateralis	—	DEFRA	>40	–
Phytophthora megasperma	—	DEFRA	>40	–
Phytophthora nemorosa	—	DEFRA	>40	–
Phytophthora palmivora	—	DEFRA	>40	–
Phytophthora parasitica	—	DEFRA	>40	–
Phytophthora pseudosyringae	—	DEFRA	>40	–
Phytophthora ramorum	—	DEFRA	>40	–
Phytophthora richardiae	—	DEFRA	>40	–
Phytophthora syringae	—	DEFRA	>40	–
Phytophthora uliginosa	—	DEFRA	>40	–
Phytophthora cambivora	—	DEFRA	>40	–

Assay specificity

In all, 30 different species of *Phytophthora* and five *Pythium* species were tested using the PTA RT-PCR TaqMan assay. Detection of PTA was observed with cycle threshold (Ct) values varying between 16.2 and 19.1. All other *Phytophthora* and *Pythium* spp. tested negative (Table 21).

Assay efficiency and limit of detection

Detection of PTA DNA from pure cultures had a limit of detection of 2.00 fg (extrapolated from Fig. 45; antilog of 0.303 = 2.00). Detection of PTA DNA from soil containing PTAwas not as sensitive as from pure cultures of PTA; the limit of detection of PTA from soil was 20 fg (extrapolated from Fig. 45; antilog of 1.302 = 20.04).

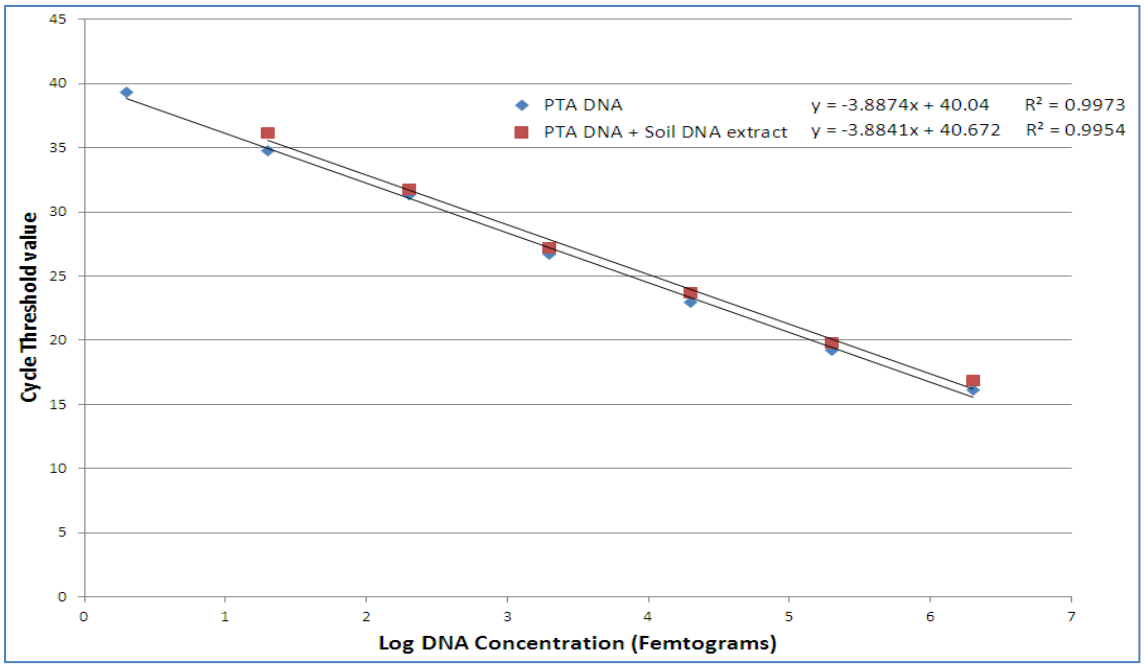


Figure 45. Assay sensitivity graph showing a liner relationship between DNA concentration and Ct value for PTA.

The cycle threshold for PTA detection of 400 oospores per 100 g of soil was 32.7 ± 0.35 (range based upon application of 95% confidence limit: 32.02–33.38) (Table 22).

Table 22. Sensitivity of TaqMan ITS PTA-specific primer

Assay (per 100 g soil)	TaqMan ITS		95% confidence limit
	Mean CT	SEM	
40,000 oospores	26.02	0.45	–
4000 oospores	29.82	0.48	–
400 oospores	32.70	0.35	± 0.68
Positive control (REB327-68)	22.74	0.01	–
Negative control	35.43	0.43	–
Water negative control	–	–	–

CT = Cycle threshold; SEM = standard error; (–) represents a negative result; 95% confidence limit estimated using sd = 0.78 (calculated from SEM of 0.35), n = 5, α = 0.05.

Table 23. Soil bioassay versus RT-PCR detection of PTA in 40 soil samples taken from under Monique's Tree

Sample	Soil baiting	RT-PCR (Ct)	Concordancy
31	Nil	100	Yes
32	Pc	35	Yes
33	Nil	38	Yes
34	Nil	35.7	Yes
35	Nil	30.3	No
36	Nil	100	Yes
37	Nil	100	Yes
38	Nil	100	Yes
39	Nil	39	Yes
40	Nil	35	Yes
41	Nil	25	No
42	PTA	30.7	Yes
43	Nil	34.2	Yes
44	PTA	31	Yes
45	Nil	34.8	Yes
46	Nil	33.9	Yes
47	PTA	25.6	Yes
48	PTA	38.3	No
49	PTA	24	Yes
50	Nil	35.6	Yes
51	PTA	24.4	Yes
52	Nil	33.2	No
53	Nil	37.9	Yes
54	Nil	100	Yes
55	Nil	28.7	No
56	Nil	27.9	No
57	Nil	100	Yes
58	Nil	32.1	No
59	Nil	33.2	No
60	Nil	37	Yes
61	Nil	100	Yes
62	Pm	100	Yes
63	PTA	100	No
64	PTA	37	No
65	Nil	36.6	Yes
66	Nil	25	No
68	PTA	36.6	No
71	Nil	30	No
79	PTA	26	Yes
80	PTA	26	Yes

N.B. Soil baiting data = the combined results from lupin and cedar baits. Red highlighting indicates PTA detections. RT-PCR PTA threshold = 33.38 (= 95% c.l.); Pc = *P. cinnamomi*; Pm = *P. multivora*

Application of assay to field samples

Conventional soil baiting recovered PTA from 11 out of the 40 samples (Table 23). The RT-PCR performed well in comparison with the soil bioassay with 16 detections of PTA (Table 23). The RT-PCR assay did not detect *P. cinnamomi* or *P. multivora,* which were detected by soil bioassay (Table 23).

The diagnostic sensitivity of the RT-PCR assay was 63.6% and its diagnostic specificity was 69% (Table 24).

For 9 soil samples (22.5%), RT-PCR picked up "false negatives" (i.e., when PTA was not detected by soil bioassay). On 4 occasions (10 %), the soil bioassay picked up "false negatives" (i.e., when PTA was not detected by RT-PCR).

Table 24. Comparison of the efficacy of soil bioassay versus RT-PCR detection of PTA

RT-PCR	Soil bioassay		Total
	+	–	
+	7	9	16
–	4	20	24
Total	11	29	40

N.B. RT-PCR diagnostic sensitivity = (7/(7+4)) ×100 = 63.6%; RT-PCR diagnostic specificity = (20/(20+9)) ×100 = 69%

9.4 Discussion

The conventional approach to *Phytophthora* diagnosis involves either the use of leaf baits to recover PTA from flooded soil samples (Dance et al. 1975) or mycological methods involving isolation from host material to *Phytophthora*-specific media, followed by morphological identification. There are, of course, some limitations to using traditional morphological identification with microscopy, as the taxonomic features upon which the systematic keys are based require human interpretation, and this can lead to mistakes. Morphological diagnosis of *Phytophthora* spp. has been greatly assisted by the use of conventional PCR – mainly through the use of the internal transcribed spacer (ITS) 1 and 2 regions of the nuclear ribosomal gene (see Cooke et al. 2000). More recently, researchers are using multiple loci from both the nuclear and mitochondrial genomes to resolve species boundaries within *Phytophthora* (e.g., Martin & Tooley 2003; Kroon et al. 2004a; Villa et al. 2006; Blair et al. 2008; Weir et al. 2012).

PCR-based and RT-PCR technologies have increasingly been favoured for rapid and accurate identification of *Phytophthora* spp. (Minerdi et al. 2008). The PCR-based "molecular tool box" provided by Schena et al. (2008) was based upon the ras-related protein gene *Ypt-1* and used nested PCR techniques to increase the sensitivity of detection. To expedite the reliable, accurate and rapid diagnosis from symptomatic and asymptomatic tissue and soil, one-step real-time protocols have been developed (e.g., Hughes et al. 2006).The present study successfully developed a specific primer for PTA using TaqMan chemistry. We successfully used this protocol to assay PTA from soil and plant material (Than et al. 2013).

Effective disease management and implementation of plant health legislation is reliant upon rapid and accurate disease diagnosis based upon recognition of symptoms in the field and identification of the cause by conventional laboratory testing. This assay may be useful in resolving "false-negative" situations when conflicting results from field surveys and complementary assays (e.g., soil bioassay; cambial tissue sampling) suggest PTA may be

present but confirmation of this cannot be validated by existing techniques. Development of a duplex assay (i.e., incorporating elements of the extended soil baiting bioassay and RT-PCR assay of the bait tissues) will help add reliability to PTA soilborne detection.

Combination assays utilising soil baiting and direct PCR of leaf bait tissues are well established (Nechwatal et al. 2001). In this duplex assay, soil baits are not surface sterilised and plated to *Phytophthora*-selective media, but are removed to an Eppendorf® tube for immediate DNA extraction and RT-PCR analysis. By including this step, the speed of PTA detection can be increased by 7 days.

Other applications of RT-PCR technology could potentially integrate generic lateral-flow devices (i.e., LFD) with RT-PCR. Tomlinson et al. (2010) have developed a rapid-detection method for *P. ramorum* and *P. kernoviae* by a 2 min DNA extraction followed by a loop-mediated, isothermal amplification (LAMP) and amplicon detection. There is the potential to use the DNA extraction protocol described by Tomlinson et al. (2010) to recover DNA from the nitrocellulose membranes contained in the LFDs, and then use the alternative TaqMan-based, PTA-specific RT-PCR analysis described in this study to determine if PTA is present.

The 10-fold reduction in sensitivity of the assay in the presence of soil (compared with pure culture assay; Fig. 45) may be due to organic products (e.g., humic acids) and organic decomposition products that are present in field-soil (e.g., Picard et al. 1992) interfering with the TaqMan reaction chemistry. Removal of these potential contaminants has been achieved using precipitation reactions and size exclusion chromatography (e.g., Matheson et al. 2010). There are opportunities to refine the sensitivity of the present assay through the integration of these types of novel sample pre-treatments.

10 Recommendations

10.1 PTA specific research recommendations

After consideration of the data and review of past and current research literature, we consider that the following areas of research are necessary to help "Keep Kauri Standing".

Biology and origins of PTA

1 Continue population evaluation, and determine haplotype diversity within PTA populations in New Zealand.
2 Develop molecular tool to track PTA movement within New Zealand.

Clarifying PTA disease aetiology and host range

1 Continue host range studies using root-based inoculation on the remaining species identified by the P&I team, namely, Kirk's daisy *Brachyglottis kirkii*, neinei *Dracophyllum latifolium*, and tawari *Ixerba brexioides*.
2 Elucidate factors governing fine-root infection, and progression of disease from fine roots to collar.
3 Histological studies of disease progression within host, and host responses to disease.
4 Study density-dependent and environmental factors controlling zoospore release and infection of fine roots.
5 Study environmental factors controlling oospore dormancy and germination.

Temporal and spatial soil inoculum dynamics of PTA in a forest stand

1 Establish further National Vegetation Survey plots in other prioritised kauri forest systems – Waipoua, Trounson, Albany, Coromandel and Hunua. Ideally, these will be placed in healthy and infested kauri forests, and monitored regularly.
2 Establish soil test pits to better characterise soils in healthy and diseased kauri forests throughout the geographic range of kauri (see above).
3 Undertake plant health analysis of taxa found from this study to be susceptible to PTA in order to confirm in-field susceptibility.

PTA detection

1 Develop a duplex assay, integrating RT-PCR detection of PTA with conventional soil bioassay, to increase the speed of the detection process.
2 Run RT-PCR concurrently with future soil bioassays to obtain specificity and sensitivity data.
3 Institute RT-PCR detection of PTA as a routine protocol for the resolution of suspected "false negative" soil-based results.

10.2 Associated research needs

Cascading plant community issues

1 Instigate studies on the consequences of PTA and *Phytophthora*-mediated selection on plant community dynamics in representative forests throughout the kauri estate.
2 Study changes in nutrient cycling (especially C:N ratios) in PTA-infested plant communities, and the consequences for C release in relation to sink-source dynamics compared with healthy kauri stands.

3 Assess hydrological changes at diseased sites in response to removal of deep-rooted, overstorey tree species across the kauri estate, and how this influences post-disease plant recruitment and succession.
4 Monitor secondary succession and parameterise the loss in ecosystem function for kauri-dependent fauna.

Other *Phytophthora* speces

1 Review by-catch data from Surveillance 2, to identify the frequency and impact (i.e., presence of trunk and/or crown decline symptoms) of other *Phytophthora* species present in kauri forest.
2 Evaluate susceptiblity of kauri to *P. kernoviae* and *P. multivora.*
3 Evaluate susceptibility of plants in the kauri forest to *P. cinnamomi, P. kernoviae, P. nicotianae* and *P. multivora*: singly and in combinations as part of future surveillance efforts.

Nursery pathway

1 Consider screening nurseries to evaluate *Phytophthora* risks.
2 Consider a nursery certification scheme to ensure that plant material is certified *Phytophthora*-free.
3 Consider a plant nursery education programme promoting industry "best-practice".
4 Consider development of a "clean-chain" for certified, disease-free plant-growth media for the New Zealand nursery industry esp. importation of plant materials (e.g., peat) and live Rhododendron from Europe and USA as potential pathways for *P. ramorum*.

11 Kauri at risk from the genus Phytophthora? – considering the wider picture

Phytophthora species have been recognised as impacting upon kauri health in the Waitakere Ranges since 1956 (Newhook 1960). The collar-rot symptoms of kauri dieback were first recognised in the 1970s (Gadgil 1974), although erroneously attributed to *P. heveae*. *P. cinnamomi* had been isolated as early as 1960 from each of four indigenous communities near Auckland: three were second-growth communities respectively dominated by (1) mānuka, (2) kauri regrowth, and (3) māmāngi–nīkau association, and the fourth was in mature forest in the Waitakere Ranges (Podger & Newhook 1971). Nine out of 10 kauri trees sampled in undisturbed virgin forest on Little Barrier Island were positive for *P. cinnamomi* (Newhook 1960). *P. cinnamomi* is widely found in natural stands and has been linked to ill-thrift and occasional tree death, especially, in regenerating kauri stands on poorly drained sites (Podger & Newhook 1971). Symptoms include severe chlorosis, canopy-thinning, "little-leaf" syndrome and crown dieback, sometimes resulting in tree death. PTA symptoms are similar, but additionally affected trees have lesions at the collar. These lesions sometimes encircle the stem and bleed copious amounts of resin (kauri gum).

Of the *Phytophthora* species associated with kauri, *P. cinnamomi, P. cryptogea* and *P. nicotianae* were likely introduced to New Zealand following human contact about 600 years ago (Beever et al. 2009). Exactly when PTA was introduced is unknown. The first known PTA-infected area was on Great Barrier Island (Compartment 47) being part of a forest logged in 1931–33, with a few old stumps still remaining in 1971 (Gadgil 1974). The affected area covered about 1.5 ha in which a few saplings and rickers from 5–30 cm DBH were dead and the rest had pale green to yerllowish foliage and thin crowns (Gadgil 1974). Earlier, "damping off" syndromes were recorded as impacting upon kauri seedling health at the Waipoua Nursery in the 1950's (Morrison 1954). So was there a pathway of transfer from Waipoua Nursery to Great Barrier Island which saw the movement of PTA-infected seedlings to Great Barrier Island and out-planted into Compartment 47 in the 1950's?

11.1 Taxonomy and origins of PTA

On the basis of genetic data, comparative morphology and host-specificity, we propose in a forthcoming publication to formally name PTA as *Phytophthora "agathidicida"* (ined.; the "*Agathis*-killing" *Phytophthora*), a new species within ITS Clade 5. In addition, another new morphospecies, previously misidentified as *P. castaneae*, has been delineated as *P. "cocois"* (the "coconut" *Phytophthora*). To date, it has been confirmed that the closely allied taxa *P. heveae* and *P. castaneae* occur in Papua New Guinea, Queensland and Taiwan. Further study of cultural, morphological and molecular variation of isolates from around the world, including, in particular, areas of occurrence of *Agathis* species (especially the Philippines, Papua New Guinea, New Caledonia, Vanuatu and Queensland) are still warranted to better clarify the original source of PTA. There are, potentially, undiscovered members of ITS Clade 5 species – including *P. "novae-guineae"* isolated from under *Agathis* in Papua New Guinea – that need further consideration. Andre Drenth (University of Queensland) now curates the culture collection of Bruce Brown who carried out *Phytophthora* research in tropical Queensland in the 1980s (see Brown 1999). Through DNA extraction and sequencing of samples from this collection it could be determined whether PTA occurs in Australia. *Agathis robusta* (Queensland kauri) seedlings were resistant to PTA (Table 8) and could be informative in understanding PTA resistance in a closely related *Agathis* species.

We propose that PTA is an invasive exotic species, but conjecture remains about its geographic origin. Further studies into the population biology of PTA are necessary to answer the question of whether or not PTA represents a "founder population" and can be confirmed as an exotic incursion. Microsatellite sequences have been obtained by pyrosequencing of multiplex-enriched libraries of PTA and representatives of the other ITS Clade 5 species. Candidate loci with dinucleotides or trinucleotides were selected and primer pairs were tested for eight PTA isolates and two isolates each of *P. heveae*, *P. castaneae* and *P. "cocois"*. The 18 most polymorphic and unambiguous loci have been selected for the development of the three multiplex PCR pools. From this analysis, nucleotide diversity and the number of multi-locus genotypes ("haplotypes") present in New Zealand can be estimated. This information will guide discussions about whether there is any evidence for the development of genetic structure in the PTA population present in New Zealand, since its putative introduction.

The other consideration is of course the distribution of the *Phytophthora* species closely allied to PTA, namely *P. heveae* and *P. castaneae.* Arentz (1986) reported both species are present in soils in Papua New Guinea, including rainforests containing *Agathis robusta* and *Araucaria cunninghamii* and *A. hunsteinii*, although without any reported linkage to disease. Brown (1999), in a study focused on the association between *P. cinnamomi* and patch death in tropical rainforests of northern Queensland (containing both *Agathis* and *Araucaria*), also reported both *P. heveae* and *P. castaneae*, with *P. heveae* more frequent at higher elevations under dead patches of rainforest. Ko et al. (2006) report both species from natural forests in Taiwan, neither in obvious association with disease. However, none of these authors provide DNA sequence data for their isolates and so the precise relationships are not clear, although the oogonial ornamentation of the Taiwanese *P. castaneae* isolates matched ICMP16915, also from Taiwan. Ko et al. (2006) pointed out that their Hawaiian *Cocos* isolates of *"P. castaneae"* (i.e., isolates H1024 and H1027) were much less ornamented than the Taiwanese isolates; and indeed some approach *P. heveae* and PTA in their lack of ornamentation, although differing from both in ITS sequence. While it is reasonable to conclude both *P. heveae* and *P. castaneae* are indigenous to eastern Asia and Australasia, whether records from further afield (Erwin & Ribeiro 1996) represent indigenous populations or recent introductions must await further study.

11.2 Clarifying the aetiology and host range of PTA

Future surveillance programmes should also assess the plant health status of the non-*Agathis* plant species in the kauri forest community. Not enough is known about disease progression and the extent of damage on the other plant species tested in this study; plants were studied only as young specimens in the glasshouse rather than in the natural forest. The application of evolutionary tools (e.g., centrifugal phylogenetic analysis; Wapshere 1974) for phytosanitary risk analysis is well established (e.g., Gilbert et al. 2012). The identification of a "phylogenetic signal" discriminated from the future host-range testing of PTA could be applied to help predict the potential host-range of novel pathogens such as *P. multivora*, and the potential pre-border threat of *P. ramorum* on the native flora on New Zealand.

11.3 Transfer of PTA infection

Roots containing oospores of PTA, when placed adjacent to kauri roots, could transfer infection. Therefore, oospores (or hyphae) in fine roots, secondary roots and root-collar debris could play a part in the survival and transfer of PTA if soil containing PTA-infected roots is accidentally moved to an uninfested kauri forest. In native forests, PTA has been recovered only from kauri; however, we have little or no knowledge of the field susceptibility of any other woody plant species growing in kauri forests. Differential staining of oospores

was used to visualise PTA in the root tissues of kauri, and oospores were also found in the diseased roots of deliberately inoculated korokio seedlings. One very powerful extension of the primer we have designed is the ability to bind it to a fluorescent tag, or marker, which can also be differentially visualised under UV light (Vandersea et al. 2006). The use of fluorescent in situ hybridisation (FISH) to differentiate *Phytophthora* hyphal structures from other root-inhabiting fungi will provide the ability to study interactive effects of PTA with other "beneficial" fungi currently being considered as biocontrol candidates (Bellgard et al. 2012).

11.4 Temporal and spatial soil inoculum dynamics of PTA in a forest stand

The temporal dynamics and spatial distribution of PTA inoculum need further examination, especially in relation to kauri phenology. Patterns of inoculum occurrence in PTA disease centres need to be studied using the drying and flooding of soil samples taken from around an infected tree. In this way the density and diversity of viable inoculum can be ascertained. Phenological studies relating host activities to pathogen dynamics could be studied via periodic sampling over 1–3 years. Application of molecular RT-PCR techniques could be used to increase the efficiency of diagnostics, by providing PTA DNA concentrations of the soil samples.

The appropriate temperature regime for sporulation and zoospore release and the relationship to the impervious nature of the underlying subsoil clay layer need to be understood. Because of the presence of *P. cinnamomi* at a depth of 70 cm, there could be opportunity for dispersal of zoospores laterally by subsurface water flow (e.g., Kinal et al. 1993).

11.5 Improving the detection of PTA

A TaqMan RT-PCR assay was developed, tested, and validated on soil and plant material. This molecular technology could be applied to assist with the resolution of "false negative" PTA results when physical disease symptoms suggest that the pathogen is present in either soil or cambium samples. The opportunity exists to integrate the RT-PCR assay with the soil-baiting technique to reduce the time needed for soil-based detection by 7 days. Additionally, the RT-PCR assay could be applied to analysis of LFD kits. Plant material (e.g., cork cambium samples) could be placed in the extraction buffer and shaken vigorously to break down the plant material. A small aliquot would be pipetted to the LFD and the reaction assessed. The LFD could then be broken open and extraction of PTA-DNA contained on the nitrocellulose membrane undertaken using the RT-PCR assay. Each of the steps could be carried out without specialist equipment and is potentially suitable for on-site use applications – with results being available in about an hour if the isothermal block is mounted on the back of a vehicle. The expertise exists within Ecogene® to undertake the "tech-transfer" needed to adapt the RT-PCR TaqMan assay into a mobile unit that could be transported in a vehicle, to enable feasibility studies to be carried out regarding the field application of this protocol.

11.6 PTA and environmental stressors

From the studies presented, it is clear that kauri dieback is a complex and challenging problem. One approach to this biologically and ecologically destructive phenomenon of kauri is to consider the disease "syndrome" as the dynamic interplay between three interrelated parameters: (a) the severity of the environment, (b) the prevalence of inoculum of the pathogen, and (c) the susceptibility of the host (Fig. 46). This conceptualisation is also a useful framework for the final concluding discussion. In the depiction below, the extent of damage to the host by disease is mediated by the severity of the environment (e.g., water

stress, nutrient stress) and the prevalence and virulence of the pathogen. The soil profiles under diseased kauri at the study site appear to have characteristics that are conducive to PTA infection; that is, they are prone to saturation, which could promote sporulation of zoospores when flooded. It is also clear that the soils are depauperate of the key plant nutrients N and P. In order to be able to generalise more broadly about the edaphic and landform factors that render sites conducive to high impacts by kauri dieback, more soil test plots need to be established in other kauri forests. Sites could include, but not be limited to, Maungaroa Ridge (Piha), the Hunua Ranges, Raetea Forest, Waipoua Forest, Trounson Forest, the Whangarei region, and Coromandel Peninsula.

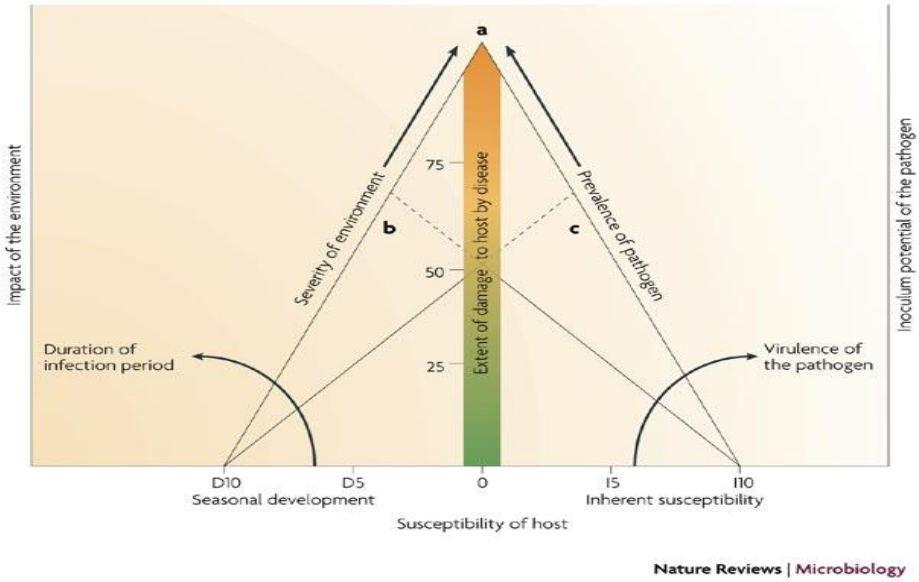


Figure 46. The disease triangle "revisited" (Scholthof 2007).

Studies investigating the role of drought stress and waterlogging on kauri physiology need to be undertaken. The susceptibility of kauri to PTA infection in relation to hydraulic conductivity (plant and soil) and stomatal conductance also needs to be understood, as this will help inform decisions around future possible interventions, for example augmenting water supplies in summer months. Also, there need to be nutritional physiological studies of the host, to see if fertilisation could help in host–disease resistance. Huber & Haneklaus (2007) consider that the nutrition of a plant determines in a large measure its resistance or susceptibility to disease, its histological or morphological structure or properties, and the virulence or ability of pathogens to survive. For example, potassium nutrition has been linked to a number of physiological and biochemical processes that have relevance for susceptibility to pathogens and insects (Amtmann et al. 2008). Silicon has been shown to alleviate abiotic and biotic stresses and increases the resistance of plants to pathogenic fungi through the activation of plant defence mechanisms (Fauteux et al. 2005). Information and data gathered as part of previous silvicultural experiments investigating kauri growth responses to artificial fertilisers should be revisited in terms of health and potential for the use of remedial fertilisation treatments in diseased stands.

Genetic resistance in host populations will also affect the spread of the disease – nothing is known about the prevalence of PTA resistance and/or tolerance in kauri populations. Determining if any resistance to PTA is present among populations across the geographic range of kauri may allow the development of resistant seedling lines, and also assist with predictions on the spread of the disease. Studies to understand the seed biology and ways of

cryostoring important kauri germplasm should be undertaken as a priority. Securing important iconic trees as tissue cultures, seedlings/progeny-line plantings in secure, offshore, PTA-free arboreta/sanctuaries should also be considered as part of the long-term security of the taxon.

11.7 Multi-species *Phytophthora* threats

Through the process of stimulating PTA activity, there is a risk that other *Phytophthora* species may also take advantage of the enhanced infection conditions. For example, Table 25 summarises unpublished by-catch data from soil analysis from Surveillance 2 (Appendix 8).

Table 25. Summary of by-catch data Surveillance 2, samples 052–103

Isolation	Number of recoveries
PTA	11
Pc	14
Pm	11
Phyt sp.	9
Pyth	6
PTA+Pc	6
PTA+Pm	1
Pc+Phyt sp.	2
Pc+Pm	1
PTA+Phyt sp.	2
No recovery	19
Σ	82

Pc = *P. cinnamomi*; Pm = *P. multivora*, Phyt sp. = *Phytophthora* sp. Pyth =- *Pythium* sp.

PTA was recovered a total of 20 times; eleven times as the only *Phytophthora* species, six times together with with *P. cinnamomi* in a soil sample, and once co-occurring with *P. multivora*. *P. cinnamomi* was recovered 14 times as the only *Phytophthora* species isolated from a soil samples, and *P. multivora* on 11 occasions. For these 52 soil samples, oomycetes were found not to be present in 19 soil samples (i.e. 36.5% of soil samples did not have any recoveries).

Although PTA has been identified as posing a particular threat to kauri, it is important to clarify whether other *Phytophthora* species are also impacting kauri in the field. Five species of *Phytophthora* are presently known from kauri (Beever et al. 2006, unpubl. report) and all may impact kauri, both individually and synergistically. These could include *P. kernoviae* first described in the UK (Brasier et al. 2005), and recovered from Trounson Park in soil under a large dead kauri. Its pathogenicity to kauri, together with that of *P. multivora*, if any, needs clarification. Ho et al. (2010) recovered *P. multivora* associated with kawakawa tree decline in the Oratia region of New Zealand. Declining trees were found in poorly drained soil, at least temporarily water-logged during winter months.

It has been recently proposed that the geographic origin of *P. multivora* is situated in the Afromontane region of eastern and southern Africa (Rea et al. 2012). Coincidently, podocarp species (e.g., *Afrocarpus*) are also native to this region of Africa (Page 1989). The plant trade is unwittingly accelerating the worldwide spread of well-known and new or undescribed *Phytophthora* species and creating novel niches for emerging pathogens (Moralejo et al. 2009). Some form of screening process to ensure that *Phytophthora* species are not

unintentionally translocated to amenity plantings or native restoration planting-sites via native potted plants needs to be considered via some form of Nursery Accreditation Scheme[15].

11.8 Cascading hierarchy of impacts

It is necessary to develop expertise to predict the likely impacts of PTA at the ecosystem level. Will kauri be completely lost from these stands? Or will a residue of "escapes" remain capable of ensuring long-term retention of species dependent on kauri, whether it be native bats living in the hollow trunks of old giants, or native orchids restricted to the humus layer under kauri. To document what is actually happening at infected sites, long-term plots need to be established at representative kauri forests throughout its geographic range. Detailed monitoring of these plots will provide the data necessary for generating a predictive kauri population model (R.E. Beever pers. obs.).

The core issues and those that cascade from PTA disease centres of kauri dieback still need to be completely articulated. PTA-mediated kauri death has the potential to affect structural composition through the selective removal of a keystone canopy species (see Shearer et al. 2009). The potential for PTA to have a wider host range than just kauri could mean a change in forest species composition (Fig. 47).

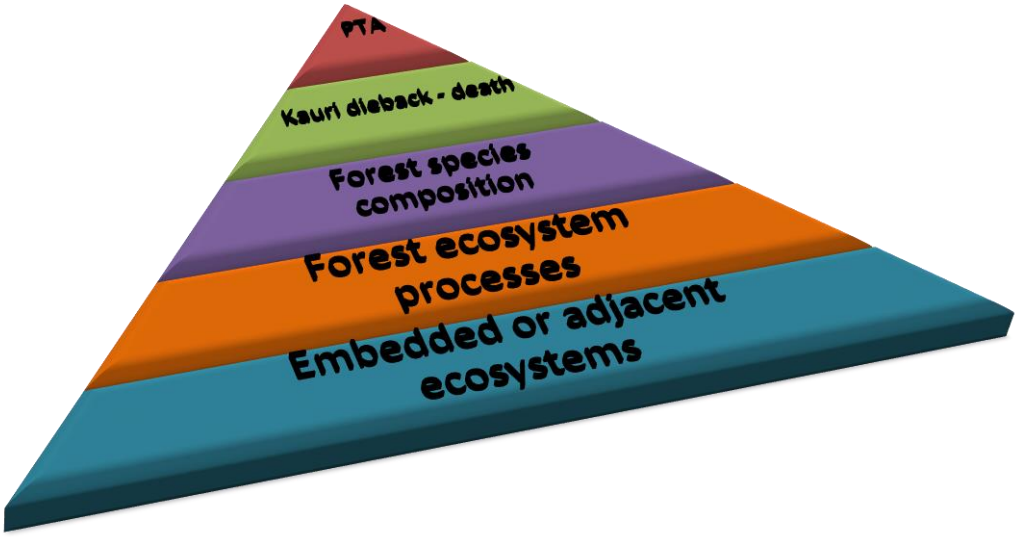


Figure 47. The triangle of cascading impacts potentially due to PTA-collar-rot of kauri (schema adapted from Lovett et al. 2006).

Any change in plant host distributions could also change host receptivity and/or the pathogen's invasion success, arrival, and establishment. The contemporaneous presence of other *Phytophthora* species may also put other plant species in the kauri forest at risk. Forest ecosystem processes such as carbon-capture, productivity, nutrient cycling, and carbon-release could also be impacted as a consequence of kauri removal. These ecosystem

[15] http://www.ngia.com.au/Category?Action=View&Category_id=125

processes can feed back to affect the other species; for example, increased light (due to canopy opening) and nutrient availability from kauri death may improve the conditions for survivors. Potential destabilisation of soil profiles as a consequence of premature kauri death and breakdown of its root systems could reduce water quality downstream, due to increased siltation of runoff. This could be of particular importance where kauri dieback occurs in water catchments.

12 Acknowledgements

This work would not have been possible without the pioneering, ground-breaking research of the late Ross Beever (Fig. 48). Through ITS analysis he made the discovery that the kauri pathogen on Great Barrier was not *P. heveae.* Together with Ian Horner, Peter Johnston and Duckchul Park, he went on to make the link between PTA and kauri collar-rot, and recognised the first clues about another taxonomic entity in Clade 5 associated with *Cocos* – this discovery was made mainly on the morphology of the oospore.

Figure 48. The "clean-team" (c. 2009), L-R: Ross Beever [deceased], Joan Webber, Margaret Dick and Tod Ramsfield (ex-Scion) emphasising the importance of phytosanitary measures to minimise the spread of PTA.

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Appendix 1. List of PTA and non-NZ isolates studied

Appendix 1A: Origin of PTA isolates used in this study

ICMP	Host	Location	Isolation year	Substrate	Other references	Sample details
16471	Kauri	Gt Barrier	1971	Soil		
17027	**Kauri**	**Gt Barrier**	**2006**	**Cambium**	**REB 316-14**	**K2**
18244	Kauri	Pakiri	2008	Cambium	REB 326-1	Bleeding lesion
18358	Kauri	Waitakere	2009	Cambium	REB 326-221	Tree RJ 1C1
18360	Kauri	Gt Barrier	2009	Cambium	REB 326-155	Tree 2 low lesion P2
18401	Kauri	Raetea	2010	Cambium	REB 327-34	Raetea Tree 2 ET lesion edge
18403	Kauri	Raetea	2010	Cambium	REB 327-39	Raetea Tree 2 along lens
18404	Kauri	Trounson	2010	Cambium	REB 327-41	Trounson Tree 1
18405	Kauri	Trounson	2010	Cambium	REB 327-42	Trounson Tree 1 tissue 3
18406	Kauri	Huia	2010	Soil	REB 327-46	Twin Peaks Track site
18407	Kauri	Waipoua	2010	Cambium	REB 327-47	TM Tree 2 tissue outer
18408	Kauri	Waipoua	2010	Cambium	REB 327-53	Gov. Grove Tissue 1
18410	Kauri	Trounson	2010	Soil	REB 327-60	Tree 2 dead roots

N.B.. Isolate ICMP 18405 comes from the same tree as isolate ICMP 18404. We wish to acknowledge the issue of the high impact DOC permit to sample Northland kauri in 2010, negotiated by Tony Beauchamp.

Appendix 1B: Non-NZ isolates studied

Isolate	Other references	Species	Substrate	Country
ICMP 16915	–	*P. castaneae*	soil	Taiwan
ICMP 18737	–	*P. castaneae*	*Castanopsis*	Taiwan
ICMP 19434	**NBRC 9753**	***P. castaneae***	***Castanea***	**Japan**
ICMP 19435	NBRC 30433	*P. castaneae*	*Castanea*	Japan
ICMP 19436	NBRC 30434	*P. castaneae*	*Castanea*	Japan
ICMP 19437	NBRC 30435	*P. castaneae*	*Castanea*	Japan
ICMP 19450	–	*P. castaneae*	soil	Taiwan
ICMP 19635	P10661	*P. castaneae*	forest soil	China
ICMP 16948	**–**	***P. "cocois"***	***Cocos***	**USA, Hawai'i**
ICMP 16949	–	*P. "cocois"*	*Cocos*	USA, Hawai'i
ICMP 19685	IMI 360596	*P. "cocois"*	*Cocos nucifera*	Ivory Coast
ICMP 16691	–	*P. heveae*	soil	Australia
ICMP 16914	IMI 131093	*P. heveae*	*Theobroma cacao*	Malaysia
ICMP 17964	–	*P. heveae*	soil	China
ICMP 19451	**CBS 296.29**	***P. heveae***	***Hevea***	**Malaysia**
ICMP 19452	CBS 954.87	*P. heveae*	*Persea*	Guatemala
ICMP 19453	ATCC 46299, CBS 958.87	*P. heveae*	*Rhododendron*	USA, NC
ICMP 19454	**CBS 124094**	***P. multivora***	**soil**	**Australia**

N.B. Designated and proposed type cultures in bold

Appendix 2. Glasshouse conditions and host-plant data

Appendix 2A: Glasshouse conditions: mean temperature (°C) and relative humidity (%), 2010–2013.

Year	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
Temp 2010	–	–	–	–	–	–	–	–	–	16.9	18.6	22.3
Temp 2011	22.9	21.4	19.7	17.4	15.8	14.9	11.47	13.0	13.8	18.8	17.8	19.3
Temp 2012	19.7	20.1	18.6	17.5	14.6	12.7	12.7	13.4	14.6	15.8	17.33	20.4
Temp 2013	22.1	21.9	20.6	17.8	14.3	–	–	–	–	–	–	–

Year	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
RH 2010	–	–	–	–	–	–	–	–	–	74.9	75	76.9
RH 2011	71	77.5	73.7	81	89	89	92.7	93	87	79	73.5	80.3
RH 2012	79	79	79	78	81	83.7	84	87	79	76.7	72	78
RH 2013	70.1	70.7	78	89.5	95.6	–	–	–	–	–	–	–

Appendix 2B: host-plant data and statistical analysis (See attached PDF).

Appendix 3. Transfer of infection

	ICMP 18403	Shoot mass (g)	Mean (fshwt g)	SD	Root mass (g)	Mean (fshwt g)	SD	Shoot length (cm)	Mean	SD	Root length (cm)	Mean	SEM
18403 1-1	Fine roots	14.36	5.174	4.351615792	7.94	2.466	2.448869127	39	22.9	11.91124585	17	12.7	5.538752367
18403 1-2	Fine roots	9.81			4.22			33			18		
18403 1-3	Fine roots	7.15			3.94			33			15		
18403 1-4	Fine roots	5.56			2.7			28			15		
18403 1-5	Fine roots	4.22			1.63			24			15		
18403 1-6	Fine roots	4.1			2.7			28			14		
18403 1-7	Fine roots	2.5			0.88			17			16		
18403 1-8	Fine roots	3.54			0.63			17			10		
18403 1-9	Fine roots	0.5			0.02			10			7		
18403 1-10	Fine roots	0			0			0			0		
Control 1-1	Fine roots	16.49	12.414	6.965915749	9.59	5.834	4.201587531	46	38.5	7.245688373	22	18.2	#NAME?
Control 1-2	Fine roots	26.93			15.65			45			20		
Control 1-3	Fine roots	6.94			2.28			35			16		
Control 1-4	Fine roots	17.94			7.01			48			19		
Control 1-5	Fine roots	7.24			3.14			32			16		
Control 1-6	Fine roots	8.24			4.12			32			19		
Control 1-7	Fine roots	6.94	0.003065212		2.28	0.01544068		35			16		
Control 1-8	Fine roots	17.94			7.01			48			19		
Control 1-9	Fine roots	7.24			3.14			32			16		
Control 1-10	Fine roots	8.24			4.12			32			19		
18403 1-1	Secondary roots	13.4	6.074	6.062410962	6.94	2.978	3.263433093	35	24.9	15.94748326	19	12.7	7.454305244
18403 1-2	Secondary roots	6.76			6.94			30			17		
18403 1-3	Secondary roots	18.59			8.33			50			23		
18403 1-4	Secondary roots	5.53			2.7			35			15		
18403 1-5	Secondary roots	8.42			2.97			33			15		
18403 1-6	Secondary roots	4.28			1.63			19			14		
18403 1-7	Secondary roots	2.88			0.22			30			12		
18403 1-8	Secondary roots	0.88			0.05			17			12		
18403 1-9	Secondary roots	0			0			0			0		
18403 1-10	Secondary roots	0			0			0			0		
Control 1-1	Secondary roots	16.49	22.672	4.628618224	9.59	13.9	3.877470539	46	45.9	0.737864787	22	20	6.20996267
Control 1-2	Secondary roots	26.93			15.65			45			20		
Control 1-3	Secondary roots	16.49			9.59			46			22		
Control 1-4	Secondary roots	26.93		0.000332772	15.65			45			20		
Control 1-5	Secondary roots	26.08			19.55	0.000214958		47			18		
Control 1-6	Secondary roots	22.15			12.09			46			19		
Control 1-7	Secondary roots	16.49			9.59			46			22		
Control 1-8	Secondary roots	26.93			15.65			45			20		
Control 1-9	Secondary roots	26.08			19.55			47			18		
Control 1-10	Secondary roots	22.15			12.09			46			19		
18403 1-1	Primary roots	0	0.927	1.997342957	0	0.5	1.064174171	0	4.4	9.32380466	0	3.111111111	5.902918299
18403 1-2	Primary roots	0			0			0			0		
18403 1-3	Primary roots	0			0			0			0		
18403 1-4	Primary roots	0			0			0			0		
18403 1-5	Primary roots	0			0			0			0		
18403 1-6	Primary roots	0			0			0			0		
18403 1-7	Primary roots	0			0			0			0		
18403 1-8	Primary roots	0			0	1.48051E-06		0			0		
18403 1-9	Primary roots	5.51		1.2905E-07	2.19			24			14		

	ICMP 18403	Shoot mass (g)	Mean (fshwt g)	SD	Root mass (g)	Mean (fshwt g)	SD	Shoot length (cm)	Mean	SD	Root length (cm)	Mean	SEM
18403 1-10	Primary roots	3.76			2.81			20			14		
Control 1-1	Primary roots	16.49	22.672	4.628618224	9.59	13.9	3.877470539	46	46.2	0.918936583	21	19.77777778	1.523883927
Control 1-2	Primary roots	26.93			15.65			45			22		
Control 1-3	Primary roots	16.49			9.59			46			19		
Control 1-4	Primary roots	26.93			15.65			48			21		
Control 1-5	Primary roots	26.08			19.55			46			18		
Control 1-6	Primary roots	22.15			12.09			47			19		
Control 1-7	Primary roots	16.49			9.59			46			22		
Control 1-8	Primary roots	26.93			15.65			45			20		
Control 1-9	Primary roots	26.08			19.55			46			18		
Control 1-10	Primary roots	22.15			12.09			47			19		
18403 1-1	Collar tissue	33.16	7.857	10.13433772	7.71	2.898	3.926254081	56	24.3	17.72035866	23	14.25	8.885850175
18403 1-2	Collar tissue	16.41	0.042587513		7.91			46			25		
18403 1-3	Collar tissue	6.44			3.3			32			20		
18403 1-4	Collar tissue	8.18			4.6	2.7527E-05		29			19		
18403 1-5	Collar tissue	5.03			2.1			23			15		
18403 1-6	Collar tissue	5.67			2.23			20			17		
18403 1-7	Collar tissue	2.39			0.9			18			16		
18403 1-8	Collar tissue	1.1			0.23			19			7.5		
18403 1-9	Collar tissue	0.19			0			0			0		
18403 1-10	Collar tissue	0			0			0			0		
Control 1-1	Collar tissue	0	21.023	8.442139079	19.55	14.896	3.926254081	46	41.2	10.44349878	0	17.2	15.2225014
Control 1-2	Collar tissue	22.15			12.09			45			20		
Control 1-3	Collar tissue	16.49			9.59			46			18		
Control 1-4	Collar tissue	26.93			15.65			47			19		
Control 1-5	Collar tissue	26.08			19.55			23			15		
Control 1-6	Collar tissue	22.15			12.09			20			17		
Control 1-7	Collar tissue	26.93			15.65			46			21		
Control 1-8	Collar tissue	16.49			9.59			45			22		
Control 1-9	Collar tissue	26.93			15.65			46			19		
Control 1-10	Collar tissue	26.08			19.55			48			21		

Appendix 4. Soils data

Appendix 4A: Soil sample analysis from Monique's Tree (March 2011)[16]

Depth (cm)	Horizon	Texture & field notes	Munsell colour H/V/C	pH (1:2)	EC (1:2) (Ds/m)	Grams water per 100 g soil	Organic C (%)	Total N (%)	NO3-N (mg/kg)	NH4-N (mg/kg)	Bray 2 P (mg/kg)	CEC (cmol(+)/kg)	Acid Oxalate Extr (%)		
													Fe	Al	Si
6–3	Oi	Coarse organic debris													
3–0	Oe	Finely divided organics	Black/Brown												
0–4	A	Clay minerals & organics	9YR/3.5/2.0	5.3	0.16	83.93	12.7	0.61	4.1	34.2	3		1.12	0.51	0.02
5–14	B	Clay, mixed, massive	10YR/3.0/4.4	4.3	0.22	67.75	6.2	0.40	2.6	19.2	1	33.3	1.22	0.53	0.05
15–24	B	Clay, mixed, massive	8YR/4.6/2.2	4.1	0.24	69.02									
25–34	B	Clay, mixed, massive	8.5YR/4.5/3.1	4	0.26	66.83									
35–44	B	Clay, mixed, massive	9YR/5.4/3.9	3.9	0.3	64.76									
45–54	B	Clay, Mottles, 2 mm d., rare	9YR/5.4/3.10; motts 8.5 YR/5.4/3.9	4.1	0.33	62.11									
55–64	B	Clay, Mottles, 0.5 - 1.5 mm, 10 % by volume	9YR/4.4/4.5; motts9R/5.3/12.7	4	0.23	70.02									
65–74	B	Clay, Mottles, 5 mm d, 5 % by volume	9YR/4.4/4.5; motts9R/5.3/12.7	4	0.26	67.85	2.1	0.14	2.0	7.0	<1		0.50	0.48	0.08

[16] Grey highlighted rows indicate the O horizon strata, with depths measured upward from the top of the A horizon.

Appendix 4B. Soil sample analysis from Joan's Tree (March 2011)[17]

Depth (cm)	Horizon	Texture & field notes	Munsell colour H/V/C	pH (1:2)	EC (1:2) (Ds/m)	Grams water per 100 g soil	Organic C (%)	Total N (%)	NO3-N (mg/kg)	NH4-N (mg/kg)	Bray 2 P (mg/kg)	CEC (cmol(+)/kg)	Acid Oxalate Extr (%)		
													Fe	Al	Si
3–0	Oi	Coarse, organic debris													
0–10	A & Oe	Organic & Clay, Much Kauri gum	9YR/3.5/2.0	5.6	0.66	87.02	11.9	0.52	7.1	26.6	3		1.18	0.47	0.04
11–20	B	Clay, Some mottles	9YR/3.4/2.9 Motts 9.5YR/4.2/5.7	5.4	0.42	65.81									
21–30	B	Clay, mottles	9.5yr/5.7/3.8 Motts 9.5YR/4.1/6.7	5.3	0.29	57.46	3.0	0.21	2.3	9.5	1	46.2	1.22	0.48	0.06
31–40	B	Clay, mottles	9.5YR/4.1/6.7 Motts 7.5YR/6.7/13.5	5	0.13	62.48									
41–50	B	Clay, mottles	9.5YR/4.1/6.7 Motts 7.5YR/6.7/13.6	4.8	0.15	64.53									
51–60	B	Mottles 50% by vol.Hard concretions, clay	8YR/4.6/2.2 Motts 7.5YR/7.0/10.5	4.8	0.18	62.74									
61–70	B	Some very large, hard concretions. Heterogeneous, Clay; sandy clay.	8YR/4.6/2.2 Motts 7.5YR/7.0/10.6	4.7	0.38	68.21	0.79	0.06	2.4	7.7	<1		0.36	0.56	0.05

[17] Grey highlighted rows indicate the O horizon strata, with depths measured upward from the top of the A horizon.

Appendix 5. Twin Peak Track data/analysis

Appenidix 5A: Tree Health Status for 2006, 2009, and 2012.

See attached PDF

Appendix 5B: Trees sampled in March 2011 to confirm PTA presence / absence.

Sample	PTA	Sample	PTA	Sample	PTA
24244	n/t	24225	Yes	24331	No
24257	n/t	24264	Yes	24280	Yes
24250	n/t	24239	Yes	24247	n/t
24249	n/t	24263	n/t	24277	No
24336	No	24261	n/t	24275	No
24234	No	24294	No	24287	No
24337	No	24295	No	24290	No
24252	n/t	24313	No	24248	n/t
24334	No	24292	No	24269	No
24255	n/t	24293	No	24271	Yes
24221	No	24235	No	24286	No
24265	Yes	24229	n/t	24282	No
24245	n/t	24335	No	24285	No
24238	No	24218	Yes	24283	Yes
24243	n/t	24330	Yes	24289	No
24251	n/t	24273	No	24274	No
24220	n/t	24345	n/t	24276	No
24339	No	24254	No	24262	n/t
24284	No	24258	n/t	24329	No
24224	No	24272	Yes	24340	Yes
24223	No	24231	n/t	24219	Yes
24253	n/t	24217	n/t	24288	No
24236	No	24344	No	24281	Yes

N.B. Yes = PTA detected, No = no PTA detected, n/t = not tested

Appendix 6. Rate-of-spread analysis

Focus	Diameter of infected foci (m)
1	7
2	3
3	11
4	19
5	13
6	5
7	3
8	6
9	6
10	12
11	3
12	1
13	8
14	8.5
15	5
16	6
17	1
18	1
19	7
20	11
Σ	136.5

Appendix 7. Soil bioassay data from 80 soil samples around Monique's tree

Sample	Lupin cotyledon	Cedar
Huia 1	PTA	Nil
Huia 2	PTA Pc	Pc
Huia 3	Nil	Nil
Huia 4	Nil	Nil
Huia 5	Nil	Nil
Huia 6	Nil	Nil
Huia 7	Nil	Nil
Huia 8	Nil	Nil
Huia 9	Nil	PTA
Huia 10	Nil	PTA
Huia 11	Nil	Nil
Huia 12	Nil	Nil
Huia 13	PTA	PTA
Huia 14	Nil	Nil
Huia 15	Pc	Nil
Huia 16	PTA	Nil
Huia 17	Nil	Nil
Huia 18	Nil	Nil
Huia 19	Nil	Nil
Huia 20	Nil	Nil
Huia 21	Nil	PTA
Huia 22	PTA	PTA
Huia 23	Nil	Nil
Huia 24	Nil	Nil
Huia 25	Nil	Nil
Huia 26	Nil	Nil
Huia 27	PTA	PTA
Huia 28	PTA	PTA
Huia 29	Nil	Nil
Huia 30	Nil	Nil
Huia 31	Nil	Nil
Huia 32	Nil	Nil
Huia 33	Nil	Nil
Huia 34	Nil	Nil
Huia 35	Nil	Nil
Huia 36	Nil	Nil
Huia 37	Nil	Nil
Huia 38	Nil	Nil
Huia 39	Nil	Nil
Huia 40	Nil	Nil
Huia 41	Nil	Nil
Huia 42	PTA	PTA
Huia 43	Nil	Nil
Huia 44	PTA	PTA

Sample	Lupin cotyledon	Cedar
Huia 45	Nil	Nil
Huia 46	Nil	Nil
Huia 47	PTA	PTA
Huia 48	PTA	Nil
Huia 49	Nil	PTA
Huia 50	Nil	Nil
Huia 51	PTA	PTA
Huia 52	Nil	Nil
Huia 53	Nil	Nil
Huia 54	Nil	Nil
Huia 55	Nil	Nil
Huia 56	Nil	Nil
Huia 57	Nil	Nil
Huia 58	Nil	Nil
Huia 59	Nil	Nil
Huia 60	Nil	Nil
Huia 61	Nil	Nil
Huia 62	Pm	Pm
Huia 63	Nil	PTA
Huia 64	PTA	PTA
Huia 65	Nil	Nil
Huia 66	Nil	Nil
Huia 67	Pm	Pm
Huia 68	PTA	PTA
Huia 69	PTA	PTA
Huia 70	Nil	Nil
Huia 71	Nil	Nil
Huia 72	PTA	Nil
Huia 73	Nil	Nil
Huia 74	Nil	Nil
Huia 75	Nil	Nil
Huia 76	PTA	PTA
Huia 77	PTA	PTA Pc
Huia 78	PTA	PTA
Huia 79	PTA	PTA
Huia 80	PTA	PTA
Σ	21 PTA; 2 Pc; 2 Pm	21 PTA; 2 Pc, 2 Pm

N.B. PTA = *Phytophthora* "taxon Agathis"; Pc = *P. cinnamomi*; Pm = *P. multivora.*

Appendix 8. By-catch data from Surveillance 2 (Samples 52–120)

Sample	Landcare Research – Lupin	Landcare Research – Cedar	Plant & Food	Scion
052	n/d	n/d	Phyt. sp.	n/t
053	n/t	n/t	n/d	n/d
054	n/d	n/d	n/d	n/t
055	n/t	n/t	Pm	n/d
056	PTA+Pc	Pc	PTA+Pm	n/t
057	n/t	n/t	Phyt. sp	Pc
058	n/d	n/d	n/t	Pc+Phyt sp.
059	n/d	n/d	n/t	n/t
060	Pm	Pm	n/t	n/d
061	n/d	n/d	n/d	n/t
062	n/t	n/t	n/d	n/d
063	n/d	n/d	n/t	n/d
064	Pc	n/d	n/d	n/t
065	n/t	n/t	n/d	n/d
066	Pc	Pc	n/t	n/d
067	Pc+Pm	Pm	Pm	n/t
068	n/t	n/t	n/d	n/d
069	Pyth	n/d	n/t	Pm
070	Pyth	Pyth	Pm	n/t
071	n/t	n/t	n/d	n/d
072	n/d	n/d	n/t	Pc
073	Pc	Pc	n/t	n/d
074	n/t	n/t	Pm	n/d
075	n/d	n/d	n/t	Pc
076	n/d	n/d	n/t	Phyt sp.
077	n/d	n/d	n/d	n/t
078	n/t	n/t	n/d	n/d
079	Pc	n/d	Phyt sp.	n/t
080	n/t	n/t	Phyt sp.	n/d
081	n/d	Pyth	n/t	PTA
082	Pc	Pc	n/d	n/t
083	n/t	n/t	n/d	PTA
084	n/d	n/d	n/t	n/d
085	Pc	PTA+Pc	n/d	n/t
086	n/t	n/t	Phyt sp.	Phyt sp.
087	Pm	Pm	n/t	Phyt sp.
088	n/d	n/d	n/d	n/t
089	n/t	n/t	n/d	n/d
090	Pc	Pc	n/t	n/d
091	n/d	n/d	n/d	n/t

Sample	Landcare Research – Lupin	Landcare Research – Cedar	Plant & Food	Scion
092	n/t	n/t	n/d	n/d
093	n/d	n/d	n/t	n/d
094	PTA	PTA	PTA	n/t
095	n/t	n/t	PTA	PTA
096	Pm	n/d	n/t	n/d
097	n/d	n/d	n/d	n/t
098	n/t	n/t	Phyt sp.	Pc+Phyt sp.
099	*Fusarium*	n/d	n/t	n/d
100	Pc	PTA+Pc	PTA	n/t
101	n/t	n/t	PTA	PTA+Pc
102	Pyth	Pyth	n/t	PTA+Phyt sp.
103	PTA	PTA	PTA+Phyt sp.	n/t

N.B. n/d = PTA not detected, Pc = *P. cinnamomi,* Pm = *P. multivora,* Phyt sp. = unknown *Phytophthora* sp., Pyth = *Pythium* sp. n/t = not tested. Phyt. sp. were not identified to species, as the quantificaiton and identification of by-catch *Phytophhtora* sp. was not carried out in Surveillance Two.