Development of an *in vitro* assay to screen *Agathis australis* (kauri) for resistance to *Phytophthora agathidicida*

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By
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Declaration

The work described in this thesis was undertaken while I was an enrolled student for the degree of Master of Science (Agriculture) at Massey University, Palmerston North. I declare that this thesis is my own account of my research and contains, as its main content, work which has not previously been submitted for a degree at any tertiary education institution. To the best of my knowledge, all work performed by others, published or unpublished, has been duly acknowledged.

Echo Herewini

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Abstract

The iconic *Agathis australis* (kauri) of New Zealand, is under serious threat from kauri dieback disease caused by the soil-borne pathogen *Phytophthora agathidicida.* Infected kauri express symptoms of root and collar rot, bleeding resins at the base of the trunk, yellowing of foliage, canopy thinning, and tree mortality. *Phytophthora agathidicida* was first associated with kauri decline in 1972, where it was initially identified as *P. heveae* however, there was some uncertainty about its significance and taxonomy. The pathogen was officially identified as a new organism in 2008 and was called *Phytophthora* taxon Agathis until its formal description as *Phytophthora agathidicida* in 2015. This pathogen is easily vectored through root to root contact and mobile zoospores. Management and research has focused on mapping pathogen distribution, reducing spread, improving detection, *ex situ* conservation and clonal production using tissue culture techniques.

In order to gain better understanding of the disease epidemiology and to develop better breeding programmes, a reliable *in vitro* resistance screening assay is required. This research focused on the development of a screening assay using detached leaves from tissue culture material as a means of accelerating screening assays compared to the more labour-intensive root inoculation assays.

Foliar inoculations and assessment techniques were initially optimised on kauri leaves from tissue culture lines. The most successful inoculation method involved placing *P. agathidicida*-colonised agar plugs on wounded detached leaves. The assay was further tested on 2 year old kauri seedlings. Variation in susceptibility across kauri genotypes and leaf age, and variation in virulence among *P. agathidicida* isolates was observed. To further investigate the impact of leaf age on lesion extension, an assay was conducted on

detached leaves from six rooted kauri saplings over 5 years of age, across three leaf age groups with *P. agathidicida*, *P. multivora*, and *P. cinnamomi*. Variation in virulence among these *Phytophthora* species was observed. Leaf necrosis was most severe with young tissue and susceptibility tended to decrease with increasing leaf age. Preliminary studies with 50 kauri clones identified different levels of susceptibility and tolerance across the different genotypes to *P. agathidicida*.

The methods developed within this study have increased our understanding of the overall response of kauri to *P. agathidicida* foliar inoculations. This study demonstrated variation in the susceptability of kauri foliage to *Phytophthora* inoculation, although no complete resistance was observed. Further work is required to determine if there is a relationship between root and leaf responses which will help establish if *in vitro* genotypic variation can accurately predict natural genotypic variation seen within kauri forests.

Keywords: Kauri, *Agathis australis*, *Phytophthora agathidicida*, *Phytophthora*, Kauri dieback, Resistance, Susceptibility, Screening assay, Pathogenicity, Virulence, New Zealand taonga species.

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Whakataka te hau ki te uru
Whakataka te hau ki te tonga
Kia mākinakina ki uta
Kia mātaratara ki tai
E hī ake ana te atakura
He tio, he huka, he hau hū
Tīhei mauri ora!

Cease the winds from the west,

Cease the winds from the south,

Let the breeze blow over the land,

Let the breeze blow over the ocean,

Let the red-tipped dawn come with a sharpened air,

A touch of frost, a promise of a glorious day.

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"Ehara taku toa, he toa takitahi, he toa takitini"

"Success is not the work of one, but the work of many"

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Chapter 1

Introduction

1.1 Overview

Many plant species and ecosystems worldwide are at major risk from a range of invasive pathogens and biosecurity threats (Stukenbrock & McDonald, 2008). This includes a wide range of important species of the genus *Phytophthora*, also known as the 'plant destroyer' (Erwin & Ribeiro, 1996). Kauri dieback, caused by the soil-borne pathogen *Phytophthora agathidicida* (Weir, Beever, Pennycook & Bellgard, 2015), is a deadly disease of native New Zealand *Agathis australis* ((D.Don) Lindl) (kauri).

Kauri dieback was first reported by Gadgil (1974) at Whangaparapara on Great Barrier Island. The causal organism was misdiagnosed as *P. heveae* (Gadgil, 1974). In 2006, the same pathogen was isolated from unhealthy kauri stands along the Maungaroa ridge track of Piha (Jamieson, Hill, Waipara, & Craw, 2011). Upon further investigation, it was identified as an undescribed *Phytophthora* species and was designated as *Phytophthora* taxon Agathis (PTA) (Beever, Waipara, Ramsfield, Dick, & Horner, 2009) and has recently been described as *P. agathidicida* (Weir et al., 2015).

Phytophthora agathidicida is a destructive pathogen, capable of killing all life stages of kauri (Horner, 1984). Symptoms of kauri dieback include root and collar rot, bleeding resins at the collar, yellowing of foliage, canopy thinning, tree mortality and localised extinctions (Beever et al., 2009). Kauri dieback, associated with *P. agathidicida*, has been identified throughout the geographic range of kauri forests in the northern North Island of New Zealand (Waipara, Hill, Hill, Hough, & Horner, 2013) (Figure 1.1). *Phytophthora agathidicida* is now present in forest plantations of Omahuta,

in the Waitakere Ranges Regional Park, in Glenbervie and Russell in Northland, on private land throughout Auckland, in Okura on Department of Conservation Reserves, Albany, Great Barrier Island, Pakiri, Waipoua Forest, and Trounson Kauri Park in Northland. In March 2014, the Ministers of Conservation and Primary Industries announced the presence of kauri dieback in native forest blocks on the Coromandel Peninsula (Kauri Dieback Management Team, 2014).

Figure 1.1. Map of the northern North Island of New Zealand showing where *Phytophthora agathidicida* has been detected (red) and undetected (yellow) in kauri stands (Kauri Dieback Management Team, 2014).

Resistance screening has proven to be a useful management tool for identifying resistant host plants to a range of *Phytophthora* species and other fungal pathogens and pests (Dorrance & Inglis, 1997; Infantino et al., 2006; Vleeshouwers et al., 1999).

Significant international examples include the identification of resistant *Chamaecyparis lawsoniana* ((A. Murray.) Parl), known as Port-Orford-Cedar (POC), to *P. lateralis* (Tucker & Milbrath, 1942) (Hansen, Hamm, & Roth, 1989; Oh, Hansen, & Sniezko, 2006), and resistant *Eucalyptus marginata* (Donn ex Smith) (jarrah), to *P. cinnamomi* (Rands 1922) which have been used to successfully restore infected forest stands (Stukely, Crane, McComb, & Bennett, 2007). Screening kauri for resistance to *P. agathidicida* infection may be an effective management tool for kauri dieback. Resistant kauri genotypes identified by an assay could potentially be used for the future production of resistant breeding lines.

1.2 Kauri

Kauri is endemic to New Zealand and belongs to the ancient family of coniferous trees, *Araucariaceae* (Henkel & Hochstetter, 1865). The genus *Agathis* (Salisb.) appeared during the Cretaceous period (Barton, Thompson, Hodder, & Edmonds, 1983) and consists of 21 species distributed in Australia (Queensland), New Caledonia, Vanuatu, Fiji, Solomon Islands, New Zealand, Sumatra, Fiji, and the Philippines (Hooker, 2011; Kirk, 1889; Whitmore, 1977). Kauri appeared during the Oligocene era (Fleming, 1979) and is known for its high-quality timber (Whitmore, 1980).

1.2.1 Kauri history

The history of kauri largely reflects the settlement of New Zealand. Prior to European arrival, indigenous Māori had a significant relationship with kauri. The trees were incorporated into everyday Māori traditions, artwork, folktales, and legends. In the early

19th century, pristine forests covered more than 1.5 million hectares of New Zealand (Halkett, 1983a). After the colonization of New Zealand by Europeans, the kauri timber and gum industries became established. Due to the appearance and working properties of kauri timber, it was highly sought after for many uses (Figure 1.2). This included spars and masts for sailing ships, constructing bridges, railways, furniture, dams, chutes for transporting logs, house and boat building, and decorative carving (Cheeseman, Hemsley, & Smith, 1914; Clifton & Harris, 1991; Kirk, 1874; Reed, 1964; Steward, 2011). A vast amount of kauri forest was also transformed into farmland. This led to the destructive exploitation of the species which has ultimately changed the upper North Island's forest landscape completely (Steward, 2011).

Figure 1.2. Giant kauri logs that were felled by Europeans in 1920 (Mahoney, 2007).

By 1987, the New Zealand government declared all remaining kauri to be protected by the Department of Conservation, including kauri growing on private land (Steward & Beveridge, 2010). Areas of kauri forest have been set aside in scientific

reserves to preserve its biological features and intrinsic forest values (Halkett, 1983a). Currently, only 7,500 hectares of primary virgin kauri forests remain. An additional 60,000 hectares of secondary kauri forests, or scrubland containing regenerating kauri, also exists (Halkett, 1983a). Much of these forests are confined to remote locations on the Coromandel Peninsula, Great Barrier Island, and on the North Auckland Peninsula (Halkett, 1980). A significant example is the Waipoua forest, located in the Northland region of New Zealand (Morrison & Lloyd, 1972). This is the largest remaining kauri stand in the country and still stands today because of its remote location.

1.2.2 Present distribution

The kauri population and its current distribution in New Zealand have been severely altered due to the high demand of kauri timber by early European settlers. Kauri forests have been greatly reduced in area over the past 200 years, where less than 1% of original kauri forests now remain (Steward & Beveridge, 2010).

The natural home of kauri is confined to the subtropical northern part of the North Island of New Zealand, latitude 34-38ºS (Hinds & Reid, 1957; Sando, 1936; Von Hochstetter & Sauter, 2013), but its geographical range includes Auckland, Coromandel Peninsula, Kawhia, Northland, to as far down south as Stewart Island (Sando, 1936). Kauri are most common in Northland and the Coromandel Peninsula, between sea level and altitudes of around 300 metres (Cockayne, 2011).

Some old-growth forests remain on Great Barrier Island and Little Barrier Island. A few stunted trees also exist as high as 800m on Mt. Moehau on the Coromandel Peninsula (Steward & Beveridge, 2010). Factors such as slow spread by seed dispersal,

mice destroying seeds (Barton et al., 1983), and climatic factors (Mitchell, 1991), are suggested reasons for the absence of kauri growing elsewhere. A low rate of seed germination and slow growing shade-tolerant seedlings, limit the ability for existing kauri stands to expand their populations throughout the mainland (Barton et al., 1983). Kauri are one of the faster-growing indigenous conifers under favourable conditions despite growing slowly in their natural habitat (Bergin & Steward, 2004). Their tolerance to poor soil sites and resistance to drought, leads to their preference for ridge-top sites on heavy residue soils (Bieleski, 1959; Sando, 1936).

1.2.3 Botanical features

Kauri are among the most ancient trees in the world (Lewington, 2012). Volume wise, they are the largest trees in New Zealand (Cockayne, 2011; Reed, 1964). Kauri are notable for their straight branchless trunks which can grow over 13 metres high (Roche, 2005). A kauri tree can reach a total height of between 30 – 60 metres (Allan, 1961; Hooker, 2011) and a stem diameter ranging from 1 – 5 metres (Steward, 2011). The maximum age of kauri is estimated to be 1700 years old (Cheeseman et al., 1914; Steward & Beveridge, 2010). In New Zealand, the largest known kauri in volume is Tāne-Mahuta, also known as Lord of the Forest (Figure 1.3), who stands tall in the Waipoua Forest.

Figure 1.3. Tāne-Mahuta – Lord of the Forest (Brownlee, 2008).

Kauri are monoecious with both male and female reproductive structures, seed, and pollen cones (Figure 1.4), able to be borne on the same tree (Morrison, 1950). Between the ages of 25 - 40 years old, seed cones begin to produce viable wind-dispersed seeds, which are released in late summer to early autumn (Halkett, 1983). The seeds do not travel further than 20 - 100 metres from the tree crowns. Between September and October, pollen produced from young kauri is shed. The pollen can be attacked by fungi and is only viable for a short amount of time (Steward & Beveridge, 2010). Leaves are often a dull-green colour and can remain on the tree for up to 15 years (Silvester & Orchard, 1999). Adult leaves are functional for 3 - 6 years and budburst occurs anytime between September and October (Bieleski, 1959; Ogden & Ahmed, 1989).

Figure 1.4. Kauri male cone (left) and female cone (right) (Wassilieff, 2007).

Kauri have extensive root systems. Peg roots descend from lateral roots and anchor the tree firmly to the ground (Steward, 2011). The fine feeder roots spread throughout the litter layer where the fine root hairs feed in the organic litter near the surface of the soil (Ecroyd, 1982). Kauri have well-developed tap root symptoms that exploit free-draining soil and are important for optimum growth, nutrient uptake, and mycorrhizal symbiosis (Bergin & Steward, 2004; Morrison & Lloyd, 1972; Padamsee et al., 2016).

Kauri is a dominant tree of lowland stands, often occurring in mixed forests (Ecroyd, 1982). Ecologically, they support many indigenous ecosystems and biodiversity, making them an important keystone species (Ecroyd, 1982). Kauri have a great influence

on surrounding forest composition and structure. Their unique ecological niche is their special ability to modify the soil (Padamsee et al., 2016; Wyse, Burns, & Wright, 2014). Kauri can grow on low nutrient to infertile soils and are efficient nitrogen and phosphorus users. They are capable of depriving competitors of nutrition in a process known as leaching (Madgwick, Oliver, & Holten-Anderson, 1982).

1.2.4 Cultural significance

Kauri are a well-recognised icon that contribute substantially to the culture and identity of New Zealand's native forests. Kauri forests pull together a range of ecosystems and landscapes and are part of New Zealand's rich and vibrant history. Visitors, scientists, and foresters worldwide appreciate these majestic trees.

Kauri have a cultural value to indigenous Māori. They are a significant taonga (keystone indigenous species) that is an integral part of Māori whakapapa (genealogy) and are repository for Mātauranga Māori (traditional knowledge) (Ecroyd, 1982; Orwin, 2012). For example, kauri have an important role in Māori traditions and creation mythologies. The most well-known mythology tells of how the mighty kauri, Tāne-mahuta, used his legs to separate sky father and earth mother to create the world (Boswijk, 2010). Kauri also hold a traditional chiefly status as "kings of New Zealand forests" and are valued by Māori as a connection to their spiritual beliefs and ancestors (Orwin, 2012).

Kauri were a valuable resource for Māori and play an important role in many aspects of Māori culture. Gum was a valuable commodity used for torches and chewing gum (Walrond, 2012). The powder from kauri soot was used as the colouring agent for

tattooing (Dieffenbach, 1843). The straight branch-free stems were first used for building sea canoes, houses (Best, 1925) and for carving (Clifton & Harris, 1991). As the felling of trees required considerable amounts of effort and time using the fire and stone tool methods, only a limited number of kauri were targeted by Māori before European arrival (Maning, 1863).

1.3 *Phytophthora* diseases worldwide

Phytophthora species are taxonomically classified as oomycetes and are often referred to as water moulds due to the importance of needing water for their reproduction cycle (Judelson & Blanco, 2005). They are eukaryotic, diploid, soil-borne, microscopic 'fungus-like' pathogens that produce a combination of asexual and sexual spores (Krull et al., 2013). *Phytophthora* is the second largest genus in the *Peronosporaceae* family and is comprised of more than 100 species (Thines, 2013).

Phytophthora pathogens have received a lot of attention due to a large number of destructive diseases they cause on a range of crops. One of the most notorious species is *P. infestans* ((Mont.) de Bary, 1876), the cause of the fatal Irish Potato Famine during the 19th Century in Europe (Ribeiro, 2013). According to Scott, Burgess, & Hardy (2013), there are at least 121 described *Phytophthora* species distributed in 138 countries. The greatest number of known *Phytophthora* species currently exists in America, followed by Australia, and the United Kingdom (Sanogo & Bosland, 2013).

Phytophthora species have the ability to infect both above and below-ground plant parts of their hosts, where they are capable of causing significant epidemics to new hosts in new environments (Cooke, Drenth, Duncan, Wagels, & Brasier, 2000). Once a

Phytophthora species has become established in a new environment, it is nearly impossible to eradicate, although successful eradications have been demonstrated in natural ecosystems under specific conditions (Dunstan et al., 2010). *Phytophthora* infection symptoms can be difficult to identify and are often expressed as secondary symptoms such as wilt and drought dieback.

Soil-borne *Phytophthora* species have developed a life cycle that allows them to produce asexual and sexual spores with characteristics that greatly contribute to their pathogenic success (Judelson & Blanco, 2005). Resting oospores are sexual reproductive spores that can survive in the soil and plant tissue for up to three years or more. Chlamydospores are asexual reproduction spores with a thickened cell-wall to aid in their role as long-term survival structures. Under favourable environmental conditions such as wet weather, chlamydospores or oospores germinate to form lemon-shaped sporangia (Jung, Vettraino, Cech, & Vannini, 2013). Indirect germination occurs when the sporangia ripen and release biflagellete zoospores (asexual spores) which swim through soil-water, or on the surface of flowing water, into a new area. Using chemical attraction, these zoospores actively locate their hosts and form mycelium which is a mass of filamentous structures known as hyphae that penetrate and infect the roots (Erwin, Bartnicki-Garcia, & Tsao, 1983). Sporangia can also germinate directly to form hyphae. More chlamydospores, oospores, and/or sporangia are then produced, thus completing its life cycle.

Phytophthora infection on *Fagus sylvatica* (L.), also known as European beech, has caused serious aerial bark lesions due to *P. cambivora* ((Petri) Buisman), *P. citricola* (Sawada) and *P. gonapodyides* ((H.E. Petersen) Buisman). The decline in European

beech illustrates the complex interactions between *Phytophthora* species, secondary pathogens, and conducive site conditions. Climatic features such as rainfall and drought are known to facilitate the disease in central Europe. This often leads to bark necrosis and facilitates the entry of secondary pathogens into the bark (Jung, 2009).

In co-evolved plant communities, many *Phytophthora* species remain benign unless the environment is modified or host physiology is changed through stress. *Phytophthora* species can undergo sexual recombination, acquiring new composites of pathogenicity traits, or they can be spread into new ecosystems where hosts have not evolved adequate resistance (Hansen, 2008). *Phytophthora* pathogens have evolved various mechanisms for dispersal including chemotactic zoospore movement towards roots (Duniway, 1976). The movement of infected plants, animals, and humans all play an important role in spreading *Phytophthora* species into new regions (Scott, Burgess, & Hardy, 2013). Within managed and natural ecosystems, it is possible for new *Phytophthora* hybrid species to form which may lead to the rapid generation of new pathogens and diseases (Érsek & Nagy, 2008).

1.4 Notable *Phytophthora* diseases

1.4.1 *Phytophthora cinnamomi*

Phytophthora cinnamomi has had a significant impact on American, Australian, and European forests, resulting in near extinction of some host species. *Phytophthora cinnamomi* was first isolated from cinnamon trees in Sumatra in 1922 (Zentmyer, 1980). It has a broad host range and is capable of causing fine root mortality as well as above-ground damage, such as bleeding cankers (Erwin & Ribeiro, 1996). This

pathogen has destroyed an extensive range of jarrah forests in south-western Australia (Stukely & Crane, 1994). There are limited practices to eradicate or limit the spread of *P. cinnamomi* however, breeding resistant American chestnuts and jarrah was developed as one form of management strategy for this pathogen (Jeffers, James, & Sisco, 2009).

Using stem inoculations, a vast amount of open-pollinated jarrah families were screened for *P. cinnamomi* resistance in pot and soil inoculation experiments in an already infected site (Stukely & Crane, 1994). Susceptible and resistant individuals were identified. Seedlings from these genotypes were later micro-propagated in tissue culture, planted in jarrah forest sites, and purposely infected with *P. cinnamomi* to validate the resistance of the clones in the field (Stukely et al., 2007). This screening programme has led to the identification of 60 jarrah resistant lines. These were made available for regenerating dieback-affected sites by either directly planting *P. cinnamomi*-resistant clones or by establishing seed orchards to supply the resistant seeds (Stukely et al., 2007).

1.4.2 *Phytophthora ramorum*

Phytophthora ramorum (Werres, De Cock & Man in 't Veld, 2001) is the causal agent of sudden oak death in North America and Europe. The pathogen was first observed in California during the mid-1990s where it was associated with *Lithocarpus densiflorus* ((Hook. & Arn.) Rehder) (tanoak) and *Quercus agrifolia* (Née) (coast live oak) mortality (Rizzo, Garbelotto, & Hansen, 2005). Infected hosts express three typical symptoms, including bleeding cankers, twig dieback, and leaf blight or necrosis (Rizzo et al., 2005). Mortality often results when the stem of a tree is infected. Many hosts are broadleaf plants and have different levels of susceptibility.

Phytophthora ramorum is also capable of infecting roots of trees without showing any visible symptoms and it can survive in the soil for a long period of time (Shishkoff, 2007). Management strategies have been implemented to help prevent *P. ramorum* establishment in coastal Californian forests. This includes the use of herbicides to prevent sprouting, host tree removal to eradicate any pathogen inoculum, and sanitizing the site by burning infected materials (Garbelotto, Schmidt, Tjosvold, & Harnik, 2003; Goheen et al., 2004).

1.4.3 *Phytophthora lateralis*

Phytophthora lateralis causes significant root disease, collar damage and dieback of POC. It was first reported in 1923 in ornamental nurseries (Balci & Bienapfl, 2013). The pathogen has since spread throughout POC's natural range in western Oregon and northern California forests (Hansen & Hamm, 1996). *Phytophthora lateralis* is commonly spread by roads next to POC forests. Vehicles often transport the pathogen uphill from where it is then washed down into streams where POC are located (Hansen et al., 2000). A number of management strategies have been employed to minimise the spread of cedar root disease, including harvesting infected trees, precommercial thinning of cedar stands, and the development of breeding resistant POC lines (Hansen, Goheen, Jules, & Ullian, 2000; Zobel, Roth, & Hawk, 1982).

The majority of POC trees in the field are susceptible to *P. lateralis* (Hansen, 2015) however, a greenhouse study conducted in 1989 identified a few POC trees demonstrating genetic resistance to *P. lateralis* (Hansen et al., 1989). Since then, an extensive programme to identify resistant genotypes from the field, with the goal of

propagating the resistant seedlings for future forest regeneration, has been established (Betlejewski et al., 2003; Hansen et al., 1989; Sniezko et al., 2002). Using stem and root dip techniques, over 10,000 POC field trees were screened. Over 1,100 trees were further analysed by comparing parent trees showing high levels of resistance to the reaction of susceptible POC control trees (Oh et al., 2006). The first generation of resistant POC seedlings are now available and are being effectively used for natural ecosystem restoration.

1.5 Notable *Phytophthora* tree diseases in New Zealand

Phytophthora species are associated with a number of plant diseases in New Zealand, posing a significant threat to agriculture, horticulture, forestry, and natural ecosystems (Scott & Williams, 2014). The origin of most *Phytophthora* species in New Zealand is unknown however, some species such as *P. cinnamomi* most likely originated outside of New Zealand (Newhook, 1970).

Many *Phytophthora* species in New Zealand are disseminated through the soil as spores or via root to root contact (Scott & Williams, 2014). Significant examples include *P. agathidicida* associated with kauri dieback (Gadgil, 1974; Weir et al., 2015), *P. cinnamomi* associated with kauri root and stem dieback (Horner, 1984) and *P. cactorum* (Lebert and Cohn) associated with apple crown rot (Alexander & Stewart, 2001). Some New Zealand *Phytophthora* species are also spread aerially including *P. kernoviae* (Brasier, Beales, Kirk, Denman, & Rose, 2005), *P. infestans* (Reeser, Sutton, & Hansen, 2013) and *P. captiosa* and *P. fallax* (Dick, Dobbie, Cooke, & Brasier, 2006).

Red Needle Cast (RNC), caused by *P. pluvialis* (Reeser, Sutton & Hansen 2013), is a significant foliar disease affecting *Pinus radiata* (D.Don) trees of all ages (Dick, Williams, Bader, Gardner, & Bulman, 2014). The recent outbreak of this disease indicates the pathogen was most likely introduced to the country (Scott & Williams, 2014). In the initial stages of RNC infection, needles develop distinct olive with black resinous bands, which eventually turn red-brown before falling off the tree (Figure 1.5). Symptoms can begin anytime between March and August. Phosphite application has proven to reduce RNC severity (Rolando, Gaskin, Horgan, Williams, & Bader, 2014) however, effective management of RNC will require a greater understanding of the long-term impact of *P. pluvialis* on *P. radiata* forests (Scott & Williams, 2014).

Figure 1.5. Field expression of red needle cast disease on *Pinus radiata* trees caused by *Phytophthora pluvialis* (Dick et al., 2014).

1.6 *Phytophthora agathidicida*

In 2008, it was declared as an unwanted organism in New Zealand (Bellgard, 2012). Since the early 2000s, increasing levels of kauri stands showing canopy thinning associated with kauri dieback have been reported (Figure 1.6). The distribution of the disease has now been confirmed throughout the geographic range of kauri forests.

Figure 1.6. Kauri stand showing canopy thinning caused by *Phytophthora agathidicida* (Weir et al., 2015).

Losing forests due to invasive *Phytophthora* species will have a great impact on many ecosystems and natural processes. This highlights the need for urgent and efficient kauri dieback disease management in order to provide the best likelihood of success in protecting one of New Zealand's most vulnerable forest systems.

1.6.1 Impact of *Phytophthora agathidicida* on kauri

Phytophthora agathidicida poses a serious threat to kauri at both the individual and population level. It has had a significant impact on kauri ecosystems and their natural processes so far. Kauri forests are a vital part of local and global carbon cycles and soil and nutrient cycles (Madgwick et al., 1982). They help preserve biodiversity and act as carbon sinks. Infected trees can cause changes in a number of natural processes including altering long-term decomposition rates and soil chemistry. Unhealthy trees also change the surrounding regenerative vegetation. This in turn, will cause long-term changes in the composition of kauri-dominated forests to forests dominated by podocarps (Van der Westhuizen et al., 2013).

The decline of kauri forests due to *P. agathidicida* has had a huge impact on Tāngata whenua (people of the land), Kaitiaki (guardians) of kauri forests and Mana whenua (the authority of a tribe over land). Non-Māori are also coming to terms with the loss. Losing a cultural icon will be unthinkable to both New Zealanders and tourists worldwide. Kauri forests are ecologically and culturally important ecosystems that provide habitats for a range of wildlife species.

1.6.2 *Phytophthora agathidicida* isolation

Since 2008, a management programme involving diagnostic field surveys was initiated to help manage kauri dieback across the natural range of kauri. Methods were developed to evaluate tree health, disease symptoms, and other possible contributing factors across private land in the Auckland region (Waipara et al., 2013). Pathogens associated with symptoms of canopy thinning and foliar dieback were isolated and identified using diagnostic sampling. *Phytophthora agathidicida* was the most frequently observed kauri

pathogen, confirming kauri dieback was widely spread across the Auckland region. Other *Phytophthora* species including *P. cinnamomi* and *P. multivora* (Scott & Jung, 2009) were also isolated from plants expressing dieback symptoms, suggesting that these additional pathogens may contribute to the overall poor health of kauri (Waipara et al., 2013).

1.6.3 Origin of *Phytophthora agathidicida*

Phytophthora agathidicida is a species within Clade 5, a group that has been poorly studied in the past (Weir et al., 2015). *Phytophthora agathidicida* currently sits alongside *P. heveae* and *P. katsurae* (Ko & Chang 1979) in the *Phytophthora* Clade 5 (Weir et al., 2015). *Phytophthora heveae* and *P. katsurae* are seemingly centred across the Pacific region and are known to come from indigenous communities in tropical forests in Australia, Taiwan, and Papua New Guinea (Bellgard, Johnston, Duckchul, & Than, 2011).

Preliminary studies have shown that there are only small genetic differences between the *Phytophthora* species of Clade 5. Molecular studies indicate *P. agathidicida* has a different oospore morphology than the other member species (Cooke et al., 2000), but it has a close genetic relationship with *P. heveae* (Beever et al., 2009; Weir et al., 2015). For this clade, host and geographic associations points to a centre of diversity in the East Asia and the Pacific region (Weir et al., 2015).

Agathis robusta (C.Moore ex F.Muell) found in Queensland, Australia, is known to be resistant to *P. agathidicida* (Bellgard et al., 2011). As there are several species of *Agathis* in the Pacific, it is possible that *P. agathidicida* may have been introduced to

New Zealand by soil. Further research is required to determine the origin of *P. agathidicida* and whether this species has been previously described. This will include comparison studies of morphological and physiological data between Clade 5 isolates collected from overseas to *P. agathidicida* isolates from New Zealand (Bellgard et al., 2011).

1.6.4 Life-cycle and spread

P. agathidicida follows the same life-cycle as other soil-borne *Phytophthora* pathogens (Section 1.3). Under favourable environmental conditions, resting oospores germinate and produce lemon-shaped sporangia (Figure 1.7). The sporangia ripen and release biflagellete zoospores (asexual spores) which swim into a new area. White hyphal structures, collectively known as mycelia, grow throughout the root system, and restrict the host from absorbing essential nutrients and water, eventually starving the tree to death. More oospores are formed within infected root tissue and are released into the soil as the roots begin to decay. The cycle of infection continues to the next plant.

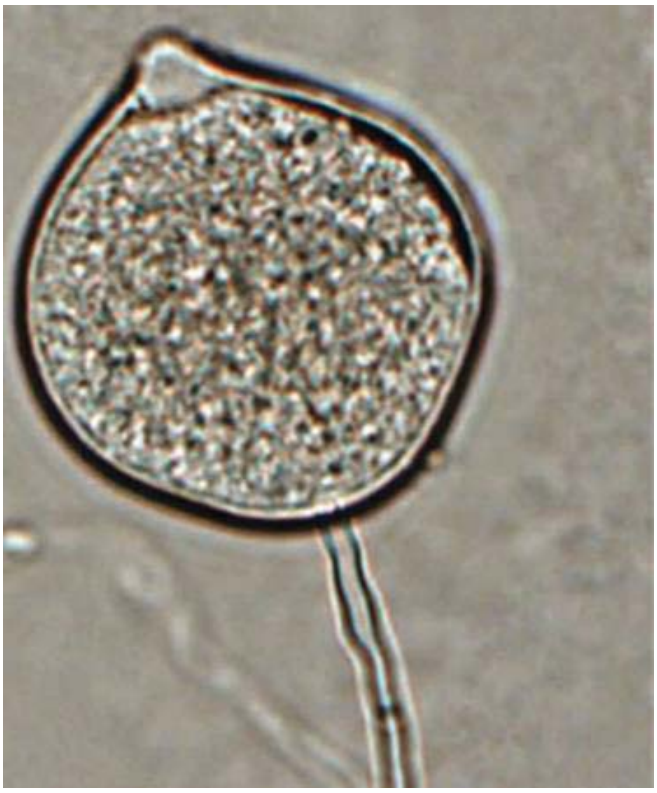

Figure 1.7. A *Phytophthora agathidicida* sporangium containing biflagellate zoospores (Weir et al., 2015).

The specific number of zoospores necessary to cause *P. agathidicida* infection is unknown at this stage (Kauri Dieback Management Team, 2014). There is a high probability that feral animals such as pigs (Krull et al., 2013) and streams (Randall, 2011) are vectors of *P. agathidicida*. At this stage, the time course for the onset of disease symptoms after primary infection occurs and the morphology of the pathogen as it infects through the root cortex, is still unknown (Bellgard, Padamsee, Probst, Lebel, & Williams, 2016).

1.7 Control measures

As a response to this devastating disease, the New Zealand Government granted funding for a long-term management programme in 2009. This programme covers research into the detection, spread, and control methods of kauri dieback (Bellgard, 2012). Several government agencies including the Department of Conservation, took on the research alongside the Tāngata-whenua-roopu, who help provide strategic advice into all aspects of kauri dieback management. Together, they worked in partnership with The Ministry of Agriculture and Forestry Biosecurity New Zealand, Plant and Food Research and Scion (Bellgard, 2012).

1.7.1 Kauri dieback management – quarantine

Kauri root systems are known to be sensitive and the disease requires a susceptible host, favourable environmental conditions, and an introduction pathway for the pathogen to cause infection (De Wolf & Isard, 2007). Any disturbances, both human and natural, can cause open wounds which provide an opportunity for infection to occur (Bellgard, 2012). Containment strategies have been set in place to ensure any activities that can damage and stress kauri root systems are kept to a minimum. One example is the Whangapoua/Hukarahi Conservation area in Coromandel which has been closed due to the identification of two *P. agathidicida* infected trees (Kauri Dieback Management Team, 2014).

Raising public awareness about the disease and good plant hygiene practices have also been established. Cleaning tools for footwear and equipment have been provided at high risk sites to limit the spread of kauri dieback (Bellgard, 2012). Ground surveys to monitor tree health, distribution of kauri dieback and associated pathogens have been

carried out at suspected sites across the Auckland region (Waipara et al., 2013) and the Hunua Ranges (Jamieson et al., 2011). The use of these surveys determined whether kauri dieback was detected or undetected in those areas. Research that improves knowledge into detecting and visualising *P. agathidicida* (Bellgard et al., 2016) and how it is spread (Beever et al., 2009; Krull et al., 2013; Randall, 2011) have also been conducted.

1.7.2 Kauri dieback management – resistance screening

The Healthy Trees, Healthy Future programme led by Scion, has been established which aims to tackle a broad range of *Phytophthora* species that have a major impact on plant systems across horticulture, forestry, and natural forest estates in New Zealand (Williams, 2013). Major examples include kauri dieback, red needle cast of radiata pine and crown rot of apples. This programme will aid the development of disease management and breeding programmes for resistance to eight *Phytophthora* species by building a library of pathogen profiles for future identification and screening purposes (Williams, 2013).

Kauri dieback is known to affect trees of all age classes (Horner & Hough, 2014; Horner & Hough, 2013; Waipara et al., 2013). There appears to be a broad vulnerability of kauri to *P. agathidicida* however, kauri dieback resistance is expected as asymptomatic trees have been identified among diseased trees in the Waitakere ranges and other kauri sites. Although there is no direct proof that kauri dieback resistance exists, asymptomatic trees were mapped frequently in amongst infected trees at more than one kauri site. This strongly hints that any resistance out there is most likely to be uncommon, possibly rare,

and randomly dispersed. An important component of kauri dieback management is screening as many host plants as possible to identify this resistance.

Screening methods can involve the use of pathogen inoculum, such as a spores or mycelia, applied to plant parts or plant organs (Lebeda & Svábová, 2010). It can also be performed on *in vitro* produced plant material. The most important characteristic of resistance screening assays is that it should mimic the infection process under natural conditions as much as possible (Lebeda & Svábová, 2010).

1.7.3 Kauri dieback management – chemical control

Chemical control using phosphite has been successfully used in the past to achieve good control of *Phytophthora* diseases in a number of hosts both nationally and internationally (Graham, 2011; Reglinski, Spiers, Dick, Taylor, & Gardner, 2009; Scott, Bader, & Williams, 2016; Tynan et al., 2001). The application of phosphite to control kauri dieback in both glasshouse seedlings (Horner et al., 2013) and ricker size kauri trees, between the ages of 30 – 50 years in the field (Horner et al., 2013), have demonstrated good control by supressing the activity of *P. agathicida* within the plant tissue.

Foliar phosphite application has been associated with minor phytotoxic effects across a diverse range of conifers and woody angiosperms native to New Zealand, including 18 month old kauri saplings, regardless of the concentration used (Scott et al., 2016). The use of phosphite can cause significant damage and mortality at high doses and there is evidence that severely diseased trees are less tolerant to phosphite injections which may accelerate their decline (Horner, Hough, & Horner, 2015). This raises the concern of the potential phytoxicity of phosphite on kauri health and its

potential ecological impacts after repeated application. The use of phosphite will need to take into account the ethical, moral, cultural, and spiritual considerations of local iwi (Patterson, 1999) therefore, the potential use of phosphite as a long-term control to save and restore infected kauri trees back to health is yet to be determined.

1.8 Foliar resistance screening

In woody plants, *Phytophthora* species are often associated with root and crown rot (Balci, Balci, MacDonald, & Gottschalk, 2008) however, screening for resistance to *Phytophthora* species using root inoculations can be costly and time-consuming. Foliar inoculations have been used in the past as an alternative to identify resistant hosts to root-infecting *Phytophthora* species. Leaf assays are often more rapid, non-destructive to the entire plant, and provide a greater reproducibility than roots (Irwin, Musial, Mackie, & Basford, 2003). Foliar inoculations have been used to screen North American oak species to seven different *Phytophthora* species, including *P. ramorum*, that are commonly isolated from rhizosphere soil in oak ecosystems (Balci et al., 2008). Susceptible oak species were identified and associations between hosts and pathogens were established. A foliar screening assay may be a good alternative to a root-based assay for identifying kauri resistance to *P. agathidicida* infection.

The disadvantages of detached leaf assays can include compromising host-defence response due to interferences in defence mechanisms caused by leaf detachment, and they do not always reflect the variability of whole plants in terms of leaf age and leaf orientation. Use of leaf material under controlled conditions may not accurately reflect what happens under natural conditions where additional pathogens and

environmental factors play a role in disease expression (Arraiano, Brading, & Brown, 2001; Denman, Kirk, Brasier, & Webber, 2005; Karjalainen, 1984; Parke, Roth, & Choquette, 2005). Foliar leaf inoculation assays may not accurately correlate to the susceptibility of other naturally infected tissue including roots, collar, or fruits, due to variations in physical structures, biochemistry, and micro-environmental conditions.

1.8.1 Koch's postulates

Robert Koch was a bacteriologist in the 19th Century who laid down the criteria for proving the pathogenicity of an organism. Manion (1981) described the four criteria for Koch's postulates:

- The suspected causal pathogen must be constantly associated with the disease.
- The suspected causal pathogen must be isolated in pure culture.
- When a healthy plant is inoculated with the cultured pathogen, the original disease must be produced.
- The same pathogen must be reisolated from the experimentally infected plant.

For each experiment in this study, Koch's postulates had to be fulfilled to confirm if *P. agathidicida* was the causal organism associated with kauri dieback disease symptoms that were expressed on the infected kauri.

1.9 Aims and objectives

The main aim of this study was to develop an *in vitro* assay to screen kauri for resistance to *P. agathidicida.*

The specific objectives of this study were to:

a) Develop a suitable method for detached foliar inoculations on kauri with *P. agathidicida*.

b) Determine if leaf inoculations are applicable to planted kauri saplings over 5 years of age.

c) Determine if there is any variation in susceptibility across kauri genotypes and variation in virulence across *P. agathidicida* isolates.

d) Determine if leaf age has an effect on kauri foliar inoculations using three *Phytophthora* species: *P. agathidicida*, *P. multivora* and *P. cinnamomi*.

Chapter 2

Comparing foliar inoculation methods on detached *Agathis australis* (kauri) leaves with *Phytophthora agathidicida*

2.1 Introduction

Resistance and susceptibility testing on whole-plants, especially on a large-scale, is often restricted to quarantine facilities that are not always available to researchers. Whole-plant testing requires glasshouse space or field areas for prolonged time periods and a strict control of inoculum levels is often required in seedling assays (Irwin et al., 2003). Experimental assays using detached leaves have proven to be a good alternative to glasshouse and field tests to predict resistant genotypes and can be a reliable indicator of whole-plant responses (Parke et al., 2005).

The epidemiology of *Phytophthora* species in native ecosystems has previously been determined in some regions. This was done by artificially infecting non-infected natural forests to confirm Koch's postulates and variation in isolate virulence on host plants (Podger, 1972). As *P. agathidicida* is an invasive pathogen in New Zealand that has been shown to cause significant impacts, including death of ancient trees and localised extinctions (Waipara et al., 2013), it is inappropriate and un-ethical to artificially infect kauri forests to research *P. agathidicida* infection. It is also virtually impossible to eradicate *Phytophthora* species once they are established in a new environment (Jung et al., 2011).

Therefore, experimental assays are required to measure variation in isolate virulence and host resistance outside of kauri forests that reflect natural virulence on hosts under forest conditions. Nationally (Dick et al., 2014; Horner & Hough, 2014; Newhook & Podger, 1972; Robin & Guest, 1994) and internationally (Ahumada, Rotella, Slippers,

& Wingfield, 2013; Jönsson, Jung, Rosengren, Nihlgård, & Sonesson, 2003; Scott et al., 2012), *in vitro* assays have been developed to research Koch's postulates (Section 1.8.1) and identify variation in isolate virulence and host resistance to *Phytophthora* pathogens.

Soil borne root diseases, including kauri dieback, are often difficult to diagnose compared to cankers, leaf, and shoot diseases of above-ground parts of trees (Scott et al., 2012). Root symptoms also tend to be more challenging to measure than aerial symptoms and their impacts are often expressed through non-specific symptoms of branch and crown dieback which may also be associated with other pathogens or insects (Hansen & Delatour, 1999; Scott et al., 2012). If there is a strong association between kauri leaf and root response to *P. agathidicida*, a leaf assay may be a good alternative to a root-based assay for identifying kauri resistance.

The first step towards developing a leaf screening assay for resistance screening for kauri dieback is to determine the most suitable inoculation method and inoculum type for foliar inoculations with *P. agathidicida* (Horner & Hough, 2014). Several authors have used detached leaf assays to identify a range of resistant host plants to *Phytophthora* species. Vleeshouwers et al. (1999) ranked resistance levels for 20 plant genotypes of *Solanum tuberosum* (L. – potato) cultivars to *P. infestans,* the causal agent of potato late blight disease. In this study, infected intact plants and detached leaves from field-grown and chamber-grown plants, produced similar results under laboratory and field conditions (Vleeshouwers et al., 1999). Similarly, Brooks (2008) used a detached leaf assay to screen taro lines for resistance to leaf blight disease caused by the foliar pathogen, *P. colocasia* (Racib.). The mean lesion diameters from this assay were highly correlated with field results, allowing for the selection of resistant taro genotypes for breeding programmes (Brooks, 2008). *Medicago sativa* (L.) (Lucerne) plants resistant to the root infecting

pathogen, *P. medicaginis* (Hansen & Maxwell, 1991), have also been identified using a detached leaf assay involving zoospore inoculum (Irwin et al., 2003).

Compared to root-based assays, detached leaves offer several advantages as described by (Irwin et al., 2003; Parke et al., 2005):

- They are non-destructive to the entire plant.
- Simple to handle.
- Cost-effective.
- Use less space.
- Provide greater reproducibility due to similar leaf size and age.
- Inoculum can be delivered more consistently.
- Quantification of disease can be more accurate.
- Visualisation is much easier as the leaves are above-ground.
- It allows for the localization of inoculation site to specific parts of the leaf.

Several authors have successfully used zoospores as the infection unit for *Phytophthora* infection on different host plants for *in vitro* resistance and susceptibility studies (Barksdale, Papavizas, & Johnston, 1984; Denman et al., 2005; Dick et al., 2014; Iwaro, Sreenivasan, & Umaharan, 1997; Robin & Guest, 1994; Widmer & Dodge, 2015). Hansen, Parke, & Sutton (2005) compared four zoospore artificial inoculation methods with *P. ramorum* infection on 49 mixed-hardwood forest seedlings to natural infection symptoms observed in south-western California and Oregon forests. Whole plants or leaves were immersed in a zoospore concentration whereby, plant-dip came closest at predicting the full range of symptoms in the field and leaf-dip inoculation provided a rapid assay and permitted a reasonable assessment of susceptibility to leaf blight (Hansen, Parke, & Sutton, 2005).

Agar colonised by *Phytophthora* mycelia has also been successfully used for *Phytophthora* infection in New Zealand. Horner & Hough (2014) successfully used mycelia agar plugs to investigate the potential pathogenicity of four commonly isolated *Phytophthora* species on kauri seedlings. In another study, agar plugs were used to identify taro genotypes with disease tolerance to taro leaf blight caused by *P. colocasiae* (Tyson & Fullerton, 2015). International studies that have used *Phytophthora* mycelia as the inoculum source to study variation in isolate virulence and host resistance include those by Haque, Martín-García, & Diez (2015) and Robin & Desprez-Loustau (1998).

The main aim of experiment 1 was to compare seven techniques: agar plug, agar and non-sterile pond water, agar and sterile pond water, cellophane, zoospore drop, dip petiole, and dip tip, to determine the most suitable foliar inoculation method for a kauri screening assay.

2.2 Materials and methods

Experimental Design

Seven foliar inoculation methods were used for this experiment (Table 2.1). Treatment variables involving mycelia included four inoculation methods, two inoculum treatments, and three kauri clones. For each of these treatments, three *P. agathidicida* isolates and 9 replicate leaves were used. Treatment variables for methods involving zoospores included three inoculation methods, two inoculum treatments, and three kauri clones. For each of these treatments, three replicate leaves were inoculated with a mixture of three *P. agathidicida* isolates. All leaves used in this experiment were set up in a complete block randomised design.

Table 2.1. Experimental design for experiment 1.

Inoculum type	Inoculation Method	Isolates	Treatment replicates	Control replicates
Mycelia	Agar plug	P.a 3687, 3709, 3770	3 clones x 9 leaves	3 clones x 9 leaves
	Agar+NSPW	P.a 3687, 3709, 3770	3 clones x 9 leaves	3 clones x 9 leaves
	Agar+SPW	P.a 3687, 3709, 3770	3 clones x 9 leaves	3 clones x 9 leaves
	Cellophane	P.a 3687, 3709, 3770	3 clones x 9 leaves	3 clones x 9 leaves
Zoospores	Zoospore drop	P.a 3687*, 3709*, 3773*	3 clones x 3 leaves	3 clones x 3 leaves
	Dip petiole	P.a 3687*, 3709*, 3773*	3 clones x 3 leaves	3 clones x 3 leaves
	Dip tip	P.a 3687*, 3709*, 3773*	3 clones x 3 leaves	3 clones x 3 leaves

P.a, *Phytophthora agathidicida*; NSPW, non-sterile pond water; SPW, sterile-pond water. *three isolates were mixed together to form a single zoospore solution.

Kauri clones

Three kauri clones from Scion's tissue culture collection were used for this experiment: 1_3, 3_3, and 3_4. A clone refers to a seedling that is genetically identical to the parent. Kauri clones were grown in jars containing charcoal media (Figure 2.1) and were amplified through tissue culture techniques, using *in vitro* germinated mature zygotic kauri embryos. Each clone was made up of many individuals known as a ramet or a shoot (Murashige, 1974) (Figure 2.2). Propagation of tissue culture clones was carried out by the tissue culture team at Scion, as described in Gough et al. (2012). Detached leaves from kauri ramets were used for this experiment.

Figure 2.1. One jar containing ramets (shoots) from a single kauri clone.

Figure 2.2 Close-up image of three kauri ramets (shoots) from one single kauri clone.

Seeds used to grow these kauri clones were sourced from Holts forest, Napier, which had been planted with seeds originating from Waipoua forest, Northland. During the 1940's, the New Zealand Forest Service implemented a planting programme in Waipoua Forest, Northland, to identify kauri (60-70 cm in diameter) that produced regular crops of seed cones. This first selection was maintained as a grafted nursery at the former Forest Service headquarters within Waipoua Forests. The initial selection was later refined to kauri that produced better than average seedlings (Halkett, 1983b). These seedlings were used to establish private kauri plantings throughout New Zealand. One of these plantings was established in Napier at Holt's forest (Halkett, 1983b), from which seeds were sourced from open-pollinated mother trees selected for their height. For this study, there were 50 kauri genotypes available that were initially derived from seeds sourced from kauri stands located at Waipoua Forest and Holt's Forest (Halkett, 1983b; Steward, 2011). The susceptibility of the three clones to *P. agathidicida* was not known.

Isolates

Three *P. agathidicida* isolates were sourced from the New Zealand Forest Research Institute culture collection, stored at Scion, Rotorua. The isolates used in this experiment were: *P. agathidicida* 3687, 3709, 3770 (Table 2.2). Isolates 3687 and 3709 were isolated from kauri soil collected in the Waipoua forest. Isolate 3770 was isolated from dead and dying kauri roots on Great Barrier Island**.** Isolates were already growing on carrot agar (CA) (Appendix B). Before inoculations were conducted, isolates were transferred to V10 agar (Appendix B) and incubated under continuous light at 20°C for three days.

Table 2.2 Isolate details for all *Phytophthora* species used within this study.

Phytophthora species	Isolate number	Host	Date Collected	Location	Substrate	Experiments
P. agathidicida	3118	*A. australis*	11/03/2009	Auckland, Waitakere Ranges Regional Park, Huia, Bush track to ridge	Isolate obtained by baiting soil with rhododendron leaf beneath kauri with root disease and butt canker.	2, 3, 4
P. agathidicida	3687	*A. australis*	1/10/2011	Waipoua Forest, Northland	Kauri soil	1
P. agathidicida	3709	*A. australis*	2011	Waipoua Forest, Northland	Kauri soil	1
P. agathidicida	3770	*A. australis*	23/03/2006	Great Barrier Island	Isolated from dead and dying kauri roots	1
P. agathidicida	3813	*A. australis*	30/01/2014	Coromandel	Kauri rhizosphere soil	2, 3, 4
P. agathidicida	3814	*A. australis*	30/01/2014	Coromandel	Kauri rhizosphere soil	2, 3, 4
P. agathidicida	3815	*A. australis*	30/01/2014	Coromandel	Kauri rhizosphere soil	3, 4
P. cinnamomi	3750	*P. radiata*	9/01/2013	Cpt 64, Golden Downs	Radiata pine soil and roots	3, 4
P. multivora	3907	*A. australis*	9/05/2014	Unknown	Kauri soil	3, 4

P, *Phytophthora*; A, *Agathis*; P, *Pinus*. Isolates were sourced from the New Zealand Forest Research Institute culture collection, stored at Scion, Rotorua, New Zealand.

Luria broth test procedure

Before this experiment was conducted, a luria broth (LB) test was carried out to determine if the *Phytophthora* cultures were contaminated with bacteria. Isolates were transfered to a 30 ml sealed Falcon tube (LabServ – ThermoFisher Albany, Auckland, New Zealand) containing 10 ml of 25 g/l concentration of LB (Eisenstark, 1967), that is known to support the growth of bacteria. The sealed Falcon tubes were agitated at 150 revolutions per minute at 30°C for 48 hours (Bio-strategy SI-300R shaker). Cultures were identified as contaminated if they produced an opaque suspension, while non-contaminated cultures remained transparent, as per the control suspensions of un-inoculated LB.

Pancaking procedure

The pancaking procedure was carried out if the *Phytophthora* cultures looked contaminated with bacteria or if they failed the above LB test. The purpose of the pancaking procedure was to clean bacterial contamination from cultures. Scion's pancake standard operating procedure was used. Using sterile technique, a fine slice of the culture from the contaminated plate was place in the middle of the top half (semicircle) of the clean plate of media. A scalpel was used to cut a D shaped area of media from the bottom half of the plate with the straight edge running through the middle of the plate. The flap (rounded edge) of media was flipped and folded over the fine scraping to ensure the contaminated slice was pancaked between layers of media. As many bacteria require air to grow, pancaking creates an airlock around the edges of the culture, restricting the growth of the bacteria through the media.

Zoospore production

For each isolate, five 3 mm diameter mycelial plugs, from three day old V10 cultures were placed in 20 ml of non-sterile soil extract (Appendix C) in Petri plates and left over night at 18°C under a fluorescent light. To induce sporangia release, the cultures were cold-shocked at 4°C for 45 minutes and then placed on a light box for another 45 minutes (Dick et al., 2014). A sporangia count was done under the dissecting microscope and the spores were counted using a haemocytometer in order to calculate the zoospore concentrations. Each of the zoospore solutions for the three isolates were mixed together to form one total zoospore concentration of 9.6 x 10^3 zoospores/ml. This was used to infect the leaves within 2 hours of being made.

2.2.1 Inoculation Methods

Agar plug

Leaves were placed into small 90 mm x 15 mm plastic Petri dishes, three leaves per dish, with the abaxial surface facing upwards on a double layer of tissue paper moistened with deionised water (Hansen et al., 2005). A 3 mm diameter cork borer was used to cut mycelial plugs from the leading edge of a colonised V10 agar culture, or a non-colonised V10 agar culture for controls. One plug was placed in the centre on the abaxial surface of each leaf, with the mycelium side always facing down on the leaf surface. The Petri dishes were incubated at 20°C for three days.

Agar and non-sterile pond water (Agar+NSPW)

The concept of this method was based on the idea that non-sterile pond water could potentially induce the development of sporangia from the mycelia growing in the agar

plugs. This in turn, would facilitate the release of zoospores that swim through the water and cause infection on the under surface of the leaves (Dick, Williams, Bader, Gardner, & Bulman, 2014). One 3 mm mycelia agar plug from each of the three *P. agathidicida* isolates, or three non-colonised V10 plugs for controls, were placed together in a Petri dish and covered with 10 ml of non-sterile pond water. Three leaves were placed into the Petri dish with the abaxial surface facing down in the water. The Petri dishes were incubated under continunous light at 20°C for three days. Leaves were transferred into clean Petri dishes with the abaxial surface always facing upwards, on a double layer of paper towels moistened with deoinised water. Leaves were incubated for a further three days under the same conditions.

Agar and sterile pond water (Agar+SPW)

As per the agar and non-sterile pond water method however, the pond water was sterilised in the autoclave. The purpose of this method was to compare both sterilised and non-sterilised pond water to see which one was better at facilitating zoospore release for *P. agathidicida* infection.

Cellophane

The purpose of this method was to infect leaves with mycelia from the test *P. agathidicida* strain, without the agar media attached. A single sterile cellophane sheet was applied to V10 agar before a 3 mm diameter plug of the *P. agathidicida* strain was placed on top of the cellophane. Nutrients from the media were absorbed through the cellophane membrane, which allowed the mycelia to grow. Three days later, the cellophane was peeled off from the media with the mycelia still attached to it. On a sterile cutting board,

3 mm diameter plugs were cut from the cellophane sheet. Leaves were placed into small Petri dishes, three leaves per dish, with the abaxial surface facing upwards onto a double layer of tissue paper moistened with deionised water. One cellophane plug was placed in the centre of each leaf with the mycelium side facing down. Non-colonised 3 mm cellophane plugs were used for the controls. The Petri dishes were transferred to plastic trays and incubated in the incubator under continuous light at 20°C for three days.

Zoospore drop

Three leaves were placed into 90 mm x 15 mm plastic Petri dishes, on a double layer of tissue paper moistened with deionised water, with the abaxial surface facing upwards (Hansen et al., 2005). Using a pipette, 10 µl of the above zoospore solution, or 10 µl of non-sterile soil extract for the controls, was placed in the centre of each leaf. Lids were placed on the Petri dishes and stored in plastic trays. Leaves were incubated under continuous light at 20°C for three days (Eshraghi et al., 2011).

Dip petiole

Leaves were placed petiole down in 1.5 ml eppendorf tubes. Using a pipette, 50 µl of the zoospore solution, or 50 µl of non-sterile soil extract for controls, was added into each eppendorf tube. Leaves were incubated in the incubator at 20°C with the lids closed for three days. Leaves were taken out of the eppendorf tubes and placed onto Petri dishes with moist paper towels, with three leaves per Petri dish. Leaves were further incubated under the same conditions for three days (Hüberli, Shearer, Calver, & Paap, 2008).

Dip tip

As per the dip petiole method, however, the tips of the leaves were suspended in 50 µl of the zoospore solution, or 50 µl of the non-sterile soil extract for controls (Hüberli, Shearer, et al., 2008).

2.2.2 Lesion assessments

Three days post-inoculation, the following lesion assessments were carried out:

- Lesion length: the lesion length on each leaf was measured with a ruler in mm. Measurements stretched from one distinct lesion end to the other.

- Area infected: the necrotic areas on the surface of each leaf were rated on a scale from 0 – 5. A rating of 0 indicated no infection, a rating of 3 indicated moderate infection, and a rating of 5 indicated severe infection covering the whole surface leaf area.

- Recovery: to determine if Koch's postulates was fulfiiled, the recovery of the pathogen from the leaf tissue was conducted for all isolate and control treatments. (Manion, 1981). Each leaf was plated onto V10-PRPH media (Appendix B) and incubated at 20°C for seven days. Plates were checked for any *Phytophthora*-like colonies emerging from the leaf segments every two days, for up to a week, and colonised leaves were marked on the bottom of the plates. To confirm their identities, colonies were checked under the microscope for characteristic *Phytophthora* hyphae or spores (Weir et al., 2015).

2.2.3 Statistical analysis

All statistical analyses in this entire study were conducted by the Massey University statistician, Zhao Xing. All statistical analyses used the Statistical Analysis System (SAS) software, version 9.4 (SAS Institute Inc., NC, USA). A Shapiro-Wilk goodness-of-fit test test, indicated lesion length and area infected were not normally distributed. The Wilcoxon signed-rank test, i.e., non-parametrical one sample t test, using the PROC Univariate procedure, compared lesion length and area infected of each treatment with the control treatments (i.e., 0). A non-parametric ANOVA Proc General Linear Model (PROC GLM) was applied to compare the mean lesion length and area infected between combination treatments. The possibility of recovering the pathogen from the leaf tissue, was estimated by a generalised logistic linear model (Proc GLIMMIX). The possibility of lesion length and area infected affecting recovering was estimated using the same model. Data with a value of 0 were not included for analysis.

2.3 Results

To compare the seven inoculation methods, the average lesion lengths and areas of infected kauri leaves were recorded (Figure 2.3, Figure 2.4).

Average lesion length and areas infected were significantly higher for the agar plug, agar+NSPW, agar+SPW, cellophane, and zoospore drop methods compared to the controls: signed-rank test: $S = 18.0 \sim 115.5$, $P < 0.01$. The exceptions were methods dip petiole and dip tip which were not significantly higher than the controls: signed-rank test: $S = 1.5 \sim 3$, $P > 0.05$. The agar plug method for isolates 3687 and 3770 produced significantly more disease symptoms than the agar+SPW, zoospore drop, and dip petiole

methods, excluding the length of the agar plug itself: non-parametrical ANOVA: $F_{10, 250} = 4.64$ and 4.90 for lesion length and area infected, respectively; $P < 0.0001$. Although not statistically significant, the dip tip method produced larger lesions than the zoospore drop and dip petiole methods, and isolate 3770 produced a larger lesion length and area infected than isolate 3709 for the agar plug method.

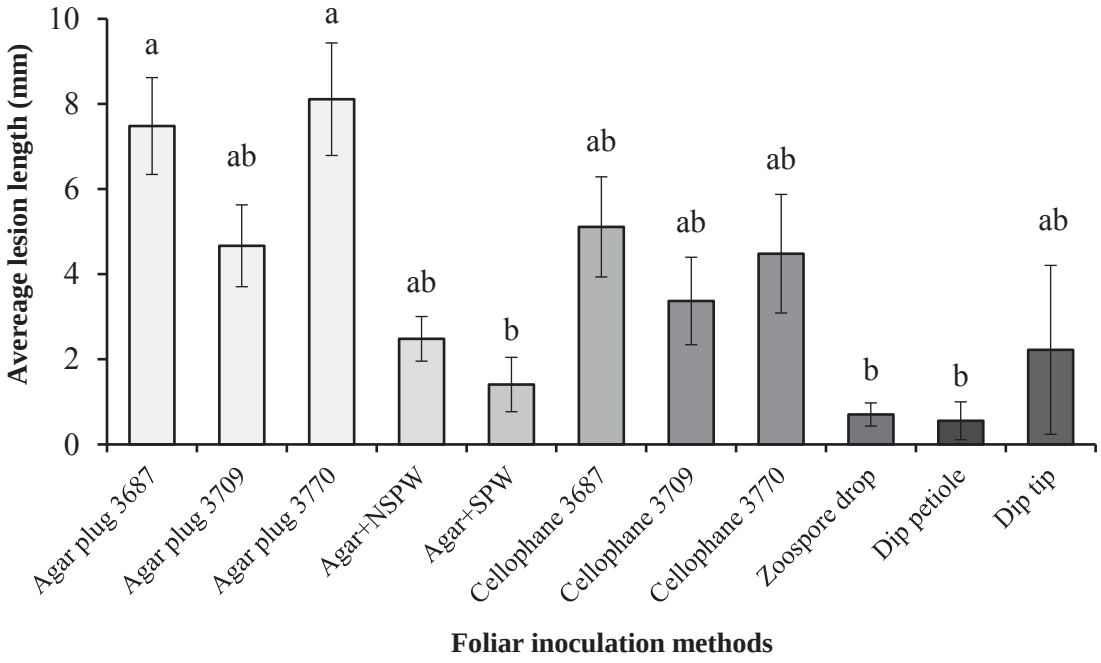

Figure 2.3. Average lesion length (mm) on *Agathis australis* (kauri) leaves. Detached leaves from kauri (three clones: 1_3, 3_3, 3_4) were inoculated with *Phytophthora agathidicida* (three isolates: 3687, 3709, 3770) using seven foliar inoculation methods. There were no lesions for control treatments. Columns with different letters are significantly different ($P < 0.05$).

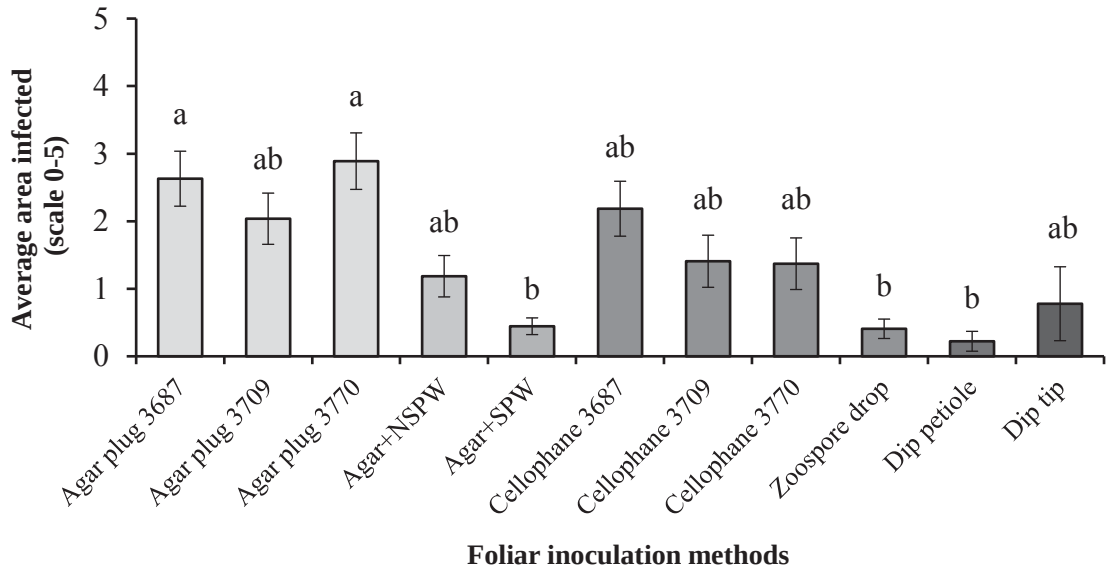

Figure 2.4. Average area infected (on a scale from 0-5) on *Agathis australis* (kauri) leaves. Detached leaves from kauri (three clones: 1_3, 3_3, 3_4) were inoculated with *Phytophthora agathidicida* (three isolates: 3687, 3709, 3770) using seven foliar inoculation methods. A rating of 0 indicates no infection, 3 indicates moderate infection, and 5 indicates severe infection covering the whole surface leaf area. There were no lesions for control treatments. Columns with different letters are significantly different ($P < 0.05$).

The positive recovery of the pathogen from foliar lesions was significantly higher in the agar plug methods for isolates 3687 and 3770, agar+NSPW and agar+SPW than all other methods (Figure 2.5), $F_{10, 250} = 8.37$, $P < 0.0001$. It also increased significantly with increasing lesion length, $F_{1, 259} = 14.43$ and area infected, $F_{1, 259} = 12.25$, $P < 0.001$ (Figure 2.6). There were no positive recoveries for control treatments.

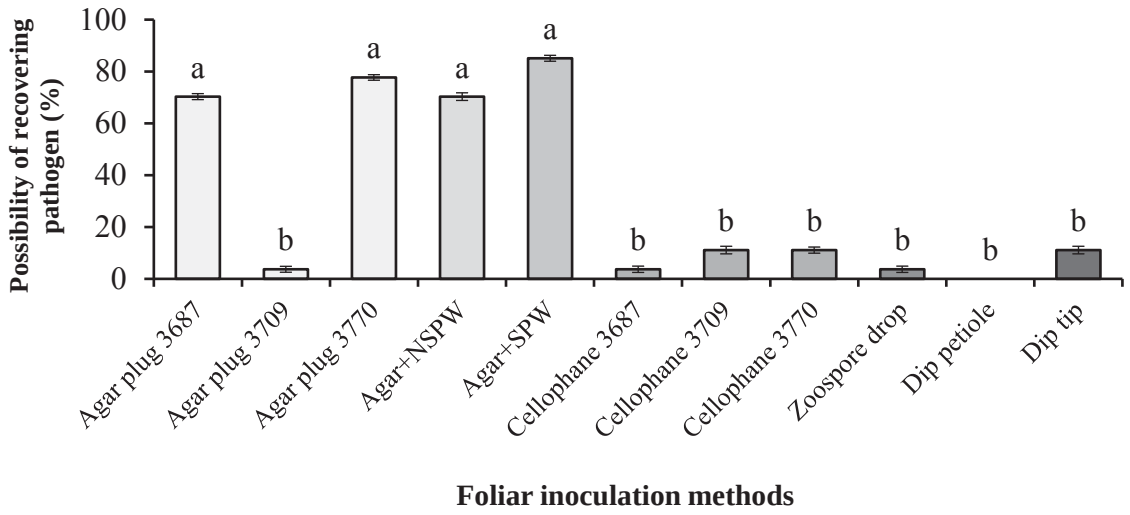

Figure 2.5. The possibility of recovering the pathogen (%) from *Agathis australis* (kauri) leaves. Detached leaves from kauri (three clones, 1_3, 3_3, 3_4) were inoculated with *Phytophthora agathidicida* (three isolates: 3687, 3709, 3770) using seven inoculation methods. There were no recoveries for control treatments. Columns with different letters are significantly different ($P < 0.05$).

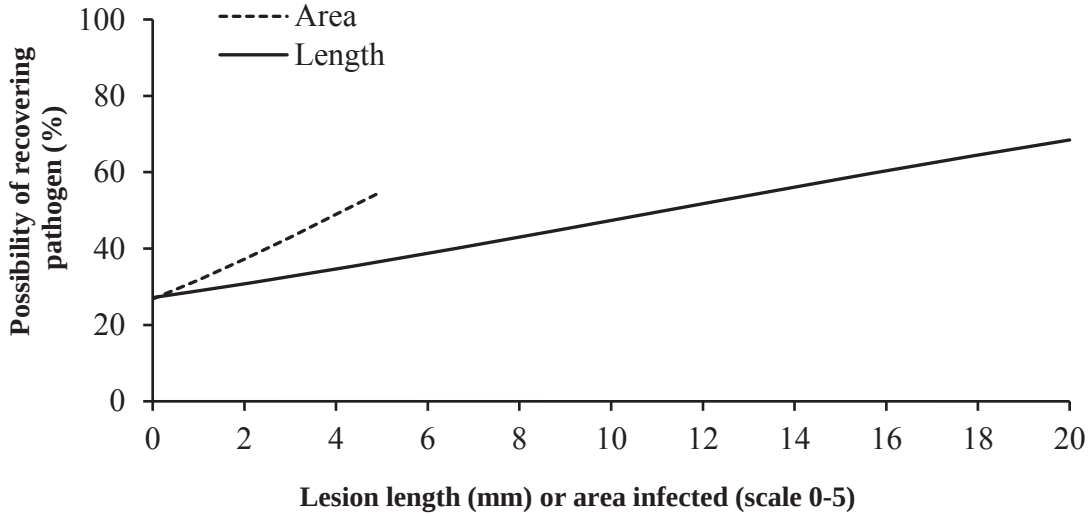

Figure 2.6. The best-fit correlation curve for the possibility of recovering the pathogen (%) from *Agathis australis* (kauri) leaves in relation lesion length (mm) or area infected (scale 0-5). Detached leaves from kauri (three clones: 1_3, 3_3, 3_4) were inoculated with *Phytophthora agathidicida* (three isolates: 3687, 3770, 3709) using seven inoculation methods.

2.4 Discussion

Using seven inoculation methods, it was shown that *P. agathidicida* can infect and invade the foliar tissue of kauri, despite being a root-infecting pathogen. Each method produced necrotic lesions at varying levels of severity. Methods utilizing mycelia as the inoculum source produced larger necrotic lesions than those using zoospores. The agar plug method caused significantly more disease than the remaining six methods, and this method was very rapid and easy to conduct. For the above reasons, the agar plug method was considered the most suitable technique for foliar inoculations of kauri. There appeared to be a positive correlation between lesion length, area infected, and the possibility of recovering the pathogen from the lesions. This was expected as there should be more biomass (oomycete growth) in larger lesions, providing more opportunities for the pathogen to grow and expand in the leaf tissue.

In experiments using other *Phytophthora* species to assess foliar susceptibility, disease incidence and severity often increased when leaves were wounded prior to inoculation (Balci et al., 2008; Denman et al., 2005; Tooley & Kyde, 2007). The cell walls and cuticles of the leaves act as major barriers to the pathogen which make it more difficult for the pathogen to penetrate through the leaf surface. Artificial wounding of leaves is often required to cause a high infection rate. As demonstrated by Balci et al. (2008), a high infection rate only resulted when wounded oak leaves were inoculated with agar plugs colonised by *P. ramorum*. A physical injury inflicted on kauri foliage can make leaves more vulnerable to invasion and should be considered for future inoculations (Dijkstra & de Jager, 2012).

Inoculation methods utilizing agar plugs, are known for their quick assessment of foliage susceptibility, capable of demonstrating variation in virulence among *Phytophthora* species (Balci et al., 2008). Using the agar plug method in this experiment, isolate 3770 appeared to be the most virulent, followed by isolates 3687 and 3709. however, isolate 3687 appeared to be the most virulent using the cellophane method. There appeared to be a low recovery rate of isolate 3709 from the lesions for both methods, suggesting that this strain was not growing well inside the host tissue. As environmental factors play an important role in disease epidemiology, the true susceptibility of kauri to different strains of *P. agathidicida* under field conditions could be considerably different (Balci et al., 2008). Therefore, care must be taken when interpreting the importance of small necrotic lesions as an indication of kauri susceptibility to *P. agathidicida* infection.

The cellophane method demonstrated that *P. agathidicida* mycelia are a suitable inoculum source for infecting kauri leaves, even without the agar plug attached however, the extent of the lesions was less than those produced by the agar plug method. It also had limitations in that the methodology was very time consuming, a disadvantage when a rapid and efficient screening assay is required. Methods involving pond water produced moderate lesions, with non-sterile water producing larger lesions than sterile pond water. As the sterile pond water was autoclaved, this may have depleted any nutrients in the water that could have potentially been used to aid in the production of zoospores.

In comparison to mycelia on agar plugs, zoospore solutions are known to produce a larger number of infective propagules, providing more opportunities for the pathogen to enter the foliar tissue (Balci et al., 2008). Despite this, the zoospore drop, dip tip, and

dip petiole methods in this experiment produced a lower disease incidence and severity than all other methods. In past studies, *P. ramorum* zoospores and sporangia produced a higher infection rate than colonised agar plugs, when testing the susceptibility of foliage of different oak species (Balci et al., 2008; Denman et al., 2005; Hansen et al., 2005; Tooley & Kyde, 2007). As zoospores need to germinate to produce mycelium, it is possible that the number of zoospores required to cause severe infection in kauri was not high enough or required more time to germinate, resulting in the low infection rate observed.

Modifying the zoospore production protocol may be necessary to increase the chances of producing a more severe infection rate in kauri leaves. Zoospore concentration has a significant influence on the number and length of lesions produced by *Phytophthora* species, as illustrated by a study conducted on *P. pluvialis* to determine the minimum zoospore concentration needed for red needle cast infection to occur. A concentration of 200 per ml or greater were needed for artificial inoculations (Ganley, Scott, & Bader, 2014). The zoospore concentration used in this experiment was very low, 9.6×10^3 per ml. This highlights how important it is for the zoospores to be produced reliably and in adequate numbers when being used for inoculation procedures (Eye, Sneh, & Lockwood, 1978).

The dip tip method produced more disease than the dip petiole and zoospore drop methods, despite the same zoospore concentration being used. This suggests that a higher infection rate occurred when the tips of the leaves were in contact with a large volume of inoculum, as opposed to a small droplet being placed on the top of the leaves. A small zoospore drop size of 50 µl could have contributed to the low disease incidence observed

in this experiment. A studied carried out on pepper fruit infection, caused by *P. capsica* (Leonian 1922), showed that droplets of 10, 50, and 100 µl with identical zoospore content (5000 zoospores/drop) did not significantly affect lesion size on mature green pepper fruit but disease incident was significantly greater with a 100 µl drop compared to the 10 and 50 µl drops (Biles, 1995). A higher zoospore concentration greater than 200 per ml and a minimum volume of 100 µl for a zoospore droplet, could be used for future kauri inoculations and compared to the results observed in this experiment. Future work to improve the zoospore production of this screening assay could include flooding the plugs of agar mycelium with clarified carrot agar dextrose (CAD) broth, a process which may help produce a good quantity of *P. agathidicida* zoospores (Dick et al., 2014).

2.5 Conclusion

Seven foliar techniques were compared to determine the most suitable inoculation method for a kauri resistance screening assay. Methods involving mycelia produced a higher infection rate than the methods involving zoospores. The agar plug method was rapid and efficient, causing significantly more disease symptoms than the remaining six methods. It also demonstrated variation in virulence among the three *P. agathidicida* isolates used. For these reasons, the agar plug method was determined as the most suitable inoculation method for a kauri resistance screening assay. It is possible that the zoospore concentration used in this experiment was not high enough to cause severe infection therefore, the use of zoospores as a potential inoculum source for kauri foliar inoculations should not be ruled out at this stage. Future work, including modifying the zoospore production process and artificially wounding kauri leaves prior to inoculation, may improve the infection rate of *P. agathidicida*.

Chapter 3

Detached foliar inoculations on *Agathis australis* (kauri) saplings over 5 years of age with *Phytophthora agathidicida*

3.1 Introduction

In vitro screening assays to identify resistant and susceptible plant genotypes, using detached plant material, have provided valuable information about the variation in resistance of kauri genotypes to *P. agathidicida*, as described in Section 2.1. In woody plants, some *Phytophthora* species are associated with stem and leaf lesions, and root and crown rot (Balci et al., 2008; Tsao, 1990). In these cases, foliar inoculations have been used to identify putatively resistant and susceptible plant species to help inform disease management (Balci et al., 2008; Denman et al., 2005; Hüberli, Lutzy, et al., 2008; Ireland et al., 2012). Although *P. agathidicida* has not been associated with any foliar disease symptoms in kauri, foliar inoculations with *P. agathidicida* have been used for pathogenicity studies; however, they have never been used for resistance screening purposes (Horner & Hough, 2014).

Detached leaves have been used to screen host material for susceptibility to a range of *Phytophthora* species including identifying highly susceptible and asymptomatic hosts of *P. ramorum*, which infects bark and leaves. This has been shown for both endemic and commercial New Zealand (Hüberli, Lutzy, et al., 2008) and United Kingdom (Denman et al., 2005) plant species. *In vitro* leaf assays have identified Australian flora that are extremely susceptible to *P. ramorum* which are now candidates for surveillance programmes in high risk incursion areas of Australia (Ireland et al., 2012). Similarly,

Balci et al. (2008) inoculated wounded detached leaves with agar plugs colonised with one of seven soil-borne *Phytophthora* species to determine their ability to infect foliage of common North American oak species and their use to estimate foliar susceptibility.

Lucerne plants resistant to the root infecting pathogen, *P. medicaginis*, have been identified by comparing detached leaf, intact cotyledon, and root responses when infected with zoospore inoculum (Irwin et al., 2003). In this study, intact cotyledon responses showed a strong association with root responses, allowing for the selection of *Phytophthora* resistant plants. Detached leaves had a weaker association with root response, despite being a reproducible assay (Irwin et al., 2003). As demonstrated by these studies, an *in vitro* detached leaf assay could be a meaningful screening tool for enabling informed management of kauri dieback disease, if the lesions obtained from foliar inoculations are representative of those from root inoculations.

Past studies on detached foliar inoculations with *P. agathidicida* have been conducted on 2-year-old glasshouse-grown kauri seedlings, but have yet to be conducted on plants older than this age**.** Horner & Hough (2014) determined the relative pathogenicity of four *Phytophthora* species associated with kauri including *P. agathidicida*, *P. cinnamomi*, *P. multivora*, and *P. cryptogea* (Pethybridge and Lafferty, 1919). Using a wounded detached leaf assay on 2-year-old kauri, *P. agathidicida* was the most aggressive *Phytophthora* species. Kauri tissue appeared to have little innate resistance to this pathogen (Horner & Hough, 2014). Further research is required to determine if leaf inoculation assays will be useful for determining the resistance of older trees to *P. agathidicida* which, if so, could be applied to the wider population of mature kauri in New Zealand forests.

Based on the results from experiment 1 (Chapter 2) agar plugs colonised by the test *Phytophthora* strain produced the most consistent lesions on unwounded detached leaves.

The aims of this experiment were to:

a) Determine if *P. agathidicida* inoculations using mycelia agar plugs were a suitable means of resistance screening on wounded detached leaves from kauri saplings over 5 years in age.
b) Determine if there was any variation in virulence across the *P. agathidicida* isolates used: *P. agathidicida* 3118, 3813, and 3814.
c) Determine if there was any variation in susceptibility to *P. agathidicida* infection across the six kauri saplings over 5 years of age.

3.2 Materials and methods

Kauri saplings

Six kauri saplings over 5 years of age, growing in Scion's Long Mile Reserve (Long Mile Rd, Rotorua, 3010), were used for this study. In April 2015, six saplings with a large proportion of leaves from the current year's shoots were selected. The family history of these saplings is outlined in experiment 1, Section 2.2. The seedlings were first grown from tissue culture material and later planted along Long-Mile Rd where they developed an extensive root system (Figure 3.1).

Figure 3.1 A stand of *Agathis australis* (kauri) saplings over 5 years of age. Saplings were growing at Scion, Long Mile Rd, Rotorua. Detached leaves from six saplings were used for this foliar inoculation study in April 2015.

Isolates

Three *P. agathidicida* isolates were sourced from the New Zealand Forest Research Institute's culture collection, stored at Scion, Rotorua: *P. agathidicida* 3118, 3813, and 3814 (Table 2.2, Chapter 2). Isolates were grown on carrot agar (CA).

Leaf layout

A split plot design was used, incorporating single leaves from six kauri saplings; three *P. agathidicida* isolates and one control treatment comprised of non-colonised CA, in 10 replicates comprising all treatments. A 6 x 4 gridline was printed onto 29.7 x 42.0 cm waterproof paper for each replicate. Each square in the grid measured 7.5 x 7.5 cm, being large enough to fit an entire leaf. Isolates and sapling source were randomised using a

random number generator. A double layer of wet paper towels measuring 6 x 6 cm was placed inside each square grid to provide moisture for infected leaves.

Harvesting of leaves

Saplings were assigned a metal tag for identification. From each sapling, 40 fully-formed current year leaves in April, 2015, were randomly selected and placed into labelled zip-lock bags. Using a water dropper, paper towels in the above mentioned grids were moistened with deionised water before the leaves were placed on top with the lower leaf surface facing upwards.

3.2.1 Inoculation method

A small surface wound was made 0.5 – 1.0 cm from the base of each leaf with a hypodermic needle (Horner & Hough, 2014). Leaves were inoculated, as per the method in Section 2.2.1. A 3 mm diameter CA plug colonised with the different isolates, cut from the leading edge of a three day old culture, was placed on top of the wound with the colonised side of the plug facing down on the leaf. A non-colonised CA plug was used for the controls. Using a wet sponge, plastic tray edges were moistened with water to create a continuous seal and covered with cling film to ensure the leaves did not dry out during the incubation period. Inoculated leaves were incubated on the laboratory shelf for six days at ambient temperature, between 20-23°C, and exposed to diurnal light variation of 12 hours of light and 12 hours of dark (Roden & Ingle, 2009). The paper towels under the leaves were re-moistened and sealed every two days with deionised water.

Scanning of leaves

Six days post-inoculation, the agar plugs were removed from the leaves. Each replicate was removed from it's plastic tray. Two small paper rulers measuring at least 2 cm was placed at the top and right-hand side of each replicate as a scale. Leaves were scanned using an Epson Expression 11000XL photo scanner at 1200 DPI. The entire replicate was scanned to obtain a high-quality image of the leaves for lesion assessments (Figure 3.2). To prevent cross-contamination, the scanner was wiped with 70% ethanol and allowed to dry completely between each scan.

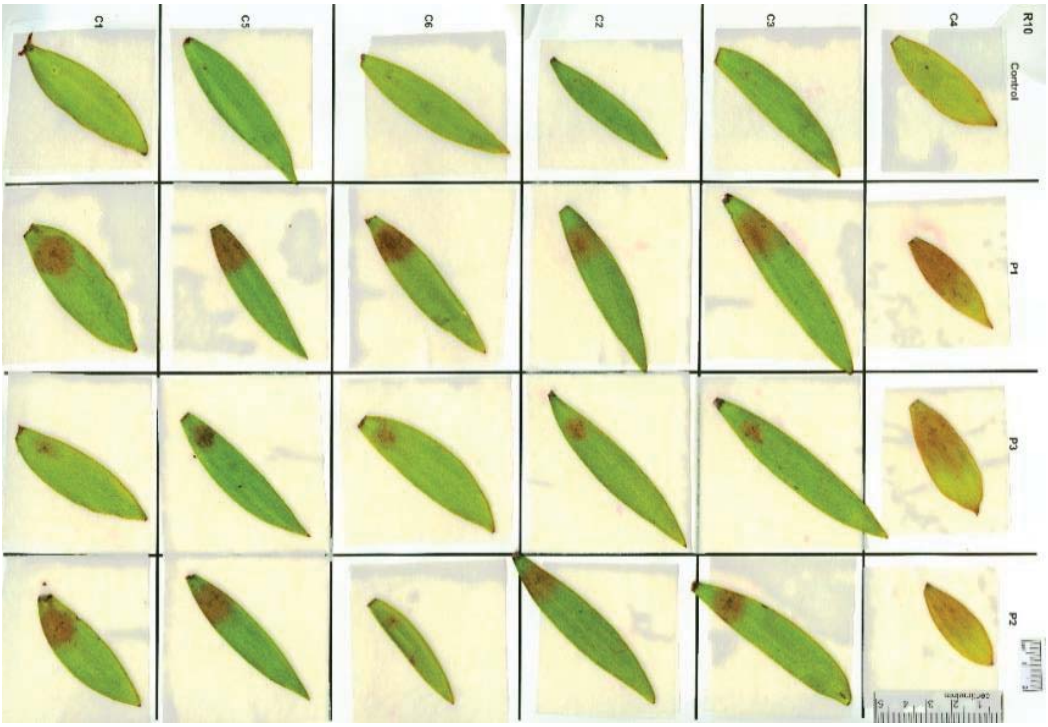

Figure 3.2. A scanned image showing the randomised experimental layout for one replicate of leaves with necrotic leaf tissue, six days post-inoculation. The replicate contains detached leaves from six *Agathis australis* (kauri) saplings (columns) over 5 years of age, inoculated with one of three *Phytophthora agathidicida* isolates (rows) using colonised carrot agar plugs, or non-colonised carrot agar plugs for the controls.

Leaf analysis

The software package, WinFOLIA PRO (Reagent Instruments, Québec, QC, Canada, Version 2013e) was used to quantify lesion area and percentage of lesioned leaf area using colour analysis, as described in WinFOLIA (2001). Colour classes were defined in WinFOLIA to differentiate between healthy and necrotic tissue, and leaf area was calibrated using standards of known area, as described in Pinkard & Mohammed (2006).

3.2.2 Lesion assessments

Six days post-inoculation, scanned images of all replicates were printed and the following lesion assessments were carried out:

- Lesion length (mm): using a ruler, the lesion length of each leaf was measured to scale from the centre of the inoculation point towards the tip of the leaf.

- Infection length (mm) and recovery: the infection length measured how far the pathogen could be recovered from leaf tissue beyond the visible external lesion margin i.e., asymptomatic infection. Recovery of the pathogen from the inoculation point was done to fulfil Koch's postulates (Manion, 1981). Each leaf was sliced into five 5 mm segments (Figure 3.3), using the methods of Shearer, Crane, Scott, & Hardy (2012). All five segments from one leaf were plated onto a plate containing CRNH media (Appendix B) and incubated at 20°C for six days. Plates were checked for any *Phytophthora*-like colonies emerging from the leaf segments every two days for up to a week. Colonies were marked on the bottom of the plate. To confirm their identities, the colonies were visually checked using a compound microscope for characteristic *Phytophthora* hyphae or spores (Weir et al., 2015).

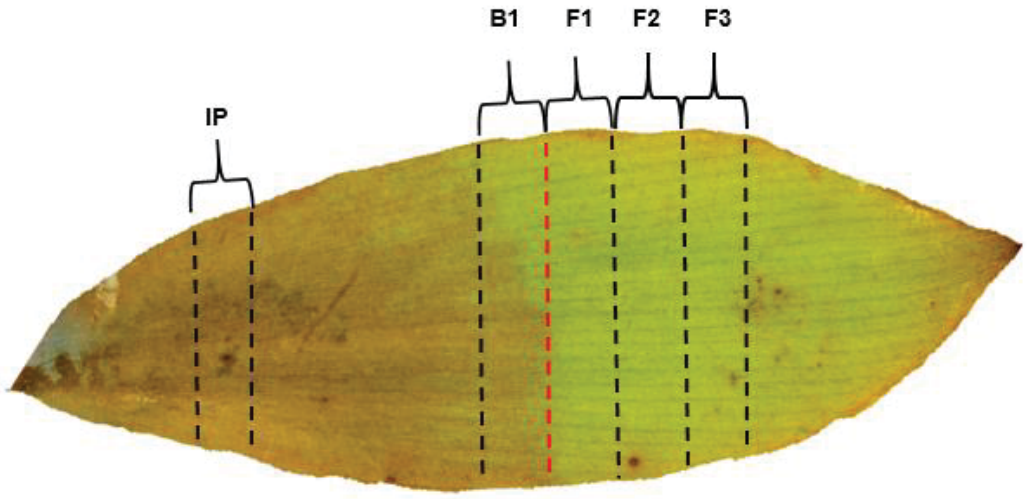

Figure 3.3 Five 5 mm leaf tissue segments that were sliced from *Agathis australis* (kauri) leaves to measure infection length (mm) and to confirm the recovery of the pathogen from the leaf inoculation point. Leaves were inoculated with one of three *Phytophthora agathidicida* isolates (3118, 3813, 3814) using colonised carrot agar plugs, or non-colonised carrot agar plugs for the controls, six days post-inoculation. The red dashed line shows the visible external lesion margin. Leaf tissue comprising of the inoculation point (IP), 5 mm back from the lesion margin (B1), and 5 mm (F1), 10 mm (F2), and 15 mm (F3) forward from the lesion margin were sliced and plated onto 10% carrot agar, ampicillin, nystatin, pimaricin, rifampicin, and hymexazol (CRNH) media for 10 replicates.

- Asymptomatic infection length (mm): infection length measurements were subtracted from the lesion length measurements to show where the pathogen was isolated from in the different sections of the leaves that were plated. This was to show whether the pathogen was present in the same section as the visible lesion length or whether the pathogen was in the asymptomatic region forward of the lesion.

- Lesion area: The WinFOLIA software was used to analyse necrotic lesion area in mm^2 and lesion area as a percentage of leaf area.

3.2.3 Statistical analysis

A Shapiro-Wilk goodness-of-fit test indicated lesion length, infection length, and lesion area were all not normally distributed. The Wilcoxon signed-rank test, non-parametric one sample t test using the PROC Univariate procedure**,** was used to compare lesion and infection length of saplings and isolate treatments with the controls. Non-parametric ANOVA Proc General Linear Model (PROC GLM) was applied to compare the mean lesion length, infection length, and lesion area between saplings and isolate treatments. The possibility of recovering the pathogen from the leaf tissue was estimated using a generalised logistic linear model (Proc GLIMMIX).

3.3 Results

All six kauri saplings infected with each of the three *P. agathidicida* isolates produced visible external disease symptoms of necrotic tissue (lesions). Small areas of necrotic tissue were observed for some of the control treatments (Figure 3.7, Figure 3.8). Sapling and isolate treatments were compared. Results were presented in bar graphs to show mean lesion length, infection length, asymptomatic infection length and lesion area (Figures 3.4 to 3.8).

Variation was observed in both lesion length and infection length between sapling and isolate treatments (Figure 3.4, Figure 3.5). Lesion and infection lengths were significantly higher for all isolates than controls: signed-rank test: $S = 22.5 \sim 27.5$, $P < 0.01$. Sapling 4 had a significantly longer mean lesion length across all isolates, suggesting it was more prone to infection. For each sapling, isolate 3813 and 3814 inoculations resulted in significantly longer lesions than isolate 3118: $F_{5, 54} = 3.63$, 9.45, and 9.69 for 3118, 3813, 3814, respectively; $P < 0.01$. The same was observed for

infection length: $F_{5, 54} = 3.19$, 11.53, and 5.31 for 3118, 3813, 3814, respectively; $P < 0.05$.

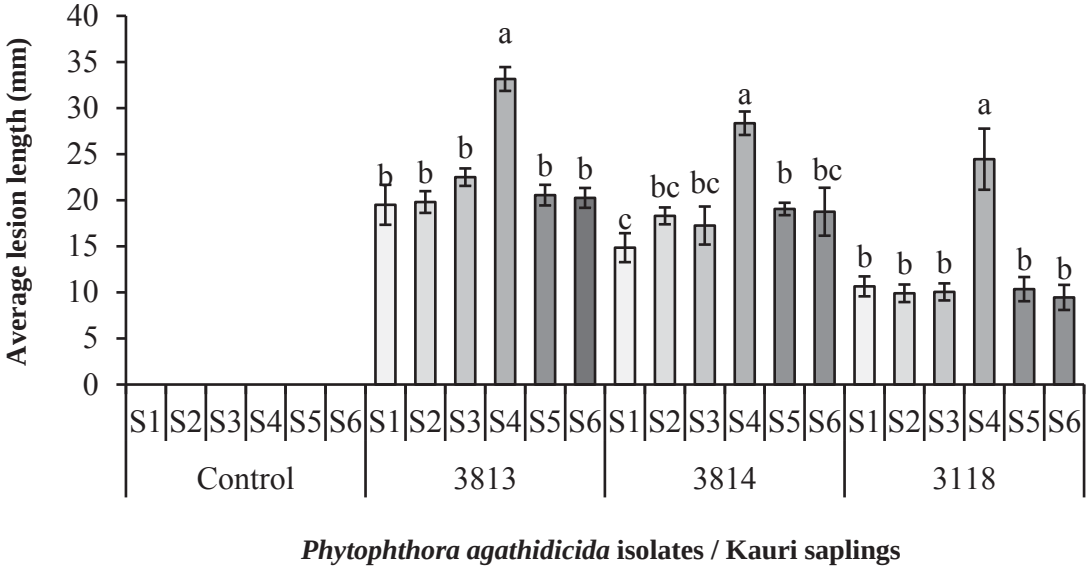

Figure 3.4. Average lesion length (mm) on detached leaves for six *Agathis australis* (kauri) saplings over 5 years of age. Six saplings (S1-S6) were inoculated with one of three *Phytophthora agathidicida* isolates (3118, 3813, 3814) or a control carrot agar plug, for 10 replicates. Columns with different letters within each isolate group are significantly different ($P < 0.05$). Control leaves had no lesions.

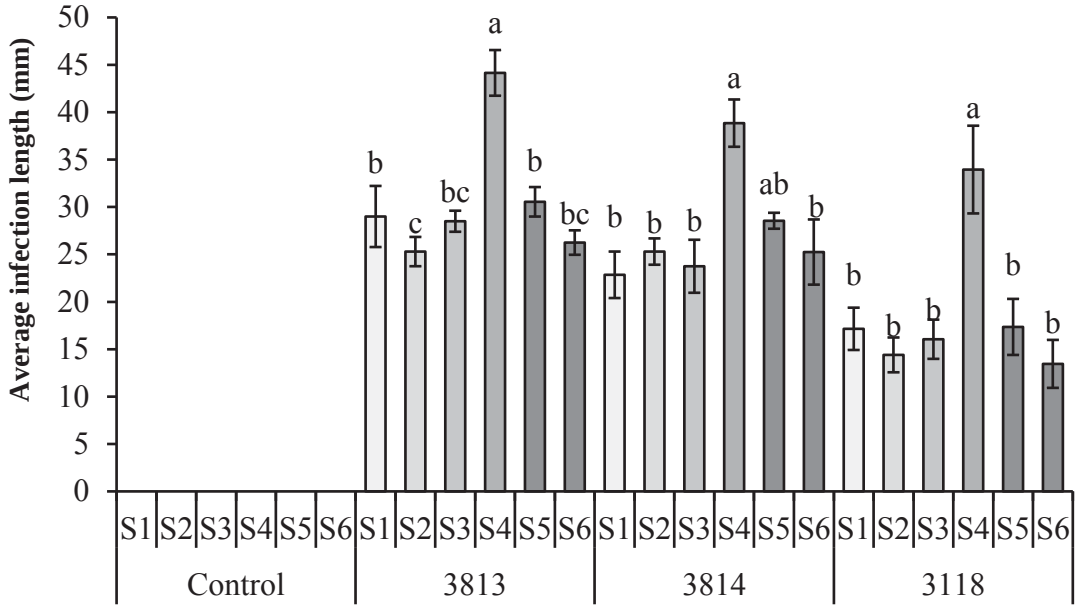

Figure 3.5. Average infection length (mm) on detached leaves from six *Agathis australis* (kauri) saplings over 5 years of age. Six saplings (S1-S6) were inoculated with one of three *Phytophthora agathidicida* isolates (3118, 3813, 3814) and a control comprising of a carrot agar plug for 10 replicates. Columns with different letters within each isolate group are significantly different ($P < 0.05$). Control leaves had no infection.

Average asymptomatic infection length was significantly greater for sapling 4 than sapling 2, sapling 3, and sapling 6 for isolate 3813, $F_{5, 54} = 4.85$, $P = 0.001$ (Figure 3.6). There was no significant differences across saplings for isolate 3814 $F_{5, 54} = 2.02$, $P > 0.05$, and isolate 3118, $F_{5, 54} = 1.44$, $P > 0.05$.

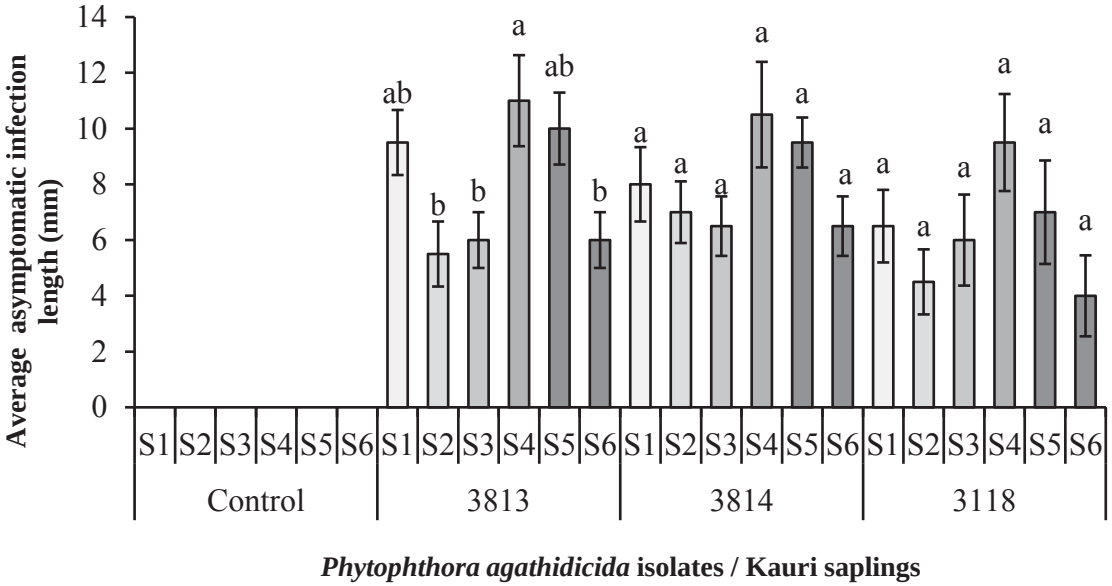

Figure 3.6. Average asymptomatic infection length (mm) on detached leaves from six *Agathis australis* (kauri) saplings over 5 years of age. Six saplings (S1-S6) were inoculated with one of three *Phytophthora agathidicida* isolates (3118, 3813, 3814) and a control comprising of a carrot agar plug for 10 relicates. Columns with different letters within each isolate group are significantly different ($P < 0.05$). Control leaves had no infection.

There was variation in lesion area, whether measured as lesion area in mm^2 or as a percentage of leaf area, across sapling and isolate treatments (Figure 3.7, Figure 3.8). For the un-inoculated control, sapling 4 had significantly larger necrotic lesion-like areas than any other sapling. Lesion areas in mm^2 for all isolates were significantly larger in sapling 4 than any other sapling, $F_{5, 54} = 7.34$ and 6.74 for 3813 and 3814, respectively; $P < 0.0001$. For the same isolates, the percentage of lesion area was also significantly larger in sapling 4 than any other sapling, $F_{5, 54} = 14.75$, 8.00 11.39, 12.27 for control, 3118, 3813, and 3814 respectively; $P < 0.0001$.

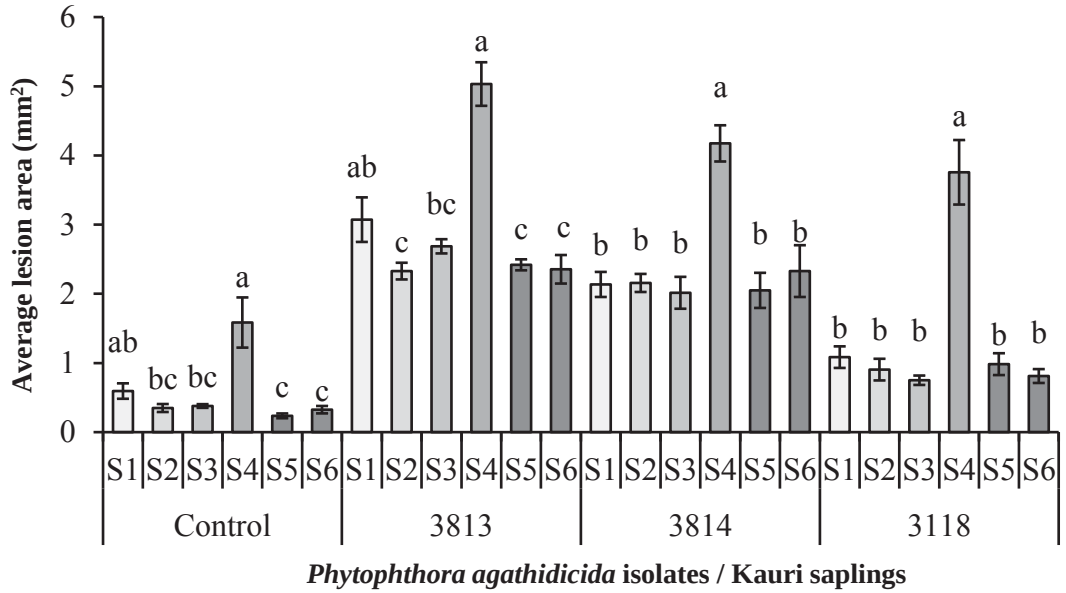

Figure 3.7. Average lesion area (mm^2) on detached leaves from six *Agathis australis* (kauri) saplings over 5 years of age. Six saplings (S1-S6) were inoculated with one of three *Phytophthora agathidicida* isolates (3118, 3813, 3814) and a control carrot agar plug for 10 replicates. Columns with different letters within each isolate group are significantly different ($P < 0.05$).

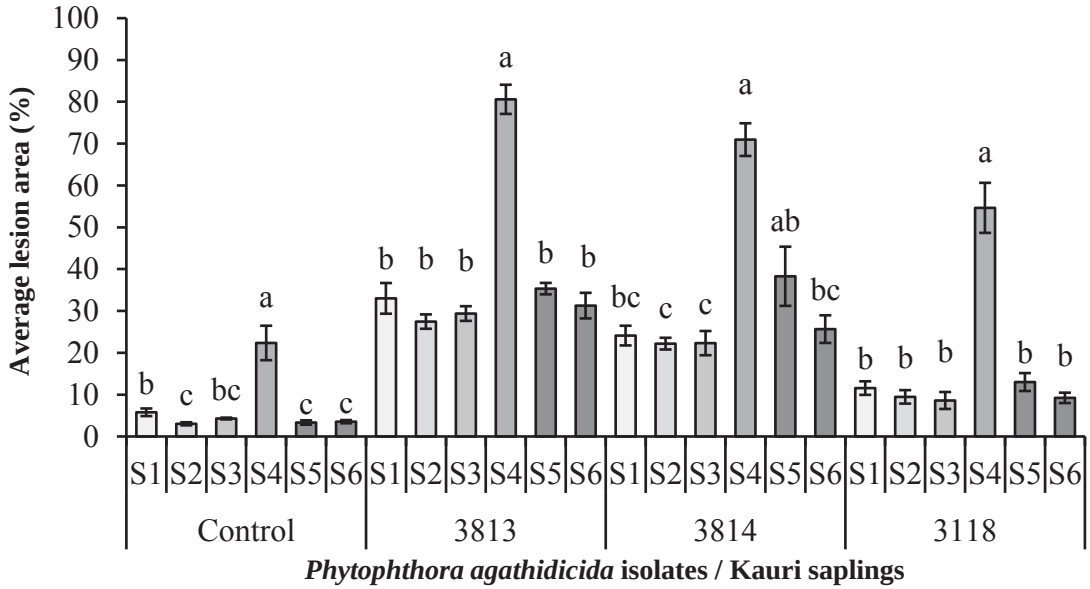

Figure 3.8. Average lesion area (%) on detached leaves from six *Agathis australis* (kauri) saplings over 5 years of age. Six saplings (S1-S76) were inoculated with one of three *Phytophthora agathidicida* isolates (3118, 3813, 3814) and a control carrot agar plug for 10 replicates. Columns with different letters within each isolate group are significantly different ($P < 0.05$).

The possibility of recovering the pathogen from the leaf tissue was compared between the six saplings and three isolates. There was ≥ 80% recovery of the pathogen with no significant differences detected across the six saplings, $F_{2,27} = 0 \sim 0.19$, P=0.8282 ~0.996, or across the three isolates, $F_{5,54} = 0 \sim 0.08$, P = 0.9956 ~1.000.

A summary of data using the combined means for the three isolates and for the six sapling treatments is presented in Tables 3.1 and 3.2. Isolates 3813 and 3814 had significantly larger measurements for lesion length, infection length, and lesion area than isolate 3118. Sapling 4 had significantly larger measurements for lesion length, infection length, and lesion area than any other sapling.

Table 3.1 A summary of mean foliar lesion assessments for the three *Phytopthora agathidicida* isolates used in experiment 2.

Isolates	Lesion length (mm)	Infection length (mm)	Lesion area (mm^2)	Lesion area (%)
3118	12.5 ± 1.49	18.7 ± 2.71	1.38 ± 0.18	17.75 ± 2.44
3813	22.6 ± 1.30	30.6 ± 1.86	2.98 ± 0.19	39.51 ± 2.51
3814	19.4 ± 1.52	27.4 ± 2.24	2.48 ± 0.24	33.93 ± 3.49

Values are the average ± SE of 10 replicates for six kauri saplings over 5 years of age. Detached leaves were inoculated with three *Phytophthora agathidicida* isolates (3118, 3813, 3814).

Table 3.2 A summary of mean foliar lesion assessments for the six *Agathis australis* (kauri) saplings used in experiment 2.

Saplings	Lesion length (mm)	Infection length (mm)	Lesion area (mm^2)	Lesion area (%)
S1	15.0 ± 1.61	23.0 ± 2.64	2.10 ± 0.22	22.90 ± 2.55
S2	16.0 ± 1.02	21.7 ± 1.59	1.80 ± 1.34	19.71 ± 1.58
S3	16.6 ± 1.31	22.8 ± 1.99	1.82 ± 0.13	20.10 ± 2.46
S4	28.7 ± 1.96	39.0 ± 3.18	4.32 ± 0.35	68.74 ± 4.46
S5	16.7 ± 1.03	25.5 ± 1.78	1.82 ± 0.16	28.88 ± 3.53
S6	16.1 ± 1.68	21.7 ± 0.75	1.83 ± 0.23	22.06 ± 2.53

Values are the average ± SE of 10 replicates for three *Phytophthora agathidicida* isolates (3118, 3813, 3814) used to infect detached leaves from six kauri saplings (S1-S6) over 5 years of age.

The correlations between lesion length, infection length, and lesion area were compared using the raw data. Figure 3.9 presents scatter plot graphs showing the correlation between the four lesion assessment methods and their corresponding R^2 values.

Lesion length and infection length (Figure 3.9 a) showed a strong positive correlation with a higher R^2 value of 0.97 compared to any other assessments. Infection length and lesion area as a percentage (Figure 3.9 e) had a weaker correlation, with a lower R^2 value of 0.81.

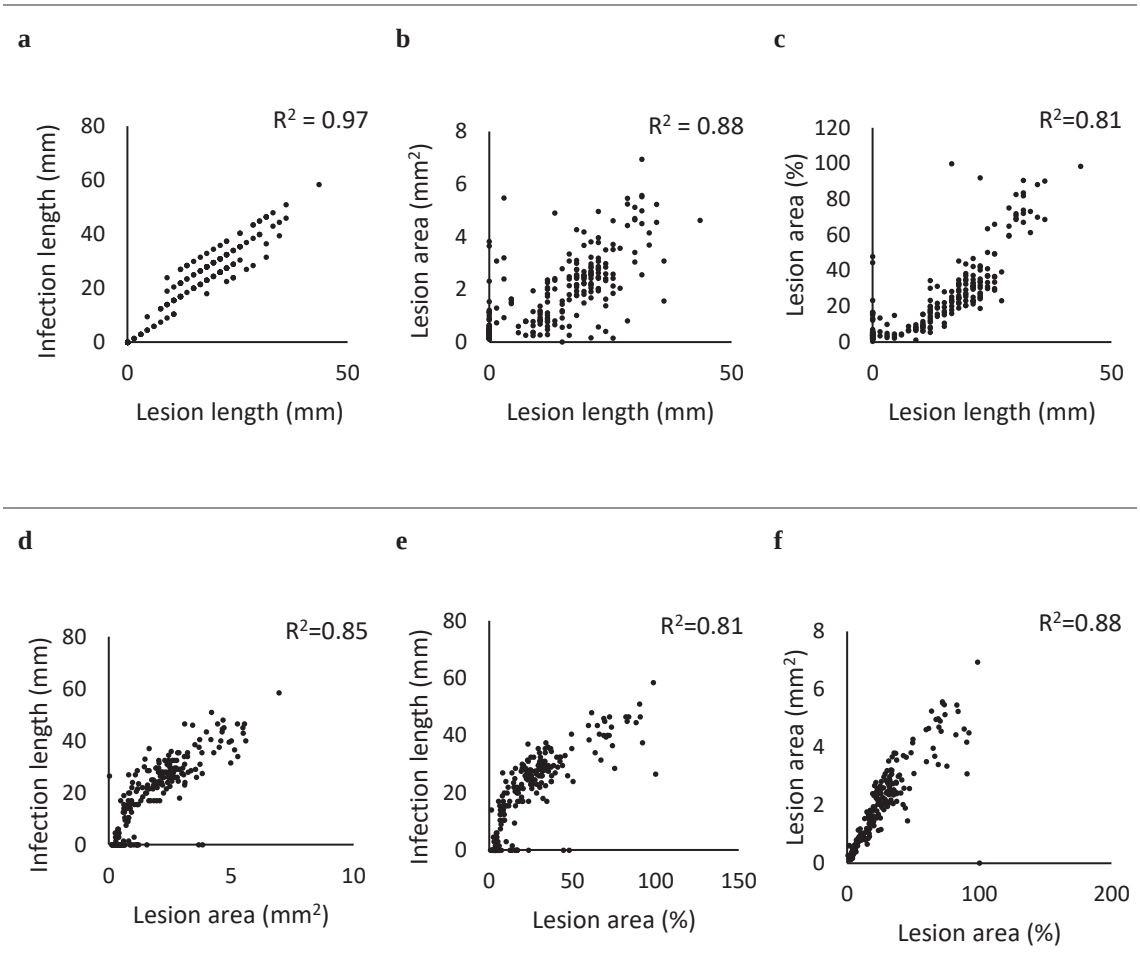

Figure 3.9. Correlation and R^2 values for lesion measurement parameters. Parameters compared were lesion length (mm) and infection length (mm) (a), lesion length (mm) and lesion area (mm^2) (b), lesion length (mm) and lesion area (%) (c), infection length (mm) and lesion area (mm^2) (d), infection length (mm) and lesion area (%) (e), and lesion area (mm2) and lesion area (%) (f).

3.4 Discussion

Across all lesion assessments, isolate 3813 produced the largest lesion and pathogen advancements for all six sapling genotypes, followed by isolate 3814 and isolate 3118. This variation may be associated with the different pathogenicity traits of the *P. agathidicida* isolates (Scott et al., 2012; Weir et al., 2015). There were no lesions for the controls except for the agar plug wound. There is not enough data to determine if the

isolates in this experiment represent the natural population of *P. agathidicida* as *P. cinnamomi*, *P. cryptogea* and *P. multivora* are also recognised as pathogens of kauri in natural stands which may play a significant role in disease development (Horner & Hough,2014). Future work, including testing a more diverse range of *Phytophthora* species associated with kauri, and incorporating more *P. agathidicida* isolates that capture the pathogen's temporal and geographic distribution is required.

Across all lesion assessments, sapling 4 produced larger measurements than any other sapling and appeared to be the most susceptible genotype to foliar inoculations. We did not however, observe any resistance that would imply that any of the other genotypes would survive infection by *P. agathidicida* under natural conditions. This could indicate a compatible response to the pathogen whereby, saplings did not detect the pathogen, or the activated defense responses are ineffective at this level of infection (Hammond-Kosack & Jones, 1996). This highlights the importance of disease tolerance in that if disease levels are low, trees can often tolerate it and survive (Manion, 1981). The limitation of this assay is that it is difficult to select seedlings with low or moderate resistance to infection. In future, it is important to choose isolates with intermediate levels of pathogenicity that can kill all susceptible genotypes, but not those with lower levels of resistance (Van Jaarsveld, Wingfield, & Drenth, 2003).

Sapling 4 had a greater proportion of immature and soft leaves than the remaining saplings, a factor which could have affected lesion size and development. Visker, Budding, Van Loon, Colon, & Struik (2003) tested different potato cultivars to determine the effects of plant age, leaf age, and leaf position on resistance against *P. infestans*. During this study, leaf position was a significant factor whereby, the resistance of a specific leaf did not change during it's lifetime (Visker et al., 2003). Leaf age also had an

effect on the susceptibility of detached leaves from Oregon trees and shrubs to *P. ramorum* (Hansen et al., 2005). In this study, leaf age correlated with leaf position and when inoculated at the highest spore concentration, immature leaves became completely necrotic compared to mature leaves which only developed tip necrosis (Hansen et al., 2005). On foliage that is young and wounded, lesion size tends to be greater on deciduous plants as confirmed by Balci et al. 2008 and Denman et al. 2005. Although, lesion size on detached leaves from evergreen plants remained the same regardless of foliar age when more aggressive *Phytophthora* isolates were used (Balci et al., 2008). Based on these previous results in other plant species, it is still unclear whether the higher susceptibility of sapling 4 is associated with a resistance response or the physiological status of the leaf tissue itself. Further work is required to determine how leaf age affects kauri response to *P. agathidicida* infection.

For one of the assessments, the control leaves of sapling 4 appeared to produce a high level of lesion area, as estimated by WinFOLIA. This is most likely an artefact of sapling 4 having green and red pigment variations that were slightly more pronounced than any other saplings and less clearly defined by WinFOLIA. During visual lesion assessment, there were no visible external lesions and hence, no recorded lesion length for the control leaves and no recovery of the pathogen from the leaf tissue. As pigment content is responsible for leaf colour and plays an important role in the physiological function of leaves (Sims & Gamon, 2002), senescing or young leaves with low photosynthetic rates tend to have a high content of anthocyanin, the red pigment (Gamon & Surfus, 1999). Similarly, leaves of plants where growth has been restricted by environmental stresses or low temperature also tend to have a high anthocyanin content (Chalker-Scott, 1999; Pietrini & Massacci, 1998). This suggests that the WinFOLIA

results for the controls may have been false positives and the software could not differentiate between necrotic lesions and the natural colour variation of kauri foliage.

Lesion length and infection length produced the strongest positive correlation, followed by disease area in mm^2 and lesion length. These relationships are expected due to the radial growth of the *Phytophthora* from the inoculation point, the dimensions of the leaf, and the growth of the pathogen along a single infection front once it hits the edges of the leaf (Anacker et al., 2008). This suggests that the lesion length and disease area assessments conducted by WinFOLIA for non-controls are accurate. Infection length produced results which showed that the pathogen can colonise the leaf up to 1 cm ahead of the lesion, suggesting that asymptomatic material cannot be assumed to be free of *P. agathidicida*. The results further suggest that this pathogen can colonise leaves.

The use of WinFOLIA is valuable in that it provided a more objective means of lesion assessment compared to visual assessment however, optimisation of the colour library is necessary. Scott et al. (2016) used WinFOLIA to analyse chlorotic and necrotic leaf area on a range of conifers and woody angiosperms exposed to foliar phosphite application. In this study, colour classes were defined as healthy, necrotic, and discoloured tissue, and each whole leaf image was tested to ensure the colour classes accurately reflected the actual damage shown in the image (Scott et al., 2016). Future work analysing lesion area on kauri leaves using WinFOLIA software will require defining and testing colour classes that can accurately differentiate between healthy, necrotic, and the natural colour variation of kauri foliage.

3.5 Conclusion

Foliar inoculations with three *P. agathidicida* isolates were performed on wounded detached leaves from six kauri saplings over 5 years of age. This experiment was conducted to determine if the assay will be useful for identifying resistance of older kauri trees to *P. agathidicida*. Using both visual and image analysis assessments, this assay demonstrated colonisation and successful infection of *P. agathidicida* in kauri leaves. The three isolates used within this study exhibited different levels of virulence within kauri tissue. Isolate 3813 appeared to be the most virulent, where as isolate 3118 appeared to be the least virulent. Variation in susceptibility was also observed among the six sapling genotypes, with sapling 4 appearing to be the most susceptible of all. Together, these results suggest that the assay is applicable to established kauri over 5 years in age however, we did not observe any resistance that would imply that these genotypes would survive infection by *P. agathidicida* under natural conditions. As *P. agathidicida* can colonise the leaf up to 1 cm ahead of the lesion, this suggests that asymptomatic material cannot be assumed to be free of the pathogen. This experiment has shown the potential application of this leaf screening assay for studying the response of kauri to *P. agathidicida* infection. Further analysis across leaf age classes, and work with root inoculations, is required to determine correlations with leaf assay results. Validating the screening procedures under natural conditions is also needed however, this could lead to ecological and ethical issues, including risk of spreading the pathogen in un-infected kauri sites.

Chapter 4

Agathis australis (kauri) foliar inoculations with three *Phytophthora* species to determine the effect of leaf age on lesion extension.

4.1 Introduction

Multiple *Phytophthora* species are known to be associated with kauri and have previously been recorded either directly from kauri or from kauri soil: *P. agathidicida*, *P. cryptogea*, *P. kernoviae* (Brasier, Beales & Kirk, 2005) and *P. nicotianae* (Breda de Haan, 1896). Other *Phytophthora* species associated with kauri include *P. cinnamomi*, which can occasionally cause disease in natural stands with poor drainage (Horner, 1984; Podger & Newhook, 1971), and *P. multivora* which has been isolated from a range of tree and shrub species including jarrah in western Australia (Scott et al., 2009). *Phytophthora multivora* has also been isolated from kauri sites during a passive surveillance programme on private land in Auckland where it was associated with sudden tree mortality (Waipara et al., 2013). *Armillaria novae-zelandiae* (Stevenson) and *A. limonea* (Stevenson) also contribute to the ill-health of kauri as either a primary or secondary pathogen in both natural and planted stands (Hood, 1989).

The relative virulence levels of *P. agathidicida*, *P. cinnamomi*, *P. multivora* and *P. cryptogea* have been tested in a recent *in vitro* pathogenicity study on detached leaves from 2-year-old kauri seedlings (Horner & Hough, 2014), as per Section 3.1. This demonstrates experimental assays using detached leaves can be useful for pathogenicity tests and as an alternative to root inoculations for *Phytophthora* resistance screening (Balci et al., 2008; Irwin et al., 2003).

Detached foliar inoculations using agar plugs colonised by a strain of *P. agathidicida* were successful at causing lesions on kauri leaves (Chapter 2). Using this inoculation method, wounded detached leaves from six kauri saplings over 5 years of age were infected with *P. agathidicida* in experiment 2, Chapter 3. In experiment 2, variation in virulence across isolates and variation in susceptibility across the six sapling genotypes was observed. Although *P. agathidicida* is naturally a root-infecting pathogen, these results suggest it can cause foliar infection. Leaf age appeared to have an impact on lesion extension in which young leaves appeared to be more susceptible than middle-aged and old leaves. As the susceptibility of foliage has been reported to differ with leaf age (Denman et al., 2005; Hansen et al., 2005), further work is required to determine the impact of leaf age on *P. agathidicida* infection and how *in vitro* genotypic variation correlates to the genotypic variation seen within kauri forests.

The main aim of this experiment was to determine how leaf age affects lesion extension using detached foliar inoculations with three *Phytophthora* species known to be associated with kauri: *P. agathidicida*, *P. multivora* and *P. cinnamomi.*

4.2 Materials and methods

Kauri seedlings

Two year old glasshouse-grown kauri seedlings were used for this experiment. The kauri were all clone number 7. Seedlings were sown at the Scion nursery on August 19th 2013. The kauri were genetically identical individuals derived from a single progenitor from Holt's Forest, as described in Section 2.2.

Isolates

Six *Phytophthora* isolates from the New Zealand Forest Research Institute culture collection, stored at Scion, Rotorua, were used for this experiment: *P. agathidicida* 3118, 3813, 3814, 3815, *P. cinnamomi* 3750 and *P. multivora* 3907 (Table 2.2, Chapter 2). Isolate *P. agathidicida* 3118 was sourced from kauri soil in the Auckland Waitakere Regional Park and isolates *P. agathidicida* 3813, 3814, and 3815 were sourced from kauri soil in the Coromandel. *Phytophthora cinnamomi* isolate 3750 was isolated from *Pinus radiata* soil and roots located in Golden Downs, Nelson. Isolate *P. multivora* 3907 was sourced from a kauri dieback survey.

Kauri seedling layout

This experiment was set up as a split plot design using one inoculation method with seven treatments; four *P. agathidicida* isolates 3118, 3813, 3814, 3815, *P. cinnamomi* isolate 3750, *P. multivora* isolate 3907, and a control non-colonised agar plug. There were 15 replicates where each replicate consisted of seven potted kauri seedlings, one for each treatment (Figure 4.1). Each replicate and the seven seedlings within each replicate were randomised using a random number generator.

Figure 4.1. Experimental layout for experiment 3. There were seven 2 year old potted *Agathis australis* (kauri) seedlings within each of the 15 replicates. Each seedling per replicate was inoculated with one of seven treatments: four *Phytophthora agathidicida* isolates 3118, 3813, 3814, 3815, one *Phytophthora cinnamomi* isolate 3750, one *Phytopthora multivora* isolate 3907, and a control comprising of a non-colonised agar plug.

Leaf age groups

Three leaf age groups were classed as either young, middle-age, or old. From each seedling, three fully-formed leaves, from each of the three leaf age groups, were harvested at the same time in May 2015. Young leaves were excised from the very tip of the current year's shoots; middle-aged leaves were excised from the middle of the current year's shoots, and old leaves were excised from the very bottom of the current year's shoots. Special attention was paid to collect similar sized leaves from similar positions of the seedling.

4.2.1 Inoculation method

Leaves were wounded and inoculated by placing CA plug cultures on the wounds, as described in Section 3.2. Inoculated leaves were exposed to diurnal light variation of 12 hours of light followed by 12 hours of darkeness for six days (Roden & Ingle, 2009). After this time, the agar plugs were removed and scanned for lesion assessments that were carried out in Section 3.2.

4.2.2 Lesion assessments

Lesion length and lesion area was conducted, as described in Section 3.2. WinFOLIA was used to measure lesion area in mm^2 and lesion area as a percentage of leaf area. The inoculation points from three replicates were plated onto CRNH media (Appendix B) to confirm Koch's postulates.

4.2.3 Statistical analysis

A Shapiro-Wilk goodness-of-fit test indicated lesion length and lesion area were not normally distributed. A non-parametrical ANOVA Proc General Linear Model (Proc GLM) was applied to compare the mean lesion length and lesion area between isolates for each leaf age group. The possibility of recovering the pathogens from young, middle-aged, and old leaf tissue was estimated using a generalised logistic linear model (Proc GLIMMIX).

4.3 Results

All *Phytophthora* species produced lesions on young, middle-aged and old leaves. Average lesion length was significantly longer in young leaves than old leaves for isolates *P. agathidicida* 3118, *P. agathidicida* 3813, *P. agathidicida* 3814 and *P. cinnamomi* 3750 (Figure 4.2). Average lesion length was slightly longer on old leaves than young and middle-aged leaves for isolates *P. agathidicida* 3815 and *P. multivora* 3907 however, there were no significant differences across the three leaf age groups for both isolates.

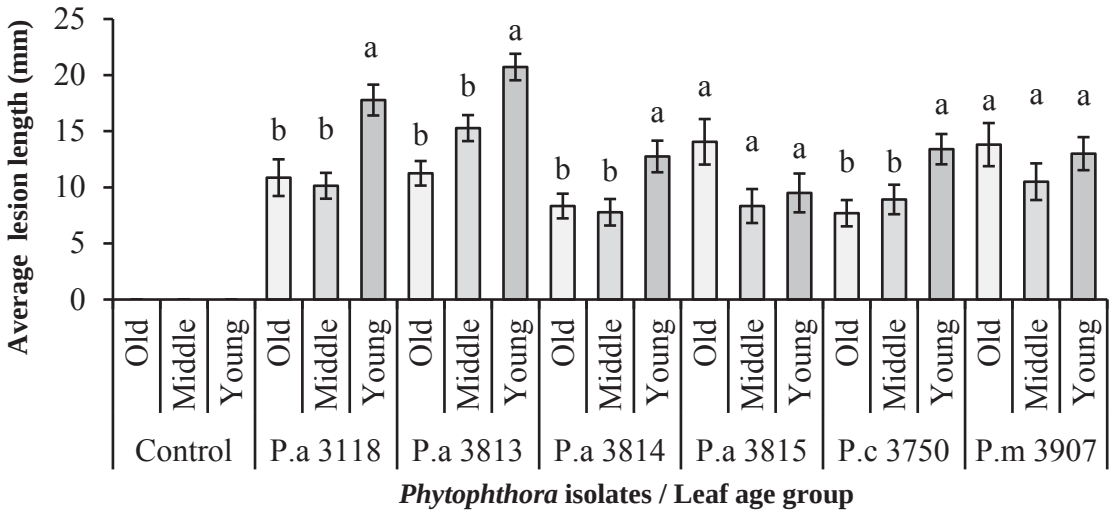

Figure 4.2. Average lesion length (mm) on young, middle-age, and old *Agathis australis* (kauri) leaves. Two-year-old kauri seedlings were inoculated with one of seven treatments: *Phytophthora agathidicida* (P.a) 3118, 3813, 3814, 3815, *Phytophthora cinnamomi* (P.c) 3750, *Phytophthota multivora* (P.m) 3907, and a control comprising of a non-colonised agar plug for 15 replicates. Columns with different letters within each isolate treatment are significantly different ($P < 0.05$). Controls had no visible external lesions.

When the average lesion area was expressed in mm^2 (Figure 4.3) or as a percentage of leaf area (Figure 4.4), all isolates, except one, showed larger lesions on younger leaves. The exception was *P. agathidicida* 3815 which showed no significant

differences across the three leaf age groups for both assessments (Table 4.1). The WinFOLIA software registered lesions on the controls for all leaf age groups.

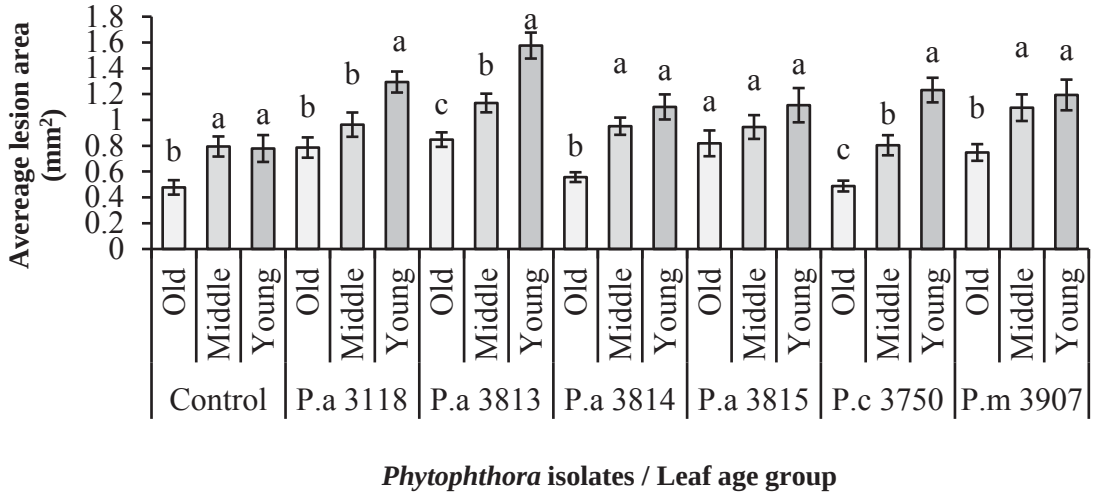

Figure 4.3. Average lesion area in mm^2 on young, middle-age, and old *Agathis australis* (kauri) leaves. Two-year-old kauri seedlings were inoculated with one of seven treatments: *Phytophthora agathidicida* (P.a) 3118, 3813, 3814, 3815, *Phytophthora cinnamomi* (P.c) 3750, *Phytophthora multivora* (P.m) 3907, and a control comprising of a non-colonised agar plug for 15 replicates. Columns with different letters within each isolate group are significantly different ($P < 0.05$).

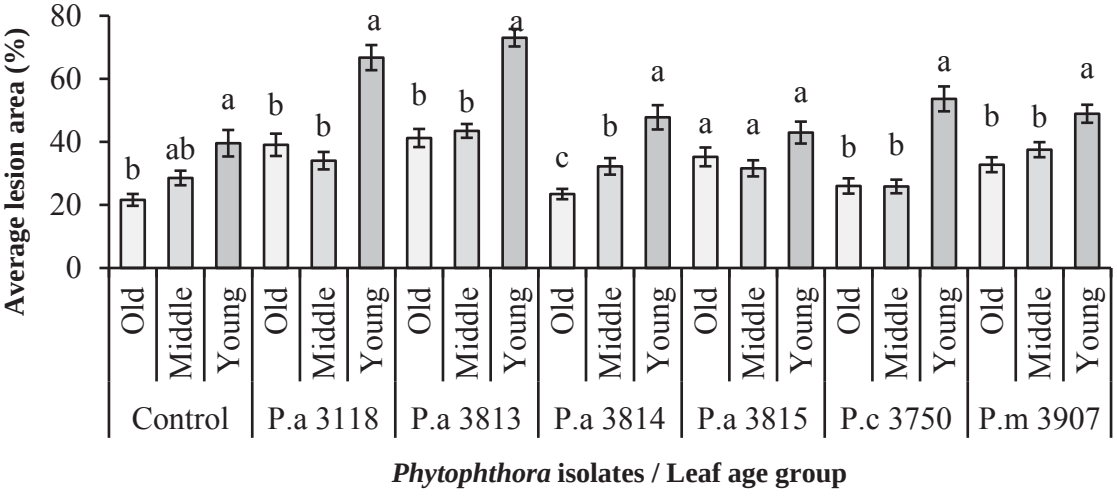

Figure 4.4. Average lesion area as a percentage of leaf area on young, middle-age, and old *Agathis australis* (kauri) leaves. Two-year-old kauri seedlings were inoculated with one of seven treatments: *Phytophthora agathidicida* (P.a) 3118, 3813, 3814, 3815, *Phytophthora cinnamomic* (P.c) 3750, *Phytophthora multivora* (P.m) 3907, and a control comprising of a non-colonised agar plug for 15 replicates. Columns with different letters within each isolate group are significantly different ($P < 0.05$).

For the possibility of recovering the pathogen from the lesion (Figure 4.5), there appeared to be differences among old, middle, and young leaves inoculated with isolate *P. agathidicida* 3815, as well as between old/middle and young leaves inoculated with isolate P. *multivora* 3907. However, the overall statistical analysis showered there were no significant differences between leaf age (all isolates) and significant differences between isolates (all leaf ages), $F_{2,132} = 0 \sim 2.85$ $P = 0.6120 \sim 1.000$.

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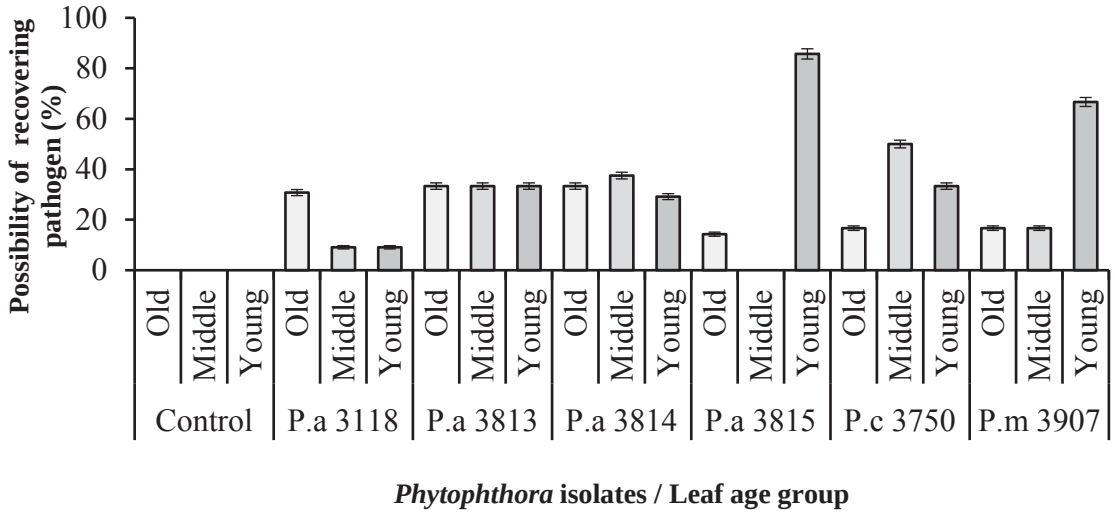


Figure 4.5. The possibility of recovering the pathogen (%) from young, middle-age, and old *Agathis australis* (kauri) leaf tissue. Two-year-old kauri seedlings were inoculated with one of seven treatments: *Phytophthora agathidicida* (P.a) 3118, 3813, 3814, 3815, *Phytophthora cinnamomi* (P.c) 3750, *Phytophthora multivora* (P.m) 3907, and a control comprising of a non-colonised agar plug. For each leaf age group, 21 isolations were made for three replicates.

A summary of F and P values for the six *Phytophthora* isolates and lesion assessments are presented in Table 4.1. A summary of data using the combined means of the six isolate treatments plus the control, and the three leaf age groups for the four lesion assessments, are presented in Table 4.2 and Table 4.3. Isolates *P. agathidicida* 3118, 3813, and *P. multivora* 3907 appeared to have a larger mean lesion length and

lesion area (%) than all other control and isolate treatments. Young leaves produced the greatest lesion measurements followed by middle-aged and old leaves respectively.

Table 4.1. A summary of F and P values using the combined means of six *Phytophthora* isolates plus a control, for three lesion assessments.

Isolates	Lesion length (mm)	Lesion area (mm^2)	Lesion area (%)
P. a 3118	F = 10.15 P < 0.05	F = 11.92 P < 0.01	F = 22.22 P < 0.01
P. a 3813	F = 17.65 P < 0.05	F = 20.94 P < 0.01	F = 40.96 P < 0.01
P. a 3814	F = 4.37 P < 0.05	F = 14.61 P < 0.01	F = 14.45 P < 0.01
P. a 3815	F = 0.1 P > 0.05	F = 1.67 P > 0.05	F = 2.74 P > 0.05
P. c 3750	F = 7.1 P < 0.05	F = 25.27 P < 0.01	F = 21.93 P < 0.01
P. m 3907	F = 2.27 P > 0.05	F = 5.44 P < 0.01	F = 9.95 P < 0.01
Control	F = 0 P > 0.05	F = 7.11 P < 0.01	F = 6.48 P < 0.01

$F_{2,132}$ and P values are of 15 replicates for seven *Agathis australis* (kauri) seedlings. Detached leaves were inoculated with one of seven treatments: *Phytophthora agathidicida* (P. a) 3118, 3813, 3814, 3815, *Phytophthora cinnamomi* (P. c) 3750, *Phytophthora multivora* (P. m) 3907, and a control comprising of a non-colonised agar plug.

Table 4.2. A summary of data using the combined mean lesion assessments of six *Phytophthora* isolates and a control.

Isolates	Lesion length (mm)	Lesion area (mm^2)	Lesion area (%)	Possibility of recovery (%)
P. a 3118	12.9 ± 1.39	1.01 ± 0.08	46.61 ± 3.43	26.0
P. a 3813	15.8 ± 1.14	1.18 ± 0.08	52.58 ± 2.61	18.0
P.a 3814	9.6 ± 1.23	0.87 ± 0.08	34.50 ± 2.70	24.0
P. a 3815	10.6 ± 1.76	0.96 ± 0.11	36.60 ± 3.00	7.0
P. c 3750	10.0 ±1.28	0.84 ± 0.07	35.17 ± 2.84	12.0
P. m 3907	12.4 ± 1.68	1.01 ± 0.10	39.73 ± 2.54	12.0
Control	3.6 ± 1.13	0.68 ± 0.08	29.91 ± 2.80	0.0

Values are the combined means ± SE of 15 replicates for seven two-year-old *Agathis australis* (kauri) seedlings. Detached leaves were inoculated with one of seven treatments: *Phytophthora agathidicida* (P. a) 3118, 3813, 3814, 3815, *Phytophthora cinnamomi* (P. c) 3750, *Phytophthora multivora* (P. m) 3907, and a control comprising of a non-colonised agar plug.

Table 4.3. A summary data using the combined mean lesion assessments of three leaf age groups.

Leaf age	Lesion length (mm)	Lesion area (mm^2)	Lesion area (%)	Possibility of recovery (%)
Old	10.0 ± 1.48	0.67 ± 0.06	31.34 ± 2.53	27.0
Middle	9.2 ± 1.27	0.94 ± 0.08	33.33 ± 2.43	32.0
Young	12.9 ± 1.36	1.18 ± 0.10	53.24 ± 3.59	40.0

Values are the combined means ± SE of 15 replicates for seven two-year-old *Agathis australis* (kauri) seedlings. Detached leaves were inoculated with one of seven treatments: *Phytophthora agathidicida* 3118, 3813, 3814, 3815, *Phytophthora cinnamomi* 3750, *Phytophthora multivora* 3907, and a control comprising of a non-colonised agar plug.

4.4 Discussion

Leaf age appeared to have an effect on lesion extension caused by three *Phytophthora* species known to be associated with kauri. Leaf necrosis was most severe with young tissue and susceptibility tended to decrease with increasing leaf age. This suggests that the physiological condition of kauri tissue associated with leaf age does influence lesion expansion. This may be due to the suberisation and lignification of leaves during aging which causes changes in leaf morphology and physiology (Vance, Kirk, & Sherwood, 1980). Young leaves tend to have soft thin cuticles that makes it easier for the pathogen to form lesions as opposed to old leaves which are hardened-off (Denman et al., 2005).

Lesion area analysis using the WinFOLIA software, showed lesion measurements for the controls (Figure 4.3, 4.4). During visual lesion assessment, there were no external lesions and hence, no recorded lesion length for the control leaves and no recovery of the pathogen from the leaf tissue across the three leaf age groups. The registered lesions from WinFOLIA may have been due to a wound response from the effects of the agar plug, causing pigment loss to the lower area of the leaves or due to differences in leaf

pigmentation across the different aged leaves (Gamon & Surfus, 1999; Sims & Gamon, 2002). Similar results were also observed in experiment 2. This highlights how variation in anthocyanin and chlorophyll leaf pigmentation can interfere with the current leaf assessments. Future research using this assay requires standardising leaf age to account for the variation in kauri leaf pigmentation.

Young leaves appeared to be more susceptible than middle-aged and old leaves. Results from this study agree with other foliar assays conducted where leaf necrosis was more severe with younger foliage, such as *P. ramorum* inoculations on a range of plant species in the United Kingdom (Denman et al., 2005; Hansen et al., 2005), and inoculations using several *Phytophthora* species associated with eastern oak species in America (Balci et al., 2008). Although leaf age had an influence on lesion extension, leaf position and plant age may also affect kauri susceptibility to *P. agathidicida* infection. Using detached leaves, Visker et al. (2003) demonstrated that leaf position was a significant factor in *Solanum tuberosum* (potato) resistance to *P. infestans* and leaf age and plant age only had minor effects. Further research requires selecting leaves of different positions i.e., apical and basal leaves, and plants of different physiology stages including seedlings, rickers between the ages of 30 – 50 years, and mature kauri with a spreading crown of leaves (Bergin & Steward, 2004), to determine if leaf age and plant age are significant factors for kauri susceptibility to *Phytophthora* infection.

All three *Phytophthora* species could colonise, spread into healthy tissue, and cause lesions on wounded leaves of different ages under controlled conditions, despite being naturally root-infecting pathogens. There appeared to be variation in virulence across the four *P. agathidicida* isolates and variation in virulence across the three *Phytophthora* species. Lesion extension (lesion length and area infected) for

P. cinnamomi and *P. multivora* were relatively lower than *P. agathidicida*, however, only one isolate for each species was used. The *P. multivora* isolate produced longer lesions than many of the *P. agathidicida* isolates. These results differ to the results of the pathogenicity study conducted by Horner & Hough (2014), in which *P. agathidicida* produced significantly greater lesion size than *P. cinnamomi* and *P. multivora*. As different *Phytophthora* isolates and host material were used, this may have resulted in the different results observed in this experiment. Whether some isolates of *P. multivora* can affect both leaves and roots of kauri is an important issue yet to be determined.

Young and middle-aged leaves produce similar lesion areas. Middle-aged and old leaves produced similar measurements as a percentage of leaf area across all treatments. This data suggests that *P. cinnamomi* and *P. multivora* produce lesions of similar sizes to those of *P. agathidicida*; however, it is possible some results may be an artefact due to the WinFOLIA measurements being affected by leaf pigmentation.

Across all lesion assessments, isolate *P. agathidicida* 3813 produced larger lesions and appeared to be the most virulent isolate of all. This supports previous results where this isolate tended to be the most virulent in experiment 2 (Chapter 3). The second most virulent appeared to be isolate *P. agathidicida* 3118, followed by isolate 3815. Isolate *P. agathidicida* 3815 did not show any significant differences in lesion length across the three leaf age groups. It did however, produce the highest percentage of recovery from young tissue but had smaller lesion measurements than the other *P. agathidicida* isolates. This may have been associated with more asymptomatic infection because of the pathogen being present forward of the external visible lesion margin. Isolate 3815 also followed the same trend that was observed with the other *Phytophthor*a species where young leaves produced the longest lesions. The least virulent

isolate appeared to be *P. agathidicida* 3814 which produced the shortest lesion length and lowest lesion area than all isolates. These results differ from experiment 2 (Chapter 3), in which isolate *P. agathidicida* 3814 appeared to more virulent than isolate *P. agathidicida* 3118. Leaf-age response to *Phytophthora* infection is important to consider during detached leaf assays to screen kauri for resistance. The differences in virulence across isolates may be mistaken for differences across treatments or genotypes however, they may in fact be caused by differences in leaf age (Visker et al., 2003).

Results from this experiment support the existing knowledge that *P. agathidicida* is an aggressive pathogen of kauri, however, some *P. agathidicida* isolates produced smaller lesions than the *P. multivora* isolates. Previous studies have demonstrated the presence of *P. agathidicida* alone in a kauri site can cause decline and tree mortality (Horner & Hough, 2014) and additional *Phytophthora* species may affect the overall health and survival of kauri which may demonstrate a synergistic attack (Horner & Hough, 2014; Waipara et al., 2013). Root inoculations using the same isolates are required to further investigate these results.

4.5 Conclusion

Phytophthora agathidicida, *P. cinnamomi*, and *P. multivora* were capable of producing lesions on wounded detached leaves from young, middle-aged, and old leaves from 2 - year-old kauri seedlings. Leaf necrosis was most severe with young leaves and foliage susceptibility tended to decrease with increasing leaf age. These results suggest that the physiological condition of kauri tissue associated with leaf age does affect lesion extension therefore, leaf-age response to *Phytophthora* infection is important to consider

during detached leaf assays for kauri resistance. Plant age and leaf position may affect kauri lesion extension and merit further investigation. Variation in virulence across the three *Phytophthora* species and across the four *P. agathidicida* isolates used within this experiment was observed. As some *P. agathidicida* isolates produced smaller lesions than the *P. multivora*, a better understanding of the variation in virulence across these isolates is required. Root inoculations using the same isolates is also needed to further validate these results.

Chapter 5

General Discussion

5.1 Overview

In forest ecosystems, fulfilment of Koch's postulates is often difficult for soil-borne root pathogens. This is because it is ecologically irresponsible and un-ethical to conduct *in situ* artificial soil infestation of non-infested healthy forest sites (Scott et al., 2009) and symptoms of decline may only be expressed a long time after the introduction of the inoculum source (Jönsson et al., 2003). For this reason, the development of an assay to screen kauri for resistance to *P. agathidicida* was conducted *in vitro,* using detached leaves from kauri seedlings propagated from tissue culture material (Gough et al., 2012).

This research has shown the potential application of an *in vitro* leaf assay to screen kauri for resistance to *P. agathidicida*. In this study, utilizing agar plugs as the inoculum source proved to be an effective method to assess the aggressiveness of *Phytophthora* species and the susceptibility of foliage. Although there were less zoospores available than needed in experiment 1, Chapter 2, zoospores as the inoculum source should not be ruled out at this stage. If enough zoospores are produced in future and the inoculation method can cause severe infection (Ganley et al., 2014; Hansen et al., 2005), it is possible that *P. agathidicida* zoospores may be more of an efficient inoculum source than mycelial plugs.

The methods developed within this study, including the techniques used to measure foliar lesions, have proven to be useful for studying the response of kauri to *P. agathidicida* infection. There appeared to be limitations with WinFOLIA, in that it was

not able to differentiate between natural kauri leaf pigmentation and necrotic lesions caused by *P. agathidicida* infection. Future inoculations using this current assay will need to be conducted at the same time of year to account for the variation in anthocyanin and chlorophyll leaf pigmentation, and for leaf age to be standardised.

Although there appeared to be variation in susceptibility across the six kauri saplings inoculated with *P. agathidicida* (Chapter 3), this may have been associated with the physiological age status of the leaf tissue itself. There was no resistance observed that would imply that these genotypes would survive infection by *P. agathidicida* under natural conditions. The interpretation of artificial inoculation results with tree species, such as kauri, is always difficult. As kauri dieback is known to affect trees of all ages, the effects of disease on juvenile tissue may differ from those on mature trees. As young seedlings are often used due to their practicality, the potential differences between the susceptibility or resistance of juvenile and mature trees must be resolved.

When leaf-age response to *Phytophthora* infection was tested on 105 glasshouse kauri seedlings (Chapter 4), leaf necrosis was most severe with young leaves and susceptibility tended to decrease with increasing leaf age. This is important to consider as an effective assay will require certainty in that the resistant genotypes identified is associated with a resistance response and not the physiological status of the leaf tissue itself. Results from this study support the existing knowledge that *P. agathidicida* is an aggressive pathogen of kauri (Horner & Hough, 2014) however, some *P. agathidicida* isolates produced smaller lesions than the *P. multivora* isolates. There is not enough data to determine if the isolates represent the natural population of *P. agathidicida* or *P. multivora*.

This is the first time an *in vitro* assay has shown variation in susceptibility across kauri genotypes. Although *ex situ* results will differ to *in situ* results since host, pathogen, and environmental conditions will vary within natural conditions (Manion, 1981), the current assay has shown good promise in that it has potential to identify kauri with resistance to *P. agathidicida.*

5.2 Future research

Until we gain more knowledge on the basal aspects of kauri dieback, we cannot effectively manage and quarantine the spread of *P. agathidicida*, let alone manage infected kauri sites. Many questions relating to the biology, aetiology, distribution, epidemiology, pathogenicity, and control of *P. agathidicida* remain unanswered. Multi host-pathogen systems biology models may help long-term management of invasive pathogens in slow growing tree species (Williams, 2013). Research in plant breeding, molecular biology and analytical chemistry will continue to be conducted as part of the effort to combat kauri dieback disease. The development of an efficient resistance screening assay is an important component of kauri dieback management. Genomic studies will be highly advantageous in that all molecular data can be correlated to levels of resistance or susceptibility in the kauri host.

Continuing from this study, a series of root inoculations on the same 105 kauri seedlings used in experiment 3 (Chapter 4) have commenced to determine the correlation between root and foliar response. As it took a long time for the clonal material to generate roots, there was not enough time to analyse the results and complete the work as part of this thesis. Follow up work has also been done on 50 kauri clonal lines grown from tissue

culture at Scion, Rotorua (Appendix A). In this work, foliar inoculations following the methods in experiment 3 and 4, were conducted using one *P. agathidicida* isolate, 3118. The experiment could not be completed in the available time-frame however, the preliminary results are shown in Appendix A which suggests variation in susceptibility to *P. agathidicida* across different genotypes of kauri.

Confirming the reliability of the lesion assessments used in this assay, specifically the use of WinFOLIA is necessary. Using a larger sample size of *P. agathidicida* isolates that capture the natural temporal and geographical range of *P. agathidicida* is highly important to confirm the relative virulence of *P. agathidicida* isolates on kauri. This includes incorporating more *Phytophthora* species known to be associated with kauri and its soil. As there appeared to be an insufficient number of zoospores produced in this study, the assay may need to be conducted using a zoospore inoculum and modifying the zoospore inoculation technique, including the concentration.

As leaf age appeared to have an impact on foliage susceptibility, it is possible whole-plant age and leaf-position can also have a significant impact and will require further investigating. In hind-sight, it may be possible that detached leaves are not the most suitable plant material for a resistance screening assay, despite its practical advantages. A range of plant materials including stems, twigs, and under-bark inoculations will need to be compared in future.

Once the development of a rapid and effective screening method has been achieved, understanding the mechanisms behind the resistance identified will be required. This includes genomic studies to identify the genetic basis of resistance in kauri to *P. agathidicida*. Genetic resistance has been very useful in other plant programmes for

native species resistant to introduced pathogens both internationally (Danti, Panconesi, Di Lonardo, Della Rocca, & Raddi, 2006; Hansen, Reeser, Sutton, & Sniezko, 2012; Stukely & Crane, 1994; Wilcox et al., 1996; Wu et al., 2007) and nationally (Butcher, Stukely, & Chester, 1984; Dungey, Williams, Low, & Stovold, 2014; Wilcox, 1982). There are large opportunities to utilise heritable genetic resistance in kauri as shown by these past genetic-based studies.

Along with efforts to understand the mechanism of resistance, future work could aim to develop effective seed production and propagation methods. This would accelerate the identification and deployment of kauri resistance. For example, tissue culture methods will be beneficial in that they will allow for a high number of resistant or tolerant seedlings to be propagated. The development of DNA marker tools and a full genome sequence of kauri would also be highly advantageous as it would rapidly increase the process of identifying truly resistant kauri genotypes and provide more confidence in the genotypes chosen. This has been done for genetic resistance to fusiform rust in southern pines and white pine blister rust in white pine in USA (Sniezko, Smith, Liu, & Hamelin, 2014). Seedlings from resistance genotypes could be micro-propagated in tissue culture and then planted in kauri forest sites to validate the resistance of the clones in the field. Eventually, true kauri resistant genotypes could be screened widely from the entire national kauri population to establish resistant breeding lines, ensuring coordinated efforts across kauri lands.

5.2.1 Moving forward with Māori

The Māori world view incorporates indigenous knowledge, values and perspectives that recognise the universe as a well-balanced natural order, embellished by the diversity of life (Harmsworth & Awatere, 2013). Māori have an intricate and holistic relationship with the natural world and all its resources, interconnected and closely intertwined by their ancestral linkage. All things existing in this whānau (family) network are dependent upon each other and have a mauri (life-form) that is linked by whakapapa (genealogy) and sustained through the act of kaitiakitanga (guardianship) (Kawharu, 2000). There is no dichotomy between humans and the natural world. Through mātauranga Māori (traditional knowledge) that has been developed over thousands of years, Māori seek to understand this entire system as one complete whole (Harmsworth & Awatere, 2013). When one part of this network is harmed or significantly affected, this in return harms the cultural and spiritual well-being of the entire lineage.

Tāngata whenua (people of the land) inherited obligations and responsibilities to ensure New Zealand's forest, freshwater, wetland, coastal, and marine ecosystems are managed and sustained for future generations to come (Marr, Hodge, & White, 2001). For many years, science and conductors of research have not respected and fully understood the holistic relationship Māori have with their taonga species, along with the mātauranga, customs and practices that govern how they see ecosystems and their ecosystem services related to that of native flora and fauna (Roberts, Waerete, Minhinnick, Wihongi, & Kirkwood, 1995). A prime example is the management of one of New Zealand's most endangered indigenous taonga plant species, the kowhai ngutukaka (Shaw, 1993).

If Māori cannot exercise their responsibilities as kaitiaki, their inner well-being and cultural identity is lost. The Treaty of Waitangi stands out as a powerful voice for Māori which guarantees chieftainship over their lands, villages, and all their treasures (Love, 2003). The Wai 262 claim seeks protection of these rights, both legally and culturally (Solomon, 2005).

Moving forward with Māori to achieve better outcomes for kauri dieback management will require establishing and implementing better frameworks that consider the different worldviews of Māori and Europeans, and the processes Tāngata whenua operate under. For example, the Te Aroturuki Process is a framework that has been designed to facilitate effective dialogue between scientists and Māori on the topic of kauri dieback management (Wilcox et al., 2008). A cross-cultural dialogue models such as this, will help achieve mutually beneficial outcomes for both iwi and scientists.

There also needs to be an appropriate ethical framework defined upfront for ongoing work in genetics. Although there have been disagreements that kaitiakitanga and legal ownership are synonymous concepts, a framework for moving forward with the Wai 262 claim has been outlined (Waitangi Tribunal, 2011). This framework has largely been ignored by the Government and their departments and institutes which has given rise to further grievances. These grievances will continue to underpin the nature and outcomes of kauri dieback management until they have been solved.

Meaningful and extensive iwi consultation, especially when regarding the use of genetic variation and genomic technologies is required. Tampering with the genetic makeup of native flora and fauna means tampering with the natural whakapapa of Māori (Greensill, 1999). Therefore, strong iwi participation is required at all stages, prioritising

their early involvement in the response to kauri dieback. The local knowledge and expertise Tāngata whenua have with kauri could feed into a larger respiratory of information among collaborators. Collectively, this could provide an opportunity to work together and form a pathway for two-way knowledge exchange, an approach that has been considered with the management of myrtle rust (Teulon et al., 2015).

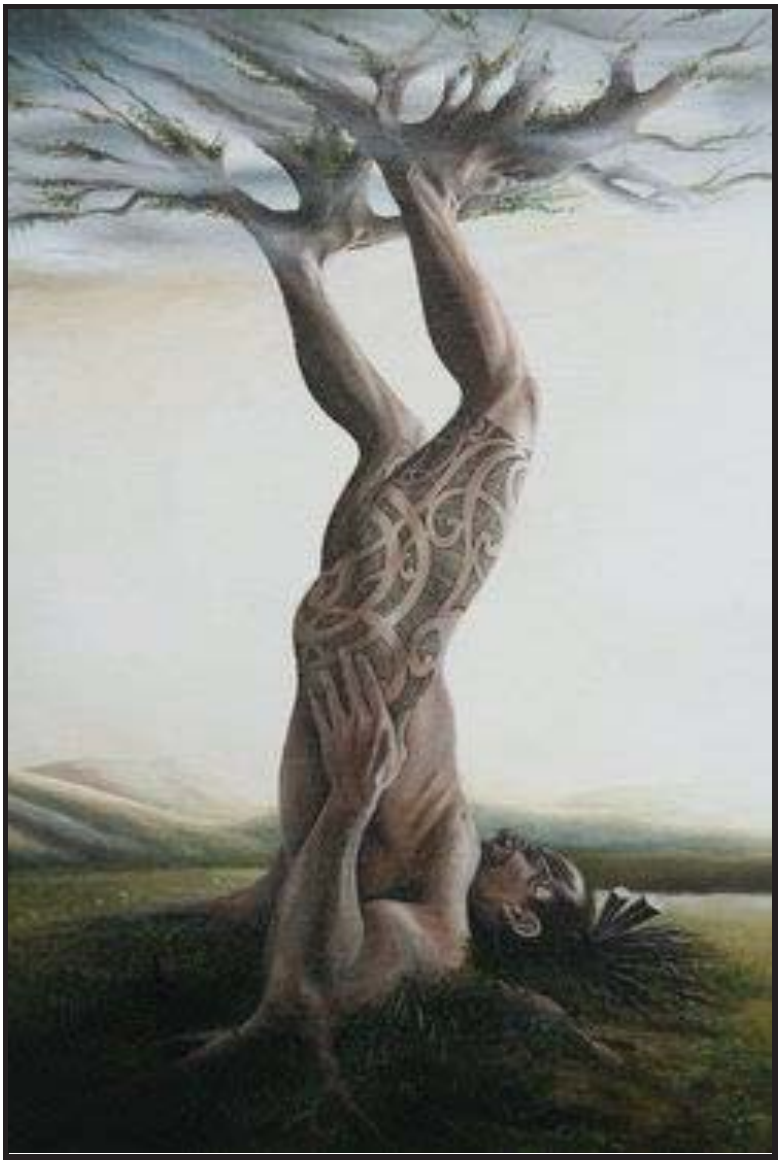
The management of kauri dieback will require continuous ethical, moral, cultural, and spiritual considerations. Moving forward with Māori is a long-term endeavour, with many learning curves to be had. Science only offers tools to complement established forest management and knowledge. As the Wai 262 claim has yet to be settled, this is a chance for the Government to settle past grievances, providing opportunities of economic development for iwi. Until then, conflict in the management of New Zealand's native flora and fauna, including that of kauri, will continue to be inevitable.

5.3 Final conclusion

This is the first time an *in vitro* screening assay has been developed for identifying kauri resistance to *P. agathidicida*. Despite *P. agathidicida* being a soil-borne root pathogen in kauri ecosystems, the development of this screening assay has demonstrated successful inoculation of detached kauri leaves, allowing for the variation in kauri susceptibility and virulence of *P. agathidicida* to be determined. This study has increased our understanding of the impact of leaf age on kauri susceptibility and on the overall response of kauri to *P. agathidicida* foliar inoculations. Although the current screening assay is only at a very early stage in the development process, it is beginning to show potential.

Urgent work is now required to determine if the lesions obtained from foliar inoculations are representative of those from root inoculations. If there is an apparent association, this foliar screening assay may be a meaningful screening tool for enabling informed management of kauri dieback disease. Further work is also required to understand the most basal aspects of kauri dieback disease, and to determine what characteristics influence the pathogenicity of *P. agathidicida* and the range of impacts on kauri ecosystems. Finding true kauri resistance will aid in the development of restoring infested kauri sites and protecting high-risk incursion areas.

Kauri dieback management provides opportunities for achieving conservation, cultural, heritage, environment, education, and production outcomes. Although this highlights the ultimate question of who holds the power to decide the management of kauri dieback, the most significant aspect of common ground between researchers of science and Māori is that we all genuinely care about the conservation of kauri and its long-term survival.



Tāne-Mahuta – Lord of the Forest (Crisp, 2007).

“Ka mau tonu ngā taonga tapu o ngā matua tupuna
Koinei ngā taonga i tuku iho, na te ātua”

“*Hold fast to the treasures of the ancestors*
For they are the treasures that have been handed down to us by God”

Appendix A.

Foliar inoculations on 50 *Agathis australis* (kauri) clones with *Phytophthora agathidicida*

Expanding on from the previous experiments conducted in this study, the screening assay was further up-scaled using 50 different kauri clonal lines grown from tissue culture. The main aim of this experiment was to determine if there was any variation in susceptibility or resistance among the 50 clonal lines. These are preliminary results (Figure A1) as the experiment could not be completed in the available time-frame, but are included here as they suggest variation in susceptibility to *P. agathidicida* across different genotypes of kauri.

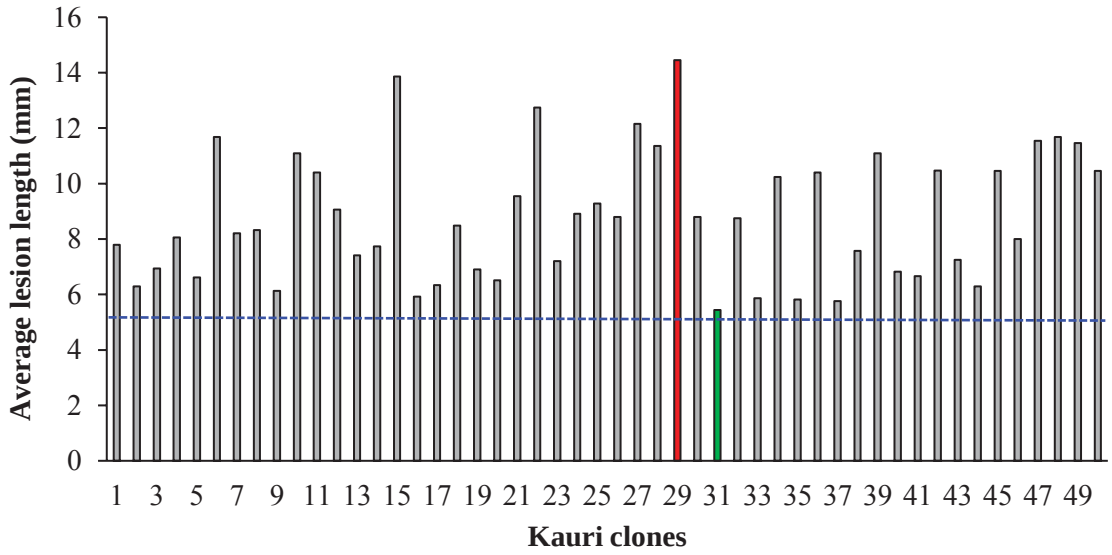


Figure A1. Preliminary results showing average lesion length (mm) for a subset of *Agathis australis* (kauri) leaves from 50 different clonal lines infected with *Phytophthora agathidicida*, isolate 3118. Wounded detached foliar inoculations were conducted using the inoculation and lesion assessment methods outlined in chapters 3 and 4. There were three leaves per seedling and 19 seedlings per clone. Of the 57 leaves per clone, 11 leaves were measured and the results shown here. The same number of leaves were used for un-inoculated controls. Lesion length for the un-inoculated control treatments 5 mm from the agar plug. Blue line is the base line from the 5mm agar plug, red bar is the largest lesion length (clone 29), and the green bar is the smallest lesion length (clone 31) out of the 50 clonal lines.

Although Figure A1 only shows preliminary results, there appears to be differences in average lesion length across the clones. Clone 29 produced the largest lesion length of 14 mm compared to clone 31, which produced the smallest lesion length of 5 mm. This variation in lesion length was typical across the other clonal lines. This suggests that there is variation in susceptibility and disease tolerance to *P. agathidicida* infection across these 50 clonal lines. Isolate 3118 was chosen for this experiment as it appeared to be the second most virulent isolate out of isolates 3813 and 3814, which were used in experiments 3 and 4. Choosing isolate 3118 meant it had intermediate levels of pathogenicity that could possibly kill susceptible genotypes, but not those with lower levels of resistance (Van Jaarsveld et al., 2003).

Further analysis, including measuring lesion length for the remaining subset of leaves and conducting WinFOLIA assessments of necrotic lesions is still required to establish the differences in disease severity across these clones. Root inoculations have commenced on the same 50 kauri clones at Scion, Rotorua however, assessments of the roots are still needed to establish if there is any apparent association between foliar and root response to *P. agathidicida.* The current preliminary results show good promise.

Appendix B.

Preparation of culture media

1. 10% Carrot agar (CA) (Dick et al., 2006)

Frozen carrots	200 g
Agar	15 g
Deionised water	1000 ml

Method:

Blend carrots with approximately 200 ml of deionised water. Filter through several layers of cheese cloth. Dissolve agar in 500 ml of deionised water. Combine filtered juice and dissolved agar and make up to volume. Autoclave using Media Cycle at a temperature of 121°C (15 psi) for 15 minutes. Once the bottle containing media is cool enough to hold, pour the plates and store in sealed containers.

2. CRNH agar (Dick et al., 2006, Jeffers & Martin 1986)

Media name:10% Carrot Agar + Ampicillin, Nystatin, Pimaricin, Rifampicin & Hymexazol

Frozen carrots	100 g
Bacto agar	15 g
Deionised water	1000 ml
Ampicillin stock 25/mg/ml	8 ml (from freezer to thaw)
Pimaricin (remove from fridge)	0.4 ml
Hymexazol stock solution 5mg/ml	10 ml (remove from fridge)
Nystatin	0.05 g (dissolved in 1-2 ml 90% ethanol in small beaker, cover with tin foil and stand).
Rifampicin	0.01 g (dissolved in 1-2 ml acetone in small beaker, cover with tinfoil and stand).

Method:

Blend carrots with approximately 200 ml deionised water. Filter through several layers of cheese cloth. Dissolve agar in 500 ml deionised water. Combine filtered juice and dissolved agar make up to volume. Autoclave using Media Cycle at a temperature of 121°C (15 psi) for 15 minutes. Once the bottle containing the media is cool to touch, add ampicillin and hymexazol stocks, pimaricin, dissolved nystatin and rifampicin solutions. Ensure all solutions are mixed thoroughly with the media. Pour and label the plates. Store in sealed containers.

3. PRPH agar (Jeffers & Martin, 1986)

Media name: ParpH Agar

Cornmeal agar	17 g
Deionised water	100 ml
Ampicillin stock solution 25 mg/ml	10 ml (from freezer thaw)
PCNB stock	5.0 ml
Rifampicin	0.01 g (dissolved in 1-2 ml acetone in small beaker, cover with tin foil and stand).
Pimaricin	0.4 ml (remove from fridge)
Hymexazol stock solution 5mg/ml	5.0 ml (remove from fridge)

Method:

Remove frozen stocks from the freezer to thaw. Dissolve agar in 500 ml deionised water. Dissolve rifampicin in 1-2 ml acetone in a small beaker, cover with tin foil and stand in laminar flow. Mix cornmeal agar and distilled water and dissolve. Autoclave for 15 minutes. Once the media bottle is cool to the touch add ampicillin, PNCB and hymexazol

stocks, pimaricin and the rifampicin solution. Ensure the stock solution is mixed with the media. Pour and label the plates. Store in sealed containers.

4. V10 agar (Černý, Tomšovský, Mrázková, & Strnadová, 2011)

Media name: 10% V8 Agar

Campbells V8 juice	100 ml
$CaCO_3$ (calcium carbonate)	1 g
Deionised water	900 ml
Agar	17 g

Method:

Mix the V8 juice and $CaCO_3$ with about 500 ml of deionised water using magnetic stirrer for 5 minutes. Filter using GFA filter paper using vacuum flask and funnel. Change filters as necessary. Make supernatant up to 1000 ml. Check pH level and adjust if necessary to pH 6. Add agar and dissolve. Autoclave using Media Cycle at a temperature of 121°C (15 psi) for 15 minutes. Once the bottle containing the media is cool enough to hold, pour the plates. Label the plates and store in sealed containers.

Appendix C.

Preparation for *Phytophthora* zoospore production

1. Sterile pond water (SPW)

One litre of pond water from Scion's garden pond (latitude: -38.161744, longtitude: 176.262842) was collected in a 2 litre schott bottle, sterilised in the autoclave at 121 °C (15 psi) for 15 minutes, and cooled to room temperature.

2. Non-sterile pond water (NSPW)

As per the sterile pond water method, except no sterilising required.

3. Non-sterile soil extract

Garden soil	200 g
Deionised water	1 litre

Ingredients were stirred vigorously for 2 minutes, then stirred vigorously again 30 minutes later and allowed to stand overnight. The solution was filtered through paper hand towels, bottled and stored in the refrigerator.

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