Characterising the growth response and pathogenicity of *Phytophthora agathidicida* in soils from contrasting land-uses

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The genus *Phytophthora* (Oomycetes, Peronosporales, Pythiaceae) is responsible for several forest declines worldwide (i.e. jarrah dieback in Australia (*P. cinnamomi*) and sudden oak death in California and Europe (*P. ramorum*)). The recently described pathogen, *P. agathidicida,* is the causal agent of dieback in remnant stands of New Zealand kauri (*Agathis australis*), and poses a significant threat to the long-term survival of this iconic species. However, what is least understood are how key physicochemical parameters (e.g. soil pH and soil organic matter) influence growth and pathogenicity of *P. agathidicida.*

This study examined the effects of three contrasting land-uses (kauri forest, grazed pasture, short-rotation pine plantation (*Pinus radiata*) on the growth and pathogenicity of *P. agathidicida* in soils sampled from Waipoua Forest, Northland, New Zealand. This was investigated usin: 1) Growth response assay, 2) Pathogenicity trait study with blue lupin (*Lupinus angustifolius*), and 3) Pasture and pine alternative host infection study. Experiment 1 found that significantly greater sporangia ($p < 0.001$) and oospore ($p < 0.01$) counts occurred within pasture and pine soils compared to kauri soils, suggesting that they favour *P. agathidicida* in the early stages of establishment. Additionally, significant increases in oospores ($p < 0.01$) over time in the pine soils potentially suggest their enhanced capacity to act as pathogen reservoirs. Furthermore, two new *Phytophthora* spp. to New Zealand (*P. pini* and *P. gregata*) were identified in this study. Experiment 2 identified non-significant land-use effects on pathogenicity traits (e.g. lesion presence, lesion length etc.) of *P. agathidicida* inoculation of blue lupin. Experiment 3 also confirmed the potential for pasture and pine to act as reservoirs for *P. agathidicida*. Overall, the findings of this study revealed that contrasting land-use affects the growth of *P. agathidicida* in soil, and further detailed study of the activity, distribution and pathogenicity of *P. agathidicida* in fragmented landscapes is warranted.

Keywords: *Phytophthora agathidicida*, Waipoua Forest, Kauri (*Agathis australis*), land-use impact, growth response, pathogenicity, alternative hosts

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Chapter 1:
Literature review

1.1 The history and current conservation status of New Zealand Kauri (*Agathis australis*)

Araucariaceae is an ancient, southern conifer plant family that was widespread globally in the Jurassic Period approximately 60 – 200 million years ago (Setoguchi *et al*., 1998), and remains part of modern forest ecosystems of Australia, South America, Melanesia, Malesia, Norfolk Island, and New Zealand (Padamsee *et al*., 2016). *Agathis australis* (kauri; Araucariaceae) is the only *Agathis* species endemic to New Zealand (Ecroyd, 1982). Kauri is also known to be one of the world's largest and longest-living conifer species, with individuals attaining trunk diameters of up to 5+m (Ecroyd, 1982), and lifespans ranging between 600 to 1700 years (Ahmed and Ogden, 1987; Wyse *et al*., 2014). The northern distribution of NZ kauri extends beyond 38°07'S towards the uppermost reaches of the North Island, where only vestiges of natural forest remain (Ecroyd, 1982). At the time of European settlement in the mid-19th century, it was reported that kauri forests once dominated more than 1,000,000 ha throughout this region (Ahmed and Ogden, 1987). Currently it is estimated that approximately 68,000 ha of regenerating and virgin kauri forest remain due to extensive deforestation in the 19th and early 20th centuries (Enright *et al*., 1999; Steward and Beveridge, 2010). This has been further exacerbated through pasture farmland and pine plantation conversions, which alongside natural fire events, have been major factors in the fragmentation and concentration of indigenous kauri forests on steep, less productive and mountainous terrain, over the last 150 years (Enright *et al*., 1999; Kauri National Park Proposal, 2011; Young and Mitchell, 1994). Despite this, the New Zealand kauri is still recognised as an iconic species of significance to all New Zealanders, and as a culturally significant taonga (treasured) species to Māori (Waipara *et al*., 2013). This is exemplified by the names given to the largest individuals, such as Tane Mahuta ('Lord of the Forest') and Te Matua Ngahere ('Father of the Forest') found in Waipoua forest, Northland; which is arguably the largest remaining mature kauri stand in New Zealand (Beever *et al*., 2009; Ecroyd, 1982).

The commercial felling of kauri trees is now prohibited (http://www.doc.govt.nz/nature/native-plants/kauri/), however these iconic trees attract up to 200,000 visitors per year to Waipoua

forest (Kauri National Park Proposal, 2011), which further illustrate their national and regional importance. Beyond the cultural and economic role of kauri, these trees are considered keystone species that drive ecosystems through creating and supporting a distinct biodiversity assemblage within kauri forests (Waipara *et al*., 2013). Mature kauri trees exert ecological effects through their considerable influence on the chemistry of the soils beneath them, altering resource distribution and consequently having a profound effect on the surrounding vegetation and invertebrate communities (Beever *et al*., 2009; Silvester and Orchard, 1999; Verkaik *et al*., 2006; Wyse *et al*., 2014). Given their diminished population and fragmented distribution throughout northern New Zealand, threats to the survival of kauri trees require thorough investigation.

1.1.1 The role of *Phytophthora agathidicida* in kauri dieback

The genus *Phytophthora* (Oomycetes, Peronosporales, Pythiaceae) represents a group of oomycetes responsible for causing serious disease and tree decline such as fruit and heart rot of Hawaiian coconuts (*P. cocois*), jarrah dieback in Australia (*P. cinnamomi*) and sudden oak death in California and Europe (*P. ramorum*) (Gruenwald *et al.*, 2008; Kroon *et al*., 2012; Podger, 1978; Uchida *et al*., 1992; Weir *et al*., 2015). The recently classified *P. agathidicida* is the primary causal agent of kauri dieback in kauri (*A. australis*) throughout lowland stands in northern New Zealand (Beever *et al*., 2009; Gadgil, 1974; Weir *et al.*, 2015). This aggressive soil borne pathogen causes collar rot in kauri trees of all ages, through infecting roots and damaging tissues that distribute nutrients within the tree (Beever *et al.*, 2009). Several symptoms arise as a result, including the characteristic root and collar rot, resin-exuding lesions, severe chlorosis, canopy thinning and widespread tree mortality (Basset *et al*., 2017; Waipara *et al*., 2013). Given the current fragmented distribution of kauri forests today and the widespread dispersal of *P. agathidicida* throughout major kauri stands (e.g. Waitakare Ranges and Waipoua forest) in northern New Zealand, this pathogen poses a significant threat to the long-term survival of this iconic tree species (Beever *et al*., 2009; Waipara *et al*., 2013).

This review initially covers the historical context of *Phytophthora* species, followed by a detailed description of kauri dieback's discovery, identity, distribution, symptoms and life cycle. The effect of abiotic and biotic factors on *Phytophthora* spp. is discussed next, followed by current management strategies employed to mitigate *P. agathidicida* spread.

1.2 History of the genus *Phytophthora*

Oomycetes are members of the recently classified eukaryotic super-group known as Chromalveolata (Adl *et al.*, 2005). They have long been considered fungal-like organisms due to morphological similarities such as the production of filamentous threads known as hyphae, which are characteristic structures of many fungi (Rossman and Palm, 2006). Despite this apparent similarity, physiological and genetic studies have identified that oomycetes are more closely related to members of Chromista, or more specifically the heterokont algae (Gunderson *et al.*, 1987; Rossman and Palm, 2006). Many species of this grouping are of considerable social, economic and ecological importance as they are major plant pathogens of key crops and already threatened indigenous species (Beever *et al.*, 2009; Kroon *et al.*, 2012; Podger, 1972; Tyler, 2007; Uchida *et al.*, 1992; Weir *et al.*, 2015). Of the multitude of oomycete species which can cause disease in plants, *Phytophthora* (Peronosporales) is likely to be the most recognisable due to the identification of *P. infestans* as the causal agent of the Irish potato famine in the 1840s (De Bary, 1876; Kinealy, 1994; Rossman and Palm, 2006). This event had a tremendous effect on human history, resulting in significant famine and population displacement; it is estimated that one million people died of starvation, and a further one and a half million people were displaced, many of whom immigrated to New York State (De Bary, 1876; Kinealy, 1994; Nowicki *et al.*, 2012). Even in modern times, *P. infestans* remains one of the most destructive pathogens of solanaceous crops such as tomato (*Solanum lycopersicum*) and potato (*S. tuberosum*). Depending on the severity of disease, plant variety and management practices, potato crop losses worldwide caused by *P. infestans* can reach as much as $6.7 billion annually (Haas *et al.*, 2009). In the United States alone, in 2009, total losses were upwards of $3.5 billion, with approximately half attributed to *P. infestans* (http://www.nass.usda.gov/Data_and_Statistics/Quick_Stats/; Nowicki *et al.*, 2012). These figures clearly highlight the major social and economic impacts these pathogens have.

According to Ersek and and Rubeiro (2010), in the 120 years separating the studies of Heinrich Anton de Bary (1876) and the monograph '*Phytophthora* species worldwide' (Erwin and Rubeiro, 1996), over 100 species of *Phytophthora* have been described. Many of these species are able to infect a broad range of hosts in many parts of the world (Erwin and Rubeiro, 1996; Gruenwald *et al.*, 2008; Lamour *et al.*, 2012; Nowicki *et al.*, 2012). For instance, *P. capsici*, which causes root, crown and foliar rot on a variety of important vegetable crops such as *Capsicum* and *Solanum* spp., has been reported in North and South America, Asia, Africa and

Europe (Lamour *et al.*, 2012; Podger, 1972). Furthermore, over 2000 plant species in Western Australia have been described to exhibit a range of susceptibility (from susceptible to highly susceptible) to *P. cinnamomi* infection (Shearer *et al.*, 2004). In particular, the loss of the canopy layer of large trees such as *Eucalyptus marginata* (i.e. Jarrah), can have devastating effects on the biodiversity of fauna within these forest ecosystems (Garkaklis *et al.*, 2012; Podger, 1972). Therefore, the enormous social, economic and ecological impact *Phytophthora* species have on threatened and already endangered plant species necessitates a greater understanding of their epidemiology, distribution and pathogenicity, in addition to the environmental factors which influence these processes.

1.3 The pathology of *Phytophthora agathidicida* and kauri dieback

1.3.1 Discovery, identification and distribution

Collar rot of kauri was first reported by Gadgil (1974), 40 years ago on Great Barrier Island, which is approximately 100km north-east off the coast of Auckland city, and was initially attributed to *P. heveae* due to morphological similarities (Beever *et al.*, 2009). Despite the putative *P. heveae* isolate being much more pathogenic than than other *Phytophthora* spp. found in association with kauri ecosystems (e.g. *P. cinnamomi, P. cryptogea, P. kernoveae* and *P. nicotianae)*, it was not considered a threat due to its apparent isolation (Beever *et al.*, 2009; Gadgil, 1974; Horner and Hough, 2013; Waipara *et al.*, 2013). With the discovery of the kauri collar rot on the mainland in 2006, an investigation into its identity and management was initiated (Beever *et al.*, 2009; Weir *et al.*, 2015). Beever *et al.* (2009) speculated that a recent migration of the disease from Great Barrier Island to the mainland was unlikely. Instead, they suggested that patches of infected areas may have remained undetected due to symptoms (e.g. bleeding lesions) resembling a common response of kauri to injury. They also argued that this lack of recognition was exacerbated in the promotion of gum production by purposeful injury to kauri (Beever *et al.*, 2009).

Advances in DNA sequencing techniques have led to a leap forward in our understanding of the phylogenetic relationships between *Phytophthora* spp. (Blair *et al.*, 2008). Using the sequences of specific genes and/or genetic loci, species complexes can be delineated based on their similarities between isolates (Kroon *et al.*, 2012). The original identification of the causal

agent as *P. heveae* was subsequently questioned as the ITS (internal transcribed spacer) sequences were found to be identical to those in *P. castaneae* rather than *P. heveae* (Beever *et al.*, 2009). However, given that morphological characteristics were very similar to those exhibited by *P. heveae* rather than *P. castaneae*, the initial classification is not surprising (Beever *et al.*, 2009; Gadgil, 1974; Weir *et al.*, 2015). As a result, the isolate was provisionally given the interim name Phytophthora "taxon Agathis" or PTA in 2009 (Beever *et al.*, 2009). It was later officially classified as *P. agathidicida* (*Phytophthora* clade 5) in 2015 as part of a taxonomic revision based on differences in genetic sequences, morphologies and host specificities to the other members of this clade, *P. castaneae, P. heveae* and *P. cocois* (Beever *et al.*, 2009; Weir *et al*., 2015).

Although six *Phytophthora* spp. have been found in association with kauri (i.e. *P. agathidicida, P. cinnamomi, P. cryptogea, P. kernoviae, P. nicotianae* and *P. multivora*), only a couple have been linked with disease symptoms in the field (Beever *et al.*, 2009; Newhook, 1959; Waipara *et al*., 2013). For instance, *P. cinnamomi* is widespread in both mature and regenerating kauri forests, and has been linked to disease symptoms (i.e. canopy loss) and occasional tree death (Beever *et al*., 2009; Newhook, 1959). Despite these observations, *P. cinnamomi* has not been attributed as the cause of widespread dieback (Beever *et al*., 2009). In contrast, size class distribution and virulence studies suggest that *P. agathidicida* is highly pathogenic against kauri trees of all ages (Beever *et al.*, 2009; Horner and Hough, 2013; Horner *et al*., 2015; Waipara *et al*., 2013). For example, detailed examination of regenerating kauri trees in the Waitakare Range by Beever *et al*. (2009) confirmed an abundance of symptoms throughout most size classes of kauri trees attributable to *P. agathidicida* (Fig. 1.1). In addition to this, Horner and Hough (2013) have observed 100% mortality of untreated two-year old kauri seedlings within four months of *P. agathidicida* inoculation. This demonstrates not only the lack of age-class restrictions to *P. agathidicda* infection, but also the rapid mortality of young kauri seedlings, further highlighting the distinct threat this pathogen poses to the long-term survival of kauri.

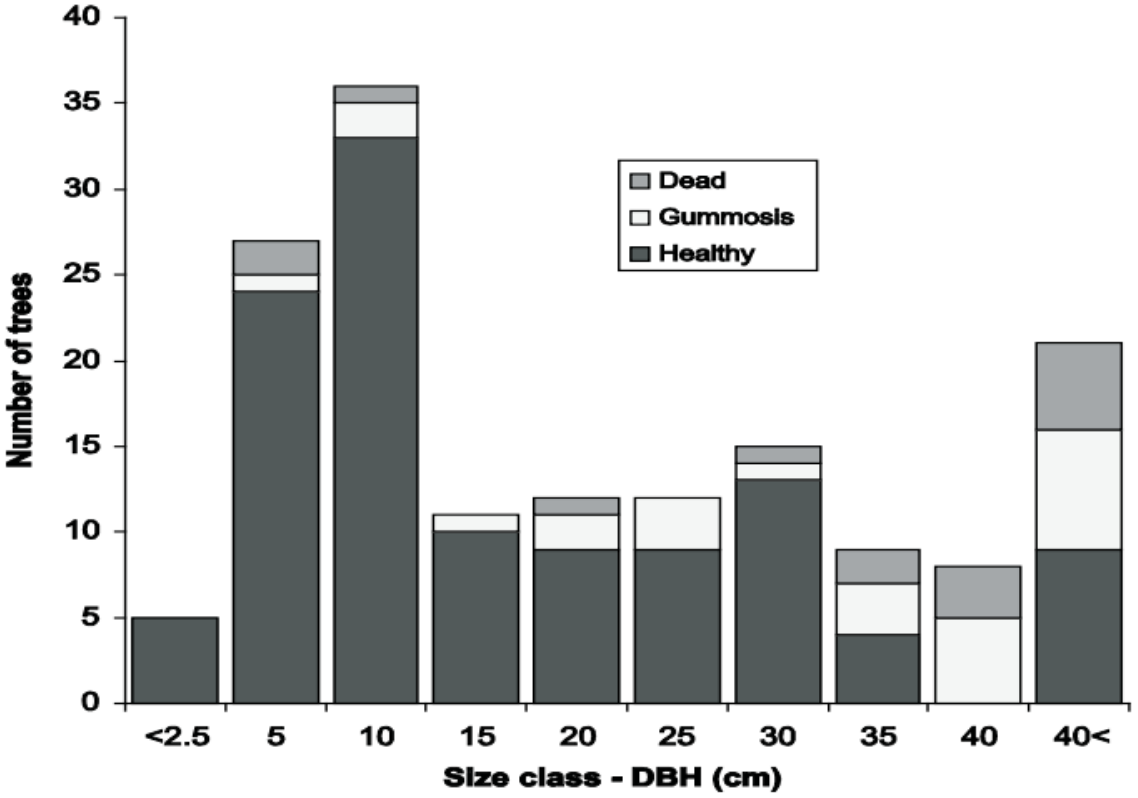


Figure 1.1 Size class distribution of *Agathis australis* (kauri) in collar rot affected forest stand in Huia, Waitakare Range. Health status recorded as: healthy, showing gummosis (lesions oozing gum) or dead. DBH = diameter at breast height (source: Beever et al., 2009).

Surveillance studies since 2006 have revealed the widespread distribution of *P. agathidicida* throughout some of the largest remaining mature kauri stands in New Zealand, including the Waitakare Ranges Regional Park (-36° 58′S, 174° 31′E) and Waipoua Forest National Park (35.6211° S, 173.5177° E) (Beever *et al*., 2009; Waipara *et al*., 2013). In addition to this the surveys identified infected regions in rural fragments, across the Rodney and Kaipara districts north of Auckland (-36.8485° S, 174.7633° E), and the last remnants of kauri on the Awhitu Peninsula (37° 16′S, 174° 40′E) (Waipara *et al*., 2013). These surveys have identified multiple pathogenic agents (e.g. *P. multivora, P. agathidicida, P. cryptogea* and *P. cinnamomi*) from symptomatic trees (Waipara *et al*., 2013). Although it is known that *P. agathidicida* results in the most severe pathogenic responses (Beever *et al.*, 2009; Horner and Hough, 2013), Waipara *et al.* (2013) notes that the discovery of multiple agents directly from infected individuals may

demonstrate synergistic attack and/or disease complexes that contribute to overall poor health of kauri at these sites, but further research is needed to elucidate these relationships. Although studies on two-year old kauri seedlings have demonstrated the high virulence of *P. agathidicida* in juvenile kauri seedlings (Horner and Hough, 2013), the question of how long the soil-borne pathogen incubates within mature kauri trees prior to the rise of symptoms, remains unanswered. The next logical step involves identifying indicator species or alternative hosts that *P. agathidicida* may infect and cause disease, to infer the pathogen's potentially more widespread distribution (Davidson *et al*., 2005). While it has been assumed that *P. agathidicida* was primarily restricted to *A. australis*, a recent study has identified several other native species found in association with kauri such as *Dracophyllum latifolium* (neinei), *D. sinclairii* (gumlands grass tree), *Astelia trinervia* (kauri grass) and *Leionema nudum* (mairehau), that display signs of infection following inoculation by *P. agathidicida in planta* (Ryder *et al*., 2016). Whether these alternative host infections reflect observations in the field remains to be confirmed, however, it would prove prudent to include these potential alternative hosts in further surveys to improve our understanding of the pathogen's distribution and dispersive potential.

1.3.2 Symptoms

As with many soil borne *Phytophthora* spp., early infection by *P. agathidicida* is via fine roots (Beever *et al*., 2010; Hardham, 2005; Nowicki *et al*., 2012). The pathogen progresses further into the plant along larger woody roots, infecting the cork cambium, although rarely invades the xylem (Beever *et al*., 2010; Bellgard *et al*., 2016). Upon reaching the trunk, *P. agathidicida* causes cankers that are characterised by the production of resin exudate, which are further associated with infection of cork cambium (Beever *et al*., 2010). Extending around the trunk, the lesion can end up girdling the tree resulting in a series of additional symptoms (Bellgard *et al*., 2013 and 2016), including the slow decline of the crown through chlorotic and necrotic symptoms developing in the canopy and ultimately tree death (Bellgard *et al*., 2013). As mentioned, the time-course between primary infection and the onset of disease symptoms in mature kauri trees is not known, although it is presumed to number in years (Bellgard *et al*., 2016). However, a recent study investigating early infection of immature kauri plants (two-year old seedlings) discovered that internal hyphal progression reached primary root structures by day 16 (Bellgard *et al*., 2016). Symptoms were expressed as desiccation of the lower leaves,

followed by constriction of the collar region by day 20, reflecting vascular dysfunction and associated shoot decline (Bellgard *et al*., 2016). In corroboration with observations by Horner and Hough (2013), these observations suggest that immature kauri seedlings display a severe and rapid onset of kauri dieback related symptoms following infection. While more mature kauri trees are also likely to be infected in sites where kauri seedlings show visible symptoms, the multi-year long infection process for these trees may potentially set up distinct size-class differences in infected sites compared to uninfected sites.

1.3.3 Life cycle

With the primary focus of kauri dieback research directed towards transmission vectors, chemical controls, detection and disease distribution maps (Basset *et al*., 2017; Beauchamp, 2013; Beever *et al.*, 2009; Horner and Hough, 2013; Krull *et al*., 2012; Lawrence *et al*., 2017; Than *et al*., 2013; Waipara *et al*., 2013), little work has focused on the role of environmental factors and their impact on the specific life cycle stages of *P. agathidicida.* As such, the best guidance comes from extrapolation off of general growth characteristics common to many *Phytophthora* species and applied to life cycle stages observed in *P. agathidicida* (Duniway, 1983; Grünwald and Flier, 2005; Hardham, 2005; Nowicki *et al*., 2012; Weir *et al*., 2015).

Compared to true fungi, the capacity for *Phytophthora* species to survive as saprophytes is limited (Judelson and Blanco, 2005). Therefore, following the decline of a colonised host, spores are required for their survival and subsequent movement to a new host (Judelson and Blanco, 2005). As with other oomycetes, *P. agathidicida* makes use of several different spores that are likely to be important in infection, disease development and survival (Beever *et al*., 2009; Weir *et al*., 2015). This includes sporangia, zoospores and oospores; chlamydospores are also recognised in some other *Phytophthora* spp., but none have been observed in previous studies of *P. agathidicida* (Weir *et al*., 2015; Weste, 1983).

Favourable environmental conditions (e.g. for *P. agathidicida* up to its optimal temperature of 21.5^{o}C and water availability) set up the potential for asexual reproduction (Fig. 1.2), where sporangia are produced from the termini of specialised hyphae called sporangiophores (Hardham, 2005; Judelson and Blanco, 2005; Weir *et al*., 2015). Sporangia are capable of germinating in two modes, either directly through production of germ tubes that penetrate and infect host tissue, or indirectly through production of motile zoospores that actively migrate

along chemotactic gradients towards host tissue and similarly produce germ tubes to initiate infection (Hardham, 2005; Judelson and Blanco, 2005; Weste, 1983). These two pathways have consistently been shown to be a function of environmental factors, notably temperature, water and host availability (Fry *et al*., 2015; Nowicki *et al*., 2012; Weste and Marks, 1987). For example, in *P. infestans*, direct germination generally occurs in moist (>75-90% relative humidity) and warm temperatures (>15^{o}C), while indirect germination occurs when temperatures are lower (<15^{o}C) (Fry *et al*., 2015; Nowicki *et al*., 2012). This dual germination strategy is advantageous as it allows *Phytophthora* species to colonise plants in diverse environments, such that motile zoospores extend the range of the pathogen beyond the resting site of sporangia (Judelson and Blanco, 2005). For example, zoospores have been observed to remain motile for several hours or even days, resulting in movement of >6 cm in water films and further in surface waters (Ristaino and Gumpertz, 2000). Therefore, this allows sporangia which are situated some distance from a plant to infect a host by motile zoospores travelling along water films suspended by stems and leaves that are in contact with soil and/or roots (Fry *et al*., 2015; Judelson and Blanco, 2005). Following this germination process, the germ tube can penetrate the host tissue and initiate infection (Fry *et al.*, 2015; Nowicki *et al*., 2012). As mentioned previously, early infection by *P. agathidicida* has been shown to occur via the fine roots, with the pathogen then progressing along larger woody roots (Beever *et al*., 2010; Bellgard *et al*., 2016). This disease progression results in the proliferation of the pathogen, both within and outside the plant root as mycelium and sporangia (Bellgard *et al*., 2016), rapidly increasing inoculum loads in soils where conditions are favourable for sporulation (Nowicki *et al*., 2012; Weste and Marks, 1987). Jung *et al*. (2013) have suggested that soil-borne *Phytophthora* pathogens (e.g. kauri dieback) have developed a distinct life cycle that is characterised by rapid periods of dissemination (i.e. motile zoospores, movement of infected plant material) onset by favourable environmental conditions, ultimately resulting in host infection. While there are distinct advantages to asexual reproduction through the extension of infection zones and rapid inoculum turnover (Fry *et al*., 2015; Judelson and Blanco, 2005; Nowicki *et al*., 2012), these features come at a price. To sense and rapidly respond to changing environmental conditions, sporangium must remain metabolically active (Judelson and Blanco, 2005). Demonstrated in *P. infestans*, studies have revealed that this depletes nutrient reserves and increases mortality risk of sporangia through desiccation and solar radiation, with viability in field conditions rarely exceeding several days (Mizubuti *et al*., 2000; Warren and Coulhoun, 1975). Although contradictory data exists, it is suggested that sexual reproduction and the development of oospores occurs under relatively dry conditions, where asexual spore types are

produced less abundantly (Duniway, 1983). As a homothallic *Phytophthora* species, sexual reproduction in *P. agathidicda* occurs with both male and female gametangia on a single isolate without the requirement of an alternate mating type (Beever *et al*., 2009; Weir *et al*., 2015). These gametangia fuse to form oospores (Fig. 1.1), where they subsequently germinate under warm, moist conditions to produce either a germ tube for direct infection, or a germ sporangium (Hardham, 2005). These thick-walled spores allow viable inoculum to survive across seasons and withstand desiccated environments detrimental to the survival of sporangia (Hardham, 2005; Judelson and Blanco, 2005; Waipara *et al*., 2013; Weir *et al*., 2015). For example, there have been reports of *P. cinnamomi* oospores remaining viable in moist soils for up to 6 years (Hardham, 2005), and *P. fragariae* oospores surviving for at least 3 years (Duncan, 1980). This exemplifies the importance of oospores as survival structures in the genus *Phytophthora*. Furthermore, with some indication that the currently utilised disinfectant trigene is ineffective against *P. agathidicida* oospores, it places further emphasis on the potentially central role of oospores in the dissemination of kauri dieback (Bellgard *et al*., 2010).

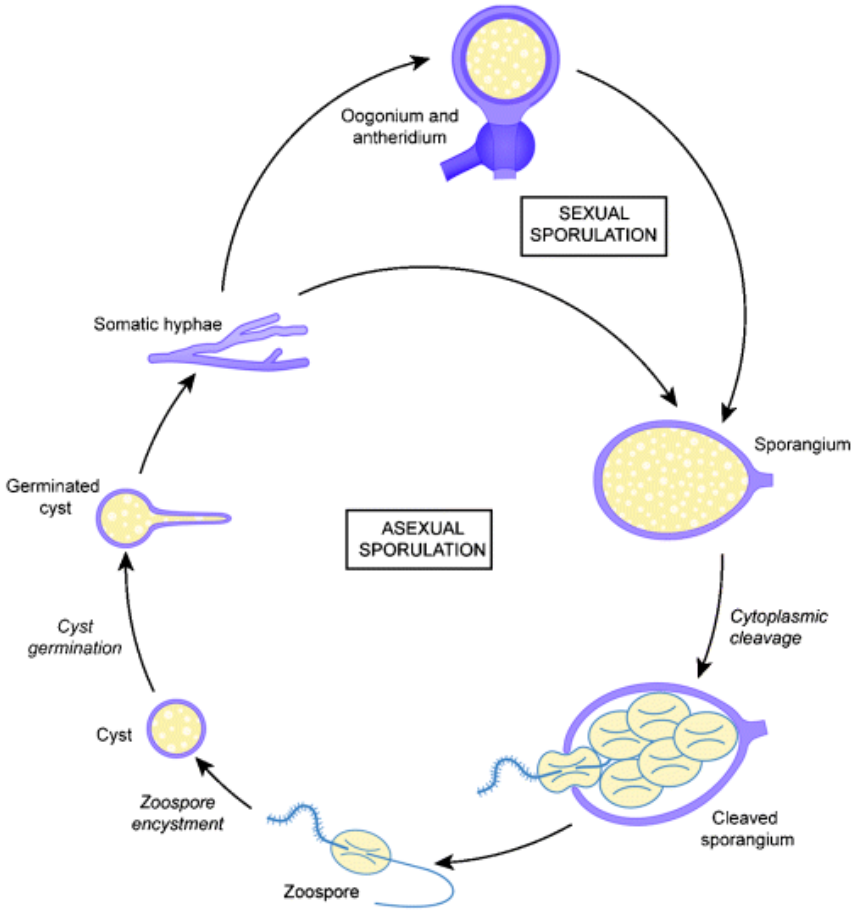


Figure 1.2 General life cycle of a *Phytophthora* species (source: Hardham, 2005).

1.4 Effect of abiotic and biotic factors on *Phytophthora*

A key aspect of plant disease epidemiology is understanding how host-pathogen interactions are formed and mediated by heterogeneous landscapes (Anacker *et al*., 2008; Shearer *et al*., 2010). With landscape impacts on disease incidence demonstrated for several *Phytophthora* species (Jung *et al.,* 2000 and 2013; Ristaino *et al*., 1993; Shearer and Dillon 1996; Shearer *et al*., 2010), understanding epidemic development in these environments is fundamental in achieving integrated and sustainable methods of long-term management for diseases caused by these pathogens (Benson *et al*., 2006; Ristaino and Gumpertz, 2000, Shearer *et al*., 2010). This has been established in characterising sources of inoculum (Benson *et al*., 2006), mechanisms of dispersal (Ristaino *et al*., 1993), and environmental influences (Anacker *et al*., 2008) in several prominent *Phytophthora* species such as *P. cinnamomi*, *P. capsici* and *P. ramorum*. In general, patterns of pathogen distribution or disease incidence arise from the complex interaction of physical (e.g. temperature and water availability), chemical (e.g. nutrient availability and pH) and biological (e.g. microbial antagonism) factors specific to landscapes or regions, with respect to the biological and ecological characteristics of the pathogen (Erwin *et al*., 1983; Ristaino and Gumpertz, 2000).

1.4.1 Abiotic factors

Physical environmental conditions such as water availability and temperature influence spore development (e.g. sporangia and oospores) in *Phytophthora* species (Duniway, 1983; Hardham, 2005; Judelson and Blanco, 2005). As described previously, the production of sporangia and oospores, and their subsequent germination are heavily dependent on these physical parameters (Duniway, 1983; Judelson and Blanco, 2005). For example, in *P. infestans* these factors impact the sporangia germination pathways; under wet conditions direct germination of asexual sporangia typically occurs in warmer temperatures (>18^{o}C), while indirect germination occurs at lower temperatures (<15^{o}C) (Fry *et al*., Nowicki *et al*., 2012).

As such, the constraints imposed by physical conditions largely determine the geographic distribution of *Phytophthora* species and their associated diseases, holding contributing chemical and biological factors constant (Duniway, 1983; Hardham, 2005; Judelson and Blanco, 2005). For example, *P. cinnamomi* poses little danger in field conditions where temperatures consistently fall below freezing, where it is unfavourable for spore production

and germination (Zentmyer, 1980). In contrast, within the Mediterranean climates of Australia where soil moisture and temperature are highly conducive for *P. cinnamomi* sporulation and survival (Duniway, 1983; Zentmyer, 1980), it has become a highly destructive pathogen for a wide range of hosts (Hardham, 2005), and in particular the jarrah forests (*Eucalyptus marginata*) of Western Australia (Zentmyer, 1980).

Soil pH is known to be a major factor that influences microbial diversity and abundance (Fierer and Jackson, 2006). In the case of *Phytophthora* spp., a study investigated the response of *P. infestans*, the causal agent of potato blight, to low soil pH (<4 pH) conditions, and observed significantly lower sporangia germination rates, which were further associated with decreased disease potential (Andrivon, 1994b). The authors suggested that this could be due to indirect effects of low soil pH resulting in the release of toxic ions or the favouring of acidophilic, lytic microorganisms that outcompete the slower growing *Phytophthora* species (Andrivon, 1994b; Judelson and Blanco, 2005; Jung *et al*., 2013). A large-scale study further demonstrated the effects of soil pH on soil-borne *Phytophthora* species in a study that investigated Central European oak (*Quercus robur*) decline and the effect of site factors on disease incidence (Jung *et al*., 2000). The authors failed to isolate any *Phytophthora* species from stands with sandy to sandy-loam soils and a mean soil-pH of <3.9 containing little oak decline. In contrast, oak stands located on sandy-loam to clay sites with a mean soil-pH >3.5 displayed significant oak decline (Jung *et al*., 2000). They hypothesised that while soil-pH likely plays a role in inhibiting sporangia/zoospore germination, the structure of soil particulates influenced the free-water period, even after heavy rainfall. In sandy soils, rapid drainage may have resulted in too short a period for sufficient moisture build-up to allow for sporulation and the release of zoospores, even at suboptimal pH values (Jung *et al.*, 2000). This illustrates that while chemical factors impact *Phytophthora* germination and/or disease incidence, the interaction with physical properties of the soil complicate the situation. This effect of low pH on germination rates is corroborated in studies performed in other prominent *Phytophthora* species such as *P. cinnamomi* (Bingham and Zentmyer, 1954) and *P. nicotianae* (McCarter, 1965), suggesting chemical characteristics of different land-uses may have an impact on germination rates of important *Phytophthora* species. However, it is necessary to note that while these physical factors may represent hard boundaries that restrict the distribution and hence the pathogenic activities of one *Phytophthora* species, they do not necessarily affect other species in the same manner (Duniway, 1983). For example, the cold climate that restricts *P. cinnamomi* distribution and pathogenicity has little impact on the activity of relatively low-temperature oomycetes such

as *P. syringae*, *P. cactorum* and *P. lateralis* (Erwin and Ribeiro, 1996). In many cases, this sets up the potential for overlap in pathogen distributions, where multiple *Phytophthora* species may be found alongside another. This is demonstrated aptly by the recovery of six *Phytophthora* species (*P. multivora, P. cryptogea, P. kernoviae, P. cinnamomi, P. nicotianae* and *P. agathidicida*) from tissue retrieved from infected kauri and/or kauri forest soil (Beever *et al*., 2009; Waipara *et al*., 2013; Weir *et al*., 2015). This has been similarly observed internationally with the recovery of eleven *Phytophthora* spp. (i.e. *P. cambivora, P. quercina, P. cinnamomi*) when characterising their diversity and association with oak decline in Italy (Vettraino *et al*., 2002).

1.4.2 Biotic factors

In terrestrial ecosystems, the rhizosphere represents the narrow zone of soil surrounding plant roots that is characterised by high microbial activity due to the availability of nutrients such as carbon, nitrogen and phosphorous (Berg and Smalla, 2009; Singh *et al*., 2004). As such, it represents a highly dynamic interface that consequently affects the biogeography and community structure of indigenous microbial populations, including *Phytophthora* species (Berg and Smalla, 2009; Drenovsky *et al*., 2004; Fierer and Jackson, 2006; Macinnis-Ng and Schwendenmann, 2015).

The composition and abundance of microbes in the soil affects the ability of plants to access nutrients (Berg and Smalla, 2009). Conversely, soil microbes are dependent on plants for carbon substrates for growth, which are commonly released as root exudates in the rhizosphere (Broeckling *et al*., 2008). These exudates are known to contain primary metabolites such as sugars and amino acids, in addition to a diverse array of secondary metabolites (Broeckling *et al*., 2008). The role of root exudates as a major driver in microbial community structure has been explored previously (Berg and and Smalla, 2009; Broeckling *et al*., 2008; Grayston and Jones, 1996). It can be reasoned that the release of easily assimilated carbon compounds has a stimulatory effect on microbial growth and activity (Grayston and Jones, 1996). This in turn has been hypothesised to favour microbes which are more competitive and/or utilise a greater range of compounds for growth (Drenovsky *et al*., 2004). Ultimately this may result in differences in the relative abundance of microbial groups and potentially restructure the microbial community.

The composition and function of root exudates varies with plant species, however, some of these secondary metabolites have been shown to selectively alter microbial populations (Bais *et al.*, 2006; Berg and Smalla, 2009; Grayston and Jones, 1996). For example, Tippet and Malajczuk (1979) discovered that species of the genus *Acacia,* such as *A. pulchella,* were found to reduce inoculum production of *P. cinnamomi* through metabolite production in the rhizosphere. This was hypothesised to be the result of the production of volatile organic compounds that are potentially fungistatic or sporostatic in nature. A later study by Whitfield *et al*. (1981) confirmed these results by identifying numerous volatile compounds from a steam-volatile extractive of *A. pulchella* roots. The extractive was found to restrict not only mycelial growth, but also suppress sporangial production and germination, and reduce zoospore germination of *P. cinnamomi in vitro* (D'Souza *et al*., 2005; Whitfield *et al*., 1981).

Belowground microbial community structure also plays a significant role in the establishment, growth and subsequent infection caused by *Phytophthora* species (Broadbent and Baker, 1974; Broadbent *et al*., 1971). This is due to the beneficial or deleterious interactions between microbial organisms that govern their growth and development within the environment (Malajczuk, 1983). Broadly speaking, the competitive saprophytic behaviour of *Phytophthora* species is low, further exacerbated by rapid mycelial degradation under natural soil conditions (Kuhlman, 1964; Malajczuk, 1983). This has been confirmed in several *Phytophthora* species, such as *P. parasitica* (Tsao and Bricker, 1963), *P. dreshleri* (Mehrotra, 1972), *P. palmivora* (Turner, 1965), and *P. cinnamomi* (Malajczuk and Theodorou, 1979). Common to all these studies is the association between mycelial lysis and spore development with intense microbial colonisation of hyphae, suggesting the impact of microbial antagonism on *Phytophthora* growth and development.

Microbial antagonism is characterised by different behaviours such as antibiosis, predation/parasitism and competition (Malajczuk, 1983). Antibiosis can be described as the production of antibiotics or toxic secondary metabolites that are deleterious to development and growth of susceptible microorganisms (Baker, 1968). For example, little leaf of pines is a disastrous disease of shortleaf (*Pinus echinata*) and loblolly (*Pinus taeda*), resulting from *P. cinnamomi* infection (Bruehl, 1987). However, cultures of the mycorrhizae fungus *Leucopaxillus cerealis* var. *piceina* have been shown to inhibit mycelial growth and zoospore germination of *P. cinnamomi* through production of the antibiotic, diatretyne nitrile, resulting in protection from zoospore infection in most mycorrhizae treated pine seedlings (Marx and

Davey, 1969). Although microbial predation/parasitism is a well-known phenomenon, parasitism of oospores is less well understood (Malajczuk, 1983). Despite this, evidence exists on the parasitism of these resting structures in *P. parasitica* by *Streptomycetes* (Honour and Tsao, 1973), *P. cinnamomi* by *Catenaria anguillulae* (Daft and Tsao, 1984) and *P. megasperma*/*P. cactorum* by a number of microorganisms ranging from chytridiomycetes to actinomycetes and bacteria (Sneh *et al*., 1977). This illustrates the potential for predation of *Phytophthora* species where they are found alongside these microbial predators.

Microbial competition has also been recognised as a major biological factor in determining *Phytophthora* growth, development and disease potential (Malajczuk, 1983). For example, the addition of *Gliocladium virens* and *Trichoderma harzianum* to organic mulch (e.g. sudangrass, *Sorghum X drummondii*) amendments were shown to improve root health of avocado roots displaying *Phytophthora* root rot (*P. cinnamomi*) by up to 31-37% and 22-25% respectively (Costa *et al*., 2000). The authors suggested that the strong cellulolytic activity of these microorganisms likely had a central role in their ability to colonise the rhizosphere of *Persea americana* and out-compete *P. cinnamomi*. Considering these antagonistic properties of various microbial groups towards *Phytophthora* growth and disease development, it becomes obvious that contrasting soils containing different microbial communities will affect disease incidence. This is likely to be further impacted by compounding chemical (e.g. nutrient availability) and physical (e.g. temperature and water availability) factors, resulting in soils that are either conducive or suppressive to *Phytophthora* growth and disease incidence (Broadbent *et al*., 1971; Schroth and Hancock, 1982).

Extensive literature exists on disease suppressive soils and their ability to shape microbial populations within them (Andrivon, 1994a, 1994b; Broadbent and Baker, 1974; Broadbent *et al.*, 1971; Schroth and Hancock, 1982). Disease suppressive soils are those in which the ability of the pathogen to cause disease in the host plant is diminished (Schroth and Hancock, 1982). While this property can be attributed to abiotic variables such as pH and nutrient availability, the presence of antagonistic microbes have also been demonstrated to alter microbial populations (Andrivon, 1994a, 1994b; Schroth and Hancock, 1982). For example, a study using soil recovered from an avocado grove in Tamborine Mt., Queensland, Australia, discovered significantly greater numbers of Actinomycetes and *Bacillus* spp. in disease suppressive soils compared to soils conducive to root rot by *P. cinnamomi* (Broadbent and Baker, 1974; Broadbent *et al.*, 1971). In other words, greater numbers of Actinomycetes and *Bacillus* spp.

were highly correlated with biological antagonism to the host pathogen, *P. cinnamomi*. The authors argue that the presence of these bacterial/fungal species inhibits sporangial formation and that any subsequent infection of avocado trees by *P. cinnamomi* is significantly reduced.

1.5 Managing the effects of *Phytophthora* spp.

Globally, there are numerous examples of *Phytophthora* species infecting and decimating forest ecosystems, including, *P. cinnamomi*, the causal agent of jarrah dieback (*Eucalyptus marginata*) in Australia and *P. ramorum*, the causal agent of sudden oak death, which has led to the decimation of oak and other tree species throughout North-Western American regions and Europe (Podger, 1972; Rizzo *et al*., 2005). Through management of these plant diseases, we can gain crucial insight into potential strategies for the conservation of kauri (Kia Toitu he Kauri, 2014). Kauri dieback management has been the focus of a partnership management program since 2009, which involves tāngata whenua (local people) from areas with natural kauri stands, Ministry for Primary Industries, Department of Conservation, Auckland Council, Northland Regional Council, Waikato Regional Council and Bay of Plenty Regional Council (Kia Toitu he Kauri, 2014). Conventional approaches to *Phytophthora* disease management has primarily focused on determining pathogen distribution, preventing pathogen-host contact, identifying potential host resistance, and utilising fungicide sprays (Scholthof, 2007; Kia Toitu he Kauri, 2014).

1.5.1 Detection tools

The reliable and accurate detection of *P. agathidicida* underpins our ability to delimit its distribution and organise successful management strategies (Than *et al*., 2013; Waipara *et al*., 2013). Conventional methods for soil based detection of *Phytophthora* species uses leaf, fruit and plant material to bait the pathogen from flooded soil samples (Martin *et al.*, 2012), or sterile techniques using *Phytophthora*-specific media to isolate the pathogen from host root samples (Jeffers and Martin, 1986). For both soil bioassays and sterile isolation techniques, the steps involved are highly time consuming and it can take up to 20 days for a definitive detection (Jeffers and Martin, 1986; Martin *et al.*, 2012). Moreover, identification keys developed by Waterhouse (1963) and subsequently revised by Stamps *et al*. (1990), are heavily based on morphological criteria. These keys are not considered to be optimal, as morphological

similarities between *Phytophthora* spp., together with natural variations within taxa, make species determination challenging (Kroon *et al.*, 2012; Martin *et al.*, 2012; Than *et al.*, 2013).

Molecular diagnostic tools which utilise PCR provide the most sensitivity for detection of *Phytophthora* and other plant pathogens (Bailey *et al.*, 2002). A recent study developed a TaqMan real-time PCR detection method to enable the rapid quantification of *P. agathidicida* within the soil (Than *et al.*, 2013). Initial tests showed the method could distinguish between *P. agathidicida* and a variety of other *Phytophthora* and *Pythium* spp. found in kauri forest soils, in addition to 26 non-target species of *Phytophthora*, demonstrating its high specificity (Than *et al*., 2013). Although DNA extraction is notoriously difficult in soil due to the presence of inhibitors such as phenolic and humic compounds (Picard *et al*., 1992), the sensitivity and specificity of this TaqMan assay is comparable to detection limits reported for real-time detection of other *Phytophthora* species (Than *et al*., 2013; Tomlinson *et al*., 2005; Schena *et al*., 2008). Results suggest this TaqMan real-time PCR detection method will allow rapid and accurate detection of *P. agathidicida*, which will facilitate delimitation of its distribution.

1.5.2 Preventing pathogen-host contact

As a soil borne oomycete, the movement of infected water, soil or plant material plays a key role in the distribution of kauri dieback (Beever *et al.*, 2009). Human activity has likely resulted in the increased distribution of this pathogen throughout kauri stands (Beever *et al*., 2009). For instance, actively injuring trees for gum production, road building, bush walking/recreational use, planting of infected nursey stock are all known examples where human activities have contributed to the spread of disease (Beever *et al.*, 2009; Hardy *et al.*, 2001; Kia toitu kauri, 2014). To mitigate the effects of human activity, certain measures have been put into place, such as upgrading high-use forest tracks via installation of boardwalks, informing the public about phytosanitary protocols, and closing track walks in high risk areas (Hardy *et al.*, 2001; Kia Toitu he Kauri, 2014). Where humans are concerned, lack of sanitation is a large contributor to dispersal of the pathogen. This is because the effectiveness of the signage accompanied by phytosanitary stations is largely determined by social compliance (Kauri Dieback Report V2, 2017). However, as mentioned previously, a recent study suggests that even the disinfectant, Trigene, is ineffective against *P. agathidicida* oospores (Bellgard *et al.*, 2010). Further research is required to understand the ramifications and impact of ineffective

disinfectants on oospores, in addition to identifying appropriate counter-measures to facilitate this gap in targeted management.

In addition to humans acting as vectors, feral pigs and livestock have also been implicated as vectors to the dispersal of kauri dieback (Beever *et al*., 2009). While studies have since shown that feral pigs can act as a high-risk pathway for *P. cinnamomi* and potentially other *Phytophthora* spp., there is inconclusive evidence for the role of feral pigs in *P. agathidicida* dissemination (Krull *et al*., 2012; Bassett *et al*., 2017). Meanwhile, field observations in unfenced kauri fragments on pastoral rural properties have shown they were often subject to soil compaction by livestock (Waipara *et al*., 2013). Frequent recovery of *P. agathidicida* from kauri rootzones exposed to soil disturbance by livestock provides inferential evidence that livestock potentially contribute to the dissemination of *P. agathidicida* (Beauchamp, 2013). Further studies are required to elucidate this relationship between livestock compaction, movement and *P. agathidicida* distribution.

1.5.3 Host resistance

Natural resistance of *P. agathidicida* in forest populations of kauri has yet to be reported in literature. With studies indicating the infectivity of *P. agathidicida* for all ages of kauri (Waipara *et al.*, 2013), its wide distribution in the Auckland region (Beever *et al.*, 2009), and its highly virulent nature in relation to other *Phytophthora* spp. (e.g. *P. multivora*, *P. cinnamomi* and *P. cryptogea*) recovered from the same soil, further study into the natural resistance of kauri dieback is urgently needed (Gadgil, 1974; Horner and Hough, 2014). Current research into host resistance is being investigated at Scion, the forestry and technology crown research institute in Rotorua (Scott. P. *Personal communications*). However, even if resistant kauri varieties were discovered soon, the long-lived nature of kauri trees preclude its immediate practical application. While there are clear benefits in having resistant host trees (Nowicki *et al.*, 2012), this represents a long-term management strategy. With the discovery of resistance, Gough *et al.* (2012) described a reliable micro propagation strategy that can be used to grow nursery populations more efficiently. Even now, this method can be used to rehabilitate natural kauri populations to retain genetic diversity in adversely affected populations.

1.5.4 Fungicide usage

Fungicides such as fosetyl-Al are known to be an effective control of many plant diseases caused by oomycetes (Smiley *et al.*, 1999). Phosphite, derived from fosetyl-Al, has been shown to be effective in controlling infection by *Phytophthora* spp. (Smiley *et al.*, 1999; Hardy *et al.*, 2001; Horner and Hough, 2013; Horner *et al.*, 2015; Gruenwald *et al.*, 2008). For example, when applied as a root drench prior to inoculation, it provided protection against invasion by *P. cinnamomi*, *P. nicotianae* and *P. palmivora* in lupin (*Lupinus* spp.), tobacco (*Nicotiana* spp.), paw-paw (*Asimina triloba*) and jarrah (*E. marginata*) (Smiley *et al.*, 1999; Hardy *et al.*, 2001). Similarly, in recent glasshouse studies on 2-year old kauri seedlings, phosphite was shown to be effective in providing protection against *P. agathidicida*; phosphite injection into the trunk resulted in 100% survival rate following soil inoculation (Horner and Hough, 2013; Horner *et al.*, 2015). Horner and Hough (2013) further reported that *P. agathidicida* was more susceptible to phosphite treatment than other tested *Phytophthora* species (e.g. *P. cinnamomi* and *P. cactorum*). In fact, *P. agathidicida* was observed to display an EC_{50} of 4.0 µg/ml phosphorous acid compared to the EC_{50} values of 25.2 and 37.9 µg/ml for *P. cinnamomi* and *P. cactorum* respectively (Horner and Hough, 2013). Despite these promising results, it should be noted that the study was undertaken using two-year old seedlings in a glasshouse and efficacy under natural conditions could not be assumed. A more recent study by Horner *et al.* (2015) followed up on these earlier results by demonstrating the marginal effectiveness of phosphite as a fungicide control on mature kauri stands in the Waitakare and Mangamuka ranges. The authors observed that average lesion expansion in 3 years was 12.7 cm in untreated and 0.4 cm in phosphite treated mature trees, indicating some protection against lesion development by *P. agathidicida*.

Despite the evidence for phosphite protection against *P. agathidicida*, there are ongoing concerns with phytotoxicity and questions about the economic viability of widespread use of phosphite as a control measure. Phytotoxic symptoms (e.g. leaf yellowing) were observed in 20% of phosphite injected individuals (Horner *et al.*, 2015). Furthermore, the prospect of applying phosphite to entire kauri forests was recognised by the authors to be untenable given the enormous costs that would be involved (Horner *et al.*, 2015). Therefore, fungicide usage (i.e. phosphite) will likely only supplement other disease management strategies currently in place to mitigate kauri dieback, with a specific focus on protecting the largest and most notable kauri individuals such as Tane Mahuta and Te Matua Ngahere.

1.6 Summary and project aims

1.6.1 Summary

The success of *Phytophthora agathidicida* as a pathogen originates not only from its effective reproduction via both sexual and asexual modes, but also in large part to its widespread distribution facilitated by human activity. This is expedited by the high level of pathogenicity of *P. agathidicida* against kauri trees of all ages. Taken together, these factors suggest that annual disease cycle of *P. agathidicida* is less influenced by the developmental stage of the plant itself, but rather the prevailing environmental conditions that direct growth and propagation of the pathogen. As such, the establishment of *P. agathidicida* in any given environment is dependent on the sum total of influences of various environmental factors on pathogen dissemination, growth and disease potential. Given the fragmentation of kauri stands by plots of pasture and pine within Waipoua forest, the potential impact that variations in these factors have on *P. agathidicida* growth, pathogenicity and host range are worth exploring.

1.6.2 Project aims and objectives

This study aims to characterise the growth and pathogenicity of *P. agathidicida* within soils of three contrasting land-uses (i.e. indigenous kauri forest, commercial pine forest and pasture) in Waipoua forest and its surrounding area. Additionally, a supplementary study of alternative hosts common to pasture and pine forest was conducted to confirm the potential for plant hosts within these systems to support *P. agathidicida* growth. This was done in light of a recent study which identified the host range of *P. agathidicida* extending to several native species found in association with kauri (Ryder *et al*., 2016).

Waipoua forest was selected as it is recognised to be one of the original mainland sites for the dissemination of *P. agathidicida* (Beever *et al*., 2009; Waipara *et al*., 2013). The three land-uses were chosen due to common observations of kauri forest fragmentation by plots of pasture and pine throughout lowland kauri stands in northern New Zealand (Enright *et al*., 1999; Kauri National Park Proposal, 2011; Sustainable Management of New Zealand's Forests, 2015).

The main objectives of this thesis:

1. How the physicochemical properties vary between the soils of these three land-uses and whether they impact the growth and reproduction of *P. agathidicida* (Chapter 2).
2. Whether the three land-use soils impacted upon the virulence of *P. agathidicida* using blue lupin (*Lupinus angustifolius*) as a model host plant (Chapter 3).
3. The colonisation and effects of *P. agathidicida* on six alternative hosts (*Trifolium repens, T. ambiguum, Lolium perenne. L. multiflorum, Pinus radiata* and *Lupinus angustifolius*) that are common to pasture and pine forests (Chapter 4).

Chapter 2:
Characterising the growth of *Phytophthora agathidicida* in soils of contrasting land-uses

2.1 Introduction

Previous research on *Phytophthora agathidicida*, the hypothesised causal agent of kauri dieback (Bellgard *et al*., 2016; Weir *et al*., 2015), has focused primarily on invasion pathways (Basset *et al*., 2017; Krull *et al*., 2012), chemical control through use of phosphite (Horner and Hough, 2013; Horner *et al*., 2015; Lawrence *et al*., 2017), molecular detection technologies (Than *et al*., 2013), and genetic sequencing for host resistance (Studholme *et al*., 2016; Weir *et al*., 2015). Although the widespread distribution, high virulence and recent re-discovery of *P. agathidicida* in 2006 has arguably necessitated this research response (Beever *et al*., 2009; Waipara *et al*., 2013), little attention has been paid to the potential impacts different land-uses have on the growth and disease expression of *P. agathidicida*. Several epidemiological studies of other *Phytophthora* species have previously demonstrated that landscape properties (e.g. soil composition) are important factors that influence disease development (Jung *et al*., 2000; 2013; Keesing *et al*., 2010; Shearer and Dillon 1996; Shearer *et al*., 1987). For example, a study which investigated the disease severity and disease centre (i.e. localised disease incidence) characteristics of *P. cinnamomi* infestations of *Banksia* Woodlands on the Swan Coastal Plain in Western Australia, discovered that a majority of disease centres (60%) occurred on leached acidic soils of the Bassendean Dune System (BDS), while no disease centres were found in soils of the Spearwood (SDS) and Quindalup Dune Systems (QDS) (Shearer and Dillon, 1996). With comparable levels of susceptible plants and disturbance from human activity between BDS and SDS/QDS (Havel, 1979), Shearer and Dillon (1996) suggested that other factors such as the chemical, biological, and physical properties of the soil in these dune systems contributed to the difference in disease incidence and severity. This has been previously reviewed in Erwin *et al*. (1983), which concluded that soil characteristics could directly/indirectly affect disease occurrence by inhibiting or favouring reproduction, survival and infection of *Phytophthora* species.

Understanding this, the current study aims to characterise the growth of *P. agathidicida* within the soils of three common land-uses (e.g. indigenous kauri forest, commercial pine forest and pasture land) collected from Waipoua Forest and its surrounding area, to identify whether land-uses can influence the establishment and disease potential of *P. agathidicida* within them. I aim to do this by making counts of spore types associated with *Phytophthora* growth, including sporangia and oospores at different developmental stages based on metrics provided by Weir *et al*. (2015). Comparison of spore counts in soils collected from each land-use over four observational days (e.g. day 1, day 2, day 4 and day 8) will provide growth curves for assessing pathogen establishment, survival and disease potential. For example, asexually produced sporangia and associated zoospores are known to be the primary infective agents and dispersal mode of *Phytophthora* spp. (Duniway, 1983; Erwin and Ribeiro, 1996). Meanwhile, sexual oospores are generally recognised as survival structures that are produced in response to environmental stressors (e.g. low moisture, low nutrients), adverse to the production and survival of asexual sporangia (Fry, 1998; Hardham, 2005). Therefore, understanding the proportion of asexual to sexual production of sporangia and oospores of *P. agathidicida* in response to land-use soils will aid in characterising the growth response of the pathogen, and by extension its disease potential. Furthermore, characterising these soils for several notable physicochemical factors such as pH, C:N, P and electrical conductivity (EC) may help identify conditions that facilitate these reproduction strategies, and therefore contribute to the development of a predictive tool for assessing the risk of *P. agathidicida* establishment and growth.

It is anticipated that the information generated from this study will contribute to site-specific management of kauri dieback, in addition to understanding the establishment characteristics of *P. agathidicida* in contrasting land-uses.

2.2 Research aim

The main aim of this study is to determine how the physicochemical properties vary between the soils of these three land-uses and whether they impact the growth and reproduction of *P. agathidicida.*

2.3 Materials and Methods

2.3.1 Study area

Waipoua Forest (-35° 38' 59.99" S, 173° 32' 59.99" E) is located on the west coast of the Northland region of New Zealand (Fig. 2.1) (McGregor, 1948). It was selected for sampling as it is recognised to be one of the original mainland sites for the dissemination of *P. agathidicida* and it is the largest remaining intact kauri forests (Beever *et al*., 2009; Waipara *et al*., 2013). Three land-uses are predominant in this area, and these are regenerating and original kauri forest stands, pasture (beef), and pine plantations (*Pinus radiata*) (Burns and Leathwick, 1996). Waipoua forest will henceforth be used as a general term including these contrasting land-uses within the Waipoua region.

Waipoua Basalt is the major geological substrate within this area (Burns and Leathwick, 1996). Landforms on this basaltic substrate are moderately dissected by gently sloping hill country with weathered clay soils, displaying imperfect to poor drainage and low fertility (Burns and Leathwick, 1996). According to the National Soils Data repository (National Soil Repository, 2017), the predominant soil type is brown granular clay; formally classified as orthic litter organic soil and acidic orthic granular soil. Although phosphorous content remains consistent between these soils, carbon/nitrogen percentages and cation exchange capacity vary substantially (National Soil Repository, 2017). For example, in orthic litter organic soils, carbon and nitrogen percentages average 32% and 0.75% respectively, compared to 9.7% and 0.48% respectively for acidic orthic granular soils.

2.3.2 Site history

According to Burns and Leathwick (1992), there are 12 variations of forest types in Waipoua Forest, reflecting different environmental and historical factors affecting floral species composition. The kauri forest under study was composed of kauri (*A. australis*), tanekaha (*Phyllocladus trichomanoides*), mamangi (*Coprosma arborea*), kanuka (*Kunzea ericoides*) and towai (*Weinmania silvicola*) (Burns and Leathwick, 1992). Both the pasture and pine forest sites are located on land that previously supported kauri (*A. australis*), taraire (*Beilschmiedia*

tarairi), towai (*W. silvicola*) and kohekohe (*Dysoxylum spectabile*), but was extensively logged between 1944 and 1948 (Burns and Leathwick, 1992).

Although formally recognised by the Crown in the Te Roroa Claims Settlement Act 2008, Te Roroa have been the kaitiaki (guardian) of Waipoua Forest and its surrounding area for nearly a thousand years (Kauri National Park Proposal, 2011). The last 50 years in Waipoua forest have seen many changes with the development of technology, new settlements and the conversion of native forest to pine plantations and farms under modern agricultural practices (Kauri National Park Proposal, 2011).

Figure 2.1 Waipoua forest situated on the west coast of the Northland region of the North island of New Zealand.

2.3.3 Sampling period

Soil samples were collected in mid-April (Autumn) 2016. In Waipoua forest, temperatures averaged between 0.6 ^{o}C to 2.2^{o}C (13.5 ^{o}C low and 21.2 ^{o}C high) higher than historical averages ("Waipoua forest local weather", 2016). In stark contrast to the above normal averages in temperature, precipitation in April 2016 was well below normal, displaying 26 mm of precipitation to the April normal of 90 mm ("Waipoua forest local weather", 2016).

2.3.4 Sample collection and processing

With permission from the Te Roroa Iwi, soil sampling was conducted towards the southern end of Waipoua forest, within the three-adjacent land-uses described above. Six replicate sampling sites within each land-use (Fig. 2.2) were chosen for uniformity (e.g. similar slope gradient and soil type among the three spatially separated land-uses), and sampled using a 30x30 cm metal quadrat and sterile trowel for the leaf litter (LL), A horizon (organic surface soil layer) and B horizon (inorganic subsoil layer). The A and B soil horizons were primarily distinguished through physical characteristics such as colour and texture. All LL found within the quadrat was removed, while up to 1 kg of soil was collected from each soil horizon and stored in re-sealable plastic bags for transport back to Scion Forest Research, Rotorua, New Zealand. Following collection and transport, samples were stored within large plastic containers in ambient temperature (22^{o}C) for 12 hours prior to conducting experimental procedures. Simultaneously, within the same kauri forest sampling area, an Auckland Council technician, Lee Hill, conducted a Kauri Dieback Survey by assessing disease symptoms and retrieving samples for *Phytophthora* baiting (Fig 2.2; Nick Waipara, *personal communications*)

Figure 2.2 GPS locations of six replicate sampling sites for indigenous kauri forest, pasture land and commercial pine forest (source: Google maps). Photo from Settlement Rd, Waipoua Forest (source: Kai Lewis).

2.3.5 Selective medium

A modified *Phytophthora*/*Pythium* selective medium (Jeffers and Martin, 1986) was used to culture the isolate of *P. agathidicida* (NZFS 3813) used in this study. This isolate was originally retrieved from Coromandel in early 2014 from soil near symptomatic kauri trees, collected by Jeanie McInnes (Biosecurity Officer - Heritage Group Waikato Regional Council) on behalf of John Beachman and Tony Beauchamp. This isolate was later stored in the National Forest Culture Collection at Scion for further use.

10% carrot agar, ampicillin, pimaricin, rifampicin, nystatin and hymexazol (CRNH) medium is a modification Scion made of the well tested selective medium pimaricin, ampicillin, rifampicin, pentachloronitrobenzene (PCNB) and hymexazol (PARPH medium; Jeffers and Martin, 1986). The selective medium contained of 10% carrot agar (Wattie's Diced Carrots 5 kg and A5306, Sigma-Aldrich) amended with 200mg/L ampicillin (DUC-A0104, Total Lab Systems), 50mg/L nystatin (DUC-N0138.0005, Total Lab Systems), 10mg/L rifampicin (DUC-R0146.0001, Total Lab Systems), 0.4 ml 2.5% aqueous suspension pimaricin (P0440, Sigma-Aldrich) and 50mg/L hymexazol (ALF044450.06, Thermofisher). The minor difference to PARPH involves the replacement of PCNB with nystatin, a similarly beneficial fungicide for suppressing the growth of background mycoflora (Morita and Tojo, 2007), and the use of 10% carrot agar as the basal nutrient.

2.3.6 Growth response assay

A single isolate of *P. agathidicida* (NZFS 3813) was cultured using CRNH selective media and incubated for 7 days at 21ºC prior to initiating the observational study. This isolate was then used to inoculate approximately 950 small 5x5 mm mira-cloth discs (475855 EMD Millipore, Sigma-Aldrich) on CRNH plates as seen in Fig. 2.3 A and B; the mira-cloth provided an inert rayon-polyester substrate upon which the pathogen can grow. These inoculated mira-cloths were confirmed to show mycelial growth and oospore presence (and lack of sporangia) prior to initiating the experiment. Approximately 55 ± 1 g of soil per land-use soil sample were transferred to appropriately labelled petri-dishes (Z692336, Sigma-Aldrich), while 10 ± 0.1 g of leaf litter were allocated to labelled petri-dishes. All 54 soil/litter samples (i.e. 18 per land-use) were replicated four times to represent each of the four observational days (e.g. 1, 2, 4 and 8 days post exposure) and saturated to water holding capacity (2.3.9). Six replicate water

controls were included per observational day, consisting of 40 ml deionised water (i.e. 54 soil/litter samples + 6 water controls = 60 samples per observational day). Four inoculated mira-cloths were subsequently submerged into each sample at equal depths and distance from one another (Fig. 2.3C). Following this, all samples were randomly allocated to 3x3 stacks of 4 in six sets of trays that were kept in uniform darkness within a temperature controlled (22^{o}C) growth room for the duration of the study (Fig. 2.3D). On observational days 1, 2, 4 and 8 after initial submersion, the mira-cloths were harvested, gently washed in sterile water to remove excess soil particulates and placed into labelled 2 ml Eppendorf® tubes containing FAA (formalin acetic acid) (Appendix Table A.4) solution to biologically fix each mira-cloth sample before further processing (Fig. 2.3E). Two mira-cloths representing each sample were selected and mounted onto dual concave microscope slides where they were stained with lacto-phenol cotton blue (Fig. 2.3F), a common staining procedure for *Phytophthora* spores (Erwin and Ribeiro, 1996; Waterhouse, 1963).

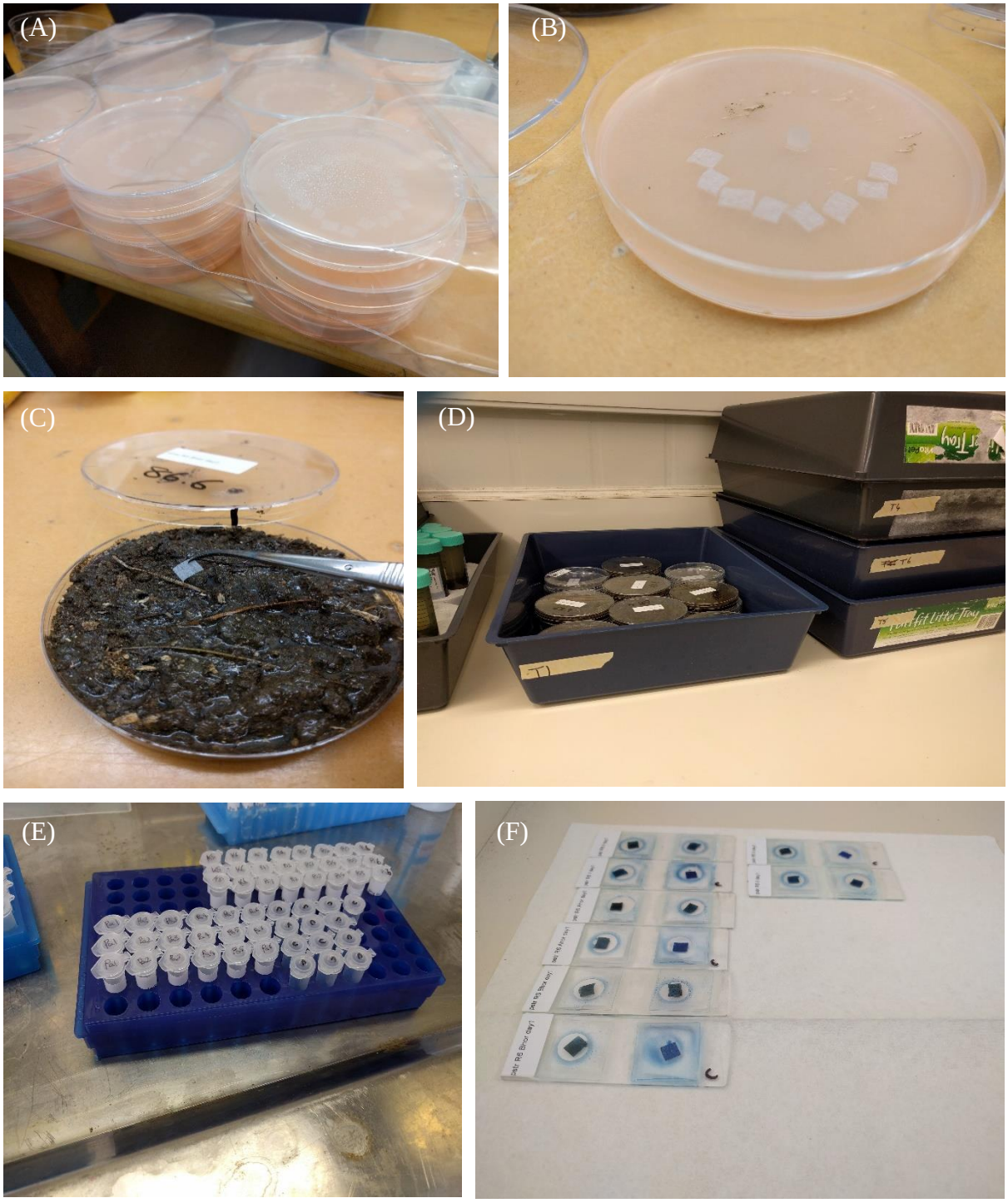
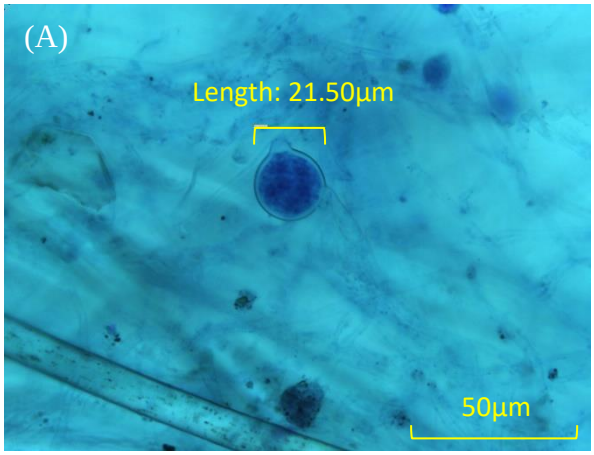


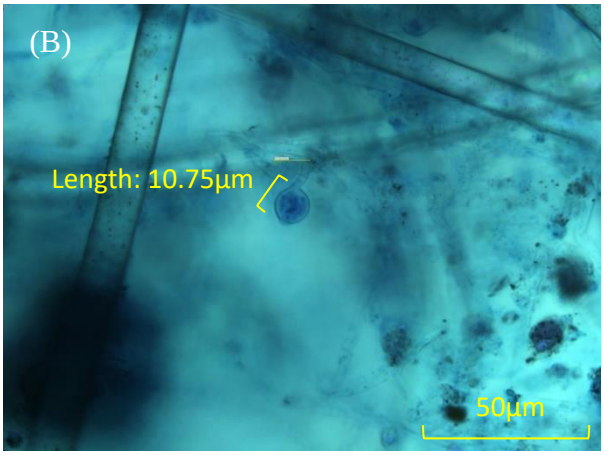
Figure 2.3 Experimental set-up of growth response study showing, A and B) Inoculated mira-cloths on CRNH medium, C) Submersion of inoculated mira-cloth in soil/litter slurry, D) Stacked samples, E) Mira-cloth samples fixed in FAA solution, and F) Stained and mounted mira-cloth samples on dual concave microscope slides.

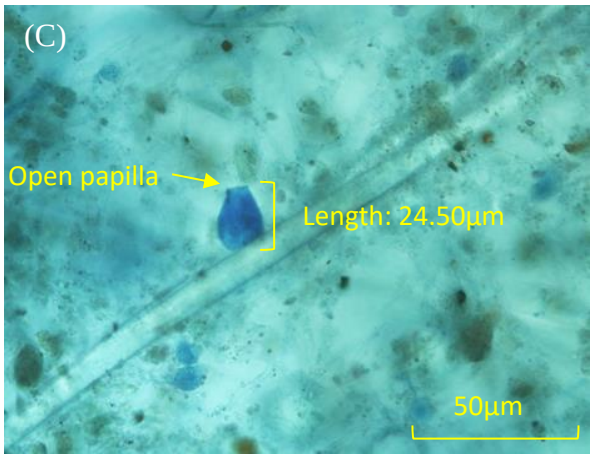
2.3.7 Spore counts

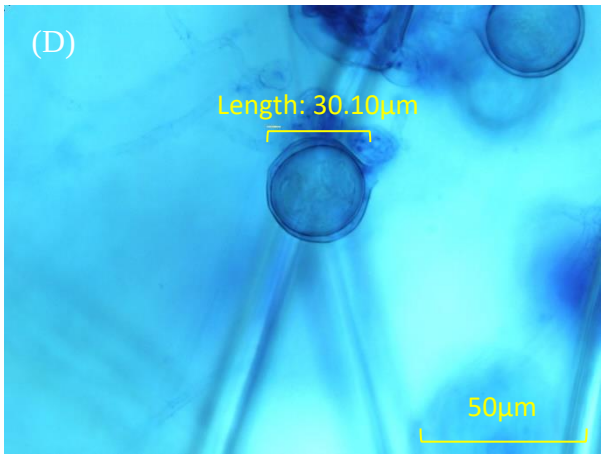
To determine the effect of land-use samples on the growth response of *P. agathidicida,* seven spore morphologies were quantified for each observational day, including mature sporangia (MS), zoospore released sporangia (ZRS), juvenile sporangia (JS), mature oospores (MO), developing oospores (DO), aborted oospore (AO) and juvenile oospores (JO). Spores were visually differentiated based on descriptions and metrics provided by Weir *et al*. (2015). Sporangia that were approximately 20 µm or more in length were defined as mature (Fig. 2.4A), while all those below this threshold were counted as juvenile (Fig. 2.4B). MS that had opened papilla were defined as ZRS (Fig. 2.4C). Oospores that had an approximate length of 25 µm and above were defined as mature (Fig. 2.4D), while smaller oospores were described and counted as juvenile (Fig. 2.4E). MO which had become aplerotic were described as AO (Fig. 2.4F), and oospores with thicker oogonial walls and coarsely granulated cytoplasms were arbitrarily designated as DO (Fig. 2.4G; P Scott, personal communication). Spore counts were assessed from four randomly chosen views of each mira-cloth under 20x magnification via light microscopy (Fig. 2.3F).

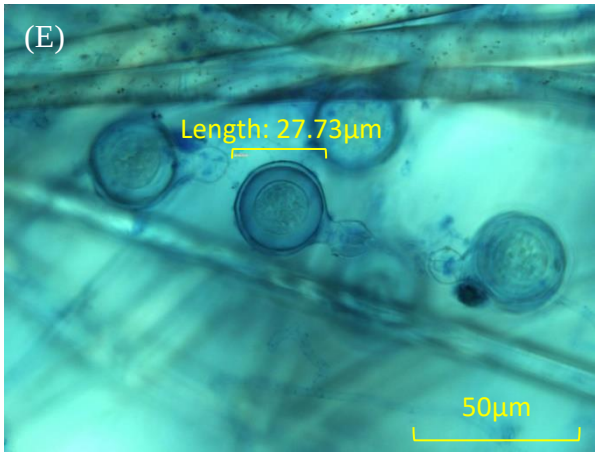
It is recognised that only spores present in the fixed microscope fields were counted and may underrepresent true spore populations present in the field.

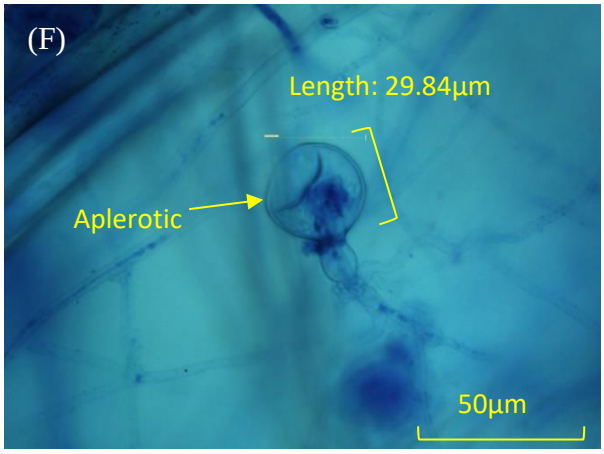

(A)
Length: 21.50µm
50µm


(B)
Length: 10.75µm
50µm


(C)
Open papilla
Length: 24.50µm
50µm


(D)
Length: 30.10µm
50µm


(E)
Length: 27.73µm
50µm


(F)
Length: 29.84µm
Aplerotic
50µm

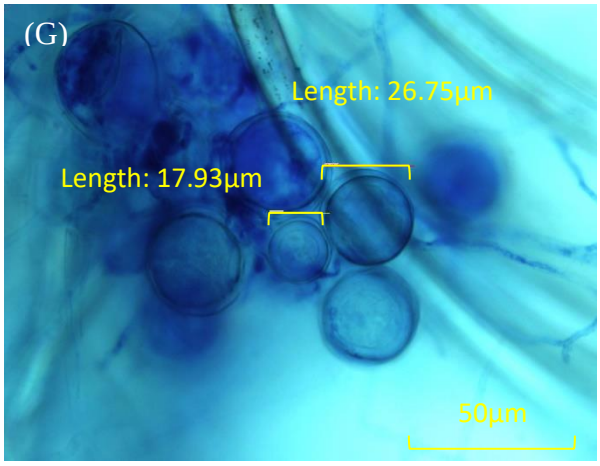


Figure 2.4 Sporangia and oospores of *Phytophthora agathidicida*. (A) Mature sporangia showing unopened papilla; (B) Juvenile sporangia; (C) Mature sporangia displaying opened papilla with zoospore release shown by consistent staining of cytosol; (D) Mature oospore; (E) Developing oospores showing thickened walls and granulated cytosol; (F) Aborted oospore; (G) Juvenile oospore shown next to mature oospores. Magnification = 100x.

2.3.8 *Phytophthora* baiting

The soil baiting bioassay and subsequent genetic analysis of isolated *Phytophthora* spp. was conducted at Scion, Rotorua. The samples retrieved for the Kauri Dieback Survey were subjected to the same baiting and genetic analysis procedures, however, these were conducted by Nick Waipara at Plant and Food Research, Auckland.

Baiting

While carrying out the growth response assay, all soil samples (A and B horizon only) were subjected to the baiting procedure outlined in Beever *et al*. (2010). The rationale behind this baiting assay was to identify potentially indigenous populations of *Phytophthora* spp. (including *P. agathidicida*) that may impact the growth response of the isolate. Samples were set-up by weighing out 175 g of soil devoid of plant litter into separately labelled 750 ml plastic containers. These were then left to dry for a period of up to three days, where on the fourth day all containers were sprayed with deionised water until wet, but not soaking. On day five approximately 540 lupin (*Lupinus angustifolius*) seeds (5 seeds per sample, in triplicate) were mixed with moist vermiculite and loosely covered with tin foil to germinate. On day eight 500 ml of deionised water were carefully poured into each sample container to avoid disturbing the soil, following which, five lupins placed into polystyrene cut-outs were set afloat making sure that no radicles touched the submerged soil beneath. After 2 days (day 10), all lupins were harvested whereby a small 1 cm segment of each root tip was excised and surface sterilized for 30 seconds in 70% ethanol, followed by two 30 second rinses in deionised water. Excised radicle segments were then cut transversely and placed onto CRNH selective medium for

culturing until day 12, and subsequent sub-culturing of growth-positive samples until day 15. This was followed by morphological assessment to the genus level using criteria outlined by Erwin and Ribeiro (1996), in order to identify *Phytophthora* presence.

Genetic analyses

Molecular identification of isolates was then conducted following the procedures cited in Beever *et al*. (2009) the Scion molecular pathology team, Rotorua. DNA was extracted from mycelium using a QIAGEN DNeasy Plant Mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The internal transcribed spacer (ITS) region of the ribosomal DNA repeat was PCR amplified using the primers ITS-4 and ITS-6 following the PCR protocol of Cooke *et al.* (2000). ITS sequences were retrieved from GenBank to represent authentic isolates. The DNA sequence data were then trimmed to (5'-CCACACC….TCTCAA-3') to anchor the alignment and aligned with Clustal X (Larkin *et al.* 2007). Gap opening and gap extension parameters were adjusted to achieve the best visual alignment.

2.3.9 Soil chemical/physical analyses

Following transport of soil samples to Scion, water holding capacity was determined by comparison of saturation to oven dry weights (Blakemore *et al*., 1987). Deep petri dishes (100 x 25 mm; Nunc®) were labelled and allocated their respective samples to a uniform volume of 40 ml. Each sample was then saturated with 250 ml tap water after transfer of deep-dishes into 750 ml takeaway containers (Better Selection®) overnight. The following day all deep-dishes were drip-dried for up to two hours and measured for saturation weight prior to transfer into a kiln for up to two days at 75ºC to determine dry weights.

Each soil sample was further analysed for pH (Mettler Toledo; Seven easy) and electrical conductivity (EC) (Mettler Toledo, Five Easy conductivity meter) according to the methods described in Blakemore *et al*. (1987). Soil samples (A and B horizon) were air dried and sieved using a 2 mm steel mesh and mixed with 25 ml or 50 ml deionised water to achieve a 1:2.5 or 1:5 solution ratio for analysis of both pH and EC respectively. Leaf litter samples were prepared by manually chopping 2.5 ± 0.05 g into 1 cm segments prior to mixing with 25 ml of deionised water to achieve a 1:10 solution ratio, which is recommended for samples with high organic matter content (Blakemore *et al*., 1987; Cornelissen *et al*., 2011). All samples were loaded onto an end over end shaker for 30 minutes at 60 turns/min and left to settle for an additional 30

minutes. Both pH and electrical conductivity meters were calibrated immediately prior to use, using pH 4/7 buffer and 1413 µS/cm standard solutions respectively. To minimize errors, the electrode was rinsed with deionised water between each sample measurement (Blakemore *et al*., 1987).

Finally, total carbon (C) and nitrogen (N) (LECO CNS-2000 element analyser, Leco Australia Pty Ltd NSW Australia) and phosphorous (P) (via Olsen P method; Olsen *et al*., 1954) content of each soil sample (exception of LL for phosphorous measurements) was determined through outsourcing to a Lincoln University based technician (C and N) and Hill Laboratories (phosphorous; 2017).

2.3.10 Statistical analyses

The results were analysed using a balanced split-plot factorial design with land-use (Kauri, Pasture and Pine) as the main-factor, and site-samples (i.e. A and B horizon, and LL) as the sub-factor. Six water controls were also included resulting in a total of 60 samples (4 land-uses (three land-uses plus water control) × 3 site-samples (A and B soil horizons, and LL) × 6 replicates per land-use). Observational day acts as another factor with levels Day 1, Day 2, Day 4 and Day 8, on which all land-use/site-sample comparisons for each spore count variable are made.

Significance testing

To test the statistical null hypothesis of no difference in physicochemical/count variables between levels of both land-use and site-samples, two-way ANOVAs were conducted (Crawley, 2012). This allowed retrieval of associated *F*-values and *p*-values of statistically significant interactions and main effects, followed by post-hoc tests via Tukey's Honest Significant Difference (HSD) at *p*-value <0.05 to identify the levels of each factor combination that were significantly different. It is necessary to note that phosphorous (P) was not determined for LL samples of any land-use, and therefore consisted of 36 samples instead of the original 54.

Variation in count data explained by environmental data

The BIOENV method in PRIMER 7 was used to link spore counts to multivariate environmental patterns (Clarke and Ainsworth, 1993; Clarke and Warwick, 2001). This

technique performs permutation tests on environmental variables to determine which variable(s) produce the highest correlations with the count data using Spearman rank correlations (Skoulikidis *et al*., 2009). As BIOENV is only able to determine which environmental variables are best correlated with the count data, a separate procedure of DistLM (Distance-based Linear Modelling) was used to describe the percent variation in count data explained by environmental variables (Clarke and Warwick, 2001). A step-wise selection process of Aikaike's Information Criterion (AIC) was used to select between the different models (Anderson *et al*., 1998). Due to the pseudo-replication inherent in analytical replication, these tests were conducted separately for each observational day as environmental measurements applied only to the 54 samples originally collected, and not the 216 sample points spread across the four observational days.

2.4 Results

2.4.1 Soil baiting

Soil samples (A and B horizon), were found to contain viable propagules of two distinct *Phytophthora* species. Subsequent genetic testing of ITS-4 and ITS-6 revealed 100% and 99% sequence matches respectively for *P. gregata* and *P. pini*. Two of the 12 pasture soil samples tested positive for these two *Phytophthora* species, while a tentative identification for *P. gregata* was made in one of the 12 kauri soil samples. In addition to this, it is worth noting that no *P. agathidicida* isolates were recovered from any soil samples, and no *Phytophthora* isolates were recovered from any pine samples. Therefore, this resulted in a 16.6%, 8.3% and 0% *Phytophthora* recovery from pasture, kauri and pine forest soils respectively.

South of the sampling sites used in this study, the Kauri Dieback Survey identified several kauri trees exhibiting dieback symptoms. Although no *P. agathidicida* was recovered from symptomatic kauri trees near the sampling sites in this study, towards the southern-most edge of the survey (Fig. 2.5), *P. agathidicida* was successfully isolated from symptomatic trees using identical molecular diagnostics as above.

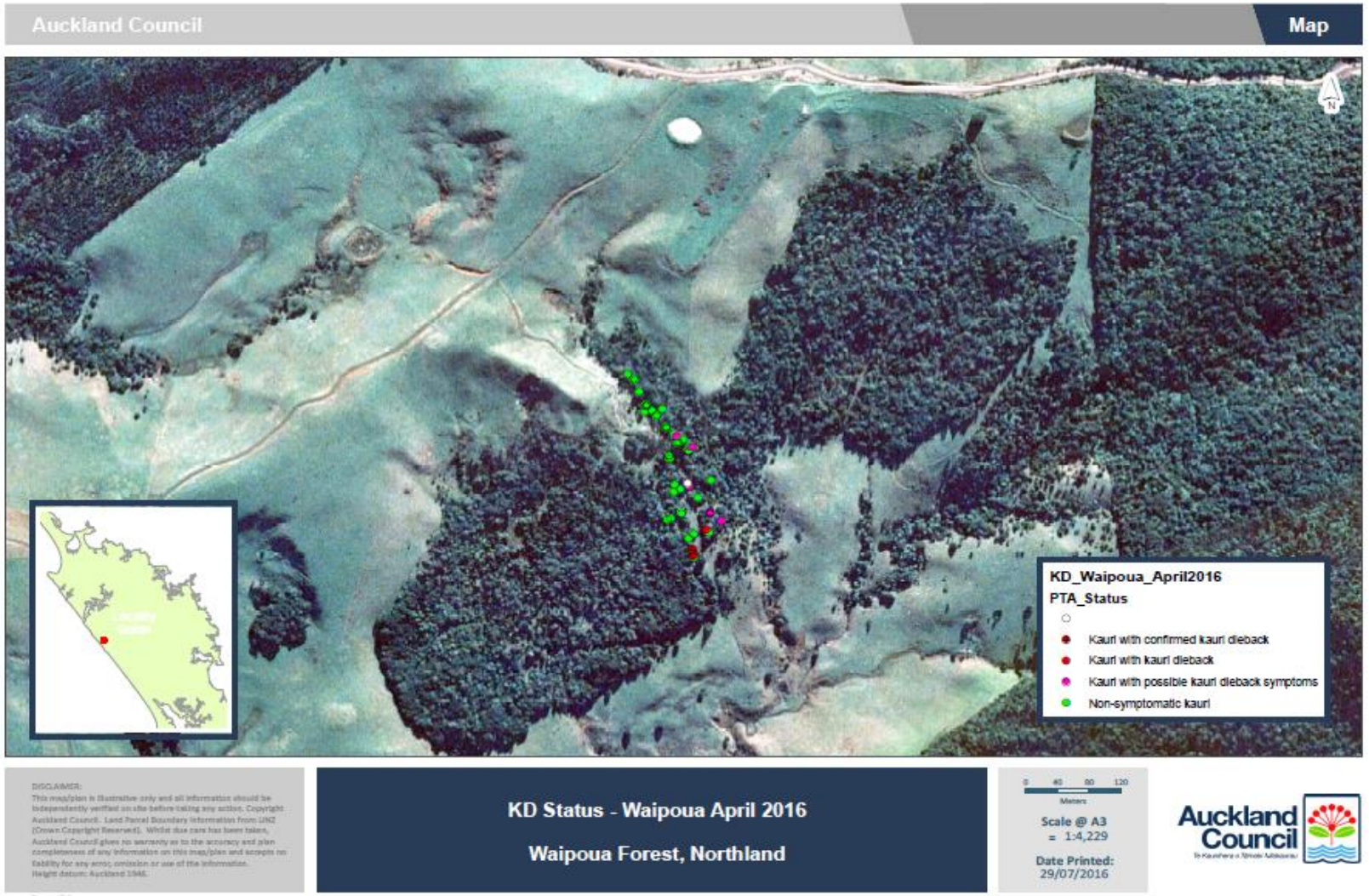


Figure 2.5 Kauri Dieback Survey of symptomatic and non-symptomatic trees in close proximity to the kauri sampling sites of this study (source: Auckland Council, Nick Waipara and Lee Hill).

2.4.2 Analysis of physicochemical data between land-uses

To test the null hypothesis of no difference in the physicochemical variables between levels of both land-use (kauri, pasture and pine) and site-samples (LL, A and B horizon), two-way ANOVAs were utilised. With the exception of pH and P content, significant interactions ($p < 0.001$) between land-use and site-sample treatments for most environmental variables were identified (Table 1). Post-hoc tests using Tukey's HSD indicated that kauri samples have significantly greater pH values than pine samples ($p < 0.05$), and pasture samples have significantly greater P content than both kauri and pine samples ($p < 0.001$) as shown by Table 2.

Table 2 also reveals the significant difference between levels of both land-use and site-samples for the remaining physicochemical variables, electrical conductivity (EC), total N%, total C% and C:N. Pasture LL shows significantly greater EC values compared to all site-sample levels of both kauri ($p < 0.001$) and pine ($p < 0.001$), however, EC values in A and B horizon are comparable to those observed in opposing land-uses. N% in LL is revealed to be significantly greater for all land-uses to their respective A and B horizons ($p < 0.001$). Both A and B horizons of kauri and pasture are observed to be significantly greater than those in the corresponding horizon levels of pine. C% of LL samples for all land-uses is significantly greater than their respective A and B horizons ($p < 0.001$), where both kauri and pine LL samples at least doubling values observed in pasture LL (Table 2). Despite this, C% values observed in kauri A and B horizon are significantly greater than those seen in pine ($p < 0.05$). C:N ratios observed in all pasture site-sample levels are significantly lower than those observed in kauri or pine ($p < 0.001$). While A and B horizons of both Kauri and Pine display similar values of C:N, kauri LL is significantly greater in C:N than pine LL, at least by a factor of 2 ($p < 0.001$).

Table 2.1 Significance table of main effects and interactions for all environmental variables including F values. Significant differences are denoted by an asterisk (* P < .05. ** P < .01. *** P < .001), while ns refers to non-significance.

Physicochemical variables	**Land-use [*F*(2,45)]**	**Site-samples [F(2,45)]**	**Interaction [F(4,45)]**
pH	3.86*	ns	ns
EC	16.5***	24.1***	3.14*
N%	25.1***	140.5***	11.4***
C%	24***	276.5***	6.5***
C:N	151***	55.1***	19.4***
	Land-use [*F*(2,30)]	**Site-samples [*F*(2,30)]**	**Interaction [*F*(2,30)]**
P content	23.33***	ns	ns

Table 2.2 Soil physicochemical properties (mean ± standard deviation) split by levels of land-use and site-samples. Values that share the same letter along each row are not significantly different under Tukey's HSD ($p < 0.05$).

	Kauri			***Pasture***			***Pine***			***Unit***
Environmental variable	A	B	LL	A	B	LL	A	B	LL	
pH	5.92±0.3^{a}	5.99±0.3^{a}	5.98±0.6^{a}	5.77±0.3^{a,b}	5.61±0.4^{a,b}	5.87±0.4^{a,b}	5.55±0.2^{b}	5.62±0.4^{b}	5.71±0.4^{b}	-
Olsen P	3±0.6^{a}	3.17±0.8^{a}	-	26.3±11.8^{b}	10.17±4.4^{b}	-	6.8±6.1^{a}	5.8±6^{a}	-	mg/L
EC	96.3±25^{c,d}	84.9±63^{d}	194.1±108^{b,c,d}	346.3±250^{b}	114.2±60^{b,c,d}	871.2±234^{a}	138.9±53^{b,c,d}	108.8±48^{b,c,d}	226.2±92^{b,c}	µS/cm
N%	0.32±0.08^{c,d}	0.29±0.09^{c,d}	0.64±0.29^{b}	0.52±0.21^{b,c}	0.3±0.06^{b,c}	1.6±0.33^{a}	0.21±0.08^{d,e}	0.13±0.02^{e}	1.23±0.18^{a}	%
C%	8.1±2.6^{c}	7.5±3.6^{c,d}	46.3±2.2^{a}	5.9±2.5^{c,d,e}	4.1±0.9^{d,e,f}	21.6±6.5^{b}	4.1±1.6^{e,f}	2.8±0.3^{f}	39.3±2.5^{a}	%
C:N	25.1±2.3^{b,c}	24.5±3.7^{b,c}	85.2±38.4^{a}	11.5±0.9^{d}	13.6±0.9^{d}	13.1±1.6^{d}	19.9±0.7^{c}	21.1±1.6^{c}	32.6±6.3^{b}	C/N

2.4.3 Spore count data analysis

The null hypothesis of no difference in all *Phytophthora* spore counts between land-use (kauri, pasture and pine) and site-sample (LL, A and B horizon) treatments, both on and across observational days (Table 2.3 and 2.4), was tested statistically using two-way ANOVA tests. Control counts were separately analysed (Tables A.1, A.2 and A.3; Fig. A.1 - 6) with the soil land-use counts, to provide insights into impacts of contrasting land-use/site-sample treatments on *P. agathidicida* spore counts.

Mature sporangia counts were significantly higher in pasture and pine land-uses than kauri on observational days 1 (p <0.05) and 2 (p <0.001), as shown in Fig. 2.6 (Table 2.3). Despite the significant difference, comparisons over days for pasture and pine indicated that it was not sustained up to day 8 (Table 2.4). In contrast, mature sporangia counts in kauri appeared to significantly decrease from day 1 to day 8 (Table 2.4). Although no significant difference was observed between land-uses for juvenile sporangia counts on any day (Table 2.3; Fig. 2.7), comparisons over days reflect patterns in mature sporangia, where both pasture and pine display a significantly greater counts (p <0.05) on observational day 2 in comparison to day 4 and 8 (Table 2.4). In contrast, mature and juvenile sporangia counts in water control samples increased as a linear function of time over the duration of the study (Appendix Table A.3; Fig. A.1 and A.2).

Between land-use comparisons of mature oospore counts were similar to the pattern observed with sporangia, in that both pasture and pine samples displayed significantly greater (p <0.01) levels of oospore production than kauri on observational day 2 (Table 2.3 and 2.4; Fig. 2.8). Furthermore, in comparison to pasture and kauri samples, it was observed that pine samples significantly increased (p <0.01) in mature oospore counts from observational day 1 to day 8 (Table 2.3 and 2.4; Fig. 2.8). Little to no significant difference (Table 2.3 and 2.4) was observed between counts for developing oospores (Fig. 2.9), aborted oospores (Fig. 2.10) and juvenile oospores (Fig. 2.11). Mature oospore counts were not significantly different between the control and any of the land-use samples (Fig. A.3; Appendix Table A.3). In contrast, significantly greater developing oospore (Fig. A.4), aborted oospore (Fig. A.5) and juvenile oospore counts (Fig. A.6) in control samples were observed compared to all land -use samples across most observational days. (Appendix Table A.1 and A.2).

Table 2.3 Two-way ANOVA of each log(+1) transformed spore count variable with soil land-uses (i.e. kauri, pasture and pine) and site-samples (i.e. A and B horizon and LL) as categorical factors, split across each observational day (i.e. day 1, 2 , 4 and 8). Significant F-values are denoted by an asterisk (* P < .05 ** P < .01 *** P < .001) while ns refers to non-significance. MS = Mature sporangia, JS = Juvenile sporangia, MO = Mature oospores, DO = Developing oospore, AO = Aborted oospore, JO = Juvenile oospore.

Observational day	Spore counts	Land-use [F(2,45)]	Site-samples [F(2,45)]	Interaction [F(4,45)]
1	MS	3.83*	ns	ns
	JS	ns	ns	ns
	MO	ns	ns	ns
	DO	ns	ns	ns
	AO	ns	ns	ns
	JO	ns	ns	ns
2	MS	11.53***	ns	ns
	JS	ns	ns	ns
	MO	5.25**	0.44	3.89**
	DO	ns	ns	ns
	AO	ns	ns	ns
	JO	ns	ns	ns
4	MS	ns	ns	ns
	JS	ns	3.29*	ns
	MO	ns	ns	ns
	DO	ns	ns	ns
	AO	ns	ns	ns
	JO	ns	ns	ns
8	MS	ns	ns	ns
	JS	ns	ns	ns
	MO	6.8**	ns	ns
	DO	3.3*	ns	ns
	AO	ns	ns	ns
	JO	ns	ns	ns

Table 2.4 Mean ± standard deviation of all count variables for each soil land-use (kauri, pasture and pine), compared across observational days (i.e. day 1, 2, 4 and 8). Values that share the same letter are not significantly different under Tukey's HSD ($p < 0.05$). MS = Mature sporangia, JS = Juvenile sporangia, MO = Mature oospores, DO = Developing oospore, AO = Aborted oospore, JO = Juvenile oospore.

Spore count	*Land-use*	*Observational day*			
		1	*2*	*4*	*8*
MS	Kauri	6±3.8^{a}	4.2±4.3^{a,b}	4.5±3.7^{a,b}	3±3.8^{b}
	Pasture	6±3.6^{a,b}	9.8±4.9^{a}	3.8±3.1^{b}	4.4±3.5^{b}
	Pine	3.6±2.5^{b}	8.6±3.6^{a}	4±2.7^{b}	4.4±3.2^{b}
JS	Kauri	11.3±7.9^{a,b}	13.9±9.9^{a}	6.1±6.2^{b}	10.2±11.5^{a,b}
	Pasture	10.4±9.7^{a,b}	18.6±10.7^{a}	11.1±13.5^{b}	8.5±7.9^{b}
	Pine	8.9±9.7^{a,b}	13.1±7.3^{a}	4.9±3.4^{b}	6.5±5.7^{b}
MO	Kauri	170.5±73.9^{a}	173.2±42.9^{a}	159.5±44.5^{a}	170.8±47.5^{a}
	Pasture	161±63.9^{b}	201±38.8^{a}	173±52^{ab}	181.6±52.1^{ab}
	Pine	172.7±49.4^{b}	216±48.4^{a}	190.4±38.5^{ab}	230.2±48.3^{a}
DO	Kauri	28.4±31.7^{a}	15.9±22.1^{a}	22.8±17.7^{a}	18.9±25.4^{a}
	Pasture	29.7±28.6^{a}	8±5.6^{a,b}	32.7±43.3^{a}	2.8±3.1^{b}
	Pine	23.4±25.3^{ab}	6.5±11.8^{b}	31.7±34^{a}	13.6±32.9^{ab}
AO	Kauri	2.9±3.6^{a}	1.9±1.7^{a}	1.6±1.8^{a}	0.8±1.4^{a}
	Pasture	2.2±2.5^{a}	1.4±1.9^{a}	1.2±1.5^{a}	0.2±0.4^{a}
	Pine	2.3±3.1^{a}	2.3±4.6^{a}	0.8±1.6^{a}	1±2.6^{a}
JO	Kauri	3.2±2.4^{a}	0.7±4.3^{a}	2.6±1.7^{a}	2.4±2.3^{a}
	Pasture	4.4±4.1^{a}	2.2±2.1^{a}	3±3.4^{a}	1.5±1.6^{a}
	Pine	2.8±2.5^{a}	1.8±1.7^{a}	2.6±2.1^{a}	1.9±1.4^{a}

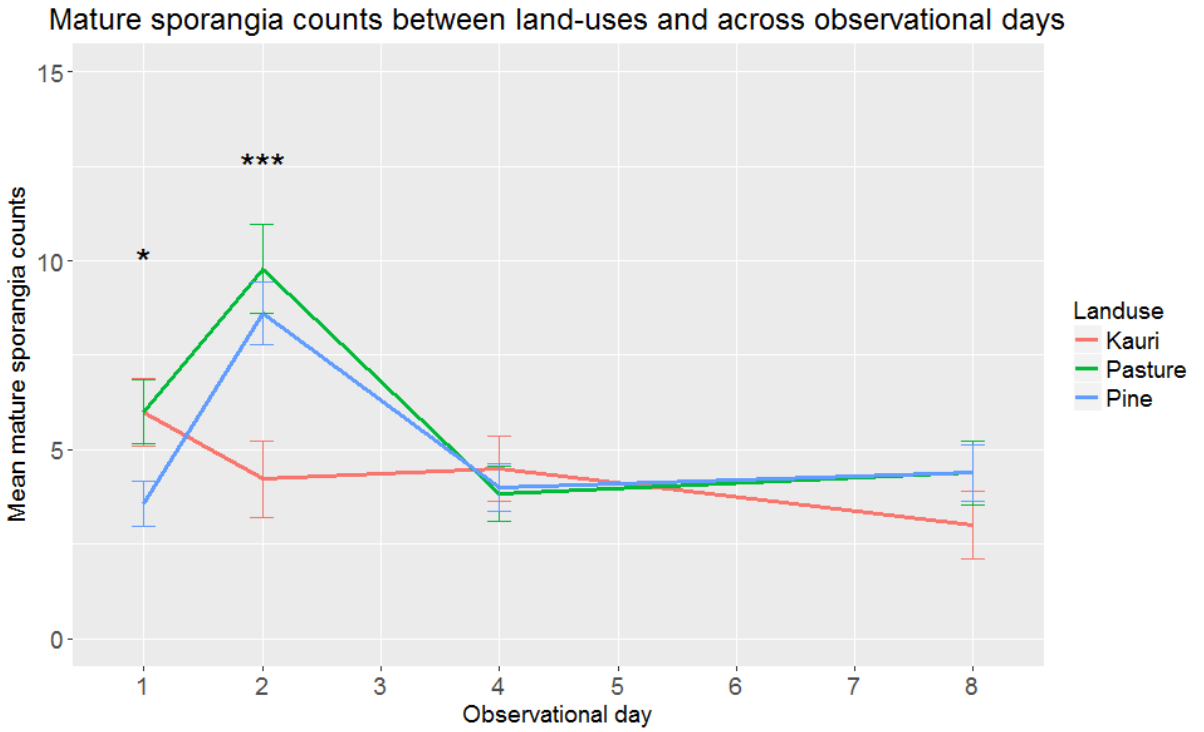


Figure 2.6 Average mature sporangia count between land-uses (kauri, pasture and pine) over time (e.g. 1, 2, 4 and 8 days). Significant differences between land-uses per observational day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.

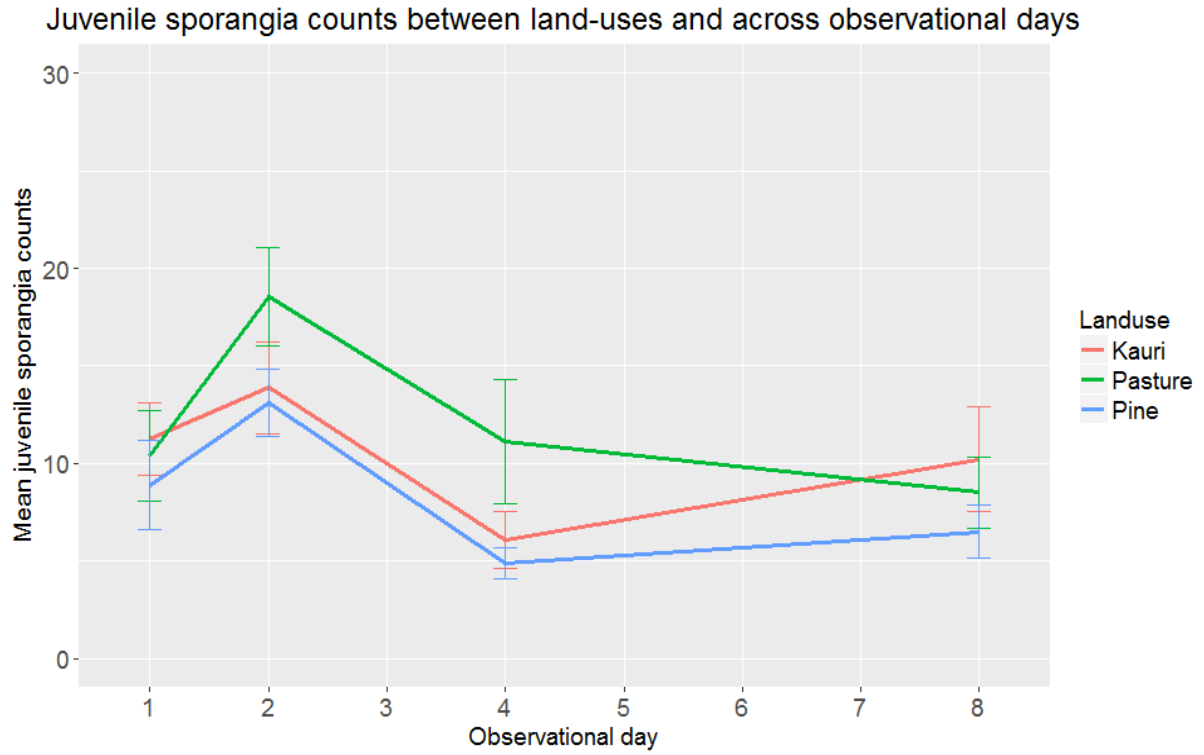


Figure 2.7 Average juvenile sporangia count between land-uses (kauri, pasture and pine) over time (e.g. 1, 2, 4 and 8 days). Significant differences between land-uses per observational day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.

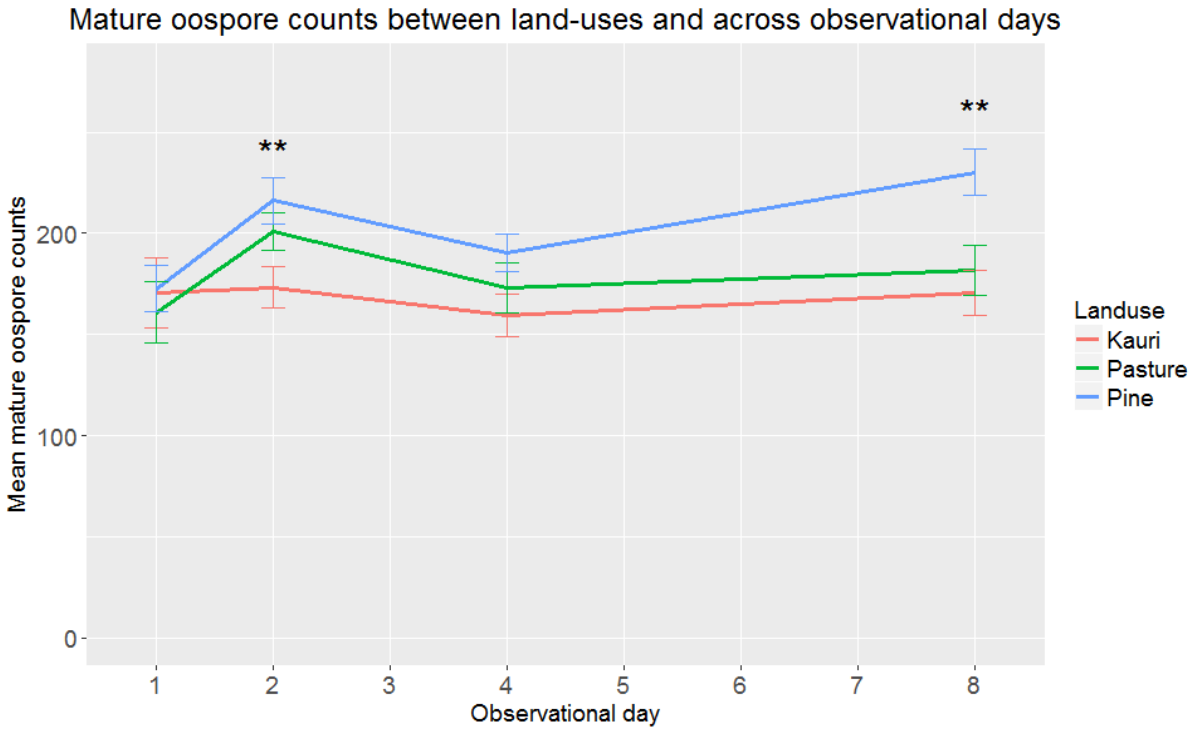


Figure 2.8 Average mature oospores count between land-uses (kauri, pasture and pine) over time (e.g. 1, 2, 4 and 8 days). Significant differences between land-uses per observational day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.

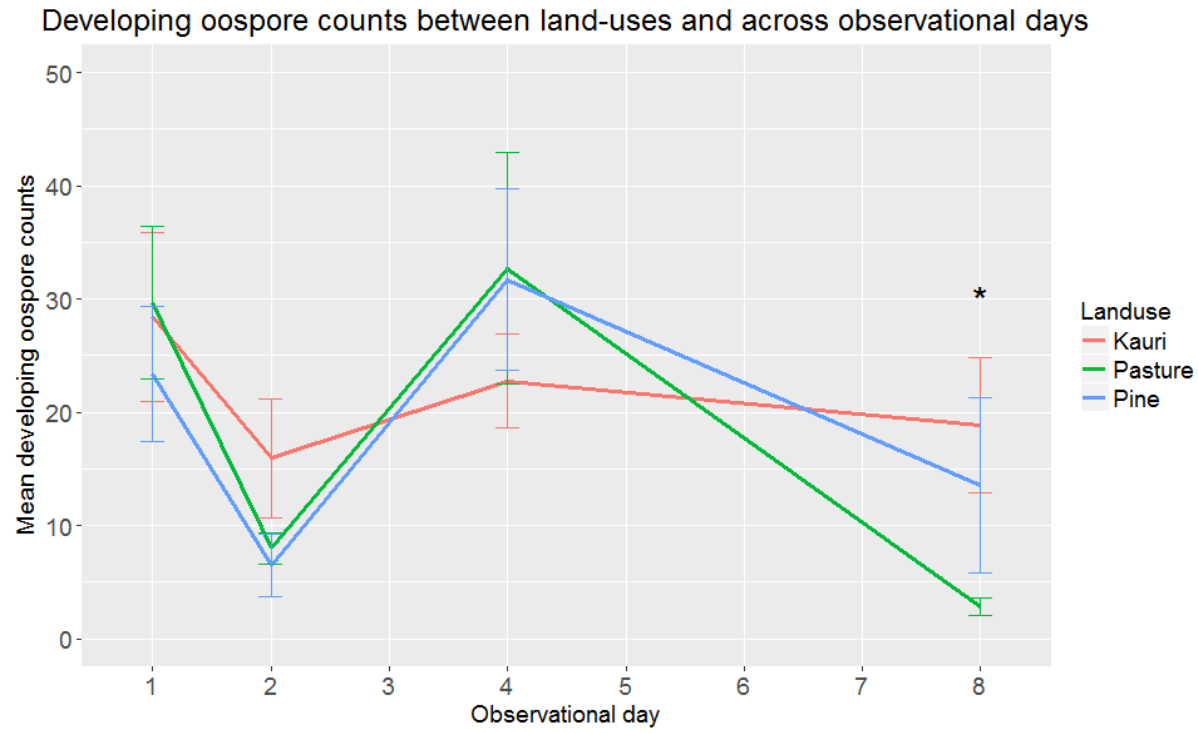


Figure 2.9 Average developing oospore count between land-uses (kauri, pasture and pine) over time (e.g. 1, 2, 4 and 8 days). Significant differences between land-uses per observational day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.

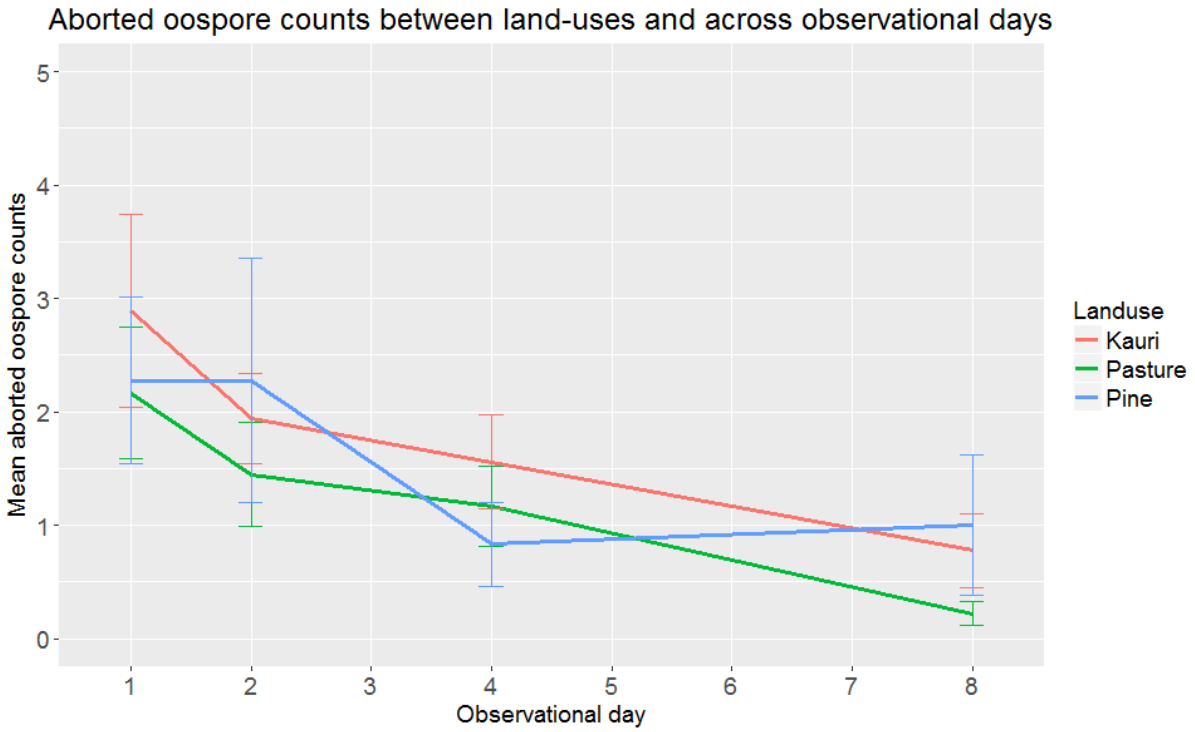


Figure 2.10 Average aborted oospore count between land-uses (kauri, pasture and pine) over time (e.g. 1, 2, 4 and 8 days). Significant differences between land-uses per observational day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.

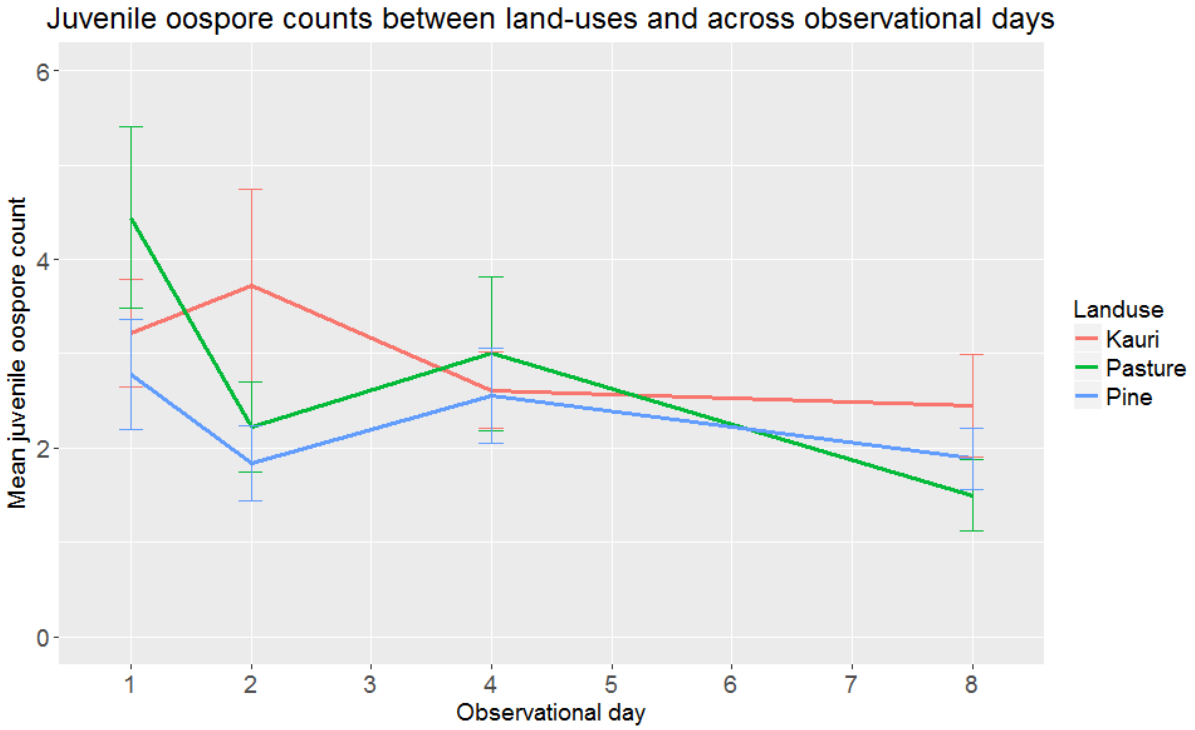


Figure 2.11 Average juvenile oospore count between land-uses (kauri, pasture and pine) over time (e.g. 1, 2, 4 and 8 days). Significant differences between land-uses per observational day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.

2.4.4 Influence of environmental variables on spore counts

As part of the BIOENV analysis, the RELATE function in PRIMER 7 (using Spearman's rank correlation) was used to identify whether the patterns in the biological count matrix were correlated with the patterns in the environmental matrix (Clarke and Ainsworth, 1993). Only observational day 2 showed any significant correlation between the two matrices (r = 0.101, significance = 3.3%). The BEST procedure found that a combination of N% and C:N were the most correlated environmental variables with the count data (r = 0.143). The following DistLM revealed that C and N provided the largest contribution of 6.6% and N% only contributed a further 0.8% (Fig. 2.12). Despite the insignificance between the other observational days and environmental data, pH, EC and N% appear to be the most frequently correlated variables which have percent contributions ranging between 0.5% and 5.6%. This seems to indicate that the environmental variables measured are not a particularly good predictor of difference in count data between land-uses.

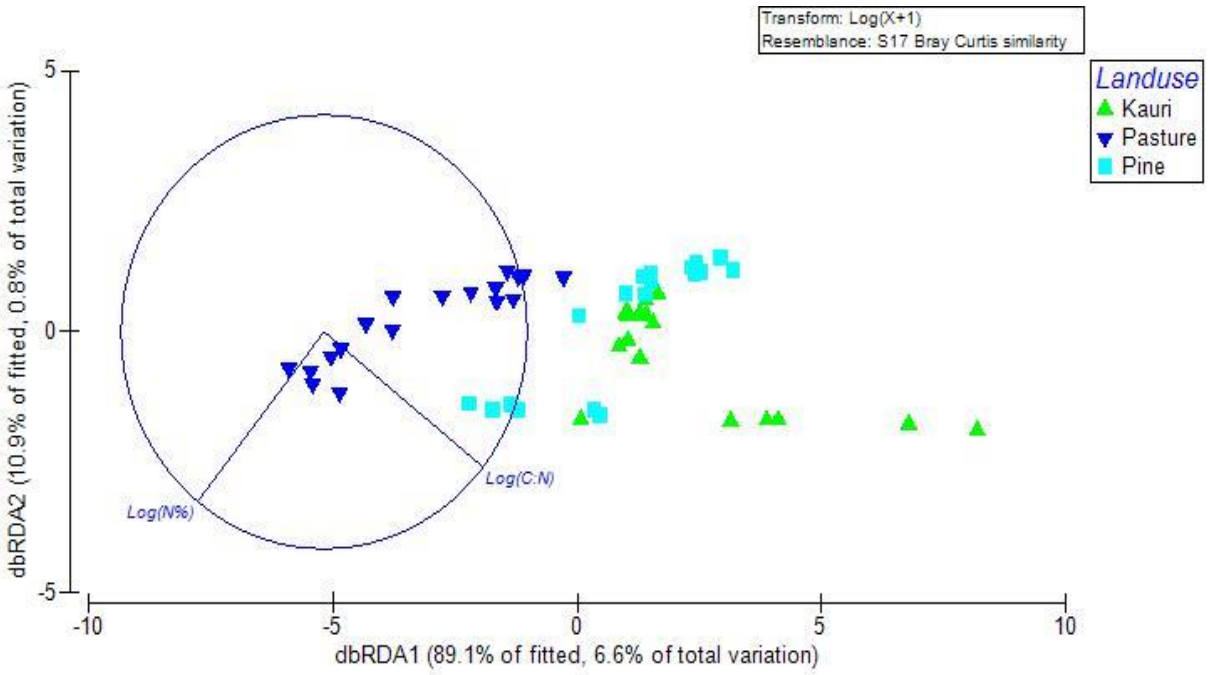


Figure 2.12 DistLM of abundance distribution among the three land-uses of all spore count variables on observational day 2. Environmental variables which best explain the distribution of abundance are reported in the graph as vectors. (resemblance matrix: Bray Curtis similarity; transformation: log(+1); correlation type: Spearman).

2.5 Discussion

2.5.1 *Phytophthora* populations associated with land-use soils

As of late 2014, thirty confirmed species of *Phytophthora* have been reported to be associated with plant diseases in New Zealand (Scott and Williams, 2014). Of these, six recorded species of *Phytophthora* found in tissue retrieved from infected kauri (*A. australis*) and/or kauri forest soil, *P. multivora*, *P. cryptogea*, *P. kernoviae*, *P. cinnamomi*, *P. nicotianae* and *P. agathidicida* (Beever *et al*., 2009; Waipara *et al*., 2013). Soil bioassays in this study have revealed two additional *Phytophthora* species (*P. gregata* and potentially *P. pini*) to be present in kauri or near-kauri ecosystems in New Zealand. Previous literature has shown these two species to be recognized soil-borne pathogens internationally (Hong *et al*., 2011; Jung *et al*., 2011). For instance, *P. gregata* has been recovered from the rhizosphere soil of several dying Australian native plants, including species of the genera *Patersonia*, *Xanthorrhoea* and *Hakea* (Jung *et al*., 2011). Similarly, *P. pini* is recognised as an established pathogen in North America and Europe where it is known to infect at least seven genera (Hong *et al*., 2011), and may potentially impact a variety of other ornamental and vegetable plants, in addition to European beech trees (Jung *et al*., 2009). Like many other *Phytophthora* spp., these appear to be opportunistic pathogens associated with sporadic but severe mortality in wet and water logged areas (Hong *et al*., 2011; Jung *et al*., 2011). It is likely that these species, among a variety of yet to be characterised *Phytophthora* spp., have always been present in New Zealand soils (P. Scott, personal communications). While it is unclear whether *P. pini* and *P. gregata* are significant contributors to plant disease in New Zealand, investigation of their potential impact on native plant species and horticultural industries internationally is warranted. Additionally, given the presence of *P. gregata* in kauri soils, it may prove prudent to include this pathogen in future research studies looking at the combined impacts of multiple pathogens present (e.g. *P. cinnamomi* and *P. agathidicida* in particular) in kauri forests.

The impact of indigenous microbial populations on the establishment and disease potential of invading *Phytophthora* species has been demonstrated previously (Erwin and Ribeiro, 1996). For example, ectomycorrhizal fungi such as *Leucopaxillus cerealis* var. *piceina* have been discovered to protect against shortleaf pine (*Pinus radiata*) onset by *P. cinnamomi* infection, by providing a physical barrier to pathogen invasion, and by secreting inhibitory antibiotics such as diatretyne nitrile (Marx and Davey, 1969; Shea and Broadbent, 1983). Similarly, the impact of *Phytophthora* spp. on *Phytophthora* caused diseases has also been examined (Lucas,

1991). For example, protection against the infection of avocado (*Persea* spp.) by *P. cinnamomi* root rot has been achieved by previous inoculation of other *Phytophthora* species (Lucas, 1991). Localised protection against *P. citricola* and *P. cinnamomi* infection was provided by initial inoculation of avocado trees by a non-pathogenic isolate of *P. nicotianae* (Dolan *et al*., 1986). The interactions between pathogenic and non-pathogenic isolates of *Phytophthora* suggest that the presence of *P. pini* and *P. gregata* in the soil samples (e.g. pasture and kauri), may have had an impact on the growth characteristics of *P. agathidicida* and potentially its disease potential. Given the lack of information surrounding the ecological interactions of *P. agathidicida,* this warrants further investigation into the interactions and impact on disease potential of *P. agathidicida* with other *Phytophthora* spp. found in association with kauri ecosystems, as identified above.

2.5.2 Kauri Dieback Survey

The kauri dieback survey provides some context to this study by demonstrating not only the presence of *P. agathidicida* within Waipoua Forest, but also near the kauri forest sampling sites used in this study. These results confirm those in previous surveys which have characterised Waipoua Forest as one of the largest remnants of kauri forests containing *P. agathidicida* (Waipara *et al*., 2013).

2.5.3 Physicochemical variable analysis

Land-use physicochemical analysis

Significance testing showed that the land-uses under study displayed contrasting differences in all physicochemical variables measured (Table 2.1 and 2.2). Differences in these properties is not surprising given the differing plant species composition in each land-use which influences the content of their respective surface litter and soil horizons (Brockerhoff *et al*., 2003; Goh and Bruce, 2005; Verkaik *et al.*, 2006). For example, kauri forests are known to be the most diverse forest type in New Zealand, containing an assemblage of woody and non-woody plant taxa such as *Alseuosmia macrophylla* (Toropapa), *Dracophyllum latifolium* (Neinei), *Leionema nudum* (mairehau) and *Astelia trinervia* (kauri grass) (Wyse, 2012). With a high

proportion of woody material and reproductive structures (e.g. 13-21% total litter-fall) making up its litter-fall (Enright, 1999), a relatively high C% can be expected, as observed in this study (Table 2.2). Similarly, in the commercial pine plantation studied, the predominant plant species was *Pinus radiata,* which is also known to result in high C% accumulation in LL due to a high proportion of woody tissues deposited on the forest floor (Carey *et al*., 1982). In contrast, pastures typically contain non-woody legume and ryegrass species; in New Zealand pastures perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) species predominate (Goh and Bruce, 2005). This sets up for substantially different C% accumulation in litter (Goh and Bruce, 2005), which is reflected in the significant difference observed between kauri and pine LL samples compared to pasture LL samples (Table 2.2). Although sharp decreases in C% are observed in A and B soil horizons for both kauri and pine (Table 2.2), greater C% values seen in kauri soil samples compared to pine is consistent with previous literature which states that differences can be due to both storage and turnover rates in their respective soils (Silvester and Orchard, 1999).

Larger N% values observed in kauri A and B horizon compared to pine can be attributed to a small but significant rate of asymbiotic N fixation in decaying leaves (Silvester, 1978). Meanwhile, greater N% values in pasture samples may be due to a combination of N fertilizer addition, urea contributions of grazing animals and biological fixation by rhizobium bacteria (Ball, 1969; Goh and Bruce, 2005). Given the comparatively high C% in kauri and pine to pasture samples, substantially different C:N values were observed (Table 2.2); kauri has at least twice the C:N values seen in all pasture samples. This pattern was also observed with respect to phosphorous (P), where significantly larger P values were observed in pasture samples relative to both kauri and pine samples. These values are likely to be influenced by phosphorous fertilizer loadings (Loganathan *et al*., 2003).

Soil electrical conductivity (EC) has been shown to be primarily a function of soil salinity, saturation percentage, water content and bulk density (Rhoades *et al*., 1989). Understanding that the predominant mechanism causing salt accumulation in agricultural soils is evapotranspiration from irrigation water, this likely contributes to the significantly greater EC values observed in pasture samples relative to non-irrigated forest soils (Corwin and Lesch, 2003; Guretzky *et al*., 2004). Although no significant difference in pH is observed between pasture and pine samples, results show that kauri soils had a significantly higher pH than soil from under pine forest (Table 2.1 and 2.2). While this appears contradictory to a wide body of

research recognising kauri soils to display low values of approximately pH 4 (Silvester and Orchard, 1999; Steward and Beveridge, 2010; Wyse and Burns, 2013), the kauri sampling site from this study primarily consisted of young (i.e. ~30 years) and ricker (i.e. aged 30-50 years) individuals which may explain this difference as they are too young to have developed significant podzols beneath them. In studies investigating mature kauri stands, low pH values have been attributed to large quantities of organic matter on the soil surface, alongside tannin-rich litter leachates that create the characteristic acidic, infertile and highly podsolised soils (Wyse, 2012; Verkaik *et al*., 2006).

Influence of physicochemical factors on *P. agathidicida* spore counts

Although there are significant differences in the physicochemical variables measured between land-uses (Table 2.1 and 2.2), the relative contributions of these edaphic soil factors to the variation explained in the spore count data suggest that they are not the most important environmental variables in determining relative spore counts of sporangia and oospores. Conversely, this suggests that these physicochemical factors are not limiting the growth of *P. agathidicida,* and may potentially impact the risk of pathogen dissemination and establishment within the three land-uses studied. Unfortunately, this further implies that these results cannot be utilised in a predictive tool to assess the relative risk of different land-uses to establishment of *P. agathidicida*. A follow-up investigation of the physiological constraints, sporulation patterns and dispersive risk of *P. agathidicida* with respect to varying concentrations of numerous environmental factors (e.g. C:N and pH) would be advantageous. This would significant inform the development of a predictive conservation tool to assess the establishment risk of *P. agathidicida* within different land-uses/sites of differing soil characteristics.

2.5.4 Count variables

Observations from this study indicate that land-uses have a significant impact on all *Phytophthora* sporangia and oospore counts compared to water control samples, on and across observational days (Appendix Table A.1 - 3; Appendix Fig. A.1 - 6). In contrast to this, significant differences between land-uses (e.g. kauri, pasture and pine) were only observed for a subset of the spore metrics (e.g. mature sporangia and oospores) quantified in this study (Table 2.3 and 2.4; Fig. 2.6 – 2.11).

Asexual sporangia

Members of the genus *Phytophthora* have been shown to enter a prolific and rapid asexual sporulation period following optimal levels of important environmental conditions such as temperature and water availability (Bernhardt and Grogan, 1982; Hardham, 2005; Judelson and Blanco, 2005). This has been observed in *P. megasperma* on apples (Duniway, 1983) and *P. infestans* on potatoes (Andrivon, 1995), where substantial numbers of sporangia (~10^5-10^6 spores/cm^2 of leaf tissue) were produced within a wetting period of one night under optimal temperature conditions. Even under these conditions, the speed and pattern of sporulation has been noted to vary among *Phytophthora* species and isolates (Bernhardt and Grogan, 1982; Erwin *et al*., 1983). For example, Gisi *et al*. (1980) reported that sporangia production of *P. palmivora* increased steadily from 1 to 4 days of incubation in saturated soil, whereas maximal sporangia production in *P. cinnamomi* occurred within 1 day, and declined thereafter.

The asexual sporulation patterns observed in these other *Phytophthora* species (e.g. *P. palmivora*) corroborate the patterns observed in the present study, where sporangia production (e.g. mature and juvenile sporangia) significantly increased as a linear function of time in water control samples over the duration of the study (Table A.3; Fig. A.1 and A.2). In contrast, soil land-use sporangia counts remained non-significant between observational days 1 and 8, clearly demonstrating a land-use effect on sporulation patterns in *P. agathidicida* compared to water controls (Table 2.4). Despite this non-significant result across observational days, between land-use comparisons identified significantly greater mature sporangia counts in both pasture and pine samples compared to kauri samples, within two days of the observational study (Fig. 2.6; Table 2.4).

This initial rise in sporulation count seems to suggest that the pasture and pine forest soil favour sporangia production in the early stages of *P. agathidicida* establishment compared to kauri soils. The implications of this finding are significant as it indicates that these former soil environments not only increase inoculum loads, but may also act as pathogen reservoirs of *P. agathidicida*. Furthermore, as sporangia and its associated zoospores are recognised as the primary infective/dispersive agents of *Phytophthora* spp., increased sporulation in these land-uses may increase the dispersive potential of the pathogen (Erwin and Ribeiro, 1996). It is understood that sporangia of most non-caducous (i.e. no separation of sporangia from sporangiophore) soil-borne *Phytophthora* species such as *P. agathidicida* are not likely to be

dispersed widely from their point of formation within the soil without external intervention (e.g. rain water, animal/insect/human vectors) (Duniway, 1983; Waterhouse, 1974). Although dispersive potential, and often disease incidence, increases with a variety of factors including season, local topography, soil-type and the presence of moving water (Duniway, 1983; Weste, 1983), these factors typically represent distances between 6 to 400 metres per annum (Weste and Marks, 1987). In contrast, a recent study of the dispersal of *P. cactorum* discovered that human activity in the form of hiking and mountain biking contributed to a dispersal of at least 60-100 metres per individual (Cushman *et al*., 2008). While the type of human activity cited in the previous study is likely to be minimal near the sampling sites used in this study due to a lack of hiking/mountain biking trails, the results of the study clearly exemplify the extreme dispersive risks associated with human activity. Moreover, given that accessibility to and within pasture sites would typically be higher than corresponding forest sites, it also implies that pasture land-uses may contribute more to the dispersive potential of *P. agathidicida*. An investigation into the role of unfenced rural pastoral properties adjacent to kauri forest sites in the dissemination potential of *P. agathidicida* is recommended.

In addition to human vectors, pathogen dissemination is likely to be further exacerbated through impacts by livestock and other animal vectors in pasture sites (Weste, 1983). For example, the frequent recovery of *P. agathidicida* from kauri root zones exposed to significant soil disturbances by livestock (Beauchamp, 2013) indicated the potential for soil-borne vectoring of this pathogen between unfenced rural kauri fragments and adjacent pastoral properties (Waipara et al., 2013). Although two studies (Basset *et al*., 2017; Krull *et al*., 2013) have been inconclusive on the dispersive potential of feral pigs for *P. agathidicida*, further studies assessing the role of livestock in *P. agathidicida* dispersal between unfenced pastoral properties adjacent to kauri forest sites are required given the results of this study.

Sexual oospores

Due to their fragility, and the energetic requirements involved in responding quickly to optimal environmental conditions, asexual sporangia are ineffective resting structures (Judelson and Blanco, 2005). Instead, thick-walled sexually produced oospores allow for viable inoculum to survive across seasons (Hardham, 2005; Judelson and Blanco, 2005; Waipara *et al*., 2013; Weir *et al*., 2015). The results of this study show that mature oospore production follow reasonably similar patterns to sporangia counts, suggesting that pasture and pine land-uses favour oospore

production of *P. agathidicida* over kauri soils in the early stages of establishment (Fig. 2.8; Table 2.4). Moreover, with a significant increase in oospore production from day 1 to day 8 (Table 2.4) in pine soils, this may imply these soils harbour greater potential to act as pathogen reservoirs given their impact on inoculum loads. As such, further investigations into the interactions between pine forest soil and sexual reproduction of *P. agathidicida* are required to elucidate this relationship.

The higher oospore production in pasture and pine forest soil within two days of the study further contributes to the concern that these soils may not only increase inoculum loads, but also act as pathogen reservoirs of *P. agathidicida.* This is exemplified by reports of *Phytophthora* oospores remaining viable for several years in moist soil (e.g. *P. cinnamomi* for up to 6 years, and *P. fragariae* for at least 3 years) (Duncan, 1980; Hardham, 2005), suggesting that once established, these soil environments may allow long-term persistence of *P. agathidicida* inoculum loads. Long-term investigations of the impact these soils have on the survival of *P. agathidicida* oospores is recommended, to understand whether this inoculum increase is an isolated or multiplicitous event.

The persistence of *P. agathidicida* in pasture and pine forest sites is likely exacerbated due to indications that the currently utilised disinfectant in phytosanitary stations, trigene, is ineffective against *P. agathidicida* oospores (Bellgard *et al*., 2010). As a consequence, this would suggest that the biosecurity measures put in place at kauri forest entrances (e.g. phytosanitary stations with trigene sprays/baths) are less effective than previously thought at restricting the movement of *P. agathidicida* from infected kauri sites to surrounding areas (Bellgard *et al*., 2010). Furthermore, given the accessibility and human/animal vector related dispersive risks associated with pasture sites, this would suggest that adjacent pastoral properties may act not only as pathogen reservoirs but also as potential incursion pathways into non-infected kauri forest sites despite placement of phytosanitary stations at the entrance; potentially further exacerbated by unfenced adjacent pastoral sites. These implications raise the possibility that oospores play a greater role in the dissemination of *P. agathidicida*, and hence potentially the disease impact of kauri dieback as a whole.

Although it is generally recognised that survival structures such as oospores are produced in response to environmental stressors (e.g. low soil moisture, low nutrients) adverse to the production of asexual sporangia, many abiotic (e.g. temperature, water availability and pH)

and biotic (e.g. microbial metabolites and antagonism) factors have been demonstrated to affect oospore formation in *Phytophthora* species (Duniway, 1983; Schmitthenner and Canaday, 1983; Weste, 1983). Further examples such as host resistance (Drenth *et al.*, 1995; Hanson and Shattock, 1998), fungicide application (Groves and Ristaino, 2000), drought conditions (Singh *et al.*, 2004) and high humidity (Cohen *et al.*, 2000), and the variable or contradictory response of sporangia/oospore formation to these stressors results in complex patterns of asexual/sexual reproduction (Duniway, 1983; Elliot, 1983; Schmitthenner and Canaday, 1983). Furthermore, the importance of these factors varies with *Phytophthora* species, and even within isolates of the same species (Ribeiro, 1983), suggesting that a *P. agathidicida* specific assay for its response to these different factors would be required.

2.5.5 Environmental drivers of *Phytophthora* spore numbers

It is known that environmental conditions influence the development of *Phytophthora* spp., and the constraints imposed by these conditions largely determine the geographic distribution of specific *Phytophthora* species and the diseases they incite (Duniway, 1983). For example, *P. cinnamomi* is not a pathogen of consequence in field conditions where temperatures consistently fall below freezing (Zentmyer, 1980). In contrast, within the Mediterranean climates of Australia where soil moisture and temperature are highly conducive for *P. cinnamomi* sporulation and survival (Duniway, 1983; Zentmyer, 1980), it has become a highly destructive pathogen for a wide range of hosts (Hardham, 2005), and in particular the jarrah forests (*Eucalyptus marginata*) of Western Australia (Zentmyer, 1980). While temperature and water availability have been demonstrated as two of the most important factors in spore development of *Phytophthora* spp. (Hardham, 2005; Judelson and Blanco, 2005), these conditions were held at an optimal constant (e.g. 21^{o}C and saturated to water holding capacity) throughout the duration of this study (Weir *et al*., 2015). This suggests that the difference in sporangia and oospore production between pasture-pine and kauri samples is associated with some other environmental and/or biological difference between the land-use sites (Duniway, 1983; Judelson and Blanco, 2005; Mitchell and Kannwischer-Mitchell, 1983).

In general, diseases caused by *Phytophthora* spp. are more severe at high soil pH values (Schmithenner and Canaday, 1983). This can largely be attributed to the inhibitory effects of low pH values have on sporangial development and subsequent zoosporogenesis (Jung *et al*., 2000; Schmithenner and Canaday, 1983). For example, Muchovej *et al*. (1980) found that

decreasing soil pH lead to an inhibition of sporangial formation of *P. capsici*, the causal agent of pepper blight in a range of *Capsicum* and *Solanum* species. In the present study, a significant difference between kauri (pH 5.96) and pine (pH 5.63) samples was observed (Table 2.2 and 2.3). However, understanding that the inhibitory effects induced by low pH values ranged between pH 3.0-4.5 for several prominent *Phytophthora* species such as *P. cinnamomi* (Bingham and Zentmyer, 1954), *P. infestans* (El Fahl and Calvert, 1976), and *P. nicotianae* (McCarter, 1965), the difference observed here may only reflect a statistical, and not a practical one concerning pH effect on sporangial development. Further investigation of the impact of varying pH gradients on *P. agathidicida* sporulation and sexual reproduction is required.

In addition to the role of physical and chemical factors, biological interactions in the form of microbial metabolite production and antagonism are also potential contributing factors to the difference in sporangia counts observed between kauri, and pasture and pine samples on observational day 2 (Malajczuk, 1983). For example, *P. cinnamomi* has been demonstrated to be dependent on metabolites from bacteria (e.g. *Pseudomonas* spp.; Ayers, 1971) and fungi (e.g. *Trichoderma* spp.; Brasier, 1975) to stimulate production of both sporangia and oospores (Pratt *et al*., 1972). Meanwhile, a study investigating soil recovered from an avocado grove in Tamborine Mt, Queensland, Australia, discovered significantly greater numbers of Actinomycetes and *Bacillus* spp. in disease suppressive soils compared to soils conducive to root rot by *P. cinnamomi* (Broadbent and Baker, 1974). In other words, greater numbers of Actinomycetes and *Bacillus* spp. were highly correlated with biological antagonism to the host pathogen, *P. cinnamomi*. The authors argued that the presence of these bacterial/fungal species inhibited sporangial formation, and that any subsequent infection of Avocado trees by *P. cinnamomi* were reduced. Therefore, further studies investigating the role of different microbial populations retrieved from each of these land-uses on the growth response of *P. agathidicida* would contribute to a better understanding of the influence soil ecology has on *P. agathidicida* adaptation and establishment characteristics.

2.6 Conclusion

Two new *Phytophthora* spp. to New Zealand (i.e. *P. pini* and *P. gregata*) were discovered in this study. The recognition of these *Phytophthora* isolates as international plant pathogens of a multitude of native and horticultural plants warrants further research into their impact on New Zealand plant species. Production of sporangia and oospores through both reproductive cycles in *P. agathidicida* are significantly impacted by different land-uses. While soils collected from pasture and pine supported significantly higher inoculum loads (e.g. mature sporangia and oospores) within two days of the observational study compared to kauri samples, differences in production patterns between all land-uses were not sustained through to days 4 and 8, suggesting the effect was short-lived. Despite this, concerns over human/animal vectoring, accessibility and the potential for these land-use sites to act as pathogen reservoirs, particularly in pasture land-use sites, warrant further research into the impact of fragmented landscapes on *P. agathidicida* transfer and epidemiology. Contrary to the sporangia counts, oospore production significantly increased over observational days in pine, indicating these soils increase inoculum loads, further increasing their capacity to act as pathogen reservoirs. Although significant differences are observed in the environmental variables between levels of both land-use (kauri, pasture and pine) and site-sample (LL, A and B horizon), they did not explain variation observed in the spore count data. This suggests that some other combination of environmental variables may need to be included for developing a predictive tool for assessing the risk of *P. agathidicida* establishment, which as demonstrated in this study, has been shown to vary across the three land-uses.

Chapter 3:
Impact of contrasting land-uses on *Phytophthora agathidicida* pathogenicity using blue lupin (*Lupinus angustifolius*) as a model plant system

3.1 Introduction

Disease potential and virulence of plant pathogens is inherently spatial, with each aspect of disease epidemiology from establishment to dispersal, influenced by spatial variations in biotic (e.g. microbial antagonists) and abiotic (e.g. temperature, moisture, slope gradient) factors between contrasting land-uses (Condeso and Meentemeyer, 2007; Duniway, 1983; Holdenrieder *et al*., 2004; Malajczuk, 1983). As such, interest in the impact of heterogeneous landscapes on pathogen epidemiology has increased (Condeso and Meentemeyer, 2007; Holdenrieder *et al*., 2004). Several papers have confirmed variations in establishment and virulence characteristics of *Phytophthora* species across heterogeneous landscapes (Anacker *et al*., 2008; Anderson *et al*., 2004; Holdenrieder *et al*., 2004; Shearer *et al*., 2010). For example, a study by Jung *et al*. (2000) studied the impact of site factors (e.g. soil pH, soil texture/composition) on *Phytophthora* (e.g. *P. quercina*, *P. cambivora* and *P. citricola*) involvement in Central European oak (*Quercus robur*) decline. They were unable to isolate any *Phytophthora* from stands with sandy to sandy-loam soils and a mean soil-pH of <3.9, which coincided with little observed oak decline in these sites. In contrast, oak stands located on sandy-loam to clay sites with a mean soil-pH >3.5 displayed significant oak decline (Jung et al., 2000). The authors hypothesised that while soil pH likely influences sporangia and zoospore germination rates as has been demonstrated previously (Schmitthenner and Canaday, 1983), the structure of the soils may also contribute to the difference in disease incidence. Soil particulates influence the free-water period, therefore, sandy soils which allow for rapid drainage may have resulted in less than optimal moisture levels to allow for sporulation and zoospore release (Jung et al., 2000). Similarly, a study investigating disease impact of *P. ramorum* on *Umbellularia californica* (large Californian hardwood), discovered that tree-to-tree susceptibility at a regional scale was superseded by environmental factors at the local scale (Anacker *et al*., 2008). This study indicated that although disease incidence was affected by

variability in *P. ramorum* pathogenicity between isolates (Hüberli *et al*., 2012), and the genetic susceptibility of *U. californica* individuals, environmental factors such as temperature, humidity and spatial position (Anacker *et al*., 2008; Davidson *et al*., 2005) were the major determinants of *P. ramorum* activity and disease expression locally.

Although these examples represent large-scale spatial studies in the field, they clearly demonstrate the importance of identifying the impact of heterogeneous landscapes on *Phytophthora* spp. dissemination and infection. In order to identify potential landscape impacts on *P. agathidicida* pathogenicity, the current study aims to characterise the pathogenicity (e.g. lesion presence, lesion length, lupin length etc) of *P. agathidicida* on blue lupin (*Lupinus angustifolius*) within the soils collected from three contrasting land-uses (e.g. indigenous kauri forest, commercial pine forest and pasture land), present in Waipoua Forest. It is anticipated that this study will aid in understanding *P. agathidicida* pathogenicity between contrasting land-uses within Waipoua forest, and help assess the potential for a large-scale spatial study.

3.2 Research aim

This study aimed to determine if soils retrieved from three contrasting land-uses in Waipoua Forest (e.g. kauri forest, pasture land and pine forest) affect *Phytophthora agathidicida* pathogenicity using blue lupins (*Lupinus angustifolius*) as an alternative host plant.

3.3 Materials and methods

3.3.1 Study area

Samples were collected from the same three land-uses (e.g. kauri forest, pasture and commercial pine forest) as in Chapter 2 (2.3.1.), in Waipoua Forest.

3.3.2 Sampling period

Soil samples were collected in late-September (Spring) 2017. In Waipoua Forest, temperatures were on average 2^{o}C (11^{o}C low and 17^{o}C high) greater than historical September averages for

the past ten years (Waipoua forest local weather, 2017). This is similar to the warmer than average climate conditions recorded from the previous sampling period in April (2016). While the average precipitation during September 2017 was 5 mm below the historical average (90mm) for this month (Waipoua forest local weather, 2017), it was substantially greater than the precipitation for the previous sampling period (2016) where 26 mm was observed.

3.3.3 Sample collection and processing

Soil samples were collected from the same six sampling sites per land-use as in Chapter 2. However, only A horizon soil samples were retrieved due to the largely insignificant differences observed between site-samples (leaf litter - LL, A and B horizon) in the *P. agathidicida* growth response study (Chapter 2). Approximately 2 kg of soil per sampling site per land-use was collected and separated into re-sealable plastic bags for two-day transport to Lincoln University, Christchurch, New Zealand. Following collection and transport, samples were stored within large plastic containers in a 4^{o}C walk-in fridge prior to initiating experimental procedures.

3.3.4 Selective medium

A mixture of 10% carrot agar, ampicillin, pimaricin, rifampicin, nystatin and hymexazol (CRNH) medium was used in this study as the *Phytophthora* selective medium. Details are reported in Chapter 2 (2.3.5).

3.3.5 Inoculum development (kauri root plugs)

Phytophthora agathidicida inoculum plugs were prepared using a modified method of Butcher *et al* (1984), where kauri roots (*Agathis australis*) were used instead of pine (*Pinus radiata*) branches. *P. agathidicida* (NZFS 3813), originally isolated from infected soil samples in Coromandel, was incubated at 21^{o}C for 7 days on CRNH selective media (Weir *et al*., 2015). Live roots of 2-year old kauri were cut into segments approximately 1-2 cm long, soaked overnight in distilled water, rinsed and transferred to six 250 ml conical flasks (100 plugs per

flask). Following the addition of distilled water to sufficiently cover the kauri plugs, they were autoclaved to 121^{o}C at 100kPA (15 psi) for 15min, then cooled to room temperature (~18^{o}C). Five 5 mm agar plugs of the incubated *P. agathidicida* isolate were then placed into the flasks containing the 100 kauri root segments and incubated at 21^{o}C for 2-3 days to ensure infection of kauri root plugs. The flasks were gently shaken on an orbital shaker (50 rpm) to oxygenate and disperse inoculum, and were then incubated for a further 1-2weeks at 21^{o}C. Following this infection period, a small sub-sample of kauri root plugs from each inoculation flask was confirmed for infection via surface sterilisation (30 seconds 70% ethanol, follow by two 30 seconds rinses sterile water) and plating onto CRNH selective medium. Light microscopy of transversely cut sections of kauri root plugs confirmed the presence of sexually produced oospores as the main inoculum; these root plugs were utilised as the inoculum source for the following pathogenicity experiments.

3.3.6 Host plant

Blue lupin (*Lupinus angustifolius*; Mitre 10 MEGA) was selected as the host plant species as previous infection and baiting bioassay studies of *Phytophthora* have long utilised blue lupin (Chee and Newhook, 1965; Pratt and Heather, 1972; Weir *et al*., 2015). Similarly, blue lupin has been previously used for baiting *P. agathidicida*, as shown by Beever *et al*. (2009), and is now the standardized bait system used across all laboratories undertaking *P. agathidicida* surveillance in New Zealand (Waipara, *Personal communication*). Lupin is an ideal model host plant because it produces thick, white susceptible radicles and has uniform, rapid and high germination rates (Chee and Newhook, 1965). Following inoculation with *Phytophthora* spp. such as *P. cinnamomi*, lesions develop rapidly and these typically appear as sharply defined brown/orange lesions (Pratt and Heather, 1972).

3.3.7 Lupin pathogenicity study

Prior to conducting the land-use soil pathogenicity study, a study was carried out to assess the pathogenicity effects of *P. agathidicida* on blue lupin seedlings under sterile growth conditions. This was in order to not only confirm *P. agathidicida* infection of blue lupin, but also to assess pathogenicity effects (e.g. height, lesion number and length) following infection. Auto-claved

coarse river sand (Drymix Coarse Sand 30 kg, Mitre 10 MEGA) was used as a growth substrate to remove any soil influence on pathogenicity. Three replicate pots of each of the two treatments, inoculated and control, were harvested on five harvest days spread over a 15 day period (e.g. HD 1 = day 3, HD 2 = day 6 etc); resulting in a total of 15 treatment and 15 control replicate 0.5 L plastic tub containers (2 isolate treatments (inoculated/control) x 3 replicates x 5 harvest days). Plastic containers each contained 250g of sterilised coarse river sand and were adjusted with 60 ml sterile water so pots were at a uniform moisture level. As the inoculum plugs were smaller than those used in Butcher *et al*. (1984), each treatment pot was thoroughly mixed with 10 inoculated kauri root plugs (instead of 4) to ensure uniform infection. Control pots were similarly treated with 10 kauri root plugs, however, all plugs were autoclaved prior to addition to sand to prevent contamination. Blue lupin seeds were pre-germinated in sterile vermiculite for 4 days prior to initiating the study. Seedlings were measured (root tip to beginning of cotyledon) and five seedlings were planted into each treatment container. Following this, all containers were allocated to five separate shelves in an incubator according to their harvest day, and subsequently randomised to a position within a 2 x 3 rectangle using a split plot randomised design in R (R Development Core Team, R version 3.4.0). For the duration of the study, a 12 hour light/dark photoperiod was maintained, while temperature was held constant at 21^{o}C. 20 ml sterile deionised water was used to top up each container every 24 hours to maintain consistent moisture levels within the sand. On each harvest day, thirty blue lupin seedlings from six treatment containers (i.e. three inoculated/non-inoculated) were removed and measured for their length (root tip to cotyledons), weight, lesion presence, lesion length and lateral root numbers. Presence of *P. agathidicida* within infected blue lupin roots and kauri root plugs was also tested following surface sterilisation and culturing on CRNH selective medium.

3.3.8 *Phytophthora* baiting

Soil samples from all three land-uses collected from Waipoua Forest were subject to a soil baiting bioassay as in Chapter 2 (2.3.8.). Initially this was conducted in Lincoln University, and then a second round of baiting was conducted in Scion, Rotorua for accurate determination of *Phytophthora* spp. within the soil samples. Identical methods were used between each site.

3.3.9 Land-use soil pathogenicity study

Following the lupin pathogenicity study, an experiment was conducted to assess the virulence and pathogenicity effects of *P. agathidicida* on blue lupins in the soils collected from the contrasting land-uses described above. Treatments consisted of six replicate soil samples (per land -use) of inoculated/control 0.5L plastic container pots for each of the five harvest days (HD). However, instead of a 15-day period used in the lupin pathogenicity study, harvest days were spread over a 10-day period (e.g. HD 1 = day 2, HD 2 = day 4 etc) as relatively high infection rates were observed from day 6 (HD 2) in the lupin pathogenicity study. This resulted in a total of 180 containers (2 isolate treatments (inoculated/control) x 3 land-use soils x 6 soil replicates x 5 harvest days). Each container contained 250g of soil from their respective land-use replicate and raised to water holding capacity using sterile deionised water for uniformity in moisture levels. As with the preliminary study, 10 inoculated or autoclaved kauri root plugs were added to each pot. Five lupin seedlings were evenly distributed within each container. Containers were then split among five shelves within an incubator according to their harvest day, and randomly assigned to a position using a similar split plot randomised design in R.

3.3.10 Measurements

Several metrics were used to assess the pathogenicity effects of *P. agathidicida* on blue lupins, including lupin length, lesion presence, lesion length, lateral root number and weight change. *Phytophthora* recovery from infected lupin roots and kauri root plugs was also undertaken.

Lupin length

Measurements of lupin length (cm), from root tip to cotyledons, were made prior to insertion into the sand/soil, and immediately following their removal on each harvest day. Comparison of these measurements was used to calculate the difference in length from day 0 to each harvest day.

Lesion presence and length

Lesions were noted as present or absent on lupin roots, and their length was recorded in mm.

Lupin weight

To account for the initial difference in lupin seedling size, measurements of wet to dry weight change were made. These measurements were only made for the land-use soil pathogenicity study. Wet weights of each seedling were measured immediately after removal from soil and compared to dry weights following transfer into an 80^{o}C kiln overnight.

Lateral root number

Number of lateral roots were counted for each lupin seedling on all harvest days.

Pathogen recovery from lupin roots

Recovery of *Phytophthora* spp. from infected blue lupin roots was recorded as either presence or absence data. However, due to the presence of indigenous populations of *Phytophthora* species in the soil samples from each land-use, this made identification and isolation of *P. agathidicida* difficult due to its comparatively slow growth rate (Beever *et al*., 2009). Therefore, pathogen recovery of any *Phytophthora* species was recorded in the soil pathogenicity study, while isolates recovered in the blue lupin pathogenicity study were recorded as *P. agathidicida* as this assay was run under sterile conditions.

Excisions of 1 cm segments from the root tips of blue lupin seedlings were surface sterilised in 70% ethanol for 15 seconds, followed by two 15-second rinses in sterile water. Excised root segments were then placed onto *Phytophthora* selective CRNH media for isolation over 3-5 days, followed by sub-culturing for a further 7 days to confirm *Phytophthora* isolation.

P. agathidicida recovery from kauri root plugs

Similar to pathogen recovery from blue lupin roots, pathogen recovery from kauri root plugs of any *Phytophthora* species was recorded in the soil pathogenicity study, while isolates recovered in the blue lupin pathogenicity study were recorded as *P. agathidicida* as this assay was run under sterile conditions.

On each harvest day, kauri root plugs were removed from each container and surface sterilised, before being placed onto selective media and cultured for confirmation of infection by *Phytophthora* species.

3.3.11 Statistical design and analysis

The land-use soil pathogenicity study used a balanced split-plot factorial design with land-uses (kauri, pasture and pine) as the primary factor, and treatment (inoculated and control) and harvest days (e.g. HD 1 = Day 2, HD 2 = Day 4 etc.) as the secondary factors. This resulted in 36 soil samples (3 land-uses × 6 replicates per land-use × 2 treatments), multiplied by five harvest days to result in a total of 180 (36 × 5 HD) sample containers. Three replicate blue lupin seedlings were placed into each sample container, for a total of 540 seedlings, with 108 blue lupin seedlings harvested on each harvest day. Measurements of blue lupin length, weight, lesion presence, lesion length, lateral root number, *Phytophthora* recovery from blue lupin roots and kauri root plugs, were made on each harvest day for all blue lupin seedlings to assess pathogenicity effects of *P. agathidicida* on blue lupins.

To test the statistical null hypothesis of no difference in pathogenicity effects of *P. agathidicida* (e.g. blue lupin length, weight, lesion length and lateral root number) on blue lupin, between levels of both land-use and treatment, two-way ANOVAs were conducted on and across all harvest days (Crawley, 2012). This allowed retrieval of associated *F*-values and *p*-values of statistically significant interactions and main effects, followed by post-hoc tests via Tukey's Honest Significant Difference (HSD) at *p*-value <0.05 to identify the levels of each factor combination that were significantly different. Tukey's HSD was used as the post-hoc test as it maintains the experiment-wise alpha level at $p < 0.05$ and accounts for multiple comparisons, assuming the model assumptions of normality, homogeneity of variance and independence are met (Crawley, 2012).

For the binary response variables lesion presence and absence, *Phytophthora* recovery from infected blue lupin roots and kauri root plugs, a logistic binary regression model was used. Harvest day, treatment and land-use were used as the explanatory categorical factors to identify overall significant differences (Crawley, 2012).

3.4 Results

3.4.1 Soil baiting

The initial soil baiting bioassay identified viable propagules of two distinct *Phytophthora* spp. similar to the previous sampling run in April 2016. However, due to inherent diagnostic difficulties in accurately identifying the species present, the samples were sent off for a second round of soil baiting and genetic analysis (via internal transcriber sequences) at Scion, Rotorua. Despite this, no *Phytophthora* spp. were recovered from these samples. Logistical and timing issues resulted in the soil samples not being available for baiting for one and a half months, which likely impacted the recovery of *Phytophthora* spp. from these samples.

3.4.2 Lupin pathogenicity study

Significant differences in lupin length (p <0.001), lesion length (p <0.001) and lateral root numbers (p <0.001) were observed across harvest days and between treatments (e.g. inoculated vs control). Inoculated samples were significant lower in lupin length and lateral root numbers, and significantly greater in lesion length, and *P. agathidicida* recovery from infected lupin roots and kauri root plugs compared to control samples. *P. agathidicida* was not recovered from any excised lupin roots or kauri root plugs from the control samples.

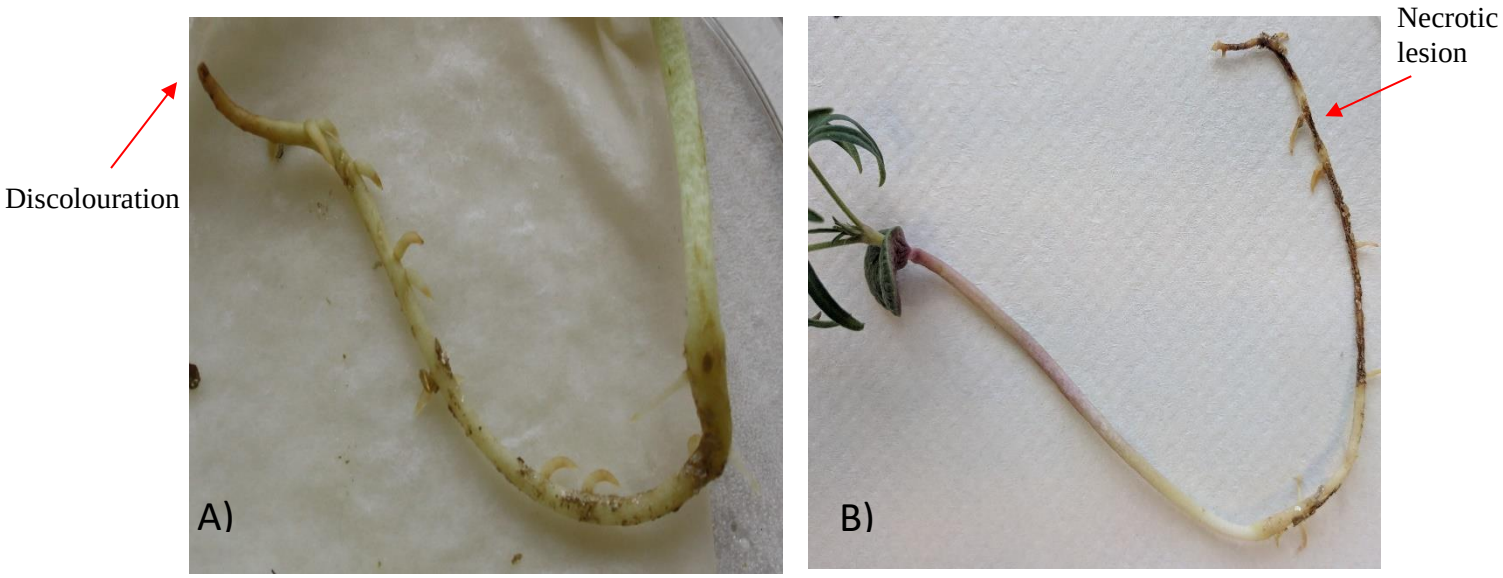


Figure 3.1: Lupin roots showing signs of infection on, A) Day 6 (HD 2) and B) Day 15 (HD 5) in the lupin pathogenicity study.

3.4.3 Land-use soil pathogenicity study

Contrary to what was observed in the lupin pathogenicity study, only lesion length displayed a significant treatment effect ($p < 0.001$); unsurprisingly suggesting that inoculated samples were significantly greater in lesion length compared to non-inoculated samples (Table 3.1; Fig. 3.4 A, B and C). Despite the non-significant treatment, land-use and interaction effects in lupin length and weight, towards day 10 (HD 5) it was observed that inoculated samples were significantly lower in blue lupin length ($p < 0.05$) and weight ($p < 0.05$) compared to control samples (Fig. A.2C and A.3 C); indicating the potential start to a difference between inoculated and non-inoculated samples in these two metrics. To account for potential differences between inoculated and non-inoculated samples, comparisons were made between harvest days and land-use for each level of treatment separately (Table 3.2). These results confirmed that the land-use effect for metrics lupin length, weight and lateral root number were non-significant; suggesting that soils from contrasting land-uses had little effect on these metrics. The only exception to this was lesion length in non-inoculated samples, where a significant ($p < 0.01$) land-use effect was observed (Table 3.2). This is likely to be associated with the negative recovery of *Phytophthora* spp. from pine soil samples relative to the positive recovery of *Phytophthora* spp. in kauri and pasture soils, demonstrated by the soil bioassay.

The logistic regression output shows that land-use is significantly associated with lesion presence, *Phytophthora* recovery from blue lupin roots and kauri root plugs (Table 3.3). As expected, the presence of lesions, and *Phytophthora* recovery from blue lupin roots increases significantly ($p < 0.001$) over harvest days (Fig. 3.5 and 3.6; Table 3.3). *Phytophthora* recovery from kauri root plugs do not significantly increase as illustrated in Fig. 7 A, B and C. Overall, all three binary metrics were significantly ($p < 0.001$) greater in inoculated samples compared to non-inoculated samples (Table 3.3). Although land-use differences did not contribute to changes in metrics lupin length, weight and lateral roots (Table 3.1 and 3.2), significantly lower lesion presence ($p < 0.05$), *Phytophthora* recovery from infected lupin roots ($p < 0.001$) and kauri root plugs ($p < 0.01$) were observed in non-inoculated pine samples compared to kauri samples (Fig. 3.6 – 3.8; Table 3.3). As with lesion length, the difference in *Phytophthora* recovery between pine samples and pasture/kauri samples, as identified in the soil bioassay, is likely to have impacted lesion presence, *Phytophthora* recovery from infected lupin roots and kauri root plugs.

Table 3.1 Two-way ANOVA of each response variable with treatments (inoculated vs non-inoculated) and land -uses (kauri, pasture and pine) as categorical factors, split across each harvest day (i.e. day 2 = HD 1, day 4 = HD 2 etc.). Significant F-values are denoted by an asterisk (* P < .05 ** P < .01 *** P < .001) while ns refers to a non-significant difference.

Dependent variables	Treatment [F(1,534)]	Land-use [F(2,534)]	Interaction [F(2,534)]
Lupin length	ns	ns	ns
Lupin weight	ns	ns	ns
Lesion length	50.2 ***	ns	ns
Lateral roots	ns	ns	ns

Table 3.2 Two-way ANOVA of each response variable with harvest days (i.e. day 2 = HD 1, day 4 = HD 2 etc.). and land -uses (kauri, pasture and pine) as categorical factors, split by levels of treatment (inoculated vs control). Significant F-values are denoted by an asterisk (* P < 0.05 ** P < 0.01 *** P < 0.001) while ns refers to non-significance.

Dependent variables	Treatment	Harvest days [F(4,255)]	Land-use [F(2,255)]	Interaction [F(8,255)]
Length difference	Inoculated	28.4 ***	ns	ns
	Non-inoculated	51.9***	ns	ns
Weight change	Inoculated	47.4***	ns	ns
	Non-inoculated	77.6***	ns	ns
Lesion length	Inoculated	17.9***	ns	ns
	Non-inoculated	5.2***	5.4**	ns
Lateral roots	Inoculated	68***	ns	ns
	Non-inoculated	85***	ns	ns

Table 3.3 Binary logistic regression analysis of lesion presence, pathogen and plug recovery data. B = logistic regression coefficients, SE B = standard errors of regression coefficients. Reference coding HD 1 (Harvest Day), non-inoculated (Treatment) and pine soil (Land-use).

Independent variables		Lesion presence				Pathogen recovery				Plug recovery			
		B	SE B	Sig.	Odds ratio	B	SE B	Sig.	Odds ratio	B	SE B	Sig.	Odds ratio
Main effects													
HarvestDay (overall)				<0.001				<0.001				ns	
	Day 2 (HD 1)	Reference											
	Day 4 (HD 2)	1.4	.46	<0.01	4.1	1.7	.33	<0.001	5.6	-	-	ns	-
	Day 6 (HD 3)	1.9	.45	<0.001	7.4	1.9	.33	<0.001	7	-	-	ns	-
	Day 8 (HD 4)	2.2	.45	<0.001	9.2	2.1	.34	<0.001	8.2	-	-	ns	-
	Day 10 (HD 5)	2.4	.45	<0.001	10.6	1.9	.49	<0.001	7	-	-	ns	-
Treatment	Non-inoculated	Reference											
	Inoculated	2.1	.46	<0.001	8.1	3.4	0.48	<0.001	30.3	7.7	1.1	<0.001	2224
Land-use (overall)				<0.05				<0.001				<0.01	
	Pine	Reference											
	Kauri	1.1	.48	<0.05	3.1	2.8	0.48	<0.001	16.2	3.2	1.04	<0.01	24.5
	Pasture	1	.49	<0.05	2.9	2.9	0.48	<0.001	17.9	2.8	1.05	<0.01	16.7
Interaction effects													
Treatment * Land-use				ns				<0.001				<0.05	
	Inoculated * Pine	Reference											
	Inoculated * Kauri	-1.2	.58	<0.05	.31	-2.7	0.58	<0.001	0.07	-3.4	1.2	<0.01	0.03
	Inoculated * Pasture	-0.95	.58	ns	-	-2.7	0.59	<0.001	0.06	-3.4	1.2	<0.01	0.03
Constant		-4.3	0.56	<0.001	.013	-4.4	0.5	<0.001	0.013	-4.6	1.1	<0.001	0.01

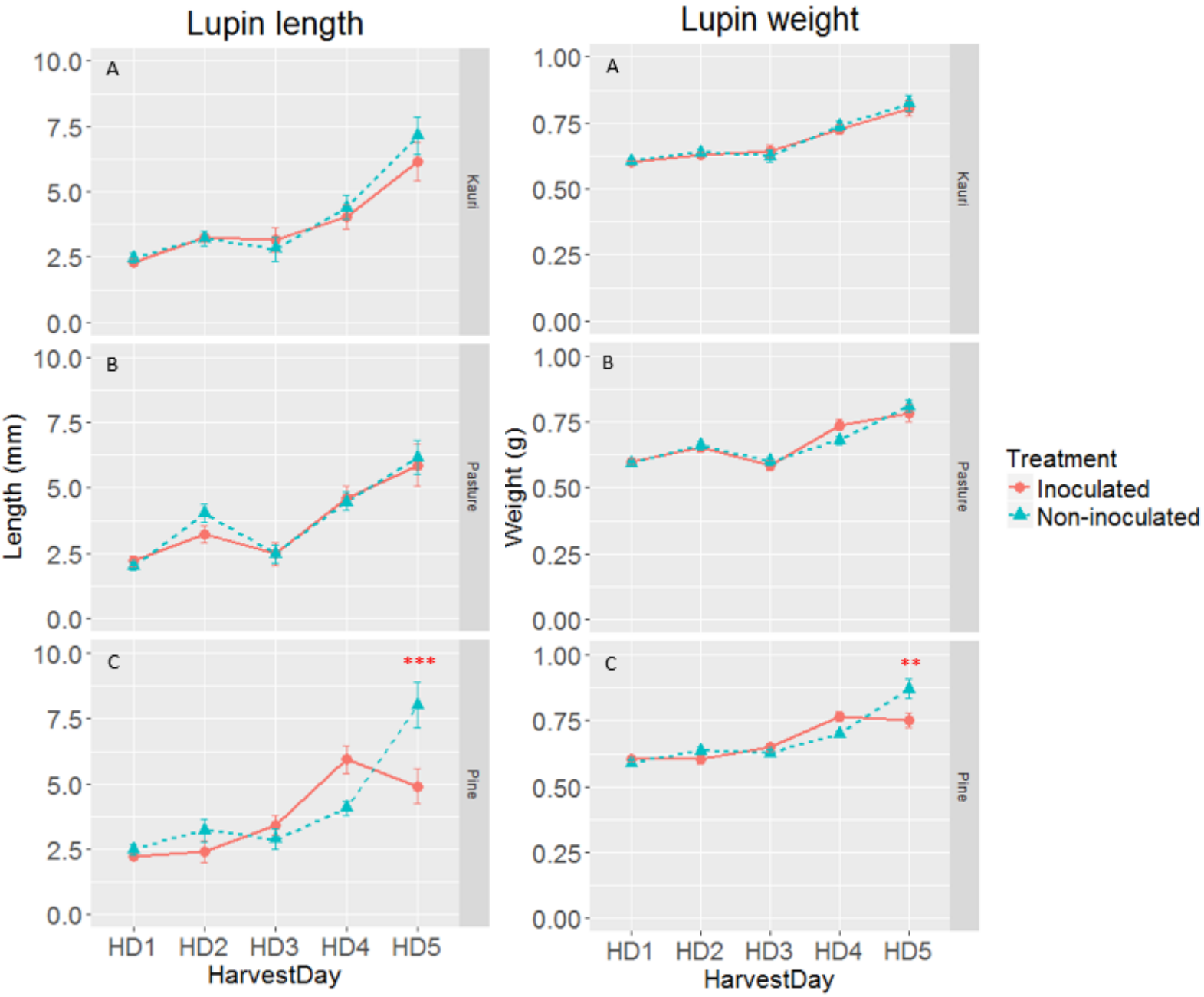


Figure 3.2 Average length difference of lupin (*Lupinus angustifolius*) seedlings between treatments (inoculated vs non-inoculated) for A) Kauri; B) Pasture; and C) Pine samples across harvest days. Significant differences between inoculated and non-inoculated treatments per harvest day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.

Figure 3.3 Average weight difference of lupin (*Lupinus angustifolius*) seedlings between treatments (inoculated vs non-inoculated) for A) Kauri; B) Pasture; and C) Pine samples across harvest days. Significant differences between inoculated and non-inoculated treatments per harvest day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.P < 0.05 ** P < 0.01 *** P < 0.001.

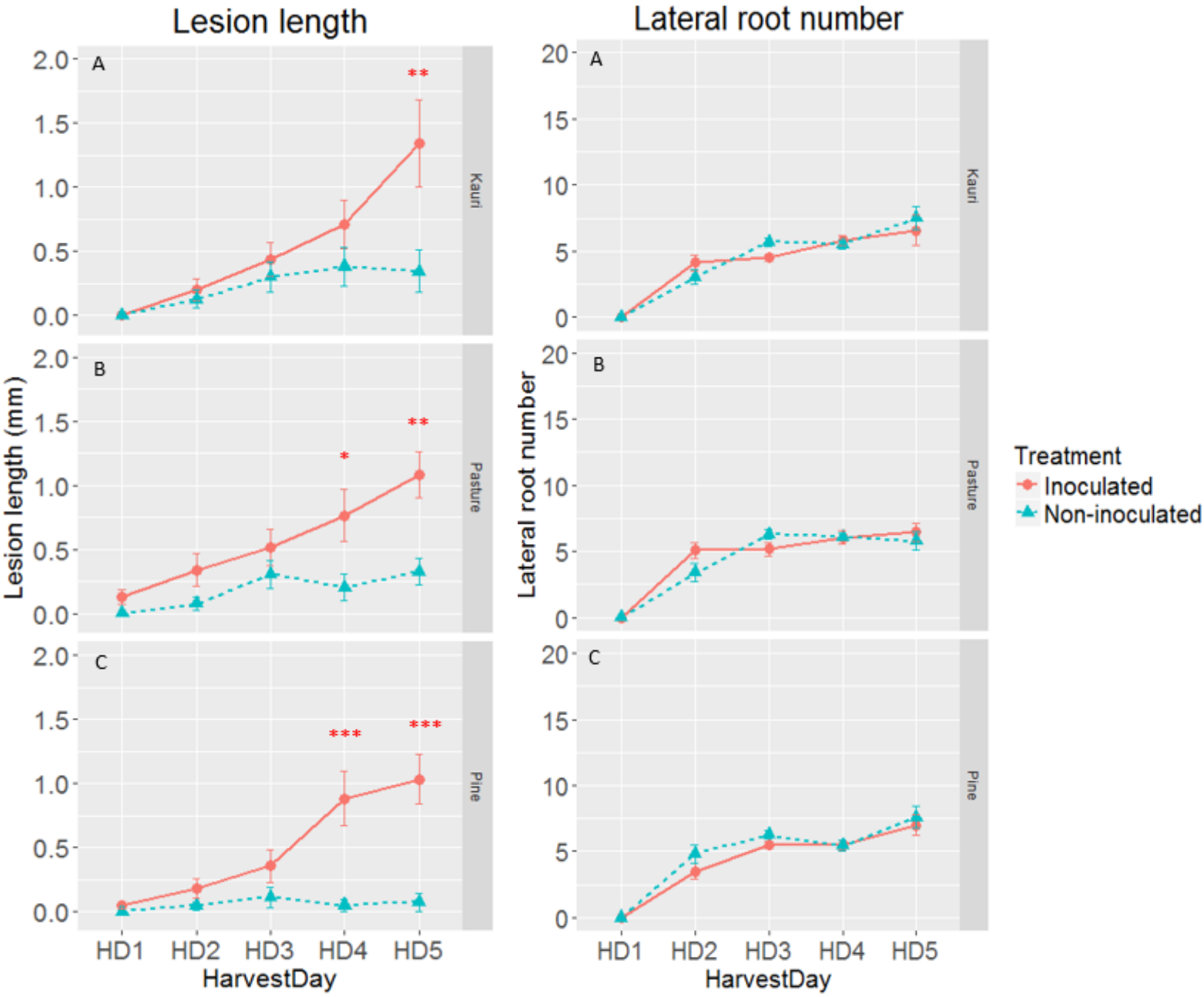


Figure 3.4 Average lesion length in lupin (*Lupinus angustifolius*) seedlings between treatments (inoculated vs non-inoculated) for A) Kauri; B) Pasture; and C) Pine samples across harvest days. Significant differences between inoculated and non-inoculated treatments per harvest day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.

Figure 3.5 Average lateral root number of lupin (*Lupinus angustifolius*) seedlings between treatments (inoculated vs non-inoculated) for A) Kauri; B) Pasture; and C) Pine samples across harvest days. Significant differences between inoculated and non-inoculated treatments per harvest day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.

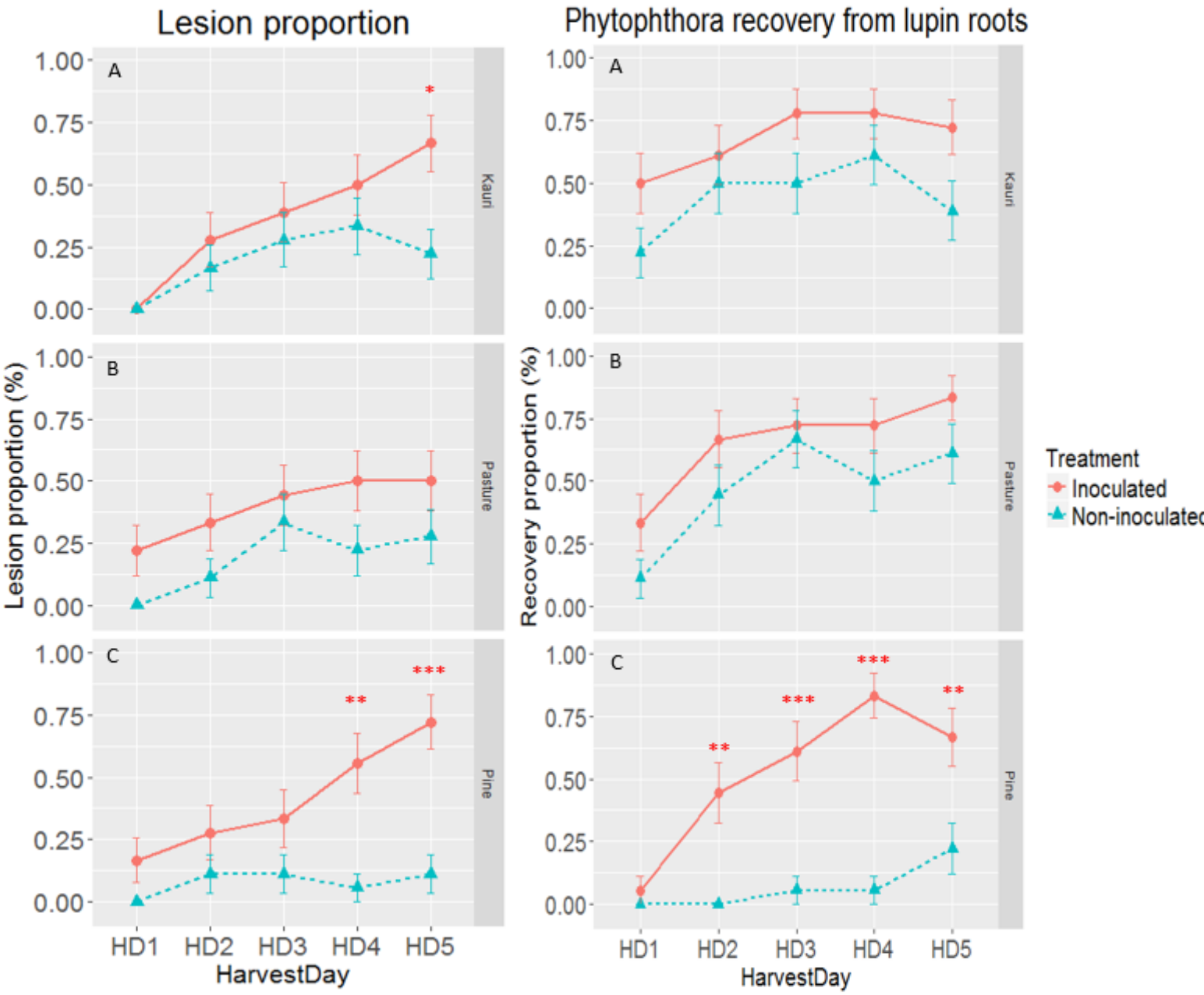


Figure 3.6 Lesion proportion in lupin (*Lupinus angustifolius*) seedling roots between treatments (inoculated vs non-inoculated) for A) Kauri; B) Pasture; and C) Pine samples across harvest days. Significant differences between inoculated and non-inoculated treatments per harvest day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.

Figure 3.7 Recovery of *Phytophthora* species from excised lupin (*Lupinus angustifolius*) roots between treatments (inoculated vs non-inoculated) for A) Kauri; B) Pasture; and C) Pine samples across harvest days. Significant differences between inoculated and non-inoculated treatments per harvest day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.

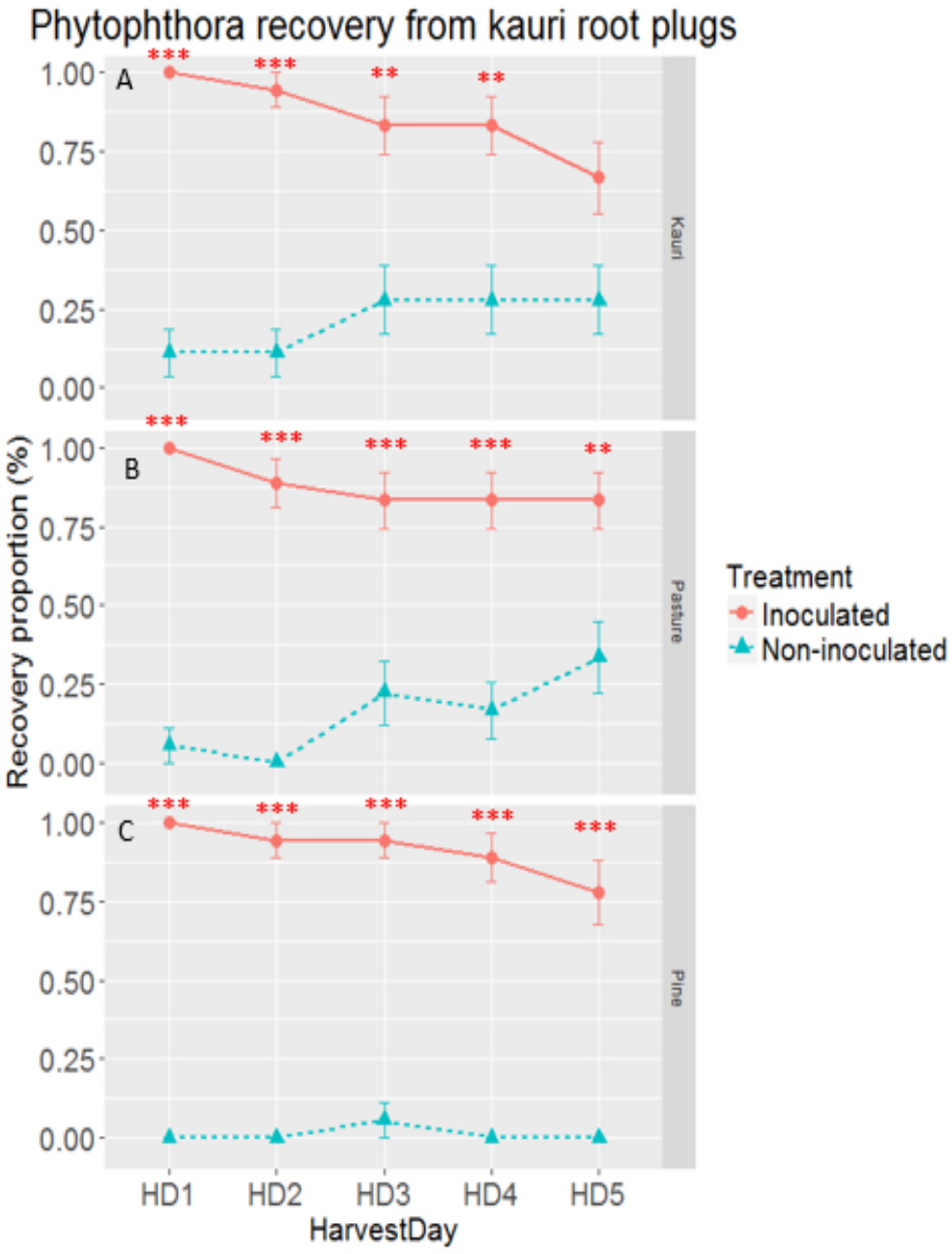


Figure 3.8 *Phytophthora* recovery from kauri root plugs between treatments (inoculated vs non-inoculated) for A) Kauri; B) Pasture; and C) Pine samples across harvest days. Significant differences between inoculated and non-inoculated treatments per harvest day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.

3.5 Discussion

3.5.1 Soil bioassay

The initial soil baiting bioassay was performed immediately following the transport of all soil samples to Lincoln University, in early October. Following the two-week baiting assay, culturing and sub-culturing, two viable *Phytophthora* species were recovered from both pasture and kauri forest soil, according to morphological criteria outlined in Erwin and Ribeiro (1996). Due to difficulties in accurately identifying the species present, the samples were sent off for a second round of soil baiting and genetic analysis at Scion This procedure was unable to identify any *Phytophthora* species present within the samples. However, logistical issues in sending the samples to Scion, resulted in the samples not being used for soil baiting until a month and a half had passed, which likely influenced these results. Therefore, the following discussion will be based on initial soil bioassay where two distinct *Phytophthora* spp. were recovered.

3.5.2 Lupin pathogenicity study

The inoculation of *P. agathidicida* (NFZFS 3813) on blue lupin (*L. angustifolius*) confirmed this isolate had a significant effect on plant growth (plant length and lateral root development) with symptoms of disease also present (lesion presence and length), and the isolate could be effectively recovered from infected lupin roots and kauri root plugs. As expected, given the sterile conditions in this study, lesion development and re-isolation of the pathogen was only achieved in the inoculated samples. These results are similar to those reported in other *Phytophthora* pathogenicity studies utilising blue lupin as hosts (Chee and Newhook, 1965; Cother, 1975; Pratt and Heather, 1972). For example, Chee and Newhook (1965) observed that inoculation of lupins with *P. cinnamomi* resulted in stunted growth and sharply defined brown or orange coloured lesions starting several millimetres behind the root tip. While lesion characteristics and timing has been shown to vary between *Phytophthora* species (Pratt and Heather, 1972), *P. agathidicida* was observed to display similar lesion traits as *P. cinnamomi* (Chee and Newhook, 1965). For example, in the current study lesions initially appeared as brown or orange patches on inoculated lupin radicles, extending from the root tip towards the collar, becoming necrotic over time (Fig. 1A and B). Despite this similarity, Chee and Newhook (1965) noted that *P. cinnamomi* was capable of causing infection within 24 hours of inoculation. This is in contrast to the preliminary study where lesion development occurred between 3-6 days (HD 1 - HD 2).

While this may reflect the slow growing nature of *P. agathidicida* (Beever *et al.,* 2009), it should be noted that differences in growth substrate can affect lesion development (Pratt and Heather, 1972). For example, Pratt and Heather (1972) observed that lesions caused by *P. cinnamomi* were obvious in soil extract after 2-3 days incubation, while only appearing after 4-5 days in inoculated distilled water. The results of this preliminary pathogenicity study have confirmed that blue lupin is an effective plant host for the assessment of *P. agathidicida* pathogenicity, suggesting further studies may capitalise on this interaction.

Given that lesion development in the following soil pathogenicity study occurred between 2-4 days compared to the 3-6 day development period under the sterile sand conditions of the lupin pathogenicity study, this would suggest that soil retrieved from the contrasting land-uses stimulated lesion development by *P. agathidicida* in blue lupin. Although numerous physical (e.g. soil pore size, temperature) and chemical (e.g. soil nutrients, pH) factors have been shown to influence *Phytophthora* sporulation patterns, and hence disease potential (Duniway, 1983; Schmitthenner and Canaday, 1983; Shearer *et al*., 2010; Weste, 1983), the potential impact of microbial communities on *Phytophthora* sporulation as described by Marx and Haasis (1965) and Chee and Newhook (1966), warrant further investigation in their role with *P. agathidicida* pathogenicity and dissemination between land-uses. Furthermore, the results of this lupin pathogenicity study have confirmed that blue lupin is an effective plant host for the assessment of *P. agathidicida* pathogenicity, suggesting this host could provide an alternative host plant to carry out further studies.

3.5.3 Land-use soil pathogenicity study

Comparisons made over harvest days revealed that blue lupins grown in inoculated soil samples retrieved from the contrasting land-uses (e.g. kauri forest, pasture, pine forest) were significantly greater in lesion presence, lesion length and *Phytophthora* recovery from infected roots and kauri root plugs compared to non-inoculated samples (Table 3.1 – 3.3; Fig. 3.2 – 3.8). These results support those observed in the lupin pathogenicity study used sterile coarse sand, where *P. agathidicida* inoculation was confirmed to be pathogenic against blue lupin (Beever *et al*., 2009).

A major finding in this study was that significant land-use effects were only observed in non-inoculated samples, within the pathogenicity metrics lesion presence, lesion length and

Phytophthora recovery from blue lupin roots and kauri root plugs (Fig. 3.2 – 3.8; Table 3.2 and 3.3). These results are likely to be associated with the lack of *Phytophthora* spp. recovery from pine soils compared to the positive isolations of two *Phytophthora* spp. from kauri and pasture samples in the initial soil baiting bioassay. The effects of this difference are explained by the significantly lower *Phytopthora* recovery from infected blue lupin roots in non-inoculated pine samples compared to non-inoculated kauri and pasture samples (Fig. 3.2 – 3.8; Table 3.2 and 3.3). Despite this difference between non-inoculated samples, it should be noted that no significant differences were detected between inoculated land-use samples (Fig. 3.2 – 3.8; Table 3.1 – 3.3), suggesting that land-use impact on *P. agathidicida* pathogenicity in blue lupin was negligible.

The lack of land-use impact on *P. agathidicida* pathogenicity contrasts with other studies which have observed an impact of landscapes on *Phytophthora* pathogenicity (Holdenrieder *et al*., 2004; Jules *et al*., 2002; Kelly and Meentemeyer, 2002). For example, a study by Moreira and Martins (2005) investigated the influence of site-factors (e.g. soil nutrients and fertility) on *P. cinnamomi* disease incidence in cork oak (*Quercus suber*) stands in Portugal, between 56 sites of varying characteristics. They identified that shallow soils with low fertility and low mineral nutrients, particularly phosphorous, were more favourable for *P. cinnamomi* infection of cork oak stands (Moreira and Martins, 2005). The authors further elaborated that these soils were more common in the south of Portugal (Algarve), where cork oak decline by *P. cinnamomi* had a high impact. Similarly, a landscape-scale study of spatial disease characteristics of *P. ramorum* on coastal live oak (*Q. agrifolia*) in California, identified proximity to forest edges to be one of the most important factors in mortality risk (Kelly and Meentemeyer, 2002). This study illustrated the role landscape fragmentation plays in influencing *P. ramorum* dissemination and disease incidence in uninfected sites through forest edge effects (Holdenrieder et al., 2004; Kelly and Meentemeyer, 2002).

Although the current study is limited in spatial replication to these previously cited studies, the known impact of various physical (e.g. temperature, moisture levels), chemical (e.g. pH, available nutrients such as C and N) and biological (e.g. microbial antagonism, predation and competition) factors on *Phytophthora* sporangia and oospore production (Duniway, 1983; Schmitthenner and Canaday, 1983; Shearer *et al*., 2010; Weste, 1983), and hence disease potential (Kelly and Meentemeyer, 2002; Moreira and Martins, 2005), was thought to contribute to a difference in *P. agathidicida* pathogenicity between the contrasting land-use soils utilised in this study.

The main physical factors influencing *Phytophthora* sporangia production and germination, soil moisture and temperature, are known to impact disease incidence between sites with significant differences in their values (Duniway, 1983). This is exemplified in several controlled field experiments that were conducted to assess the effects of different irrigation techniques on the yield of chilli peppers (*Capsicum annuum*) and disease incidence by *P. capsici*, the causal agent of pepper blight in a range of *Capsicum* and *Solanum* spp. (Biles *et al*., 1992; Ristaino *et al*., 1992; Xie *et al*., 1999). For example, Xie *et al*. (1999) demonstrated that increased water availability through furrow irrigation (i.e. small water channels running parallel to crop lines) compared to drip irrigation methods, led to an increase in disease incidence of approximately 30%, and decreased chilli yield by up to 55% in *P. capsici* inoculated field sites.

In general, *Phytophthora* diseases are often more severe in higher soil pH values compared to lower pH (Erwin and Ribeiro, 1996; Schmitthenner and Canaday, 1983). Mucheovej *et al*. (1980) demonstrated this in a greenhouse study of green pepper (*C. frutescens*), where increasing pH values led to a significant increase in disease incidence by *P. capsici*. The authors suggested that lowering soil pH impacted *P. capsici* mycelium and sporangium formation, resulting in the lower disease rates observed.

Biological antagonism is also known to play a role in influencing disease incidence of *Phytophthora* species (Erwin and Ribeiro, 1996; Malajczuk, 1983). For example, among its numerous hosts, *P. cinnamomi* is the causal agent of little leaf of shortleaf (*Pinus echinata*) and loblolly (*Pinus taeda*) pine (Bruehl, 1987). Biological antagonism through antibiosis by the mycorrhizal fungus *Leucopaxillus cerealis* var. *piceina* has been shown to inhibit mycelial growth and zoospore germination of *P. cinnamomi* through production of the antibiotic, diatretyne nitrile, resulting in protection from zoospore infection in mycorrhizae treated pine seedlings (Marx and Davey, 1969).

These studies illustrate only a few of the various physical, chemical and biological factors capable of impacting *Phytophthora* disease incidence. Combined with the complex interactions that may take place, these environmental factors are likely to have contributed to the non-significant land-use impact on *P. agathidicida* pathogenicity in blue lupin observed in this study.

A potentially major confounding factor in this experiment was the thorough mixing of inoculum plugs into each treatment and land-use sample pot. This is because it is known that physical soil factors such as structure and texture affect zoospore dispersal of *Phytophthora* species (Duniway, 1983). For example, a study by Duniway (1976) found that zoospore dispersal of *P. cryptogea* was substantially reduced in soils that contained fewer large pores suitable for zoospore motility beyond its point of formation within the soil. Therefore, removal of this physical component to *P. agathidicida* dispersal through mixing may have skewed infectivity rates between the different land-use soils by not accounting for variability in soil pore sizes.

3.6 Conclusion

Both the lupin pathogenicity study and land-use soil pathogenicity study confirmed that *P. agathidicida* inoculation on blue lupin (*L. angustifolius*) had a significant effect on selected pathogenicity traits (lesion presence, *Phytophthora* lupin recovery and kauri root plug recovery) measured in this study. The faster lesion development within land-use soils compared to sterile coarse sand (e.g. 2 - 4 days and 3 - 6 respectively) may potentially reflect a stimulatory effect of microbial communities on *P. agathidicida* growth and pathogenicity, which requires further investigation. Land-use effects in inoculated samples were non-significant, suggesting that soils retrieved from contrasting land-uses had a negligible impact on *P. agathidicida* pathogenicity in blue lupin. Despite this, land-use was observed to display a significant impact on lesion presence, length and *Phytophthora* recovery in lupin roots and kauri root plugs between non-inoculated samples. This was associated with the positive isolations of *Phytophthora* spp. from kauri and pasture soils compared to the negative recovery from pine soils. Furthermore, a complex interaction of physical, chemical and biological factors may have potentially contributed to this non-significant difference in pathogenicity between inoculated land-use samples. A potential confounding factor identified in this study may be the thorough mixing of inoculum plugs into each land-use soil sample. A separate study investigating *P. agathidicida* zoospore motility and subsequent pathogenicity in soils containing contrasting soil pore sizes is recommended. It is anticipated that this would provide essential information in understanding localised pathogen dispersal within soils between contrasting land-uses.

Chapter 4:
Identification of *Phytophthora agathidicida* host-range in grazed pasture and commercial pine plantations

4.1 Introduction

In the genus *Phytophthora*, host range can be highly variable with species such as *P. cinnamomi* and *P capsici* infecting a broad range of hosts in multiple plant families (Lamour *et al*., 2012; Shearer *et al*., 2004), while others such as *P. medicaginis* and *P. sojae* displaying a narrow host range primarily restricted to single families (Erwin and Ribeiro, 1996). Although *Agathis australis* (New Zealand kauri) is the only known host of *P. agathidicida* in the field (Beever *et al*., 2008; Waipara *et al*., 2013), *Phytophthora* species rarely have a single plant host (Erwin and Ribeiro, 1996). Recent research by Ryder *et al*. (2016) has shown that *P. agathidicida* is capable of infecting and causing lesions in several native plant species found in association with kauri forests such as neinei (*Dracophyllum latifolium*), gumlands grass tree (*D. sinclairii*), rewarewa (*Knightia excelsa*), mairehau (*Leionema nudum*), mingimingi (*Leucopogon fasciculatus*) and toru (*Toronia toru*). This suggests that *P. agathidicida* may potentially have a much greater host range than previously anticipated.

The last two centuries have seen many changes in the landscape within and around Waipoua forest (Kauri National Park Proposal, 2011). In particular, the last 50 years have had an enormous impact, where land drainage, new settlements and native forest conversions to pine plantations and farms has resulted in a significant degree of Waipoua Forest landscape fragmentation (Kauri National Park Proposal, 2011). As such, this has had a profound influence on the plant species composition between contrasting land-uses such as pasture land and pine plantations. For instance, pastures typically contain legume and ryegrass species; in New Zealand pastures, perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) species predominate (Goh and Bruce, 2005). Unsurprisingly, in commercial pine plantations, the predominant plant species are pine (*Pinus radiata*).

In terms of plant disease epidemiology, host range/density is one of the most important factors determining invasion success and establishment in new infection sites (Moreira and Martins,

2005). Given the indication that *P. agathidicida* displays a wider host range than initially thought (Ryder *et al.*, 2016), identifying the host range and susceptibility of plant hosts in sites adjacent to fragmented kauri forests is important. The objective of this chapter is to determine the susceptibility and infection characteristics of *P. agathidicida* (NZFS 3813) on a range of hosts specific to pasture and pine forests. This will involve identifying the optimal growth timing for each host (i.e. white clover (*Trifolium repens*), Caucasian clover (*T. ambiguum*), perennial ryegrass (*Lolium perenne*), annual ryegrass (*L. multiflorum*) and pine (*Pinus radiata*)), followed by a plant infection study whereby the root tip of each host will be inoculated and subsequently excised every two days (over a ten-day period) to identify infection rates by *P. agathidicida.*

It is anticipated that this study will provide insights into the potential for these host species to act as alternative hosts, and to the level of infection by *P. agathidicida*. Furthermore, this study will also inform the potential of these alternative hosts to act as indicator species to identify disease fronts of *P. agathidicida*.

4.2 Research Aim

The main aim of this study was to determine the infection characteristics of six key agricultural plant species (*Trifolium repens, T. ambiguum, Lolium perenne. L. multiflorum, Pinus radiata* and *Lupinus angustifolius*) to *P. agathidicida.*

4.3 Materials and Methods

4.3.1 Host selection

Given indications that *P. agathidicida* displays a wider host range than initially thought (Ryder *et al*., 2016), selection of plant hosts was based on those common to pasture and pine forest. As such, hosts will include white clover (*Trifolium repens*), Caucasian clover (*T. ambiguum*), perennial ryegrass (*Lolium perenne*), annual ryegrass (*L. multiflorum*) and pine (*Pinus radiata*), from pasture and pine forest land-uses respectively. Due to the known virulence of *P. agathidicida* on blue lupins (*Lupinus angustifolius*), this host was included as a positive control.

4.3.2 Plant growth trials

Preliminary growth trials were conducted to determine germination and growth rates of the fast-growing plant hosts (i.e. white clover, Caucasian clover, perennial ryegrass, annual ryegrass and blue lupin) to inform growth timings of the subsequent plant infection study. Vermiculite (Vermiculite Grade 4, Ausperl NZ Shop) was autoclaved to 121^{o}C at 100 kPA (15 psi) for 15 min, then cooled to room temperature (~18^{o}C). Sterilised vermiculite was used to fill 750 ml rectangular plastic containers, for a total of 15 containers (5 plant species × 3 measurement days). Approximately 100 ml deionised water was used to water all containers until wet, but not soaking. Twenty host seeds were sown at equal distances from one another in each of the host container replicates, for a total of 300 seeds or 60 seeds per plant host (20 seed replicates × 5 plant species × 3 measurement days). Following this, all containers were randomly allocated to a position on three separate shelves (e.g. 5 containers per shelf) within a temperature controlled (21^{o}C) incubator under a 12 hr light/dark photoperiod (Sanyo Versatile Environmental Test Chamber - MLR 351) for the duration of the two-week growth trial. Deionised water (30 ml) was used to water each container every 48 hours to maintain moisture levels. Length measurements were made destructively on each of the three measurement days (e.g. day 6, day 10 and day 14) over the 14 day growth trial. This involved collecting 10 replicate seedlings of each plant species, gently rinsing and measuring from the root tip to the cotyledon tip. Germination of each species was assessed between day 1 – 5 and on each measurement day.

To prepare pine seeds for germination and growth, they were initially stratified to release seeds from dormancy by placing them into deionised water overnight, draining the water and transferring to a 4^{o}C fridge for a period of two weeks (Bi and Turvey, 1994). Sixty pine seeds were then germinated in separate plastic growing pots containing soil potting mix (Number 8 potting mix, Mitre 10), over the course of three months due to their comparatively slow growth (Bi and Turvey 1994). Their length was similarly measured from root to short tip.

4.3.3 Media

A mixture of 10% carrot agar, ampicillin, pimaricin, rifampicin, nystatin and hymexazol (CRNH) medium was used in this study as the *Phytophthora* selective medium. Details are reported in Chapter 2 (2.3.5). A non-selective CAH medium was also prepared for sub-culturing of *P. agathidicida* from the CRNH medium. Each litre of CAH medium preparation included

15g bacteriological agar, 100 ml carrot solution (i.e. 100 g frozen carrots blended with 100 ml deionised water and sieved), and 900 ml deionised water.

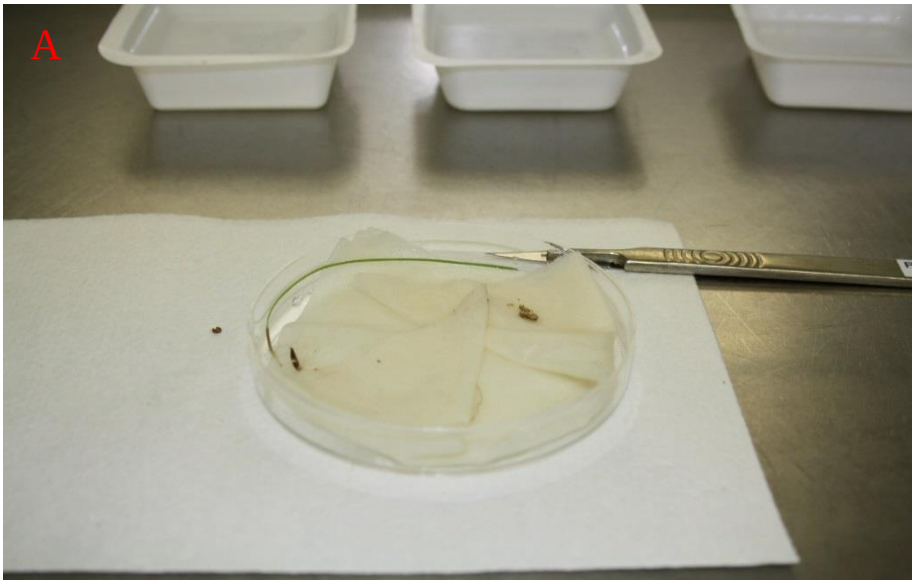
4.3.4 Plant infection study

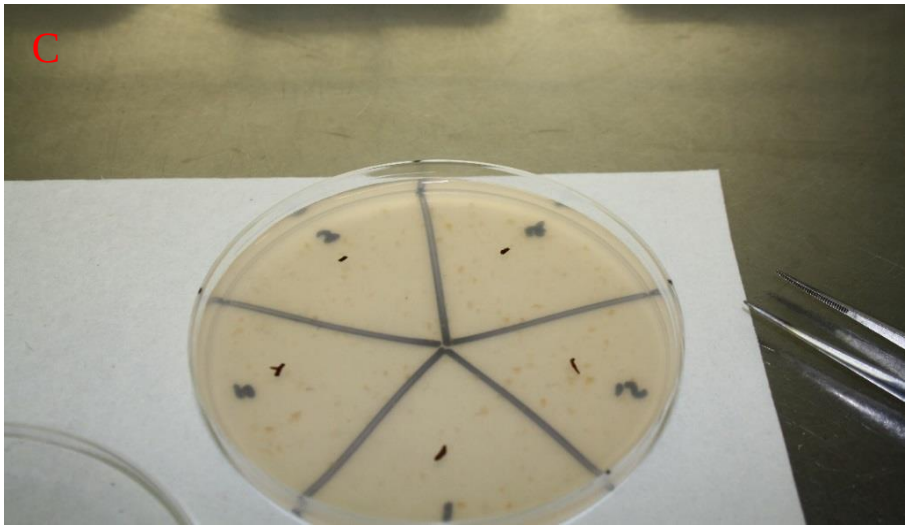
The following plant infection study was based on a similar study by the Healthy Trees Healthy Future (HTHF) program led by Scion (Whakapapa Lines of Kauri Partners update, 2017), the forestry and technology Crown Research Institute in Rotorua, New Zealand (Nari Williams, *Personal communications*). This was conducted to identify the susceptibility of each plant species to *P. agathidicida*, the pathogens growth through the root, in addition to any symptoms following infection.

A single isolate of *P. agathidicida* (NZFS 3813) was cultured on CRNH selective medium for 7 days at 21ºC, and then transferred to CAH medium for three days prior to initiating the plant infection study. Simultaneously, according to data collected from the host growth trials, hosts were germinated in plastic containers with sterile vermiculite. Six replicate seedlings were used for each host, treatment (inoculated vs non-inoculated) and harvest day (e.g. HD 1 = day 2, HD 2 = day 4 etc.), for a total of 360 seedlings (6 hosts × 6 host replicates × 2 inoculation treatments × 5 harvest days). Germinated seedlings were gently extracted from each container, rinsed and transferred into an inoculation cartridge consisting of a 90 mm Petri dish (Nunc® petri dishes, Sigma-Aldrich). For both blue lupin and pine seedlings, a small hole was melted to allow for their stems to extend beyond the inoculation cartridge. Paper towels moistened with deionised water were used to line the inoculation cartridges before placing each plant inside (Fig. 4.1 A). The root tips were inoculated with a 5 mm agar plugs of three-day old *P. agathidicida* culture (NZFS 3813), where gentle pressure was applied to squash the agar plug without damaging the root tip itself. Following this, each inoculation cassette was sealed with plastic tape, blocked according to their harvest day and randomly allocated to an 18 × 4 position within an 8 cm deep tray; inoculation cassettes were placed vertically to fit (Fig. 4.1 B). Each tray was randomly placed onto separate shelves within a growth incubator (Sanyo Versatile Environmental Test Chamber - MLR 351) where a 12-hour light/dark photoperiod was maintained, and temperature was held constant at 21ºC. On each of the five harvest days (e.g. HD 1 = day 2, HD 2 = day 4 etc.), 72 seedlings (12 per plant species) were harvested and inspected for chlorotic/necrotic symptoms.

All plant species were surface sterilised in 70% ethanol for 30 seconds followed by two rinses in sterile deionised water. After being dabbed dry, 5 cm of root (starting from root tip) from each plant seedling was excised into 1 cm sections (i.e. section 1 = 1 cm, section 2 = 2 cm),

using sterile technique, a sharp scalpel, forceps and a ruler. These sections were split in half (5 mm root segments), where one half was plated onto *Phytophthora* selective CRNH medium (Fig. 4.1 C), while the other was placed into a 2 ml Eppendorf® tube containing FAA (formalin acetic acid) fixative for microscopic observation (Fig. 4.1 D). Isolation of *P. agathidicida* from sequentially plated root segments gave a measurement of growth through the root for each plant species. Microscopic observations of transversely cut root segments were conducted to confirm *P. agathidicida* infection.





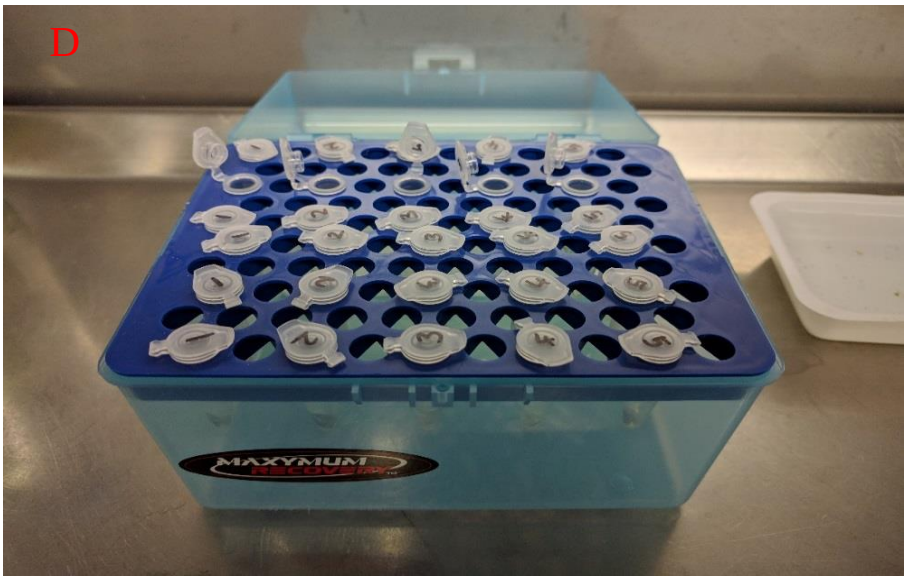


Figure 4.1 Experimental set-up of plant infection study; A) Set-up of inoculation cassettes; B) Randomly allocated inoculation cassettes blocked by harvest day; C) Alternative 5 mm root sections (per 1 cm root) placed onto CRNH *Phytophthora* selective media; D) Alternative 5 mm root sections transferred to 2 ml Eppendorf® tubes containing FAA solution.

4.3.5 Statistical analyses

The plant infection study used a factorial design with plant species (e.g. white clover, Caucasian clover, annual ryegrass, perennial ryegrass, blue lupin and pine) and harvest days (e.g. HD 1 = day 2, HD 2 = day 4 etc.) as the categorical factors assessing the infection rates (e.g. percent *P. agathidicida* infection of 5 cm excised roots) between levels of each factor. With six replicate seedlings for each species, treatment and harvest day combination, this resulted in a total of 360 seedlings (6 plant species × 6 replicate seedlings × 2 inoculation treatments × 5 harvest days).

To test the null hypothesis of no difference in *P. agathidicida* root infection between plant species, two-way ANOVAs were conducted (Crawley, 2012). This allowed retrieval of associated *F*-values and *p*-values of statistically significant interactions and main effects, followed by post-hoc tests via Tukey's Honest Significant Difference (HSD) at *p*-value <0.05 to identify the levels of each factor combination that were significantly different. Tukey's HSD was used as the post-hoc test as it maintains the experiment-wise alpha level at $p < 0.05$ and accounts for multiple comparisons, assuming the model assumptions of normality, homogeneity of variance and independence are met (Crawley, 2012).

4.4 Results

4.4.1 Plant growth trials

Caucasian clover was on average 12.8 cm ± 2.3 cm at the end of a two-week growing period, consistently showing longer growth than white clover which was on average 8 cm ± 1.3 cm by the end of the study (Table 4.1). White clover had higher germination rates than Caucasian clover on day 1 – 5, however, this levelled off by day 6 onwards (Table 4.2). Perennial ryegrass length was on average 21.1 cm ± 2.3 cm, compared to annual ryegrass length of 21.7 cm ± 4.1 cm by the end of the study (Table 4.1). Annual ryegrass had higher germination rates than perennial ryegrass on day 1 – 5, however, as with the clover species this levelled off by day 6 (Table 4.2). Blue lupin length was on average 17.3 cm ± 2.3 cm by the end of the study, with a high germination rate of 90% by day 6. The germination timing of each fast-growing host was staggered so that all plant species were between 5 – 10 cm in length at the start of the plant infection study (e.g. Blue lupins were grown for 7 days, Caucasian/white clover grown for 10 days and Annual/perennial ryegrass for 5-6 days). This ensured there was sufficient root length available for excision.

Pine seedlings were germinated in a soil potting mix over the course of three months; they reached a height of 17.4 ± 1.7 cm, growing approximately 5.8 cm every month. Germination rates were approximately 85%, consistent with the germination rates of the other species (Table 4.2).

Table 4.1 Average length ± sd for hosts on each measurement day (e.g. Day 6, Day 10 and Day 14).

	Day 6		Day 10		Day 14	
Host	**Mean**	**sd**	**Mean**	**sd**	**Mean**	**sd**
Caucasian clover	5.7	1.6	7.4	1.7	12.8	2.3
White clover	5.1	1.0	6.75	1.1	8	1.3
Perennial ryegrass	7.8	2.3	16.2	3.0	21.1	2.3
Annual ryegrass	11.05	2.1	17.8	3.0	21.65	4.1
Blue lupin	6.7	1.3	12.5	1.8	17.3	2.3

Table 4.2 Species germination rates across day 1-5 and each measurement day (e.g. Day 6, Day 10 and Day 14).

Hosts	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 10**	**Day 14**
Caucasian clover	0	0	20%	40%	65%	80%	80%	80%
White clover	0	5%	30%	55%	80%	85%	85%	85%
Perennial ryegrass	0	0	0	10%	40%	75%	85%	85%
Annual ryegrass	0	0	5%	45%	70%	80%	90%	90%
Blue lupin	0	0	30%	60%	80%	90%	90%	90%

4.4.2 Infection of plant species by *Phytophthora agathidicida*

To test the null hypothesis of no difference in *P. agathidicida* infection between levels of both harvest day and plant species, a two-way ANOVA was used. Significant interactions between harvest days and hosts (p <0.001), and main effects for harvest days (p <0.001) and hosts (p <0.001) were observed for *P. agathidicida* infection within excised root segments (Table 4.3). Comparisons made between plant species on each harvest day separately showed significant differences (p <0.001) on day 2 – 6 (HD 1 – 3) and day 8 (HD 4) (p <0.01), as illustrated by Fig. 4.2.

Post-hoc test using Tukey's HSD indicated that annual (ARG) and perennial ryegrass (PRG) displayed significantly greater percentage infection of roots than all other plants on day 2 (HD 1) and day 4 (HD 2) (p <0.001) and most other hosts on day 6 (HD 3) (p <0.01) (Table 4.4). For example, *P. agathidicida* infection of 5 cm excised root segments were between 75 – 95% (or 4 – 4.5 cm) in ARG and PRG, compared to the 35 – 50% (or 2 – 2.5 cm) infection observed in other hosts on day 2 and 4 (HD 1 and 2) (Table 4.5). Pine root infection was significantly lower (p <0.01) than most other plant species on day 2 (HD 1), however, from day 4 - 10 (HD 2 – 5) they were similar to those found in most other plants (with the exception of ARG and PRG) (Table 4.4). At the end of the study, all plants were displaying 100% infection of the 5 cm root segments excised, and were not significantly different from one another. This suggests that despite the initial difference in infection rates, *P. agathidicida* was capable of not only infecting all plant species, but doing so rapidly. Microscopic observations of root segments confirmed these results (Fig. B.1 A – F). It should be noted that the isolate of *P. agathidicida* utilised in this study was not recovered from any excised root segments from non-inoculated hosts (Table 4.3). Despite the high level of root infection by *P. agathidicida* in all plants by day 10 (HD 5), only blue lupins and pine seedlings developed visible symptoms (Fig. 4.3 A and B; Fig. B.2 A - D). This suggests that despite their susceptibility to *P. agathidicida* infection, not all plant species were liable to disease symptoms. Infected lupin roots developed brown lesions of varying intensity near the radical tips, which later developed into necrotic lesions marked by soft dark tissue (Fig. 4.3 A). In addition to this, lupin cotyledons were often wrinkled and desiccated. Infected pine roots displayed light brown colouration, sometimes accompanied by white lateral roots extending from the root tip towards the stem (Fig. 4.3 B). Some foliar symptoms were observed in pine seedlings; however, these were also observed in non-inoculated samples (Fig. 4.3 C), suggesting these may be the result of mechanical damage or water stress from the nature of the study (e.g. no additional water added beyond day 0, close packing of inoculation cassettes). Despite this, the softness in root tissue for both lupins and pine indicated the cellular integrity of infected root was severely compromised.

Table 4.3 Significance table of percent *P. agathidicida* infection of root segments between levels of harvest day (i.e. day 2 = HD 1, day 4 = HD 2 etc.) and host (i.e. lupin, pine etc.).

	Treatment	Harvest Day [F(4,150)]	Host [F(5,150)]	Interaction [F(20,150)]
% presence	Inoculated	166***	47***	7.4***
	Non-inoculated	NA	NA	NA

Table 4.4 Percentage *P. agathidicida* infection of total 5 cm excised root segments for each host across harvest days (i.e. day 2 = HD 1, day 4 = HD 2 etc.).

	Blue lupin	Pine	Annual ryegrass	Perennial ryegrass	White-clover	Caucasian-clover
Day 2 (HD 1)	53.3^{b}	33.3^{a}	73.3^{c}	83.3^{c}	53.3^{b}	46.7^{ab}
Day 4 (HD 2)	70^{a}	53.3^{a}	96.7^{b}	96.7^{b}	70^{a}	53.3^{a}
Day 6 (HD 3)	83.3^{ab}	73.3^{a}	100^{c}	100^{c}	96.7^{bc}	80^{a}
Day 8 (HD 4)	87^{ab}	89^{ab}	100^{b}	100^{b}	96.7^{ab}	86.7^{a}
Day 10 (HD 5)	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}

Table 4.5 *Phytophthora agathidicida* infection distance within excised roots of all hosts across harvest days (i.e. day 2 = HD 1, day 4 = HD 2 etc.).

	Day 2 (HD 1)	Day 4 (HD 2)	Day 6 (HD 3)	Day 8 (HD 4)	Day 10 (HD 5)
Blue lupin	2.5 ± 0.5	3.5 ± 0.4	4.5 ± 0.4	≥ 5	≥ 5
Pine	1.5 ± 0.5	2.5 ± 0.5	3.5 ± 0.5	4.5 ± 0.3	≥ 5
Caucasian-clover	2.5 ± 0.5	3 ± 0.5	4 ± 0.4	4.5± 0.3	≥ 5
White-clover	2.5 ± 0.5	3.5 ± 0.5	4.5 ± 0.4	≥ 5	≥ 5
Perennial ryegrass	3.5 ± 0.4	4.5 ± 0.2	≥ 5	≥ 5	≥ 5
Annual ryegrass	4 ± 0.4	4.5 ± 0.3	≥ 5	≥ 5	≥ 5

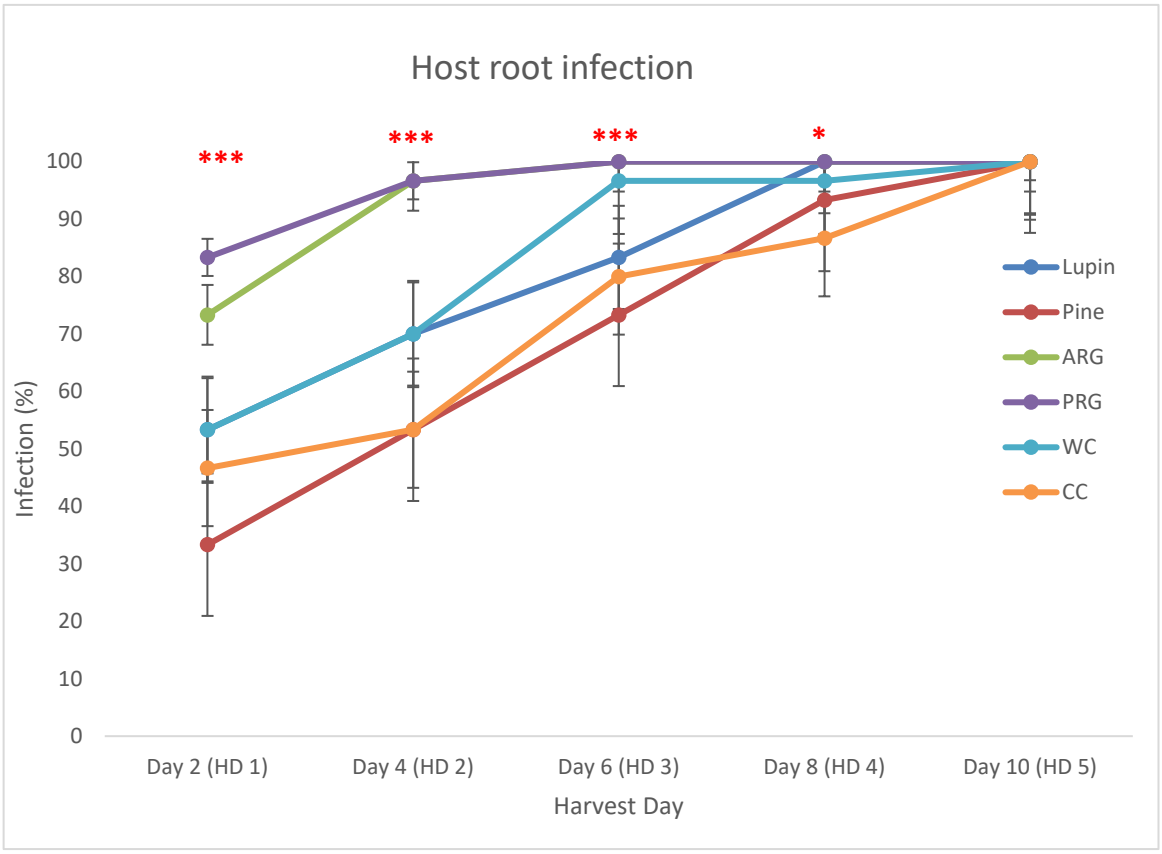


Figure 4.2 Percentage *P. agathidicida* infection of excised 5 cm root segments from each plant species (e.g. Lupin = Blue lupin, Pine = Pine, ARG = Annual Ryegrass, PRG = Perennial Ryegrass, WC = White Clover, and CC = Caucasian Clover) on all harvest days (i.e. day 2 = HD 1, day 4 = HD 2 etc.).

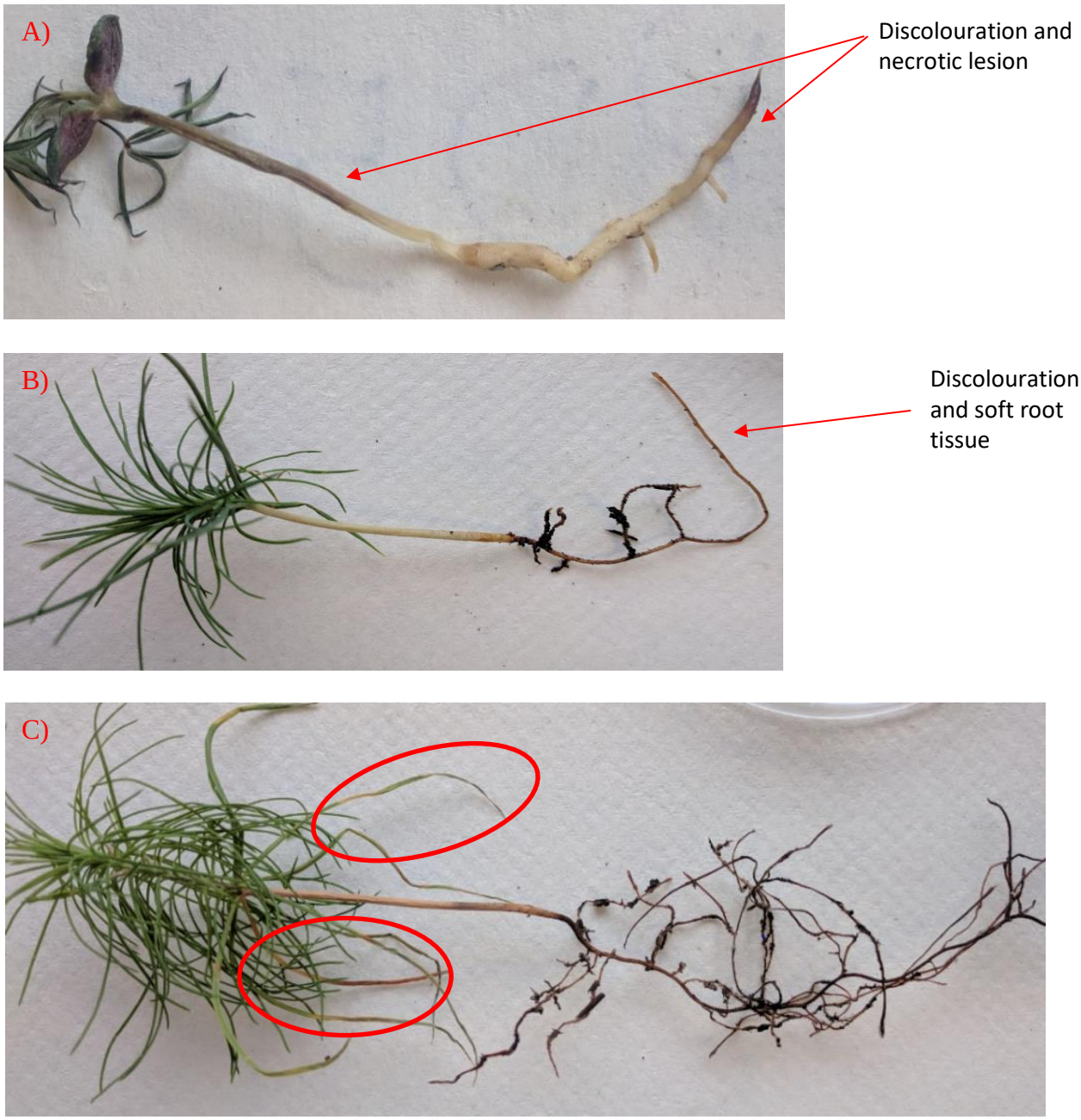


Figure 4.3 A) *Phytophthora agathidicida* inoculated lupin on day 10 (HD 5) showing visible symptoms on radical and collar; B) *Phytophthora agathidicida* inoculated pine seedling on day 10 (HD 5) showing visible symptoms on roots; C) Non-inoculated pine seedling on day 10 (HD 5) displaying signs of mechanical damage.

4.5 Discussion

Host range is an important factor in plant disease epidemics, as susceptible hosts can provide crucial infection points that allow establishment within new sites (Rizzo *et al*., 2002; Tian and Babadoost, 2004). Additionally, they may provide refuge during periods unsuitable for dissemination of the pathogen, and conversely, a source of inoculum for pathogen dissemination, thereby acting as pathogen reservoirs within infected sites (Erwin and Ribeiro, 1996). For example, a study that investigated *Phytophthora cinnamomi* within cork (*Quercus robur*) and hoalm oak (*Q. rotundifolia*) stands within Portugal, identified susceptible plant species such as *Cistus* spp., *Calluna vulgaris* and *Ulex* spp., that played important roles in inoculum production and dissemination (Moreira and Martins, 2005). Similarly, host density has been shown to influence plant disease incidence and severity (Plantegenest *et al*., 2007). This has been documented in forest (Gilbert *et al*., 1994; Condeso and Meentemeyer, 2007) and grassland ecosystems (Knops *et al*., 1999; Mitchell *et al*., 2002). For example, a study investigating the effects of landscape heterogeneity on sudden oak death, caused by *Phytophthora ramorum*, discovered that landscape-scale configuration, local host composition and density were highly related to the severity of sudden oak death (Condeso and Meentemeyer, 2007). The authors suggested that greater disease severity was likely the results of an increase in available inoculum reservoir from susceptible hosts surrounding sampling plots (Condeso and Meentemeyer, 2007). Therefore, understanding the range of susceptible hosts for a given pathogen is an important component for plant disease management.

Observations from this study indicate that *P. agathidicida* can colonise the roots of all plant species tested (e.g. white clover (*Trifolium repens*), Caucasian clover (*T. ambiguum*), perennial ryegrass (*Lolium perenne*), annual ryegrass (*L. multiflorum*) and pine (*Pinus radiata*)) (Fig. 4.2; Table 4.3 – 4.5). Combined with reports that *P. agathidicida* can infect several native plant species (e.g. neinei (*Dracophyllum latifolium*), gumlands grass tree (*D. sinclairii*) etc.) found in association with kauri (Ryder *et al*., 2016), the results suggest that *P. agathidicida* has a larger host-range than initially thought, spanning plants prevalent in different land-uses. Given the understanding that greater numbers of susceptible host species may act to increase inoculum loads within infected sites (Condeso and Meentemeyer, 2007; Moreira and Martins, 2005), the results of this study suggest that pine and pasture land-use hold the potential to act as pathogen reservoirs for *P. agathidicida*. This potential is further exacerbated with the recognition that both pasture and pine forest soils were observed to increase sporangia and oospore production within the early stages of establishment (Chapter 2).

Understanding that host density influences plant disease incidence and severity (Plantegenest *et al*., 2007), studies have identified host density thresholds, below which plant pathogens are unable to invade susceptible populations due to a lack of hosts (McCallum *et al*., 2001; Otten and Gilligan, 2006; Plantegenest *et al*., 2007). This is not likely to play a major role in either land-uses pasture and pine due to the high densities of host plants within these sites. However, understanding the relationship between host density, host susceptibility and fragmentation of indigenous kauri forests by contrasting land-uses would likely provide crucial insights into kauri dieback transfer and management.

As mentioned in Chapter 2, the accessibility of these land-uses likely plays a role in *P. agathidicida* dissemination, given the impact of human and livestock vectors on *Phytophthora* dispersal (Basset *et al*., 2017; Cushman *et al*., 2008; Krull *et al*., 2013). However, the establishment and successive dispersal from these land-uses is dependent on several factors, including the presence of susceptible hosts and prevailing environmental conditions (e.g. biological, physical and chemical) within the soil (Duniway, 1983; Erwin and Ribeiro, 1996; Schmitthenner and Canaday, 1983; Weste, 1983). With the presence of susceptible hosts in these two land-uses, it would seem that any barriers to establishment within these sites are not related to hosts, but rather the pre-disposing environmental conditions within the soil. Therefore, investigations of *P. agathidicida* infection rates on host species within land-use soils is recommended to identify whether localised environmental conditions impact host susceptibility.

The comparatively rapid and high infection rates of both ryegrass species (Table 4.4) by *P. agathidicida*, suggest the use of these hosts as indicator species may initially be considered. However, indicator species are typically characterised by not only high susceptibility, but also visibility of symptoms (Burgess *et al*., 2009). For example, Moreira and Martin (2005) recommended the use of *Cistus populifolius* for *P. cinnamomi* detection due to its high susceptibility and rapid mortality rate upon infection. Despite rapid infection by *P. agathidicida* throughout the roots of both ryegrass and clover species, no visible symptoms were apparent on the roots or cotyledons (Fig. B.2 A and B). Pine and lupin seedlings on the other hand displayed signs of infection on both the roots and foliage (Fig. 4.3 A, B and C). Despite this, foliar damage on pine seedlings were also observed in some control samples, and may potentially be a result of mechanical damage or water stress due to the nature of the study. Given that pine plantations made up 90% of the 1.75-million-hectare plantation area in New Zealand, and contributed approximately NZD $4.4 billion to New Zealand's GDP (The Forest

Owners Association, 2016), *P. radiata* clearly represents an important species in New Zealand. Further experiments utilising growth pots and an inert soil medium such as vermiculite or perlite would allow more long-term assessments of pine seedling foliar damage to *P. agathidicida* infection.

4.6 Conclusion

Host range is an important aspect of plant disease epidemiology, due to their contributions to pathogen survival, dissemination and disease severity. As such, characterising host range and pathogenicity is critical for disease management. This study has demonstrated that *P. agathidicida* is capable of infecting six plant species (e.g. Caucasian clover, white clover, perennial ryegrass, annual ryegrass, pine and blue lupin) common to the two land-uses, pasture and pine forest. Combined with indications that soil conditions under these land-uses contribute to increased sporulation patterns in *P. agathidicida* (Chapter 2), these results suggest these land-uses may act as pathogen reservoirs. Given the artificial nature of this study, further investigations into the infection of these hosts within their respective land-use soils is recommended to identify whether local environmental conditions impact host susceptibility, pathogenicity effects, and any potential ramifications this may have on commercial pine production.

Chapter 5:
Conclusions and Recommendations for Future Research

5.1 Conclusions and key results

This study aimed to identify the impact of contrasting land-uses (e.g. indigenous kauri forest, pasture and commercial pine forest) from Waipoua Forest, on the growth and pathogenicity of a single isolate of *P. agathidicida* (NZFS 3813). In addition to this, given the results of Ryder *et al*. (2016) which discovered a wider host-range for *P. agathidicida*, a supplementary experiment examined the colonisation rate of several potential hosts (*Trifolium repens, T. ambiguum, Lolium perenne. L. multiflorum, Pinus radiata* and *Lupinus angustifolius*) common to both pasture and pine forest.

The main objectives of this thesis were to determine:

1. How the physicochemical properties vary between the soils of these three land-uses and whether they impacted the growth and reproduction of *P. agathidicida.*
2. Whether the three land-use soils impacted upon the virulence of *P. agathidicida* using blue lupin (*Lupinus angustifolius*) as a model host plant.
3. The effect of *P. agathidicida* infection on six alternative hosts (*Trifolium repens, T. ambiguum, Lolium perenne. L. multiflorum, Pinus radiata* and *Lupinus angustifolius*) that are common to pasture and pine forests

5.1.1 Contrasting land-use soils impact *Phytophthora agathidicida* growth and reproduction. Two new *Phytophthora* spp. to New Zealand identified

Investigations into the growth response of *P. agathidicida* in soils collected from three contrasting land-uses (i.e. indigenous kauri forest, pasture, commercial pine forest) present in and around Waipoua Forest identified significant differences in sporangia (asexual) and oospore (sexual) counts between pasture/pine samples from kauri samples, within two days of initiating the growth response experiment. These results suggest that soils from pasture/pine favoured asexual and sexual reproduction in the early stages of *P. agathidicida* establishment, potentially contributing to their capacity to act as pathogen reservoirs. However, this effect was short-lived as sporangia production patterns were not sustained from day four to eight of the study. Contrary to what is observed in sporangia counts, oospore production significantly

increased over the course of the study in pine samples, indicating these soils increase inoculum loads, enhancing their capacity to act as pathogen reservoirs. Combined with indications that the currently utilised disinfectant in phytosanitary stations, trigene (Sterigene), is ineffective against *P. agathidicida* oospores (Bellgard *et al*., 2010), these results suggest the potentially central role of oospores in the survival of this soil-borne pathogen in land-use areas other than kauri forest sites. Despite these results, this study was conducted over a short time frame and may not reflect the growth response under longer exposure, so further investigation is required. Two new *Phytophthora* spp. (*P. pini* and *P. gregata*) were recovered from pasture and kauri soils respectively. Their recognition as international horticultural and native plant pathogens warrants further investigation into the impact they may have on New Zealand plant species.

5.1.2 *Phytophthora agathidicida* pathogenicity was not different between contrasting land-use soils

Both the lupin pathogenicity study under sterile conditions and the land-use soil pathogenicity study confirmed that *P. agathidicida* (NZFS 3813) inoculation on blue lupin (*Lupinus angustifolius*) had a significant effect on several pathogenicity traits (e.g. lesion presence, lesion length, *Phytophthora* recovery from lupin root and kauri root plugs) measured in this study. However, it was observed in the land-use soil pathogenicity study that land-use effects in inoculated samples were non-significant, suggesting that contrasting land-use soils had a negligible impact on *P. agathidicida* pathogenicity on blue lupin. Despite this, non-inoculated samples displayed significant differences in lesion presence, length and *Phytophthora* recovery from lupin roots and kauri root plugs; with pine samples showing significantly lower values for all metrics than both kauri and pasture samples. This was likely associated with the results of the soil baiting assay where *Phytophthora* spp. were isolated from kauri and pasture but not pine soils.

5.1.3 *Phytophthora agathidicida* capable of infecting hosts common to grazed pasture and commercial pine forest

Given the findings of Ryder *et al*. (2016) that *P. agathidicida* can infect and cause lesions in several native plant species found in association with kauri forests (e.g. nei nei, gumlands grass tree and rewarewa), an alternative host study assessing *P. agathidicida* colonisation in plant hosts common to both pasture and pine land-uses was conducted. This study demonstrated that *P. agathidicida* (NZFS 3813) can infect all six hosts (e.g. white clover - *Trifolium repens*, Caucasian clover - *T. ambiguum*, perennial ryegrass - *Lolium perenne*, annual ryegrass *L.*

multiflorum, pine - *Pinus radiata* and blue lupin - *Lupinus angustifolius*) common to pasture and pine forest. Although all hosts displayed high levels of colonisation towards day 10 (HD 5), annual and perennial ryegrass were observed with significantly greater (p <0.001) infection rates (percentage 70-90%) than all other hosts for the first two harvest days (day 2 and 4). Combined with indications that pasture and pine sites increase sporangia and oospore production in the early stages of establishment (Chapter 2), these results suggest that these land-uses may contribute to their potential as pathogen reservoirs of *P. agathidicida*.

5.2 Recommendations for Future Research

Based on the research described in this thesis, there are several areas that warrant further investigation:

High priority

- Given concerns over human/animal vectoring, accessibility and the potential for pasture and pine sites to act as pathogen reservoirs, further research into the role of fragmented landscapes on *P. agathidicida* transfer and epidemiology is required. For example, it would be useful to investigate the role of unfenced pastoral properties adjacent to kauri forest sites in the dissemination and disease incidence of *P. agathidicida*.
- Due to the limited spatial scale of the land-use soil pathogenicity study, a separate large-scale study incorporating components from previous *Phytophthora* pathogenicity studies (Kelly and Meentemeyer, 2002; Moreira and Martins, 2005), such as plant composition, landscape topology and disease distribution would significantly aid in understanding *P. agathidicida* epidemiology between contrasting land-uses. For example, in the study by Moreira and Martins (2005), sampling was carried out in 56 cork oak (*Quercus suber* and *Q. rotundifolia*) stands throughout Portugal. Soil bioassays were used to identify *P. cinnamomi* distribution, canopy foliage and tree decline were assessed, soil samples were analysed for chemical characteristics (e.g. pH, phosphorus content), and topographical (e.g. geographic location and slope) data were collected. This resulted in a thorough assessment of *P. cinnamomi* associated disease stands of cork oak, linked to specific soil characteristics (e.g. low fertility and nutrients) and different sites (e.g. higher *P. cinnamomi* recovery from south-facing and sloped/valley sites). Performing a similar study with *P. agathidicida* would help identify site-specific characteristics of contrasting land-uses associated with *P. agathidicida* distribution and

disease incidence. This would inform long-term management strategies by identifying, and potentially ranking, disease risk in similar sites.

- The discovery of two internationally recognised *Phytophthora* spp. new to New Zealand, *P. pini* and *P. gregata*, in pasture and kauri soils respectively, warrants further investigation into the potential impact they have on New Zealand plant species. For example, *P. pini* is recognised as an established pathogen in North America and Europe where it is known to infect at least seven genera (Hong *et al*., 2011), and may potentially impact a variety of other ornamental and vegetable plants, in addition to European beech trees (Jung *et al*., 2009). Given that pure and mixed beech forest comprise almost three-quarters (4.3 million hectares) of remaining New Zealand native forest (Smale *et al*., 2012), the potential for *P. pini* to infect and impact these forests requires serious consideration.

Medium priority

- Due to the capacity of *P. agathidicida* to infect all hosts considered in the alternative host study, further research into the relationship between host density in these sites, landscape fragmentation and *P. agathidicida* distribution would likely provide insights into pathogen dissemination and management.
- Lesion development in the land-use soil pathogenicity study was observed to occur between 2-4 days compared to the 3-6 day development period under sterile conditions, indicating land-use soils stimulated lesion development by *P. agathidicida* in blue lupin. While numerous physical (e.g. temperature, moisture content) and chemical (e.g. soil nutrients, pH) factors have been shown to influence *Phytophthora* sporulation and germination patterns, and hence disease incidence (Duniway, 1983; Schmitthenner and Canaday, 1983; Shearer *et al*., 2010; Weste, 1983), the potential impact of biological factors (e.g. microbial antagonism and secondary metabolites) as described by Marx and Haasis (1965) and Chee and Newhook (1966), necessitates further investigation. This may involve first identifying symptomatic and non-symptomatic *P. agathidicida* infected sites using disease monitoring (Waipara *et al*., 2013) and molecular diagnostic tools (Scibetta *et al*., 2012), followed by a meta-genomic approach to characterise the microbial communities common to each site (Riesenfeld *et al*., 2004). Finally, a bioassay screen for microbial antagonists to *P. agathidicida* sporulation and germination may be considered (Rajkumar *et al*., 2005).

Low priority

- With the recovery of several *Phytophthora* spp. (e.g. *P. agathidicida, P. cinnamomi, P. multivora* and *P. cryptogea*) from kauri forest soils, in addition to their known pathogenicity against excised kauri leaves and stems (Horner and Hough, 2014; Waipara et al., 2013), the potential role of disease complexes contributing to infection of kauri warrants further investigation. Given the recovery of *P. gregata* in kauri soils in this study, it may prove prudent to include this pathogen in future surveys to assess the role this pathogen plays in kauri dieback.

5.3 Concluding remarks

Current trends in Waipoua Forest indicate that land-use change will continue in many forms, dependent on the prevailing economic circumstances. Understanding land-use fragmentation and the impact this has on soil borne pathogen growth, pathogenicity and host-range is crucial when attempting to mitigate these effects on kauri forests. This is particularly relevant for *P. agathidicida*, which has only recently been characterised, and where focus has been directed towards short-medium term control measures such as identifying invasion pathways, chemical (phosphite) control, molecular detection and genetic sequencing for host resistance. This study is a small step in the quest to characterise the impacts of heterogeneous landscapes on *P. agathidicida* to inform long-term management decisions in reducing the deleterious effects of kauri dieback.

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Appendix A:

Supplementary data for Chapter 2

Appendix Table A.1 Two-way ANOVA of each spore count variable with all land-use (i.e. kauri, pasture, pine and controls) and site-samples (i.e. A and B horizon and LL) as categorical factors, split across each observational day. Significant F-values are denoted by an asterisk (* P < 0.05 ** P < 0.01 *** P < 0.001) while ns refers to non-significance. MS = Mature sporangia, JS = Juvenile sporangia, MO = Mature oospores, DO = Developing oospore, AO = Aborted oospore, JO = Juvenile oospore.

Observational day	*Spore counts*	*Land-use* [*F*(3,60)]	*Horizon* [*F*(2,60)]	*Interaction* [*F*(6,60)]
1	MS	6.9***	ns	ns
	JS	9.5***	ns	ns
	MO	ns	ns	ns
	DO	14.4***	ns	ns
	AO	15.8***	ns	ns
	JO	3.8*	ns	ns
2	MS	10.1***	ns	ns
	JS	29.7***	ns	ns
	MO	4.7**	ns	2.81*
	DO	55.4***	ns	ns
	AO	24.4***	ns	ns
	JO	9.5***	ns	ns
4	MS	6.2***	ns	ns
	JS	ns	ns	ns
	MO	ns	ns	ns
	DO	4.3**	ns	ns
	AO	15.2***	ns	ns
	JO	6.9***	ns	ns
8	MS	18.9***	ns	ns
	JS	ns	ns	2.27*
	MO	4.9**	ns	ns
	DO	22.7***	ns	ns
	AO	36.9***	ns	ns
	JO	31.3***	ns	ns

Appendix Table A.2 Statistical descriptives (mean ± standard deviation) of all log(+1)-transformed count variables for each observational day, compared across all land-use treatments (i.e. kauri, pasture, pine and control). Values that share the same letter are not significantly different under Tukey's HSD ($p < 0.05$). MS = Mature sporangia, JS = Juvenile sporangia, MO = Mature oospores, DO = Developing oospore, AO = Aborted oospore, JO = Juvenile oospore.

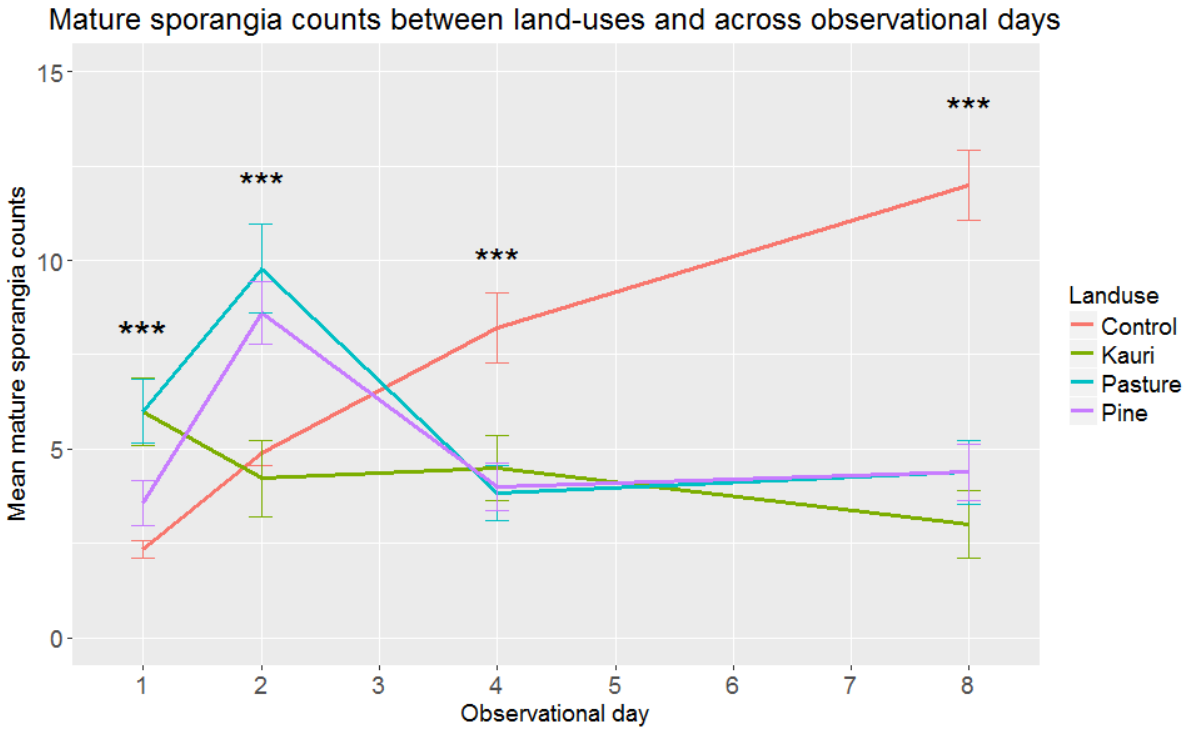
Observational day	*Spore count variables*	*Kauri*	*Pasture*	*Pine*	*Control*
1	MS	6 ± 3.8^{a}	$6\pm3.6^{a,b}$	$3.6\pm2.5^{b,c}$	2.3 ± 0.9^{c}
2	MS	4.2 ± 4.3^{a}	9.8 ± 4.9^{c}	$8.6\pm3.6^{b,c}$	$4.9\pm1.4^{a,b}$
4	MS	4.5 ± 3.7^{a}	3.8 ± 3.1^{a}	4 ± 2.7^{a}	8.2 ± 3.9^{b}
8	MS	3 ± 3.8^{a}	4.4 ± 3.5^{a}	4.4 ± 3.2^{a}	12 ± 3.9^{b}
1	JS	11.3 ± 7.9^{a}	10.4 ± 9.7^{a}	8.9 ± 9.7^{a}	2.5 ± 1.1^{b}
2	JS	13.9 ± 9.9^{a}	18.6 ± 10.7^{a}	13.1 ± 7.3^{a}	4.2 ± 1.5^{b}
4	JS	6.1 ± 6.2^{a}	11.1 ± 13.5^{a}	4.9 ± 3.4^{a}	6.7 ± 4.1^{a}
8	JS	10.2 ± 11.5^{a}	8.5 ± 7.9^{a}	6.5 ± 5.7^{a}	7.6 ± 2.1^{a}
1	MO	170.5 ± 73.9^{a}	161 ± 63.9^{a}	172.7 ± 49.4^{a}	171.2 ± 13.2^{a}
2	MO	173.2 ± 42.9^{a}	201 ± 38.8^{b}	216 ± 48.4^{b}	$175.7\pm35.8^{a,b}$
4	MO	159.5 ± 44.5^{a}	173 ± 52^{a}	190.4 ± 38.5^{a}	170.9 ± 31.3^{a}
8	MO	170.8 ± 47.5^{a}	181.6 ± 52.1^{a}	230.2 ± 48.3^{b}	$198.8\pm43.3^{a,b}$
1	DO	28.4 ± 31.7^{a}	29.7 ± 28.6^{a}	23.4 ± 25.3^{a}	120.1 ± 10^{b}
2	DO	15.9 ± 22.1^{a}	$8\pm5.6^{a,b}$	6.5 ± 11.8^{b}	111.9 ± 11.1^{c}
4	DO	22.8 ± 17.7^{a}	32.7 ± 43.3^{a}	31.7 ± 34^{a}	54.9 ± 24^{b}
8	DO	18.9 ± 25.4^{a}	2.8 ± 3.1^{b}	$13.6\pm32.9^{a,b}$	60.7 ± 41.2^{c}
1	AO	2.9 ± 3.6^{a}	2.2 ± 2.5^{a}	2.3 ± 3.1^{a}	9.1 ± 2.4^{b}
2	AO	1.9 ± 1.7^{a}	1.4 ± 1.9^{a}	2.3 ± 4.6^{a}	8.8 ± 3.6^{b}
4	AO	1.6 ± 1.8^{a}	1.2 ± 1.5^{a}	0.8 ± 1.6^{a}	5 ± 2.8^{b}
8	AO	0.8 ± 1.4^{a}	0.2 ± 0.4^{b}	$1\pm2.6^{a,b}$	6.3 ± 3^{c}
1	JO	$3.2\pm2.4^{a,b}$	$4.4\pm4.1^{a,b}$	2.8 ± 2.5^{a}	5.6 ± 2.2^{b}
2	JO	0.7 ± 4.3^{a}	2.2 ± 2.1^{a}	1.8 ± 1.7^{a}	5.7 ± 1.8^{b}
4	JO	2.6 ± 1.7^{a}	3 ± 3.4^{a}	2.6 ± 2.1^{a}	6.4 ± 3.1^{b}
8	JO	2.4 ± 2.3^{a}	1.5 ± 1.6^{a}	1.9 ± 1.4^{a}	11.8 ± 4.3^{b}

Appendix Table A.3 Statistical descriptives (mean ± standard deviation) of all log(+1)-transformed count variables for each observational day for control samples. Values that share the same letter are not significantly different under Tukey's HSD ($p < 0.05$). MS = Mature sporangia, JS = Juvenile sporangia, MO = Mature oospores, DO = Developing oospore, AO = Aborted oospore, JO = Juvenile oospore.

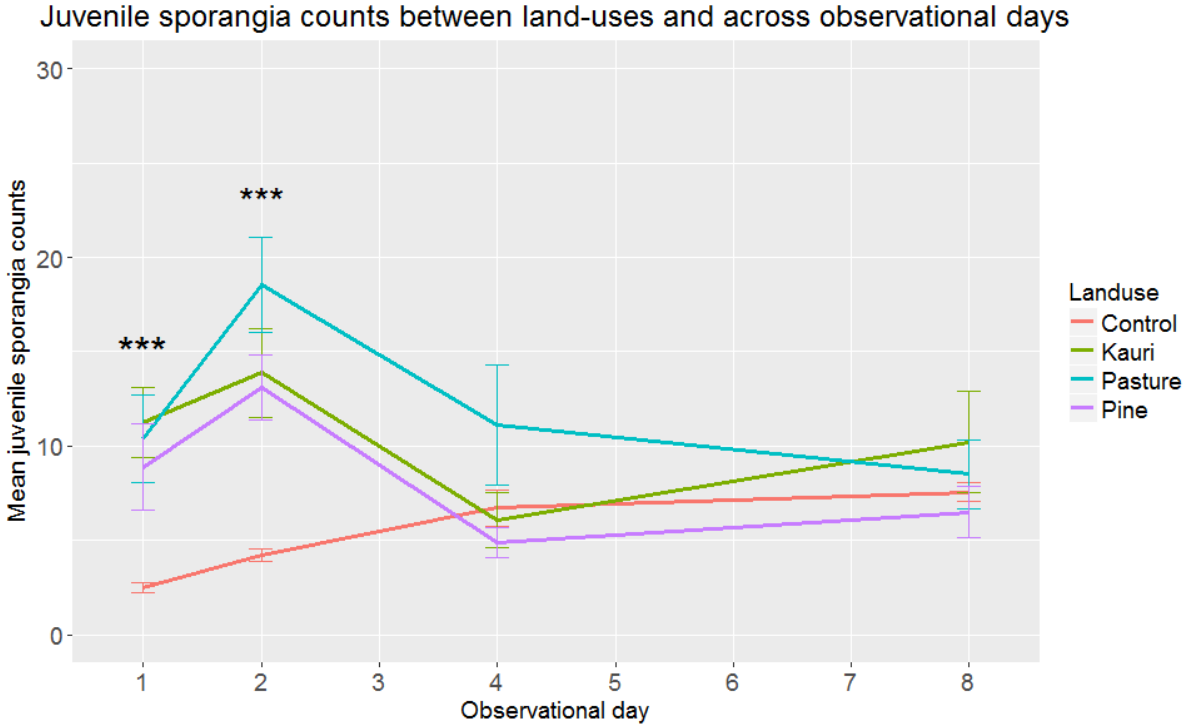
Spore count	*Land-use*	*Observational day*			
		1	*2*	*4*	*8*
MS	Control	2.3±0.9^{a}	4.9±1.4^{ab}	8.2±3.9^{bc}	12±3.9^{c}
JS	Control	2.5±1.1^{a}	4.2±1.5^{ab}	6.7±4.1^{ab}	7.6±2.1^{b}
MO	Control	171.2±13.2^{a}	175.7±35.8^{a}	170.9±31.3^{a}	198.8±43.3^{a}
DO	Control	120.1±10^{a}	111.9±11.1^{a}	54.9±24^{b}	60.7±41.2^{b}
AO	Control	9.1±2.4^{a}	8.8±3.6^{a}	5±2.8^{a}	6.3±3^{a}
JO	Control	5.6±2.2^{a}	5.7±1.8^{a}	6.4±3.1^{a}	11.8±4.3^{b}

Appendix Table A.4 Formalin-Acetic Acid Solution (FAA) 100 ml stock.

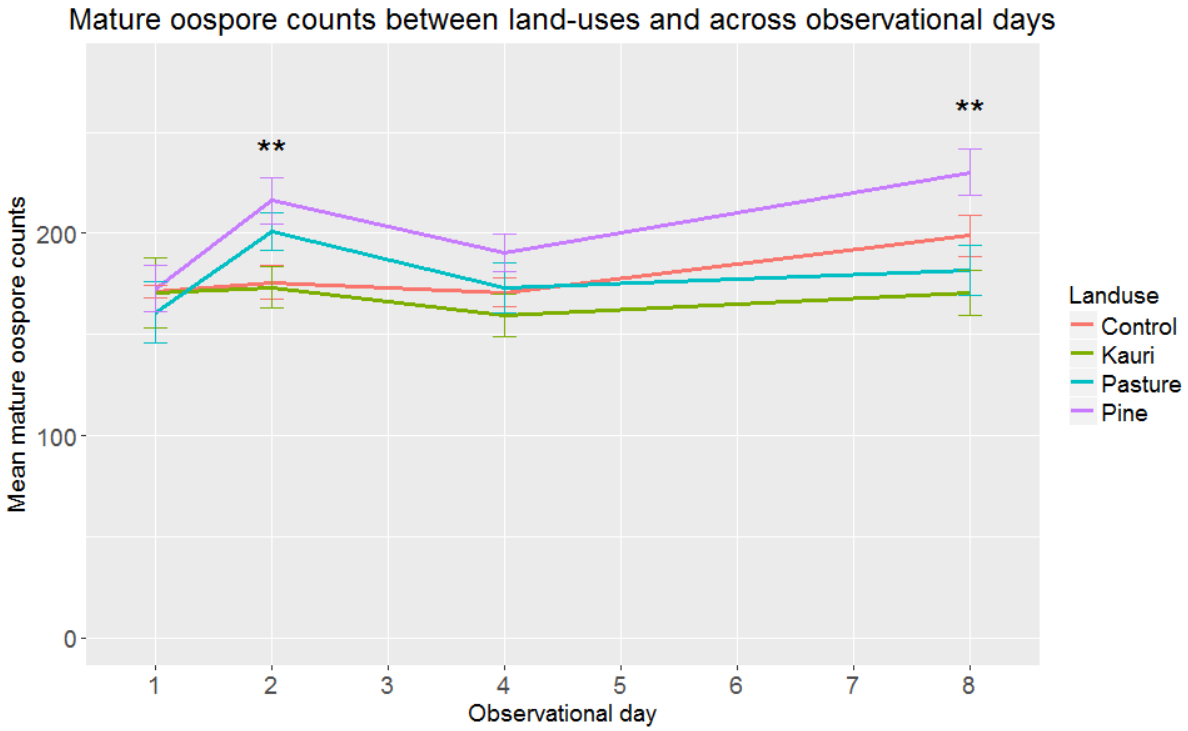
Mix together:
Formaldehyde (37-40%) -10 ml
Distilled water - 85 ml
Acetic acid - 5 ml



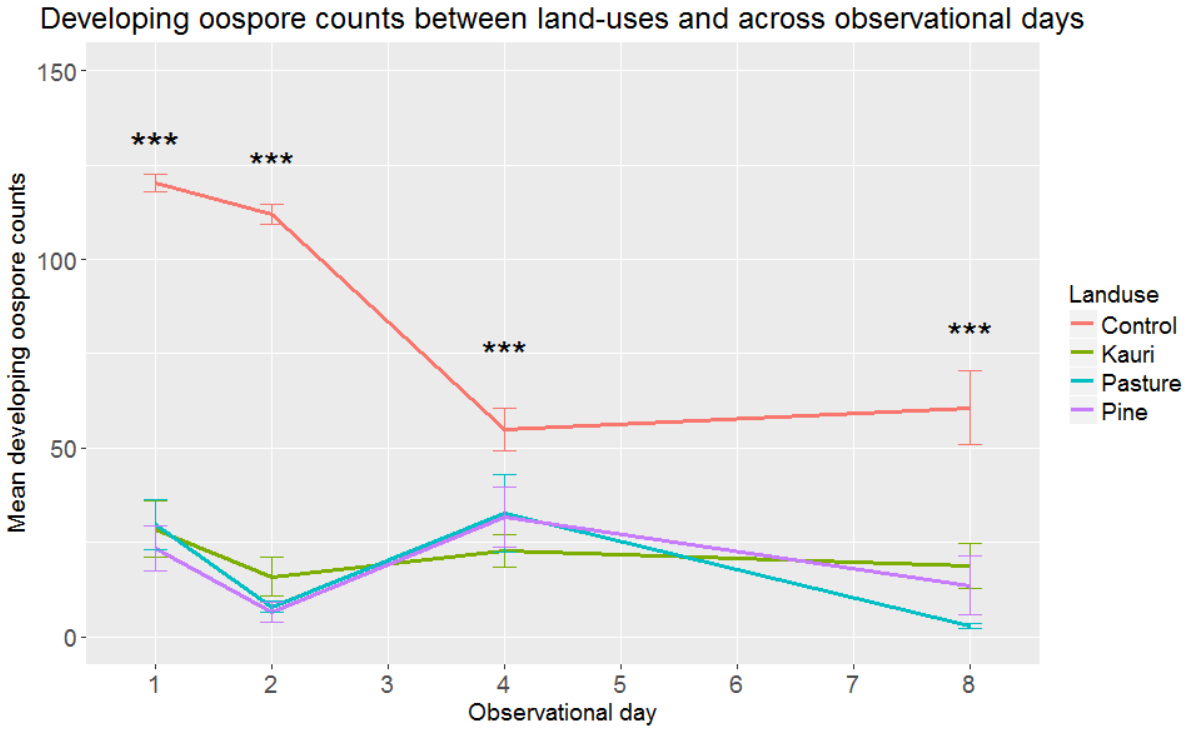
Appendix Figure A.1 Mean counts of mature sporangia between land-uses (kauri, pasture, pine and control) over observational days (day1, 2, 4 and 8). Significant differences between land-uses per observational day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.



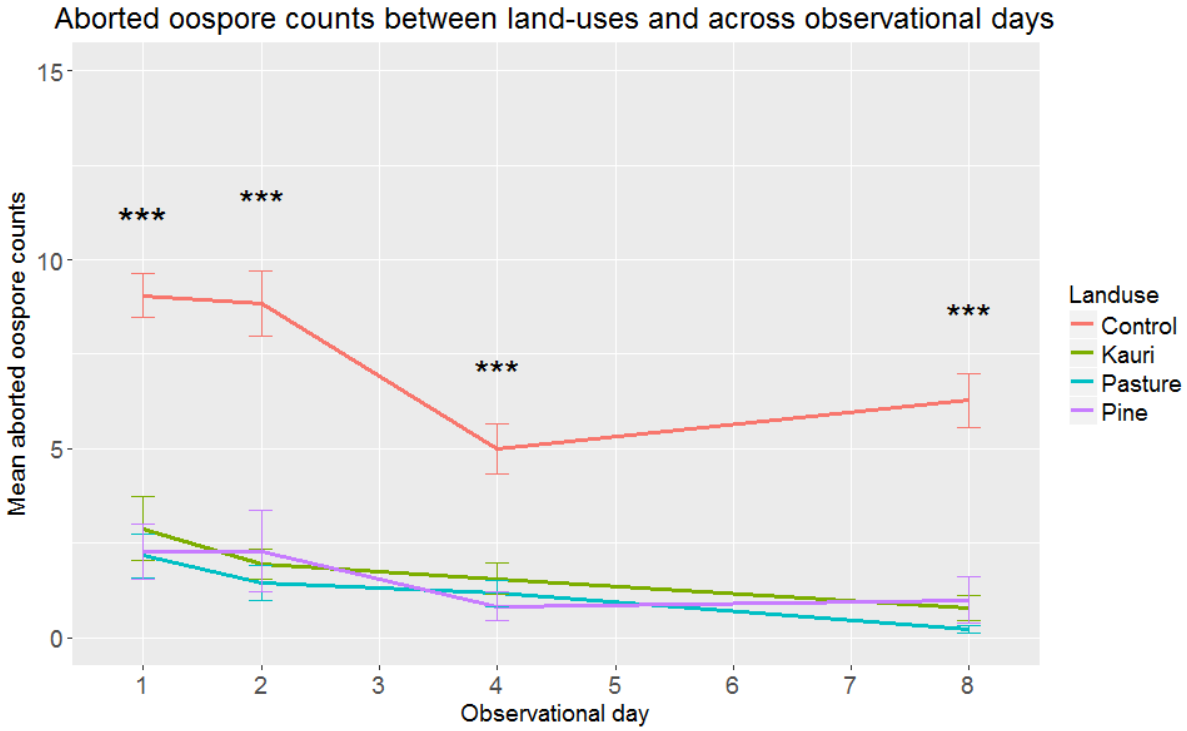
Appendix Figure A.2 Mean counts of juvenile sporangia between land-uses (kauri, pasture, pine and control) over observational days (day1, 2, 4 and 8). Significant differences between land-uses per observational day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.



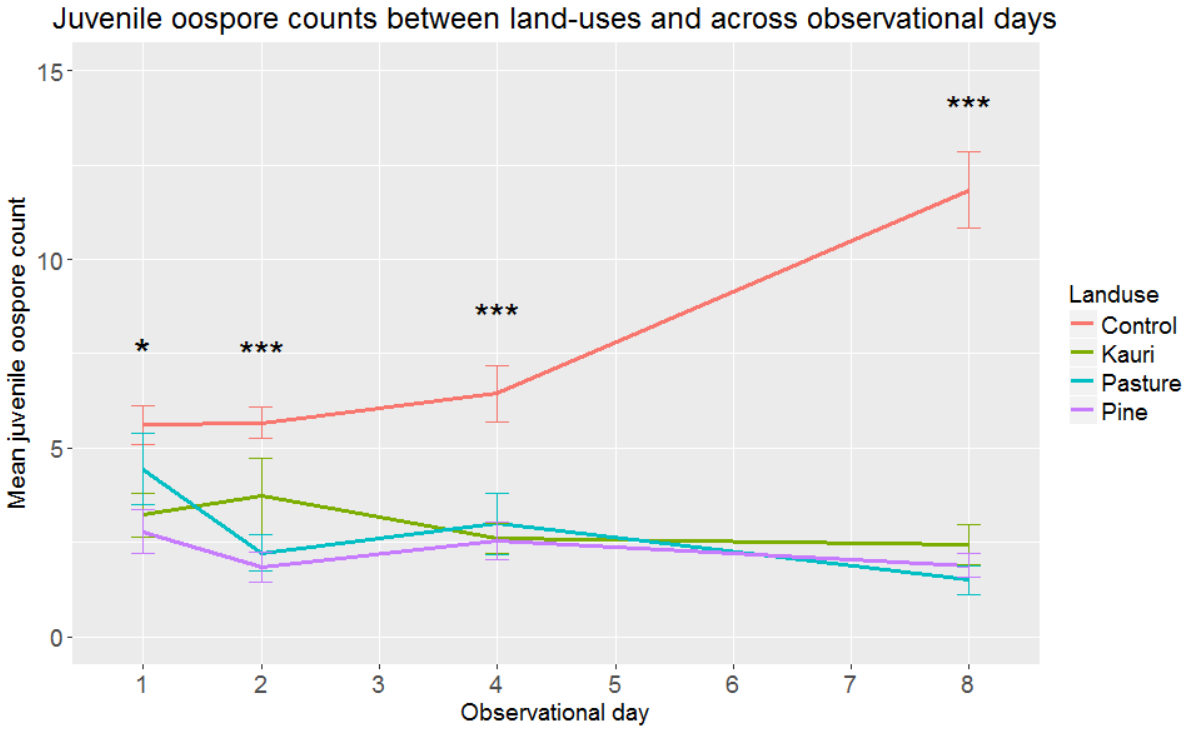
Appendix Figure A.3 Mean counts of mature oospores between land-uses (kauri, pasture, pine and control) over observational days (day1, 2, 4 and 8). Significant differences between land-uses per observational day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.



Appendix Figure A.4 Mean counts of developing oospores between land-uses (kauri, pasture, pine) over observational days (day1, 2, 4 and 8). Significant differences between land-uses per observational day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.



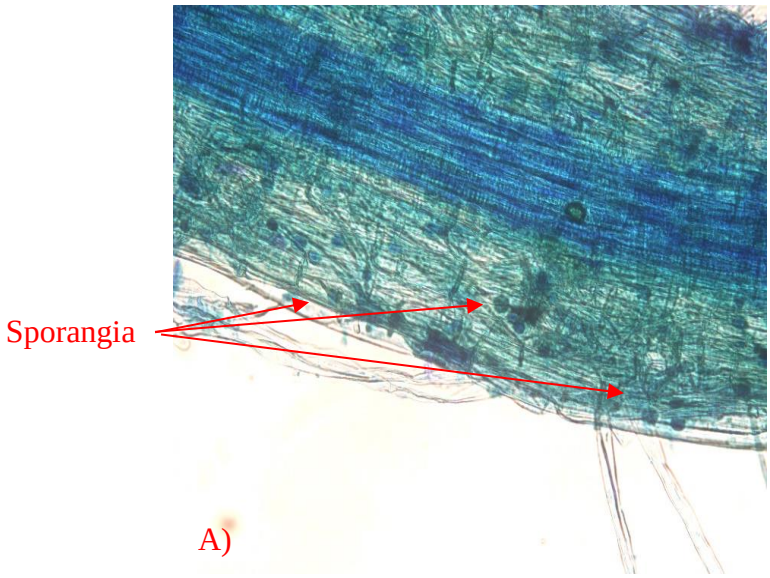
Appendix Figure A.5 Mean counts of aborted oospores between land-uses (kauri, pasture, pine) over observational days (day1, 2, 4 and 8). Significant differences between land-uses per observational day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.

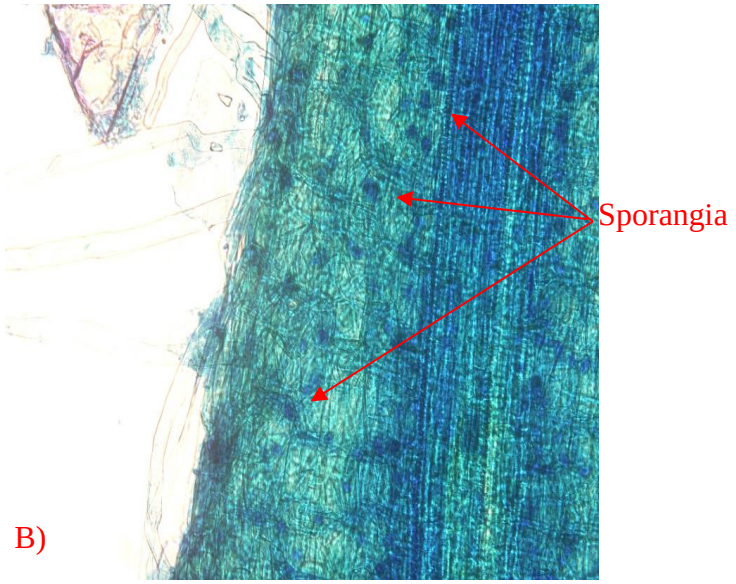


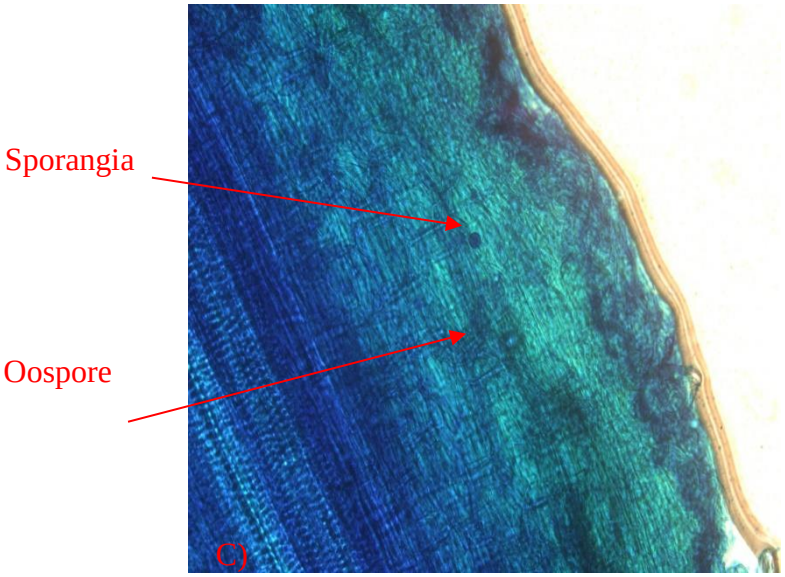
Appendix Figure A.6 Mean counts of juvenile oospores between land-uses (kauri, pasture, pine) over observational days (day1, 2, 4 and 8). Significant differences between land-uses per observational day are indicated by * P < 0.05 ** P < 0.01 *** P < 0.001.

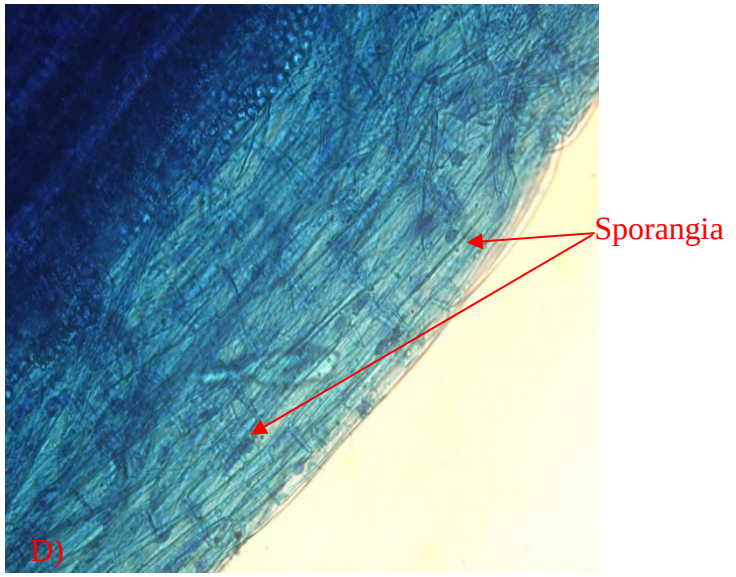
Appendix B:

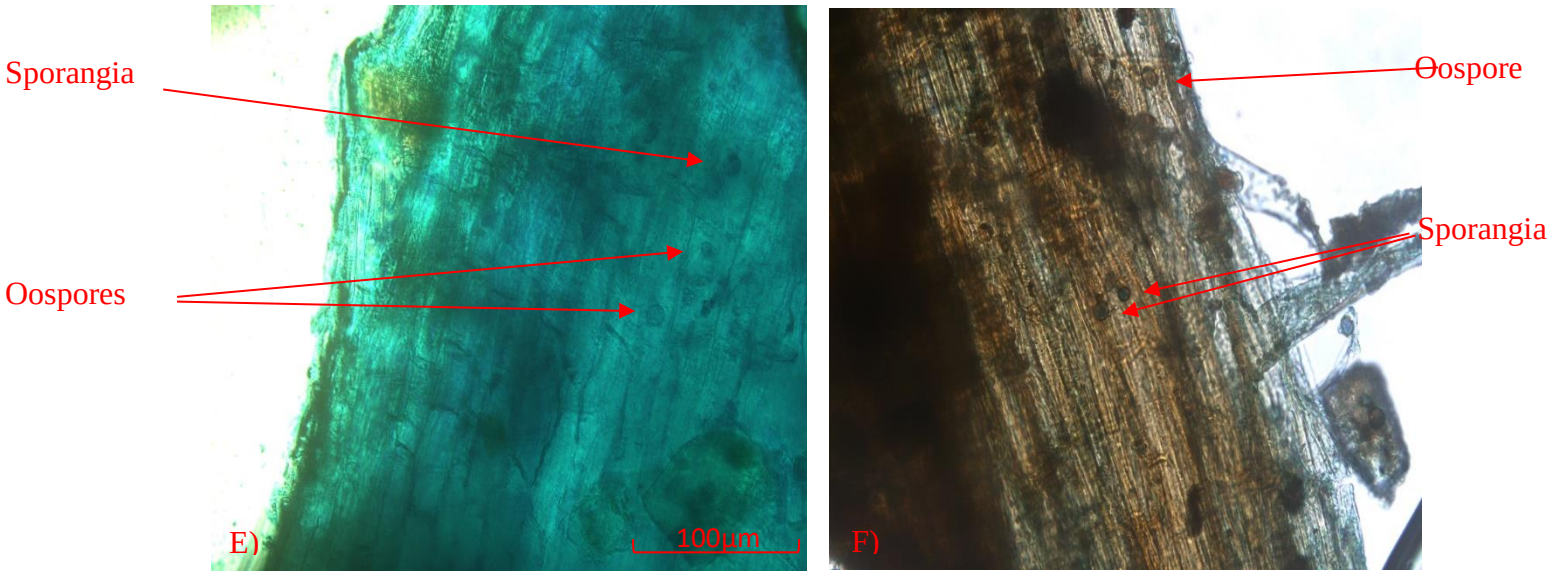
Supplementary data for Chapter 4



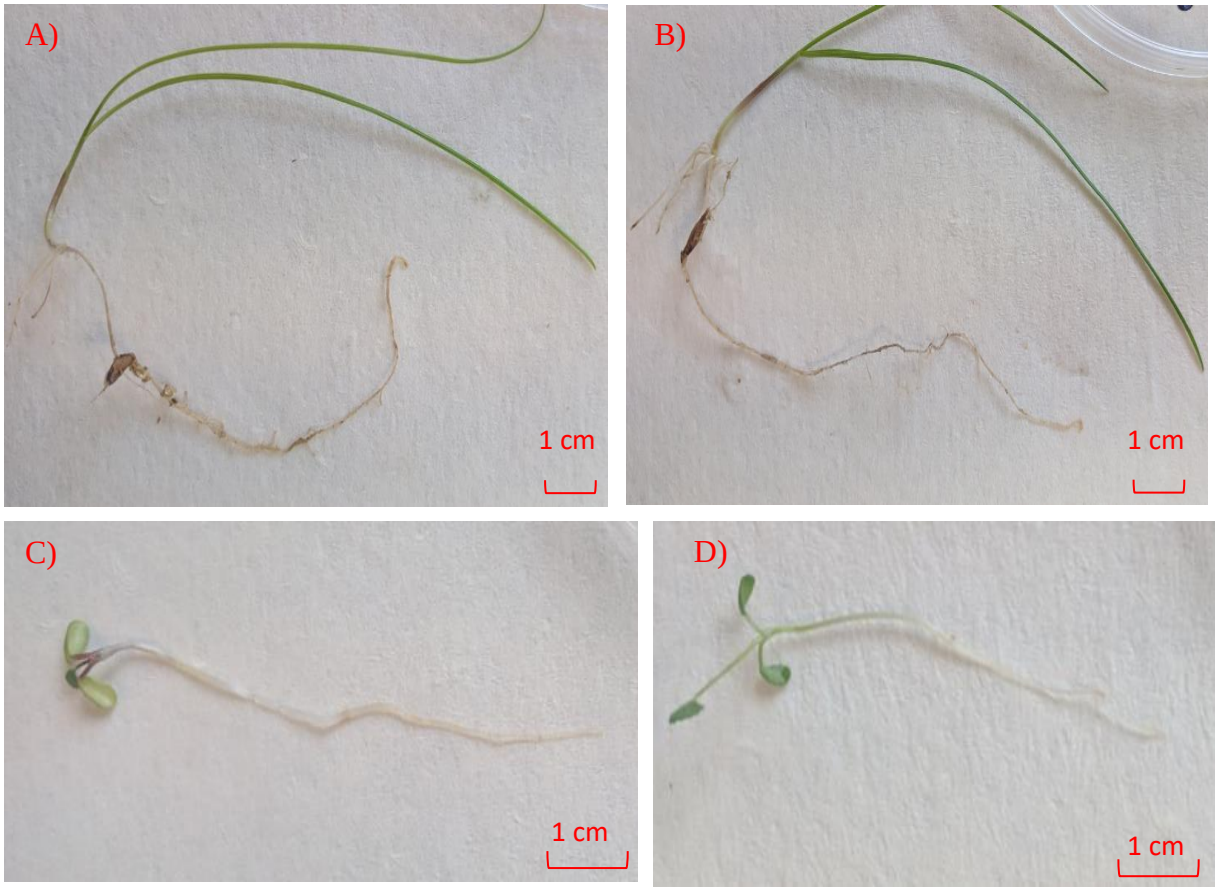








Appendix Figure B.1 All six hosts showing infection by *Phytophthora agathidicida* within section 5 (5 cm from root tip) A) Annual ryegrass (*Lolium multiflorum*) on day 10 (HD 5) showing numerous sporangia; B) Perennial ryegrass (*L. perenne*) on day 10 (HD 5) showing numerous sporangia; C) Caucasian clover (*Trifolium ambiguum*) on day 10 (HD 5) showing some sporangia/oospore; D) White clover (*T. repens*) showing several sporangia; E) Lupin (*Lupinus angustifolius*) showing oospores/sporangia; F) Pine (*Pinus radiata*) root displaying several sporangia/oospore. 200x magnification.



Appendix Figure B.2 A) *Phytophthora agathidicida* inoculated annual ryegrass (*Lolium multiflorum*) on day 10 (HD 5); B) Inoculated perennial ryegrass (*L. perenne*) on day 10 (HD 5); C) Inoculated Caucasian clover (*Trifolium ambiguum*) on HD 5; D) Inoculated white clover (*T. repens*) on day 10 (HD 5)