# Determining the risk of phosphite tolerance in *Phytophthora* species in New Zealand and the United States: a case study on the implications of long-term use of phosphite to control *Phytophthora cinnamomi* in avocado (*Persea americana*)

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**SHANNON RENEE HUNTER**


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# Abstract

*Phytophthora* species cause many major plant disease epidemics worldwide, impacting horticulture, plant trade and production planting industries through the loss of plants and the costs arising from disease management. *Phytophthora* species have specialised structures which enhance their dispersal, survival and infection. These include motile swimming spores, caducous sporangia and resting spores. Nurseries and orchards use integrated management to control phytophthora diseases, including cultural practises, biological control, resistant host plants and chemical control. The use of fungicides is a very effective control method; however, *Phytophthora* species can develop resistance to fungicides after prolonged exposure. For example, phosphite tolerant isolates of *Phytophthora cinnamomi* have been found in avocado orchards in Australia and South Africa.

Phosphite is widely used in nurseries and orchards in New Zealand as a preventative and control treatment. The avocado industry largely relies on it for the control of avocado root rot caused by *P. cinnamomi*. Phosphite is most commonly applied to avocado trees using passive trunk injections but can also be applied as foliar sprays, soil drenches, high-pressure injections and capsule implants.

To assess phosphite tolerance of *P. cinnamomi* isolates from New Zealand avocado orchards, a high-throughput optical density (OD) assay was developed to measure mycelial growth inhibition in the presence of phosphite. The OD assay was used to screen 24 *P. cinnamomi* isolates from four orchards never treated with phosphite and 32 isolates from eight orchards treated with phosphite for 15 – 37 years. Four isolates had increased tolerance to phosphite and two were intermediately tolerant. These six isolates were from phosphite managed orchards and five were isolated from unhealthy trees.

To test if *in vitro* tolerance would be expressed *in planta*, three tolerant and three sensitive isolates were tested for their ability to colonise phosphite treated lupin (*Lupinus angustifolius*) roots. The tolerant isolates were more extensive colonisers of lupins treated with 5 and 10 g/L phosphite. In the presence of phosphite the tolerant isolates were able to produce more sporangia and release viable swimming zoospores more often.

Internationally important *Phytophthora* species from culture collections in New Zealand and the United States were assessed for sensitivity to phosphite and a subset of 32 isolates from the Berkeley collection was tested for mefenoxam sensitivity. The causal agent of kauri (*Agathis australis*) dieback, *P. agathidicida*, was found to be highly sensitive to phosphite while the possibly New Zealand native *P. kernoviae*, was found to be relatively tolerant. Intraspecific variation was found in lineages of *P. ramorum*, the causal agent of sudden oak death in the United States and the United Kingdom, NA2 was more tolerant than NA1. One *P. megasperma* isolate was more tolerant to mefenoxam compared to the other isolates tested. Of nine *P. cinnamomi* isolates from avocado orchards in southern California, one was relatively more tolerant to phosphite.

This study has shown phosphite tolerant isolates of *P. cinnamomi* have developed in phosphite managed avocado orchards in New Zealand and interspecific variation in sensitivity to fungicides is present across *Phytophthora* species in New Zealand and the United states.

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# Introduction

## 1.1 Phytophthora

*Phytophthora* (Oomycetes: Peronosporales: Pythiaceae) is a genus of microscopic plant pathogens which are responsible for many major plant disease epidemics (Erwin & Ribeiro, 1996; Kroon *et al.*, 2012). It is estimated that there are likely to be between 200 and 600 *Phytophthora* species, most of which are yet to be formally described (Braiser, 2007).

Once a *Phytophthora* species has colonized an organic matrix, such as root fragments, vascular tissue or leaves, and has formed resting structures it can easily survive for many years and be spread long distances (Collins *et al.*, 2012; Crone *et al.*, 2013c; Erwin & Ribeiro, 1996; Jung *et al.*, 2013) by anything vectoring soil, such as snails (Alvarez *et al.*, 2009) and pigs (Li *et al.*, 2014). The global trade of plants provides an international highway for *Phytophthora* species to be transported on (Braiser, 2008a; Scott *et al.*, 2013). Introduced *Phytophthora* species are often able to infect more host species more virulently because native plants have not evolved natural defences (Scott *et al.*, 2013). An infamous example of an introduced *Phytophthora* species causing severe disease is *Phytophthora cinnamomi* in Australia, where it is able to infect over 2000 plant species and caused severe damage to the Jarrah forests (Podger, 1972; Shearer *et al.*, 2007; Shearer *et al.*, 2004).

*Phytophthora* species infect host plants through the roots, trunk or foliage (Oßwald *et al.*, 2014) and disease symptoms can vary widely. Symptoms of phytophthora diseases include damping-off, chlorosis, dieback, root rot, and the rotting of other organs (Akino, Takemoto, & Hosaka, 2014). *Phytophthora* species which infect the host though the root system often cause damage to the fine feeder roots which affect the plant's ability to conduct water resulting in canopy wilting and leaf curling. For example *P. cinnamomi* infects the roots of jarrah (*Eucalyptus margintina*), resulting in declining tree crowns (Davison, 1997).

In some phytophthora trunk diseases, prominent bleeding trunk cankers and lesions develop. For example, *P. ramorum* causes lethal stem cankers on oaks and tanoaks (*Notholithocarpus densiflorus*) resulting in a 'sudden death' (Rizzo et al., 2002), these are recognised as dead end hosts. Cankers can appear up to 10 meters from the base of the tree and bleed a dark brown coloured sap (Garbelotto, Svihra, & Rizzo, 2001). This outstanding symptom causes the death of the tree over a period of months and also makes it more susceptible to other pathogens. In contrast, on Bay Laurel (*Umbellularia californica*) *P. ramorum* is predominately a foliar pathogen, is less virulent and causes leaf spots (Hüberli & Garbelotto, 2012). Bay laurels act as a reservoir for the pathogen and are necessary for transmission between oak trees in Californian forests (Davidson *et al.*, 2005).

There are more than 30 *Phytophthora* species known to be associated with disease in New Zealand horticultural, native and plantation/agricultural systems, most of which are probably introduced (Hansen, 2008; Scott & Williams, 2014). These diseases threaten ecosystem health and human welfare. The geographic isolation of New Zealand and progressive tightening of biosecurity measures have contributed to the exclusion of many important invasive *Phytophthora* species including *P. ramorum*, which is listed as a national biosecurity threat. *Phytophthora ramorum* has the potential to infect native and commercially important species in New Zealand (Hüberli *et al.*, 2008).

It is hypothesised that *P. kernoviae* is a native species to New Zealand as it does not appear to be a serious pathogen to indigenous flora of New Zealand (Gardner *et al.*, 2015), however this has not yet been confirmed (Ramsfield *et al.*, 2009). *Phytophthora kernoviae* is a serious pathogen in the United Kingdom, where it causes disease and bleeding stem lesions on ornamental plants including *Fagus sylvatica* (Brasier *et al.*, 2005; Braiser, 2008b). Isolates of *P. kernoviae* from the UK are more virulent than isolates from New Zealand, based on the degree of damage to *Rhododendron pointicum*, *Magnolia stellata* and *Annonoa cherimola*, inoculated with sporangia from six isolates from each geographical region (Widmer, 2015). More recently *P. kernoviae* has been isolated from necrotic lesions on fallen leaves of *Drymis winteri* from forests in southern Chile (Sanfuentes *et al.*, 2016). New Zealand and Chilean Valdivian rain forests share flora from the genera Winteraceae so it is possible *P. kernoviae* evolved with such

plants and this explains its location in New Zealand and Chile (Sanfuentes *et al.*, 2016).

Several important phytophthora diseases occur in New Zealand that threaten tourism, forestry and horticulture. Tourism may suffer if movement within affected forests is significantly reduced or restricted to prevent spreading the disease. For example, tracks in kauri (*Agathis australis*) forests are closed to reduce foot traffic and protect remaining kauri trees which suffer a dieback disease caused by *P. agathidicida* (Beever *et al.*, 2007; Gadgil, 1974). As kauri is a keystone species, the whole ecosystem is under threat of being destabilised if too many kauri die off (Beever et al., 2007).

New Zealand's most important forestry plantation species, Monterey pine (*Pinus radiata*), is threatened by Red Needle Cast (RNC) caused by *P. pluvialis* (Ganley et al., 2014). Pine trees that experience reoccurring disease events each year can experience growth declines which may affect the size of the tree and wood quality (Ganley *et al.*, 2014). Fortunately it has been determined that the possibility of transporting *P. pluvialis* on exported logs is negligible (Hood *et al.*, 2014) and it is possible to select resistant *P. radiata* genotypes to deliver healthier trees (Dungey *et al.*, 2014). Also, copper oxychloride, phosphite and metalaxyl-M have potential to protect commercially planted *P. radiata* (Rolando *et al.*, 2017).

*Phytophthora* diseases of horticultural species present the most serious economic threat, because seasonal yields are impacted resulting in massive economic losses. The most well-known epidemic caused by a *Phytophthora* species is potato (*Solanum tuberosum*) late blight, caused by *P. infestans* (Akino *et al.*, 2014; Fry, 2008). This disease caused the Irish potato famine in the mid-19$^{th}$ century and continues to be the most important disease of potatoes worldwide. Another example comes from *Phytophthora cinnamomi*, which is responsible for the most serious disease of avocado (*Persea Americana*) worldwide (Dann *et al.*, 2013). It has been estimated that losses in avocado crops caused by *P. cinnamomi* exceed US$40 million annually, in California alone (Ploetz, 2013). Avocado root rot is the major limiting factor for production in Australia, south Africa and California and has eliminated commercial production in many areas in Latin America (Ploetz *et al.*, 2002). The avocado industry worldwide largely relies on the application of the chemical fungicide phosphite to manage the disease.

Food security is a major concern for the future as the world's population is growing. Global food production needs to increase by 50% by 2050 to meet the projected demand (Chakraborty & Newton, 2011). Plant pathogens, such as *Phytophthora*, are responsible for significant losses of crop harvests and threaten global food security (Strange & Scott, 2005). It is predicted that climate change will alter the spread, reproduction and severity of many *Phytophthora* pathogens and their associated diseases. For example, it is predicted that global warming and climate change will increase *P. cinnamomi* disease problems (Burgess *et al.*, 2017a). Fungicide efficacy is important to maintain if global food demands are to be met when faced with the threats of climate change and effective pathogens like *Phytophthora.*

### 1.1.1 Phytophthora root rot of avocado

#### *1.1.1.1 Avocado*

Internationally, avocado (family Lauraceae of the order Laurales) is a commercially important crop with production levels exceeding 3.5 million tons of fruit (Schaffer *et al.*, 2013). Countries with the highest annual avocado exports are Chile (100,000 ton), and Mexico (380,000 ton) (Schaffer *et al.*, 2013). The avocado industry has developed rapidly in the last 150 years (Bost *et al.*, 2013), most recently this is attributed to the health qualities of the fruit being increasingly recognised (Schaffer *et al.*, 2013). The market for processed avocado products is growing, providing further opportunity for market increases.

Avocado was first brought to New Zealand in 1920 (White, 2001). In New Zealand, a record high of 43,375 tons of avocados was produced in 2016-17 (New Zealand Avocado Annual Report, 2017). New Zealand has approximately 1350 avocado growers with a total planted area of more than 4000 hectares (NZ Avocado Growers Association, 2016). Most of the orchards are located in the Bay of Plenty and Northland regions in the North Island.

Avocado was domesticated at least three times. These events were geographically separated and resulted in the three recognised races of avocado, the Mexican (*P. americana* var. *drymifolia*), Guatemalan (*P. americana* var. *guatemalensis*) and West Indian (*P. americana* Mill.var. *americana*) races (Chen *et al.*, 2009). These races have remained genetically and morphologically distinct and have been

spread to ecologically appropriate areas to be cultivated (Bost *et al.*, 2013). The Guatemalan race is the most important for world trade followed by the Mexican race to a lesser extent (Schaffer *et al.*, 2013). The West Indian race grows well in tropical lowland countries and plays an important role in upgrading the diets of local people (Chanderbali *et al.*, 2013).

Hybridisation between races can occur as there are no sterility barriers. The 'Hass' cultivar, regarded as pure Guatemalan but containing Mexican alleles (Davis *et al.*, 1998), is responsible for 90% of the export trade (Crane *et al.*, 2013). In New Zealand the dominant cultivar is 'Hass', while 'Fuerte' is usually grown as a pollinator in 'Hass' orchards. New cultivars are bred in an attempt to improve aspects of avocado production (Crane *et al.*, 2013).

Avocado are subject to many pests and diseases, however avocado root rot caused by *P. cinnamomi* is the most important disease of avocado worldwide (Dann *et al.*, 2013; Schaffer *et al.*, 2013). *Phytophthora cinnamomi* has been speculated to be of south-east Asian origin in the sub-tropical region spanning Indonesia, Malaysia, Taiwan and New Guinea with high diversity also identified throughout South Africa (Engelbrecht *et al.*, 2017). However, no evidence for long-term association with avocado exists, even though it is a key pathogen of avocado production globally (Schaffer *et al.*, 2013).

#### *1.1.1.2 Avocado root rot biology*

Phytophthora root rot of avocado was first reported in 1929 in Puerto Rico (Tucker, 1929). The disease was thought to be a 'water injury' and was called melanorhiza (Horne, 1934). Trees of all ages are attacked and eventually die if the disease is not managed (Dann *et al.*, 2013). The causal agent, *P. cinnamomi*, is a soil borne species with an extensive host range, estimated to be in excess of 5000 species (Cahill *et al.*, 2008; Jung *et al.*, 2013). Hosts range from horticultural species, such as avocado, pineapple, peach and chestnut (Hardham, 2005) to large forest trees such as jarrah in Australia (Davison, 2015).

Several other species of *Phytophthora* have been found associated with avocado; however, in New Zealand only *P. cinnamomi* has been recorded (Table 1.1).

**Table 1.1** *Phytophthora* species recorded as being associated with *Persea americano*. From: https://nt.ars-grin.gov/fungaldatabases/fungushost/fungushost.cfm

| Species | Location |
|---|---|
| *P. boehmeriae* | Mexico |
| *P. cactorum* | California, Indonesia, Peru, Spain, United States |
| *P. cambivora* | California, Madagascar, South Africa |
| *P. capsici* | United States |
| *P. cinnamomi* | Argentina, Australia, Barbados, Bolivia, Brazil, California, Chile, China, Costa Rica, Cuba, El Salvador, Fiji, Florida, Guatemala, Hawaii, Honduras, Indonesia, Italy, Malaysia, Mexico, New Guinea, New Zealand, Panama, Papua New Guinea, Peru, Puerto Rico, South Africa, Taiwan, United States, Venezuela, Virgin Islands |
| *P. cinnamomi* var. *cinnamomi* - (*P. cinnamomi*) | United States |
| *P. citricola* | Australia, California, Guatemala, Mexico, United States |
| *P. citrophthora* | California, United States |
| *P. heveae* | Guatemala |
| *P. megasperma* | Greece |
| *P. mengei* | California, Guatemala, Mexico |
| *P. nicotianae* | China, Cuba, French Polynesia, United States |
| *P. nicotianae* var. *nicotianae* - (*P. nicotianae*) | Papua New Guinea |
| *P. palmivora* | China, Florida, Honduras, Philippines, Thailand |
| *P. palmivora* var. *palmivora* - (*P. palmivora*) | Philippines, United States, West Africa |
| *P. parasitica* - (*P. nicotianae*) | Florida, United States |
| *Phytophthora* sp. | Malaysia |

*Phytophthora* species are well adapted to a wide range of hosts and environments and they have a variety of reproductive structures associated with dispersal, survival and infection (Erwin and Ribeiro 1996). In avocado the phytophthora infection cycle begins when the pathogen first attaches itself to the host and then infects it. The pathogen spreads throughout host plant tissue while obtaining nutrients to survive and continue reproduction. Spores are produced within host tissue and released into the environment thus starting the disease cycle again (Figure 1.1).

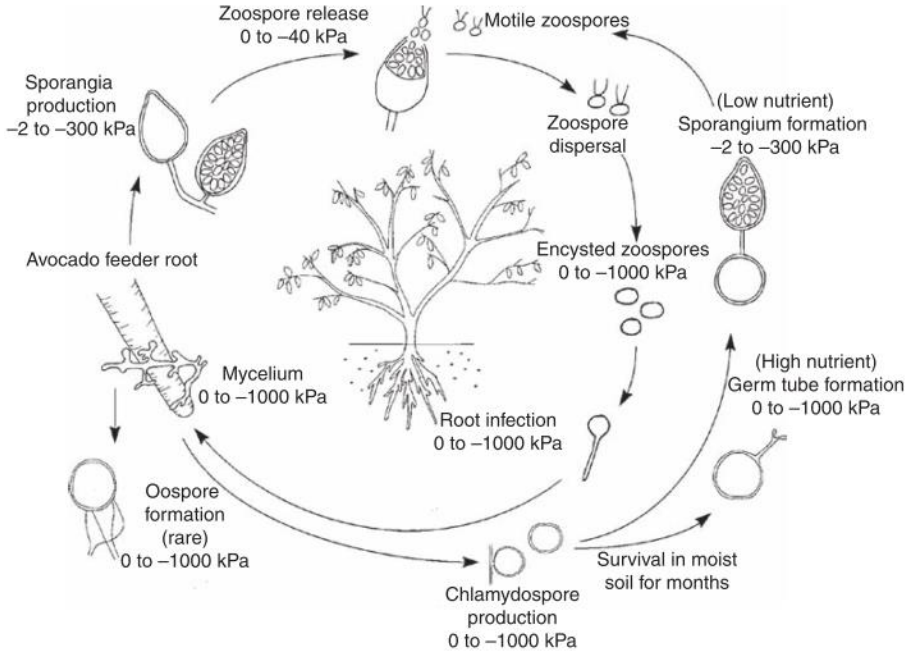


**Figure 1.1** Disease cycle of *Phytophthora cinnamomi* causing avocado root rot. From Dann et al. (2013).

Some *Phytophthora* isolates have been grown for years in pure culture through vegetative hyphal growth, however, the development of spores in nature is fundamental to the survival of the pathogen. *Phytophthora* species cannot survive long as saprophytes because they are quickly decomposed or inhibited by surrounding antagonistic bacteria, fungi and soil microbiomes once they are outside of the colonized organic material (Bae *et al.*, 2016; Costa *et al.*, 2000; Erwin & Ribeiro, 1996; Judelson & Blanco, 2005). Spores produced by *Phytophthora* include the sexual oospore and the asexual chlamydospores, sporangium, and motile zoospore (Judelson & Blanco, 2005).

Chlamydospores and oospores function as resting spores, these are important for survival and dispersal. They are formed within infected plant material including roots, stems, twigs and leaves, and released to the environment as the tissue decays. *Phytophthora cinnamomi* is usually introduced to an area through transport of resting spores in soil.

*Phytophthora cinnamomi* chlamydospores range from 31 – 50 µm in diameter and are formed readily in the lab (Dann *et al.*, 2013). They are more resistant to microbial attack than hyphae (Dann *et al.*, 2013) and can persist in the environment for several years until the conditions are suitable to germinate. *Phytophthora cinnamomi* chlamydospores are important for survival during dry

conditions between rain events in natural ecosystems in Australia; however, oospores and hyphae in infected plant material are most significant for long-term survival (Jung *et al.*, 2013).

All species produce sexual oospores which are thick-walled and durable and remain viable between growing seasons of hosts (Judelson & Blanco, 2005). Oospores are a significant inoculum source for homothallic species (selfed oospores) and occur less often in heterothallic species because two mating strains need to cross (Judelson & Blanco, 2005). *Phytophthora cinnamomi* is a heterothallic species with two mating strains of hyphae, A1 and A2 (Shepherd *et al.*, 1974), however the A2 mating strain which dominates in avocado orchards worldwide can produce selfed oospores (Coffey, 1992).

*Phytophthora cinnamomi* resting spores germinate when soil temperatures are above 15˚C and soil moisture is sufficient, producing several germ tubes or a single sporangium. Sporangia of *P. cinnamomi* are non-caducous (they remain attached to the hyphae), non-papillate and elliptical to ovoid (Dann *et al.*, 2013). Sporangia of other species may be caudacous, with the sporangia able to break off and spread in the environment (Erwin & Ribeiro, 1996). Caducous sporangia may be disperse aerially for a range of species including *P. ramorum* (Rizzo *et al.*, 2002) and *P. infestans* (Cooke *et al.*, 2013). Non-caducous or only partially-caducous sporangia cannot be spread by large distances in moist air and spread occurs predominately by rain-splash of disseminating zoospores.

Sporangia germinate in two ways, directly by the emergence of hyphae through the sporangial wall or the papilla of the sporangia opens and motile zoospores swim out into the environment (Figure 1.1). Inside a *P. cinnamomi* sporangium, up to 40 zoospores develop (Dann *et al.*, 2013). Zoospores locate the avocado fine feeder roots by following nonspecific chemoattractants exuded by the plant from root tips (Judelson & Blanco, 2005). *Phytophthora cinnamomi* zoospores may exhibit auto-aggregation at high densities (Figure 1.2 A) (Hardham & Blackman, 2018) potentially increasing the chance of successful infection (Savory *et al.*, 2014). Zoospores eventually encyst, losing their flagella and discharging several compounds which enable the zoospore to stick to its potential host (Figure 1.2 B) (Hardham & Blackman, 2018).

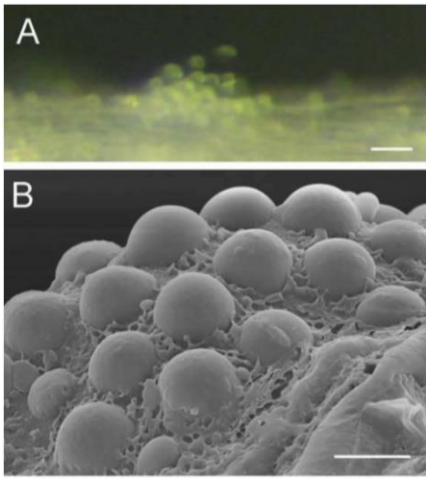


**Figure 1.2** Auto-aggregation and adhesion of *Phytophthora cinnamomi* zoospores on the plant surface. (A) Motile zoospores aggregation on the surface of a plant root. (B) A cluster of cysts embedded in mucin-like material secreted during encystment on a root surface. From Hardham and Blackman (2018).

The cyst produces a germ tube which swells into an appressorium (Judelson & Blanco, 2005) (Figure 1.3).

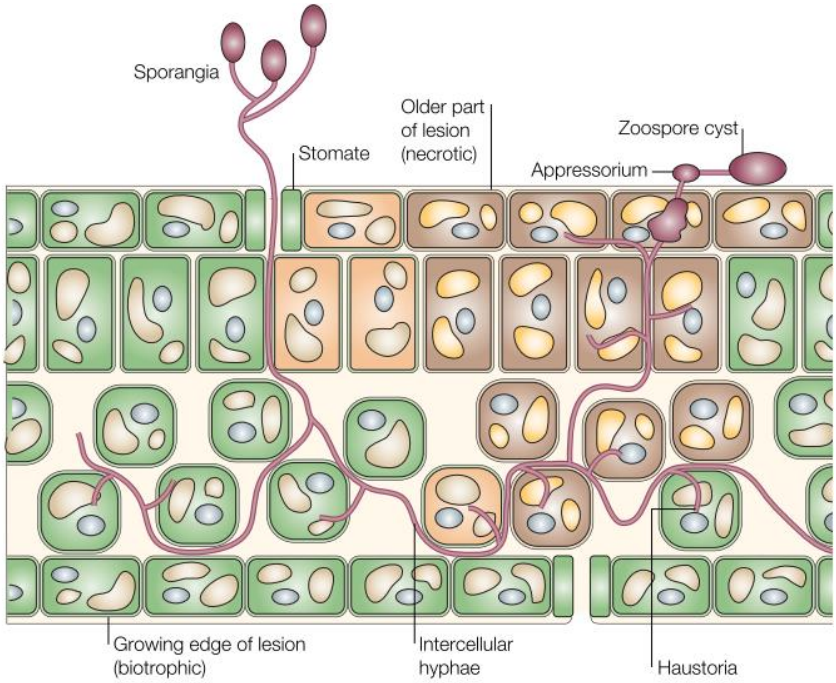


**Figure 1.3** *Phytophthora* infection process diagram. A zoospore encysts on the host tissue surface, produces a germ tube which swells to an appressorium and breaches the epidermis. From Judelson et al (2005).

Using thigmotropism (touch) and chemotropism the germ tube finds a suitable infection point on the host. Through a combination of mechanical pressure and cell-wall-degrading enzymes it breaches the epidermis (Hardham & Blackman, 2018). *Phytophthora* have an initial biotrophic stage, growing intracellularly and producing haustoria (Figure 1.3), followed by a necrotrophic stage during which it

feeds on dead plant tissue (Oßwald *et al.*, 2014). Distinct changes in *Phytophthora* and host transcriptomes are associated with early infection and the transition from biotrophy to necrotrophy (Jupe *et al.*, 2013; Zuluaga *et al.*, 2016).

*Phytophthora* have evolved ways to overcome plant defence responses, manipulate plant cells and cause infection. One important example is the secretion of an array of effectors into host cells (Kamoun, 2006; Oßwald *et al.*, 2014). Avocado plants can halt infection by zoospores by inducing host defence responses if it is receptive to effector proteins secreted by *P. cinnamomi* (Hardham & Blackman, 2018). The defence response starts with a hypersensitive-like response (Davison, 2011) in which cells near the infection site are programmed to die. Systemic Acquired Resistance (SAR) may be induced, making the tree more resistant to further infections by *P. cinnamomi* (Oßwald *et al.*, 2014).

Avocado root rot infection is mostly limited to the fine feeder roots, they become black and brittle and die (Zentmyer, 1980). The disease can progress through the fine feeder root system affecting the ability of the tree to uptake water. This results in a depletion of carbohydrates and a disturbance in the uptake of minerals and the distribution of nutrients in the tree (Dann *et al.*, 2013). Trees at this stage show visible symptoms of canopy decline including chlorotic leaves which are often wilted with necrotic tips. The tree suffers a reduced canopy because of reductions in vegetative growth and less fruit is produced as their health declines (Figure 1.4 A).

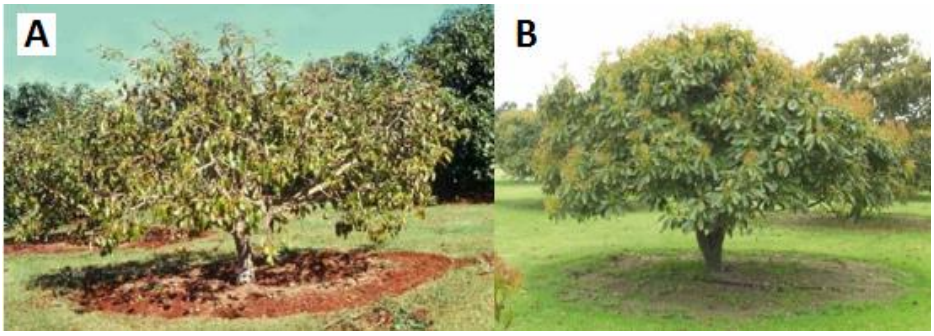


**Figure 1.4** (A) Avocado tree with Phytophthora root rot (From: http://www.camtacgroup.com/?page_id=56), (B) A healthy avocado tree (From http://www.waimeanurseries.co.nz/our-products/fruit-trees/avocados/hass/).

The reduced water up-take can result in soil around the tree becoming water soaked (Dann *et al.*, 2013). Avocado are a flood sensitive species (Reeksting *et al.*, 2016), and flooding exacerbates the effects of avocado root rot because

*Phytophthora* species can readily sporulate and rapidly infect new tissues in water logged soil (Dann *et al.*, 2013). Tree health declines much faster in flooded orchards.

The optimum temperature for disease development is between 19-25˚C, with the upper limit around 30˚C and the lower at 12˚C (Dann *et al.*, 2013). *Phytophthora cinnamomi* is able to grow well at temperatures that are lower than the optimal growth temperatures of avocado, when infection occurs at low temperatures root rot can be severe (Dann *et al.*, 2013).

To continue the infection cycle, sporangia are formed on root surfaces and chlamydospores form prolifically in root tissue, eventually being released to soil as the tissue decays (Dann *et al.*, 2013). These inoculum sources infect other trees in the orchard and can be spread to previously uninfected sites.

### 1.1.2 Detection of *Phytophthora* species

Identifying phytophthora disease symptoms is difficult as they are often complex and are rarely unique to a *Phytophthora* species or the genus. For example, root necrosis associated with soil borne *Phytophthora* species, initially expresses as general drought or water logging symptoms. Often the first visible symptoms of disease are signs the pathogen has significantly progressed in the plant, for example the canopy decline symptom in avocado trees with avocado root rot. Phytophthora pathogens reduce plant health making it more susceptible to invasion by other pathogens, this complicates detection as *Phytophthora* species can be masked.

Furthermore, some *Phytophthora* species have been shown to infect hosts asymptomatically (Crone *et al.*, 2013a). For example, in jarrah forests in Western Australia, *P. cinnamomi* survives in areas where susceptible perennial woody species, namely jarrah, are absent by infecting annual and perennial herbaceous plant species asymptomatically (Crone *et al.*, 2013b). This method of infection is important for survival and dissemination.

For plant species which are sourced from nurseries, phytophthora disease symptoms are often suppressed through broad-scale chemical control agents. Once the plants are planted out and the chemical control agents are diluted or degraded, disease symptoms will likely appear (Brasier, 2008b). This delayed

expression of disease symptoms makes it difficult to track the origin of many *Phytophthora* species that are spread over large distances through the plant trade.

*Phytophthora* species are detected by stream and soil baiting, plating infected material and/or molecular identification (Erwin & Ribeiro, 1996; Martin *et al.*, 2012). Soil and stream baiting are used to monitor the distribution and disease progression of *Phytophthora* species in environmental surveys. Stream baiting involves floating plant material in mesh bags in streams for several days and then plating the baited plant material in *Phytophthora* selective media. Stream baiting methods have been widely used when searching for biosecurity threats (Eyre & Garbelotto, 2015; Hansen & Delatour, 1999; Hwang *et al.*, 2010; Hwang *et al.*, 2008; Reeser *et al.*, 2011; Remigi *et al.*, 2007; Stamler *et al.*, 2016). Phytophthora zoospores can be baited from soil by floating plant material on flooded soil samples. Soil baiting is useful for confirming *Phytophthora* presence in areas, such as orchards and nurseries, suspected to have disease. *Phytophthora* species may also be collected through direct isolation from symptomatic plant material such as lesions and roots. Cultured isolates are identified morphologically or using Deoxyribonucleic acid (DNA)-based methods (Cooke *et al.*, 2007).

It is possible for samples of plant tissue which are symptomatic or asymptomatic to produce false negative identification. This occurs when mycelia are present in plant material, yet do not grow out when that plant tissue is plated onto artificial growth media. Hüberli *et al.* (2000) showed that washing the plant material in sterile deionised water and re-plating it, induces *Phytophthora* to grow. It is hypothesised that the *Phytophthora* is present in the plant material as dormant structures and that washing the plant material may wash away phenolic compounds which act as inhibitory substances to pathogens (Hüberli *et al.*, 2000)

As technology has developed, it has become possible to analyse populations of *Phytophthora* present in plant, water and soil samples by analysing environmental DNA (eDNA) using high-throughput sequencing methods. This was first explored using Polymerase Chain Reaction (PCR) based methods, such as PCR-restriction fragment length polymorphism (RFLP) (Drenth *et al.*, 2006) and real-time quantitative PCR (Huang *et al.*, 2010). More recently a DNA-based identification method, called meta-barcoding, has been trialled (Català *et al.*, 2017; Prigigallo *et al.*, 2015). Meta-barcoding involves the combination of DNA based identification

and high-throughput DNA sequencing of eDNA and has been shown to detect a larger number of species compared to traditional baiting methods (Burgess *et al.*, 2017b; Català *et al.*, 2017). However, while several species can be found in a DNA analysis of soil, many soil borne species cannot be easily isolated into pure culture. These new tools provide insights into the *Phytophthora* population diversity associated with a diseased tree or an ecosystem.

*Phytophthora cinnamomi* can be isolated from soil and roots around avocado trees using a soil baiting procedure or by plating colonized tissue. It is possible to identify *P. cinnamomi* using morphology alone because it has distinctive characteristics such as coralloid hyphae and abundant globose hyphal swellings and chlamydospores (Hardham, 2005). Sporangia can be prompted to form when the pathogen is starved of nutrients in water, they appear at the hyphal tips. Molecular identification of *P. cinnamomi* can be performed using oomycete specific primers, such as ITS4 and ITS6 which target the Internal transcribed Spacer (ITS) regions and produce an amplicon of approximately 900bp (Cooke *et al.*, 2000), and then sequencing the product. Two sets of *P. cinnamomi* specific primers, LPV2/LPV3 (Kong *et al.*, 2003) and LPC2/RPC3 (O'Brien, 2008), target the multigene family, putative storage protein LPV (Marshall et al., 2001).

### 1.1.3 Classification of *Phytophthora* species

The genus *Phytophthora* is in the kingdom Chromista and phylum Oomycota. The distinguishing feature of oomycetes is the presence of oogonia and oospores (Erwin & Ribeiro, 1996). The genus consists of 10 phylogenetically defined clades containing 124 described species (Martin *et al.*, 2014). *Phytophthora cinnamomi* is in clade 7b (Martin *et al.*, 2014). More new species are being found due to an increase in the sophistication of tools, the occurrence of larger scale and more frequent surveys (Martin *et al.*, 2012) and molecular based phylogenetic revisions (Weir *et al.*, 2015).

Interspecific hybridisation is an important process in *Phytophthora*, contributing to environmental adaptions, speciation and the rapid generation of new pathogens (Kroon *et al.*, 2012). Hybrid *Phytophthora* species have been demonstrated *in vitro* (Donahoo & Lamour, 2008; Ersek *et al.*, 1995; Goodwin & Fry, 1994) and naturally (Brasier *et al.*, 2004; Man In 't Veld *et al.*, 1998). Hybrids are created when *Phytophthora* species are brought together into the same area, offering the

opportunity for sexual reproduction between previously isolated species. These novel interactions are stimulated through trade, globalization and nursery plant production processes (Scott *et al.*, 2013). Some hybrid species appear to be more aggressive than the parent species (Man In 'T Veld *et al.*, 2007) however, many more occur which are unstable within the environment (Hüberli *et al.*, 2013; Nagel *et al.*, 2013). With increasing plant trade worldwide the creation of new hybrids may pose a threat to plant heath and the plant trade industry (Brasier *et al.*, 1999).

## 1.2 Avocado root rot management

It is typically impossible to eradicate an established *Phytophthora* species. It may be attempted in nurseries by cutting and burning infected host plants and creating a buffer zone around the infection area (Frankel, 2008). For this to be successful it is necessary that all infected plant material is burned. It is virtually impossible to eradicate *P. cinnamomi* from soil in an avocado orchard (Drenth & Guest, 2004), yet growers can try to reduce the levels of inoculum. One method is soil solarisation, this involves covering soil and roots with thin plastic to increase temperatures in the soil, it is most useful in countries with hot sunny, cloud free summer days (Dann *et al.*, 2013). It is necessary to implement strategies to manage phytophthora root rot diseases as it is so difficult to eradicate. Orchards and nurseries use cultural practises, resistant host plants, and biological and chemical control to control avocado root rot (Drenth & Guest, 2004; Dunne *et al.*, 2011; Frankel, 2008).

### 1.2.1 Cultural practices

All *Phytophthora* associated diseases are influenced by pathogen virulence, host susceptibility and the surrounding environmental factors including soil health and salinity, temperature, rainfall, inoculum density and soil water potential, as well as neighbouring land use (Manion, 1981). The effectiveness of control strategies is largely determined by the ability of *Phytophthora* spores to survive, either as saprophytes or as dormant structures (Drenth & Guest, 2004). A range of cultural practices are used to manage phytophthora diseases, including avocado root rot, in nurseries and orchards. These include but are not limited to quarantine, heat treating new seeds and soil, cleaning equipment, managing irrigation and

drainage, mulching, using suppressive soils, fertilisers, organic amendments and companion planting.

Diseased nursery stock is commonly responsible for the spread of plant diseases nationally and internationally (Parke & Grünwald, 2012). For example, the introduction of the causal agent of sudden oak death, *P. ramorum*, to the United States can be largely attributed to trade in ornamental plants (Grünwald *et al.*, 2012). Preventing the introduction of a *Phytophthora* species to orchards and nurseries is made particularly difficult by the common occurrence of asymptomatic host plants. For example, 70% of asymptomatic potted plants from two large retail European nurseries tested positive for *Phytophthora* species using a *Phytophthora*-specific TaqMan MGB Probe (Migliorini *et al.*, 2015). It is likely the movement of *Phytophthora* species will continue, threatening agricultural and ornamental crops and forests (Parke & Grünwald, 2012).

Sourcing disease free material from nurseries is the first step to avoid phytophthora root rot in avocado orchards. In California and Australia nurseries can be accredited to show they follow procedures to exclude *P. cinnamomi* (Ernst *et al.*, 2013). As a precautionary step, orchards and nurseries can quarantine new plants for several weeks to monitor disease development.

Nurseries can heat treat new seeds to prevent introducing phytophthora and strict hygiene practises combined with close monitoring of growing seedlings should prevent phytophthora establishing (Dann *et al.*, 2013). The source of soil for potting media should be carefully selected and heat treated before use (Drenth & Guest, 2004). *Phytophthora ramorum* can survive in potting media and soil for up to 1 year when introduced as chlamydospores and six months as sporangia (Linderman & Davis, 2006). The pathogen's presence in soil allows it to become a source of primary inoculum for foliar infections (Tjosvold *et al.*, 2009) and it has the potential to be disseminated geographically without being detected visually (Linderman & Davis, 2006). However, correct compost management practices results in phytophthora inoculum being killed in the composting process (Swain *et al.*, 2006). It is important to manage finished compost carefully because it can become reinfected with *Phytophthora* (Swain & Garbelotto, 2015).

Simple hygiene practices can be very effective at minimising inoculum spread in nurseries and orchards. For example, storing potted nursery plants on tray tables elevated from the ground and cleaning equipment (Drenth & Guest, 2004).

*Phytophthora* is a water mould that relies on water to spread. Careful monitoring of the irrigation scheme is important because over- and under-watering plants can make plants predisposed to infection by *Phytophthora* (Dann *et al.*, 2013). Water logged conditions allow *Phytophthora* species to thrive, for example the Jarrah dieback epidemic in Western Australia cause by *P. cinnamomi* was proliferated by waterlogging in the forests (Davison, 1997). Irrigation water can contain *Phytophthora* species (Ghimire *et al.*, 2011); this is more of a concern where water is recycled (Bush *et al.*, 2003). Within horticultural production, soil management plays a key role in disease development. Soils that have good internal and surface drainage are preferred locations for avocado orchards.

Mulching tree crops helps regulate soil moisture and temperature however more water is retained which can favour *Phytophthora* disease development (Downer *et al.*, 2002). Increased soil salinity is a major predisposing factor to phytophthora diseases (DiLeo *et al.*, 2010; Mcdonald, 1982; Sanogo, 2004). Mulches stabilise soil salinity by reducing fluctuations in soil moisture levels and add nutrients to soil as they mineralise (Downer *et al.*, 2002). In avocado orchards, mulching with suitable material such as avocado pruning's, aged hardwood chips, aged or composted pine bark, improves vigour of trees and promotes microflora antagonistic to *P. cinnamomi* (Dann *et al.*, 2013).

Suppressive soils may be of use in controlling phytophthora diseases. The principle cause of suppressiveness is an increase in microbial populations of antagonistic bacteria, fungi and actinomycetes (Drenth & Guest, 2004; McDonald *et al.*, 2007). Soil characteristics including hydrology, nutrition and soil structure also contribute to suppressiveness. One soil type in California, Somis-1, has shown consistent degradation of *P. cinnamomi* hyphal mats and low pathogen populations while maintaining good tree health (McDonald *et al.*, 2007). Suppressive soils can also inhibit the germination of chlamydospores and some may contain microorganisms which parasitise oospores (Drenth & Guest, 2004).

The use of fertilisers and plant strengtheners to control phytophthora diseases is not straightforward. Some organic and synthetic fertilisers and synthetic resistance inducers showed effectiveness for reduced susceptibility in tomato plants to *P. infestans* (Sharma et al., 2012). Calcium fertilisers induced a decrease in chlamydospore viability in soil and reduced foliar and root symptoms on Holm oak (*Quercus ilex*) caused by *P. cinnamomi* (Serrano et al., 2012). However, increasing nitrogen and potassium ($N_{150} + K_{40}$ kg/ha) applied through urea and muriate of potash, resulted in greater disease severity in taro (*Colocasia esculenta*) caused by *P. colocasiae* (Das et al., 2003).

*Phytophthora* are inhibited by organic amendments including alfalfa meal, cottonwaste, soybean meal, wheat straw and manure (Drenth & Guest, 2004). Chicken manure effectively reduces *P. cinnamomi* growth (Aryantha *et al.*, 2000), however the ammonia and organic acids released are also toxic to avocado (Dann *et al.*, 2013). It is best to incorporate manures into soil a few months prior to planting to reduce the potential negative effects on the plants.

Companion planting can reduce the impact of phytophthora diseases. For example in Australia, banana and avocado are planted together in an orchard because banana trees reduce soil water after heavy rain and provide mulch (Drenth & Guest, 2004). Several species of legumes, including *Acacia extensa, A. stenoptera, A. alata* and *A. pulchella,* were found to be suppressive of *P. cinnamomi* in Western Australia (D'Souza *et al.*, 2005).

### 1.2.2 Host resistance

The most important form of control is the selection of resistant genes by plant breeders (Ballvora *et al.*, 2002; Leister *et al.*, 1996); however; this provides a direct selective pressure through which pathogens can overcome the resistance (Fry & Goodwin, 1997). Therefore, where single gene (qualitative) resistance is adopted, an arms race exists between plant breeders and the pathogen which is not sustainable in the long run. In contrast, quantitative resistance lasts longer because it is mediated by multiple interacting genes that slow down the development of disease (Engelbrech & Van Den Berg, 2013). Artificial selection for host resistance is only relevant to important plants such as those used in horticulture and plantations and those of significant cultural value because it is a long and costly process.

The durability of the resistance that is imposed on the host plant is important; durability is defined as the persistence of resistance efficiency when resistant cultivars are used over long periods, in large areas and in the presence of the target pathogen (Johnson, 1981). The resistance of a host is one of the most important evolutionary forces acting on the pathogen, directly influencing population structure because virulence is a key driving force in host-pathogen coevolution (Zhan *et al.*, 2002). As a result the question of pathogen response to manipulated host resistance through plant breeding has received considerable attention (Zhan *et al.*, 2002).

Researchers have found *P. infestans* strains can adapt to the resistance in potato cultivars bred for resistance. Flier *et al.* (2003) found *P. infestans* was able to adapt regardless of the level of quantitative resistance. Montarry *et al.* (2006) found that a directional selection for virulence in *P. infestans* is superimposed when race-specific resistance is introduced in potato. Furthermore, Andrivon *et al.* (2007) found *P. infestans* populations adapt to locally dominant cultivars when they studied partially resistant host cultivars from France and Morocco. This adaptation occurred irrespective of the resistance levels of the cultivars, indicating that *P. infestans* can overcome polygenic, quantitative resistance (Andrivon *et al.*, 2007). These results are concerning for all phytophthora pathosystems as the adaptive ability of phytophthora may render partial host resistance nondurable and unstable. Conversely, Forbes *et al.* (2005) claim quantitative resistance is stable in 14 potato genotypes including the most widespread cultivar, Bintje, for which it was suggested that increased aggressiveness in *P. infestans* was selected for in trials by Montarry *et al.* (2006) and Andrivon *et al.* (2007). We have learnt from potato late blight that resistant potato plants can lead to the selection of increased pathogenicity in *P. infestans.*

In comparison with the vegetatively propagated annual crop potato, there has been some success in breeding resistance in root stocks of the long-term perennial tree crop avocado. Breeding avocado root stocks resistant to phytophthora root rot has been a major focus of breeding programs since the discovery of the disease in 1942 (Crane *et al.*, 2013) and programs exist in Australia, the United States, South Africa, Spain and the Philippines (Lahav & Lavi, 2009). Historically, breeding for resistance has involved inoculating seedlings sourced from specific breeding

blocks or from sources where avocado is found, such as Central America (Douhan *et al.*, 2011). Using these methods, the Californian breeding program has found several resistant root stocks, including 'Duke 7', 'Steddom', 'Uzi' and 'Zentmyer' (Menge *et al.*, 2012). 'Duke 7' was discovered in the 1950s and became commercially available in 1975 (Crane *et al.*, 2013). It was highly successful in its resistance to avocado root rot and used worldwide to combat the disease (Zentmyer, 1980). Resistant cultivars discovered in South Africa include 'Dusa', which was significantly more tolerant to phytophthora root rot with 'Hass' grafted on compared to 'Duke 7' and 'Latas' which is not commercially marketed due to the success of 'Dusa' (Crane *et al.*, 2013). The genetic diversity of Steddom, Uzi, Zentmyer, Dusa and Latas is large, suggesting the mechanisms of resistance is not shared among tolerant rootstocks (Douhan *et al.*, 2011). It is highly likely that the resistant rootstock cultivars have many genes at various levels that enable them to withstand avocado root rot (Engelbrech & Van Den Berg, 2013). This is more durable and sustainable than single gene resistance.

The main rootstock used in New Zealand is 'Zutano', a Mexican and Guatemalan hybrid, which has low tolerance to phytophthora root rot but is noted for its tolerance to cold and saline conditions (Crane *et al.*, 2013).

### 1.2.3 Biological control

Biological control is particularly important organic orchards and nurseries. It involves promoting or introducing microrganisms antagonistic to *Phytophthora* species. Mulching soil is an indirect form of biological control because they support larger and more diverse populations of microorganisms compared to un-mulched soils (Downer *et al.*, 2001). It is thought these microorganisms, including bacteria, fungi, and actinomycetes, inhibit *Phytophthora* pathogens through competition, parasitism and antibiosis (Downer *et al.*, 2002).

The Ashburner method of biological control (Broadbent & Baker, 1974) was designed to maintain healthy avocado trees in *P. cinnamomi* infested soil. It involves adding large amounts of plant residues from cover cropping, mulch, chicken manure, and dolomite to support soil health and stimulate suppressive microbes. Recently coarse mulch and gypsum (Messenger *et al.*, 2000) were added to the system to provide an oxygen rich environment and suppress phytophthora growth, respectively (Dann *et al.*, 2013).

Specific fungi antagonistic to *Phytophthora* have been identified. For example *Trichoderma harzianum* and *Gliocladium virens* were efficient antagonists of *P. cinnamomi,* and they could efficiently colonise mulches which provides a potential method to deliver biocontrol agents into soils (Costa & Menge, 2000). Furthermore, the application of *Trichoderma* species to soil around avocado trees in the field can encourage the growth of arbuscular mycorrhizal fungi (Lara-Chávez *et al.*, 2013), some species of which may promote vegetative growth (Da Silveira *et al.*, 2002).

Endophytic fungi have been shown to increase protection against *Phytophthora,* for example cocoa (*Theobroma cacao*) was protected through direct antagonism between the endophytic fungi and the *Phytophthora* species (Arnold *et al.*, 2003). There is the potential to inoculate seedlings with beneficial endophytes in nurseries before planting the crops out in orchards so they are protected in the field (Drenth & Guest, 2004). Not all fungal associations are beneficial in combating phytophthora diseases, for example avocado seedlings inoculated with mycorrhizae were affected more severely by phytophthora root rot than seedlings without mycorrhizae (Davis *et al.*, 1978). These results were not surprising because avocado trees in orchards have arbuscular mycorrhizae and are still prone to root rot infection (Davis *et al.*, 1978).

Bacteria also exhibit antagonistic behaviour to *Phytophthora* species. Xiao *et al.* (2002) found antibiotic-producing *Streptomyces* were able to significantly reduce root rot severity in alfalfa and soybean by inhibiting growth of the causal agents *P. medicaginis* and *P. sojae,* respectively. This was also tested in phytophthora blight in red-peppers caused by *P. capsici*, a culture broth of *Streptomyces halstedii* was effective at supressing growth of the *Phytophthora* (Joo, 2005). *Phytophthora drechsleri*, the causal agent of damping-off of cantaloupe, was effectively inhibited by fluorescent *Pseudomonas* bacterial isolates *in vitro* and *in vivo* (Tabarraei *et al.*, 2011).

Rhizobacteria associated with avocado trees from the *Bacillis* genus show antagonistic activity against *P. cinnamomi,* they could effectively inhibit growth by up to 25% (Guevara-Avendaño *et al.*, 2017). Individual isolates of bacterial species from soil suppressive to *P. cinnamomi* around 15-year old avocado trees did not act antagonistically to *P. cinnamomi* (Stirling et al., 1992). However, a

leachate from the soil caused lysis of *P. cinnamomi* mycelium, showing the effectiveness of suppressive soils and how the combination of multiple antagonists works effectively to inhibit *P. cinnamomi* (Stirling *et al.*, 1992).

It is likely that simply adding individual species antagonistic to *P. cinnamomi* around avocado trees will not be effective at reducing avocado root rot (Guest, 2004). The overall improvements to soil health and organic matter amendments involved in the Ashburner system already promotes fungi and bacteria antagonistic to *P. cinnamomi* and likely provides good conditions for their continued effectiveness.

### 1.2.4 Chemical control

Chemical control methods are limited for phytophthora diseases because many fungicides that are effective against most true fungi do not work against oomycete pathogens, including *Phytophthora* (Dann *et al.*, 2013). New chemicals are often tested for their efficacy to control *Phytophthora* species and their diseases (Elliott *et al.*, 2015; Garbelotto *et al.*, 2009; Lawrence *et al.*, 2017; Lucero *et al.*, 2014; Qi *et al.*, 2012; Weiland *et al.*, 2009). Most recently, Benzethonium chloride was identified as a promising chemical control as it was effective at inhibiting mycelial growth, zoospore germination and zoospore motility of *P. agathidicida* and *P. cinnamomi* (Lawrence et al., 2017). In order for chemical control to continue to be effective, proper use is vital to prevent the development of fungicide resistant *Phytophthora* isolates. The control agent also needs to be translocatable to the root tips, not phytotoxic and not environmentally toxic. Two groups of fungicides most commonly used to protect plants from phytophthora disease are phenylamides and phosphonates.

Phenylamides are xylem translocated and usually applied as soil drenches (Guest & Grant, 1991). They act directly on the pathogen by inhibiting RNA synthesis which results in a reduction in growth and sporulation of the pathogen (Cohen & Coffey, 1986). This site-specific mode of action exposes them to the development of fungicide resistances. Populations of *P. infestans* developed resistance to metalaxyl after one year of application (Davidse *et al.*, 1981; Dowley & O'Sullivan, 1981; Russell, 1995). Resistance to mefenoxam has developed in isolates of *P. capsici* from bell peppers (Parra & Ristaino, 2001).

## 1.3 Phosphite

Phosphite is the anionic form of phosphonic acid ($HPO_3^{2-}$). The term 'phosphite' will be used to refer to salts of phosphonic acid ($H_2PO_3$). Fosetyl-a is degraded to phosphonic acid in plant tissue (Table 1.2) (Guest & Grant, 1991) and is the active ingredient in the fungicide Aliette®. This study will focus on phosphite as it is used extensively to protect avocado trees from *P. cinnamomi*.

Phosphite contains a P-H bond and is found in Fosetyl-a and phosphonic acid. Phosphite is a reduced form of orthophosphate ($PO_4^{3-}$). Orthophosphate is intimately involved with cellular bioenergetics and metabolic regulation and it is an important structural component of macromolecules, such as nucleic acids (McDonald *et al.*, 2001). Orthophosphates are the sole phosphorous-containing nutrient important for optimal plant growth and development and are widely used in fertilisers to improve the yield of many crop species (McDonald *et al.*, 2001).

### 1.3.1 Phosphite application

Phosphite can be applied as a soil drench, foliar spray, trunk paints, cartridge tree implants or trunk injections. In Western Australia, where *P. cinnamomi* infects thousands of host plants, natural ecosystems are sprayed to run-off using spray backpacks, trailer mounted spray equipment and as an aerial application using an ultra-low volume mist (Hardy *et al.*, 2001). Phosphite is used extensively in the avocado industry. It is applied to avocado trees most commonly with passive injections into the xylem; however, air pressurised injection guns can also be used to force the phosphite into the tree rapidly. Once injected into the xylem, the phosphite moves rapidly and systemically throughout the plant. Injection sites are evenly spaced around the trunk of the tree because there is no lateral movement of phosphite. Passive trunk injections are the most common method used in New Zealand (West, 2017). Most product manufacturers and consultants recommend injecting 20ml of a 15% - 20% (w/v) solution per metre of canopy diameter with syringes spaced every 20 cm around the trunk (West, 2017).

The most effective time to apply phosphite is in late spring when shoot growth has matured and again at the end of the summer flush (Dann *et al.*, 2013). At these stages, fine feeder roots, where the infection starts, are a strong sink in the tree (Dann *et al.*, 2013). In New Zealand most orchards inject phosphite once a year in

spring to all the trees in the orchard followed by a second injection in unhealthy trees in autumn (West, 2017).

### 1.3.2 Phosphite translocation

Phosphite is phloem and xylem mobile and thus has movement upwards and downwards in plants (Ouimette & Coffey, 1990). Phosphite injected in to the trunk xylem is transported to the leaves in the transpiration stream, and then trapped in the phloem and translocated in association with photo-assimilates in a source-sink relationship (Guest & Grant, 1991). Phosphite accumulates in plant tissue at a rate directly related to the application rate (Smillie *et al.*, 1989). This source-sink nature of translocation allows phosphite to be applied as a preventative and curative treatment (Guest & Grant, 1991). Phosphite is able to be injected directly into tree trunks because it is water soluble. The timing of year and day is important for phosphite application because the rates of transpiration and the seasonal sink dynamics directly impact translocation. Although phosphite is not metabolised by plants (Guest & Grant, 1991; McDonald *et al.*, 2001), it is slowly lost from the plant through harvesting of fruit, dilution through plant growth, loss from senescing organs and through root exudation (Dann *et al.*, 2013).

### 1.3.3 Mode of action of phosphite

Phosphite works in two ways to provide relief from phytophthora diseases. Firstly, it actively reduces growth and sporulation of *Phytophthora* species, while also stimulating plant defence responses, to ultimately prevent colonisation at the infection court (Guest *et al.*, 2010; Smillie *et al.*, 1989). Phosphite has been shown to result in some harmful secondary compounds (Stasikowski, 2012), inhibit enzyme activity (Stehmann & Grant, 2000) and alter gene regulation (King *et al.*, 2010) of *Phytophthora* which results in inhibition of hyphal growth and degradation of cell walls (King *et al.*, 2010).

*Phytophthora* readily take up the phosphite compound, probably because it resembles phosphate and is transferred through many of the same cell wall transporters (Stasikowski, 2012). Inside *Phytophthora* hyphae, phosphite is not directly utilised in any functional metabolic process, rather it is metabolized into a range of compounds (Stasikowski, 2012) including pyrophosphate which accumulates within the pathogen cells (Barchietto *et al.*, 1992; Griffith *et al.*,

1990; Niere *et al.*, 1990). Pyrophosphate is actively translocated throughout the pathogen where it accumulates in the growing margins and can reach high concentrations. Pyrophosphates can naturally occur in *Phytophthora* at very low concentrations, but at high concentrations they are particularly toxic as they are not metabolised (Niere *et al.*, 1994). When pyrophosphates are applied to any oomycete they cause similar retarded growth rates and toxicity as if applying phosphite. Phosphite inhibits inorganic pyrophosphatase (Martin *et al.*, 1998), the enzyme which breaks down pyrophosphate which is why pyrophosphates are able to accumulate to toxic levels.

Stehmann and Grant (2000) demonstrated that phosphite inhibits the activities of several enzymes in the glycolytic and oxidative pentos-phosphate pathways in clarified *Phytophthora* extracts. Phosphite likely disrupts metabolism by competing with phosphate as an allosteric regulator at sites on several enzymes (Martin *et al.*, 1998; Stehmann & Grant, 2000). When phosphate is low, the phosphite inhibition is exaggerated, for example when conducting inhibition tests in low phosphate media (Guest & Grant, 1991).

Phosphite causes hyphal distortions and degradation of cell walls of *P. cinnamomi* and has an overall adverse effect on hyphal growth (King *et al.*, 2010). King *et al.* (2010) found changes in gene expression in *P. cinnamomi* as a result of phosphite presence. A total of 43 transcripts in the mycelium of *P. cinnamomi* were altered in their expression levels and 33 of these could be identified (King *et al.*, 2010). These transcripts are mostly involved with cell wall synthesis, gluconeogenesis, and transport (King *et al.*, 2010).

Phosphite also stimulates plant defence responses and provides protection through the induction of host defence responses, however the complex mechanisms underlying this are still being explored (Berkowitz *et al.*, 2013; Jost *et al.*, 2015). A close relationship exists between the amount of phosphite at an invasion site and the extent to which defence genes of the plant are expressed (Jackson *et al.*, 2000). For example, the concentration of phosphite in the roots of jarrah affected the mechanism of protection against *P. cinnamomi* (Jackson et al., 2000). When the concentration was high the phosphite acted directly on the pathogen inhibiting its growth before it established in the host, and when concentrations were low,

host plant defence responses were stimulated where the pathogen was attempting to infect.

Phosphite primes host plants for a fast and effective defence response to *Phytophthora* infection by increasing the transcription of defence genes (Eshraghi *et al.*, 2011). Defence responses that are enhanced include callose deposition and hydrogen peroxide production (Eshraghi *et al.*, 2011). Burra *et al.* (2014) identified three classes of transcripts as the core of the host response, including those related to defence, wounding and oxidative stress. Massive phosphite induced transcriptomic changes in the host begin three hours after phosphite treatment.

It is further hypothesised that the presence of phosphite stimulates *Phytophthora* to produce elicitins (Perez *et al.*, 1995) or inhibit the production of suppressors (Guest & Grant, 1991). Host plants can prevent infection by inducing their defence responses when they recognise the elicitins. Evidence for this indirect action has been shown in *Eucalyptus marginata* (Jackson *et al.*, 2000) and *Xanthorrhoea australis* (Daniel *et al.*, 2005).

Phosphite also has a direct effect on the host plant regardless of the presence of the pathogen (McDonald *et al.*, 2001). Phosphate availability is a major factor limiting growth, development and productivity of plants. In the absence of phosphate, plants exhibit deficiency symptoms including anthocyanin accumulation, enhanced root growth and increased root to shoot ratio (Varadarajan *et al.*, 2002). The ability to acquire phosphate increases in phosphate starved plants. These responses are directed by a coordinated expression of genes in response to phosphate starvation. Phosphate transporters are expressed, these are able to transport phosphite because of structural similarities (Guest & Grant, 1991), however plants are not able to metabolise phosphite into phosphate (McDonald *et al.*, 2001), so it accumulates and persists in plant tissue.

Phosphite supresses phosphate starvation responses (Varadarajan *et al.*, 2002). For example even at relatively low concentrations phosphite was shown to disrupt processes involved in regulation of the phosphate starvation response in phosphite-starved *Brassica napus* seedlings by downregulating the induction of enzymes such as phosphatase, phosphoenolpyruvate phosphatase, inorganic

pyrophosphate-dependent phosphofructokinase and the high-affinity phosphate translocator (Carswell *et al.*, 1997). It also inhibited root growth in *B. nigra* seedlings (Carswell *et al.*, 1996).

Phosphite has a negative effect on phosphate-starved host plants because the plants downregulate phosphate-starvation responses when they are still phosphate starved. Some people argue that phosphate should be applied to trees treated with phosphite to overcome the effects of phosphite interfering with the phosphate-starvation response (Varadarajan *et al.*, 2002). This concept has recently been trialled in Bull Banksia (*Banksia grandis*) and jaraah (*Eucalyptus marginata*) to control *P. cinnamomi* (Scott *et al.*, 2015). Here there was no significant variation in the suppression of *P. cinnamomi* lesion development between the phosphite and the combined phosphite and phosphate treatments; however, treatment impacts on plant growth was not assessed. Further work is required to determine the impacts of combined phosphite and phosphite treatments on disease suppression and plant growth; however, the persistence of phosphite induced disease suppression may be supressed by excess phosphate that dilutes the effective concentration of phosphite ions. Further work is required to ensure that plant productive industries do not supress the effectiveness of phosphite for disease control and phosphate for growth enhancement through dual application.

Phosphite cannot effectively kill *Phytophthora* species, it merely acts as a 'band aid' solution by being able to prevent and reduce the spread of infections. It is an effective management tool when it is applied regularly. However, phosphite resistant *Phytophthora* isolates can develop with prolonged exposure (Dobrowolski *et al.*, 2008) yet it is difficult to predict when this will happen and how it will impact long term disease.

### 1.3.4 Phosphite resistance

Phosphite sensitivity is determined by (i) an ability to exclude phosphite (Griffith *et al.*, 1993), (ii) a difference in the sensitivity of the internal metabolic state (Griffith *et al.*, 1993) and possibly (iii) an ability to remove phosphite from hyphae (Dunstan *et al.*, 1990). It is not yet known which genes are responsible for phosphite resistance in tolerant isolates of *Phytophthora* species.

Phosphite sensitivity is assessed using mycelial growth assays, two common methods involve measuring radial growth on solid media (Duvenhage, 1994; Kaiser *et al.*, 1997; Wilkinson *et al.*, 2001b) and dry weight from liquid media. Ma and McLeod (2014) compared the two methods using *P. cinnamomi* isolates and Ribeiros Modified Media (RMM) and found isolates were more sensitive in liquid than agar, which is likely due to more of the mycelium being in direct contact with the phosphite. Phosphite sensitivity is likely more accurately measured using dry weight rather than radial growth because mycelial density is accounted for (Guest & Grant, 1991; Ma & McLeod, 2014; Wilkinson *et al.*, 2001b).

The amount of phosphite needed to inhibit mycelial growth by 50% is predicted from plotting the inhibition values against the logged phosphite treatments. This is known as the Effective Concentration (EC) 50% and is used to compare the sensitivity of different isolates.

Mycelial inhibition by phosphite is exaggerated in low phosphate media due to the effect of phosphate starvation (Guest & Grant, 1991). Comparing mycelial growth experiment results is difficult between experiments. It is not surprising that results from *in vitro* tests in low phosphate media do not always correlate with *in vivo* results because phosphate levels are higher in plants (Guest & Grant, 1991). More work is required to determine how sensitivity observed *in vitro* relates to sensitivity *in planta*. This requires a better understanding of how, when and where phosphite concentrations vary within the plant.

The underlying phosphite tolerance of *Phytophthora* species differs (Coffey & Bower, 1984a; Niere *et al.*, 1994) as does the underlying phosphite sensitivity of isolates within a species, even without previous exposure to phosphite. Of 66 *P. cinnamomi* isolates from various host plants in Western Australia with no previous phosphite exposure, six isolates were tolerant (classified as being 4% promoted to 20% inhibited at 5 µg/ml and 31-76% inhibited by 50 µg/mL), 54 were intermediately tolerant (classified as 30 – 63% and 73 – 94% inhibited at 5 and 50 µg/mL respectively) and were six sensitive (classified as those inhibited 59 – 64% and 94 – 100% by 5 and 50 µg/mL, respectively) (Wilkinson *et al.*, 2001b).

Isolates of *P. cinnamomi* obtained from avocado trees treated with phosphite for a prolonged period from orchards in South Africa (Duvenhage, 1994; Ma & McLeod, 2014) and Australia (Dobrowolski *et al.*, 2008) had decreased sensitivity to phosphite. Ma and McLeod (2014) assessed the tolerance of 50 *P. cinnamomi* isolates from avocado, tolerant isolates were classified as those inhibited ≤20% at 30 µg/mL and ≤30% at 100 µg/mL in a radial growth assay on RMM. Nine isolates were classified as tolerant on media with 1mM phosphate, 13 on 7 mM phosphate and 19 on 15 mM phosphate. Duvenhage (1994) reported *P. cinnamomi* isolates obtained from trees continuously treated with phosphite for a prolonged period tended to be less inhibited by phosphite *in vitro* compared to isolates from untreated trees based on the inhibition of mycelial growth on Corn Meal Agar (CMA) plates. Dobrowolski *et al.* (2008) assessed the ability of phosphite to inhibit growth of *P. cinnamomi* mycelium *in planta* using *Leucadendron* sp., lupin seedling roots and *Eucalyptus sieberi* cotyledons. *P. cinnamomi* isolates obtained from avocado orchards treated with phosphite for 2 – 8 years were more extensive colonisers of phosphite-treated plants. While decreased sensitivity to phosphite has been found, widespread resistance has yet to be reported.

In New Zealand, avocado orchards have been using the phosphite injection method for up to 45 years. These orchards proved an ideal model system to study the development of resistance in *P. cinnamomi* due to prolonged exposure to phosphite. The phosphite sensitivity of *P. cinnamomi* isolates from New Zealand avocado orchards has not yet been explored.

## 1.4 Aims of this thesis

The main aim of this research was to assess the phosphite sensitivity of *P. cinnamomi* isolates from New Zealand avocado orchards in response to the discovery of isolates with decreased sensitivity in Australian (Dobrowolski *et al.*, 2008) and South African (Duvenhage, 1994; Ma & McLeod, 2014) orchards. This study provides the first comprehensive study of phosphite sensitivity of *P. cinnamomi* isolates from New Zealand. Given the high numbers of isolates to be assessed *in vitro* it was necessary to first develop a high-throughput assay to assess phosphite sensitivity which could detect inhibition at comparable rates to traditional methods such as radial growth and dry weight tests.

Phosphite has two modes of control, direct inhibitory activity on *Phytophthora* growth and the stimulation of host defence responses (Guest & Grant, 1991). It was of interest to assess the ability of phosphite tolerant and sensitive isolates to colonise phosphite treated plant material. An additional assessment of their ability to produce sporangia in the presence of phosphite was included.

The underlying phosphite sensitivity of *Phytophthora* species has been shown to differ (Coffey & Bower, 1984a; Niere *et al.*, 1994) however it is only possible to compare the sensitivity of isolates tested in the same experiment. The phosphite sensitivity of several internationally important *Phytophthora* species was assessed in New Zealand and in California, USA.

To achieve these aims this thesis presents four experimental chapters with the following objectives:

1) Develop a high-throughput method to assess *in vitro* phosphite sensitivity
2) Assess the *in vitro* phosphite sensitivity in *Phytophthora cinnamomi* isolates which have been exposed to large doses of phosphite for over 25 years.
3) Test the most resistant and most sensitive *P. cinnamomi* isolates identified *in vitro* for expression of resistance *in planta* and in the production of sporangia
4) Investigate the underlying phosphite sensitivity of other internationally important *Phytophthora* species

# Chapter 2

# Development of a high throughput optical density assay to determine fungicide sensitivity of oomycetes

## 2.1 Abstract

An assay was developed to screen *Phytophthora* species for fungicide sensitivity which uses microtiter plates and optical density measurements for high-throughput capability and unbiased, automated measurements. The efficacy of the optical density assays (OD) to measure phosphite sensitivity in *Phytophthora* species was compared to two widely used methods, radial growth (RG) and dry weight (DW) assays. Six isolates of each of *Phytophthora cinnamomi, P. multivora* and *P. pluvialis* (three of which were previously exposed to phosphite and three with no phosphite history) were screened for phosphite sensitivity using the three assays. Mycelial growth measurements were taken on days 6, 14 and 15 for the OD, DW and RG assays, respectively. Mycelial growth inhibition at 15, 80, 200 and 500 µg/mL phosphite relative to growth on the control was used to predict effective concentration values for 50% growth reduction (EC50). The species varied in their tolerance to phosphite with *P. cinnamomi* being the least sensitive followed by *P. multivora* and *P. pluvialis*. No significant differences in tolerance were found between isolates within the same species using any method. Growth of the three species was more sensitive to phosphite in the DW assays than the RG and OD however the DW assay lacks sensitivity compared to the OD and is open to greater variation in measurements because dry weights of mycelia are minor compared to filter paper weights, increasing the chances of error. The OD assay offers a fast method to enable an inventory of chemical resistance. It has application for other oomycete species and also true fungi. It may be particularly advantageous for slow growing species as it requires less time for the experiment compared to the RG and DW methods.

## 2.2 Introduction

*Phytophthora* species are a genus of plant pathogens which cause disease on horticultural, ornamental, and forest plants worldwide. They are very successful plant pathogens and there are currently no methods to completely eradicate them

from an area once they are established. However, there are several types of chemicals that are effective at controlling phytophthora diseases, including phenymalides and phosphite. Chemical control is widely used to protect agricultural crops from phytophthora diseases, resulting in frequent reapplication of chemicals. Management of chemical control is very important because it is possible for *Phytophthora* species isolates become tolerant to fungicides. For example, tolerance to the phenymalide fungicide metalaxyl (Dowley & O'Sullivan, 1981) has been found in *Phytophthora infestans*, the causal agent of potato (*Solanum tuberosum*) late blight. Isolates of *P. cinnamomi* have been shown to have decreased sensitivity to phosphite after prolonged exposure (Dobrowolski *et al.*, 2008; Duvenhage, 1994; Ma & McLeod, 2014). Phosphite is a widely used fungicide for controlling many phytophthora diseases which affect horticultural crops, planted forest species and culturally and ecologically important species (Hardy *et al.*, 2001b). Phosphite is also used extensively in nurseries and botanical gardens to manage diseases caused by oomycete pathogens, including establishment and damping off pathogens within the genus *Pythium* and foliar, soil and water-borne species within the genus *Phytophthora*.

Understanding the current level of resistance to fungicides in true fungi and oomycete plant pathogens is vital to ensure that treatments which reduce sporulation, growth and spread of plant pathogens continue to be effective into the future. This is most pertinent to the agricultural industry, particularly horticulture, as we face increasing food production demands worldwide due to population growth and climate change. Other important industries including silviculture, viticulture and conservation also use chemicals to control plant pathogens that threaten them.

The sensitivity of *Phytophthora* species and other fungal plant pathogens to fungicides has been studied *in vitro* using mycelial growth tests. These techniques have been used to quantify chemical suppression of mycelial growth *in vitro* by measuring radial growth (RG) and dry weight (DW). RG involves measuring linear growth of cultures on agar amended with fungicide and has been used for true fungi (Chapman *et al.*, 2011; Chen *et al.*, 2008; LaMondia, 2014; Patón *et al.*, 2017) and *Phytophthora* species (Bashan *et al.*, 1990; Coffey & Bower, 1984a; Duvenhage, 1994; Garbelotto *et al.*, 2009; Horner & Hough, 2013; Keinath &

Kousik, 2011; Qi *et al.*, 2012; Wilkinson *et al.*, 2001b). DW involves weighing dried mycelium of cultures grown in liquid media amended with fungicide and has been used for fungi (Datta *et al.*, 2016; Özer *et al.*, 2010; Tremblay *et al.*, 2003) and *Phytophthora* species (Fenn & Coffey, 1984; Ma & McLeod, 2014).

There are advantages and disadvantages of using these different techniques. RG assays can be used to obtain time series measurements and provides an opportunity to assess the dose response of culture morphology. However, linear growth measurements do not reflect variation in mycelial density (Guest & Grant, 1991). DW assays provides a more accurate measure of growth inhibition; however, as mass can only be determined on dried material, it is not possible to measure growth variation over time or determine when growth is limited by resource availability. It is likely that the DW assay provides a more accurate measure of growth inhibition as linear growth measurements can be misleading because while linear growth may not be affected the density of the mycelium can be greatly reduced (Guest & Grant, 1991; Ma & McLeod, 2014). Ma and McLeod (2014) found *P. cinnamomi* isolates were more sensitive to phosphite in liquid medium than solid medium. They concluded that the relative sensitivity of the isolates was influenced by both the phosphite concentration and media hardness due to a higher surface area of mycelia being exposed to the phosphite in the liquid media. While the DW assay may be more accurate and informative than the RG assay for determining growth inhibition, it is also more time consuming. Furthermore, dry weights of mycelia are very small which increases the chance of error, especially when they are dried on filter paper which weighs substantially more than mycelia.

Optical density (OD) measurements are a commonly used measure in bacteriology for quantification of bacterial suspensions. Recently OD been used to quantify the growth of fungal isolates, within multiwell plates containing fungicide amended liquid media (Akhavan *et al.*, 2017; Cox *et al.*, 2009; Frac *et al.*, 2016; Rampersad, 2011; Seyran *et al.*, 2010; Wedge *et al.*, 2013) and to assess *Phytophthora* zoospore germination (Elliott *et al.*, 2015; Kuhajek *et al.*, 2003). However, OD measurements have not been used previously to measure the sensitivity of *Phytophthora* mycelium to chemical control agents such as phosphite.

Mycelium is the most relevant propagule to use when testing the impacts of systemic fungicides, such as phosphite, on *Phytophthora* species because mycelium grow through host plants and will be exposed to systemic fungicides. Phosphite is also used to protect host plants which are already infected with *Phytophthora*, therefore it is necessary that it is affective on mycelium. Furthermore, it is difficult to get a truly homogenous solution of zoospore inoculum because zoospores often aggregate at the surface of a liquid (Cameron & Carlile, 1977), the solution would be a heterogeneous suspension resulting in high standard deviations in optical density values. *Phytophthora* mycelium is aseptate therefore it is not possible to use a homogenised mycelial solution as an inoculum source because hyphal fragments may not be able to survive (Kuhajek *et al.*, 2003). Therefore, it is necessary to inoculate with a standardised amount of mycelial mat from a *Phytophthora* culture when inoculating with mycelia.

The RG and DW assays have been used widely for testing tolerance to phosphite in *Phytophthora* species but the OD assay has never been used to test hyphal growth inhibition in *Phytophthora* species as a response to phosphite treatment. The specific objectives of this study were: 1) to determine if the OD method can detect the same levels of variation in sensitivity as the RG and DW assays between species; and 2) to analyse the effects of phosphite on hyphal growth of three *Phytophthora* species, including *P. cinnamomi, P. multivora* and *P. pluvialis*.

## 2.3 Methods

### 2.3.1 *Phytophthora* isolates and media used

*Phytophthora* isolates from the New Zealand Forest Research Institute Culture Collection (NZFS) (Table 2.1) were maintained in water vials at 4˚C on carrot (CAD) agar (Appendix A) (Brasier *et al.*, 2003). Isolates were cultured onto a modified Ribeiros' Minimal Medium (RMM) (Appendix A) (Ribeiro *et al.*, 1975), modified as outlined below. The glucose concentration was 9.0 g/L and β-sitosterol was omitted. MES hydrate buffer (2-(N-morpholino) ethanesulfonic acid) was added at a final concentration of 0.03 M and the pH adjusted to 6.2 with KOH 3M.

### 2.3.2 Experimental design

The impact of five phosphite treatments (0, 15, 80, 200 and 500 µg/mL) on the growth of 18 *Phytophthora* isolates, comprised of six isolates of three species (Table 2.1), was compared using three methods. The phosphite sensitivity of *Phytophthora* isolates was determined by (i) measuring radial growth in phosphite amended agar media, (ii) by measuring hyphal growth in phosphite amended liquid nutrient media by measuring hyphal dry mass and (iii) by measuring optical density during growth in liquid media. For all three methods, phosphite and isolate treatments were arranged in a split plot randomised replicated block design. Each experiment contained six replicates for each isolate at each phosphite concentration.

### 2.3.3 Phosphite amendment

The phosphite used was Agri-Fos® 600 (Agrichem, Yatala QLD, Australia), a commercial fungicide containing 600 g/L phosphorous acid, present as mono- and di- potassium phosphonate. The phosphite was filtered using 0.22 µm pore filters (Millex®-GV, Millipore Corporation, Bedford, MA, USA) then added to autoclaved media after it had cooled to approximately 50˚C.

**Table 2.1** *Phytophthora* isolates used to compare the radial growth (RG), dry weight (DW) and optical density (OD) methods for measuring dose response.

| Species | NZFS number | Date Collected | Host | Location | Location type | History of phosphite application |
|---|---|---|---|---|---|---|
| ***Phytophthora cinnamomi*** | 4446 | 27/02/2017 | *Persea americana* (Avocado) | Bay of Plenty | Avocado orchard | Yes |
| | 4447 | 27/02/2017 | *P. americana* | Bay of Plenty | Avocado orchard | Yes |
| | 4449 | 27/02/2017 | *P. americana* | Bay of Plenty | Avocado orchard | Yes |
| | 3030 | 1/10/2008 | *Agathis australis* (Kauri) | Auckland | Park | No |
| | 3953 | 21/07/2014 | *Vitex lucens* (Puriri) | Auckland | Beachfront park | No |
| | 2960 | 13/12/2007 | *Araucaria heterophylla* (Norfolk Island pine) | Auckland | Reserve | No |
| ***Phytophthora multivora*** | 3732 | 24/10/2012 | *Magnolia* sp. | Wellington | Botanical gardens | Yes |
| | 3740 | 24/10/2012 | *Magnolia* sp. | Wellington | Botanical gardens | Yes |
| | 3796 | 30/09/2013 | *A. australis* | Auckland | Nursery | Yes |
| | 3766 | 11/06/2013 | *Araucaria bidwillii* | Napier | Reserve | No |
| | 3842 | 31/01/2014 | *Pinus radiata* (Monterey pine) | Gisborne | Pine plantation | No |
| | 3797 | 9/07/2013 | *A. heterophylla* | Mount Maunganui | Beachfront park | No |
| ***Phytophthora pluvialis*** | 4057 | 10/10/2014 | *P. radiata* | Tokoroa | Nursery | Yes |
| | 4058 | 9/10/2014 | *P. radiata* | Tokoroa | Nursery | Yes |
| | 4059 | 10/10/2014 | *P. radiata* | Tokoroa | Nursery | Yes |
| | 4317 | 18/07/2016 | *P. radiata* | Bay of Plenty | Pine plantation | No |
| | 4340 | 19/10/2016 | *P. radiata* | Gisborne | Pine plantation | No |
| | 4368 | 26/08/2016 | *P. radiata* | Nelson | Pine plantation | No |

### 2.3.4 Radial growth assay

Radial growth (RG) of *Phytophthora* isolates was measured in 90 mm plastic Petri dishes containing 20 mL of phosphite amended RMM agar at 17.5˚C. Plates were inoculated with a 5 mm diameter agar plug from the growing margin of 5 day old colonies on RMM agar. Radial growth rate was recorded from two colony diameters measured at right angles 15 days after inoculation. Microscopic examinations were made of the cultures after 15 days to check for any abnormal or anomalous growths on the phosphite amended agar.

### 2.3.5 Dry weight assay

Dry weight (DW) growth experiments were conducted in 90 mm x 25 mm petri dishes containing 35 mL of amended RSM broth. One 3 mm diameter RMM agar plug from the growing margin of 5 day old colonies was used for inoculum. Non-colonized RMM agar plugs were used as the control across all treatments. Plates were stored at 17.5˚C for 14 days. Whatmans grade 1 (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) 7.0 cm filter paper were dried at 70˚C for 24 hours and weighed individually before the mycelium was vacuum filtered onto it. The filter paper and mycelium were then dried at 70°C for 24 hours. The filter paper and mycelium were weighed and the weight of the mycelium was determined by subtracting the weight of the relevant piece of filter paper. The weight of the control plugs was calculated in the same manner and were subtracted from the weights of the mycelium.

### 2.3.6 Optical density assay

Optical density (OD) growth experiments were conducted in 24 well microtiter plates (Corning, New York, United States), each well had 2 mL of liquid RMM. The inoculum was a 2 mm mycelial plug from the growing margin of a 5-day old colony in liquid RMM medium. Plugs were cut with a 2 mm borer and tweezers were used to put the plugs into the wells. One control well, containing uninoculated medium, for each treatment was included in each replicate block. OD was measured at 620 nm two, four, six and 14 days after inoculation using the Polar Star Galaxy Microplate Reader (BMG Lab Technologies, Offenburg, Germany). Six days was the best indicator of growth rate and used for further analysis. In each well 32 measurements were taken at consistent locations within each well and the average $OD_{620}$ was used as the final value. The average $OD_{620}$

value of the control wells was subtracted from the $OD_{620}$ measurements for the isolates.

### 2.3.7 Data analysis

Percentage inhibition of each isolate at each of the phosphite concentrations was calculated as a percentage of the growth in the absence of phosphite. The inhibition data was then converted to proportion data by dividing the inhibition value by 100. Data analysis was carried out in R version 3.4.2 (R Core Team, 2016). Graphical model validation tools were used to check the model assumptions of variance homogeneity and normality (plots of standardised residuals vs. fitted and explanatory variables and quantile-quantile plots). To determine whether the effect of phosphite treatment on the proportions of inhibition in the three methods was distinct from each other, the proportions of inhibition were analysed using the logit transformation. We fitted a Generalised linear model (GLM) using a logit link included method, species and log-phosphite treatment as covariates. All main effects and interactions were tested. The three-way interaction was not significant, isolate was insignificant in each species and was dropped from the model. There was no evidence of nonlinearity or heteroscedasticity. Tukey's Honestly Significant Difference (HSD) tests (Tukey, 1949) were used to obtain estimates and comparisons of the tests.

EC50 values describe the point at which the growth of mycelium is reduced to 50% relative to the control. EC50 values were calculated using by plotting percentage inhibition against $\log_{10}$ phosphite concentration and extracting the phosphite concentration (x axis) at 50% growth inhibition (y axis) (Bower & Coffey, 1985; Fenn & Coffey, 1984). Data were examined for homogeneity of variance using Levene's test (Levene, 1960) and normality using the Shapiro-Wilk test (Shapiro & Wilk, 1965). The EC50 values were square root transformed to satisfy the assumptions of ANOVA. Two Way ANOVA was used to compare differences in the EC50 values of the species for the different assay methods and species. Post-hoc pairwise comparisons using a Tukey's HSD test (Tukey, 1949) were used to compare sensitivities between the species.

## 2.4 Results

### 2.4.1 Tolerance of *Phytophthora* species to phosphite

There was a strong interaction between method and phosphite treatment-dose at p <0.001), and the variable species was statistically significant at p <0.001. All three species were significantly different to each other in their tolerance to phosphite (p<0.001). The slopes for log phosphite in the OD and DW assays were statistically distinct from each other at p<0.001, but there was no difference in the slopes of log phosphite between the OD and RG assays (Figure 2.1). The results also indicate that there was a difference in the slopes of log phosphite between the DW and RG assays at p<0.001 (Figure 2.1).

The average EC50 of the *P. cinnamomi* isolates was higher than *P. multivora* and *P. pluvialis* in each of the three methods (Table 2.2).

**Table 2.2** Average EC50 (µg/mL) value for *Phytophthora cinnamomi, P. multivora* and *P. pluvialis* from the optical density (OD), radial growth (RG) and dry weight (DW) assays with standard error in the brackets.

| Species | DW | RG | OD |
|---|---|---|---|
| *P. cinnamomi* | 26.0 (± 3.3) | 22.5(± 4.0) | 23.5 (± 2.3) |
| *P. multivora* | 22.6 (± 4.0) | 8.5 (± 2.9) | 11.4 (± 1.3) |
| *P. pluvialis* | 3.4 (± 2.1) | 2 (± 0.8) | 4.5 (± 1.4) |

This data was analysed using a Two-Way ANOVA. There was no significant interaction between species and method (p=0.1). There was a marginally significant difference between the average EC50s for the species produced by the RG and DW assays (p=0.06). There was a significant difference in the EC50s of the three species (p<0.0001). The EC50s of *P. cinnamomi* were significantly higher than that of *P. multivora* (Tukey's HSD p=0.002) and *P. pluvialis* (p<0.0001). The EC50s of *P. multivora* were significantly higher than that of *P. pluvialis* (p<0.0001).

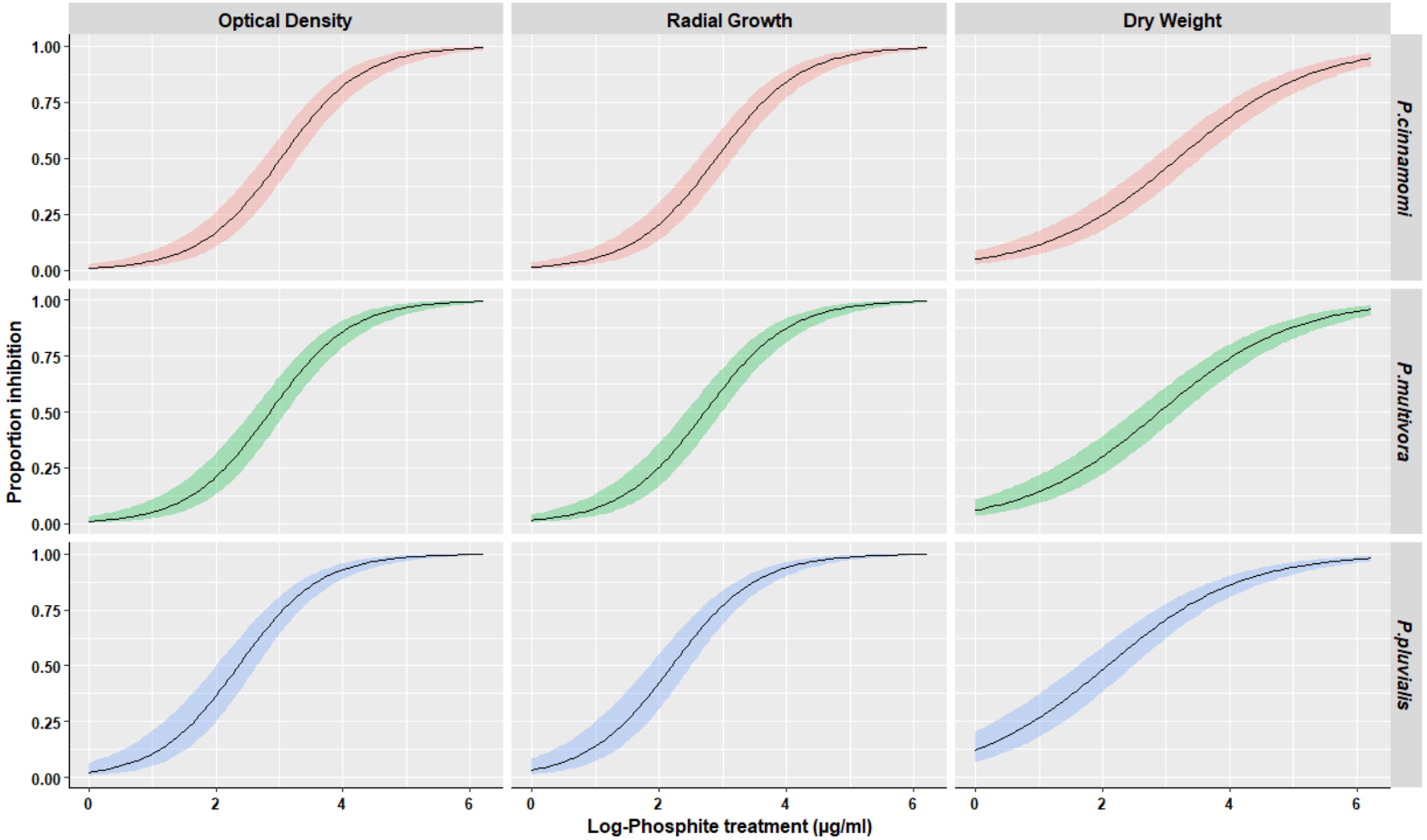


**Figure 2.1** Average growth inhibition (%) of the six *Phytophthora cinnamomi*, six *P. multivora* and six *P. pluvialis* isolates grown on modified Ribeiro's Minimal Media (RMM) amended with 15, 80, 200 and 500 µg/mL phosphite using the radial growth (RG), dry weight (DW) and optical density (OD) methods

### 2.4.2 Morphological observations

All of control plates of the *P. multivora* isolates in the radial growth assay had abundant oospores and one isolate, 3797, produced oospores on the 15 µg/mL plates. The morphology of isolate 3797 was an exaggerated rosette-like growth on the 15 µg/mL plates compared to the control plates. No oospores were observed in any of the *P. multivora* plates on 80, 200 and 500 µg/mL phosphite concentrations and the hyphae was very densely aggregated for all isolates compared to the control plates. *P. cinnamomi* and *P. pluvialis* hyphae became denser as the phosphite concentration increased and hyphal swellings became more abundant. *P. cinnamomi* and *P. pluvialis* did not readily produce oospores on any of the treatments. No *P. multivora* oospores were observed in any of the liquid cultures for the dry weight assay. Coralloid hyphal growth and hyphal swellings of *P. cinnamomi* and *P. pluvialis* became more abundant in liquid cultures.

## 2.5 Discussion

A new high-throughput method was developed for screening fungicide tolerance of *Phytophthora* species using optical density measurements of mycelial growth inhibition. The OD assay is as good at measuring phosphite inhibition as the RG assay and it has the added benefit of scalability and it is easier to take repeat measurements over time using the OD method (Figure 2.1). The growth of the three species was more inhibited in the DW assay compared to both the RG and OD assays (Figure 2.1). Previous work has also found *Phytophthora* isolates were more inhibited in liquid RMM than solid RMM and hypothesised this was because the mycelia were greater contact with the phosphite in the liquid medium (Ma & McLeod, 2014). The DW method is open to greater variation and has lower sensitivity to change in growth due to the very low dry weights of cultures and challenges weighing these accurately. This was also reflected in the EC50 analysis with RG and OD showing a differential response for *P. multivora* while DW was not able to differentiate the response between *P. cinnamomi* and *P. multivora* (Table 2.2).

*Phytophthora cinnamomi* was the most tolerant to phosphite followed by *P. multivora* and then *P. pluvialis* (Table 2.2). None of the three assay methods detected any significant differences in the inhibition of isolates of the same

species with and without phosphite histories. The *P. cinnamomi* isolates were recovered from soil and roots from randomly selected trees in an orchard which manages avocado root rot using phosphite. Phosphite tolerant isolates are more likely to be isolated from unhealthy trees (Chapter 3). The specific phosphite application history of the *P. multivora* and *P. pluvialis* isolates is unknown however they were isolated from areas where phosphite was used (Table 2.1).

While there was no significant difference in the tolerance of the *P. multivora* isolates only one isolate was able to produce oospores in the presence of the phosphite (15µg/mL phosphite). This has implications for the development of resistant isolates because oospores are sexual structures that allow for the selection of desirable traits, such as phosphite resistance, to be passed on because phosphite does not kill *Phytophthora*. The production of dense hyphal aggregations by *P. cinnamomi* and *P. pluvialis* in response to phosphite is likely a survival strategy, as hyphal aggregations can be long term survival propagules (Jung *et al.*, 2013).

The OD assay was faster, allowing measurement six days after inoculation in contrast to the DW and RG assays which were measured 14 and 15 days after, respectively. The OD method provides the opportunity for increased screening of larger numbers of isolates concurrently. OD microplates are a downscaled version of petri dishes that decrease the amount of time and resources required for an experiment. The smaller scale and shorter growing time may also decrease the chance of contamination. The time required to test fungicide sensitivity in fast growing species of fungi and oomycetes can be greatly reduced compared to using the radial growth and dry weight assays especially when nutrient rich media are used. This could be useful in cases where fungicide sensitivity of isolates in nurseries or production crops is suspected and finding out the true level of resistance quickly is necessary for implementing disease management plans.

With the OD method and an automated plate reader it is also possible to take frequent time series measurements, allowing a whole growth response curve to be captured (Frac *et al.*, 2016). This enables researchers to identify the exponential growth, fungistatic, resource and space limitation phases of growth. These details can be used to guide fungicide sensitivity studies, by informing the time that optical density measurements should be taken in order to capture the fungistatic

activity phase for each inhibition assay. Costs are also reduced when using optical density assays because media requirements are reduced.

Photometric evaluation provides an unbiased, automated three-dimensional analysis of mycelium growth (Kuhajek *et al.*, 2003). Optical density measurements may be advantageous for studying slow growing fungi as the scale can be downsized from 9 cm petri dishes to 96-well microtiter plates containing 300 µL per well, reducing the time required for sufficient colony growth. *Phytophthora* species are relatively fast growing; therefore, the optical density assessment could be a very rapid and accurate option for studying fungicide sensitivity. While hyphae may not grow homogenously in the wells, this can be accounted for by multiple OD reads per well and taking an initial measurement immediately after inoculation which the final result can be blanked against. Variation in OD measurements within a well can be accounted for by setting the OD reader to take more measurements and/or by taking repeated measurements over time.

Having an efficient, cost-effective method to assess fungicide resistance in *Phytophthora* species is a valuable tool for protecting industries affected by phytophthora diseases by informing chemical control management. By using the optical density method to screen for fungicide sensitivity, it is possible to screen more isolates to more fungicides or fungicide concentrations faster. It is also possible to analyse growth patterns of different isolates in the presence of different phosphite concentrations using frequent measurements. These comparisons are not possible using the dry weight or radial growth assays.

When testing *Phytophthora* for resistance to the systemic fungicide phosphite, mycelium is the most relevant propagule to use because mycelium grows through host plants and will be exposed to systemic fungicides. Phosphite is also used to protect host plants which are already infected with *Phytophthora*, therefore it is necessary that it is effective on mycelium. Furthermore, it is difficult to get a truly homogenous solution of zoospores inoculum because zoospores often aggregate at the surface of a liquid (Cameron & Carlile, 1977) resulting in a heterogeneous suspension and high standard deviations in optical density values.

The radial growth assay does not enable changes in hyphal density and morphology to be quantified. In this study, the growth of the mycelium was not uniform for any of the three species using RG, both in the baseline controls and in response to the phosphite treatments. These variations in growth were obvious morphologically but difficult to quantify by measuring radial growth. The DW and OD assays were able to overcome variations in colony growths through the nature of their measurements. Growth of *Phytophthora* cultures in liquid media has the further benefit of complete immersion of the mycelium in the solution being tested, allowing the diffusion of metabolites more readily than in solid media. Growth in liquid media may therefore provide a more consistent exposure to the test solution in contrast to growth on solid media (Ma & McLeod, 2014). It is easier to get more accurate measurements of mycelial density in liquid using the OD method compared to the DW method. The plate reader takes the measurements in the OD method and in the DW method both filter paper and dried mycelium are weighed. The filter papers were weighed individually and ranged from 297 – 349 mg while the mycelium weighed from 0 – 130 mg, the inclusion of the filter paper increases chance of error and makes it more difficult to accurately measure the mycelium weight.

It is advantageous to conduct preliminary trials with a range of fungicide concentrations for which the optical density is measured frequently until the mycelium are spatially limited. This will ensure the whole growth phase is capture to accurately determine when to measure fungistatic activity. The timing of measurements and the size of the wells will differ for species relative to how fast they grow or if they are known to be resistant isolates.

## 2.6 Conclusions

The OD assay resulted in comparable inhibition curves to the RG assay, probably the most widely used measure of fungicide sensitivity in true fungi and oomycete plant pathogens. Contrasts between the growth response curves with each method indicate that the growth of the *Phytophthora* species was more inhibited in the DW assay compared to the RG and OD. It is challenging to accurately weigh the dried mycelium because the weights are so small (the maximum was 130 mg) and this loss in sensitivity was also reflected in the EC50 analysis as the DW method was not able to differentiate the response between *P. cinnamomi* and *P. multivora*.

Optical density measurements can be performed as a high-throughput assay to screen more isolates, replicates and treatments, faster than the other two methods. It enables more data points to be collected and hence all growth phases can be captured to inform when it is best to measure fungicide sensitivity. It may be particularly useful when testing fungicide sensitivity in slow growing species as the process could be sped up significantly by using small welled microplates. It is also relatively inexpensive and less labour intensive. Using this method, *Phytophthora cinnamomi, P. multivora* and *P. pluvialis* showed varying levels of phosphite sensitivity at the species level with *P. cinnamomi* being the least sensitive followed by *P. multivora* and *P. pluvialis.* Comparable results were produced with all three methods with the optical density method offering greater throughput and thus capacity for pathogen toxicology analyses.

# Chapter 3

# Phosphite sensitivity of *Phytophthora cinnamomi* from New Zealand avocado orchards

## 3.1 Abstract

To test the hypothesis that prolonged exposure to phosphite leads to decreased sensitivity to phosphite in isolates of *Phytophthora cinnamomi* from avocado orchards, 24 isolates from four orchards (7-30 years old) never treated with phosphite and 32 isolates from eight orchards treated with phosphite for 15 – 37 years were screened for sensitivity using the optical density (OD) method (Chapter 2). The inhibitory effect of phosphite on mycelial growth was tested over six concentrations of phosphite (0, 15, 40, 80, 200 and 500 μg/mL) in modified Ribeiro's Minimal Medium (RMM) liquid. *Phytophthora cinnamomi* isolates formed a continuum in their sensitivity to phosphite, EC50s from 11.9 – 124.3 μg/mL phosphite, but could be subjectively divided into sensitive (50 isolates), intermediate (2 isolates), and tolerant (4 isolates) groups. Intermediately tolerant and tolerant isolates were from orchards which manage avocado root rot with phosphite. Five of the six isolates with increased tolerance were isolated from unhealthy trees. Selection for decreased sensitivity to phosphite *in vitro* has occurred in isolates of *P. cinnamomi* from New Zealand avocado orchards that have used phosphite for a prolonged period. It is hypothesised that tolerant isolates are often closely associated with unhealthy trees because they receive additional treatments of phosphite. Furthermore, the use of phosphite keeps trees alive that would have otherwise died, allowing *P. cinnamomi* to continue to grow and reproduce where it would have likely been outcompeted as a saprophyte or antagonised by other microorganisms had the tree died.

## 3.2 Introduction

Avocado root rot is the most important disease of avocado worldwide. In many New Zealand avocado orchards, phosphite fungicides are used to manage the disease. Phosphite is a phosphonate, in the form of phosphorus acid as mono- and di-potassium salts. Phosphite has a complex mode of action, it works directly on the pathogen by inhibiting hyphal growth and preventing sporulation while also

stimulating host defence responses in the plant (Guest & Grant, 1991; Smillie *et al.*, 1989). Phosphite is phloem mobile so it is also transported down to the root system of the tree which is the infection point for the causal agent of avocado root rot, *Phytophthora cinnamomi*. It accumulates in plant sink tissues because it is not metabolised by the plant (McDonald *et al.*, 2001).

Phosphite can be applied as a foliar spray, root drench, in pill form, and as passive or high-pressure trunk injections. Of growers surveyed at the 2015 annual NZ grower forum, over 90% use phosphite and the most commonly used method is passive injections (over 60%) followed by high-pressure injections (about 18%) (West, 2017). In the passive injection method, the rate of phosphite uptake is determined by the rate of transpiration in the tree, as phosphite is injected into the xylem and there is no lateral movement of phosphite so syringes need to be evenly spaced around the tree. High-pressure stem injections allow for faster processing time because the application rate is not dependent on the rate of transpiration, it only takes 3 – 4 minutes per tree. Directions on the label of high-pressure product Stemshot AV-1 (Chengeta Crop Care, New Zealand) recommends applying of 5 mL per metre of canopy of undiluted product (400 g/L phosphite) at a rate of 3 mL per stroke. For trees with a diameter smaller than 750 mm, the product is applied in a single 10 mm diameter hole drilled through the tree to within 50mm from the opposite surface. If the diameter is larger than 750 mm then a second hole is drilled 300 mm above and perpendicular to the first.

Phosphite became widely available in 1985 in Australia and was cheaper than Fostyl-Al (Aliette®) which was main fungicide used previously. Orchard growers imported phosphite to New Zealand as a ‘fertiliser’ in 1985 to test it on trees heavily infected with root rot in the far North (West, 2017). Trees were injected 40 days after the start of vegetative leaf flush in mid-summer using a 15% solution in 20 mL syringes space 115mm around the trunk. The first results were almost miraculous, with sickly trees recovering to become healthy and highly productive. After a decade or two of use, root rot issues arose again and growers were advised to increase the concentration to 20% into twice as many holes and start injecting twice a year in March and August (West, 2017). This higher concentration appeared to impact the trees ability to heal from the drill holes, with the solution often spilling out. Growers in South Africa also found a concentration

of 20% caused damage to the wood and sealing the injection holes did not reduce the damage (Blakey & Wolstenholme, 2014). As the lower parts of the trunk filled with holes injection sites had to move up into the limbs. At this stage some orchards started to use high pressure application methods as tree health continued to decline. It was not until tests of the phosphite levels in the root material was carried out that growers realised the sick trees were actually suffering from phosphite toxicity. The recommended phosphite concentration in roots is 25 ppm, the sick trees had levels of phosphite as high as 600 ppm (West, 2017).

New Zealand consultants currently recommend injecting trees once a year, coinciding with the main root flush in March to June (West, 2017). The recommendation of 25 ppm in roots requires testing of root samples to measure the concentration and over 80% of the growers surveyed at the 2015 annual NZ grower forum do not test for phosphite levels (West, 2017). Most products recommend injecting 20 mL of a 15% - 20% (w/v) solution per metre of canopy diameter with syringes spaced every 20 cm around the trunk however actual practice varies across growers (West, 2017). In Australia the recommended application rate is 20%, with 15 mL per metre of canopy (Whiley, 1999).

*Phytophthora* isolates can become tolerant to phosphite with prolonged exposure (Dobrowolski *et al.*, 2008). This has been shown clearly *in vitro* many times (Bower & Coffey, 1985; Duvenhage, 1994; Kaiser *et al.*, 1997; Wilkinson *et al.*, 2001b). *Phytophthora cinnamomi* isolates from avocado orchards in Australia (Dobrowolski *et al.*, 2008) and South Africa (Ma & McLeod, 2014) had increased tolerance to phosphite after 3 – 8 and 10 years exposure, respectively. While New Zealand has a proud history of *Phytophthora* research (Chee & Newhook, 1965a; Dick *et al.*, 2006; Hüberli *et al.*, 2008; Newhook, 1959; Stewart & McCarrison, 1992), with initial interest being in *P. cinnamomi* (Chee & Newhook, 1965b, 1965c, 1965d; Podger & Newhook, 1971) due to the impacts it has in Australian jarrah (*Eucalypts margintina*) forests, no comprehensive study of *P. cinnamomi* phosphite sensitivity in New Zealand avocado orchards has been conducted. This study is an initial step in that direction.

Phosphite sensitivity is measured using mycelial growth tests to measure growth inhibition in the presence of phosphite. The inhibition values calculated are used to predict the amount of phosphite required to inhibit growth by 50% relative to

the control, this is known as the Effective Concentration 50 (EC50). Predicting EC50 is a commonly used measure of sensitivity and is used to compare sensitivity between isolates in the same study. It is difficult to compare sensitivities between studies due to differences in methods and medium.

The main aim of research in this chapter is to test the phosphite sensitivity of *P. cinnamomi* isolates from eight New Zealand avocado orchards that have managed avocado root rot with phosphite for 15 – 37 years and four orchards which have never used phosphite to test the hypothesis that prolonged exposure leads to decreased sensitivity to phosphite. It was first necessary to conduct a survey of orchards to obtain isolates of *P. cinnamomi.*

## 3.3 Methods

### 3.3.1 Survey of *Phytophthora* from avocado orchards

#### *3.3.1.1 Collection of* Phytophthora *species*

Two sample collections from avocado orchards were conducted, the first occurred in September 2016 as part of a project for a university paper and will be referred to as the '2016 collection', the second collection occurred in January to February of 2017 and will be referred to as the '2017 collection'.

The 2016 collection involved collecting 1000 g of soil and fine feeder roots from the base of one unhealthy avocado tree from orchards 1 – 6 (Table 3.1).

**Table 3.1** Details of the orchards surveyed for *Phytophthora* isolates. Two separate collections at six orchards each occurred in 2016 and 2017.

| Orchard | Location | Date Sampled | Phosphite management | Orchard age (years) |
|---|---|---|---|---|
| 1 | Kaitaia, Northland | 1/09/2016 | All trees injected annually in Autumn with sick trees given an additional injection in Spring | 17 |
| 2 | Whangarei, Northland | 1/09/2016 | Inject annually in Autumn | 15 |
| 3 | Waihi, Coromandel | 1/09/2016 | No phosphite application | 7 |
| 4 | Kaitaia Northland | 1/09/2016 | Annually injected with multiple foliar application as well | 15 |

| Orchard | Location | Date Sampled | Phosphite management | Orchard age (years) |
|---|---|---|---|---|
| 5 | Kaitaia, Northland | 1/09/2016 | Injected once or twice a year annually | 15 |
| 6 | Kaitaia, Northland | 1/09/2016 | Injected twice a year since 1990 (27 years) | 33 |
| 7 | Katikati, Bay of Plenty | 27/01/2017 | Injected for 37 years. All trees injected in February with 133 g/L phosphite using 20 mL syringes. Declining trees injected again in autumn. Use HiPk® | 45 |
| 8 | Katikati, Bay of Plenty | 27/01/2017 | No phosphite application | 12 |
| 9 | Waihi, Coromandel | 15/02/2017 | No phosphite application | 7 |
| 10 | Te Puke, Bay of Plenty | 15/02/2017 | Injected for 32 years. Regularly inject every year with sick trees injected twice a year. Uses Agrifos 600® | 41 |
| 11 | Katikati, Bay of Plenty | 27/02/2017 | Injected for 20 years, first 14 years used passive method and last 6 years used STEMSHOT AV-1® high pressure injections. Only symptomatic trees injected each year, about 30% of the orchard. | 25 |
| 12 | Tauranga, Bay of Plenty | 27/02/2017 | No phosphite application | 30 |

Orchards 7 – 12 were sampled in 2017, three of which have a history of phosphite use and three which have no phosphite history (Table 3.1). In the 2017 collection, 16 trees were randomly selected for sampling from each orchard. The trees in Orchard 11 (Table 3.1) were randomly selected from those trees which are injected regularly because only 30% of the trees in the orchard are injected each year. Soil and fine feeder roots were collected at four points around the tree along the North, South, East and West axes one metre from the trunk. The soil was mixed together and a 1000 g subset was collected. All soil samples were stored at room temperature for up to seven days before commencing baiting (Scott *et al.*, 2009).

*3.3.1.2 Isolation of* Phytophthora *species*

A commonly used soil baiting procedure was used to isolate *Phytophthora* species from the soil samples (Chee & Newhook, 1965a; Jung *et al.*, 1996; Marks & Kassaby, 1972; Zentmyer *et al.*, 1960) . In a plastic container 140 x 85 x 70 mm, 15-20 mm of soil and fine feeder roots were placed and slowly flooded with 400 mL of distilled water. Two leaves of a cultivated *Rhododendron* species and five Himalayan cedar (*Cedrus deodara*) needles were floated on top of the water. The lid was sealed at three corners, leaving one open for air flow to maintain an aerobic environment.

Three days after commencing baiting the leaves and needles were removed, washed with tap water, and patted dry with paper towels. In a sterile environment, four 10 $mm^2$ pieces containing lesions were cut from the leaves. If no lesions were apparent two 10 $mm^2$ pieces were taken from either side of the centre vein. The Rhododendron pieces and four of the cedar needles were surface sterilised in 70:30 % (vol/vol) ethanol:sterile water and washed in two rinses of sterile distilled water (30 sec for each step). The plant material was dabbed dry with paper towels and plated at an angle onto CRNH (Appendix A) agar on separate plates.

The plates were stored at 17.5˚C in the dark and monitored for *Phytophthora*-like growth by examining them under an inverted dissecting microscope. Growths were sub-cultured onto carrot (CAD) agar (Appendix A) agar and grown at 17.5˚C in the dark. *Phytophthora cinnamomi* isolates were identified morphologically. Cultures were stored at 17.5˚C in the dark until required for further work. Any *Phytophthora* species unidentifiable using morphology were analysed using molecular analysis (section 3.3.1.3).

If less than three *P. cinnamomi* isolates were obtained from an orchard in the first round of soil baiting then the soil baiting was repeated for those samples from which no isolate was collected. Soil baits were not repeated a third time.

*3.3.1.3 Molecular analysis of Phytophthora isolates*

*Phytophthora* isolates which could not be identified based on morphology alone were identified using molecular techniques. Deoxyribonucleic acid DNA was extracted using the REdExtract-N-Amp Plant PCR Kit (Sigma-Aldrich) according to the manufacturer's instructions. Three agar plugs from the growing margins of

the isolates were plated onto CAD agar overlaid with sterile cellophane (Appendix A) for 5 days. The mycelium was scraped into a 1.5 mL Axygen Max Recovery tube containing 100 µl of Extraction solution. The samples were vortexed briefly and then incubated at 90˚C for 10 minutes. Dilution Solution (100 µl) was added and the tubes were vortexed again briefly. A sample of DNA was used immediately for PCR and the remainder stored at -20˚C.

Polymerase Chain Reactions (PCR's) were run using oomycete specific primers ITS6 (5'-GAAGGTGAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Cooke *et al.*, 2000) to amplify a region of the Internal Transcribed Spacer (ITS) gene, and OomCoxI-Levup (5'-TCAWCWMGATGGCTTTTTTCAAC-3') and FM85mod (5'-RRHWACKTGACTDATRATACCAAA-3') to amplify a region of the Cytochrome c oxidase subunit I gene (Robideau *et al.*, 2011). PCR was performed using the HOT FIREPol® GC Master Mix (Solis BioDyne) according to the manufacturer's instructions. Each 20 µL PCR reaction contained 12.4 µL of PCR grade water, 4.0 µL HOT FIREPol® GC Master Mix (5×), 0.6 µL of each primer (10 µM), 0.4 µL $MgCl_2$ (25nM) and 2 µL DNA. Thermal cycling conditions consisted of an initial denaturation step of 94˚C for 15 min, then 35 cycles of 94˚C (30 s), annealing at 55˚C (40 s), and 72˚C (1 min) and a final extension step of 72˚C (10 min). A portion (5 µ1) of the amplified products was electrophoresed in a 1.5% agarose gel in Tris-Acetate-EDTA (TAE) buffer. The presence of a single bright band, 862-941bp for ITS4 and ITS6 and 700-750 bp for OomCoxI-Levup and FM85mod, for each sample was a check for successful amplification. For the isolate 4460 both PCRs were run using the ITS and Cox primers and the ITS PCR only was conducted for the isolate 4470. All DNA samples were stored at -20˚C.

DNA sequencing was performed in-house at Scion, Rotorua, using a 3500 Series Genetic Analyzer (Applied Biosystems®) using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®). Sequencing was performed in both directions with the ITS4 and ITS6 primers for both the isolates 4470 and 4460. DNA sequence analysis was performed using Geneious software (version 9.0.5). Chromatograms were used to check the base-calling in each sequence. Sequences were compared to those in the GenBank database using BLASTn (Altschul *et al.*, 1990).

### 3.3.2 Phosphite sensitivity of *P. cinnamomi* isolates

*3.3.2.1 Experimental design*

This study examined the phosphite sensitivity of 56 *P. cinnamomi* isolates, from New Zealand avocado orchards, to six phosphite concentrations (0, 15, 40, 80, 200 and 500 µg/mL) using the Optical Density (OD) method developed in Chapter 2 of this thesis. Six replicates for each isolate at each phosphite concentration were included.

*3.3.2.2* Phytophthora cinnamomi *isolates*

The *Phytophthora cinnamomi* isolates used were from the 2016 collection and 2017 collection. Five days prior to the experiment the isolates were sub-cultured into liquid Ribeiro's' Modified Medium (RMM) (Appendix A) (Ribeiro *et al.*, 1975) in 90 mm petri dishes, modified as outlined below. The glucose concentration was 9.0 g/L and β-sitosterol was omitted. MES hydrate buffer (2-(N-morpholino) ethanesulfonic acid) was added at a final concentration of 0.03 M and the pH adjusted to 6.2 with KOH 3M. The inoculum plates were stored at 20˚C in the dark. The media contained 7.35 mM phosphate (Appendix A).

*3.3.2.3 Phosphite amendment*

The phosphite used was AgriFos® 600 (Agrichem, Yatala QLD, Australia), a commercial fungicide containing 600 g/L phosphorous acid, present as mono- and di- potassium phosphonate. The phosphite was filtered using 0.22 µm pore filters (Millex®-GV, Millipore Corporation, Bedford, MA, USA) then added to autoclaved media after it had cooled to approximately 70˚C.

*3.3.2.4 Phosphite medium*

Growth experiments were conducted in 24-well microtiter plates (Corning, New York, United States) containing 2 mL of liquid RMM amended with phosphite. The wells were randomised for inoculation in each replicate block. The inoculum was a 2 mm mycelial plug from the growing margin of a 5-day old colony in liquid RMM. Control wells, containing uninoculated medium, were included for each concentration in each replicate block. Optical density ($OD_{620}$) measurements at 620 nm were taken 13 days after inoculation using the Polar Star Galaxy Microplate Reader (BMG Lab Technologies, Offenburg, Germany), 32

measurements were taken per well at consistent locations within each well and the average $OD_{620}$ was used as the final value.

### 3.3.3 Data analysis

Percentage inhibition of each isolate at each phosphite concentration was calculated as a percentage growth relative to growth in the absence of phosphite. EC50 values were calculated from linear regression lines obtained by plotting the percentage inhibition of mycelial growth against $\log_{10}$ concentration of phosphite (Bower & Coffey, 1985; Fenn & Coffey, 1984). Data were examined for homogeneity of variance using Levene's test (Levene, 1960) and normality using the Shapiro-Wilk test (Shapiro & Wilk, 1965) in R version 3.4.2 (R Core Team, 2016). The EC50 values were log transformed to satisfy the ANOVA assumptions of normality and homogeneity of variances. Two Way ANOVA was used to test the effect of the different phosphite histories and year of collection on the tolerance grouping. Tukey's Honestly Significant Difference (HSD) test (Tukey, 1949) was used to assess the differences between the EC50 values of the tolerance groups (sensitive, intermediate and tolerant). A Fisher's exact test (Fisher, 1922) was conducted to test the hypothesis that there is an association between the phosphite history of an isolate and the phosphite tolerance level *in vitro*. Phosphite tolerant and intermediately tolerant isolates were grouped together as 'increased tolerance' in the Fisher's exact test.

## 3.4 Results

### 3.4.1 Survey of *Phytophthora* species from avocado orchards

One *P. cinnamomi* isolate per orchard was retained from orchards 1 – 6 and all isolates (2 to 16 per orchard) were retained from orchards 7 – 12, resulting in 24 isolates with no previous exposure to phosphite and 32 isolates with previous exposure (Table 32).

**Table 3.2** List of pure *Phytophthora cinnamomi* cultures retained from the soil baiting of the samples from the orchard surveys. NZFS is the Forest Research Culture Collection number at Scion, New Zealand.

| Sample population | Orchard | NZFS | Phosphite history |
|---|---|---|---|
| 2016 | 1 | 4353 | Yes |
| | 2 | 4342 | Yes |
| | 3 | 4357 | No |
| | 4 | 4359 | Yes |
| | 5 | 4469 | Yes |
| | 6 | 4373 | Yes |
| 2017 | 7 | 4409 | Yes |
| | 7 | 4410 | Yes |
| | 7 | 4411 | Yes |
| | 7 | 4412 | Yes |
| | 7 | 4413 | Yes |
| | 8 | 4414 | No |
| | 8 | 4415 | No |
| | 8 | 4416 | No |
| | 8 | 4417 | No |
| | 8 | 4418 | No |
| | 8 | 4419 | No |
| | 8 | 4420 | No |
| | 8 | 4421 | No |
| | 8 | 4422 | No |
| | 8 | 4423 | No |
| | 8 | 4424 | No |
| | 8 | 4425 | No |
| | 9 | 4426 | No |
| | 9 | 4427 | No |
| | 10 | 4428 | Yes |
| | 10 | 4429 | Yes |
| | 10 | 4430 | Yes |
| | 10 | 4431 | Yes |
| | 10 | 4432 | Yes |
| | 10 | 4433 | Yes |
| | 11 | 4434 | Yes |
| | 11 | 4435 | Yes |
| | 11 | 4436 | Yes |
| | 11 | 4437 | Yes |
| | 11 | 4438 | Yes |
| | 11 | 4439 | Yes |
| | 11 | 4440 | Yes |
| | 11 | 4441 | Yes |
| | 11 | 4442 | Yes |
| | 11 | 4443 | Yes |

| Sample population | Orchard | NZFS | Phosphite history |
|---|---|---|---|
| | 11 | 4444 | Yes |
| | 11 | 4445 | Yes |
| | 11 | 4446 | Yes |
| | 11 | 4447 | Yes |
| | 11 | 4448 | Yes |
| | 11 | 4449 | Yes |
| | 12 | 4450 | No |
| | 12 | 4451 | No |
| | 12 | 4452 | No |
| | 12 | 4453 | No |
| | 12 | 4454 | No |
| | 12 | 4456 | No |
| | 12 | 4457 | No |
| | 12 | 4458 | No |
| | 12 | 4459 | No |

Several *Phytophthora*-like growths were observed from the one soil sample baited from orchards 1 – 6 and of the sixteen soil samples baited from orchards 7 – 12 at least 10 *Phytophthora*-like growths were observed from all orchards except orchard 9 (Table 3.3).

**Table 3.3** Number of *Phytophthora*-like growths observed and pure cultures of *Phytophthora cinnamomi* from the one soil bait from the 2016 collection and the 16 soil baits from the 2017 collection.

| Year collected | Orchard | Phosphite history | Number of *Phytophthora*-like growths observed | Number of pure isolates of *Phytophthora cinnamomi* |
|---|---|---|---|---|
| 2016 | 1 | Yes | 8 | 1 |
| | 2 | Yes | 3 | 1 |
| | 3 | No | 6 | 1 |
| | 4 | Yes | 7 | 1 |
| | 5 | Yes | 6 | 1 |
| | 6 | Yes | 2 | 1 |
| 2017 | 7 | Yes | 10 | 5 |
| | 8 | No | 13 | 12 |
| | 9 | No | 4 | 2 |
| | 10 | Yes | 15 | 6 |
| | 11 | Yes | 16 | 16 |
| | 12 | No | 14 | 9 |

Two of the 16 soil samples from orchard 9 yielded an isolate of *P. cinnamomi* in the first series of soil baiting, after baiting the other 14 soil samples again, the *Phytophthora* isolate 4470 was obtained but could not be identified

morphologically as no distinguishing characteristics were present and morphology keys in Erwin and Ribeiro (1996) could not be followed. Three isolates, 4460, 4461, and 4462, of a *Phytophthora* species isolated from orchard 10 were morphologically identical but could not be identified, therefore one isolate, 4460, was sequenced.

#### *3.4.1.4 Molecular analyses*

A BLAST search with the sequences of isolates 4460 and 4470 in GenBank produced the following results. With the ITS sequences isolate 4460 from orchard 10 showed a 100% similarity with *P. citricola* P1815 but also a 100% similarity with *P. palmivora* and isolate 4470 from orchard 9 showed a 100% similarity with *Phytophthora* sp. ex *Drimys winteri* but also a 97% similarity with *P. kernoviae* NZFS2707. A BLAST search of the Cox sequences of the 4460 isolate showed a 100% similarity with *P. citricola* CH98U121C. Isolate 4460 was concluded to be a *P. citricola* and isolate 4470 was concluded to be a *P. kernoviae*.

### 3.4.2 Phosphite sensitivity of *Phytophthora cinnamomi* isolates

#### *3.4.2.1 Tolerance of 56* Phytophthora cinnamomi *isolates to phosphite*

The EC50 values of the *P. cinnamomi* isolates ranged from 11.9 to 124.3 µg/mL phosphite (Table 3.4).

**Table 3.4** EC50 values (µg/mL phosphite) for the 56 *Phytophthora cinnamomi* isolates in response to phosphite and their tolerance class from Figure 3.1

| Collection year | Isolate | Orchard phosphite management | EC50 | Tolerance class |
|---|---|---|---|---|
| 2017 | 4413 | Yes | 11.9 | Sensitive |
| 2017 | 4427 | No | 13.4 | Sensitive |
| 2017 | 4424 | No | 13.72 | Sensitive |
| 2017 | 4420 | No | 19.53 | Sensitive |
| 2017 | 4441 | Yes | 19.63 | Sensitive |
| 2017 | 4437 | Yes | 20.21 | Sensitive |
| 2017 | 4443 | Yes | 20.57 | Sensitive |
| 2017 | 4458 | No | 22.22 | Sensitive |
| 2017 | 4442 | Yes | 23.04 | Sensitive |
| 2017 | 4438 | Yes | 24.08 | Sensitive |
| 2017 | 4426 | No | 24.79 | Sensitive |
| 2017 | 4410 | Yes | 25.77 | Sensitive |

| Collection year | Isolate | Orchard phosphite management | EC50 | Tolerance class |
|---|---|---|---|---|
| 2017 | 4439 | Yes | 27.74 | Sensitive |
| 2016 | 4357 | No | 28.01 | Sensitive |
| 2017 | 4431 | Yes | 28.03 | Sensitive |
| 2017 | 4444 | Yes | 28.53 | Sensitive |
| 2017 | 4436 | Yes | 29.04 | Sensitive |
| 2017 | 4434 | Yes | 29.88 | Sensitive |
| 2017 | 4433 | Yes | 30.1 | Sensitive |
| 2017 | 4452 | No | 31.08 | Sensitive |
| 2017 | 4429 | Yes | 31.38 | Sensitive |
| 2017 | 4440 | Yes | 31.79 | Sensitive |
| 2017 | 4445 | Yes | 32.12 | Sensitive |
| 2017 | 4419 | No | 32.17 | Sensitive |
| 2017 | 4435 | Yes | 32.76 | Sensitive |
| 2017 | 4432 | Yes | 33.65 | Sensitive |
| 2017 | 4430 | Yes | 34.17 | Sensitive |
| 2017 | 4425 | No | 34.39 | Sensitive |
| 2017 | 4416 | No | 35.25 | Sensitive |
| 2017 | 4409 | Yes | 37.44 | Sensitive |
| 2017 | 4459 | No | 37.59 | Sensitive |
| 2017 | 4428 | Yes | 39.02 | Sensitive |
| 2017 | 4414 | No | 41.76 | Sensitive |
| 2017 | 4422 | No | 42.16 | Sensitive |
| 2017 | 4448 | Yes | 42.18 | Sensitive |
| 2017 | 4417 | No | 43.54 | Sensitive |
| 2017 | 4456 | No | 43.56 | Sensitive |
| 2017 | 4450 | No | 44.5 | Sensitive |
| 2017 | 4454 | No | 45.01 | Sensitive |
| 2017 | 4451 | No | 45.44 | Sensitive |
| 2017 | 4415 | No | 46.63 | Sensitive |
| 2017 | 4412 | Yes | 49.1 | Sensitive |
| 2017 | 4423 | No | 50.63 | Sensitive |
| 2017 | 4453 | No | 56.66 | Sensitive |
| 2017 | 4446 | Yes | 58.17 | Sensitive |
| 2017 | 4447 | Yes | 58.73 | Sensitive |
| 2017 | 4457 | No | 65.48 | Sensitive |
| 2017 | 4421 | No | 65.53 | Sensitive |
| 2017 | 4418 | No | 67.52 | Sensitive |
| 2017 | 4449 | Yes | 71.66 | Sensitive |
| 2016 | 4353 | Yes | 77.05 | Intermediate |
| 2016 | 4373 | Yes | 80.5 | Intermediate |
| 2016 | 4469 | Yes | 105.69 | Tolerant |

| Collection year | Isolate | Orchard phosphite management | EC50 | Tolerance class |
|---|---|---|---|---|
| 2016 | 4342 | Yes | 108.44 | Tolerant |
| 2017 | 4411 | Yes | 115.76 | Tolerant |
| 2016 | 4359 | Yes | 124.3 | Tolerant |

There was no significant difference in the EC50 values of the isolates from phosphite managed and phosphite-free orchards (p=0.5). There was a significant difference in the EC50 values of the isolates collected in 2017 compared to those collected in 2016 (p<0.0001). There was a significant difference in the EC50 values of the isolates in the difference tolerance groups (p<0.0001). There was a significant difference in the EC50 values of the tolerant and susceptible isolates (Tukey's HSD p=0.01). There was no significant difference between the EC50 values for the intermediate isolates and the sensitive or tolerant isolates (p>0.05).

The four isolates, 4469, 4342, 4411 and 4359 had the highest EC50 values (Table 3.4) and are grouped together as tolerant isolates when the inhibition values from the two highest phosphite concentrations (200 and 500 µg/mL phosphite) are plotted against each other (Figure 3.1). The two isolates 4343 and 4373 grouped as intermediate tolerance (Figure 3.1) have predicted EC50 values of 77.05 and 80.50, respectively, which are the next highest EC50 values after the tolerant isolates (Table 3.4).

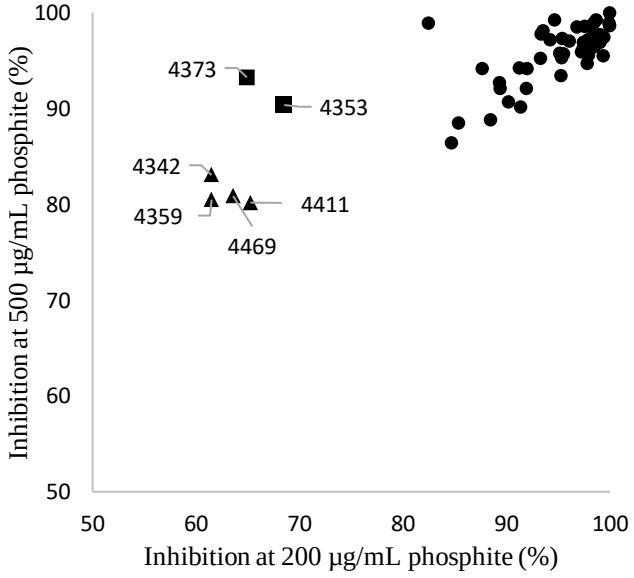


**Figure 3.1** Average inhibition of each of the 56 *Phytophthora cinnamomi* isolates grown in liquid Ribeiros Modified Media (RMM) containing 200 and 500 µg/mL phosphite. Tolerant isolates (triangles), intermediate (squares) and susceptible isolates (circles) are indicated. Tolerant and intermediate isolates are labelled with their NZFS number.

The isolates grouped as tolerant and intermediate are from phosphite managed orchards (Table 3.2 and Figure 3.1). Tolerant isolates were 62 – 65% and 80 – 83% inhibited by 200 and 500 µg/mL phosphite, respectively (Figure 3.1). Intermediate isolates were 65 – 69% and 90 – 93% inhibited by 200 and 500 µg/mL phosphite, respectively. Sensitive isolates were 83 – 100% and 86 – 100% inhibited by 200 and 500 µg/mL phosphite, respectively. A significant association between the phosphite history of an isolate and the level of tolerance to phosphite was found (Fisher's exact test p= 0.03).

Three phosphite tolerant isolates with the highest EC50 values (Table 3.2) and three sensitive isolates with low EC50s were selected for further study (Chapter 4 of this thesis) (Table 3.5). At least one isolate from each of the 2016 and 2017 collections was chosen for each category.

**Table 3.5** Three phosphite resistant and three sensitive *Phytophthora cinnamomi* isolates selected for further study.

| NZFS | Phosphite resistance | EC50 (µg/mL phosphite) | Collection year | Previous phosphite exposure |
|---|---|---|---|---|
| 4424 | Sensitive | 13.72 | 2017 | No |
| 4458 | Sensitive | 22.22 | 2017 | No |
| 4357 | Sensitive | 28.01 | 2016 | No |
| 4342 | Tolerant | 108.44 | 2016 | Yes |
| 4411 | Tolerant | 115.76 | 2017 | Yes |
| 4359 | Tolerant | 124.3 | 2016 | Yes |

## 3.5 Discussion

Tolerance to phosphite was detected (Figure 3.1 and Table 3.4) and it was clearly associated with orchards that used phosphite as the two intermediately tolerant and four tolerant isolates were from avocado orchards with a history of prolonged phosphite use (>15 years). Furthermore, all the isolates collected from unhealthy trees in phosphite managed orchards in 2016 were either intermediately tolerant or tolerant to phosphite (Table 3.4). It is possible the isolates from unhealthy trees were exposed to phosphite more often because some orchard growers treat sick treats with phosphite more often than healthy trees (Table 3.1).

*Phytophthora cinnamomi* was isolated from every orchard sampled and at least one isolate from each orchard was measured for phosphite sensitivity in the OD mycelial growth assay (Table 3.2). Pure cultures were not isolated for every

*phytophthora*-like growth observation (Table 3.3), this was most often due to *Pythium* species out competing the *Phytophthora* on the CRNH (Appendix A) plates. Considering that in the 2017 collection the soil samples were from randomly selected trees rather than symptomatic trees, observing 72 *Phytophthora*-like growths in the 96 samples was a high rate of *Phytophthora* species presence (75%) (Table 3.3).

The collections in 2016 and 2017 gave a suitably sized population for carrying out the *in vitro* phosphite sensitivity experiment as 32 isolates with phosphite exposure and 24 without were obtained. There does not appear to be any obvious pattern in the number of *P. cinnamomi* isolates obtained from the phosphite managed orchards compared to the orchards with no phosphite use (Table 3.3). However, the youngest orchard, orchard 9, had the lowest incidence of *P. cinnamomi* (2 out of the 16 samples) and baiting was repeated for 14 of the samples. The site was on a hill and therefore may have relatively good drainage. Water-logging is known to exacerbate conditions for phytophthora root rot disease progression (Dann *et al.*, 2013). Furthermore, the orchard is 7 years old which may contribute to the low incidence of *P. cinnamomi* due to populations not having enough time to become established.

Two other *Phytophthora* species were collected from the survey, three isolates of *P. citricola* from orchard 10 and one isolate of *P. kernoviae* from orchard 9. *Phytophthora citricola* was first found to cause a stem canker on avocado in California in 1964 (Zentmyer & Jefferson, 1974). It continues to cause problems for the avocado industry in California in at least 5% of orchards however it is thought to be present in up to 90% of the orchards (Marais *et al.*, 2002). Phosphite has been trialled for controlling canker spread and was found to be efficient (El-Hamalawi *et al.*, 1995). *Phytophthora citricola* does not cause major problems in New Zealand orchards in comparison to *P. cinnamomi* root rot disease. *Phytophthora kernoviae* is not known to cause any major diseases to New Zealand flora and may be indigenous (Gardner *et al.*, 2015).

EC50 is a measure used to predict the amount of phosphite necessary to inhibit mycelial growth by 50% relative to the control (Fenn & Coffey, 1984; Wilkinson *et al.*, 2001b). The sensitivity of the isolates formed a continuum (Figure 3.1 and Table 3.4). The tolerant group of isolates, 4342, 4411, 4469 and 4359, based on

their inhibition values at 200 and 500 µg/mL (Figure 3.1) had the highest predicted EC50 values (Table 3.4). The isolates classified as intermediate (Figure 3.1) had the next highest EC50 values (Table 3.4). Previous exposure to phosphite did not result in increased phosphite tolerance for every isolate, as some isolates without previous exposure to phosphite had higher predicted EC50 values than some isolates with previous exposure (Table 3.4). It is not possible to determine how long an isolate has been in an avocado orchard and therefore exposed to phosphite, however it is likely the isolates identified as being intermediately tolerant and tolerant to phosphite (Figure 3.1) had been exposed to phosphite for an extended period of time.

It is difficult to compare the tolerance of isolates across studies due to differences in media and phosphite concentrations tested and the effect of phosphate starvation on *Phytophthora* growth. The tolerant isolates from the current study were inhibited 61.5 – 65.3% by 200 µg/mL phosphite (Figure 3.2) and had EC50s from 105.69 – 124.30 (Table 3.3). In comparison, six tolerant isolates with no previous exposure to phosphite were inhibited 31 -71% by 50 µg/mL phosphite and EC50s ranged from 9 – 48 with one isolate having an EC50 of 148 µg/mL phosphite (Wilkinson *et al.*, 2001b). The Wilkinson *et al.* (2001b) study used RMM agar amended with 50 and 100 µg/mL phosphite, while the current study used RMM liquid and tested up to 500 µg/mL phosphite. It has been hypothesised that isolates are more sensitive to phosphite in liquid than in agar because the mycelium is in greater contact with phosphite in liquid media than solid Ma and McLeod (2014). Therefore the tolerant isolates identified in the current study may be more tolerant than their EC50 values suggest compared to isolates from Wilkinson *et al.* (2001b).

*Phytophthora* are phosphate starved in low phosphate media and thus EC50 values are exaggerated (Guest & Grant, 1991). Ma and McLeod (2014) assessed the impact of phosphate concentration on the inhibitory effect of phosphite to *P. cinnamomi* isolates from avocado orchards with different phosphite histories. Fifty *P. cinnamomi* isolates were screened for phosphite sensitivity in a radial growth assay on RMM agar across three phosphate concentrations of 1, 7 and 15 mM phosphate (Ma & McLeod, 2014). They identified 37, 28 and 24 sensitive isolates (classified as being inhibited >60% at 100 µg/mL) at 1, 7 and 15 mM

phosphate, respectively. In comparison, more tolerant isolates were identified as the phosphate concentration increased because they were not phosphate starved. Nine, 13 and 19 isolates were tolerant (defined as <20% and <30% inhibition at 30 and 100 µg/mL phosphite respectively) on 1, 7 and 15 mM phosphate, respectively. The tolerant isolates in the current study were inhibited 12 – 28% at 40 µg/mL and 43 – 44% at 80 µg/mL in liquid RMM containing 7.35 mM phosphate. Ma and McLeod (2014) found isolates were more sensitive in liquid than agar RMM. It is possible the isolates from the current study, which grew in liquid RMM, are of a similar tolerance level to the tolerant isolates from Ma and McLeod (2014) on RMM agar with 7 mM phosphate based on their inhibition at similar phosphite concentrations.

The level of phosphate is different in plants (Ma & McLeod, 2014) and therefore isolates identified as being tolerant *in vitro* should be tested for their tolerance *in planta*. This is also important because phosphite not only inhibits *Phytophthora* growth but it also stimulates host defence responses. So, to be tolerant *in planta* *Phytophthora* have to overcome the inhibitory effect of phosphite and the plant's defensive responses. A subset of the phosphite sensitive and tolerant isolates (Table 3.5) identified in the current study will be used to analyse the *in-planta* tolerance of the isolates (Chapter 4) and to study the genes being expressed in *P. cinnamomi* when exposed to phosphite using transcriptomic analysis.

In the current study isolates with decreased sensitivity to phosphite were consistently found on unhealthy trees treated with phosphite. It is hypothesised that tolerant isolates may be associated with unhealthy trees often because the trees receive additional treatments of phosphite to manage avocado root rot. The use of phosphite keeps trees alive that would have otherwise died, allowing *P. cinnamomi* to continue to grow and reproduce where it would have likely been outcompeted as a saprophyte or antagonised by other microorganisms had the tree died. Furthermore, phosphite does not impact the viability of selfed/homothallic oospores produced by the A2 *P. cinnamomi* mating strain which dominates in avocado orchards worldwide (Coffey, 1992) and oospore production is only inhibited by high concentrations of phosphite (McCarren et al., 2009b). If *P. cinnamomi* infects and spreads successfully through the fine feeder roots of phosphite treated avocado trees, and produces sexual oospores, this provides the

opportunity for the traits related to phosphite tolerance to be passed on as phosphite does not kill *Phytophthora*, threatening the continued effectiveness of phosphite to manage avocado root rot.

It is proposed that the best management practice for treating a tree with avocado root rot is to combine several methods of control (such as mulching and promoting soil drainage). This may reduce the necessity to treat the unhealthy trees with phosphite more often than healthy trees and avoid the development of phosphite tolerant isolates which can then be spread through orchards. If a tree is still declining from root rot after being treated with phosphite the tree could be removed to avoid promoting phosphite tolerance in the *P. cinnamomi* isolates causing disease.

To conduct a targeted survey of resistant isolates of *P. cinnamomi* it would be advisable to sample trees along a scale of sick to healthy sampling both healthy and symptomatic roots below and above lesion margins if possible. Orchards that inject sick trees multiple times should be included. It is likely that a targeted survey of symptomatic trees in orchards managed with phosphite will result in a higher number of isolates with increased phosphite resistance.

## 3.6 Conclusions

The *P. cinnamomi* isolates from New Zealand avocado orchards screened in this study could be subjectively separated into sensitive, intermediate and tolerant groups, and the predicted EC50 values correlated with the groupings (Table 3.4). The four tolerant and two intermediately tolerant isolates were from phosphite managed orchards. This provides evidence that phosphite tolerance develops in isolates with prolonged exposure to phosphite. Furthermore, 5 of the 6 isolates, with increased tolerance (intermediate and tolerant group), were isolated from unhealthy trees. From the random sampling regime in the 2017 collection only one tolerant isolate was found from 96 soil samples. It is therefore suggested that targeted surveys of trees with avocado root rot symptoms in phosphite manged orchards will provide the most likely opportunity to isolate phosphite tolerant *Phytophthora* isolates. Integrated management using cultural practises, biological control alongside chemical control should help to avoid the development of phosphite tolerant isolates of *P. cinnamomi* and repeat phosphite treatments on unhealthy trees should be used with caution.

# Chapter 4

# *In planta* phosphite tolerance and sporangia production of *Phytophthora cinnamomi* isolates of known tolerance

## 4.1 Abstract

To test the hypothesis that isolates of *Phytophthora cinnamomi* showing decreased sensitivity to phosphite in mycelial growth tests will express decreased sensitivity *in planta*, six isolates of known levels of *in vitro* resistance were used to inoculate the root-tip of phosphite treated lupin seedlings. They were also assessed for their ability to produce sporangia and viable zoospores in the presence of phosphite *in vitro*. The three isolates with high phosphite resistance *in vitro* were more extensive colonisers of phosphite-treated lupin roots than three known susceptible isolates. Asymptomatic infection was measured by plating fragments of the root taken from above the lesion margin; the tolerant isolates colonised significantly further above the lesion margin than the sensitive isolates. The tolerant isolates produced more sporangia and zoospores in the presence of phosphite than the susceptible isolates. Isolates from phosphite managed avocado orchards identified as being tolerant to phosphite in mycelial growth tests were more tolerant to phosphite *in planta* than susceptible isolates. These tolerant isolates also demonstrated decreased sensitivity to phosphite in the production of sporangia and consequently zoospore release, life stages fundamental to the spread of disease between trees in avocado orchards.

## 4.2 Introduction

Isolates of *Phytophthora cinnamomi* have been shown to develop resistance after prolonged exposure to phosphite. Isolates of *P. cinnamomi* from South African avocado orchards were shown to be less inhibited by phosphite than isolates obtained from trees not treated with phosphite (Duvenhage, 1994). Similarly, the tolerance of *P. cinnamomi* isolates from various hosts in Australia to phosphite was variable (Wilkinson *et al.*, 2001b). Assessing *in vitro* tolerance using mycelial growth inhibition assays is a useful screening for large sample populations. After identifying isolates that have increased resistance to phosphite based on *in vitro* mycelial growth studies it is then of interest to assess the effectiveness of

phosphite to suppress mycelium inside the host and also other life stages of *Phytophthora*. It is possible that isolates identified as having increased resistance *in vitro* do not exhibit this resistance in plant material treated with phosphite because phosphite stimulates host defence responses (Guest & Grant, 1991; Wilkinson *et al.*, 2001b). Phosphite is a systemic fungicide and should be effective at suppressing mycelial growth of susceptible isolates within host plant cells. It is also important to determine the efficacy of phosphite to control various life stages of *Phytophthora* species, particularly those responsible for dispersal, including sporangia and accompanying zoospores and survival, including hyphae and resting spores.

Resistant *P. cinnamomi* isolates from Australian avocado orchards were able to colonise a greater length of phosphite treated lupin roots compared to isolates without a history of phosphite exposure (Dobrowolski *et al.*, 2008). Assays using model species, such as lupin, are often employed in pathology research (Dobrowolski *et al.*, 2008) because it is much more difficult to set up experiments with large perennial plants, such as avocado, due to the costs and space involved. Avocado plants are also relatively slow growing and this study was time limited to one year of research.

Phosphite affects the production of sporangia in a range of *Phytophthora* species and generally a lower phosphite concentration is required to reduce sporangia production than necessary to reduce mycelial growth *in vitro* (Coffey & Joseph, 1985; Dolan & Coffey, 1988; Garbelotto *et al.*, 2009; Greenhalgh *et al.*, 1994; Guest & Grant, 1991). The sporangia production response to phosphite can vary greatly among isolates (Garbelotto *et al.*, 2009). Phosphite may be effective at lowering the risk of sporulation but not eliminating it. For example, Wilkinson *et al.* (2001a) found phosphite reduced, but did not prevent, the production of viable zoospores of a known phosphite tolerant isolate (MP94-17) from infected Bull Banksia (*Banksia grandis*) and Jarrah (*Eucalyptus marginata*) seedlings. However the EC50 of this isolate was 9 µg/mL phosphite, lower than the most sensitive isolate in Chapter 3, and it was inhibited 79% on 50 µg/mL phosphite relative to the control (Wilkinson *et al.*, 2001b). The tolerant isolates identified in Chapter 3 which were inhibited between 20 – 28 and 43 – 44 % by 40 and 80 µg/mL phosphite, respectively, and had much higher EC50s (Table 4.1).

The objective of this study was to test the hypothesis that *P. cinnamomi* isolates which have increased resistance to phosphite *in vitro* will also have increased resistance *in planta* relative to phosphite sensitive isolates. This study also aimed to assess the ability of tolerant and sensitive *P. cinnamomi* isolates to produce sporangia and release viable zoospores in the presence of phosphite.

## 4.3 Methods

This study examined the impact of phosphite on infection potential *in planta* and the ability to produce sporangia of known *in vitro* tolerant and sensitive isolates of *Phytophthora cinnamomi* (Table 4.1)

**Table 4.1** Details of the three phosphite tolerant and three sensitive *Phytophthora cinnamomi* isolates used in this study and their phosphite exposure history (Chapter 3).

| NZFS | Phosphite resistance | EC50 (µg/mL phosphite) | Sample population | Previous phosphite exposure |
|---|---|---|---|---|
| 4424 | Sensitive | 13.72 | 2017 | No |
| 4458 | Sensitive | 22.22 | 2017 | No |
| 4357 | Sensitive | 28.01 | 2016 | No |
| 4342 | Tolerant | 108.44 | 2016 | Yes |
| 4411 | Tolerant | 115.76 | 2017 | Yes |
| 4359 | Tolerant | 124.30 | 2016 | Yes |

The isolates collection in 2016 are from unhealthy trees and those collected in 2017 are from randomly selected trees (Chapter 3).

### 4.3.1 Lupin root inoculation

Lupin seeds (*Lupinus angustifolius*) were germinated on damp paper towels in the dark at 20˚C. Once the roots were 1 cm long, they were transferred into modified seedling pouches (‘cyg', mega International, St. Paul, MN, USA). Each pouch comprised of an inert transparent pouch and wicking media, within a modified growth system (Dierking & Bilyeu, 2008; Ryba-White *et al.*, 2001). Seedling pouches were modified by joining two pouches together to increase the length and cutting 5-mm drainage holes at the base of the bottom pouch, to allow free drainage. After 14 days, the plants were inoculated with an agar plug from the growing margin of 5 day old plates which was put under the root tip through a small hole made in the bag. The location of the root tip at the time of inoculation was marked on the bag in permanent marker. Thirty lupins were inoculated with each of the six *P. cinnamomi* isolates. After 24 hours, lupins were sprayed to run

off with 0, 5, and 10 g/L phosphite solution so there were ten plants inoculated with each isolate per treatment and left to air dry at a 45˚ angle with the plant downwards for 3 hours. The plants were kept at 20˚C out of direct sunlight with a 12h light/12 h dark cycle and watered to run off daily. On day 10, the lupins were harvested – preliminary trials showed root colonisation was could be differentiated between isolates on this day. Lesion length was measured before the roots were surface sterilised by dipping them in 70% ethanol and blotting dry. Three 1 cm sections from above and three below the upper lesion margin were cut and placed onto CRNH medium (Appendix A) to determine if *P. cinnamomi* had colonised the root section. The experimental design included 10 replicates per treatment randomised in a split plot design.

### 4.3.2 Inhibition of sporangia formation

The efficacy of phosphite to inhibit the formation of sporangia was tested for the 6 *P. cinnamomi* isolates. Carrot (CAD) agar (Appendix A) was inoculated with the 6 isolates and 5 days later a 5 mm plug was taken from the actively growing margin and placed into the well of 24 well plate (Corning, New York, United States) so that the upper surface of the plug was on its side (perpendicular to the bottom of the well). Five treatments were tested, including 0, 15, 30, 60 and 120 µg/ml phosphite. Plugs were submerged in 1.2 mL of pond water from the Scion (filtered with two layers of paper towels to remove large debris) amended with phosphite to obtain the appropriate phosphite concentration. Pond water was collected from the Scion pond (latitude: -38.161744, longitude: 176.262842) two weeks prior to use and was stored at 4˚C in the dark. Plates were incubated at 20˚C in the light. After 24 hours, the water was changed. Mature, full sporangia produced on the upper surface of the inoculum plug were counted at 4x magnification 48 hours after inoculation (Figure 4.1). The experimental design included six replicates per treatment randomised in a split plot design.

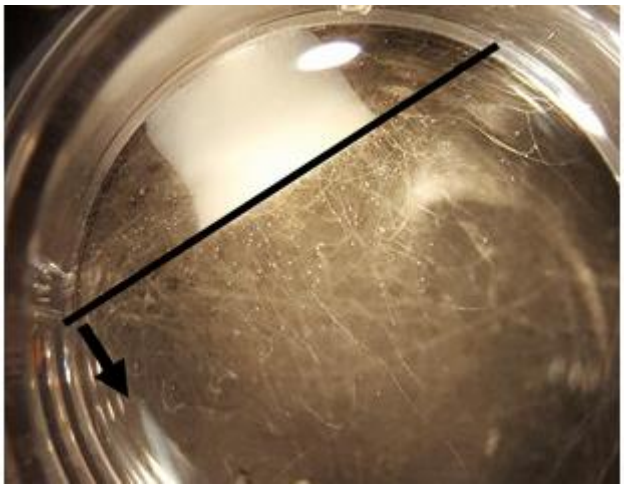

**Figure 4.1** Photo of a well in a 24 well plate containing a 5 mm inoculum plug of CAD on its side with mature sporangia produced after 48 hours. Sporangia on the side of the line with the arrow were counted.

### 4.3.3 Zoospore observation

For each replicate well a record was taken of whether viable (swimming) zoospores were present or absent.

### 4.3.4 Data analysis

Data analysis was done using the program R version 3.2.4 (R Core Team., 2016). Data were examined for homogeneity of variance using Levene's test (Levene, 1960) and normality using the Shapiro-Wilk test (Shapiro & Wilk, 1965). Frequent observation of zero growth skewed that data and meant non-parametric tests were more appropriate. Differences among groups with unequal variances and/or without a normal distribution were examined with the Kruskal-Wallis non-parametric test (Kruskal & Wallis, 1952) followed by pairwise comparisons with Dunn's test (Dunn, 1964) when the difference between lesion lengths were significant. Median lesion lengths were presented because the median is less influenced by outliers than the mean.

## 4.4 Results

### 4.4.1 Lupin root inoculation

Phosphite was effective at controlling lesion development for the isolates with no phosphite history as the lesions were smaller on phosphite treated roots than untreated roots (Figure 4.2). Phosphite was not very effective in controlling lesion development by the tolerant isolates as the lesions lengths were not very different on the phosphite treated lupins to those produced on the control (Figure 4.2).

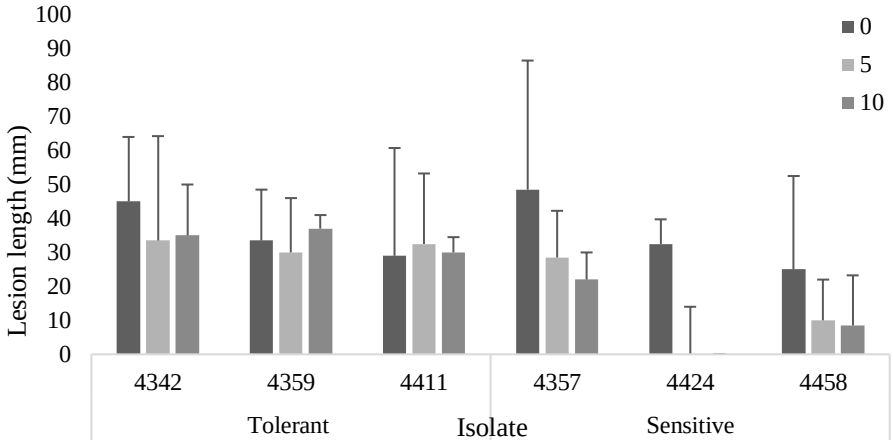


**Figure 4.2** Median lesion length (mm) formed on lupin roots for the three tolerant and sensitive *Phytophthora cinnamomi* isolates across three phosphite treatments (0, 5 and 10 g/L). Error bars are interquartile ranges.

The phosphite treatment had a significant effect on the length of lesions produced, as the distribution of the phosphite treatment groups were dissimilar from one another (Kruskal-Wallis test p-value<0.0001). Overall, there was a significant difference between the lengths of lesions produced on the control and 5 g/L treatment (Dunn's Test p= 0.0002) and the control and 10 g/L treatment (p<0.0001). There was no significant difference in the lesion lengths produced on lupins treated with 5 and 10 g/L phosphite (p= 0.4). Tolerant isolates produced longer lesions compared to those without a phosphite history (Figure 4.2 and Table 4.1) (Kruskal-Wallis test p-value<0.0001).

All isolates were able to infect the lupin roots above the lesion margins across the three phosphite treatments (Figure 4.3).

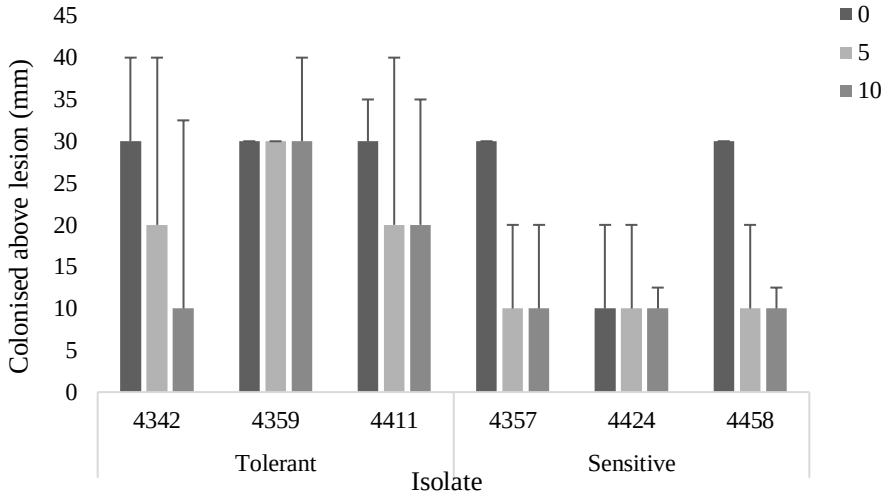


**Figure 4.3** Median distance colonised by the three tolerant and three sensitive isolates of *Phytophthora cinnamomi* above the lesion margins on lupin roots treated with phosphite (0, 5 and 10 g/L). Error bars are interquartile ranges.

The distance above the lesion margin that was colonised by mycelium, as determined by plating three 10 mm fragments of the root, was significantly affected by the phosphite concentration as the distribution of lesion length in the phosphite treatment groups were dissimilar from on another (Kruskal-Wallis test p-value<0.001). Colonisation above the lesion margin was also significantly affected by the previously determined phosphite sensitivity as the distribution of lesion length in the tolerance groups were dissimilar from one another (Kruskal-Wallis test p-value=0.0005). There was a significant difference in the average distance colonised above the lesion margin for all isolates between the control treatment and two phosphite treatments 5 g/L and 10 g/L (Dunn's Test p=0.002 and p=0.0003, respectively). There was no significant difference in the distance colonised above the lesion on lupins treated with 5 and 10 g/L phosphite (p= 0.5).

### 4.4.2 Inhibition of sporangia formation

Tolerant isolates produced more sporangia on in the presence of phosphite compared to the sensitive isolates (Figure 4.4) (Kruskal-Wallis test p-value<0.0001).

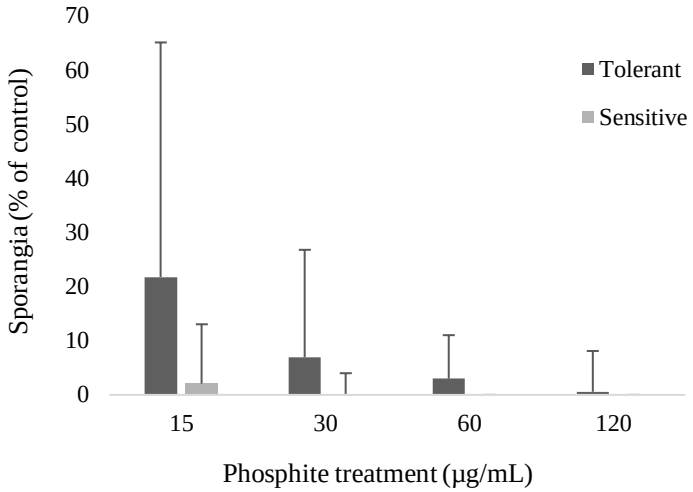


**Figure 4.4** Median number of sporangia of *Phytophthora cinnamomi* produced by the tolerant and sensitive isolates in filtered pond water amended with different amounts of phosphonic acid (Agrifos600®,Agrichem, Yatala QLD, Australia), expressed as percentage of sporangia produced on unamended media. Numbers shown are the median values from six replicates of three isolates for the tolerant and sensitive groups. Error bars are interquartile ranges.

The phosphite treatment had a significant effect on the number of sporangia produced, as the distributions between the treatment groups were dissimilar to one another (Kruskal-Wallis test p-value<0.0001).

### 4.4.3 Zoospore observations

All six replicates of the control treatment for every isolate had swimming zoospores at 48 hours (Table 4.3).

**Table 4.2** Number of replicate wells (n= 6) of the three tolerant and sensitive *Phytophthora cinnamomi* isolates that had viable zoospores at 48 hours in filtered pond water amended with 0, 15, 30, 60 and 120 µg/mL.

| Phosphite | Tolerant | | | Sensitive | | |
|---|---|---|---|---|---|---|
| (µg/mL) | 4342 | 4411 | 4359 | 4357 | 4424 | 4458 |
| **0** | 6 | 6 | 6 | 6 | 6 | 6 |
| **15** | 6 | 4 | 6 | 4 | 3 | 2 |
| **30** | 6 | 2 | 6 | 2 | 2 | 1 |
| **60** | 6 | 0 | 4 | 2 | 0 | 0 |
| **120** | 3 | 0 | 3 | 0 | 0 | 0 |

The zoospore release data was analysed using a logistic Generalised linear model (GLM) as it is binomial data. The proportion of zoospores produced in the presence of phosphite was higher for the tolerant isolates compared to the sensitive isolates ($p<0.0001$). It was estimated that the odds of the tolerant isolates producing zoospores in the presence of phosphite was between 4.24 to 26.77 times the odds of the sensitive isolates producing zoospores in the presence of phosphite.

## 4.5 Discussion

The results indicate that isolates identified as having decreased sensitivity to phosphite *in vitro* (Chapter 3) also have decreased sensitivity *in planta* (Figure 4.2). The tolerant isolates with a prolonged exposure to phosphite were more extensive colonisers of the lupin roots than the isolates with no phosphite history, as they produced longer lesions (Figure 4.2) and they were able to colonise further above the lesion margin asymptomatically than the sensitive isolates (Figure 4.3). This level of tolerance also extended to sporulation, with the tolerant isolates producing more sporangia and viable oospores in the presence of phosphite (Figure 4.4 and Table 4.2).

Dobrowolski *et al.* (2008) also found isolates of *P. cinnamomi* with a prolonged exposure to phosphite were able to colonise a greater length of lupin root compared to isolates without previous exposure to phosphite. The same trend was evident in *Leucadendron* 'Safari Sunset' plants inoculated with a mycelial plug

placed on the stem of the plant (Dobrowolski *et al.*, 2008). These results show that the host–pathogen interaction was not restricted to the leucadendron experiment and lupin seedlings are a good model species to study *P. cinnamomi.*

In the current study, the lupins did not die in the 10 days after inoculation. This may be attributed to the indirect effect of phosphite on the plant host defence response. However, it is possible mortality may have occurred with more time as the roots were substantially colonised. When the roots were cut an obvious lesion was apparent in the central xylem and phloem channels of the roots when the lesion was not yet visible on the surface of the root. This asymptomatic infection above the lesion margin was assessed by plating three 10 mm root fragments from above the upper lesion margin. While the tolerant isolates colonised further above the lesion margins than the sensitive isolates in lupin roots (Figure 4.3), more work needs to be done to understand if this occurs regularly and in avocado trees.

The higher concentration of phosphite, 10 g/L was more effective at inhibiting colonisation of the lupins by the sensitive isolates compared to the control and the 5 g/L treatment (Figure 4.2). Phosphite was not very effective in controlling lesion development by the tolerant isolates as the lesions lengths were not very different on the phosphite treated lupins to those produced on the control (Figure 4.2). There appears to be no advantage in applying the higher concentration of 10 g/L compared to 5 g/L to reduce the growth of the tolerant isolates as the lesions lengths were not reduced further by 10 g/L (Figure 4.2). When phosphite is applied as a foliar spray to avocado trees it is recommend to be applied at a rate of 5 g/L based on the Agrifos® 600 (Agrichem, Yatala QLD, Australia) label. The results from the lupin assay suggests that if only foliar sprays are used this concentration may not be effective at controlling colonisation by tolerant isolates. Phosphite injections as the primary method of application in orchards may be more effective at developing the required concentration of phosphite in the roots. Further work using woody plant material is needed to investigate the pattern of movement and final concentration of phosphite in host tissues; the current study was limited by the use of 14-day old lupin seedlings in a contained environment.

This study showed for the first time that isolates with known tolerance to phosphite based on mycelial growth *in vitro* and *in planta* also expressed this decreased sensitivity to phosphite in the production of sporangia. The tolerant

isolates in the current study were able to produce sporangia on all the phosphite treatments and produced 22% of the number of sporangia produced in the control at 15 µg/mL phosphite (Figure 4.4). In contrast sporangia production by the sensitive isolates was inhibited by 98% in 15 µg/mL phosphite (Figure 4.4). The inhibition of sporangia production by the sensitive isolates was similar to *P. cinnamomi* isolates from avocado orchards isolated prior to 1985 and never exposed to phosphite, they were inhibited by approximately 90% by 5 µg/mL (Coffey & Joseph, 1985). In comparison, the average sporangia production of 11 *P. ramorum* isolates in the presence of 337 µg/mL phosphite was 16.5% of the amount produced in the control (Garbelotto *et al.*, 2009). However, *P. ramorum* produces abundant sporangia in laboratory assays and they are generally less sensitive to phosphite than *P. cinnamomi* in mycelial growth assays (Garbelotto *et al.*, 2009; Wilkinson *et al.*, 2001b). No studies have quantified sporangia produced by tolerant isolates of *P. cinnamomi* in the presence of phosphite however, the results from this study suggest isolates with known tolerance in mycelial growth tests *in vitro* and *in planta* will express this decreased sensitivity to phosphite in sporangia production and produce more sporangia than sensitive isolates in the presence of phosphite.

The production of *P. cinnamomi* zoospores in response to phosphite has been assessed using isolates of unknown tolerance from avocado (Coffey & Joseph, 1985) and an isolate from jarrah (*Eucalyptus marginata*) known to be relatively tolerant to phosphite (Wilkinson *et al.*, 2001a). The 'tolerant' isolate from Wilkinson *et al.* (2001b) is not very tolerant in comparison to the tolerant isolates in the current study. The isolate used by Wilkinson *et al.* (2001a) had an EC50 of 9 µg/mL and was inhibited 79% by 50 µg/mL phosphite relative to growth in unamended RMM agar, in comparison the tolerant isolates in the current study were inhibited 20 – 28 and 43 – 44% by 40 and 80 µg/mL phosphite, respectively, and had much higher EC50s (Table 4.1) (Chapter 3). The 'tolerant' isolate from Wilkinson *et al.* (2001a) produced 0.04 and 0.09 zoospores/mL on 5 and 10 g/L phosphite treated jarrah plants, respectively, and 1.75 zoospores/mL on untreated plants. A 39 % and 59 % reduction in zoospore release was observed at 2 and 6 µg/mL phosphite, respectively, for the isolates with unknown tolerance Coffey and Joseph (1985). While the level of zoospore release was not quantified in the

current study, two tolerant isolates, 4342 and 4359, were recorded as having motile zoospores in three of the six replicate wells at 120 µg/mL while none of the sensitive isolates produced viable zoospores at this concentration (Table 4.2). It is likely that the tolerant isolates from the current study are able to release zoospores at higher phosphite concentrations than both the sensitive isolates in this study and the isolates tested by Coffey and Joseph (1985). Phosphite appears to reduce the production of viable zoospores for tolerant and susceptible isolates but does not prevent the production of zoospores by tolerant isolates even at high concentrations (e.g. 120 µg/mL phosphite) (Table 4.2).

Zoospores are only viable is they are able to swim to host material and then germinate and infect it, only the first stage was assessed in the current study. The zoospores produced in the presence of phosphite by the 'tolerant' *P. cinnamomi* isolate from (Wilkinson *et al.*, 2001a) were able to infect untreated *Pimelea ferruginea* leaves though significantly less baits were infected by zoospores from phosphite treated plants compared to control plants (Wilkinson *et al.*, 2001a). Phosphite is used as a preventative treatment in avocado orchards so it is of interest to know if phosphite reduces the germination of zoospores which would lower the chances of infection. While this has not been assessed in *P. cinnamomi*, zoospores of *P. ramorum* were able to germinate on full strength corn meal agar amended with 45, 67.5, 108 and 135 µg/mL phosphite (Garbelotto *et al.*, 2009). Three isolates were tested for which 5.4, 16.3 and 16.4 % zoospores germinated on the 135 µg/mL phosphite treatment relative to the control. Zoospores are a primary infection structure in *Phytophthora* species and phosphite is able to reduce the production (Table 4.2) (Coffey & Joseph, 1985; Wilkinson *et al.*, 2001a) and germination (Garbelotto *et al.*, 2009) of zoospores. However, this study has shown it is less effective at preventing the production of zoospores by isolates of known tolerance. Further work is necessary to understand if zoospores of tolerant *P. cinnamomi* isolates are able to germinate in the presence of phosphite and infect phosphite treated plant material more effectively than phosphite sensitive isolates.

Another stage important in the life cycle of *P. cinnamomi* is the production of resting spores, including chlamydospores and oospores, which are a key inoculum source that is vectored in soil. The production of chlamydospores and oospores by

the phosphite tolerant and sensitive isolates was not assessed in the current study. The production of chlamydospores by the *P. cinnamomi* isolates of unknown tolerance from Coffey and Joseph (1985) were reduced to 50% with 15 – 44 µg/mL phosphite and germination of chlamydospores produced in the absence of phosphite was unaffected by 0, 1, 10 and 50 µg/mL. In contrast, phosphite simulated the production of chlamydospores in tolerant and sensitive isolates of *P. cinnamomi* and induced dormancy in sensitive isolates (McCarren *et al.*, 2009a). Dormancy is a survival strategy which allows the pathogen to survive unfavourable conditions, such as high concentrations of phosphite. If phosphite stimulates the production of chlamydospores and induces dormancy, this has implications for disease management as inoculum load may build up for future disease outbreaks.

Oospores are sexual spores which offer the opportunity for isolates to evolve and desirable traits, such as phosphite resistance, to be selected for. The production of heterothallic oospores (crossing of A1 and A2 strains) of *P. cinnamomi* isolates from avocado orchards with unknown phosphite tolerance were inhibited by 60 – 78 % at 1 µg/mL phosphite (Coffey & Joseph, 1985). In contrast, homothallic/ selfed oospores produced by phosphite tolerant and sensitive *P. cinnamomi* isolates of the A2 mating strain were only inhibited by high phosphite concentrations (100 µg/mL phosphite) and phosphite did not impact the viability of the oospores produced (McCarren *et al.*, 2009b). Phosphite is applied in late spring to facilitate the accumulation of phosphite in fine feeder roots as it is a strong sink in the tree at this time (Dann *et al.*, 2013). Phosphite accumulates in plant tissue at the sink locations because it is phloem translocated and not metabolised by plants (Guest & Grant, 1991; McDonald *et al.*, 2001). If *P. cinnamomi* infects and spreads successfully through the fine feeder roots of phosphite treated avocado trees, and produces selfed oospores, this allows for the selection of phosphite tolerant isolates which can threaten the continued effectiveness of phosphite to manage avocado root rot.

## 4.6 Conclusion

Isolates identified in Chapter 3 as tolerant to phosphite *in vitro* were able to colonised phosphite treated plant material more extensively than sensitive isolates, including asymptomatic infection above the lesion margin. This decreased sensitivity was expressed in sporangia production and consequent zoospore release. The expression of phosphite tolerance across several life stages is concerning for the continued effectiveness of phosphite to manage phytophthora diseases. The key question is whether the observed incremental decrease in sensitivity will eventually lead to the appearance of isolates that are fully resistant to phosphite.

# Chapter 5

# Phosphite sensitivity of *Phytophthora* species from New Zealand and the United States

## 5.1 Abstract

*Phytophthora* species vary in their tolerance to chemical fungicides and often intraspecific differences are observed in isolates with and without previous exposure to the fungicide. The *Phytophthora* species tested from New Zealand and Berkeley, USA, culture collections were characteristic in their sensitivities to phosphite. The EC50 values of the isolates tested in New Zealand ranged from 0.00 – 2900 µg/mL phosphite. *Phytophthora agathidicida* was extremely sensitive to phosphite while *P. kernoviae* was relatively tolerant. The EC50 values from the Berkeley isolates ranged from 0.0 to 546.3 µg/mL phosphite. The *P. ramorum* NA2 and EU1 lineages appeared to be more tolerant to phosphite than the NA1 lineage while *P. nemorosa* was highly sensitive to phosphite Non-uniform growth was observed on the 10% V8 agar plates used for the mycelial growth inhibition testing at Berkeley. In some instances, the diameter of the colony was larger on phosphite amended media than the controls. It is likely this is a survival response in the *Phytophthora* whereby it grows more sparsely in response to phosphite. This may be an effective strategy in phosphite treated plants where phosphite accumulates in high concentrations. One isolate of *P. megasperma* was found to be less sensitive to mefenoxam than the other isolates tested however it was not tolerant compared to previous studies. One isolate of *P. cinnamomi* was more tolerant to phosphite than the other eight isolates tested from the Riverside collection. This study has shown that both intraspecific and interspecific variation in tolerance to fungicides exist in important *Phytophthora* species from New Zealand and the United States.

## 5.2 Introduction

Fungicide resistance in *Phytophthora* species is a great concern for nursery, horticulture and plant production industries. Resistance develops after prolonged exposure to the fungicide. Increased tolerance to some fungicides has been shown, for example *P. cinnamomi* isolates from avocado orchards had increased tolerance

to phosphite (Duvenhage, 1994; Ma & McLeod, 2014) and *P. erythroseptica* isolates from potato tubers developed tolerance to Mefenoxam (Venkataramana *et al.*, 2010).

Phosphite is used to control *Phytophthora* diseases in nurseries, orchards and planted and native forest systems. The use of phosphite in nurseries raises the risk of creating phosphite resistant *Phytophthora* species and then releasing them into the wider environment when the nursery plants are sold. Of more of a concern is the use of phosphite to manage *Phytophthora* diseases in the field over a prolonged period of time may lead to the selection of phosphite resistant *Phytophthora* isolates *in-situ*. Fungicide sensitivity is difficult to compare across studies, experimental conditions vary including phosphate conditions and lower phosphate concentrations exaggerate phosphite inhibition. Sensitivity is compared between isolates using EC50 values (the amount of fungicide needed to inhibit mycelial growth by 50%). Most phosphite sensitivity studies with *Phytophthora* to date have focussed on a one or two *Phytophthora* species of interest (Coffey & Joseph, 1985; Duvenhage, 1994; Garbelotto *et al.*, 2009; Horner & Hough, 2013; Ma & McLeod, 2014; Wilkinson *et al.*, 2001b). Coffey and Bower (1984a) tested the sensitivity of eight *Phytophthora* species to phosphite and found they could differentiate phosphite sensitivity at the species level, for example all isolates of *P. citricola*, *P. citrophthora* and *P. cinnamomi* were extremely sensitive to phosphite.

Mefenoxam sensitivity was also of interest to study for a particular subset of isolates from restoration sites and nurseries in California, USA, because it is used in the nursery from which the restoration plants were sourced. Mefenoxam is an isomer of Metalaxyl, both are systemic phenylamide fungicides which selectively inhibit oomycetes ribosomal RNA synthesis by affecting the activity of their RNA polymerases (Cohen & Coffey, 1986). Phenylamide fungicides have strong inhibitory activities against mycelial growth and sporulation however due to the site specific mode of action, resistance to metalaxyl (Dowley *et al.*, 2002; Timmer *et al.*, 1998; van Jaarsveld *et al.*, 2002) and mefenoxam (Hu & Li, 2014; Hu *et al.*, 2008; Hwang & Benson, 2005; Lamour & Hausbeck, 2000; Parra & Ristaino, 2001; Venkataramana *et al.*, 2010) has been detected in several *Phytophthora* species.

The aim of this study was to conduct sensitivity assays for *Phytophthora* species in New Zealand and California under the same conditions so that sensitivity could be compared at both the species and isolate levels. Comparisons of sensitivity were made only within experimental groups which were tested using the same method at the same time.

## 5.3 Methods

This study examined the phosphite sensitivity of nine *Phytophthora cinnamomi* isolates from the University of California Riverside collection, seven *Phytophthora* species from the Scion culture collection in New Zealand, and eleven species from the University of California, Berkeley collection (Table 5.1). Sensitivity to phosphite was tested across five concentrations including 15, 40, 80, 200 and 500 µg/mL and a control treatment. An additional test for mefenoxam sensitivity across six concentrations 0, 0.1, 1, 5, 10 and 100 µg/mL, was conducted for five species from the Berkeley collection (Section 5.3.3.2).

**Table 5.1** *Phytophthora* isolates used in this study. No information was available for the blank cells.

| Culture collection | Species | Isolate | Host | Substrate | Location | Location type | Date collected | Clade/ Lineage |
|---|---|---|---|---|---|---|---|---|
| Scion, New Zealand | *Phytophthora multivora* | 3871 | *Agathis australis* | Soil | | | 9/05/2014 | |
| | | 3913 | *A. australis* | Soil | | | 9/05/2014 | |
| | | 3866 | *A. australis* | Soil | | | 9/05/2014 | |
| | *P. kernoviae* | 3680 | *Pinus radiata* | Needles | Bay of plenty | | 17/05/2011 | |
| | | 3610 | *P. radiata* | Needles | Auckland | | 22/06/2011 | |
| | | 4053 | *P. radiata* | Pine needles used at bait station | Bay of Plenty | | 9/10/2014 | |
| | | 4470 | *Persea americana* | Soil and roots | Coromandel | Avocado orchard | 15/02/2017 | |
| | *P. citricola* | 4460 | *P. americana* | Soil and roots | Bay of plenty | Avocado orchard | 15/02/2017 | |
| | | 4461 | *P. americana* | Soil and roots | Bay of plenty | Avocado orchard | 15/02/2017 | |
| | | 4462 | *P. americana* | Soil and roots | Bay of plenty | Avocado orchard | 15/02/2017 | |
| | *P. cactorum* | 4040 | *P. radiata* | Branch | Nelson | | 14/08/2014 | |
| | | 4071 | *P. radiata* | Root Collar | Bay of plenty | Nursery | 29/09/2014 | |
| | | 4037 | *P. radiata* | Root Collar | Nelson | | 14/08/2014 | |
| | *P. pluvialis* | 4234 | *P. radiata* | Needle | Marlborough | | 12/08/2015 | |
| | | 4019 | *P. radiata* | Pine needles used at bait station | Rangitikei | | 25/08/2014 | |

| Culture collection | Species | Isolate | Host | Substrate | Location | Location type | Date collected | Clade/ Lineage |
|---|---|---|---|---|---|---|---|---|
| Scion, New Zealand | *P. agathidicida* | 3118 | *A. australis* | Soil | Auckland | | 11/03/2009 | |
| | | 3815 | *A. australis* | Rhizosphere soil | Coromandel | | 30/01/2014 | |
| | | 3813 | *A. australis* | Rhizosphere soil | Coromandel | | 30/01/2014 | |
| | *P. cinnamomi* | 3784 | *P. radiata* | Soil and roots | Marlborough | | 19/01/2013 | |
| | | 3750 | *P. radiata* | Soil and roots | Marlborough | | 9/01/2013 | |
| | | 3034 | *P. radiata* | Pinus radiata cuttings | Bay of plenty | | 26/06/2008 | |
| Riverside, California, USA | *P. cinnamomi* | R_1 | *P. americana* | Soil and roots | California | Avocado orchard | | |
| | | S2W_1 | *P. americana* | Soil and roots | California | Avocado orchard | | |
| | | W38_1 | *P. americana* | Soil and roots | California | Avocado orchard | | |
| | | 2109 | *P. americana* | Soil and roots | California | Avocado orchard | 2013 | A2 |
| | | 2110 | *P. americana* | Soil and roots | California | Avocado orchard | 2013 | A2 |
| | | 2113 | *P. americana* | Soil and roots | California | Avocado orchard | 2013 | A2 |
| | | 2114 | *P. americana* | Soil and roots | California | Avocado orchard | 2013 | A2 |

| Culture collection | Species | Isolate | Host | Substrate | Location | Location type | Date collected | Clade/ Lineage |
|---|---|---|---|---|---|---|---|---|
| Riverside, California, USA | *P. cinnamomi* | 2117 | *P. americana* | Soil and roots | California | Avocado orchard | 2013 | A2 |
| | | 2120 | *P. americana* | Soil and roots | California | Avocado orchard | 2013 | A2 |
| Berkeley, California, USA | *P. cryptogea* | MBP-B-DIAU1.1 | *Diplacus aurantiacus* | root bait | Marin, California | Restoration site | | |
| | | CCWN-295B-DIAU5.1 | *D. aurantiacus* | root bait | Santa Cruz, California | Nursery | | |
| | | MBP-B-DIAU4.1 | *D. aurantiacus* | root bait | Marin, California | Restoration site | | |
| | | CCW-DP-DIAU-ROOTS.1 | *D. aurantiacus* | root isolation | Santa Cruz, California | Nursery | | |
| | | NPC-79B-MIAU.1 | *D. aurantiacus* | root bait | San Francisco, California | Restoration site | | |
| | | ENPN122-DP-DIAU12.1 | *D. aurantiacus* | root isolation | Santa Cruz, California | Nursery | | |
| | | ENPN123-DP-DIAU13.2 | *D. aurantiacus* | root isolation | Santa Cruz, California | Nursery | | |

| Culture collection | Species | Isolate | Host | Substrate | Location | Location type | Date collected | Clade/ Lineage |
|---|---|---|---|---|---|---|---|---|
| Berkeley, California, USA | *P. cryptogea* | ENPN80-B-DIAU10.2 | *D. aurantiacus* | root bait | Santa Cruz, California | Nursery | | |
| | *P. multivora* | FOFU-C2-CETH.1 | *Ceanothus thrysifloru* | root bait | San Francisco, California | Nursery | | |
| | | NPC-47B-CETH.1 | *C. thrysiflorus* | root bait | San Francisco, California | Restoration site | | |
| | | MA-33B-FRCA.1 | *Frangula californica* | root bait | Marin, California | Restoration site | | |
| | | MA-60B-FRCA.1 | *F. californica* | root bait | Marin, California | Restoration site | | |
| | *P. crassamura* | PLRA-SFPUC.1 | *Platanus racemosa* | bark canker | Alameda, California | Restoration site | | |
| | | PLRA-DRYSOIL1A.1 | *P. racemosa* | root bait | Alameda, California | Restoration site | | |
| | | TEVA-59B-ALRU.1 | *Alnus rubra* | root bait | Marin, California | Nursery | | |
| | | TEVA-326B-JUEF.1 | *Juncus effusus* | root bait | Marin, California | Nursery | | |

| Culture collection | Species | Isolate | Host | Substrate | Location | Location type | Date collected | Clade/ Lineage |
|---|---|---|---|---|---|---|---|---|
| Berkeley, California, USA | *P. crassamura* | SM-45B-FRCA.1 | *F. californica* | root bait | San Mateo, California | Restoration site | | |
| | | SM-39B-FRCA.1 | *F. californica* | root bait | San Mateo, California | Restoration site | | |
| | | FOR-OUT-06B.1 | | root bait | San Francisco, California | Nursery | | |
| | | MA-85B-SOIL.1 | | root bait | Marin, California | trail stock area | | |
| | *P. megasperma* | MBP-DIAU10-DPSTEM.1 | *D. aurantiacus* | bark canker | Marin, California | Restoration site | | |
| | | MBP-DIAU5-DPSTEM.1 | *D. aurantiacus* | bark canker | Marin, California | Restoration site | | |
| | | MBP-DIAU4-DPSTEM.1 | *D. aurantiacus* | bark canker | Marin, California | Restoration site | | |
| | | MBP-B-DIAU10.1 | *D. aurantiacus* | root bait | Marin, California | Restoration site | | |
| | *P. cactorum* | 7-HR.1 | *Heteromeles arbutifolia* | root bait | Orange, California | Nursery | | |

| Culture collection | Species | Isolate | Host | Substrate | Location | Location type | Date collected | Clade/ Lineage |
|---|---|---|---|---|---|---|---|---|
| Berkeley, California, USA | *P. cactorum* | 7-HP.1 | *H. arbutifolia* | root bait | Orange, California | Nursery | | |
| | | PNPN-C-39FRCA.1 | *F. californica* | root bait | San Francisco, California | Nursery | | |
| | | 10-SP.1 | *Salix lasiolepis* | root bait | Orange, California | Nursery | | |
| | | AKWA 7912.1 | | soil or canopy drip | Alaska | | | |
| | | 117R | *Alnus rubra* | root isolation | Lincoln, Oregon | Riparian zone | | |
| | | MP19 | *Pseudotsuga menziesii* | | Oregon | | | |
| | *P. cambivora* | MP21 | Almond | | Chico, California | | 1980 | |
| | | MP28 | Apple | | Ulster, New York | | 1983 | A1 |
| | | L.170.B.HEAR | | | | | | |
| | | FOR.61B.HEAR | | | | | | |
| | | NPL.22B.HEAR | | | | | | |

| Culture collection | Species | Isolate | Host | Substrate | Location | Location type | Date collected | Clade/ Lineage |
|---|---|---|---|---|---|---|---|---|
| Berkeley, California, USA | *P. cinnamomi* | MC11 | *Abies concolor* | | Eldorado, California | Nursery | | A2 |
| | | P.2021 COFFEY | *Camelia japonica* | | California | | | A1 |
| | | P.3662 COFFEY | *Araucaria sp.* | | Papua New Guinea | | | A2 |
| | | P.6377 COFFEY | | soil | Taiwan | | | A2 |
| | | P.6493 COFFEY | *Rhodendron sp.* | | China | | | A1 |
| | *P. nemorosa* | P.106 | *Umbellularia californica* | | Marin, California | State park | | |
| | | P.113 | *U. californica* | | Marin, California | State park | | |
| | | P.114 | *U. californica* | | Marin, California | State park | | |
| | | P.115 | *U. californica* | | Marin, California | State park | | |
| | | 1050 Hansen1 | *Lithocarpus densiflora* | | Oregon | | 30/07/1998 | |
| | | 2052.2 Hansen2 | *L. densiflora* | | Oregon | | 9/10/1997 | |

| Culture collection | Species | Isolate | Host | Substrate | Location | Location type | Date collected | Clade/ Lineage |
|---|---|---|---|---|---|---|---|---|
| Berkeley, California, USA | *P. nemorosa* | 2059.4 Hansen6 | *L. densiflora* | | Oregon | | 17/10/1997 | |
| | | 5104 Hansen22 | *Myrtle* | Myrtle leaf | Oregon | | 9/12/1997 | |
| | *P. lateralis* | PL-9 | *Chamaecyparis lawsoniana* | | Del Norte, California | | 25/08/2004 | |
| | | PL-25 | *Taxus brevifolia* | | Del Norte, California | | 25/08/2004 | |
| | | Pl-28 | | | Del Norte, California | | 25/08/2004 | |
| | | PL-31 | | soil | Del Norte, California | | 25/08/2004 | |
| | | PL-34 | *Taxus brevifolia* | | Del Norte, California | | 25/08/2004 | |
| | | PL-47 | *T. brevifolia* | | Del Norte, California | | 25/08/2004 | |
| | | PL-54 | *T. brevifolia* | | Del Norte, California | | 25/08/2004 | |
| | *P. syringae* | MP-12 | *Rhododendron* sp | | Santa Cruz, California | Nursery, Scotts Valley | 1/04/2002 | |
| | | MP-15 | *Rhododendron* sp | | Santa Cruz, California | Nursery, Scotts Valley | 1/04/2002 | |
| | | KDA_RT9 | | | | | | |

| Culture collection | Species | Isolate | Host | Substrate | Location | Location type | Date collected | Clade/ Lineage |
|---|---|---|---|---|---|---|---|---|
| Berkeley, California, USA | *P. syringae* | SM15FEB_5CRP | | | | | | |
| | | SM15APR_B0V | | | | | | |
| | | SM15FEB_HOP | | | | | | |
| | | BSP2014_502 | | | | | | |
| | *P. ramorum* | SI-556 | | stream monitoring | | | 2012 | EU1 |
| | | SI-592 | | stream monitoring | | | 2012 | EU1 |
| | | SI-595 | | stream monitoring | | | 2012 | EU1 |
| | | MR-59 | *Rhododendron* 'Colonel Coen' | Isolated from asymptomatic plants | Sacramento, Colorado | Nursery | 2005 | NA2 |
| | | MR-64 | *Rhododendron* 'Colonel Coen' | Isolated from asymptomatic roots | Sacramento, Colorado | Nursery | 2005 | NA2 |
| | | MR-69 | *Rhododendron* 'Colonel Coen' | Isolated from asymptomatic plants | Sacramento, Colorado | Nursery | 2005 | NA2 |

| Culture collection | Species | Isolate | Host | Substrate | Location | Location type | Date collected | Clade/ Lineage |
|---|---|---|---|---|---|---|---|---|
| Berkeley, California, USA | *P. ramorum* | MR-88 | *Rhododendron* 'Colonel Coen' | Isolated from asymptomatic plants | Sacramento, Colorado | Nursery | 2005 | NA2 |
| | | MR-126 | *U. californica* | | Marin, California | Angel island | 2005 | NA1 |
| | | MR-180 | *U. californica* | | Marin, California | State park | 2005 | NA1 |
| | | MR-268 | | | Humboldt, California | | 2005 | NA1 |
| | | MR-270 | | | Humboldt, California | | 2005 | NA1 |
| | | MR-187 | *U. californica* | | Monterey, California | Big Sur/Deetjen's Inn | 2005 | NA1 |
| | | MR-196 | *U. californica* | | Monterey, California | Big Sur/Deetjen's Inn | 2005 | NA1 |
| | | 1461 | *U. californica* | | San Mateo, California | | 2005 | NA1 |

### 5.3.1 Riverside isolates

*Phytophthora* isolates from the University of California, Riverside culture collection were maintained in water vials at 22˚C on 10% V8 agar (Appendix A). Isolates were cultured onto 10% V8 agar.

#### *5.3.1.1 Phosphite amendment*

The phosphite used was Agri-Fos® Systemic Fungicide (Agrichem, Yatala QLD, Australia), a commercial fungicide containing 400 g/L phosphorous acid, present as mono- and di-potassium phosphonate. The phosphite was filtered using 0.22 µm pore filters (Millex®-GV, Millipore Corporation, Bedford, MA, USA) then added to autoclaved media after it had cooled to approximately 50˚C.

#### *5.3.1.2 Phosphite medium*

Growth experiments were conducted in 24 well microtiter plates (Corning, Kennebunk, ME, United States) containing 2 mL of 10% V8 broth amended with phosphite. The wells were randomised for inoculation in each replicate block. The inoculum was a 4 mm mycelial plug from the growing margin of a three day old colony in 10% V8 liquid. A media control well was included for each treatment on each plate. Measurements of Optical Density at a wavelength of 620nm ($OD_{620}$) were taken 4 days after inoculation using the Infinite® F200 PRO plate reader (Tecan, Männedorf, Switzerland), 36 measurements were take per well at consistent locations and the average $OD_{620}$ was used as the final value. Plates were stored at 22˚C in the dark.

### 5.3.2 New Zealand isolates

*Phytophthora* isolates from the New Zealand Forest Research Institute Culture Collection (NZFS) (Table 5.1) were maintained in water vials at 4˚C on carrot agar (Brasier *et al.*, 2003). Isolates were cultured onto a modified Ribeiro's Minimal Medium (RMM) (Ribeiro *et al.*, 1975), modified as outlined below. The glucose concentration was 9/0 g/L and β-sitosterol was omitted. MES hydrate buffer (2-(N-morpholino) ethanesulfonic acid) was added at a final concentration of 0.03 M and the pH adjusted to 6.2 with KOH 3M. The inoculum plates were stored at 20˚C in the dark.

*5.3.2.1 Phosphite amendment*

The phosphite used was Agri-Fos® 600 (Agrichem, Yatala QLD, Australia), a commercial potassium phosphate fungicide containing 600 g/L phosphorous acid, present as mono- and di-potassium phosphonate. The phosphite was filtered using 0.22 µm pore filters (Millex®-GV, Millipore Corporation, Bedford, MA, USA) then added to autoclaved media that had cooled to approximately 50˚C.

*5.3.2.2 Phosphite medium*

Growth experiments were conducted in 24 well microtiter plates (Corning, New York, United States) containing 2 mL of RMM amended with phosphite. The wells were randomised for inoculation in each replicate block. The inoculum was a 2 mm diameter mycelial plug from the growing margin of a five-day old colony in RMM broth. A media control well was included on each plate. $OD_{620}$ measurements were taken 13 days after inoculation using the Polar Star Galaxy Microplate Reader (BMG Lab Technologies, Offenburg, Germany), 32 measurements were take per well at consistent locations and the average $OD_{620}$ was used as the final value.

### 5.3.3 Berkeley isolates

*Phytophthora* isolates from the University of California, Berkeley culture collection were maintained in water vials at 4°C on 10% V8 agar. Isolates were cultured onto 10% V8 agar. There were 79 isolates screened for phosphite susceptibility and 32 of these isolates were also tested for mefenoxam sensitivity.

*5.3.3.1 Phosphite sensitivity*

*Phosphite amendment*

The phosphite used was Agri-Fos® Systemic Fungicide (Agrichem, Yatala QLD, Australia), a commercial fungicide containing 400 g/L phosphorous acid, present as mono- and di-potassium phosphonate. A stock solution containing 100 g/L phosphite was filter sterilised using 0.22 µm pore filters (VWR International, Radnor, Pennsylvania, USA) then added to autoclaved media when it had cooled to approximately 50˚C.

*Phosphite medium*

Growth experiments were conducted in 90 mm petri dishes (Thermo Fisher, San Francisco, California, United States) containing 20 mL of 10% V8 agar amended with phosphite. The inoculum was a 5 mm mycelial plug from the growing margin of a five day old colony on 10% V8. Two perpendicular measurements of the colony diameter were taken 5 days after inoculation, the diameter of the plug was subtracted to get the diameter of the mycelium. A second measurement of the diameter of the *P. lateralis* isolates was taken 7 days after inoculation because they were slow growing, the day 7 measurement was used for the analysis.

*5.3.3.2 Mefenoxam sensitivity*

*Mefenoxam amendment*

The Mefenoxam used was Subdue Maxx® (Syngenta, Basel, Switzerland), a commercial mefenoxam fungicide containing 220 g/L mefenoxam. A stock solution containing 10g/L mefenoxam was filter sterilised through 0.22 µm pore filters (VWR International, Radnor, Pennsylvania, USA) then added to autoclaved media when it had cooled to approximately 50˚C.

*Mefenoxam medium*

Growth experiments were conducted in 90 mm petri dishes (Thermo Fisher, San Francisco, California, United States) containing 20 mL of V8 10% agar amended with mefenoxam. The inoculum was a 5 mm mycelial plug from the growing margin of a five day old colony on 10% V8. Two perpendicular measurements of the colony diameter were taken 4 days after inoculation. Isolate MBP-B-DIAU10.1 overgrew on the control plate by day 4 so daily growth rates were calculated using the day three measurement for the control and the day four measurements for the phosphite treatments. The diameter of the plug was subtracted to get the diameter of the mycelium.

## 5.3.4 Data analysis

Percentage inhibition of each isolate at each of the phosphite concentrations was calculated as a percentage of the growth in the absence of phosphite. The EC50 was calculated by plotting percentage inhibition against $\log_{10}$ phosphite concentration and using the equation from a logarithmic trendline (Bower &

Coffey, 1985; Fenn & Coffey, 1984). The phosphite sensitivity data cannot be reliably compared between the three experimental groups of isolates (New Zealand, Riverside and Berkeley) because the methods and media differ.

Data analysis was done using the program R version 3.2.4 (R Core Team, 2016). Data were examined for homogeneity of variance using Levene's test (Levene, 1960) and normality using the Shapiro-Wilk test (Shapiro & Wilk, 1965). Differences among groups with unequal variances were examined with the Kruskal-Wallis non-parametric test (Kruskal & Wallis, 1952) followed by a post-hoc Dunn test (Dunn, 1964) when the difference between sensitivities of species were significant.

## 5.4 Results

The results are analysed by location (Riverside, New Zealand and Berkeley) because the experiments were conduct separately and the methods vary slightly.

### 5.4.1 Riverside isolates

The *P. cinnamomi* isolates from avocado orchards from the Riverside collection were tested for phosphite sensitivity in 10% V8 liquid media, a nutrient rich and high phosphate media, which resulted in high EC50 predictions ranging from 366.6 – 8515.8 phosphite (Table 5.2).

**Table 5.2** Predicted EC50 values for phosphite for the nine *Phytophthora cinnamomi* isolates from Riverside.

| Isolate | EC50 (µg/mL phosphite) |
|---|---|
| RR1 | 366.6 |
| 2120 | 388.6 |
| 2109 | 441.2 |
| 2114 | 465.5 |
| 2117 | 503.7 |
| 2113 | 593.6 |
| S2WR1 | 663.4 |
| W38R1 | 824.2 |
| 2110 | 8515.8 |

Isolate 2110 had an EC50 10-fold higher than the next lowest isolate (Table 5.2) and when the inhibition values from the two highest treatments, 200 and 500 µg/mL, were plotted against each other, 2110 stands out as more tolerant to phosphite than the other isolates (Figure 5.1).

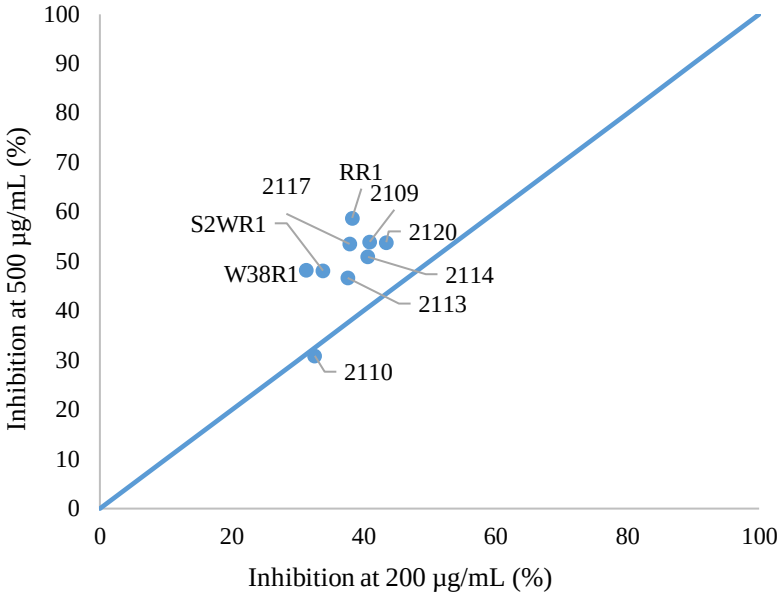


**Figure 5.1** Average inhibition of each of the nine *Phytophthora cinnamomi* isolates from Riverside grown in Ribeiro's Modified Media liquid containing 200 and 500 µg/mL phosphite. A 1:1 reference line is included.

### 5.4.2 New Zealand isolates

EC50 values of the New Zealand isolates ranged from 0.00 – 2903.46 (Table 5.3).

**Table 5.3** Predicted EC50 values for the *Phytophthora* isolates from the New Zealand experimental group treated with phosphite in the optical density assay.

| Species | Isolate | EC50 (µg/mL phosphite) |
|---|---|---|
| *P. agathidicida* | 3815 | 0.00 |
| *P. agathidicida* | 3118 | 0.00 |
| *P. agathidicida* | 3813 | 0.00 |
| *P. cactorum* | 4037 | 0.00 |
| *P. pluvialis* | 4019 | 21.13 |
| *P. cinnamomi* | 3784 | 24.66 |
| *P. cinnamomi* | 3750 | 29.46 |
| *P. multivora* | 3866 | 34.83 |
| *P. cinnamomi* | 3034 | 36.15 |
| *P. pluvialis* | 4234 | 49.35 |
| *P. multivora* | 3913 | 50.28 |
| *P. kernoviae* | 3680 | 59.20 |
| *P. cactorum* | 4040 | 63.02 |
| *P. citricola* | 4461 | 107.32 |
| *P. multivora* | 3871 | 109.74 |
| *P. citricola* | 4460 | 110.21 |
| *P. kernoviae* | 3610 | 174.33 |
| *P. citricola* | 4462 | 263.07 |
| *P. kernoviae* | 4470 | 1024.44 |
| *P. kernoviae* | 4053 | 2903.46 |

The *P. kernoviae* isolates 4470 and 4053 were inhibited by 49% and 38%, respectively, at 500 µg/mL phosphite, resulting in large EC50 predictions (Table 5.3). The *P. agathidicida* isolates were extremely sensitive to phosphite, their EC50 values were 0 µg/mL phosphite (Table 5.3) and their growth was inhibited by over 97% on 15 µg/mL phosphite relative to the control (Figure 5.2).

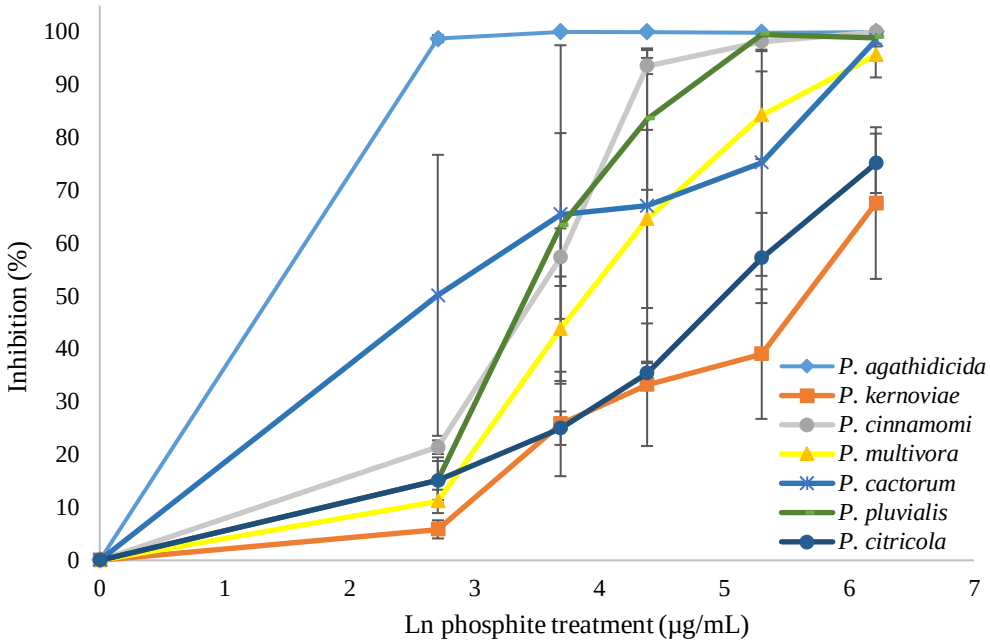


**Figure 5.2** Average inhibition response curves for each *Phytophthora* species from the New Zealand experimental group. Used the natural logarithm of phosphite concentration (µg/mL).

The EC50 values for the different species from New Zealand were significantly different as the distributions of the species were dissimilar from one another (Kruskal-Wallis test p-value = 0.02).

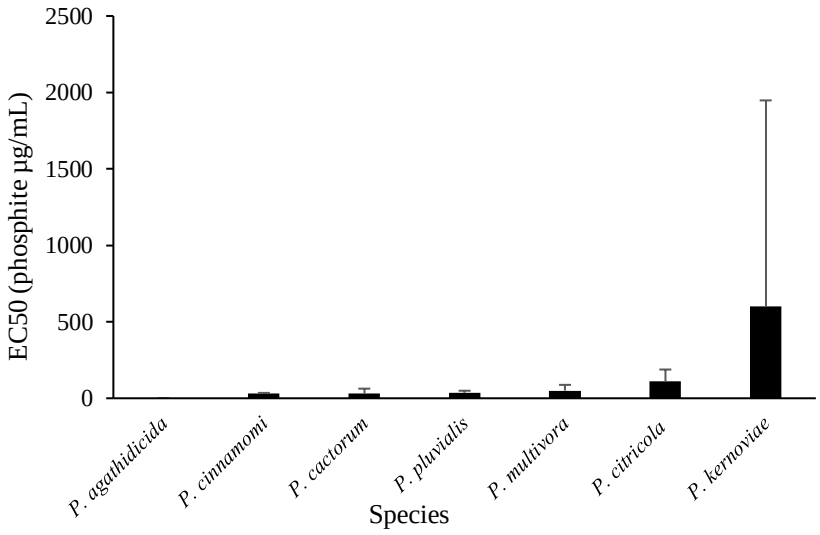


**Figure 5.3** Median EC50 values predicted for each *Phytophthora* species screened for phosphite sensitivity from New Zealand. Error bars are interquartile ranges.

There was a significant difference in the distribution of the EC50 values between *P. agathidicida* and *P. kernoviae* (Dunn test p-value = 0.02).

*Phytophthora agathidicida* was highly sensitive to phosphite on all concentrations and was significantly more inhibited than every other species on the 15, 40 and 80 µg/mL treatments (Figure 5.3).

### 5.4.3 Berkeley isolates

#### *5.4.3.1 Phosphite sensitivity*

The predicted EC50 values of the 77 isolates tested from the Berkeley collection formed a continuum ranging from 0.0 to 546.3 (Table 5.4).

**Table 5.4** Predicted phosphite EC50 values for phosphite for the 77 Berkeley isolates from the radial growth assay on RMM agar. Measurements for *Phytophthora lateralis* were taken 7 days after inoculation, every other species was measured on day 5. Clade and lineage included for *P. ramorum* and *P. cinnamomi* isolates for which the information was available.

| Species | Isolate | EC50 | Clade/lineage |
|---|---|---|---|
| *P. nemorosa* | 5104 Hansen22 | 0.0 | |
| *P. nemorosa* | 2052.2 Hansen2 | 0.1 | |
| *P. nemorosa* | 2059.4 Hansen6 | 0.4 | |
| *P. nemorosa* | P.106 | 0.5 | |
| *P. nemorosa* | P.114 | 0.6 | |
| *P. lateralis* | PL-47 | 0.9 | |
| *P. lateralis* | PL-9 | 1.0 | |
| *P. nemorosa* | P.113 | 1.1 | |
| *P. cinnamomi* | MC11 | 1.4 | A2 |
| *P. multivora* | FOFU-C2-CETH.1 | 1.7 | |
| *P. multivora* | NPC-47B-CETH.1 | 1.9 | |
| *P. lateralis* | Pl-28 | 2.1 | |
| *P. syringae* | SM15FEB_HOP | 2.3 | |
| *P. lateralis* | PL-34 | 2.4 | |
| *P. ramorum* | MR-187 | 2.6 | NA1 |
| *P. nemorosa* | 1050 Hansen1 | 3.0 | |
| *P. multivora* | MA-33B-FRCA.1 | 3.1 | |
| *P. cryptogea* | NPC-79B-MIAU.1 | 3.9 | |
| *P. cinnamomi* | P.6377 COFFEY | 4.1 | A2 |
| *P. cinnamomi* | P.3662 COFFEY | 4.2 | A2 |
| *P. cryptogea* | ENPN122-DP-DIAU12.1 | 4.9 | |
| *P. cryptogea* | ENPN80-B-DIAU10.2 | 5.4 | |
| *P. cinnamomi* | P.6493 COFFEY | 5.8 | A1 |
| *P. cryptogea* | CCWN-295B-DIAU5.1 | 6.0 | |
| *P. syringae* | SM15FEB_5CRP | 6.9 | |

| Species | Isolate | EC50 | Clade/lineage |
|---|---|---|---|
| *P. crassamura* | PLRA-SFPUC.1 | 7.0 | |
| *P. cryptogea* | MBP-B-DIAU1.1 | 7.8 | |
| *P. cryptogea* | ENPN123-DP-DIAU13.2 | 7.9 | |
| *P. multivora* | MA-60B-FRCA.1 | 8.1 | |
| *P. syringae* | KDA_RT9 | 9.1 | |
| *P. cactorum* | 7912.1 | 9.9 | |
| *P. cactorum* | 7-HR.1 | 11.3 | |
| *P. syringae* | MP-12 | 11.6 | |
| *P. cactorum* | PNPN-C-39FRCA.1 | 13.2 | |
| *P. megasperma* | MBP-B-DIAU10.1 | 13.3 | |
| *P. cambivora* | MP21 | 13.6 | |
| *P. ramorum* | MR-126 | 18.1 | NA1 |
| *P. syringae* | SM15APR_B0V | 25.8 | |
| *P. cactorum* | MP19 | 26.4 | |
| *P. cactorum* | 10-SP.1 | 26.4 | |
| *P. nemorosa* | P.115 | 27.6 | |
| *P. cactorum* | AKWA | 27.6 | |
| *P. cryptogea* | CCW-DP-DIAU-ROOTS.1 | 30.2 | |
| *P. cactorum* | 7-HP.1 | 30.8 | |
| *P. ramorum* | MR-268 | 36.9 | NA1 |
| *P. cinnamomi* | P.2021 COFFEY | 38.1 | A1 |
| *P. crassamura* | TEVA-59B-ALRU.1 | 45.5 | |
| *P. cryptogea* | MBP-B-DIAU4.1 | 50.8 | |
| *P. lateralis* | PL-54 | 57.3 | |
| *P. syringae* | BSP2014_502 | 66.5 | |
| *P. cambivora* | FOR.61B.HEAR | 69.7 | |
| *P. syringae* | MP-15 | 73.0 | |
| *P. megasperma* | MBP-DIAU5-DPSTEM.1 | 81.0 | |
| *P. megasperma* | MBP-DIAU4-DPSTEM.1 | 83.6 | |
| *P. crassamura* | SM-45B-FRCA.1 | 89.2 | |
| *P. ramorum* | SI-595 | 95.5 | EU1 |
| *P. crassamura* | MA-85B-SOIL.1 | 98.4 | |
| *P. lateralis* | PL-31 | 102.3 | |
| *P. crassamura* | PLRA-DRYSOIL1A.1 | 107.9 | |
| *P. cactorum* | 117R | 113.6 | |
| *P. ramorum* | 1461 | 116.5 | NA1 |
| *P. cambivora* | L.170.B.HEAR | 117.3 | |
| *P. megasperma* | MBP-DIAU10-DPSTEM.1 | 118.0 | |
| *P. cambivora* | NPL.22B.HEAR | 118.9 | |
| *P. crassamura* | SM-39B-FRCA.1 | 124.6 | |
| *P. ramorum* | MR-196 | 154.2 | NA1 |
| *P. ramorum* | MR-270 | 158.0 | NA1 |
| *P. crassamura* | TEVA-326B-JUEF.1 | 164.0 | |
| *P. cambivora* | MP28 | 165.8 | |
| *P. ramorum* | MR-59 | 190.4 | NA2 |

| Species | Isolate | EC50 | Clade/lineage |
|---|---|---|---|
| *P. ramorum* | MR-88 | 191.7 | NA2 |
| *P. ramorum* | SI-556 | 192.0 | EU1 |
| *P. lateralis* | PL-25 | 273.8 | |
| *P. crassamura* | FOR-OUT-06B.1 | 275.9 | |
| *P. ramorum* | SI-592 | 348.4 | EU1 |
| *P. ramorum* | MR-69 | 351.6 | NA2 |
| *P. ramorum* | MR-64 | 546.3 | NA2 |

The four *P. multivora* isolates tested were all very sensitive to phosphite with predicted EC50 values less than 8.1 µg/mL (Table 5.4). Seven of the eight *P. nemorosa* isolates were highly sensitive to phosphite, with predicted EC50 values less than 3.0 µg/mL, the other isolate had an EC50 of 27.6 µg/mL (Table 5.4). The phosphite EC50 values for the different species from Berkeley were significantly different as the distributions of the species were dissimilar from on another (Kruskal-Wallis test p-value<0.0001) (Figure 5.5).

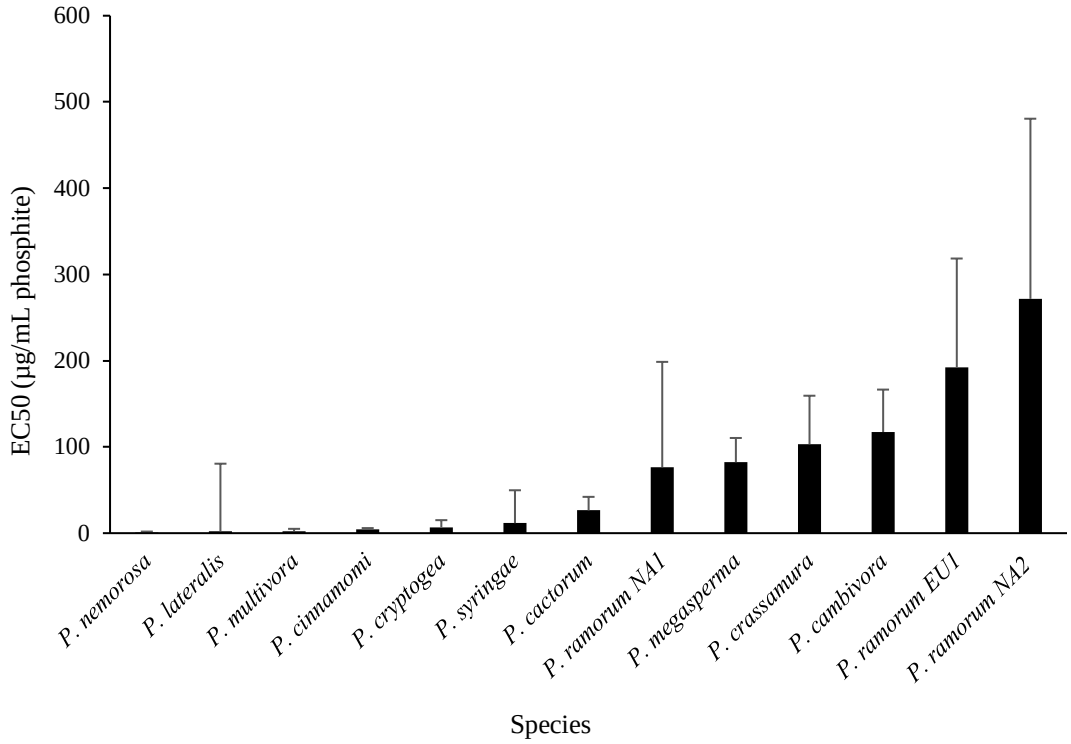


**Figure 5.4** Median EC50 value predicated for each species screened for phosphite sensitivity from Berkeley. Error bars are interquartile ranges. *P. cambivora* isolate MP21 was excluded. *Phytophthora lateralis* median EC50 from day 7 growth, all other species from the day 5 growth.

Post-hoc comparisons were analysed using Dunn Test. There was a significant difference in the EC50 values between *P. ramorum* NA2 and *P. multivora* (p=0.02) and a marginally significant difference between *P. ramorum* NA2 and *P.*

*cinnamomi* (p=0.06). The sensitivity of *P. nemorosa* was significantly different to several species including *P. ramorum* NA2 (p =0.0002), *P. ramorum* EU1 (p=0.01), *P. cambivora* (p=0.02) and *P. crassamura* (p =0.002) (Figure 5.5).

Growth was 'promoted' on some concentrations of phosphite for five *P. crassamura*, one *P. cactorum*, one *P. cambivora*, two *P. lateralis*, two *P. syringae*, and seven *P. ramorum* isolates. This was generally observed as the mycelium growing less densely in response to the presence of phosphite and the culture having a larger diameter than that of the control. For example, the diameter of the colony of *P. ramorum* isolate MR-64 was larger than the colony on the control on 15, 40 and 200 µg/mL phosphite (Figure 5.5).

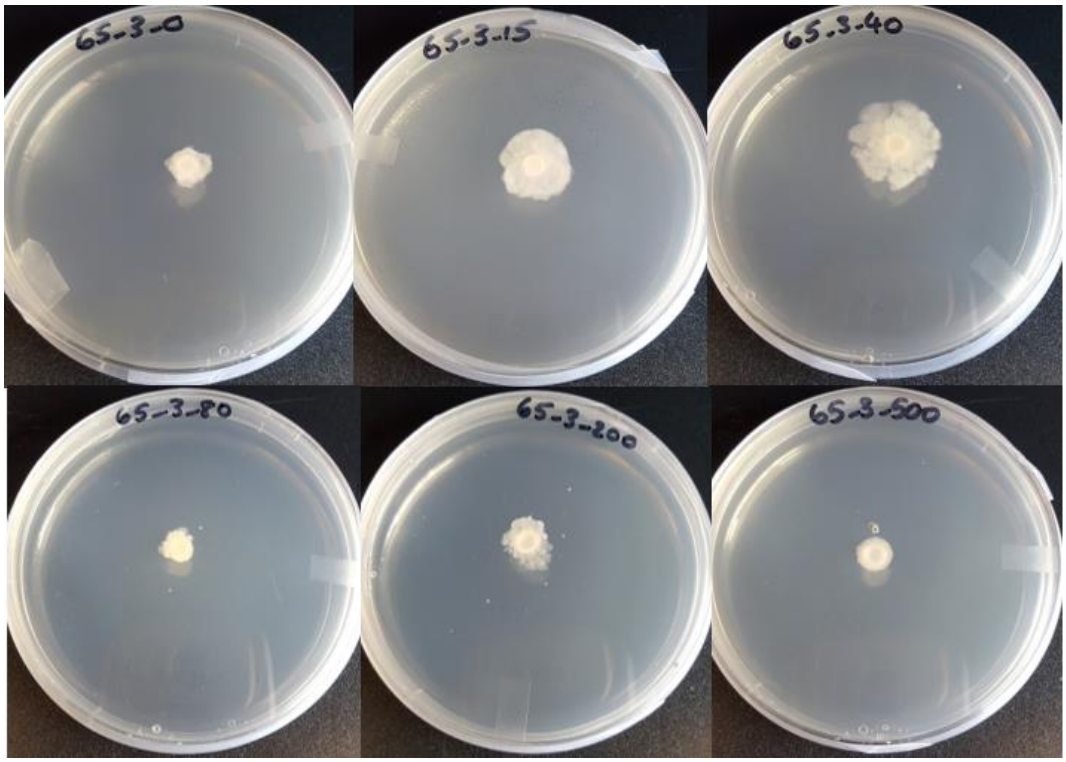


**Figure 5.5** Growth on 10% V8 media of *P. ramorum* isolate MR-64 at 0, 15, 40, 80, 200 and 500 µg/mL (From left to right). Growth was promoted on 15, 40 and 200 µg/mL relative to the control and inhibited on 80 and 500 µg/mL.

*5.4.3.2 Mefenoxam sensitivity*

*Phytophthora cactorum* was the species most sensitive to mefenoxam, the eight isolates had the lowest EC50 values at nearly zero (Table 5.5).

**Table 5.5** Predicted EC50 values for the 32 *Phytophthora* isolates tested for mefenoxam sensitivity from Berkeley in a radial growth assay.

| Species | Isolate | EC50 |
|---|---|---|
| *P. cactorum* | 10-SP.1 | <0.0001 |
| *P. cactorum* | MP19 | <0.0001 |
| *P. cactorum* | 7912.1 | <0.0001 |
| *P. cactorum* | 117R | <0.0001 |
| *P. cactorum* | AKWA | <0.0001 |
| *P. cactorum* | 7-HR.1 | <0.0001 |
| *P. cactorum* | 7-HP.1 | <0.0001 |
| *P. cactorum* | PNPN-C-39FRCA.1 | <0.0001 |
| *P. multivora* | FOFU-C2-CETH.1 | <0.0001 |
| *P. multivora* | NPC-47B-CETH.1 | <0.0001 |
| *P. crassamura* | SM-45B-FRCA.1 | <0.0001 |
| *P. multivora* | MA-33B-FRCA.1 | <0.0001 |
| *P. multivora* | MA-60B-FRCA.1 | <0.0001 |
| *P. crassamura* | FOR-OUT-06B.1 | <0.0001 |
| *P. crassamura* | SM-39B-FRCA.1 | <0.0001 |
| *P. crassamura* | MA-85B-SOIL.1 | <0.0001 |
| *P. crassamura* | TEVA-326B-JUEF.1 | <0.0001 |
| *P. cryptogea* | CCWN-295B-DIAU5.1 | <0.0001 |
| *P. cryptogea* | MBP-B-DIAU4.1 | <0.0001 |
| *P. crassamura* | PLRA-DRYSOIL1A.1 | <0.0001 |
| *P. cryptogea* | MBP-B-DIAU1.1 | <0.0001 |
| *P. crassamura* | TEVA-59B-ALRU.1 | <0.0001 |
| *P. cryptogea* | CCW-DP-DIAU-ROOTS.1 | <0.0001 |
| *P. cryptogea* | ENPN123-DP-DIAU13.2 | 0.0003 |
| *P. crassamura* | PLRA-SFPUC.1 | 0.0004 |
| *P. cryptogea* | NPC-79B-MIAU.1 | 0.0007 |
| *P. megasperma* | MBP-DIAU5-DPSTEM.1 | 0.0007 |
| *P. cryptogea* | ENPN122-DP-DIAU12.1 | 0.0009 |
| *P. cryptogea* | ENPN80-B-DIAU10.2 | 0.0011 |
| *P. megasperma* | MBP-DIAU4-DPSTEM.1 | 0.0018 |
| *P. megasperma* | MBP-DIAU10-DPSTEM.1 | 0.0022 |
| *P. megasperma* | MBP-B-DIAU10.1 | 0.1578 |

The *P. megasperma* isolate MBP-B-DIAU10.1 had a predicted EC50 value 73-fold that of the isolate with the next highest value (Table 5.5) and appears to be more tolerant than the other isolates when the inhibition at 0.1 and 1 are plotted against each other (Figure 5.6).

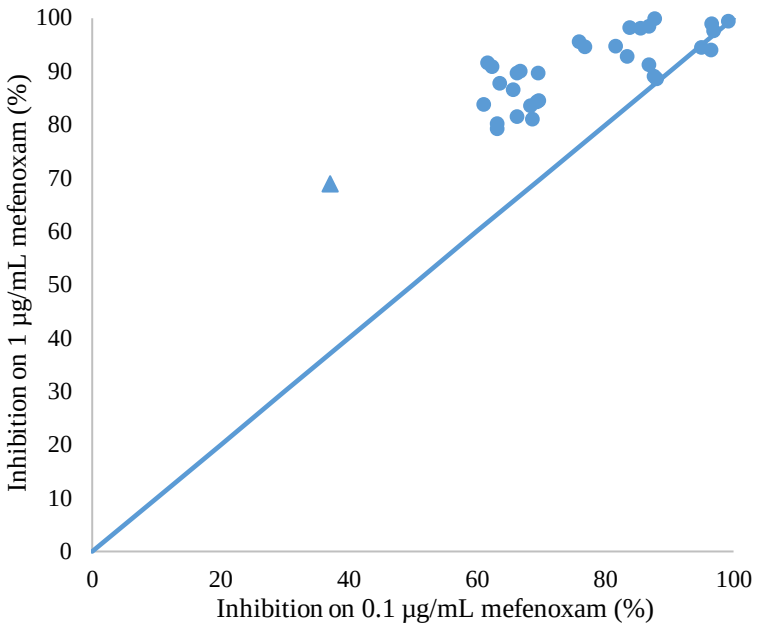


**Figure 5.6** Average inhibition of the 32 *Phytophthora* isolates from Berkeley grown on 10% V8 agar containing 0.1 and 1 µg/mL mefenoxam. The triangle is *P. megasperma* isolate MBP-B-DIAU10.1.

On average *P. megasperma* isolate MBP-B-DIAU10.1 was inhibited 37 and 69% on 0.1 and 1 µg/mL mefenoxam, respectively.

The mefenoxam EC50 values for the different species from Berkeley were significantly different as the distributions of the species were dissimilar from one another (Kruskal-Wallis test $p<0.0001$) (Figure 5.7).

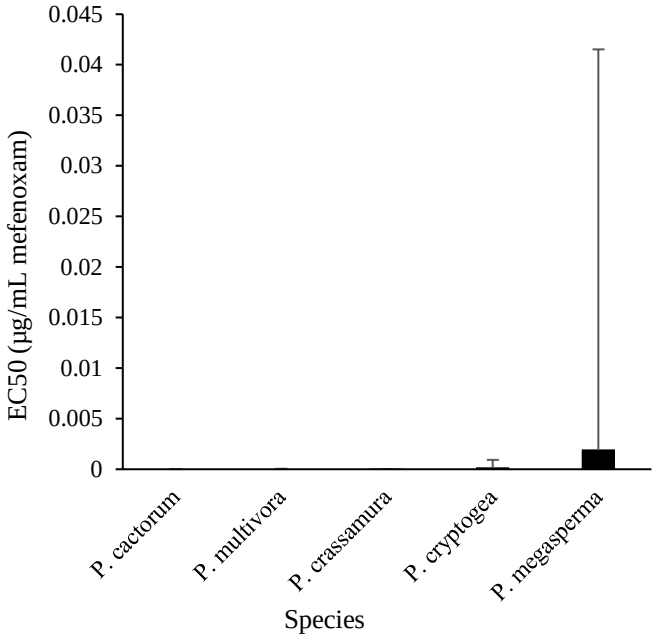


**Figure 5.7** Median EC50 prediction for each *Phytophthora* species tested for mefenoxam sensitivity in 10% V8 agar excluding the tolerant isolate *P. megasperma* MBP-B-DIAU10.1. Error bars are interquartile ranges.

The Dunn Test was used for post-hoc analysis after the Kruskal Wallis test. There were significant differences between the EC50 values of *P. cactorum* and several species including *P. crassamura* (p=0.04), *P. cryptogea* (p=0.0004) and *P. megasperma* (p<0.0001) (Figure 5.7). There was a significant difference between *P. megasperma* and *P. multivora* (p=0.03).

The pattern of sensitivity in the species shown by the median EC50 values (Figure 5.7) can also be observed in the average inhibition for each species on the concentrations 0.1, 1, 5 and 10 µg/mL mefenoxam (Figure 5.8).

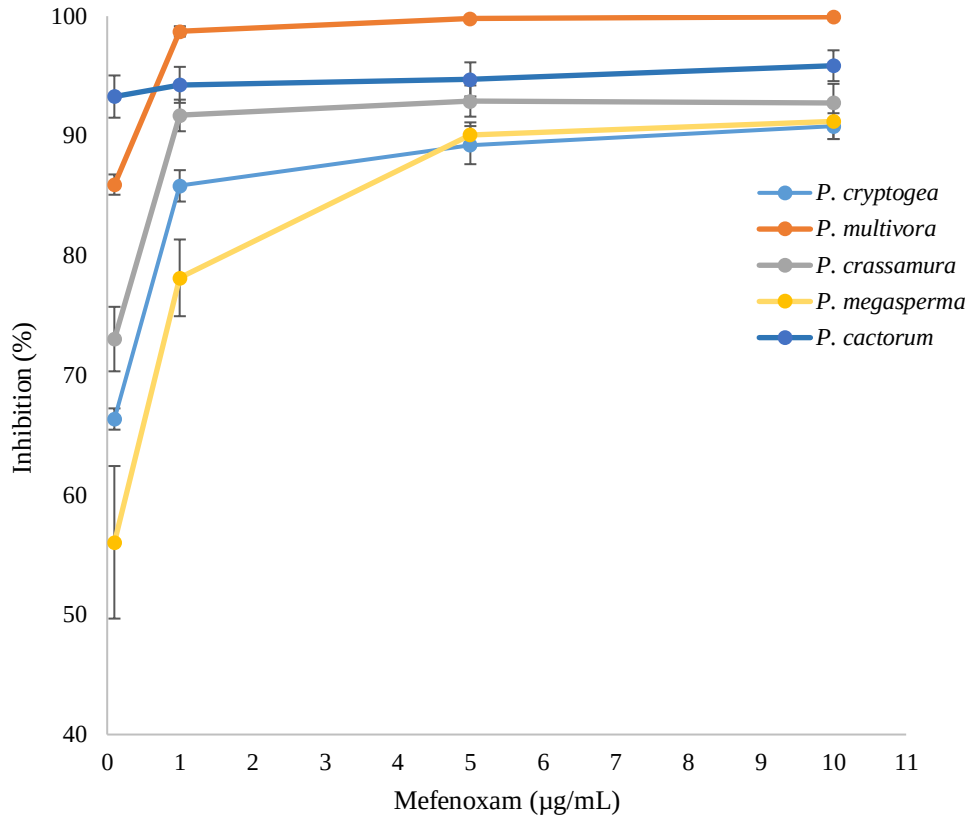


**Figure 5.8** Average inhibition for each *Phytophthora* species on 10% V8 agar amended with 0.1, 1, 5 and 10 µg/mL mefenoxam. Error bars are standard error values.

## 5.5 Discussion

Variability among *Phytophthora* species and isolates in response to chemicals is not uncommon (Coffey & Bower, 1984a; Garbelotto *et al.*, 2009; Ma & McLeod, 2014) and is not necessarily linked to previous exposure (Wilkinson *et al.*, 2001b). Two methods were used in this study, the OD screening of the New Zealand and Riverside isolates and the radial growth measurements for the Berkeley isolates. Both methods were able to detect variation in sensitivity

between isolate and species of *Phytophthora*. The *Phytophthora* species tested from the collections in New Zealand and Berkeley, were characteristic in their sensitivity to phosphite and mefenoxam (Figures 5.1, 5.3 and 5.4).

The *P. cinnamomi* isolates from Riverside were not highly inhibited by the phosphite in the liquid 10% V8 medium (Table 5.3 and Figure 5.3). This is likely because phosphate levels are higher in V8 media and phosphite inhibition is exaggerated by phosphate starvation, thus the EC50 values from Riverside are much higher than the *P. cinnamomi* isolates from New Zealand which were grown on RMM (Table 5.2 and 5.3). However, there does appear to be one *P. cinnamomi* isolate, 2110, which is more tolerant to phosphite compared to the other isolates from Riverside (Table 5.3 and Figure 5.3). No information is known about the phosphite management at the orchards the isolates were obtained from so no comment can be made on the effect of prolonged exposure resulting in tolerance in isolate 2110 (Table 5.1).

Isolates of *P. cinnamomi, P. cactorum,* and *P. multivora* were screened for phosphite sensitivity in New Zealand and Berkeley. The *P. multivora* isolates form Berkeley were more sensitive than the *P. cinnamomi* and *P. cactorum* isolates tested (Figure 5.4). In comparison, for the isolates from New Zealand, *P. cinnamomi* was the most sensitive followed by *P. cactorum* and then *P. multivora* (Figure 5.3). This observed difference in the pattern of sensitivity between the species from the two collections is likely due to the history of the isolates. The *P. cactorum* isolates form Berkeley were isolated from a nursery and from a restoration site which sourced its plants from a nursery (Table 5.1). It is possible these had been previously exposed to phosphite in the nursery.

The A1 and A2 mating types of *P. cinnamomi* from Berkeley showed no clear difference in their tolerance to phosphite however one A1 isolate, P.2021 COFFEY, did have a higher EC50 value than the rest (38.1 µg/mL phosphite) (Table 5.4), but due to the low sample number it is not possible to say this is due to the mating type. Furthermore Coffey and Bower (1984a) and Wilkinson *et al.* (2001b) tested both mating types and concluded there was no significant difference in the phosphite sensitivity of the two types.

*Phytophthora agathidicida* has only been found in New Zealand, causing a dieback disease of kauri. The isolates tested were extremely sensitive to phosphite (Table 5.2 and Figures 5.1 and 5.1). This correlates with other phosphite testing of *P. agathidicida* for which EC50 of 4.0 µg/mL was predicted using a radial growth assay on V8 agar (Horner & Hough, 2013). The slightly higher EC50 prediction from Horner and Hough (2013) compared to the results from the current study are likely due to difference in media as RMM is a low phosphate media which exaggerates phosphite inhibition. Furthermore the *P. agathidicida* isolates in the current study were grown in liquid media which has been suggested to result in higher sensitivity to phosphite due to the mycelium being in higher contact with phosphite in liquid media compared to solid media (Ma & McLeod, 2014).

The *P. citricola* isolates used from the New Zealand collection were isolated from soil in an avocado orchard (Orchard 10 from Chapter 3). This orchard as managed avocado root rot, caused by *P. cinnamomi,* with phosphite by injecting the trunk of every tree with 150 g/L of Agri-Fos® 600 (Agrichem, Yatala QLD, Australia) every year for 32 years, with additional injections into unhealthy trees (Chapter 3). The predicted EC50 values for the *P. citricola,* isolates, 4470, 4471 and 4472 were 110.21, 107.32 and 263.07 µg/mL, respectively (Table 5.2) and they were inhibited between 11 – 22% on 15 µg/mL. In 1984, the phosphite sensitivity of *P. citricola* isolates from avocado orchards were assessed by measuring radial growth on RMM plates, the predicted EC50 of one isolate was 7.0 µg/mL phosphite (Table 1 in Coffey & Bower, 1984) and four of the five isolates were inhibited by more than 50% at 5 µg/mL (Coffey & Bower, 1984a). This was when Aliette®, the commercial fungicide containing Fosteyl-a (the aluminium salt of phosphonate) (Cohen & Coffey, 1986), was available (Guest & Grant, 1991) and before commercial products containing phosphorous acid ($H_3PO_3$) existed. It is likely the *P. citricola* isolates from the current study were more tolerant to phosphite compared to those in the study by Coffey and Bower (1984a) probably because of their long history of exposure to phosphite in a phosphite managed orchard.

The only other time *P. citricola* isolates have been screened for phosphite sensitivity was by measuring radial growth on 25% V8 agar so the results are not comparable to RMM media, however, the EC50s ranged from 34 – 67 µg/mL

(Weiland *et al.*, 2009). Weiland *et al.* (2009) also screened, *P. cactorum* isolates which had EC50 values from 166 – 238 μg/mL. It is interesting that the *P. citricola* isolates had such low EC50s on the 25% V8 media and the *P. cactorum* isolates had very high EC50s when the New Zealand *P. citricola* isolates in the current study were more tolerant than the *P. cactorum* (Table 5.2). It is difficult to say if this is only due to the phosphite history of the *P. citricola* isolates in the current study or if the two species respond differently to high phosphate concentrations such as those in V8 media.

Two of the *P. kernoviae* isolates tested had relatively high EC50 values, over 1000 μg/mL phosphite (Table 5.2). Isolate 4470 was isolated from soil from a sick tree in an organic avocado orchard (Orchard 9 in Chapter 3). The other isolate with a high EC50 was isolated from pine needles at a bait station in Kapenga forest in the Bay of Plenty (Table 5.1). *Phytophthora kernoviae* is not known to cause any major diseases to New Zealand flora and may be indigenous (Gardner *et al.*, 2015) but does cause disease on ornamental species in the United Kingdom where it behaves as an invasive organism in its spread and disease progression (Brasier *et al.*, 2005).

While measurement of mycelial diameter is a relatively easy method to determine inhibition, it may not provide a realistic measure and this was observed in cultures of *P. ramorum* and *P. crassamura* for which growth was 'promoted' on lower concentrations of phosphite. It is difficult to determine if the cultures that were 'promoted' to grow on phosphite were more tolerant or if it was a survival strategy to move away from the phosphite because mycelial density is not accounted for in radial growth measurements. In this situation the use of dry weight or optical density measurements would have more accurately captured the true inhibition because density could be accounted for. It is also relevant that the growth was promoted on 10% V8 media because it is probably easier for the *Phytophthora* to alter growth when in nutrient and phosphate rich media as it is not phosphate starved.

The *P. ramorum* isolates in this study from the NA2 lineage had a higher median EC50 compared to those from NA1 lineage and the EU1 lineage came close to NA2 (Figure 5.4). The NA lineages cause sudden oak death in the United States and the EU lineages cause disease in Europe. Recently it has been shown that

NA2 was more aggressive on detached wounded Rhododendron leaves followed by EU1, EU2 and then NA1 (O'Hanlon *et al.*, 2017) this follows the same pattern of phosphite tolerance in the current study so perhaps there is something about the aggressiveness of a lineage that allows it to grow in the presence of phosphite easier. It has been shown previously that *P. ramorum* was generally less sensitive to phosphite (Garbelotto *et al.*, 2009) compared to *P. cinnamomi* (Wilkinson *et al.*, 2001b) and the results from the Berkeley isolates in the current study support this. Recovery of trees infected with *P. cinnamomi* upon treatment with phosphite has been reported (Cohen & Coffey, 1986) and it is used abundantly in avocado orchards to control phytophthora root rot caused by *P. cinnamomi.* In comparison the direct fungistatic effects of phosphite on *P. ramorum* may be modest (Garbelotto *et al.*, 2009)

Phenylamide fungicides are heavily relied upon to control potato late blight caused by *P. infestans,* but insensitivity to the fungicide has been observed many times in *P. infestans* isolates (Davidse *et al.*, 1981; Dowley *et al.*, 2002; Dowley & O'Sullivan, 1981; Mukalazi *et al.*, 2001). Metalaxyl became commercially available in Ireland in 1977 and quickly became the most widely used fungicide used to control potato late blight. By 1980, resistant isolates of *P. infestans* were confirmed (Dowley & O'Sullivan, 1981). In response, phenylamide fungicides were withdrawn from the market in 1980 and were not reintroduced until 1985. The incidence of resistance in *P. infestans* strongly followed the intensive applications from 1977-1980 and 1985 (Dowley *et al.*, 2002). The occurrence of phenylamide resistant isolates is a major concern.

Isolates of five *Phytophthora* species from a nursery and restoration sites that used nursery plants were assessed for their sensitivity to mefenoxam (Table 5.1), it is possible these isolates would have been exposed to mefenoxam at the nursery. This is the first time isolates of *P. multivora* and *P. crassamura* have been assessed for sensitivity to a phenylamide fungicide. However, *P. megasperma* (Coffey & Bower, 1984b), *P.* cactorum (Hill & Hausbeck, 2008; Weiland et al., 2009) and *P. cryptogea* (Hwang & Benson, 2005) isolates have been previously screened. All isolates were relatively sensitive to mefenoxam as the average EC50 values are lower than the lowest treatment of 0.1 μg/mL mefenoxam (Figure 5.4 and 5.5). The four *P. multivora* isolates were highly sensitive to mefenoxam as all

were inhibited 100% on 10 µg/mL mefenoxam. *Phytophthora crassamura* is a new species which was found on Norway spruce (*Picea abies*) in a nursery and in the rhizosphere of Phoenicean juniper (*Juniperus phoenicea*) in a wetland and a forest in Italy (Scanu *et al.*, 2015). The *P. crassamura* isolates in this study were from a nursery and a restoration site which used nursery plants and were sensitive to mefenoxam, they were inhibited by over 62% on the lowest treatment of 0.1 µg/mL mefenoxam (Table 5.1).

*Phytophthora megasperma* was the most tolerant species to mefenoxam (Figures 5.4 and 5.5) and isolate MBP-B-DIAU10.1 was 73-fold more tolerant than the next highest (Table 5.5). The tolerant *P. megasperma* isolate in the current study was inhibited 37% at 0.1 µg/mL mefenoxam while the other three isolates were inhibited between 61 – 63% and the same concentration. In comparison, *P. megasperma* isolates with no previous exposure to metalaxyl varied in their sensitivity with 9.2 – 100.0 % inhibition of radial growth at 0.1 µg/mL metalaxyl (Coffey & Bower, 1984b). Isolates from Douglas fir were much less sensitive to metalaxyl than those from alfalfa and soybean, being inhibited 36 – 41% on 0.1 µg/mL metalaxyl.

The *P. cactorum* isolates from Berkeley in the current study were highly sensitive to mefenoxam (Table 5.5 and Figures 5.4 and 5.5), with EC50 values below 0.0001 µg/mL mefenoxam (Table 5.7). Metalaxyl is used to control phytophthora root rot of ginseng (*Panax quinquefolium*), caused by *P. cactorum.* Eighty-five, 48 and 98 % of isolates collected from Wisconsin gardens, Michigan gardens and from greenhouse seedlings grown from seeds from a commercial garden, were tolerant to mefenoxam based on the definition of tolerance as the ability to grow >10 % relative to the control on 100 µg/mL mefenoxam (Hill & Hausbeck, 2008). Isolates from strawberry plants with crown rot caused by *P. cactorum,* were highly tolerant to mefenoxam, they grew 73 to 89% of that on nonamended media on 100 µg/mL mefenoxam (Jeffers *et al.*, 2004). In comparison the *P. cactorum* isolates in the current study were highly sensitive to mefenoxam as they grew 0 – 5% on 100 µg/mL mefenoxam.

The *P. cryptogea* isolates in the current study grew 3 – 13% on 100 µg/mL mefenoxam compared to growth on non-amended media. These isolates can be considered very sensitive to mefenoxam in comparison to isolates from

symptomatic plants on production locations which could grow more than 50% (relative to growth on control media) on 100 µg/mL mefenoxam (Hwang & Benson, 2005).

## 5.6 Conclusions

The *Phytophthora* species differed in their sensitivity to phosphite and mefenoxam. From New Zealand, *Phytophthora agathidicida* was extremely sensitive to phosphite while *P. kernoviae* was relatively tolerant. From Berkeley *P. ramorum* NA2 and EU1 lineages appeared to be the most tolerant to phosphite and *P. nemorosa* was highly sensitive. One isolate of *P. megasperma* was found to be less sensitive to mefenoxam than the other isolates tested however it was not tolerant compared to previous studies. The isolates of *P. cryptogea* and *P. cactorum* were much more sensitive to mefenoxam compared to tolerant isolates from previous studies. One isolate of *P. cinnamomi* from Riverside was more tolerant to phosphite than the other eight despite them all being from avocado orchards. This study has shown that both intraspecific and interspecific variation in tolerance to fungicides exist in *Phytophthora* species.

# Chapter 6
# Conclusions

## 6.1 Overview

The main aim of this thesis was to test the hypothesis that isolates of *Phytophthora cinnamomi* can become tolerant to phosphite after prolonged exposure. A second key aim of this thesis was the analysis of the phosphite tolerance of several internationally important *Phytophthora* species.

Firstly, a high through-put method to screen for fungicide sensitivity in *Phytophthora* species was developed using an optical density plate reader (Chapter 2), which was subsequently used to screen the phosphite sensitivities of 56 *P. cinnamomi* isolates from eight avocado orchards with a phosphite history and four without. Four and two isolates of *P. cinnamomi* from orchards with a history of prolonged exposure to phosphite were grouped as tolerant and intermediately tolerant to phosphite, respectively. Three tolerant isolates and three sensitive isolates were used to inoculate lupin (*Lupinus angustifolius*) seedlings treated with phosphite (Chapter 4). The isolates identified as being more tolerant to phosphite *in vitro* were also more tolerant to phosphite *in planta* as they were more extensive colonisers of phosphite-treated lupin seedlings and could grow further past the lesion margin asymptomatically than the sensitive isolates. These results align with the work by Dobrowolski *et al.* (2008) who found *P. cinnamomi* isolates from phosphite treated sites were able to colonise treated plant material more effectively than isolates with no previous exposure to phosphite. The tolerant isolates were also able to produce more sporangia and viable zoospores than the sensitive isolates in the presence of phosphite. Phosphite appears to reduce the production but not prevent the production of sporangia and zoospores by tolerant isolates (Chapter 4) (Wilkinson et al., 2001a).

Phosphite is used to treat many phytophthora diseases, particularly those affecting nurseries and crop species, and is often tested for its efficacy to treat emerging phytophthora diseases in native ecosystems and forest. The phosphite sensitivity of *Phytophthora* species has been shown to vary (Coffey & Bower, 1984a). In response, a second key aim of this thesis was to assess the phosphite sensitivity of

seven *Phytophthora* species from the culture collection at Scion, Rotorua, NZ, and 11 species from the University of California, Berkeley, USA, collection. A third group of nine *P. cinnamomi* isolates from Avocado orchards in Southern California were tested from the University of California, Riverside, USA collection. The sensitivity to phosphite and the chemical fungicide mefenoxam of 32 isolates in the Berkeley collection, isolated from nurseries and restoration sites (using nursery sourced plants), were also assessed. Mefenoxam is the active ingredient in Ridomol Gold® (Syngenta, Basel, Switzerland) and is used to protect plants from phytophthora diseases. As the methods and timing of experiments varied between the three experimental groups (NZ, Berkeley and Riverside), sensitivity was not directly compared between experiments. EC50 values, which represent the concentration required to inhibit mycelial growth by 50% relative to the growth in the absence of phosphite, were used to compare sensitivity of isolates.

From the NZ group, a possible native species (Gardner *et al.*, 2015), *P. kernoviae,* was the most tolerant to phosphite based on the average EC50 values for each species (Chapter 5). *Phytophthora kernoviae* was followed by three isolates of *P. citricola* obtained from a phosphite managed orchard from the survey in Chapter 3. The species *P. multivora, P. pluvialis, P. cactorum* and *P. cinnamomi* were intermediate in their sensitive to phosphite with EC50s all under 100 µg/mL phosphite. Despite the risk of selecting for tolerance, this study has shown how effective phosphite can be in inhibiting growth of certain species, such as *P. agathidicida,* as all three isolates were inhibited by over 97% at 15 µg/mL phosphite. *Phytophthora agathidicida* was previously shown to be very sensitive to phosphite in comparison to *P. cinnamomi* and *P. cactorum* in trials testing the efficacy of phosphite to protect kauri trees from the dieback disease it causes (Horner & Hough, 2013). The isolates used in the current study and the trials by Horner and Hough (2013) were different and thus the results show a consistent response across independent studies and strong potential for the use of phosphite to manage kauri dieback. Phosphite has two modes of action, the direct inhibitory effect on *Phytophthora* species growth and also the stimulation of host defence responses in treated plants (Grant *et al.*, 1990; Guest & Grant, 1991; Smillie *et al.*, 1989). It is possible that for species such as *P. agathidicida* for which phosphite is

extremely inhibitory, the likelihood of phosphite tolerant isolates developing is lower because they are so sensitive to phosphite already and the combined effect of host defences may prevent tolerant isolates developing. However, this remains to be tested as the application of phosphite in response to kauri dieback is still in its infancy (Horner & Hough, 2013; Horner *et al.*, 2015).

*Phytophthora* species not present in New Zealand including *P. ramorum*, *P. crassamura, P. lateralis,* and *P. nemorosa* were assessed from the Berkeley culture collection. *Phytophthora ramorum* isolates of the NA2 and EU1 lineages stood out as the most tolerant and *Phytophthora nemorosa* was the most sensitive species to phosphite of the species tested at Berkeley species (Chapter 5). For the species tested in Berkley, a much lower concentration of mefenoxam was required to inhibited mycelial growth by 50% compared to phosphite. All species tested for mefenoxam were relatively sensitive and only one isolate, *P. megasperma* isolate MBP-B-DIAU10.1, was inhibited by less than 50% on the lowest concentration of 0.1 µg/mL mefenoxam. This isolate was fast growing compared to the other isolates, tt grew to cover a 90 mm diameter plate in just 3 days. However, this faster growth did not correlate with resistance to phosphite as it was the most sensitive of the *P. megasperma* isolates to phosphite, with an EC50 of 13.3 µg/mL.

Of the nine isolates of *P. cinnamomi* screened at Riverside, one isolate (2110) appeared to be more tolerant to phosphite than the other isolates tested (Chapter 5). While all the isolates were obtained from avocado orchards in southern California, no information regarding the regime of phosphite or chemical control of the orchards was available for this study.

It was not possible to test the isolates from New Zealand and the United States in the same experiment due to quarantine restrictions precluding the import of isolates into New Zealand. *Phytophthora ramorum* especially is on the New Zealand Ministry for Primary Industries (MPI) unwanted organism register. It is difficult to directly compare the phosphite sensitivities of *Phytophthora* isolates which are measured in different experiments. While *P. multivora, P. cactorum* and *P. cinnamomi* were screened in both NZ and the USA, they cannot be compared directly as they each have very different hosts and histories. However,

at Berkeley the *P. cactorum* isolates were most tolerant followed by *P. cinnamomi* then *P. multivora* while the isolates from New Zealand showed the opposite pattern with *P. multivora* being the most tolerant followed by *P. cinnamomi* then *P. cactorum.* It is likely these patterns were greatly influenced by the histories of the isolates, for example the *P. cactorum* isolates from Berkeley may have been exposed to phosphite in a nursery.

The findings from this study have implications for the use of phosphite to manage phytophthora diseases in both horticultural and forest systems. This work demonstrates that tolerance can develop in isolates with prolonged exposure to phosphite which is of specific concern for the avocado industry where the efficacy of treatment may decline with ongoing phosphite use. This in turn may lead to further intensification in the application of phosphite which presents a considerable risk for higher levels of resistance to treatment. Phosphite does not kill *Phytophthora* so its use provides an environment which selects for the most tolerant isolates with each subsequent phosphite application.

The isolates obtained from unhealthy trees in phosphite managed orchards were classified as either intermediate or tolerant in the *in vitro* screening (Chapter 3). It is hypothesised that once infection is established, repeated treatment with phosphite provides an ideal environment to directly select for phosphite tolerant isolates. In contrast to annual cropping cycles, tree crops including avocado maintain these isolates within their infected root systems providing ongoing opportunities for selection over time. Given that avocado trees with avocado root rot tend to succumb to infection and die in the absence of active management, treatment with phosphite may provide an ideal opportunity for the build-up of resistant isolates which may have declined naturally in competition with other saprophytic microbes had the tree died or been managed with more organic practices. This leaves many orchardists in the conundrum of treating despite the likelihood of selecting for resistance. It is therefore recommended that an integrated approach of both cultural and chemical management be undertaken to reduce the reliance on phosphite application and minimise the risk of developing phosphite-tolerant *P. cinnamomi* isolates.

In addition to avocado, this research has implications across the many horticultural species impacted by soil-born species of *Phytophthora.* In many

cases, plants are treated with phosphite within nursery production and are transplanted with pathogens which are likely to already be under selection pressure for phosphite resistance. Of further consideration is the use of phosphite on plants either destined for restoration plantings or already established within natural ecosystems (Simamora *et al.*, 2018). While there are numerous cases of phosphite being highly effective for the long-term management of disease in natural ecosystems (Barrett & Rathbone, 2018), its responsible use is paramount for long-term disease management in natural ecosystems where options for building carbon and microbial diversity in a sustained manner are limited.

Another key outcome of this study is the development of a high through-put method for screening fungicide sensitivity in oomycete plant pathogens in the form of an optical density assay. This method may be used for assessing and contrasting the tolerance of species and isolates under phosphite management. It is also an efficient way to assess the sensitivity of isolates previously exposed to phosphite or other forms of chemical, biological or physical inhibition. As the cultures are grown in liquid media in wells, enabling unimpeded three-dimensional mycelial growth that can be quantified photometrically, more accurate measurements of mycelial density can be obtain compared to radial growth measurements on agar plates. This method was demonstrated to be a much faster method than measuring the dry weight of cultures grown in liquid media. The optical density method is scalable with potential application in screening the efficacy of many fungicide treatments and isolates in the same experiment.

## 6.2 Future directions

Phosphite is used to protect numerous plants affected by *Phytophthora* species, including horticultural crops and endangered plant species (Hardy *et al.*, 2001) and is also being looked to as a control method for new phytophthora diseases such as kauri dieback (Horner & Hough, 2013; Horner *et al.*, 2015). The results from this study showing selection for *P. cinnamomi* isolates with decreased sensitivity to phosphite from phosphite managed avocado orchards are concerning although complete resistance was not observed. These results support previous work which has shown decrease sensitivity to phosphite in *P. cinnamomi* with prolonged use of the fungicide (Dobrowolski et al., 2008; Duvenhage, 1994; Ma

& McLeod, 2014) but is the first demonstration of this in New Zealand and specifically within avocado. It is proposed that the sensitivity of *P. cinnamomi* isolates treated with phosphite for a prolonged period must be monitored closely. If truly resistant isolates of *P. cinnamomi* develop in an avocado orchard, they could spread through orchards easily as many vectors are available such as contract avocado pickers and machinery that travel between orchards. Measures minimising the spread of pathogen isolates between growing regions could therefore be considered for future-proofing the industry should higher levels of resistance appear over time.

The high through-put optical density assay developed in this study not only provides the opportunity to screen phosphite exposed isolates for their current sensitivity but also to screen many species and isolates of *Phytophthora*. To truly understand the underlying resistance of a *Phytophthora* species, the assessment of many isolates is recommended given that they can vary greatly even without previous exposure to phosphite, as shown in Chapter 3 of this thesis and by Wilkinson *et al.* (2001b).

Further work needs to be carried out to assess the *in-planta* tolerance of isolates determined to have decreased sensitivity to phosphite, of most relevance would be to use the plants they naturally cause disease on such as *P. cinnamomi* and avocado. Avocado plants were not used in this study because they were expensive and it is difficult to obtain enough plants that are definitely *Phytophthora*-free.

To meet the demand of the world's population by 2050, global food production must increase by 50% (Chakraborty & Newton, 2011) and crop destroying species of *Phytophthora* threaten current and future global food security (Bebber & Gurr, 2015). Furthermore, climate change is likely to alter the spread and severity of many *Phytophthora* pathogens and their associated diseases (Pautasso *et al.*, 2012). There are a range of fungicides available to protect crops however fungicide efficacy will be vital if we are to meet future demands as climate changes.

Global movement of *Phytophthora* species and other plant pathogens occurs largely due to increased trade in plant produce, whole-plants and the resulting introduction of a species to a new area with devastating impacts on specific

species and entire ecosystems. The optical density assay developed here may therefore have direct application in dealing with a new pathogen. In such cases, it may be advantageous to quickly screen fungicides to identify one which can most effectively treat the pathogen and manage its spread.

Temporary suppression of disease symptoms, particularly in nurseries is concerning because supposedly 'disease-free' material is out-planted resulting in spread of previously suppressed pathogens (Parke & Grünwald, 2012). *Phytophthora* isolates from such nurseries should be monitored for fungicide sensitivity to avoid promoting and then releasing tolerant isolates. The temporary suppression of symptoms poses a threat to biosecurity. If a plant is symptomless and granted entry, then it could lead to a new pathogen establishing.

Very little is known about the mechanism of action of phosphite induced inhibition in *Phytophthora* or how isolates are resistant to phosphite. If we understand how tolerant isolates behave differently to sensitive isolates then it would be easier to deal with resistant isolates in the field. Limited knowledge is available of phosphite induced changes at the gene level in the pathogen. On-going work which builds on that presented here will investigate transcriptional responses in three tolerant and susceptible *P. cinnamomi* isolates from this research (Chapter 4). The isolates have been exposed to 0, 30 and 60 µg/mL phosphite for four hours and gene expression will be analysed to compare differences between the phosphite tolerant and sensitive isolates.

## 6.3 Final summary

This is the first time isolates of *P. cinnamomi* from New Zealand avocado orchards have been tested for their sensitivity to the widely used fungicide phosphite. The results from this study showed isolates had developed decreased sensitivity to phosphite, supporting work with *P. cinnamomi* from avocado orchards in Australia (Dobrowolski *et al.*, 2008) and South Africa (Duvenhage, 1994; Ma & McLeod, 2014). These results are concerning for the avocado industry worldwide as phosphite is largely relied upon to manage avocado root rot. It is also concerning for the management of other phytophthora diseases with phosphite. This study has increased our understanding of the phosphite sensitivity of various *Phytophthora* species in New Zealand and California, USA.

The continued monitoring of *Phytophthora* isolates from sites treated with phosphite, such as avocado orchards, will improve our understanding of the development of fungicide resistance. By monitoring for fungicide resistance, we will hopefully avoid the development of fully resistant isolates.

# Appendix A.

# Preparation of culture media

---

**1. Modified Ribeiro's Minimal Media (RMM)** (Ribeiro *et al.*, 1975)

| | |
|---|---|
| Glucose | 9.0 g |
| Asparagine | 0.1 g |
| Potassium Nitrate ($KNO_3$) | 0.15 g |
| Monopotassium phosphate ($KH_2PO_4$) | 1.0 g |
| Magnesium sulphate heptahydrate ($MGSO_4$-7H2O) | 0.5 g |
| Calcium chloride ($CaCl_3$) | 0.1 g |
| Microelement stock solution | 1 mL |
| Iron stock solution | 1 mL |
| Thiamine-HCl stock solution | 1 mL |
| MES Hydrate | 5.86 g |
| Agar | 17 g |

Micro element stock solution

| | |
|---|---|
| Sodium molybdate dihydrate ($NaMoO_4$) | 41.1 mg |
| Zinc sulphate heptahydrate ($ZnSO_4$-7H2O) | 87.8 mg |
| Copper sulphate pentahydrate ($CuSO_4$-5H2O) | 7.85 mg |
| Manganese sulphate monohydrate ($MnSO_4$-H2O) | 15.4 mg |
| Sodium borate ($Na_2B_4O_7$) | 0.5 mg |

Iron Stock solution

| | |
|---|---|
| Iron chloride hexahydrate ($FeCl_3$-6H2O) | 44.44 mg |
| Ethylenedinitrilo tetracetic acid (EDTA) | 2.6 g |
| Potassium hydroxide (KOH) | 1.5 g |

Thiamine-HCL stock solution

| | |
|---|---|
| Hydrochloric acid (HCl) | 30 mg |
| 25% ethanol (pH 3.5) | 300 mL |

***Method***

The first 6 ingredients were weighed out disolved in deionised water using a magnetic stirrer. The three stock solutions were added and the solution was made up to the final volume with deionised water. MES Hydrate buffer was added and dissolved. To make agar, RMM was amended with 17g of Bacto agar per 1000mL before the pH was adjusted. The pH was adjusted to 6.2 with $6_N$ KOH before autoclaving at 121˚C for 15 minutes. The media contained 7.35 mM phosphate in the form of monopotasium phosphate.

2. **10% Carrot agar + Ampicillin, Pimaricin, Rifampicin, Nystatin and Hymexazol (CRNH)** (Dick *et al.*, 2006; Jeffers & Martin, 1986)

| | |
|---|---|
| Frozen carrots | 100 g |
| Agar | 15 g |
| Ampicillin stock solution (25 mg/ml) | 8 mL |
| Nystatin (dissolved in 1-2 mL 90% ethanol) | 0.05 g |
| Rifampicin (dissolved in 1-2 mL acetone) | 0.01 g |
| Pimaricin | 0.4 mL |
| Hymexazol stock solution (5 mg/mL) | 10 mL |

***Method***

Blend carrots with approximately 200 mL deionised water. Filter through several layers of cheese cloth. Dissolve agar in 500 mL deionised water in the microwave on high for 3 minutes. Combine filtered juice and agar and make up to volume with deionised water. Autoclave for 15 minutes at 121˚C. Once the bottle has cooled to approximately 50˚C, add the ampicillin and hymexazol stocks, pimaricin and the dissolved nystatin and rifampicin solutions. Ensure all solutions are mixed thoroughly in the media. Pour the plates.

3. **10% Carrot agar (CAD)** (Dick *et al.*, 2006)

| | |
|---|---|
| Frozen carrots | 100 g |
| Agar | 15 g |
| Deionised water | 1000 mL |

***Method***

Blend carrots with approximately 200 mL deionised water. Filter through several layers of cheese cloth. Dissolve agar in 500 mL deionised water in the microwave on high for 3 minutes. Combine filtered juice and agar and make up to volume with deionised water. Autoclave for 15 minutes at 121˚C.

4. **10% Carrot agar (CAD) with cellophane**

***Method***

Make CAD agar. Place cellophane circles (already cut to fit plates) into deionised water. Microwave for 10 minutes. Lay one cello circle onto each agar plate aseptically.

5. **10% V8 Agar** (Erwin & Ribeiro, 1996)

| | |
|---|---|
| Campbells V8 juice | 100 mL |
| Calcium carbonate ($CaCO_3$) | 1 g |
| Deionised water | 900 mL |
| Agar | 17 g |

***Method***

The V8 juice and $CaCO_3$ are mixed with 500 mL deionised water on a magnetic stirrer for 5 minutes. The solution is filtered using filter paper and vacuum flask and funnel, changing filters as necessary. The supernatant is made up to 1000 mL with deionised water. To make agar, the agar is first dissolved in deionised water and this is used to make up the supernatant to the final volume. The pH was adjusted to 6.0 using $6_N$ KOH before autoclaving. Autoclave for 15 minutes at 121˚C.

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