Biosecurity New Zealand
Tiakitanga Pūtaiao Aotearoa

19207 Biological Control of *Phytophthora agathidicida:* Desk-top literature review

Final Report

Biosecurity New Zealand Technical Paper No: 2019/14

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ISBN No: 978-1-98-859495-8 (online)
ISSN No: 2624-02033 (online)

June 2019

New Zealand Government

Ministry for Primary Industries
Manatū Ahu Matua

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Executive Summary

Phytophthora agathidicida is characterised by its life history of hemi-biotrophy (i.e. using kauri root tissue to survive and complete its life cycle). Once kauri trees have died due to kauri dieback, the roots remain infected by *Phytophthora agathidicida.* This leaves behind a legacy of root-inhabiting survival structures, which for *P. agathidicida* are oospores in the root cortical cells and sporangia on the root surface (Figure 1). Infection occurs at the root–pathogen interface, and therefore control of kauri dieback needs to be targeted in the rhizosphere. Biological control holds some promise for being able to deliver such a targeted intervention.

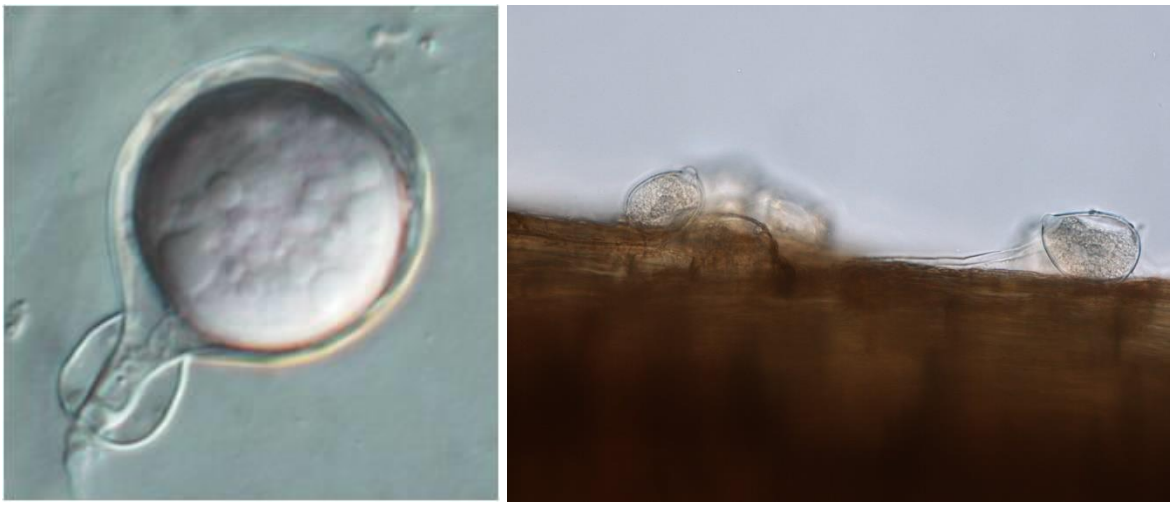

Figure 1. Oospore of *Phytophthora agathidicida* (37µm in diameter) (image by M.A. Dick) and sporangia (23 µm) of *P. agathidicida* forming on the surface of a kauri root (image by C.M. Probst).

The term “biological control” has been applied to the use of “organic” or “natural” products extracted or fermented from a biological source. While such products may mimic the function of the biological source, *non-living* agents are referred to as *biopesticides* and *biofertilisers*. For the purposes of this review, biological control refers to introduced or resident *living* micro-organisms. By this definition, a biological control agent (BCA) is used to suppress the activities and populations of one or more plant pathogens.

The remit of this literature review is to provide the Kauri Dieback Programme with the background information necessary to make informed decisions on the value of conducting research into the use of biological control and alternative natural treatments for the treatment of kauri dieback.

There is a body of literature addressing the microbes that interact with those self-fertile *Phytophthora* species that can produce oospores (e.g. *P. agathidicida*). However, very few of the microbes studied have been operationalised to a forest-scale intervention. Most of the research is focused on horticultural, high-end food crops such as capsicum and understanding the mode of action of the BCA against *Phytophthora capsica, P. cactorum* and *P. cinnamomi*, with little consideration given to non-target impacts on native/indigenous symbiotic micro-organisms.

The focus of the research efforts on BCAs has been on competitive saprophytes, facultative plant symbionts and facultative hyper-parasites. Ten to 12 organisms, mainly bacteria and ascomycetes, are the focus of biological control research efforts (e.g. *Bacillus*, *Burkholderia*, *Lysobacter*, *Trichoderma*, *Paecilomyces*, *Gliocladium*). These organisms are easy to culture in synthetic media and readily produce spores, and so they have been attractive for biological control studies because their populations are easily manipulated. The other major focal group in biological control research is the mycorrhizal fungi, with an emphasis on *Pisolithus* and *Glomus* spp.

Two groups of BCAs were studied by the Auckland Botanic Gardens: mycorrhizal fungi and *Trichoderma*. Mycorrhizal fungi are a group of symbiotic fungi that live inside or enclose the roots of plants. In New Zealand kauri, there is a suite of native, indigenous arbuscular mycorrhizal (AM) fungi and dark septate endophytic (DSE) fungi that inhabit the roots and root nodules of healthy kauri. Overall, the addition of the mycorrhizal formulation enhanced kauri growth, but the mode of action remains unknown, because there was no *post hoc* analysis of the kauri root colonisation with or without the mycorrhizal inoculation in the presence/absence of the root pathogen.

Trichoderma spp. (Hypocreales, Ascomycota) are aggressive parasites of other fungi, which also naturally occur with living and dead kauri roots. Overall the addition of Trichoflow (*Trichoderma atroviride* LU182) enhanced the growth of kauri. However, the mode of action is not known, because no *post hoc* analysis was made of the kauri seedlings after inoculation with *P. agathidicida* with or without the *Trichoderma* product.

New Zealand leads the world in the application of *Trichoderma* research to managing broad-acre crop diseases in the Pacific and Southeast Asia. The only forest-scale soil treatment of *Phytophthora* has been carried out with *Trichoderma.* This demonstrated that the BCA can directly protect the host plant, but also reduces the amount of secondary inoculum produced. Therefore, there is considerable hope that the knowledge of these BCAs is transferable to kauri forest once the potential risk of non-target impacts on native kauri root microbiota is evaluated.

Soil-focused BCA products are generalists and have been demonstrated to attack both pathogenic organisms and potentially beneficial microbes in the soil. Therefore, it is necessary to overcome the following constraints before BCAs for soil-borne tree diseases in native kauri forests are adopted.

- Kauri's native soil endophyte bioprotection ecology and biology needs to be understood so that the potential non-target impacts of introduced, exotic, generalist mycoparasites can be evaluated, to see if they have any flow-on effects to the native microbial biodiversity.
- There is the potential for spill-over from introduced AM inocula to kauri and other native podocarps, such that these introduced species may displace native endophytes.
- There is also evidence that exotic ectomycorrhizal species can invade (and have invaded) podocarp forest in New Zealand from pine forests independent of their original pine host. Therefore, the potential risk of exotic ectomycorrhizal inoculum (e.g. *Pisolithus*) to invade and displace kauri mycorrhiza in kauri forests needs to be evaluated. Also, with our unique suite of endemic *Pisolithus* spp. in New Zealand, any *Pisolithus* species considered for forest-scale introduction would be classified as a New Organism.
- The unknown side-effects of nutrient enrichment on kauri growth and nutrient-cycling processes (carbon–nitrogen) through the broad-acre use of biofertilisers will prevent their immediate application and uptake until pot- and glasshouse trials are carried out to understand the role of nutrient enrichment of kauri growth.

1 Introduction

1.1 Background

Phytophthora diseases pose a threat to forest ecosystems around the world. In the context of global climate disruption and the global movement of plant products, there is an increase in the risks posed by this unique group of chromists (Hansen 2015). Once tree roots are infected, there is the enduring soil- and root-based legacy of the prevalence of long-lived survival structures, such as oospores of *Phytophthora*, residing in the roots (e.g. Fichtner et al. 2011).

For *Phytophthora agathidicida* there is evidence of this hemi-biotrophic tendency, with oospores forming readily in the roots of deliberately infected kauri seedlings and sporangia forming on the surface of kauri roots (Bellgard et al. 2016; Figure 2). Oospores play a key role in perpetuating *Phytophthora* diseases in the infested forest site, as the infected roots remain in the soil after the host has died, and remain viable and able to transfer infection to healthy kauri seedlings (Bellgard et al. 2013). Little is known about the factors controlling the activity of oospores in kauri forest. What little knowledge we have comes from indirect soil bioassays (e.g. the extended soil bioassay). In this protocol, we aim to activate oospores contained in kauri roots by using a drying and wetting phase, as the use of alternate wetting and drying cycles has been shown to be effective in stimulating germination of *Phytophthora* oospores (Sneh et al. 1977).

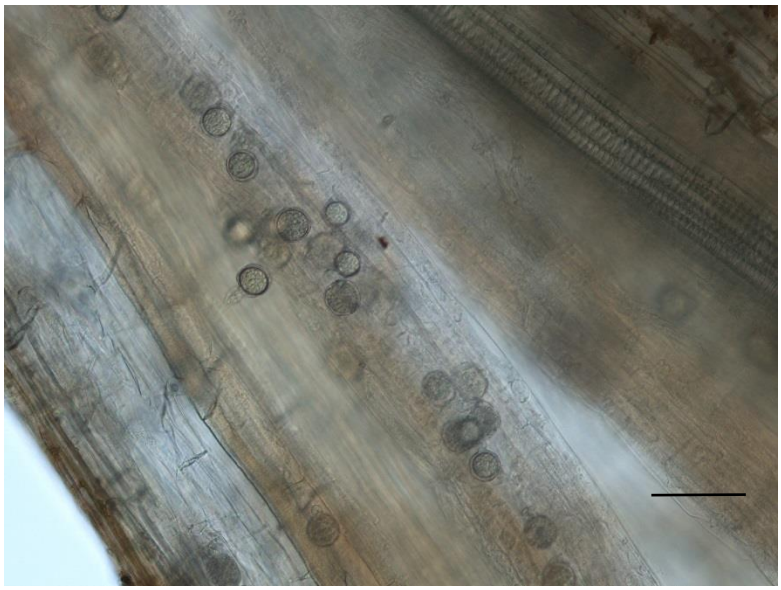

Figure 2. Oospores of *Phytophthora agathidicida* formed in the cortical cells of roots of kauri seedlings deliberately inocuated with *P. agathidicida* as part of a screening study (photograph by C.M. Probst), scale bar 70 µm.

Currently the control programme for kauri dieback involves trunk injection with phosphite. However, there are issues associated with relying solely on phosphite for the long-term management of kauri dieback, as this remedial application is intensive and difficult to up-scale for treatment over landscape and forest scales. The disease needs to be controlled at the forest level to maintain the quality and abundance of habitat of the kauri forest. However, the usual agronomic and horticultural practices that rely on fertilisers and pesticides are not available for broad-scale application to a forest habitat due to constraints such as unforeseen non-target impacts and the social licence to operate. Any potential control methods also need to take into consideration the side-effects of the implementation pathway to get the control agent into the root-infection zones (i.e. through the surface humus layer that accumulates under kauri trees and into the soil, and into the roots of kauri infected with oospores).

The growing awareness of the environmental pollution, chemical residues in surface water, and unexpected side-effects on vertebrates (including genetic defects) associated with some agrochemicals has led to political pressure to remove the most hazardous chemicals from the market

(Pal & McSpadden Gardener 2006). Also, the geographical distribution of kauri, the spread of kauri dieback and the latent phase of disease expression may preclude successful application of chemical treatments because of the scale of the necessary applications. The term “biological control” (and the abbreviated term “biocontrol”) in plant pathology applies to the use of biological antagonists, usually microbial, to suppress diseases, as well as the use of host-specific pathogens to control weed populations. More broadly, the term has been applied to the use of “organic” or “natural” products extracted or fermented from a biological source. While such products may mimic the function of the biological source, non-living agents are referred to as “biopesticides” and “biofertilisers” (Chandler et al. 2011).

For the purposes of this review, “biological control” refers to the purposeful use of introduced or resident living micro-organisms. A biological control agent (BCA) is used to suppress the activities and populations of one or more plant pathogens. This may involve the use of microbial inoculants to suppress *P. agathidicida*, or managing soils to promote the activities of native soil- and plant-associated organisms that modulate the ability of a soil system to suppress disease (e.g. Jaiswal et al. 2017). BCAs therefore offer a promising and environmentally friendly method to control plant pathogens. However, applications are still limited because of the lack of consistency of BCAs when they are applied in the field (Gerbore et al. 2014).

1.2 Microbial interactions

Micro-organisms, like other living entities, interact with each other as they compete for resources such as space and food. Odum (1953) originally summarised some of the types of interactions between micro-organisms and provides a framework to explain how and why biological control works. Elements of these “assumptions” have been ratified through more recent research.

- *Mutualism* is an association between two or more taxa where both partners benefit. The relationship is characterised by a high level of specificity between the partners (e.g. mycorrhizae; see section 3).
- *Photo-cooperation* is another form of beneficial partnership, but without the inter-dependency, and typifies the life history of most microbial BCAs (Pal & McSpadden Gardener 2006).
- In contrast, *antagonism* between organisms results in a negative outcome for one or both entities.
- *Competition* within and between species can result in decreased growth, activity, fecundity and/or nutrition of the competing entities. Biological control can occur when non-pathogenic strains compete for space and resources with pathogenic strains in and around a host plant (e.g. biological control of *Botrytis cinerea*; Wang et al. 2018).
- *Parasitism* is a form of partnership in which the host is exploited by the parasite over a prolonged period. The parasite in some cases is smaller and has a much shorter life cycle than the longer-lived hosts. Hyper-parasites like *Darluca filum* (Biv.) Berk. are an example of a BCA against rust fungi like the blackberry rust *Phragmidium violaceum* (Schultz) G. Winter (Yuan et al. 1998).
- *Predation* refers to the killing of one organism by another for nutrition and is typified by the nematode-trapping fungi (Klironomos & Kendrick 1995).

Some of these types of interaction are potentially occurring simultaneously within a kauri root, and/or closely adjacent to a kauri root, with the combination of the consequences of these interactions determining the effects on the kauri tree.

1.3 Mechanisms of biological control

As will be clear from the above discussion, biological control can result from a diverse array of interactions between a range of organisms. In all cases the objective of biological control is to antagonise plant pathogens by inhibiting their growth, activity, fecundity, and/or infective capability. Direct antagonism involves intimate physical contact between the pathogen and the BCA. Hyper-parasitism by obligate (restricted to living as a parasite, with no saprotrophic ability) parasites could be considered the most direct form of antagonism (e.g. *Darluca filum*, a hyper-parasite of rust fungi).

Indirect antagonisms result from a range of mechanisms that do not involve sensing or targeting a pathogen, but rather constrain the life history of the pathogen through an indirect mode of action.

Stimulation of plant host defence pathways by a non-pathogenic BCA is the most indirect form of antagonism (Pal & McSpadden Gardener 2006). Antibiotics are another form of indirect antagonism, as these microbial toxins, at low concentrations, can poison or kill plant pathogens (e.g. Smith et al. 1993). To be effective, antibiotics must be exuded in sufficient quantities near the pathogen to result in a biocontrol effect.

Other extra-cellular secretions can also decrease pathogen growth and/or fecundity. For example, many micro-organisms secrete lytic enzymes that can hydrolyse a wide variety of polymeric compounds, including those composing cell-wall materials such as chitin, proteins, cellulose, hemi-cellulose and DNA (Bohlin et al. 2010). Bacteria are characterised by their ability to synthesise small, high-affinity, iron-chelating compounds under low iron conditions to assist with growth and reproduction (Neilands 1995).

1.4 Aims

The remit of this literature review is to provide the Kauri Dieback Programme with the background information necessary to make informed decisions on the value of conducting research into the use of biological control and alternative natural treatments for the control of kauri dieback. The review addresses the following questions.

1.4.1 General questions

1 Is there any laboratory and field research that has shown biological control agents (BCAs) and other natural products/remedies that are effective (or not) against *Phytophthora* species (*Phytophthora* oospores) and/or improve (or not) the growth and overall health of the plant host?
2 How are BCAs and natural products applied in the environment: under what circumstances were they applied (e.g. forest, field/plantation trials, greenhouse/nursery, laboratory) and over what scale (e.g. landscape deployment or spot treatments)?
3 Are the tools practical and feasible to use in a natural ecosystem?
4 What are the potential non-target impacts that will need to be considered in a natural ecosystem?
5 What are the potential human health impacts of introducing a BCA or a natural product/remedy?
6 What is the social acceptability of introducing a BCA or a natural product/remedy in a natural ecosystem?
7 What are the gaps in knowledge and barriers that may prevent the tools being deployed in a natural ecosystem and being effective against *Phytophthora agathidicida* and/or improving the health of kauri?
8 Can BCAs survive in kauri soil?
9 What is the feasibility of using biocontrol products as a bio-activator to break the *Phytophthora* oospore dormancy?

These general questions are addressed in section 3.

1.4.2 Specific questions

1 Is there any scientific literature that suggests that the active ingredients of Trichoflow®, Unloc®, and Mycormax® could potentially improve tree health or show a degree of efficacy against *Phytophthora* species?
2 Is there any literature to suggest that Trichoflow, Unloc and Mycormax could survive in kauri soil (via inoculation of natural soils)?
3 Is there any literature to suggest that the active ingredients of Trichoflow, Unloc and Mycormax will have any non-target impacts?
4 What is the label use of these three products, and are there any implications/barriers in applying them to a natural ecosystem?

These specific questions are addressed in section 3.1.

2 Methodology

2.1 Literature review

We implemented a systematic review process to address the general and specific questions posed by the Planning and Intelligence Team of the Kauri Dieback Programme to enable them to make informed decisions on the value of conducting research into the use of biological control against kauri dieback.

Our literature review had four steps:

1 searching databases for titles and abstracts
2 selecting articles for inclusion in the review process,
3 classifying the selected articles on the basis of application (i.e. forest, field/plantation, greenhouse/nursery, laboratory)
4 synthesising and validating the articles according to whether the studies have been carried out in a natural ecosystem context.

Our review does not focus on the replicability of the studies, but rather is a comparative and functional synthesis to illuminate contextual issues associated with the potential application of BCA technology to control kauri dieback. The products are assessed based on their forest-based practicality and feasibility, the potential non-target impacts on forest biota, the potential risks to humans and wildlife, and their acceptability and ability to gain a social licence to operate.

The review involved searching the following databases:

- Google
- CAB Abstracts
- The University of Auckland Library.

The primary search terms were:

- Phytophthora
- Biological control
- Mycormax
- Trichoflow
- Unloc.

The initial search yielded 94 items. We reviewed the 53 papers that were relevant, and assessed their relevance, resulting in 53 studies that we categorised according to:

1 author
2 year
3 country of study
4 host organism
5 *Phytophthora* species
6 experimental context (laboratory, greenhouse, field)
7 direct/indirect effects
8 biological control organism
9 impacts
10 success
11 salient notes.

2.2 Expert consultation

Direct consultation was carried out with experts in mycology and new organisms. The scientists consulted were:

- Dr Peter Johnston, Manaaki Whenua – Landcare Research (MWLR) – invasive potential of the exotic ectomycorrhizal (EM) fungus *Amanita muscaria*
- Dr Clarke Ehlers, Environmental Protection Authority – new organism assessments and the status of *Pisolithus tinctorius*

- Teresa Lebel, Botanic Gardens of Melbourne – the presence of EM fungi *Pisolithus* and *Scleroderma* in New Zealand
- Dr Peter Buchanan, MWLR – current taxonomy of *Scleroderma cepa.*

3 Results

3.1 Results of review

The results of the review are shown in the Appendix and summarised in Table 1. Overall, we found only 11 published studies that had an in-field evaluation of biological control efficacy. More than 75% of the studies demonstrated "laboratory proof of concept", 77% demonstrated efficacy of biological control against *Phytophthora* species in the greenhouse, and 20% tested and evaluated the efficacy of biological control of *Phytophthora* species in a field environment. No studies focused on oospore deactivation of *Phytophthora*.

Table 1. Results of literature review, stratified according to application context and the principal functional BCA group.

Category	Field/forest	Greenhouse	Laboratory
Ascomycete	5	15	19
Arbuscular mycorrhizal fungi (AMF)	2	4	1
Bacteria	1	13	16
Actinomycete	0	4	3
Other	3	6	3

The other key features that stand out from a meta-analysis of the reviewed papers are:

- the most intensively studied crop is capsicum (>20 studies)
- the most studied *Phytophthora* species for biological control is *P. capsici* (host = capsicum, >20 studies), followed by *P. cactorum* and *P. cinnamomi*
- the most studied BCAs are *Trichoderma* spp. (>20 studies).

3.2 Mycorrhizae

3.2.1 Introduction to mycorrhizal functional diversity

Most biological control has focused on a limited number of bacterial and fungal genera due to the ease with which they are cultured. Many microbes are difficult to manipulate in culture, but mycorrhizae form mutualistic partnerships involving an association between a fungus (myco-) and a plant root (-rhizo). These include mycorrhizal fungi like *Pisolithus* and *Glomus* species, which have been shown to limit infection by plant pathogens.

These associations between plant and fungus are symbiotic and almost entirely mutualistic in that both fungus and plant benefit (Bellgard & Williams 2011). The benefits seem predominantly connected to improved nutrition of the host and the infecting agent, but extend to attenuation of hormonal balance, physical protection, chemical protection, and modification of other rhizosphere organisms that influence competition for substrates.

Currently, mycorrhizae are categorised into seven structural types of equal taxonomic rank: arbuscular mycorrhizae (AM), orchid mycorrhizae, ericoid mycorrhizae, ectomycorrhizae (ECM), ectendo-mycorrhizae, arbutoid mycorrhizae, and monotropoid mycorrhizae (Bellgard & Williams 2011). Arbuscular mycorrhizae have turned out to be much more diverse in structural features than previously thought and are associated with two families of trees: Araucariaceae and Podocarpaceae. In contrast, there is much structural homology exhibited among ecto-, ectendo-, arbutoid and monotropoid mycorrhizae, and together they comprise a distinct ECM lineage. Imhof (2009) proposes orchid mycorrhizae as a third, distinct lineage from AM and ECM. Mycorrhizae, particularly AM fungi, have also been associated with antagonistic effects on soil-borne pathogens (e.g. *Phytophthora*) (Bellgard & Williams 2011).

The major taxonomic revision of the fungi constructed by Hibbett and Matheny (2009) resulted in significant revision of the traditional phylum Zygomycota. The sub-phylum Zygomycotina and class Zygomycetes have been discontinued, and the phylum Glomeromycota now encompasses the AM fungi. This revision is one of the most significant findings in mycorrhizal research in the last 10 years. The AM fungi are considered primitive because of their simple spores, lack of sexual reproduction, the relatively few species of these fungi, and their association with a wide diversity of plants (Bellgard & Williams 2011).

ECM fungi include at least 6,000 species, primarily of Basidiomycetes but with some Ascomycetes (Brundrett 2002), as well as hypogeous (below-ground) fungi forming ECM relationships (Castellano & Beever 1994; Smith & Read 2008). It is likely that a rapid diversification of the Basidiomycetes occurred in the Cretaceous period, as plants with ECM became increasingly important (Brundrett 2002). The large Basidiomycete ECM families Amanitaceae, Boletaceae and Russulaceae probably arose then, and are still major ECM partners associated with species such as kānuka and mānuka in Australia and New Zealand (Bellgard 1991; Davis 2008), *Eucalyptus* (Bowen 1981), and *Pinus* (Marais & Kotzé 1976). In contrast to two related families, Cupressaceae and Pinaceae, the Araucariaceae do not form ECM (McKenzie et al. 2002).

3.2.2 Review of existing data on kauri mycorrhizae

Padamsee et al. 2016

New Zealand kauri is the only species of the endemic Araucariaceae family, and like the other southern conifers (Podocarpaceae) possesses AM fungi housed in spherical root nodules (Figure 3).

Figure 3. Amber-, golden-, and chocolate-coloured root nodules of kauri. Un-suberised nodules are highlighted by arrows.

AM fungi associated with kauri roots have co-evolved with kauri for many millions of years, since the Devonian period (Brundrett 2002). Padamsee et al. used light, scanning and electron microscopy to characterise colonisation, and 454-sequencing to identify the AM fungi associated with kauri roots and root nodules. Representatives of the Glomeromycota were identified via high-throughput sequencing, and some of these have not been previously known to science (Padamsee et al. 2016).

Subsequent research conducted at MWLR identified another mycorrhizal form. These mycorrhizae are classified as dark septate endophytes (DSE) and were observed growing out from kauri root nodules that had been collected from the field, had their surface disinfested, and allowed to grow on water agar (Figure 4). The fungi were identified as belonging to the taxonomic grouping called the Helotiales (class: Leotiomycetes) within the division Ascomycota. The isolated fungi were grown into pure cultures and used in challenge bioassays against *P. agathidicida.* Several of the isolates recovered

from kauri root nodules showed strong antagonism against *P. agathidicida* (Figure 4) (unpublished data, L. Jackson).

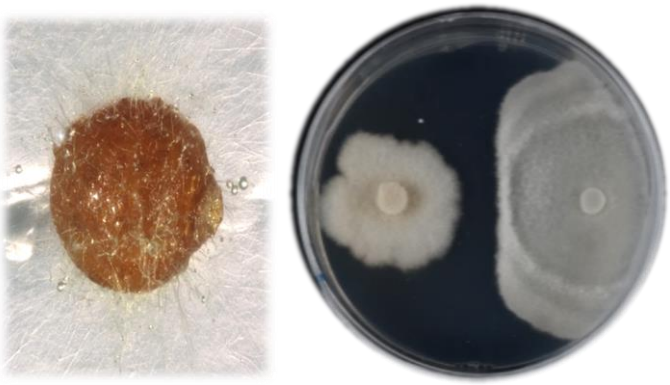

Figure 4. Left: dark septate endophyte (DSE) hyphae growing out from a kauri nodule. Right: *Pezicula* sp. on the left-hand side of the plate, inhibiting *P. agathidicida* in a pure colony challenge bioassay (both images by L. Jackson).

3.2.3 Active ingredients of exotic formulations and tree health improvement identified from the Auckland Council report

Background

The Auckland Council evaluated the potential for 10 commercial products to inhibit the growth (via an *in vitro* laboratory assay) and pathogenic effects of *P. agathidicida* via a pot trial (Auckland Council 2017). The results of the *in vitro* study identified that at 4 days' growth, *P. agathidicida* was enhanced by Mycormax® (AM plus ECM fungi) and Unloc® (biofertiliser). Trichoflow® (*Trichoderma atroviride*, a mycoparasite) was observed to reduce growth of *P. agathidicida* after 2 days of growth.

With respect to the pot trials, overall kauri health and "bio-protection" was demonstrated to be enhanced by Unloc, Trichoflow and Mycormax (Auckland Council 2017). This result is contrary to the result obtained from the *in vitro* study, which showed that Unloc enhanced growth of *P. agathidicida* in plate culture studies. The criteria used to evaluate bio-protection potential for these studies was dry matter accumulation after treatment compared to an untreated control. The effect of the potting mix and its interaction with the applied products needed to be controlled for, because porosity, water-holding capacity, texture and bulk density all play a role in the behaviour of soil micro-organisms.

Critique of Auckland Council report

The observed growth enhancement of kauri seedlings in the presence of *P. agathidicida* identified from the Auckland Council report is difficult to interpret with respect to the unique effects enabled through bio-protection versus side-effects (e.g. nutrient enrichment) of the product formulations. The principal issue is the lack of data on the presence or absence of the microbial additives after the experiment. For example, Unloc enhanced kauri seedling growth due to a presumed biofertilisation effect. Similarly, through the addition of Mycormax there was an enhanced seedling growth response, but there was no assessment of root colonisation after the treatment to identify the mode of action. There was also no evaluation of the mycorrhizal status of the kauri seedlings before or after the treatment with Mycormax, so it cannot be determined whether it was the inoculum (2%) that had an effect, or the other "inert" products in the Mycormax formulation (98%).

Finally, Trichoflow was also associated with enhancement of seedling growth, but without data on the presence of the *Trichoderma* sp. at the end of the experiment it may not be evidence of bio-protection, but of some other ingredient in the BCA formulation. Further discussion about each of these three beneficial BCAs is presented in the next section.

Trichoflow

Trichoderma spp. (Hypocreales, Ascomycota) are aggressive parasites of other fungi (Druzhinina et al. 2018). Species of *Trichoderma* can also feed on dead fungi (mycophagy) and efficiently degrade plant material (Druzhinina et al. 2011). *Trichoderma* species are one of the major "by-catch" soil fungi recovered from bait tissues used in the conventional soil bioassay for *P. agathidicida* (unpublished data, SE Bellgard). Earlier glasshouse studies in New Zealand have shown that *Trichoderma* formulations containing *Trichoderma atroviride* and *T. virens* can enhance kauri seed germination and increase plant height compared to control treatments (Hill & Chinno-Valle 2015).

Trichoderma species have been reported to control soil-borne diseases caused by *Phytophthora* in containerised systems (Widmer 2014), and this was the effect observed in the research carried out by Auckland Council. This product is usually applied in the nursery/greenhouse, where the BCA is added to propagation media before sowing or setting cuttings to establish the *Trichoderma* population prior to germination or root initiation.[1] Application is enhanced with an additional organic formulation (e.g. Nitrosol Organic®), which is utilised as a food resource to boost the *Trichoderma* population.[2] Studies on the behaviour of *P. cinnamomi* in soil have identified *Trichoderma* as an antagonist involved in the stimulation of oospores, lysis and parasitism of hyphae (Malajczuk & Theodorou 1979) and chlamydospore production, which is one of the principal inoculum sources in *P. cinnamomi* (Kelley 1977).

Unloc

Biofertilisers like Unloc keep the soil environment rich in micro- and macro-nutrients via nitrogen fixation, phosphate and potassium solubilisation or mineralisation, release of plant-growth-regulating substances, production of antibiotics, and biodegradation of organic matter contained in soil (Bhardwaj et al. 2014). According to the manufacturer, other soil conditioning amendments contained in Unloc cause changes in the surface tension of hydroscopic water in soil by freeing the hydrogen bond and releasing the colloidal particles to move and create micropores. The manufacturer claims that Unloc stimulates massive amounts of oxygen and nutrients for beneficial bacteria and fungi such as AM (Auckland Council 2017). The major nutrients contained in Unloc are nitrate, calcium, ammonium, potassium and carbon sources – all essential nutrients for plant and microbial growth. Application requires a mix of 1 part concentrate to 200 parts of water, with a maximum recommended application of 30 L per hectare.

Mycormax

Mycormax contain spores of both ECM and AM fungi (Auckland Council 2017). The ECM species is listed as *Pisolithus tinctorius* (15,300 colony-forming units per gram, cfu/g) (Auckland Council 2017). The current taxonomic determination of the active ingredient in living microbial products is very important, as their taxonomy is intrinsically linked to the determination of whether the microbe(s) are present or not in New Zealand. For example, *Scleroderma cepa* is a mycorrhizal fungus, which was present in Northland, Auckland, Coromandel, Waikato, Bay of Plenty and Taupō when Gadgil compiled the *Fungi of New Zealand* Volume IV (Gadgil 2005). However, it is now clear from molecular data that *S. cepa* is a species agglomeration, and that *S. cepa* s.l. is probably not present in New Zealand. With respect to the sachets of Mycormax, *Pisolithus tinctorius* was determined to be a "New Organism" based on the findings of a formal section 26 determination carried out on behalf of MPI (personal communication, C. Ehlers, Environmental Protection Authority, August 2018). Therefore, the ramifications of this determination will impinge on the future release status of this product.

With respect to the taxonomic position of the AM fungi, the nomenclature has also changed dramatically since 1990, when the order Glomales was first proposed by Morton and Benny (1990)

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1 TrichoProtection™ product information sheet 2018. A living barrier protecting against plant pathogens.

2 TrichoProtection™ product information sheet 2018. A living barrier protecting against plant pathogens.

(see Imhof 2009). Therefore, establishing the minimum expectation of certified, independent validation of the microbial composition of the product that makes up the constituent of the labelled product is a necessary step in the risk evaluation process and the determination of New Organism status.

The role of ECM in limiting infection by *Phytophthora* (mainly *P. cinnamomi*) has been reported for a wide range of tree hosts and fungal symbionts (Malajczuk 1983). The mode of action of ECM against *Phytophthora*, enabling a reduction in *Phytophthora* inoculum, has been attributed to one or a combination of the following factors:

- alteration of the root exudation pattern influencing zoospore root-sensing behaviour
- reduction in the stimulation of germination of chlamydospores and oospores
- zoospores germinating faster and more vigorously at the growing tip of non-mycorrhizal roots, which shows the bio-protection afforded my mycorrhizal colonisation
- possible secretion of antibiotics by the ECM fungi
- provision of a mechanical barrier to the pathogen in the form of a fungal mantle that may be important in protecting the root
- antagonistic mycoflora inhabiting the surface of the ECM fungal mantle, contributing to another form of microbial antagonism, with hyphae-associated bacteria causing lysis of *P. cinnamomi* hyphae (Malajczuk 1983).

The AM species in Mycormax are reported to be *Glomus intraradices* (now *Rhizophagus irregularis*) and *G. mosseae* (now *Funneliformis mosseae*), both at a concentration of 25 cfu/g. Both species are ubiquitous soil fungi that are globally distributed and two of the most important and most commonly occurring plant-associated fungi in many parts of the world (Schwartz et al. 2006). AM appear to have little or no effect on disease development, except for one positive case in which reduced disease severity was attributed to the presence of AM (Davis & Menge 1980). The tolerance of citrus seedlings to *P. parasitica* was attributed to mycorrhizal seedlings having the ability to compensate for the loss of potential growth nutrients (mainly phosphorus) in decaying roots by absorbing more nutrients from the remaining healthy roots (Davis & Menge 1980).

Also, the Mycormax product claims to improve soil structure by producing humic compounds and glomalin – a glycoprotein produced abundantly on hyphae and spores of AM fungi in soil and in roots. Glomalin was discovered in 1996 by Sara F. Wright, a scientist at the US Department of Agriculture, and has been found to contribute to carbon and nutrient cycling in deeper soils (Wang et al. 2017), but no studies are available for New Zealand. Humic compounds are organic compounds that are found in humus. Humus is the major organic fraction of soil, peat and coal. It is the non-living (so it is not covered in detail in this literature review), finely divided organic matter in soil derived from microbial decomposition of plant and animal residues. Humus contains about 60% carbon, 6% nitrogen, and smaller amounts of phosphorus and sulphur, which improve the health of both soil and plants (Stevenson 1972).

3.2.4 Any evidence for forest applications

Trichoflow is formulated for nursery-scale application. Unloc is formulated for horticultural and broad-acre applications, with examples including kiwifruit vines and grass pastures. Mycormax is formulated for horticultural application, with crops including apples, pears, avocados, lettuce, kiwifruit, strawberries and tomatoes. There is no evidence for forest applications of Trichoflow, Unloc or Mycormax.

3.2.5 Any evidence of non-target impacts

There is evidence that microbial mutualists have been moved outside of their native ranges and have had unexpected, non-target impacts (Schwartz et al. 2006). Species of mutualists with the potential to be widely introduced include mycorrhizal fungi. The global movement of EM fungi has been analysed, with a total of 83 species reported as being introduced to New Zealand (Vellinga et al. 2009). Some introduced EM fungi have been found to persist with introduced hosts but not to spread to native hosts (Vellinga et al. 2009). These fungi are usually associated with trees planted for forestry (e.g. in Chile; Garrido 1986). There are also examples of introduced EM fungi that persist with exotic hosts and subsequently spread to native hosts. In New Zealand, *Amanita muscaria* s.l. has spread from exotic oaks, birches and pines to native *Nothofagus* species (Johnston et al. 1998). Also, probably two other basidiomycetes have naturalised into the native vegetation in New Zealand (Johnston et al. 2018).

The potential risks of invasive competitive exclusion of native endophytes through the introduction of commercial AM formulations have been reviewed by Schwartz et al. (2006). Both the AM fungal species present in Mycormax (*Glomus intraradices* [*Rhizophagus irregularis*] and *Glomus mosseae* [*Funneliformis mosseae*]) were identified as being present in the rhizosphere of kauri roots from the study of Padamsee et al. (2016). However, the relative population levels of AM fungi that currently exist in the kauri rhizosphere, how AM fungi vary across time and space, and the environmental interactions modulating the population dynamics of the soil community need to be researched before any component of the symbiotic mycoflora is augmented.

Before any consideration is given to augmenting the kauri soil with exotic formulations of AM fungi, the present levels of AM fungi associated with kauri must be carefully quantified, and work done on how the addition of an exotic blend will alter the balance of the indigenous AM populations. Also, consideration needs to be given to the potential for non-target impacts on other AM-dependent tree species present in the kauri forest (e.g. members of the Podocarpaceae, of which 17 species are endemic to New Zealand, such as rimu, *Dacrydium cupressinum*; Dickie and Holdaway 2011). These co- and sub-dominant canopy species also have their own array of mycorrhizal associates that are part of their natural root ontogeny and have co-evolved here in New Zealand with their tree hosts, in relative isolation from other species of AM fungi (Baylis et al. 1963; Russell et al. 2002).

3.2.6 Label use of Trichoflow, Unloc and Mycormax

Trichoflow is formulated for nursery-scale application and has been shown to increase the yield of bell peppers grown in soil (e.g. Bal & Altintas 2006). It is a wettable powder formulation for application as a soil drench in the root zone. Recommendations are given for the wetting of propagation media before sowing or setting cuttings to establish a *Trichoderma* population prior to germination or root initiation. Repeat applications 14 and 28 days are suggested. Recommended application rates for seedlings and cuttings are 500 g/200 L as a soil drench. Post-planting, the recommended dosage is a rate of 1 kg per 5,000 m^2 per month (as applied in irrigation, for permanent-bed roses). No specific recommendation of a “spreader” additive to assist with dispersal was provided, as this can also have ramifications for the biology of the microbes and the efficacy of the product.

Unloc 500 is formulated for large-scale application. The recommended dosage rate is 10 L of concentrate per hectare, and it needs to be “watered in well, to enable Unloc 500 to reach the plants’ roots”. Maximum application of the product is 30 L/ha. The company recommends that “first time” users contact the supplier for the recommended dosage rates for specific applications. The product is supplied in 1,200 L containers with a view to broad-acre application.

Mycormax is formulated for horticultural application (e.g. all vegetable crops, apples, pomes and stone fruits, kiwifruit and grapes). For tree crops, the recommended rate is 2–4 kg/ha, to be applied in spring and repeated annually. Overall, only Unloc has been trialled at the field scale and is supplied in a package that could treat 40 ha (at the recommended maximum rate of 30 L/ha).

3.2.7 Bacterial antagonists

In addition to *Trichoderma* spp., the other group of microbes that were found to have good *in vitro* efficacy against *P. agathidicida* were Fulzyme® and Terracin®, with both products based on *Bacillus* species (Auckland Council 2017). Due to their large populations in soil, bacteria constitute a significant microbial component, influencing the behaviour of soil-borne *Phytophthora* propagules (Malajczuk 1983). *Bacillus* species are known to produce antibiotics *in vitro* that are antagonistic to *Phytophthora* species (Ji et al. 2013). *B. subtilis* (e.g. Fulzyme®) has also demonstrated inhibition of sexual and asexual reproductive structures of *Phytophthora* (Berger et al. 1996).

B. amyloliquefaciens (e.g. Terracin) is a spore-forming soil rhizobacterium that colonises and reproduces well near plant roots (Siemering et al. 2016), which is the infection court of zoospores of *Phytophthora* species, and in most experiments a positive correlation between hyphal lysis and soil bacterial populations has been observed (Malajczuk 1983). Antibiotic production *in vitro* by bacteria antagonistic to *Phytophthora* species is well recognised in species of *Bacillus*, *Rhizobium*, *Flavobacterium*, and *Pseudomonas* species (Malajczuk 1983). Also, *B. subtilis* is known to help

stimulate the production of cytokinin[3] *in planta* (personal communication, S. Casonata, plant pathologist, November 2018).

B. amyloliquefaciens growth begins underneath the epithelial cells of the primary plant roots and then spreads along the root surface. *B. amyloliquefaciens* controls soil pathogens by competing with them for nutrients such as iron, and by producing antibiotics or bacteria-destructive lytic enzymes (Arguelles-Arias et al. 2009). *B amyloliquefaciens* contributes to biofertilisation by the production of extracellular phytases[4]. There is evidence suggesting that *B. amyloliquefaciens* also improves the overall health of the plant by producing indole-3-acetic acid[5] (IAA). In *B. amyloliquefaciens*, the biosynthesis of IAA is responsible for plant growth promotion that is dependent on the presence of tryptophan, which is one of the main compounds present in plant root exudates.

It has also been found that this *Bacillus* species initiates a host–plant defence response, and it appears there is an up-regulation of the genes controlling systemic resistance. Many *Bacillus* strains can also induce systemic resistance in the host plant if they regulate the jasmonic acid[6] pathway (Ji et al. 2013). Hence, once "infected", the plant can defend itself from pathogenic organisms by inducing defence mechanisms resulting from the bacterial infection.

3.3 Examples of BCA applied in a natural forest ecosystem

There were very few examples of BCA applied in a natural forest ecosystem. Of the 53 articles reviewed, only one trialled a BCA in a forest setting for forest disease management. Widmer et al. (2018) studied the effects of a BCA on management of the sudden oak death pathogen, *P. ramorum.* They found that *T. asperellum* (Isolate 04-22) not only protected roots of *Viburnum* but also reduced secondary inoculum production from already protected roots. There are, however, caveats when interpreting this promising data. The target species for bioprotection was not the terminal host (i.e. tan and live oaks), but rather an understorey species, and so bioprotection of the terminal host still needs to be field validated.

The other field trials identified from this review all pertained to an agro-forestry setting. The closest affinity to a forest setting would be the Cacao trials, carried out in forest using *Trichoderma* (e.g. Villamizar-Gallardo et al. 2017).

3.4 Non-target impacts

The ecology of plant-associated microbes is currently the subject of much revolutionary research via the auspices of meta-genomic analysis and direct extraction and characterisation of microbial DNA through PCR amplicon surveys (Nesme et al. 2016). The technology exists to answer questions such as: which micro-organisms are linked to which kauri forest soils, and how do microbial assemblages interact and influence one another synergistically or antagonistically? However, gaining these insights for the kauri ecosystem forest fragments requires urgent, concerted investment and collaboration to achieve the knowledge in the timeframe necessary to support short-term interventions to protect remnant forest fragments.

Non-target risk analysis studies need to focus on answering the following questions:

- Under what circumstances and levels do BCAs exert their suppressive capacity on *P. agathidicida*?
- How do indigenous and introduced populations respond to different levels of BCAs?

[3] Cytokinin is a class of plant hormones that promote cell division.

[4] Phytases are any type of phophatase enzyme that catalyse the hydrolysis of indigestible organic forms of phosphorus

[5] Indole-3-acetic acid is the most common naturally occurring plant hormone of the auxin class.

[6] Jasmomic acid (JA) is a plant hormone that plays a central role in plant defence against herbivores. JA induces the presytemin gene in addition to various defence-related genes.

- What are the interactive benefits of the application of multiple BCAs introduced in unison (e.g. the addition of Trichoflow with Unloc)?
- What are the interactive components and dynamics of kauri host defence induction in relation to phosphite treatment and beneficial microbes (indigenous and exotic)?

3.5 Potential human health impacts

The guidance of the OECD is that biopesticides should be authorised if they pose minimal or zero risk (OECD 2003), defined as follows:

> *the microorganism and its metabolites pose no concerns of pathogenicity or toxicity to mammals and other non-target organisms which will likely be exposed to the microbial product; the BCA does not produce a known genotoxin[7]; all additives in the microbial manufacturing product and in the end-use formulations are of low toxicity and suggest little potential for human health or environmental hazard.*

The registration data required by regulators for biopesticides were designed originally for the risk assessment of chemical pesticides, and included: mode of action, toxicology, allergenicity, eco-toxicology, and host range testing (Chandler et al. 2011). This has led to significant technical and market-entry barriers for the uptake of biopesticides in the UK.

3.6 Social acceptability of introducing BCA

Societal acceptance has been achieved for the use of biological control for the management and control of exotic weeds in the conservation estate (Hayes et al. 2013). To obtain the social licence to operate with BCAs in the kauri forest, there must be robust scientific data demonstrating their efficacy and highlighting the potential side-effects. Demonstration of applications of BCAs for nursery and contained experimentation would be acceptable if all side-effects and education are provided as part of the normalisation process.

BCAs may also achieve social licence to operate if the intervention does not impinge upon the wider environment of interacting species. The necessary safeguards would need to include:

- no risk of non-target impacts on plants, soil microbes, or micro- and meso-fauna by accidental release of the product
- no risks to humans
- no aesthetic damage to the forest.

If efficacy and safety can be demonstrated in nursery applications, there should be no social barriers to the use of BCA for nursery amendments as part of plant production potting media.

3.7 Gaps in knowledge and barriers to entry

Ecological

There is no knowledge of the functional significance of indigenous mycorrhizal associations and whether they are providing some level of bio-protection. One working hypothesis is that the indigenous kauri mycoflora provides some level of unquantified protection for the tree. But the question is: are the co-evolved defences ultimately overwhelmed by a combination of the pathogen's activity and the environmental conditions (soil water conditions in summer), which has tipped the balance? In the absence of this knowledge, there is a need for careful assessment documenting the need for augmentation of natural mycorrhizal populations before any field releases of exotic BCA AM inoculum.

[7] A genotoxin is a chemical agent that damages the genetic information contained within a cell, which can lead to mutations.

Secondly, before considering the use of exotic AM inocula, the efficacy of augmenting or increasing the concentrations of local indigenous AM species should be assessed. Experimentation on how to increase and enhance the activities of indigenous microbial associations should be carried out. Also, it needs to be determined whether there are uncharacterised microbes that can act as BCAs if their concentration/prevalence were preferentially increased or augmented through strategic nutritional amendments.

Interactions between BCAs are not well understood. There are examples of suppression of the efficacy of *Trichoderma harzianum* by the mycelium of the AM fungi *G. intraradices* (Green et al. 1999). It was observed that the growth and phosphorus uptake of the external mycelium of the AM fungi were not affected by the antagonistic fungus *T. harzianum*, but that *T. harzianum* was adversely affected by the AM fungi *G. intraradices.*

Biosecurity

In relation to biosecurity aspects of phytosanitary benchmarks, only certified, sterile cultures of AM inoculum should be allowed for importation. Currently the production of the AM inoculum is carried out overseas (in California, USA). There are risks associated with unexpected “hitch-hikers”, which can inhabit the surface of hyphae and spores of mycorrhizal fungi (e.g. mycorrhizal helper bacteria; Frey-Klett et al. 2007; Labbé et al. 2014). Confirmation of the living entity displayed on the package needs to be confirmed prior to any permitting, as well as any other microbes that may have accidentally been included in the formulation due to a lack of quality assurance during fermentation. It should be the responsibility of the proponent seeking permission to import the BCA to provide validated data that supports the identity of the organisms in the BCA according to the most up-to-date taxonomy (e.g. Imhof 2009). There are potential biosecurity risks associated with misinterpretation of the current taxonomic identity of the microbes in the imported products because taxonomic uncertainty can lead to delays in the EPA’s determination of the risk status under section 26.

Technical

Firstly, how feasible is it going to be to get the BCA to the kauri root–pathogen interface for the duration necessary to have a negative impact on *P. agathidicida*? Field trials are currently underway testing the efficacy of five agri-products on private properties in Auckland (Auckland Council 2017). The results of these field trials need to be evaluated, because they may provide some insight into the application technology that needs to be operationalised if these BCAs are going to be considered for landscape-scale applications.

Secondly, which kauri forest fragments would most benefit from BCA application: diseased forest (as a remedial treatment to kill soil-borne inoculum), or healthy forest (as a prophylactic treatment), or both? Paired-plot, field-level evaluations are necessary to study host responses, but also the changes in the below-ground microbial abundances in relation to the application of the remedial BCA need monitoring, both before and after treatments.

Thirdly, what is the level of BCA necessary to achieve efficacious control of the pathogen, and how does this relate to kauri tree health? Also, how does the bioprotection afforded by exotic BCAs compare to the natural level of bioprotection currently being afforded by the native soil symbionts? It may be more appropriate to use native symbionts.

Legal

In New Zealand, the EPA evaluates new organisms, including BCAs. For example, in 2015 the EPA ruled under a section 26 determination that *Pisolithus tinctorius* is a new organism (personal communication, C. Ehlers, EPA Principal Advisor, August 2018). This ruling has implications for the potential applications of Mycormax in the environment. It has been suggested by the EPA that any future potential importer of BCA products seek independent advice from the EPA to determine the

"status" of the living entity in their BCA formulations. Also, products in New Zealand may have to be evaluated under the ACVM[8] registration process.

If an organism is present in New Zealand, there is no need for a section 26 determination, and for a BCA there is the possibility of controlled and/or uncontrolled field release if the organism is considered low risk (personal communication, C. Ehlers, EPA, August 2018). If, for example, the BCA is a generalist mycoparasite (e.g. *Trichoderma* spp.), with a broad host range, then there may be some controls imposed over the application of the product.

In Canada, pesticide registration is a complex process that involves the evaluation of ingredients, extensive testing to determine risks posed to human health and the environment, and an assessment of the pesticide's value, which is determined by assessing its efficacy. The Pest Management Regulatory Agency will only register a pesticide if there is sufficient scientific evidence to show that the product does not pose unacceptable health or environmental risks, and that it possesses value in its use. A registration is typically granted for a term of 5 years and is then subject to renewal. Pesticides regulated include: anti-microbial biocides (including preservatives, pool and spa products, and sanitisers), biopesticides (including microbial biocides and biochemical biocides), and conventional products (including agricultural products such as fungicides, herbicides and insecticides).

New European Union legislative packages relating to integrated pest management (IPM) have provided significant incentives to incorporate biopesticides into crop protection (Chandler et al. 2011). However, the focus is crop protection, not forestry or conservation applications. The new legislation gives specific status to non-chemical and natural alternatives to conventional chemical pesticides and requires them to be given priority wherever possible. Under their schedule, biopesticides, because they have no residues, are considered lower risk and are granted initial approval for 15 years rather than the standard 10 years (Chandler et al. 2011). One requirement for low-risk substances is that the half-life in the soil should be less than 60 days, and this may cause problems for the application of some microbial biopesticides, which may require longer exposure times to achieve maximum efficacy.

Social

Social licence to operate has been achieved for the use of exotic fungi and insects for the control of exotic weeds. This has included the release of BCAs into the conservation estate. The use of BCAs for managing soil pathogens in the conservation estate represents a new area of endeavour, however, and will require consultation with iwi and the public to convey the risks and rewards of applying this new control technology to the kauri forest. Ultimately, the EPA may be engaged if the organism is considered a new organism.

3.8 Can biological agents survive in kauri soil?

This question forms the basis of a separate literature review, and is one that cannot be easily answered due to our lack of knowledge of the form and function of the indigenous mycoflora associated with the rhizosphere of kauri. From the data of Padamsee et al. (2016), there are indigenous, long-time, co-evolved AM fungi associated with kauri roots and root nodules, belonging to the Archaeosporaceae and six other families of AM fungi. There are also dark septate endophytes (DSE), which inhabit the root nodules (unpublished data, L. Jackson, February 2016). Therefore, any amendment to enrich the AM mycoflora of kauri roots will be in competition with a diverse community of indigenous symbiotic fungi.

There are also the native species of *Trichoderma*, which are considered to represent a mixture of ancient indigenous lineages, more recent natural introduction, and species introduced because of human-mediated dispersal (Braithwaite et al. 2017). Any potential forest-scale application of "exotic" *Trichoderma* species needs to consider the potential for species displacement/competition, as these ecological niches are currently occupied by native species.

[8] Agricultural Compounds and Veterinary Medicines NZ Food Safety

On a global scale, data are available for EM fungi, with some studies demonstrating that introduced EM fungi are rapidly replaced by indigenous fungi (Vellinga et al. 2009). Most of the data are from forestry applications, where exotic EM fungi were introduced with the out-planted forest seedlings, but the seedlings were quickly colonised by the indigenous EM fungal associates that had the higher resident population levels, and potentially filled a larger number of microbial soil and root niches. However, evidence from Africa and Madagascar suggests that the development of eucalyptus plantations has resulted in an increase in exotic fungal species being potentially invasive in these areas due to the widespread introductions of the exotic host tree species (Ducousso et al. 2012). This observation is consistent with the results from New Zealand and the observed invasion of native *Nothofagus* forest by the exotic pine symbiont *A. muscaria*, which invades without its exotic host, and has the potential to competitively displace native, co-evolved hypogeous fungi (Johnston et al. 1998; Dickie et al. 2016).

3.9 Feasibility of using BCAs to break oospore dormancy

3.9.1 Oospore formation, dormancy and germination

Phytophthora agathidicida is a soil-borne, hemi-biotrophic plant pathogen that completes its life cycle and survives as oospores in root debris, which is ultimately broken down by soil meso-fauna and soil microflora (Bellgard et al. 2016). Oospores are sexual reproductive spores that result from the fertilisation of the oogonium (female) by an antheridium (male). Oospores of *P. agathidicida* are thick-walled, globose, and retain their viability after cool storage at 10°C for at least 9 years (unpublished data, S.E. Bellgard, November 2018). It is assumed that oospores of *P. agathidicida* have some form of endogenous dormancy but can be stimulated to germinate under high soil water conditions to produce sporangia (Figure 5).

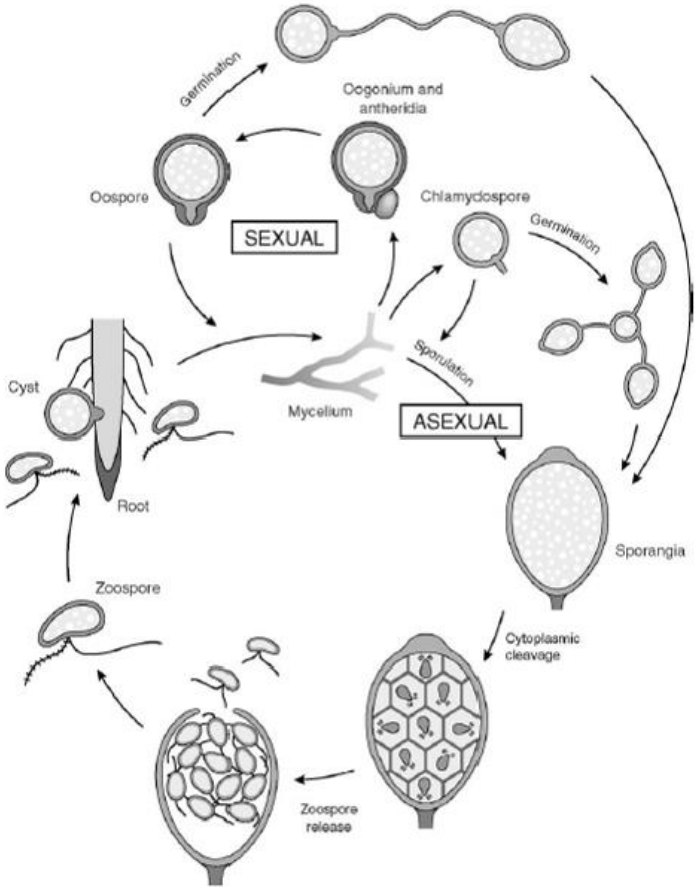

Figure 5. Generalised life cycle of homothallic *Phytophthora* sp. showing the oospore germinating to produce a sporangium (image contributed by Prof. Adrienne Hardman).

3.9.2 Survival of oospores

Although oospores are long-lived in soil, they are parasitised by various micro-organisms (Erwin & Ribeiro 1996). The following organisms have been associated with parasitism of oospores of *Phytophthora.*

- Oomycetes: *Pythium* sp. hyphae invade *Phytophthora* oospores and produce their own small oospores inside the parasitised oospore.
- Hyphomycetes:
  - *Humicola fuscoatra* hyphae can penetrate the *Phytophthora* oospores, consume the contents of the oospores, and form aleuriospores[9] in or near the parasitised oospore.
  - *Fusarium oxysporum* can invade oospores and produce chlamydospores within the parasitised oospores.
- Chytridiomycetes: *Rhizidiomycopsis japonicas* zoospores encyst on the oospore wall and invade the oospore lumen by rhizoids.
- Actinomycetes: *Actinoplanes missouriensis* hyphae lyse the oospore wall.
- Bacteria: e.g. *Pseudomonas* spp. completely digest the cytoplasm and oospore wall (Sneh et al. 1977).

3.9.3 BCAs and the ability to trigger oospore germination

Oospores are thick-walled structures that can survive for considerable periods and become propagules that initiate disease in host plants when conditions become favourable for their germination and growth (Malajczuk 1983). *Trichoderma* were first reported to stimulate oospore formation in *Phytophthora* by Brasier in 1978. *Trichoderma* have also been associated with the stimulation of oospore production in heterothallic *Phytophthora cinnamomi* using a single mating type (Reeves & Jackson 1972; Johnson & Heather 1982). So, there is empirical evidence to demonstrate that BCAs can stimulate oospore formation while also playing the role of mycoparasite.

The factors controlling *P. agathidicida* oospore germination remain unknown. However, evidence from the flooding phase of the conventional soil bioassay implicates water availability (Beever et al. 2010). This in turn implicates a range of aqueous-phase microbial metabolites, suggesting that wetting and aqueous-phase connectedness are crucial for resource distribution and longer-range transport of micro-organisms. Feedbacks between microbial activity and the immediate environment are responsible for the emergence and stabilisation of soil structure – the scaffolding for soil ecological functioning of healthy forest ecosystems (Tecon & Or 2017).

[9] Aleuriospores are asexual spores in certain fungi, produced terminally by septation.

4 Discussion

Phytophthora agathidicida is a soil-borne, hemi-biotrophic plant pathogen that completes its life cycle and survives as oospores in kauri roots. After the roots die, necrotrophic fungi and soil meso-fauna break down the roots and the oospores are released into the soil (Bellgard et al. 2016). Oospores of *P. agathidicida* are thick-walled, globose, and can survive in a dormant state for at least 9 years at 10°C (unpublished data, S.E. Bellgard). It is assumed that oospores of *P. agathidicida* have some form of endogenous dormancy but can be stimulated to germinate under high soil water conditions to produce sporangia. Evidence of this mechanism forms the basis of the extended soil bioassay for *P. agathidicida*; i.e. the stimulating and flooding phase of the extended soil bioassay is used to activate the dormant oospores contained in the kauri roots in the soil sample (Beever et al. 2010).

Multiple laboratory assays have demonstrated that microbes are able to directly parasitise oospores and mycelium. Microbes have also been shown to stimulate oospore formation and germination, so there is *a priori* evidence to support the hypothesis that microbial BCAs can influence and/or modulate the expression and intensity of kauri dieback disease. However, this demonstrated efficacy is based on laboratory and glasshouse studies, which control for the influence of background microbial activity. This "background" microbial influence of the indigenous micro-organisms, which are symbiotically associated with the roots and root nodules of kauri, represents an unknown, non-target impact risk. This risk needs to be evaluated before any forest-scale introduction of broad-spectrum microbial BCAs to kauri forest soils to manage or control kauri dieback.

Indigenous AM fungi of kauri are unique to New Zealand and have co-evolved with kauri and the kauri ecosystem (Padamsee et al. 2016). The DSE of kauri observed in association with kauri roots have demonstrated antagonism against *P. agathidicida* in paired kill-plate studies. These endophytic root fungi belong to the Helotiales (Ascomycetes), and have been associated with enhanced nitrogen uptake in ericaceous plants and the unrelated grass *Deschampsia flexuosa* (Zijlstra et al. 2005). Therefore, without an understanding of the function and seasonal presence or absence of the DSE of kauri roots, no application of a broad-spectrum mycoparasite into a natural forest ecosystem should be considered.

Many *Trichoderma* species grow in the rhizosphere and are capable of penetrating and internally colonising plant roots (Mukherjee et. al 2012). Non-target impacts of broad-spectrum mycoparasites like *Trichoderma* are difficult to predict. There is evidence to suggest competitive interactions can occur between different functional groups of symbiotic root endophytes (e.g. AM fungi mycelium suppressing the efficacy of *T. harzianum*; Green et al. 1999).

The feasibility of getting the BCA to the kauri root–*Phytophthora* pathogen interface for the necessary duration to allow the biocidal effect to attain its optimal efficacy remains unknown. There is very little empirical evidence in the literature of such an intervention at a forest scale. The only example of the application of a non-specific mycoparasite (*T. asperellum*) at the field scale for the management of the sudden oak death pathogen *P. ramorum* demonstrated the type of disease suppression necessary for kauri dieback control and management: "*T. asperellum* provided biocontrol and decreased the amount of the secondary inoculum of *P. ramorum* from diseased roots" (Widmer et al. 2018).

The biological control of weeds has achieved national uptake in New Zealand, mainly because of the long-established history of the technology and the specificity of the BCAs. Steps towards achieving social licence to use biological control for kauri dieback will also need to demonstrate specificity, and the absence of non-target impacts that negatively impact the native microbial, co-evolved root symbionts. Prospective BCAs should demonstrate an "end-to-end" solution, with definitive, multi-gene data supporting the taxonomic identity and certification of the purity of the product formulation, and field-validated data of the efficacy of the product as applied in a natural ecosystem.

Following is a list of other important topics that need to be understood and researched as part of ongoing, adaptive management.

1 The ecology of the plant-associated microbes already associated with kauri roots:
   a What are the components and dynamics of kauri host defence induction?
   b What determines successful colonisation and expression of biological traits?
   c How do native and introduced microbial populations respond to different management practices?

d Under what conditions do kauri's natural biological agents exert their suppressive capacities?
e How are *P. agathidicida* and its antagonists distributed in the kauri root environment?

2 Application strategies of the prospective strains to get the active ingredient to the pathogen–root interface for a suitable duration of exposure:
   a How can exotic formulations be used to enhance the activities of kauri's own known biological agents?
   b Will the formulation of microbes alter their bio-protective efficacy?
   c Can more effective strains be added to the rhizosphere for a fixed-term effect?

3 Understanding what bio-protection our own novel strains have and the local modes of action:
   a Which signal molecules of plant and microbial origin regulate the expression of the biocontrol traits of different kauri symbionts?
   b Which of the novel DSE fungal strains are compatible with other individual agents?
   c What other genes and gene products involved in pathogen suppression in kauri roots are affected by AM mycorrhizae and DSE?
   d Can previously uncharacterised/unculturable microbes of kauri act as biological control agents?

4 Practical integration of intervention into standard forest management practices:
   a Can effective biocontrol–kauri combinations be developed by foresters for restoration plantings in off-shore island refugia?
   b Which biocontrol strategies best fit with other adaptive management system components (e.g. phosphite trunk injections)?
   c Which forests can most benefit from BCA for disease management: infested forest (applying BCA as a remedial treatment) or healthy forest (applying BCA as a prophylactic treatment to stimulate host-immunity/resilience), or applying the BCA before and after disease expression?

5 Acknowledgements

The authors would like to thank the contract management team, E. Delahunty and P. Pye, and the editing team, Dr S. Casonato, R. Prebble, and A. Austin. The principal author wishes to acknowledge the late Dr R.E. Beever for his sterling leadership and encouragement to pursue the mycorrhizal associations of kauri. Dr L. Jackson and Dr M. Padamsee are acknowledged for the supply of the original unpublished data on the DSE of kauri.

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Appendix 1 – Systematic review of literature of biological control of *Phytophthora*

Separate XLS spreadsheet.