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11748 Temperature treatment protocol for deactivating oospores of *Phytophthora agathidicida*.

Interim Report

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Prepared for Planning & Intelligence, Kauri Dieback Programme

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Figure 1 Oospores of *Phytophthora agathidicida* in cortical cells of kauri root.

Executive Summary

Oospores of *Phytophthora agathidicida* are formed in the roots of kauri. The infected roots are capable of initiating the progression of kauri dieback (KD). Therefore minimising impacts of KD involves reducing the accidental spread of the pathogen contained as oospores in kauri roots, root fragments or soil.

Heat deactivation of the oospores of *P. agathidicida* has been previously demonstrated, with 60-70°C for 4-hours reducing by 95% the viability of oospores of *P. agathidicida* embedded in mesh.

Our current project aims to standardise the temperature X time combinations necessary to deactivate oospores of *P. agathidicida* in a range of soil types and volumes and in the roots of kauri. The project is divided into three parts; A) *in vitro* temperature tolerance of *P. agathidicida* oospores, B) temperature X time combinations for soil-based deactivation, and C) temperature X time combinations for root- and plant-based deactivation of oospores of *P. agathidicida.*

Part A: In *in vitro* studies, *P. agathidicida* mycelia did not survive when exposed to -14°C for 24 h or more, or 35°C or higher for 24 h or more. Oospores were more tolerant of temperature extremes, with one out of 10 isolates surviving exposure to -14°C for 48-hours, and all isolates surviving at 35°C for 48 h. However, no isolates survived for more than 24 h at 40°C or above. The pattern of growth, with emergence only from older portions of the culture, reflects survival of oospores but not hyphae during the cool-heat treatment process. The focus of future experiments at the lower threshold will remain around -15°C, but for longer periods. At the upper temperature threshold, the focus will be between 35 and 50°C, and also include longer durations.

Part B: White millet is a suitable organic substrate in which oospores of *P. agathidicida* can form. Infected millet inoculum is capable of initiating colonies of *P. agathidicida.* There exists a lag-time between the outer heating environment and the transfer of cold/heat to the central “core” of a 500g sample of soil. This so-called acclimation time was 5.5 hours to achieve -15°C and 4.5 hours to get to achieve 42°C. Acclimation times will be calculated for each of the soil types (and volumes), and a safety factor will be derived for cooling and/or heating of soils to account for the acclimation time, and to ensure that the core of the sample achieves the necessary temperature to deactivate oospores of *P. agathidicida.*

Te Roroa have agreed to allow us access to sample soils from KD infected sites identified by DoC. A DoC concession permit has been granted by the Hamilton Permissions Branch of DoC.

Part C: The next round of trials will be carried out *in vitro* on *P. agathidicida*-colonised kauri roots. We expect oospore thermal tolerance to be slightly greater in oospores formed within kauri tissue, so given the results in the current experiment we will target -15°C or colder for 48 h or more at the lower end of the scale, and 35-50°C for 48 h or more at the upper end of the scale.

1 Introduction

1.1 Kauri Dieback

Phytophthora agathidicida was first reported from a stand of unhealthy kauri at Kaitoke Creek near Whangaparapara on Great Barrier Island (Gadgil 1974) and was recorded as *Phytophthora heveae*. Symptoms of disease in the Kaitoke Creek stand included yellowing of foliage, canopy thinning and lesions on the lower trunk, which sometimes encircled the stem and were bleeding copious amounts of resin (kauri gum). Lesions were also found on the main roots. *P. agathidicida* was recovered from the stem lesions, roots and the soil. It was also found in soil in a second stand of kauri on Great Barrier Island at Kaiaraara where the trees had healthy crowns and no sign of gummosis on the stems. In pathogenicity tests *P. agathidicida* has been demonstrated to be capable of rapidly killing kauri seedlings (3-8 years old) (Gadgil 1974; Beever et al. 2010; Bellgard et al. 2013).

Understanding of the epidemiology of the disease is limited. We know little about the length of time for a visible lesion to develop after infection has taken place, or the influence of site factors on survival of *P. agathidicida* in soil or how infection is transferred between trees and within an infected stand of trees. What has been confirmed from kauri root-material, produced in pot-trials, is that oospores of *P. agathidicida* are formed in the roots of kauri (Fig. 1) (Bellgard et al. 2016) and that infected roots are capable of initiating infection (Bellgard et al. 2013). Therefore, oospores contained in roots potentially play a role in the ability of the pathogen to remain viable and represent a long-term survival strategy for the pathogen in the kauri forest ecosystem.

The goal of the Kauri Dieback Program (KDP) is to reduce the impacts of *P. agathidicida* by controlling the spread of the pathogen and to undertake measures to limit its effect on individual trees and ecosystems (e.g. through the use of phosphite injections). An important part of this process is to ensure that there is no viable inoculum of *P. agathidicida* in any material before it enters or is removed from kauri field sites. Material could include bark and wood, or mud and soil washed from footwear, equipment and vehicles used in track maintenance. Propagules of *P. agathidicida* can include mycelium, sporangia, zoospores, and oospores (Bellgard et al. 2016). Mycelium and zoospores have been found to be readily killed by exposure to hypochlorite and other biocides such as tertiary amine (e.g. Trigene II Advance; SteriGENE®) and potassium peroxymonosulphate (e.g. Virkon S) (Bellgard et al. 2010). Chemicals such as disinfectants and biocides were not effective at deactivating oospores (Bellgard et al. 2010). Comparatively, pH and heat may be useful control mechanisms. Dick and Kimberley (2013) found that pH (pH levels 9 and 10 for 24 and 48 hour treatment reduced viability to levels below all other treatments) and heat could be effective at deactivating oospores of *P. agathidicida* (4-h treatment at 60°C and 70°C reducing activity by 95%).

Oospores are the most persistent of the propagules types produced by *Phytophthora* spp. and the main survival propagule in soil for many species, especially those that do not produce chlamydospores (Erwin & Ribeiro 1996). *P. agathidicida* completes its life cycle in the roots of kauri – which is a characteristic of its hemi-biotrophic life history. When conditions become favourable, oospores germinate and develop mycelia or produce sporangia. Typically, oospores will survive environmental conditions that are lethal to mycelia and sporangia. For example, the mycelium of *P. kernoviae* ceases to grow at 26°C and is killed after several hours at 35°C but oospores required temperatures from 50 - 60°C for periods of several hours (Widmer 2011) before all are propagules were killed. Germination of oospores

is influenced by oospore age, nutrition, temperature, light, chemical treatments, and potential unrecognised factors. Oospores are usually formed in diseased plant tissue, both on infected plants and in fragments of decaying tissue on the ground and in soil (Erwin & Ribeiro 1996). They may be found in soil after fungal degradation of infected plant tissue. Experimentally, sporangia and mycelium of *P. kernoviae* may survive in soil by forming oospores (Widmer 2011).

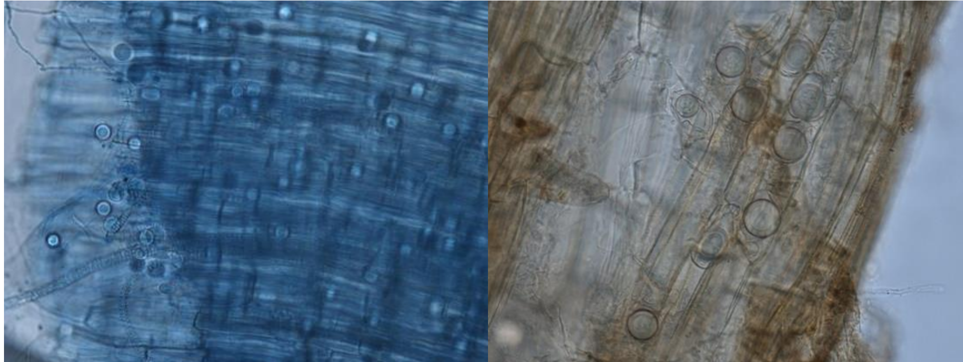

Figure 2: Oospores of *P. agathidicida* formed in the roots of kauri seedlngs deliberately inoculated with *P. agathidicida.*

Dick and Kimberley (2013) proposed that for practical purposes, the application of heat is likely to be the most effective and practical treatment for deactivation of *P. agathidicida* oospores in samples of contaminated soil collected from equipment or footwear. Results of their work indicated that temperatures of 60 - 70°C applied to wet soil or through a steam applicator for periods of 4 hours would result in total kill. Dick and Kimberley (2013) also suggested that a "safety factor" could be applied to the temperature X time combination; but they did not specify the percentage which would be appropriate. At temperatures higher than 70°C, they expected that shorter treatment periods are likely to be effective (Dick and Kimberley 2013).

1.2 Use of heat for sterilising soil

The rhizosphere soil is the ultimate reservoir of infected roots and oospores of *P. agathidicida.* Soils and plant growing medium are treated with chemicals or steam to free them from disease causing organisms (Allan et al. 1981). While chemical treatment is preferred for field use, steam is the accepted method in glasshouse-horticulture and nursery industries, because:

a) Steam is the best generally accepted method of treatment

b) Penetration and effectiveness are more easily measured (with a thermometer) than they are with chemical treatments

c) Soil can be treated with steam within 30-60 cm of living plants without injuring them

d) There are no risks of residues which may be toxic to some plants as steam is water in its vapour phase (Dion and Parker 2013).

Steam sterilisation has been used for more than a century to sterilise items that can withstand moisture or high temperature (Dion and Parker 2013).

1.3 Sterilisation principles

The effects of temperature–time combinations during composting on 64 plant pathogens (including fungi, plasmodiophoromycetes, oomycetes, bacteria, viruses and nematodes) were reviewed by Noble & Roberts (2004). For all but five pathogens a peak temperature of 64–70°C and duration of 21 days, were sufficient to reduce numbers to below the detection limits of the tests used. Noble & Roberts (2004) commented that shorter periods and/or lower temperatures than those quoted in these tests may be satisfactory for eradication, but this had not always been examined in detail in composting systems. The eradication of pathogens from organic material is not solely a result of the heating process but also due to the production of toxic compounds, the lytic activity of enzymes formed in the compost and to microbial antagonism. Coventry et al. (2002) suggested that some of the compounds produced in the early stages of the compost process would stimulate the resting stages of fungi into germination and these would therefore become more susceptible to elimination. Notwithstanding the contribution of these processes it is the heat generated during the thermophilic high temperature phase of aerobic composting that is the most important factor in the death of micro-organisms (Bollen & Volker, 1996).

1.4 Stages of the Project Plan

The project is divided into three phases, each building upon the findings of the previous study to increase experimental efficiency (Table 1);

- Expt A: *In vitro* thermal tolerance of *Phytophthora agathidicida* (PA) oospores (-14°C to +50°C)
- Expt B: Efficacy of low (-20°C to 5°C), moderate (20°C to 30°C) and high temperature (>50°C) on oospore activity in soil (up to 10 kg) across a range of soil types and time variables
- Expt C: Efficacy of moderate heat (30°C to 50°C) on oospore activity in biological materials (in vitro) using wet heat and time combinations under controlled lab conditions.

Table 1: Stages of the project

Experiments	Completion	Output
Part A	Jun 2018	In vitro thermal tolerance of *P. agathidicida*
Part B	Dec 2018	Heating and cooling thresholds for thermal deactivation of *P. agathidicida* oospores in soil
Part C	Dec 2019	Heating and cooling thresholds for thermal deactivation of *P. agathidicida* oospores in plant materials

This report provides a progress report of research carried out on Part A and Part B of the Project Plan (Appendix 1).

2 Part A: In vitro thermal tolerance of *P. agathidicida*

2.1 Introduction

As a first step to understanding the thermal limits of oospores of *P. agathidicida*, experiments were carried out to determine the variation (if any) that exists in the "population" of isolates of *P. agathidicida* recovered from different geographical and historical areas of kauri dieback.

2.2 Materials and Methods

Ten *P. agathidicida* isolates were selected from throughout its known geographic range, as summarised in Table 2.

Table 2: Name and source of *P. agathidicida* isolates studied in heat tolerance

	H-series	ICMP	Geographic source
A	H257		Raetea, Mangamuka Ranges, Northland
B	H261		Titirangi, Waitakere Ranges, Auckland
C	H263		Oratia, Waitakere Ranges, Auckland
D	H270		Great Barrier Island
E	H654		Kerikeri, Northland
F	H698	20275	Whangapoua, Coromandel
G	H691	17021	Maungaroa, Waitakere Ranges, Auckland
H	H692	18407	Waipoua Forest, Northland
I	H693	18363	Awhitu, South Auckland
J	H697	18404	Trounson Forest Park, Northland

The ten *P. agathidicida* isolates were grown on V8-juice agar plates (ø85 mm) for 5 days, then a 3-mm plug was extracted and inoculated onto the extreme edge of a fresh V8-juice agar plate (ø85 mm). Plates were incubated in the dark for 14 days (18°C) before exposure to the various test temperatures, with the culture margin marked on the bottom of the plate every 4 days. After 14 days the cultures had grown almost completely across the agar plates. The pure-culture contained a range of stages from new mycelium on the leading edge of the colony to old hyphae with mature oospores near the original point of inoculation. Oospore initiation was noted on 2–3 day-old mycelia, and oospores appeared to be mature after 7–10 days.

The colonised plates were then placed in incubators at the various test temperatures (Table 3) and incubated for either 4, 24 or 48 hours. Temperature was recorded every 5 min throughout the incubation using EL-USB-2+ data loggers accurate to +/- 0.5°C (Table 3). After their allotted incubation time, plates were removed, retained on the lab bench for 1 h, then portions were sub-cultured onto fresh V8-agar plates. This was done by cutting a 5-mm-wide strip of agar from the colony leading-edge to the oldest portion, including the plug. These strips were divided into colony age classes based on the growth marks previously made on the bottom of the plate, resulting in strip portions that were 0–2, 2–6, 6–10 and 10–14 days old (reflecting the culture age at the commencement of the heat treatment). The 10–14-day-old strip also contained the inoculation plug, so would have contained tissue approximately 16 days old. These strips were placed in sequence along the central axes of the agar plate, so that plated

mycelial age could be determined in subsequent re-growth assessments. There were four replicates for each Isolate X Temperature X Time combination.

Following heat treatment and subbing to fresh V8, plates were incubated at 20°C and assessed after 2, 4, 7, 14 and 21 days. Mycelial growth was recorded, noting the age of the strip from which any growth emerged.

Table 3: Machine / instrument errors of incubators

Target temp. (°C)	Actual mean (°C)	Range (°C)
-14	-14.2	-15 to -13
-5	-4.0	-4.5 to -4.0
1	-0.18	-0.5 to 2
20	20.0	19.5 to 20.5
30	30.0	29.5 to 30.5
35	35	35
40	39.6	39.5 to 40.0
45	44.5	44.5
50	49.0	48.5 to 49.5

2.3 Results and Discussion

Data on culture regrowth following incubation at various temperatures and times are presented in Figure 2. At 20°C, regrowth was rapid (within 2 days) and complete in all portions of the cultures. Towards the temperature extremes, regrowth was either delayed or prevented.

The thermal tolerance of mycelia covered a narrower temperature range than that of oospores. This was the case at both upper and lower temperature thresholds. Mycelia did not survive freezing at -14°C for 24 h or more, as demonstrated by the failure of 0–6-day-old portions of the culture to regenerate following incubation (Figure 2A). Older portions of the cultures (containing oospores) were able to regrow after 24 h at -14°C, although the regrowth was sporadic and not present in all replicates. There was very little regrowth following 48 h at -14°C, even from the older portions of the culture, with only two of the four replicates from Isolate D (Great Barrier) showing any regrowth, and no regrowth in any of the other isolates (Fig. 2).

At the upper end of the temperature range, mycelia survived 35°C for 4 h, but not for 24 or 48 h (Figure 2A). At the same temperature, regrowth from older parts of the culture containing mature oospores occurred in all isolates following 24 or 48 h incubation (Figure 2B), although emergence of colonies was delayed by a week or more (Figure 3).

At 40°C, there was no regrowth from younger parts of the culture (Figure 2A), even after just 4 h incubation. There was some survival of most isolates in the oospore-bearing parts of the colonies after 4 h incubation at 40°C, although very sporadic re-growth of mycelium from cultures was noted only after 2 to 3 weeks incubation at 20°C,. There was no regrowth from any parts of the culture when incubated for 24 or 48 h at 40°C, nor after 4 h of exposure at 45°C (Figure 2). Similarly, there was no growth after incubation at 50°C (data not shown).

From the results obtained, there were few obvious differences in thermal tolerance of the various *P. agathidicida* isolates. Isolate ‘A’ may have a slightly lower upper threshold the other isolates, and isolate ‘D’ may have a slightly lower threshold, but otherwise there is little of interest. Future experiments will probably be done with just two or three isolates, so these should include isolate ‘D’ and any of the others except ‘A’.

Fig 2A: 0-6 day old culture

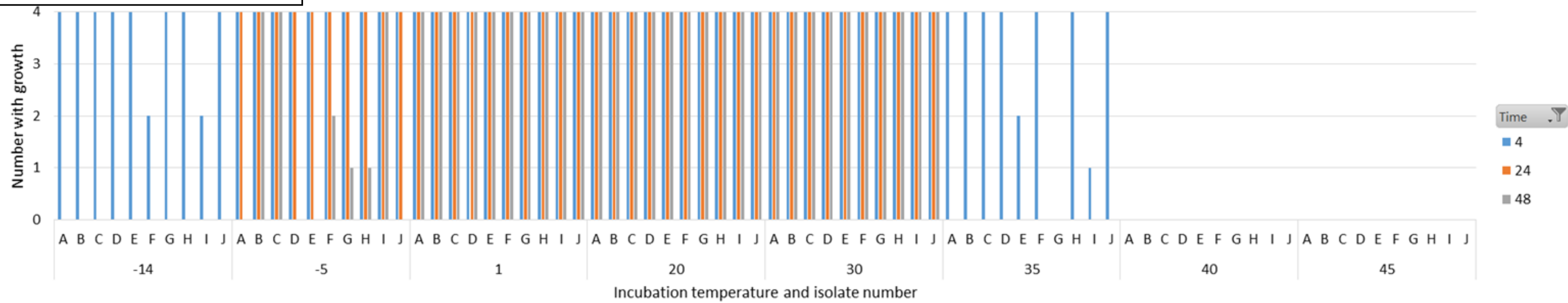

Fig 2B: 6-14 day old culture

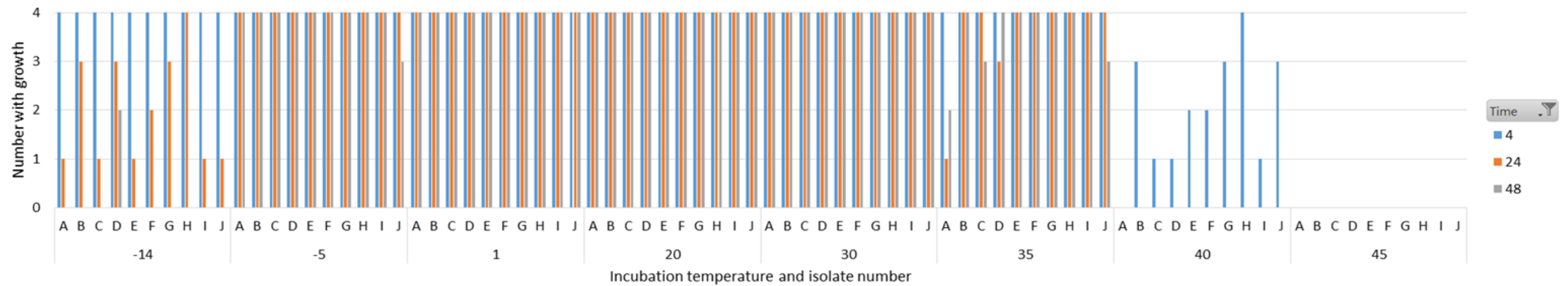

Figure 3: . Out-growths from *P. agathidicida* oospore-cultures (A-J) that regrew on V8 agar after exposure to various temperatures for 4, 24 or 48 h. A: regrowth from 0 to 6-day-old portions of the culture; B: regrowth from 6 to 14-day-old portions of the culture.

Figure 4: Emergence of *P. agathidicida* colonies from sections of cultures previously incubated at 35°C for 48 h. The plated strips are, respectively from top to bottom, cut from 0–2, 2–6, 6–10 and 10–14 day-old portions cultures incubated at 35°C, and subsequently allowed to regrow at 20°C.

Further trials will be carried out *in vitro* on *P. agathidicida*-colonised kauri roots. We expect oospore thermal tolerance to be slightly greater in oospores formed within kauri tissue, so given the results in the current experiment we will target -15°C or colder for 48 h or more at the lower end of the scale, and 35-50°C for 48 h or more at the upper end of the scale.

3 Part B: Heat deactivation of oospores in soil

3.1 Introduction

Dick and Kimberley (2013) proposed that for practical purposes, the application of heat is likely to be the most effective and practical treatment for deactivation of *P. agathidicida* oospores in samples of contaminated soil collected from equipment or footwear. We began our studies of temperature deactivation using a standardised sand/loam substrate, at a fixed water content, and varying the volumes. The following summarises the progress made in the experimental process; 1) millet seed inoculum, 2) acclimation temperatures for a standardised substrate and 3) iwi consultation for the collection of field soil from KD positive sites in Waipoua Forest.

3.2 Millet inoculum

The three isolates; H261, H270, H697 (ICMP#18404), were received from Plant Food Research under movement permit MA18489. The three isolates were grown and colonies established into white millet seed and allowed to grow at 20°C for six weeks.

After six weeks, oospores of *P. agathidicida* were observed in the “germ” and in the seed coat of the millet seeds inoculated with *P. agathidicida* (Fig. 4).

Colonies of *P. agathidicida* were observed growing from millet inoculum plated to *Phytophthora*-selective media after 4-days. The uninoculated-control millet did not have any out-growths when plated to *Phytophthora*-selective media.

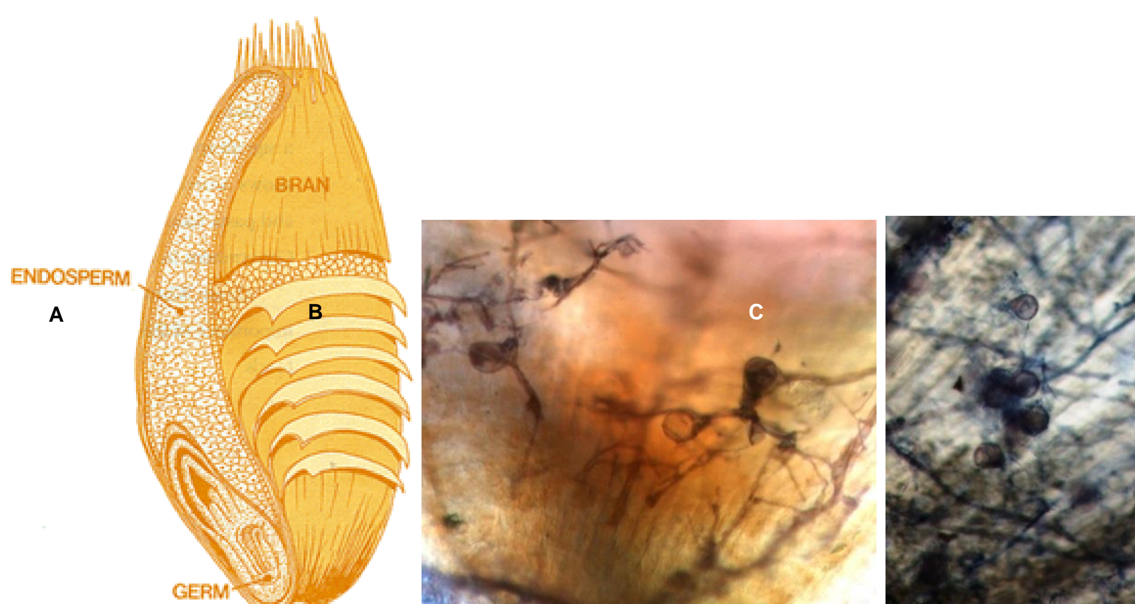

Figure 5: 4A: Stylised depiction of millet grain, 4B: Oospores of *P. agahtidicida* and sub-tending hyphae associated with the “germ” section of an infected millet grain, 4C: Oospores of *P. agathidicida* associated with the seed coat of the millet inoculum (stained with Trypan Blue).

3.3 Acclimation time studies

The change of energy between two systems (bodies) due to their different temperatures is called heat transfer. The heat transfer from one to the other body takes place spontaneously, from the warmer (higher temperature) to the colder body (lower temperature). If there are no other causes, the state of the observed systems will change until heat balance is established. In principle, there are three thermal properties of soil that contribute to the variation that exists between different soil types; specific heat, thermal conductivity and thermal diffusivity[1]. In order to assess the time taken for a standard mass of substrate to reach the desired temperature, an experiment was set-up to measure the internal "core" temperature, as the 500 g of soil was cooled or heated from 18°C. The cooling and heating times for 500 g samples of sand/loam (at a water content of 11.4%), to reach the lower (-15°C) and upper temperature (42°C), thresholds were 9.5 h and 4.5 h respectively (Table 4).

Table 4: Acclimation times for 500 g sanld/loam at 11.4% water content all starting at 18°C

Temperature (°C)	Acclimation time (hours)	Temperature differential (°C)
-15	5.5	33
18	0.1	0
+42	4.5	24

These acclimation times, need to be added to the lethal exposure times, when either cooling or heating is considered for thermal deactivation of oospores of *P. agathidicida.* Acclimation times will be calculated for a range of soil types / volumes, including the field collected soil volumes, to ensure that the "core" temperature of the sample attains the necessary treatment heat for the correct duration (MPI 2017).

3.4 Iwi consultation with Te Roroa

Manaaki Whenua approached Te Roroa about the potential for sampling soil from confirmed kauri dieback diseased sites in Waipoua Forest. We provided an overview of the research that we are engaged with PFR and the implications for on-ground risk minimisation of materials contaminated with oospores of the kauri dieback pathogen.

Te Roroa support this research effort (Appendix 2). If this research can be operationalised, then a standardised heat treatment protocol could be used to handle the safe, local-disposal of contaminated soil waste. This would in turn increase their ability to reduce impacts of kauri dieback by reducing the risk of accidental inoculum spread in soil and plant materials.

The Department of Conservation have issued a Research and Collection Authorisation (69218-GEO) for the collection of soil from positive Kauri Dieback sites in January, 2019.

[1] http://mech.utah.edu/~pardyjak/efd/SoilHeatTransfer.pdf

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Appendix 1 – Final Project Plan

Background and aim

MPI requires research undertaken on the effectiveness of heat and cold temperatures in deactivating oospores of *Phytophthora agathidicida* (PA) in infected soil, growing media and kauri seedlings (i.e. vector pathways). This will establish temperature treatment standard protocols for the management of vector pathways to mitigate the spread of kauri dieback.

Project Breakdown

Work Item	*Rationale*	*Timeline*	*Budget*	*Service provider*
Expt A In vitro thermo-tolerance of PA oospores (-14°C to +45°C)	This experiment will establish a baseline temperature profile for PA survival across a range of isolates. It will enable refinement of the range of lethal test-temperatures.	Nov 17-Apr 18	$20,000	PFR; Ian Horner, Matthew Arnet, Mary Horner
Expt B Efficacy of cold (-15°C to 5°C), moderate (20°C to 30°C) and lethal temperature >50°C) on oospore mortality in soil (up to 10 kg) across a range of soil types and time variables	B1: Cold-, moderate- and the lethal temperatures will be applied to 1% millet inoculum in 500 g aliquots of soil at a fixed moisture regime.	May 18-Aug 18	$28,500	MW-LR; Stan Bellgard, Elsa Paderes, Chantal Probst, Zane McGrath
	B2: Naturally infected soil and four different texture classes (1, 3, 5, &10 kg) will be treated at the temp X time combinations determined from Expt B-1	Sep 18-Dec 18	$29,750	
Expt C Efficacy of moderate heat (30°C to 50°C on oospore mortality in biological materials (in vitro) using wet heat and time combinations under controlled lab conditions	C1-3: The results from Expt B will refine the temp X time needed to deactivate infected kauri roots (C1), naturally infected roots (C2), roots and potting mix (C3)	Jun 18-Sept 18	$48,500	PFR; Ian Horner, Matthew Arnet, Mary Horner
	C4-5: The heat-treatment of forest soil and roots (C4) and kauri seedlings (C5) will be informed by the previous studies.	Jul 18-Dec 18	$37,500	

Methodology

1 **Temperature monitoring:** Temperature will be monitored *in situ* using Thermochron Temperature Data Loggers® (iButtons) and EL-USB-2+ Data Loggers. DS1922L iButtons and EL-USB-2+ data loggers are both quoted as having a level of accuracy of +/- 0.5°C.
Temperature inside various depths of soil cores will be measured with a Testo 962 Temperature Meter, which has a quoted accuracy of 0.3°C (-20°C +70°C).

2 **Source of *Phytophthora agathidicida* (PA) isolates: Isolates** of PA recovered from kauri, will be sourced from International Collection of Micro-organisms from Plants (ICMP). The isolates have been selected to cover the geographic range of kauri dieback e.g. (subject to availability);

	H-series	**ICMP**	**Geographic source**
A	H257		Raetea, Mangamuka Ranges, Northland
B	H261		Titirangi, Waitakere Ranges, Auckland
C	H263		Oratia, Waitakere Ranges, Auckland
D	H270		Great Barrier Island
E	H654		Kerikeri, Northland
F	H698	20275	Whangapoua, Coromandel
G	H691	17021	Maungaroa, Waitakere Ranges, Auckland
H	H692	18407	Waipoua Forest, Northland
I	H693	18363	Awhitu, South Auckland
J	H697	18404	Trounson Forest Park, Northland

The concentration of the contaminated materials will be dependent upon the Experiment:

- Expt A: In vitro cultures, 5-day old (mycelium) and 14-day old cultures (mature oospores).
- Expt B1-2: Millet inoculum will be added at a rate of 0.2% (i.e. 1 g / 500 g soil). Artificial inoculum will be added to the natural infested soil aliquots used in Expt B2 (held at 20°C) to act as a positive control.
- Expt C: PA either present/absent: microscopy will be used to determine PA infection before treatments.

3 Positioning and recovery of contaminated material:

- Infected millet will be mixed into the soil using a surface decontaminated portable, barrel mixer.
- Post-hoc assessments will involve recovering millet grains from the "soil sample and plating to *Phytophthora*-selective media. Colonies will be sequence analysed (*ßeta*-tubulin) to confirm PA presence.

4 Assessment of oospore viability:

- Inoculated millet will be plated to *Phytophthora*-selective media and the proportion of viable oospores assessed as a baseline prior to any experimental treatments (using viability stain).
- In all cases, the "positive control" will be inoculated millet held at 20°C. Post-hoc studies on the viability of deactivated millet will be confirmed via viability staining. Root pieces will be plated to selective media and bioassayed with three cycles of drying and repeated wetting.

5 Levels of detection probability (sensitivity/specificity):

- Level of detection probability will be calculated to determine the number of millet seeds that need to be recovered after the experiment to ensure there are no further viable entities present in the soil sample.
- Via a preliminary calibration study, the recovery efficiency of increasing number of millet grains will be determined from a positive control, spiked with a known number of infected millet seeds (20%), held at 20°C, with 6% water content.

6 Experimental design:

Expt A: In vitro Thermal tolerance of oospores of *P. agathidicida*

- The number of replicates: 3 plates per isolate per temperature per time
- The number of positive and negative controls: No negative controls. Positive controls will be comparable plates held at 20°C
- Types of variables tested = isolate (10), temperature (9); -14°C, -5°C, 1°C, 20°C, 30°C, 35, 40°C, 45°C & +50°C, time (3); 4 hours, 24 hours 48 hours.
- The assessment of variables on treatment efficacy = isolate x temperature x time
- Statistical analysis (dependent upon temperature responses).
- Testing methods used to detect the presence of PA (incl. sensitivity and specificity of test) = Plates removed from incubator and left on laboratory bench for 1 hour prior to plating to fresh plates of V8-juice agar.

Expt B-1: Temp X time 500 g soil at field capacity[2] study;

- The number of replicates = 5 per temp X time
- The number of positive and negative controls = 5 +ve controls (held at 20°C and 5 –ve controls (uninoculated millet)
- Types of variables tested;
  - Three PA isolates (H261, H270, H697)
  - Time; 2, 4, 24, 48 h, 72 h, 1, 3 & 4 weeks (8 treatments) [plus for 3-4 weeks for moderate temperatures]
- Temperature: -15°C to 5°C (cold), 20°C to 30°C (moderate), and >50°C (high).
- The assessment of variables on treatment efficacy;
  - Time
  - Temperature.
- Statistical analysis (detection sensitivities; confidence intervals);
- ANOVA will be used to analyses the data and provide confidence intervals.
- Testing methods used to detect the presence of PA (incl. sensitivity and specificity of test); Direct plating of millet extracted from the soils to *Phytophthora* selective media, viability staining, post hoc recovery of the isolate and DNA-sequence confirmation of the resulting cultures.

Expt B-2: Temp X time soil type study

- The number of replicates = 5 per temp X time X 4 soil type combination (natural colonised PA soils; Clay; Loam; mud-water slurry).
- The number of positive and negative controls = 5 +ve controls (held at 20°C) and 5 –ve controls (uninoculated millet)
- Types of variables tested;
  - Time- 2 time periods (from Expt B-1; 2 treatments),
  - Temperature - Test temperatures (from Expt B-1; 2 treatments),
  - Water content; 20%, 30%, 50% (3 treatments).
- The assessment of variables on treatment efficacy;
  - Time,
  - Temp,
  - Water content,
  - Soil texture, and
  - Bulk density[3] of soil (1, 3, 5 and 10 kg)
- Statistical analysis (detection sensitivities; confidence intervals);
- ANOVA will used to analyse the data and provide confidence intervals.
- Testing methods used to detect the presence of PA (incl. sensitivity and specificity of test) = Direct plating of millet seeds to *Phytophthora*-selective media, and ITS-sequence analysis of cultures to confirm presence of PA. Bulk densities will be carried out on final soil samples.

Expt C-1 – C-3: Nursery plants and growing media;

- These experiments will be a progressive series from autoclaved kauri roots → naturally colonised roots → bulk colonised roots and potting mix, all inoculated with a blend of PA

[2] Field capacity is the amount of soil moisture or water content held in the soil after excess water has drained away and the rate of downward movement has decreased.
[3] Bulk density is the weight of soil in a given volume and will be ascertained for 4 soil texture classes; up to 10 kg.

isolates H261, H270, H697. PA oospore colonisation will be determined by microscopic examination.

- The test temperatures and heat exposure times will be determined following results from Expt. B. We will include a longer exposure time (i.e. 3-4 weeks) at -15°C and + 35-40°C to assess whether this will result in complete kill. PA survival will be determined by direct plating to V8-enriched, *Phytophthora*-selective agar, and by baiting using a modified soil bioassay detection protocol.
- At least 50 colonised root pieces will be assessed at each temperature X time combination. Inoculated roots/media will be held at 20°C as a positive control.

Expt C-4: Forest collected roots and kauri seedlings;

- Soil and roots will be collected from three PA-infested kauri sites. Each soil will be homogenised in a concrete mixer before assigning subsamples to treatments
  - The number of replicates: 5 replicates per soil per temperature per time.
  - The number of positive controls: 5, held at 20°C.
  - Types of variables tested: survival in different soils, temperatures x exposure times as determined in Expts C1 to C3.
- The assessment of variables on treatment efficacy: soil x temperature x time
  - Statistical analysis (detection sensitivities; confidence intervals): ANOVA
  - Testing methods used to detect the presence of PA (incl. sensitivity and specificity of test): soil bioassay for PA, repeated at least once for all negative samples

Expt C-5: Kauri tree survival related to temp x time

Container-grown kauri seedlings (1-3 years) will be exposed to various heat-treatments, related to temp X time profiles generated from above studies with *P. agathidicida*. The aim is to find a temp X time window for kauri survival but not *P. agathidicida*. It is anticipated that temperatures between 40 and 55°C will be the foci, with time durations of 12 to 72 h, with longer durations at moderate temperature (e.g. 40°C if results from Experiments B and C1-4 indicate a possible window of opportunity). Following heat treatment, seedlings will be returned to the glasshouse where they will be grown under optimal conditions (20-25°C, well-watered and fertilised), for 3-5 weeks to determine tree survival.

7 Landowner consultation:

Consultation with Tom Donovan (Te Roroa) has identified the PA infected site in the Waipoua Valley. A DoC concession has been submitted to the Hamilton Permissions Branch to samples 15 Kauri Dieback positive sites in Waipoua Forest.

8 Project management:

Monthly up-dates will be made between PFR and Landcare about milestone achievements and to identify any potential barriers. MW-LR tracks job budgets via a project management system, which provides monthly summaries of job costs against set budgets for each work item.

9 Time line and workflow and synergies / dependencies:

The **project breakdown** (at the start of the document), gives a summary of the timeline. We propose that the Project Plan remain as a Final Draft, until we have competed Expt A, and

have provided the summary report to P&I. We then can finalise the temp X time combinations based upon the thermo-tolerance experimental data.

Preparatory stage: November-December, 2017

The start of the work-flow will commence in November with the formulation of the Project Plan between MPI and the contractors. In November, the selection of the isolates for the study, and the recovery of them from ICMP will be commenced, and the transfer permit developed to get the isolates to PFR laboratory.

Stage 1: Development of Thermo-tolerance, November 2017-June, 2018

Expt A is to be carried out by PFR, and the lethal upper and lower temperatures will inform the choice of cold, moderate and lethal temperatures for the soil study. Also three PA isolates will be chosen for the remainder of the studies (H261, H270, H697), provided in a progress report at the end of June, 2018. The interim, draft progress report will include; (i) Final Project Plan, (ii) summary of the *in vitro* temperature study work, (iii) validation of the millet inoculum, (iv) results of iwi consultation and (iv) the recommended temp X time combinations to be used for Expt B&C.

Stage 2: Efficacy of cold temperatures on oospore mortality in standardised soil. May-Aug 2018

Expt B-1 will begin after the completion Expt A, as the test lethal temperatures, and the three PA isolates will be chosen for incorporation into the millet inoculum. Preparation of millet inoculum takes six weeks.

The consultation and permitting for collection of soil (esp. DoC) has been submitted. The consultation for access to 15 diseased sites for soil and root collection started with a hui with Te Roroa Centre of Excellence in May, 2018. We have a Mana Enhancing Agreement in place with Te Roroa for the collection of seeds from the Waipoua Valley. Tom Donovan (Waipoua Forest Trust) will assist with the field-collection in the Waipoua Forest. We have preliminary engagement completed with Auckland Council, and we have three previously sampled PA-positive sites; i) Lower Kauri Track (Auckland City Walk), ii) Huia (Twin Peaks Track and iii) Maungaroa Ridge. The rahui which currently exists does not prevent KD research but will necessitate an additional cost not budgeted as part of the preliminary project plan.

Expt C1-3 will also commence after Expt A, as the test lethal temperatures will be utilised. Infected kauri roots will take time to develop (3-4 weeks). Standardised commercial potting mix will be used for this experiment, with this stage reported in a Progress Report at the end of September 2018.

Stage 3: Efficacy of moderate heat on oospore mortality in infected soil, and biological materials September - March 2019.

Expt B-2: The results of the soil study Expt B-1, will inform the temperature regime necessary for the deactivation of oospores contained in roots and soil potting medium, with a progress report provided at the end of September, 2018.

Expt C-4-5 will commence after Expt C1-3, and will be informed by the outputs of Expt B-2, with the collected finding summarised in a progress report 31st December, 2018.

A draft phase 1 report summarising the findings of the entire project is due to MPI on the 28th February, 2019, with; Executive Summary, Background, Methods, Results, the MPI having until 16th March, 2019 for comments, with the final report due 28th March, 2019.

Appendix 2: Te Roroa Letter of Support

Department of Conservation
Permissions Team
Private Bag 3072
Hamilton 3240

Attention: Permissions Team

RE: Support for soil sampling in Waipoua Forest for oospore deactivation study

This letter is to confirm that Manaaki Whenua Landcare Research NZ Ltd, has approached Te Roroa about the potential for sampling soil from confirmed kauri dieback diseased sites in Waipoua Forest. It is our understanding that Stan Bellgard, together with Plant and Food Research have been engaged by the KDP to study the ability of heat to deactivate oospores of the kauri dieback pathogen.

Te Roroa support this research effort. If this research can be operationalised, then a standardised heat treatment protocol could be used to handle the safe, local-disposal of contaminated soil waste. This would in turn increase our ability to reduce impacts of kauri dieback by reducing the risk of accidental inoculum spread in soil and plant materials.

Naku noa, na,

Snow Tane

General Manager

TRDG