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17748 Temperature treatment protocol for deactivating oospores of *Phytophthora agathidicida*

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by Ian Horner, Matthew Arnet, Plant & Food Research, Havelock North.
Stan Bellgard, Chantal Probst, Quentin Paynter, John Claydon, Manaaki Whenua – Landcare Research

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Reviewed by:	Approved for release by:
Beccy Ganley Science Team Leader Plant & Food Research	Chris Jones Portfolio Leader Managing Invasives Manaaki Whenua – Landcare Research
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Executive summary

Background

Oospores of *Phytophthora agathidicida* are formed in the roots of New Zealand kauri. The infected roots are capable of initiating the progression of kauri dieback. Therefore, minimising the impacts of kauri dieback 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 a temperature of 60–70°C for 4 h reducing the viability of oospores of *P. agathidicida* embedded in an artificial mesh matrix by 95%.

Project

Our current project aims to standardise the temperature × time combinations necessary to deactivate oospores of *P. agathidicida* in a range of soil types and volumes and in the roots of kauri plants. The project is divided into three parts:

A. in vitro temperature tolerance of *P. agathidicida* oospores

B. temperature × time combinations for soil-based deactivation

C. temperature × time combinations for root- and plant-based deactivation of oospores of *P. agathidicida*.

Part A

In in vitro studies on V8-agar plates, *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 h, and all isolates surviving at 35°C for 48 h. However, no isolates survived for more than 24 h at 40°C or above, or for more than 4 h at 45°C. The pattern of growth, with emergence only from older portions of the culture, reflects the survival of oospores but not hyphae at the temperature extremes.

Part B

Temperature × time combinations for soil-based deactivation: white millet is a suitable organic substrate in which oospores of *P. agathidicida* can form, and the infected millet inoculum is capable of initiating colonies of *P. agathidicida*.

There is a lag-time between the outer heating environment and the transfer of cold/heat to the central core of a 500 g sample of soil. This so-called ‘acclimatisation time’ was between 3 and 5.5 h across all soil types. As the water content of the soil sample increased, the efficacy of freezing attenuated, especially in sand and loam soil types. However, it was observed that freezing was more effective in sand at the highest water content. For the smaller size classes of 1–2 kg, freezing at –20°C, effectively eliminated oospore activity in the sand and loam, but this effect attenuated above 2 kg. There is a significant interaction between mass and water content, and, overall, freezing of spores did not affect their oospore wall or ‘granular’ texture (pre-germination) of the oospores.

Heating to 50°C, across all soil types and size classes, at between 20 and 30% water content, for 72 h, effectively eliminated *P. agathidicida* oospore viability. The effectiveness of heating was only compromised at a water content of 40%, in loam-type soils. Because a soil’s thermal conductivity is significantly influenced by its saturation, too much water changes the heat diffusivity of soil. Therefore, water (20–30%) is critical for the conductivity of heat to deactivate oospores of *P. agathidicida* in soil, but greater than 40% water content undermines the thermal diffusivity of the soil, thus reducing the thermal efficacy of heat treatment.

Part C

When autoclaved kauri roots were colonised in vitro by *P. agathidicida*, then exposed to –15°C for periods from 1 to 21 days, there was a steady decline in recovery of the pathogen with increasing time. However, there was still some survival and regrowth of *P. agathidicida* after 21 days. A subsequent experiment with colonised kauri roots and potting mix indicated that temperatures of –15°C and –20°C for 7 to 21 days prevented the direct re-isolation of *P. agathidicida*, although the pathogen was readily detected from repeated baiting of the treated samples. From these experiments, it appears that freezing infected material to –15°C and –20°C does reduce the viability of *P. agathidicida*, without necessarily eliminating it.

When autoclaved kauri roots were colonised in vitro by *P. agathidicida*, then exposed to 40°C for 24 h, the pathogen could not be detected by direct plating onto agar, or in four rounds of bioassay baiting. However, there was one detection after pooled samples were re-baited for a fifth round of the bioassay. There was no detection following 40°C incubation for times longer than 24 h. Similar results were obtained when kauri seedling roots and potting mix colonised in the glasshouse were exposed to high temperatures. At 40°C for 24 h there was a low amount of re-isolation of live *P. agathidicida*, but none after 48 h. At 45°C, 24 h was sufficient to kill *P. agathidicida* in roots across all replicates without exception.

P. agathidicida could survive 35°C for a few days, but trials with autoclaved inoculated kauri roots and inoculated seedling roots/potting mix showed substantially reduced survival after 7 days and no survival after 14 days.

When naturally colonised forest soil was exposed to high temperature treatments, survival thresholds were at lower temperatures and times than in the previous artificial inoculation studies. *P. agathidicida* was readily detected in all control samples incubated at 20°C, but could not be detected by repeated baiting from samples incubated for 24 h or more at 37.5, 40 or 42.5°C. A single detection was made after repeated baiting of a sample incubated at 35°C for 4 days, but there were no detections following incubation at this temperature duration of 7 days or longer. This slightly lower detection could reflect the effects of microbial activity on weakened *P. agathidicida* propagules, or could simply be a factor of much lower inoculum numbers in natural soil samples.

In a preliminary study to determine the heat tolerance of kauri seedlings and whether there is potential for heat treatment to eradicate *P. agathidicida* from nursery plants without killing the tree, 2- to 3-year-old kauri seedlings were exposed to temperatures determined to be near the thermal limits of the pathogen. No trees survived at 45°C for 24 h, or at 42.5°C for 48 h. Trees survived 40°C for 24 or 48 h, although there was some leaf damage. Survival was good for up to 4 days at 37.5°C and at 35°C for 14 days. Thus, there is potentially a window for treatment between 35°C for 7-plus days and 40°C for up to 2 days, where *P. agathidicida* could be killed, without killing the plant.

A more extensive study is required, exploring these limits with larger numbers of trees, and with trees exposed to a longer period of pre-infection with *P. agathidicida*. Variables such as fluctuating heat and pre-acclimatisation, and optimising plant xylem-water levels, to enable the prediction of the thermal tolerance of trees of different age- and size classifications, should also be investigated.

1 Background

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. *Phytophthora agathidicida* was recovered from the stem lesions, roots and 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, the influence of site factors on the 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 (Figure 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 Programme 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. using phosphite injections). An important part of this process is to ensure 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, mud and soil washed from footwear or equipment, and vehicles used in track maintenance.

Propagules of *P. agathidicida* can include mycelia, sporangia, zoospores and oospores (Bellgard et al. 2016). Mycelia 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). However, disinfectants and biocides are not effective at deactivating oospores (Bellgard et al. 2010). Comparatively, pH and heat may be useful control mechanisms. Dick and Kimberley (2013) found that a pH of 9 and 10 for 24 and 48 h treatments reduced viability to levels below all other treatments. Furthermore, when heat treated for 4 h at 60°C and 70°C, oospore activity was reduced by 95%.

1.2 *Phytophthora* oospores

Oospores are the most persistent propagule types produced by *Phytophthora* spp. and are 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. However, consistent success with germinating oospores in the laboratory has not been achieved (Förster et al. 1982). It is generally assumed that oospores of homothallic species germinate more rapidly and in greater abundance than those of heterothallic species (Zentmeyer & Erwin 1970).

Baumer (1980) reported 5–10% germination in soil, while Kuan and Erwin (1982) reported up to 20%. Germination of oospores is influenced by oospore age, nutrition, temperature, light, chemical treatments, and potentially a further set of currently unrecognised physiological factors. Oospores are usually formed in diseased plant tissue, both on infected plants and in fragments of decaying tissue (Erwin & Ribeiro 1996). They may be found in soil after fungal degradation of infected plant tissue. Typically, oospores will survive environmental conditions that are lethal to mycelia and sporangia. For example, the mycelium of *P. kernoviae* ceased to grow at 26°C and was killed after several hours at

35°C, but oospores required temperatures from 50 to 60°C for periods of several hours (Widmer 2011) before all propagules were killed.

The germination of oospores has been described previously, and involves the protoplasm becoming granular in appearance (Figure 1). The inner oospore wall erodes, the ooplast disappears, and the oospore swells, filling the whole oogonium (Figure 2).

Figure 1: Oospores of P. agathidicida formed in the roots of kauri from a glasshouse trial, showing the granular appearance of the oospore (width of each oospore approx. 34 µm). Note the granular texture of the lower oospore.

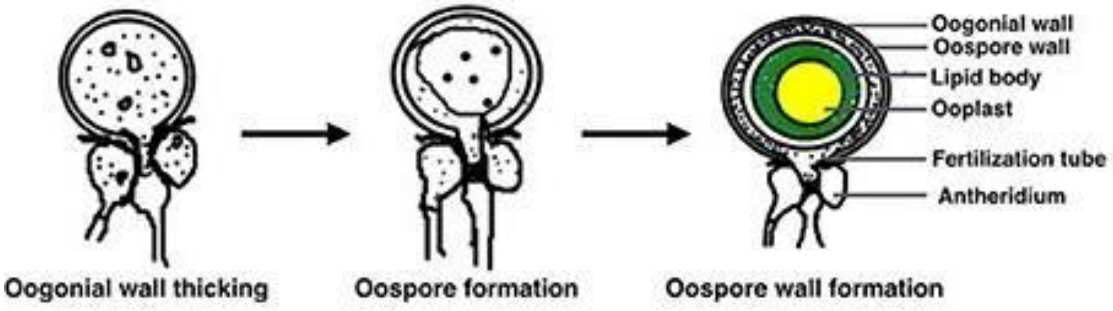


Figure 2: Steps in oospore formation showing the walls and internal structures (Guo et al. 2017).

1.3 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 media 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:

- steam is the best at penetration and effectiveness are more easily measured (with a thermometer) than they are with chemical treatments

- soil can be treated with steam within 30–60 cm of living plants without injuring them
- there are no risks of residues that may be toxic to some plants (Dion & Parker 2013).

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

1.3.1 Sterilisation principles

The effects of temperature–time combinations during composting on 64 plant pathogens (including fungi, oomycetes, bacteria, viruses and nematodes) were reviewed by Noble and Roberts (2004). For all but five pathogens, a peak temperature of 64–70°C and a duration of 21 days were sufficient to reduce numbers to below the detection limits of the tests used. Noble and 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 is also due to the production of toxic compounds (e.g. 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).

1.3.2 Heat transfer

The change of energy between two systems (bodies) due to their different temperatures is called heat transfer. The heat transfer from one body to the other takes place spontaneously, from the warmer (higher temperature) to the colder body (lower temperature). If there are no other factors, the state of the observed systems will change until heat balance is established. In practice, there are three properties of soil that contribute to their thermal variation: specific heat, thermal conductivity, and thermal diffusivity (Ghuman & Lal 1985).

Specific heat

Specific heat is the amount of heat required to raise the temperature of 1 gram of a substance by 1°C. The specific heat of some common substances is provided in Table 1. For example, the specific heat of dry soil is about one-fifth that of water.

Table 1: The specific heat of some common materials

Category	Cal/gram.°C
Sand, quartz	0.19
Dry soil	0.19
Wet soil	0.35
Wet mud	0.60
Wood	0.41
Water	1.00

Source: Gvozdkova & Arkhangelskaya 2014.

Note: the specific heat of water has the highest value of the materials tested.

Thermal conductivity

The thermal conductivity (often denoted by λ) of a substance is its ability to conduct heat. Heat moves along a temperature gradient, from an area of high temperature and high molecular energy to an area with a lower temperature and lower molecular energy. Across most soil types the thermal conductivity of soil increases with water content up to 22% (Roxy et al. 2014) (Table 2).

Table 2: Thermal properties of two soil samples: 0% and 100% saturation, at room temperature

Saturation	Temperature (°C)	Thermal conductivity (W/m. K)
0	25.8	0.364
100	21	1.59

Source Hamdhan & Clarke 2010

A soil's thermal conductivity is significantly influenced by its saturation and dry density. Saturation describes the amount of moisture contained in a soil, while dry density refers to the mass of soil particles per unit volume (Hamdhan & Clarke 2010). An increase in either the saturation or dry density of a soil will result in greater thermal conductivity.

Thermal diffusivity

In heat transfer analysis, thermal diffusivity is the thermal conductivity divided by the density and the specific heat capacity at constant pressure. It measures the rate of transfer of heat from the hot end to the cold end. It has the SI-derived unit of m^2/s. Thermal diffusivity is usually denoted as α.

There is an inter-relationship between thermal properties of soils related to the: 1) specific heat of water, 2) thermal conductivity, and 3) thermal diffusivity. This is highlighted by the fact that saturated soils (i.e. 100%) have the highest conductivity. However, increasing the water content to this level changes the density of the substance, thereby influencing its thermal diffusivity. Thus, it is hypothesised that water content and water texture will be the key factors in determining the temperature necessary to deactivate oospores of *P. agathidicida* in soil.

1.4 Stages of the project

The project is divided into three phases, each building on the findings of the previous study to increase experimental efficiency (Table 3). This technical report provides the research in the format of a scientific paper, with Background, Methods, Results, Discussion and Recommendations sections. The three sets of experiments form the basis of the sub-sections in each of the sections. The research carried out was in accordance with a co-created project plan.

Table 3: Stages of the project

Experiment	Completion	Output
Part A	June 2018	*In vitro* thermal tolerance of *P. agathidicida:* –14°C to +50°C – Plant & Food Research
Part B	May 2019	Heating and cooling thresholds for the thermal deactivation of *P. agathidicida* oospores in soil – Manaaki Whenua – Landcare Research
Part C	June 2019	Heating and cooling thresholds for the thermal deactivation of *P. agathidicida* oospores in plant materials – Plant & Food Research

1.5 Iwi consultation

Manaaki Whenua – Landcare Research (MWLR) approached Te Roroa, an iwi from the region between the Kaipara Harbour and the Hokianga Harbour in Northland, about the potential for sampling soil from confirmed kauri dieback diseased sites in Waipoua Forest. We provided an overview of the research we are engaged in with Plant & Food Research and the implications for on-the-ground risk minimisation of materials contaminated with oospores of the kauri dieback pathogen. Te Roroa provided a letter of support for the research effort and for the collection of soil from their ngahere (forest).

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 the impacts of kauri dieback by reducing the risk of accidental inoculum spread in soil and plant materials.

The Department of Conservation has issued a Research and Collection Authorisation (69218-GEO) for the collection of soil from positive kauri dieback sites from December 2018 until February 2019. The Authorisation was issued without any cost incurred for the permitting process, with the Department expecting a copy of the final report, once issued for release by the Ministry.

2 Materials and methods

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

Ten *P. agathidicida* isolates were selected throughout the geographical range where it is known to be associated with kauri dieback, as summarised in Table 4.

Table 4: Source of the *P. agathidicida* isolates used in the *in vitro* thermal tolerance study

Isolate code	H-series	Geographical origin
A	257	Raetea, Mangamuka Ranges, Northland
B	261	Titirangi, Waitakere Ranges, Auckland
C	263	Oratia, Waitakere Ranges, Auckland
D	270	Great Barrier Island
E	654	Kerikeri, Northland
F	698	Whangapoua, Coromandel
G	691	Maungaroa, Waitakere Ranges, Auckland
H	691	Waipoua Forest, Northland
I	693	Āwhitu, South Auckland
J	697	Trounson Forest Park, Northland

The 10 *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 (20°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 mycelia 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 from –14°C to 50°C (Appendix 1) and incubated for either 4, 24 or 48 h. Temperature was recorded every 5 min throughout the incubation using EL-USB-2+ data loggers accurate to +/–0.5°C (Appendix 1). 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, 3–6, 7–10 and 11–14 days old (reflecting the culture age at the commencement of the heat treatment). The 11–14-day-old strip also contained the inoculation plug, and therefore 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 regrowth assessments. There were four replicates for each isolate × temperature × 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. Machine errors of the incubators used in this experiment are presented in Appendix 1.

2.2 Part B: Heat deactivation of oospores in soil

2.2.1 Isolates and millet inoculum production

The three isolates, H261, H270, and H697 (ICMP#18404), were received from Plant & Food Research, Havelock North, under movement permit MA18489, and grown on clarified V8 agar for 3 days at 18°C. The millet inoculum of *P. agathidicida* was prepared by placing blocks of agar colonised with the isolates for 6 weeks at 20°C on sterilised millet seeds thoroughly moistened with V8 juice broth (Vettraino et al. 2001; Jeffers 2006).

After 6 weeks, millet seeds were removed from the flasks and fixed in FAA solution (formaldehyde-acetic acid-alcohol). The seeds were boiled in 10% potassium hydroxide and stained with trypan blue in lactic acid glycerol solution. The seeds were de-stained in lactic acid glycerol solution (without stain), and squash preparations were viewed under a Nikon compound light microscope. Images were captured using a Nikon Fi3 colour camera (RW-Nikon-1482) and NIS-Elements Br photographic software.

2.2.2 Soil acclimatisation time studies

Replicates of three different soil types were set up to measure the time necessary for the internal 'core' temperature to reach the desired upper and lower temperatures. Water was added to five replicates of 500 g of four soil types: 1) commercial propagation sand, 2) Waipoua loam, 3) Waipoua clay, and 4) Huia dam composite. An iButton was wrapped in parafilm and placed in the middle of each of the soil samples, and the samples were cooled from 18°C to –14°C, or heated from 18°C to 45°C in a soil drying oven overnight. The following day the soil samples were brought back to room temperature, and the iButtons were removed and downloaded.

Time taken to achieve desired temperatures was analysed by ANOVA across the four soil types using an orthogonal design on GENSTAT.

2.2.3 Temp × time 500 g study (B-1)

The time and temperature necessary to deactivate oospores of *P. agathidicida* in millet grains was tested using 500 g replicates of propagation sand, with five per temperature × time. The number of positive controls held at 18°C was five, along with the negative controls, using uninoculated millet. The three *P. agathidicida* isolates (H261, H270, and H697) were combined for this study at a rate of 20%/L. The times assessed were 2, 4, 24, 48, and 72 h, plus 1, 3 and 4 weeks (eight treatments), at temperatures ranging from –15°C to 5°C (cold), 25°C to 30°C (moderate), and >50°C (high).

The assessment of the effect of time and temperature on the viability of *P. agathidicida* oospores contained in millet was evaluated through plating to selective media. Statistical analysis was carried out on the viability data using an orthogonal design on GENSTAT to determine the effect of time on the exposed temperature for a standardised soil type.

The viability of oospores contained in millet was assessed using the vital stain Thiazolyl Blue following the method of Widmer (2011). This involved removing the seed coat of the millet grains and incubating them in the vital stain for 3 h before examination under the Nikon compound microscope. Fifty seeds were examined in all cases.

2.2.4 Temp × time soil type study (B-2)

Soil sampling

Three different soils types recovered from Waipoua forest were used to understand the time and temperature necessary to deactivate oospores of *P. agathidicida* contained in millet grains. The field collection sites from the Waipoua Forest were provided by Te Roroa, based on previous surveillance efforts. The three locations and the soil types are provided in Table 5. The sites were sampled under

a high impact permit from the Department of Conservation on two occasions: once in December 2018 and then again in February 2019, with direct supervision from kaitiaki (guardian) T. Donovan and/or T. Putuawa.

The other natural soil was from the Waitakere Ranges Regional Park and represents a composite of soil sampled from around three trees over a period of 1 year in 2010. The study site was located along the Twin Peaks Track, Huia dam (GPS E2649586; N6466012).

Table 5: Waipoua forest study sites

Category	GPS
Sand	E1647886, N6054499
Loam	E1650451, N6055136
Heavy clay (clay)	E1651749, N6054496

Approximately 80 kg of soil was collected from around three trees at each location. The soils were bulked together, and due to the nature of the sampling they were not kept intact. The soils were 'double bagged' in autoclave plastic bags, placed in 20 L plastic drums, and sealed with the lid on tightly. The drums were transported back to MWLR, where they were stored at 10°C. Three hundred gram sub-samples of the fresh soil were oven dried at 105°C overnight, and then re-weighed. Water content was calculated as a percentage of the gravimetric loss of water (Rayment and Lyons 2011; all soil methods available at: https://www.landcareresearch.co.nz/resources/laboratories/environmental-chemistry-laboratory/services/soil-testing/methods).

Experimental design

The temperature × time soil study utilised five replicates for each soil mass of 1, 2, and 5 kg, except for the 10-kg weight class; for this weight class, there was only a single replicate. There were four soil types assessed: 1) sand, 2) loam, 3) clay, and 4) composite. There were five positive and five negative controls (held at 18°C), except for the 10-kg weight class. The temperatures tested were –20°C and +50°C over a period of 3 days at a water content of 25%. Statistical analyses were carried out on the oospore viability count data, with types of substrate (sand, loam, clay and composite), four levels of water content (10%, 20%, 30% and 40%), and three temperatures (control, –20°C, and +50°C) using an orthogonal ANOVA.

2.3 Part C: Heat deactivation of oospores in kauri roots and soil

2.3.1 Part C1 – inoculated autoclaved kauri roots

Using the results from Part A as a guide, three isolates were selected for subsequent experiments. These were isolates B (H261), D (H270) and J (H697) (Table 4).

Kauri feeder roots collected from glasshouse-grown seedlings were cut into 1–2 cm sections, double autoclaved and inserted into V8 agar plates. These were incubated at 20°C for 2 weeks to ensure no contaminants emerged. Plates were then inoculated with one of the three test isolates, and incubated at 20°C for 4 weeks to allow root tissue colonisation and oospore development. Microscopic examination of sample roots after 2 weeks confirmed prolific oospore development (Figure 3).

After 4 weeks of incubation, the agar plates containing the colonised roots were placed in incubators and exposed to the various temperature × time combinations outlined in Table 6. For each isolate at each temperature × time combination there were 24 colonised root pieces, with a total of 2,088 roots across the experiment (72 roots for each temperature × time).

After incubation, these roots were removed from the agar plates and aseptically cut in half. One-half was re-plated onto a fresh V8 agar plate, incubated at 20°C, and checked regularly for the

emergence of *P. agathidicida* colonies. Initial checks were daily, then weekly, followed by monthly for up to 10 months.

The second portion of each of the 1,044 roots was placed in a 20-mL cell well containing 10-mL distilled water and baited with a cedar needle. After 4 days of incubation the cedar needle bait was plated onto *Phytophthora*-selective agar, enriched with PARP (pentachloronitrobenzene as terraclor 100 mg/L, ampicillin 250 mg/L, rifampicin 10 mg/L, and pimaricin 6 mg/L), to allow *P. agathidicida* colony emergence. Roots were then placed in fresh water and re-baited with a fresh cedar needle, which was plated onto PARP after 4 days of incubation.

Roots were then air-dried for 1–2 days, re-flooded, and baited with a fresh needle for 4 days, after which the baits were plated. This was repeated for a total of three cycles. Once a root tested positive it was not re-baited. Any roots from which *P. agathidicida* had not been baited after three cycles were then pooled (for the same isolate × temperature × time combination) and rebaited a further five times.

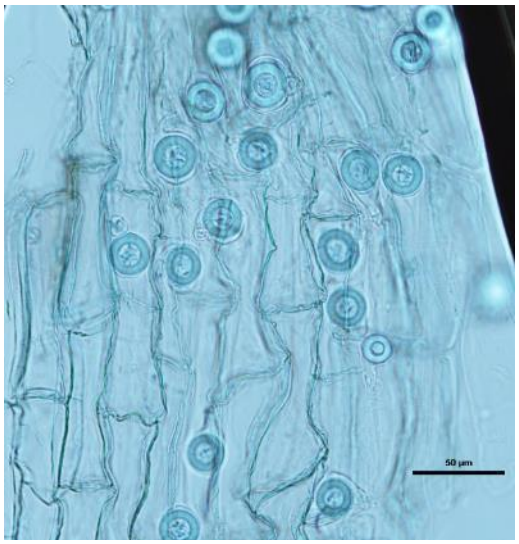


Figure 3: Micrograph of autoclaved kauri roots plated on V8 juice agar. Two weeks after inoculation with *Phytophthora agathidicida,* prolific oospore development was observed within kauri roots.

Table 6: Incubation temperatures and duration of treatment of *Phytophthora agathidicida*-inoculated autoclaved kauri roots. There were 72 root pieces for each tested temperature × time combination, indicated by an 'X'; '-' indicates that this temperature × time combination was not tested

Temperature (°C)			Incubation time (days)					
Target	Actual mean	Actual range	1	2	4	7	14	21
–15	*	*	X	X	-	X	X	X
20	*	*	-	X	-	X	-	X
35	34.8	34.5–35.5	X	X	X	X	X	X
40	40.1	39.0–40.5	X	X	X	X	X	X
45	45.0	43.0–45.5	X	X	X	X	X	-
50	49.4	48.5–50.5	X	X	X	X	-	-

* There were no continuous temperature loggers at 20°C and –15°C. Periodic checks showed the temperature was always within 1°C of the target temperature.

2.3.2 Parts C2 and C3 – infested kauri roots and potting mix

Two-year-old kauri seedlings growing in potting mix in PB2 bags were inoculated with a blend of three isolates of *P. agathidicida* (B [H261], D [H270] and J [H697]; Table 4). Inoculum was prepared by blending *P. agathidicida*-colonised V8 agar plates, colonised oats, and mycelial balls produced in liquid culture, to create a thick sludge. Approximately 50-mL of this sludge was poured into each of the three holes made with a metal spike to a depth of 10 cm in each planter bag. Seedlings were then flooded for 48 h to facilitate zoospore production and root colonisation, drained, then subsequently kept well-watered and grown at 15–20°C in a temperature-controlled glasshouse. Six weeks after inoculation, sample roots were surface-sterilised and placed onto *Phytophthora*-selective media to confirm *P. agathidicida* colonisation and oospore development before proceeding with the heat treatment.

Clumps of the seedling roots and potting mix were destructively harvested, using a sharp knife to cut portions of roughly 4 × 5 × 5 cm. These clumps were put into 100-mL glass jars with screw-top lids. The jars were placed into incubators at various temperature × time combinations (Table 7). There were four replicate jars for each temperature × time combination.

Following temperature treatment, root portions approximately 1 cm long were excised from the root mass from each jar, surface sterilised in 50% ethanol for 30 s, rinsed in distilled water, and plated onto *Phytophthora*-selective media plates. A total of 10 roots were plated from each jar, totalling 40 for each temperature × time combination. Emergence of *Phytophthora* colonies was recorded regularly for up to 4 weeks.

The remaining roots and potting mix in the jars were tested for the presence of live *P. agathidicida* using the brief and extended baiting process. Samples were fresh-baited (i.e. no drying step) with five cedar needles for 5 days. Needles were then plated onto *Phytophthora*-selective medium and emergent *Phytophthora* colonies were recorded for 7 days (Figure 4). Following baiting, samples were drained, and air-dried for 2–3 days before re-moistening and processing using the extended baiting procedure. Following this, samples were drained then flooded with fresh water and re-baited for two more cycles. Following the fourth cycle of baiting, samples that had consistently tested negative were pooled (within a temperature × time category) and rebaited for a further three cycles.

Table 7: Incubation temperatures and duration of treatment of *Phytophthora agathidicida*-inoculated kauri roots and potting mix. There were four 100 mL jars of colonised roots and potting mix for each tested temperature × time combination, indicated by an 'X'; '-' indicates that this temperature x time combination was not tested

Temperature (°C)			Incubation time (days)					
Target	**Actual mean**	**Actual range**	**1**	**2**	**4**	**7**	**14**	**21**
–20	*	*	-	-	-	X	X	X
–15	*	*	-	-	-	X	X	X
20	19.5	19.0 – 20.5	X	-	-	X	X	X
35	35.5	35.5	X	X	X	X	X	X
40	39.9	39.5 – 40.0	X	X	X	-	-	-
45	45.0	44.9 – 45.0	X	X	-	-	-	-

* There were no continuous temperature loggers at –20°C and –15°C. Periodic checks showed the temperature was always within 1°C of the target temperature.

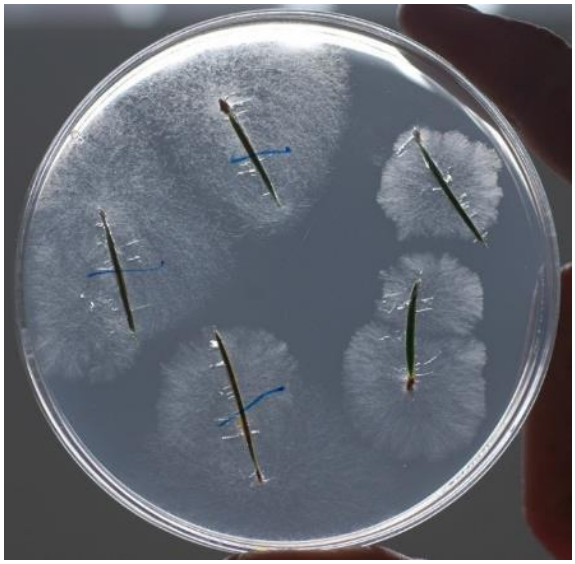


Figure 4: Cedar needles plated onto *Phytophthora* selective medium following baiting of heat-treated kauri roots and potting mix. The emergence of *P. agathidicida* can be seen from three needles (blue marks). The other colonies are not *Phytophthora*.

2.3.3 Part C4 – naturally colonised forest soil

Soil was collected from within the kauri root zones on three *P. agathidicida*-positive sites, with soils described respectively as 'sandy', 'light loam' and 'heavy clay'. The soils were a subset of those used by MWLR in Part B of the wider project.

The soil was thoroughly mixed, and a sample was tested to confirm *P. agathidicida* presence before proceeding. Soil water was adjusted so that it was thoroughly moistened but not saturated. Portions weighing approximately 150 g were placed in 500 mL plastic jars with lids, then incubated at the temperature and time combinations outlined in Table 8. A prior pilot study confirmed that the soil within the jars equilibrated to the desired test temperatures within approximately 2 h. There were three replicate jars for each soil × temperature × time combination (i.e. nine replicates for each temperature × time).

Following incubation at the prescribed temperature and time, samples were returned to room temperature (c. 20°C) then tested using the standard extended baiting process used for detecting *P. agathidicida*. This involved air-drying for 3–4 days, moistening for 4 days, flooding and baiting with cedar needles and lupines for 2–3 days, plating baits onto *Phytophthora*-selective media, and sub-culturing *Phytophthora*-like colonies to V8 agar for microscopic examination and identification.

Following removal of the baits, the flood water was drained off, the soil was again air-dried (7 days) and the extended baiting process was repeated. Samples that tested negative for both extended baiting cycles were then pooled for each soil × temperature × time combination, then dried and the extended baiting repeated for a further cycle.

Table 8: Incubation temperature and duration of treatment of forest soil and roots naturally colonised by *Phytophthora agathidicida*, collected from three sites. Nine 150 g soil samples were tested in temperature × time combinations, indicated by an 'X'; '-' indicates that this temperature × time combination was not tested

Temperature (°C)			Incubation time (days)				
Target	Actual mean	Actual range	1	2	4	7	14
20	21.1	20.3–22.1	-	-	-	-	X
35	35.3	35.0–35.6	-	-	X	X	X
37.5	37.1	36.9–37.4	X	X	X	X	X
40	40.1	39.4–41.3	X	X	X	-	-
42.5	43.2	42.5–43.3	X	X	-	-	-
45	45.4	45.1–46.0	X	X	-	-	-

2.3.4 Part C5 – kauri seedling survival at high temperatures

Two- to three-year-old kauri seedlings grown in PB2 bags in a 20°C controlled-temperature glasshouse was exposed to various temperature × time combinations (Table 9). The test temperatures were determined following a brief pilot study to help adjust critical temperatures. There were four seedlings for each temperature × time combination. All seedlings had active growth at the time of treatment. Incubators were in a PC2 laboratory, so seedlings were double bagged to minimise the risk of mites and other problem organisms contaminating the system. The bagging also prevented dehydration of seedlings during the heat treatment.

All incubators were unlit. Checks of soil temperature at the centre of each bag showed that soil temperatures were within 1°C of target temperatures within 2 to 3 h of placement in the incubator.

Following heat treatment, seedlings were removed from the incubator and returned to the 20°C glasshouse for 11 weeks to assess seedling survival and growth.

Table 9: Incubation temperature and duration of treatment of kauri seedlings. There were four 2- to 3-year-old seedlings for each tested temperature × time combination, indicated by an 'X'; '-' indicates that this temperature x time combination was not tested

Temperature (°C)			Incubation time (days)				
Target	Actual mean	Actual range	1	2	4	7	14
20	21.1	20.3–22.1	-	-	-	-	X
35	35.3	35.0–35.6	-	-	-	X	X
37.5	37.1	36.9–37.4	-	X	X	X	X
40	40.1	39.4–41.3	X	X	-	-	-
42.5	43.2	42.5–43.3	X	X	-	-	-
45	45.4	45.1–46.0	X	-	-	-	-

3 Results

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

Data on culture regrowth following incubation at various temperatures and times are presented in Figure 5. 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, and the pattern of growth, with emergence only from older portions of the culture, reflected survival of oospores but not hyphae.

The thermal tolerance of mycelia covered a narrower temperature range than that of oospores. This was the case at both the 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- to 6-day-old portions of the culture to regenerate following incubation (Figure 5A). Older portions of the cultures (containing oospores) could regrow after 24 h at –14°C, although the regrowth was sporadic and not present in all replicates. There was very little regrowth after 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 (Figure 5B).

At the upper end of the temperature range, mycelia survived 35°C for 4 h, but not for 24 or 48 h (Figure 5A). At the same temperature, regrowth from older parts of the culture containing mature oospores occurred in all isolates following 24 or 48 h of incubation (Figure 5B), although emergence of colonies was delayed by a week or more (Figure 6).

When exposed to temperatures of 40°C there was no regrowth from younger parts of the culture (Figure 5A), even after just 4 h of incubation. There was some survival of most isolates in the oospore-bearing parts of the colonies after 4 h of incubation at 40°C, although re-emergence of cultures was noted only after 2 to 3 weeks of incubation at 20°C and was very sporadic. There was no regrowth from any parts of the culture when incubated for 24 or 48 h at 40°C, nor after just 4 h of exposure at 45°C. 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 than the other isolates, and isolate D may have a slightly lower low threshold, but otherwise there was little of interest.

The results from this trial were used to set parameters and select isolates for subsequent studies. It was expected that thermal tolerance might be slightly greater in oospores formed within kauri tissue, so given the results in the current experiment, temperatures of –15°C or colder for 48 h or more at the lower end of the scale, and 35–40°C or above for 48 h or more at the upper end of the scale should be targeted. Isolate D, with a possibly slightly wider thermal tolerance, should be included in future trials, plus any other isolates except A, which had a slightly narrower tolerance.

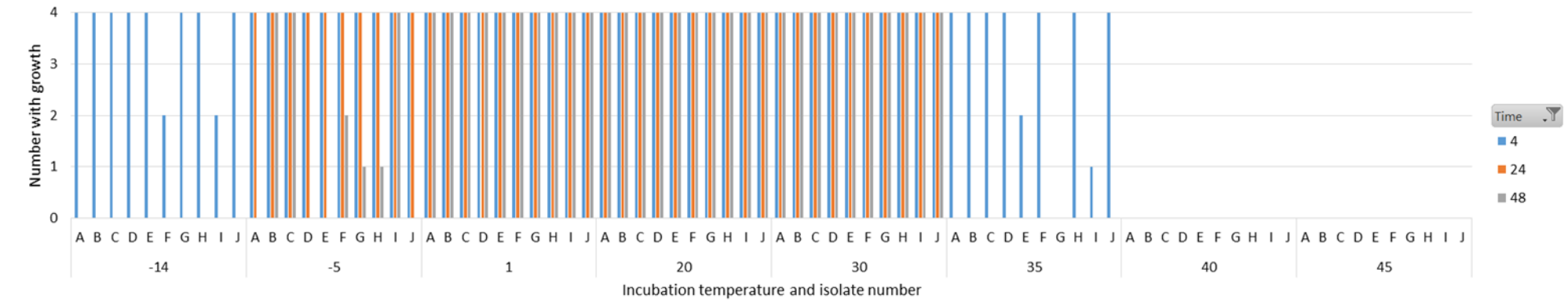
3.2 Part B: Heat deactivation of oospores in soil

3.2.1 Oospore formation in millet

Oospores of *P. agathidicida* were observed in the 'germ-region' and in the seed coat of the white millet seeds inoculated with *P. agathidicida* (Figure 7). Colonies of *P. agathidicida* were observed growing from millet inoculum plated to *Phytophthora*-selective media after 4 days. The un-inoculated control millet did not have any out-growths when plated to *Phytophthora*-selective medium.

Significantly, *P. agathidicida* can complete its life cycle on white millet grains by producing hyphae and viable oospores in the grain, and these are able to be a source of *P. agathidicida* infection.

A.



B.

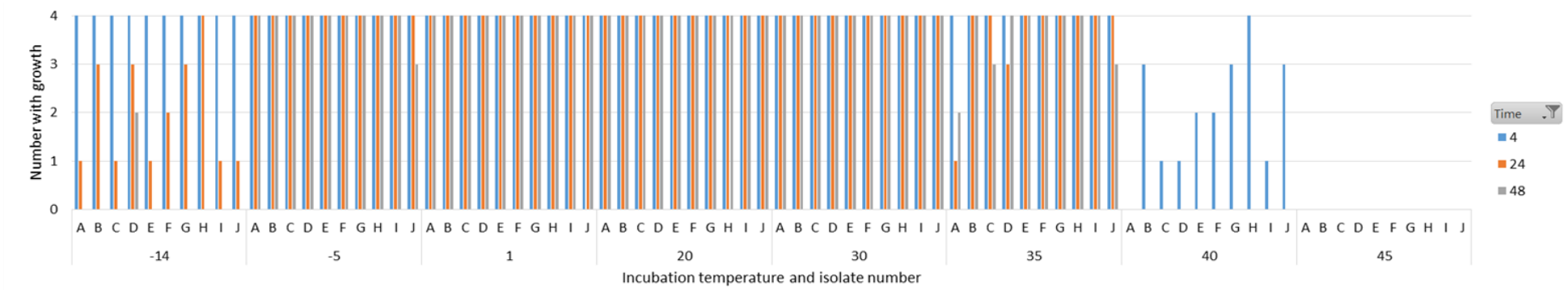


Figure 5: Out-growths from *P. agathidicida* oospore-cultures (A–J) on V8-juice agar after exposure to various temperatures for 4, 24 or 48 h. A: regrowth from 0 to 6-day-old portions of the culture; B: regrowth from 7 to 14-day-old portions of the culture.

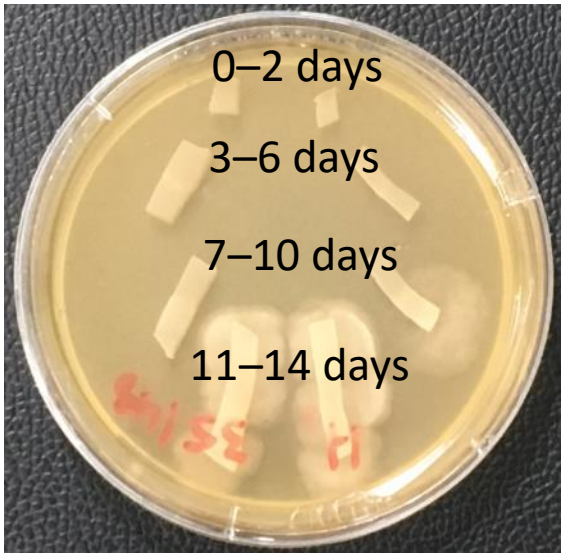


Figure 6: Emergence of *P. agathidicida* colonies from colonised agar previously incubated at 35°C for 48 h. The plated strips are from top to bottom: 0–2, 3–6, 7–10 and 11–14 day-old cultures incubated at 20°C. Note the emergence of colonies from only the older oospore-bearing portions of the culture.

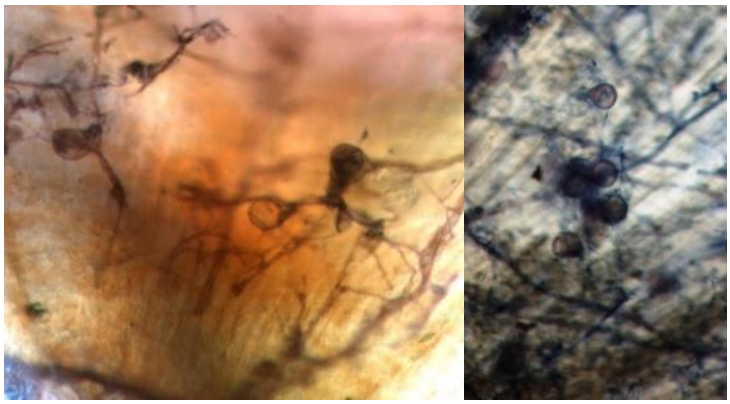


Figure 7: Oospores of *P. agathidicida* in the endosperm of white millet seed. Micrograph on RHS oospores and subtending hypahe stained blue.

3.2.2 Soil properties

The soil properties for the soil types selected for the heat study are presented in Table 10. The classification of the soil fractions is according to the New Zealand Soil Classification System. The commercial propagation sand was composed of three grades: sand, no clay fraction, and 22% silt content (Table 10). In comparison, the Waipoua loam soil had a high percentage of medium and fine sand, with small percentages of silt and clay. The Waipoua clay soil and the Huia dam composite soil had small percentages of the sand fractions and higher proportions of silt and clay (Table 10).

Table 10: Soil texture properties for selected soils for heat studies

Client	Particle Size Distribution - Fine Earth Fraction (New Zealand Classification)				
ID	Coarse Sand (2.0 - 0.6mm) (%, w/w)	Medium Sand (0.6 - 0.2mm) (%, w/w)	Fine Sand (0.2 - 0.06mm) (%, w/w)	Silt (0.06 - 0.002mm) (%, w/w)	Clay (< 0.002mm) (%, w/w)
Propagation sand	27	36	15	22	0
Waipoua loam	1	42	45	6	6
Waipoua clay	8	5	5	39	43
Huia Composite	7	9	13	35	36

3.2.3 Acclimatisation times of different soil types

The cooling and heating times for 500 g samples of four types of soil (propagation sand, Waipoua loam, Waipoua clay and Huia composite), at a water content of 10%, to reach the lower (–15°C) and upper (45°C) temperatures, are presented in Table 11, with a representative temperature trace provided in Appendix 2.

Acclimatisation time was statistically significantly different between soil types (Table 11). The propagation sand cooled significantly slower, and heated significantly faster than the clay, but slower than loamy substrates (Table 11). The Waipoua clay and Huia composite took the longest to heat, followed by sand and loam (Figure 8). These acclimatisation times will need to be taken into consideration for future experiments, as the rates of heating and cooling of soils differs according to the texture of the four different soil types.

Table 11: Mean acclimatisation times for 500 g aliquots of four soil types, at 10% water content and starting at 18°C to be cooled to –15°C and heated to 45°C.

Soil type	Time to reach –15°C (h)	Time to reach 45°C (h)
Propagation sand	$5.440 \pm 0.053_A$	$4.5 \pm 0.090_C$
Waipoua loam	$5.140 \pm 0.053_B$	$3.5 \pm 0.090_D$
Waipoua clay	$5.060 \pm 0.053_B$	$5.1 \pm 0.090_E$
Huia composite	$5.060 \pm 0.053_B$	$4.9 \pm 0.090_E$

Note: 1) Values followed by the same letter in a column are not significantly different. $LSD_{-15°C} = 0.16$ ($P = 0.05$); [2] $LSD_{45°C} = 0.28$ ($P = 0.05$); 2) The "std.error" is the standard deviation of the coefficient point estimate in the GLM, and is therefore the same in each column.

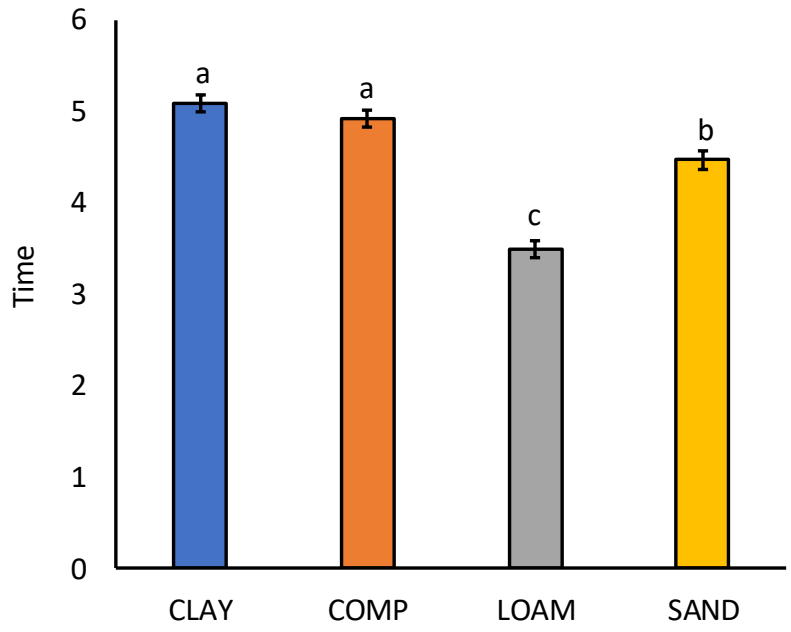


Figure 8: Heat acclimatisation time for four soil types (500 g aliquots; time in hours) to reach the desired temperature of 45°C. Values followed by the same letter are not significantly different.

3.2.4 Temp × time 500 g study (B-1)

The results of the time and temperature studies on *P. agathidicida* oospores in millet, in a standardised 500 g commercial propagation sand, are presented in Table 12. Temperature trace showing temperature profile over time). The results show that oospores remained viable for 4 weeks when held at the moderate soil temperature (Table 12) (Appendix 3: Temperature × time temperature traces of propagation sand).

Table 12: Mean % oospore viability (± s.e.m.) counts in millet grains in 500 g of propagation sand at a water content of 25%. Data are from microscopic examination using vital stains

Time	Low (–15°C)[1]	Moderate (25°C)	High (55°C)[2]
2 h	48.0 ± 0.57	48.0 ± 0.57	48.0 ± 0.57
4 h	43.2 ± 0.57	43.2 ± 0.57	38.0 ± 0.57
24 h	41.4 ± 0.57	41.0 ± 0.57	27.4 ± 0.57
48 h	38.40 ± 0.57	36.0 ± 0.57	9.0 ± 0.57
72 h	38.0 ± 0.57	36.0 ± 0.57	0
1 week	26.20 ± 0.57	35.2 ± 0.57	0
2 weeks	26.2 ± 0.57	25.4 ± 0.57	0
4 weeks	9.6 ± 0.57	29.2 ± 0.57	0

N.B. Respective LSD values for columns; [1] LSD = 0.16 (*P* = 0.05); [2]LSD = 0.28 (*P* =0 .05).

At the lower temperature threshold, –15°C did not eliminate oospore viability after 4 weeks (Table 12). After 4 weeks, the viability had dropped by a factor of five times, but some oospores remained viable, with the oospore wall remaining intact, and the oospores had a granular texture, which was interpreted as being in the pre-germination phase (Figure 10).

At the higher temperature of 55°C, oospore viability was not completely extinguished after 48 h (Table 12). To achieve 100% elimination of viability, required between 48 and 72 hours exposure to 55°C

(Table 12). Oospore viability staining differentiated active oospores from de-activated oospores, with viable oospore walls stained light pink (Figures 9 and 10). Oospores from the 'moderate' and 'lower' temperature treatments retained the integrity of the inner oospore wall, and the 'granular' appearance of the oospore (Figures 9 and 10). In contrast, the oospores recovered from the 55°C treatment (after 72 h) were empty, and the inner oospore wall was degraded or absent (Figure 11).

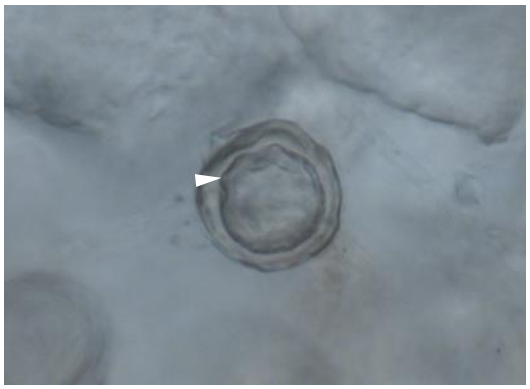


Figure 9: Oospore from moderate temperature treatment, with arrow showing intact oospore wall (approx. width 34µm)

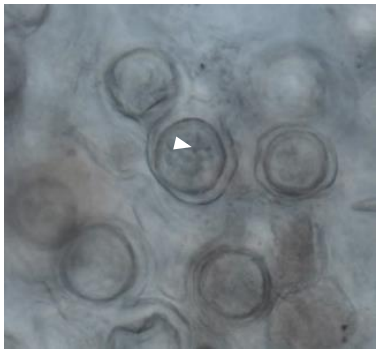


Figure 10: Oospores from –15°C treatment. Note the granular nature of the arrowed oospore (pre-germination) (approx. width 34µm).

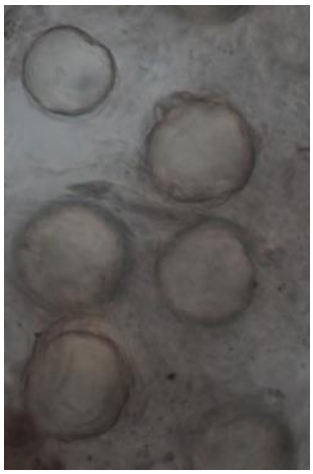


Figure 11: Deactivated, empty oospores from the 55°C treatment (after 72 h) without an inner oospore wall (approx. width 34µm).

3.2.5 Temp × time soil type study (B-2)

Substrate, water content and temperature all significantly affected the viability of oospores ($P < 0.0001$). The data from the temperature treatment on four soil types at four water contents (500 g) are presented in Table 13. For the –20°C treatment, oospore deactivation was achieved at 30% water content for clay, and 10 and 30% water content for sand (Table 13). As the water content of the soil sample increased, the efficacy of freezing increased, especially in sand and loam soil types (Table 13). Freezing at 40% water content was more effective at deactivating oospores than at 20% water content (Table 13).

For all soils, at all water contents, incubation at 18°C, only reduced oospore activity by 6.8% i.e. thermal deactivation at 18°C was not an effective treatment (Table 13).

For 20–40% water content, oospore viability counts at 50°C were all zero, except for a very low count for loam (Table 13) (Appendix 4: Temperature × time trace for sand over four water contents). The uniformity of the heating response in deactivating oospores of *P. agathidicida* was more consistent than that of the freezing treatment.

Table 13: Effects of temperature × water content (500 g aliquots) of four soil types on oospore viability counts. Data are from microscopic examination using vital stains

Temperature treatment	Soil type	10% water content[A]	20% water content[B]	30% water content[C]	40% water content[D]
-20°C	Clay	2.8 ± 0 16	2.2 ± 0.163	0	0.4 ± 0.12
	Composite	7.6 ± 0.12	2.8 ± 0.163	3.4 ± 0.115	0
	Loam	4.4 ± 0.16	3.6 ± 0.163	2.2 ± 0.163	2.4 ± 0.16
	Sand	0	5.4 ± 0.115	0	2.6 ± 0.23
18°C	Clay	95.5 ± 2.5	93.5 ± 2.3	92.5 ± 2.6	92.5 ± 2.8
	Composite	95.2 ± 1.5	93.2 ± 1.8	92.2 ± 3.3	92.2 ± 1.8
	Loam	98.2 ± 1.2	92.2 ± 2.2	92.2 ± 5.4	93.2 ± 1.8
	Sand	96.4 ± 1.3	98.4 ± 2.3	92.4 ± 5.4	93.4 ± 1.8
50°C	Clay	2.2 ± 0.16	0	0	0
	Composite	1.6 ± 0.12	0	0	0
	Loam	11.4 ± 0.16	0	0	0.8 ± 0.12
	Sand	5.67 ± 0.23	0	0	0

Note. Respective LSD values for the four columns: LSD A = 0.9212, LSD B = 0.3225, LSD C = 0.4561, LSD D = 0.6450.

The data from the study of the interaction between increasing mass and soil type at –20°C, 18°C and 50°C are presented in Table 14. At –20°C, increasing mass decreased the efficacy of freezing across all soil types (Table 14). For the smaller size classes of 1–2 kg, freezing at –20°C effectively eliminated oospore activity in the sand and loam, but this effect attenuated above 2 kg (Table 14). Oospore viability responses were the same as those observed from the earlier assessments. Freezing of spores did not affect their oospore wall or 'granular' texture (pre-germination) of the oospores. These oospores exhibited a pink colour in the oospore wall.

When held at 18°C the mass of the soil type had no effect on oospore viability (Table 14).

At 50°C oospore viability across all soil types and size classes was eliminated (Table 14). Heating impacts associated with 50°C were like those observed previously at 55°C, with heating resulting in degradation of the oospore wall, which resulted in the appearance of the oogonia being empty.

Table 14: Effect of mass × temp × soil type (1, 2, 5 and 10 kg) on percentage oospore viability, as determined using vital stains. Temperature exposure was for 3 days

Soil type[1]	Mass[2]	Viability count –20°C[3]	Viability count 18°C[3]	Viability count 50°C[3]
Clay[B]	1 kg	6.4[A]	98.6[A]	0
	2 kg	16[B]	96.4[A]	0
	5 kg	14.4[B]	94.6[B]	0
	10 kg	**18c**	**96.5**	**0**
Comp[B]	1 kg	8[A]	92.5[A]	0
	2 kg	25.6[B]	92.5[A]	0
	5 kg	14.8c	96.5[B]	0
	10 kg	**16**	**94.2**	**0**
Loam[A]	1 kg	0[A]	96.5[A]	0
	2kg	4.4[B]	98.2[A]	0
	5 kg	6.4[B]	93.4[B]	0
	10 kg	**8**	**97.8**	**0**
Sand[A]	1 kg	0[A]	98.7[A]	0
	2 kg	0[A]	95.8[A]	0
	5 kg	3.6[B]	98.8[A]	0
	10 kg	**8**	**92.1**	**0**

Notes:

[1] Soil types followed by the same letter are not significantly different (LSD = 2.73; $P = 0.05$) for oospore viability.

[2] $n = 5$ for all size classes except for 10 kg size class, where n = 1 (therefore, no LSD value).

[3] Values in the column followed by the same letter are not significantly different (LSD = 2.48; $P = 0.05$).

3.3 Part C: Deactivation of oospores in kauri roots and soil

3.3.1 Part C1 – inoculated autoclaved kauri roots

Results of the emergence of *P. agathidicida* from roots following heat exposure are presented in Table 15 and Figure 12. *P. agathidicida* was re-isolated by plating and/or baiting from 100% of roots incubated at 20°C for 2 or 7 days, allowing us to assume that all roots were colonised at the start of the experiment. At 40°C, 45°C and 50°C no *P. agathidicida* was recovered by direct plating or after three cycles of baiting, even at the lowest incubation time of just 24 h. However, it was detected in one sample (isolate J) when the residual roots from the 40°C x 1 day treatment were pooled and rebaited for a fourth time. There were no other detections at 40°C or above, in up to eight baiting cycles.

At 35°C, compared with the 20°C control, there was a reduction in detection following 1 to 7 days of incubation. There was no detection following the 14- and 21-day treatments, even after eight baiting cycles.

At –15°C the percentage of roots from which *P. agathidicida* could be recovered steadily declined with increasing incubation time. However, after 21 days at –15°C *P. agathidicida* was still alive in at least one-third of the roots (Table 15).

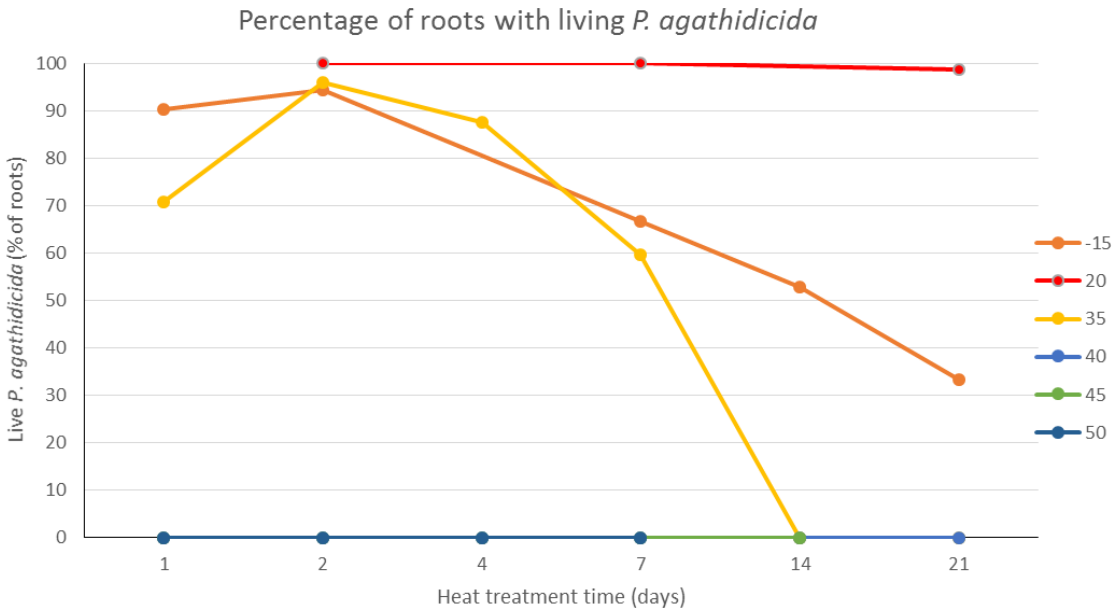


Figure 12: Survival of *Phytophthora agathidicida* in inoculated autoclaved kauri roots following exposure to various temperature × time combinations. Survival data were pooled for each root such that a root was deemed 'positive' if *P. agathidicida* was detected on either the agar plate or in any of the first three baiting cycles. There were 72 roots assessed for each temperature × time combination.

Table 15: Percentage recovery of *Phytophthora agathidicida* from inoculated autoclaved roots following incubation at various temperatures and times. Roots were either plated directly onto V8 agar (A), or flooded and baited with cedar needles three consecutive times (B). C = combined data for detection by either plating or baiting from a given sample. Data are percentage recovery, from 72 roots treated for each time × temperature combination; '-' indicates that this temperature × time combination was not tested

Temperature (°C)	Incubation time (days)					
	1	2	4	7	14	21
A. V8 plate						
–15	44	68	-	31	19	8
20	-	100	-	100	-	99
35	57	81	83	53	0	0
40	0	0	0	0	0	0
45	0	0	0	0	0	-
50	0	0	0	0	-	-
B. Cedar bait						
–15	90	94	-	65	47	31
20	-	97	-	97	0	93
35	68	92	65	25	0	0
40	0*	0	0	0	0	0
45	0	0	0	0	0	-
50	0	0	0	0	-	-
C. V8 plate or cedar bait						
–15	90	94	-	67	53	33
20	-	100	-	100	-	99
35	71	96	88	60	0	0
40	0*	0	0	0	0	0
45	0	0	0	0	0	-
50	0	0	0	0	-	-

* *P. agathidicida* was detected in one sample when the residual roots from the 40°C × 1-day treatment were pooled and rebaited for a fifth time.

3.3.2 Parts C2 and C3 – infested kauri roots and potting mix

Summaries of *P. agathidicida* isolations from root samples directly plated following various heat exposures are presented in Table 16A. Baiting results are presented in Tables 16B and 16C, and in Figure 13.

Detection of *P. agathidicida* in the root fragments directly plated on agar was substantially lower than detection by baiting of the bulk samples. This presumably reflects the substantially greater volume of root and soil material targeted in the latter.

For samples incubated at 20°C for various times, three to ten root fragments out of the 40-plated yielded *P. agathidicida* (Table 16A), indicating an acceptable background degree of colonisation of the potted seedling roots. Baiting of the bulk samples showed that all replicate jars contained viable *P. agathidicida* (Table 16B).

Baiting of samples incubated at –20°C or –15°C for 7, 14 or 21 days showed that live *P. agathidicida* was still present in all replicate jars. Total needle baits infected were comparable to those of 20°C controls. However, none of the 240 root pieces randomly selected from these jars and directly plated

onto agar yielded *P. agathidicida*, suggesting that the inoculum levels (i.e. oospores) had been substantially lowered, or had gone into dormancy.

Supporting this observation is the anecdotal evidence, which indicated that the first cycle of baiting yielded substantially lower positives than subsequent cycles (data not shown).

At 35°C, direct plating of roots again indicated reduced viability of the inoculum, although baiting results showed that all replicate jars retained live inoculum after 1, 2 or 4 days of incubation. After 7 days at 35°C, live *P. agathidicida* could only be detected in one out of four jars, and no living inoculum could be detected after 14 or 21 days of incubation.

There was a low rate of survival after 1 day at 40ºC, but no survival after 2 days; 45ºC was lethal after just 1 day, with no detection by either plating or repeated baiting.

Table 16: Recovery of *Phytophthora agathidicida* from inoculated kauri roots and potting mix following incubation at various temperatures and times. A: sample roots directly plated onto V8 agar – data are numbers of roots testing positive for *P. agathidicida*, out of 40 plated. B and C: cedar needle baiting of bulk root and potting mix samples, with four replicate samples tested in four successive baiting cycles with five baits/sample in each cycle. Data are number of sample jars testing positive out of four tested (B), and total number of cedar needles colonised out of 90 plated (C); '-' indicates that this temperature × time combination was not tested

Temperature (°C)	Incubation time (days)					
	1	2	4	7	14	21
A: Positive roots/40 plated						
–20	-	-	-	0	0	0
–15	-	-	-	0	0	0
20	3	-	-	4	10	8
35	0	0	3	0	0	0
40	0	0	0	-	-	-
45	0	0	-	-	-	-
B: Positive baits/four replicates						
–20	-	-	-	4	4	4
–15	-	-	-	4	4	4
20	4	-	-	4	4	4
35	4	4	4	1	0	0
40	3	0	0	-	-	-
45	0	0	-	-	-	-
C: Positive cedar needles/90 plated						
–20	-	-	-	37	45	46
–15	-	-	-	40	38	32
20	52	-	-	45	61	33
35	53	36	43	5	0	0
40	17	0	0	-	-	-
45	0	0	-	-	-	-

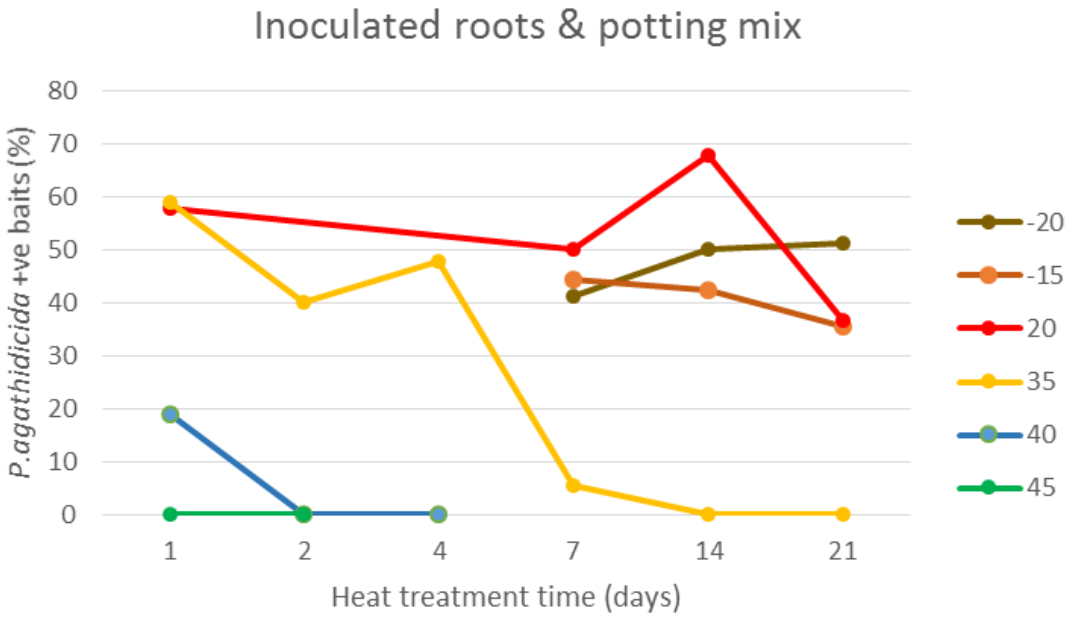


Figure 13: Positive (+ve) bait recovery of *P. agathidicida* from inoculated kauri roots and potting mix following incubation at various temperatures and times. Data are the percentage of cedar needle baits colonised by *P. agathidicida* in four successive baiting cycles with five baits per sample in each cycle.

3.3.3 Part C4 – naturally colonised forest soil

The results of baiting for *P. agathidicida* from naturally colonised forest soil following heat exposure are presented in Table 17. For the control, soil was incubated at 20°C. *P. agathidicida* was detected in all nine samples, indicating a thorough distribution of the pathogen throughout the bulk soil sample. Detection following the various heat regimes selected was very low. After 35°C for 4 days, no *P. agathidicida* was detected in the first two rounds of baiting. However, when the residual samples were bulked and rebaited, one soil (Waipoua loam) yielded *P. agathidicida*.

There was no detection following 7 or 14 days of incubation at 35°C. At 37.5°C, 40°C and 42.5°C there was no detection of *P. agathidicida*, even after just 1 day of incubation[1].

3.3.4 Part C5 – kauri seedling survival at high temperatures

Data for kauri seedling survival following heat treatment are presented in Table 18. At 20°C all seedlings survived and had some active growth at the time of final assessment, 11 weeks after treatment. However, three of the four seedlings were dormant at the apex.

No seedlings survived at 45°C for 1 day and at 42.5°C for 2 days. Although three seedlings survived 42.5°C for 1 day, most of the growing tips (on apex and branches) were killed.

No trees died at 40°C for 1 or 2 days, or at 37.5°C for 2 or 4 days, and most seedlings were actively growing at the final assessment. However, there was leaf damage noted on all the seedlings at 40°C, and on some seedlings at 37.5°C for 2 or 4 days. There was some tree mortality when seedlings were incubated at 37.5°C for 7 or 14 days.

[1] Residual soils have been stored, and could potentially be retested in the future.

Table 17: Recovery of *Phytophthora agathidicida* from naturally colonised kauri forest soils following incubation at various temperatures and times. Data are cumulative across three soil types tested and are the number of samples testing positive out of nine samples tested in two successive baiting cycles; '-' indicates that this temperature × time combination was not tested

Temperature (°C)	Incubation time (days)				
	1	2	4	7	14
20	-	-	-	-	9
35	-	-	0*	0	0
37.5	0	0	0	0	0
40	0	0	0	-	-
42.5	0	0	-	-	-

* *P. agathidicida* was detected in one sample when the residual soils from the 35°C × 4-day treatment were pooled and rebaited for a third time.

At 35°C for 7 days, one seedling died, but all seedlings survived the 14 days' exposure at this temperature.

Table 18: Kauri seedling survival following incubation at various temperatures and times. Data are from four seedlings tested for at each temperature × time combination, and are, respectively, number of seedlings alive/actively growing/dead, at the final assessment 11 weeks after treatment; '-' indicates that this temperature × time combination was not tested

Temperature (°C)	Incubation time (days)				
	1	2	4	7	14
20	-	-	-	-	4/4/0
35	-	-	-	3/3/1	3/2/0
37.5	-	4/2/0	4/1/0	3/3/1	2/1/2
40	4/2/0	4/4/0	-	-	-
42.5	3/0/1	0/0/4	-	-	-
45	0/0/4	-	-	-	-

4 Discussion

From the results obtained there were few obvious differences in thermal tolerance among the various *P. agathidicida* isolates screened as part of this first stage of the project. Isolate A may have a slightly lower upper threshold than the other isolates, and isolate D (H270) may have displayed a slightly lower low thermal threshold, but otherwise there were few differences to report. The selection of the three test isolates for subsequent experiments encompassed the thermal- and geographical range of the distribution of *P. agathidicida.*

Freezing

At lower thermal limits, with *P. agathidicida* agar cultures containing mature oospores, freezing to –14°C for 24 to 48 h killed all mycelia and substantially reduced recovery from the remaining oospores, but a small number could survive and germinate. Tolerance of freezing was greater with oospores produced in autoclaved kauri roots. Although there was a steady decline in recovery after increasing duration of freezing at –15°C, living propagules could still be detected in bioassays after 21 days of exposure. Propagules produced in living kauri seedlings and potting mix seemed even more tolerant, with repeated baiting roots and potting mix achieving high recovery rates, even after 21 days at –15 or –20°C. But the lack of recovery by direct plating of infected roots demonstrated that the pathogen had been at least; i) partially suppressed, ii) diminished in population and/or iii) entered dormancy.

Similar trends were noted in trials with bulk soils exposed to –15 or –20°C. Cooling to –15°C for 2 h halved the activity of *P. agathidicida* oospores, and only after 4 weeks were 90% of the oospores deactivated. Cryostorage is used for the long-term storage of fungi and *Phytophthora* species at –80°C, so it might be expected that freezing would not eliminate *P. agathidicida* viability. But the cryostoring process has safeguards to ensure that ice-crystals are not formed in the cell cytoplasm. Our results indicate that *P. agathidicida* propagule survival is certainly reduced by moderate freezing, Without the cryofreezing safeguards, and by snap-freezing, we may be able to increase the impact of freezing injury. The rapid cooling could initiate ice crystal formation inside the ooplast, which could cause swelling or rupturing, increasing the efficacy of cold shock to thermally-deactivate oospores of *P. agathidicida* (Simione 1992).

High temperatures

At high temperature extremes, the agar plate studies demonstrated that mycelia did not survive 35°C for 24 h, but that some portions of the culture containing oospores could survive for up to 48 h at the same temperature. None of the 10 isolates could be regrown after incubation at 40°C or higher for 24 h or more. At 45°C no isolates survived 4 h of exposure.

Similar trends were observed with inoculum produced in either autoclaved kauri roots or in living kauri roots and potting mix. After 24 h of exposure at 40°C, *P. agathidicida* could not be detected by direct plating, and only a very small amount could be detected by repeated baiting. Treatment for 48 h at 40°C, or 24 h at 45°C, eliminated any detection of *P. agathidicida* by re-isolation or repeated baiting. A temperature of 35°C appears lethal to *P. agathidicida* provided there is sufficient incubation time, with substantially reduced detection after 7 days and no detection after 14 days.

When naturally colonised forest soils were exposed to high temperature treatments, survival thresholds were at lower temperatures and times than in the previous artificial inoculation studies. *Phytophthora agathidicida* was readily detected in all control samples incubated at 20°C, but could not be detected by repeated baiting from samples incubated for 24 h or more at 37.5, 40 or 42.5°C. A single detection was made after repeated baiting of a sample incubated at 35°C for 4 days, but there were no detections following incubation at this temperature duration of 7 days or longer. This slightly lower detection could reflect the effects of microbial activity on weakened *P. agathidicida* propagules, or could simply be a factor of much lower inoculum numbers in natural soil samples. Similar trends were noted with the bulk soils of various textures, volumes and water content, where 50°C or 55°C were generally lethal within 48 h.

The previous study of Dick and Kimberley (2013) found that 4 h at 60–70°C was capable of deactivating oospores of *P. agathidicida.* The mode of action of heat deactivation appears to be the 'melting of the bonds that hold proteins together' (Leuenberger et al. 2017). Walls of hyphae and sporangia of *Phytophthora* species consist chiefly of β-glucan, arranged in three layers (Tokunaga & Bartnicki-Garcia 1971). Less than 10% of the oospore wall was found to be composed of cellulose, while proteins comprise about 12% of the oospore wall; the most abundant are arginine and glycine (Lippman et al. 1973). The peptide bonds (amide), which are the linkages that form polypeptide chains, are heat-labile above 41°C, and so this represents the 'lethal thermal threshold' for amino acids, which comprise the hyphal or oospore wall of *P. agathidicida*.

Soil texture, volume and water content

Soil texture had an impact on heat transfer. The soils used as part of this study provided considerable textural variation, especially clay content. The propagation sand had three size classes of sand, but no clay. Waipoua forest provided soils with an increasing amount of silt and clay, and the Huia dam composite soil sample was similar in texture to the Waipoua forest clay soil. The acclimatisation times for the four soil types varied significantly, based on textural composition. Loam-type soils heated the fastest, followed by sand and then clay-type soils. Overall, the acclimatisation time (to 45°C) across all soils was between 3.5 h (loam) and 5 h (clay). These times need to be added to the lethal exposure times when heating is considered for thermal deactivation of oospores of *P. agathidicida*, to ensure the core temperature of the sample attains the necessary treatment heat for the correct duration.

Clayey soils display unique thermal properties because of the way they interact with water molecules. Soils with smaller particles (i.e. silt and clay) have a larger surface area than those with larger, sand-sized particles (e.g. propagation sand). The larger surface area allows a clay soil to hold more water. This is because clay particles are so tiny that the numerous small pore spaces make water move slowly, whereas sandy soils have good drainage but low water- and nutrient-holding capacity (e.g. Dhindsa et al. 2016). This results in clay soils taking longer to transfer heat.

As the water content of the soil sample increased, the efficacy of freezing increased across all soil types. It was observed that freezing was more effective in sand at the highest water content. This may be because the water trapped in the voids in the soil formed ice crystals along the 'frost front' that may have been responsible for cell damage (Simione 1992). The nature of the freezing may have some influence on the viability of oospores. Slow cooling leads to freezing external to the cell before intercellular ice forms (Simione 1992). As ice forms outside the cell, water is removed from the extracellular environment, and an osmotic imbalance occurs across the cell. However, snap-freezing may increase the impact, as rapid cooling could ensure water remains inside the cell, enabling increased-damage to occur due to ice crystal nucleation within the ooplast (Simione 1992).

In a similar way, for the smaller size classes of 1–2 kg, freezing at –20°C effectively eliminated oospore activity in the sand and loam, but this effect attenuated above 2 kg. This result is in keeping with the higher heat diffusivity of loam and sand. There is a significant interaction between mass and water content, and overall, freezing of oospores did not affect their wall or 'granular' texture (pre-germination).

Heating to 50°C, across all soil types and size classes, at between 20 and 30% water content, for 72 h, effectively eliminated *P. agathidicida* oospore viability. The competency of heating was only compromised at a water content of 40% in loam-type soils. These soil types, like sandy soils, have a higher heat diffusivity than heavier-textured clayey soil. At this high-water content, the presence of water in loamy soils increases the specific heat of the soil, compared to a dry, clayey soil of the same mass/volume, which means it is going to take longer to heat all the way through.

There is an interplay between water content and thermal conductivity of soil, based on sand/clay content. A soil's thermal conductivity is significantly influenced by its saturation, but too much water changes the heat diffusivity of soil, because the thermal conductivity of a volume of material is influenced by the density and specific heat capacity of the material. In this situation, water (20–30%) is critical for the conductivity of heat to deactivate oospores of *P. agathidicida*, but greater than 40% volumetric water content undermines the thermal diffusivity of the soil (Roxy et al. 2014), thus

reducing the thermal efficacy of heat treatment to deactivate oospores of *P. agathidicida*. However, this can be mitigated by increasing the energy input or exposure time required to ensure the heating effect penetrates to the core of the sample.

Kauri seedling treatment

In a preliminary study to determine the heat tolerance of kauri and whether there is potential for heat treatment to eradicate *P. agathidicida* from nursery plants without killing the tree, 2- to 3-year-old kauri seedlings were exposed to temperatures determined to be near the thermal limits of the pathogen. No seedlings survived at 45°C for 24 h, or 42.5°C for 48 h. Seedlings survived 40°C for 24 or 48 h, although there was some leaf damage. Survival was good for up to 4 days at 37.5°C and at 35°C for 14 days. Although seedling numbers in this trial were not large, and none of the variables such as seedling age, condition, fluctuating temperatures and pre-conditioning of seedlings were investigated, the trial showed there is a potential window for treatment. Between 35°C for 7-plus days and 40°C for up to 2 days there appears to be a zone where *P. agathidicida* could be killed without killing the seedling. A more extensive study is required, exploring these limits with larger numbers of seedlings, and with seedlings to a longer period of pre-infection with *P. agathidicida*. The impact of factors such as fluctuating heat and pre-acclimatisation to improve thermal tolerance of seedlings should also be investigated, plus variables such as tree age, condition, growth phase and xylem / whole-plant water-content.

5 Recommendations

1 Treatment standards for the deactivation of *P. agathidicida* oospores in soil, plants and growing media.

a ***Soil treatment protocol:*** Heating moist soil (20-30% water content) to 50°C, across all soil types, for 72 h, will effectively eliminate *P. agathidicida* oospore viability.

b ***Plant treatment protocol:*** This needs refinement (see below), but there is potentially a window for treatment between 35°C for 7-plus days and 40°C for up to 1–2 days when *P. agathidicida* could be killed without killing the tree. This cannot be operationalised until further work is carried out.

c ***Growing medium treatment protocol:*** The MPI standard for soil samples up to 10 kg is heat treatment of soil to either 100°C for 25 minutes (40% relative humidity [RH]), or 85°C for at least 15 hours (40% RH) (MPI 2017). From our investigation, achieving a core temperature in the middle of the sample (at optimum water content of 20-30%), for 72 h at 50°C, should be efficacious for deactivating oospores of *P. agathidicida* from all soil types of any volume.

d ***Guidelines to support operationalisation***

- *Calibrate:*
  - Safeguards need to be established for the safe and legal transfer of plant and soil materials contaminated with the unwanted organism *P. agathidicida* from the site of operations to the receiving facility (the facility).
  - The nominated facility will need to be certified under the appropriate plant and soil, rock, gravel import and health standards.
  - The nominated facility will need to demonstrate its ability to contain the contaminated material up to the capacity of the consignment (e.g. for consignments >1,000 kg) prior to processing.
  - The nominated facility will need to demonstrate the efficacy of its heating instrument to log time × temperature to achieve the necessary deactivation time (72 h) × temperatures (50°C) in the centre of the soil sample at the optimum water content (20-30%) (Figure 14).

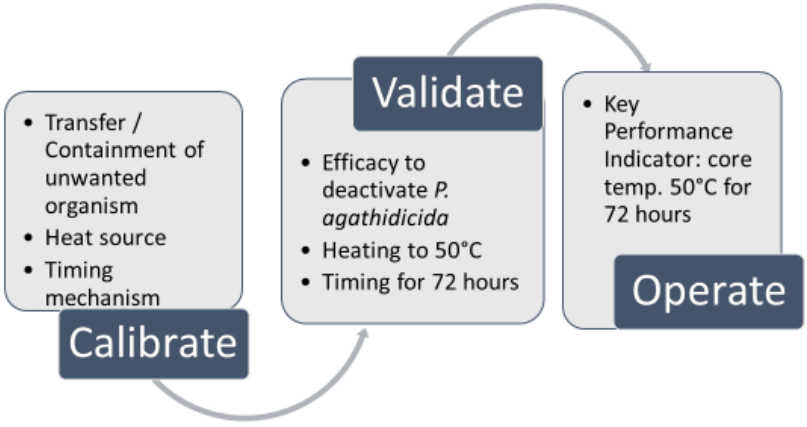


Figure 14: Steps towards operationalisation of the heat deactivation protocol for oospores of *P. agathidicida*.

- *Validate*
  - The nominated facility needs to develop, and have MPI sign-off for, its receiving, storage and disposal protocols for plant and soil material contaminated with the unwanted organism, *P. agathidicida.*
  - The facility needs to demonstrate its efficacy to deactivate oospores of *P. agathidicida* up to the maximum mass of the expected consignment(s).
  - Post-hoc analysis is needed to confirm deactivation of the inoculum.

- Post-hoc analysis of time × temperature profiles are needed at the centre of the consignment, and throughout the test consignment (Figure 14).

- *Operate (suggested only)*
  - The facility should demonstrate consistency in achieving 50°C for 72 h at the core of the sample at the optimum water content of 20-30%.
  - There should be QA/QC electronic and hard-copy logging of the processing operations.
  - Any failure to achieve the necessary time × temperature will require re-processing of the sample.
  - Six-monthly external, compliance auditing of the facility will be needed to monitor efficacy and compliance (Figure 14).

e List of risks associated with using these protocols

These include:

- the efficacy of heating systems to measure internal core temperatures
- estimation of soil water content to assist with optimal hydration of samples prior to treatment
- lack of operator-diligence in relation to the pre- and post-treatment risks of the unwanted organism, and transfer control safeguards and protocols.

2 Ongoing research: Phase 2: Research glasshouse trials of pasteurisation of *P. agathidicida*-infected plants

a Rationale

- From the pilot study with heat treatment of kauri seedlings, there appears to be a window of temperature × time combinations where *P. agathidicida* spores will be killed but kauri trees will survive.
- This observation warrants further research. There is possibly not much margin for error, and many variables need to be explored to refine any potential nursery treatments.

b Variables to test

These should include:

- tree age
- growth stage (active v dormant)
- extended pre-exposure time to infection
- physiological measures such as xylem water potential, leaf conductance
- hardening off before treatment
- gradual vs. sudden increase in temperatures (pre-conditioning)
- fluctuating vs. stable temperatures (e.g. 40°C days, 35°C nights for prolonged periods.

c Material to test

Materials include:

- kauri seedlings
- *P. agathidicida*-infected roots, soil and potting media
- *P. agathidicida*-infected living seedlings

d Measurements

Measurements to be carried out are:

- tree survival and health (pre- and post-treatment)
- physiological parameters (pre- and post-treatment)
- pathogen survival (pre- and post-treatment.

e Estimate of probable costs

Based on the current research effort, an estimate of probable costs is provided. The summary provided below is not an exact evaluation of the costs to undertake this phase of the project, and should permission be granted to proceed, then the proposed costs will need to be reviewed

based on personnel availability, exact trial parameters, number of kauri family lines tested, and number of replicates as part of the co-development of any future “project plan”.

Task	Personnel	Approx. cost ($000)
Inoculum production	MWLR	5-10
Infesting kauri seedlings with differing pre-exposure times to disease	MWLR	25-75
Infesting kauri roots	PFR	35-50
Heat treatments	PFR	45-60
Health/PA kill assessments	PFR	55-70
Reporting	Team	35-50

f Risks / barriers that may affect likelihood of successful implementation of Phase 2

- Kauri seedlings need to be available.
- The fine thermal tolerance window between pathogen deactivation and plant mortality may not be achievable on a large scale, due to the high proportion of plant mortality associated with the stringent boundaries of the prescribed treatment.

6 References

Allan P, Lamb D, Charlton D 1981. Sterilisation and pasteurisation of soil mixes. South African Avocado Growers' Association Yearbook 4, pp. 124–127.

Baumer JS1980. Effect of sitosterol on the plasma membrane composition of *Phytophthora megasperma* f.sp. *glycine* and inoculum density-disease incidence relationships of Phytophthora root rot of alfalfa. PhD dissertation, University of California, Riverside.

Beever RE, Bellgard SE, Dick, MA, Horner IJ, Ramsfield TD 2010. Detection of *Phytophthora* taxon Agathis (PTA): Final report. Prepared for MAF, Biosecurity New Zealand on behalf of the Kauri Dieback Joint Agency.

Bellgard SE, Padamsee M, Probst CM, Lebel T, Williams SE 2016. Visualising the early infection of *Agathis australis* by *Phytophthora agathidicida* using microscopy and fluorescent *in situ* hybridisation. Forest Pathology. doi: 10.1111/efp.12280.

Bellgard SE, Paderes EP, Beever RE 2010. Comparative efficacy of disinfectants against *Phytophthora* 'taxon Agathis' (PTA). Paper presented at 5th IUFRO Conference Phytophthora in Forests and Natural Ecosystems: Phytophthora diseases in forest trees and natural ecosystems, Rotorua, New Zealand, 7–12 March 2010.

Bellgard SE, Weir, BS, Pennycook, SR, Paderes EPP, Than DJ, Anand N, Beever RE† 2013. Specialist research into the biology, pathology, ecology and detection of PTA. Landcare Research Report.

Bollen GJ, Volker D 1996. Phytohygenic aspects of composting. In: de Bertoldi M, Sequi P, Lemmes B, Papi T Blackie eds. The science of composting: European Commission International Symposium. London, Academic & Professional. Pp. 233–246.

Coventry E, Noble R, Mead A, Whipps JM 2002. Control of allium white rot (*Sclerotium cepivorum*) with composted onion waste. Soil Biology & Biochemistry 34: 1037–1045.

Dhindsa HS, Sharma RD, Kumar R 2016. Role of fly ash in improving soil physical properties and yield of wheat (*Triticum aestivum*). Agricultural Science Digest 36(2): 97–10.

Dick M, Kimberley MO 2013. Deactivation of oospores of *Phytophthora* 'taxon Agathis'. MPI Contract No. 15775. Rotorua, New Zealand Forest Research Institute Limited.

Dion M, Parker W 2013. Steam sterilisation principles. Pharmaceutical Engineering 30(6): 1–8.

Erwin DC, Ribeiro OK 1996. Phytophthora diseases worldwide. St Paul, MN, APS Press. http://dx.doi.org/10.1017/S001447979825109XErwin and Ribeiro 1996

Förster H, Ribeiro OK, Erwin DC 1982. Factors affecting oospore germination of *Phytophthora megasperma* f.sp. *medicaginis.* Phytopathology 73: 442–448.

Gadgil PD 1974. *Phytophthora heveae*, a pathogen of kauri. New Zealand Journal of Forestry Science 4: 59–63.

Ghuman BS, Lal R 1985. Thermal conductivity, thermal diffusivity, and thermal capacity of some Nigerian soils. Soil Science 19(1): 74–80.

Guo T, Wang XW, Shan K, Sun WX, Guo LY 2017. The loricrin-like protein (LLP) *of Phytophthora infestans* is required for oospore formation and plant infection. Frontiers in Plant Science 8(February): 142.

Gvozdkova A, Arkhangelskaya T 2014. Thermal diffusivity of peat, sand and their mixtures at different water contents. Geophysical Research Abstracts Vol. 16. EGU2014-8494.

Hamdhan IN, Clarke BG 2010. Determination of thermal conductivity of course and fine sand soils. Proceedings of the Geothermal Congress, Bali, Indonesia, 25–29 April 2010.

Jeffers SN 2006. Identifying species of *Phytophthora.* Clemson University. http://fhm.fs.fed.us/sp/sod/misc/culturing_species_phytophthora.pdf

Kuan T-L, Erwin DC 1982. Effect of matric potential on Phytophthora root rot of alfalfa. Phytopathology 72: 543–548.

Leuenberger P, Ganscha S, Kahraman A, Cappelletti V, Boersema PJ, von Mering C, Claassen M, Picotti P 2017. Cell-wide analysis of protein unfolding reveals determinists of thermostability. Science 335(6327): eeai 7825.

Lippman E, Erwin DC, Bartnicki-Garcia S 1973. Isolation and chemical composition of oospore-oogonium walls of *Phytophthora megasperma* var. *sojae*. Journal of General Microbiology 80: 131–141.

MPI 2017. Import Health Standard, Soil, Rock, Gravel, Sand Clay and Water, 1 July 2015. Imports and Exports, MPI, Wellington.

Noble R, Roberts SJ 2004. Eradication of plant pathogens and nematodes during composting: a review. Plant Pathology 53: 548–568.

Rayment GE, Lyons DJ. 2011. Soil Chemical Methods – Australian Soil and Land Survey Vol. 3. CSIRO Publishing Collingwood, Victoria.

Roxy MS, Sumithranand VB, Renuka G 2014. Estimation of soil moisture and its effect on soil thermal characteristics at Astronomical Observatory, Thiruvananthapuram, south Kerala. Journal of Earth System Science 123(8): 1793–1807.

Simione FP 1992. Key issues relating to the genetic stability and preservation of cells. J. Parent. Sci. and Technology 46: 226–232.

Tokunaga J, Bartnicki-Garcia S 1971. Cyst wall formation and endogenous carbohydrate utilisation during synchronous encystment of *Phytophthora palmivora* zoospores. Archives of Microbiology 79(4): 283–292.

Vettraino AM, Natili G, Anselmi N, Vannini A 2001. Recovery and pathogenicity of *Phytophthora* species associated with a resurgence of ink disease in *Castanea sativa* in Italy. Plant Pathology 50: 90–96.

Widmer T 2011. Effect of temperature on the survival of *Phytophthora kernoviae* oospores, sporangia and mycelia. NZ Forest Science 42S, S15-S23.

Zentmeyer GA, Erwin DC 1970. Development and reproduction of *Phytophthora.* Phytopathology 60: 1120–1127.

Appendix 1: Machine (incubator) and measurement (thermometers) errors

Plant & Food Research incubator

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

MWLR

Thermochron iButton

DS1922L iButtons and EL-USB-2+ data loggers are both quoted as having a level of accuracy of +/-– 0.5°C

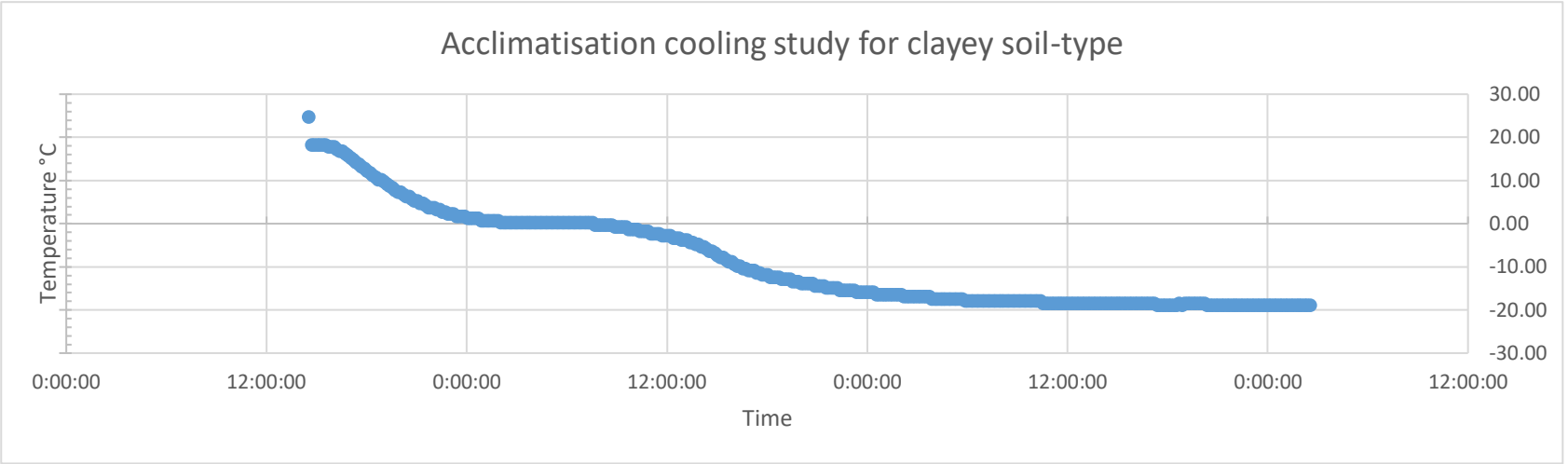
Digital thermometer

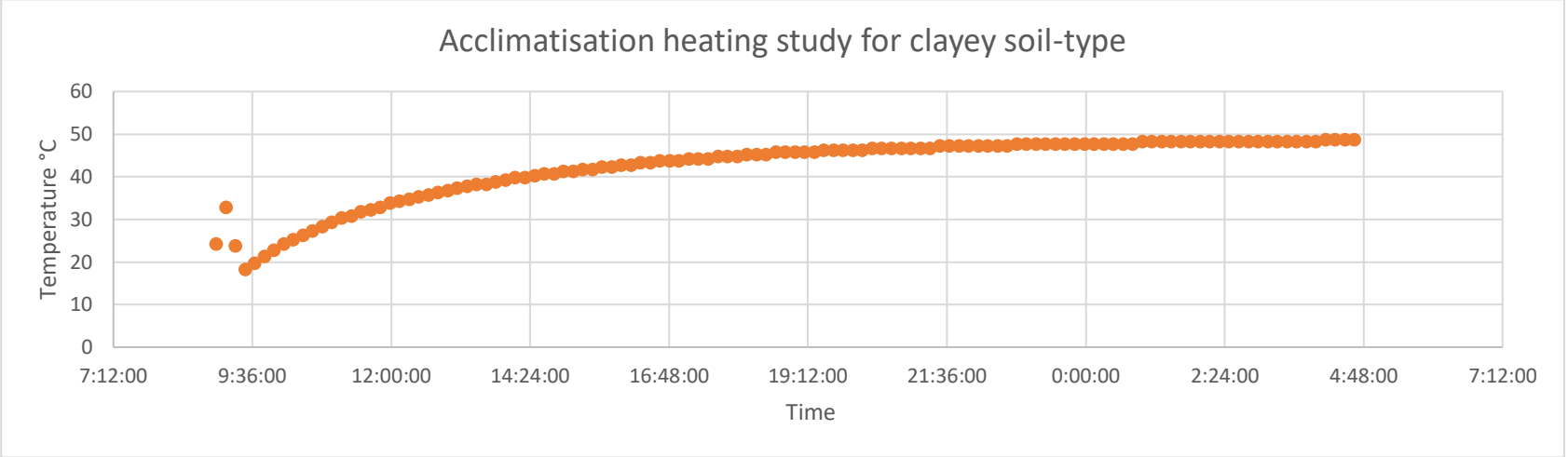
0.5°C between –20°C and 70°C.

Incubators

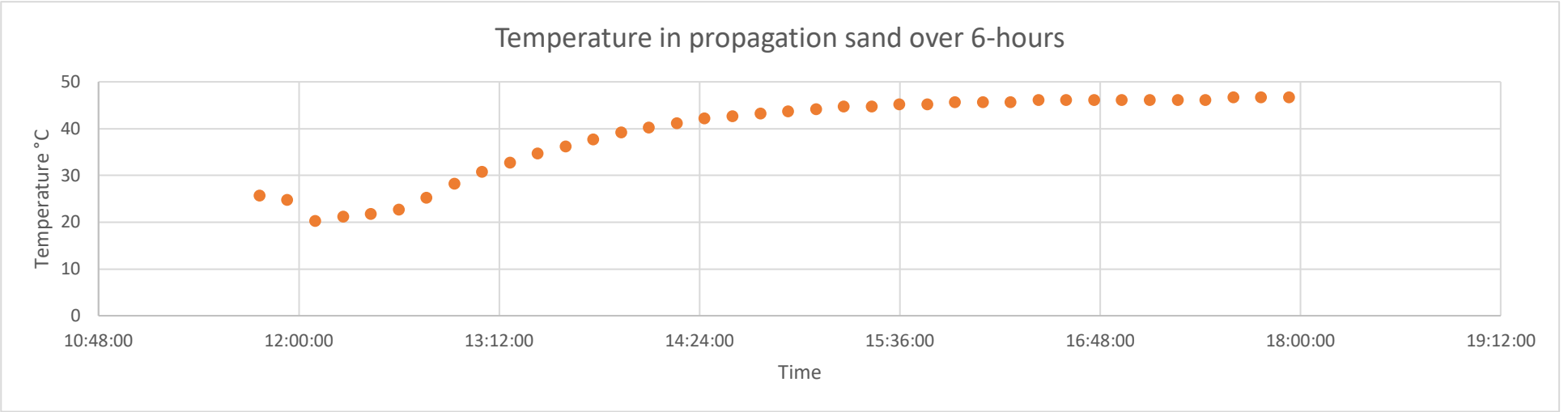
Target temp. (°C)	Actual mean (°C)	Range (°C)
–20	–19.2	–19 to –21
18	18.9	19.5 to 20.5
50	50.0	48.5 to 55.5

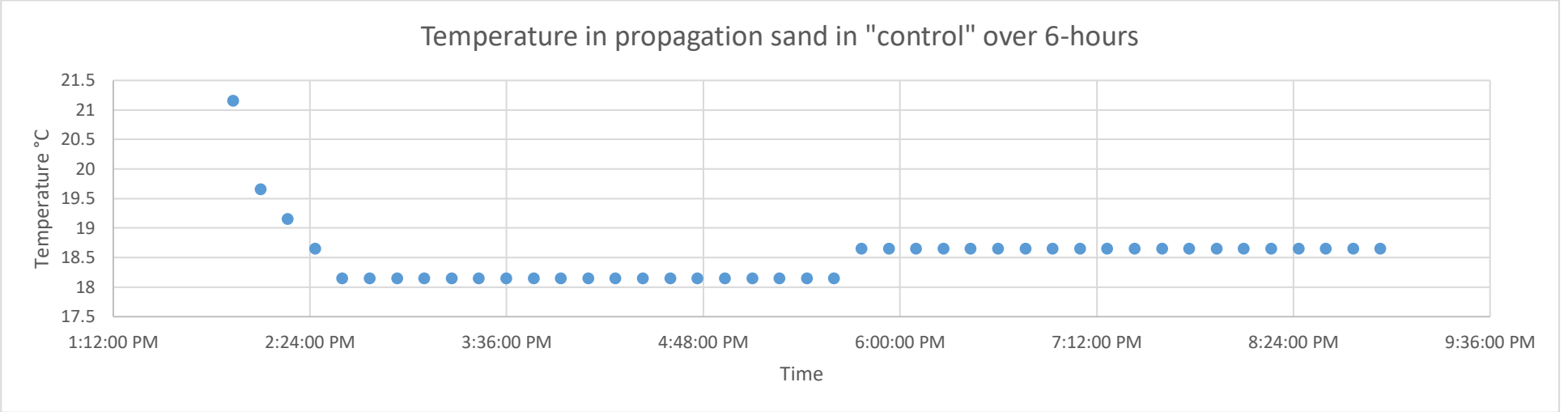
Appendix 2: Soil acclimatisation temperature trace for clay soil type (cooling and/or heating)



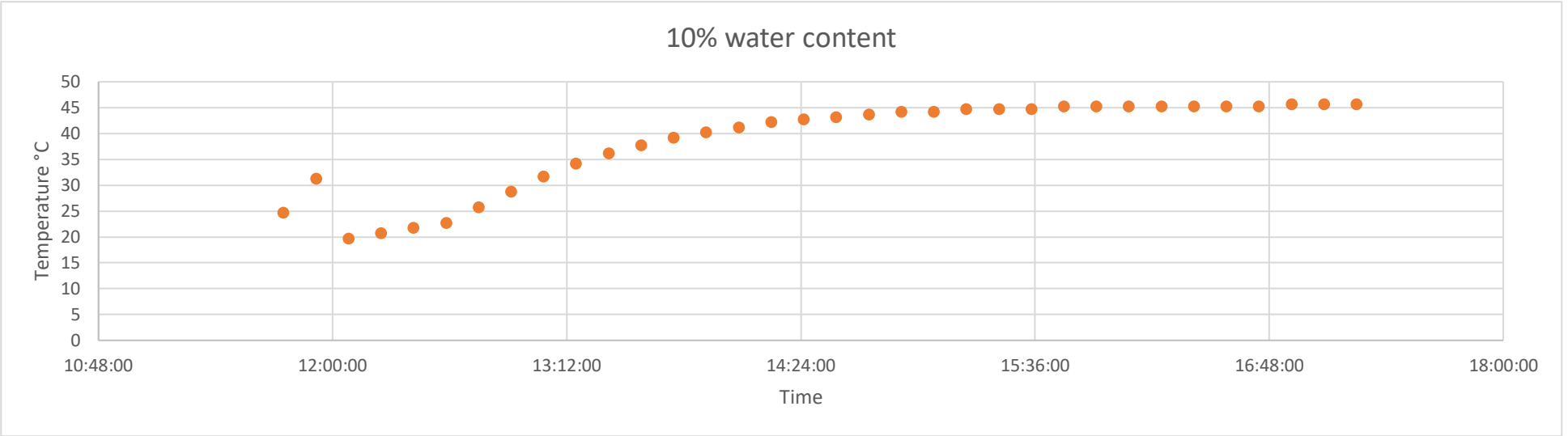


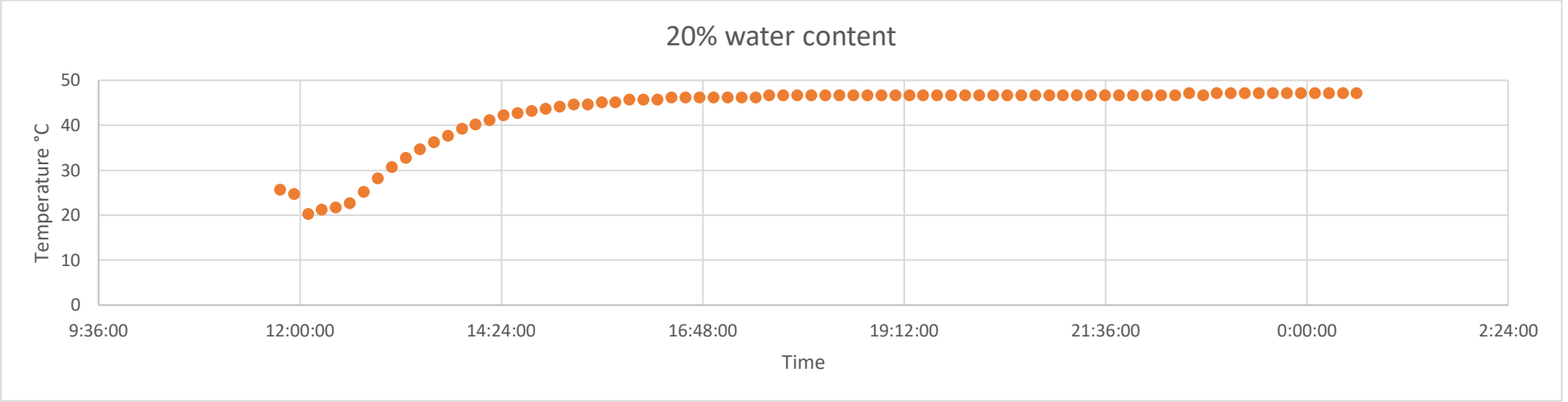
Appendix 3: Temperature × time temperature traces of propagation sand

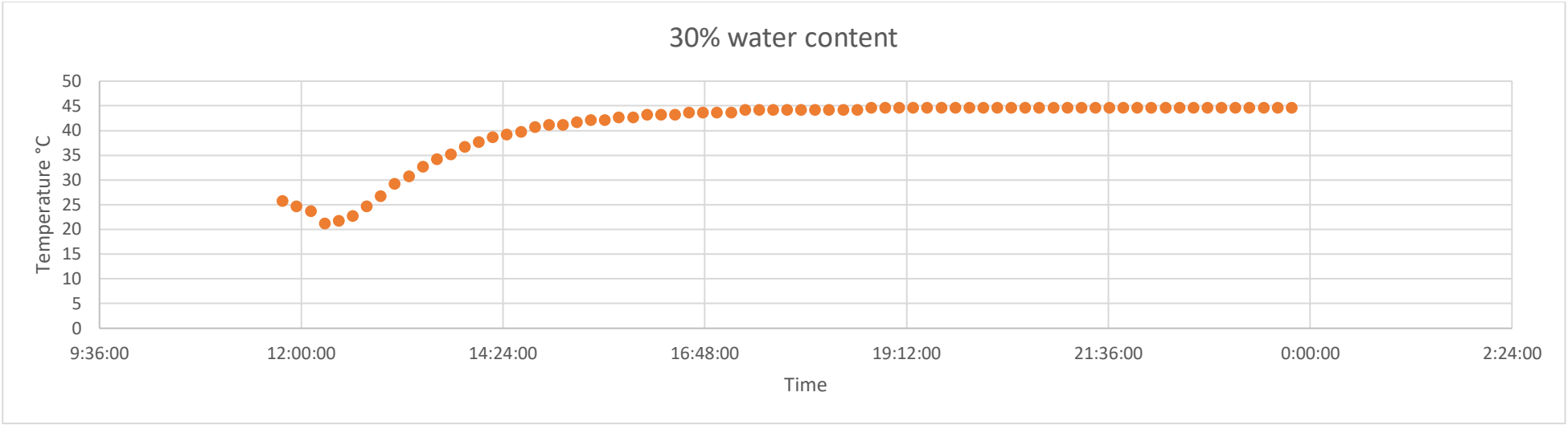


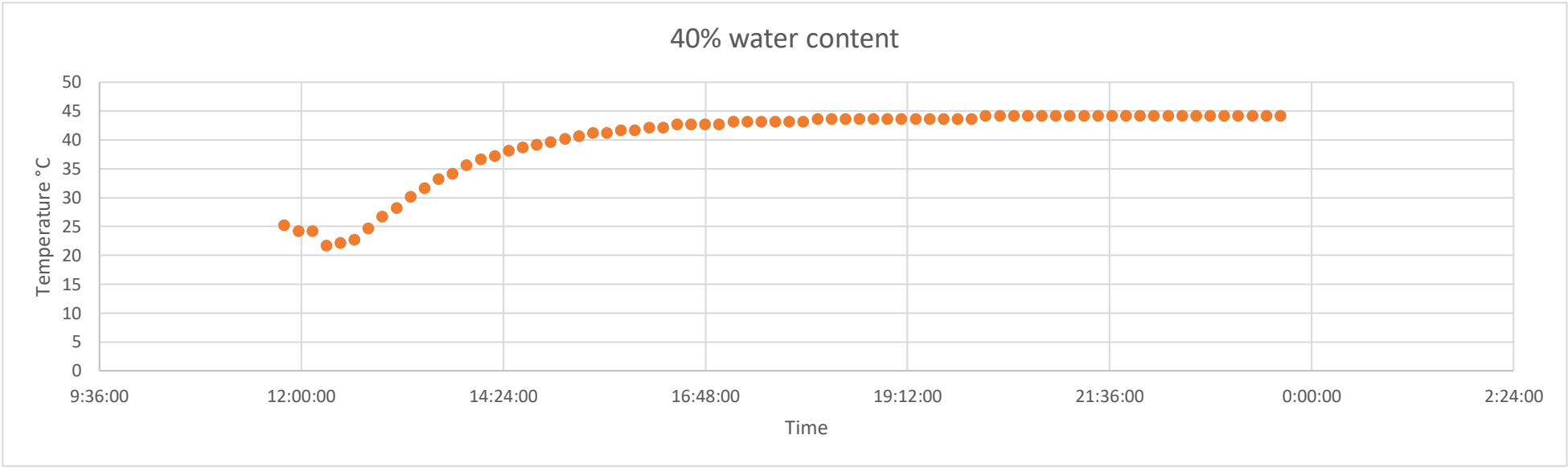


Appendix 4: Temperature × time trace for sand over four water contents






30% water content
Temperature °C
50
45
40
35
30
25
20
15
10
5
0
9:36:00
12:00:00
14:24:00
16:48:00
19:12:00
21:36:00
0:00:00
2:24:00
Time


40% water content
Temperature °C
50
45
40
35
30
25
20
15
10
5
0
9:36:00
12:00:00
14:24:00
16:48:00
19:12:00
21:36:00
0:00:00
2:24:00
Time