CLIENT REPORT (Confidential)

Deactivation of Oospores of *Phytophthora* Taxon Agathis – Phase 2

REPORT INFORMATION SHEET

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EXECUTIVE SUMMARY

Report Title: Deactivation of Oospores of *Phytophthora* taxon Agathis – Phase 2
Author: Nari Williams

The problem

Effective methods to deactivate *Phytophthora* taxon Agathis (PTA) oospores, the most persistent of the discrete propagules types produced by *Phytophthora* spp., are needed so measures can be taken to help prevent the dissemination of infective material and ultimately slow the spread of PTA.

This project

This project validates the Dick and Kimberly (2013) PTA oospores deactivation study. In addition to the work done by Dick and Kimberly (2013) it includes a post treatment storage period, allowing for enzyme activity to stabilise prior to viability staining, and a comparison to analysis by quantitative PCR. The findings are applied to assess the effectiveness of oospore deactivation by heating.

Key Results

Oospore viability decreased substantially with temperature when assessed by tetrazolium bromide staining, oospore germination and re-isolation by baiting. Baiting analysis provided the most conclusive assessment of viability. It is unclear whether tetrazolium bromide staining and oospore germination are good indicators of oospore viability, due to a high degree of subjectivity and uncertainty associated with assessment of this methods. Quantitative PCR showed a decrease in the detection of PTA with incubation time regardless of the incubation temperature and for the sandy soil only; there was a decrease in PTA detection with temperature.

Implications of Results for Client

Heating contaminated materials to above 50^{o}C for prolonged periods will substantially reduce the risk of spreading PTA. Studies using oospores that are cultured in the laboratory need careful consideration for extrapolation to naturally contaminated soil, water and plant material. It is not recommended that qPCR is used as a method to infer oospore viability.

Further Work

Consideration for future studies should be given to recent developments in the understanding of the survival strategies of *Phytophthora* in plant roots. Jung et al (2013) reported the development of stromata in host roots as a key survival strategy of *P. cinnamomi*. Similar structures have been recently observed being formed by PTA in the roots of kauri in greater abundance to oospores (Bellgard, Williams, Probst, Padamsee, & Lebel, 2014). These observations further support future studies on soil sterilization and hygiene should being carried out on naturally infested soils and root fragments rather than spores produced in vitro.

Further consideration should be given to the use of solarisation bags as a method of decontamination in remote areas with further studies investigating the role of microbial antagonism, maintenance of anoxic conditions and starvation from fresh host material within these bags.

Deactivation of Oospores of *Phytophthora* taxon Agathis – Phase 2

Nari Williams

March 2015

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1. BACKGROUND

The goal of the Kauri Dieback Programme (KDP) is to slow the spread of *Phytophthora* taxon Agathis (PTA) and to undertake measures to limit its effect on individual trees and on ecosystems. An important part of this process is to ensure that there is no viable inoculum of *Phytophthora* taxon Agathis (PTA) in any material before it enters or is removed from kauri field sites. Material could include mud and soil washed from footwear, equipment and vehicles used in track maintenance. Oospores are the most persistent of the discrete propagules types produced by *Phytophthora* spp. and a key survival propagule in soil for many species, especially those that do not produce chlamydospores (Erwin & Ribeiro 1996). When conditions become favourable, oospores germinate and develop mycelia or produce sporangia. Typically oospores will survive environmental conditions of desiccation and heat that are lethal to other *Phytophthora* propagules.

Once oospores are mature they may remain viable but in a dormant state for several years. Conditions that break dormancy and promote germination at any one time may not lead to the germination of all of the spores. Hence determination of whether material such as plant tissue or soil contains viable inoculum requires methods other than plating onto agar medium and evaluating colony formation. The ability of those oospores characterised as alive but dormant to survive and germinate at a later stage remains an issue for the development of mitigating procedures when managing *Phytophthora* diseases. At low-moderate temperatures dormant spores in soil have been demonstrated experimentally to germinate after many months albeit at low percentages (Widmer 2010; 2011).

Preliminary work has been carried out to investigate means of deactivating PTA oospores as reported by Margaret Dick and Mark Kimberly (2013; Client Contract MPI 15775/Scion Output 50797). From which it was recommended that the tetrazolium bromide staining method employed should be validated by repeat analysis of selected treatments employing both oospore staining and a bioassay for viability. A further recommendation was to store treated spores for a period post treatment to allow for enzyme activity to stabilise prior to viability staining. The recent publication of quantitative PCR methods for detecting PTA provides an alternate means of monitoring inoculum viability in culture and soil (Than et al., 2013). Such PCR based approaches have been used for other species of *Phytophthora* to monitor inoculum densities within soil and provide an efficient means of monitoring inoculum densities across a range of experimental applications (Hussain, Lees, Duncan, & Cooke, 2005).

This project validates the analysis of Dick and Kimberly (2013), compares these results to analysis by quantitative PCR and applies these findings to the assessment of oospore deactivation by heating.

2. METHODS

2.1 Oospore culturing

Oospores of PTA isolates (NZFS 3681, 3687 and 3709) were cultured on carrot agar for 3 days before small blocks taken from the leading edge of the colony were placed in petri dishes of sterile clarified V8 broth. Plates were sealed with plastic film and incubated at 20^{o}C in the dark for 8 weeks as described by Dick and Kimberly (2013). A spore suspension was produced by macerating in an an ULTRA-TURRAX® Tube Drive for 60 seconds at maximum power to separate the oospores from the mycelium.

2.2 Tetrazolium bromide staining

Tetrazolium bromide staining followed the protocol used by Dick and Kimberly (2013). In brief, mesh sections imbedded in oospores were immersed in a 1% solution of tetrazolium bromide for 24 hours at 34^{o}C. Following this each section was examined under a microscope and the number of oogonia of different shades (unstained, black, purple, pink) was recorded. Due to the subjectivity of assessment, each mesh was assessed by two staff members independently with both observing at least 100 oospores from each mesh sample.

2.3 Oospore germination

Oospore germination was observed by rinsing the treated oospores from the mesh. The mesh pieces were then incubated in concave microscope slides in water for 6 weeks at ambient temperature. Oospore germination was observed at 2, 4 and 6 weeks following immersion in water on the microscope slides, looking for the production of a mycelium germ tube from the oospore. As with the tetrazolium bromide stained spores, these were assessed by two staff members in parallel to account for the subjectivity of the assessment. Each of the two assessors observed at least 100 spores in five replicates per treatment.

2.4 Viability bioassay

Germination analysis by water baiting involved placing the heat treated mesh into 250 ml plastic containers flooded with 120 ml of sterile pond water. Three cedar needles and three rhododendron leaf pieces were floated on the surface of each dish. The pieces of rhododendron were made with a 10 mm diameter cork borer. All bait containers were incubated at room temperature on the laboratory bench. Baits were harvested after three days, blotted dry and placed onto PARP agar. Fresh baits were added and harvested at three-daily intervals up to 12 days. A positive control was run in parallel with a 5 mm^2 PTA culture grown on VTOM media was placed in a container of sterile pond water which was baited as described above.

2.5 Quantitative PCR

DNA was extracted from three replicates of 1 cm^2 mesh per treatment (see section 2.7) using a modified version of the method published by Ramsfield et al. (2013). Positive controls were

meshes with 1×10^3 oospores. Negative controls were meshes without oospore inoculation. The mesh samples were added to 5 ml polyethylene tubes, containing one 9.5 mm and 4 mm stainless steel balls, and 800 µl of buffer CSL-VF, 200 µl buffer PPS (FastDNA kit, MP Biomedicals, OH, USA). Material was lysed on a 2000 Geno/Grinder™ (SPEX Certiprep, NJ, USA) for two cycles of 1400 strokes per minute for 20 s. Tubes were centrifuged at 3200 ×g for 10 min. Supernatant was removed and DNA extracted using the FastDNA kit from step five of the manufacturers' instructions. Real-time PCR was performed on triplicate reactions for each soil sample as described previously by Than et al. (2013). Strain NZFS3772 genomic DNA was used as a positive control and a negative control (no template DNA) was included for each series of reactions. As per the published method, a TaqMan® Environmental Master Mix 2.0 and Internal Positive control kit (Life Technologies) were used. Real-time PCR was performed on an Eco Real-time PCR instrument (Illumina).

2.6 Study 1: Validation of killing oospores by heating.

This study analysed four heat treatments which were analysed using tetrazolium bromide staining, oospore germination and recovery by baiting. The oospores were passed through sterile cheesecloth to remove the bulk of the mycelial strands from the oospore suspension. Oospores were re-suspended to a concentration of 1×10^3 oospores per ml and mounted on 25 µm nylon mesh (Millipore) to a final spore density of 1×10^3 oospores per cm^2. The mesh filters were cut into 1 cm square sections and allocated to temperature treatments at random.

Mesh sections containing oospores were placed into 1.5 ml microcentrifuge tubes and immersed in 1 ml of water. The tubes were placed on heat blocks at 22, 50, 60 and 70^oC for 4 hours. Replicate sets of oospores were produced. One set was analysed immediately, using the three methods described above. The second set were left for two weeks at room temperature to enable enzyme activities to decline in dying spores and were then analysed using the three methods. Spores were assessed for viability by staining with tetrazolium bromide, direct observation of oospore germination and baiting. All treatments were replicated 5 times within treatment blocks in a randomized block design.

2.7 Study 2: Analyze PTA decay within homogenized soil mixtures containing known quantities of oospores assessing wet heat.

Oospore suspensions were prepared as described above, however the suspension was further clarified by filtering through a nested sieve with a 75 and 25 µm sieves. Oospores were rinsed from the sieve and re-suspended to 1×10^3 oospores per ml before being mounted on a 25 µm mesh as described above in study 1.

Three soils (sand, forest loam and forest clay) were dried overnight at 40^oC to remove excess moisture, sieved through a 2 mm sieve and thoroughly homogenized. The forest loam and clay soils were taken from left over kauri forest soils submitted for diagnostic analysis during the ad-hoc sampling programme. Soil (1 gram) was portioned into 2 ml micro-centrifuge tubes and 1 ml of sterile deionized water was added to each tube and mixed to form a soil-slurry. Six 1 cm^2 pieces of mesh, each imbedded with 1×10^3 oospores were added to each tube and submerged into the slurry mixture.

The soil slurries were heated at 22, 40, 50, and 60^{o}C, for 15 minutes, 2 hours and 4 hours, respectively in a heat block. The mesh samples were recovered from the soil slurry and separated into two sets of three meshes for parallel qPCR, tetrazolium staining and bioassay validation. Samples for PCR analysis were stored at -20^{o}C prior to DNA extraction. The bioassay, tetrazolium staining and qPCR were carried out as described above. One replicate set of positive control meshes was run in parallel to the experiment to confirm the viability of the oospores prior to exposure to soil. All treatments were replicated 5 times within treatments randomized in a complete block design.

2.8 Study 3: Determine the temperature reached by solarization of water bags left in full sun and determine the efficacy of killing oospores.

Solarisation bags (black plastic bags/camp shower) containing the recommended volume of 18 L water and fitted with an electronic thermocouple temperature probe were exposed to full sun continuously from 6 June 2014. Two replicate bags were each set up on the ground, a cement slab and insulated polystyrene board to assess the impact surface may have on the peak temperature and heat retention due to sun exposure (Figure 1). The temperature within each bag, ambient temperature, solar radiation and relative humidity data were captured hourly using a Campbell CR1000 data logger.

Figure 1: Weather station and monitoring of temperature potential of solar radiation for degradation of *Phytophthora* taxon Agathis oospores. Bags A and F were maintained on cement slabs, B and E on polystyrene boards and C and D were kept on the ground. The temperature within each bag, ambient temperature, solar radiation and relative humidity data were captured hourly using a Campbell CR1000 data logger.

Statistical analysis

Oospore germination and viability assessment with tetrazolium bromide were analysed using a generalised linear mixed effects model (GLMM) with binomial error distribution and logit link function was used to analyse the proportion germination data (HFD, A, A, & B., 2013; R Core Team, 2014). The model contained incubation temperature; immediate and delayed (2-week) analyses, and plating time after treatment (one day and two weeks post treatment), assessment time (2, 4, 6 weeks after plating) and their interaction as fixed effects and assessor (technician) and assessment time as random terms to account for individual bias and the repeated measures nature of the data. The significance of the fixed terms was assessed using a backwards selection procedure based on likelihood ratio testing (Zuur, Leno, Walker, Saveliev, & Smith, 2009). For the viability assessment a log likelihood based McFadden R^2 was calculated for the model predictions.

Due to the binomial nature of the baiting analyses used for the viability bioassays, a bias-reduced generalised linear model with binomial errors and logit link was used to analyse the viability bioassay data (Kosmidis, 2013). The model contained incubation temperature, immediate and delayed (2-week) analyses, days of baiting and their interaction as explanatory variables. The analysis of deviance model output was used to assess the significance of the fixed term. . For study 2 the model contained incubation temperature, soil, assessment time and their interaction as explanatory variables. In both studies 1 and 2, the analysis of each deviance model output was used to assess the significance of the fixed term.

The oospore qPCR data were analysed using a generalised least squares model (GLS) fitted by restricted maximum likelihood (REML) (R version 3.1.2, R Core Team 2015, R-package nlme, Pinheiro et al. 2015). The model contained incubation time and temperature and soil type as well as their interaction as explanatory variables. The significance of the explanatory variables was assessed using a backwards selection procedure based on likelihood ratio testing (Zuur et al. 2009). Model validation plots (standardised residuals vs. fitted values, quantile-quantile plot) were used to assess the model assumption of variance homogeneity and normality. The residual plot indicated slight variance heterogeneity, which was modelled using a power variance structure using the fitted values as variance covariate and incubation time as grouping variable. The quantile-quantile plot did not show strong deviations from normality. Graphical exploratory data analysis showed that the internal positive control always had Cq values ranging between 20 and 35 cycles suggesting no inhibition of amplification.

3. RESULTS

3.1 Study 1: Validation of killing oospores by heating.

Tetrazolium bromide staining

Tetrazolium bromide staining was performed on paired samples of heat treated meshes the day following heat treatment and two weeks post treatment. Irrespective of the assessment period, oospore viability at 22°C was around 90%, and declined significantly with increasing temperature (Figure 2). Oospore viability showed a greater decline in stained, viable oospores at 50, 60 and 70°C the assessment two weeks post treatment, compared to the assessment one day post temperature treatment resulting in a significant incubation temperature × assessment time interaction ($\chi 2 = 4.55$, df = 1, P = 0.033). More than 60% of oospores remained stained in each of the mesh sections treated at 50°C.

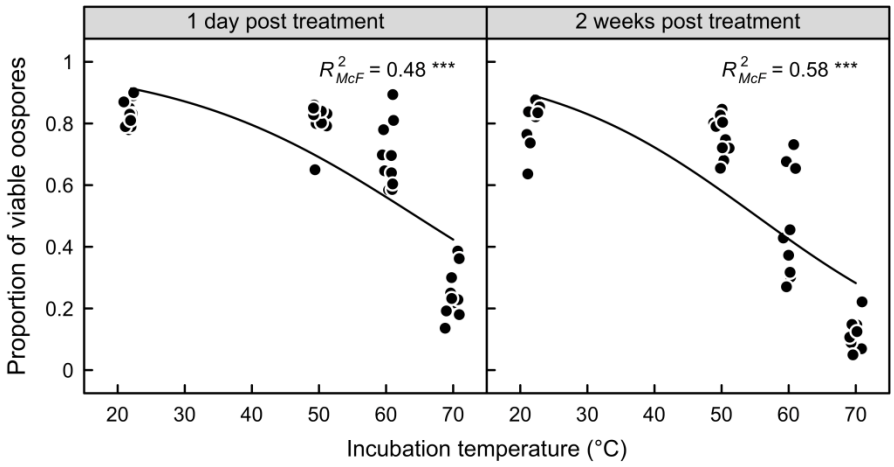

Figure 2: Assessment of oospore viability. Portion of oospores incubated at 22, 50, 60, and 70°C that were stained pink/red by tetrazolium bromide, indicating oospore viability, either one day (1 day post treatment) or two weeks (2 weeks post treatment) post heat treatment. Oospores that were tested two weeks post treatment were left at room temperature to allow enzyme activities to decline. Solid lines represent predicted values from a binomial generalized linear mixed effects model, n = 10, R2McF: McFadden pseudo-R2.

Oospore germination

Oospores immediately plated after the incubation treatment, showed on average 60 – 75% germination after exposure to 22°C depending on assessment date, and this proportion declined with increasing incubation temperature to ca. 20 – 40% at 70°C. (Figure 3, left panels). The proportion of spores germinated increased slightly over time for up to 6 weeks post treatment for spores maintained in water. In contrast, oospores stored for 2 weeks following treatment before being mounted in water started off with 22 – 32% germination at the lowest incubation temperature and this proportion remained largely constant with increasing incubation temperature (Figure 3, right panels). Direct comparison between the spores placed directly into water following heat treatment and those allowed to desiccate for 2 weeks in ambient conditions shows a significant temperature × post treatment desiccation ×assessment date interaction ($\chi^2 = 36.62$, $df = 2$, $P < 0.001$).

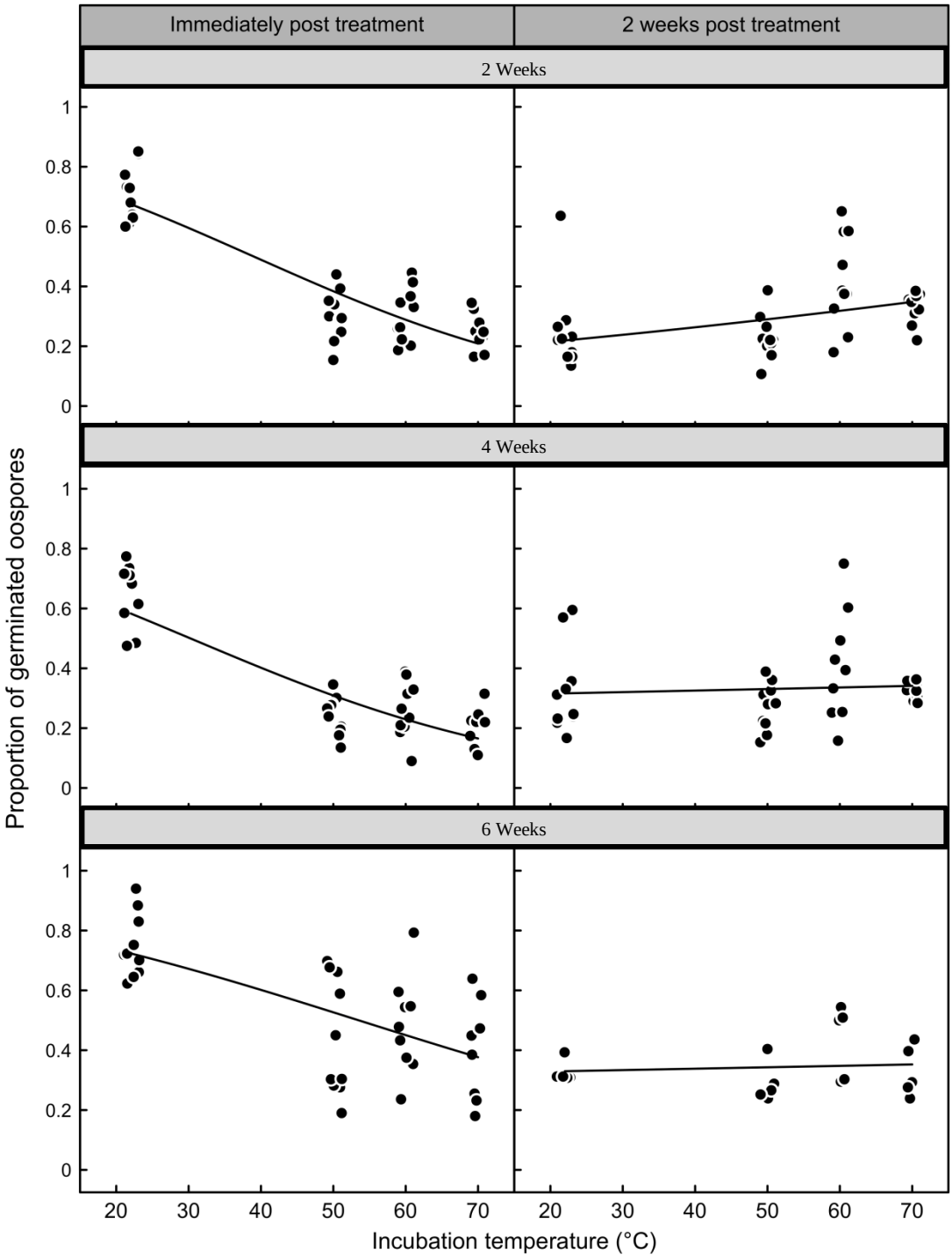

Figure 3: Assessment of oospore germination assessments. Portion of germinated oospores incubated at 22, 50, 60, and 70°C. Two replicate sets of oospores were tested. One replicate was assessed at 2, 4 and 6 weeks immediately after heat treatment (immediately post treatment). The other replicate was left at room temperature to allow enzyme activities to decline for two weeks after heat treatment (2 weeks post treatment) and was then assessed at 2, 4 and 6 weeks after room temperature storage. Solid lines represent predicted values from a binomial generalized linear mixed effects model. The interactions of temperature × plating date × assessment date was significant ($\chi 2 = 36.62$, df = 2, $P < 0.001$).

Direct observation of oospore germination was obscured in this study by residual fragments of mycelium which accounted for the inability to rule out post treatment germination of oospores in up to 30% of spores (Figure 4).

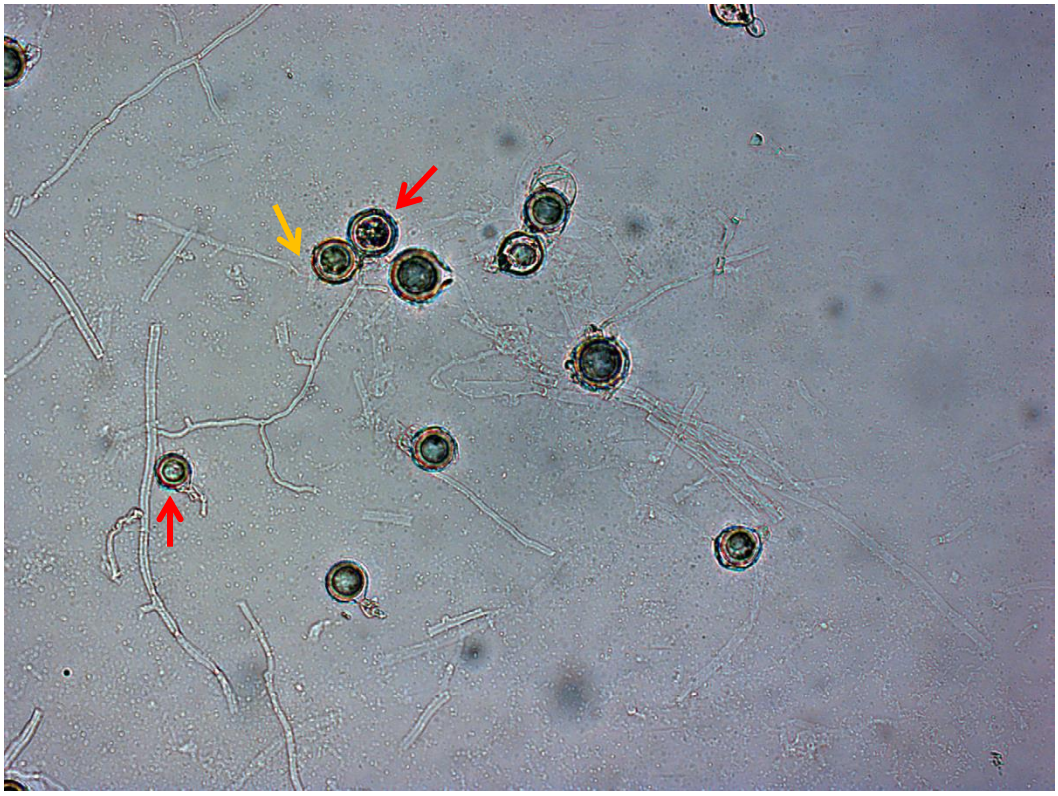

Figure 4: Germination of oospores four weeks after heat treatment at 50°C. Spores indicated with a red arrow have germinated with fresh mycelium protruding from the spore. The orange arrow indicates a spore which has germinated with the germination tube just out of focus.

Viability bioassay

Re-isolation of PTA using a baiting bioassay showed consistent recoveries from samples held at 22^{o}C with nearly nearly 100% oospore isolates were derived from suspensions set up immediately after the incubation treatment (Figure 5). This high initial proportion rapidly decline in a sigmoid fashion with increasing temperature as anticipated for a logistic model, resulting < 10% isolates at 50°C incubation temperature regardless of the number of days of baiting (Figure 5, left panels). No isolates were recovered from mesh that was allowed to desiccate for two weeks post treatment after 3 days of baiting. However, this apparent induced dormancy was overcome after 6 days of baiting with similar isolate recoveries to water immersion post treatment. Fresh bait material was replaced every three days in the baiting bioassay, whether this contributed to the breaking of dormancy is unknown.

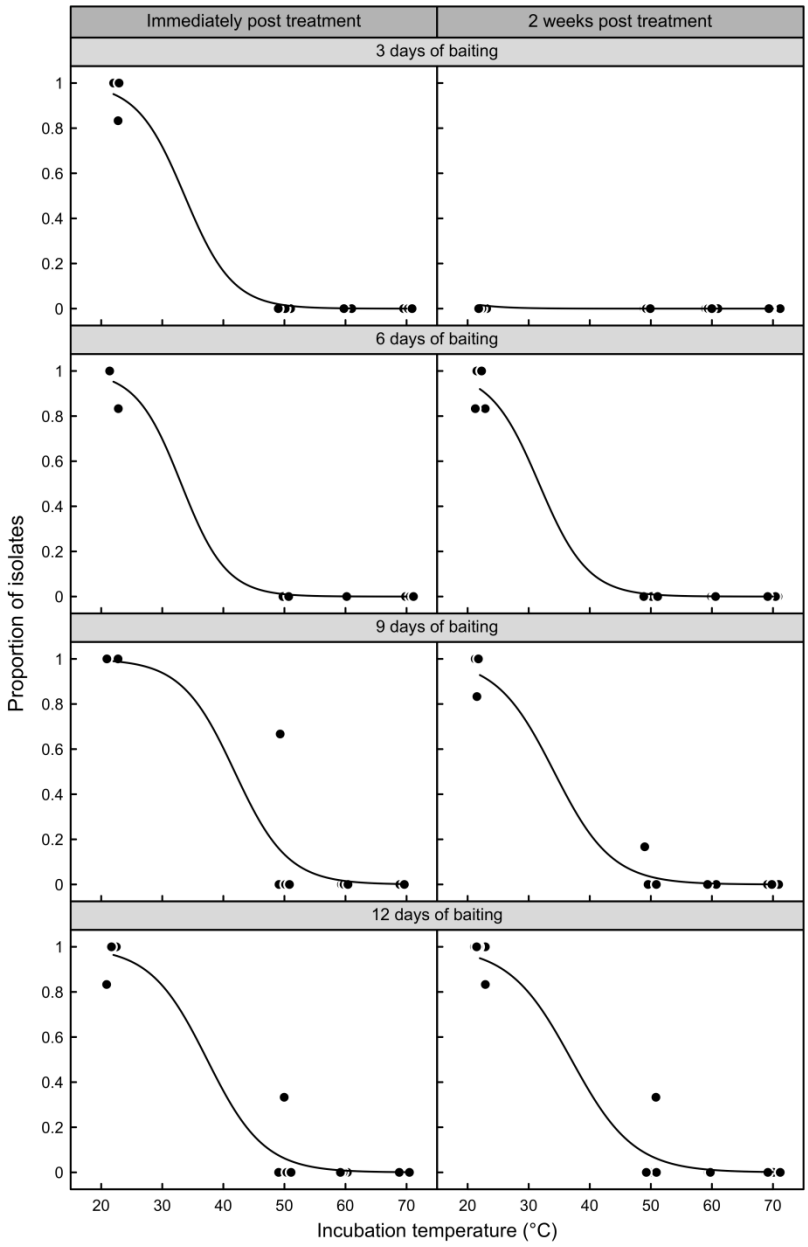

Figure 5: Proportion of *Phytophthora* taxon Agathis oospore isolates recovered by baiting 3, 6, 9 and 12 days post treatment at 22, 50, 60 and 70°C. Analysis was carried out on fresh samples (Immediately post treatment) and on a second set of samples stored at room temperature for two weeks prior to analysis (2 weeks post treatment). Solid lines represent predicted values from a binomial generalized linear mixed effects model, *n* = 10.

3.2 **Study 2:** Analyze PTA decay within wet homogenized soil mixtures containing known quantities of oospores with heat.

The results from study 1 showed that delayed assessment (oospores left for two weeks at room temperature, did not significantly impact oospore viability, compared with viability of oospores tested immediately post heat treatment, but potentially increased the induction of oospore dormancy. Due to these results, analysis in study 2 was carried out immediately after heat treatment.

Tetrazolium bromide staining

In contrast to study 1 results, oospore staining in study 2 was considerably lower and less variable across this experiment (Figure 6). Oospore viability in soil treated at 22 and 40^{o}C was either similar or slightly increased over time, but was reduced to negligible levels following heating for 4 hours at 50 and 60^{o}C.

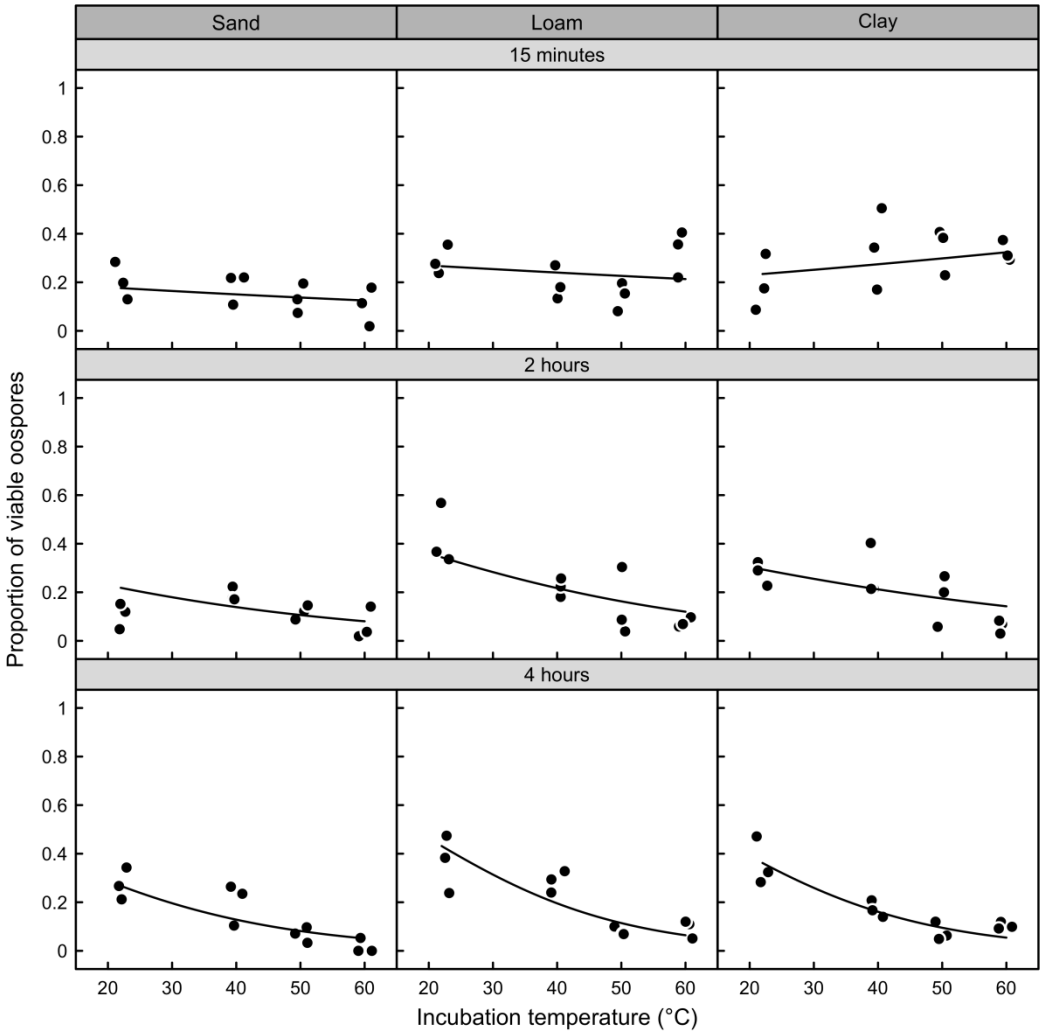

Figure 6: Assessment of oospore viability. Portion of oospores that were stained pink/red to indicate viability following heat treatment at 22, 40, 50 and 60°C for 15 minutes, 2 hours and 4 hours in sand, loam and clay soil slurries.

Viability bioassay

Baiting analysis for up to 12 days recovered PTA only from oospores incubated in sand at 22^{o}C with no re-isolations from any other samples (Figure 7).

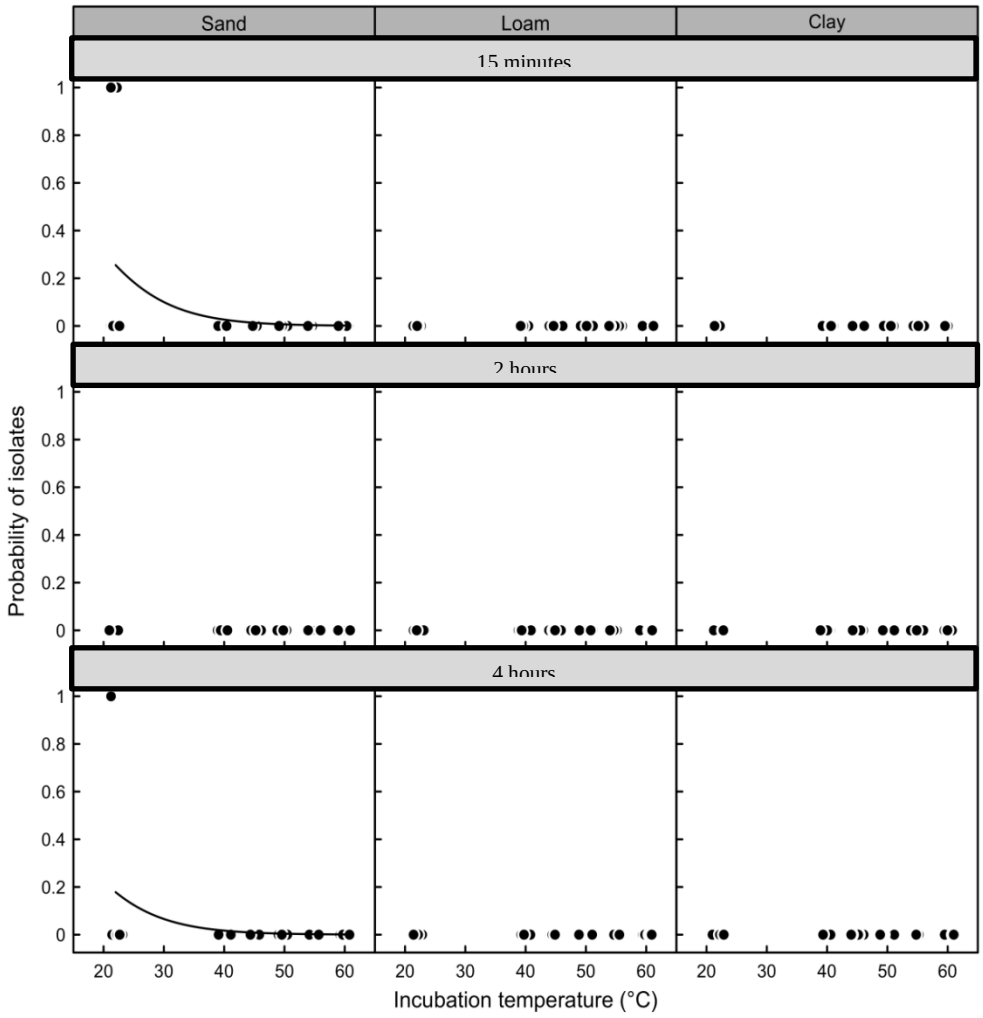

Figure 7: Rate of re-isolation of PTA from mesh samples incubated to 22, 40, 50 and 60°C in sand, loam and clay soils for 15 minutes, 2 hours and 4 hours. Isolation results were pooled for samples baited for 3, 6, 9 and 12 days.

Quantitative PCR

A statistically significant incubation time (Likelihood ratio = 30.38, $P > 0.001$) and soil type × incubation temperature interaction (Likelihood ratio = 9.19, $P = 0.01$) were detected with the qPCR analysis (Figure 8). The amount of DNA detected by qPCR decreased with increasing incubation time, as shown by increasing Cq values. For soil × temperature interaction, the detection of PTA from sandy soil decreased with increasing temperature incubation (Figure 8, left-hand column), supporting results observed with the tetrazolium bromide staining and viability bioassays. However, the detection of PTA from loam and clay derived samples remained largely unaffected by incubation temperature (Figure 8, middle and right-hand columns).

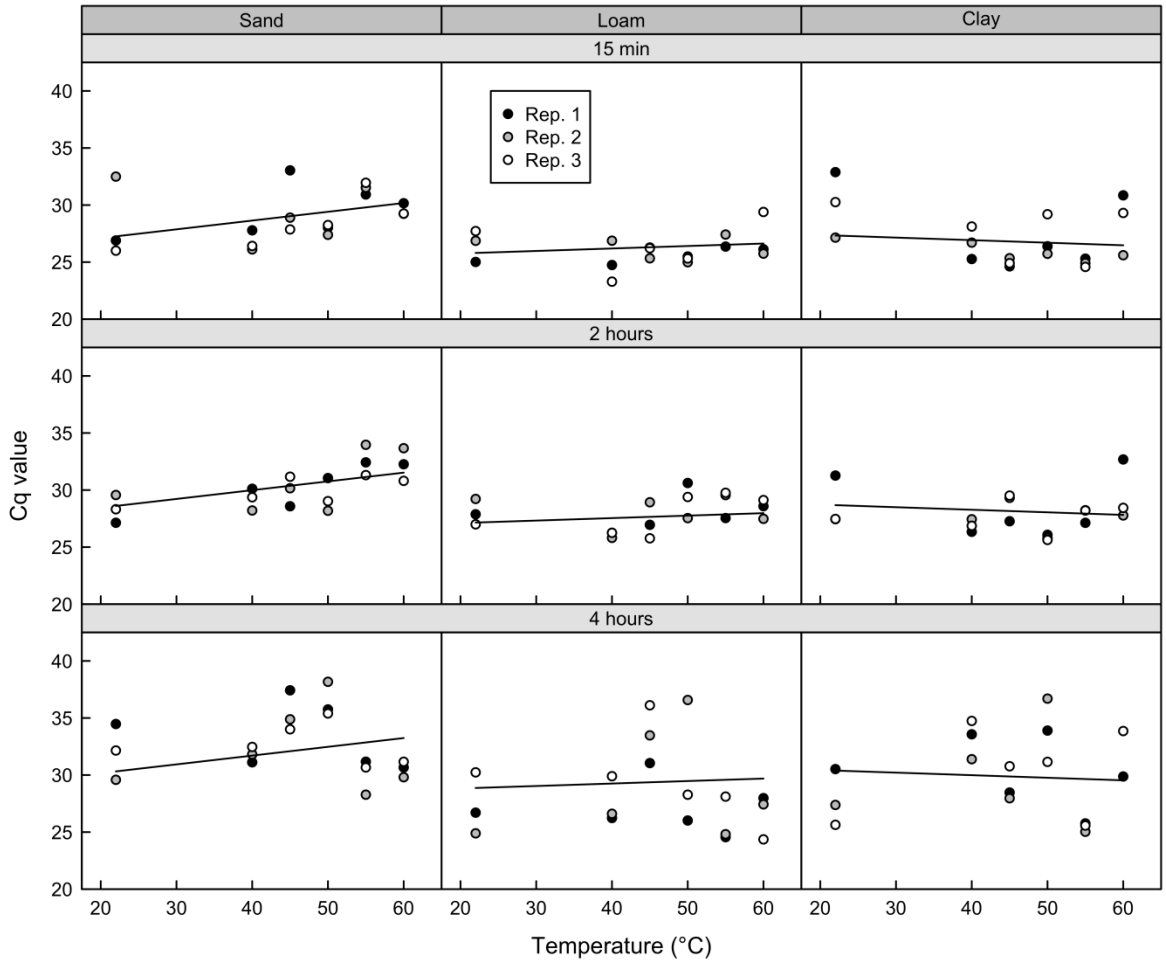

Figure 8: Amplification (Cq value) of PTA from mesh samples incubated to 22, 40, 50 and 60°C in sand, loam and clay soils for 15 minutes, 2 hours and 4 hours using qPCR. High Cq values indicate a low presence of DNA in the sample.

3.3 Study 3: Temperature potential of water bags

Water bags containing temperature probes have shown daily fluctuations in temperature of between 15-20^{o}C. For the majority of the period monitored, temperatures remained at temperatures that would not be expected to be adverse for PTA survival based on the results presented in Study 1. During summer, temperatures within the bags in the original orientation of black side facing the sun reached a maximum 39.28^{o}C on an insulated polystyrene base where as the bags on the ground and cement slabs reached a maximum of 37.37^{o}C and 36.61^{o}C, respectively (Figure 9).

Upon receipt of an updated recommendation from the manufacturer, three of the bags were inverted (from 13 January 2015) so that the clear side was exposed. This resulted in a significant increase in the temperature of the water within those bags. With the insulating polystyrene barrier, the water in the bag reached 49.23^{o}C, the cement 46.16^{o}C and the bag on the ground got to 46.85^{o}C (Figure 10).

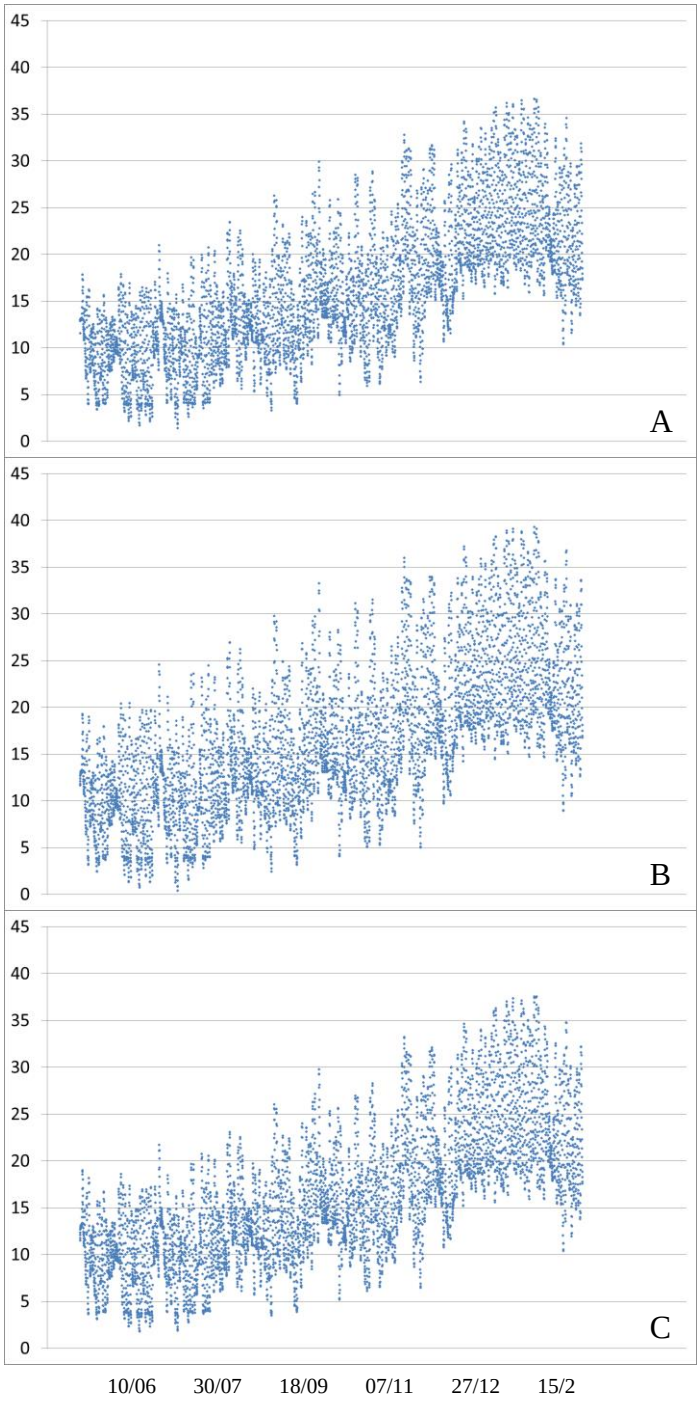

Figure 9: Temperature of solarization bags measured hourly between 6 June 2014 and 20 February 2015 with the black surface of the bag facing up when laid on (A) cement, (B) polystyrene and (C) ground.

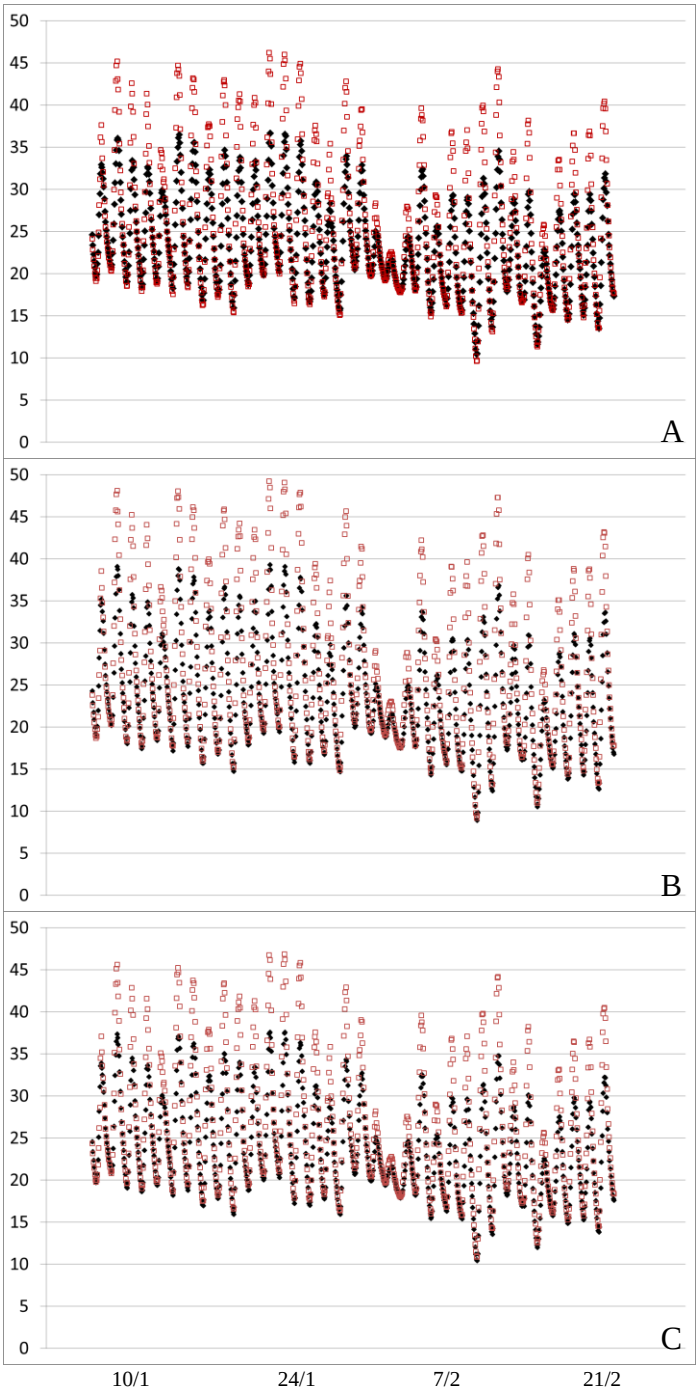

Figure 10: Temperature of solarization bags measured hourly between 13 January and 20 February 2015 with black (■) and clear (□) surfaces facing up when laid on (A) cement, (B) polystyrene and (C) ground.

4. DISCUSSION

Oospores are the sexual spore of heterothallic species of *Phytophthora* and a key resting spore stage of homothallic species. In both cases, the spores are characterized by thick walls, resistance to desiccation and ability to enter a dormant phase which is more resistant to degradation under conditions of increased heat and external stressors. Assessment of viability of these spores is notoriously difficult to study as the triggers for breaking dormancy are not well understood. Researchers are therefore limited to a series of metabolic and germination based assays that provide indications of viability with a combination of approaches used to provide confidence of each observed trend.

The primary goal of this series of experiments was to validate the analyses carried out by Margaret Dick and Mark Kimberly (2013; Client Contract MPI 15775/Output 50797) by performing parallel assessment of viability using a baiting and germination assay. With each of the methodologies employed, a significant reduction in oospore vitality, germination and re-isolation was observed at temperatures above 50^{o}C.

The base-line viability and re-isolation of oospores used in study 2 was significantly lower. This may be due to several factors: the additional sieving of spores through the 75 μm sieve may have selected for fully mature and dormant oospores, it may have inadvertently selected for damaged spores and/or the un-sieved spores may have been buffered by the presence of mycelial fragments in study 1. The additional sieving was however required to enable accurate quantification of the amount of PTA present in each analysis and to improve the consistency of viability assessments based on the observations in study 1. Despite these factors, exposure to soil slurries at 22 and 40^{o}C increased the level of spore staining in each of the soils tested indicating an up-regulation of spore metabolism in the presence of soil biological and chemical factors. This is consistent with other studies which have shown oospore activation is increased due to a range of biological and enzymatic stimuli (Dick & Kimberley, 2012).

Analysis of PTA survival with qPCR analysis was variable. The results showed that the longer the oospores were incubated the less PTA DNA detected. However, as there was no interaction between time and temperature, these results suggest there was no change in oospore viability when the oospores were heated. It is likely that the decrease in PTA DNA detection over time is due to degradation by compounds present in the soil mixtures and it appears the influence of temperature on DNA degradation is minimal. In keeping with the tetrazolium bromide staining and viability bioassay results, the detection of PTA from sandy soil decreased with increasing temperature incubation. This was the only soil type where heating showed a decrease in PTA detection. Although there was variation in the amount of PTA DNA detected, this cannot be interpreted as a reduction in oospore viability. DNA can be amplified from non-viable oospores as long as the DNA has not degraded. The degradation rates for DNA in soil are variable and influenced by many environmental factors. It should also be noted that a Cq cut-off threshold has not been applied to this data for indicating positive or negative detection (McMullen and Petter, 2014, Bustin et al. 2009), and instead the overall trend of Cq values was analysed. The qPCR analysis was run in parallel to the work presented by McDougal et al. (2014) with the base-line and sensitivity analyses presented in that report.

One of the biggest issues with the tetrazolium bromide analysis is the reliance on visual assessment and subjective assessments of staining as an assessment of metabolic activity. The assessors of these trials were unable to discern faintly stained oospores with confidence which is likely to have contributed to the higher base-line viability assessments in both studies 1 and 2 compared to those presented by Dick and Kimberley (2013). To remove this subjectivity, oospores should be produced in far higher concentrations to enable the utility of independent spectrophotometric analyses. Recent developments in metabolic assays have shown significant improvements in the analysis of dormant life stages of microscopic spores and arthropod eggs and should be considered for future assays (Phillips, Iline, Richards, Novoselov, & McNeill, 2013; Richards et al., 2013). Preliminary analysis of PTA oospores with this assay indicates that this is a viable option for assessing oospore viability (Nari Williams and Craig Phillips, data not presented).

The results presented here demonstrate the high variability between batches of oospores produced under laboratory conditions raising a key issue of the relevance of such studies in improving the management of these pathogens. Under natural conditions, oospores are predominantly produced within the root and vascular cells of host plant tissues. While suspensions of spores may be produced in the laboratory and lend themselves to analyses with a range of assays, oospores produced in vitro are a poor proxy for investigating soil hygiene and disinfection (Etxeberria, Mendarte, & Larregla, 2011). *Phytophthora* survival studies currently underway for *P. cinnamomi* are utilizing inoculated plant roots as a more natural source of oospore inoculum (Giles Hardy, pers com. November 2014).

Solarization was considered in this study as a means of decontamination in remote areas. For the majority of the study period, the temperature within the bags remained suitable for PTA survival. In addition to heat, the role of microbial antagonism, maintenance of anoxic conditions and starvation from fresh host material within these bags should be considered in their potential application for containing and decontaminating equipment in remote areas (Butler et al., 2012). These factors were not tested here, but based on research demonstrating the low rates of survival of *Phytophthora* species in the absence of host material (Crone, 2012; Crone, McComb, O'Brien, & Hardy, 2013) and poor saprophytic potential of *Phytophthora* in the presence of microbial competition, this may warrant further investigation.

Conclusions

Oospore viability was observed to decrease substantially with temperature when assessed by tetrazolium bromide staining, oospore germination and re-isolation by baiting. Baiting analysis provided the most conclusive assessment of viability with both tetrazolium bromide staining and oospore germination open to considerable subjectivity and uncertainty. Similarly, the use of qPCR to determine viability was not appropriate as PTA DNA could be amplified from non-viable oospores, as long as the DNA had not degraded substantially.

Consideration for future studies should be given to recent developments in the understanding of the survival strategies of *Phytophthora* in plant roots. Jung et al (2013) reported the development of stromata in host roots as a key survival strategy of *P. cinnamomi.* Similar structures have been recently observed being formed by PTA in the roots of kauri in greater abundance to oospores (Bellgard et al., 2014). These observations further support future studies on soil sterilization and hygiene should being carried out on naturally infested soils and root fragments rather than spores produced in vitro.

Further consideration should be given to the use of solarisation bags as a method of decontamination in remote areas with further studies investigating the role of microbial antagonism, maintenance of anoxic conditions and starvation from fresh host material within these bags.

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6. REFERENCES

Bellgard, S., Williams, S., Probst, C., Padamsee, M., & Lebel, T. (2014). *Visualisation of early infection by Phytophthora "taxon Agathis" in the roots of 2-year old kauri Agathis australis plants*. Paper presented at the IUFRO Working Party 7.02.09 Phytophthora in Forests and Natural Ecosystems. , Esquel, Argentina

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry, 55*(4), 611-622. doi:DOI 10.1373/clinchem.2008.112797

Butler, D. M., Kokalis-Burelle, N., Muramoto, J., Shennan, C., McCollum, T. G., & Rosskopf, E. N. (2012). Impact of anaerobic soil disinfestation combined with soil solarization on plant–parasitic nematodes and introduced inoculum of soilborne plant pathogens in raised-bed vegetable production. *Crop Protection, 39*, 33-40.

Crone, M. (2012). *Persistence of Phytophthora cinnamomi in nature: Biotrophic growth and presence of stromata, oospores and chlamydospores in annual and herbaceous perennial plant species.* Murdoch University.

Crone, M., McComb, J. A., O'Brien, P. A., & Hardy, G. E. S. J. (2013). Survival of Phytophthora cinnamomi as oospores, stromata, and thick-walled chlamydospores in roots of symptomatic and asymptomatic annual and herbaceous perennial plant species. *Fungal biology, 117*(2), 112-123.

Dick, M. A., & Kimberley, M. (2012). Deactivation of oospores of Phytophthora taxon Agathis: Scion Client Report MPI 15775.

Etxeberria, A., Mendarte, S., & Larregla, S. (2011). Thermal inactivation of Phytophthora capsici oospores. *Revista Iberoamericana de Micología, 28*(2), 83-90.

HFD, S., A, N., A, M., & B., B. (2013). GlmmADMB package. (0.6.7.1 edn). See http://glmmadmb.r-forge.r-project.org/.

Hussain, S., Lees, A., Duncan, J., & Cooke, D. (2005). Development of a species-specific and sensitive detection assay for Phytophthora infestans and its application for monitoring of inoculum in tubers and soil. *Plant Pathology*, *54*(3), 373-382.

Jung, T., Colquhoun, I., & Hardy, G. S. J. (2013). New insights into the survival strategy of the invasive soilborne pathogen Phytophthora cinnamomi in different natural ecosystems in Western Australia. *Forest Pathology*, *43*(4), 266-288.

Kosmidis, I. (2013). brglm: Bias reduction in binary-response Generalized Linear Models. *R package version 0.5-9*.

McDougal, R., Bellgard, S. E., Scott, P. M., & Ganley, R. J. (2014). *Comparison of a real-time PCR assay and a soil bioassay technique for detection of Phytophthora taxon agathis from soil*. Scion (New Zealand Forest Research Institute, Ltd.). SIDNEY #53789.

McMullen, M., & Petter, F. (2014). Report of the EPPO workshop on setting Ct cut-off values for real-time PCR. *Bulletin OEPP/EPO Bulletin*, *44*(2), 165. doi:10.1111/epp.12121

Phillips, C. B., Iline, I. I., Richards, N. K., Novoselov, M., & McNeill, M. R. (2013). Development and Validation of a Quick Easily Used Biochemical Assay for Evaluating the Viability of Small Immobile Arthropods. *Journal of Economic Entomology*, *106*(5), 2006-2019. doi: 10.1603/EC13028

Pinheiro J, Bates D, DebRoy S, Sarkar D and R Core Team (2015). *nlme*: Linear and Nonlinear Mixed Effects Models. R package version 3.1-119.

R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

Ramsfield, T. D., Power, M. W. P., & Kimberley, M. O. (2013). The relationship between pruning and the incidence of *Neonectria fuckeliana* in *Pinus radiata*. *New Zealand Journal of Forestry Science*, *43*, 1-6.

Richards, N., Winder, L., Iline, I., Novoselov, M., McNeill, M., & Phillips, C. (2013). A biochemical viability assay is compatible with molecular methods for species identification. *New Zealand Plant Protection*, *66*, 29-33.

Than, D. J., Hughes, K. J. D., Boonhan, N., Tomlinson, J. A., Woodhall, J. W., & Bellgard, S. E. (2013). A TaqMan real-time PCR assay for the detection of Phytophthora 'taxon Agathis' in soil, pathogen of Kauri in New Zealand. *Forest Pathology*, *43*(4), 324-330. doi: Doi 10.1111/Efp.12034

Zuur, A. F., Leno, E. N., Walker, N., Saveliev, A. A., & Smith, G. M. (2009). *Mixed effects models and extensions in ecology with R*. New York.: Springer.