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Phytophthora agathidicida inoculum deactivation: Determining the source of persistent *Phytophthora agathidicida* inoculum within soils

Nari Williams and Matt Arnet

June, 2020















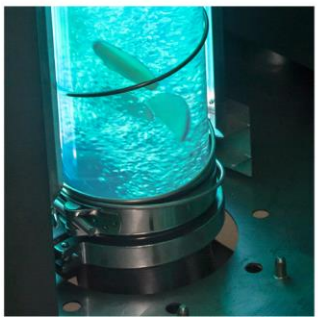


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Contents

Executive summary

Phytophthora agathidicida inoculum deactivation: Determining *the* source of persistent Phytophthora agathidicida inoculum within soils

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Adherence to hygiene protocols is important in mitigating the spread of kauri dieback caused by *Phytophthora agathidicida*. Wash-down stations for pedestrians, vehicles and heavy machinery are a key tool in minimising the risk of pathogen spread associated with kauri dieback. There are a rage of disinfectants available for decontaminating surfaces and variable volumes of contaminated soil and water. This study presents one component of a larger programme of work commissioned by the Ministry for Primary Industries to establish consistent protocols for the use of disinfectants in a range of kauri dieback management applications. Assessing the efficacy of surface and volumetric disinfection treatments used in the decontamination of equipment and footwear entering and exiting kauri dieback disease risk areas requires that the reservoir of *P. agathidicida* inoculum is first determined.

The aim of this study was to identify the primary inoculum sources of *P. agathidicida* within soil and determine whether it is present as free-living spores or contained within small decaying root fragments. This study largely replicates the methodology of Jung et al., (2013) investigating the survival of *Phytophthora cinnamomi* in naturally infested soils.

The presence of *P. agathidicida* propagules within the soil was investigated by direct plating of fine roots and organic fragments onto selective agar from 10 *P. agathidicida*-infested soil samples to identify the source of persistent *P. agathidicida* inoculum within each soil.

Phytophthora agathidicida was readily recovered from the fine root fraction of all soils assessed specifically from surface-sterilised root fragments ranging between 3 and 5 mm in length. Fine organic matter was confirmed as the origin of a high proportion of *P. agathidicida* colonies.

The laboratory work was cut short by the COVID-19 pandemic. The observations from the initial method optimisation and proof-of-concept stage of the proposed work are presented but the trial needs to be replicated to clarify the proportion of organic fragments harbouring *P. agathidicida* within naturally infested soils.

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1 Introduction

Adherence to hygiene protocols is important in mitigating the spread of kauri dieback. Wash-down stations are a key tool in minimising the risk of pathogen spread associated with kauri dieback, however contaminated soil and water on machinery, vehicles, bicycles and nursery production all present distinct risks for pathogen spread that need to be mitigated. There are a range of disinfectants available for decontaminating surfaces and large volumes of contaminated soil and water.

Kauri dieback is caused by the soil-borne pathogen, *Phytophthora agathidicida* (Weir et al., 2015). Within the genus *Phytophthora*, oospores are recognised as being both a means of sexual recombination and persistent survival structures that allow the pathogen to persist for extended periods within the soil (Erwin & Ribeiro, 1996). While oospores can be produced in axenic culture for experimental purposes, their growth, maturation, viability and persistence vary between media in response to available sugars and fatty acids and are therefore strongly influenced by their culturing environment (Ribeiro et al., 2018, Forster et al., 1983). Cultured oospores are therefore a poor proxy for testing the survival of *P. agathidicida* within soil.

Oospores are naturally produced following infection and mycelial penetration into the host plant's cells and have been observed in abundance in kauri within days of infection by *P. agathidicida* (Bellgard et al., 2018). Previous studies have focused on testing the viability of *P. agathidicida* oospores, but the observations of Bellgard et al. (2018) and the biology of infection suggest that the primary reservoir of inoculum is not free-living oospores within the soil, rather mycelium, stromata and oospores contained within fine roots and organic matter. This persistent inoculum source is, in turn a reservoir for the production of shorter lived sporangia and zoospore inoculum produced in the presence of available water.

In addition to oospores, hyphal aggregations known as stromata have been observed to be produced within host roots by several species of *Phytophthora* including *P. agathidicida* (Bellgard et al., 2016, Crone, 2012, Crone et al., 2013, Jung et al., 2013). To date, little attention has been given to this reservoir of *P. agathidicida* inside decaying root fragments, yet it is fundamental to determining the efficacy of surface and volumetric disinfection treatments.

The aim of this study was to identify the primary inoculum sources of *P. agathidicida* within soil and determine whether it is present as free-living spores or contained within small decaying root fragments. This study large replicates the methodology of Jung et al., (2013) who investigated the survival of *Phytophthora cinnamomi* in naturally infested soils. This report presents the observations from the initial method optimisation and proof-of-concept stage of the proposed work. Further replication of this trial and quantification of the proportion of organic fragments from which *P. agathidicida could be isolated* were not completed due to the COVID-19 pandemic preventing completion of the laboratory work and subsequent funding cuts of this programme of research by MPI.

2 Methods

2.1 Activation of *P. agathidicida* inoculum within soils

Ten forest soils were selected that had previously tested positive for *P. agathidicida* by baiting analysis but diagnostic analysis had not isolated any other species of *Phytophthora* (Table 1). Each soil was submitted as part of routine testing for *P. agathidicida* for which the sample details are confidential but not important in the context of the analysis presented here. Each soil had been stored for several weeks following baiting analysis. During which time, transient inoculum is likely to have degraded leaving more persistent *P. agathidicida* propagules. Each soil was mixed thoroughly and passed through a graduated sieve to remove all fragments greater than 2 mm diameter. A sample of approximately 20 ml of soil placed in a flask, flooded with 180 ml of sterile distilled water, stirred and incubated for 48 h at 20°C to stimulate germination of resting structures (Jung et al., 2013).

A slurry of suspended soil–debris was taken from the flask of flooded soil and further diluted 1:1 with distilled water For each soil, 5 ml aliquots of the diluted suspension were poured onto the surface of five replicate 90 mm diameter Petri dishes containing selective PARPH agar (Erwin & Ribiero, 1996) and incubated for 48 hours at 20°C in the dark. Following incubation the water was discarded and the plates were rinsed with sterile distilled water using water streamed from a 10 ml pipette to wash away any particulates not adhered to the plate surface. Plates were then inverted and incubated for a further 24 hours. Each plate was thoroughly examined under a compound microscope for developing colonies of *P. agathidicida* and the origin of 100 colonies identified by microscopic observation to determine if they originated from free-living oogonia, oospores, sporangia, zoospores or organic fragments. Observations were made with compound microscope at 20 x optical magnification. The proportion of organic fragments associated with colonies was also quantified by direct observation at 20x magnification. While the trial was set up to assess 10 soils, only five were assessed before colony growth prevented the identification of the source of each colony.

Three representative isolates of *P. agathidicida* per soil were sub-cultured from emergent colonies onto PARPH and subsequently V8 agar (Erwin & Ribiero, 1996) to confirm their identity.

2.2 Direct plating of fine root material

The same ten soils used in 2.1 were used for direct plating of fine root material. In accordance with the methods described by Jung et al., (2013), for each soil, fifty fine root fragments of less than 2 mm diameter and between 3 and 10 mm in length were washed thoroughly in sterile distilled water, surface disinfected for 10 s in 70% ethanol, blotted dry on a paper towel and plated onto selective PARPH agar using tweezers and sterile technique. Ten root fragments were plated onto each of five replicate plates for each of the 10 soils. Agar plates were incubated at 20°C in the dark for 72 hours and examined daily for developing colonies of *P. agathidicida*. Three representative isolates per soil were sub-cultured from emergent colonies onto PARPH and subsequently onto V8 agar to confirm their identity by morphological examination.

The trial was repeated twice with two weeks between experimental set ups. Only the root fragment assessment was made on the second trail due to the COVID-19 shut down.

3 Results

P. agathidicida was isolated from 6.5% of fine root fragments plated across the 10 soils but ranged from 2–21% of the root fragments plated (Table 1). From the soil slurry, oogonia were observed very infrequently with only six colonies were observed associated with potentially free-living oogonia from the soil slurry across all of the soils tested. The maturity of the oogonia was not noted at the time and requires closer examination to confirm whether these were produced in culture or were present within the soil. Those photographed during this preliminary assessment were immature oogonia (Figure 1a), suggesting they were produced in culture. No colonies originating from clearly mature oospores, indicative of being present in the soil, were noted in this preliminary assessment.

Colonies originating from zoospores were observed in abundance and were found to be the origin of > 84% of colonies in five of the soils assessed (Table 1, Figure 1 b and c). Due to the large number of zoospores present, only five of the soils were assessed before the plates were too overgrown to distinguish the origin of each colony; no assessments were able to made on the five soils that were completely overgrown (Table 1). .In one sandy soil (S3902), only zoospores were found to be the origin of the colonies within the soil slurry, with the plates devoid of fine organic fragments. *P. agathidicida* did however grow from fine root fragments recovered from this soil. This suggests that the zoospores observed in the slurry plates had originated from infected root fragments. While total colony forming units were not assessed across the plates, those from the S3902 soil only had between 12 and 44 *P. agathidicida* colonies per plate. The other soils had well in excess of 100 colonies per plate.

Of the total colony forming units observed, the majority were associated with zoospores with only 16% observed to have originated from organic fragments. Given the 48 + 24 hour aqueous incubation of the soil slurry, it is most probable that zoosporogenesis was induced within the experiment, with the zoospores originating from inoculum within the root fragments in the primary incubation, or from the finer organic fragments in the secondary incubation on the PARPH plate. Direct observation of organic fragments found that between 85 and 100% of these were the origin of *Phytophthora*-like colonies. It must be noted that the incubation and rinsing protocol using Phytophthora-selective agar positively selected for fine organic fragments retained by hyphal growth so this is likely to be an over-representation of the total organic fraction within the soil. While it is likely that species other than *P. agathidicida* were present, three isolates were sub-cultured onto PARPH agar from each soil. From these, two or three of the resultant colonies were confirmed as *P. agathidicida* for each of the four soils from which isolates were subbed. This assessment should be followed up by confirming the identity of a greater number of sub-cultured colonies either by morphological or molecular analysis.

Fine root fragments were confirmed to be a source of *P. agathidicida* with 6.5% of fragments raging between 3 and 5 mm producing colonies of the pathogen. In two of the soils, 20% of these fine root fragments were shown to harbour the pathogen (Table 1). This observation shows the active inoculum potential of fine root fragments within the soil.

Table 1. Initial assessment to identify the origin of *P. agathidicida* within naturally infested kauri forest soil.

	% Root Fragments with *P. agathidicida*			Soil Slurry			
Soil (PFR)	Assessment 1	Assessment 2	Mean %	% colonies associated with oogonia (Whole plate)	% Zoospore origin (Whole plate)	% Organic matter origin (Whole plate)	% Organic matter associated with colony growth
S3902	4	6	5	0	100*	0	0
S3903	0	2	1	0	92.4	7.6	100 (3)**
N3864	4	2	3	0	89.2	10.8	92.8 (2)
S3912	2	4	3	0	86.4	13.6	84.8 (2)
S3906	0	2	1	1.2	84	16	89.6 (2)
N3844	0	2	1	Plates overgrown by the time they were to be assessed due to high levels of colony formation. The specific source of each colony could not be confidently noted or sub-cultured for identification.			
S3880	12	30	21				
S3899	24	16	20				
S3900	14	2	8				
S3909	4	0	2				

* Very little organic matter observed on the plate. Maximum of 44 observed colonies per plate, all originated from zoospores. ** The numbers within the brackets indicate the number of sub-cultured colonies of the three taken across each soil that was confirmed as *P. agathidicida.*

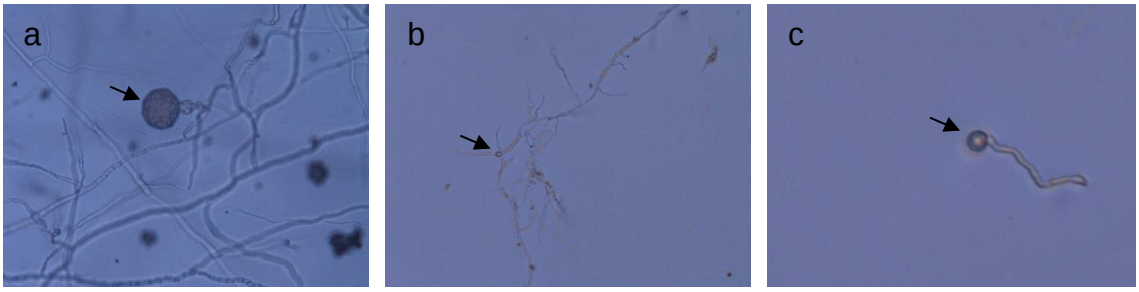

Figure 1: *P. agathidicida* colony associated with an immature oospore (a) and zoospore (b and c). In each case, the spore from which the colony originated is marked with an arrow. Images were captured under 40x (a) and 10x (b and c) magnification, and have been cropped for clarity.

4 Discussion

Phytophthora agathidicida was isolated from the fine root fraction of each of the 10 soils assessed in this trial, with the pathogen recovered from surface-sterilised root fragments ranging between 3 and 5 mm in length. Zoospores were observed in abundance on the plates incubated with the fine soil slurry, while oospores were very rarely observed and made up only 1.2% of the colonies from one of the soils tested. Fine organic matter was confirmed as the origin of a high proportion of *P. agathidicida* colonies. However, a greater number of direct isolations from organic fragments need to be confirmed to provide statistical support for this observation.

Given the presence of *P. agathidicida* infected root fragments within the primary incubation, there is a high probability that zoosporogenesis occurred during incubation, leading to an over-representation of colonies originating from zoospores. Based on the biology of *P. agathidicida* it is likely that this represents a transient source of inoculum rather than a persistent, long-lived inoculum source. It does,

however show how readily zoospores are produced from primary inoculum sources in the presence of available water under suitable environmental conditions. However, the objective of this project was to confirm the origin of persistent inoculum of *P. agathidicida* within soil to confirm the target of surface and volumetric disinfectant applications. To do this we really need to focus on the fine roots and organic fragments. In a second attempt to do this, the primary incubation was reduced to 24 hours and the plates were dried down for the secondary incubation. Despite this, the plates were similarly dominated by colonies originating from zoospores. The observations of the second round of root plating are included here, but the soil slurry plates were not assessed due to the COVID-19 shut down.

Although not fully replicated, this preliminary assessment has confirmed that *P. agathidicida* is present in both the fine root fragments and fine organic matter within infested kauri forest soils. These observations have critical significance for disinfection, as target treatments must penetrate these sources of inoculum to effectively mitigate risks of *P. agathidicida* spread. Further refinements of the approach taken here should take a direct plating approach to eliminate the primary incubation in water. This will eliminate the influence of colonies originating from zoospores and will give a direct measure of the proportion of fine organic fragments from which *P. agathidicida* originates within forest soils. The information from these observational studies will inform the design of the disinfection trials to follow.

5 Recommendations

We recommend this work is carried on as originally plan and in accordance with the scheduled programme of work (Table 2).

Table 2: Break down of activities to progress *Phytophthora agathidicida* inoculum deactivation work.

Activity		Milestone	Funding
1	July 2020	Identification of the source of persistent *P. agathidicida* inoculum within naturally infested soil. Repeat the analysis of inoculum source by individually plating of the ultra-fine organic fragments to enable the significance of this inoculum source to be quantified.	$26,650
2	Aug 2020	Tier 1 Treatment Efficacy trials (concentration x time) with Chlorine, Sterigene, Ethanol, steam and boiling which specifically target the persistent inoculum source of *P. agathidicida* (fine roots and organic matter) from naturally infested soils.	$68,185
3	Sept 2020	Develop proposal and identify academic panel for a PhD project to investigate the activation and persistence of *P. agathidicida* within soil	In kind
4	Sept 2020	Progress report, incorporating outcomes from 1 to 4 above with recommendations for Tier 2 and 3 experiments.	$5,150
		Total	**$99,985**

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