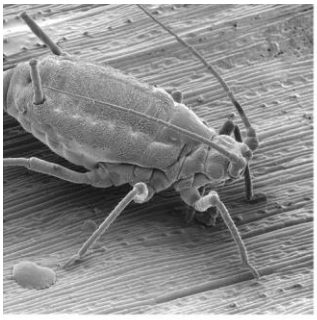
PFR SPTS No. 12254

Testing transmission of *Phytophthora agathidicida* in pig faeces

Horner IJ, Hough EG

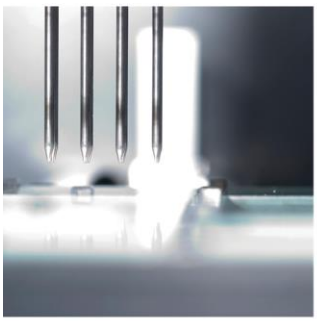
October 2015





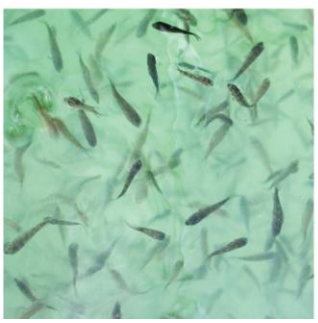
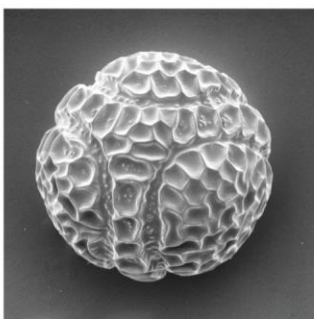










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Testing transmission of *Phytophthora agathidicida* in pig faeces

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October 2015

Feral pigs could potentially spread *Phytophthora agathidicida* (PTA), the causal organism of kauri dieback. The pathogen could possibly be vectored either in contaminated soil on hooves and fur, or through the digestive system following ingestion of contaminated root material. The current study investigated whether *P. agathidicida* in contaminated plant material could survive passage through the pig gut.

Millet/oat grains and kauri feeder roots were artificially infected with *P. agathidicida*, then fed to pigs. Faeces were collected every 24 h for 7 days and undigested millet/oat seed and kauri roots were sampled and rinsed. Portions of these were directly plated onto *Phytophthora*-selective media and other portions were flooded and baited. None of the 265 millet seeds direct plated or 535 seeds baited yielded *P. agathidicida* or any other *Phytophthora*. Similarly, none of the plated or baited oat grains yielded any *Phytophthora*.

Of the 580 kauri root fragments (totalling 7.3 m in length) that were direct plated onto selective agar, none yielded *P. agathidicida* or any other species of *Phytophthora*. One sample that was baited yielded *P. agathidicida*, out of a total of 308 root fragments (totalling 3.9 m in length) that were baited. This single detection of live *P. agathidicida* was from a 4-mm long fragment of kauri root that was collected from pig faeces passed within 24 h of feeding. No detections were made from any other samples.

For each faecal collection from each pig at each sampling time, a sample was mixed with potting mix and applied to a pot with a kauri seedling. These seedlings were grown for 6 months, then assessed for *P. agathidicida* symptoms. No such symptoms developed and *P. agathidicida* could not be isolated from any roots or from baited roots and soil.

This work demonstrated that passage of live *P. agathidicida* through the pig gut is possible, but that the frequency and longevity of survival is low. It appears that the conditions within the pig gut are not conducive to long-term *P. agathidicida* survival, and that survival beyond 24 h is unlikely. Most of the kauri root material took more than 24 h to pass through the gut, with only very small fragments passing within this period.

These finding are in contrast to those of Li et al. (2013), who found that *P. cinnamomi* readily survived passage through the pig gut. In comparison to *P. cinnamomi*, it is likely that the poor survival of *P. agathidicida* in the pig gut reflects its relative intolerance of high temperatures. In laboratory studies, cultures of PTA containing numerous oospores were unable to be regenerated after 24 h at 37°C, whereas *P. cinnamomi* survival was high.

In conclusion, this study demonstrated that although it is possible for *P. agathidicida* to survive the pig gut, this probably occurs only in root fragments that pass through very rapidly. Transmission of *P. agathidicida* in infected soil on the outside of pigs is probably a greater risk.

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1 INTRODUCTION

Kauri Dieback caused by *Phytophthora agathidicida* is a problem in some New Zealand kauri forests, causing tree decline and death. In many forests its distribution is limited, but there is a risk that it could be spread by feral animals, either as infected soil on feet or in the digestive system. *Phytophthora cinnamomi* has been shown to survive passage through the digestive system of pigs (Li et al. 2013), demonstrating that this pathogen could potentially spread great distances before being deposited. The current work is to determine whether *P. agathidicida* could potentially survive passage through the pig digestive system. The first step was to determine suitable sampling and detection techniques for extracting *P. agathidicida* from pig faeces, followed by feeding of infested material and attempting to extract the pathogen at various times afterwards.

2 PILOT STUDY 1

2.1 Methods

Two *P. agathidicida* isolates extracted from kauri soil were used in this study. Cultures were grown on V8 agar plates and on autoclaved oat grains. To obtain colonised kauri roots rapidly, feeder roots of glasshouse-grown kauri seedlings were autoclaved then imbedded in V8 agar plates. These plates were then inoculated with plugs of *P. agathidicida*. After 1 month roots were checked under the microscope to confirm *P. agathidicida* colonisation and oospore production within roots.

Domestic pig faeces samples were collected from an Auckland abattoir and shipped to the Hawke's Bay laboratory. Samples of *P. agathidicida*-colonised kauri feeder roots and oat grains were inserted in the pig faeces and left to incubate at 20°C. After 24 h, 15-20 root fragments (approximately 5-10 mm long) and oat grains were extracted from the faeces, surface-sterilised in 50% ethanol for 30 seconds, and then plated onto *Phytophthora*-selective agar. Comparable oat grains and roots that had not been incubated in pig faeces were plated at the same time.

The pig faeces containing the remaining *P. agathidicida*-colonised kauri roots and oat grains were divided into two portions. From the first portion, five samples, each approximately 80 ml were put into 500-ml containers, flooded with distilled water, and baited with freshly germinated lupins and fresh cedar needles (fresh baiting). From the second portion, five 80-ml samples were air-dried on paper towels for 5 days, placed in 500-ml containers then moistened with distilled water for 4 days, then flooded and baited with lupins and cedar needles as above. After 48 h of exposure, lupin and cedar baits were plated onto *Phytophthora*-selective media (extended baiting).

To help to determine potential regimes for storage of faeces samples in future experiments, *P. agathidicida*-colonised oats, kauri feeder roots and V8-agar plates were incubated at -10°C, 1°C or 20°C for 10 days. Samples were then plated onto V8 agar, or moistened and held at 20°C for 1 day, then flooded and baited with lupins and cedar needles for 3 days, with subsequent plating of these baits onto *Phytophthora*-selective agar.

2.2 Results

Results from Pilot Study 1 are presented in Table 1. Oat grains and roots used in the study were heavily colonised by *P. agathidicida*, as demonstrated by the high re-isolation rate when these were plated directly onto agar.

When the *P. agathidicida*-colonised oats and roots were incubated in pig faeces for 24 h and then extracted from the faeces and directly plated onto agar, all the root fragments and a majority of the oat grains yielded viable *P. agathidicida*. This indicated that *P. agathidicida* can survive within plant tissue in the pig faeces environment.

However, when faeces samples containing viable *P. agathidicida* in oat grains or kauri roots were run through the standard *Phytophthora* baiting systems, *P. agathidicida* was not isolated. This was the case with both baiting of the fresh sample and when the samples were air-dried, moist-incubated and baited, as per the standard *P. agathidicida* isolation procedure.

These results indicate that the pig faeces environment does not allow some step in the baiting procedure, either oospore germination, sporangial production or zoospore release and colonisation of baits. This would not be a problem in a natural environment, as the faeces would break down, releasing dormant oospores or colonised tissue fragments into the soil for subsequent germination. But for our extraction and detection work from the feeding trials, we needed a detection technique that could work with the bulk fresh sample.

Results of the temperature storage trial indicated that *P. agathidicida* survival following freezing was poor, no matter what the inoculum form, so this was not an option for sample storage and handling (Table 2). Even at 1°C the survival was slightly lower than at room temperature, so a decision was made to store samples at room temperature and process them as soon as received.

Table 1. *Phytophthora agathidicida* extraction from colonised oats and kauri roots by direct plating or baiting following incubation for 24 h on the laboratory bench or in fresh pig faeces.

Incubation conditions	Inoculum type	Extraction technique	Number plated	Number positive	Percentage positive
Lab. bench	oats	Direct plate	10	6	60
Lab. bench	roots	Direct plate	10	9	90
Pig faeces	oats	Direct plate	16	11	69
Pig faeces	roots	Direct plate	17	17	100
Pig faeces	oats	Fresh bait - lupin	15	0	0
Pig faeces	oats	Fresh bait - cedar	25	0	0
Pig faeces	roots	Fresh bait - lupin	15	0	0
Pig faeces	roots	Fresh bait - cedar	25	0	0
Pig faeces	oats	Extended bait - lupin	15	0	0
Pig faeces	oats	Extended bait - cedar	25	0	0
Pig faeces	roots	Extended bait - lupin	15	0	0
Pig faeces	roots	Extended bait - cedar	25	0	0

Table 2. Survival of *Phytophthora agathidicida* inoculum in agar, oats or kauri roots following incubation for 10 days at various temperatures.

Incubation conditions	Inoculum type	Extraction technique	Number plated	Number positive	Percentage positive
-10°C	agar	Direct plate	10	0	0
-10°C	oats	Direct plate	10	0	0
-10°C	roots	Direct plate	6	0	0
1°C	agar	Direct plate	5	5	100
1°C	oats	Direct plate	10	9	90
1°C	roots	Direct plate	6	5	83
20°C	agar	Direct plate	5	5	100
20°C	oats	Direct plate	5	5	100
20°C	roots	Direct plate	5	5	100
-10°C	agar	Bait	8	1	13
-10°C	oats	Bait	8	0	0
-10°C	roots	Bait	4	0	0
1°C	agar	Bait	8	3	38
1°C	oats	Bait	5	0	0
1°C	roots	Bait	4	3	75
20°C	agar	Bait	9	8	89
20°C	oats	Bait	5	2	40
20°C	roots	Bait	5	4	80

3 PILOT STUDY 2

3.1 Methods

Following the failure to extract *P. agathidicida* when baiting from pig faeces containing *P. agathidicida*-infected material, a number of studies were carried out to manipulate the faeces sample to determine if this would improve baiting success. Various modifications of the baiting system were set up in 250-ml plastic cups, with five replicates of each treatment, as follows:

1. Faeces diluted with 1 part faeces, 5 parts sterilised garden soil (by volume), then flooded with 5 parts water
2. Faeces diluted with 1 part faeces, 5 parts vermiculite (by volume) then flooded with 5 parts water
3. Five times the water volume (i.e. 1 part faeces, 25 parts water)
4. Acidification with acetic acid
5. Water control.

The pH of the solutions was 5.55, 6.60, 5.69 and 5.78 for Treatments 1, 3, 4 and 5, respectively.

P. agathidicida inoculum, as either colonised oat grains or mycelial mats, was added to each of the various suspensions 2 h after setting them up. After a further 2 h, Himalayan cedar (*Cedrus deodara*) needle baits were floated on the surface of the water. Three days after baiting the baits were removed and plated to selective agar. Fresh baits were floated on the water surface and plated after a further 2 days.

3.2 Results

Results indicated that dilution of *P. agathidicida*-contaminated pig faeces with soil, vermiculite, or water, or acidification of the samples using acetic acid failed to improve baiting efficacy to a sufficient degree to give confidence in the ability to detect *P. agathidicida* where it was present (Table 3). From this result, it was decided that baiting from bulk faeces samples would not be used as the standard detection procedure in the main pig feeding trials, and the focus would instead be on direct plating and baiting of washed substrate fragments.

Table 3. Pig faeces baiting efficiency following amendment with various substrates. Pig faeces samples were modified by dilution with soil, vermiculite or water, or acidified using acetic acid before inoculating with *Phytophthora agathidicida* (mycelial mats or oat grains) then baiting with cedar needles for 3 days, followed by a further 2 days.

Incubation conditions	Inoculum type	Extraction technique	Number plated	Number *P. agathidicida* positive	Percentage *P. agathidicida* positive
5x soil	Mycel. mat	Bait cedar d0-d3	5	0	0
		Bait cedar d3-d5	5	0	0
	Oat	Bait cedar d0-d3	5	1	20
		Bait cedar d3-d5	4	1	25
5x vermiculite	Mycel. mat	Bait cedar d0-d3	4	0	0
		Bait cedar d3-d5	5	0	0
	Oat	Bait cedar d0-d3	5	0	0
		Bait cedar d3-d5	4	0	0
5x water	Mycel. mat	Bait cedar d0-d3	5	0	0
		Bait cedar d3-d5	5	0	0
	Oat	Bait cedar d0-d3	5	1	20
		Bait cedar d3-d5	4	0	0
Acidified	Mycel. mat	Bait cedar d0-d3	5	0	0
		Bait cedar d3-d5	5	0	0
	Oat	Bait cedar d0-d3	4	3	75
		Bait cedar d3-d5	5	0	0
Control – no faeces	Mycel. mat	Bait cedar d0-d3	5	5	100
		Bait cedar d3-d5	5	4	80
	Oat	Bait cedar d0-d3	5	4	80
		Bait cedar d3-d5	5	4	80

4 PIG FEEDING TRIALS

4.1 Methods

4.1.1 Inoculum preparation

Oats and millet: Oat and millet seeds were soaked overnight in distilled water, autoclaved, then inoculated using *P. agathidicida*-colonised V8-agar cubes. Jars containing this inoculum were incubated for 3 weeks before being supplied for the feeding trials. Colonised oat and millet seeds were then mixed in even proportions and weighed into 150-g portions for feeding to pigs.

Kauri roots: Six two-year-old kauri seedlings growing in planter bags of potting mix were inoculated with *P. agathidicida*. A metal probe was used to make two 10-cm deep holes in the potting mix, then these holes were filled with inoculum. The inoculum consisted of *P. agathidicida*-colonised oats and colonised V8-agar plates, macerated with distilled water to a thick porridge consistency. Following inoculation, trees were held in saturated conditions for 48 h to facilitate zoospore production and root infection. Following inoculation and the initial 48 h flooding, seedlings were kept well watered but free draining until they were required. Every two weeks for a 10-week period a new batch of seedlings was inoculated in a similar manner. This was to ensure that a range of roots colonised for different times were available for the pig feeding trials. Twelve weeks after the first seedlings were inoculated, roots were prepared for pig feeding. Seedlings from a range of inoculation times were harvested, avoiding trees that had already died. Above-ground portions were discarded and as much potting-mix as possible washed from the roots. The washed feeder roots were bunched and roughly cut to 1-2 cm lengths using secateurs. The nature of the fibrous root systems meant that such cutting resulted in a wide range of root lengths, from 1 mm to about 50 mm. Most of the root fragments were between 1 and 3 mm diameter. All cut roots were pooled into one container, then divided evenly into 80-g portions for feeding to each pig.

Inoculum was sent by courier to the Massey University Institute of Veterinary, Animal and Biomedical Sciences in Palmerston North the day before feeding was scheduled. Samples of each inoculum type were retained for later comparison of *P. agathidicida* recovery.

4.1.2 Pig preparation, feeding and faecal collection

Thirteen trial pigs, weighing 15-18 kg were acclimated at the Massey University Institute of Veterinary, Animal and Biomedical Sciences farm on a normal diet for 7 days before inoculum feeding began. The day before feeding the inoculum, pigs were fed a slightly reduced volume of feed to ensure they were hungry and would consume the inoculum diet. The inoculum (both oat/millet and roots) was mixed with the pigs' normal food to aid consumption. Six pigs were fed 150 g of colonised oats and millet and six pigs were fed 80 g of colonised kauri roots. One pig was fed a mixture of 150 g uncolonised oats/millet and 80 g uncolonised kauri roots as a control. In all cases, the inoculum was mixed with 700 g of their normal diet.

Colostomy bags were attached to pig rears to collect faeces. These were changed every 12 h. Faeces for each 24 h period up to 168 h were pooled for each pig, and then sieved using tap water. Course material (>1 mm diameter), including oat and millet seeds, root fragments and coarser parts of the pigs' normal diet were retained on the sieve, and sent to the PFR laboratory in Havelock North for further analysis. A full description of the pig preparation, feeding and faeces collection is given in the Massey University report (Wolber et al. 2015).

4.1.3 Processing of faeces

Plating and baiting: Samples received in the PFR laboratory contained coarse fragments of the pig's normal diet, and fragments of the three types of inoculum. A sample of oat, millet and root fragments were removed from the faeces using forceps, then rinsed in sterile distilled water. One portion of these were surface sterilised in 50% ethanol, then directly plated onto *Phytophthora*-selective agar (PARPH). Up to 20 oat and millet seeds or 30 kauri root fragments were plated from each faecal sample. The remaining portion of rinsed seeds or roots were placed in 23-mm diameter cell wells. Up to 10 cells, each with five seeds, and up to 30 cells with root fragments were then flooded with sterile distilled water. Cedar needle baits were then floated on the surface of each cell and incubated at 20°C. After 4 days baits were removed, surface sterilised in 50% ethanol and plated onto selective media. Cells were then re-baited with fresh cedar needles, incubated for a further 5 days, then plated as above. Agar plates were monitored for 7 days, and any *Phytophthora*-like colonies were sub-cultured onto V8 agar for identification.

The control samples that were retained in the laboratory for validation of inoculum viability were plated and baited using the same techniques list above.

Tree inoculation: A 5-10 g sample of sieved faeces from each pig at each sample time was retained for tree inoculation. These were pooled into 0-48, 48-96, 96-144 and 144-168 h samples for each of the six pigs in each inoculum type (oats/millet or kauri roots). Each pooled sample was divided into two, with half mixed with potting mix, placed in a PB1½ planter bag, and a clean 6-month-old kauri seedling placed on top, taking care to avoid direct contact between the roots and the faeces. The other portion was inserted into a hole made around the root system of a 2- to 3-year-old nursery-grown kauri seedling in a PB3 planter bag. All seedlings were then flooded for 24 h, then grown under normal watering conditions in a glasshouse maintained at 18 to 25°C. Periodically seedlings were flooded for 24 h to facilitate zoospore production and infection. Seedlings were monitored weekly for 26 weeks, looking for any sign of infection symptoms.

After 26 weeks, all 6-month-old seedlings (now 12 months old) were carefully harvested. Roots were washed and scored for signs of root disease. Samples of roots (10 per seedling) were surface sterilised and plated onto *Phytophthora*-selective agar, and other samples (with attached soil) were flooded and baited for up to 7 days with lupin radicles and cedar needles. After 2 days baits were surface-sterilised and plated onto *Phytophthora*-selective agar which was subsequently checked for *Phytophthora*-like colonies. Sample were re-baited with cedar needles for a further 5 days as above.

To sample the faeces-inoculated 3-year-old seedlings after 26 weeks, a portion of roots was cut from each planter bag, and plated or baited as described for the 6-month-old seedlings. The seedlings were retained in the glasshouse for a further 3 months and re-sampled as above.

Trees for the inoculation trials were from two sources: the 2- to 3-year-old seedlings were obtained from the Scion nursery in Rotorua, and held in the PFR glasshouse for 6 months before inoculation with faeces. The 6-month-old seedlings were grown from seed in the PFR glasshouse, taking extreme care about hygiene to guarantee no *Phytophthora* contamination in advance of the faeces inoculation.

4.2 Results

4.2.1 Faecal sample analysis

The numbers of millet seeds plated and baited from faecal samples at the various time intervals are presented in Table 4. Millet seeds were clearly discernible and plentiful in the 24-h sample and up until 96 h. A few were discernible in the 120-h and 144-h samples, and none in the 168-h sample. The numbers plated at the various times reflect the ease of locating the millet seeds within the samples. For example, in the 48-h sample the full complement of seeds was readily detected and plated or baited. At other times, millet seeds were more difficult to locate and lower numbers were plated.

Oat grains were mostly digested after passage through the pig gut, and only a small number were plated or baited.

Table 4. Number of millet seeds plated or baited after extraction from pig faecal samples collected various times after feeding *Phytophthora agathidicida*-inoculated millet seed.

	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Number of millet seeds plated	70	120	49	22	2	2	0
Number of cells baited (5 millet seeds/cell)	35	60	10	2	0	0	0

A few small (mostly <6 mm long) kauri root fragments were visible in the 24-h samples (Tables 5 & 6). The 48- to 96-h samples contained large numbers of root fragments of a range of sizes. Numbers of roots declined from 48 h onwards, but there were still kauri roots clearly visible in the 168-h samples. Most of the root fragments in faecal samples collected 120 h or more after feeding were relatively large (average >15 mm long).

Table 5. Number and length of kauri root fragments plated after extraction from pig faecal samples collected various times after feeding *Phytophthora agathidicida*-inoculated kauri roots.

Plated roots	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Root fragment count	40	178	130	89	63	57	23
Total length (mm)	222	2036	1480	1166	996	996	398
Maximum length (mm)	15	26	45	24	40	33	35
Minimum length (mm)	2	2	2	3	6	6	8
Average length (mm)	5.55	11.4	11.4	13.1	15.8	17.5	17.3

Table 6. Number and length of kauri root fragments baited after extraction from pig faecal samples collected various times after feeding *Phytophthora agathidicida*-inoculated kauri roots.

Baited roots	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Root fragment count	36	149	84	39	0	0	0
Total length (mm)	181	2035	946	716	0	0	0
Maximum length (mm)	21	38	25	38	-	-	-
Minimum length (mm)	1	3	2	8	-	-	-
Average length (mm)	5.0	13.6	11.3	18.4	-	-	-

4.2.2 *P. agathidicida* detection by plating or baiting

None of the 265 millet seeds direct plated or the 535 seeds baited yielded *P. agathidicida* or any other *Phytophthora*. Similarly, none of the plated or baited oat grains yielded any *Phytophthora*.

Of the 580 kauri root fragments (totalling 7.3 m in length) that were plated onto selective agar, none yielded *P. agathidicida* or any other species of *Phytophthora*.

Of the 308 root fragments (totalling 3.9 m in length) that were baited, only one sample yielded *P. agathidicida*. This sample was from the second baiting (Day 4-9) of a 4-mm long fragment of kauri root that was collected from pig faeces passed within 24 h of feeding.

The control samples that were retained in the laboratory for validation of inoculum viability readily yielded *P. agathidicida*, with re-culturing from 90% of millet seeds and 40% of kauri roots.

4.2.3 Kauri seedling inoculation

After sampling for the plating and baiting assays described above, many of the kauri roots and millet seeds had been removed from the faecal samples. However, roots and millet seed were still visible in most samples, particularly those collected up to 72 h.

None of the kauri seedlings died in the 26 weeks following inoculation with pig faeces collected at various times after feeding trial pigs with *P. agathidicida*-inoculated kauri roots or millet/oat seeds. No seedlings demonstrated any symptoms indicative of *P. agathidicida* infection. There were no observable differences in the root health of control seedlings or seedlings inoculated with potentially contaminated pig faeces.

None of the plated or baited roots of the 6-month-old seedlings yielded *P. agathidicida* or any other *Phytophthora*. None of the plated or baited roots of the 2- to 3-year-old seedlings yielded *P. agathidicida*. However, six isolates of *P. cinnamomi* and seven isolates of *P. multivora* were extracted from roots of 3-yr-old trees, both from trees inoculated with pig faeces and from uninoculated trees. It is assumed that the source of these *Phytophthora* species was low-level contamination in the nursery which supplied the trees, as these species have been detected in seedlings from the same supplier in the past. The fact that no *Phytophthora* was detected in the 6-month-old seedlings grown in quarantine conditions and tested at the same time supports this assumption.

5 DISCUSSION

This work demonstrated that passage of live *P. agathidicida* through the pig gut is possible, but that the frequency of survival is very low. Despite plating and baiting of large numbers of *P. agathidicida*-colonised millet seed and kauri roots after passage through the pig gut, only one detection of the pathogen was made. This was from a root fragment that passed through the gut within 24 h, and the pathogen emerged only after repeated baiting of the sample. This result indicates that the conditions within the pig gut are not conducive to *P. agathidicida* survival, and that survival beyond 24 h is unlikely. Most of the kauri root material took more than 24 h to pass through the gut, with only very small fragments passing within this period.

These finding are in contrast to those of Li et al. (2013), who found that *P. cinnamomi* readily survived passage through the pig gut. In comparison to *P. cinnamomi*, it is likely that the poor survival of *P. agathidicida* in the pig gut reflects its relative intolerance of high temperatures.

In separate work, we demonstrated that mature cultures of *P. agathidicida* containing numerous oospores could not be regenerated after 24 h of incubation at 37°C. In contrast, survival of *P. cinnamomi* cultures under similar conditions was 100%. Although such cultures may differ from the survival structures that may be formed within kauri roots, this result gives some insight into the contrast between the poor survival of *P. agathidicida* passaging though the pig gut in the current study with that of *P. cinnamomi* in the study of Li et al. (2013).

In conclusion, this study demonstrated that although it is possible for *P. agathidicida* to survive the pig gut, this probably occurs only in root fragments that pass through very rapidly. Transmission of *P. agathidicida* in infected soil on the outside of pigs is probably a greater risk.

6 REFERENCES

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