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# A TaqMan real-time PCR assay for the detection of *Phytophthora* 'taxon Agathis' in soil, pathogen of Kauri in New Zealand

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## Summary

Kauri Agathis australis, an iconic tree of New Zealand, is under threat from an introduced disease-causing pathogen provisionally named *Phytophthora* 'taxon Agathis' (referred to as PTA). This soilborne, Pythiaceous species belongs to the Chromista and causes a collar rot resulting in yellowing of the foliage and thinning of the canopy, which eventually causes death of the infected tree. The management and containment of this pathogen requires rapid and reliable detection in the soil. The current method for soil detection utilizes a soil bioassay involving lupin baits and soil flooding in a process that takes between ten and twenty days. We describe a real-time PCR assay based on TaqMan chemistry for the specific detection of PTA, which targets the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA. This TaqMan real-time PCR assay could be used with DNA extracted directly from bulk soil samples to enable rapid quantification of PTA within soil. The detection limit was 2 fg of PTA DNA from pure culture, or 20 fg in the presence of DNA extracted from soil. The assay was validated using soil samples taken from a PTA-infested site and soil spiked with a known concentration of oospores. We conclude that the TaqMan real-time PCR assay offers a more time-efficient method for detection of PTA in soil than existing methods.

## 1 Introduction

The origin of *Phytophthora* 'taxon Agathis' (PTA), the causal agent of the collar rot of Kauri, *Agathis australis,* remains uncertain (Beever et al. 2009). Kauri, a coniferous tree belonging in the genus Araucariaceae, is an iconic and dominant tree in forests in northern New Zealand. According to Beever et al. 2009, five *Phytophthora* species have been recorded from Kauri forest soils; *P. cinnamomi*, *P. cryptogea*, *P. kernoviae*, *P. nicotianae* and PTA.

PTA causes large 'resin bleeding' lesions, collar rot, yellowing and thinning of the foliage and tree death and has been isolated from the lesions and soil samples around infected Kauri (Beever et al. 2009). It was originally misidentified as *P. heveae* in 1974 (Gadgil 1974). Subsequently, it has been established that PTA has different oospore morphology than *P. heveae,* and via ITS-analysis, it is matched to *P. katsurae sensu stricto* – an isolate recovered from forest soil in Taiwan, but not recorded in New Zealand (Beever et al. 2009). However, it is clear that PTA has a different oospore wall morphology to *P. katsurae s.s.* (Fig. 1). Importantly, PTA shares its place in Clade 5 with *P. heveae* and *P. katsurae* (Cooke et al. 2007).

PTA poses a threat to the long-term conservation of Kauri at the local and national level and also to those proposing to plant kauri in plantations (Steward and Beveridge 2010). Because of the difficulties and uncertainty about its origins and distribution, it is imperative that we can accurately identify its presence in soil.

Conventional approaches to soil-based detection of *Phytophthora* species use leaf, fruit and other plant parts as baits to recover the pathogen from flooded soil samples (Newhook 1958; Martin et al. 2012) or sterile isolation techniques to recover the pathogen from host root material on *Phytophthora*-specific media (Jeffers and Martin 1986; Ho 1987; Kato et al. 1990). The steps involved in soil bioassays and conventional sterile techniques are lengthy and require precise timing to obtain pure mycelial cultures. The current protocol developed for soil-based detection of PTA can take 20 days, which includes air-drying the soil, flooding with water, adding the baits and finally plating the baits (Horner and Wilcox 1996). Traditional morphological identification schemes are not considered to be optimal, as morphological plasticity within discrete taxa can be considerable, making unknown species determinations challenging (e.g. Kuan and Erwin 1980). Dormant or dead spores may exist but are not detectable. Moreover, morphological identification requires skill and knowledge of taxonomy, evident from the initial misidentification of PTA as *P. heveae.*

Identification, detection and phylogeny of *Phytophthora* spp. have been assisted through the use of species-specific polymerase chain reaction (PCR) technologies (Minerdi et al. 2008). Useful nuclear loci include ribosomal ITS (Cooke et al. 2007), β-tubulin (Kroon et al. 2004a; Bilodeau et al. 2007a; Blair et al. 2008), translation elongation factor 1-α (Kroon et al. 2004a; Blair et al. 2008), elicitin (Bilodeau et al. 2007a,b), 60S ribosomal protein L10, enolase, heat shock protein 90, tigA gene fusion protein (Blair et al. 2008) and ras-related protein gene ypt 1 (Cooke et al. 2007; Schena et al. 2008). Species separation has also been achieved using mitochondrial multiple copy gene regions such as cox-1 (Kroon et al. 2004a,b), cox-1 and cox-2 spacer (Martin and Tooley 2003), nadh1 (Kroon et al. 2004a) and nad5 (Ivors et al. 2004). Technical limitations related to post-amplification procedures and cross-contamination have seen real-time PCR replace conventional gel-based PCR for high throughput diagnostic applications, including detection of a number of species of *Phytophthora* (Schena et al. 2004; Hughes et al. 2006).

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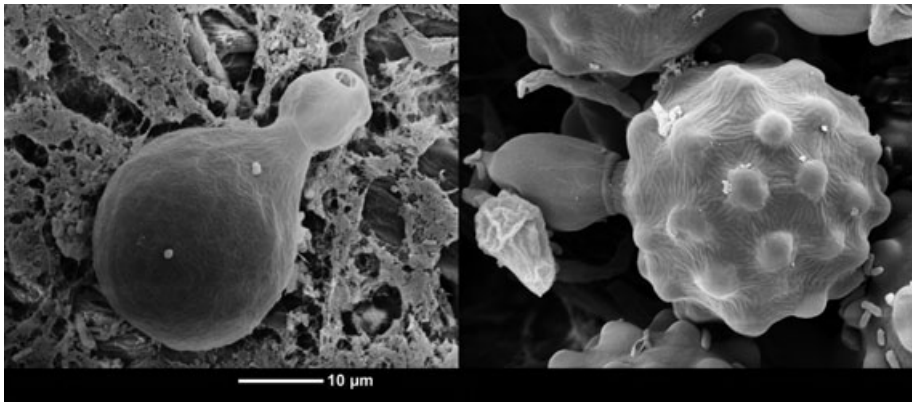


*Fig. 1.* Left: Scanning electron microscope (SEM) image of PTA oogonium, Right: SEM image of *P. katsurae* oogonium.

The reliable detection of PTA underpins our ability to delimit the distribution of the pathogen throughout Kauri forests. The aim of this study was therefore to develop a taxon-specific, TaqMan, real-time PCR assay for detection of PTA suitable for use in the routine testing of soil.

## 2 Materials and methods

### 2.1 Maintenance of isolates and oospore production

Thirteen isolates from Landcare Research ICMP collection and 26 from Fera (The Food and Environment Research Agency); Three PTA, three species of *Pythium* and 31 species of *Phytophthora* were used for the various experiments. The isolates shown in Table 1 were grown and maintained on potato dextrose agar (PDA) at 18°C under 12 h of light and 12 h of dark until required for DNA extraction. Suspensions of PTA oospores were produced by taking agar plugs from the margin of 10-day-old cultures grown on PDA. These plugs were transferred to sterile, non-ventilated Petri dishes containing 20 ml of clarified V8 juice broth (10% clarified V8 juice sterilized at 121°C for 15 min, 0.02 M $CaCO_3$, pH adjusted to 4.5). Plates were incubated in the dark for 30 days at 20°C until the formation of oospores was observed. The mycelial mat was then thoroughly macerated twice, centrifuged at 5000 × ***g*** and the supernatant discarded. There was no viable mycelium left after the second maceration. After discarding the supernatant, the pellet was resuspended in water and vortexed for 5 min at 10 000 rev $min^{-1}$. Oospores were stored in sterile, reverse osmosis water at 4°C until required.

### 2.2 Extraction of DNA from cultures and soil

DNA was extracted from pure mycelial cultures grown on PDA for 10 days by scraping off mycelia using a sterile pipette tip and DNA extracted using the X-tractor Gene robot (Qiagen X-tractor Tissue kit). For determination of assay sensitivity, DNA concentration was estimated using a QuantiFluor single-tube fluorometer (Promega). DNA was stored in elution buffer (Qiagen X-tractor Tissue kit) at −40°C until required.

Soil was air-dried for 2 days then passed through a two millimetre sieve to remove gravel and coarse organic debris. All PTA-free soil samples were collected from Landcare Research Tamaki garden. This is a brown sandy load soil used for native amenity planting. The top 15 cm of soil was used. DNA extraction from soil followed a modified methodology of Woodhall et al. (2012). All centrifugation steps were performed at room temperature. Soil samples (100 g) were weighed into screw-topped containers (Nalgene) containing ten surface-sterilized one inch stainless steel ball bearings. The sample was mixed with 200 ml of 2% soil CTAB buffer (120 mM sodium phosphate buffer pH8, 2% CTAB, 1.5 M NaCl) and 9 ml of antifoam B emulsion (Sigma) by vigorous manual shaking for 5 min. The ground sample extract was transferred into a sterile 50 ml, screw-cap tube and centrifuged at 5000 × ***g*** for 5 min. 20 ml of cleared extract was transferred to a sterile 50-ml tube with 2 ml 5 M ammonium acetate solution, vortexed for 20 seconds, placed on ice for 10 min, then centrifuged at 12000 × ***g*** for 5 min. The cleared extract was transferred to a sterile 50-ml tube with 15 ml isopropanol and 800 µl acid-washed silica particles (Sigma), then placed in a rack and horizontally rocked at 70 revs $min^{-1}$ for 10–15 min. The sample was centrifuged at 12000 × ***g*** for 5 min and the supernatant was discarded. The silica pellets were resuspended in 2 ml tissue lysis buffer (DXT from Qiagen X-tractor Tissue kit) and were shaken at 70 revs $min^{-1}$ for 5 min at 65°C, then centrifuged at 12000 × ***g*** for 5 min. One ml of the supernatant was transferred into a clean 1.5-ml tube and purified with the X-tractor Gene robot according to the manufacturer's protocol.

### 2.3 Conventional PCR and sequencing

Sequences of four gene regions were determined for the isolates indicated in Table 2. These isolates represent *Phytophthora* and *Pythium* that have been isolated from soil in Kauri forests, and therefore likely to be similar in sequence to PTA.

*Table 1. Phytophthora* and *Pythium* isolates used for development and specificity testing of the TaqMan real-time PCR assay.

| Species | Isolate reference | Culture collection | Result | Isolate origin | Collection date | Host |
|---|---|---|---|---|---|---|
| *Phytophthora* 'taxon Agathis' | REB327-68 | LCR | + | Huia, NZ | April 2010 | Kauri |
| *Phytophthora* 'taxon Agathis' | REB327-73 | LCR | + | Waitakere, NZ | 2010 | Kauri |
| *Phytophthora* 'taxon Agathis' | ICMP18407 | LCR | + | Waipoua Forest | March 2010 | Kauri |
| *Phytophthora boehmeriae* | cc2273 | Fera | – | N/A | N/A | N/A |
| *Phytophthora botryosa* | cc2201 | Fera | – | N/A | N/A | N/A |
| *Phytophthora cactorum* | cc1254 | Fera | – | N/A | N/A | N/A |
| *Phytophthora cambivora* | cc1221 | Fera | – | N/A | N/A | N/A |
| *Phytophthora cinnamomi* | REB326-68 | LCR | – | Huia, NZ | December, 2008 | Stream bait |
| *Phytophthora cinnamomi* | cc1226 | Fera | – | N/A | N/A | N/A |
| *Phytophthora citricola* | REB326-61 | LCR | – | Huia, NZ | December, 2008 | Stream bait |
| *Phytophthora citricola* | REB326-67 | LCR | – | Huia, NZ | December, 2008 | Stream bait |
| *Phytophthora citricola* | cc1531 | Fera | – | N/A | N/A | N/A |
| *Phytophthora citrophthora* | cc1705 | Fera | – | N/A | N/A | N/A |
| *Phytophthora cryptogea* | cc1708 | Fera | – | N/A | N/A | N/A |
| *Phytophthora cryptogea* | REB326-60 | LCR | – | Huia, NZ | December, 2008 | Stream bait |
| *Phytophthora europaea* | cc2159 | Fera | – | N/A | N/A | N/A |
| *Phytophthora gonapodyides* | cc2196 | Fera | – | N/A | N/A | N/A |
| *Phytophthora heveae* | cc1700 | Fera | – | N/A | N/A | N/A |
| *Phytophthora hibernalis* | cc2198 | Fera | – | N/A | N/A | N/A |
| *Phytophthora ilicis* | cc2194 | Fera | – | N/A | N/A | N/A |
| *Phytophthora insolita* | cc2274 | Fera | – | N/A | N/A | N/A |
| *Phytophthora kernoviae* | cc2286 | Fera | – | N/A | N/A | N/A |
| *Phytophthora lateralis* | cc2199 | Fera | – | N/A | N/A | N/A |
| *Phytophthora megasperma* | cc2158 | Fera | – | N/A | N/A | N/A |
| *Phytophthora nemorosa* | cc2193 | Fera | – | N/A | N/A | N/A |
| *Phytophthora palmivora* | cc1706 | Fera | – | N/A | N/A | N/A |
| *Phytophthora parasitica* | cc1709 | Fera | – | N/A | N/A | N/A |
| *Phytophthora pseudosyringae* | cc2192 | Fera | – | N/A | N/A | N/A |
| *Phytophthora ramorum* | cc2267 | Fera | – | N/A | N/A | N/A |
| *Phytophthora richardiaea* | cc2190 | Fera | – | N/A | N/A | N/A |
| *Phytophthora rubi* | cc2106 | Fera | – | N/A | N/A | N/A |
| *Phytophthora sp.* | REB326-69 | LCR | – | Huia, NZ | December, 2008 | Stream bait |
| *Phytophthora syringae* | cc2239 | Fera | – | N/A | N/A | N/A |
| *Phytophthora uliginosa* | cc2156 | Fera | – | N/A | N/A | N/A |
| *Pythium sp.* | REB326-70 | LCR | – | Huia, NZ | December, 2008 | Stream bait |
| *Pythium sp.* | REB326-73 | LCR | – | Huia, NZ | December, 2008 | Stream bait |
| *Pythium sp.* | REB326-74 | LCR | – | Huia, NZ | December, 2008 | Stream bait |
| *Pythium sp.* | REB326-75 | LCR | – | Huia, NZ | December, 2008 | Stream bait |
| *Pythium sp.* | REB326-77 | LCR | – | Huia, NZ | December, 2008 | Stream bait |

LCR, Landcare Research Ltd; Fera, The Food and Environment Research Agency; N/A, Not applicable; NZ, New Zealand.
Positive results (+) have Ct of $< 40$; Negative results (–) are $\geq 40$. Mean Ct values are for duplicate reactions. Isolates from Fera are not available for the public.

The gene regions selected were NADH dehydrogenase subunit 1 (NADH1), translation elongation Factor 1α (TEF), β-tubulin (βTub) and rDNA internal transcribed spacer (ITS). NADH1, TEF and βTub genes were amplified using the primers and method described in Kroon et al. (2004a).The rDNA ITS region was amplified using primers ITS6 and ITS4 (White et al. 1990). Successful amplifications were then confirmed by visualization of the PCR by electrophoresis. The PCR products were then sequenced using the ABI Genetic Analyser 3130xl sequencing machine (Applied Biosystems).

### 2.4 TaqMan primers and probe for real-time PCR

Alignments of sequences of PTA (target sequence) and non-target *Phytophthora* and *Pythium* isolated from Kauri forest were constructed using Geneious Pro software (Biomatters Ltd) and used as the basis for primers and probe design. Initial examination of alignments of NADH, TEF, βTub and ITS indicated that the ITS region displayed the highest variability between target and non-target species, so this region was selected for the design of primers and probe specific to PTA. The GenBank accession numbers of sequences used for the alignment on which primer design was based are shown in Table 2. Primer Express 3 software (Applied Biosystems) was used to design the primers and probe for the TaqMan assay (Table 3).

### 2.5 TaqMan real-time PCR and assay specificity

The assay was tested against all the isolates collected from Kauri forests and an additional 26 *Phytophthora* isolates from Fera. A TaqMan, real-time PCR was carried out on a Rotor-Gene 6000 instrument (Qiagen) using TaqMan Environmental

*Table 2.* GenBank accession numbers of the ITS sequences used in the design of the TaqMan primers and probe.

| Species | Isolate reference | GenBank accession number | Closest species | % Homology |
|---|---|---|---|---|
| *Phytophthora cinnamomi* | REB326-68 | JX122743 | *Phytophthora cinnamomi* | 100% |
| *Phytophthora multivora ex citricola* | REB326-61 | JX122741 | *Phytophthora multivora* | 100% |
| *Phytophthora multivora ex citricola* | REB326-67 | JX122742 | *Phytophthora multivora* | 100% |
| *Phytophthora cryptogea* | REB326-60 | JX122740 | *Phytophthora cryptogea* | 100% |
| *Phytophthora sp.* | REB326-69 | JX122744 | *Phytophthora constricta* | 84% |
| *Phytophthora* 'taxon Agathis' | REB327-68 | JX122749 | *Phytophthora sp. ICMP 16471* | 100% |
| *Phytophthora* 'taxon Agathis' | REB327-73 | JX122750 | *Phytophthora sp. ICMP 16471* | 100% |
| *Phytophthora* 'taxon Agathis' | ICMP18407 | JX122751 | *Phytophthora sp. ICMP 16471* | 100% |
| *Pythium sp.* | REB326-70 | JX122745 | *Pythium vexans* | 99% |
| *Pythium sp.* | REB326-73 | JX122746 | *Pythium vexans* | 100% |
| *Pythium sp.* | REB326-74 | JX122747 | *Pythium vexans* | 99% |
| *Pythium sp.* | REB326-75 | - | *Pythium intermedium* | 97% |
| *Pythium sp.* | REB326-77 | JX122748 | *Pythium senticosum* | 99% |

*Table 3.* Sequences of TaqMan real-time PCR primers and TaqMan probe targeting the ITS gene region, for PTA detection.

| Primer/Probe | Sequence (5′-3′) | 5′ base position in sequence accession JX122751 for PTA |
|---|---|---|
| PTA_ITS_F2 | AACCAATAGTTGGGGGCGA | 41 |
| PTA_ITS_R3 | GACGAGCTCTATCATGGCGAG | 102 |
| PTA_ITS_Probe[1] | GGCGGCTGCTGGCTTTGGCT | 67 |

PTA, *Phytophthora* 'taxon Agathis'.
[1]Labelled with 5′ reporter FAM (6-carboxyfluorescein) and 3′ quencher BHQ1 (Black Hole Quencher 1).

Master Mix (Applied Biosystems). Each 15 µl reaction contained primers at a final concentration of 350 nM and probe at 80 nM and DNA at 1 µl DNA. The reaction conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 61°C for 60 s. Potential false negatives due to PCR inhibition were monitored using an internal positive control from Applied Biosystems (TaqMan Exogenous Internal Positive Control Reagent using VIC probe). Negative controls containing water instead of DNA and positive controls consisting of PTA genomic DNA (REB327-68) were included in all TaqMan real-time PCR runs. Reactions were carried out in triplicate except where otherwise stated.

### 2.6 Assay sensitivity

Assay sensitivity was determined by testing a 10-fold dilution series of PTA DNA (REB327-68) ranging from 2 ng/µl to 0.2 fg/µl. DNA concentrations were determined using a fluorometer. The average amount of DNA in 100 g of soil sample is about 180 ng, averaged from extractions of five soil samples. To investigate the effects of inhibitors carried over in DNA extraction from soil, the same dilution series was tested with the addition of 180 ng DNA extracted from PTA-free soil to each reaction. Four replicates reactions were used to test each dilution.

### 2.7 Oospore detection in artificially inoculated soil

PTA-free soil was collected from Landcare Research property garden and prepared for DNA extraction as described above. A 10-fold dilution series of PTA oospores (Isolate ICMP 18407) was prepared. Mean oospore density was estimated by 10 counts using a haemocytometer. Known numbers of oospores were added to 100 g samples of dry soil, ranging from 40 000 to 400 oospores per sample. Samples were subjected to DNA extraction; unspiked soil from the same source was used as the extraction negative control. DNA was extracted from five replicate samples at each oospore dilution, and each extract was tested by TaqMan real-time PCR in triplicate.

### 2.8 Application of assay to field samples

The TaqMan real-time PCR assay was validated using field soil samples collected from the Kauri forests in the North Island of New Zealand. DNA was extracted from ten soil samples and tested for PTA using the TaqMan real-time PCR assay. Of the ten samples, four samples were previously known to be positive for PTA using the conventional lupin baiting technique, two were soil samples spiked with oospores, used as positive controls, and four were collected from PTA-free sites, used as negative controls.

## 3 Results

### 3.1 TaqMan real-time PCR assay development and specificity

The ITS gene region was selected for having the most variability in the target sequence compared with the non-target sequences. This enabled more options to design the primers and probe. The other gene targets were too similar to the target sequence. Concatenated sequences from the two primers and probe were subjected to BLAST search on the NCBI database. Results show 100% match to PTA, *Phytophthora katsurae* and *Phytophthora novaeguineae.* Neither of the other two *Phytophthora,* however, have been recorded in New Zealand and therefore dismissed as a potential source of cross-reactivity. When tested against all non-target isolates, the ITS TaqMan real-time PCR assay only amplified DNA from the target PTA culture; no amplification was observed for any of the 36 non-target isolates tested (Table 1).

### 3.2 Assay sensitivity

When testing DNA extracted from a culture of PTA, the limit of detection of the assay was approximately 2 fg, as shown in Fig. 2, and the relationship between DNA concentration and Ct value was linear between 2 ng and 2 fg of DNA per reaction ($R^2$ = 0.997) There was a small but statistically significant increase in the limit of detection (a reduction in sensitivity) in the presence of soil DNA extract to 20 fg PTA DNA ($F$ = 4.2 > 2.5 at p = 0.05).

### 3.3 Oospore detection in artificially infected soil

Oospores were successfully detected in artificially spiked soil at various concentrations ranging from 400 oospores to 40,000 oospores (Fig. 3). The relationship between the number of oospores and Ct value was linear with very high efficiency ($R^2$ = 0.9938) Standard errors range between 0.35 and 0.48.

### 3.4 Application of assay to field samples

Results of testing using the TaqMan real-time PCR assay concurred with the results obtained using the conventional lupin baiting bioassay. Observed Ct values for naturally infested soil ranged from 217 to 55 fg. Oospore-spiked soil used as a positive control for this experiment showed detection. No detection, however, was recorded in the negative controls.

## 4 Discussion

A TaqMan real-time PCR-based detection assay has been developed for *Phytophthora* 'taxon Agathis' using TaqMan chemistry, targeting the rDNA ITS region. The specificity and sensitivity of the assay have been shown to be suitable for the detection of PTA in the presence of various *Phytophthora* and *Pythium* species occurring in Kauri forest soils and 26 other non-target species of *Phytophthora.*

The PTA-specific TaqMan assay using the ITS region proved to be very sensitive, detecting as low as 2 fg of PTA DNA extracted from pure culture. The ITS region in a Eumycotan fungi has between 60 and 220 copies per genome (Phytophthora database 2012), and this assay is highly sensitive. In the presence of soil extract, the limit of detection was

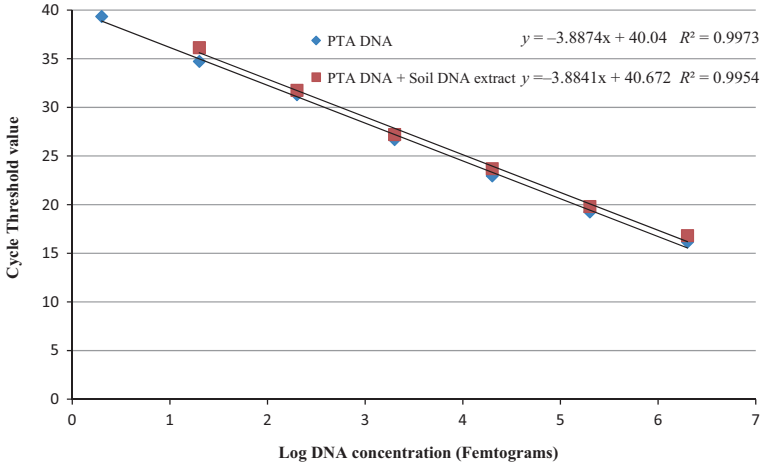


*Fig. 2.* Standard curve showing the linear relationship between DNA concentration and Ct value. PTA DNA dilutions were also tested in the presence of 180 ng DNA extracted from PTA-free soil. Ct values shown are mean values for four replicate reactions. Standard errors were between 0.03 and 0.2.

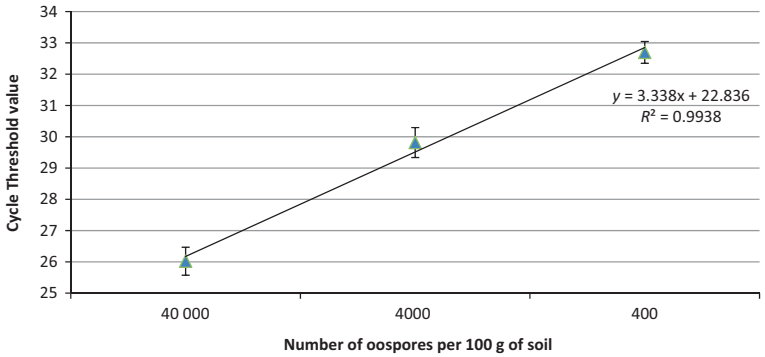


*Fig. 3.* Standard curve showing the linear relationship between the number of oospores in artificially inoculated soil and the Ct value.

observed to be approximately 20 fg PTA, suggesting that soil extract contains inhibitors. DNA extraction is notoriously hard from soil due to the presence of inhibitors (Picard et al. 1992) such as phenolic and humic compounds, which can reduce the PCR efficiency. Nevertheless, sensitivity of the PTA assay is comparable to the detection limits reported for real-time PCR detection of other *Phytophthora* species (Tomlinson et al. 2005; Schena et al. 2008). Initial testing of field samples suggests that the assay, when used in conjunction with the DNA extraction method described, is suitable for detection of PTA at levels found in naturally infested soil. All TaqMan real-time PCR results concurred with the results obtained using the conventional soil baiting bioassay; in contrast to the bioassay, the PCR-based approach will also detect PTA in samples where PTA oospores are dormant or where the viability of the pathogen is compromised.

Consideration should be made of how the newly developed TaqMan real-time PCR assay can be best incorporated into routine testing procedures. We envisage the development of an assay where bait tissues can be transferred directly from flooded soil to the TaqMan real-time-PCR detection assay. This may provide better sensitivity compared with detection directly from bait tissues recovered from flooded soil. This tool also provides insights into frequency of 'false negatives', due to its higher detection sensitivity.

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