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| **Project title:** | Raspberry: Detection and quantification of *Phytophthora rubi* in soil and plant tissue |
| **Project number:** | SF 130 |
| **Project leader:** | Jeff Peters/James Woodhall, Fera |
| **Report:** | Final Report, September 2014 |
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| **Key staff:** | James Woodhall, Fera<br>Kate Perkins, Fera<br>Jeff Peters, Fera |
| **Location of project:** | Fera |
| **Industry representative:** | Tim Place, Place UK Ltd |
| **Date project commenced:** | 1 April 2012 |
| **Date project completed (or expected completion date):** | 5 September 2014 |

## *DISCLAIMER*

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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

## *AUTHENTICATION*

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

James Woodhall
Molecular Plant Pathologist
Fera

Signature ..... Date .....

Christine Henry
Team Leader
Fera

Signature ..... Date .....

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## *GROWER SUMMARY*

### Headline

- A new real-time species specific PCR assay has been designed for *Phytophthora rubi* in plant material and field soils, which is specific for the target pathogen and does not detect *P. fragariae* or other related Phytophthora species.

### Background and expected deliverables

Soil-borne *Phytophthora rubi* (previously known as *Phytophthora fragariae* var. *rubi*) can infect raspberry and cause wilting, leading to the death of otherwise long-lived plants. Other species of *Phytophthora* can also cause root rot in raspberry, but *P. rubi* causes the most common and serious form of rot (Kennedy and Duncan, 1991). Raspberry root infection by *Phytophthora rubi* leads to root rot and cane death. In the absence of effective host resistance, control is focused on cultural practices and agrochemical use. The pathogen is spread either via infected planting material or through contaminated soil. Therefore, ability to rapidly detect pathogen at low levels would be a key component of a management strategy.

It is currently possible to detect *P. rubi* in plant material using conventional and molecular methods. However, there is no direct soil test to quantify the pathogen because existing PCR assays for *P. rubi* also detect other *Phytophthora* species (i.e. *P. fragariae*). Recent HDC-funded research (Project SF 97) developed quantitative polymerase chain reaction (abbreviated to qPCR) tests that enabled the detection and quantification of *V. dahliae* DNA in soil. This project will build on the knowledge gained from SF 97 to develop a method for quantifying DNA levels of *P. rubi* in sample material.

The aim of the current work is to develop a real-time quantitative PCR assay for *P. rubi* to allow the rapid detection in planting material and soil. The outcome would be a rapid specific assay for the detection of *P. rubi* in soil and plant material.

## Summary of the project and main conclusions

### Objective 1 – To develop and validate a new molecular assay for the quantification of *Phytophthora rubi*

Sequences for rDNA ITS, rDNA IGS, *cox* I, *cox* II and the ras-related *Ypt*-1 genes were either directly sequenced or obtained from GenBank for a range of *Phytophthora* isolates. All sequences, with the exception of those originating from *cox* I, were either identical or highly similar, with too few sequence differences present to enable the design of species specific qPCR assays for *P. rubi*. A real-time PCR assay was therefore designed to the *cox* I sequence. Despite relatively few sequence differences being present the assay was highly specific and did not cross react with a range of other *Phytophthora* species. The technical limit of quantification of the assay was 6.6 pg/µl, which is approximately 10 to 100 times less sensitive than assays designed to multi-copy genes.

### Objective 2 – To investigate detection thresholds for *P. rubi* in host tissues and soils

The assay was able to detect *P. rubi* in both artificially (spiked) and naturally infested soil and plant material. In artificially infested material, the assay could detect as little as 4 mg of *P. rubi* hyphae in 50g soil but was not able to detect *P. rubi* in one 50 g soil sample spiked with 1.5 mg of hyphae. For plant material, the assay was able to detect 15 mg of *P. rubi* hyphae in a 2 g sample of raspberry crown and root material. In both cases there was a linear relationship between the weight of spiked hyphae of *P. rubi* and amount of *P. rubi* DNA detected using the assay. The assay successfully detected *P. rubi* in seven from 11 plant samples tested and two from nine soil samples tested, demonstrating detection with naturally infested samples.

### Objective 3 – To promote to growers and breeders the proposed commercial availability of a rapid quantitative DNA assay for *Phytophthora rubi* in soils and plant tissue and to highlight the potential application of soil thresholds for grower selection of planting material

The assay can detect *P. rubi* in plant material and highly infested soil material. The assay will be used in parallel with established conventional testing for *P. rubi* in plant material at Fera over the next 12 months to ensure consistency with existing conventional tests, with the aim of launching a commercial service in 2015 if the validation is successful.

## Financial benefits

With a *P. rubi* specific assay the presence of the pathogen in plant and soil material can be accurately determined. Accurate knowledge of the presence of *P. rubi* will inform decisions about selection of plant material and suitable planting sites. The qPCR assay can also be used reactively to rapidly confirm the presence of *P. rubi* in symptomatic plants in outbreak situations. qPCR could give a result in several days as opposed to isolation which can take several weeks.

## Potential further work

- Sequence data from next generation sequencing work initiated at Fera with Defra funding will be analyzed to identify species specific multi-copy loci that can be used to design more sensitive nested qPCR assays for *P. rubi*.
- The assays developed in this project will be used in parallel with conventional testing for *P. rubi* at Fera for 12 months prior to full launch of a molecular screening service for soils of soft fruit crops. This will be launched with assays for other soft fruit pathogens including *V. dahliae* and *Phytophthora cactorum* for a comprehensive soil screening package.
- Droplet digital PCR approaches will be examined at Fera. This has the potential for much greater levels of sensitivity than qPCR or nested qPCR.
- No knowledge exists on the levels of inoculum of *P. rubi* required for disease to occur (thresholds). Field and/or glasshouse experiments are required to determine this and also to find if it varies with different ages of planting material, soil type, plant variety and environmental conditions.
- Knowledge on how *P. rubi* survives in the soil is also required. Does it survive as hyphae, spore or in organic matter? This knowledge will help determine an accurate unit to be used in the threshold discussed above but also could be used to improve assay sensitivity. For example if the pathogen survives in organic matter, organic matter could be separated from the soil, effectively concentrating the pathogen in the organic matter subsample, enabling higher levels of *P. rubi* DNA to be recovered.

## Action points for growers

- Growers submitting plant material for testing for *Phytophthora* can ask for additional molecular testing for *P. rubi* using the new assay.

- Growers wishing to provide soil material for testing should contact James Woodhall by email (james.woodhall@fera.gsi.gov.uk). Soil samples should consist of at least 500 g. Ideally soil samples should consist of at least 20 sub-samples taken over one hectare.

# *SCIENCE SECTION*

## Introduction

### *Industry situation*

The expected minimum five year life of raspberry and blackberry plantations can be severely shortened when roots become infected by *P. rubi* (previously known as *P. fragariae* var. *rubi*), leading to root rot and cane death. The area of primocane-fruiting raspberries has been increasing in the UK and the varieties grown tend to be susceptible to *P. rubi*. In the absence of effective host resistance control is focused on cultural practices and agrochemical use. The pathogen is spread either via infected planting material or by planting clean canes in contaminated soil. Therefore, the ability to rapidly detect the pathogen in soil would be a key component of a management strategy.

Fumigants such as Basamid (dazomet) and chloropicrin can be used before planting, but there is concern over their future availability. Planting in substrate in pots or troughs can help to avoid wilt and root rot problems, but this remains effective only if the irrigation water is not contaminated by spores. Fungicide application to the substrate of either Shirlan (fluazinam - EAMU 2168/2003) or Paraat (dimethomorph - EAMU 2777/2007) is usually applied against *Phytophthora* in spring before primocane growth - the effect is principally protectant.

The availability of a soil test to detect soil-borne pathogens could stop unnecessary disinfestation in soils where there are no target pathogens present. In addition, soils where *P. rubi* has been detected could be avoided in favour of soils that have no inoculum or are below any thresholds for disease.

### *Recent research*

Real-time, or quantitative, PCR assays (qPCR) for testing soils prior to planting for specific soil-borne *Verticillium* species using DNA extracted from large volumes (up to 1 kg) of soil have been successfully developed (SF 97). The techniques were initially developed during the Potato Council-funded potato diagnostics project (R253) and utilize pre-extraction processing buffers to remove reaction inhibitors and an automated DNA isolation system to isolate total DNA. These tests provide results within a few days (rather than 6-8 weeks for *V. dahliae* microsclerotia). PCR detects DNA in both dead and live cells, but microbial DNA deteriorates quickly in the environment under normal conditions and so fungicide/fumigant killed pathogens would rarely be detected.

Detection of *V. dahliae* using qPCR has been previously achieved down to levels correlating with 0.5 microsclerotia / g soil and the assay is still being improved in order to attain a detection level of <0.5 microsclerotia / g soil. It is already possible to detect below 1 microsclerotia / g soil by testing multiple soil extractions, although this increases the cost of the test. Some observations suggest that achieving low detection rates may not, however, be as important for *P. rubi* in raspberries.

Raspberry growers commonly request soil testing for *Phytophthora* from Fera's Plant Clinic. Currently it is not possible to specifically detect or quantify *P. rubi* in soil. Previously developed assays cannot distinguish between *P. fragariae* and *P. rubi*. Therefore, it was suggested that a new *P. rubi* specific real time PCR assay is developed to detect and quantify *P. rubi* from difficult matrices such as soil.

The aim of the current work was to develop a real-time PCR assay for *P. rubi* to allow the rapid detection in planting material and soil. Intended outcomes were a rapid, cost-effective assay for *P. rubi* detection and thresholds for detection in plant and soil.

## Materials and methods

### Objective 1: To develop and validate a new molecular assay for the quantification of *Phytophthora rubi*

#### *General*

A working collection of 27 isolates representative of *P. rubi, P. fragariae* and closely related *Phytophthora* isolates was obtained from culture collections at Fera, University of California – Riverside (UCR), James Hutton Institute (JHI) and Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre (CBS) - see Table 1. Isolates were grown on carrot piece agar at room temperature in 90 mm petri-dishes. Long term storage of isolates was on 5 mm agar plugs placed in sterile distilled water in 30 ml glass tubes at 4°C. DNA was extracted from mycelia taken from 1 week old cultures macerated using a micro-pestle in a 1.5 ml tube and purified using a Wizard Magnetic DNA Purification System for Food (Promega UK, Southampton) according to the manufacturer's instructions. DNA from cultures was checked to be of suitable quality by undertaking a conventional PCR with ITS4 and ITS5 primers (White *et al*., 1990).

Table 1. Isolate code, species, source, original habitat and origin of isolates used in this study

| Isolate code | Species | Source* | Other codes for isolate | Original habitat | Geographical origin |
|---|---|---|---|---|---|
| 138 | *Phytophthora alni* | CBS | CBS 117375 | Alder | Hungary |
| 93 | *Phytophthora cactorum* | Fera | | *Vaccinium* | UK |
| 108 | *Phytophthora cambivora* | Fera | | Reservoir water | UK |
| 1707 | *Phytophthora cambivora* | JHI | P208 | Not known | UK |
| 1693 | *Phytophthora cambivora* | CBS | CBS 376.61 | *Andromeda floribunda* | Poland |
| 2085 | *Phytophthora cinnamomi* | JHI | CIN5 | Not known | Not known |
| 367 | *Phytophthora cinnamomi* | Fera | | *Chaemacyparis* | UK |
| 1221 | *Phytophthora cinnamoni* | Fera | | Not known | UK |
| 1226 | *Phytophthora cinnamoni* | Fera | | Not known | The Netherlands |
| 133 | *Phytophthora citricola* | Fera | | *Rhododendron* | UK |
| 368 | *Phytophthora cryptogea* | Fera | | *Chaemacyparis* | UK |
| Pcc46 | *Phytophthora europea* | UCR | | Not known | Not known |
| 931 | *Phytophthora fragariae* | Not known | | Strawberry | Not known |
| 947 | *Phytophthora fragariae* | Not known | | Strawberry | Not known |
| CBS 209.46 | *Phytophthora fragariae* | CBS | IMI 181417 | Strawberry | UK |
| CBS 309.62 | *Phytophthora fragariae* | CBS | ATCC 11107 | Strawberry | UK |
| Pcc35 | *Phytophthora megasperma* | UCR | | Not known | Not known |
| 1659 | *Phytophthora ramorum* | Fera | | Peat mixture | UK |
| 1566 | *Phytophthora ramorum* | Fera | | *Rhododendron* | UK |
| 159 | *Phytophthora rubi* | Fera | | Raspberry | UK |
| 1217 | *Phytophthora rubi* | Fera | | Raspberry | Not known |
| 1218 | *Phytophthora rubi* | Fera | | Raspberry | UK |
| 1219 | *Phytophthora rubi* | Fera | | Raspberry | Not known |
| 2106 | *Phytophthora rubi* | Fera | | Raspberry | France |
| CBS 967.95 | *Phytophthora rubi* | CBS | ATCC 90442;IMI 355974 | Raspberry | UK |
| CBS 109892 | *Phytophthora rubi* | CBS | | Raspberry | UK |
| 1699 | *Phytophthora syringae* | CBS | CBS 364.52 | *Prunus armeniaca* | New Zealand |

*CBS= Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, The Netherlands, JHI = James Hutton Institute, Dundee, UK, UCR=University of California, Riverside

### *Sequencing and design of primers*

Sequences were obtained either by DNA sequencing at Fera or from previously deposited sequences on GenBank for *Phytophthora rubi* and closely related species for the following genes: rDNA internal transcribed spacer region (ITS), rDNA intergenic spacer region (IGS), ypt1 (RAS) gene, cytochrome oxidase subunit 1 (*cox* I) gene and cytochrome oxidase subunit II (*cox* II) gene.

For each gene, sequences were aligned using ClustalW (Larkin et al., 2007). Species specific sequence regions were identified and Primer Express 2.0 software (Life Technologies, Warrington, UK) was used to design specific TaqMan® primers and probes for qPCR. A standard real-time qPCR assay and also a single-tube nested real-time qPCR assay were designed to the *cox* I gene.

### *qPCR*

qPCR was carried out in 96 plates using the ABI Prism 7500HT Sequence Detector System (Applied Biosystems). Environmental Master Mix 2.0 (Life Technologies) was used for all samples. Primers and probes (MWG Biotech, Germany) were added to a final concentration of 300 nM and 100 nM respectively. DNA samples consisted of 2 µl for plant and fungal material or 5 µl for soil material. The remaining volume was made up to a final volume of 25 µl with molecular biology grade water. Template DNA (5 µl) was spiked into each reaction. Cycling conditions for standard qPCR consisted of 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. For single tube nested PCR, reaction conditions were 50°C for 2 min, 95°C for 5 min, and 10 cycles of 95°C for 15 s and 65°C for 30 s and 40 cycles of 95°C for 15 s and 54°C for 20 and 72°C for 30 s. Two (technical) replicate reactions were used for fungal DNA originating from pure culture. With DNA originating from soil and plant samples, three technical replicates were undertaken for each DNA sample.

## Objective 2: To investigate detection thresholds for *P. rubi* in host tissues and soils

### *DNA extraction from plant and soil material*

Hyphae were harvested from fully colonized agar plates of *Phytophthora rubi* (isolate 1218) and blotted dry with Miracloth (Millipore) and paper towels. The hyphae were then macerated using a scalpel and hyphae of various weights between 1.5 mg to 290 mg were used to spike 13 plant samples. Plant samples consisting of plant material from the crown and roots were excised from the plant using a scalpel. For each plant used in the experiment unspiked material was also extracted from each plant to ensure that all samples used were healthy and free from *P. rubi*. For soil *P. rubi* hyphae material of various weights between 1.5 mg and 635 mg was used to spike 23 soil samples. One additional soil sample was extracted without any hyphae added as a negative control.

Plant material was extracted by placing samples in Homex bags which were frozen in liquid nitrogen then ground to a fine powder using a rubber mallet. CTAB buffer (3 ml) was added to each grinding bag and ground to a paste-like consistency using the Homex flatbed

grinder. Once the plant material had settled (approx. 5 minutes), as much of the supernatant as possible was transferred into a labeled 2 ml tube and centrifuged at 10,000 g for 5 minutes. Cleared lysate (700 µl) was transferred to fresh 2 ml tubes containing 200 µl of chloroform and mixed by vortexing. The samples were centrifuged for 5 minutes at 13,000g. The top 500 µl of the aqueous layer was transferred into the first well of the Kingfisher tube set also containing 500 µL isoproponol plus 50 µL magnetic beads. DNA was extracted using the Wizard® Magnetic DNA Purification System for Food (Promega) in conjunction with a Kingfisher ML magnetic particle processor (Thermo Electron Corporation). The extractions were completed using the gDNA program including the optional heating stage on the Kingfisher ML. Samples were eluted into 200 µL Tris-EDTA (pH8) buffer and stored at –30°C until required.

The Fera standard soil extraction method was used throughout, using a CTAB buffer plus a Kingfisher ML DNA purification step as described in Woodhall et al. (2012). A sample size of 50 g was used for all soil extractions. To test whether the assay was effective on actual samples nine raspberry soils and 11 plant samples were tested to determine levels of *P. rubi* using the standard *cox* I qPCR assay developed in this project.

## Results

### Objective 1: To develop and validate a new molecular assay for the quantification of *Phytophthora rubi*

### *Sequencing and selection of suitable sequence for primer design*

DNA sequences for a range of *Phytophthora* isolates were successfully obtained for rDNA ITS, rDNA IGS, *cox* I, *cox* II and the ras-related *Ypt*-1 gene. Neighbour-joining trees were constructed to verify the discrimination between the species for rDNA ITS (Figure 1), rDNA IGS (Figure 2), *cox* I (Figure 3), *cox* II (Figure 4) and the ras-related *Ypt*-1 gene (Figure 5). Only *cox* I was able to resolve the two species (Figure 3) and this was the gene used originally to propose that *P. rubi* and *P. fragariae* were distinct species (t' Veld, 2007). The other neighbour joining trees show the sequences were unable to differentiate between *P. rubi* and *P. fragariae* or in the case of the rDNA IGS region between *P. rubi* and *P. cambivora*.

Sequence differences in the five loci indicated that the *cox* I gene would be most suitable for *P. rubi* specific primer design. The *cox* I gene was 680 base pairs long, a comparison of the sequence for *P. fragariae* and *P. rubi* indicated that there were 13 bp differences which could be suitable for species specific primer design. The remaining loci appeared to be

more similar between *P. rubi* and *P. fragaraie* and contained no suitable primer/probes sites.

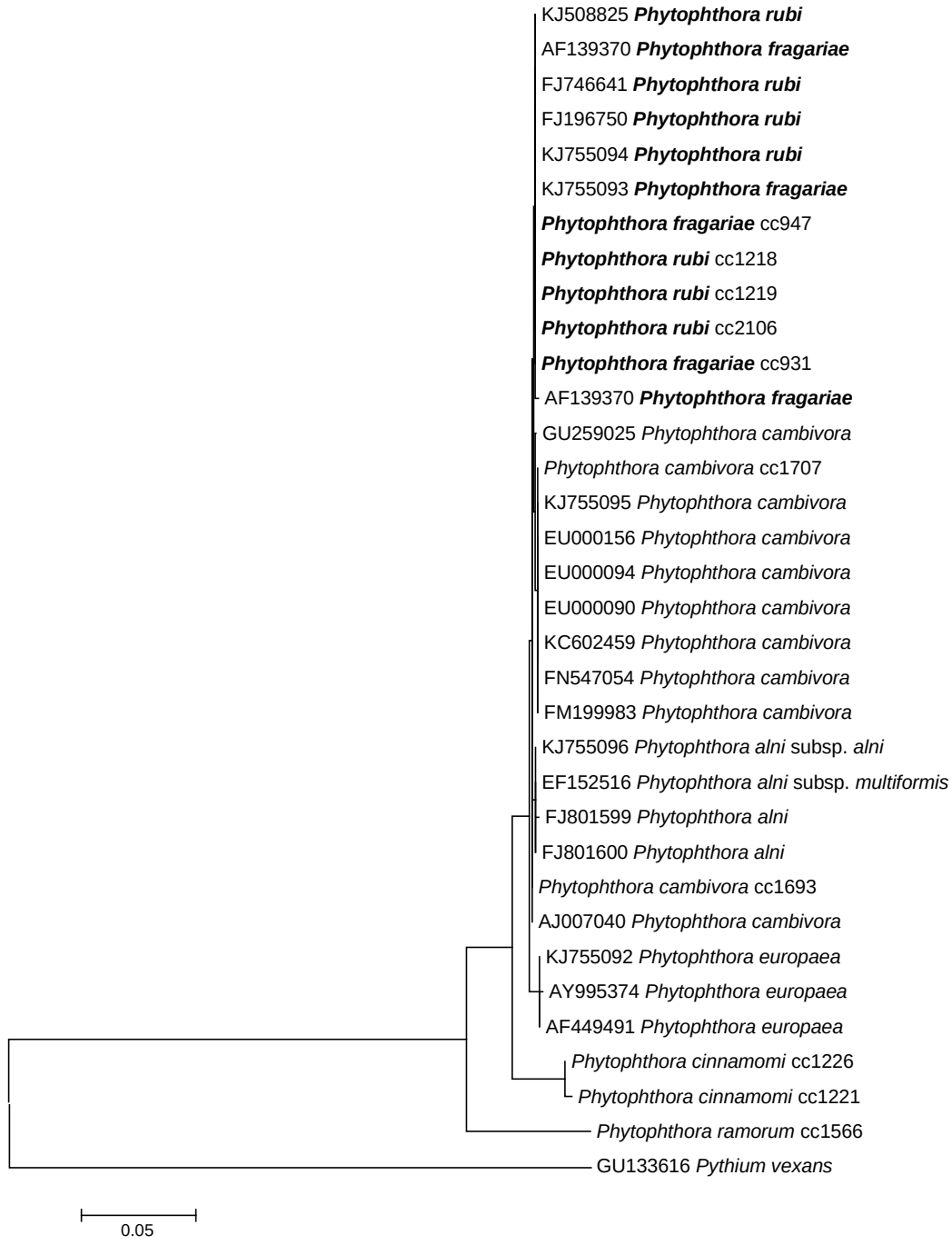


Figure 1. A neighbour-joining tree of the ITS sequences of *Phytophthora* species closely related to *P. rubi. Pythium vexans* was used as an out group. Bar indicates two base changes per 100 nucleotide positions. Isolates of *P. rubi* and *P. fragariae* are in bold.

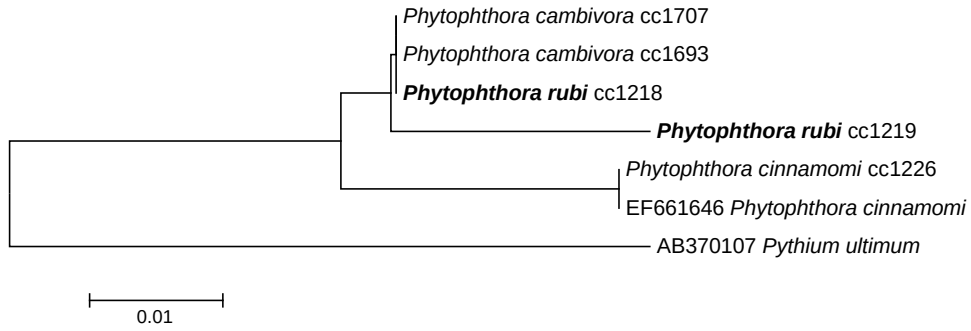


Figure 2. A neighbour-joining tree of the rDNA IGS1 sequence of *Phytophthora* species closely related to *P. rubi. Pythium ultimum* was used as an out group. Bar indicates two base changes per 100 nucleotide positions. *P. rubi* isolates are in bold.

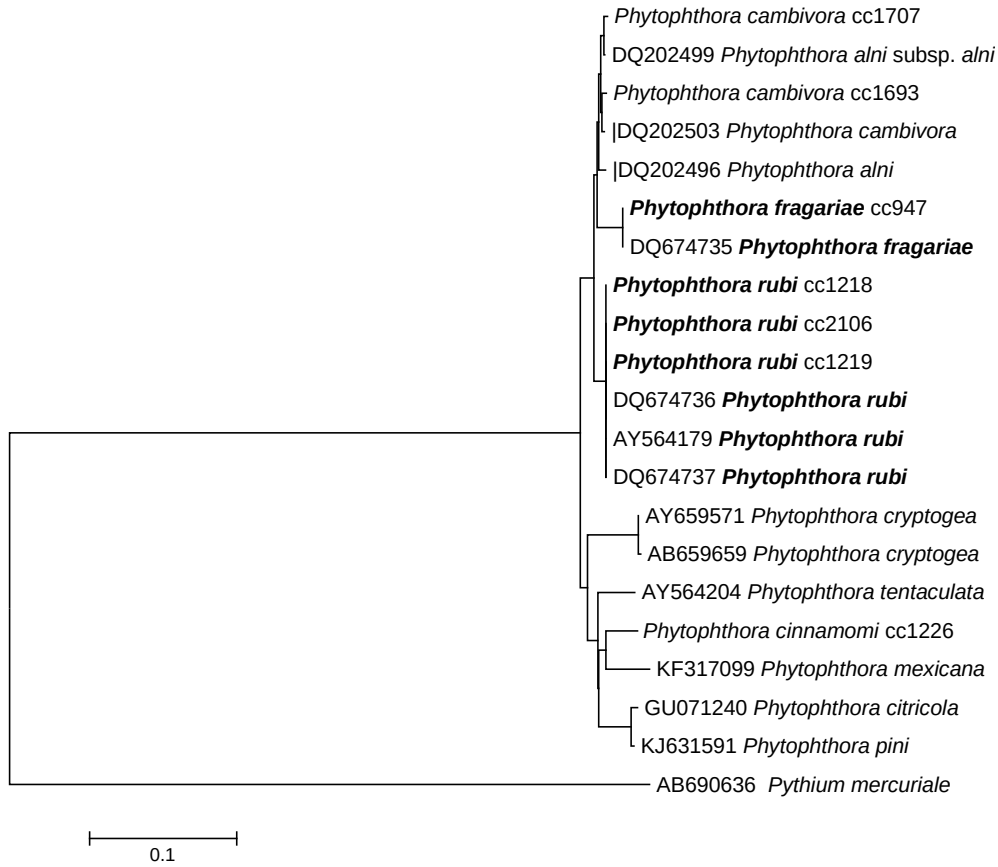


Figure 3. A neighbour-joining tree of the *cox* I sequences of *Phytophthora* species closely related to *P. rubi. Pythium mercuriala* was used as an out group. Bar indicates two base changes per 100 nucleotide positions. Isolates of *P. rubi* and *P. fragariae* are in bold.

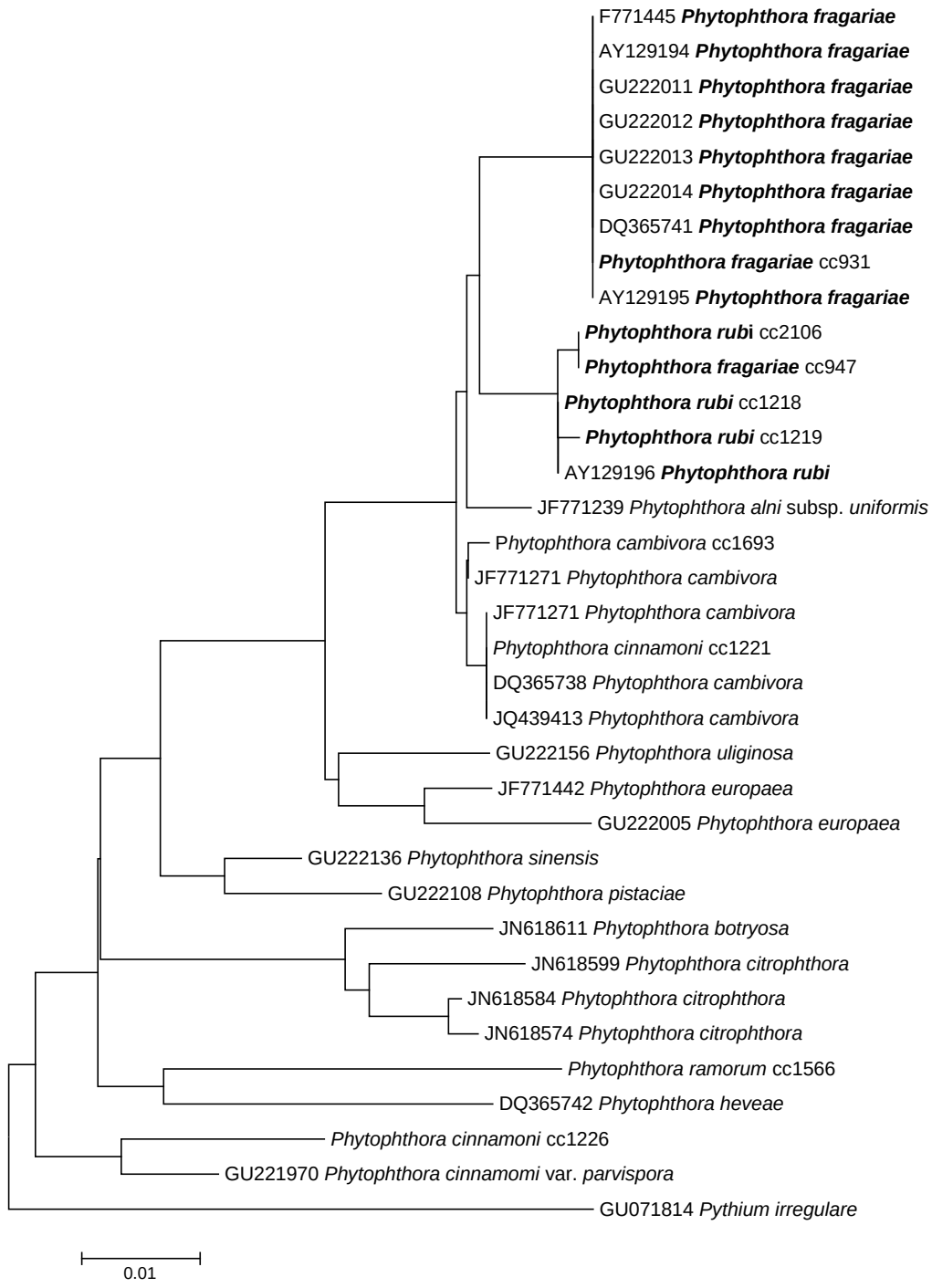


Figure 4. A neighbour-joining tree of the *cox* II sequence of sequence of *Phytophthora* species closely related to *P. rubi. Pythium irregulare* was used as an out group. Bar indicates two base changes per 100 nucleotide positions. Isolates of *P. rubi* and *P. fragariae* are in bold.

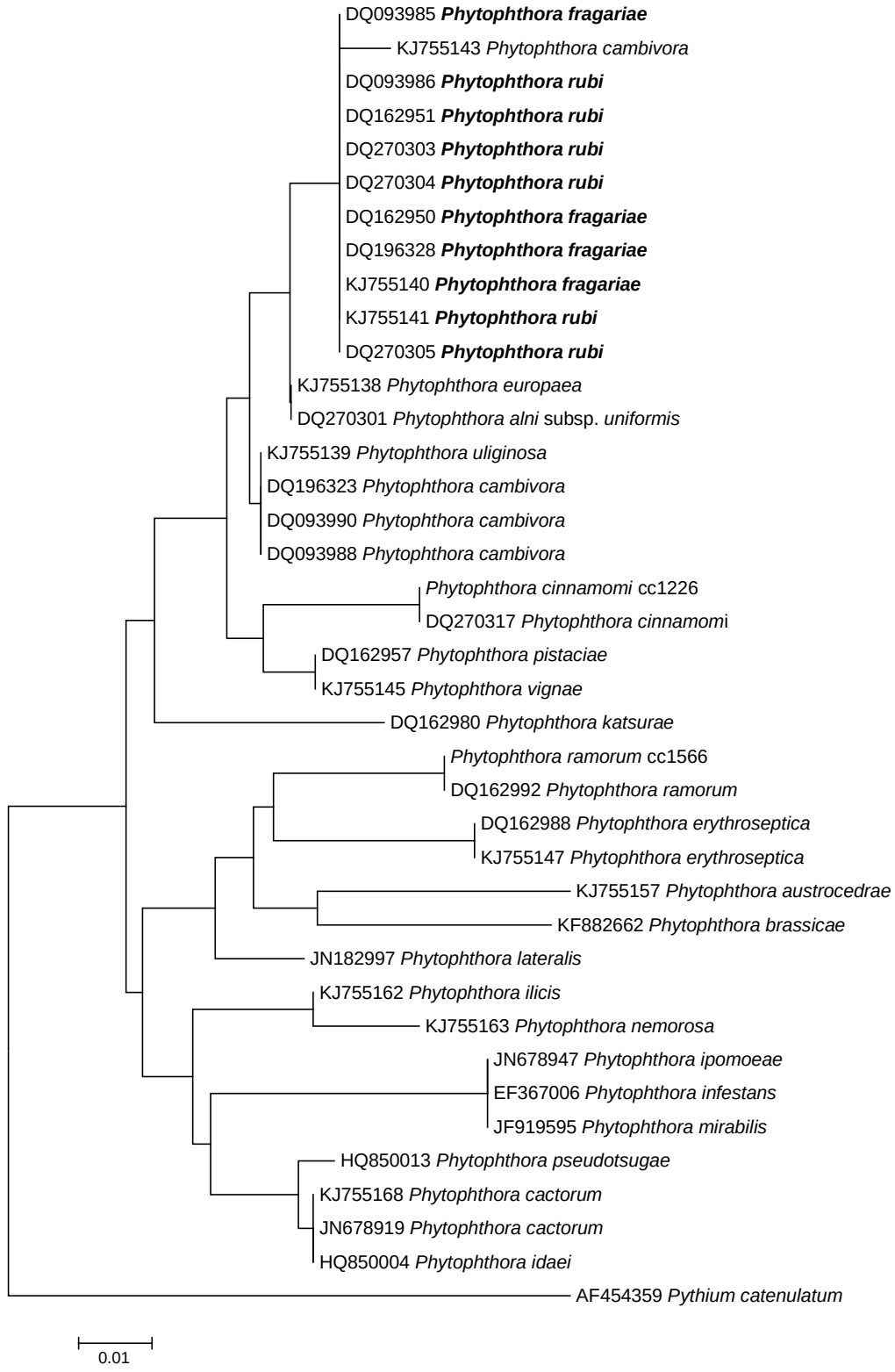


Figure 5. A neighbour-joining tree of the *ypt1* sequence of *Phytophthora* species closely related to *P. rubi. Pythium catenulatum* was used as an out group. Bar indicates two base changes per 100 nucleotide positions. Isolates of *P. rubi* and *P. fragariae* are in bold.

### *Primer design*

Potential areas for primer design were identified in the *cox* I gene. Two assays, a standard real-time PCR assay and a nested real-time PCR with putative specificity to *P. rubi,* were designed, although a common probe was used for both (Table 2). Specificity was built into the primers and, due to few sequence differences in the primers, synthetic DNA base pairs known as locked nucleic acid (LNA) bases were used to enhance specificity.

Table 2. Details of sequences for all *Phytophthora rubi* primers and probes used in this study.

| Primer/Probe | Sequence[1] |
|---|---|
| Standard Forward | TTTATTATTAACTTTACCCGTATTGGCA |
| Standard Reverse | CCCCCGGAAGGATCATAAA |
| Nested Inner Forward | TTATTAACTTTACCCGTATTGGCA |
| Nested Inner Reverse | CCCCCGGAAGGATCATA |
| Nested Outer Forward | TTTATTTGTTTGGTCTGTATTAATTACAGCATTTC |
| Nested Outer Reverse | AAAACCAAAATAAATGTTGATATAGTACAGGATCC |
| FAM-MGB Probe (both assays) | AATGTTATTAACTGATAGAAATTTAAATAC |

[1]Underlined base denotes locked nucleic acid (LNA)

Initial testing on a subset of isolates showed that the standard qPCR assay was specific for *P. rubi* (Table 3). The assay was therefore tested against all isolates in Table 1 and no amplification was observed with DNA from isolates of other *Phytophthora* species. The nested qPCR assay was 10 to 100 times more sensitive than the standard *P. rubi* assay based on differences in Ct value. However, this assay was not specific, as similar Cts were observed with DNA originating from other *Phytophthora* species, including *P. fragariae*. The standard *P. rubi* assay had a technical sensitivity limit of detection of 6.6 ng/µl and a reaction efficiency of 92%. Recommended reaction efficiency for qPCR assays is between 90 and 110%.

Table 3. Ct values observed with standard qPCR and nested qPCR assays

| Isolate | Species | Standard qPCR assay (Ct value) | Nested qPCR assay (Ct value) |
|---|---|---|---|
| 1217 | *Phytophthora rubi* | 28.3 | 19.8 |
| 2106 | *Phytophthora rubi* | 31.5 | 27.2 |
| 931 | *Phytophthora fragariae* | No Ct value | 31.0 |
| 138 | *Phytophthora alni* | No Ct value | 23.5 |
| 1699 | *Phytophthora syringae* | No Ct value | 33.3 |
| 133 | *Phytophthora citricola* | No Ct value | 31.8 |
| 2085 | *Phytophthora cinnamomi* | No Ct value | 24.3 |
| 108 | *Phytophthora cambivora* | No Ct value | No Ct value |
| Pcc35 | *Phytophthora megasperma* | No Ct value | No Ct value |

**Objective 2: To investigate detection thresholds for *P. rubi* in host tissues and soils**

The standard qPCR assay was able to detect *P. rubi* in both spiked and naturally infested soil and plant samples. In artificially spiked material, the assay could detect as little as 4 mg of *P. rubi* hyphae in soil but was not able to detect *P. rubi* in one soil sample spiked with 1.5 mg of hyphae. This was similar with spiked plant material; no detection was possible at the lowest spiked level of 1.5 mg in 2 g plant material. However, for both soil (Figure 6) and plant material (Figure 7) a linear relationship was observed between the weight of spiked *P. rubi* material and the level of *P. rubi* (DNA) detected using the assay. The assay successfully detected *P. rubi* in seven from 11 plant samples tested and two from nine soil samples tested, demonstrating that detection with naturally infested samples is possible.

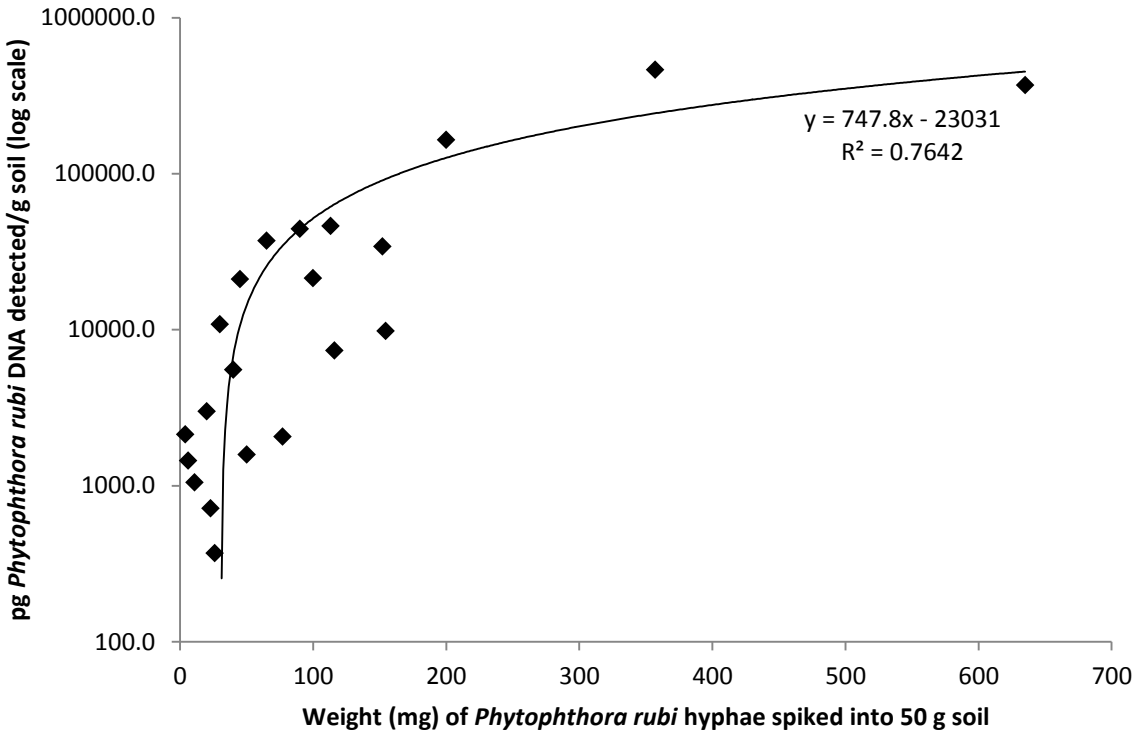


Figure 6. Relationship between *Phytophthora rubi* DNA detected (as pg DNA per g soil) and weight of *P. rubi* hyphae spiked into the 50 g soil sample.

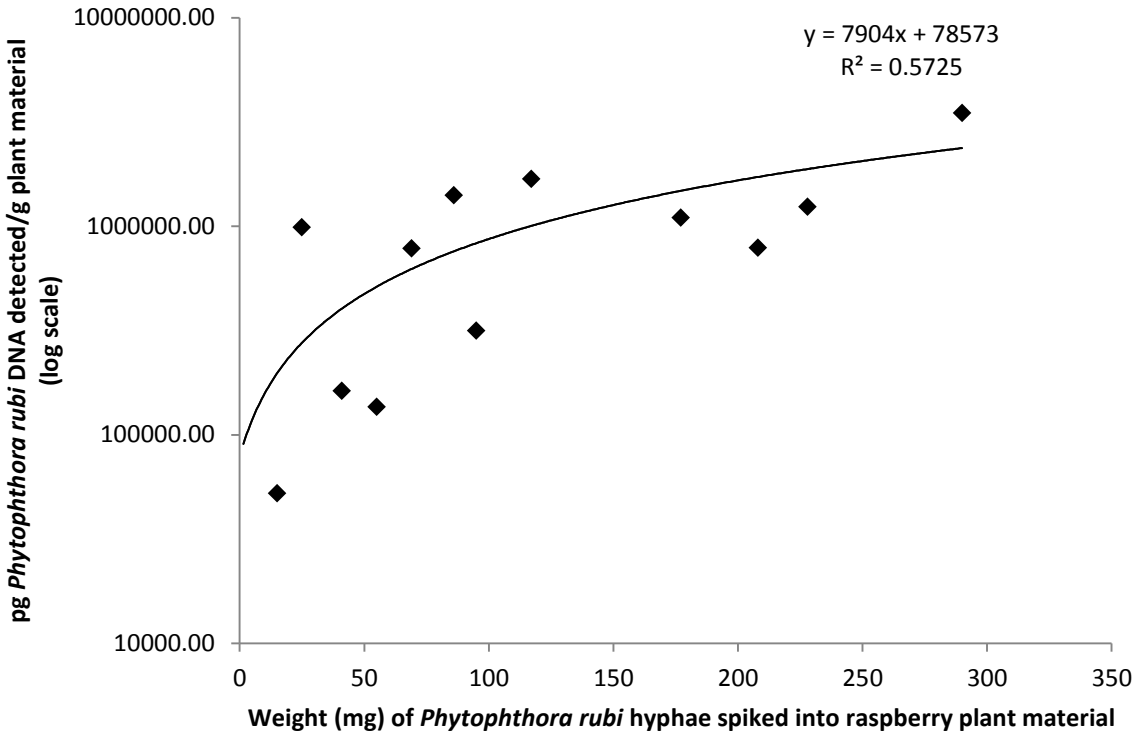


Figure 7. Relationship between *Phytophthora rubi* DNA detected (as pg DNA/g plant material) and weight of *P. rubi* hyphae spiked into the 2 g plant samples.

## Discussion

### Objective 1: To develop and validate a new molecular assay for the quantification of *Phytophthora rubi*

The sequences of all loci analysed showed that *Phytophthora rubi* and *P. fragariae* are closely related and few differences exist between the two species. The *cox* I gene was identified as the best locus for developing a species-specific assay, although this gene still had relatively few sequence differences compared to genes used for other qPCR assays. From *cox* I, a standard qPCR (TaqMan) assay was designed. This assay was specific for *P. rubi* and could detect *P. rubi* in a range of spiked and naturally infested soils samples. A single tube nested qPCR assay was also designed and, whilst this significantly boosted sensitivity, this also detected a range of related *Phytophthora* species and was therefore unsuitable for use. In this instance specificity was lost with single tube nested PCR, as the target was a highly conserved gene with few sequence differences between the target and other species. Using a single tube nested PCR with unique gene sequences, with no homologs present in related non-target species, could be one approach to vastly increase the sensitivity of assays.

Another approach is droplet digital PCR (ddPCR), which works by partitioning a single qPCR mixture (with probes) into water-in-oil droplets numbering up to 20,000. The florescence signal intensity is monitored for each droplet and each droplet is assigned as positive or negative and the absolute quantity of target DNA molecules is then determined using Poisson statistics (Kim et al., 2014). There are three key advantages of ddPCR over qPCR:

- The technology does not require the preparation of a standard curve (or reference DNA) for quantification (Hindson et al. 2011).
- Greater tolerance to PCR inhibitors than qPCR (Morisset et al. 2013).
- Increases in sensitivity. Kim et al. (2014) demonstrated a ten-fold increase in sensitivity for the detection of soil bacteria compared to standard qPCR.

However, ddPCR does require specific ddPCR equipment, more expensive reagents and throughput is ten-times lower than standard qPCR.

**Objective 2: To investigate detection thresholds for *P. rubi* in host tissues and soils**

The species specific qPCR assay was able to detect the pathogen in a range of artificially and naturally infested soil and plant material. It was encouraging to see the assay successfully detect the pathogen in several naturally infested plant and soil samples. In this study, the assay was unable to detect the lowest level spiked into soil, which was 1.5 mg of *P. rubi* hyphae in 50 g soil. This may not be of relevance since the threshold of *P. rubi* required to cause disease is considered to be relatively high (Jeff Peters, Personal Communication). Further work is needed to determine the levels of *P. rubi* required to cause disease under a range of different environmental conditions with plants of different ages and variety.

Although the assay is less sensitive than other assays for soil-borne plant pathogens, considering the strict limitations placed upon assay design by only being able to use a highly conserved gene, the sensitivity compares relatively favourably. For example, the detection limit is only twenty times less sensitive than a recent assay developed for *Rhizoctonia solani* AG3-PT based on multi-copy rDNA ITS sequences which can detect 0.2 mg of fungal material (as sclerotia) spiked into a soil sample (Woodhall et al., 2013).

It is expected that next generation sequencing (NGS) approaches will generate new sequence data which will enable a more efficient assay to be designed. Recently draft genome sequences have been published on GenbBank. Once these sequences are finalised they could be used to identify a novel sequence only present in *P. rubi.* This will remove the limitations imposed by designing from highly conserved sequences. A single tube nested assay could be designed to such a novel sequence. NGS sequencing could therefore enable a highly sensitive and specific assay to be designed. Once finalised genome sequences are available, nested qPCR assays will be designed at Fera for a range of *Phytophthora* species associated with soft fruit and validated with the material generated in this project.

Another approach to lower the threshold for detection could be combining a standard species-specific assay with a baiting step. Test soil could be planted with bait plants from which, after a period of incubation, DNA could be extracted and tested with the species-specific assay developed in this project. The bait plants would stimulate growth of *P. rubi* present in the soil and viable propagules of *P. rubi* would colonise the bait plants. Baiting therefore, through a form of biological amplification, can increase the

sensitivity of the assay. However, a disadvantage to a baiting approach is that it considerably increases the time taken to undertake the assay by up to several weeks. However, the detection of exclusively viable material is often seen as an advantage in some instances. Another barrier to using baiting would be obtaining a reliable continued supply of healthy, *Phytophthora*-free, raspberry plants.

**Objective 3 – To promote to growers and breeders the proposed commercial availability of a rapid quantitative DNA assay for *Phytophthora rubi* in soils and plant tissue and to highlight the potential application of soil thresholds for grower selection of planting material**

The Plant Clinic staff at Fera have been trained in the DNA extraction methods and interpreting the results of the assay. The assay will be used in parallel with the existing conventional test for *P. rubi* for a period of 12 months on a range of samples received by the Fera Plant Clinic. If this validation is successful it is envisaged that a soft fruit soil molecular screening package will be launched to test the soil for key *Phytopthora* species (*P. rubi, P. fragariae, P. cactorum*), *V. dahliae* as well as for key nematode species.

## References


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