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Project leader:	Jeff Peters, Fera
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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.

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GROWER SUMMARY

Headline

- A novel real-time PCR assay has been designed for *Phytophthora rubi.*

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, 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 by planting clean canes in contaminated soil. Therefore, being able 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 real-time PCR assays for *P. rubi* cross-react with other *Phytophthora* spp (i.e. *P. fragariae*). Specific conventional PCR assays for *P. rubi* have been developed by JHI/SASA for use on plant material but are not suitable for testing soils. Recent HDC-funded research (SF 97) developed quantitative polymerase chain reaction (abbreviated to QPCR) tests that enabled the detection and quantification of *V. dahliae* and *V. albo-atrum* DNA in soil. The proposed project aims to 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 QPCR primer set for *P. rubi* to allow the rapid detection in planting material and soil. Outcomes would be a quick and inexpensive assay for *P. rubi* detection and an initial indication of how soil DNA levels affect disease development in the main raspberry varieties.

Summary of the project and main conclusions

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

A molecular, real-time PCR, test has been developed for *Phytophthora rubi*. The sequencing work required to develop the PCR test showed that *P. rubi* and *P. fragariae* are closely related and few sequence differences exist between the two species. One gene, the Cox1 gene, shows promise as a target for discriminating between the two species. Two assays have been designed, one for *P. fragariae* and another for *P. rubi.* Initial results suggest specificity is possible. However, with few differences in the DNA sequences, the first attempt at an assay has produced assays of relatively low sensitivity which would not be suitable for use for soil diagnostics. Further primer sites within the Cox1 gene will be determined along with a nested real-time PCR approach which should increase both specificity and sensitivity.

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

The research is still on-going. So far, infected plant tissue is detected at higher levels than soils. Work is ongoing to increase the detection of *P. rubi* in soil.

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 promotion of the new real-time PCR to *P. rubi* will be done during the second year of the project.

Financial benefits

It is not possible to estimate the potential financial benefits from this work until the project is completed. Benefits may arise from:

a) Reduced losses from *P. rubi*;

b) Increased accuracy in the identification of *P rubi* present in soft fruit soils;

c) Development of a rapid pre-plant soil test for *P. rubi*:

Action points for growers

- A new molecular test has been developed that discriminates between *P. rubi* and *P. fragariae* thus suggesting that a novel diagnostic assay will be available for detecting soil-borne *P. rubi*.
- Further validation work is required in year 2 to ensure that the test is suitable for commercial testing.

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, being able to rapidly detect pathogen at low levels would be a key component of a management strategy.

Fumigants such as Basamid 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) SOLA 2168/2003, or Paraat (dimethomorph) SOLA 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 soil disinfestation in soils where there are no target pathogens. In addition, soils where *P. rubi* has been detected could be avoided in favour of soils that have no inoculum or are below any established thresholds.

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 binding system to capture 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 microbe DNA deteriorates quickly in normal conditions and so fungicide/fumigant killed pathogens would rarely be detected.

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

Raspberry growers commonly request soil testing for *Phytophthora* from FERA's Plant Clinic. Currently, it is not possible to detect or quantify *P. rubi* in soil. Although detecting *Phytophthora fragariae* is possible for strawberry growers, this relies on having a supply of clean strawberry bait plants. As a *P. rubi*-specific PCR test has not yet been developed, it is not possible to be certain that raspberry cane bait material is free from pathogen. Existing end-point and real-time primers developed by JHI and FERA, respectively cannot distinguish *P. fragariae* and *P. rubi*. Therefore, it was proposed that a new real time assay is developed by FERA as part of this project to specifically detect and quantify *P. rubi* from difficult matrices such as soil.

The aim of the current work is to develop a QPCR primer set for *P. rubi* to allow the rapid detection in planting material and soil. Outcomes would be a quick and inexpensive assay for *P. rubi* detection and an initial indication of how soil DNA levels affect disease development in the main raspberry varieties.

Materials and methods

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

General

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 1.5ml 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).

Design of primers

Sequences were obtained from GenBank for *Phytophthora rubi* and closely related species for the following genes:

- rDNA internal transcribed spacer region
- ypt1 (RAS) gene
- Elongation factor 1 gene
- NADPH gene
- cytochrome oxidase subunit 1 (COX1) gene

For each gene, sequences were aligned using ClustalW (Larkin et al., 2007) in Mega5 (Tamura et al., 2011). Species specific sequence regions were identified and Primer Express 2.0 software (Applied Biosystems, Warrington, UK) was used to design specific TaqMan® primers probes.

Real-time PCR

Real-time PCR (TaqMan®) was carried out in 96 plates using the ABI Prism7500HT Sequence Detector System (Applied Biosystems). Environmental Master Mix 2.0 (Applied Biosystems) was used for all samples. Primers and probes (MWG Biotech, Germany) were added to a final concentration of 300 nM and 100 nM respectively. The remaining volume was made up to 20 µl with molecular biology grade water. 5 µl of template DNA was spiked into each reaction. Cycling conditions 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. Two replicate reactions were done for each sample.

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

Eight raspberry farms across the UK with histories of Phytophthora root rot were visited in May 2012. Crops were from a range of varieties and could be tunnel or open field. Two plantations were visited at each farm. At each location a stool, including the roots and lower part of the canes but not showing any foliar effects of root rot, was sampled adjacent to a stool showing obvious wilting. The wilted stool was then also dug up and collected. One kg of soil was taken from around the root zone of each stool and bagged separately (a total of 32 samples) in order to provide a number of samples with potentially higher and lower soil infestation levels of *Phytophthora*. Plant tissue samples were tested in the laboratory, while fresh, for the presence of *Phytophthora* spp. using a lateral flow device, and tissue was also examined for the presence of oospores. Plant material confirmed as *Phytophthora* spp. was

taken for molecular diagnosis to species. Cold stored soil samples taken from around plants that have been identified as having *Phytophthora rubi* are to be utilised in the molecular quantification of this species in each sample.

To date, 11 raspberry tissue showing symptoms of root rot and eight soils taken from around suspected infected plant roots were tested to determine levels of *P. rubi*. Plant material was extracted using the Fera material method for extracting *Phytophthora* plant method (based on CTAB/GITC buffer chemistry). Samples in Homex bags were frozen in liquid nitrogen then ground to a fine powder using a rubber mallet. Three ml of CTAB buffer 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 centrifuge tube and centrifuged at 10,000g for 5 minutes. Seven hundred μl of the cleared lysate was transferred to fresh 2 ml tubes containing 200μl of chloroform and mixed by vortexing until the mixture turned to a milky liquid. The samples were centrifuged for 5 minutes at 13,000g.

The top 500μl of the aqueous layer was transferred into the first well of the Kingfisher tube set. The aqueous supernatant (cleared lysate) from each sample was transferred into the first well of the Kingfisher tube set containing 500 uL isoproponol plus 50 uL magnetic beads. DNA was extracted using the Wizard® Magnetic DNA Purification System for Food (Promega, FF3750) 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 uL TE buffer and stored at –30°C until required.

A bespoke soil extraction method using CTAB buffer plus a Kingfisher ML DNA purification step was used (Budge et al, 2009). Replicated samples of 50g soils were used throughout.

Real-time PCR quantification

Extracts (typically 1–5 ng uL-1) were stored at -20°C until required. All real-time PCR assays were set up as duplicate 25 uL reactions using Brilliant QPCR Master Mix (Stratagene, Cat. No. 929549) following manufacturer's protocols. All sets of primers were used at 300 nM and probes at 100 nM. TaqMan® PCR reactions were carried out within the ABI Prism7900HTSequence Detector System (PE Biosystems) beginning with 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°Cfor 15 s and 60°Cfor 1 min.

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 real-time PCR assay for *P. rubi* will be promoted to growers during year 2.

Results

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

Primer design

Sequences differences in the five genes indicated that the COX1 gene would be most suitable for *P. rubi* specific primer design. A neighbour joining tree was constructed to verify the discrimination between the species (Figure 1). This 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. This gene was the gene used originally to propose *P. rubi* and *P. fragariae* were distinct species (t Veld, 2007).

The remaining genes appeared to be more similar between the two species. The ypt1 gene only differing in 1 bp in 459 bp between the two species, rDNA ITS differed 2 in 830 bp, EF1 differed in 8 from 930 bp and NADPH, 10 bp difference in a 760 bp gene.

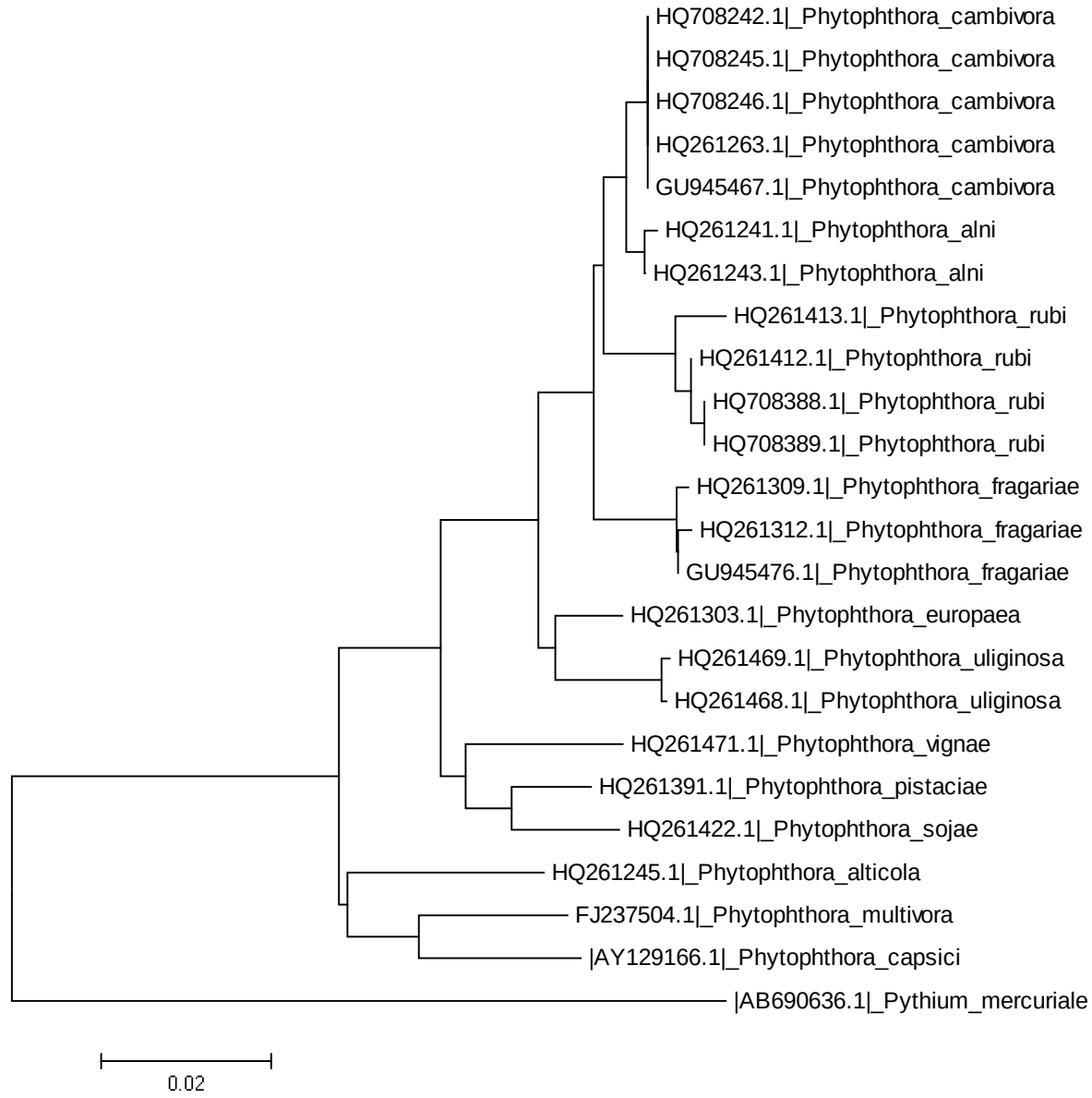

Figure 1. A neighbour-joining tree of *Phytophthora* species closely related to *P. rubi. Pythium mercuriala* was used as an out group. Bar indicates two base changes per 100 nucleotide positions.

By visually assessing the sequence, potential areas for primer design were identified in the COX1 gene. A 28 bp forward primer site was identified, where in the last four base pair two base pair differences were observed between *P. rubi* consensus sequence and *P. fragariae* (Table 1). A FAM-MGB probe common for both the *P. fragariae* assays and *P. rubi* was also designed (5'-AATGTTATTAACTGATAGAAATTTAAATAC-3'). Table 2 shows the corresponding reverse primers for *P. rubi* and *P. fragariae.*

Table 1. Forward primer sequences and complementary sequence for *P. rubi* and *P. fragariae* used in the study

***P. rubi* forward primer**	**TTTATTATTAACTTTACCCGTATTGGCA**
***P. rubi* consensus sequence**	**ATTACAGCATTTCTTTTATTATTAACTTTACCCGTATTGGCAGG**
***P. fragariae* consensus sequence**	**ATTACAGCATTTCTTTTATTACTAACTTTACCGGTATTAGCTGG**
***P. fragariae* forward primer**	**ACAGCATTTCTTTTATTACTAACTTTACCGG**

Underlined bases denote where LNA have been incorporated into the primer. Highlighted bases show bases different between *P. rubi* and *P. fragariae*.

Table 2. Reverse primer sequences and complementary sequence for *P. rubi* and *P. fragariae* used in the study

***P. rubi* reverse primer**	**CCCCCGGAAGGATCATAAA**
***P. rubi* consensus sequence**	**GAGGATCCCCCCCCCCGGAAGGATCATAAAAAG**
***P. fragariae* consensus sequence**	**GAGGATCTCCCCCACCAGAAGGATCATAAAAAG**
***P. fragariae* reverse primer**	**CCCCCACCAGAAGGATCATAA**

Underlined bases denote where LNA have been incorporated into the primer. Highlighted bases show bases different between *P. rubi* and *P. fragariae*.

The primers and probes were ordered from MWG biotech and used in a real-time PCR with *P. rubi* (isolates 1219, 947 and 1218) and *P. fragariae* DNA (931). DNA from other *Phytophthora* isolates were also included (*P. cambivora, P. ramorum, P. cinnamomi* and *P.*

lateralis). Both assays appeared specific, no Cts were observed in wells where non-target DNA was present. The average Ct observed for the *P. fragariae* assay set with the *P. fragariae* DNA sample was 23.78. Ct was significantly higher for the *P. rubi* samples (38). For both assay the delta Rn values were also relatively low.

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

To date, 11 plant samples were tested using the existing Fera *P. fragaeiae* (not published) and the new *P. rubi* assay developed in this project. The Fera *P. fragariae* assay detected nine out of 11 positive raspberry plant material extracts (Table 3). The plant tissues are likely to have been affected by *Phytophthora* species. These were assumed to be *P. rubi* but it is possible that another species of Phytophthora was present that reacts with the *P. fragaeiae* test. In contrast, the new *P. rubi* assay detected seven out of 11 positive raspberry samples. The *P. fragaeiae* assay reacted with the *P. fragaeiae* and *P. rubi* controls. Therefore, the *P. fragaeiae* assay cross-reacts with both pathogens. The *P. rubi* assay reacted only with the *P. rubi* control (the *P. fragaeiae* assay was negative).

Table 3. Putative *Phytophthora rubi* samples collected from raspberry crops.

		sample	***Plant***	***Plant (frag)***	***Plant (new)***
	1	21211050	11050	+ve	+ve
	2	21211224	11224	+ve	-ve
	3	21211226	11226	+ve	+ve
	4	21213088	13088	+ve	+ve
	5	21209466	9466	+ve	-ve
	6	21209467	9467 root	+ve	+ve
	7	21209703	9703	+ve	+ve
	8	21209704	9704	-ve	-ve
	9	21209719	9719	-ve	-ve
	10	21209720	9720	+ve	+ve
	11	21209424	9424	+ve	+ve
	12	21209423	-	na	na
	13	21211227	-	na	na
	14	21211225	-	na	na
control		*P. fragariae* +ve		+ve	-ve
control		*P. rubi* +ve		+ve	+ve

Table 4. Putative *Phytophthora rubi* samples collected from raspberry soils.

	sample	***soil***	***Plant (frag)***	***Soil (new)***
1	21211050	la	nt	nt
2	21211224	-	na	na
3	21211226	-	na	na
4	21213088	5b	-ve	-ve
5	21209466	6b	+ve	-ve
6	21209467	7a	+ve	-ve
7	21209703	8a	+ve	+ve
8	21209704	-	na	na
9	21209719	-	na	na
10	21209720	-	na	na
11	21209424	11a	+ve	-ve
12	21209423	12a	+ve	-ve
13	21211227	13a	+ve	-ve
14	21211225	15a	+ve	+ve
	P. fragariae +ve		+ve	-ve
	P. rubi +ve		+ve	+ve

To date, nine soil samples were tested using the existing Fera *P. fragaeiae* (not published) and the new *P. rubi* assay developed in this project. The Fera *P. fragariae* assay detected seven out of eight positive raspberry soil extracts that are likely to have *Phytophthora* species affecting raspberry (Table 4). The new *P. rubi* assay detected two out of eight positive raspberry soil samples. As with the plant material, The *P. fragaeiae* assay reacted with the *P. fragaeiae* and *P. rubi* controls. Therefore, the *P. fragaeiae* assay cross-reacts with both pathogens. The *P. rubi* assay reacted only with the *P. rubi* control (the *P. fragaeiae* assay was negative).

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 real-time PCR assay for *P. rubi* will be promoted to growers during year 2.

Discussion

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

The sequences of the five genes investigated showed that *Phytophthora rubi* and *P. fragariae* are closely related and few differences exist between the two species. The Cox1 gene shows promise as a target for designing a species-specific assay. Two new assays have been designed, one for *P. fragariae* and another for *P. rubi.* Initial results suggest specificity is possible. However, with few differences in the DNA sequences, the first attempt at an assay has produced assays of relatively low sensitivity which would not be suitable for routine use with predictive diagnosis from soil. Further primer sites within the Cox1 gene will be determined along with a nested real-time PCR approach which should increase both specificity and sensitivity.

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

The *P. rubi* assay developed as part of SF 130 was selective and did not detect *V.* fragariae. Further work is required to increase the sensitivity of the *P, rubi* to levels found using the existing Fera *P.* fragariae assay which detects both *P. rubi* and *P. fragariae.*

References

Budge GE, Shaw MW, Colyer A, Pietravalle S, Boonham N (2009). Molecular tools to investigate *Rhizoctonia solani* distribution in soil. *Plant Pathology* **58**, 1071-1078.

Kennedy D.M. & Duncan J.M. (1991). Methods for assessing the resistance of raspberry genotypes to *Phytophthora* root rot. Plant Pathology 40, 387-394.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution. 28,:2731–2739.

Veld, W. (2007). Gene flow analysis demonstrates that *Phytophthora fragariae* var. *rubi* constitutes a distinct species, *Phytophthora rubi* comb. nov. Mycologia 99(2): 222-226

White TJ, Bruns T, Lee S, Taylor JW, 1990. Amplification and direct sequencing of fungal

ribosomal RNA genes for phylogenetics. *In:* Innis MA, Gelfand DH, Sninsky JJ, White TJ, *eds.* PCR Protocols: A Guide to Methods and Applications. *New York, USA: Academic Press,* 315–22.