Project title:	**Towards a better understanding of the biology and genetics of *Phytophthora rubi* and *Phytophthora fragariae***
Project number:	CP 173
Project leader:	Eleanor Gilroy, The James Hutton Institute (JHI), Dundee
Report:	Annual report, October 2019
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Key staff:	Aurelia Bezanger (PhD student), Steve Whisson, Lydia Welsh, Ingo Hein
Location of project:	The James Hutton Institute, Dundee
Industry Representative:	Ross Mitchell, Castleton Fruit Ltd, Laurencekirk
Date project commenced:	October 2017

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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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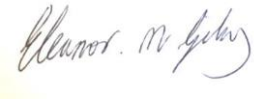
Signature  Date .....23/10/18.....

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GROWER SUMMARY

Headlines

- Progress is being made to improve our understanding of the biology and genetics of *Phytophthora rubi* in raspberry and *Phytophthora fragariae* in strawberry.

Background and expected deliverables

Phytophthora rubi (raspberry root rot) and *Phytophthora fragariae* (strawberry red stele) are currently poorly understood and understudied pathogens causing significant economic and environmental impact on soft fruit production in the UK. At the moment, there are no effective chemical control measures and the best control strategy relies on prevention, good conditions of the cultures and destruction of infected plants on which the pathogen depends for reproduction. However, this leaves the soil contaminated and unusable for future crop production. Consequently, the industry has been forced into pot-based annual or short-term production in substrate, but this only reduces, rather than solves the problem. In order to find suitable, reliable and durable fighting strategies, the pathogen first needs to be understood. This project aims at understanding the biology and genetics of *P. rubi* and *P. fragariae.*

Summary and the project's main conclusions

Hydroponics raspberries

In this project, raspberries have successfully been grown in hydroponics, using a Nutrient Film Technique (NFT – figure 1). This involves the use of rockwool plugs, soaked in nutrient solution at a correct pH (5.2 – 5.8), where raspberry cuttings were placed after being dipped in rooting hormone. Once roots appeared and grew enough, the plugs were transferred onto the NFT channel. Roots and above parts of the plants continued to grow healthily, and the root mat developed shoots that were placed inside rockwool media before being re-introduced into the hydroponics rotation once they grew sufficiently.

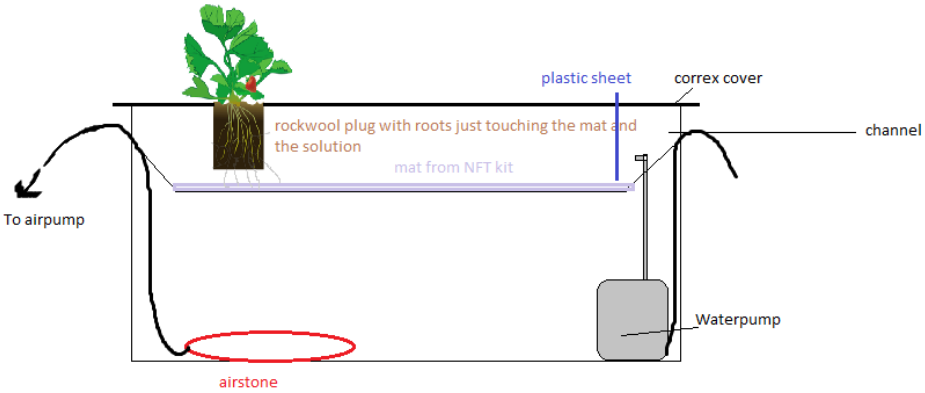

Figure 1: Schematic representation of the NFT set up used in this study.

Cutting are produced on a regular basis. Raspberry plants left to grow over the winter produced fruits (figure 2).

Figure 2: Raspberry fruits produced by plants left to grow over the winter

We tested that plants grown in hydroponics could go through dormancy and recovered, while being kept in hydroponic conditions: plants were moved in their hydroponic tank to a cold glasshouse (non-heated, outdoor temperature in October/November) for 3.5 weeks. When plants started to show fruits and buds (figure 3), they were moved to a 4°C store - hydroponics unplugged.

a.

b.

Figure 3: a. fruits and b. buds seen on the plants after 3 weeks stored in the cold glasshouse

After 7 weeks in the cold store, plants in the hydroponic tank were brought back to normal temperatures progressively: the temperature was increased weekly from 10°C, 15°C to 18/20°C. During that storage, buds broke open (figure 4), showing good recovery of plants grown and kept in hydroponics during and after induced dormancy.

Figure 4: Broken buds on plants in usual glasshouse (25C) after being stored at 4C for 6-7 weeks and in a glasshouse where the temperature was regularly raised, from 10C to 18-20C for 3 weeks

P. rubi isolation from canes

A protocol adapted from Stewart et al., 2014, was used in this study, using Italian selection media with antibiotics (PCNB, pimaricin, rifampicin, nystatin, hymexazol and ampicillin) rather than CMA-PARP media (Stewart et al., 2014; Cooke, personal communication). Once hyphal growth was isolated, it was morphologically checked to narrow down to the *Phytophthora* genus. After being sub-cultured on Rye agar, DNA was extracted and the cox I region was sequenced along positive controls as to confirm that the isolate was indeed *P. rubi*. This method was proven successful when field isolates of *P. rubi* were taken from the Bullion field, outside JHI (Dundee) and from a farm in Aberdeenshire. These isolates were added to the JHI culture collection. Table 1 lists the different isolates used for various studies in this project.

Table 1: Details on the 12 main isolates of *P. rubi* and *P. fragariae* used in this project

Species	Isolate	Date isolated	Country isolated from	Region isolated from	Host	Cultivar	Race
P. rubi	SCRP333	1985	Scotland	Essendy, Perthshire	Rubus idaeus	Glen Clova	Race 3
P. rubi	SCRP1202	2010	The Netherlands		Rubus idaeus	Red raspberry	Unknown
P. rubi	SCRP1208	2017	Scotland	South Bullion field, Invergowrie	Rubus idaeus	Autumn Treasure x Glen fyne	Unknown
P. fragariae	BC1	1991	Canada		Fragaria×ananassa		Race 1 (CA1)
P. fragariae	BC16	1992	Canada		Fragaria×ananassa		Race 2 (CA3)
P. fragariae	NOV9	1986	Canada		Fragaria×ananassa		Race 3 (CA2)
P. rubi	SCRP1213	2018	Scotland	Aberdeenshire	Rubus idaeus	Glen Dee	Unknown
P. rubi	SCRP324	1991	Scotland	Cranslea	Rubus idaeus	Glen Clova	Race 1
P. fragariae	SCRP245	1945	England	Kent	Fragaria×ananassa		Unknown
P. rubi	SCRP249	1985	Germany	Munich	Rubus idaeus	Sohonemann	Unknown
P. rubi	SCRP296	1993	Scotland	East Loan	Rubus idaeus?	Glen Coe?	Unknown
P. rubi	SCRP339	1985	France	Pyrenees Atlantiques	Rubus idaeus	Meeker?	Race 3

P. rubi infection and life cycle

Transgenic P. rubi

P. rubi was successfully transformed using a protocol adapted from Judelson et al. (1991), to express green and red fluorescent proteins, respectively eGFP and tdTomato.

Sporangia and zoospores production

Sporangia and zoospores were successfully produced for both *P. rubi* and *P. fragariae,* as described in the 2018 report, using soil water and Petri's solution.

Infection in hydroponic conditions

Transgenic *P. rubi* expressing tdTomato red fluorescent protein was used in a hydroponic infection. Sporangia and zoospores were successfully produced, similarly to the wild types as described in the 2018 report. Zoospores were used to infect roots of raspberries grown in hydroponics – no soil and easily accessible and assessed. Roots were dipped in zoospore solution for 6 hours before being transferred to a hydroponic tank (using the Deep-Water Culture method, where plant roots are suspended in distilled water, with an air pump and air stone for access to oxygen). Infection state was assessed regularly: at 1-day post-inoculation (dpi), 3dpi, 7dpi, 11dpi, 22dpi and 30dpi, and confocal images using a fluorescent microscope were taken as well as root samples for future RNA extraction.

Figure 5 shows different live stages observed with the fluorescent microscope: from hyphae growing in the root and the central vascular cylinder to sporangia releasing zoospores, the transgenic pathogen showed every infection stage of the cycle in hydroponic conditions.

This infection study showed that it was possible to study the life cycle of root pathogens, such as *P. rubi*, in hydroponics conditions, and that the pathogen completed its full life cycle.

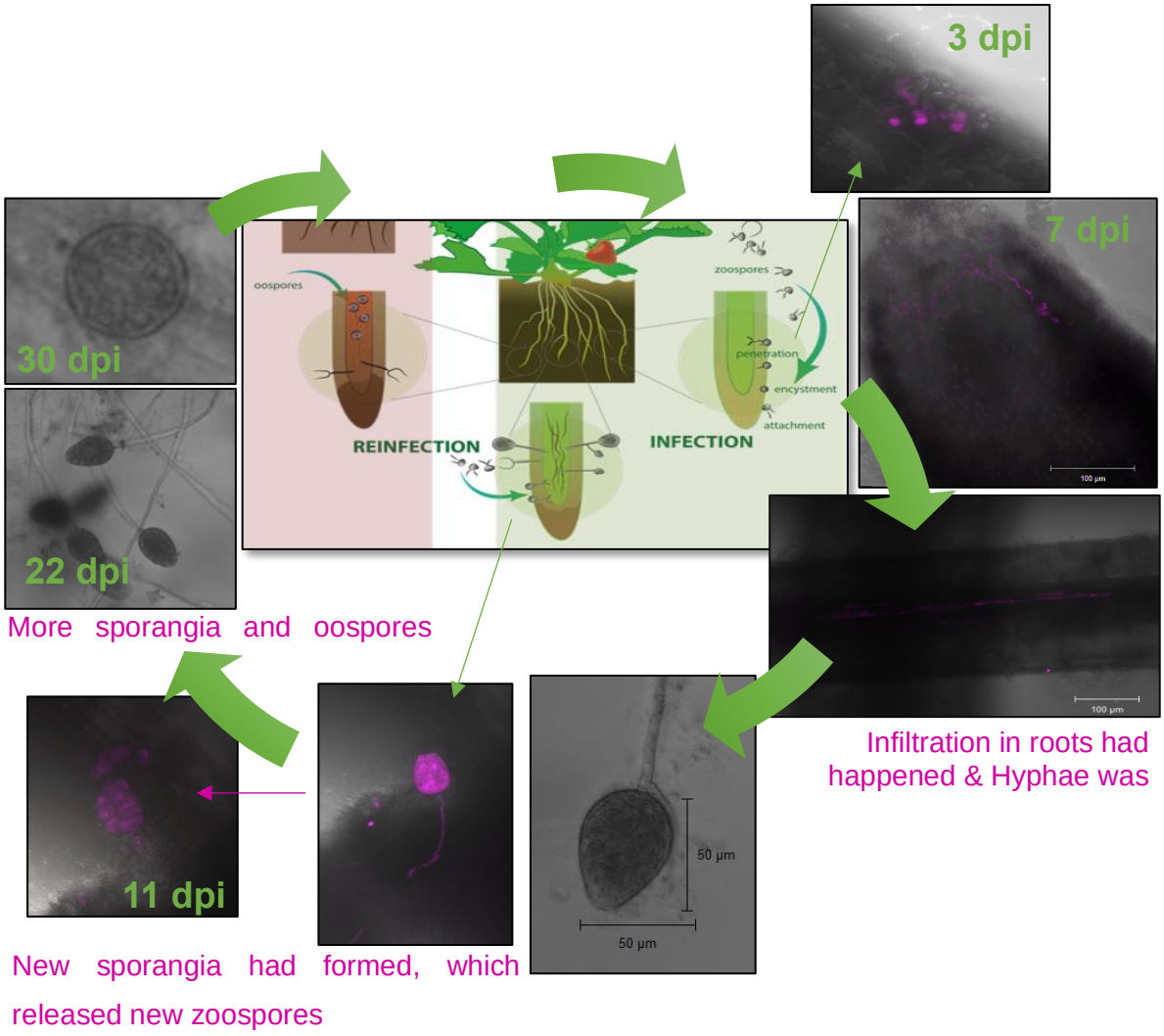

Figure 5: Infection assay using hydroponic raspberries and transgenic *P. rubi* SCRP333-tdT. Photos taken with Zeiss 710 confocal microscope show red fluorescent hyphae growing in the roots. This figure shows that the transgenic *P. rubi* went through its whole infection cycle while in hydroponic conditions.

P. rubi phenotypic diversity study

Effects of the temperature

Several lab isolates and field isolates of *P. rubi* and *P. fragariae* were grown at 2 different temperatures, 15˚C and 18˚C – see figure 6. Statistical analysis showed that the *P. rubi* lab isolates grew significantly better at 18˚C whereas the *P. rubi* field isolates grew similarly under both temperatures. *P. fragariae* isolates also grew significantly better at 18˚C.

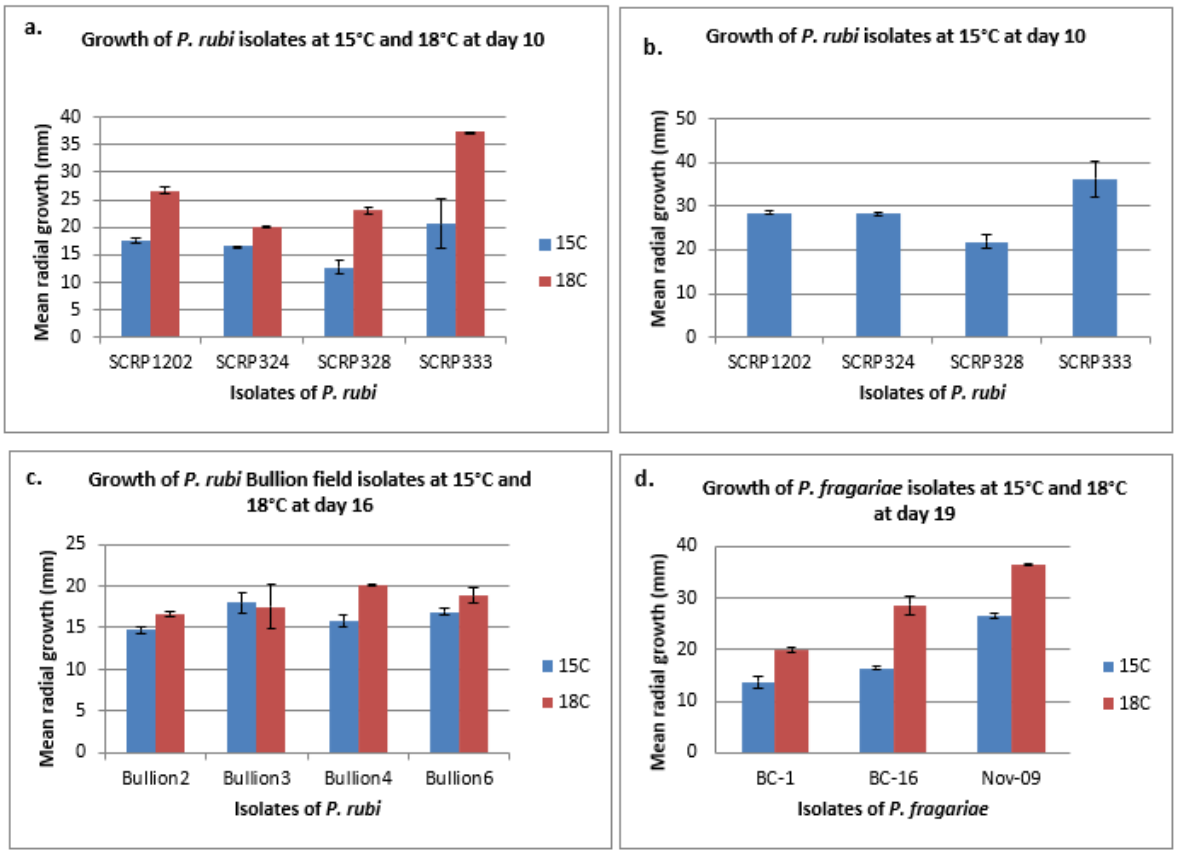

Figure 6: Chart graphs showing *P. rubi* and *P. fragariae* isolates growth under different conditions, at 15˚C and 18˚C. a. Chart showing the mean radial growth of P. rubi SCRP isolates at day 10 for the two temperatures. b. Chart showing the mean radial growth of P. rubi SCRP isolates at day 16 at 15˚C. c. Chart showing the mean radial growth of P. rubi Bullion field isolates at day 16 for the two temperatures. d. Chart showing the mean radial growth of P. fragariae isolates at day 19 for the two temperatures

Chemical screening

Twelve isolates of *P. rubi* and *P. fragariae* were investigated in a chemical screening assay, in order to assess the phenotypic diversity and the relevance of current chemical treatments. Isolates were grown in agar incorporated with different doses of chemicals. Fluazinam, active ingredient of Shirlan (fungicide used against potato blight - *Phytophthora infestans* - and tuber blight in potatoes) and Dimethomorph, active ingredient of Paraat (fungicide used against crown rot - *Phytophthora cactorum* - in strawberries and root rot - *Phytophthora spp*. - in raspberries and blackberries) were used for the study, at 4 different doses: 0ppm, 0.1ppm, 1ppm and 10ppm; the conventional application dose of these chemicals being between 1 and 10 ppm. Diametral mycelial growth was measured regularly and statistical analyses (ANOVA) were carried out. Figure 7 shows the mean diametral growth (percentage of untreated) for *P.*

rubi and *P. fragariae* isolates after 7 days. Figure 8 illustrates the dose response of 3 different *P. rubi* isolates at day 9.

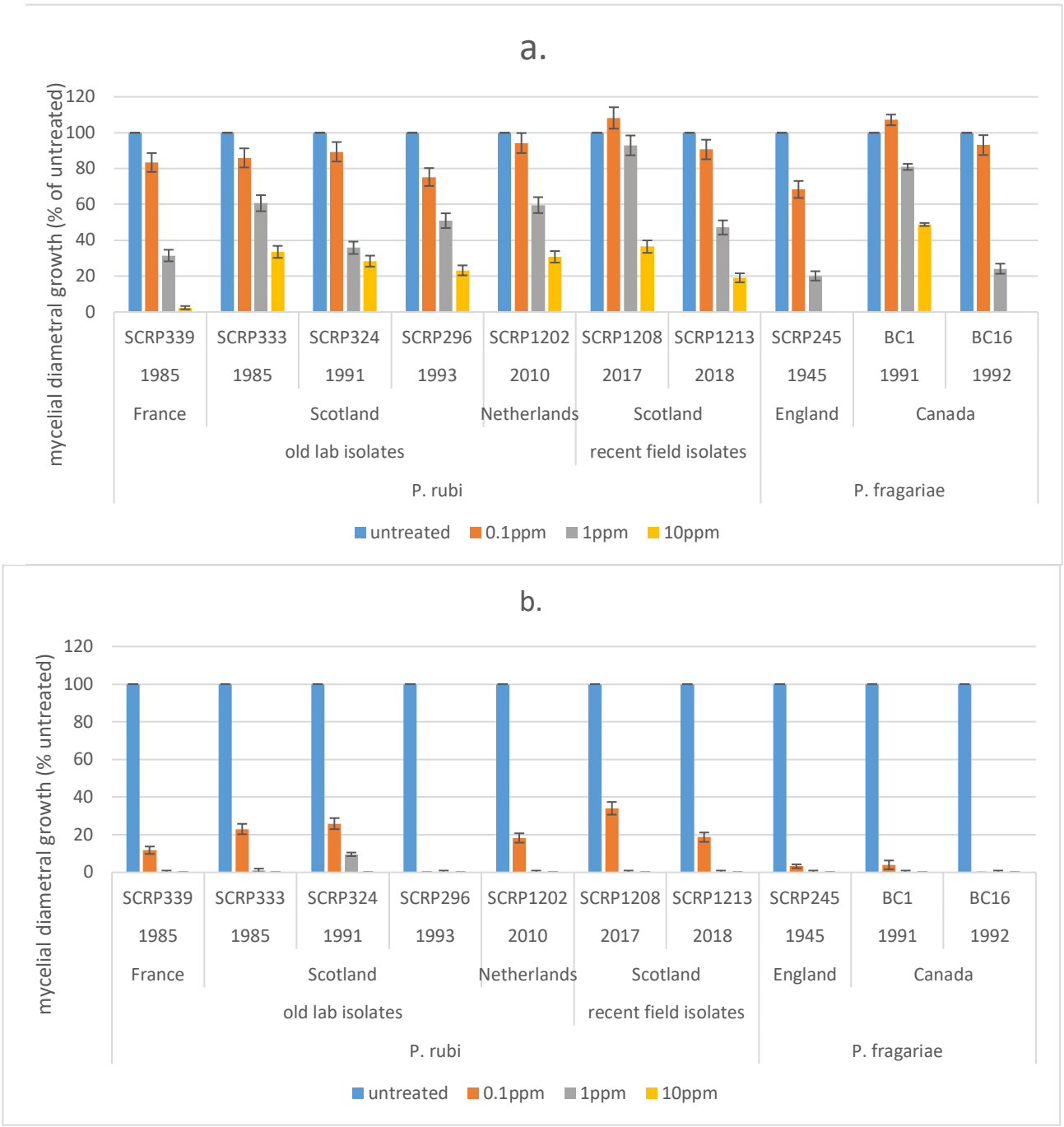

Figure 7: Mean diametral growth (percentage of untreated) of *P. rubi* and *P. fragariae* isolates grown on media incorporated with a. Fluazinam and b. Dimethomorph for 7 days

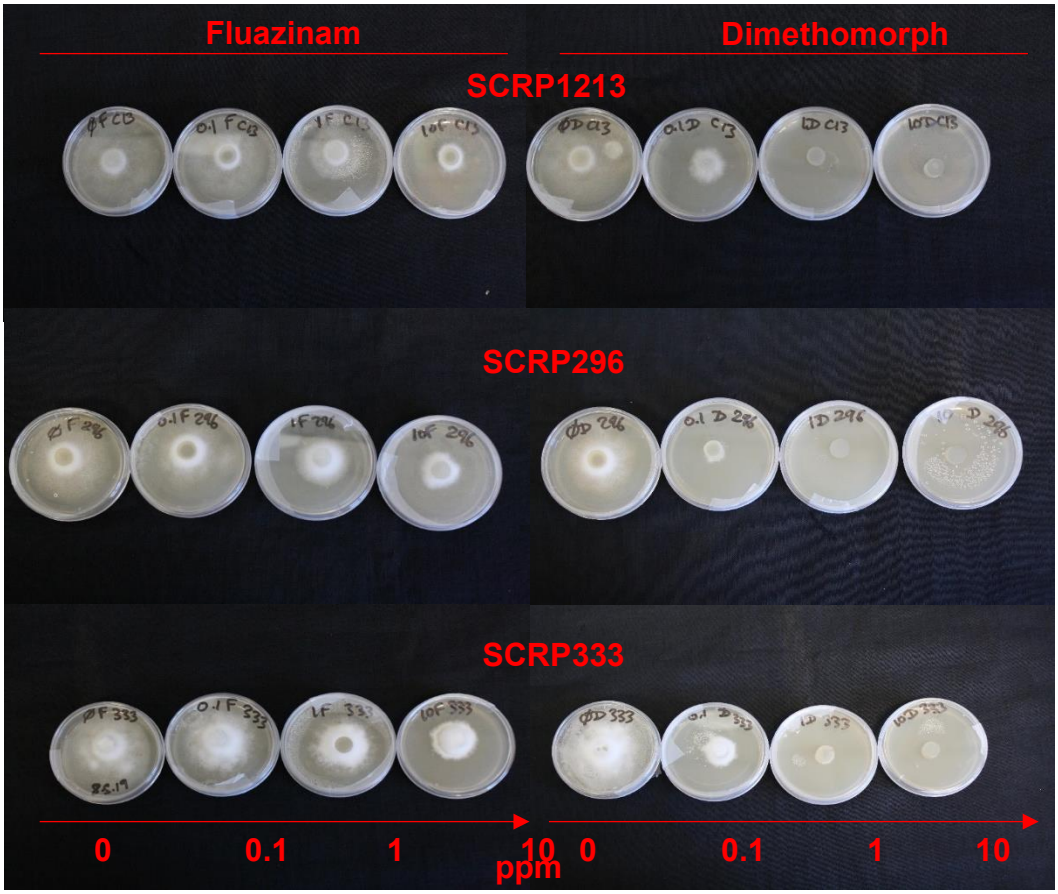

Figure 8: Photos of 3 different *P. rubi* isolates growing on plates incorporated with Fluazinam and Dimethomorph at a range of doses (from 0 to 10ppm). Photos taken at day 9

All isolates tested were sensitive to Fluazinam and Dimethomorph as incorporated media always lead to reduced growth compared to the controls. Results show that dose and chemical have a significant effect ($p<0.05$) on *P. rubi* and *P. fragariae* growth. Higher doses resulted in reduced pathogen growth and 10ppm of chemical incorporated into the growing media lead to significantly reduced or absent growth of the pathogen. Logically, Dimethomorph, commonly used against *Phytophthora* in raspberries and strawberries, was more efficient than Fluazinam, developed for potato blight; and recent field isolates of *P. rubi* show good sensitivity to the former chemical. In terms of fluctuations between growth of isolates exposed to the chemicals, very little difference was observed. Strains of *P. rubi* from the same country isolated more than 30 years apart reacted in the same way to both treatments (from Scotland: SCRP333 1985, SCRP296 1993, SCRP1208 2017, SCRP1213 2018); although SCRP1208, a recent isolate, seems less sensitive to Fluazinam than other *P. rubi.* Strains isolated in the same year from different countries (SCRP333, Scotland, 1985 and SCRP339, France, 1985) also grew similarly when exposed to the treatments. Likewise, different strains of *P. fragariae* showed comparable growth when treated, with BC1 strain (race 1, from 1991 Canada) being less sensitive to fluazinam than other *P. fragariae.*

***P. rubi* and *P. fragariae* genotypic diversity study: Pathogen Enrichment Sequencing (PenSeq)**

New bio-informatics technologies are more and more popular. Target Enrichment Sequencing will be used in this project and aims at studying the genetic diversity of *P. rubi* and *P. fragariae*, both inter and intra-specifically. This method enables the massively parallel identification of presence/absence and sequence polymorphisms in avirulence genes, which is a prerequisite for predicting host resistance durability. The Target Enrichment Sequencing relies on amino acid sequences, called “baits”, used to target predicted effectors (pathogenic proteins). In this study, a bait library was designed for RXLR effectors, Crinkler effectors, pathogenicity genes, and fungicide targets genes. Enrichment was performed on 12 isolates (see figure 9 for PenSeq principle and table 1 for list of isolates used in this study). Quantitative PCR was carried out on enriched and non-enriched samples and showed that the enrichment worked. Samples are ready to be sequenced to investigate genetic diversity in the next few months.

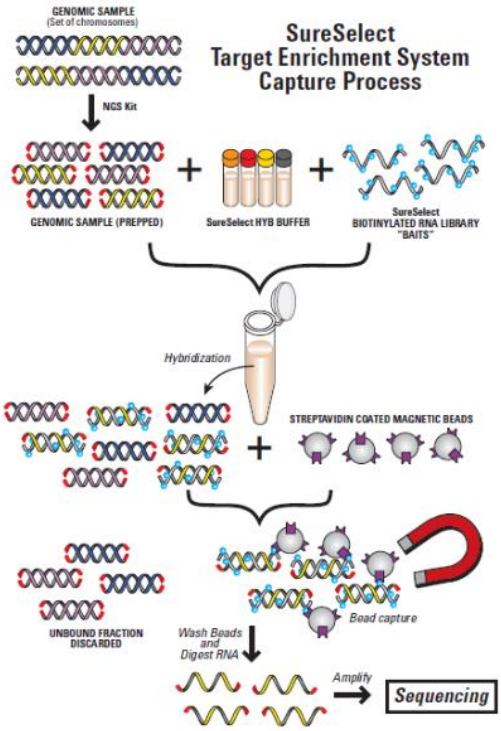

Figure 9: Target Enrichment Sequencing principle

Main conclusions

- In this project, raspberries have successfully been grown in hydroponics, and have successfully undergone and recovered from induced dormancy
- A method to isolate *P. rubi* from canes, rather than roots, has been successfully developed, and produced new recent field isolates to use in this project through various studies
- More insights into the phenotype and behaviour of *P. rubi* were gained when a hydroponic infection was carried out, using a transgenic *P. rubi* expressing a red fluorescent protein. At day 7, infiltration of the roots had happened & hyphae was

colonizing the central vascular cylinder. At day 11, new sporangia had formed, which released new zoospores. The infection cycle was complete, and more sporangia and oospores were produced in the following days, up to day 30.

- *In vitro* experiments investigated the effects of temperature (15˚C and 18˚C) and chemicals (using active ingredients of Shirlan and Paraat) on *P. rubi* and *P. fragariae.* Phenotypic diversity was shown amongst isolates grown under different temperatures. All isolates tested were sensitive to the chemicals, as incorporated media always lead to reduced growth compared to the controls.
- Genetic diversity is studied using a recently developed method, called target enrichment sequencing (PenSeq). Quantitative PCR proved that this method could be used on 12 isolates of *P. rubi* and *P. fragariae.* Future work will focus on sequencing he enriched sample to focus on the inter and intra-species diversity.

Financial Benefits

In some crops, the loss due to *Phytophthora* species is estimated at 40% of production and valued at $300 billion worldwide. *P. rubi*, causing raspberry root rot, has devastated the UK raspberry production, with over 80% of the field production that has been lost to the disease. This pathogen causes major economic and environmental damages but is very poorly understood. This project aims to gain further insights into the pathogen biology and genetics, as a foundation for further research on raspberry root rot. There are no immediate financial benefits.

Action Points

The project was not designated to produce immediate recommendations to growers.

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SCIENCE SECTION

Introduction

Raspberry consumption has been shown to have multiple health benefits, from being associated with lower cardiovascular disease risks to improving brain function, the berries' popularity is increasing. However, like any crop they are subject to attacks by a variety of pests and diseases. One major raspberry disease is *Phytophthora* root rot (PRR), primarily caused by *Phytophthora rubi*, an oomycete pathogen related to the infamous potato blight agent. Although the raspberry pathogen first appeared in the 1930s, serious outbreaks only occurred in Europe and in the UK in the 1980s. Once established, the disease could be locally spread to other growers through infested soil or water, and oospores could remain dormant in the soil for up to 10 years. As field tolerance to PRR is only seen in a few raspberry cultivars, most of which are not used commercially, highly susceptible cultivars are planted, contributing to the rapid progress of the disease across fields, regions and ultimately countries. Presently, more than 70% of the UK's soil-based raspberry production is affected by PRR, forcing growers to adopt different growing systems, such as pot-based culture. Unfortunately, there is a lack of effective chemical and control treatments for PRR. Most treatments pose the risk of being overcome by resistance from *P. rubi*, as they only rely on a single active compound with a specific mode of action. Thus, *P. rubi* poses a major economic and environmental problem for the soft fruit industry. Although it appeared in the UK decades ago, there are still knowledge gaps regarding the biology, genetics and infection lifecycle of *P. rubi*. This project aims at closing this gap by investigating *P. rubi* phenotype and genotype. The main aim of this study is to characterise *P. rubi,* causing raspberry root rot, as there are still large gaps in our understanding of the disease. Characterising a pathogen means studying its phenotype and genotype, and when possible, drawing a parallel analysis between the two. In order to achieve this, several key objectives will be addressed in this project:

- Raspberry plants will be grown in a hydroponic system and a rotation will be put in place, where new plants can be grown regularly. A suitable hydroponic set up will allow clear assessment and access to the roots prior to and for infection assays. This growing technique will open doors to study numerous root pathogens.
- *P. rubi* and *P. fragariae* phenotypes will be studied for resistance to chemical treatments and against different temperatures, by conducting *in vitro* assays.
- The host range of *P. rubi* will be assessed using a detached-petiole inoculation method from Li et al. (2017) – see annual report October 2018.
- Transformation will be carried out on several isolates of *P. rubi* using green and red fluorescent proteins (eGFP and tdTomato) to allow detailed study of infection in roots

in real time. Transgenic *P. rubi* will allow real-time assessment of the infection with imaging giving more information about location, position, state and shape of the pathogen. This method was considered easier than other techniques such as immunofluorescence, where getting antibodies into the plants can be difficult. Furthermore, following pathogens in woody tissue can be hard with techniques such as trypan blue staining, hence the transformation experiments are of greatest interest. This method will be completed by RNASeq data from samples taken at key time points of infection and screening for life markers, for different infection stages.

- *P. rubi* and *P. fragariae* genetic diversity will be studied using bioinformatics tools to identify potential effectors encoded and expressed by their genomes. This work will assess the differences between the two very closely related species but also differences within each species, at the isolate level. This could indicate whether there are specific proteins responsible for host choice or give an indication about the variance between isolates of one species, comparing strains isolated in the 1980s (lab reference strains) against strains from recent field isolations.

This work will feed back into further studies on raspberry root rot (*P. rubi*) and strawberry red stele (*P. fragariae*) diseases. It will provide scientific knowledge to underpin the horticultural industry through pre-breeding and development of novel pathogen control strategies.

Materials and methods

Hydroponics

Raspberry cultures

Cultures of raspberry (*Rubus idaeus*) cultivars Glen Dee, Glen Fyne, Glen Moy and Latham were obtained from Alison Dolan at The James Hutton Institute. Some cultivars expressed the Rub118b marker, that had been associated with *Phytophthora* root rot (PRR) resistance (Graham et al., 2011). Table 1 summarize the status of the cultivars used in this study and the presence/absence of the Rub118b marker. Glen Moy, the most susceptible cultivar to PRR, and Latham, the most resistant one, were used as reference cultivars certified Pre-Basic (High Health), the highest grade in the EU Certification Scheme for fruit plants. All raspberry plants were grown in autoclaved compost and kept in a greenhouse with 16 hours lighting, during which the temperature was set to 20˚C, followed by 8 hours with no artificial lights and with a set temperature of 18˚C.

Table 1: Resistance status and presence of Rub118b marker in the four raspberry cultivars used in this study

Raspberry cultivar	Resistance status (to root rot)	Rub118b marker?	Resistance scale (to root rot)
Latham	Resistant/tolerant	Yes	Most resistant
Glen Fyne	Susceptible	Yes	
Glen Dee	Susceptible	No	
Glen Moy	Susceptible	No	Most susceptible

Summary of hydroponics

Hydroponic raspberries were grown using the NFT method (figure 1) as described in the annual report 2018. Nutrients used were Kristalon Red (NPK 1:1:3, from Yara Tera), Solufeed (NPK 1:1:1, from Solufeed), Maxicrop (original seaweed extract, from MaxiCrop), CaNO3 (Sigma Aldrich) and a Clonex mist solution (natural root stimulator leaf spray, Growth Technology). Number of weeks for cuttings to root as well as percentage of plugs rooting were recorded to assess the effects of cultivars and nutrients on the efficiency of hydroponics.

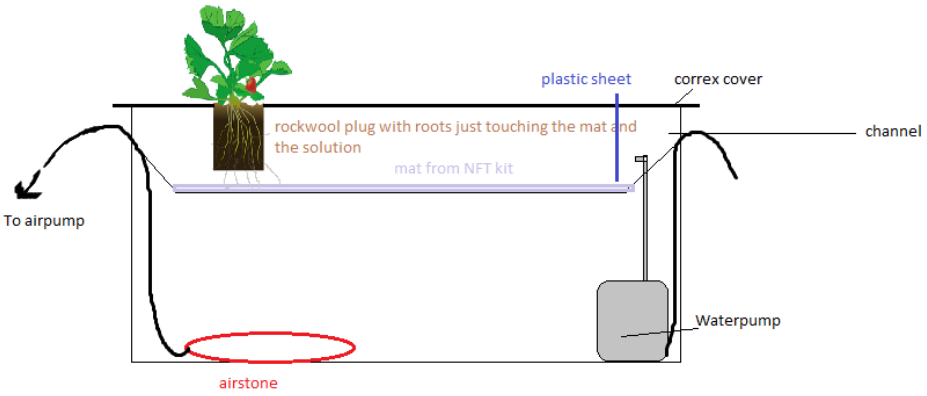

Figure 1: Schematic representation of the NFT set up used in this study.

Cultures of Phytophthora rubi and Phytophthora fragariae

Eight isolates of *P. rubi* and four isolates of *P. fragariae* were the main strains used for this project. In order to get recent field isolates, a protocol adapted from Stewart et al., 2014 was used, with Italian selection media containing antibiotics (PCNB, pimaricin, rifampicin, nystatin, hymexazol and ampicillin) instead of CMA-PARP media (Stewart et al., 2014; Cooke, personal communication). Once hyphal growth was isolated, it was morphologically checked to narrow down to the *Phytophthora* genus. After being sub-cultured on Rye agar, DNA was extracted and the cox I region was sequenced alongside positive controls to confirm that the

isolate was indeed *P. rubi*. This method has proven successful when field isolates of *P. rubi* were taken from the Bullion field, outside JHI (Dundee) and from a farm in Aberdeenshire. These isolates were added to the JHI culture collection. Table 2 lists the different isolates used for various studies in this project.

Table 2: Details on the 12 main isolates of *P. rubi* and *P. fragariae* used in this project

Species	Isolate	Date isolated	Country isolated from	Region isolated from	Host	Cultivar	Race
P. rubi	SCRP333	1985	Scotland	Essendy, Perthshire	*Rubus idaeus*	Glen Clova	Race 3
P. rubi	SCRP1202	2010	The Netherlands		*Rubus idaeus*	Red raspberry	Unknown
P. rubi	SCRP1208	2017	Scotland	South Bullion field, Invergowrie	*Rubus idaeus*	Autumn Treasure x Glen fyne	Unknown
P. fragariae	BC1	1991	Canada		*Fragaria×ananassa*		Race 1 (CA1)
P. fragariae	BC16	1992	Canada		*Fragaria×ananassa*		Race 2 (CA3)
P. fragariae	NOV9	1986	Canada		*Fragaria×ananassa*		Race 3 (CA2)
P. rubi	SCRP1213	2018	Scotland	Aberdeenshire	*Rubus idaeus*	Glen Dee	Unknown
P. rubi	SCRP324	1991	Scotland	Cranslea	*Rubus idaeus*	Glen Clova	Race 1
P. fragariae	SCRP245	1945	England	Kent	*Fragaria×ananassa*		Unknown
P. rubi	SCRP249	1985	Germany	Munich	*Rubus idaeus*	Sohonemann	Unknown
P. rubi	SCRP296	1993	Scotland	East Loan	*Rubus idaeus?*	Glen Coe?	Unknown
P. rubi	SCRP339	1985	France	Pyrenees Atlantiques	*Rubus idaeus*	Meeker?	Race 3

P. rubi infection and life cycle

In order to image *P. rubi's* life cycle in real-time in raspberry roots, genetically modified isolates expressing a fluorescent protein were used in a hydroponic infection assay, where clean intact roots could be sampled regularly. The fluorescence signal should be simple to pick-up within root tissues using appropriate microscope. When looking into woody tissues such as roots, using fluorescing strains should be easier and more detailed than staining methods, like chloral hydrate-acid fuchsin clearing-straining, or trypan blue. These methods are proven difficult when working with roots and are preferably used to stain specific structures (sporangia, oospores) and should be used in conjunction with fluorescence to provide a more detailed insight into the infection process. In this study, we combine fluorescent hyphal progression with other staining techniques (described below).

Transgenic P. rubi

Although numerous studies have focused on transformation for other *Phytophthora* species (Judelson, 1993, Judelson et al., 1993, Judelson and Michelmore, 1991, Judelson et al., 1991, Judelson et al., 1992, van West, 1999, Dunn et al., 2013, McLeod et al., 2008, Wu et al., 2016, Wang et al., 2017, Kamoun, 2000), nothing was found in the literature on *P. rubi* transformation.

In this study, the transgenes of interest encode fluorescent proteins, specifically eGFP (enhanced Green Fluorescent Protein) and tdTomato (Tandem Dimer Tomato or tdT). Plasmid vector pTOR (Genbank Accession EU257520) was used for transformation with fluorescent proteins previously cloned in between the ClaI and SacII restriction sites (figure 2). The pTOR vector included resistance genes to ampicillin and geneticin antibiotics, used for regeneration and selection of mutants. *Escherichia coli* (*E. Coli*) containing the pTOR vector described above and the gene of interest (eGFP or tdTomato) were grown and plasmids purified using Qiagen Plasmid Maxi Prep protocol and kit. Two transformation methods were tested for *P. rubi*, both adapted from a *P. infestans* protoplasts and PEG (polyethylene glycol) transformation protocol from Judelson et al. (1991) which uses sporangia. However, *P. rubi*, along with other *Phytophthora* spp. from the same clade (clade 7), cannot produce sporangia in sterile conditions, but requires a soil-solution, made of compost-extract (see paragraph about sporangia production). Absence of contamination being mandatory for transformation, mycelium cultured in sterile conditions was used instead of sporangia as starting material for the protoplasts-PEG transformation. The first method used isolate grown on rye agar plates, while the second method, newly developed to adapt to the poor growth of *P. rubi* on agar, used isolates grown in liquid lima bean media, where the mycelia thrived (all media incorporated with ampicillin antibiotic). Regenerated colonies were re-isolated onto rye agar containing ampicillin and geneticin (used for selection) at an appropriate dose, determined by a dose-response analysis where *P. rubi* was grown on doses of geneticin ranging from 0 to 10µg/mL at 18˚C for two weeks. Geneticin dose for the recovery of *P. rubi* isolate SCRP333 was 3µg/mL. Fluorescence for candidate transformants was checked using a Zeiss LSM710 fluorescent confocal microscope.

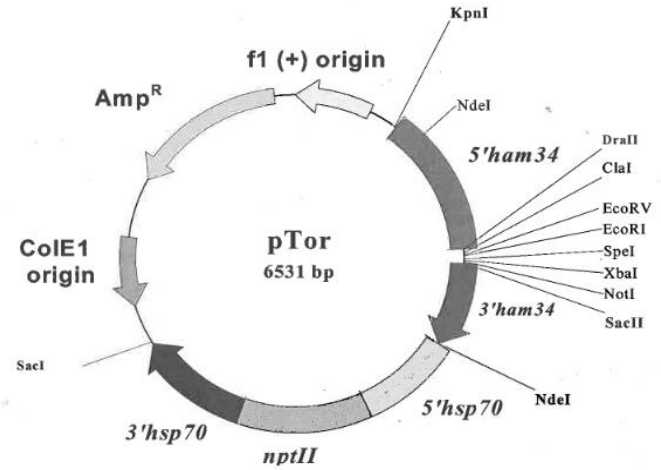

Figure 2: Map of the pTOR vector

Sporangia and zoospores production

Sporangia and zoospores were produced for both *P. rubi* and *P. fragariae,* as described in the 2018 report, using soil water and Petri's solution. Similarly, sporangia and zoospores were also obtained for the transformed *P. rubi* isolate SCRP333_tdT (SCRP333 isolate expressing the red fluorescent protein tdT or tdTomato) under the same conditions than the ones used for the wild type isolates.

Infection in hydroponic conditions

Raspberry cultivars Glen Dee, Glen Moy and Latham were used for the infection assay. They were grown in a glasshouse with the NFT method described previously, making the roots easily accessible for assessments during infection assays. Sporangia of a transgenic *P. rubi* strain expressing a red fluorescent protein (tdTomato) were successfully produced and their fluorescence checked using the Zeiss LSM710 microscope. A 30 minutes incubation at 4˚C was carried out to induce a cold shock and help in the release of zoospores. Shocked sporangia were then placed in a 4L tray and Petri's solution was poured in to induce zoospore release. Study of the life cycle on susceptible cultivars used Glen Moy and Glen Dee, grown in hydroponics. Roots of 4 raspberry plants per cultivar were 'damaged' by pressure applied onto the root, to aid the infection process, prior to being dipped in the zoospore solution for 6 hours. After the 6 hours-inoculation window, plants were transferred to a tank of 25L distilled water (Deep-Water Culture hydroponic method) with an air pump and air stone to provide oxygen to the water, which was turned on after 4 to 5 days – to avoid encystment of zoospores. A control tank with non-infected plants was set up using 2 replicates of each cultivar. Samples were collected regularly: at 1-day post-inoculation (dpi), 3dpi, 7dpi, 11dpi, 22dpi and 30dpi, and confocal Z-stack images using the Zeiss LSM710 microscope were

taken. Root samples for each cultivar were collected as well: they were snap-frozen in liquid nitrogen and stored at -80˚C for future RNA extraction.

RNA extraction

RNA of samples collected during the time-course of the infection assays will give us information about *P. rubi's* transcriptome, and the expression (expressed, non-expressed, down regulated or up regulated) of specific genes, determining key stages of the infection and life cycle (such as haustoria formation, biotrophic *vs* necrotrophic phases etc.).

Several RNA extractions methods were trialled in order to find a suitable one for the assay– due to the difficulty of getting good quality RNA, especially from woody tissue like roots due to the presence of secondary metabolites, such as polyphenols and polysaccharides, which often interfere with or degrade RNA (Azevedo et al. 2003; Mattheus et al. 2003; Sharma et al. 2003; Singh et al. 2003).

- Adapted Qiagen Rneasy Plant Mini Kit with Ambion Plant RNA Isolation Aid and β-mercaptoethanol.
- TriReagent (with chloroform/ isopropanol)

Root staining

As described above, using multiple microscopy-observation tools enables us to maximize the details of the infection and the *Phytophthora* structures observed (hyphae, sporangia, oospores). Furthermore, there is no selection (geneticin) in the hydroponic tanks during infection, due to concerns on raspberry plants not reacting well to such a strong chemical compound (possible extra damages to the roots, not due to the pathogen itself, would make the phenotyping and observations of infection more difficult). Therefore, it was found that the transgenic *P. rubi* (SCRP333_tdT) was losing its fluorescence under hydroponics-selection-free conditions, after 10 to 20 dpi. In order to verify that the infection occurred and that reproduction structures were indeed produced, other staining methods were carried out. Roots samples were regularly collected through the infection course and were stained using a Trypan Blue protocol adapted from Koske and Gemma (1989). Stained roots were observed under the microscope.

P. rubi phenotypic diversity study

Phenotypic studies are important to characterize an under-studied species, by informing about the phenotypic diversity of the pathogen. Do all isolates of one species behave similarly to environmental conditions such as different temperatures, or to chemical treatments? Are there any visible evolution of the pathogen – to temperature raise or chemical application – with recent field isolates behaving differently than older isolates kept in lab conditions for

years? To answer these questions, this project focussed on two phenotypic studies: a temperature study, in which several *P. rubi* isolates were grown under different temperatures (15˚C and 18˚C); and a chemical screening assay, where *P. rubi* isolates were grown on media incorporated with fungicides.

Effects of the temperature

As well as intra-species diversity information, the temperature assay could indicate whether *P. rubi* would thrive under warmer climates, and if perhaps the recent field isolates are better suited for higher temperatures and global warming.

P. rubi SCRP1202, SCRP324, SCRP328, SCRP333, SCRP333_tdT, Bullion 2, Bullion 3, Bullion 4, Bullion 6 and *P. fragariae* BC-1, BC-16, NOV-9 isolates were grown at 15˚C and 18˚C in the dark (details in annual report 2018). Plates were regularly checked, and radial growth measured. ANOVA and Tukey tests were carried out using R.

Fungicide screening

Fungicide screening also constitutes a useful survey for growers, indicating whether *P. rubi* field isolates have became less sensitive to chemicals or if these treatments are relevant across all isolates *vs* only a few – in which case a field diagnostic of responsible isolate(s) would be necessary for efficient control of the disease.

Twelve isolates (see table 2) of *P. rubi* and *P. fragariae* were investigated in a fungicide screening assay, in order to assess the phenotypic diversity and their sensitivity to current chemical treatments. Isolates were grown on rye agar with ampicillin in the dark, at 18°C for 12 days. After which, equal sized-plugs (9mm circular) were removed from the actively growing outer margin of the colony and transferred onto rye agar media incorporated with different doses of chemicals ordered from Sigma Aldrich, in a 50mm petri dish. Fluazinam, active ingredient of Shirlan (fungicide used against potato blight) and dimethomorph, active ingredient of Paraat (fungicide used against crown rot in strawberries and root rot in raspberries and blackberries) were used for the study, at 4 different doses: 0ppm, 0.1ppm, 1ppm and 10ppm. The conventional application dose of these chemicals in the field is between 1 and 10 ppm. Diametral mycelial growth was measured regularly for up to 15 days, although days 7 and 9 were best suited to represent mycelial growth (Saville et al., 2015, Groves and Ristaino, 2000). Statistical analyses (ANOVA) were carried out using GenStat and R.

P. rubi and _P. fragariae_ genotypic diversity study: Pathogen Enrichment Sequencing (PenSeq)

Bio-informatics technologies are constantly evolving. Target Enrichment Sequencing will be used in this project to study the genetic diversity of *P. rubi* and *P. fragariae*, both inter and intra-specifically. This method enables the parallel identification of presence/absence and sequence polymorphisms in avirulence genes, which is a prerequisite for predicting host resistance durability. The Target Enrichment Sequencing relies on amino acid sequences, called “baits”, used to target predicted effectors (pathogenic proteins). Figure 3 shows the principle of Target enrichment sequencing: specific genes are targeted with baits and enriched. This technique is particularly useful when looking at a subset of genes, in our case, pathogenicity genes, which represent less than 1% of *P. rubi’s* total genome.

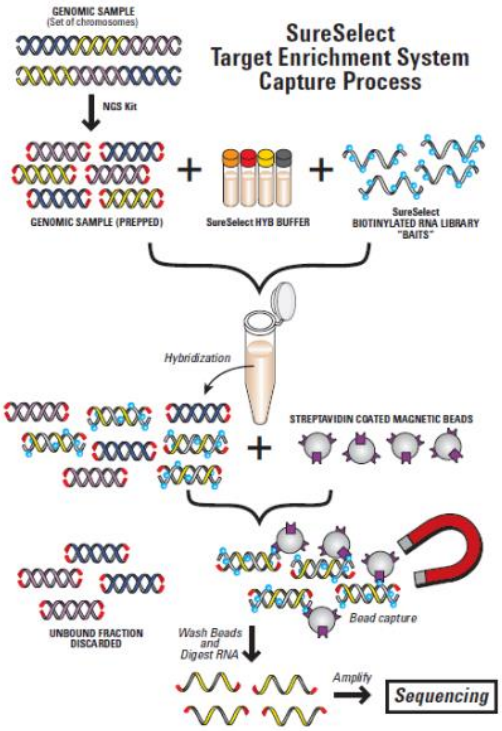

Figure 3: Target Enrichment Sequencing principle (image from Agilent)

Baits library

A bait library for PenSeq was designed by gathering sequences, or partial sequences, of genes of interest for the two species *P. rubi* and *P. fragariae*. This includes RXLR and CRN effectors, apoplastic effectors, fungicide target genes, genes involved in pathogenicity, and housekeeping genes including single copy genes using data from NIAB-EMR, from literature (Blair et al., 2008, Schena et al., 2007, Peters and Woodhall, 2014, Loos et al., 2006) and using NCBI. The compiled list was sent to Arbor Bioscience to design and produce the baits from the nucleotide sequences – see table 3. More than 185,000 sequences, covering a gene space of approximately 5 million nucleotides per isolate were provided for bait design, which produced 50,234 filtered baits.

Table 3: Average number of baits designed for targeted genes for *P. rubi* and *P. fragariae*

Apoplastic effectors	10788
RxLR effectors	2279
CRN effectors	121
Single copy genes	5
Fungicide targets genes	4
Pathogenicity genes	6
Housekeeping genes	7

Isolates collection

Twelve isolates were chosen for the first run of PenSeq, including 8 *P. rubi* and 4 *P. fragariae*, mixing locations, years of isolations and races (table 2). DNA was extracted using a method developed by Lydia Welsh (JHI) the protocol combined a phenol-chloroform DNA extraction method with a QIAGEN DNeasy Plant Mini Kit.

Target Enrichment Sequencing

For the Target Enrichment Sequencing of *P. rubi,* 500ng DNA was used per isolate. Library preparation was carried out using the NEBNext Ultra II, FS DNA Library Prep Kit for Illumina protocol (Biolabs). Hybridization and the rest of the enrichment was carried out using the SureSelect Hybridization protocol (Agilent).

Designing primers for qPCR to validate PenSeq

In order to verify the enrichment, several primers were designed for housekeeping and RxLRs genes, present in one or both species tested (*P. rubi, P. fragariae*) – see table 4. Primers were designed using the Taqman criteria (summarized in table 5).

Table 4: Primers designed for conventional and qPCR enrichment validation

Gene	**Role**	**Primer name**	**Primer sequence (5' - 3')**	**Designed by**
betatubulin	housekeeping	BetatubAB_F	AGCACGAAGGAGGTTGATGA	Aurelia Bezanger
		BetatubAB_R	GCCTTACGACGGAACATAGC	

Cox I	housekeeping	coxIAB_F	GGGCGCATCACATGTTTACT	Aurelia Bezanger
		coxIAB_R	CCTCCCCATAAAGTTGCTAACC	
g40916	RxLR	g40916AB_F	TCATTGAGCACTTAAACGAGAAGA	Aurelia Bezanger
		g40916AB_R	TCGGTCTGCTTGGTCGTAAG	
Ubiquitin	housekeeping	PsUbi_F	AAGACCCTGACTGGCAAGAC	Eleanor Gilroy
		PsUbi_R	TCCTTGTCCTGGATCTTTTGCT	

Table 5: primer design criteria

Taqman Probe and Primer design criteria
Tm: 58°C -60°C with difference between the two primers <2°C
% GC: 20-80%
Length of oligo: 9 to 40 bases
Maximum of GC at the 3' end (out of 5 last): 2
Amplicon length: 50 to 250 (50 to 150 ideally)
Self-Dimer/ Hairpin / Cross-Dimer: 0 to -7 kcal/mol

Quantitative PCR set-up

Quantitative PCR was performed using Sybr Green. Primers were used at a final concentration of 300nM. Primers were tested on enriched and non-enriched samples. Non-enriched samples were taken from DNA extracted from BC16 (*P. fragariae*) and SCRP333 (*P. rubi*). For more accuracy, dilutions of the DNA samples were made (1 in 10) and assessed using Qubit and results were used to make 3 different DNA concentrations for the assay: 10ng, 1ng and 0.5ng. Analysis of the qPCR was performed using ANOVA and Tukeys tests with GenStat.

Results

Hydroponics

Three cultivars of raspberry (Latham, Glen Dee, and Glen Moy) were successfully grown in hydroponics, offering a range of resistance and presence/absence of the Rub118b marker, which has been associated with resistance against root rot in raspberries – see table 1. The hydroponics data is shown for January 2019 as an example (Figure 4) and shows that since being introduced in October 2018, Glen Dee shows much better potential as a *Phytophthora*-susceptible raspberry cultivar to be grown in hydroponics and used in infection assay (successful infection in February/ March 2019). The data also indicates that efficacy of hydroponic as a growing method depends greatly on the cultivar. It was also demonstrated that the hydroponic efficacy varies with the time of year (recent data, not shown). Overall, Kristalon Red and MaxiCrop are better fertilisers to use for Latham whereas with Glen Dee the use of NPK 1:1:1 or Kristalon Red are recommended over MaxiCrop.

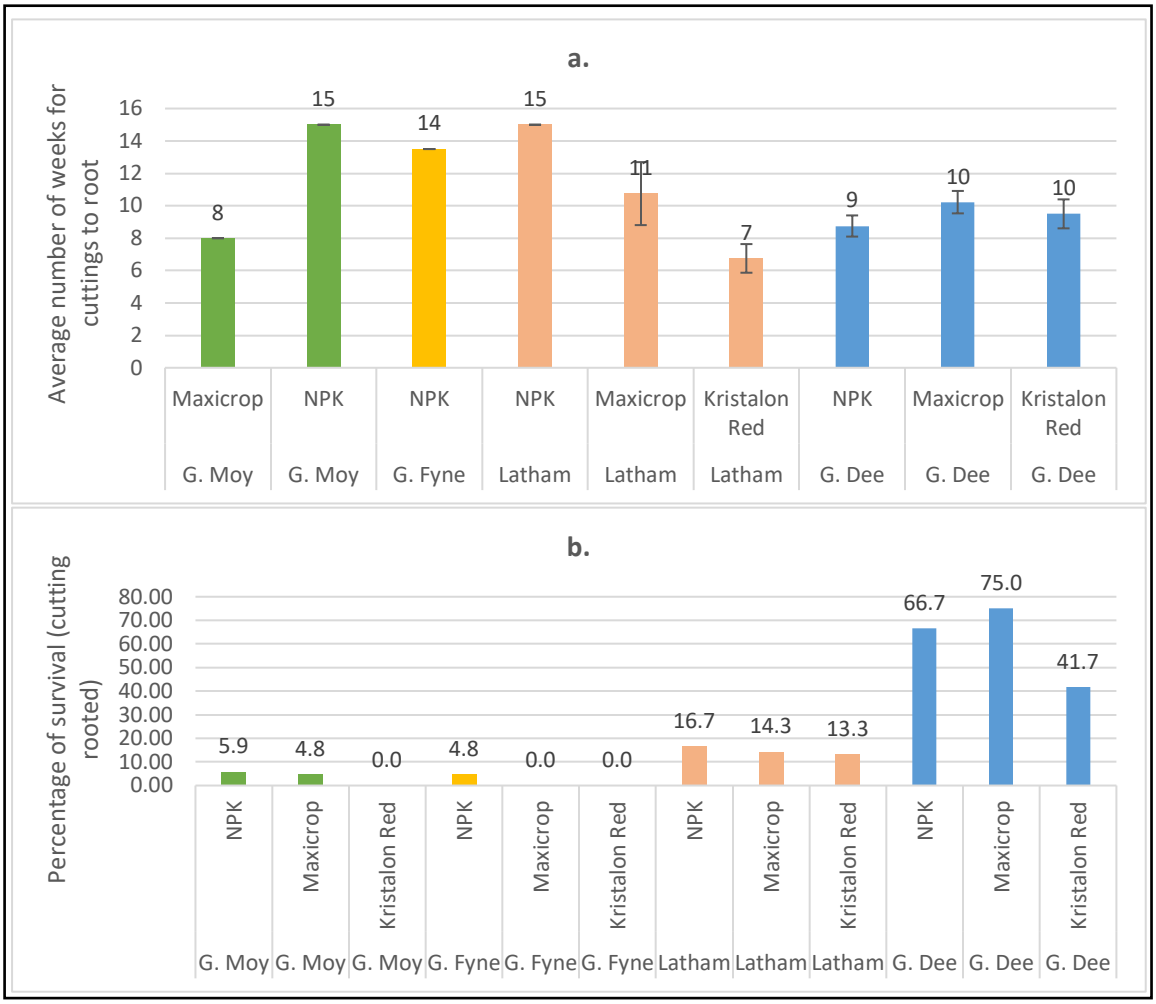

Figure 4: a. average number of weeks before rooting for cuttings taken in January 2019 per cultivar and nutrient. b. percentage of cuttings rooting (as opposed to dying) per cultivar and nutrient.

P. rubi infection and life cycle

Life cycle of P. rubi

P. rubi was successfully transformed to express green and red fluorescent proteins, respectively eGFP and tdTomato (figure 5). Sporangia and zoospores were successfully produced with both strains similarly to the wild types as described in the 2018 report (figure 6 shows sporangia and zoospores from transgenic *P. rubi* SCRP333_tdT) and tdTomato sporangia were used in an infection assay using hydroponic raspberries. Figure 7 shows different live stages observed with the fluorescent microscope: from hyphae growing in the root and the central vascular cylinder to sporangia releasing zoospores, the transgenic pathogen showed every infection stage of the cycle in hydroponic conditions.

This infection study showed that it was possible to study the life cycle of root pathogens, such as *P. rubi*, in hydroponics conditions, and that the pathogen completed its full life cycle.

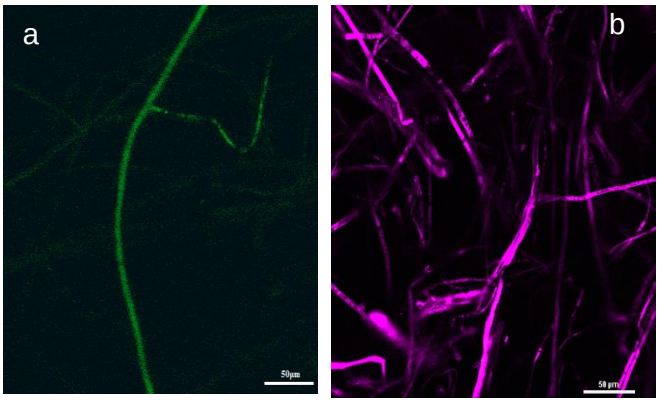

Figure 5: *P. rubi* reference isolate SCRP333 expressing green fluorescent marker eGFP (a) and red fluorescent marker tdTomato (b)

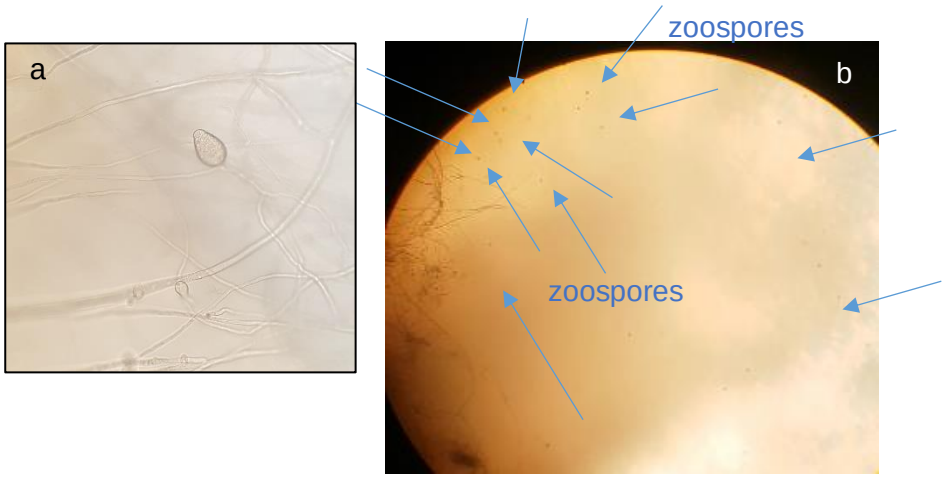

Figure 6: Transgenic *P. rubi* SCRP333_tdT successfully produced sporangia (a) and zoospores (b – pointed by blue arrows) under the same conditions than for the wild type

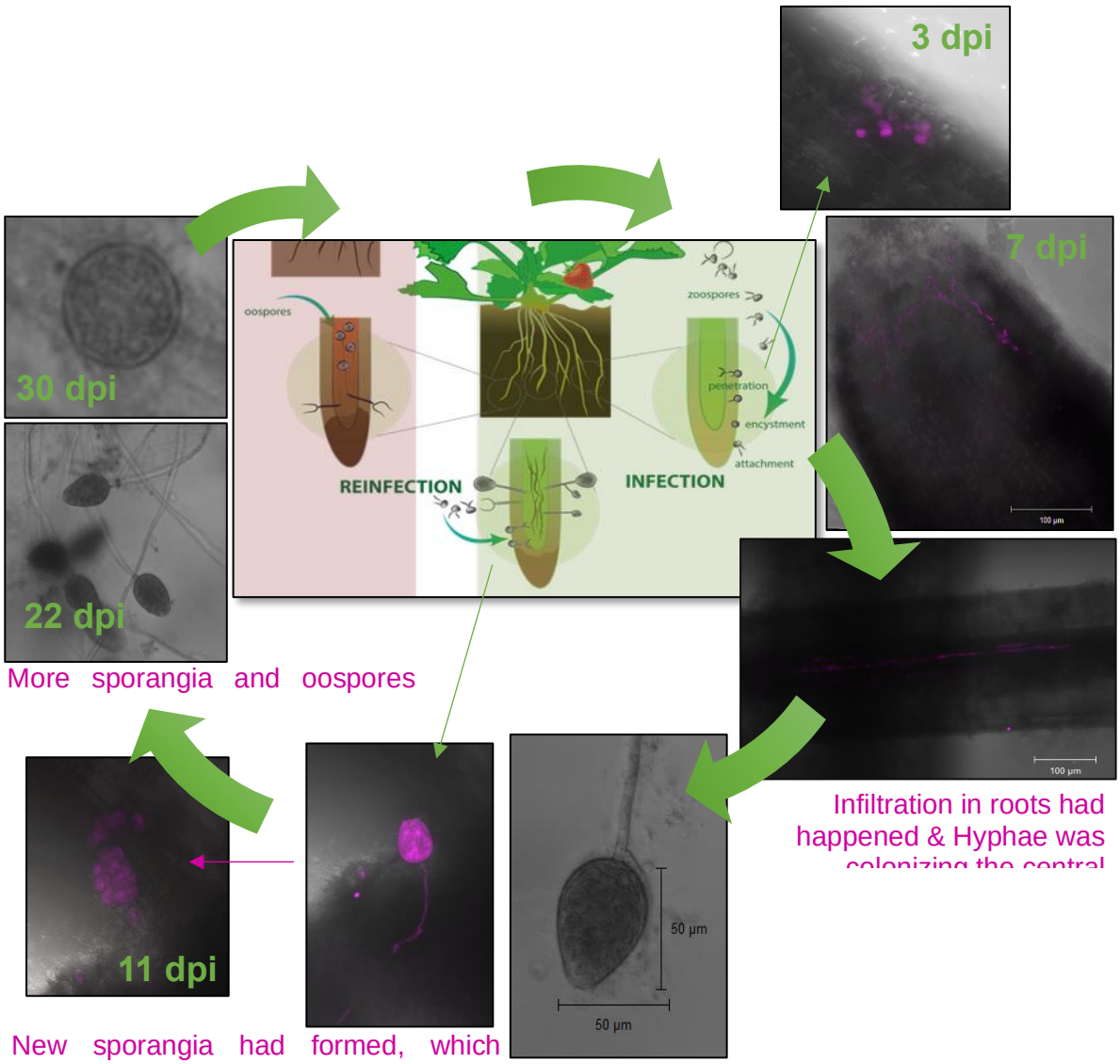

Figure 7: Infection assay using hydroponic raspberries and transgenic *P. rubi* SCRP333-tdT. Photos taken with Zeiss 710 confocal microscope show red fluorescent hyphae growing in the roots. This figure shows that the transgenic *P. rubi* went through its whole infection cycle while in hydroponic conditions.

Root staining

Stained roots are currently being assessed. Figure 8 shows an example of a Glen Moy root stained with trypan blue in a previous hydroponic infection assay carried out in this project.

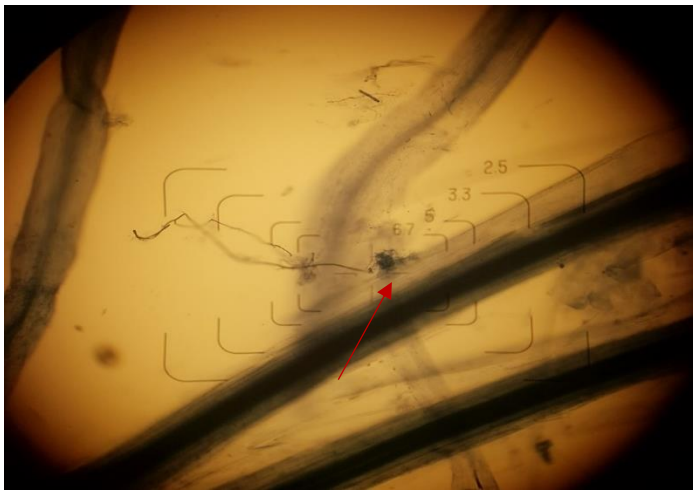

Figure 8: Photo of structure observed under microscope (zoom) showing hyphae penetration of a Glen Moy root. Root sample was stained with Trypan Blue. Sample was taken from pot infection on

P. rubi phenotypic diversity study

Effects of the temperature

Statistical analysis showed that the *P. rubi* lab isolates grew significantly better at 18˚C whereas the *P. rubi* field isolates grew similarly under both temperatures. *P. fragariae* isolates also grew significantly better at 18˚C (see figure 9).

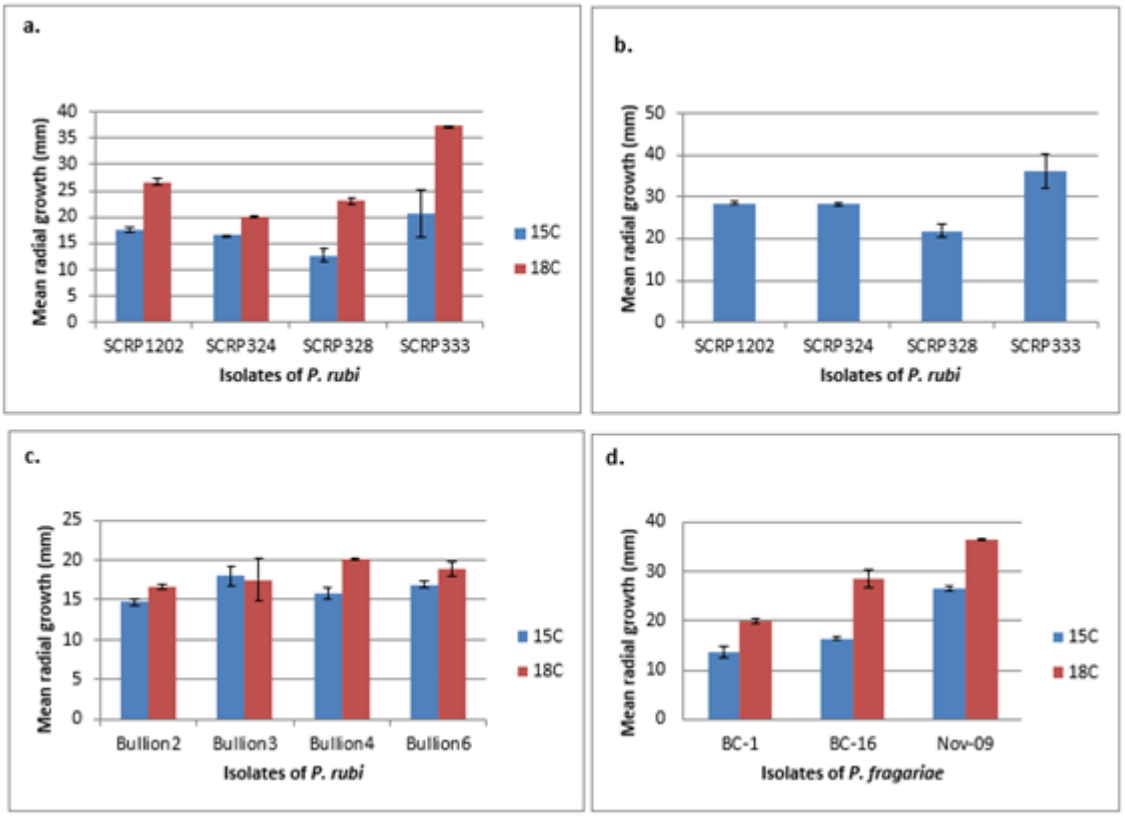

Figure 9: Chart graphs showing *P. rubi* and *P. fragariae* isolates growth under different conditions, at 15˚C and 18˚C. a. Chart showing the mean radial growth of *P. rubi* SCRP isolates at day 10 for the two temperatures. b. Chart showing the mean radial growth of *P. rubi* SCRP isolates at day 16 at 15˚C. c. Chart showing the mean radial growth of *P. rubi* Bullion field isolates at day 16 for the two temperatures. d. Chart showing the mean radial growth of *P. fragariae* isolates at day 19 for the two temperatures

Fungicide screening

Figure 10 shows the mean diametral growth (reported as a percentage of the untreated) for *P. rubi* and *P. fragariae* isolates after 7 days on media incorporated with Fluazinam and Dimethomorph. Figure 11 illustrates the dose response of 3 different *P. rubi* isolates at day 9. All isolates tested were sensitive to fluazinam and dimethomorph as incorporated media consistently lead to significantly reduced growth compared to the controls. Results show that dose and chemical have a significant effect ($p<0.05$) on *P. rubi* and *P. fragariae* growth. Higher doses resulted in reduced pathogen growth and 10ppm of chemical incorporated into the growing media lead to significantly reduced or absent growth of the pathogen. The data suggests that, dimethomorph, commonly used against *Phytophthora* in raspberries and strawberries, was more effective than fluazinam, developed for potato blight; and recent field isolates of *P. rubi* show good sensitivity to the former chemical. In terms of variability between growth of isolates exposed to the chemicals, very little difference was observed.

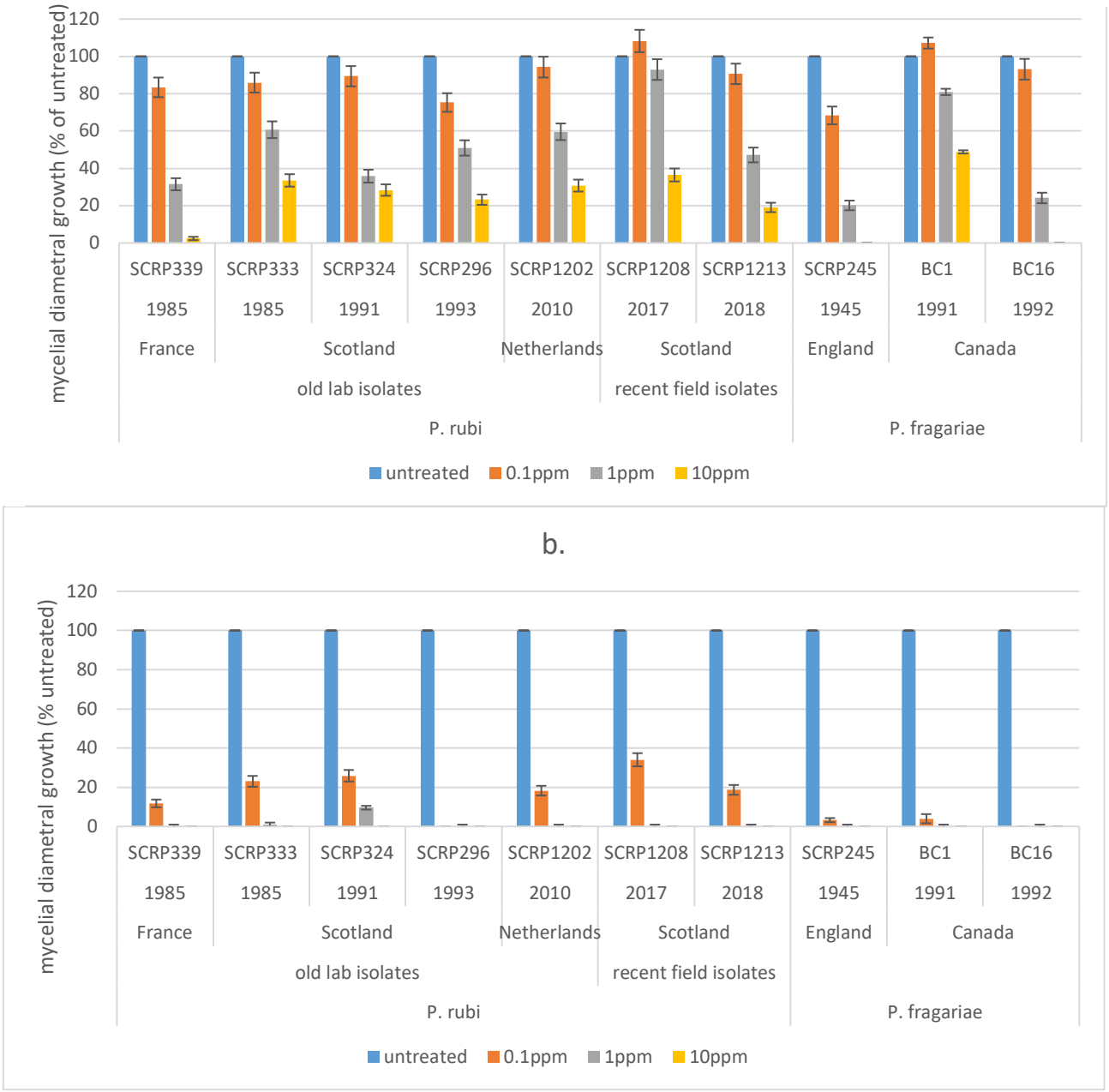

Figure 10: Mean diametral growth (percentage of untreated) of *P. rubi* and *P. fragariae* isolates grown on media incorporated with a. Fluazinam and b. Dimethomorph for 7 days

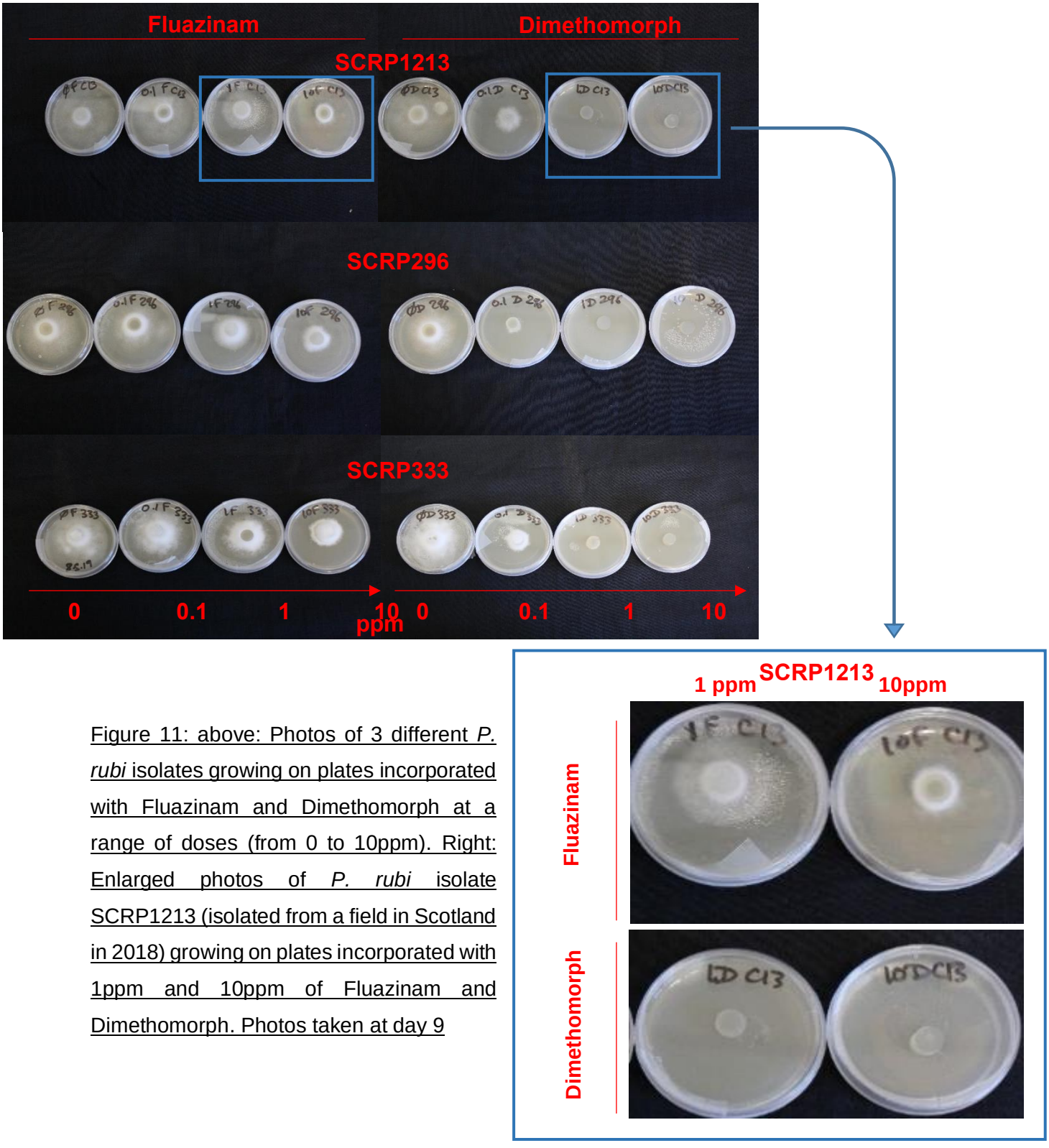

Figure 11: above: Photos of 3 different *P. rubi* isolates growing on plates incorporated with Fluazinam and Dimethomorph at a range of doses (from 0 to 10ppm). Right: Enlarged photos of *P. rubi* isolate SCRP1213 (isolated from a field in Scotland in 2018) growing on plates incorporated with 1ppm and 10ppm of Fluazinam and Dimethomorph. Photos taken at day 9

Strains of *P. rubi* from the same country isolated more than 30 years apart reacted in the same way to both treatments (from Scotland: SCRP333 1985, SCRP296 1993, SCRP1208 2017, SCRP1213 2018); although SCRP1208, a recent isolate, seems less sensitive to fluazinam than other *P. rubi.* Strains isolated in the same year from different countries

(SCRP333, Scotland, 1985 and SCRP339, France, 1985) also grew similarly when exposed to the treatments. Likewise, different strains of *P. fragariae* showed comparable growth when treated, with BC1 strain (race 1, from 1991 Canada) being less sensitive to fluazinam than other *P. fragariae* isolates tested in this study.

P. rubi **and** ***P. fragariae*** **genotypic diversity study: Pathogen Enrichment Sequencing (PenSeq)**

qPCR

Quantitative PCR produced melting curves that revealed cleared primer-dimers for the Ubiquitin primers (Figure 12) which were therefore dismissed for the rest of the qPCR study. Results from the qPCR found that the enriched sample always had a significantly lower Ct compared to the non-enriched for any of the 3 concentrations (10ng, 1ng and 0.5ng) and any of the 2 genes screened (beta-tubulin and 40916 effector) – figure 13 shows amplification plots for the two different genes and figure 14 shows the mean Ct. Tables 6 and 7 show statistical analysis carried out with GenStat, looking at effect of enrichment and DNA concentrations on amplification (Ct).The cycle difference between enriched and non-enriched varied between 3.8 cycles and 7.1 cycles, giving a fold change between 13.6 and 140.2. This shows the success of the enrichment and allow us to carry on with sequencing of the 12 isolates.

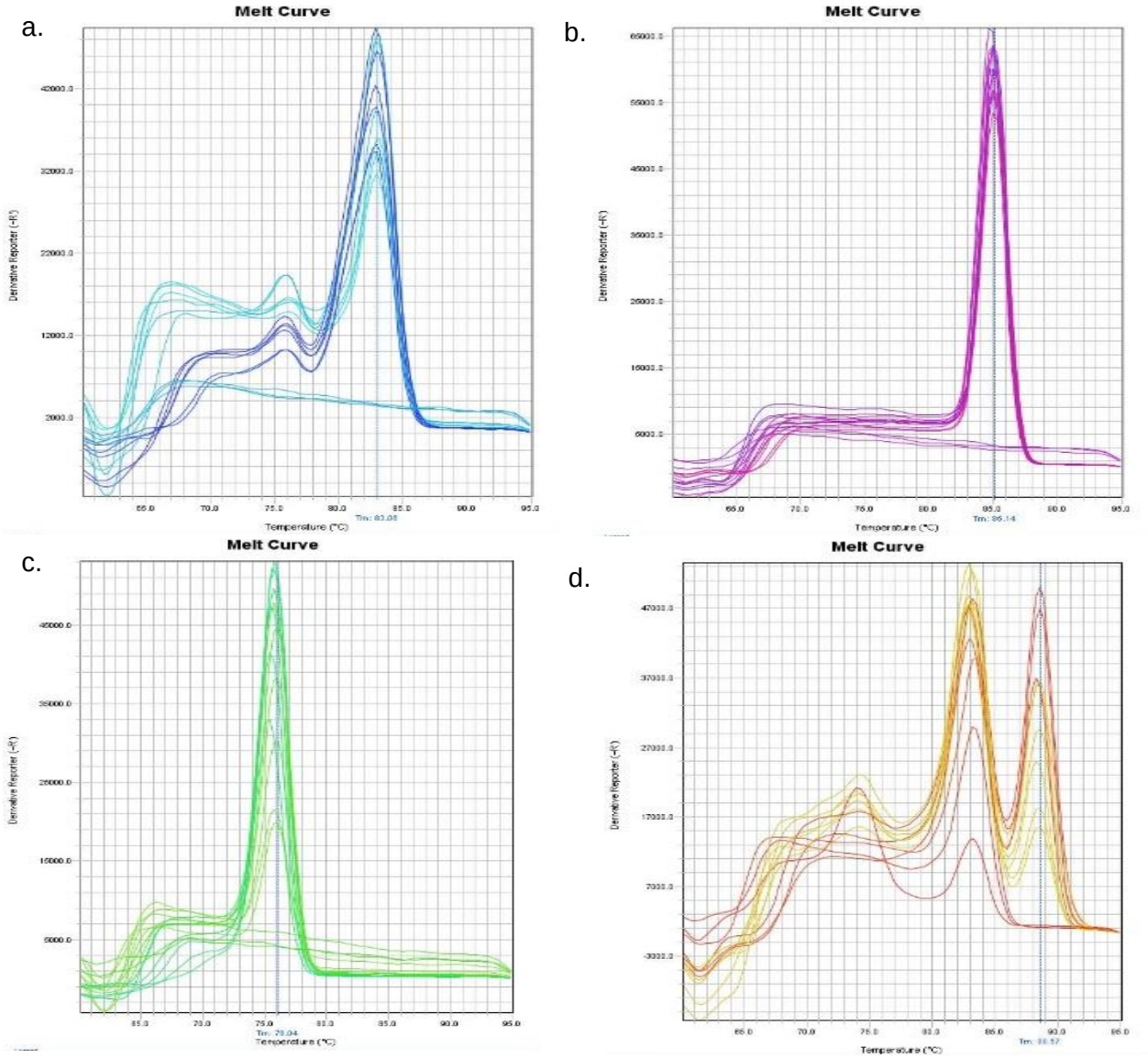

Figure 12: qPCR melting curves for a. 40916, b. beta-tubulin, c. cox I and d. ubiquitin primers

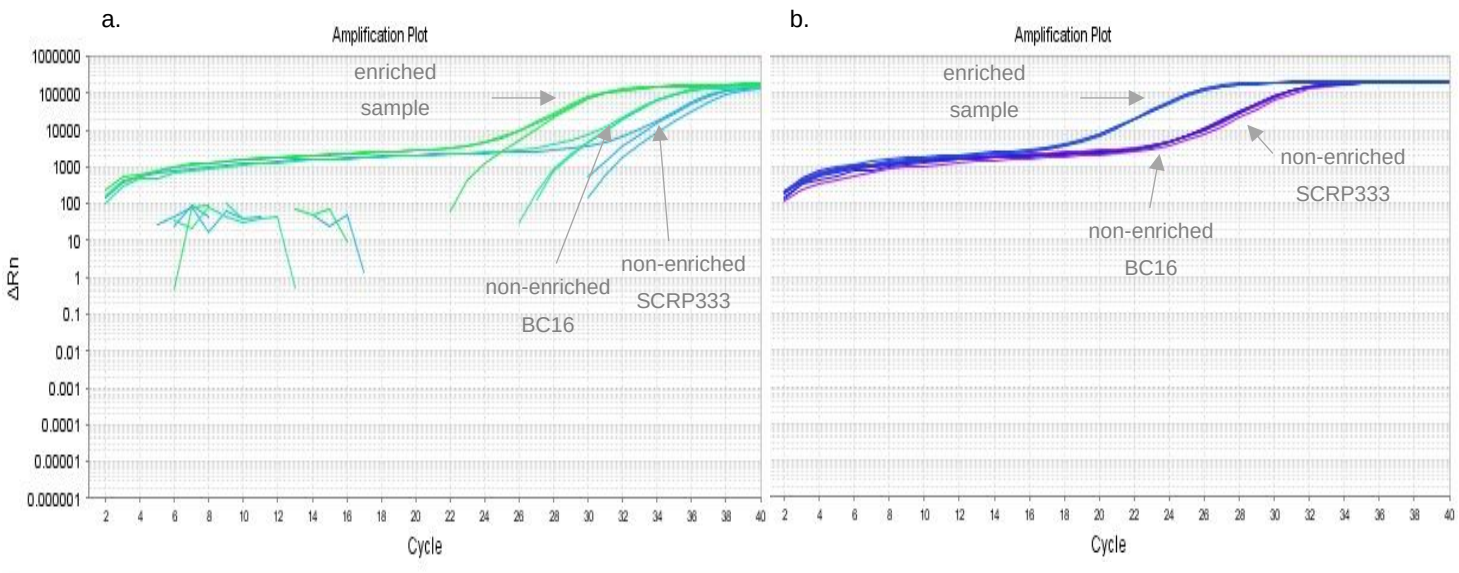

Figure 13: qPCR amplification plots. a. 40916 effector gene, DNA concentration 0.5ng. b. beta-tubulin gene, DNA concentration 10ng.

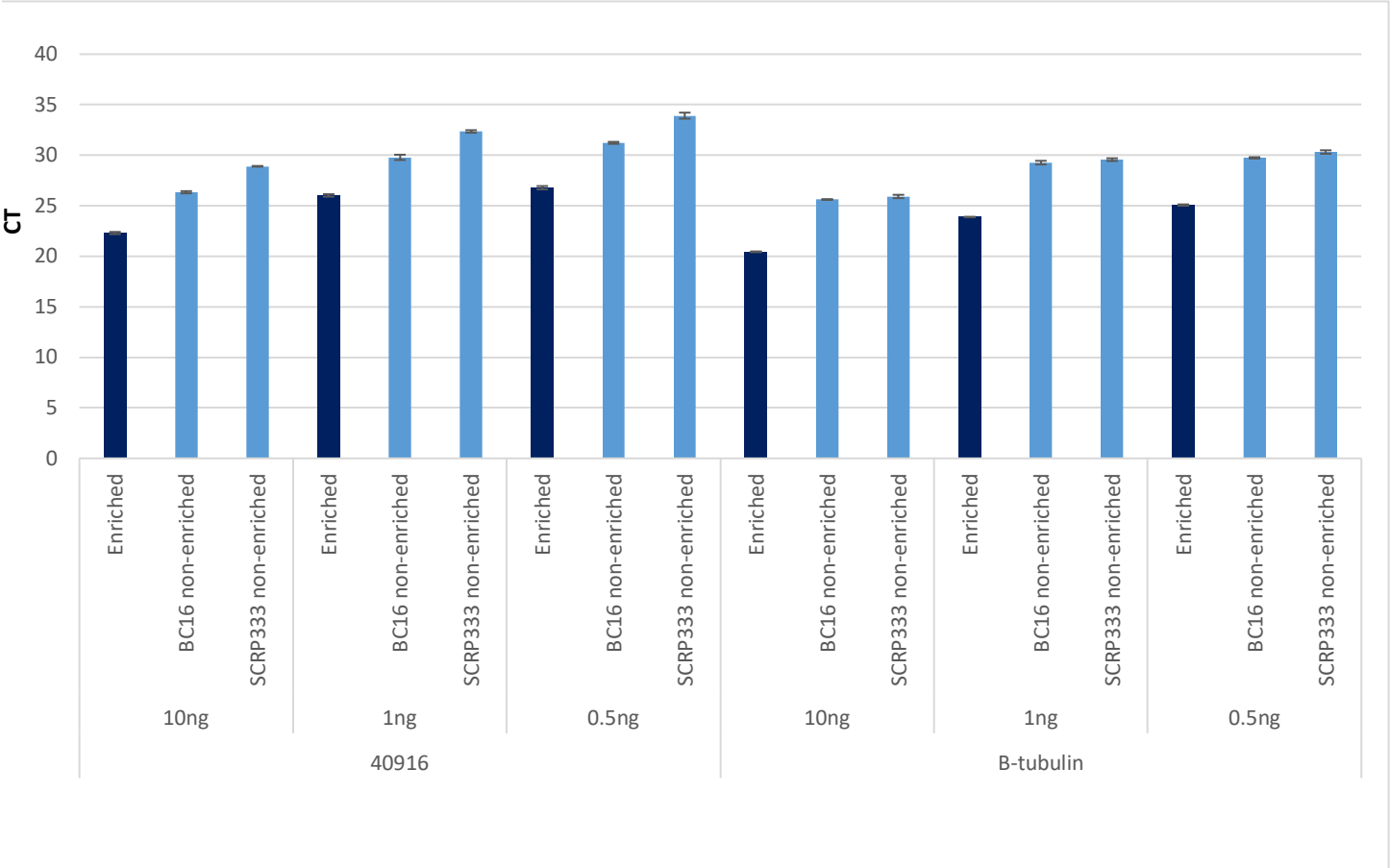

Figure 14: qPCR mean Ct values for enriched (pull of 12 isolates, *P. rubi* and *P. fragariae*) vs non-enriched (*P. fragariae*, BC16 or *P. rubi*, SCRP333) samples

Table 6 (left): Statistical analysis, using ANOVA and Tukeys tests (GenStat) to compare significant differences between mean Ct for enriched and non-enriched samples at different concentrations and for the two genes screened.

Statistical analysis of effect of enrichment		DNA concentration		
gene	sample	10ng	1ng	0.5ng
40916	Enriched	a	a	a
	BC16	b	b	b
	SCRP333	c	c	c
Beta-tubulin	Enriched	a	a	a
	BC16	b	b	b
	SCRP333	b	b	b

Table 7 (right): Statistical analysis, using ANOVA and Tukeys tests (GenStat) to compare significant differences between mean Ct for different DNA concentrations of enriched and non-enriched samples for the two genes screened.

Statistical analysis of effect of concentration		DNA concentration		
gene	sample	10ng	1ng	0.5ng
40916	Enriched	a	b	c
	BC16	a	b	c
	SCRP333	a	b	c
Beta-tubulin	Enriched	a	b	c
	BC16	a	b	b
	SCRP333	a	b	b

Discussion

Hydroponic cultures of raspberry

While raspberries are important and are becoming more and more popular fruit due to their numerous health benefits; growing techniques such as hydroponics are also used more often because of the associated environmental benefits. There is very little research carried out on growing raspberries hydroponically, although it would be interesting to determine what crops are suitable for hydroponics systems. The objective behind hydroponics in this study was to provide easy access to the roots, with minimum damage, to follow disease progression. This can be transposed to numerous studies (root pathogens/nematodes; root colonization or morphology etc.), where roots of hydroponically grown raspberries can be assessed and monitored easily.

This study showed that hydroponic cultures of raspberry cultivars Glen Moy, Glen Dee, Latham and Glen Fyne using the Nutrient Film Technique worked and produced a rotation for the NFT system. However, percentage of rooting cuttings varied greatly between cultivars, with Glen Moy and Latham showing the least satisfying results while Glen Dee was promising.

P. rubi infection and life cycle

This study shows that infection assays using transgenic *P. rubi* in hydroponic were effective and constitute a good method to study root pathogens in conditions allowing clear access and assessment of roots. Transformation did not impact the ability of *P. rubi* to form reproduction and infection structures such as sporangia and zoospores and these were used to infect roots of raspberry plants grown under the nutrient film technique. This gave us an idea of time points to sample and where key infection stages had happened, such as colonization of the central vascular cylinder of the root, formation of sporangia, release of zoospores, production of oospores etc.

P. rubi phenotypic diversity study

Phenotypic diversity studies showed that isolates behaved differently to a change of temperature, with older lab isolates growing significantly better at 18˚C, while field isolates grew similarly at 15˚C and 18˚C. This shows the potential of *P. rubi* to adapt to different and possibly higher temperatures, which could have impacts on the disease prevalence in a future where global warming is a threat. The 'Future work' section discusses the next steps in this project in order to investigate this adaptation and its limits.

Isolates of *P. rubi* from different years and countries grew similarly in media incorporated with chemicals. Dimethomorph showed a better control of the disease compared to Fluazinam across all isolates tested, indicating no diversification. Dimethomorph acts on the biosynthesis of the cell wall and is used in Paraat, commonly applied in diseased raspberry fields. Paraat was released in 2011 while Shirlan (using Fluazinam, acting on the respiration) was released in 1995. This demonstrates the importance of chemical treatments evolution and improvement, along with *in vitro* and field testing of these chemicals, in order to achieve satisfactory levels of control with lower doses of chemicals and less applications. However, rotations of fungicides with different modes of action, as well as good practice combination are an essential component of control.

P. rubi and *P. fragariae* genotypic diversity study: Pathogen Enrichment Sequencing (PenSeq)

Quantitative PCR assays demonstrated the potential for target enrichment sequencing to be used on *P. rubi* and *P. fragariae* isolates. Sequencing of the enriched sample is on-going and

diversity studies, looking at how similar/different isolates are between years and countries will be carried out.

Conclusions

Characterization of *P. rubi* and *P. fragariae* is a very important tool to help understand the biology and genetics of the pathogens. By studying both the phenotype and the genotype of the pathogens, we can draw a parallel analysis between the two and expand our comprehension of the diseases, thus giving us the prerequisite to fight them.

- In this project, raspberries have successfully been grown in hydroponics, and have successfully undergone and recovered from induced dormancy. Details on best cultivars and fertilisers to use for NFT were narrowed down.
- A method to isolate *P. rubi* from canes, rather than roots, has been successfully developed, and produced new recent field isolates to use in this project through various studies
- More insights into the phenotype and behaviour of *P. rubi* were gained when a hydroponic infection was carried out, using a transgenic *P. rubi* expressing a red fluorescent protein. At day 7, infiltration of the roots had occurred and hyphae were colonizing the central vascular cylinder. By day 11, new sporangia had formed, which released new zoospores. The infection cycle was complete, and more sporangia and oospores were produced in the following days, up to day 30.
- *In vitro* experiments investigated the effects of temperature (15˚C and 18˚C) and chemicals (using active ingredients of Shirlan and Paraat) on *P. rubi* and *P. fragariae.* Phenotypic diversity was shown amongst isolates grown under different temperatures. All isolates tested were sensitive to the chemicals, as incorporated media always lead to reduced growth compared to the controls.
- Genetic diversity is currently studied using a recently developed method, called target enrichment sequencing (PenSeq). Quantitative PCR proved that this method could be used on 12 isolates of *P. rubi* and *P. fragariae.* Future work will focus on sequencing he enriched sample to focus on the inter and intra-species diversity.

Future work

Hydroponic raspberries

Maintain hydroponics cultures of raspberries, finding the best conditions under which raspberries grow in hydroponics and artificially put the plants through dormancy to verify their fruiting abilities and further validate the NFT as a growing method for raspberries

***P. rubi* infection and life cycle**

- Repeat the hydroponic infection to get a collection of confocal photos for both susceptible and resistant cultivars.
- Capture infection of roots – using a combination of methods (fluorescent isolate, trypan blue, calcofluor, analine blue etc.)
- Investigate resistance mechanism for Latham: molecular resistance (R genes) or physiological resistance (better root vigour)?
- Test out several RNA extractions methods in order to find a suitable one for the assay – due to the difficulty of getting good quality RNA, especially from woody tissue like roots due to the presence of secondary metabolites, such as polyphenols and polysaccharides, which often interfere with or degrade RNA. Extract RNA from different time points for the successful infection: 1dpi, 3dpi, 7dpi, 11dpi and 14dpi.
- Design primers for life markers such as CDC14 (cell cycle regulator expressed during sporulation), HMP1 (haustoria, marker of biotrophic phase and feeding), INF1(elicitin expressed during necrotrophy in *P. infestans*) and for a few RxLRs, which should be upregulated early to suppress defences and promote biotrophy. Assess and optimize primers
- Perform qPCR using cDNA from the RNA collected at different infection time points, screening for the life markers and RxLRs

Phenotypic diversity of *P. rubi* and *P. fragariae*

- Finish the screening of all 12 PenSeq isolates with a few main chemicals with different mode of actions (Fluazinam, Dimethomorph, Propamocarb, Fluopicolide, Metalaxyl-M principally)
- Extend the temperatures screened to 21˚C, 25˚C and 28˚C. Since 18˚C showed better results for mycelial growth, sporangia production should also be checked at 15˚C, 18˚C, and 21˚C.

Genetic diversity of *P. rubi* and *P. fragariae*

The enriched sample of the 12 isolates will need to be sequenced and analyses regarding the intra and inter-species diversity will be performed.

Knowledge and Technology Transfer

- Poster presentation, London conference (from field to clinic), April 2018.
- Poster presentation, University of Dundee post-graduate student retreat, September 2018

- Poster presentation, BCPC (British Crop Protection Council) conference (Cambridge), October 2018
- Poster presentation, AHDB annual students conference, November 2018
- Poster presentation, University of Dundee annual symposium, March 2019
- SoapBox Edinburgh, “small matters in science!”, June 2019
- Presentation of research at the SEFARI competition (2nd prize for talk and presentation), June 2019
- Poster presentation at MPMI (Molecular Plant-Microbe Interactions) international conference, Glasgow, July 2019
- Exchange of protocols with ADAS for cane isolation, infection, reproduction structures identification; videos of zoospores release and infection

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