Grower Summary

CP 173

Towards a better understanding of the biology and genetics of Phytophthora rubi and Phytophthora fragariae

Annual report February 2019

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*
Previous report:	*Annual report, October 2018*
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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PhD Student
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Report authorised by:
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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.

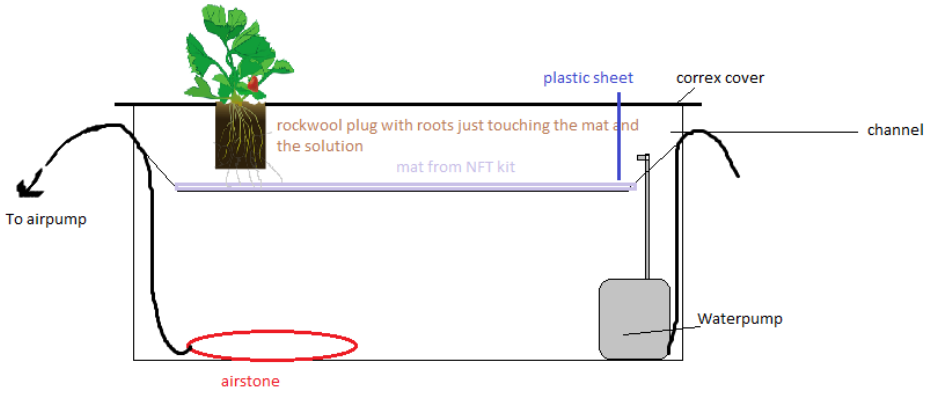

Figure 1: Schematic representation of the NFT set up used in this study.

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.

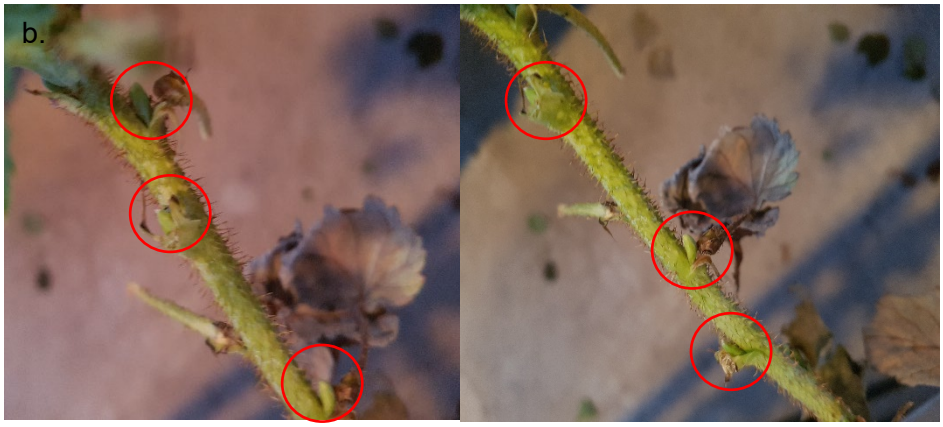

Figure 3: a. fruits and b. buds seen on the plants after 3 weeks stored in the cold glasshouse

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.

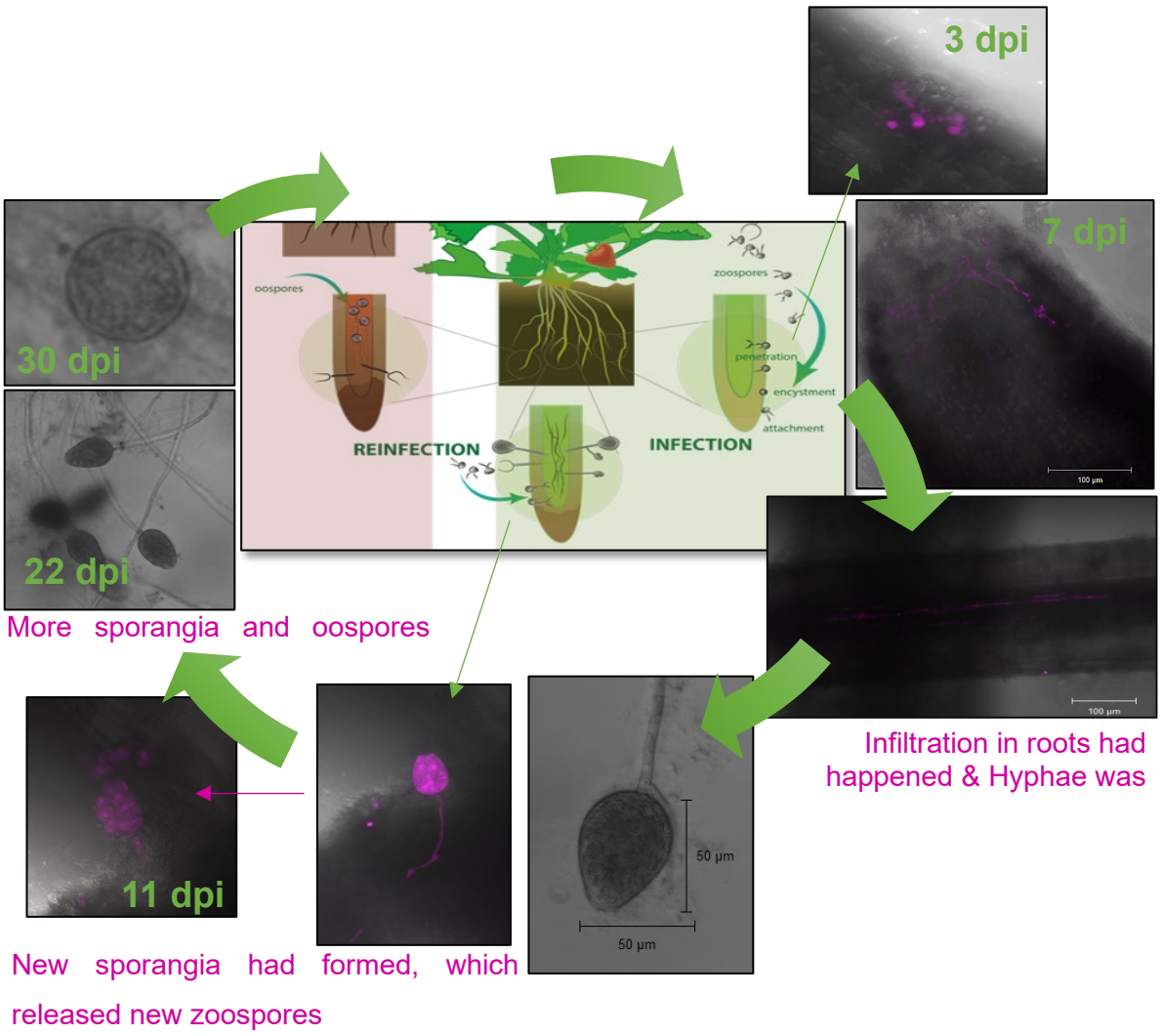

Figure 5: Infection assay using hydroponic raspberries and transgenic *P. rubi* SCRP333-tdT. Photos taken with Zeiss 710 confocal microscope show red fluorescent hyphae growing in the roots. This figure shows that the transgenic *P. rubi* went through its whole infection cycle while in hydroponic conditions.

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.

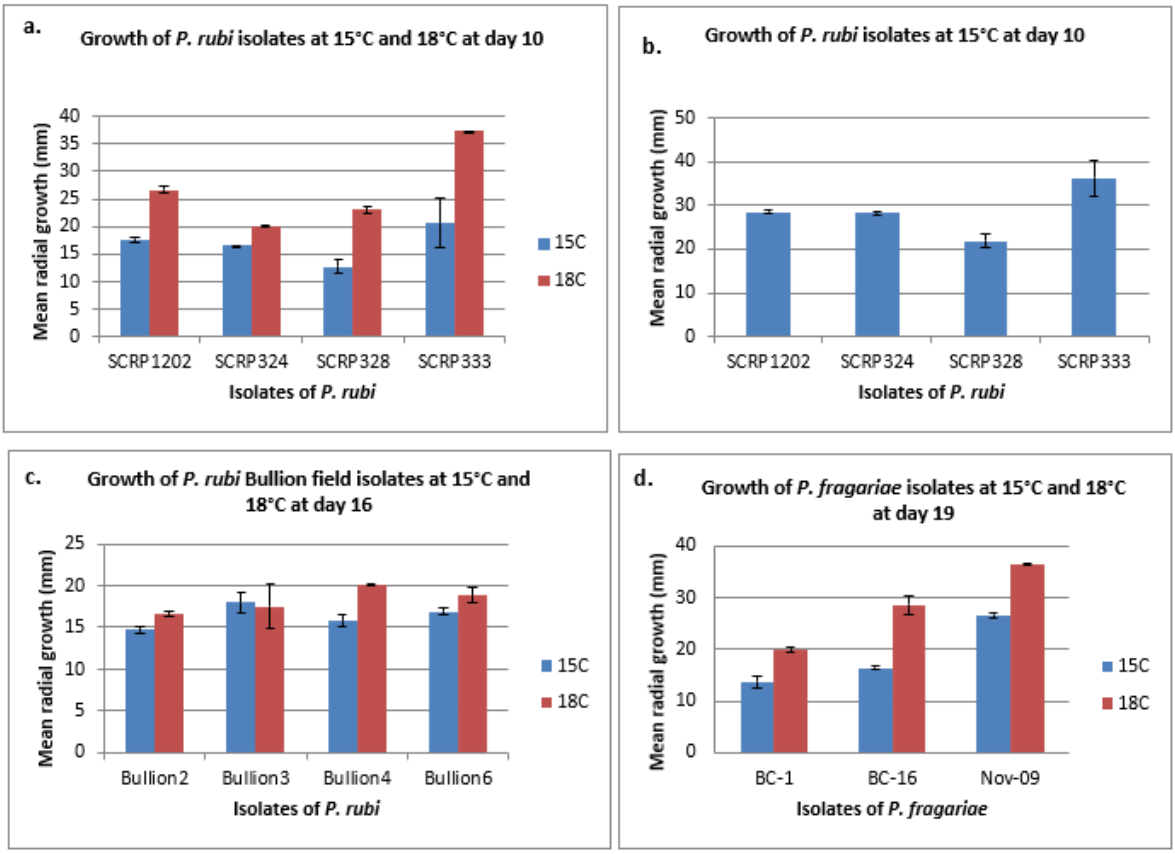

Figure 6: Chart graphs showing *P. rubi* and *P. fragariae* isolates growth under different conditions, at 15˚C and 18˚C. a. Chart showing the mean radial growth of P. rubi SCRP isolates at day 10 for the two temperatures. b. Chart showing the mean radial growth of P. rubi SCRP isolates at day 16 at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

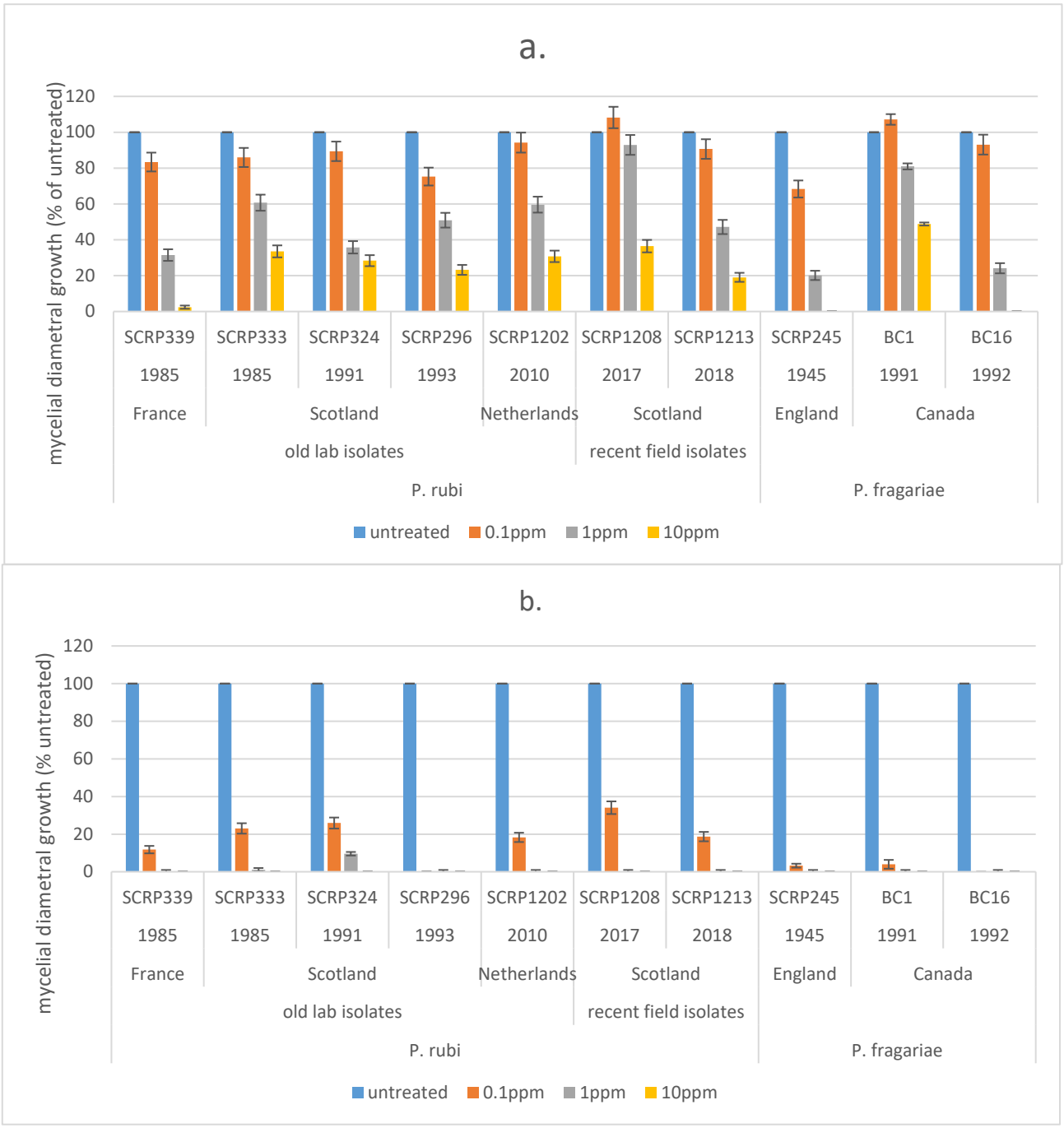

Figure 7: Mean diametral growth (percentage of untreated) of *P. rubi* and *P. fragariae* isolates grown on media incorporated with a. Fluazinam and b. Dimethomorph for 7 days

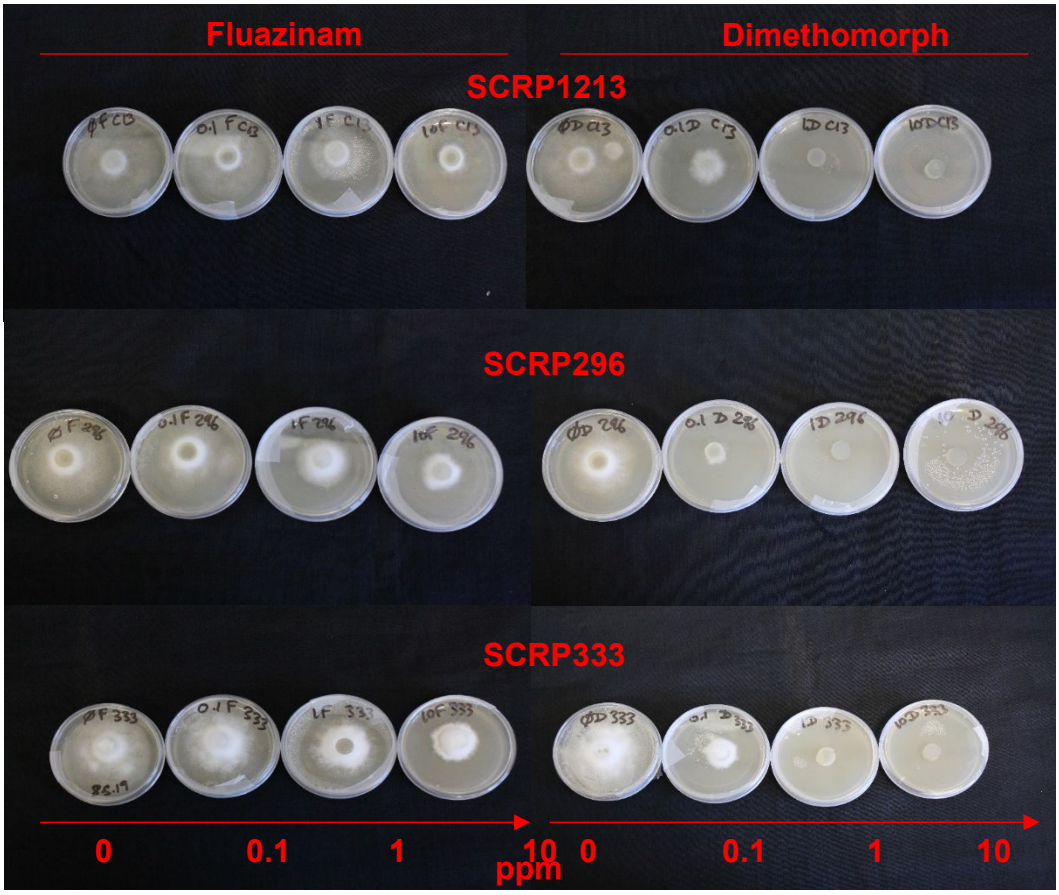

Figure 8: Photos of 3 different *P. rubi* isolates growing on plates incorporated with Fluazinam and Dimethomorph at a range of doses (from 0 to 10ppm). Photos taken at day 9

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.

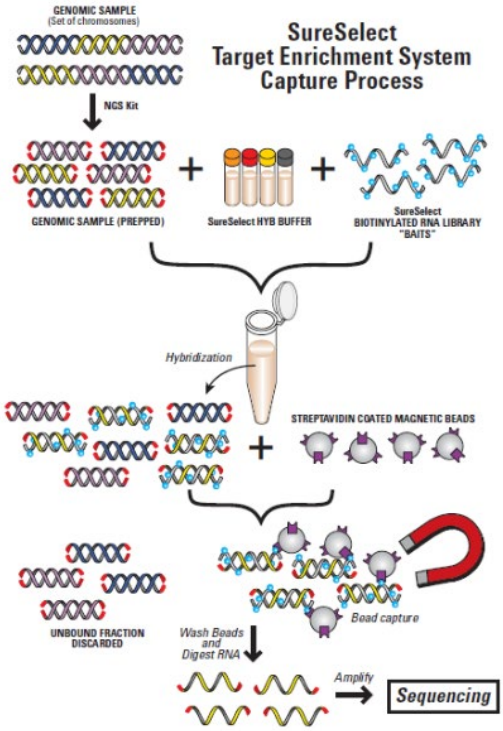

Figure 9: Target Enrichment Sequencing principle

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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