Project title:	**Towards a better understanding of the biology and genetics of *Phytophthora rubi* and *Phytophthora fragariae***
Project number:	CP173
Project leader:	Eleanor Gilroy, The James Hutton Institute (JHI), Dundee
Report:	Annual report, October 2018
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Key staff:	Aurelia Bezanger (PhD student , author of the report), Steve Whisson, Lydia Welsh, JHI
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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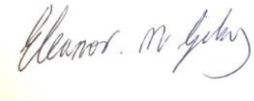
Signature  Date .....23/10/18.....

CONTENTS

GROWER SUMMARY

Headline

- New insights into the biology of raspberry root rot

Background and expected deliverables

Phytophthora rubi (raspberry root rot) and *Phytophthora fragariae* (strawberry red core/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. The best control strategy relies on prevention 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 of the project and main conclusions

Hydroponic raspberries

In this project, raspberries have successfully been grown in hydroponics, using a Nutrient Film Technique (NFT). After dipping raspberry cuttings in a rooting hormone, they were stuck in rockwool plugs and soaked in nutrient solution at a correct pH (5.2 – 5.8). Once roots appeared and grew long enough, the plugs were transferred onto the NFT channel. Healthy roots and shoots continued to grow, and the root mat developed shoots that were placed inside rockwool media before being re-introduced into the hydroponics rotation once they grew sufficiently. Raspberry plants will be left to grow over the winter and fruiting will be assessed. Cuttings are produced on a regular basis.

P. rubi isolation from canes

In work to isolate *P. rubi* from cane material rather than roots, a protocol adapted from Stewart et al., 2014, used 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 from canes, it was morphologically checked to narrow it 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 to confirm that the isolate was indeed *P. rubi*. This method was proven successful when field isolates of *P. rubi* were taken from a field in Dundee. The method was repeated twice using samples collected in East Scotland. Preliminary morphological assessment suggested *P. rubi* and molecular confirmation is on-going.

P. rubi phenotype study

Effect of temperature

Several lab isolates and field isolates of *P. rubi* and *P. fragariae* were grown at 15˚C and 18˚C. 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.

Sporangia and zoospores production

Of the 14 different types of sporulation solution tested, a soil water solution worked best in producing *P. rubi* sporangia and zoospores. The less sterile the solution, the more sporangia were produced, implying that this bacterial metabolite is highly related to the success of *P. rubi* reproduction. Swimming zoospores were successfully released. *P fragariae* kept producing full sporangia and releasing zoospores over a course of 20 days, demonstrating the extended period during which the pathogen can produce reproduction and infection structures.

Petiole inoculation for rapid resistance screening

A method adapted from Li et al., 2017 was tested for a rapid screening for resistance in raspberry cultivars. The method used raspberry petioles (rather than setting infection on roots which can be more difficult and time consuming) from Glen Moy and Latham cultivars. Petioles were set in Eppendorf tubes containing *P. rubi* mycelia slurry and put in a sealed box where the humidity was kept at an optimum level. Petioles and leaves were monitored for 3 weeks to assess symptoms. Eleven days post inoculation was found to be best to observe symptom differences, where Glen Moy leaves showed yellowing and decay sooner than the Latham ones. This method needs to be further explored in order to test for other potential *P. rubi* hosts (tayberry, blueberry etc.)

Bio-informatics and baits library design

New bio-informatics technologies are more and more popular. The Target Enrichment Sequencing will be used in this project and aims to study the 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. This library will help in assessing the pathogen’s diversity.

Main conclusions

So far in this project:

- Raspberries have successfully been grown in hydroponics
- A method to isolate *P. rubi* from canes, rather than roots, has been successfully developed
- More insights into the phenotype and behaviour of *P. rubi* were gained by *in vitro* experiments (effects of temperature and reproduction structures of *P. rubi* and *P. fragariae*)
- A method has been tested for a rapid screening for resistance in raspberry cultivars and potential *P. rubi* hosts
- A baits library was designed to be used in a bio-informatics assay to study *P. rubi* and *P. fragariae* effectors

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 field production having been lost to the disease. This pathogen causes major economic and environmental damage 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 for growers

This project seeks to inform longer-term research of *Phytophthora rubi* and is unlikely to deliver direct action points for growers.

References

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STEWART, J. E., KROESE, D., TABIMA, J. F., LARSEN, M. M., FIELAND, V. J., PRESS, C. M., ZASADA, I. A. & GRÜNWALD, N. J. (2014). Pathogenicity, fungicide resistance, and genetic variability of *Phytophthora rubi* isolates from raspberry (*Rubus idaeus*) in the western United States. Plant Disease, 98, 1702-1708

SCIENCE SECTION

Introduction

Raspberries 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 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 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. Ongoing work aims at screening cultivars of raspberries and other *Rubus* species to identify sources of resistance to the pathogen. 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 to cover regarding the biology, genetics and infection lifecycle of *P. rubi*.

Materials and methods

Raspberry cultures

Cultures of raspberry cultivars Glen Moy and Latham were obtained from Alison Dolan at The James Hutton Institute and are certified Pre-Basic (High Health), which is the highest grade in the EU Certification Scheme for Fruit Plants thus ensuring they were free of infection by *P. rubi* prior to research work commencing. Glen Moy is known to be the most susceptible cultivar to PRR whereas Latham is the most tolerant. Glen Moy and Latham were used as reference cultivars. Cultivar Glen Fyne was also used and provided by Alison Dobson but was not certified Pre-Basic. All raspberry plants were 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.

Hydroponics

Summary of hydroponics equipment and chemicals

Nutrient Film Technique (NFT) hydroponics kits containing the reservoir, channel, correx plastic cover, water pump and fibre mat were supplied by ProGrow Hydroponics UK. The different sizes of rockwool plugs and the rockwool transfer blocks were also supplied by ProGrow Hydroponics. Fertilizers and nutrients used were Kristalon Red (Yara Tera), an NPK 1:1:1 nutrient and Maxicrop. Corbel fungicide was used to treat raspberries against powdery mildew.

All raspberry plants, cuttings and hydroponics tanks were kept in a greenhouse with 16 hours lighting, at 20˚C, and 8 hours with no artificial lights at 18˚C.

Starting with cuttings in rockwool plugs

Four different assays were conducted to get cuttings to root in rockwool plugs. The first assay started in December 2017 (Dec assay), the second one in February / March 2018 (Feb assay), the third assay was carried out in May / June 2018 (May assay) and the last one in August 2018. Rockwool was used as a substrate to grow raspberry cuttings. Two different sizes of rockwool plugs were used. Before use, rockwool plugs were soaked overnight in a weak nutrient solution with the pH adjusted between 5.5 and 6.2 using phosphoric acid. Three different nutrient solutions were tested. Some rockwool plugs were soaked in tap water with and without the pH adjusted. Cuttings were taken from raspberry cultivars, mostly Glen Moy and Latham but also Glen Fyne. Soft wood and clear stem were used as cuttings, with 2-3 internodes. The bottom part of the stem of the fresh cutting was dipped into Clonex, a rooting hormone, before being anchored into the rockwool plug. Rockwool plugs with fresh cuttings were placed in a mist unit, where they were watered through mist (misting for 15 sec every 20 min in daylight hours). In the Dec assay, some cuttings were placed in sand under a mist unit (on a 20 mins cycle) for 4 weeks, until they developed a callous, after which they were placed in the rockwool plug under the same conditions described above.

Transferring the rooted cuttings into the hydroponics tank

Hydroponics tanks were filled (16L) with water where a weak nutrient solution was added. The pH was then adjusted to be between 6.0 and 6.5 using phosphoric acid. The water pump, airstone, and airpump were added (Figure 1). The fibre mat provided in the kit was cut and placed into the channel to help the nutrient solution spread more homogeneously.

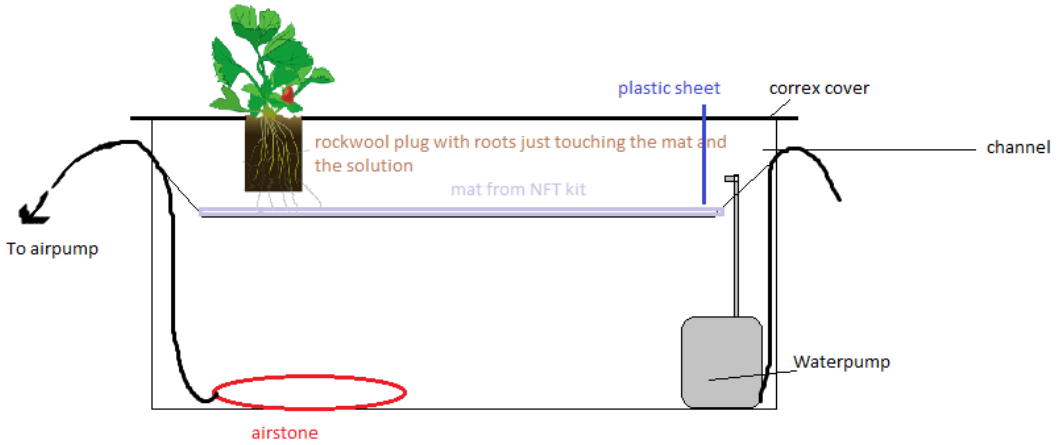


Figure 1: Schematic representation of the NFT set up used in this study. The figure shows a water pump being used to circulate the nutrient solution onto the channel placed above the reservoir and an airpump and airstone used or maximizing oxygen content. The correx cover is a plastic cover which provides support for the rockwool (holes are cut to let the plug in) and provides shade to the roots. The mat provided with the NFT kit helps to spread the nutrient solution more homogeneously onto the channel.

Holes of appropriate size were cut in the correx cover in order to put the rockwool plugs in, so that the roots would be dipped in the nutrient solution. A slit was cut in the correx cover, near the water pump outlet and another plastic/correx sheet was placed in so that the water would disperse across the sheet and spread more homogeneously into the channel. Once the cuttings had rooted through the plug, they were transferred to the hydroponic tank. There were two ways to move them to the NFT tanks. First, the plug was moved directly to the channel, so that the roots were touching the mat in the channel and had access to the nutrient solution. Secondly, the plug was moved to a bigger rockwool block (known as transfer block), that had previously been soaked in nutrient solutions. Blocks were placed in the mist unit for a week and then moved to the NFT channel to encourage root growth. Once they have rooted through the transfer block, the cuttings were moved in the NFT channel, directly resting on the mat, and the correx cover was used to provide shade for the roots by cutting holes for the above parts of the cuttings. Finally, a black cover was placed on top of the cuttings, with holes to let the stems and leaves out, in order to provide extra shade for the roots and minimize light exposure.

Growing raspberry in hydroponics

When shoots were produced from the hydroponics plant root mat, they were transferred into rockwool soaked in a weak nutrient solution. The rockwool was then placed either in the mist unit or on the channel of an NFT tank set up with a weak nutrient solution.

Cultures of *Phytophthora* species

Phytophthora rubi and Phytophthora fragariae isolates

Eight different isolates of *P. rubi* and four isolates of *P. fragariae* were used for this study. Details are given in Table 5. Stocks for isolates SCRP249, SCRP245, SCRP333, SCRP324, SCRP328, SCRP1202, BC-1, BC-16, NOV-9, BF 2, BF 3, BF 4 and BF 6 were kept on slopes of Rye agar with ampicillin (antibiotic) at the James Hutton Institute. The other isolates listed in Table 1 were kept at NIAB-EMR. Agar slopes were kept at 18˚C in the dark until sufficient growth appeared, after which they were moved to 4˚C in the dark.

Table 1: Table of all *P. rubi* and *P. fragariae* isolates used in the study

Species	Isolates (**bold**: main isolates used throughout this study; others used mainly for bio-informatics)	Race	Host	Country (and Region) originally isolated from	Date originally isolated	Origin of isolation / Location	Other code found in literature
Phytophthora rubi	**SCRP249**	Unknown	*Rubus idaeus*	Germany	1985	NIAB-EMR[3]	R37[1]
	SCRP333	Race 3		Scotland	1985	The James Hutton Institute[1] cultures stock	R49[4], P822[5]
	SCRP324	Race 1		Scotland	1991		R200[4], P823[5]
	SCRP328	Race 1		France (Cote d'Or)	1989		R188[4], 1043.89[6]
	SCRP1202	Unknown		The Netherlands	Unknown	Niklaus J. Grünwald[2],	Pd0101050015038

						American strain	
	BF 2			Scotland	2017	Isolated from raspberry field in Dundee	
	BF 3						
	BF 4						
	BF 6						
	BF 8						
	SCRP247			Wales	1984	The James Hutton Institute[1] cultures stock	R3[4]
	SCRP256			Wales	1986		R59[4]
	SCRP258			England	1986		R62[4]
	SCRP266			Scotland	1986		R86[4]
	SCRP268			Ireland	1986		R98[4]
	SCRP272			Isle of Man	1988		R121[4]
	SCRP274			Norway	1988		R128[4]
	SCRP278	Unknown		USA	1987		R134[4]
	SCRP281			Canada	1987		R142[4]
	SCRP288			Denmark	1989		R183[4]
	SCRP290			France	1989	Alain Baudry	R186[4]
	SCRP295			Holland	1993		R214[4]
	SCRP296			Scotland	1993	The James Hutton Institute[1] cultures stock	R220[4]
	SCRP301			Norway	Unknown	Olsson	CH137 (Olsson)
	SCRP307			Sweden	Unknown	Olsson	CH151 (Olsoon)
	SCRP313			Australia	Unknown	McGregor	Knox2 (Cooke)
	SCRP322	Race 1		Norway	1989		R189[4]
	SCRP335	Race 3		USA	1987		R136[4]
	SCRP336	Race 3		Canada	1987		R138[4]
	SCRP339	Race 3		France	1985	Alain Baudry	R185[4]
Phytophthora fragariae	**SCRP245**	Unknown	*Fragaria* × *ananassa*	England (Kent)	1945	The James Hutton Institute[1]	168[4]

						cultures stock	
	BC-1	CA1			1991		
	BC-16	CA3			1992		
	BC-23	CA5			1992		
	NOV-9	CA2			1986		
	NOV-5	CA1		Canada	1992	NIAB-EMR[3]	
	NOV-27	CA2			1986		
	NOV-71	CA2			1986		
	NOV-77	CA5			1994		
	A4	US4			Unknown		
	ONT3	CA4			1990		

[1] The James Hutton Institute, Errol Road, Dundee, DD2 5DA, Scotland

[2] Horticultural Crops Research Laboratory, USDA-ARS, Corvallis, OR 97330, USA

[3] NIAB EMR, New Rd, East Malling, West Malling, ME19 6BJ, England

Media and conditions of culturing

For each experiment, different media were used to culture *P. rubi* and *P. fragariae* (Table 2). Media was used with (selective media) or without (basic media) antibiotics. For every experiment, cultures were placed in the dark, at 18˚C, except for the temperature comparison assay, where cultures were placed at both 15˚C and 18˚C.

Table 2: Details of media and conditions of culturing for the different experiments carried out in this study

Experiment name	**Media used to grow *P. rubi* and *P, fragariae* cultures**
Stocks	Rye agar with ampicillin (Sigma)
Temperature comparison	For BF isolates, Rye agar with ampicillin and vancomycin (Melford Laboratories)
	For SCRP1202, SCRP333, SCRP245, SCRP324, SCRP328, rye agar with ampicillin
	For SCRP333_tdT*, Rye agar with ampicillin and geneticin (Sigma) (at 10µg/ml) **SCRP333_tdT is SCRP333 isolate successfully transformed with the fluorescent tdTomato protein*
	For isolates from NIAB-EMR: BC-1, BC-16 and NOV-9, Rye agar with ampicillin and pimaricin (Sigma)
Sporulation	FBA (French Bean Agar)
Cane isolation	- Italian media (V8 juice, water and calcium carbonate,pH=7) with pentachloronitrobenzene or PCNB (Sigma), pimaricin, rifampicin (Sigma), nystatin (Melford Laboratories), hymexazol (VWR) and ampicillin - Rye agar with ampicillin and pimaricin
Petiole inoculation - cultures	- Liquid Lima Bean with ampicillin

Temperature assay

P. rubi SCRP1202, SCRP324, SCRP328, SCRP333, SCRP333_tdT, BF 2, BF 3, BF 4, BF 6 and *P. fragariae* BC-1, BC-16, NOV-9 isolates were grown on 90mm diameter plates of Rye agar containing different antibiotics. Isolates were incubated at 15˚C and 18˚C in the dark. Plates were regularly checked, and radial growth measured along two axes. The experiment was stopped when some isolates reached the edge of the plate and data of the previous measurement was used for statistical analysis. ANOVA and Tukey tests were carried out using R.

Isolation from field (cane isolation)

Infected canes were collected from a raspberry field (cultivar Glen Dee) in April and August 2018. More canes collected in Oxfordshire in August 2018 by Ruth D'Urban Jackson at RSK-ADAS were sent to the James Hutton Institute. Isolation from canes was carried out using a protocol adapted from Stewart et al., 2014, 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 to confirm for *P. rubi*.

***P. rubi* sporangia and zoospores production**

Cultures of *P. rubi* SCRP333 were set up using three plugs of cultures on French Bean Agar (FBA) plates. Plates were incubated at 18°C in the dark for a week. Sporangia were produced using 14 different types of sporulation solutions and a control (tap water) (Table 3). After a week of growth, ten plugs of cultures were placed into a 140mm diameter Petri dish, covered with the different solutions and incubated at 15°C in the dark. Zero, one or two changes of the solutions were done in the following 24 hours according to the "sterility" of the solution (the more sterile, the more changes were performed). Plates were then left to grow a further four days under the same conditions. Four days later, sporangia were counted by observation under a microscope.

Once sporangia were counted, sporulation solutions were pipetted off and Petri's solution was added to the plates to release zoospores. Petri's solution was renewed regularly.

Table 3: Table detailing the types of sporulation solutions used to produced *P. rubi* sporangia and zoospores

Sporulation solution name	Solution content	Solution sterilization method
NFT1.ff	Water from hydroponic tanks	Filtered twice through 0.2µm filter
NFT1.f		Filtered once through 0.2µm filter
NFT1		None
NFT1.a		Autoclaved
RW.ff	Rain water	Filtered twice through 0.2µm filter
RW.f		Filtered once through 0.2µm filter
RW		None
RW.a		Autoclaved
SW.fpf	Soil water (compost and water) made before infection	Filtered twice through filter paper and once through 0.2µm filter
SW.f		Filtered once through filter paper and once through 0.2µm filter
SW		None
SW.a		Autoclaved
LW.SW.f_1	Soil water (compost and water) made month before infection and frozen	Filtered once through filter paper and once through 0.2µm filter. Sporulation solution was made by Lydia in June 2017 and frozen. Solution was changed once
LW.SW.f_2		Filtered once through filter paper and once through 0.2µm filter. Sporulation solution was made by Lydia in June 2017 and frozen. Solution was changed twice
Tap H2O	Tap water – negative control	none

Once sporangia were counted, the sporulation solution was pipetted off and Petri's solution (KCl 1mM, Ca $(NO_3)_2$ 2 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1 mM) was added to the plates to release zoospores. Petri's solution was renewed regularly. Plates were incubated at 15°C in the dark after each Petri's solution change.

***P. fragariae* sporangia and zoospores production**

Cultures of *P. fragariae* NOV-9 were set up using three plugs of cultures on Rye Agar (FBA) with antibiotics plates. Plates were incubated at 18°C in the dark for two weeks. Sporangia were produced using 4 different types of sporulation solutions: NFT1, NFT1.f, SW and SW.f (Table 7 in section 2.5.1.). After two weeks of growth, ten plugs of cultures were placed into a 140mm diameter Petri dish, covered with the different solutions and incubated at 15°C in the dark. Sporulation solutions were changed twice. Plates were then left to grow a further three days under the same conditions. Three days later, sporangia were counted by observation under a microscope.

Once sporangia were counted, the plates were left for a further 6 days in the dark at 15°C; after which they were placed for 30mins at 4°C. Then, sporulation solutions were pipetted off and Petri's solution was added to the plates to release zoospores. Petri's solution was renewed regularly.

Petiole inoculation

A petiole inoculation was carried out following the method from Li et al. (2017) (See Figure 2).

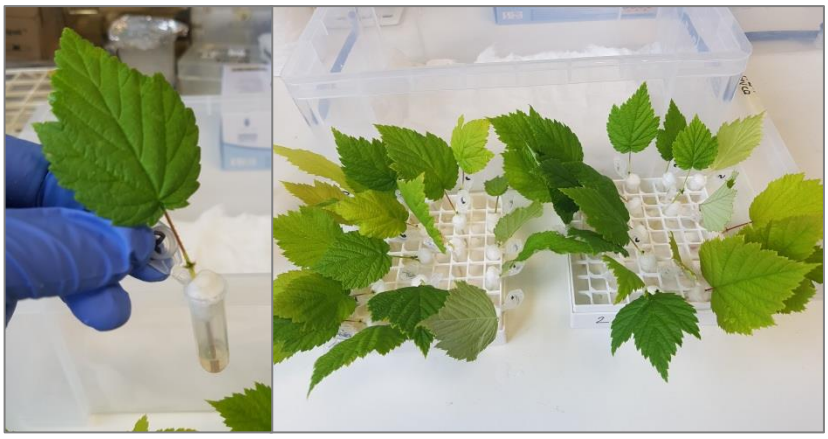


Figure 2: Photos of petiole inoculation set up on raspberry cultivars according to the Li et al. (2017) protocol.

Petioles were taken from high health Latham and Glen Moy plants grown in the glasshouse. *P. rubi* isolates SCRP333 and BF 4 were grown for 10 days at 18°C in the dark in liquid Lima

Bean. After which the mycelia were mixed in a blender to make mycelial slurry. A syringe was used to inject 0.5mL mycelial slurry to the bottom of a 2mL Eppendorf tube. Controls were set up using 0.5mL of liquid Lima Bean in Eppendorf tubes. Petioles were inserted into the tubes containing the mycelial slurry. Seven detached petioles were analysed for each cultivar. Wet cotton wool was used to fix the petioles in the tubes and to seal them. The tubes were placed in a cryogenic storage box, which was then placed in a plastic box containing a water-soaked cotton wool layer at the bottom in order to maintain a moist environment. The box was then closed and sealed in an autoclaved bag before being incubated at 19°C. Control tubes used petioles incubated in liquid lima bean. Petioles from the raspberry cultivars Glen Moy and Latham were used for this study. Petioles were examined for symptoms at 4, 5, 7, 9, 11 and 16 days post-inoculation.

Bioinformatics

Target Enrichment Sequencing

The Target Enrichment Sequencing (PenSeq) will be used in this project and aims at studying the diversity of *P. rubi* and *P. fragariae*, both inter and intra-specifically. This method enables the hugely parallel identification of presence/absence and sequence polymorphisms in avirulence genes, which is a prerequisite for predicting host resistance durability. Importantly, we have shown that this method can be applied to infection derived cDNA samples to prioritise analyses of effectors that are expressed. Target Enrichment Sequencing relies on amino acid sequences, called “baits”, used to target predicted effectors (pathogenic proteins).

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, Ioos et al., 2006) and using NCBI. A list of effectors (RXLRs, CRNs and apoplastic) for eleven different *P. fragariae* isolates (see table) and three different *P. rubi* isolates (SCRP249, SCRP324 and SCRP333) was provided by NIAB-EMR. The compiled list was sent to Arbor Bioscience to design and produce the baits from the nucleotide sequences.

Statistical analyses

All statistical analyses were carried out using R and R studio software. Differences were considered significant when p-value was lower than 0.05 (95% confidence interval). When

the p-values were lower than 0.01, differences were considered highly significant, and when p-value was lower than 0.001, they were very highly significant. ANOVA (Analysis of Variance) and Tukey HSD tests were performed.

Results

Hydroponic cultures of raspberry

Rockwool plug substrates for cuttings

Results from the December assay showed that fresh soft-wood cuttings dipped into Clonex rooting hormone worked best, as opposed to cuttings put into sand for callous formation. The rockwool plug pH was found to be best when set up between 5.5 and 6.2, although rockwool plugs soaked in water with a pH of 3.5 still showed good rooting results. Both MaxiCrop and water-soaked plugs allowed developments of roots. Figure 3.a shows the time (number of weeks) between cuttings being taken and set in rockwool plugs and cuttings rooted; ready to be moved into NFT tanks for the December assay. Figure 3.b shows the ratio of cuttings being used in hydroponics (that have rooted) on total cuttings taken.

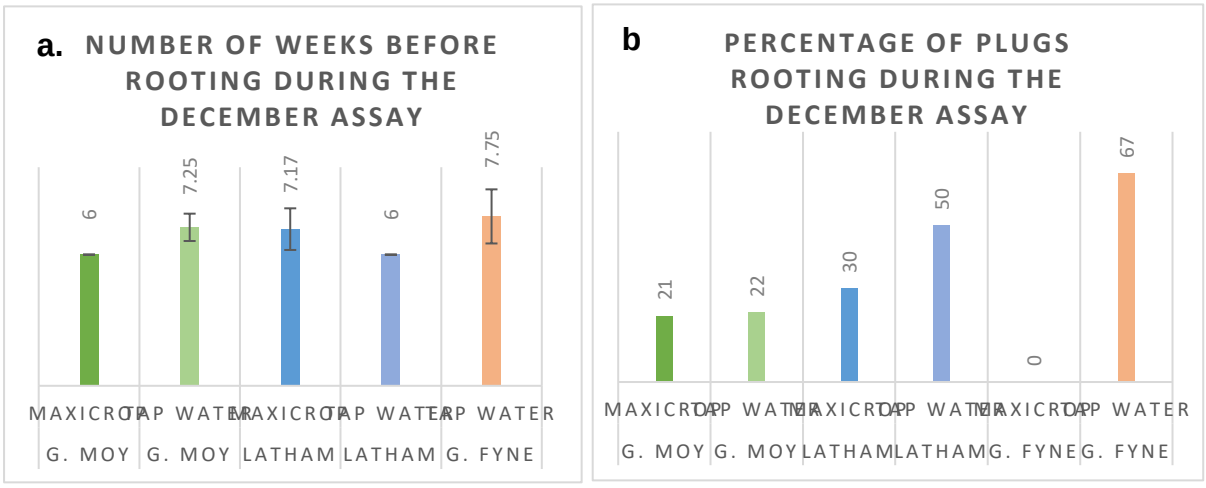


Figure 3: Chart showing data for the December hydroponics assay. a. Number of weeks it took for cuttings to root. b. Percentage of cuttings that survived to produce roots and were able to be used in hydroponics tanks. Figures show data per cultivar and nutrient solution used to soak the rockwool plugs.

The February assay used Kristalon Red and 1:1:1 NPK fertiliser, as these were the ones used in the NFT tanks. MaxiCrop was avoided as an NFT reservoir feed as it is algae based and build up was to be avoided. Both Kristalon Red and NPK allowed root development but the results were not as good as for the December assay (Figures 4.a and 4.b.). Cuttings taken in February and March were found to be smaller than the December ones, therefore other assays in May/June and later in August were carried out taking bigger cuttings and the results are currently being analysed.

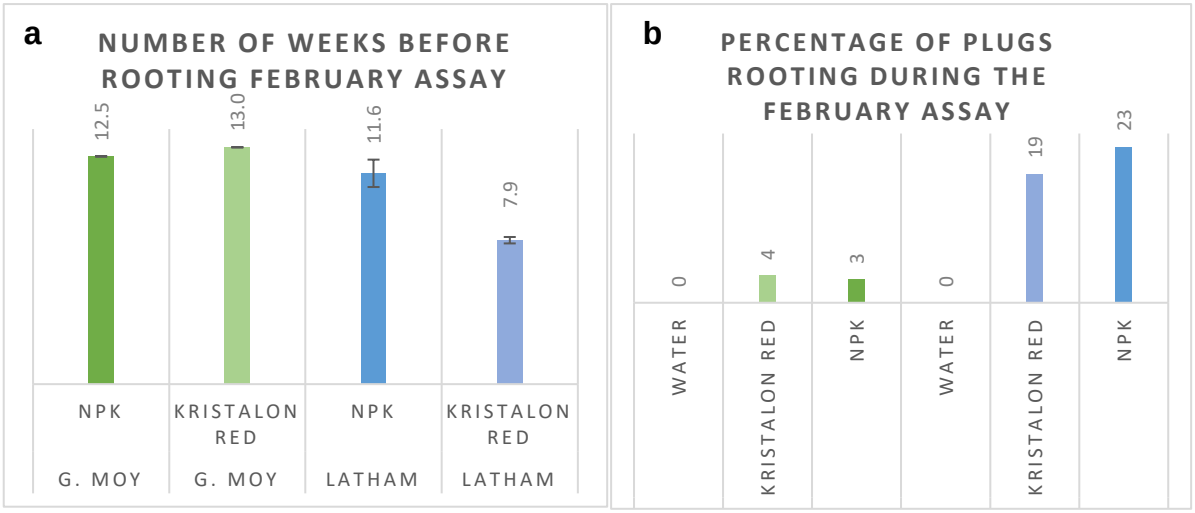


Figure 4: Chart showing data for the February hydroponics assay. a. Number of weeks it took for cuttings to root. b. Percentage of cuttings that survived to produce roots and were able to be used in hydroponics tanks. Figures show data per cultivar and nutrient solution used to soak the rockwool plugs.

Both assays (Dec and Feb) showed that growing raspberry in hydroponics from cuttings grown in rockwool substrate was possible. Roots produced from the plants once transferred to NFT tanks looked very healthy and vigorous (Figure 5). More cuttings were taken in June and July and results are on-going.

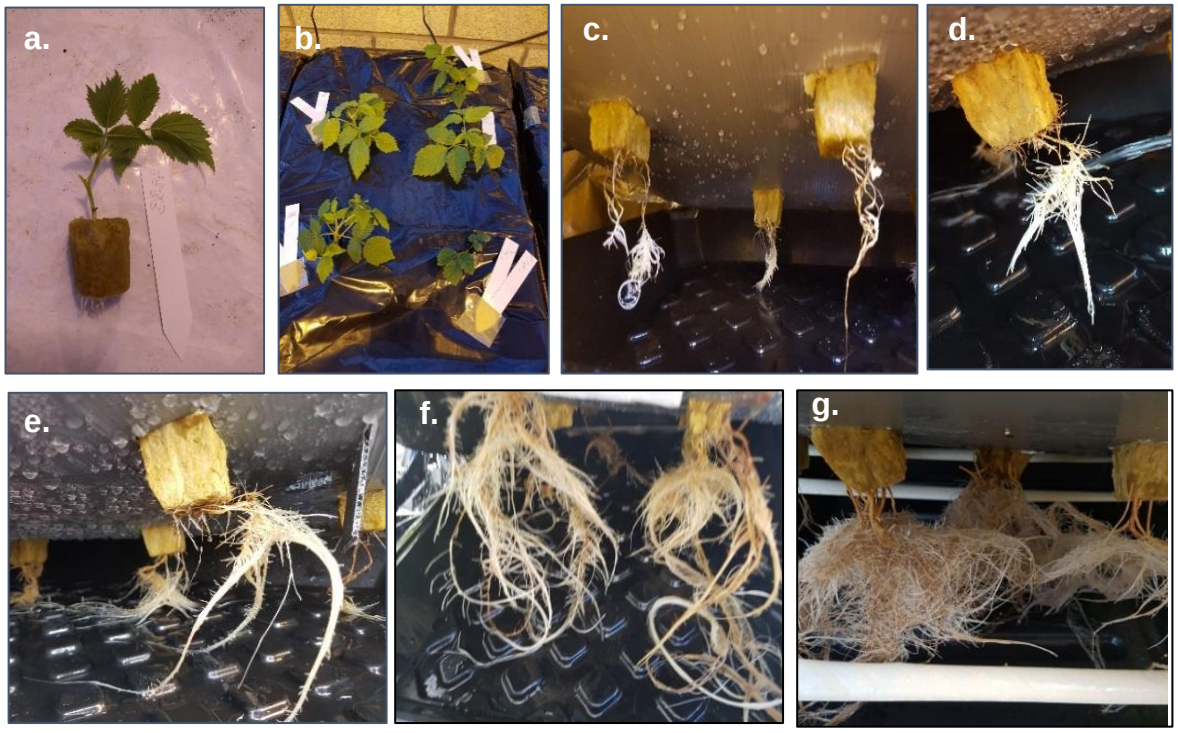


Figure 5: Progress of hydroponic-raspberry cultures. a. First rooted plug 31.01.18. b. Hydroponic tank on 12.02.18. c. Rooted raspberries on the 12.02.18. d. Rooted raspberries

on the 15.02.18. e. Rooted raspberries on the 26.02.18. f. Roots on the 26.03.18. g. Roots before infection on the 11.04.18

Raspberry plants in rockwool blocks

Some rockwool plugs, when roots appeared, were transferred into blocks and put in the NFT channel. This was to allow growth of the raspberry plant and compare with growth in smaller rockwool plugs put in NFT. Results showed that roots appeared through the rockwool block one to three weeks after the small rockwool plug had been transferred. Roots developed very well in the NFT on the blocks, forming a root mat (Figure 6). The foliar parts of the raspberry also grew well, producing new growth that was taken for cuttings (Figure 7). These cuttings were treated like the previous ones (see 2.2.) and one started to show roots through the plug after five weeks. The root mat in the NFT channel started to develop small raspberry shoots (Figure 6), which were put in nutrient-soaked rockwool plugs as described in 2.2.4. The survival of these shoots is still being assessed.

Figure 6: Photo of the rockwool block in the hydroponic tank channel. Photo a shows the root mat formed. Photo b shows the shoots emerging from the root mat.

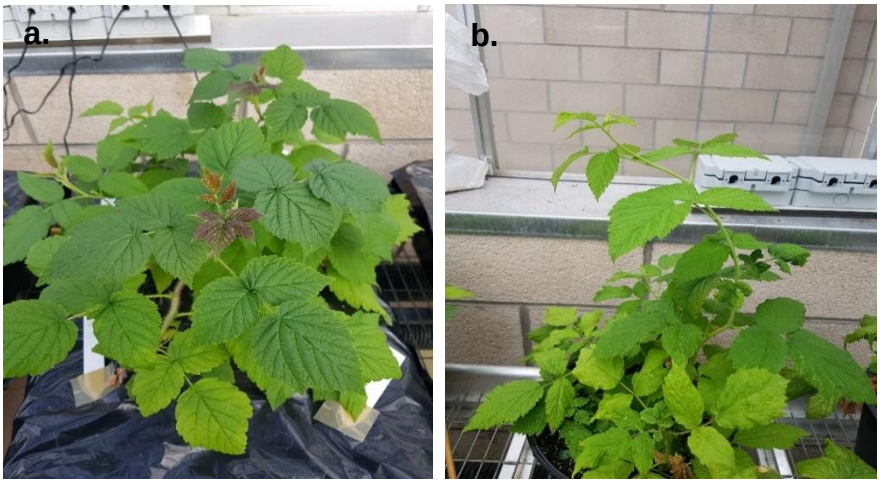


Figure 7: Image of the foliage from hydroponic tank using rockwool transfer blocks. Photo a was taken on the 29.03.18 and photo b was taken on the 12.06.18.

This demonstrates the feasibility of growing raspberry plants in hydroponics and of the implementation of a rotation.

***P. rubi* and *P. fragariae* temperature comparison assay**

Figures 8.a to d. show the radial growth for isolates at the day where statistical analysis was carried out. Final measurements for *P. rubi* isolates from JHI was taken at day 10, as a few days after, most of the isolates kept at 18˚C had reached the edge of the Petri dish (Figure 8.a). However, isolates grown at 15˚C grew slower, and final data was taken at day 16, to assess growth differences between isolates at one temperature (Figure 8.b). Final measurements for BF isolates were taken at day 16, as these strains grew slower than the lab isolates (Figure 8.c). The growth of *P. fragariae* isolates from NIAB-EMR was measured until day 19 (Figure 8.d). As SCRP333-tdT (transformants expressing the tdTomato gene) was grown on a different media from the other SCRP isolates, containing geneticin, only a comparison between the two temperature could be performed, and growth was not statistically compared to other SCRP isolates. Similarly, BF isolates were not statistically compared to SCRP isolates.

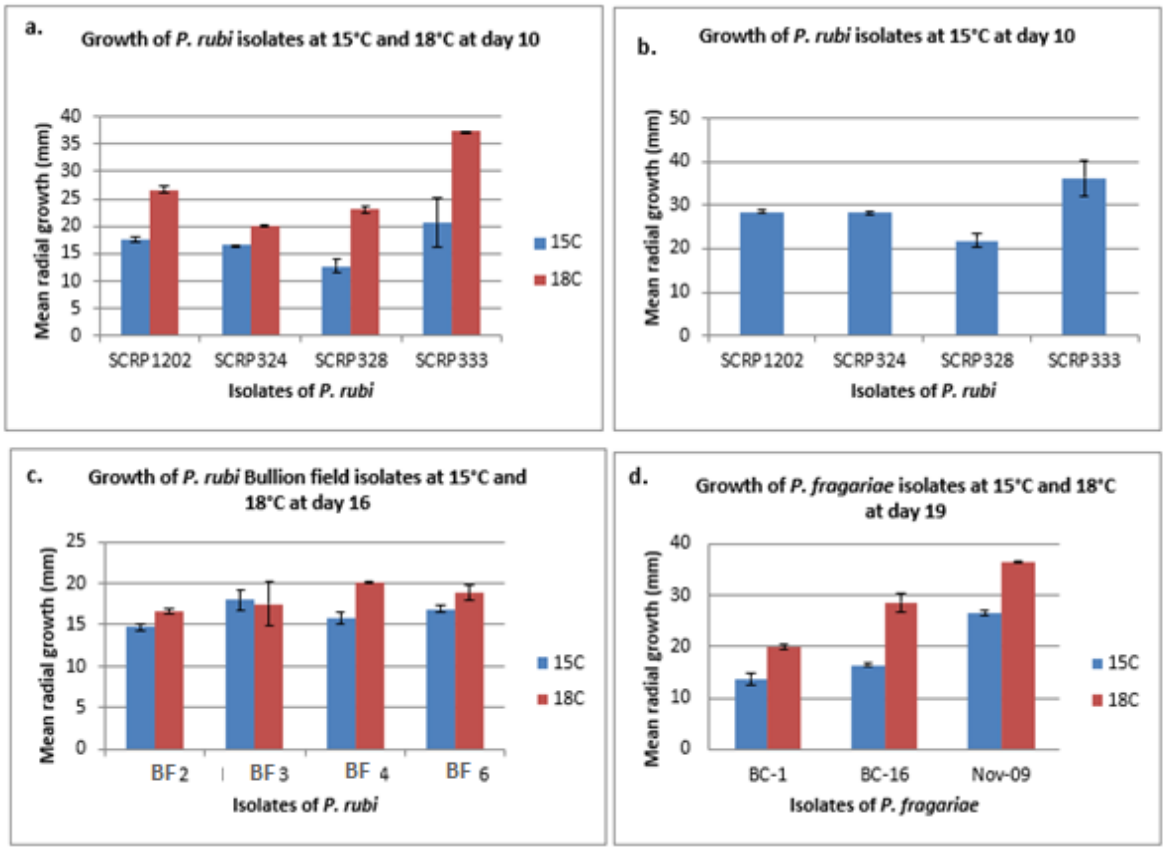


Figure 8: Chart graphs showing *P. rubi* and *P. fragariae* isolates growth under different conditions, at 15˚C and 18˚C. a. Chart showing the mean radial growth of *P. rubi* SCRP isolates at day 10 for the two temperatures. b. Chart showing the mean radial growth of *P. rubi* SCRP isolates at day 16 at 15˚C. c. Chart showing the mean radial growth of *P. rubi* BF 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

Statistical analysis showed that there was no significant effect of the temperature on the growth of the BF isolates. However, the temperature had a very highly significant effect (p value < 0.001) for the SCRP isolates (SCRP1202, SCRP324, SCRP328, SCRP333 and SCRP333_tdT), which grew faster at 18˚C. This is not surprising as BF isolates have been recently isolated from fields, where the temperature would have been lower, while SCRP strains are lab strains and have been kept at 18˚C. SCRP333 (race 3) grew significantly better than the other SCRP isolates at 18˚C.

There was a highly significant effect (p value < 0.001) of temperature on the *P. fragariae* isolates, where the growth was significantly higher at 18˚C for every isolate. At any temperature (15˚C or 18˚C), NOV-9 (race CA2) grew significantly faster than the two other isolates, showing a very highly significant effect (p value < 0.001) of the isolate.

Isolation from infected canes - April & August 2018

Unfortunately, cane isolation from samples collected in April 2018 did not yield any new isolates of *P. rubi*. A further sampling was carried out in August 2018, where raspberry canes showed obvious root rot symptoms (purplish lesion at the base of the cane, Figure 9) as opposed to the April sampling, where typical symptoms were hard to see. Typical *Phytophthora* growth was observed on plates with canes. Sub-culturing on rye agar prior to sequencing is in progress.

Figure 9: Raspberry canes (cultivar Glen Dee) sampled showing typical root rot symptoms: parts of the canes that are just above ground show purplish lesions

Isolation from canes sent by RSK-ADAS is in progress, although canes were very dry to begin with and close to senescence.

***P. rubi* sporangia and zoospores production**

Sporangia were successfully produced using a variety of sporulation solutions. Figure 10 shows the sporangia count for SCRP333 in different sporulation solutions. Figure 11 shows sporangia observed through the microscope.

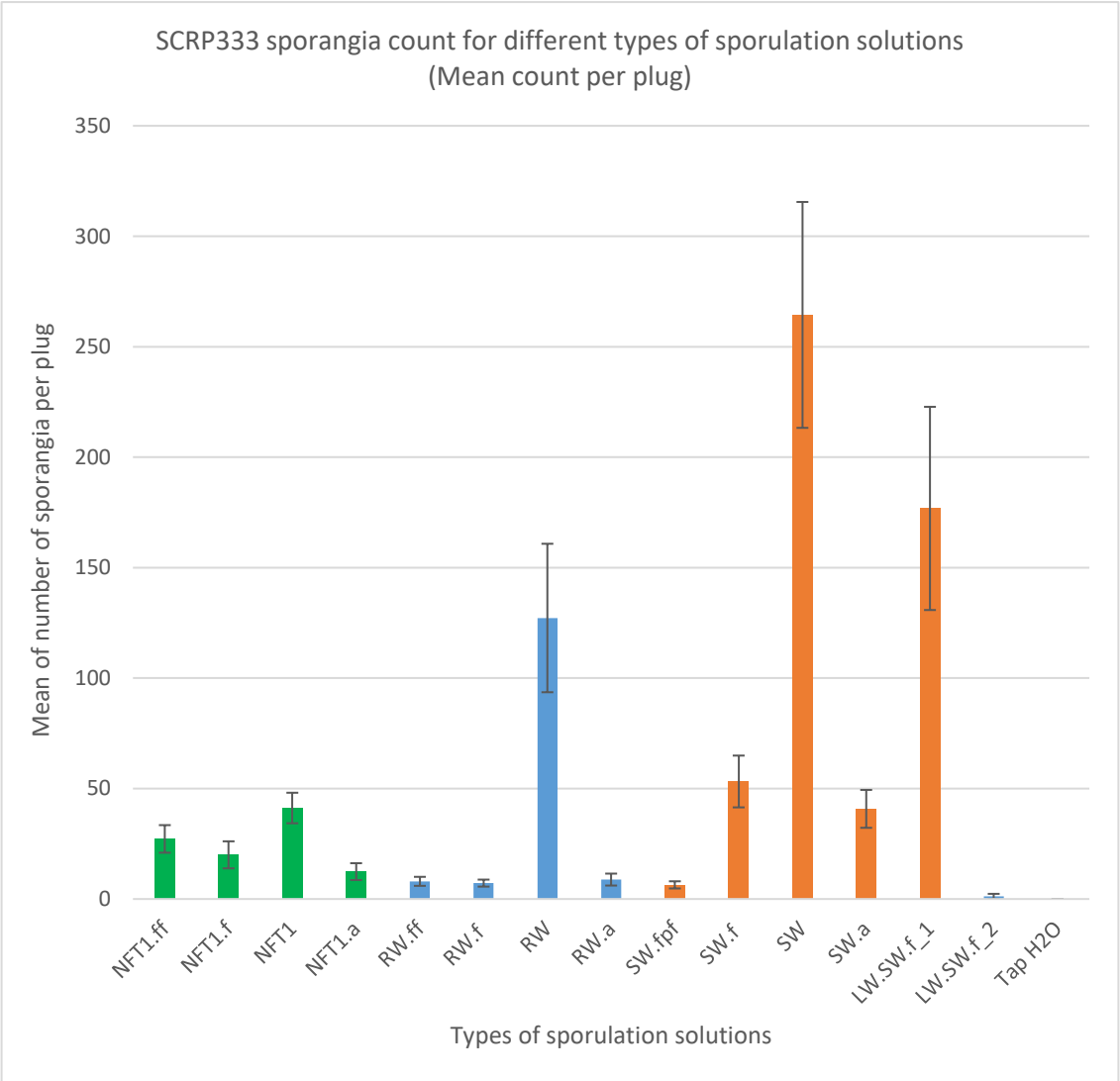
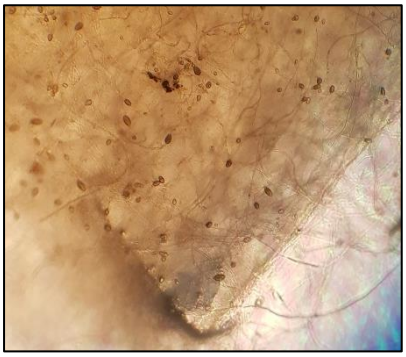


Figure 10: Results of the sporangia count (mean count per plug) per sporulation solution

Figure11: *P. rubi* SCRP333 sporangia through the microscope (for soil water SW). x16



The best results were observed when using soil waters, either not filtered or filtered once but changed twice in 24 hours during set up.

Results showed that the more “sterile” the solution is, the fewer sporangia were produced, confirming previous observations for *P. rubi* by Lydia Welsh (Personal Communication).

Similar results have been observed with other *Phytophthora* species, such as *P. cinnamomi* (Chee and Newhook, 1966).
Using water from the NFT tank was also successful at producing sporangia, although it did not result in the highest rates of sporulation.

Zoospores were successfully released using Petri's solution and transferred to the propagator and pots for infection.

P. fragariae sporangia and zoospores production

Sporangia were successfully produced for *P. fragariae* using a variety of sporulation solutions (Figure 12). The best results were observed when using soil waters, compared to the solution from hydroponics tanks.

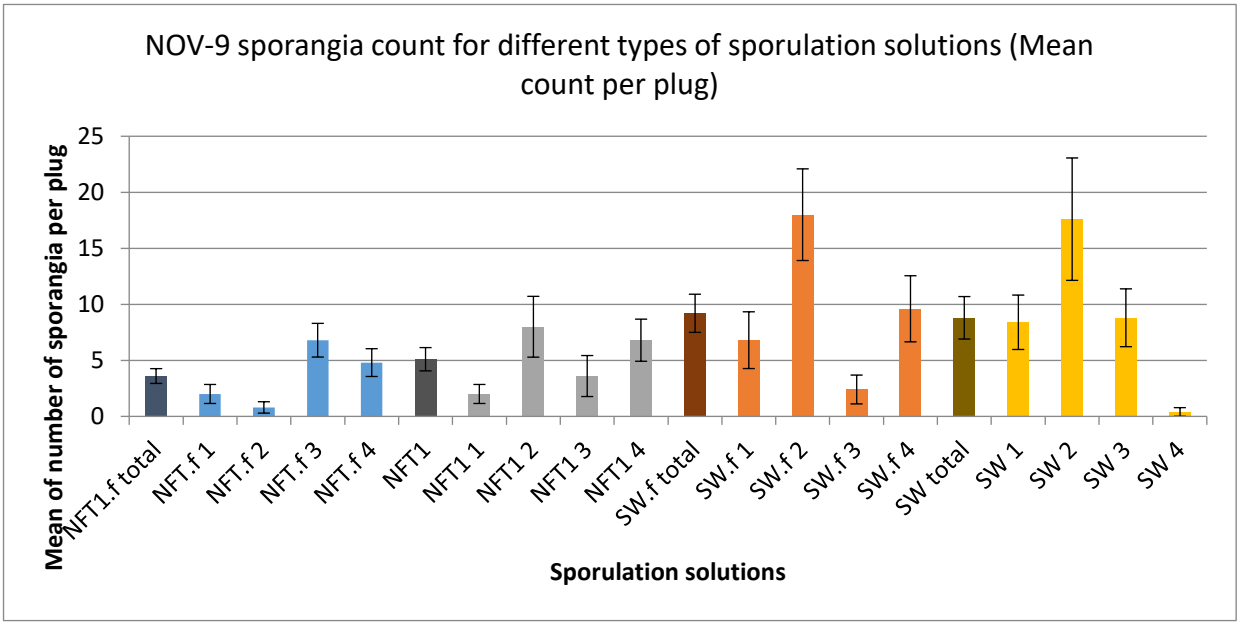


Figure 12: Results of the sporangia count (mean count per plug) per sporulation solution

Zoospores release was regularly checked before each Petri's solution change. *P.* fragariae isolate NOV-9 successfully released zoospores over the course of the experiment. Full sporangia and swimming zoospores were observed regularly, even as far as 4 days after the last change of Petri's solution. This shows that *P. fragariae* was capable of producing sporangia under the same conditions than *P. rubi*, using soil water, and that sporangia could release zoospores over a period of 19 days.

Petiole inoculation

Eleven days post inoculation seem to be the best time to observe any differences between cultivars on the inoculated petioles. When inoculated, Glen Moy petioles turned yellow quicker

than Latham petioles, for both *P. rubi* isolates SCRP333 and BF (Figure 13). However, when the petioles weren't inoculated (controls), no differences between the two cultivars was observed. For any of the cultivar, no obvious difference in symptoms was noted between inoculated and non-inoculated (controls).

Figure 13: Photos of raspberry petioles 11 days after inoculation with SCRP333. The top row shows 5 photos of petioles from the very susceptible cultivar Glen Moy whereas the bottom row shows 5 photos of petioles from the more tolerant cultivar Latham

Bioinformatics

A bait library for PenSeq was designed for the two species *P. rubi* and *P. fragariae* using RXLR and CRN effectors, apoplastic effectors, fungicide target genes, genes involved in pathogenicity, and housekeeping genes including single copy genes (see Methods for details). The compiled list was sent to Arbor Bioscience to design and produce the baits.

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 environmental benefits associated. Hydroponic cultures avoid soil-borne pathogens, which will lead to a reduced use of pesticides. Hydroponic systems are associated with a better controlled environment, which can also lead to a reduction in pesticides applications for any types of pests and pathogens. Moreover, hydroponic cultures use less land, and it is estimated that similar yields can be obtained using 1/5th of the space compared to traditional culturing. There is also less land erosion with hydroponics since there are no tilling. Finally, hydroponics cultures use less water, as it is recycled and not drained or washed off in the soil. 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 monitor 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, Latham and Glen Fyne using the Nutrient Film Technique worked and produced a rotation for the NFT system. However, it was very difficult to narrow down the best conditions to produce enough shoots, and the ratio of cuttings rooted and used in NFT / cuttings made were quite low. However, the May assay for cuttings shows promising results as the death rate observed so far is very low.

Cuttings kept in rockwool plugs seemed better to use for infection studies as the roots grew very well and did not tangle. It was also a quicker method to grow plants than using the transfer rockwool blocks, as roots needed to grow through the blocks before being transferred to the NFT tank. Plants grown in blocks were found to be better to grow bigger raspberry plants, where above ground parts would develop well, producing material for new cuttings (e.g. for a new rotation). Therefore, plants set up in rockwool plugs would be used for infections whereas plants set up in rockwool blocks would be used for long term plant growth and rotation management. Different cultivars should be kept in different tanks, especially when using the blocks, as the roots formed a mat, and shoots produced from this mat might have been from either of the cultivars. When grown, there were obvious phenotypic

differences between Glen Moy and Latham, but it might not be easy to differentiate other cultivars if grown in one NFT on blocks.

It was also noted that some of the High Health plants started to develop powdery mildew, which slowed down the cutting assays, as they had to be cut back. Plants were treated with Corbel fungicide to control the disease.

P. rubi and *P. fragariae* temperature comparison assay

Isolates of *P. rubi* that had been held under lab conditions grew better in lab conditions whereas isolates from the field, used to colder temperatures, grew similarly at 15˚C or 18˚C. This shows adaptability of *P. rubi* to environmental conditions. This adaptability and differences with isolates kept in lab conditions will be further explored using bio-informatics techniques and Target Enrichment Sequencing. However, this also means that the ability of lab isolates to infect at lower temperatures could be affected.

Isolation from canes

Isolation from canes collected in April 2018 was unsuccessful. During sample collection, infected canes were difficult to identify. The canes might have not been infected enough and/or it might have been too early in the growing season to isolate *P. rubi* from the canes. The most noticeable symptoms are usually wilting canes from early spring to late summer, and purplish lesion at the bottom of the canes just above ground. The August sampling was more successful, although sequencing is in progress and identity of the organism needs to be confirmed, infected canes were easier to identify, showing both wilting and purplish lesions, which is the part that was sampled. This shows that timing for canes sampling is key when isolating *P. rubi* from field.

Petiole inoculation for rapid resistance screening

Infections using the petiole inoculation method from Li et al. (2017) lead to differences in terms of decaying timescale, with the cultivar most susceptible to root rot showing yellowing of leaves sooner. This was observed with both *P. rubi* isolates, SCRP333 and BF 4. However, no differences in symptoms were observed between controls and inoculated petioles. This experiment should be repeated using a surface sterilisation method for the petioles used, as to minimize side effects that could influence the decaying of the leaves.

Conclusions

Characterization of *P. rubi* and *P. fragariae* is very important to understand the biology and genetics of the diseases. 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, using the Nutrient Film Technique and a rotation from cuttings to grown plants has been initiated

- *P. rubi* was successfully isolated from infected canes at different location over 2017/2018
- *P. rubi* and *P. fragariae* lab isolates grew significantly better at 18˚C, suggesting that they could have got used to lab conditions whereas field isolates grew similarly at 15˚C and 18˚C, showing the adaptability potential of the pathogen.
- *P. rubi* and *P. fragariae* both produce sporangia using “soil water”, showing that a bacterial metabolite is highly related to the success of *P. rubi* and *P. fragariae* reproduction. Swimming zoospores were successfully released for both species and *P fragariae* kept producing full sporangia and releasing zoospores over a course of 20 days, demonstrating the extended period during which the pathogen can produce reproduction and infection structures.
- A method has been tested for a rapid screening for resistance in raspberry cultivars and showed differences to *P. rubi* infection between Glen Moy (most susceptible) and Latham (most tolerant) cultivars
- A baits library was designed to be used in a bio-informatics assay to study *P. rubi* and *P. fragariae* diversity

In this study, phenotypes have been assessed via various experiments which unlocked certain answers and raised other research questions.

Future work

- Repeat most experiments to validate the results
- Conduct a chemical screening assay *in vitro* for *P. rubi* using active ingredients and common fungicides
- Continue sampling using the cane isolation method in order to 1) validate the method and 2) obtain more isolates from different location and time
- 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

- Conduct infection assays in the lab
- Transform *P. rubi* with fluorescent proteins
- Sequence *P. rubi* and *P. fragariae* isolates for PenSeq and carry out
- the diversity analysis
- Set up infection assays

Knowledge and Technology Transfer

Poster Presentation, London conference (from field to clinic) April 2018.

Exchange of protocols with ADAS for cane isolation

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