Project title:	**Novel ways of managing tree crop fungal diseases: Using precision diagnostic technologies to tailor disease management strategies**
Project number:	CTP_FCR_2018_1
Project leader:	Thomas Heaven, University of Lincoln
Report:	Annual report, Oct 2019
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Key staff:	Dr Andrew Armitage Prof Matthew Goddard
Location of project:	NIAB EMR
Industry Representative:	[Megan McKertcher, Worldwide Fruit]
Date project commenced:	01 October 2018

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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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CONTENTS

GROWER SUMMARY

Headline

Collection, culture and DNA extraction methods for studying apple scab and powdery mildew genetics have been established and optimised.

Background

Apple is among the most important fruit crops globally, ranked third in terms of annual production at 84.6 million tonnes (FAOSTAT 2014). Commercial apple cultivation is hampered by its susceptibility to a number of fungal pathogens. The most serious among these is apple scab caused by the pathogen *Venturia inaequalis*, where production losses can be as high as 70% in heavily affected orchards (Biggs 1990; MacHardy 1996). It is thought that apple producers in the eastern United States spend as much as 18.6 million USD per year on scab control alone (Cox 2015). Apart from scab the most common fungal disease found in UK apple cultivation is apple powdery mildew caused by *Podosphaera leucotricha* (Lesemann and Dunemann 2006). Currently, growers use a range of integrated pest management strategies to control this disease but remain heavily dependent upon fungicide use, applying pesticides throughout the season to limit damage to their crops (Roßberg 2003). However, this practice is unsustainable as fungicide resistant varieties have begun to emerge in both pathogen populations and increased restrictions on fungicide use are expected in the near future (Brent and Hollomon 1995; Lesemann et al. 2006).

Genomic resources for these two pathogens lags behind those available for the apple host or for other pathogenic fungi. There has long been significant interest in developing new varieties of apple combining the fruit quality of commercial cultivars with the superior disease resistance of wild species. However, whilst host resistance genes have been identified, corresponding pathogen virulence and avirulence genes, which confer disease or host defence respectively, remain unknown. Additionally the genetics behind fungicide resistance are not known. The objective of this project is to improve understanding of virulence in apple scab and mildew by identifying putative virulence and avirulence genes that determine isolate host specificity as well as mutations leading to fungicide resistance. This will inform future management strategies and breeding efforts.

Summary

In the first year of this PhD, techniques for collection, culture and DNA extraction from apple scab and powdery mildew were refined. The establishment of these methods will facilitate sequencing experiments to be performed in coming years beginning with the generation of the first apple powdery mildew genome and transcriptome assemblies. Subsequent comparison of genomes and transcriptomes will enable identification of virulence and fungicide resistance factors in the two pathogens.

- Samples of Scab were collected from indicator trees known to carry resistance genes
- Samples of mildew were collected from susceptible trees
- The identity of samples was confirmed by sequencing of the ITS region for each.
- Several methods for extracting DNA from the two pathogens were trialled for their effectiveness and DNA extractions performed on a number of isolates

Financial Benefits

This report summarises the work carried out in the first year of a four-year project, and so there are no direct financial benefits yet. Upon completion this project will provide key information for the precision diagnosis of the two diseases to strain level allowing the effects of management strategies to be tracked and modified to increase yield.

Action Points

- There are no grower actions points at this stage of the project

SCIENCE SECTION

Introduction

Apple scab (*Venturia inaequalis*) and powdery mildew (*Podosphaera leucotricha*) are key pathogens of apple cultivation and both are known to be highly host specific. This host specificity is thought to arise from each pathogen's ability to suppress or avoid recognition by the hosts immune system. Fungi secrete toxins and proteins to aid infection. These are termed effectors. However, some of these effectors may be recognised by the host plant if it carries certain resistance genes. This results in effector triggered immunity. Fungi may gain or lose effectors to avoid recognition by the plant, allowing them to overcome resistance.

The ability of an isolate to overcome known resistance genes in a host is used to define different races in a pathogen population. A race structure is well established in the apple scab pathosystem and there is also evidence for the existence of a race structure in the mildew population (Bowen et al. 2011). New patterns of virulence may occur due to the disruption of genes via the insertion of a transposon or as a result of mutation.

Resistance against some classes of fungicide have been associated with mutations in specific target regions, for example the CYP51A1 gene has been associated with resistance to demethylase inhibitor fungicides (Villani et al. 2016). For other modes of action the source of resistance is unclear. It is thought that resistance may arise through alterations in the fungicide binding site, increased production of a targeted protein or an improved ability of fungi to remove toxins (Koenraadt et al. 1992; Hamamato et al. 2000; Deising et al. 2008). Detection of resistance often still relies upon microbiological methods for many fungicide classes.

To better inform disease management strategies of growers there is an immediate need for improved diagnostic testing for diseases. Specifically, a test is needed that provides a quick diagnosis at a low cost to growers, ideally simultaneously for a panel of diseases commonly found in orchards. This test also needs to be precise enough to identify key pathogen characteristics such as resistance to certain fungicides and virulence. One approach is a diagnosis by sequencing method which identifies genetic markers associated with resistance and virulence traits. Such a diagnostic could also be used to better track the effects of different planting decisions and agronomic practices, improving disease management over the longer term.

This project will collect samples of *P.leucotricha* and *V.inaequalis* that display different virulence and fungicide resistance characteristics and perform sequencing of the genome and transcriptome of these. In the first-year initial samples have been taken and methods for collection, culture and DNA extraction trialled for the two organisms.

Project aims:

- To generate a first draft genome and transcriptome for apple powdery mildew (*P.leucotricha)*
- To identify key virulence factors in resistance breaking strains of apple scab *(V.inaequalis)*
- To identify genetic markers for fungicide resistance in *V.inaequalis*
- To develop a 'diagnosis by sequencing' method for *V.inaequalis* and *P.leucotricha*

Materials and methods

Established methods for collection, culture and extraction of *V.inaequalis* were utilised, in the case of *P.leucotricha* different techniques were trialled. **For collection**; scraping material from infected leaves, vacuum pumping material from leaves in the lab, use of a portable vacuum in the field and washing of infected leaves in the field. It was found that washing collected the most material for time spent sampling. **For culture**; shoot cultures of the cultivar 'gala' were established growing in both high and low nutrient media and inoculated with mildew sample, however these cultures grew extremely slowly and were unsuitable for bulking up of fungal material. **For DNA extraction**; kits from several manufacturers were used including the nucleospin plant 2 kit from Macherey and Nagel, Mag-bind plant DNA kit from omega bio-tek and genomic-tip kit from Qiagen, these all yielded very low concentrations of DNA. The most effective extraction method found was a C-TAB extraction method which provided better DNA concentrations of poor purity. Numerous modifications were tried with this extraction method in an attempt to improve the quality of extractions.

In the interests of brevity only those methods found to be most effective are given in detail below.

P.leucotricha Sampling:

Heavily infected leaves were removed from the tree and vigorously shaken by hand in 50 ml falcon tubes containing 40 ml distilled water, washed leaves were then removed using forceps and more infected leaves from the same tree washed in the same water. This was repeated until there were no more heavily infected leaves available from a given tree or

water remaining in the falcon tube dropped below 20 ml. Conidial suspensions were then centrifuged at 5000 g for 5 mins and water supernatant removed via stripette, followed by another 5min centrifugation at 5000 g and removal of any remaining water by pipette. Samples were then freeze dried overnight and the fully dried conidial powder transferred to 1.5 ml Eppendorf tubes stored at -80 ºC.

V.inaequalis Sampling and Culture:

Scab was collected from the field by using cork borers to excise single scab lesions from infected leaves, the resulting leaf discs were kept at -20 ºC for long term storage. In order to bulk up individual isolates from these stocks the fungus was cultured from single spores grown on PDA plates containing 60 ppm rifamycin in 24 hour darkness at 17 ºC. A leaf disc collected from a given cultivar was defrosted and placed into a 2 ml Eppendorf tube with 300 µl of sterile water, the disc was then vortexed for 1 min to wash off conidia. An optical microscope was used to confirm the presence of spores suspended in the water. 50 µl of spore suspension was then spread onto a plate. Another 300 µl of sterile water was then added to the remaining suspension and 50 µl of the more dilute spores spread onto a second plate. Germination was observed under a dissecting microscope and a scalpel was used to excise individual germinated spores onto fresh individual plates, these were sealed with parafilm and left to develop for 6 weeks. For collection of mycelium the cultures were transferred to cellophane disc plates. These were prepared by pipetting 1 ml of sterile water onto standard PDA + rifamycin plates before spreading a cellophane disc flat across the agar surface. Plugs of mycelium were taken from uncontaminated plates mixed with 1ml of sterile water, and spread over the top of the cellophane discs. These plates were then sealed with parafilm and the cultures grown until the disc was covered by mycelium, at this point sub culturing and mycelial harvest took place. Mycelium was harvested by scraping from the surface of a given cellophane disc and freeze drying in 1.5 ml Eppendorf tubes overnight. These stocks were then stored at -20 ºC until use for either DNA extraction or recovering growing cultures of contaminated isolates.

DNA Extraction:

DNA extractions were based upon the CTAB extraction protocol of Schwessinger and McDonald (2017) which was progressively modified to obtain high quality DNA from mildew. Premade buffers were combined to form lysis buffer (Buffer A; 0.35 M sorbitol, 0.1 M Tris-HCl, 5 mM EDTA pH 8 Buffer B; 0.2 M Tris-HCl, 50 mM EDTA pH 8, 2 M NaCl, 2% CTAB Buffer C; 5% Sarkosyl N-lauroylsarcosine sodium salt Buffer D; PVP40 10% Buffer E PVP10 10%) in the ratios 5:5:2:1:1, 10 µL (10kU) RNase A was added to this. Fungal samples were ground in a geno/grinder with 2 ball bearings inserted into 2 ml Eppendorf tubes at 1500 rpm in 20 s bursts for a total of 2 mins chilling with liquid nitrogen between

bursts. They were then incubated at room temperature for 30 mins in 1.5 ml lysis buffer whilst being inverted, ball bearings were left in the tubes to increase mixing, 20 µL of proteinase K was then added and incubation continued for another 30 mins. Following this samples were cooled on ice for 5 mins before 250 µl 5 M potassium acetate was added and cooling continued for an additional 5 mins. Samples were then centrifuged for 12 mins at 5000 g and the supernatant removed to safe seal tubes. Washing was then carried out by the 1:1 addition of Phenol:Chloroform:Isoamyalcohol 100 mM Tris-EDTA pH 8 (P:C:I), samples were mixed by inversion for 2 hours and centrifuged for 10 mins at 4000 g before transfer of the supernatant to a fresh tube. This wash was repeated three times per sample, this was followed by a fourth wash using Chloroform:Isoamylalcohol (C:I) in place of P:C:I. DNA precipitation was performed with 200 µl of Sodium Acetate (3 M pH 5.2) and 800 µl Isopropanol, added to approximately 1 ml of supernatant and mixed by inversion at room temperature for 10 mins. DNA was pelleted by centrifugation at 8000 g for 30 mins. The supernatant was discarded and the pellet washed three times by resuspension in 1.5 ml of 70% ethanol, centrifugation at 13000 g for 5 mins and discarding of the supernatant. Following the final wash step remaining ethanol was allowed to evaporate for 30 mins before the DNA pellet was dissolved in 100 µL of 10 mM Tris pH 8.5 at room temperature overnight. DNA was then re-precipitated using 20 µl Sodium Acetate (3 M pH 5.2) and 1.5 ml 100% ethanol overnight at - 20 °C. Following this the previous washing steps were repeated; x3 P:C:I, x1 C:I, isopropanol precipitation, x3 70% ethanol followed by dissolution using the same method.

The quality of extracted DNA was assessed initially using a Nanodrop 1000 spectrophotometer (Thermo Scientific), if these results were in the target range then quality was further assessed using a Qubit 2.0 fluorometer (Invitrogen) and using these results 100 ng of DNA was run on a 1.5% agarose gel with GelRed to assess contamination with RNA and finally these results were compared to a qubit RNA reading.

ITS sequencing:

The ITS region of isolates was amplified. PCR was performed using 12.5 µL KAPA Hifi Hotstart Ready Mix, 4 µL water, 3.75 µL ITS-1 primer, 3.75 µl ITS-4 primer and 1 µl DNA diluted 1:10 with water. PCR was performed on a Veriti thermal cycler (Applied biosystems) using the following cycle: an initial 95 °C for 3 min; 35 cycles of 98 °C for 20 s, 60 °C for 15 s and 72 °C for 2 mins; then a final extension at 72 °C for 2 mins. PCR products were visualised on a 1.5% agarose gel with GelRed before being purified using a monarch PCR & DNA clean-up kit (5 µg) following the manufacturer's instructions. Purified PCR products were submitted for sequencing by Eurofins genomics LIGHTrun tube service.

The resulting sequences were aligned to a reference ITS region (Locus EU035437 for scab and JQ999954 for mildew) downloaded from NCBI using the program Geneious V10.0.2. Alignments generated in Geneious were exported to the program Mega X which was used to determine the best genetic distance model for tree building, in each case this was the Jukes-Cantor model. Phylogenies were then generated using the tree function in Geneious.

Results

Podosphaera leucotricha

P.leucotricha was sampled directly from infected leaves in the orchards at NIAB EMR (Table 1). These infections matched the expected field symptoms of mildew and when viewed under a microscope conidial chains of the expected morphology were visible. Samples were taken from trees of the same cultivar in the same field, however numerous leaves were used from different trees. As a result it is likely that each sample is made up of several clonal isolates and that the conidia in each sample are not genetically homogeneous. Nevertheless these represent the best samples that we have available for extraction to date, and are useful for optimising DNA extraction methodologies and generating an initial draft genome.

Initial extractions from *P.leucotricha* were heavily contaminated with polysaccharides, indicated by a low 260:230 ratio when tested by nanodrop. The protocol was eventually optimised sufficiently to give 260:230 readings in the desired range of >2 (Table 2).

Table 1. *P.leucotricha* field samplings: Results of mildew samplings, including the cultivar, the field/polytunnel at NIAB EMR and specific trees sampled from as well as the resulting weight collected.

***P.leucotricha* Collections**				
Host Cultivar:	Location:	Tree:	Freeze dried Weight (g):	Collected Date:
Mixed cultivars	P28	Mixed	0.3604	25/04/2019
M1 (E)	Old Genebank	A1	0.075	24/05/2019
Idared	Old Genebank	D1	0.0671	02/06/2019
Loop Spy (4x)	Old Genebank	C29/30	0.0275	02/06/2019
M18	Old Genebank	A39/40	0.0647	02/06/2019
MM106	Old Genebank	B13/14	0.057	02/06/2019
Gala	New Genebank	R2/T24-26	0.0655	03/06/2019
Idared	New Genebank	R2/T42,44	0.0745	03/06/2019
Jonathan	New Genebank	R3/T5-7	0.0567	03/06/2019

Table 2. *P.leucotricha* DNA extractions: The highest quality DNA extractions achieved from *P.leucotricha* from each cultivar sampled, including host cultivar, nanodrop and Qubit quality results.

***P.leucotricha* DNA Extractions:**				
Cultivar:	260/280:	260/230:	Qubit DNA (ng/ul):	Qubit RNA (ng/ul):
Idared	1.83	1.44	16.5	31.2
Loop Spy (4x)	1.83	1.91	9.5	22.8
M1 (E)	1.86	1.97	9.6	13.5
Jonathan	1.80	1.49	14.9	13.6
Idared	2.00	2.00	99	160
Gala	1.78	1.78	33	54.4
M18	2.10	2.36	54.6	200
MM106	2.06	2.30	99.8	100

An initial investigation into the variation between the cultured isolates was performed by sequencing and comparison of the ITS region. This also served to definitively identify the samples taken as *P. leucotricha*, apple powdery mildew. An alignment with other *Podosphaera* species demonstrated that the *P. leucotricha* isolates were most closely related to each other with the most similar other species being strawberry mildew, *Podosphaera aphanis* (Figure 1)

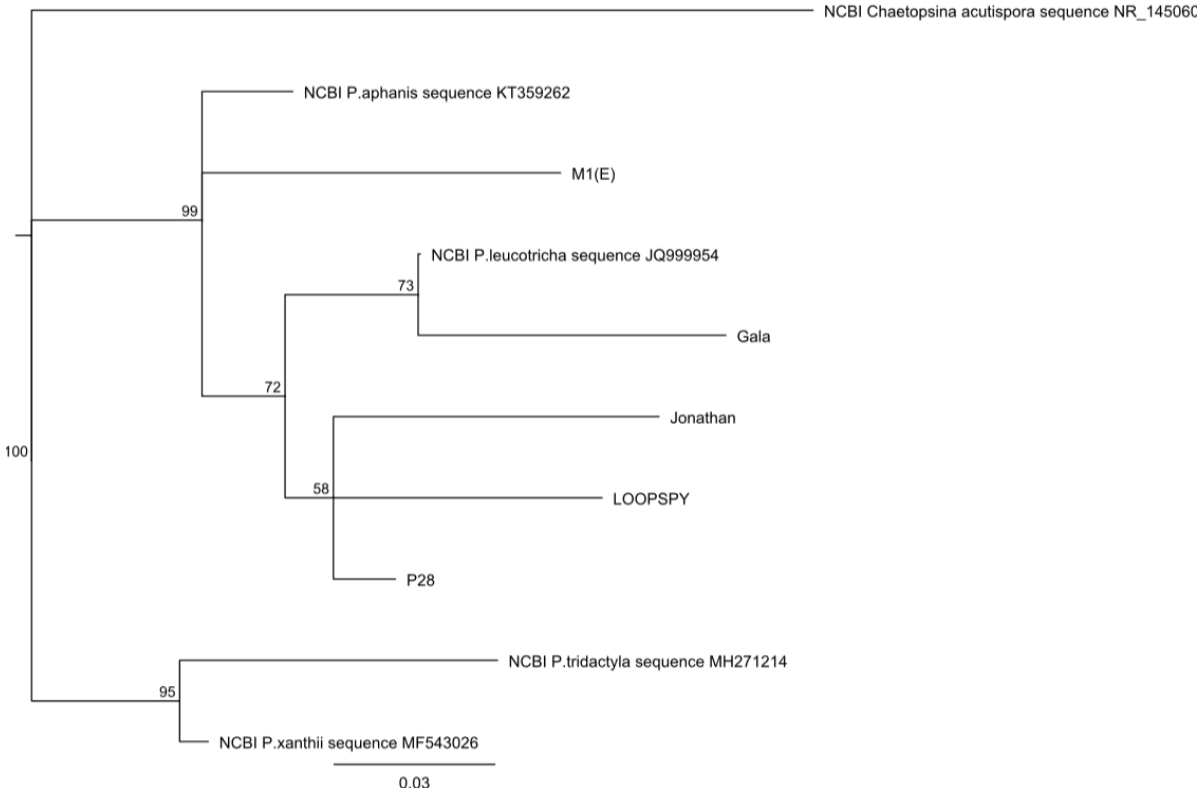

Figure 1. *P.leucotricha* ITS Dendrogram: Phylogenetic analysis of the nucleotide sequences of the ITS region for 5 *P. leucotricha* samples and closely related species; *Podosphaera aphanis* (strawberry powdery mildew), *Podosphaera tridactyla* (plum powdery mildew), *Podosphaera xanthii* (cucurbits powdery mildew) with *Chaetopsina acutispora* used as an outgroup species. Alignments were used to generate a phylogenetic tree using the neighbour joining method with nodes showing support from 100 bootstrap replications.

Venturia inaequalis

DNA extractions were also performed for 21 different single spore cultures of *V.inaequalis* isolates taken from 13 different cultivars of apple. These included indicator trees with different resistance genes (Table 3).

Table 3. *V.inaequalis* DNA extractions: Results of DNA extractions from *V.inaequalis* samples including host cultivar, host resistance gene compliment, weight of freeze dried material used in the extraction and resulting nanodrop and Qubit quality results.

***V.inaequalis* DNA Extractions:**							
Sample ID:	Cultivar:	R gene:	Starting Weight (g):	Nanodrop Conc (ng/ul):	A260/280:	A260/230:	Qubit DNA (ng/ul):
05/172	Worcester	Susceptible	0.0256	564	1.94	1.8	94.6
05/268	Bramley	Susceptible	0.0218	464	1.83	1.32	57.8
05/278	Bramley	Susceptible	0.0507	564	1.84	1.49	322
06/035	Cox	Susceptible	0.021	412	1.95	1.72	64.6
05/337	Worcester	Susceptible	0.0374	913	1.94	1.34	24.4
06/097	Gala	Susceptible	0.0232	416	1.78	1.49	30.2
T49	Tina	Susceptible	0.0269	56	2.04	1.91	73.2
K86	Katy	Susceptible	0.0241	151	1.88	1.83	47.2
K94	Katy	Susceptible	0.0313	327	2.08	1.31	72
V15	Vicky	Susceptible	0.0419	822	2.12	2.08	15.3
V16	Vicky	Susceptible	0.0089	264	2.08	2.18	26.8
S162	Spartan	Susceptible	0.0204	165	2.05	2.09	54.8
RF54	Red Falstaff	Susceptible	0.0146	360	2.07	2.09	9.6
RF68	Red Falstaff	Susceptible	0.0584	272	1.91	1.36	103
GD74	Golden Delicious	Rvi1	0.0161	502	2.03	2.11	12.3
GD133	Golden Delicious	Rvi1	0.0213	482	2.06	2.23	14.5
MF0	MxFloribunda 821	Rvi7	0.0172	475	2.07	2.21	25
MF2016	MxFloribunda 821	Rvi7	0.0315	261	2.03	1.78	42
Q710	Q71	Rvi3	0.0433	311	1.79	1.5	94.4
B450	B45	Rvi8	0.0214	869	2.01	1.75	15.5
B451	B45	Rvi8	0.0183	141	1.95	1.85	29

An initial investigation into the variation between the cultured isolates was performed by sequencing and comparison of the ITS region. Analysis of the ITS region revealed only three SNPs within the *V. inaequalis* second spacer region. A phylogenetic tree was constructed using these sequences along with other members of the *Venturia* genus, with a member of the neighbouring *Valsaria* genus used as an outgroup (Figure 2). The different *V.inaequalis* isolates form a clade within this tree (bootstrap value 74%).

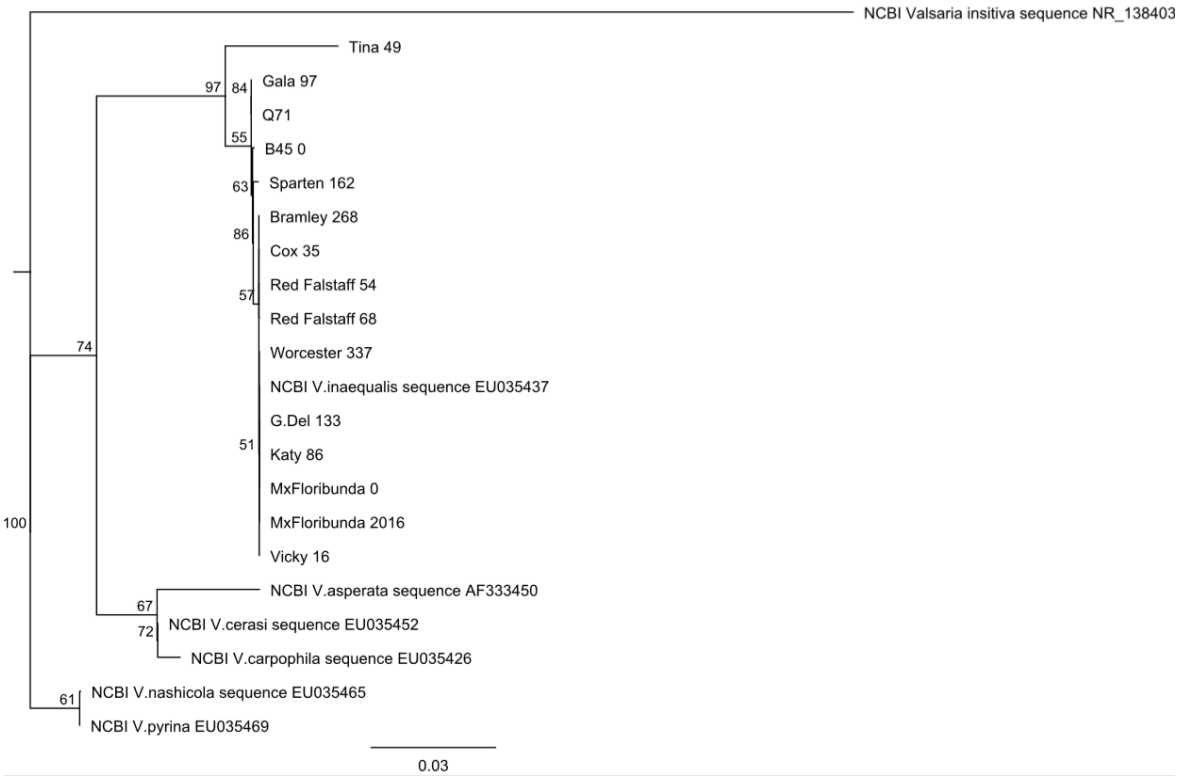

Figure 2. *V.inaequalis* ITS Dendrogram: Phylogenetic analysis of the nucleotide sequences of the ITS region for 15 *V. inaequalis* samples and closely related species from the Venturia genus with *Valsaria insitiva* used as an outgroup species. Alignments were used to generate a phylogenetic tree using the neighbour joining method with nodes showing support from 100 bootstrap replications.

Discussion

The ITS sequencing results confirm that the samples collected are indeed *V.inaequalis* and *P.leucotricha* however the variation within this region is not sufficient to distinguish between isolates from different cultivars. Sequencing of the whole genomes or transcriptomes will be required so that comparisons can be made between isolates with different virulence and fungicide resistance characteristics. This will reveal key genetic factors underlying these traits. For *V.inaequalis* the methods that we have trialled should be sufficient for this. However our experience extracting DNA from *P.leucotricha* to date suggests that obtaining large enough quantities of high quality DNA will be difficult. Having established methods for the collection of *V.inaequalis* and *P.leucotricha* samples this year will allow for the collection of the maximum amount of material next season. However, the amount of *P.leucotricha* that can be collected from field samples is still low. In contrast to many fungal pathogens *P.leucotricha* is a biotroph meaning that it can only survive on host plants and cannot be grown in culture. We have therefore modified our original plans based on our DNA extraction trails and will now focus on producing first a transcriptome and then one initial genome for *P.leucotricha* , pooling collected material from numerous cultivars**.** In order to extract material from a single strain in the future reliable methods for culturing large quantities of mildew from a single spore will first need to be developed.

Conclusions

- *V.inaequalis* and *P.leucotricha* sampling methods have been established
- Initial samples of *V.inaequalis* and *P.leucotricha* have been collected
- Culture methods for *V.inaequalis* have been established
- DNA extraction methods have been optimised

Knowledge and Technology Transfer

The student attended and presented posters at:
NIAB Poster day 2019 – Poster presentation at NIAB poster day 2019 in Cambridge, with the CTP.

MBPP 2019 – poster presentation at Molecular biology of plant pathogens conference in Norwich.

BSPP presidential meeting 2019 – poster presentation at British society for plant pathology annual presidential meeting in Bristol.

References

Biggs, A.R. (1990) Apple scab. In: Jones, A. L. and Aldwinckle, H. S. ed., *Compendium of Apple and Pear Diseases*. 1st ed. St Paul. MN: APS Press, pp. 6-9.

Bowen, J.K., Mesarich, C.H., Bus, V.G.M., Beresford, R.M., Plummer, K.M. and Templeton, M.D. (2011) Venturia inaequalis: the causal agent of apple scab. *Molecular Plant Pathology* 12(2), pp. 105-122.

Brent, K.J. and Hollomon, D.W. (1995) *Fungicide resistance in crop pathogens: how can it be managed?* 2nd ed. Brussels: Fungicide Resistance Action Committee.

Cox, K.D. (2015) Fungicide Resistance in Venturia inaequalis, the Causal Agent of Apple Scab, in the United States. In: Ishii, H. and Hollomon, D. W. ed. *Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management,* 1st ed. Tokyo: Springer Japan, pp. 433-448.

Deising, H.B., Reimann, S. and Pascholati, S.F. (2008) Mechanisms and significance of fungicide resistance. *Brazilian Journal of Microbiology* 39(2), pp. 286-295.

FAOSTAT (2014) FAO Statistics Division [Online]. Available at: http://faostat.fao.org/ [Accessed: 18 October 2018].

Hamamato H., Hasegawa K., Nakaune R., Lee Y.J., Makizumi Y., Akutsu K., Hibi T. (2000) Tandem repeat of a transcriptional enhancer upstream of the sterol 14 alpha-demethylase gene (CYP51) in Penicillium digitatum. *Appl Environ Microbiol* 66(1), pp. 3421–3426.

Koenraadt H., Somervile S.C., Jones A.L. (1992) Characterization of mutations in the beta-tubulin gene of benomyl resistant field strains of Venturia inaequalis and other plant pathogenic fungi. *Phytopathology* 82(1) pp. 1348–1354.

Lesemann, S.S., Schimpke, S., Dunemann, F. and Deising, H.B. (2006) Mitochondrial heteroplasmy for the cytochrome b gene Controls the level of strobilurin resistance in the apple powdery mildew fungus Podosphaera leucotricha (Ell. & Ev.) E.S. Salmon. *Journal of Plant Diseases and Protection* 113(6), pp. 259–266.

Lesemann, S. and Dunemann, F. (2006) Neue Erkenntnisse zur Biodiversität des Apfelmehltau-Erregers. *Gesunde Pflanzen* 58(2), pp. 117–123.

MacHardy, W.E. (1996) *Apple scab: biology, epidemiology, and management.* St.Paul, Minn:

APS.

Roßberg, D. (2003) NEPTUN 2001-Erhebung von Daten zum tatsächlichen Einsatz chemischer Pflanzenschutzmittel im Obstbau, im Hopfen und in Erdbeeren [Online] Available at: https://papa.julius-kuehn.de/index.php?menuid=41 [Accessed 01 September 2019]

Villani, S. M., J. Hulvey, Hily, J.M. and Cox K.D. (2016). Overexpression of the CYP51A1 Gene and Repeated Elements are Associated with Differential Sensitivity to DMI Fungicides in Venturia inaequalis. *Disease Control and Pest Management.* 106(6), pp. 562-571.

