Project title:	The molecular basis of pathogenicity of *Neonectria ditissima.*
Project number:	CP 141
Project leader:	Richard Harrison, NIAB EMR. Robert Jackson, Reading University.
Report:	Annual report, first year.
Previous report:	NA
Key staff:	Antonio Gomez Cortecero, NIAB EMR.
Location of project:	NIAB EMR
Industry Representative:	Tony Harding. Worldwide fruit. Acorn House, Unit 68-69, John Wilson Business Park, Harvey Drive, Chestfield, Whitstable, Kent, CT5 3QT.
Date project commenced:	Oct 2015
Date project completed (or expected completion date):	Oct 2018

DISCLAIMER

While the Agriculture and Horticulture Development Board seeks to ensure that the information contained within this document is accurate at the time of printing, no warranty is given in respect thereof and, to the maximum extent permitted by law the Agriculture and Horticulture Development Board accepts no liability for loss, damage or injury howsoever caused (including that caused by negligence) or suffered directly or indirectly in relation to information and opinions contained in or omitted from this document.

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.

[Name] ANTONIO GOMEZ

[Position] PHD STUDENT

[Organisation] NIAB EMR

Signature ..... Date 31/10/16

[Name]

[Position]

[Organisation]

Signature ..... Date .....

Report authorised by:

[Name] RICHARD HARRISON

[Position] HEAD OF DEPT

[Organisation] NIABEMR

Signature ..... Date 31/10/16

[Name]

[Position]

[Organisation]

Signature ..... Date .....

CONTENTS

GROWER SUMMARY

Headline

- It is likely that rapid progress can be made in identifying the genetic basis of resistance to *N. ditissima*.

Background and expected deliverables

Control of canker caused by the fungus *Neonectria ditissima* is a serious problem for apple growers in the UK and in temperate regions. The majority of modern varieties are highly susceptible to canker and in extreme cases do not survive establishment in the orchard. There are a lack of effective control methods currently available for canker. This is due to the pathogen's lifestyle, inhabiting woody tissue year-round, rather than an annual infection-reinfection cycle common to many foliar pathogens. Host resistance is a promising avenue to pursue for canker resistance, although deployment of host resistance in breeding programmes is slow, due to the long breeding cycle in apple.

Research into other host pathogen interactions shows that a dual strategy of understanding host resistance and pathogen avirulence and how the two are linked is key to the deployment of durable resistance into the field. Nevertheless, little is known about the pathogen at the molecular level. This project is focused on dissecting components of the pathogen's genome that modulate virulence in order to understand how virulence is controlled and whether there are specific differences in host resistance response to isolates of differing virulence.

This work will provide fundamental insights into the molecular basis of pathogenicity in *Neonectria ditissima.* It is hoped to identify candidate genes important in virulence in the pathogen, which could lead to novel opportunities for control by targeted disruption of the pathogen.

Summary of the project and main conclusions

The results of the first year of this project are summarised as follows:

- Canker is genetically similar across the globe, this is consistent with the theory that *N. ditissima* spread from Europe to other regions of the world on imported apple plant material. This provides some hope that any resistance found is likely to be effective worldwide.
- Results to date do not support the existence of distinct pathogen races of *N. ditissima*. It may therefore be possible that any resistance found will be broad spectrum and as a consequence potentially durable.

- Different artificial tests for susceptibility to canker were performed on a range of cultivars. Future work needs to be carried out to compare the results presented in this study with trees grown outside in an orchard setting, inoculated using several different wounds (leaf scars, pruning cuts, picking wounds) to determine whether the methods developed as part of this PhD can be used for rapid selection in breeding programmes.
- The most resistant cultivar to *N. ditissima* was found to be 'Robusta 5'. This is a representative of a species that is distinct from M. x domestica. Little is known about natural M. x robusta species, since much of the material that is present in Europe was collected in Northern China.
- *N. ditissima* is not reported as a significant pathogen of apple in China, indicating that the mode of resistance in M. x robusta versus the cultivated apple M. x domestica may be of distinct evolutionary origin. This gives the opportunity to study different mechanisms of resistance. This is beneficial as some modes of resistance may be more successful than others.
- Of the commonly grown cultivars, Golden Delicious was found to be the most resistant.
- Unfortunately, crossing Golden Delicious with other cultivars displaying some resistance, didn't consistently result in resistant progeny. This suggests breeding for resistance using Golden Delicious may be difficult.

Financial benefits

At this stage of the project, no financial benefits have been delivered, but if the project successfully identifies genetic resistance to apple canker, the results of the project will be used in future breeding programmes to develop varieties which offer genetic resistance to *Neonectria ditissima*, thereby overcoming the loss of trees during establishment and production of apple orchards.

Action points for growers

- No action points have been developed at this stage in the project.

SCIENCE SECTION

Introduction

European canker (caused by *Neonectria ditissima)* is one of the most destructive diseases of apple and pear. The fungus attacks trees in the orchard, causing cankers and dieback of young shoots, resulting in loss of fruiting wood and increases pruning costs (Swinburne, 1975). Apple canker can be particularly damaging in young orchards where in the first few years of orchard establishment up to 10% of trees can be lost annually as a result of trunk cankers. In some regions of the world (i.e. Northern Europe) *N. ditissima* also causes a fruit rot that can result in losses as high as 10% or more in stored fruit. The rot, which is often found at the fruit stalk end, is difficult to spot on the grading line, but becomes obvious during marketing leading to rejection of fruit consignments (Xu and Robinson, 2010).

Much is known about the epidemiology of the disease in the orchard (see Figure 1 for a graphical depiction of the lifecycle). The fungus produces two spore types, conidia (imperfect/asexual spores) and ascospores (sexual/perfect spores). Conidia are generally produced within the first year of canker formation when the temperature increases in the spring and summer and are spread throughout the season by rain splash. By contrast, ascospores are mainly produced by old canker lesions during the autumn, winter and spring and are discharged by rain and wind, or splash-dispersed. Both spore types enter through wounds, either natural such as bud-scale scars, leaf scars, fruit scars or artificial such as pruning wounds. Thus inoculum and points of entry on the tree are available all year round (Amponsah et al., 2015) and the only limiting factor is rain, which is essential for spore production, spread, germination and infection (Xu et al., 1998). Autumn leaf fall is usually the main infection period and consequently wet autumns are usually followed by a high incidence of shoot dieback due to canker development in the following spring and summer. Previous studies have shown that cankers in young orchards originate either from infection that occurred in the nursery and remained symptomless until planting out in the orchard, or from inoculum spreading in from adjacent infected orchards. The disease is most destructive in young trees infected with canker, as latent infections appear as systemic infection and trunk cankers several years after planting (McCracken et al., 2003). Factors that affect canker expression are not understood but possibly relate to stress (cold, drought, water-logging, fertiliser and herbicide applications). New cultivars being planted in the UK such as ‘Scifresh’, ‘Cameo’, ‘Kanzi’, ‘Zari’, ‘Rubens’ and older cultivars such as ‘Gala’ and ‘Braeburn’ are all very susceptible to *N. ditissima* and the development of systemic canker in young orchards leading to tree loss is a significant problem, with severe financial loss particularly in modern intensive planting systems (Weber, 2014). In the UK, in some cultivars, the development of systemic

canker in young orchards has been so devastating that extensive replanting of trees has been required and in some cases whole orchards have been grubbed. In contrast to the orchard, the epidemiology of *N. ditissima* in the nursery is not understood and infected trees are rarely seen in nursery production so it is assumed that the disease is present as a latent infection (McCracken et al., 2003).

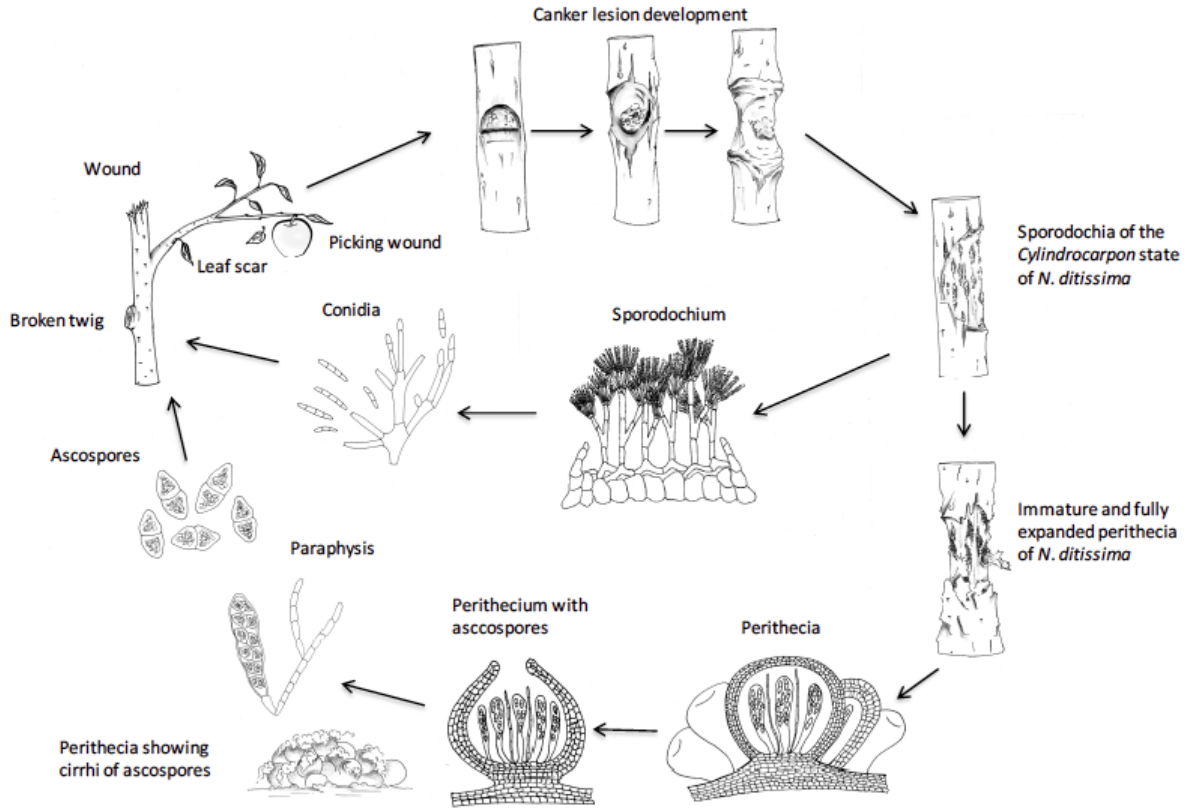

Figure 1. Life cycle of *Neonectria ditissima*- after Agrios 1997.

Currently canker is controlled in the orchard by a combination of cultural methods to remove canker lesions and the use of protectant fungicides. Effective fungicides are limited; generally copper fungicides are used at autumn leaf fall and before budburst to protect leaf scars and bud-scale scars however copper is no longer permitted for use as a fungicide in several countries including the UK and the Netherlands. Previously, products based on carbendazim were applied during the spring and summer to prevent tree and fruit infection in this period. This product is no longer approved for use in the EU. Most nurseries are similarly treated to control possible infection spreading in from outside by routine fungicide sprays. However, this approach does not seem to prevent the fungus from invading the tree and remaining as a latent infection, and may indeed promote the establishment of asymptomatic infection.

For the apple *N. ditissima* pathosystem, very little is known about the pathogenicity factors of the pathogen or the resistance mechanisms of the host. Recent work using the cultivar 'Royal Gala' has demonstrated that there are strains of *N. ditissima* that are almost non-pathogenic and others that are pathogenic, though it is not yet known whether the nearly non-pathogenic isolates are more pathogenic on other cultivars (Scheper et al., 2015). It is also unknown how resistance may be expressed in different tissues of the host e.g. wood vs. fruit. It may be that resistance mechanisms are localised at the leaf scar, an area that is vulnerable to pathogen attack, as many reports have shown variation in susceptibility of leaf scar infections (Alston, 1970; Amponsah et al., 2015).

*Malus s*pecies and apple cultivars show variation in susceptibility to *N. ditissima* (Alston, 1970; Ghasemkhani et al., 2015; Van De Weg, 1987; van de Weg, 1989) though most modern varieties are susceptible. Variations in disease susceptibility may partly be a result of disease escape e.g. the speed of wound healing in relation to *N. ditissima* infection has been shown to differ between cultivars (Xu et al., 1998).

No specific molecular resistance mechanisms have yet been reported to *N. ditissima.* It is therefore unknown whether basal defences are consitutively higher in resistant cultivars, or whether the strength or breadth of downstream induced resistance responses contributes to quantitative variation in resistance to *N. ditissima*. It is important to understand not only the genetic architecture of resistance (and the tissues in which it is expressed), but also the mechanism by which the pathogen is detected by the host.

The objective of this study was to identify the molecular basis of pathogenicity of *Neonectria ditissima.* Here I present three rapid screening tests to assess the response to *N. ditissima* in different apple scion and seedlings from different crosses. In addition, genomic resources were generated for further gene expression analysis to identify and characterise pathogenicity effectors genes of *N. ditissima*. Lastly, I present preliminary data from a survey of the phylogenetic relationships global samples of *N. ditissima* to understand whether there are significant differences between geographically distinct populations.

Materials and methods

De novo assembly of *N. ditissima* genome.

Recently we published a draft genome of a UK *N. ditissima* isolate (Gomez-Cortecero, Harrison, & Armitage, 2015).

Isolate R09/05 was obtained in 2005 from a canker-infected wood of *Malus domestica* cv. “Cox” in Kent, United Kingdom. A single ascospore culture of R09/05 was used for the genome sequencing. DNA libraries were sequenced using 300-bp reads on an Illumina MiSeq machine, resulting in 5,389,629 paired-end reads. The sequencing depth was estimated to be 42X. *De novo* assembly was performed using SPAdes version 3.1.0.

The same isolate was sequenced using Pacbio sequencing technologies and combined with the previous draft genome. *De novo* assembly of the Pacbio reads was performed using Canu and the error rate polished using the illumine reads. A hybrid assembly of both sequencing types of reads were performed using SPAdes version 3.1.0 . Consensus sequences of Spades and Canu assemblies were assembled together.

RNAseq data from the same isolate was aligned to the genome and gene prediction was carried out using Braker. Additional genes were added to Braker gene predictions, using CodingQuary in pathogen mode to predict additional regions.

Putative pathogenicity and effector related genes were identified within Braker gene models. Augustus gene models was used for the identification of effectors and secreted proteins and Hmmscan was used to identify secreted CAZY enzymes.

Locus identification for phylogenetic analysis

Existing primer sets from Marra and Corwin (2009), Shivas and Tan (2009), Grafenhan *et. al* (2011) and Armitage *et. al.* (2015) were BLASTed to the R09/05 genome sequence in order to identify loci (Gomez-Cortecero et al., 2015). Extracted regions were then BLASTed to the N305S21 and N324S12 *N. ditissima* genomes (Deng et al., 2015). Hits to the three genomes were examined for polymorphism and primers that contained no polymorphic sites between isolates with an amplicon length of approximately 500bp were designed.

DNA extraction and PCR amplification

Mycelium of isolates of *N. ditissima* gathered from the UK, Netherlands, Belgium, New Zealand and Brazil (Table 1) were grown in YPD liquid media (20g Bacto peptone, 10g yeast extract, 950mL of water, 50mL of 40% w/v glucose). The culture flask was incubated in a shaker at a constant 20°C at 120 rpm for 1 week. Cultures were then centrifuged at 5000g and the supernatant removed. The mycelium was washed with 10ml of sterile water and the supernatant removed after centrifugation. Liquid nitrogen was used to freeze the mycelium and 100mg of wet weight was homogenized using ball bearings and a tissue lyser for 2 minutes at 15Hz. For DNA extraction the Macherey-Nagel NucleoSpin Plant II kit was used following a modified manufacturer’s protocol.

Table 1: *N. ditissima* isolates used in this study with host of isolation and contributor**.**

Isolate accession	CV	Origin	Year of isolation	Contributor
R09/05	Cox	Kent, UK	2005	Angela Berrie, EMR, UK
HG199	Gala	Kent, UK	1999	Angela Berrie, EMR, UK
HG23	Gala	Kent, UK	1999	Angela Berrie, EMR, UK
HG187/B	Gala	Kent, UK	1999	Angela Berrie, EMR, UK
TL109	Cox	Kent, UK	1999	Angela Berrie, EMR, UK
TL88	Gala	Kent. UK	1999	Angela Berrie, EMR, UK
M46/A	Various	Kent, UK	1990's	Angela Berrie, EMR, UK
R28/15	Gala	Hampshire, UK	2015	Angela Berrie, EMR, UK
R36/15	Jonagold	Belgium	2006	Tom Smets, PCF, B
R37/15	Jonagold	Belgium	1999	Tom Smets, PCF, B
R38/15	Golden Delicious	Belgium	2006	Tom Smets, PCF, B
R40/15	Kanzi	The Netherlands	2015	Marcel Wenneker, WUR, NL
R41/15	Wellant	The Netherlands	2015	Marcel Wenneker, WUR, NL
R42/15	Elstar	The Netherlands	2015	Marcel Wenneker, WUR, NL
R43/15	Junami	The Netherlands	2015	Marcel Wenneker, WUR, NL
R44/15	Rubens	The Netherlands	2015	Marcel Wenneker, WUR, NL
R45/15	Elstar	The Netherlands	2015	Marcel Wenneker, WUR, NL
R46/15	Jonagold	The Netherlands	2015	Marcel Wenneker, WUR, NL
R47/15	Delcorf	The Netherlands	2015	Marcel Wenneker, WUR, NL
R48/15	Natyra	The Netherlands	2015	Marcel Wenneker, WUR, NL
NB8/15	Royal Gala	Santa Catarina, Brazil	2015	Hugo Medeiros , EPAGRI, BR
NB9/15	Royal Gala	Santa Catarina, Brazil	2015	Hugo Medeiros , EPAGRI, BR
LDPL01	Golden Delicious	Taranaki, New Zealand	2009	Reiny Scheper, PFR, NZ
LDPK01	Brookfield Gala	Lower Moutere, New Zealand	2009	Reiny Scheper, PFR, NZ

The PCR reaction mixture contained 1µl of gDNA (5ng/µl), 0.5 µM of each primer, 0.625U of Taq polymerase, 0.2 mM of dNTPs, 1X PCR buffer and water to a final volume of 25 µl. The resulting PCR products were purified using the Macherey-Nagel NucleoSpin Gel and PCR clean-up kit following the manufactures protocol.

Alignment and Population analysis

Sequenced ABI reads were imported into Geneious 9.0.4 software (www.geneious.com) and forward and reverse reads were aligned and consensus called. Construction of combined SNP and microsatellite haplotypes was carried out manually (Figure 2).

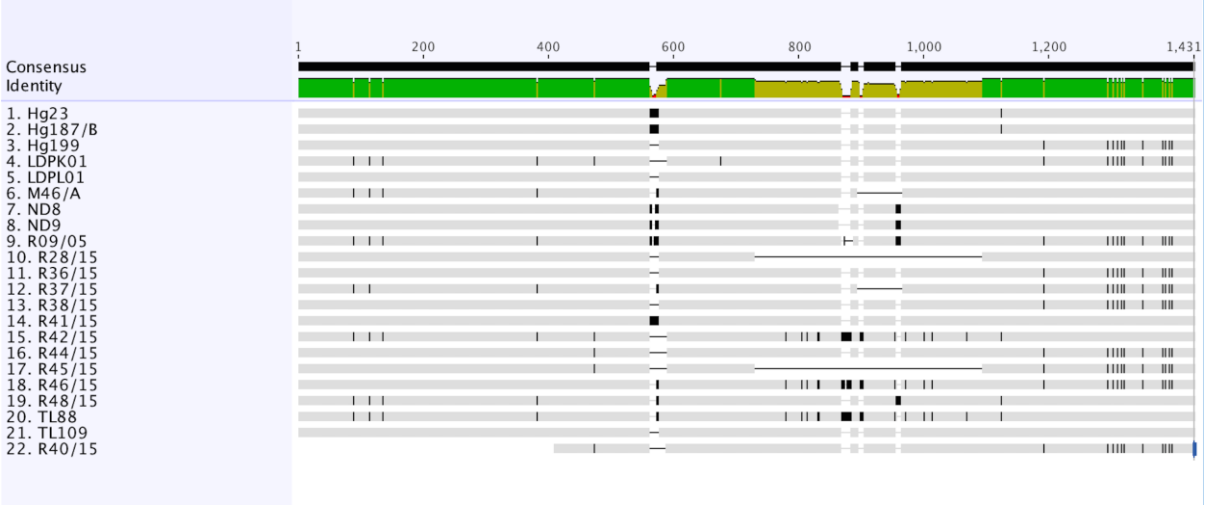

Figure 2. Concatenated consensus alignments of four polymorphic locus.

Inoculum preparation

Three isolates were used in the pathogenicity experiments; R09/05, Hg199 and R28/15 (Table 1).

The isolates were sub-cultured onto SNAY media for 13-15 days. A spore suspension was prepared using sterile water and conidia were release from sporodochia using a plastic spreader.

Cultivar cut shoot test

Dormant one-year old shoots with a length of approximately 50cm were collected from mature trees of ‘Aroma’, ‘Beauty of Bath’, ‘Cox’s Orange Pippin’, ‘Gala’, ‘Gloster 69’, ‘Golden Delicious’, ‘Grenadier’, ‘Idared’, ‘M9’, ‘Robusta 5’ and ‘Wolf River’. Shoots were inoculated with a spore suspension of two *N. ditissima isolates,* Hg199 and R09/05 along with a water control and placed into a controlled environment cabinet. The inoculation protocol was adapted from van de Weg (1989) (van de Weg, 1989). The experiment was divided between two growth cabinets, within which were trays containing cut shoots of cultivars, inoculated at three points (pseudo-replicates) with one of two *N. ditissima* isolates or a water control (not included in this analysis). Lesion length was recorded using digital callipers at 12, 16, 22, 27, 31 and 35 days post-infection. The Area Under Disease Progress Curve (AUDPC) was calculated using the agricolae package (de Mendiburu, 2014), using R version 3.2.2 (Team, 2015).

This experiment was repeated in January 2016, using a subset of cultivars inoculated with isolates R28/15, Hg199 and R45/15. The protocol differed slightly since instead of three inoculations per shoot, a single inoculation was carried out to allow lesion expansion in highly susceptible cultivars to be accurately recorded at the later stages of the experiment (14, 18, 21, 27, 34, 39, 45, 49 and 54 days post-inoculation). The data are presented from day 34, to facilitate comparison with the 2015 experiment.

Apple seedling test

6-months old potted apple seedlings were inoculated with a spore suspension of one *N. ditissima* isolate, the R09/05. The same inoculation protocol adapted from van de Weg (1989) (Van de Weg, 1989) was used. Three leaves from each plant were removed; either the fifth, seventh and ninth (or fourth, sixth and eighth) leaves depending upon the size of the plant. The corresponding axillary bud was also removed. Within five minutes of cutting, 3µl of a conidial suspension of a single *N. ditissima* isolate was placed onto the wound with an automatic micropipette.

Seedlings were fully randomised and divided into sets of 88 and placed on two benches either side of the glasshouse. A subset of 16 seedlings (all genetically non-identical) from eleven bi-parental crosses (total 176 seedlings) were used in this test. Lesion length was recorded and AUDPC calculated.

Leaf scar inoculation potted tree test

Dormant one-year-old shoots from mature trees of ‘Aroma’, ‘Golden Delicious’, ‘Gala’, ‘Gloster 69’, ‘Grenadier’, ‘Robusta 5’, ‘M9’, ‘E93-79’, ‘E202-6’ and ‘Idared’ were grafted onto M9 rootstocks in February 2015 (UK). Trees were moved to a glasshouse one day before inoculation, at the end of October 2015. Temperature varied in the glasshouse from 10°C to 25°C and no additional lights were used during the experiment. On each tree, five leaves were removed randomly along the tree leaving approximately the same distance among them. An inoculum volume of 10µl of spore suspension or water control was applied to each leaf scar. The position of the trees in the glasshouse and the order of inoculation was randomised in five different sets with one tree per cultivar, inoculating four sets with a single *N. ditissima* isolate and one with water. After five weeks, trees were moved outside keeping the same randomized design. The first symptoms of infection appeared 70 days post inoculation and lesion length was recorded using digital callipers at approximately fortnightly intervals. The experiment was ended at 115 days post inoculation.

Results

Secreted effectors proteins identification.

The genome of the R09/05 isolate of *Neonectria ditissima* was assembled into 44.75 Mb in 43 contigs (>1000 bp), with an N50 metric of 1.98 Mb, largest scaffold of 6.45 Mb and G+C content of 50.91%. Braker gene prediction supplemented with Codingquary predicted 13,490 genes.

The pathogen secretes proteins, called effectors, to modulate the host cells response suppressing the defenses and allowing the colonization. Putative effectors genes were identified within the *N. ditissima* genome. EffectorP software predicted 167 genes of secreted effectors proteins. In addition, 732 Carbonhydrate-Active Enzymes genes (CAZymes), of which 288 were secreted proteins, were identified. CAZymes are responsible for the synthesis, degradation and modification of the carbohydrates of the cell wall having an important role in the pathogenicity of *N. ditissima.*

Both, secreted effector proteins and CAZymes are distributed throughout the genome (Figure 3).

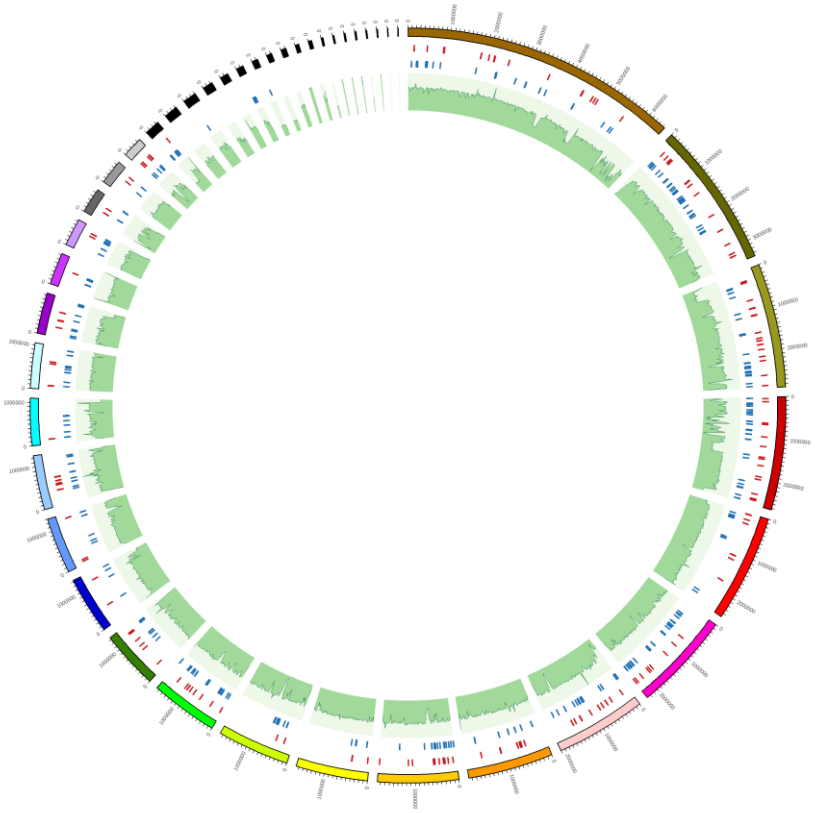

Figure 3: Architecture of the genome of *Neonectria ditissima.* Concentric rings from outer to inner refer to: **1**. R09/05 genome assembly. **2**. Predicted secreted effector proteins (Red). **3**. Predicted Carbohydrate-active enzymes (Blue). **4**. Alignment of Illumina reads of the Hg199 isolate. Diagram created using circos.

Population analysis of *N. ditissima* reveals only slight evidence for geographically structured populations

Little is known about the extent or patterns of nucleotide diversity of *N. ditissima*, or whether there are any patterns of isolation by distance on a local or a global scale. In order to study this, isolates of N ditissima gathered from the UK, Netherlands, Belgium, New Zealand and Brazil were evaluated at four single copy loci found to be polymorphic in the three recently published N. ditissima reference genomes (Deng et al., 2015; Gomez-Cortecero et al., 2015). For each locus, between 20 and 22 isolates were evaluated.

The number of segregating SNP sites varied between 2 and 12 and estimates of π, a measure of nucleotide diversity ranged by approximately an order of magnitude (0.002-0.018), depending upon the locus (Table 2). In all but one case, Tajima's *D* (a comparison of the scaled mean number of pairwise differences and the number of segregating sites) revealed no evidence for selective or demographic processes acting on the chosen loci. However, in the case of the CDP gene, where a clear haplotype containing 11/12 SNPs can be seen, there is a significantly positive measure of Tajima's D, indicative of non-neutral patterns of nucleotide polymorphism.

Gene	Samples	Sites	Sites for SNP	Segregating	π (average pairwise differences per site)	$\hat{\theta}_W$ (Watterson's theta-segregating sites)	Tajima's D (SNP)	Haplotypes (SNP)	Private SNP haplotypes (origins)	Haplotypes incl Microsat	Private SNP and microsatellite haplotypes (origins)	Evidence for recombination
ACL1	21	409	409	4	0.00447	0.00272	1.84 (ns)	3	Belgium	NA	Belgium	No
CDP	22	339	339	12	0.01794	0.00971	2.97 (p<0.01)	3		NA		No
NDCAA4_prox	22	321	297	2	0.00155	0.00185	-0.37 (ns)	3		7	Brazil, Netherlands, New Zealand, UK	No
NDCAA11_sub	20	362	278	9	0.00841	0.00913	-027 (ns)	3	Netherlands	7	Brazil,UK and Netherlands	No

Table 2. Population genetics statistics for a global sample of *N. ditissima*

The number of SNP haplotypes was the same (3) in each locus under study and only a two private SNP haplotypes were found, one in the Netherlands, in a single individual, for the CAA11_sub locus and one in a Belgian isolate for the ACL1 locus, indicating that most polymorphism is shared between populations (Table 2). Including both SNP and microsatellite variation (for which the mutation rate per cell division may be over twice as high $7x10^{-8}$) in the analysis of private haplotypes reveals that despite the small sample size, distinct private haplotypes could also be detected in UK, Netherlands, Brazilian and New Zealand samples. Across all samples no evidence for recombination could be detected using the four-gamete test (Hudson & Kaplan, 1985).

Differences in partial resistance to canker among cut shoots of apple cultivars

It is widely known that cultivars vary in their susceptibility to canker, though the exact molecular mechanism is unknown. In order to further study the response of cultivars to different inocula, different infection methods and at different physiological conditions, a pathogenicity screen using two UK isolates (R09/05 and Hg199) was carried out first, using dormant cut shoot material (van de Weg, 1989). This test allows colonization rate to be calculated and compared between isolates and cultivars. After inoculation, lesions progressed vertically along the shoots. The symptoms consisted of a sunken and necrotic bark area around the inoculation point, the progress of which was measured in a non-destructive manner with calipers. These symptoms were noticeable after 12 days after inoculation in the cut-shoot test. Cut shoot tests revealed abundant variation in resistance

and susceptibility to *N. ditissima*, but little variation in isolate pathogenicity. This variation in the response among the cultivars was consistent regardless of the differences in the inoculation pressure between the isolates.

For apple scion material, it was found that the species *Malus* x *robusta* c.v. 'Robusta 5' had the highest level of resistance in the cut shoot tests (Figure 4), followed by the known resistant cultivar 'Golden Delicious'. At the other end of the resistance spectrum, the known susceptible cultivars 'EMLA-'M9' (a rootstock) and 'Cox' were highly susceptible (Figure 4). Intermediate levels of resistance were seen for other reported field-resistant or tolerant material, including 'Aroma', 'Beauty of Bath' and 'Grenadier'. Somewhat surprisingly the field-susceptible cultivar, 'Gala' was found to be more resistant than expected to *N. ditissima* infection using this method. Based on its reported parentage ('Golden Delicious' x 'Kidd's Orange Red'- the latter reported to be a 'Delicious' x 'Cox' cross), it has both resistant and presumed susceptible material in its pedigree indicating the potential for at least partial resistance, consistent with the performance of 'Golden Delicious' and 'Cox' in this test).

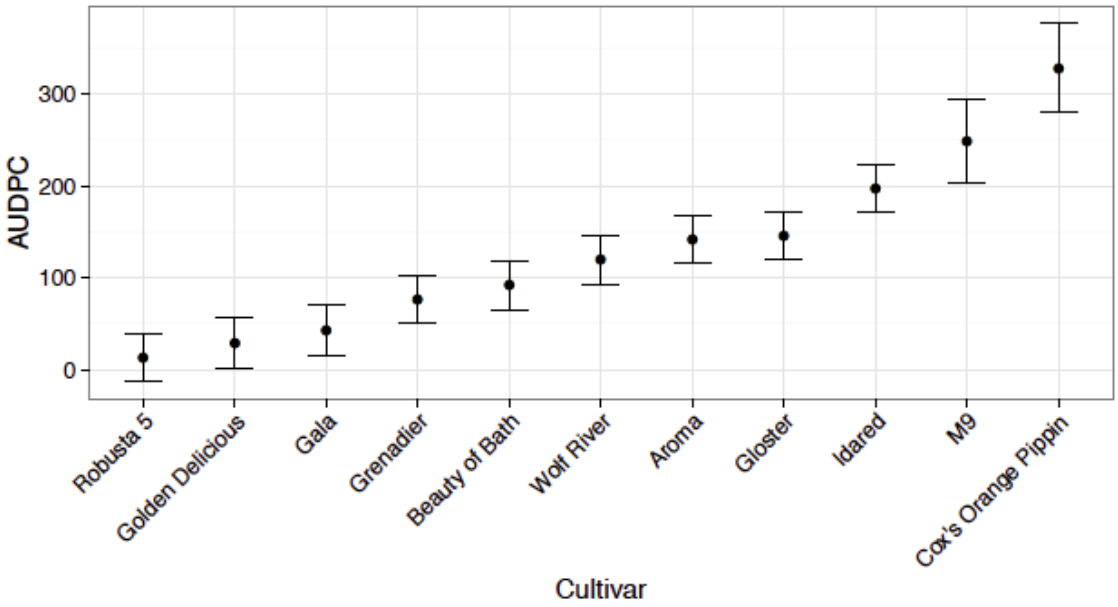

Figure 4. Cut-shoot inoculation in 2015. Mean Area under disease progress for inoculated cut shoots of common apple scion material calculated 35 days post infection (shown with standard errors).

Repetition of this experiment in 2016 with three isolates of *N. ditissima* revealed similar results, with 'Gala' and its offspring 'Scifresh' and 'Scilate' ('Gala' x 'Braeburn') all showing low levels of lesion spread and no cultivar by isolate interaction (Figure 5).

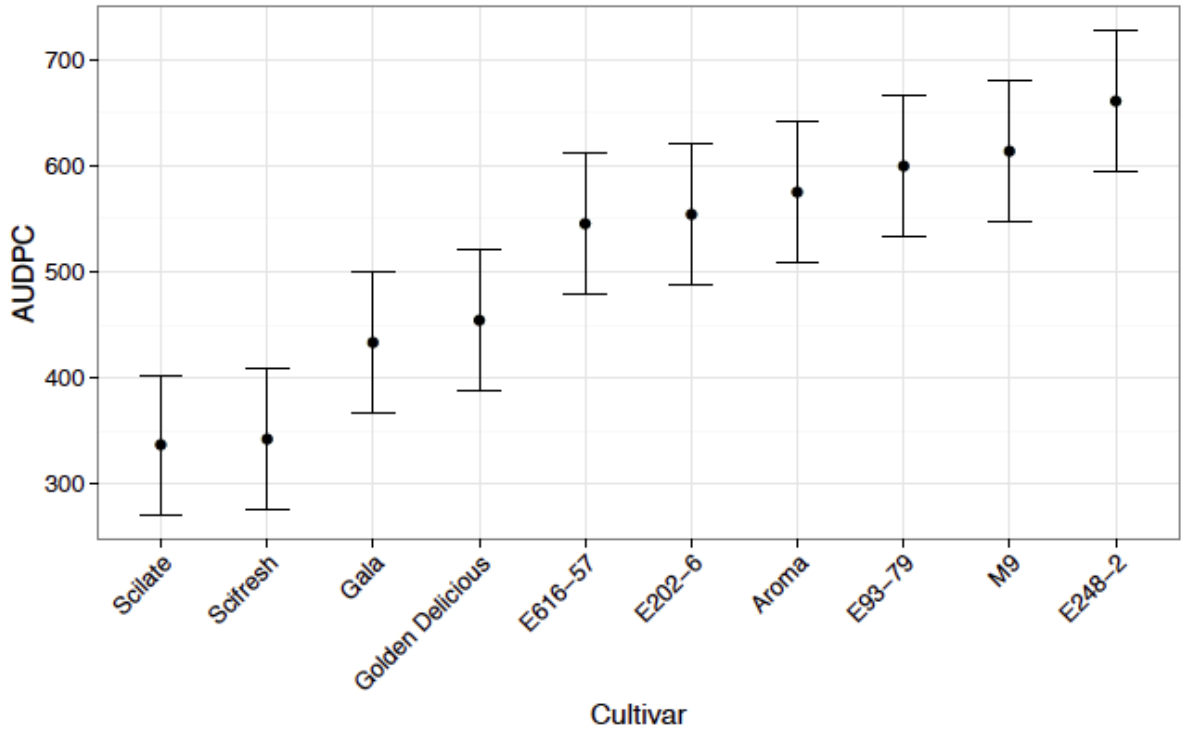

Figure 5. Cut-shoot inoculation in 2016. Mean Area under disease progress for inoculated cut shoots of common apple scion material calculated 34 days post-inoculation (shown with standard errors).

Differences in partial resistance to canker determined by leaf scar inoculation

Alongside cut shoot tests, leaf scar infection tests were carried out (Alston, 1970; Amponsah et al., 2015; Scheper et al., 2015). Again, the species level accession 'Robusta 5' demonstrated high levels of resistance (Figure 6). As with previous reports, 'Gala' was extremely susceptible in this pathogenicity test, with high levels of colonization after inoculation with the same isolate of *N. ditissima* as used in the cut shoot test (Scheper et al., 2010). 'Gloster 69' and 'E202-6' also showed high levels of susceptibility. Intermediate levels of resistance were seen in 'Golden Delicious', 'Idared', 'Aroma', 'M9', 'Grenadier' and 'E93-79'.

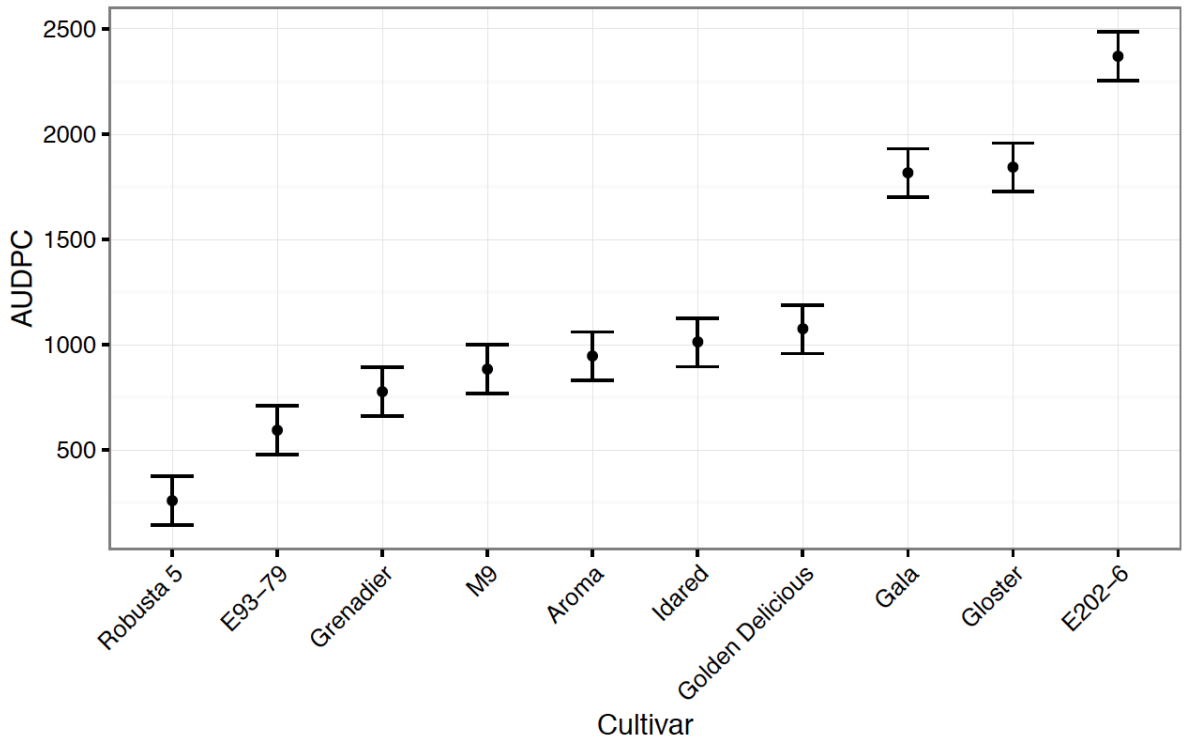

Figure 6. Mean Area under disease progress for inoculated leaf scars of common apple scion material calculated 153 days post infection (shown with standard errors). The rootstock M9 is also included as a qualitative comparison.

Seedling tests indicate a complex genetic basis for resistance

In order to further test the resistance responses of different parental material with respect to variation in colonization rate, following wound inoculation and the manner in which resistance is transmitted, crosses were made between parents, many of which were tested in the cut shoot test. Examination of the AUDPC values revealed that segregation patterns varied and crosses with both highly resistant offspring (MDX053 and MDX051 having the lowest median AUDPC values) and highly susceptible offspring (MDX057, MDX068) were observed (Table 3).

Cross Number	Female parent	Male parent	Population median (AUDPC)	Population IQR (AUDPC)	Cross combination resistance based on cut-shoot R-resistant, I-intermediate, S-susceptible , U-unknown (F x M)
MDX051	Gala	Santana	28.58	101.5	R x U
MDX052	Aroma	Gala	60.41	108.8	I x S
MDX053	Aroma	Fuji	25.92	148.41	I x U
MDX054	Aroma	Golden Delicious	181.42	248.95	I x R
MDX057	Gloster 69	Idared	266.61	227.03	I x S
MDX060	E248-2	E616-57	234.06	185.85	U x U
MDX061	E93-79	Gala	180.53	251.23	U x S
MDX063	E202-6	Golden Delicious	244.59	292.96	U x S
MDX064	Gala	3760	85.8	242.69	U x R
MDX065	Gala	3762	105.05	158.53	S x U
MDX068	Grenadier	Golden Delicious	253.39	251.96	I x R

Table 3. Cross combinations tested in the seedling test and population mean and standard deviation of the AUDPC values.

The segregation patterns that were observed were complex and some resistant parents showed poor transmission of resistance into the progeny. For example, crosses involving ‘Golden Delicious’, even when crossed with other moderately resistant parental lines (e.g. ‘Aroma’, MDX054 and ‘Grenadier’, MDX068) showed higher median levels of disease progress (Median AUDPC 181.42) than crosses involving the same parental material (e.g. Aroma) crossed to more susceptible material (e.g. ‘Gala’, MDX052- Median AUDPC 60.41), though significant differences were only observed in a single pairwise non-parametric Kruskal Wallis test between MDX052 and MDX068, but not MDX052 x MDX054 and MDX054 x MDX068.

Discussion

Our data, although incomplete, presents a pattern of SNP diversity consistent with the notion that there is broad similarity between geographically isolated populations and that much of the genetic diversity seen in the European population of *N. ditissima* are also seen in South American and Oceanic populations. At the SNP level there is little information about recent geographic isolation, as there is no clear pattern of private allelic variation with geographic origin. These data are consistent with the idea that *N. ditissima* spread from Europe to other regions of the world on imported apple plant material. Despite the small sample size, it was possible to detect with the aid of more rapidly evolving microsatellite loci evidence for some distinct patterns of polymorphism in UK, Netherlands, Brazilian and New Zealand populations of *N. ditissima.*

Interestingly, despite *N. ditissima* being a sexually reproducing species, with a year-round reproductive potential no evidence for recombination could be detected in our sequenced isolates. Further data are needed to study the genome-wide patterns of recombination to determine whether the patterns observed from the loci used in this study are general to the whole genome. The levels of nucleotide diversity vary widely and due to the clear haplotype structure of some loci there are signatures of non-neutral processes (evidenced by significantly positive Tajima's D values). A key question to address in the future is whether these patterns are present across the whole genome; if so this would be indicative of demographic processes influencing patterns of nucleotide diversity. Alternatively, it could be that different populations of *N. ditissima* have expanded their host-ranges onto apple to create hybrid recombining populations. It is important to undertake population-level analyses as genome-wide association studies of pathogenicity may be confounded by high levels of ancestral population structure. The finding that much of the common variation at putatively neutrally evolving loci is present in UK populations suggests that if resistance is found that is consistent across multiple variable UK isolates, that the same pattern may hold in other populations.

The finding from both preliminary cultivar cut shoot tests, that there is no isolate by cultivar interaction, suggests that the host response is consistent, regardless of the isolate that is inoculated. To confirm these initial observations, a further study of more isolates is required. All higher order interactions were non-significant, indicating that there may be a relatively simple pattern of host response which is not influenced by an isolate race-structure, consistent with previous reports (van de Weg, 1989).

While our results do not support the existence of distinct pathogen races, this has no bearing on whether the resistance that has been identified may be durable or not (as this is primarily determined by the capability of the pathogen to overcome specific defence or recognition mechanisms). However, it may suggest that resistance is targeting conserved factors in the pathogen and therefore the resistance present in the tested cultivars may be broad spectrum and thus has the potential to be durable. It is interesting to note that the most resistant cultivar 'Robusta 5' is a representative of a species that is distinct from *M.* x *domestica*. Little is known about natural *M.* x *robusta* species, since much of the material that is present in Europe was collected in Northern China. It is described as a hybrid species, though this is only by morphology (Forsline et al., 2002). What is interesting to note is that *N. ditissima* is not reported as a significant pathogen of apple in China, indicating that *M.* x *robusta* may be a non-host and therefore that the mode of resistance in *M.* x *robusta* versus the cultivated apple *M.* x *domestica* may be of distinct evolutionary origin. It is therefore important to study multiple origins of resistance, as some may be more durable than others, or pyramiding combinations of different alleles may offer greater resistance by combining multiple mechanisms of resistance.

The seedling test that was carried out revealed that resistance sources differed in their transmission characteristics. Most striking was the observation that crosses involving 'Golden Delicious', found to be highly resistant in cut shoot tests, had a greater level of susceptibility when crossed to resistant material, than supposed resistant x intermediate / susceptible crosses. This could be explained if the nature of the resistance sources differed among cultivars, i.e. if the resistance from 'Golden Delicious' was recessive, or if susceptibility factors in some cross combinations lead to resistance that is non-additive. These preliminary results suggest that the likelihood of transmission of resistance varies between resistant parental material and that some parental material appears to be superior to others in ability to donate resistance, despite slightly lower overall resistance in the cut shoot tests (i.e. moderately resistant 'Aroma' versus highly resistant 'Golden Delicious'). This part of the study highlights the importance of trial evaluation of seedling populations prior to embarking upon QTL studies and the importance of considering the mode and mechanism of resistance and the way in which it is phenotyped in breeding programmes.

We have identified the presence of multiple effector genes and enzymes throughout the genome of *N. ditissima.* Nevertheless, a deeper understanding of genes related with the pathogenicity is needed. Some regions in our genome have a high representation of effectors and CAZy enzymes genes. Future work will be focused to narrow down this though a gene expression analysis during the time course of an infection. Then we will seek overexpressed genes as possible candidates pathogenicity genes.

With the recent publication of new *N. ditissima* genome sequences (Deng et al., 2015; Gomez-Cortecero et al., 2015) and the increasing amount of genomic information available for apple (Antanaviciute et al., 2012; Bianco et al., 2014; Bink et al., 2014; Velasco et al., 2010) it is likely that rapid progress can be made in identifying the genetic basis of resistance to *N. ditissima*.

Conclusions

It is still unclear whether the methods that have been tested in this study are of direct relevance to the orchard situation. Therefore it is entirely possible that a newly developed cultivar that is resistant according to these tests turns out to be susceptible in the field. It is clear that both resistance to colonization and initial infection are important components of field resistance. Some cultivars that we have studied, such as 'Gala' appear to have consistently high levels of resistance to colonisation in cut shoot tests and yet are often considered to be field susceptible and indeed in whole-tree leaf scar tests are much more susceptible. It should be noted that in both types of pathogenicity test 'Robusta 5' displays low levels of infection and subsequent colonization. This suggests that the cut shoot and leaf scar tests are querying different components of resistance and that for strong resistance, low levels of colonisation and lesion expansion in both tests are required. In order to be considered to be field resistant, trees must have low disease incidence when several wound types are inoculated; a small lesion size when infection does occur; low spore production from lesions; negligible internal (latent) growth of the pathogen.

It is also important to consider the role of abiotic stresses in modulating plant resistance. It is unclear at present, when issues with drainage in the orchard occur or other changes in tree health, or nitrogen applications, whether the resistance status of some trees may alter more than others.

Future work needs to be carried out to compare the results presented in this study with trees grown outside in an orchard setting, inoculated using several different wounds (leaf scars, pruning cuts, picking wounds) to determine whether the methods developed in this paper can be considered to be sufficient for rapid selection in breeding programmes.

Future work

Extend and deploy an accurate and quantifiable automated pathology test to measure quantitative differences in pathogenicity of different *N. ditissima* isolates and host responses. Different plant models and inoculation methods will be used to assess the responses of apple cultivars in different humidity temperature combinations in order to identify the optimal conditions for disease development.

Once a pathogenicity test has been established and a set of isolates of differing virulence determined, a set of experiments to understand the expression of virulence factors and the response of both resistant and susceptible hosts will be carried out. This will involve a gene expression analysis during the timecourse of infection on apple plant material, sampling host tissue containing fungal material during the course of an infection. RNA sequencing reads of

both the host and the pathogen will be aligned to the *N. ditissima* genome sequence and differentially expressed transcripts will be sought.

This will allow the identification of specific genes that are upregulated in the pathogen and the host during different stages of infection. This is extremely important, as identification of the specific genes controlling host defence and pathogen virulence need to be identified in order to understand how host accessions and pathogen strains differ.

Once a comprehensive list of genes important to pathogenicity have been identified from genomic and transcriptomic comparisons, validation of gene function will be attempted by knockout of key gene targets in the pathogen through homologous recombination-mediated gene deletion and targeted transformation. The pathogenicity test will be repeated and differences in the virulence of deletion versus wild-type lines will be assessed in differentially resistant hosts.

Knowledge and Technology Transfer

25th November 2015 – The 2nd EMR PhD student poster exhibition. Poster presentation.

26th May 2016 - Postgraduate Symposium at University of Reading. Project presentation.

18th August 2016 – Poster presentation at University of Reading Microbiology research day. Awarded with the poster prize.

12th-13th September 2016 - BSPP Presidential Meeting. Poster presentation.

17th-19th October 2016 – 2016 International Academic Conference at Nanjing Agricultural University. Project presentation. Awarded with the first prize in the presentation competition.

17th-19th October 2016 – The 3rd International Horticulture Research Conference at Nanjing. Poster presentation. Awarded with the first prize in the poster competition.

References

Alston, F. (1970). Response of apple cultivars to canker, Nectria galligena. *Annu. Rep. East Malling Res. Stn. 1969* A53, 147–8.

Amponsah, N. T., Walter, M., Beresford, R. M., and Scheper, R. W. A. (2015). Seasonal wound presence and susceptibility to Neonectria ditissima infection in New Zealand apple trees. *New Zeal. Plant Prot.* 68, 250–256.

Antanaviciute, L., Fernandez Fernandez, F., Jansen, J., Banchi, E., Evans, K. M., Viola, R., et al. (2012). Development of a dense SNP-based linkage map of an apple rootstock

progeny using the Malus Infinium whole genome genotyping array. *BMC Genomics* 13, 203. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22631220 [Accessed July 26, 2012].

Armitage, A. D., Barbara, D. J., Harrison, R. J., Lane, C. R., Sreenivasaprasad, S., Woodhall, J. W., et al. (2015). Discrete lineages within Alternaria alternata species group: Identification using new highly variable loci and support from morphological characters. *Fungal Biol.* 119, 994–1006. doi:10.1016/j.funbio.2015.06.012.

Bianco, L., Cestaro, A., Sargent, D. J., Banchi, E., Derdak, S., Di Guardo, M., et al. (2014). Development and validation of a 20K single nucleotide polymorphism (SNP) whole genome genotyping array for apple (Malus × domestica Borkh). *PLoS One* 9, e110377. doi:10.1371/journal.pone.0110377.

Bink, M. C. A. M., Jansen, J., Madduri, M., Voorrips, R. E., Durel, C.-E., Kouassi, A. B., et al. (2014). Bayesian QTL analyses using pedigreed families of an outcrossing species, with application to fruit firmness in apple. *Theor. Appl. Genet.* 127, 1073–90. doi:10.1007/s00122-014-2281-3.

Deng, C. H., Scheper, R. W. A., Thrimawithana, A. H., and Bowen, J. K. (2015). Draft Genome Sequences of Two Isolates of the Plant-Pathogenic Fungus Neonectria ditissima That Differ in Virulence. *Genome Announc.* 3, e01348-15. doi:10.1128/genomeA.01348-15.

Dryden, G. H., Nelson, M. A., Smith, J. T., and Walter, M. (2016). Postharvest foliar nitrogen applications increase *Neonectria ditissima* leaf scar infection in apple trees. *New Zeal. Plant Prot.* 69, 230–237.

Forsline, P. L., Aldwinckle, H. S., Dickson, E. E., Luby, J. J., and Hokanson, S. C. (2002). *Collection, Maintenance, Characterization, and Utilization of Wild Apples of Central Asia*. , ed. J. Janick Oxford, UK: John Wiley & Sons, Inc.

Ghasemkhani, M., Garkava-Gustavsson, L., Liljeroth, E., Nybom, H., Bernier, L., Hubbes, M., et al. (2016). Assessment of diversity and genetic relationships of Neonectria ditissima:

the causal agent of fruit tree canker. *Hereditas* 153, 7. doi:10.1186/s41065-016-0011-3.

Ghasemkhani, M., Liljeroth, E., Sehic, J., Zborowska, A., and Nybom, H. (2015). Cut-off shoots method for estimation of partial resistance in apple cultivars to fruit tree canker caused by Neonectria ditissima. *Acta Agric. Scand. Sect. B — Soil Plant Sci.* 65. doi:10.1080/09064710.2015.1016101.

Gomez-Cortecero, A., Harrison, R. J., and Armitage, A. D. (2015). Draft Genome Sequence of a European Isolate of the Apple Canker Pathogen Neonectria ditissima. *Genome Announc.* 3, 10–11. doi:10.1128/genomeA.01243-15.

Gräfenhan, T., Schroers, H.-J., Nirenberg, H. I., and Seifert, K. A. (2011). An overview of the taxonomy, phylogeny, and typification of nectriaceous fungi in Cosmospora, Acremonium, Fusarium, Stilbella, and Volutella. *Stud. Mycol.* 68, 79–113.

Hudson, R. R., and Kaplan, N. L. (1985). Statistical properties of the number of recombination events in the history of a sample of DNA sequences. *Genetics* 111, 147–64. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1202594&tool=pmcentrez&rendertype=abstract [Accessed February 7, 2016].

Librado, P., and Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25, 1451–2.

Marra, R. E., and Corwin, J. A. (2009). Isolation and characterization of codominant markers for the perennial canker fungal pathogen Neonectria ditissima. *Mol. Ecol. Resour.* 9, 906–909. doi:10.1111/j.1755-0998.2008.02410.x.

McCracken, A. R., Berrie, A., Barbara, D. J., Locke, T., Cooke, L. R., Phelps, K., et al. (2003). Relative significance of nursery infections and orchard inoculum in the development and spread of apple canker (Nectria galligena) in young orchards. *Plant Pathol.* 52, 553–566.

de Mendiburu, F. (2015). agricolae: Statistical Procedures for Agricultural Research. R

package version 1.2-3. http://CRAN.R-project.org/package=agricolae.

Scheper, R. W. A., Fisher, B. M., and Wood, P. N. (2010). Pathogenicity of field and laboratory-grown inoculum of Neonectria galligena on potted apple trees. in *New Zealand Plant Protection*, 280.

Scheper, R. W. A., Frijters, L., Fisher, B. M., and Hedderley, D. I. (2015). Effect of freezing of Neonectria ditissima inoculum on its pathogenicity. *New Zeal. Plant Prot.* 68, 257–263.

Shivas, R. ., and Tan, Y. . (2009). A taxonomic re-assessment of Colletotrichum acutatum, introducing C. fioriniae comb. et stat. nov. and C. simmondsii sp. nov. *Fungal Divers.* 39, 111–112.

Swinburne, T. R. (1975). European canker of apple (Nectria galligena). *Rev. Plant Pathol.* 54, 787–99.

Team, R. C. (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Velasco, R., Zharkikh, A., Affourtit, J., Dhingra, A., Cestaro, A., Kalyanaraman, A., et al. (2010). The genome of the domesticated apple (Malus × domestica Borkh.). *Nat. Genet.* 42, 833–9. doi:10.1038/ng.654.

Weber, R. W. S. (2014). Biology and control of the apple canker fungus Neonectria ditissima (syn. N. galligena) from a Northwestern European perspective. *Erwerbs-Obstbau* 56, 95–107. doi:10.1007/s10341-014-0210-x.

Van De Weg, W. E. (1987). Note on an inoculation method to infect young apple seedlings with Nectria galligena Bres. *Euphytica* 36, 853–854. Available at: http://link.springer.com/10.1007/BF00051869 [Accessed January 26, 2015].

Van de Weg, W. E. (1989). Screening for resistance to Nectria galligena Bres. in cut shoots of apple. *Euphytica* 42, 233–240. Available at: http://link.springer.com/10.1007/BF00034459 [Accessed January 26, 2015].

Xu, X.-M., and Robinson, J. D. (2010). Effects of fruit maturity and wetness on the infection of apple fruit by Neonectria galligena. *Plant Pathol.* 59, 542–547. doi:10.1111/j.1365-3059.2009.02232.x.

Xu, X., Butt, D. J., and Ridout, M. S. (1998). The effects of inoculum dose , duration of wet period , temperature and wound age on infection by Nectria galligena of pruning wounds on apple. 511–519.

Zhang, W., Fraiture, M., Kolb, D., Löffelhardt, B., Desaki, Y., Freddy, F. G., et al. (2013). Arabidopsis RECEPTOR-LIKE PROTEIN30 and Receptor-Like Kinase SUPPRESSOR OF BIR1-1 / EVERSHED Mediate Innate Immunity to Necrotrophic Fungi. *Plant Cell* 25, 4227–4241. doi:10.1105/tpc.113.117010.

