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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

- This project aims to provide fundamental insights into the molecular basis of pathogenicity in *Neonectria ditissima,* the causative agent of apple canker.

Background and expected deliverables

European canker, caused by the phytopathogenic fungus *Neonectria ditissima*, is one of the most destructive diseases of apple and pear. In the orchard, this fungus is able to infect a wide range of apple varieties causing canker and die back of young shoots, resulting in significant losses of fruiting wood. This pathogen has been reported in many apple-producing regions of the world, being especially common in the North-Western European countries. Modern varieties suffer most and in extreme cases do not survive establishment in the orchard. Canker control is difficult to achieve due to the pathogen's lifestyle which is able to infect trees all year-round through wounds, either natural such as bud-scale scars, leaf scars and fruit scars or artificial such as pruning wounds.

Resistance breeding is underway in many global breeding programmes, but nevertheless, a total resistance to canker has not yet been demonstrated in either fruit or woody tissue. There is no known race structure of the pathogen and the global level of genetic diversity of the pathogen population is unknown. Plant resistance is a promising alternative to largely ineffective cultural control, but is time consuming to deploy due 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 in 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. The identification of candidate genes important in virulence in the pathogen could lead to novel opportunities for control by targeted disruption of the pathogen.

Summary of the project and main conclusions

It is widely known that cultivars vary in their susceptibly to canker, though the exact molecular mechanism is unknown. Pathogen variability and host responses can be assessed through screening tests *in planta*, to identify the optimal conditions for disease development. Host responses to different inoculum, different infection methods and at different physiological conditions are being compared in this project. The previous report presented the results of some pathology tests based on the assessments of host responses and discussed the inconsistencies observed in test performance compared with field observations. In this report, the differences in the pathogenicity of several isolates of *N. ditissima* are compared.

During the infection process, the pathogen secretes proteins, called effectors, to modulate the host cells' response suppressing defence and allowing the colonisation. The genome of *N. ditissima* facilitates the identification of putative effectors and pathogenicity genes through bioinformatics analysis of the secretome and through comparisons to other pathogens. In this report, the scientists annotated a previously sequenced *N. ditissima* genome assembly using RNA-Seq data and have now improved this assembly using PacBio long-reads sequencing, allowing them to present an updated analysis of the predicted secretome of this pathogen. This analysis showed a full repertoire of pathogenicity genes, composed of secreted effector proteins and enzymes involved in the cell degradation wall, distributed throughout the genome.

Differences in isolates' pathogenicity might be associated with their gene content. Therefore, analysis and comparison of the genome sequences of isolates differing in virulence might aid the identification of specific regions in the genome related with the virulence. On the other hand, a gene expression analysis will be carried out during the time course of an infection sequencing RNA from infected host tissue. This will allow the identification of the specific genes controlling host defences and pathogen virulence. The identification of specific candidate genes controlling pathogen virulence will allow a better understanding of the mechanism of infection.

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.

Identifying the nature of resistance to *N. ditissima* has been challenging in the past. Previous tests in apple seedlings from different crosses revealed that resistance responses to *N.*

ditissima are complex and involved multiple resistance genes, with epistatic interaction modulating inheritance. Phenotyping resistance responses of an offspring from a single bi-parental population, 'Golden Delicious' x 'M9', through previously defined screening tests, will be useful to explore genetic variation in resistance. With this data, the scientific team will be able to carry out a QTL mapping associated with the resistance to *N. ditissima*.

Financial benefits

- This research project is unlikely to offer any immediate financial benefits to growers, but may lead to the development of novel opportunities for the long-term control of apple canker.

Action points for growers

- No direct action points for growers are likely to be delivered by this project but the results will be used in developing novel methods of management and control of apple canker in future.

SCIENCE SECTION

Introduction

European canker, caused by the necrotrophic fungus *Neonectria ditissima* (Tul. & Tul.) Samuels & Rossman (synonym *N. galligena*), is one of the most destructive diseases of apple and pear. The fungus attacks trees in the orchard, causing canker lesions on the woody tissues, girdling and killing branches, resulting in loss of fruiting wood and increases pruning costs (Swinburne, 1975). Apple canker can be particularly damaging in young orchards where, in some years, up to 10% of trees can be lost annually in the first few years of orchard establishment as a result of trunk cankers.

Neonectria ditissima belongs to the phylum Ascomycota, class Sordariomycetes, subclass Hypocreomycetidae, order Hypocreales and to the family Nectriaceae, group possessing a *Neonectria* perfect (ascosporic) and a *Cylindrocarpon heteronema* imperfect (conidial) state (Castlebury et al., 2006; Chaverri et al., 2011). The taxonomic history of the pathogen is somewhat complex, having altered in name repeatedly over the past 150 years. Studies based largely on host range and morphology have at various times divided the original pathogen (named *Neonectria ditissima* Tul. and C. Tul, Tulasne & Tulasne, 1865) into two separate species, *Nectria ditissima* and *Nectria galligena* (Bres.) (Cayley, 1921) and later renamed *Neonectria galligena* (Bres.) before returning to its original name some 10 years ago (Castlebury et al., 2006).

Apple canker has been reported from Australia, New Zealand, Chile, Japan, South Africa, North America and Europe (Beresford & Kim, 2011; Weber, 2014; Xu & Robinson, 2010; Grove, 1990). A previous study by Beresford and Kim (2011) showed that at least five months per year with certain temperatures (>8 h at 11-16 °C) and a minimum of days with rainfall (>30%) are required for this pathogen to cause severe symptoms.

Phylogenetic studies have revealed that European and American populations appear to have a significant level of nucleotide divergence at ß-tubulin and RPB2 loci (Castlebury et al., 2006), indicating that the populations may be allopatrically isolated. American populations of *N. distissima* have been shown to contain abundant within-population diversity (Plante et al., 2002), which led to the hypothesis that America is the center of origin of *N. ditissima*. However, as stated by Castlebury, without further sampling in Europe this cannot yet be confirmed (Ghasemkhani et al., 2016).

Much is known about the epidemiology of the disease in the orchard (see **Figure 1** for a graphical depiction of the lifecycle). *N. ditissima* enters the shoots and branches of the tree through wounds, either natural such as bud-scale scars, leaf scars, fruit scars or artificial such

as pruning wounds (Dewey & Swinburne, 1995; Dubin & English, 1970; Swinburne et al., 1975; Weber, 2014). The hyphae of *N. ditissima* attack xylem vessels, tracheids and fibers (Crowdy, 1949). Once the xylem vessels have been colonized the disease symptoms are extended vertically from the entry point and may affect the whole tree. It has been observed a high amount of fungal hyphae in the infection sites which decrease further away from these sites (Sakamoto et al., 2004). The first symptoms of an infection consist of a sunken areas of bark tissue which becomes necrotic and gradually the epidermis turns pale brown and flakes off (Weber, 2014). These symptoms can be commonly observed during the spring time.

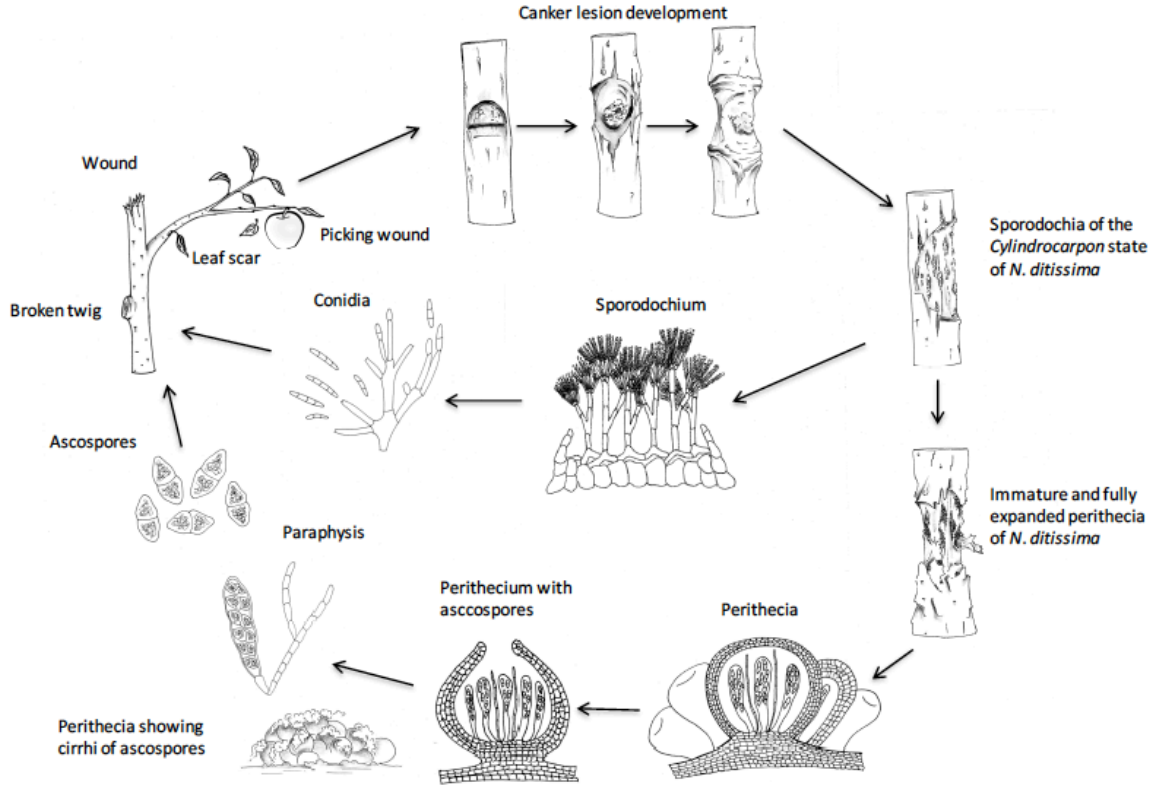

Figure 1: Life cycle of *Neonectria ditissima*- after Agrios 1997.

After a few weeks, characteristics white conidial pustules are developed on the surface of the dead bark. This is the asexual stage of this pathogen, cylindrocarpon heteronema, which produces two kinds of conidia. Macroconidia are ellipsoidal with rounded ends, and often 5-septate (**Figure 2a**). By contrast, microconidia are aseptate or 1-septate and shorter than macroconidia (**Figure 2b**) (Ghasemkhani, 2012). Previous reports argue that whereas macroconidia are extremely infectious, the epidemiological role of microconidia is unknown (Zeller, 1926; Weber, 2014).

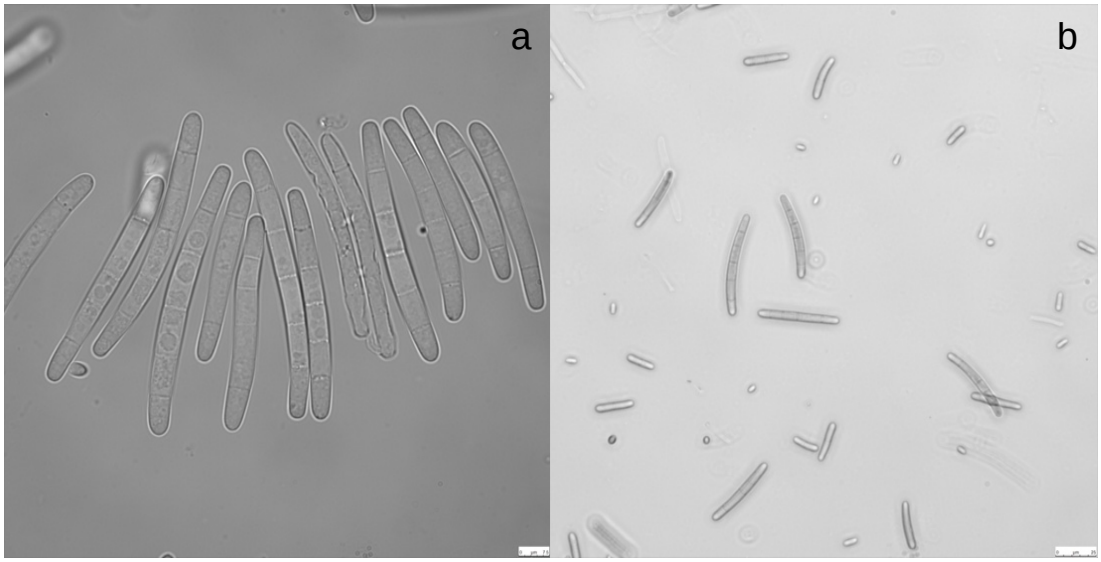

Figure 2: Conidia from a culture grown of SNAY media of the *Neonectria ditissima* isolate Hg199. **a.** Macroconidia 100x. **b**. Diversity of conidia (macroconidia and microconidia) 40x.

Much later, red bright perithecia appear mainly on the surface or edge of old canker lesions during autumn, winter and spring (Crowdy, 1949; Swinburne et al., 1975). Ascospores contained into the perithecium are discharged by rain and wind, or splash-dispersed. These are the sexual stage of *N. ditissima.*

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 conidia and ascospores may initiate the infection. Thus, inoculum and points of entry on the tree are available all year round (Amponsah et al., 2015; Weber, 2014) in some countries 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 (Swinburne et al., 1975) and consequently cool and rainy weather conditions during the season are usually followed by a high incidence

of shoot dieback due to canker development in the following spring and summer (Latorre et at., 2002).

Factors that affect canker expression are not understood but possibly relate to stress (cold, drought, waterlogging and herbicide applications), or fertilizer applications. For example, post-harvest foliar nitrogen applications increased leaf scar infections six- to nine-fold in New Zealand orchards (Dryden et al., 2016). The physiology of the tree may also strongly influence its susceptibility to canker. Thus young trees which have a higher share of vegetative growing shoots are often more strongly affected than older, more slowly growing trees (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 (Jones & Aldwinckle, 1990). Weber & Hahn (2013) reported severe occurrence of trunk cankers in a young orchard within 6 weeks, despite exceptionally dry weather throughout that period. They concluded that the nursery material must have been infected by *N. ditissima* without any visible symptoms at the time of delivery to the farmer (Weber & Hahn, 2013). The ability of *N. ditissima* to cause latent infections had previously been suggested by Brown et al. (1994). The development of molecular methods aimed in the detection of *N. ditissima* in latent infected trees (Langrell, 2002; Langrell & Barbara, 2001) lead to the conclusion that trees infected during the nursery stage might develop canker for up to three years subsequently (McCracken et al., 2003).

In some regions of the world (i.e. Northern Europe) *N. ditissima* also causes a fruit rot that can result in significant 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 & Robinson, 2010). Fruit infection can occur any time between flowering and harvest in wet climates, but the most important period is either at blossom or early fruitlet development. The spores infect through entry points such as the calyx, lenticels or wounds caused by insects. Symptoms characterised by circular and sunken necrotic area on the surface of the fruit, known as eye rots, may become visible in the tree when the fruit reaches half its maximum size. Nevertheless, symptoms are more commonly observed after harvest or during long-term storage as a result of latent infections, generally from December onwards in the Northern hemisphere. Factors affecting the expression of latent infections in fruit are not understood (Xu & Robinson, 2010).

Approximately 50% of apples produced in the UK are cultivars derived from two progenitor apple varieties, Cox and Gala, both of which are susceptible to fungal canker. Indeed, these newer varieties such as ‘Scifresh’, ‘Cameo’, ‘Kanzi’, ‘Zari’, ‘Rubens’ and older cultivars such as ‘Braeburn’ are all extremely susceptible to *N. ditissima.* Similarly, many common rootstocks

like M9, which is still the predominant, are highly susceptible. Particularly in modern planting systems, where intensive planting of canker-susceptible cultivars in combination with canker-susceptible rootstocks, has led to large problems in orchard establishment and an increase of financial losses.

Currently canker is controlled in the orchard by a combination of cultural methods to remove canker lesions and the use of protectant fungicides. The timing of pruning might play an important role for an efficient control. Pruning in winter the most extended and practical way to remove infected tissue because canker lesions are easily visible (Weber, 2014). Nevertheless, outside the growing season, the pruning wounds remain susceptible to infection for longer periods being that dormant trees will not be able to develop a protective callus layer. Effective fungicides are limited; generally copper fungicides were used at autumn leaf fall and before budburst to protect leaf scars and bud-scale scars. On the other hand, products based on carbendazim were applied during the spring and summer to prevent tree and fruit infection in this period. However, since the withdrawal of these fungicides due to the EU legislation, effective control has been absent. The lack of effective control methods renewed the interest in use natural host resistance, which has been an area of research for many years.

Apple cultivars vary in level of partial resistance towards apple canker (Alston, 1970; Borecki & Czynczyk, 2013; Kraehmer & Schmidle, 1979; Krüger, 1983; Van de Weg, 1989 and 1992). Variations in disease susceptibility may partly be a result of disease escape e.g. the speed of wound healing in relation to *N. ditissima* susceptibility has been shown to differ between cultivars (Xu et al., 1998). It might 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).

Despite of the importance of *N. ditissima* and its current high profile within the apple industry, very little is known about its pathogenicity factors or the resistance mechanisms of the host. It is 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*. Variation in loci implicated in basal resistance, for example allelic variation in clusters of germin-like proteins, have been implicated in quantitative resistance in other systems, indicating that both the complement of basal defence genes and the strength of the induced responses are important (Manosalva et al., 2009).

Many studies have been carried out using a range of quantitative pathogenicity tests to phenotype tree resistance. The results of our leaf scar inoculation test revealed that there were large differences in resistance responses, which often corresponded to field performance

of the cultivars. Well known field resistant cultivars showed high levels of resistance, while field susceptible cultivars showed high susceptibility on this test. Interestingly, Golden Delicious and M9, resistant and susceptible respectively in the field, showed similar levels of moderate resistance.

Alongside the leaf scar test, cut shoots tests were carried out on a range of cultivars and using three different isolates of *N. ditissima*. Similarly, some discrepancies were observed between the results of this tests compared with field responses and with the performance of the same cultivar in both tests. 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 the leaf scar test are much more susceptible. Conversely, E93-79 exhibits rapid colonization in wound inoculated cut shoot tests, but low susceptibility to leaf scar infection.

This suggests that while these tests are able to discriminate between resistant and susceptible cultivars in the most part, the mechanism of effective field resistance is not fully being queried in them. It should be noted that in both types of pathogenicity test 'Robusta 5' displays low levels of infection and subsequent colonization.

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. 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 these methods can be considered to be sufficient for rapid selection in breeding programmes.

Host response was consistent regardless of the isolate that is inoculated in the cut shoot test. Due to the little variety of isolates inoculated, only three on this test, it was not possible to determine differences in pathogenicity among this isolates of *N. ditissima*. A previous report of van de Weg (van de Weg, 1989), indicate that there is a relatively simple pattern of host response which is not influenced by an isolate race-structure. Nevertheless, 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 (Scheper et al., 2015). Further study of a bigger number of isolates is needed to confirm this initial observation and to identify if differences in pathogenicity are associated with gene content.

Several pathological and molecular studies have explored the necrotrophic fungal lifestyle and its infection strategies. While biotrophic pathogens have develop complex mechanism to obtain nutrients from living host tissues, necrotrophic pathogens, like *N. ditissima,* rely on an

arsenal of lytic and cell wall degrading enzymes to damage the host tissue and acquire nutrients from dead cells to complete their lifecycle. Nevertheless, recent studies have demonstrated that necrotrophic fungi have a more sophisticated infection process (Kim et al., 2015; Liu et al., 2009). The pathogen encodes secreted proteins known as effector which suppress the immune response and allow the colonization of the host. Various effectors have been characterized in necrotrophic fungi, such as *Alternaria alternata* (Tsuge et al., 2013), *Pyrenophora tritici-repentis* (Ciuffetti et al., 2010) and *Botritys cinerea* (Staats at al., 2007), revealing a wide and diverse repertoire depending on host range and lifestyle.

Similar effector proteins might be present in the *N. ditissima* genome but this remains unsolved. The generation of a high quality genome will facilitate the identification of the full effector complement and their physical location within the genome. Understanding the effector compliment of *N. ditissima* provides opportunity to aid R gene identification and characterisation. Nevertheless, a deeper functional characterization of those genes involved in the pathogenicity is needed to understand how the fungus interferes with the host. Sequencing the transcriptome of *N. ditissima* during the infection of apple plants and comparing with our reference genome, obtained from an axenic culture, and with the genomic information available for apple (Velasco et al., 2010, Daccord et al., 2017, Antanaviciute et al., 2012; Bianco et al., 2014; Bink et al., 2014) it is likely to identify the corresponding pathogenicity factors that may be manipulating host defences.

The mechanism by which the pathogen is detected by the host it is still unknown. In a classical gene-for-gene system, loss or mutation of genes or motifs within proteins in the pathogen, that the plant uses to recognize and activate defences (*R*-gene mediated resistance), results in a loss of resistance (Jones & Dangl, 2006). This model applies equally to major gene resistance or a quantitative gene-for-gene model. Work done with *Phytophthora infestans* late-blight and the cultivated potato, *Solanum tuberosum* is a current example of quantitative gene-for-gene resistance which is dependent upon recognition of multiple RxLR-containing pathogen effector genes (Rietman et al., 2012). Similar examples of quantitative *R*-gene mediated resistance have been reported in *Oryza sativa-Magnaporthe* interactions (Y. Liu et al., 2011) and non-host resistance in pepper against *P. infestans* (Lee et al., 2014).

It is likely that *R* genes may also underpin resistance to *N. ditissima*, coupled to a MAMP-triggered immune response, i.e. basal defence below the level required to activate the hypersensitve response (HR) as reported for the SCFE1/RLP30 interaction with the necrotrophic pathogen *Sclerotinia sclerotiorum* and *Arabodpsis thaliana* (Zhang et al., 2013). Alternatively resistance could also follow an inverse gene-for-gene model (Fenton et al,, 2009), whereby resistance genes act as factors that the pathogen may exploit to activate HR deliberately, as in the case of *Botrytis cinerea* and other necrotrophic pathogens, in order to

provide a nutrient source for the pathogen (Govrin & Levine, 2000). In this case, loss of recognition by *R* genes would lead to lack of HR and therefore a loss of pathogenicity. Either of these is a possiblity and is one of the fundamental questions that remains to be addressed in this pathosystem.

Identify the nature of resistance to *N. ditissima* has been challenging in the past. This might be explained with the results of our previous apple seedling test, which revealed that resistance responses to *N. ditissima* are complex and involved multiple resistance genes, with epistatic interaction modulating inheritance. In order to correctly and accurately identify resistance sources and study the interactions between them, we will generate phenotypic and genotypic data for an offspring from a single bi-parental population, 'Golden Delicious' x 'M9'. This cross involves a high susceptible cultivar, 'M9', with a resistant, 'Golden Delicious'. Screening tests at different physiological conditions and different infection methods were carried out. With this data, we will be able to carry out a QTL mapping associated with the resistance to *N. ditissima* using a Bayesian approach.

In addition, on this report we present preliminary results of a pathogenicity test of ten isolates of *N. ditissima* measuring the disease expansion in a susceptible host, in order to determine differences in virulence. These isolates, which have different origin, will be sequenced and differences in their gene content will be correlated with their performance on this test.

A high-quality reference genome of *N. ditissima* has been produce combining our previous highly fragmented genome (Gomez-Cortecero, 2015) with the innovative PacBio long-reads sequencing, allowing us to present an updated analysis of the predicted secretome of this pathogen. Visualisation the physical location of these genes in the genome of *N. ditissima* indicated that secreted carbohydrate active enzymes and predicted effectors are distributed throughout the genome. Some regions of the genome show high concentration of these genes and may represent important pathogenicity regions. Analysis of RNAseq data during infection will help narrow down regions important in pathogenicity. This will allow the identification of specific genes that are upregulated in the pathogen and the host during different stages of infection. This work is still ongoing, however on this report I describe the protocol of inoculation, sampling and RNA extraction.

The identification of the specific genes controlling host defence and pathogen virulence are needed 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.

Materials and methods

DNA Extraction

A sterile toothpick was used to scrape young mycelia of *N. ditissima* from an agar plate and to inoculate a flask with 20 ml of YPD liquid media (20g Bacto peptone, 10g yeast extract, 950mL of water, 50mL of 40% w/v glucose). The flask was closed with a cotton gauze and covered with aluminium foil. 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.

Two single-ascospore isolates of *N. ditissima* were used for the whole-genome sequencing. Isolate R09/05 was obtained in 2005 from a canker-infected wood of *Malus domestica* cv. “Cox” in Kent, United Kingdom. DNA extraction was performed on freeze-dried mycelium using a GenElute plant DNA miniprep kit (Sigma-Aldrich). The manufacturer’s protocol was modified by doubling the volume of lysis solutions used, performing an RNase digestion step, and using twice the volume of precipitation solution.

Isolate Hg199 was obtained in 1999 from a canker-infected wood of *Malus domestica* cv. “Gala” in Kent, United Kingdom. Isolate Ag04 was obtained in 2016 from a canker-infected wood of *Pyrus* cv. “Conference” in Kent, United Kingdom. Isolate R45/15 was obtained in 2015 from a canker-infected wood of *Malus domestica* cv. “Elstar” in The Netherlands. DNA was extracted from freeze-dried mycelium using the Macherey-Nagel Nucleospin Plant II kit following a modified manufacturers protocol.

Genome sequencing and assemblies

Isolate R09/05 was sequenced using PacBio long read sequencing technology by the Earlham Institute in Norwich, United Kingdom. PacBio reads for *N. ditissima* isolate R09/05 were assembled using Canu and polished using illumina MiSeq reads in Pilon to correct erroneous SNPs and InDels (Koren et al., 2016; Walker et al., 2014). RepeatModeler, RepeatMasker and transposonPSI were used to identify repetitive and low-complexity regions within the assembly (http://www.repeatmasker.org, http://transposonpsi.sourceforge.net).

Libraries of every additional isolate of *N. ditissima* were prepared using a different method. DNA was sheared to 550bp using the Covaris M220 with microTUBE-50 (Covaris 520166) and size selected (target 550bp) using the Blue Pippin (Sage Science). Illumina libraries were constructed using either Illumina TruSeq LT kit (FC-121-2001), or with a PCR-free method

using NEBNext End Repair (E6050S), NEBNext dA-tailing (E6053S) and Blunt T/A ligase (M0367S) New England Biolabs modules with slight adaptations to the protocols. Libraries were sequenced using Illumina Miseq v3 2x 300bp PE (MS-102-3003). Adaptor sequences and low-quality data were removed from the MiSeq reads using fastqc-mcf. *De novo* assembly of MiSeq data was performed using SPAdes version 3.1.0 and analysed using Quast (Gurevich et al., 2013).

The R09/05 assembly was used as a reference to map raw sequencing reads from other isolates of *N. ditissima* sequenced isolate. Alignment was performed using Bowtie2 v.2.2.4 (Langmead & Salzberg, 2012).

Quality of genome assemblies was assessed by looking for the gene space in the assemblies using cegma (Parra et al., 2009).

RNA extraction and Gene prediction

RNA was extracted from fresh fungal material of the isolates R09/05 and Hg199. Fungal cultures on YPD liquid media were washed with sterile water and flash freeze under liquid nitrogen. The RNA extraction protocol was adapted from the Macherey-Nagel RNA isolation kit, replacing the lysis buffer with TRIzol reagent during the grinding step.

Illumina libraries were constructed using Illumina TruSeq RNA Library Prep kit v2 (RS-122-2001) and sequenced using Illumina Miseq v3 300bp paired end reads (MS-102-3003).

RNAseq reads from each isolate were aligned to genomes using Tophat v.2.1.0 to aid training of gene prediction programs (Trapnell et al., 2009). An initial RNAseq alignment was used to estimate “mean insert size” and “fragment length distribution” of RNAseq reads using Cufflinks. Tophat alignments re-run using these parameters. Gene prediction was performed on softmasked genomes using Braker1 v.2 (Hoff et al., 2016), a pipeline for automated training and gene prediction of AUGUSTUS 3.1 (Stanke & Morgenstern, 2005). Additional genes were added to Braker gene predictions, using CodingQuary v.2 (Testa et al., 2015) in pathogen mode. Functional annotations were determined for gene models using InterProScan-5.18-57.0 (P. Jones et al., 2014) and through identifying homology between predicted proteins contained in the SwissProt database (Apweiler et al., 2004) using BlastP (E-value > $1x10^{-100}$). Furthermore, putative pathogenicity and effector related genes were identified within Braker gene models through prediction of signal peptides using SignalP v.4.1 and removing those proteins which were predicted to contain transmembrane domains using TMHMM v.2.0 (Krogh et al., 2011). EffectorP v1.0 was used to identify secreted proteins that had length, net charge and amino acid content typical of fungal effectors (Sperschneider et al., 2016). Secreted

Carbohydrate active enzymes were identified through HMM searches (HMMER3) models from the CAZY database (Lombard et al., 2014).

Inoculum preparation

Inoculum of *N. ditissima* used for all pathogenicity experiments was obtained from single ascospore cultures. Ten isolates were used in the pathogenicity experiments; Hg199, Ag02, Ag06, R6/17-2, R6/17-3, R37/15, R39/15, R41/15, R42/15 and R45/15 (**Table 1**).

Table 1: Isolate name, origin and contribution

Isolate accession	CV	Origin	Year of isolation	Contributor
HG199	Gala	Kent, UK	1999	Angela Berrie, EMR, UK
Ag02	Jazz	Kent, UK	2016	Antonio Gomez, EMR, UK
Ag05	E830-102	Kent, UK	2016	Antonio Gomez, EMR, UK
R6/17-2	Bramley	Kent, UK	2017	Robert Saville, EMR, UK
R6/17-3	Bramley	Kent, UK	2017	Robert Saville, EMR, UK
R37/15	Jonagold	Belgium	1999	Tom Smets, PCF, B
R39/15	Unknown	Belgium	2006	Tom Smets, PCF, B
R41/15	Wellant	The Netherlands	2015	Marcel Wenneker, WUR, NL
R42/15	Elstar	The Netherlands	2015	Marcel Wenneker, WUR, NL
R45/15	Elstar	The Netherlands	2015	Marcel Wenneker, WUR,NL

The isolates were sub-cultured onto SNAY media (1 g potassium dihydrogen phosphate, 1 g potassium nitrate, 0.5 g magnesium sulphate, 0.5 g potassium chloride, 0.2 g glucose, 0.2 g sucrose, 1 g yeast extract, 20 g agar made up to 1 litre with distilled water). Plates were incubated in 16/8 h light/dark regime at 22ºC for 13-15 days. On the day of inoculation, each plate was flooded with 3 ml of sterile water and conidia were release from sporodochia using a plastic spreader. Mixed spore (macro and microconidia) suspension was prepared from each isolate.

Macroconidia in the suspension were counted using a haemocytometer. The isolate, Hg199, was used for both screening for resistance experiments in the MDX132 population and for the gene expression experiment with a concentration of 1×10^5 conidia ml^{-1}. For the isolates pathogenicity test the conidia concentration of all the isolates was adjusted at $1x10^5$ conidia ml^{-1}.

Host resistance response of ‘Golden Delicious’ x ‘M9’.

This experiment will assess the host response for an offspring from a single bi-parental population, ‘Golden Delicious’ x ‘M9’ through two different inoculation methods, cut shoots and potted trees, using a single *N. ditissima* isolate. Individual seedlings of this population were grafted onto M9 rootstocks in May 2016 (UK) in four replicate plants.

2 replicas of 176 individual genotypes were used for a screening test in glasshouses. Each replica was placed in a chilled glasshouse at least one week before the inoculation. The temperature varied in the glasshouses from 15ºC to 21ºC. To ensure a minimum level of humidity of 80% RH during the experiment, misting lines were hung under the benches. These were placed on a timer, spraying for 1 min at 5 minutes interval during the first four days post-inoculation and then 1 min at 10 minutes during the rest of the experiment. Each replica was inoculated one month apart. The first one was inoculated in September 2016 and the second in October 2016. Additional lights in the glasshouses were used during November and December due to the reduced hours of light.

The inoculation method was similar to the seedling test. Five leaves were inoculated with 3µl of conidial suspension at $2.5x10^5$ conidia ml^{-1} concentration. White petroleum jelly was placed on the wound and removed 7-9 days post-inoculation. Plants were fully randomized and placed on two benches either sided on the glasshouse. The order of inoculation was randomized again into eight different sets and eight different inoculum tubes from a common source, to avoid prolonged used of a single tube. Lesion size was recorded with digital calipers at different time intervals. In total, seven assessments in the first glasshouse and six in the second were carried out, up to 72 and 66 respectively, days post-inoculation.

From the remaining two replica plants, dormant cut shoots were collected in February 2017. Shoots were wrapped in moist paper and kept at 4ºC in darkness for nine days. Three days before inoculation, shoots were placed in a chilled glasshouse with temperature, light and humidity conditions identical to the potted trees experiment. Shoots were immobilised at their base in Oasis floral foam, which was placed into a tray containing water, adapted from van de Weg (1989) (van de Weg, 1989). Each replica was fully randomized in three trays containing 60 shoots per tray.

Two axillary buds on each shoot were inoculated. Buds were prepared by cutting just below the bud, a little below the second abscission layer (but without removing the bud). The width of the incision was approximately 2-3mm. The chosen buds were usually the eighth and fourteenth counting basipetally (from the apex to the base of the shoot), unless the size of the shoot or the internode distance were very small. In those cases, buds were chosen randomly leaving, as far as possible, a big area for the disease development. The spore suspension

was prepared the same day of the inoculation and an inoculum volume of 3µl was applied to the wound within five minutes of making the wound. Again, to avoid prolonged use of a single tube of inoculum six different inoculum tubes were used, one per tray, and the order of tray inoculation was randomised.

Lesion length was recorded using digital callipers at 15, 22, 30, 37, 44, 52 and 59 days post-infection. The Area Under Disease Progress Curve (AUDPC) was calculated using the agricolae package (de Mendiburu, 2015), using R version 3.2.2 (Team, 2015).

N. ditissima isolates pathogenicity test

Potted M9 rootstocks were used for this test. M9 has been reported as a susceptible cultivar in our previous pathology test. Plants will be inoculated with a conidial suspension from 10 different isolates of *N. ditissima*, along with a water control, in a chilled glasshouse. This experiment was divided in twelve blocks. Each block contains one replica of each treatment and plants were fully randomized within each block.

For the inoculation, two leaves from each plant will be removed with the corresponding axillary bud. Distance between inoculation points will be at least 25 cm with a maximum of 30 cm. This variation depends on the internode distance. The distance between the 1st inoculation point to the apex and between the 2nd inoculation point to the bottom of the plant will be at least 15 cm. Plant size varies between 55 and 90 cm. Inoculation points were prepared by cutting just below the bud wound, a little below the second abscission layer; the width of the incision was approximately 2-3 mm. Within five minutes of cutting, 3µl of a conidial suspension was placed onto the wound with an automatic micropipette. Inoculum was prepared fresh and adjusted to the desired concentration just before the inoculation. Due to the large number of isolates, this will be done in sets of three isolates. Inoculated wounds were covered with white petroleum jelly within five minutes of the droplet being absorbed which was removed five days later with a tissue. Lesion size was recorded with digital callipers every week after the first signs of infection, in this case ten days after infection.

Gene expression analysis

Cut shoots of ‘Golden Delicious’ and ‘M9’ were grafted onto M9 rootstocks in March 2017 (UK). Trees were moved to a glasshouse four days before inoculation, at the end of July 2017. Temperature varied in the glasshouse from 15°C to 25°C and no additional lights were used during the experiment. To ensure a minimum humidity level of 80% RH, misting lines were

hung over the trees spraying for 1 minute at 10 minute intervals. One each tree, two leaves and the corresponding axillary bud were removed using a scalpel. Buds were chosen randomly, one on the bottom and one on the top, leaving approximately the same distance on each plant. An inoculum volume of 3µl of spore suspension or water control was applied onto the wound with an automatic micropipette. Inoculated wounds were covered with white petroleum jelly and removed after 5 days later with a tissue. Samples were collected 25 days post-inoculation. For the infected plants, a piece of stem was collected at approximately 0.5 cm of distance from the leading edge of each symptomatic tissue. A second stem sample was collect at 0.5 cm from the previous one. From the control plants, samples were collected 0.5 cm of distance from the inoculation point. All the samples were flash frozen in liquid nitrogen right after being collected.

The RNA extraction protocol was adapted from Yu et al., 2012. High-quality RNA samples will be sequenced and reads aligned to the *N. ditissima* reference genome and to the apple genome. New expressed transcript will be sought for the identification of regions in the genome related with the pathogenesis of the fungus.

Results

Genomic resources of *N. ditissima*

A high quality reference genome was generated for the pathogenic isolate R09/05 using PacBio sequencing technology. The genome assembly yielded 70 contigs and a total length of 45.88 Mb (see **Table 2** for assembly statistics). while de-novo assembly of Hg199, using Illumina MiSeq sequencing technologies, yielded 849 contigs (<500 bp) and a total length 45.4 Mb.

Table 2: Reference genome assembly statistics produced with Quast

	R0905_canu.contigs
# contigs (>= 0 bp)	70
# contigs (>= 1000 bp)	70
Total length (>= 0 bp)	45888346
Total length (>= 1000 bp)	45888346
# contigs	70
Largest contig	4130569
Total length	45888346
GC (%)	50.77
N50	1241248
N75	660033
L50	12
L75	25
# N's per 100 kbp	0.00

The quality of our assembly can be assessed using CEGMA (Core Eukaryotic Genes Mapping Approach) (Parra et al., 2009). This computational method identifies the percentage of 248 ultra-conserved Core Eukaryotic Genes (CEGs) presents in our assembly, which can be used to estimate the genome completeness. In the R09/05 assembly, 95.16% of CEGs were present.

Gene prediction performed on the softmasked genome by Braker1 v.2 (Hoff et al., 2016) and supplemented with Codingquary resulted in 13766 genes predicted in R09/05 and 13761 in the Hg199 assembly.

Generic effector-prediction approaches based upon identification these secreted proteins with an effector-like structure (small secreted cysteine rich proteins), secreted carbohydrate active enzymes (CAZymes) and identification of secondary metabolite synthesis genes were used to investigate effector enrichment throughout the *N. ditissima* reference genome. Predicted secreted effector proteins resulted in 170 putative genes. In addition, 721 CAZymes, of which 287 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**).

MiSeq reads from the isolates Hg199, R45/15 and Ag04 were mapped into the R09/05 genome, giving 96.22%, 95.38% and 94.51% of alignment rate, respectively.

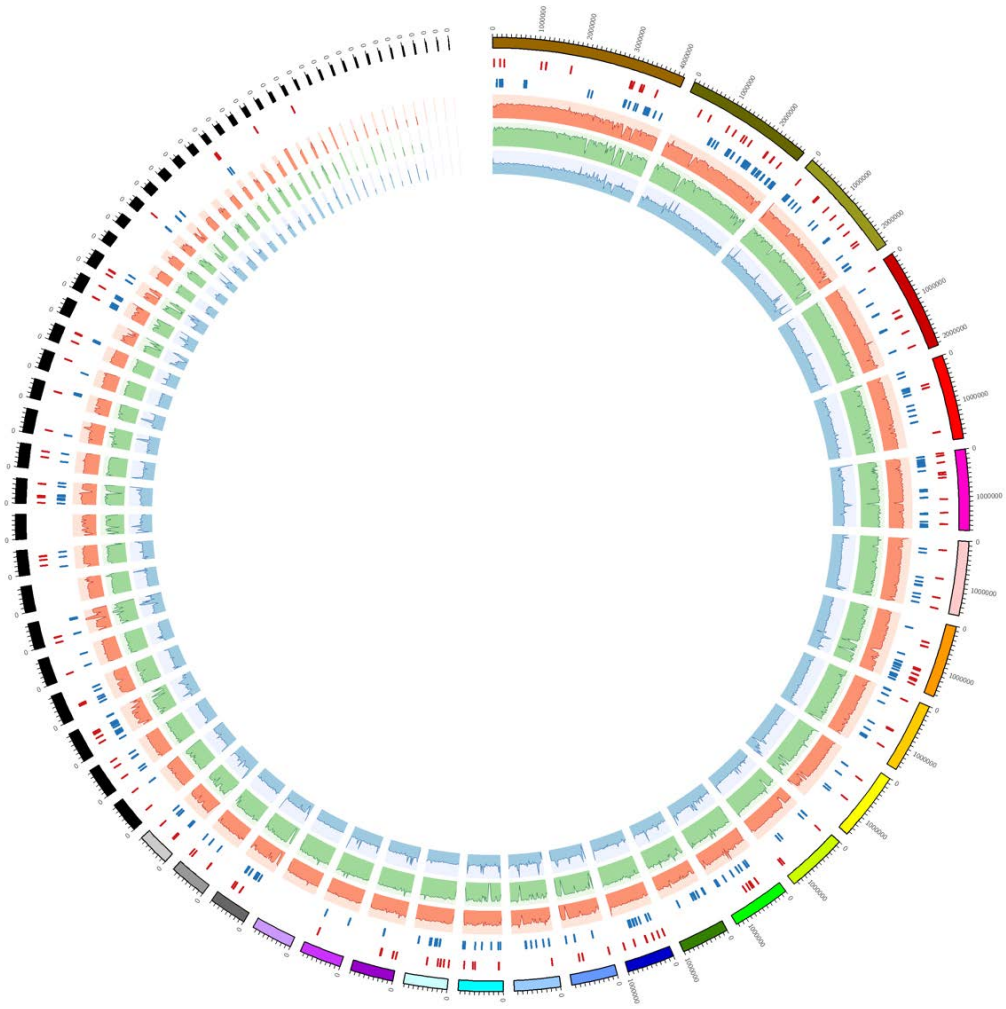

Figure 3: Genome architecture of *N. ditissima*. Concentric rings from outer to inner refer to: **1.** Genome assembly of R0905. **2.** Predicted secreted effector proteins (Red). **3.** Predicted Carbohydrate-active enzymes (Blue) **4.** Illumina reads of the Hg199 isolate. **5.** Illumina reads of the R45/15 isolate. **6.** Illumina reads of the Ag04 isolate. Diagram created using circos.

Identification of differentially expressed genes associated with the response to apple canker

In order to identify genes that are differentially expressed during the disease progression in apple, an analysis of the transcriptome from different sections of the plant material will be carried out. The resistant cultivar Golden Delicious and the susceptible cultivar M9 were used for this experiment. A total of 18 samples RNA samples from infected and control plants and 3 RNA samples from fungal mycelium growing in an axenic culture were used for RNA-seq.

Reads will be mapped to our *N. ditissima* reference genome and to the apple reference genome sequence (Velasco et al., 2010, Daccord et al., 2017) to identify differentially expressed genes. The results of this experiment are not available yet.

The nature of resistance to *Neonectria ditissima*

The genetic basis of resistance to *N. ditissima* will be explored in an offspring from a single bi-parental population. Resistance responses were assessed with respect to variation in colonization rate following artificial inoculations of cut shoots and potted trees. Significant symptoms development was seen 12 and 15 days after inoculation in the potted trees and in the cut shoots, respectively. Examination of the AUDPC values followed a normal distribution in the potted tree test. On the other hand, in the cut shoot experiment bigger AUDPC values were observed, with a few genotypes exhibiting an extremely high susceptibility to infection (**Figure 4**). Generally, bigger lesions were observed in the cut shoots experiment for most genotypes. The data are presented from day 54 in the potted tree experiment and 52 in the cut shoot test, to facilitate the comparison between inoculation methods. Along with this data, genotypic data will be generated in order to identify source the resistance on this population.

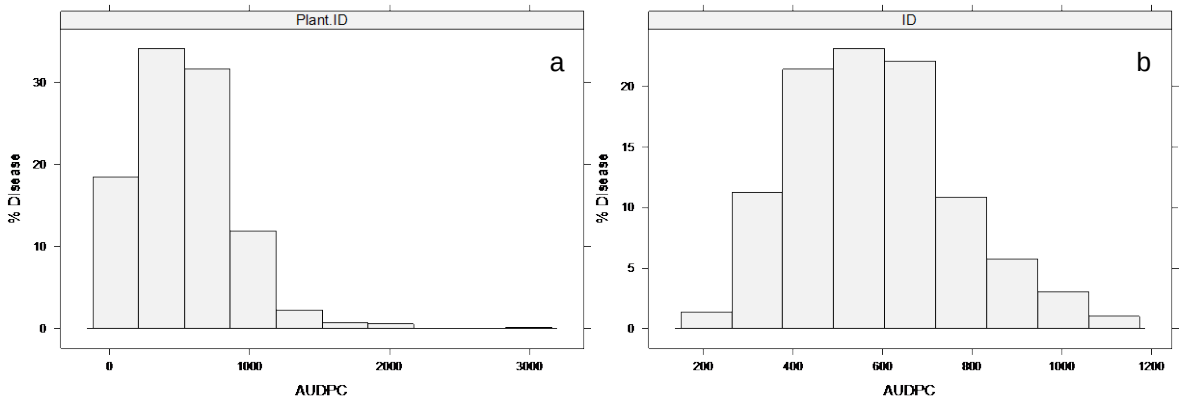

Figure 4: Histogram of AUDPC values for inoculated 'Golden Delicious' x 'M9' population. Segregation pattern observed was slightly different between tests. **a.** Cut shoot test. **b**. Potted tree experiment.

Differences in pathogenicity among *Neonectria ditissima* isolates

In order to identify differences in pathogenicity of *N. ditissima* strains, a screening test was carried out using potted trees of a common susceptible host. This test allows colonization rate to be calculated and compared between isolates. After the inoculation, lesions progressed

vertically along the shoots. The symptoms, consisted of a sunken and necrotic bark area around the inoculation point, were noticeable after 10 days.

This experiment is ongoing at the moment of writing this report. Therefore, preliminary results are presented for a total of 36 days after the inoculation. It was found that there are slight differences among the most pathogenic isolates on this test. Eight of them exhibits similar and higher levels of colonization rate at this stage (**Figure 5**). Again, no significant differences were observed between the isolates Hg199 and R45/15, both tested in one of our previous cut-shoot test. At the other end, the isolate R37/15 shows almost non-pathogenic behaviour. Intermediate levels of colonization were observed for the isolate R6/17-2.

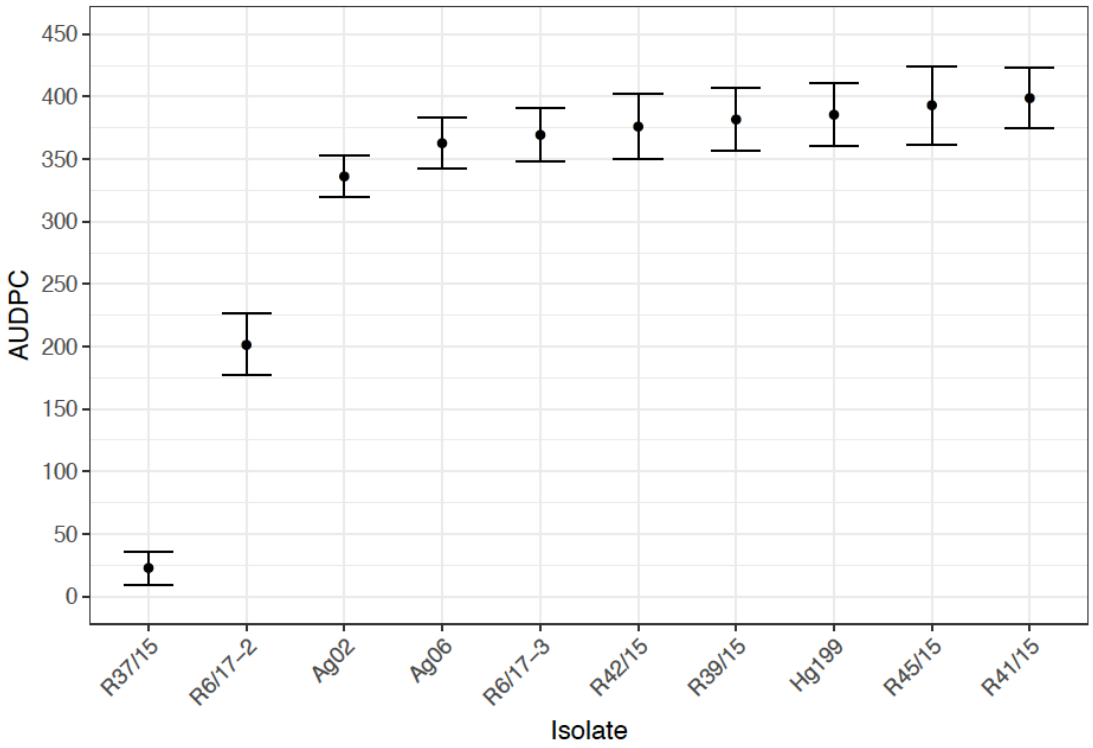

Figure 5: Mean Area under disease progress for isolates inoculated on M9 plants and calculated 36 days post-inoculation (shown with standard errors).

Discussion

The casual agent of European Canker, *Neonectria ditissima,* has become a globally important plant pathogen of apple causing significant losses across all major apple-producing countries in the world. Many studies have been carried out using a range of quantitative pathogenicity test to phenotype tree resistance. Nevertheless, there is a lack of knowledge of this pathogen at molecular level. Through examining the genome and analysing the transcriptome during the time course of an infection and by comparison with other well-studied pathogens, we aim

to identify candidate pathogenicity genes and confirm that they are responsible for the plant's resistance response.

Three draft genome sequences of *N. ditissima* have been already published (Deng et al., 2015; Gomez-Cortecero et al., 2015). A variety of novel sequencing technologies are currently under development, aiming to improve the number of bases sequenced reducing costs. To improve the genome assembly of our previously sequenced R09/05 isolate, the innovative long-read PacBio system was used. This sequencing technology, along with specifics bioinformatics tools for the genome assembly, allows the generation of a high quality reference genome of this pathogen. Compared with our previous genome assembly, the new genome sequence generated is less fragmented with a higher percentage of completeness score.

After the assembly process, RNAseq data from this isolate was used as part of our pipeline for gene prediction. This process identifies the regions of DNA that encode genes, filtering repetitive and non-coding regions. Although the genome of R09/05, generated with Pacbio long-reads sequencing, is less fragmented than the Hg199 a similar number of genes were predicted in both genomes, 13766 genes predicted in R09/05 and 13761 in the Hg199 genome.

Then, we have identified specific genes with known roles in the pathogenicity in other necrotrophic fungi. Effectors proteins are characterized to possess signal peptides for secretion, no trans-membrane domain, small size and are enriched for cysteines residues (Kim et al., 2015; Liu et al., 2009; Sperschneider et al., 2016). With the aid of bioinformatics tools, effectors genes were predicted through the identification of conserved amino acids motifs. In addition, we have identified Carbohydrate Active Enzymes responsible for the cell wall degradation (Kikot et al., 2009). From 13766 genes identified in the R09/05 isolate genome, 170 match the description of an effector gene and 721 CAZymes were identified, of which 287 were secreted proteins. This inventory of effector genes and enzymes are distributed throughout the genome of *N. ditissima*. Nevertheless, a deeper functional characterization of those genes involved in the pathogenicity is needed to understand how the fungus interferes with the host.

Transcriptome analysis, comparing gene expression level between fungal infection and growth on medium, can be used to provide insights into the pathogenicity mechanism used to invade the apple. A similar study revealed that the response of apple cultivars inoculated with *N. ditissima* involves the up-regulation of genes associated with detoxification, lignification, phosphorylation and pathogen defence (Ghasemkhani, 2015). More specifically, some genes related with a strong defence function were identified only in resistant cultivars. Nevertheless, no specific pathogenicity genes have been identified on the pathogen. With this data, we will

be able to compare differently regulated genes in the resistant and in the susceptible cultivars. Through comparison to other similar pathogen we aim to classify these genes depending on its function and in their metabolic pathways that they are involved.

Once a list of candidates genes is generated, a functional characterization need to be carried out to confirm their role during the infection. Genetic manipulation of *N. ditissima* needs to be established in the near future to make functional characterization possible.

Despite the pathogenicity test of *N. ditissima* isolates is not over yet, our preliminary results revealed that there are strains clearly less pathogenic than the majority. Looking into the gene content and comparison of these isolates offer an alternative strategy to elucidate the source of pathogenicity. An earlier study with another member of the Sordariomycetes, *Colletotrichum*, ilustrates that changes in gene content are associated with major changes of host range or pathogenicity (Baroncelli et al., 2016). This plasticity in the fungal genome was particularly observed in genes encoding proteins with known roles in plan-pathogen iteraction, higher in number in broad host range species. Therefore, through the analysis of our data we could correlate the colonization rate with these genes in the *N. ditissima* genomes. Although it is not yet known whether the nearly non-pathogenic isolates are more pathogenic on other cultivars. This highlights the importance of identiying and characterizing the source of pathogenicity of *N. ditissima* and how these changes have been adaptated in the global population. This experiment will continue for the next few weeks and new measures of disease progression will be collected.

Conclusions

During this year we aimed to explore in detail the source of pathogenicity of *N. ditissima*. The implementation of updated bioinformatics tools allowed to refine and improve the genome assembly of one UK isolate of this pathogen. In the near future, the new generation of sequencing technologies, like Nanopore sequencing, will be used to improve this genome assembly and for the generation of a new highly pathogenic strains of *N. ditissima*. Gene expressed during the infection analysed will be used to investigate biological processes involved in the pathogenicity and recognition by the host. Functional characterization of potential candidates genes requires the transformation of *N. ditissima* to determine the role in pathogenesis and will be addressed in the near future. It is not clear yet if significant differences will be observed in the gene content of the isolates of tested on this report. Nevertheless, previous work in other necrotrophic pathogens indicate that this is possible. High pathogenic isolates with a broad host range might possess a bigger arsenal of pathogenicity genes. Therefore, it is important to study, not only the role of these genes and

how are recognized by the host, but also how this changes in the gene content have been adapted in the global population of *N. ditissima.*

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.

16th-17th November 2016 – AHDB Crops Student Conference. Poster presentation.

28th February 2017 – EMR Association/AHDB Tree Fruit Day at NIAB EMR. Project presentation.

29th-30th March 2017 – Molecular Biology of Plant Pathogens Conference at Durham University. Poster presentation.

13th June 2017 – PhD Symposium at University of Reading. Project presentation.

16th-20th July 2017 – The 4th International Horticulture Research Conference at NIAB EMR. Poster presentation.

Glossary

AUDPC	Area under the disease-progression curve
InDels	Insertion of deletion of bases in the genome
mRNA	Messenger RNA
PCR	Polymerase chain reaction
QTL	Quantitative trait loci
RNA-Seq	RNA sequencing
SNPs	Single nucleotide polymorphisms

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