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Location of project:	NIAB EMR, East Malling, UK
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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

- New findings have been made about the latent infection of *Neonectria ditissima* in apple trees

Background and expected deliverables

European apple canker (also known as Nectria canker), caused by the fungus *Neonectria ditissima*, affects both apple trees in the orchard and fruits post-harvest. In the UK, the increased planting of susceptible cultivars such as Gala and the lack of registered products for disease control, has resulted in canker becoming more serious in recent years. Evidence suggests that the pathogen can infect the tree during the propagation phase in nurseries and enter a latent phase, later developing into cankers on young trees once they are transplanted in the orchard. In some years, up to 10% of trees in newly planted orchards can be lost to canker annually. The industry is keen to focus research into:

- developing alternative control methods and
- developing a diagnostic strategy to test nursery stock material in nursery certification schemes.

The development of diagnostic methods requires good knowledge of the infection process and of disease anatomy. Currently, there are substantial gaps in our understanding of *N. ditissima* biology, which hinders the implementation of a reliable sampling strategy for diagnostics. In particular, during the asymptomatic phase of infection, we don't know whether the fungus resides in the infection site as a latent pathogen, or instead grows inside the plant's woody tissues at distance from the entry point. This peculiar lifestyle, characterised by the ability to internally colonise a plant without causing any symptoms of infection, is known as 'endophytism'.

One of the aims of this present project is to assess whether *N. ditissima* is endophytic, and to provide the basic knowledge to develop reliable sampling strategies in diagnostic technology.

In developing innovative strategies for canker control, biocontrol agents have been considered as an interesting alternative to traditional crop protection products. Most biocontrol agents are 'epiphytic', so they inhabit the surface of plants, and their efficacy as pathogen antagonists is therefore strongly dependent on environmental conditions, such as UV, humidity and temperature. However, as a part of their complex microflora (referred to as 'the plant microbiome'), plants also host fungal and bacterial endophytes. Studies have shown

that some of these microorganisms interact with plant pathogens and can facilitate or antagonize them. Therefore, together with the host genetics they can modulate disease expression, and account for part of the field resistance to Nectria canker which is observed across the commercial apple cultivars.

The second aim of this present project is to explore the endophytes associated with different apple cultivars, representative of different resistance classes, and to investigate the correlation between the cultivar endophyte profile and its resistance level.

Summary of the project and main conclusions

To pave the way for the development of reliable diagnostic tools for European apple canker in nursery stock material, different methods have been evaluated to detect *N. ditissima* in asymptomatic plant tissues. In this project, we developed a serological technique called Enzyme-Linked Immunosorbent Assay (ELISA) for the detection of the pathogen. The ELISA is based on *N. ditissima*-specific monoclonal antibodies that can recognise the pathogen's proteins (antigens), is quick and straightforward and can be used to develop Lateral Flow Devices (LFD), which are already available as diagnostic tools for a number of fungal, bacterial and viral plant diseases. *N. ditissima* was also successfully detected in asymptomatic tissues with a method based on the detection of the pathogen's DNA called Real-Time Polymerase Chain Reaction (qPCR). Overall, he results obtained with the two techniques showed good correlation, however the ELISA appeared prone to false negatives. Further studies are required outside this current project to address the issue and develop a reliable serological technique. The DNA-based method appeared superior in specificity and sensitivity and it was chosen to carry out the disease anatomy study (see below).

To study disease anatomy during the asymptomatic phase of the infection, we have established a system to simulate a natural infection, and then assessed the localisation of the pathogen in the plant over time with the DNA-based method. Young shoots on apple trees (Royal Gala and Queen Cox) grown in an orchard at NIAB EMR were artificially inoculated via leaf scars with a spore suspension of *N. ditissima*. Plants were monitored periodically for symptom expression, and the inoculated shoots were sampled at different time points, before disease symptoms were expressed at high levels. The asymptomatic wood material was assessed for presence or absence of the pathogen by extracting the total DNA from plant tissues, and then detecting *N. ditissima*-specific DNA. The assay was performed on plant material sampled from the inoculated leaf scars and from portions of the shoots at distance from the inoculated leaf scars. Data collection is currently ongoing. However our initial results suggest that:

- Three months after the inoculation, *N. ditissima* is only found in the asymptomatic leaf scars; the pathogen was not detected in the asymptomatic internodes, at 1-1.5 cm from the inoculation point, suggesting that no internal colonisation of the plant tissues took place in this time interval.
- After a canker lesion had developed (i.e. during the symptomatic phase), the pathogen could be found in the cankered tissue, and in the asymptomatic wood at 1-1.5 cm from the lesion, suggesting that it had spread into the apparently healthy sapwood.

A field experiment has been set-up to study the fungal and bacterial endophyte species associated to different apple cultivars representative of different disease-resistance classes. Eight different scion cultivars, grafted onto two different rootstocks, were planted in orchards at two different sites in Kent. Samples will be collected from the trees, total DNA will be extracted and DNA-sequencing technologies (Next Generation Sequencing, NGS) will be employed to identify the fungal and bacterial groups associated to plant tissues. Samples will be collected from leaf scars, which is considered the most relevant infection site for *N. ditissima* in the UK, allowing to study the endophytes which directly interact with the fungus at the entry point. We will determine the different endophyte groups and their relative amount in the different scion x rootstock combination, i.e. the cultivar-specific endophyte profiles. Moreover, we will determine whether the different profiles are correlated with the resistance level of the different cultivars assessed.

Main conclusions so far

Neonectria ditissima was detected in artificially inoculated, asymptomatic apple trees. Initial findings suggested that:

- Up to three months after the inoculation, the fungus may latently reside in the infected wound.
- Following symptom expression, spread of the pathogen in asymptomatic sapwood can occur within several centimetres from the lesion edge.

Financial benefits

Typical modern fruit wall orchards are established using around 2,800 trees per hectare. The trees cost around £5 per tree, but including wire and cane supports, they cost £7 per planting station (personal communication Nigel Jenner, Avalon Produce). With susceptible cultivars such as Gala, it is not uncommon to lose 10% of young trees to canker in the first year after establishment. This is equivalent to 280 trees costing £1,400 per hectare. These trees must

be replaced which incurs additional labour costs and slows the establishment rate of the new orchard.

Developing new procedures to diagnose the presence of canker and systems for control will help to reduce the numbers of affected trees being planted and eradicate the additional expense required to replace diseased trees.

Action points for growers

The guidelines currently published on the Apple Best Practice Guide (available in the AHDB Horticulture website) provide advice to achieve effective disease management by a combination of pruning, chemical control and cultural control. Based on the literature review and on our results so far, the following additional suggestions can be given for the removal of cankers by pruning:

- Paring back of canker lesions is not recommended. Pruning should be performed instead; in fact, the fungus can be localised in every tissue between the bark and the hardwood in the lesions, therefore removal of cankered bark does not ensure removal of the infection.
- Pruning should be performed as soon as possible after a canker lesion appears. *N. ditissima* is apparently able to colonise the sapwood and move away from an actively growing lesion, without producing any symptoms.

SCIENCE SECTION

Introduction

Nectria canker (also known as European apple canker), caused by the fungus *Neonectria ditissima* (Tul. & C. Tul) Samuels & Rossman, is a major disease of apple (*Malus pumila*) tree and fruit. In the UK, losses occur at every stage of production. *N. ditissima* infects the plant via wounds and causes cankers and dieback of shoots in the field, leading to loss of fruiting wood and increased pruning costs. It also causes fruit rots in the field and post-harvest (Saville 2014). Cankers on trunks in young orchards can be especially serious because they can girdle the stem and kill the whole plant, causing up to 10% of trees to be lost annually, during the early phase of orchard establishment (Saville 2014; Weber 2014). In North-western Europe, except for dry periods in summer or freezing conditions in winter, *N. ditissima* inoculum is available all year round, and many types of wound are present on the host throughout the year, as potential infection sites. Therefore, European apple canker represents a problem all year round (Saville, 2014; Weber, 2014). In the UK, autumnal leaf scars are widely considered the main entry points for the pathogen (Swinburne, 1975). Apple cultivars show different degrees of susceptibility to the canker, and total resistance has not been found yet (Weber 2014). In the UK, most of both the well-established and the newly introduced varieties (such as Jazz, Braeburn, Rubens, Cameo, Kanzi and Zari) are particularly susceptible (Saville 2014). Disease management almost entirely relies on pruning, as chemical control options are essentially limited to apple scab fungicides (e.g. Captan), that can only provide partial protection against apple canker (Saville 2014). Copper fungicides, which provided good long-term protection during the dormant season (Weber 2014), are no longer allowed since 2015. As a result of the lack of registered products for disease control, and due to the increasing planting of susceptible cultivars in the UK, apple canker incidence is increasing and is becoming more and more important (Saville 2014).

The results of the “Millennium Trial” (McCracken et al. 2003) support the hypothesis that *N. ditissima* is able to infect the plant in the nursery, and then enter a latent phase during which no symptoms are expressed. Once the young trees are transplanted into orchards, this latent infection can manifest, with devastating effects on young trees. These observations led to the hypothesis that the fungus can inhabit plant tissues as an endophyte, i.e. that it can grow within the plant without producing any sign of infection. Confirmation of such endophytic status and a diagnostic procedure for the early detection of latent infections in nursery stock material are therefore needed to implement management strategies. According to Saville (2014), the two main challenges in the development of molecular tools for the diagnosis of *N. ditissima* are: the difficulty in purifying the DNA extracted from plant woody tissues, which

contain PCR inhibitor compounds, and the need for sampling strategies which can ensure representative results. Ghasemkhani et al. (2016) showed that the fungus grows in every tissue layer between the bark and the pith in infected tissue, and several authors have reported that *N. ditissima* can spread within the xylem of infected trees beyond the border of the canker lesion (Crowdy, 1949; Kennel, 1963; Dewey, Li and Swinburne, 1995; Weber and Hahn, 2013). However, no anatomy study so far addressed the asymptomatic phase of the infection. To implement a reliable sampling procedure, it is necessary to understand exactly how and where the pathogen resides within the trees following wound infection. As for the detection tools, a qPCR assay (Ghasemkhani, Holefors, *et al.*, 2016) and a *N. ditissima*-specific monoclonal antibody (Dewey, Li and Swinburne, 1995) have been made available and can be optimised to detect the pathogen in asymptomatic tissues. In the present work, we further optimised these serological and DNA-based methods, and we used them in a disease anatomy study of the *N. ditissima* asymptomatic infection.

Different apple tree cultivars show different degrees of field resistance to the pathogen (Garkava-Gustavsson et al. 2016; Ghasemkhani et al. 2015; Gomez-Cortecero et al. 2015; Saville 2014; Scheper et al. 2018; van de Weg 1989; Weber 2014), but the genetic bases of this resistance are not known yet (Garkava-Gustavsson et al. 2016). Several studies have shown that endophytes, i.e. plant associated fungi and bacteria which inhabit the internal plant tissues without causing any apparent infection, can confer their plant hosts disease resistance. For example, artificial inoculation of plants with bacterial endophytes was shown to reduce the severity of different plant diseases and to limit parasite damage (Berg and Hallmann 2006; Kerry 2000; Ping and Boland 2004; Sturz et al. 2000). Fungal endophytes instead have been shown to influence the severity of disease symptoms (Arnold et al. 2003; Freeman and Rodriguez 1993), either decreasing (pathogen antagonism) or increasing it (pathogen facilitation) (Busby et al. 2016), and thus they have been referred to as “modifiers of plant disease” (Busby et al. 2016). Our hypothesis is that endophytic microorganisms of apple tree are accountable for at least part of the observed field resistance to European apple canker. Therefore, cultivars representative of different resistance classes might harbour different endophytic communities, or in other words, might be characterised by different endophyte profiles. A pilot study has been already carried out at NIAB EMR with a meta-barcoding approach, and the profiles of the analysed apple cultivars were shown to cluster based on the degree of susceptibility to *N. ditissima* (Saville and Xu, unpublished data). It is the aim of this present work to further these studies. Should one or more microbial species be found significantly associated with a higher level of field resistance, these could be further tested *in vitro* and *in planta* for antagonism against *N. ditissima*. One of the long-term goals

of this study is the implementation of strategies for the manipulation of the apple tree microbiome with the aim of growing disease-resistant plants.

Materials and methods

Assessment of different detection methods for *N. ditissima* asymptomatic infection

On 28th and 29th November 2017, apple tree rootstocks propagated in stool beds at NIAB EMR, East Malling, were inoculated via bud scars with a conidial suspension of *N. ditissima* strain Hg199 (Gomez-Cortecero et al. 2016). Mock inoculations with sterile distilled water (SDW) were also performed as negative controls. The bud was carefully removed with a fingernail and the inoculum or SDW were applied to the wound. Inoculated stems were cut and harvested on 14th December 2017 and moved into a container, where they were kept in sand buckets at 18°C with 100% humidity. On 10th April 2018, all stems which had developed canker symptoms were measured, then 10 cm long stem sections including the inoculated bud scars were cut from symptomatic, asymptomatic and mock inoculated stems and stored at -20°C until processed as detailed below.

Symptomatic and asymptomatic rootstock samples were sprayed with ethanol and wiped, then processed as follows. The surface bark was removed. Fine stem shavings (2-3) were taken from the leading edge of cankers on the symptomatic shoots, and from the inoculated bud scar on the asymptomatic ones. A scalpel blade was used to cut the shavings and cleaned by immersing in ethanol and flaming prior to processing each sample. Both bark and layers of the underlying wood were collected. Shavings were cut down into small fragments (1 to 2 mm long) and then prepped for subsequent ELISA and real-time qPCR to assess presence/absence of *N. ditissima*. Briefly, shavings (10 to 40 mg *c*. in 2.0 ml tubes) were freeze-dried over 24 hours, added with clean tungsten beads, flash-frozen in liquid nitrogen and ground in a Geno/Grinder2010 at 1,500 rpm for 1 min. Subsequently, they were added with Phosphate Buffered Saline (PBS) in the proportion of 5 ml PBS per gram of dry plant material mass, shaken in the Geno/Grinder2010 (1,500 rpm, 1 min) and allowed to sit at room temperature for 30 min. Samples were then centrifuged and 50 µl supernatant was collected, diluted 1:6 in PBS and then used in the ELISA. To serve as positive and negative controls, shavings of disease-free plant material (Gala grafted on M9, collected in a polytunnel at NIAB EMR) added with *N. ditissima* mycelium (grown for 3 weeks on PDA, collected, freeze-dried over 24 hours, flash-frozen in liquid nitrogen, added with metal beads, ground in the Geno/Grinder2010, suspended in PBS 5:1 ml:g and added to plant material in the proportion of 0.1, 0.01 and 0.005 g:g) or sterile distilled water (100 µl) respectively, were prepped as detailed above and used in the assay. The sample remaining after collecting the supernatant was subjected to DNA extraction with the DNeasy Plant Mini Kit (QIAGEN, The Netherlands),

following the manufacturer's instructions. To serve as positive control in the real time qPCR assays, DNA of *N. ditissima* Hg199 (Gomez-Cortecero et al. 2016) grown on a cellophane membrane on PDA, was extracted with the same kit. DNA was quantified spectrophotometrically (Nanodrop™ 1000, ThermoFisher Scientific, USA).

ELISAs, were performed following the protocol developed by Dewey et al. (1995), with some modifications. Assays were carried out in 96 well MicroWell™ MaxiSorp™ flat bottom plates (Sigma-Aldrich, USA). Phosphate Buffered Saline with 0.5% Tween20 (PBST) was used as dilution buffer and for washings between incubation steps. Wells were coated in antigens by incubating 100 µl sample or controls overnight at 4°C. Wells were washed with PBST and incubated with 100 µl 1B10 hybridoma supernatant, followed by 200 µl goat anti-mouse IgG conjugated to biotin (Sigma-Aldrich) diluted 1:1000, and then by 200 µl streptavidin conjugated to horseradish peroxidase (Sigma-Aldrich) diluted 1:4000. All incubations were carried out at 30°C for 45 min. Finally, wells were incubated for 20 min in the dark with 3,3,5,5′-Tetramethylbenzidine (TMB) liquid substrate (Sigma-Aldrich). The reaction was stopped by adding H_2SO_4 1M and the absorbance at 450 nm was measured by means of a spectrophotometer (Anthos HTII; Anthos Labtec Instruments, Austria).

N. ditissima gDNA was detected in total DNA extracted from plant samples by real time qPCR using the primer set Bt-fw135/re284 (Ghasemkhani, Holefors, et al. 2016). The real-time qPCR was carried out in a CFX96 Touch™ Real Time PCR Detection System (Bio-Rad, USA), as described by the authors. Amplification was carried out with two technical replicates per reaction, and the qPCR plate included a standard curve, comprising 7 serial 1:10 dilutions (in triplicate) of *N. ditissima* gDNA, starting from 10 ng.

Data were analysed and charts were plotted with the software R studio.

Disease anatomy of the asymptomatic leaf scar infection

Apple trees (Royal Gala and Queen Cox), grown in an orchard at NIAB EMR, East Malling, were artificially inoculated via leaf scars with a high dose (6,000 conidia/wound) and a low dose (600 conidia/wound) of conidia of *N. ditissima* strain Hg199. Symptoms expression was monitored across the season, symptomatic and asymptomatic plant material was sampled at different time points after the inoculation and real time qPCR was used to assess the asymptomatic colonisation of plant tissues. Trees were 22 years old at the beginning of the experiment. In October-November 2017, before leaf fall, healthy shoots were selected, 10 to 15 leaves were hand-removed (as described in Amponsah et al. 2015 and Dubin and English 1974) starting from the tip of the shoot, and the two different doses of inoculum were immediately applied to the artificial leaf scars. Each tree was inoculated with both inoculum

doses, on different shoots. Shoots were covered in moistened plastic bags for 24 h to ensure successful establishment of the infection (Dubin and English 1974). As a negative control, mock inoculations with sterile distilled water were also performed. Three inoculation experiments were carried out on 24th and 27th October and 10th November 2017. Temperature and moisture were recorded during the experiment by the meteorological station at NIAB EMR. Every two weeks, trees were monitored for apple canker symptoms expression, and lesion incidence was recorded. At 13, 21 and 28 weeks after the inoculation, two shoots treated with two different inoculum doses were sampled from each tree. Secateurs were sterilised with isopropanol (IPA) soaked wipes before performing each cut. Shoots were conserved at -20°C until further processed.

Shoots were washed in 0.5% Tween20 and gently brushed with a toothbrush for 1 min, then soaked in 0.025% sodium hypochlorite (5.0% vol:vol commercial bleach) for 1 min to remove epiphytic DNA (Prince and Andrus 1992), and finally rinsed twice for 1 min in sterile distilled water. Shoots were air dried in sterile conditions and then dissected and pooled as detailed below and shown in **Table 1**.

Table 1. Pools (identification numbers in brackets; 1-8) of bark and wood samples collected from leaf scar-inoculated shoots; symptom presence/absence is reported for each sample pool.

	Disease-free sample pools		**Diseased sample pools**	
Tissue	**Nodes**	**Internodes**	**Nodes**	**Internodes**
Bark	Asymptomatic (1)	Asymptomatic (3)	Symptomatic (5)	Asymptomatic (7)
Wood	Asymptomatic (2)	Asymptomatic (4)	Symptomatic (6)	Asymptomatic (8)

On each asymptomatic shoot, 5 to 10 inoculated leaf scars (asymptomatic nodes) were selected and a 5-10 mm long segment of bark tissues around the leaf scar were dissected with the aid of a scalpel blade. Then, shavings (less than 1 mm thick) were taken of the underlying woody tissues. The same procedure was repeated at the two internodes above and below the inoculated nodes, at a distance comprised between 10 and 15 mm from the node sample. Tissues collected from asymptomatic shoots were then pooled ('disease-free sample pools') as follows: bark from nodes (pool 1, **Table 1**), wood from nodes (pool 2), bark from internodes (pool 3) and wood from internodes (pool 4). On each shoot that had developed canker symptoms, asymptomatic nodes and internodes were sampled and pooled as described above, and additional 'diseased sample pools' were collected. These included: bark and wood from symptomatic nodes (pools 5 and 6, **Table 1**) and bark and wood from

asymptomatic internodes adjacent to cankered nodes (pools 7 and 8); symptomatic internodes were not sampled. Finally, individual (i.e. non-pooled) asymptomatic bark and wood samples were collected in the internode at 15 mm from the basal inoculated leaf scar, either symptomatic or asymptomatic (*not shown in Table 1*). Scalpel blades were cleaned with ethanol and flame-sterilised prior to process each sample pool. All samples were collected in 5.0 or 2.0 ml tubes (depending on the amount of plant material) and stored at minus 80°C until further processed. Samples were flash-frozen in liquid nitrogen and ground in the GenoGrinder2010, then added with PBS, shaken and let sit at room temperature for 30 min. Total DNA extraction was performed using the DNeasy Plant Mini Kit (QIAGEN, The Netherlands) following the manufacturer's instruction. To serve as positive control in the subsequent real time qPCR assays, DNA of *N. ditissima* strain Hg199, grown on a cellophane membrane on PDA, was extracted with the same kit. All DNA was quantified spectrophotometrically (Nanodrop™ 1000, ThermoFisher Scientific, USA), diluted to 2 ng/µl and then run in a PCR with primers ITS1 and ITS4 (White et al. 1990) to check whether DNA extraction was successful. PCR was performed in a PTC-220 DYAD™ PCR Engine (BIO-RAD, USA) and PCR products were analysed on 1.5% agarose gel. *N. ditissima* gDNA was detected by real-time qPCR using the primer set Bt-fw135/re284 (Ghasemkhani, Holefors, et al. 2016). The real-time qPCR was carried out in a CFX96 Touch™ Real Time PCR Detection System (Bio-Rad, USA), as described by (Ghasemkhani, Holefors, et al. 2016).

Data were analysed and charts were plotted with the software R studio.

Results

Assessment of different detection methods for *N. ditissima* asymptomatic infection

Spore germination test of *N. ditissima* inoculum on microscope slides resulted in 79% (28th Nov) and 66% (29th Nov) germination after 24 hours. The results of the ELISA on artificially inoculated rootstocks are shown in **Figure 1**.

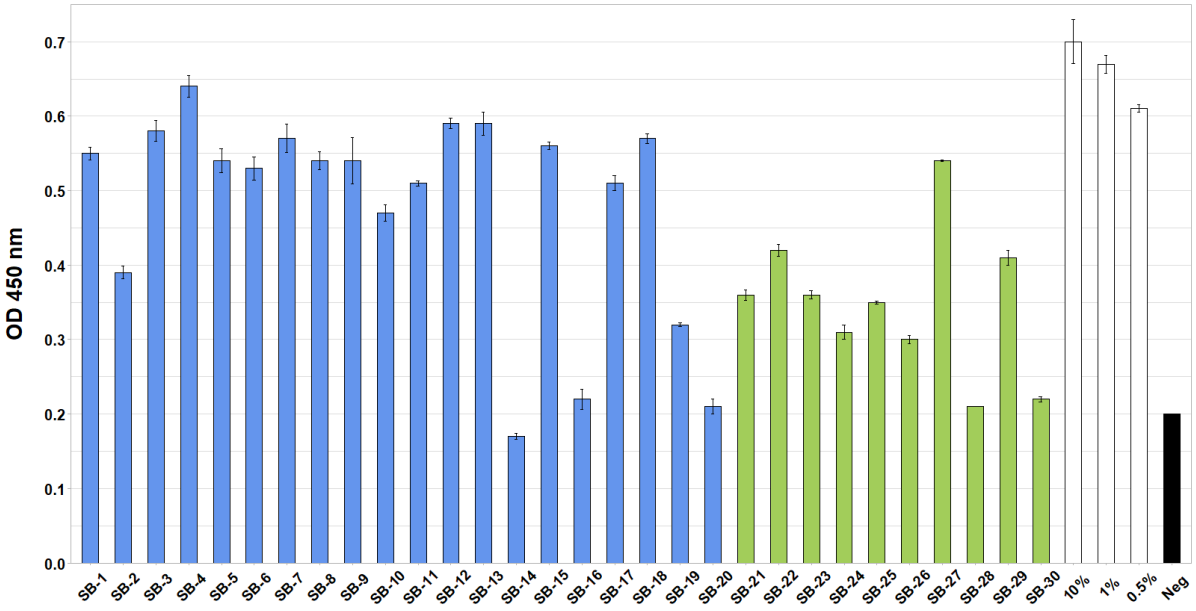

Figure 1. ELISA with hybridoma supernatant 1B10 tested against artificially inoculated rootstock samples. OD_{450nm} is shown for symptomatic samples (SB-1 through SB-30; blue bars: symptomatic samples; green bars: asymptomatic samples), positive controls (0.1, 0.05, 0.01 and 0.005 g mycelium per g plant material; white bars) and negative control (disease-free plant material added with SDW; black bar) is shown; data represent the average of 2 technical replicates; error bars represent standard deviation.

In the ELISA, *N. ditissima* antigens were detected (OD_{450nm} greater than the positive control) across all samples, except for SB-14, SB-16, SB20 (symptomatic), SB-28 and SB-30 (asymptomatic). In the real time qPCR assay, *N. ditissima* DNA was detected in all samples (data not shown); all samples showed a Cq lower than the limit of detection (Cq = 37.74) reported by Ghasemkhani, Zborowska, et al. (2016). A comparison between the results of the ELISA and the qPCR is presented in **Table 2**. All assessed samples were positive detected in the qPCR. However, only 85% of symptomatic samples and 80% of asymptomatic samples were positive to *N. ditissima* in the ELISA.

Table 2. Counts of rootstock samples positive to *N. ditissima* in the ELISA and the qPCR and total number of assessed symptomatic and asymptomatic samples; percentage of positive samples is reported in brackets.

	ELISA	qPCR	Total assessed
Symptomatic	17 (85%)	20 (100%)	20
Asymptomatic	8 (80%)	10 (100%)	10

Disease anatomy of the asymptomatic leaf scar infection

The germination percentages of inoculum used on 24th and 27th October and 10th November 2017, assessed on PDA, were 91%, 97% and 94%, respectively. Symptoms expression across the season is shown in **Figure 2**, **3** and **4** for the trees inoculated on 24th and 27th October and 10th November 2017, respectively.

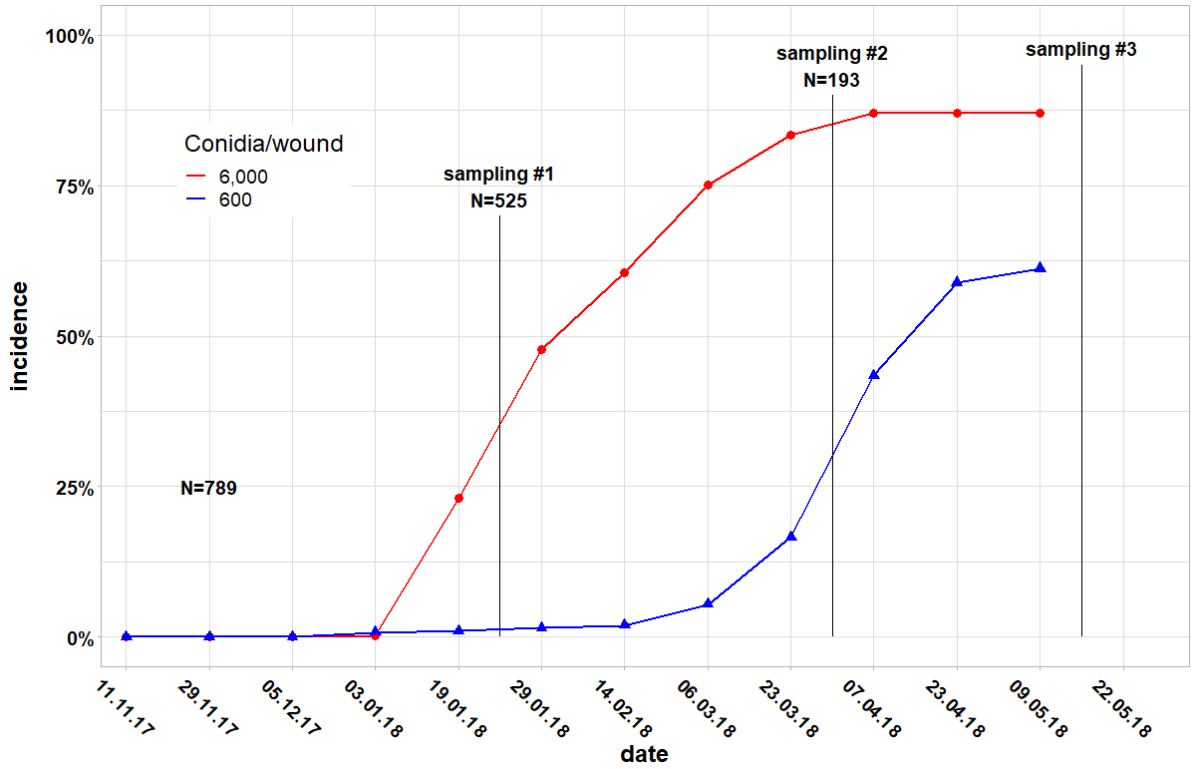

Figure 2. Cumulative incidence of canker symptoms on apple trees of cvs. Royal Gala and Queen Cox inoculated via leaf scar with two different doses of conidia (600 and 6,000 conidia/wound) on 24th October 2017; N: number of inoculated leaf scars assessed for symptom expression (decreasing after each sampling); sampling #1: 13 weeks after the inoculation (wai), sampling #2: 21 wai, sampling #3: 28 wai; data are pooled for the two cultivars.

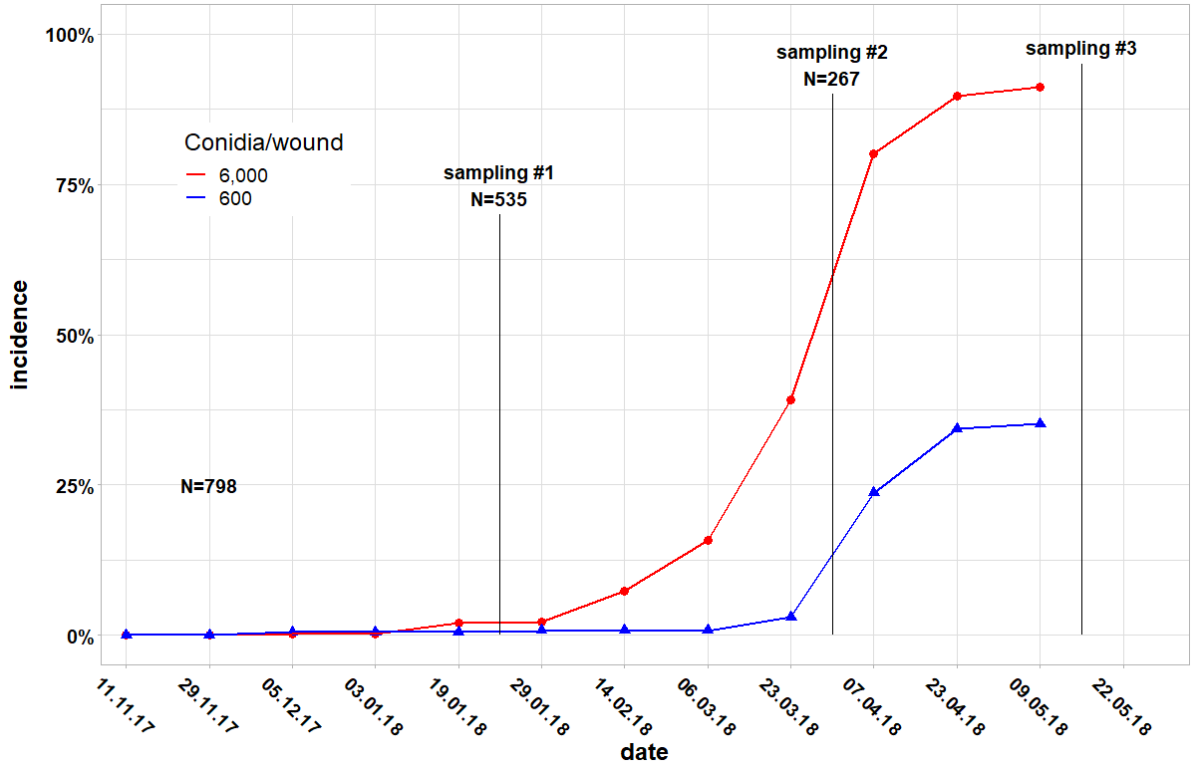

Figure 3. Cumulative incidence of canker symptoms on apple trees of cvs. Royal Gala and Queen Cox inoculated via leaf scar with two different doses of conidia (600 and 6,000 conidia/wound) on 27th October 2017; N: number of inoculated leaf scars assessed for symptom expression (decreasing after each sampling); sampling #1: 13 weeks after the inoculation (wai), sampling #2: 21 wai, sampling #3: 28 wai; data are pooled for the two cultivars.

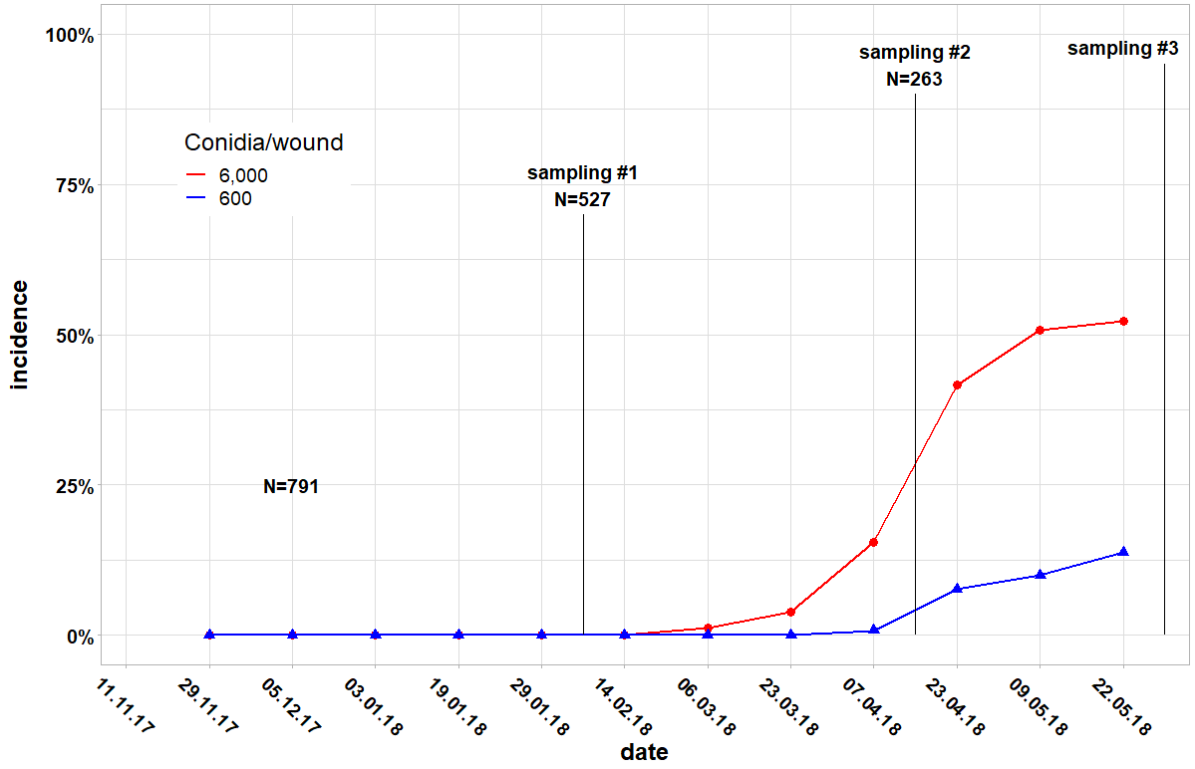

Figure 4. Cumulative incidence of canker symptoms on apple trees of cvs. Royal Gala and Queen Cox inoculated via leaf scar with two different doses of conidia (600 and 6,000 conidia/wound) on 10th November 2017; N: number of inoculated leaf scars assessed for symptom expression (decreasing after each sampling); sampling #1: 13 weeks after the inoculation (wai), sampling #2: 21 wai, sampling #3: 28 wai; data are pooled for the two cultivars.

Symptoms developed slower on shoots inoculated with the low inoculum dose (600 conidia/leaf scar) than on those inoculated with the high inoculum dose (6,000 conidia/leaf scar); at every sampling point, symptoms expression was higher on shoots inoculated with the low dose. To assess the effect of incubation time on the asymptomatic colonisation of plant tissues at 13, 21 and 28 weeks after the inoculation, shoots inoculated with the low dose and collected at three sampling points were subjected to DNA extraction and real time qPCR. Additionally, to assess the effects of the inoculum dose on the asymptomatic colonisation of plant tissues, shoots inoculated with the high dose and collected 13 weeks after the inoculation were analysed. At the time of writing, dissection, pooling, DNA extraction and subsequent real time qPCR has been completed only for two Royal Gala and two Queen Cox shoots inoculated with the low dose and two Royal Gala and two Queen Cox shoots inoculated with the high dose, all collected 13 weeks after the inoculation. The results of the real time qPCR on this sample subset are shown in **Table 3**.

Table 3. Bark and wood samples positive to real time qPCR assay against *N. ditissima*, the total number of replicates per each sample is shown in brackets; all samples were collected 13 weeks after the inoculation. Data for Royal Gala and Queen Cox cultivars are pooled; data for low and high dose are pooled.

Type	Position	Tissue	Positive samples (total replicates)
Asymptomatic individual and pooled samples	node	bark	8 (8)
		wood	0 (8)
	internode	bark	0 (8)
		wood	0 (8)
	15 mm from canker	bark	0 (8)
		wood	0 (8)
Diseased individual and pooled samples	node	bark	1 (1)
		wood	0 (1)
	internode	bark	0 (1)
		wood	1 (1)
	15 mm from canker	bark	NA
		wood	NA

On asymptomatic shoots, all node bark samples were positive to the real time qPCR assay; all node wood samples and internode samples (both bark and wood) were negative instead. Only one shoot (cv. Royal Gala) of the assessed ones had developed canker symptoms at one of the inoculated leaf scar: node bark and internode wood were positive, whereas node wood and internode bark were negative.

Discussion

Assessment of different detection methods for *N. ditissima* asymptomatic infection

By comparing the results of the ELISA and the real-time PCR, it appeared that the serological method was more prone to false negatives than the DNA-based one. *N. ditissima* antigens were not detected by the ELISA in two asymptomatic samples and, more worryingly, in three symptomatic samples. On the other hand, such samples were all positive to the real-time PCR. The false negatives could be due to the interaction of soluble components of the plant matrix on the antigen and/or the antibody. Understanding these interactions and devising a

solution to exclude the plant matrix effects would allow to improve and validate the ELISA method for use research and diagnostics, however this lies beyond the research goals of this current project. Our results suggested a higher sensitivity of the real-time PCR, and this method was therefore chosen to carry out the disease anatomy study.

Disease anatomy of the asymptomatic leaf scar infection

To assess whether *N. ditissima* can asymptomatically colonise plant tissues at distance from infected infection sites, orchard trees were inoculated via leaf scars with the pathogen, symptomatic and asymptomatic plant material was sampled during the latent stage of the infection and real-time qPCR was used to detect the pathogen at the infection site (leaf scar) and at distance from it. Symptoms expression progressed differently across the three experiments, possibly reflecting the effects of different environmental conditions (temperature and relative humidity) at the time of inoculation and/or during incubation; meteorological data are not available for this experiment at the time of writing. These differences in symptom expression may also be explained by local factors (e.g. water availability, soil type and microclimate) affecting canker susceptibility of trees planted in different orchard plots (Saville 2014).

In our experimental design, asymptomatic plant material (i.e. inoculated nodes and asymptomatic internodes) was necessary to assess the endophytic colonisation of plant tissues by *N. ditissima*, whereas symptomatic plant material (i.e. symptomatic nodes) served as positive control for the real time qPCR assay. By using two inoculum doses (low dose: 600 conidia/wound; high dose: 6,000 conidia/wound), the timing of symptoms expression allowed to collect both symptomatic and asymptomatic plant material at the time of sampling. In fact, canker symptoms were expressed quicker on shoots inoculated with the high dose of inoculum (6,000 conidia/wound); moreover, disease incidence was higher on shoots inoculated with the high dose of inoculum at every assessment.

At the time of writing, the partial results collected showed that three months after the inoculation, *N. ditissima* could only be found at the entry site (i.e. the infected leaf scars) across all asymptomatic shoots analysed. In particular, the fungus was detected in the bark tissues, which include the vascular bundles connecting the leaf scar to the stem, and never in the wood tissues of the stem. On the other hand, the pathogen was never detected in asymptomatic internodes. These early findings suggested that during the asymptomatic phase of the disease *N. ditissima* is not capable of endophytic colonisation of plant tissues at distance from the infected leaf scar. Based on these results, it is not possible to infer any conclusions on the endophytic status of the pathogen: the fungus could reside in the bark

layers of the leaf scar in the form of latent spores, or it could locally colonise plant tissues. After symptoms were expressed, the pathogen was detected in the asymptomatic wood in the internode adjacent to a symptomatic leaf scar, suggesting asymptomatic colonisation of the sapwood during the symptomatic phase of the infection. Despite the dataset is very limited at the time of writing (only one symptomatic shoot has been analysed), this observation confirms results previously published by Dewey et al. (1995) and Weber 2014.

Future Work

Endophytes associated with apple tree cultivars

A field experiment has been set-up to study the endophytic species species associated to different apple cultivars representative of different disease-resistance classes, and to explore the biological control potential of the apple tree fungal and bacterial endophytes. Eight different scion cultivars (resistant: Robusta 5, Golden Delicious, Grenadier; susceptible: Royal Gala, Breaburn, Jazz, Kanzi, Rubens), grafted onto two different rootstocks (resistant: M116; susceptible: M9 337), were planted in orchards at two different sites in Kent. Samples will be collected from the trees, total DNA will be extracted and DNA-sequencing technologies (meta-barcoding based on Next Generation Sequencing, NGS) will be employed to identify the fungal and bacterial groups associated to plant tissues. Samples will be collected from leaf scars, which represent the epidemiologically relevant infection site for *N. ditissima* in the UK, and the analysis will therefore focus on the endophytes directly interacting with the fungus at the entry point. We will determine the different endophyte groups and their relative amount across the different scion x rootstock combinations planted at the two sites, i.e. the cultivar-specific endophyte profiles and the rootstock and environment effects on such profiles. Finally, we will determine whether the cultivar-specific profiles are correlated with the resistance level of the respective cultivar.

Conclusions

- A DNA-based assay (real-time PCR) and a serological method (ELISA) were compared for their sensitivity in the detection of *N. ditissima* in asymptomatic apple tree tissues. Real-time PCR showed higher sensitivity and appeared to be the best available tool for the detection of the latent infection.
- Following artificial inoculation of leaf scars, *N. ditissima* was detected in asymptomatic plant material sampled from the inoculation point, three months after the inoculation; it was not possible to detect the pathogen at 1.0-1.5 cm from the infected leaf scar.

- Following artificial inoculation of leaf scars, *N. ditissima* was detected in asymptomatic sapwood adjacent to a canker lesion, suggesting spread of the pathogen via vascular tissues had occurred.

Knowledge and Technology Transfer

1. 26/04/2017: oral presentation at the Postgraduate Symposium 2017 at the School of Biological Sciences, Royal Holloway Univerisity of London, Egham Surrey
2. 13/09/2017: oral presentaion at AHDB Fruit Agronomists' Day, NIAB EMR, East Malling, Kent
3. 20/09/2017: oral presentation at British Tomato Conference 2017, Kenilworth, Warwickshire
4. 01/11/2017: oral presentation at the Third International Workshop on Apple Canker and Replant Disease, NIAB EMR, East Malling
5. 20/11/2017: oral presentation at 'Your Plant Science', PhD outreach event at NIAB, Cambridge
6. 24/04/2018: oral presentation at the Postgraduate Symposium 2018 at the School of Biological Sciences, Royal Holloway Univerisity of London, Egham Surrey

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