Project title:	Understanding endophytes to improve tree health: colonisation of trees by the apple canker fungus and perspectives for biological control
Project number:	CP161
Project leader:	Xiangming Xu, NIAB EMR
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The results and conclusions in this report are based on an investigation conducted over a four-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

Headlines

- Internal colonisation of apple trees by the canker fungus, *Neonectria ditissima*, did not occur in the absence of symptoms on the plant, whereas it was observed after canker lesions started to develop.
- Several apple tree-associated microorganisms showed different relative abundance in canker-resistant apple cultivars compared to susceptible cultivars, thus representing interesting candidates for the development of biological control strategies against the pathogen.

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, Kanzi, Jazz, Rubens and Braeburn and the limited availability of registered products for disease control, has resulted in canker becoming more serious in recent years. Moreover, disease management is difficult because after infecting the tree, *N. ditissima* may enter a long latent phase during which no symptoms occur. This represents a serious problem especially in newly established orchards whereby infections which occurred during the propagation phase in the nursery can remain asymptomatic until the young trees are transplanted in the orchard. In these instances, canker lesions typically develop on the main stem or the rootstock, leading to tree grubbing and sometimes causing heavy losses. In some years, over 10% of trees in newly planted orchards can be lost to canker.

The current project set out to address two key areas of research which the industry is interested in:

- developing a diagnostic strategy to test nursery stock material in nursery certification schemes and
- developing alternative control strategies for European apple canker.

Effective diagnosis of a latent infection requires a sensitive and specific detection tool and a targeted sampling strategy. The latter requires an understanding of disease anatomy and of the relationship between the growth of *N. ditissima* in plant tissues and symptom development. These aspects of the pathogen's biology are not fully understood yet. Some observations led to the speculation that the pathogen may extensively spread within the tree

during the asymptomatic phase of the infection. Therefore, one of the aims of this present project was to test the hypothesis that *N. ditissima* can colonise the plant tissues at distance from the site of infection before any visual symptoms can be observed.

Bacterial and fungal biocontrol agents offer ever increasing control options against plant pathogens, which will reduce the reliance on chemical crop protection products. However, until now they have been poorly exploited in relation to European apple canker. Most commercialised biocontrol agents are 'epiphytic', which means they inhabit the surface of bark, leaves and fruits. Their efficacy as pathogen antagonists is strongly dependent on environmental conditions influencing their establishment and survival, such as UV, humidity and temperature. Effective control of European apple canker requires protection of autumnal leaf scars, whose infection can lead to high canker incidence and serious damage to production the following spring. However, environmental conditions at leaf fall are often sub-optimal for the establishment of epiphytic biocontrol agents. Plants also host fungal and bacterial microorganisms associated with their internal tissues, called 'endophytes', which are less affected by environmental factors. It is known that endophytes can interact synergistically or antagonistically with plant pathogens, ultimately affecting disease outcome (incidence and severity). There are differences in the endophyte communities found across different commercial apple cultivars: it is possible that such differences contribute, together with the host genetics, to the different levels of canker resistance observed across cultivars. A second aim of this present project was to compare bacterial and fungal endophytes across canker-resistant and -susceptible cultivars, to identify microorganisms with a biocontrol potential against *N. ditissima*.

Summary of the project and main conclusions

To address the first aim of this project, we studied the ability of *N. ditissima* to grow in the apple tree without producing symptoms. We artificially infected apple trees with a spore suspension of the pathogen via different types of wound (leaf scars and pruning wounds), and then we assessed the distance the pathogen had colonised from the entry point to the healthy-looking part of the branch, both prior to and after canker symptoms had developed.

Firstly, we inoculated shoots on apple trees with a spore suspension of the fungal pathogen via pruning wounds and leaf scars. We used trees of the commercial cultivars Royal Gala and Queen Cox, grown in an orchard at NIAB EMR, East Malling, Kent. Then, we monitored disease incidence over time, and sampled symptomatic and asymptomatic shoots at different time points. Finally, we assessed the localisation of the pathogen at the entry site, i.e. the asymptomatic or cankered infected wound on asymptomatic or symptomatic shoots,

respectively, as well as at several distances along the shoot. In the case of leaf scar inoculations, samples were taken from the infection point and up to a distance of 10-15 mm, whilst in the pruning wound inoculations samples were collected from the inoculation point and up to a distance of 40 mm. We used a highly specific DNA-based method to detect *N. ditissima*-specific DNA in plant samples. Our findings suggested that:

1. *N. ditissima* can remain latent for several months in the infected wounds before cankers are formed; notably, we found latent infections up to four and seven months after the inoculation in pruning wounds and leaf scars, respectively.
2. During such latent period, the pathogen does not spread in the tree, but it remains confined at the infected wound.
3. However, once visual symptoms occur, *N. ditissima* can grow beyond the limits of the canker lesion into the adjacent, apparently healthy portions of stem and branches; in this study, the pathogen was detected up to 15 mm from the edge of the symptomatic tissue.

To address the second aim of this project, we studied the bacterial and fungal endophytes associated with the apple tree. Notably, we assessed how the scion cultivar, the rootstock cultivar and the site of planting determine the diversity of endophytes, and we compared endophytes associated with different scion cultivars differing in their respective canker-resistance. We focussed on the endophytes which are found at the leaf scar, as this the main natural infection route for the pathogen.

Eight scion cultivars were grafted onto two different rootstocks at a common nursery (Frank P Matthews), then planted in two orchards in Kent. Samples were collected from leaf scars of young shoots in autumn 2018. A DNA-based method (Next Generation Sequencing, NGS) was used to determine fungal and bacterial endophytes and their relative abundance in different samples. Our findings suggest that:

1. Overall, apple tree endophytes were mainly influenced by the site of planting and by the scion cultivar.
2. Some endophytes displayed higher or lower abundance across cultivars differing in canker-resistance.

The different relative abundance of some endophytes indicates that they may act as antagonists or facilitators of the pathogen and thus reduce or aggravate, respectively, disease incidence and severity. Based on these findings, further studies are being carried out (BBSRC project BB/P007899/1) to investigate their potential and how this can be exploited against

European apple canker. Moreover, our results suggest that orchard management practices and cultivation history may influence the diversity of endophyte associated with apple. In fact, different plant species may host different endophyte populations, which contribute to build-up of environmental inoculum that can in turn colonise apple trees. Similarly, it is known that specific plant hosts can act as *N. ditissima* reservoir, thus acting as pathogen inoculum source. Therefore, practices such as weed control and hedgerow management may help to achieve successful manipulation of endophytes, as well as effective disease control, by shaping the local microbial inoculum. Our results pave the way for the development of alternative strategies for European apple canker control.

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 greatly reduce the additional expense required to replace diseased trees.

Action points for growers

Successful European apple canker management needs to combine different approaches including systematic pruning of cankers, removal of heavily infected trees and timely fungicide applications. Removal of canker lesions is essential to achieve efficient disease control, as it reduces the source of infection in the orchard.

- Pruning should be performed as soon as cankers become visible on the plant. In fact, once symptoms appear the pathogen might spread internally in shoots and branches and reach healthy parts of the tree.
- To account for the possibility of internal spread of the pathogen, pruning cuts to remove canker lesions should be performed at a minimum distance of 10 cm from the canker edge.

- Paring off cankered bark on main stem or scaffold branches is not recommended, as the pathogen may survive in the underlying wood and produce a new canker lesion; pruning should be performed instead.

SCIENCE SECTION

Introduction

European apple canker (also known as Nectria canker), caused by the fungus *Neonectria ditissima* (Tul. & C. Tul) Samuels & Rossman, is a major disease of apple (*Malus* x *domestica*, syn. *M. pumila*) tree and fruit on a global scale. *N. ditissima* infects the plant via wounds, either natural or artificial, and causes cankers and dieback of shoots in the orchard, leading to loss of fruiting wood and increased pruning costs. In the UK, autumnal leaf scars are widely considered the main entry points for the pathogen (Swinburne, 1975). *N. ditissima* can also cause fruit rots in the orchard and post-harvest (Saville, 2014; Weber, 2014). In North-western Europe, except for dry periods in summer or freezing conditions in winter, pathogen 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). Different apple tree cultivars show different degrees of field resistance to the pathogen (Ghasemkhani *et al.*, 2015; Garkava-Gustavsson *et al.*, 2016; Gomez-Cortecero *et al.*, 2016), but the genetic bases of this resistance are not known yet. In the UK, most of both the well-established and the newly introduced varieties (such as 'Jazz', 'Braeburn', 'Rubens', 'Cameo', 'Kanzi' and 'Zari') are highly susceptible (Saville, 2014).

N. ditissima may cause asymptomatic infections lasting up to three years (McCracken *et al.*, 2003). The fungus can infect the plants in the nursery and then remain latent until the young trees are transplanted into orchards, when symptoms can eventually appear. Cankers in newly established orchards can be especially serious. Young trees affected on the main stem may become girdled and die. Moreover, trees affected on the trunk or scaffold branches must be uprooted to prevent disease spread in the orchard. European canker may cause up to 10% of trees to be lost annually, during the early phase of orchard establishment (Saville & Olivieri, 2019). It has been hypothesised that during the incubation phase the fungus can colonise the internal plant tissues beyond the infection site without causing any symptoms, i.e. that *N. ditissima* can grow in the plant as a systemic endophyte. If confirmed, the asymptomatic colonisation of internal plant tissues would have important consequences on disease management. Ghasemkhani *et al.* (2016) showed that after symptoms have appeared, the fungus can grow in the lesion throughout all stem tissues, from the bark to the pith. Several authors found *N. ditissima* within the xylem of cankered trees beyond the leading edge of the lesion (Crowdy, 1949; Dewey *et al.*, 1995; Weber & Hahn, 2013). However, no study so far has assessed fungal growth in the plant in the absence of visual symptoms. Such study would require a specific and sensitive detection tool for *N. ditissima*, as well as a

sampling strategy to ensure biologically representative results. A qPCR assay (Ghasemkhani *et al.*, 2016a) and a *N. ditissima*-specific monoclonal antibody (Dewey *et al.*, 1995) have been developed for the detection of *N. ditissima* in plant material. These methods could be optimised to track the pathogen spread in asymptomatic plant tissues.

Disease control is mainly achieved by pruning out cankers, thus removing the inoculum source from the orchard. Chemical control can help reduce inoculum production on lesions, as well as the risk of new infections. However, pruning is costly and time-consuming. Moreover, several pesticides previously registered for use in commercial apple orchards have now been banned or subjected to restrictions of use, with important consequences on European apple canker management. For example, Carbendazim is highly effective against *N. ditissima*, but is no longer permitted. Tebuconazole represents an alternative, but can only be applied post-harvest. Copper fungicides are undergoing increasing restrictions (Saville & Olivieri, 2019). Currently, partial canker control can be achieved utilising fungicides against apple scab (*Venturia inaequalis*) and powdery mildew (*Podosphaera leucotricha*), such as dodine, dithianon and captan (Weber, 2014; Berrie, 2016). Recently, SDHI fungicides and phosphite, either alone or applied in combination with dithianon, also showed some efficacy (Berrie, 2016).

A valuable alternative to conventional disease control products is represented by biological control agents (BCAs). Most commercial BCAs are developed from epiphytic microorganisms, inhabiting the plant surface. However, interest is growing in the possibility of harnessing the biocontrol potential of endophytes. Endophytic fungi and bacteria inhabit the internal plant tissues without causing any infection symptoms and can confer their hosts disease resistance against a wide range of pathogens. Artificial inoculation of plants with bacterial endophytes can reduce the severity of different plant diseases (Sturz *et al.*, 2000; Berg & Hallmann, 2006). Fungal endophytes have been shown to influence the severity of disease symptoms (Freeman & Rodriguez, 1993; Arnold *et al.*, 2003), either decreasing (pathogen antagonism) or increasing disease (pathogen facilitation). Therefore, they have been described as ‘modifiers of plant disease’ (Busby *et al.*, 2016).

Specific apple endophytes associated to different apple cultivars may contribute to determine the cultivar canker resistance level. In a pilot study carried out at NIAB EMR, the diversity of fungal and bacterial endophytes of apple was studied with a meta-barcoding approach across four different cultivars, including two canker-resistant and two susceptible genotypes. The endophyte community structure was shown to cluster based on the degree of susceptibility to apple canker (Saville & Olivieri, 2019). Therefore, apple endophytes might provide a reservoir of novel BCAs against *N. ditissima*.

This project aimed to:

1. Compare different high-throughput methods for detecting asymptomatic *N. ditissima* in apple tree stem tissues.
2. Study the asymptomatic colonisation of shoots by *N. ditissima* prior to and after visual symptoms occurred.
3. Using a meta-barcoding approach, (a) dissect the effects of scion genotype, rootstock genotype and environment on the apple tree endophyte diversity, (b) determine whether cultivar resistance is associated with the endophyte community structure and (c) identify fungal and bacterial endophytes associated with canker resistance.

2. Materials and Methods

2.1 Comparison of high-throughput methods for *N. ditissima* infection

The aim of this set of experiments was to develop a high-throughput method to assess *N. ditissima* colonisation of plant tissues during the asymptomatic stage of the infection. A monoclonal antibody raised against *N. ditissima* was used to develop an ELISA for the detection of the pathogen. The ELISA was then validated with symptomatic plant material and the assay was compared with a culture dependent method. Finally, the ELISA and a *N. ditissima*-specific qPCR assay were compared for their sensitivities and reliability of detecting symptomatic and asymptomatic *N. ditissima* in plant samples.

2.1.1 Monoclonal antibody validation and ELISA setup

Mouse monoclonal antibodies were raised against *N. ditissima* at the Institute of Science and Environment, University of Worcester as part of AHDB project TF223. As the source of antigen, a living isolate of *N. ditissima* strain R09/05 was supplied by NIAB EMR, East Malling, UK. At the end of 2015, the supernatants of six hybridoma cell lines, each containing a different monoclonal antibody of class IgM showing high reactivity to *N. ditissima* soluble antigens, were transferred to NIAB EMR and stored at -20°C.

An ELISA protocol was developed by Ching Yuk Wong and Robert J. Saville, NIAB EMR, and used to compare the supernatants for their cross-reactivity to purified antigens of other plant pathogenic fungi which are commonly found in apple, namely *Fusarium lateritium, Monilinia laxa*, *Colletotrichum acutatum, Botryosphaeria obtusa*, *Nectria cinnabarina* and *Phomopsis* sp./*Diaporthe* sp.. The supernatant with the highest specificity for *N. ditissima* was selected for further testing. Soluble antigens of *N. ditissima* strains R09/05 and R28/15 and of the other apple-associated fungi were prepared as described below. Fungi, conserved in a living collection at NIAB EMR, were grown in Potato Dextrose Broth (PDB; Oxoid, Basingstoke, UK, prepared according to the manufacturer's instructions). Mycelium was harvested, washed three times with distilled water, freeze-dried and ground with a mortar and pestle. Ground mycelium was re-suspended in Phosphate Buffered Saline (PBS; Sigma-Aldrich, St. Louis, USA, prepared according to the manufacturer's instructions), at the ratio of 1 ml every 10 mg of freeze-dried mycelium. To discard the insoluble components of the mycelium, the suspension was centrifuged in a microfuge at 13,000 rpm for 2 min and the supernatant was collected. The amount of total soluble proteins was determined spectrophotometrically (Nanodrop™ 1000, ThermoFisher Scientific, USA). The soluble mycelial fractions were stored at -20°C.

The ELISA protocol was developed following Dewey et al. (1995), with some modifications. Assays were carried out in 96 well MicroWell™ MaxiSorp™ flat bottom plates (Sigma-Aldrich, USA). All samples and reagents were diluted in PBS. All working volumes were 100 µl per well, and wells were washed four times after each incubation with 200 µl PBST (PBS added with 0.5% Tween20) for 1 min. Wells were coated in antigen by incubating 100 µl of 10 µg/ml soluble mycelial fraction at 4°C overnight. Wells were washed with PBST and incubated with a 1:10 dilution of the hybridoma supernatants, followed by a 1:1000 dilution of goat anti-mouse IgG conjugated to biotin (Sigma-Aldrich), and then by a 1:4000 dilution (250 ng/µl) of streptavidin conjugated to horseradish peroxidase (Sigma-Aldrich). 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 100 µl of H_2SO_4 1M and the absorbance at 450 nm (OD_{450nm}) was measured with a spectrophotometer (Anthos HTII; Anthos Labtec Instruments, Austria). For each sample, the test was run in duplicate, and a "background control" was included to control for background noise caused by non-specific interactions, as described in Dewey et al. (1995). The incubation step with the hybridoma supernatant was not carried out for the background control and the well was left empty instead. Absorbance was normalised by subtracting the OD_{450nm} of the background control from the OD_{450nm} of its respective sample.

2.1.2 – ELISA validation with symptomatic plant samples and comparison with culture-dependent method

Inoculum preparation. A conidial suspension of *N. ditissima* strain Hg199 (Gomez-Cortecero *et al.*, 2016) was cultured on SNAY (Sugar Nutrient Agar and Yeast medium; 1.0 g potassium dihydrogen phosphate, 1.0 g potassium nitrate, 0.5 g magnesium sulphate heptahydrate, 0.5 g potassium chloride, 0.2 g glucose, 0.2 g sucrose, 1.0 g yeast extract and 20 g of Agar Technical no. 2, Oxoid Ltd, Basingstoke, UK, made up to 1 litre with distilled water; Nirenberg 1976) at room temperature for 3 weeks. On the day of inoculation, conidia were harvested by washing the plate with 3 ml of sterile distilled water and then filtered through sterile cheesecloth. The concentration of conidia was determined by haemocytometer and then adjusted by diluting with sterile distilled water. For each inoculum prepared, either macroconidia (two or more cells) only or macro- plus microconidia (unicellular) were counted as detailed below. Fresh inoculum was prepared for each inoculation experiment and used within 4 hours. To assess inoculum germination, the conidial suspension was cultured at a concentration of 10^4 conidia/ml in Petri dishes filled with a 2 mm thick layer of PDA (Oxoid Ltd) and the percentage of germinated conidia recorded after 12 h.

Apple trees cv. ‘Royal Gala’ on MM106 rootstock grown in 5 l pots were inoculated via leaf scar, produced with a scalpel blade, with 3 µl inoculum ($2x10^4$ conidia/ml), approx. 60 conidia/wound. Macro- or micro-conidia were counted. As a negative control, shoots were inoculated with sterile distilled water. Inoculations were done in October 2016 and trees were kept in a polytunnel at NIAB EMR, East Malling, UK. Symptomatic shoots were collected 8.5 months after the inoculation. All cuts were performed with sterilised secateurs. In the same experiment, naturally infected cankered branches were also collected from apple trees cv. ‘Royal Gala’ on M9 rootstock grown in an orchard at NIAB EMR. Naturally infected plant material was between 3 and 4 years old. Two naturally infected branches, thereinafter referred to as *branch A* and *B*, and one artificially inoculated branch, referred to as *branch C*, were sampled. Samples were assayed in parallel with both ELISA and isolation of *N. ditissima* on selective medium.

Samples were processed as detailed below. Two consecutive 3 mm long transverse sections were excised at the leading edge of canker, at 10 and at 40 mm from the lesion by means of a backsaw and a mitre box. Additionally, transverse sections were also taken at 70 mm from the canker on *branch C*. Pictures of branch A, B and C and details of the respective transverse sections sampled are shown in Figure 2.1.1, 2.1.2 and 2.1.3 respectively. To evaluate whether the pathogen spreads differentially in different types of tissues, the bark and the underlying wood tissues of each section were dissected by means of a scalpel blade and a dissection needle and separately processed.

At each sampling distance, bark and sapwood from the section closer to the lesion were analysed by ELISA. The assay was performed as described in Section 2.1.1. To coat wells of the ELISA plate in antigens, bark and wood subsamples were cut into small fragments with an approximate width of 1 mm, the fragments were pooled and immediately incubated in wells with 100 µl PBS at 4°C overnight. Soluble mycelial fractions of *N. ditissima* and of a non-target fungus (*F. lateritium* or *N. cinnabarina*) at the concentration of 10 µg/ml were included as positive and non-target control, respectively. For all samples, two technical replicates plus a background control were included in the ELISA. Tools were wiped with 70% ethanol before processing each sample.

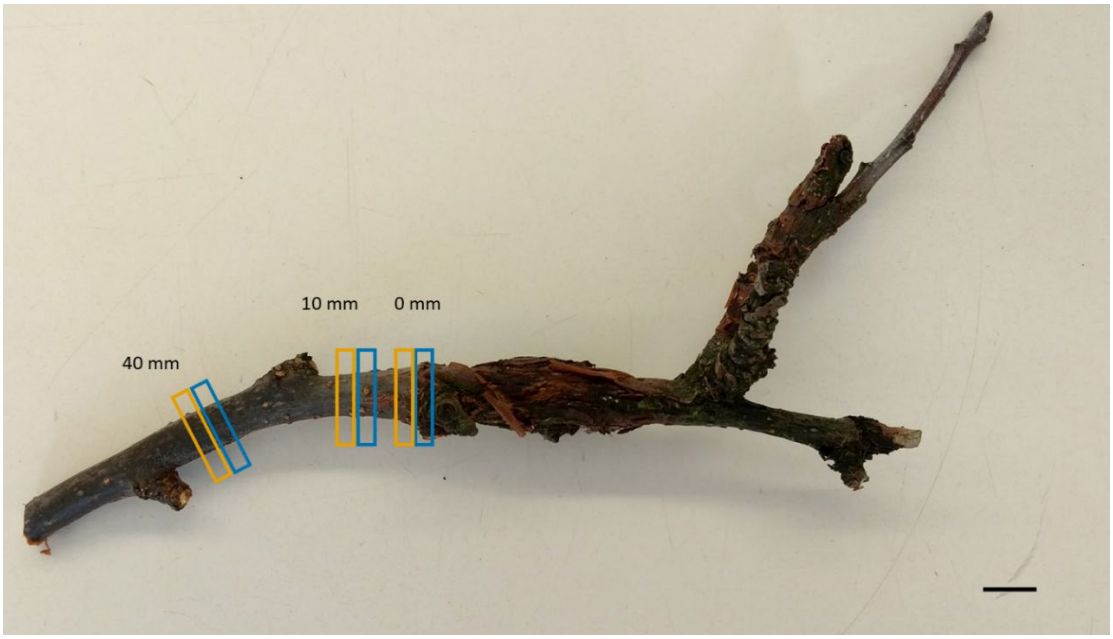

Figure 2.1.1 - Branch A; transverse sections excised for isolation (orange rectangles) or ELISA (blue rectangles) are shown; bar = 10 mm.

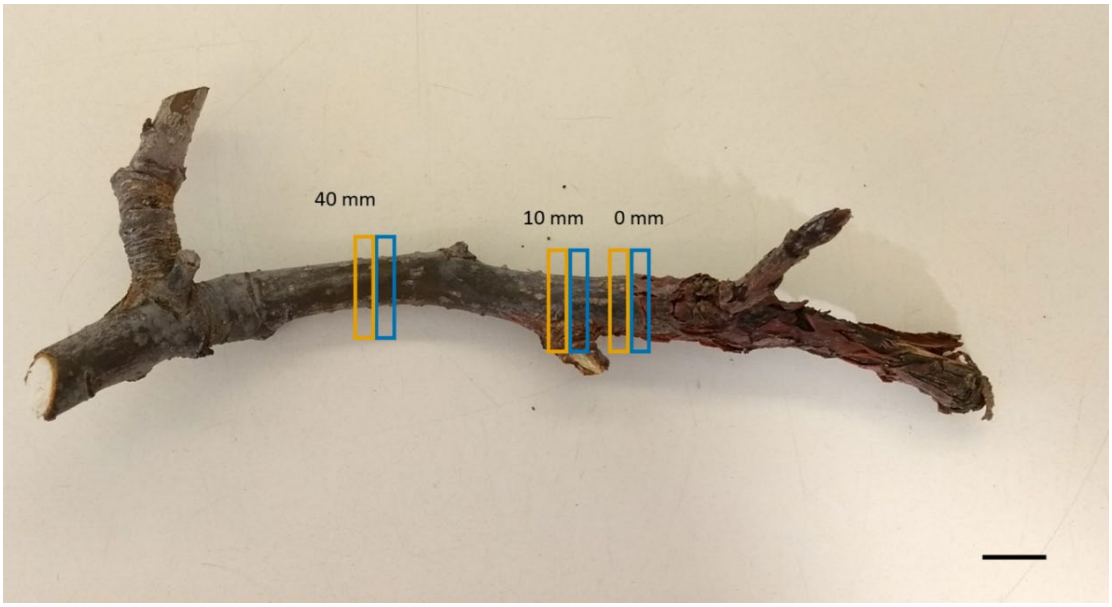

Figure 2.1.2 - Branch B; transverse sections excised for isolation (orange rectangles) or ELISA assay (blue rectangles) are shown; bar = 10 mm.

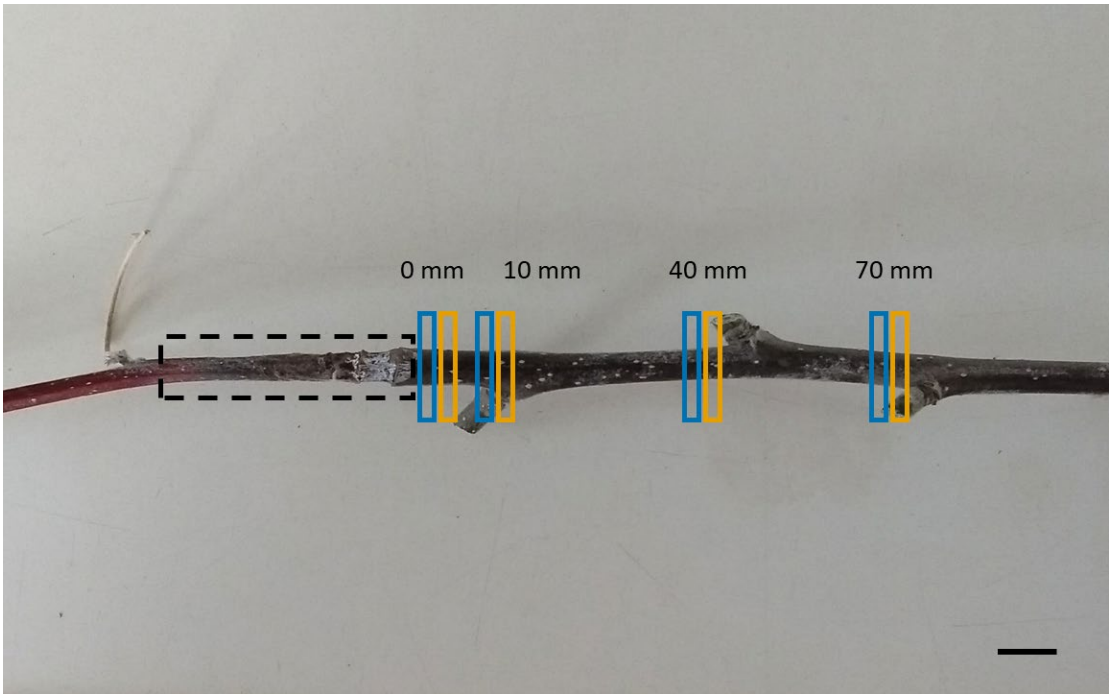

Figure 2.1.3 - Branch C; transverse sections excised for isolation (orange rectangles) or ELISA assay (blue rectangles), as well as canker (black dashed rectangle) are shown; bar = 10 mm.

At each sampling distance, bark and wood from the section distal from the canker lesion were surface sterilised to remove epiphytes. A modified technique based on method number 3 described in Schulz et al. (1993) was used, with minor modifications. Briefly, samples were immersed in 70% ethanol for 30 s, followed by sterile deionised water for 30 s, 5% bleach (0.25% free sodium hypochlorite) for 60 s, 70% ethanol for 30 s and finally sterile deionised water for 120 s. Samples were then pat-dried on sterile blotting paper and plated onto PDA amended with iprodione (Rovral ® WG, BASF plc, Cheadle, UK) 40 ppm and rifamycin 20 ppm, to prevent the isolation of bacteria and common plant-associated fungi, such as *Botrytis* spp. and *Penicillium* spp.. Petri dishes were incubated at room temperature and without parafilm for up to 2 weeks or until *N. ditissima* growth was observed. The fungus was identified based on colony morphology on the selective medium and microscopic observation of conidia.

Data analysis was performed with R 3.6.1 (R Core Team, 2019). To ensure normal distribution of residuals, absorbance values were transformed with natural logarithms. One-way analysis of variance followed by Tukey's HSD test with $\alpha = 0.05$ were used to compare the OD_{450nm} values. Normality of residuals was checked with the Shapiro-Wilk test with significance level 0.05. The R package 'agricolae' (de Mendiburu, 2020) was used to carry out HSD test. All samples that were significantly different from the negative control were considered positive.

2.1.3 - Comparison between ELISA and qPCR for the detection of N. ditissima asymptomatic infection

In October 2017, apple trees cvs. 'Royal Gala' and 'Queen Cox' on M9 rootstock, grown in an orchard at NIAB EMR, were inoculated via leaf scars and pruning wound. Inoculum was prepared as described above (Section 2.1.2), macro- or micro-conidia were counted. Leaves were hand-removed from healthy one year shoots, then 5 µl of inoculum $1.2x10^5$ conidia/ml (c. 600 conidia/wound), were applied to each resulting leaf scar within 5 minutes of leaf removal. Shoots were covered in moistened plastic bags for 24 h to maintain high humidity conducive to infection (Dubin & English, 1974). As a negative control, shoots were inoculated with sterile distilled water. Symptomatic and asymptomatic shoots were collected at 21 and 28 weeks after the inoculation (*wai*) and stored at -80°C until processed.

Transverse sections of shoots, consisting of either symptomatic or asymptomatic plant material, were sampled from the inoculated leaf scars and the adjacent internodes at 10-15 mm from the leaf scar. Overall, 4 symptomatic and 24 asymptomatic sections were collected, and for each, bark and wood tissues were dissected and processed independently as subsamples.

Samples were freeze-dried over 48 hours, then added with metal beads, flash-frozen in liquid nitrogen and ground with a GenoGrinder2010 (Spex® Sample Prep, Stanmore, UK) at 1,500 rpm for 2 min. Subsequently, samples were re-suspended in sterile 0.01 M Phosphate Buffer Saline (PBS, Sigma-Aldrich, St. Louis, USA), prepared according to the manufacturer's instructions, in the proportion of 5 ml for each 1 g dry weight and shaken in the Geno/Grinder2010 at 1,500 rpm for 1 min. Re-suspended samples were centrifuged at 2,000 g for 10 min, then 50 µl supernatant were collected for use in the ELISA. The remaining sample was homogenised with the vortex and 100 to 150 µl sludge were collected for DNA extraction. Supernatant and sludge were stored at -80°C until processed.

Total DNA was extracted from re-suspended plant samples using the DNeasy Plant Mini Kit (QIAGEN, Manchester, UK) following the manufacturer's instruction. To serve as positive control in the subsequent end point PCR, as well as in the qPCR (see below), genomic DNA (gDNA) of *N. ditissima* strain Hg199, grown in a Petri dish on a cellophane membrane on PDA for 2-3 weeks, was extracted with the same kit. All DNA was quantified using a spectrophotometre (Nanodrop™ 1000, ThermoFisher Scientific, USA). Samples were run in an end point PCR with ITS1 and ITS4 primers (White *et al.*, 1990) to check whether DNA extraction was successful. PCR was performed in a PTC-220 DYAD™ PCR Engine (BIO-RAD, USA). All reactions were run using 4 ng of DNA template in a total volume of 12.5 µl. Each reaction contained 0.05 µl MolTaq (Molzym, Bremen, Germany), 1.25 µl MolTaq buffer

10x, 0.25 µl of $MgCl_2$ 100mM, 1.0 µl dNTPs 2.5mM, 1.25 µl of each primer 2µM and 2.0 µl of 2.0 ng/µl DNA template. PCR conditions were as follows: 94ºC for 5 min, followed by 30 cycles of 94ºC for 1 min, 53ºC for 1 min and 72ºC for 2 min, with a final extension of 72ºC for 7 min. PCR products were analysed on 1.5% agarose gel and samples were excluded from downstream analysis when an ITS1-4 band was not detected.

N. ditissima gDNA was detected by qPCR using the primer set Bt-fw135/re284 (Ghasemkhani *et al.*, 2016a). The qPCR was carried out in a CFX96 Touch™ Real Time PCR Detection System (Bio-Rad, USA). All reactions were carried out with 10 ng DNA template in a total volume of 20 µl, using 0.2 ml 96-well white PCR plates (ThermoFisher Scientific, USA). Each reaction contained 10 µl SensiFAST™ SYBR® No-ROX Kit (Bioline, UK), 0.6 µl of each primer 10 µM and 5.0 µl of 2.0 ng/µl DNA template. PCR conditions were as follows: 95ºC for 3 min, followed by 40 cycles of 95ºC for 5 sec and 60ºC for 5 sec. Melting curve analysis was used to check for non-specific amplification products. One technical replicate was run per sample and a *N. ditissima* gDNA standard curve was included with the following quantities of DNA: 0.1, 0.01, 0.001 and 0.00025 ng.

The limit of detection (LOD) of the qPCR assay, i.e. the lowest amount of genomic DNA standard which produced a detectable amplification product in 95% of replicates (Burns & Valdivia, 2008), was determined based on three repetitions of the standard curve with the following points: 0.1, 0.01, 0.001 and 0.00025 ng of *N. ditissima* gDNA. Samples were considered to contain *N. ditissima* in the qPCR test when the Cq of the sample was lower than the Cq of the standard corresponding to the LOD.

The sample supernatants collected for the ELISA were first diluted 5:1 with PBS, then incubated on the plate at 4ºC overnight. The ELISA was carried out as described above (Section 2.1.1), with some modifications to prevent non-specific interactions and reduce background noise. All washes were performed with PBSTwC (PBS added with 0.5% Tween20 and 0.1% casein). All reagents were diluted with PBSTwC. After samples were incubated overnight, the wells were washed and then blocked with 1% casein (Sigma Aldrich) for 30 min at 30ºC prior to incubation with the hybridoma supernatant. The anti-mouse antibody conjugated to biotin and the HRP conjugated to streptavidin were used at the concentration of 1:500 and 1:1000, respectively. Standard samples were prepared by mixing and homogenising known amounts of *N. ditissima* mycelium (grown in PDB over three weeks, then harvested, washed with sterile water, freeze dried, ground with metal beads in the Geno/Grinder2010 and re-suspended in PBS) and freeze-dried apple shoot shavings (collected from healthy apple trees cv. ‘Royal Gala’, kept in a disease-free polytunnel at NIAB EMR). Fungal and plant materials were mixed, freeze-dried, homogenised, centrifuged at

2,000 g for 10 min and supernatant was collected. Supernatant was stored at -20°C and diluted 5:1 in PBS prior to incubation on the ELISA plate at 4°C overnight. The assay was repeated twice and the standards were: 100, 50, 10, 5, 1, 0.5 and 0 mg dry fungal biomass per gram of dry plant material in the first assay, and 1, 0.5, 0.01 and 0 mg/g in the second assay.

Data analysis was performed with R 3.6.1 (R Core Team, 2019). The qPCR standard curve was estimated by linear regression of the quantitation cycle (Cq) values on the $\log_{10}$-transformed DNA standard amount. Amplification efficiency (E) was calculated with the formula *E = -1+10^(-1/slope)*, where "slope" is the slope of the standard curve. The ELISA standard curve was estimated by linear regression of the natural log-transformed OD_{450nm} on the natural log-transformed fungal biomass concentration. To quantify the magnitude of agreement between the two methods, the percentage of agreement as well as the Cohen's Kappa coefficient were calculated as described in Viera and Garrett (2005). The sample mass corresponding to the subsample volume of tested in the ELISA (100 µl) was 4 mg for all samples. The sample mass corresponding to the DNA mass assayed in the qPCR (10 ng) varied across samples, and was calculated with the formula:

$$mass_{qPCR}(mg) = \frac{assayed\ DNA\ (ng)}{eluted\ DNA\ (ng)} \times extracted\ subsample\ vol\ (ml) \times sample\ density\ (\frac{mg}{ml})$$

$$mass_{qPCR}(mg) = \frac{10\ ng}{eluted\ DNA\ (ng)} \times 0.125\ ml \times sample\ density\ (\frac{mg}{ml})$$

where the eluted DNA (ng) was depended on the DNA extraction efficiency and sample density (mg/ml) was recalculated for each sample after aliquoting 50 µl supernatant for ELISA testing.

2.2 - *Neonectria ditissima* internal colonisation of apple shoots inoculated via leaf scar and pruning wound

The goal of this set of experiments was to assess whether *N. ditissima* can colonise internal tissues of the apple shoot away from the infection point in the absence of symptoms on the bark surface, either before and after canker symptoms appear at the entry site. Two different wound types were inoculated and two different techniques (microscopic and DNA-based methods) were employed.

General approach. Three different experiments were carried out. In all experiments, *N. ditissima* was artificially inoculated on apple shoots, either via leaf scars or pruning wounds. Shoot sections were then collected at the entry site as well as at a set distance, both before and after visual symptoms occurred. Finally, the sections were assessed for presence of the pathogen. In all experiments, bark and wood tissues were dissected and assessed separately. All experiments were carried out at NIAB EMR, East Malling, UK.

Experiment 1: potted trees were inoculated via pruning wounds and the pathogen was tracked increasing distance from the cut surface using re-isolation on a selective medium.

Experiment 2: orchard trees were inoculated via leaf scars and the pathogen was detected at the inoculated node and at the adjacent internodes by means of a *N. ditissima*-specific qPCR assay (Ghasemkhani *et al.* 2016a).

Experiment 3: orchard trees were inoculated via pruning wounds and the pathogen was detected at the cut surface and at increasing distance from the entry point using the same method as in Experiment 2.

Inoculum preparation. Inoculum was prepared as described in Section 2.1.2.

Experiment 1. To track the spread of *N. ditissima* at increasing distance from the infection site over time, apple trees of the cultivars ‘Discovery’, ‘Queen Cox’ (on M26 rootstock), ‘Royal Gala’ and ‘Golden Delicious’ (on M9 rootstock), grown in 10 l pots, were inoculated via fresh pruning wounds with a conidial suspension of the pathogen. The inoculum dose was 10,000 macroconidia/pruning wound. Then, destructive sampling of inoculated twigs was carried out at 2, 4, 8 and 16 weeks after the inoculation (*wai*). Re-isolation of the pathogen was performed at 10, 30 and 70 mm. In total, 12 trees (3 per cultivar) were used in the experiment. Plants were arranged in a randomised block design: there were three blocks and each block included one tree per cultivar. On each tree, six lateral twigs were pruned, of which four were inoculated and two were mock-inoculated as a negative control. At each time point, one inoculated twig per tree (three inoculated twigs per cultivar) and one mock-inoculated twig

per cultivar were sampled. At least one mock-inoculated twig per block was collected at each time point.

Trees were grown in compost (Sinclair, Ellesmere Port, UK) amended with Met52 Granular Bioinsecticide (Fargro, Arundel, UK) and Osmocote® Exact Protect fertilizer (Israel Chemicals Ltd.) and were 3 years old at the beginning of the experiment. One month before the inoculation (March 2017), plants were transferred into a polytunnel, where they were kept for the whole duration of the experiment. In April 2017, lateral twigs (1 to 3 years old) were pruned by cutting 10 mm above a bud. Each cut surface was inoculated by applying two 10 µl drops of inoculum at the concentration of about $5x10^5$ macroconidia/ml to the cut surface, at the interface between cambium and sapwood. Microconidia were not counted for this experiment. Within 1 min of inoculation, the inoculum was completely absorbed by wood and bark tissues. As a negative control, two twigs on each tree were mock-inoculated with sterile distilled water on fresh pruning wounds. Pots were periodically weeded and watered, spray treatments were applied twice at the full field rate against powdery mildew with myclobutanil (Systhane ® 20EW, Dow AgroSciences Ltd, Cambridge, UK), two and three months after inoculation, and against woolly aphid with pirimicarb (Aphox ®, Syngenta UK Limited, Fulbourn, UK), two weeks before inoculation and three months after inoculation. Neither of the two pesticides affects *N. ditissima.* Temperature and humidity during the experiment were logged by an EL-USB-2 Data Logger (Lascar Electronic Ltd, Whiteparish, UK). On sampling dates, twigs were cut 10 cm below the inoculated pruning wound with sterilised secateurs. To prevent spread of diseases between trees, the secateur was sterilised with 70% iso-propyl alcohol (IPA) wipes (Basan, VWR).

Immediately after sampling, *N. ditissima* was isolated from inoculated branches as described below. On each branch, 5 mm thick transverse sections spanning from 5 to 10 mm, 35 to 40 mm and 65 to 70 mm from the pruning wound were excised. Bark and wood were dissected with a scalpel and a dissection needle and treated as subsamples of the same section. All tools were sterilised before processing each sample. To remove epiphytic microorganisms, plant material was surface sterilised using a modified technique based on method number 3 described in Schulz et al. (1993), and described in Section 2.1.2. Samples were then air dried in the laminar flow hood. Bark and wood samples were plated on PDA amended with 40 ppm fungicide iprodione (Rovral® WG, BASF plc, Cheadle, UK) and 20ppm rifamycin (Rifamycin SV sodium salt, Sigma-Aldrich) (McCracken *et al.*, 2003), to prevent the isolation of other common fungi and bacteria. Absence of epiphytic *N. ditissima* was checked with imprint Petri dishes obtained by gently pressing samples on PDA for 5 seconds before plating them on the selective medium. Both isolation and imprint Petri dishes were incubated in ambient room

conditions without parafilm for 2 weeks to allow for *N. ditissima* growth. Isolates with the morphology on the selective medium resembling *N. ditissima* were sub-cultured onto PDA and incubated at room temperature for three weeks, mycelium was then collected and stored at -20^{o}C for DNA extraction and subsequent ITS-based identification.

Experiment 2. Orchard apple trees cvs. 'Royal Gala' and 'Queen Cox' on M9 rootstock were artificially inoculated via fresh leaf scars with a conidial suspension. Plant material was sampled from symptomatic and asymptomatic nodes (leaf scars) as well as from neighbouring asymptomatic internodes (10 to 15 mm away from the inoculated leaf scar) and assessed by *N. ditissima*-specific qPCR.

To ensure that the timing of symptom expression allowed both symptomatic and asymptomatic plant material at the time of sampling, two doses of pathogen inoculum (approx. 600 and 6,000 conidia/leaf scar, thereafter referred to as "high dose" and "low dose", respectively) were used after Dubin and English (1974). Plant material was sampled at 13, 21 and 28 *wai*. Moreover, to increase the probability of detecting the pathogen, each shoot was inoculated at multiple (10) adjacent leaf scars, and plant material from inoculated leaf scars of the same status (i.e. asymptomatic or symptomatic) was pooled prior to analysis. The internode samples were similarly treated.

Trees were inoculated on three occasions: October 24th and 27th and on November 10th 2017. Trees were planted in the orchard in January 1995 and were 22 years old at the beginning of the experiment. In total, 18 'Royal Gala' and 18 'Queen Cox' trees were used in the three inoculations; these trees were arranged in a randomised block design over six blocks, corresponding to six orchard plots, each block including three plants per cultivar. On each occasion, two blocks were inoculated.

Before leaf fall, eight healthy current season shoots were selected on each tree; six shoots (three shoots per dose) were inoculated with the pathogen, and two shoots were mock-inoculated with sterile distilled water. Thus, in each experiment, there were 18 shoots per dose, and 12 shoots for mock inoculation per cultivar. On each shoot, 10 consecutive leaves were hand-removed starting from the tip of the shoot, and a 5 µl droplet of inoculum ($1.2x10^6$ or $1.2x10^5$ (macro- plus micro-) conidia/ml for the high and the low dose, respectively) was immediately applied to each leaf scar. Macro- or micro-conidia were counted for this experiment, and the proportion of macroconidia in the inoculum was recorded for each of the three experiments. Within five minutes, the inoculum droplets were completely absorbed and shoots were covered in moistened plastic bags for 24 h to maintain high moisture conditions conducive to infection (Dubin & English, 1974). As a negative control, shoots were mock-inoculated with 5 µl sterile distilled water.

The orchard was managed according to the standard agronomic practices. Notably, the active ingredients with fungicidal activity applied from October 2017 to August 2018 included captan, pyrimethanil, fluxapyroxad (applied twice), difenoconazole, dithianon, potassium phosphonate, meptyldinocap, cyflufenamid and bupirimate (applied once). Of these, captan and dithianon have known effects against *N. ditissima* (Webster *et al.*, 2001; Saville & Olivieri, 2019). Temperature and moisture were recorded by the local meteorological station at NIAB EMR. Every two weeks, every inoculated (including mock-inoculated) leaf scar was monitored for canker symptoms. At 13, 21 and 28 *wai*, one shoot per dose was sampled from each tree: thus, a total of 36 shoots per dose within cultivar were sampled at each sampling time point. Shoots were sampled by cutting 6 cm below the inoculated leaf scar at the base of the shoot using sterilised secateurs and then conserved at -20°C until further processed.

Shoot transverse sections were collected from inoculated nodes and adjacent internodes, bark and wood tissues from the same section were treated as subsamples, and plant material was pooled according to the anatomical part (node vs internode) and the tissue type (bark and wood), as detailed below. Shoots were washed by gently brushing in 0.5% Tween20 for 60 s, then soaked in 5% bleach (0.25% sodium hypochlorite) for 15 s to remove fungal epiphytic DNA (Prince & Andrus, 1992), rinsed twice in sterile distilled water for 60 s, air dried in the laminar flow cabinet. Bark and wood of nodes and internodes were then dissected. Segments of bark, 5-10 mm long, centred on the leaf scar and spanning over half the girth of the shoot, were removed with a scalpel blade. Once the bark was peeled off, shavings were taken of the underlying woody tissues. A similar procedure was repeated on the internodes, at a distance comprised between 10 and 15 mm from the inoculated nodes; however, 5-10 mm bark rings encircling the whole transverse section of the shoot were sampled, followed by wood shavings from the underlying tissues.

Overall, eight different types of sample pools were collected.

On each shoot, tissues collected from asymptomatic nodes and adjacent internodes were pooled into four asymptomatic groups (node bark, node wood, internode bark and internode wood). Similarly, if symptoms were present, tissues collected from symptomatic nodes and the adjacent asymptomatic internodes were pooled into four ‘cankered sample pools’. Symptomatic internodes were not sampled, thus all internode samples were asymptomatic. Clean scalpel blades were used to process each sample pool. Additionally, to assess *N. ditissima* spread in plant tissues at increasing distance from the entry site over time, bark and wood were dissected from a 10 mm long transverse section located 15 mm below the most proximal inoculated leaf scar. Clean scalpel blades were used to process each sample. All samples were stored at -80°C until homogenised for DNA extraction.

Experiment 3. Fresh pruning wounds of orchard apple trees of cvs. 'Royal Gala' and 'Queen Cox' on M9 rootstock were artificially inoculated with a conidial suspension of *N. ditissima*, then plant material was sampled from symptomatic and asymptomatic shoots and the pathogen's asymptomatic colonisation was assessed at increasing distance from either the entry site (asymptomatic shoots) or the leading edge of canker (symptomatic shoots) by qPCR.

The inoculum dose was 300 conidia/pruning wound. Trees were grown in the same orchard as described in Experiment 2. The orchard was managed according to the standard agronomic practices as described in Experiment 2.

Overall, 15 'Royal Gala' and 15 'Queen Cox' trees arranged in a randomised block design were inoculated. There were five blocks, each one corresponding to an orchard plot with three trees per cultivar. On each tree, six shoots per dose were inoculated with the pathogen, and two shoots per tree were mock-inoculated with sterile distilled water as negative controls. Therefore, for each cultivar 90 shoots were inoculated, and 30 shoots were mock-inoculated.

On March 14th, 2018, before bud break, eight healthy current season shoots were selected on each tree and pruned by performing a slanting cut 10 mm above a bud using sterilised secateurs. Then, two 10 µl droplets of *N. ditissima* inoculum at a concentration of $1.4x10^4$ (micro- and macro-) conidia/ml were immediately applied to the cut surface at the interface between the bark and wood layers. Macro- or micro-conidia were counted for this experiment, and the proportion of macroconidia in the inoculum was recorded. Within one minute, the inoculum droplets were completely absorbed. Two pruned shoots were mock-inoculated with sterile distilled water. Temperature and moisture were recorded by the local meteorological station at NIAB EMR. Every two weeks, each inoculated (including mock-inoculated) pruning cut was monitored for canker symptoms. At 12 *wai* and 24 *wai*, one shoot per tree was sampled by cutting at 20 cm from the pruning wound surface using sterile secateurs and conserved at -20^{o}C until processed as detailed below. Shoots were processed to remove epiphytic DNA as described above (Experiment 2), air dried in sterile conditions and then dissected.

Bark and wood were collected from transverse sections of the shoots described below. On asymptomatic shoots, 5 mm long rings of bark girdling the whole transverse section of the shoot were dissected at increasing distances from the cut surface, namely at 10 mm (spanning from 7 to 12 mm), 20 mm (17 to 22 mm), 30 mm (27 to 32 mm) and 40 mm (37 to 42 mm). Once the bark was removed, shavings were taken of the underlying wood tissues. If canker symptoms had developed at the inoculated cut, 5 mm long bark rings were dissected at the leading edge of the canker lesion (from -2 to +3 mm across the leading edge) and at

increasing distances from the canker, namely at 10 mm (7 to 12 mm), 20 mm (17 to 22 mm) and 30 mm (27 to 32 mm); once the bark was removed, shavings were taken of the underlying wood tissues. Clean scalpel blades were used to process each sample pool. All samples were stored at -80°C until prepared for DNA extraction.

Experiment 1: DNA extraction and identification of N. ditissima *axenic cultures by end-point PCR.* In Experiment 1, genomic DNA was extracted from axenic fungal cultures following a method for rapid fungal DNA extraction described in Cenis (1992). Briefly, frozen fungal mycelium was mixed with 50 µl of lysis buffer and crushed using a micro-pestle attached to a mini-homogeniser, then supplemented with a further 150 µl of lysis buffer and vortexed; 150 µl of sodium acetate 3M was added, the suspension was stored at -20°C for 10 min and then centrifuged at 14,000 rpm for 10 min; supernatant was mixed with an equal volume of isopropanol and DNA was precipitated by centrifuging at 14,000 rpm for 10 min. The pellet was washed once with chilled ethanol, air dried and suspended in TE buffer. To confirm the isolates were *N. ditissima*, DNA was run in a PCR with a *N. ditissima*-specific primer set (Ghasemkhani et al. 2016). PCR was carried out in a T100™ Thermal Cycler (Bio-Rad, California, USA) with 20 ng of template DNA in a total volume of 25 µl. Each reaction contained 0.05 µl MolTaq (Molzym, Bremen, Germany), 2.5 µl MolTaq buffer 10x, 2.0 µl dNTPs 2.5mM, 0.5 µl $MgCl_2$ 100 mM and 2.5 µl of each primer 10µM. PCR conditions were as follows: 95°C for 5 min, followed by 35 cycles at 95°C for 15 sec, 60°C for 30 sec and 72°C for 15 sec, with a final extension at 72°C for 5 min. PCR products were analysed on a 1.5% agarose gel.

Experiment 2 and 3: Detection of N. ditissima *in plant samples by qPCR.* Samples were prepared, DNA was extracted and fungal ITS was amplified as described in Sections 2.1.3. Samples were considered to contain *N. ditissima* in the qPCR test when: (1) the Cq of the sample was lower than the Cq of the standard corresponding to 0.001 ng of *N. ditissima* genomic DNA (limit of detection, LOD, of the assay in this present study, as defined by Burns & Valdivia, 2008) in the same qPCR run, and (2) the results were consistent across two technical replicates of the qPCR assay.

2.3 - Meta-barcoding study of fungal and bacterial endophytes across apple cultivars differing in *N. ditissima* susceptibility

This experiment was carried out to assess whether specific endophytes differ across European apple canker resistant and susceptible cultivars, and whether these differences correlate with the resistance/susceptibility level of the cultivar. Therefore, the differences between endophytic communities associated with canker-resistant and canker-susceptible

apple cultivars were determined, then the contribution of scion genotype, rootstock genotype and environment on their composition were dissected.

Leaf scar tissues were sampled in autumn, immediately before leaf fall occurred, to target the microorganisms that might co-localise with *N. ditissima* during the early stage of the infection. Total DNA was extracted from plant samples, bacterial and fungal DNA was amplified by means of PCR and a meta/barcoding was utilised to characterise endophyte communities.

Experimental design and sampling. Three canker resistant cultivars ('Grenadier', 'Golden Delicious' and 'Robusta 5') and five susceptible cultivars ('Gala', 'Braeburn', 'Jazz', 'Kanzi', 'Rubens'), grafted on M116 (reduced canker susceptibility) and M9 337 (canker susceptible) rootstocks and planted out at two different sites in Kent, UK, were used in this study. Therefore, there were 32 treatments: 8 scions x 2 rootstocks x 2 sites. At both planting sites, trees were arranged in a randomised block design. Trees were grafted at the Frank P Matthews nursery in Worcestershire, UK (Berrington Court, Tenbury Wells) in winter 2016-17, kept in the same greenhouse at the nursery until autumn 2018, and finally transplanted in the orchards in Maidstone (Friday St Farm, latitude 51°12'51.6"N, longitude 0°36'41.9"E) and Canterbury (Perry Farm, Wingham, latitude 51°17'01.0"N, longitude 1°14'06.6"E) during winter in early 2018.

Orchards were subjected to fungicide, pesticide and herbicide treatments and irrigation according to the standard commercial management practices. Plant material was sampled in October 2018, before leaf fall had occurred. A total 15 leaf scars were collected from 5 shoots of the same tree (including the main stem) and pooled before proceeding to DNA extraction. Four biological replicates, namely one tree from each of selected four blocks, were sampled for each treatment.

Five 15-20 cm long segments were collected from different shoots of each tree using secateurs, and stored at -20^{o}C until further processed. On each shoot, three leaf scars were dissected as described below. Using a scalpel blade, the petiole and bud were removed, then a transverse cut was performed through the bark layers below a leaf scar and a longitudinal cut was performed between the leaf scar and the stem, from the upper surface to the base of the leaf scar. As a result, a 4 x 1 mm fragment of the leaf scar was excised from the shoot. Samples were stored at -80^{o}C until DNA extraction.

Figure 2.3.1 – Position of experimental apple trees within the orchards in (**a**) Maidstone and (**b**) Canterbury. Bar: 50 m. Source of images: Google Maps.

DNA extraction. Samples were freeze-dried over 24 hours, flash-frozen in liquid nitrogen and ground in a Geno/Grinder2010 (SPEX SamplePrep, Stanmore, UK) at 1,500 rpm for 90 s. Completely powdered samples were then re-suspended in sterile PBS (Phosphate Buffer Saline, Sigma-Aldrich, St. Louis, USA) 0.01 M, prepared according to the manufacturer's instructions, in the proportion of 5 ml : 1 g dry weight, and shaken in the Geno/Grinder2010

at 1,500 rpm for 1 min to ensure thorough homogenisation, then stored at -80ºC until DNA extraction was performed. DNA was extracted from 100-120 µl aliquots of re-suspended samples using the DNeasy Plant Mini Kit (QIAGEN, Manchester, UK) following the manufacturer's instructions. DNA quality and quantity was checked spectrophotometrically (Nanodrop™ 1000, ThermoFisher Scientific, USA).

Library preparation. The amplicon library was prepared as described in Tilston et al. (2018). The primers EkITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') (Gardes & Bruns, 1993) and Ek28R (5'-AT ATG CTT AAG TTC AGC GGG-3') (corresponding to 3126T in Sequerra et al. (1997)) were used to amplify the non-overlapping variable internal transcribed spacer (ITS) regions of ITS1 and ITS2 in the fungal genome. The primers Bakt_341F (5'-CCT ACG GGN GGC WGC AG-3') and Bakt_805R (5'-GGA CTA CHV GGG TAT CTA ATC C-3') (Herlemann *et al.*, 2011) were used to amplify the V4 variable region of the 16S rDNA. Both primer sets were modified at the 5' end with adaptors: TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG (forward primer adaptor) and GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA (reverse primer adaptor). All ITS and 16S PCRs were run in triplicate per each sample, and technical replicates were then pooled. PCRs were carried out in a T100™ Thermal Cycler (Bio-Rad, California, USA) with 4 ng of template DNA in a total volume of 13 µl. All reactions contained 0.05 µl MolTaq (Molzym, Bremen, Germany), 1.25 µl MolTaq buffer 10x, 1.0 µl dNTPs 2.5mM, 0.25 µl $MgCl_2$ 100 mM and 1.25 µl of each primer 2µM. ITS PCR conditions were as follows: 95ºC for 5 min, followed by 30 cycles at 94ºC for 30 sec, 52ºC for 45 sec and 72ºC for 60 sec, with a final extension at 72ºC for 7 min. 16S PCR conditions were as above, but the annealing temperature was 55ºC and the number of cycles was 25. PCR products were viewed on 1.5% agarose before proceeding to clean-up and indexing.

PCR clean-up, sample indexing and sequencing. Equal volumes of fungal and bacterial amplicons were pooled according to individual plant samples and then PCR clean-up was performed with the Agencourt AMPure XP kit (Beckman Coulter™, ThermoFisher Scientific) following the manufacturer's instructions. Sample indexing was carried out by ligating Illumina compatible adapters using the Nextera® XT Index Kit v2 (Illumina, USA) and the KAPA HiFi HotStart ReadyMix kit (KAPA Biosystems, Roche, USA). All reactions were carried out in a 50 µl volume with 25 µl KAPA reaction mix 2x, 5 µl of both forward and reverse index primers and 5 µl of cleaned-up DNA sample. Index PCR clean-up was performed with the Agencourt AMPure XP kit, then quantity and quality of the cleaned-up DNA were checked using a Nanodrop™ 1000 spectrophotometer (ThermoFisher Scientific) and DNA was diluted to 10 ng/µl. Subsequently, DNA concentration was checked with a Qbit2.0 fluorometer (Life Technologies, ThermoFisher Scientific) and samples were pooled into a 4 nM library. The

library was denatured with 0.1M NaOH and diluted to 12 pM according to the manufacturer's protocol (16S Metagenomic Sequencing Library Preparation protocol, Illumina (2019)), then it was combined with a denatured PhiX library (PhiX Control v3, Illumina) at an equimolar concentration at a rate of 20% v/v to increase heterogeneity of the samples. Sequencing was carried out on an Illumina MiSeq instrument using the 300 bp paired-end protocol (16 indexing cycles and 301 sequencing cycles, total 618 cycle run) and the MiSeq v3 reagent cartridge and kit (Illumina).

Bioinformatic analysis

Sequence processing was performed according to the methods and pipelines described by Deakin et al. (2018). FASTQ reads were demultiplexed into bacterial (16S) and fungal (ITS) datasets based on their primer sequences. Reads with any ambiguous positions in the primer region or non-matching forward and reverse primers were discarded. Demultiplexed sequences were then filtered according to length and quality criteria to generate the operational taxonomic units (OTUs) and the representative DNA sequence for each OTU. Finally, all (unfiltered) reads were aligned with the OTU sequences to generate an OTU frequency table. All operational taxonomic unit (OTU) processing was carried out using the UPARSE v11.0 OTU clustering pipeline (Edgar, 2013), unless specified otherwise.

Generation of OTUs and assignment of taxonomic rank. Paired-end 16s reads had an overlap of c. 160 nucleotides (NT) and were merged with a minimum merged sequence length of 300 NT and 5% maximum difference in overlap. Merged reads shorter that 300 NT or containing adapters contamination were excluded from further analysis, and the remaining merged reads were trimmed by 17 bp at the left end and 21 bp at the right end, to remove forward and reverse primers respectively. Merged sequences were then filtered for quality with maximum expected error threshold of 0.5 per sequence (Edgar & Flyvbjerg, 2015) before proceeding to OTU clustering.

The expected distance between forward and reverse fungal ITS primer was greater than the maximum MiSeq merged read length (600 bp), hence reads could not be merged. Only forward reads (ITS1) were used in the analysis. Sequences were truncated at 250 NT, all reads shorter than 250 NT or containing adapters contaminations were discarded, then the forward primer sequences were removed from the remaining reads. Sequences were quality filtered with maximum expected error threshold of 1 per sequence before proceeding to OTU clustering.

Filtered 16S and ITS reads were dereplicated to obtain unique sequences, then unique sequences with fewer than 2 occurrences overall (i.e. singletons) were discarded for the

purpose of generating OTUs. The remaining sequences were clustered into OTUs at the level of 97% similarity and a representative sequence was generated for each OTU. Chimeras were identified and removed by the clustering algorithm. Finally, all unfiltered sequences were aligned with the OTU representative sequences at the level of 97% similarity to generate ITS and 16S OTU frequency tables.

Taxonomic predictions were made with the SYNTAX algorithm (Edgar, 2016) by aligning the OTU representative sequences with the reference databases and "RPD training set v16" for 16S (Cole *et al.*, 2014) and "UNITE v8.0" for ITS (Nilsson *et al.*, 2018; UNITE Community, 2019).

Statistical analyses

General approach. All statistical analyses were carried out with R 3.6.1 (R Core Team, 2019). For each sample, the cumulative number of fungal reads per OTU was used to check the depth of sequencing. To minimise the effects of sampling, including sequencing depth and OTU composition, OTU count data were normalised for library size using the median-of-ratio method implemented in DESeq2 (Love *et al.*, 2014). Before Principal Component Analysis (PCA), normalised counts were further transformed with the DESeq2 variance stabilising transformation (VST) method to make data approximately homoskedastic (Anders & Huber, 2010). After normalisation and transformation, chloroplast and mitochondria V4 rDNA (identified by BLASTn, Zhang *et al.*, 2000) were excluded from all downstream analyses. All analyses were performed separately for 16S and ITS data.

Exploratory data analysis. To dissect the main effects of experimental factors on the microbial community structure, PCA was first carried out on the VST transformed data. Analysis of variance (ANOVA) was performed on the first four principle components (PCs) to assess the contribution of experimental factors to the proportion of variance in the PC scores. The model included orchard (Maidstone or Canterbury), spatial location of the trees within each orchard (block nested within orchard), susceptibility level of the cultivar (susceptible or resistant), scion genotype within susceptibility level ('Grenadier', 'Golden Delicious' and 'Robusta 5' within Resistant and 'Gala', 'Braeburn', 'Jazz', 'Kanzi' and 'Rubens' within Susceptible cultivars), rootstock genotype (M116 or M9 337) and the scion:rootstock interaction, respectively.

Diversity indices. Normalised OTU counts were rounded prior to calculation of alpha (α) diversity indices to allow computation of indices based on species singletons and doubletons. To estimate α diversity, species richness indices (observed species and Chao1) and diversity indices (Shannon and Simpson) were calculated with the R package phyloseq v1.30.0

(McMurdie & Holmes, 2013). Alpha diversity indices were analysed by permutational ANOVA, as implemented in the R package lmPerm v2.1.0 (Wheeler & Torchiano, 2016), to assess the effects of orchard, block within orchard, susceptibility level, scion within susceptibility, rootstock and the scion : rootstock interaction, respectively. Statistical significance was calculated by permutation test (5,000 permutations) based on sequential sum of squares. Beta (β) (Bray-Curtis) diversity was calculated from normalised OTU counts. To investigate the global structure of bacterial and fungal communities, non-metric multidimensional scaling (NMDS) of the Bray-Curtis indices was carried out with the R package Vegan 2.5-6 (Oksanen *et al.*, 2019). Permutational multivariate analysis of variance (PERMANOVA), as implemented in Vegan 2.5-6 in the function "adonis", was carried out to assess the effects of orchard, location within the orchard (block), susceptibility, scion within susceptibility, rootstock and scion:rootstock interaction on the Bray-Curtis indices. Statistical significance was calculated by permutation test with 9,999 permutations based on sequential sum of squares.

Differential OTU abundance. Comparison of OTU relative abundance in relation to the cultivar susceptibility level was performed in R with DESeq2. As shown by McMurdie and Holmes (2014), this package is more suitable for differential analysis of microbiome data than other methods based on data normalisation with proportion or rarefaction. DESeq2 analyses raw OTU counts and compares species relative abundances between groups using generalised linear modelling, assuming a negative binomial distribution for residuals (Anders & Huber, 2010; Love *et al.*, 2014). To account for library size (sequencing depth and OTU composition of samples), count data were normalised with the median-of-ratios method. Prior to differential abundance analysis, DESeq2 performs independent filtering of OTUs according to several criteria. These include the overall abundance level and the variance in abundance across samples. DESeq2 uses a Wald test to test for significance of differentially abundant OTUs with the Benjamini-Hochberg (BH) method (Benjamini & Hochberg, 1995) used to adjust the p-value. The differences between two treatments are expressed as Log_2 of the fold-change (Log_2FC) of normalised counts. Before running DESeq2, the block factor was recoded to include the orchard factor, resulting in a block factor with 8 levels that accounted for all differences between and within orchards. The fitted models were: (1) scion, recoded block and rootstock for bacteria and (2) scion, recoded block, rootstock and scion : rootstock interaction for fungi. OTUs that were differentially abundant in resistant genotypes compared to the susceptible genotypes were identified by contrasting resistant scion cultivars ('Robusta 5', 'Golden Delicious' and 'Grenadier') and susceptible ones ('Rubens', 'Gala', 'Kanzi', 'Jazz' and 'Braeburn'). Statistical significance was determined at the 5% level (BH adjusted).

Taxonomic predictions for differentially abundant OTUs were filtered with a SYNTAX confidence threshold of 0.7. BLASTn 2.10.1+ (Zhang *et al.*, 2000) was used to check predictions with confidence value lower than 0.7 against NCBI's non-redundant nucleotide collection. BLASTn hits were accepted when they proved unambiguous based on the query coverage (100%), percent identity and e-value. Taxonomic predictions were accepted up to the taxon level on which there was agreement between BLASTn and SYNTAX. Putative lifestyle and ecological functions were obtained from available peer-reviewed literature.

3. Results

3.1 - Comparison of high-throughput methods for *N. ditissima* detection

3.1.1 - Monoclonal antibody validation and ELISA setup

The supernatant of hybridoma cell line 1B10 showed high affinity for *N. ditissima* and low cross-reactivity with the other antigens (Figure 3.1.1). The hybridoma cell line 1B10 showed high specificity for *N. ditissima* antigens, with readings for non-target antigens always below 0.2 OD_{450nm}.

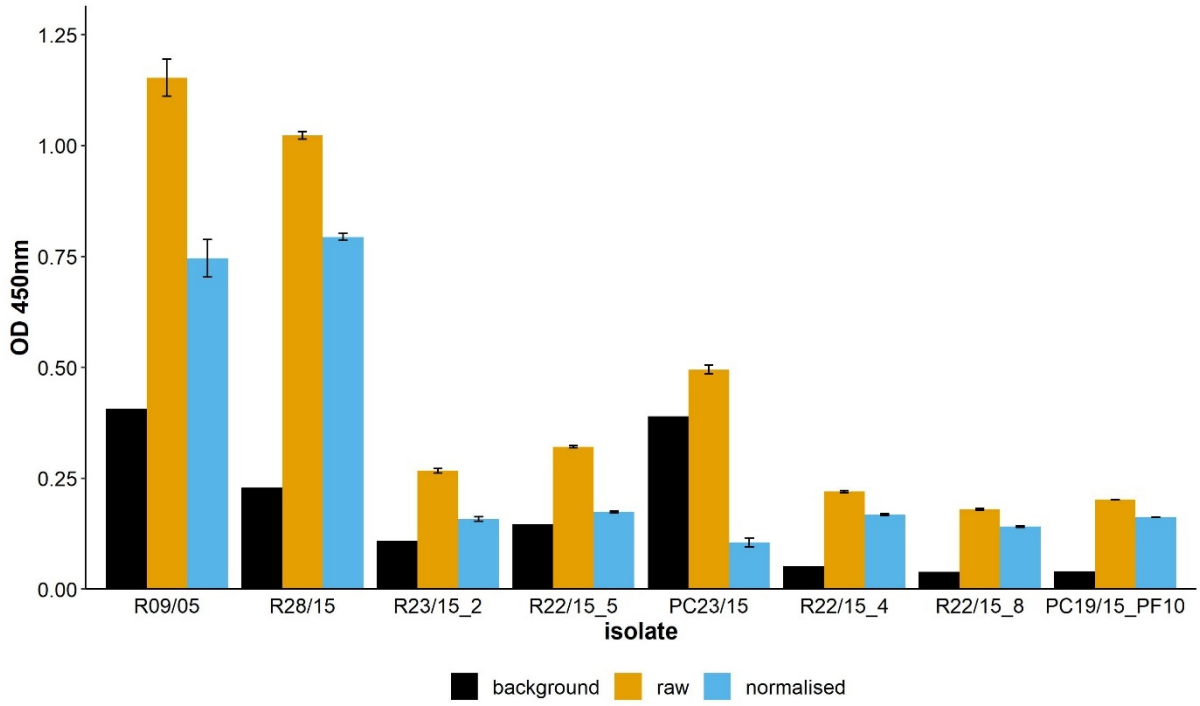

Figure 3.1.1 - ELISA with the hybridoma supernatant 1B10 tested against fungal antigens (10 µg/ml). R09/05 and R28/15: *N. ditissima*; R23/15_2: *Fusarium lateritium*; R22/15_5: *Monilinia laxa*; PC23/15: *Botryosphaeria obtusa*; R22/15_4: *Nectria cinnabarina*; R22/15_8: *Colletotrichum acutatum*; PC19/15_PF10: *Phomopsis* sp./*Diaporthe* sp.. Background, raw (non-normalised) and normalised OD_{450nm} is shown for each sample. Error bars represent standard error based on two replicates.

3.1.2 - ELISA validation with symptomatic plant samples and comparison with culture-dependent method

As determined by one-way ANOVA, there was a statistically significant effect of sample distance from canker on absorbance (OD_{450nm}) for naturally infected branches A and B ($F(7,18) = 32.57$, $p < 0.001$) and for artificially inoculated branch C ($F(5,14) = 68.96$, $p < 0.001$). Bark and wood subsamples from branch A, B and C were pooled by sample (i.e.

transverse section) prior to statistical analysis, and absorbance data were transformed with the natural logarithm prior to analysis. Non-transformed OD_{450nm} and Tukey's HSD test results are shown in Figure 3.1.1 (branches A and B) and Figure 3.1.2 (shoot C). *N. ditissima* antigen was detected at the leading edge of lesion (0 mm) on branch A and branch B. It was not possible to assess the bark at the canker edge on branch B, as the OD_{450nm} of the background was higher than the OD_{450nm} of the sample replicates, resulting in a negative normalised value. Therefore, the bark subsample was removed and only the wood subsample was analysed. Using the culture dependent method on branch A, *N. ditissima* was isolated from wood and bark at the leading edge of the lesion (0 mm), whereas on branch B the fungus was not successfully isolated at any distance from the canker. On shoot C, *N. ditissima* antigen was detected at the leading edge of lesion (0 mm) and at 10 mm from the canker. However, it was not possible to isolate it at any distance from the canker on shoot C.

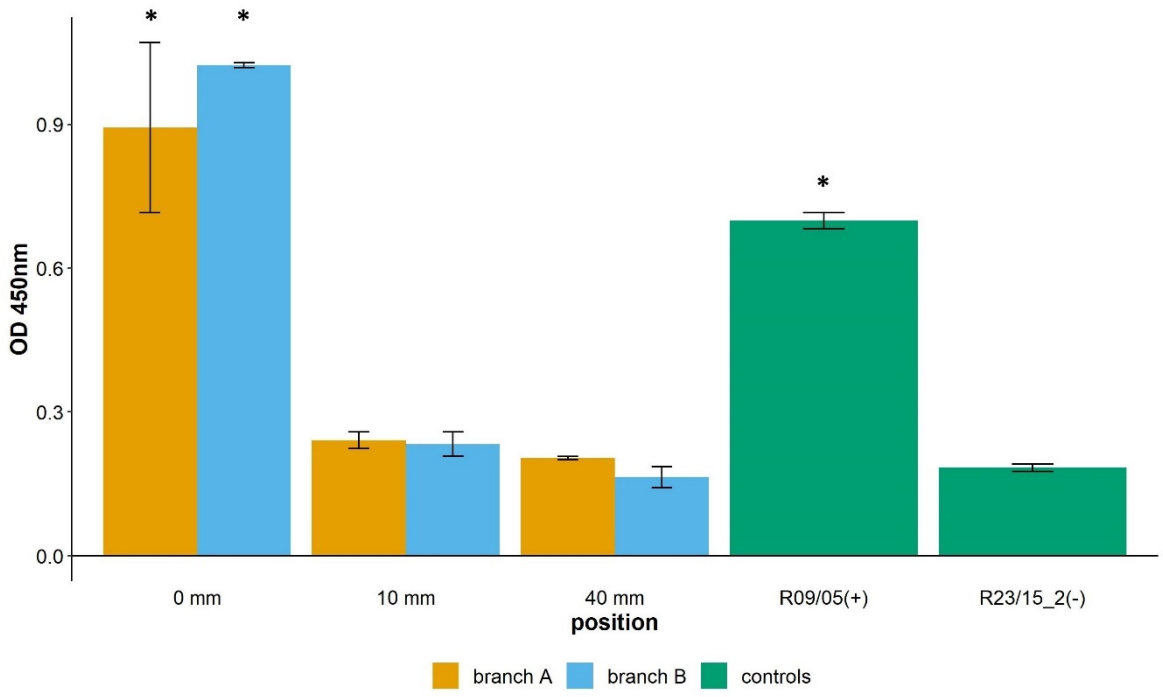

Figure 3.1.1 - Hybridoma supernatant 1B10 tested against shavings from sections collected at different distance from canker (0, 10 and 40 mm) on naturally infected branches A (yellow bars) and B (blue bars); bark and wood subsamples were assessed separately in the ELISA, but pooled by section prior to analysis. Non-transformed absorbance is reported. *N. ditissima* R09/05 (positive control) and *F. lateritium* R23/15_2 (negative control) antigens were used at the concentration of 10 µg/ml. Data represent the average of four technical replicates, except for branch B at 0 mm and controls, where two replicates were used instead. Error bars: standard error of the mean. Starred samples (*) are statistically different

from the negative control following Tukey's HSD test ($p<0.05$) on natural log-transformed absorbance.

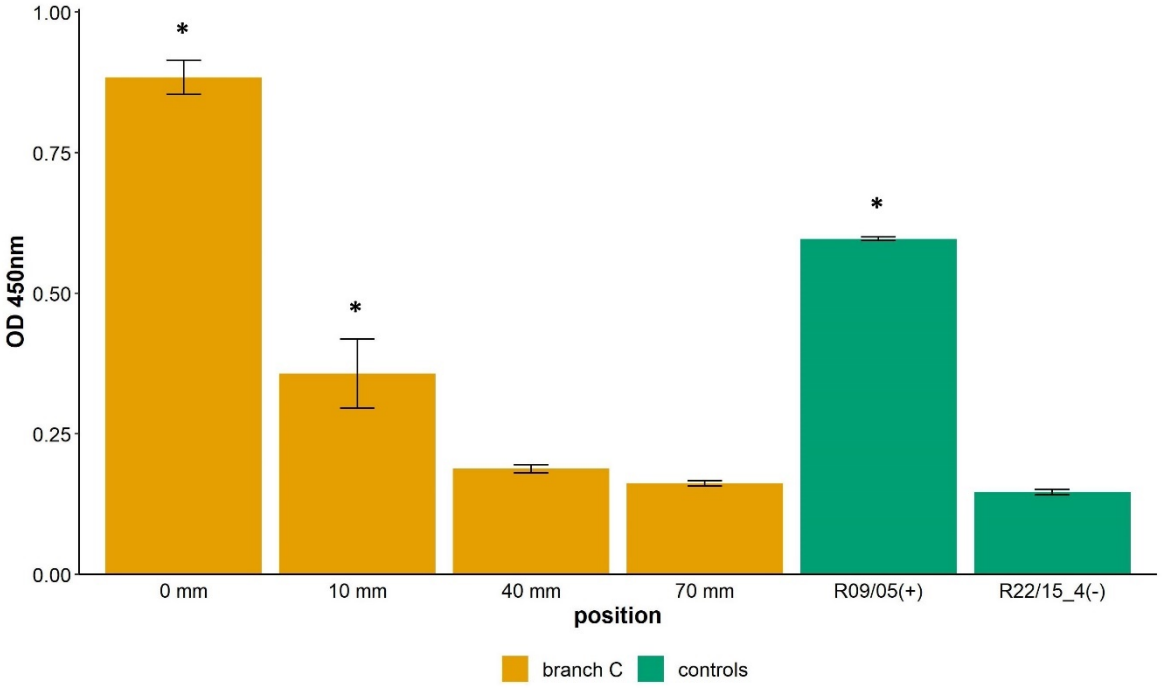

Figure 3.1.2 - Hybridoma supernatant 1B10 tested against shavings from sections collected at different distance from canker (0, 10, 40 and 70 mm) on artificially inoculated branch C; bark and wood subsamples were assessed separately in the ELISA, but pooled by section prior to analysis. Non-transformed absorbance is reported. *N. ditissima* R09/05 (positive control) and *N. cinnabarina* R22/15_4 (negative control) antigens were used at the concentration of 10 µg/ml. Data represent the average of four technical replicates, except for controls for which two replicates were used instead. Error bars: standard error of the mean. Starred samples (*) are statistically different from the negative control following Tukey's HSD test ($p<0.05$) on natural log-transformed absorbance.

3.1.3 - Comparison between ELISA and qPCR for the detection of N. ditissima asymptomatic infection

Germination of *N. ditissima* conidia after 24 h was 97%. The LOD of the qPCR assay, based on three repetitions of the DNA standard series, was 0.001 ng gDNA. This corresponded to about 20 *N. ditissima* genomes and generated an average Cq of between 33.2 and 34.6, depending on amplification efficiency. The R^2 ranged between 0.98 and 0.99 and amplification efficiency ranged between 90% and 97%. The ELISA standard curves are

reported in Figure 3.1.3 (first assay) and Figure 3.1.4 (second assay). The R^2 were 0.93 and 0.76, respectively.

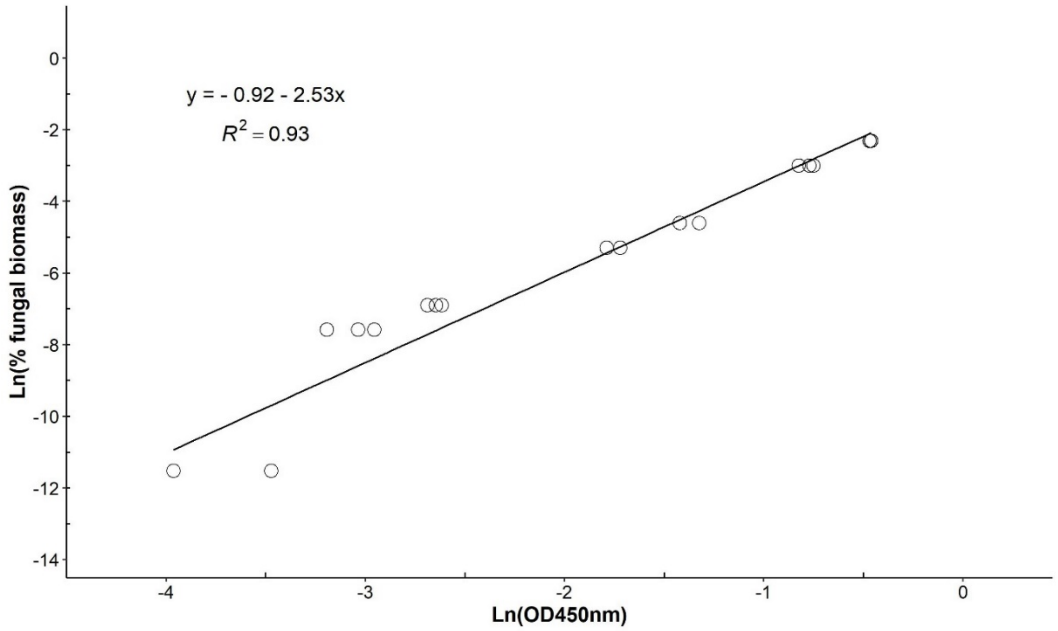

Figure 3.1.3 - Standard curve for the first ELISA. Regression line equation and R^2 are reported. Ln = natural logarithm.

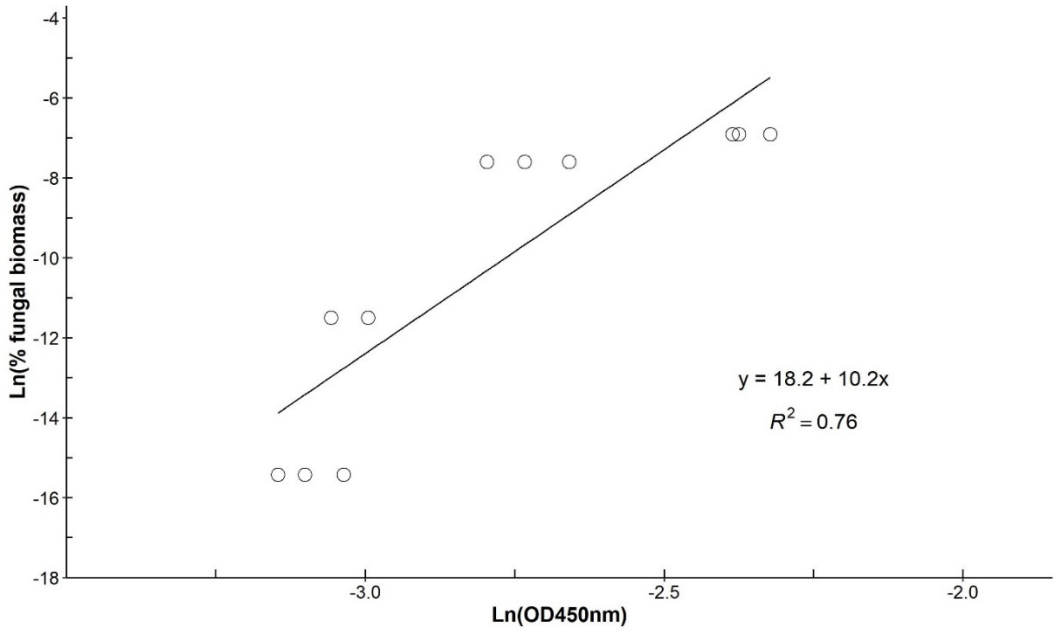

Figure 3.1.4 - Standard curve for the second ELISA. Regression line equation and R^2 are reported. Ln = natural logarithm.

Samples positive or negative for both the ELISA and the qPCR are reported in Table 3.1.1. Observed agreement between the two assays was 80.4% and Cohen's Kappa coefficient was 0.58, which corresponded to a "moderate" agreement according to the scale reported by Landis and Koch (1977).

Table 3.1.1 - Plant samples positive to ELISA and qPCR.

	ELISA +	**ELISA -**	**total**
qPCR +	14	1	15
qPCR -	10	31	41
total	24	32	56

All symptomatic samples were positive in both ELISA and the qPCR test. Conversely, the 11 samples for which the two assays disagreed were asymptomatic; one of these was positive for the qPCR and negative for the ELISA whilst the opposite result was found for the other 10. Seven of the ten samples which were negative in the qPCR and positive in the ELISA had $Ln(OD_{540nm})$ lower than -2.50 and fell in the lower end of the standard curve. Overall, a smaller fraction of the sample was assayed in the qPCR, ranging from 0.04 to 2.44 mg (average 0.58 mg). This corresponded to a ratio between the sample masses tested in the qPCR and the ELISA in the range of 0.01 to 0.61 (average of 0.15).

3.2 - *Neonectria ditissima* internal colonisation of apple shoots inoculated via leaf scar and pruning wound

Experiment 1. The germination test showed that 97% of macroconidia had germinated after 6 hours. Growth of *N. ditissima* was not observed on imprint plates. The results of *N. ditissima* re-isolation from the apple shoots artificially inoculated via pruning cut at 2, 4 and 8 *wai* are summarised in Table 3.2.1.

N. ditissima was not isolated from any of the mock-inoculated shoots. Inoculated shoots sampled 2 *wai* were all asymptomatic, while 4 and 7 out of the 12 samples collected 4 and 8 *wai*, respectively, developed canker symptoms; all these cankers extended up to the first transverse section excised for re-isolation (10 mm), but they never reached the second section (40 mm). At every time point, *N. ditissima* was isolated from the wood at 10 mm from the inoculated pruning cut, isolation was successful in 22 out of 25 asymptomatic shoots and in all 11 cankered shoots at the cut surface. These correspond to 12, 10 and 11 positive samples out of the 12 samples at 2, 4 and 8 *wai*, respectively. *N. ditissima* was not isolated from three asymptomatic shoots (one each from 'Golden Delicious' and 'Queen Cox' 4 *wai*, and one from 'Queen Cox' 8 *wai*). At all time points, *N. ditissima* was not re-isolated at 40 or

70 mm from the pruning cut. Re-isolation of *N. ditissima* at 16 *wai* was hindered by yeast contaminants in the selective medium. Despite this, the pathogen could be isolated at 10 mm from the pruning cut surface from the wood of three asymptomatic samples (one 'Royal Gala', one 'Golden Delicious' and one 'Queen Cox') and from the wood and bark of one symptomatic 'Royal Gala' sample (data not shown). Re-isolation of the pathogen was not possible from all other asymptomatic samples (1) and symptomatic samples (6) collected at 16 *wai*, at any distance from the pruning cut surface.

Table 3.2.1 - *N. ditissima* internal colonisation of potted trees inoculated via pruning cuts. Number of samples with positive re-isolation of *N. ditissima* at different time points and at increasing distance from the inoculated cut surface; positive samples are highlighted in bold and the respective data are also shown separately for bark and wood subsamples; total number of replicates is shown in brackets; data for 'Royal Gala', 'Discovery', 'Queen Cox' and 'Golden Delicious' are pooled.

	Weeks after inoculation:	**2 *wai***		**4 *wai***		**8 *wai***	
Section[a]	**Symptoms (No/Yes):**	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**
	Total (sample)	**12(12)**	-	**6(8)**	**4(4)**	**4(5)**	**7(7)**
5-10 mm	**Bark (sub-sample)**	0(11)[b]	-	0(8)	0(4)	0(4) [b]	0(7)
	Wood (sub-sample)	12(12)	-	6(8)	4(4)	4(5)	7(7)
35-40 mm	**Total (sample)**	0(12)	-	0(8)	0(4)	0(5)	0(7)
65-70 mm	**Total (sample)**	0(12)	-	0(8)	0(4)	0(5)	0(7)

a: length of transverse section measured from the pruning cut surface; **b:** subsample set had one missing replicate.

Experiment 2. Germination of conidia after 24 h was 91% in the first inoculation (24th October 2018), 97% in the second inoculation (27th October) and 94% in the third inoculation (10th November). The proportion of macroconidia in the inoculum, the inoculation date and the

average daily temperature and relative humidity ranges over a period of 14 days after the inoculation are given in Table 3.2.2.

Table 3.2.2 - Macroconidia content of the inoculum, inoculation date and meteorological data for the leaf scar inoculation experiments.

Experiment no.	Inoculation date	Macroconidia in the inoculum (%)	Average daily temperature range (°C) [a]	Average relative humidity range (%) [a]
1	24th Oct 2017	2.0	3.9 - 15.5	78 - 99
2	27th Oct 2017	0.5	3.0 - 11.3	77 -97
3	10th Nov 2017	0.2	3.4 - 13.7	75 - 98

[a]: average daily temperatures and relative humidity are calculated over a period of 14 days after the inoculation.

Cumulative incidence in the three replicate experiments is shown in Figure 3.2.1. Symptoms developed faster on trees inoculated with the high dose in all three inoculations. At 21 *wai*, disease expression rate (indicated by the slope of the curve) was highest on trees inoculated with the low dose in the first inoculation, and on trees inoculated with both doses in the second and third inoculation. At 28 *wai* disease expression had become slower in all inoculations.

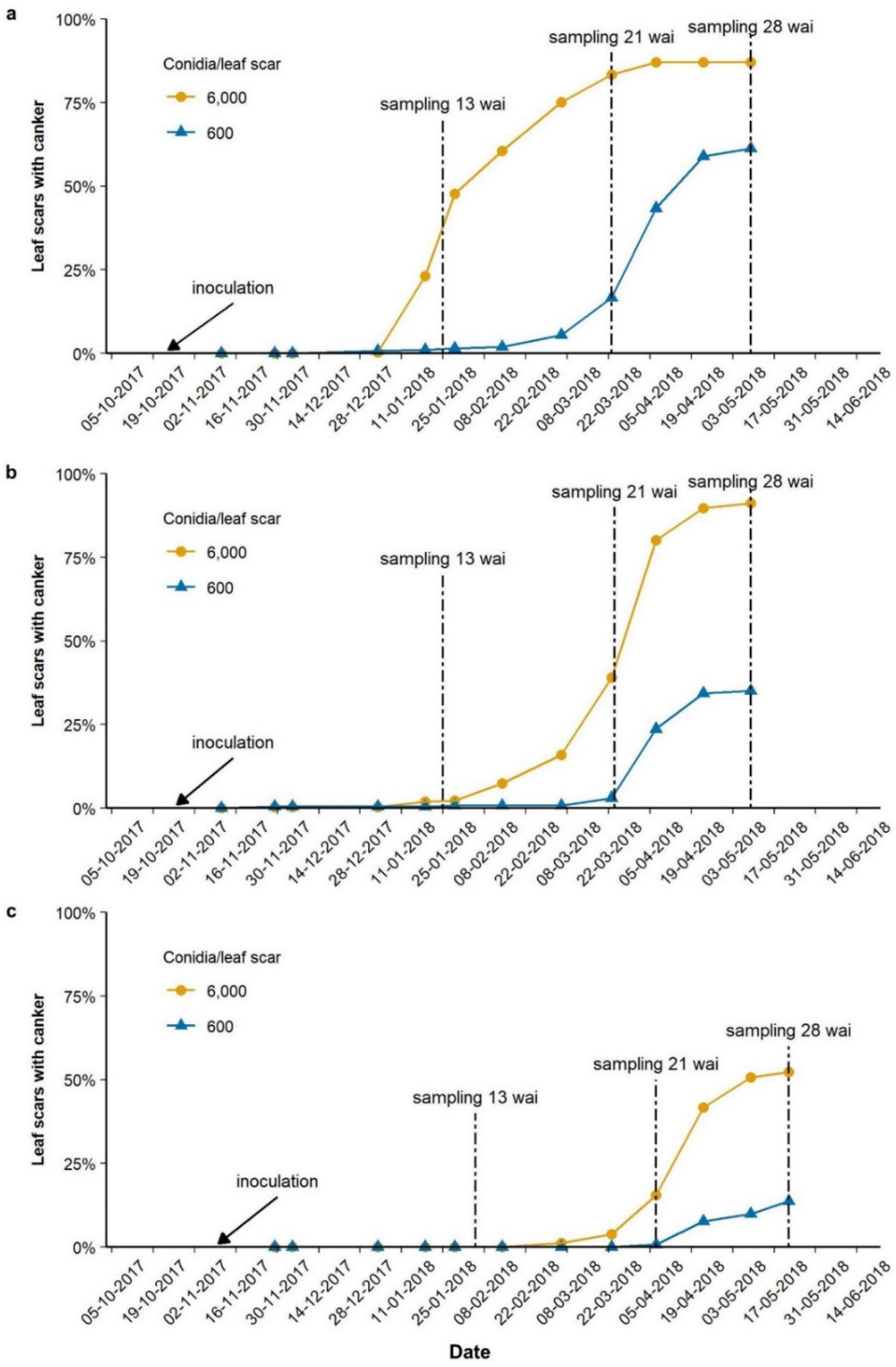

Figure 3.2.1 - Cumulative incidence of canker symptoms on apple trees inoculated via leaf scar with two different doses of conidia on 24th October 2017 (a), 27th October 2017 (b) and 10th November 2017 (c); data are pooled for 'Royal Gala' and 'Queen Cox'.

Of the 188 DNA samples, 185 yielded an amplicon band and were therefore included in the downstream analysis. The results of *N. ditissima*-specific real time qPCR assays on total

DNA of samples collected from shoots inoculated via leaf scars are summarised in Table 3.2.3.

Table 3.2.3 - *N. ditissima* asymptomatic colonisation of orchard tree shoots inoculated via leaf scar: number of samples positive to real time qPCR assay against *N. ditissima*; positive samples are highlighted in bold and the respective data are also shown separately for bark and wood subsamples; total number of replicates is shown in brackets; data for 'Royal Gala' and 'Queen Cox' cultivars are pooled; data for low and high dose are pooled.

			Weeks after the inoculation		
Type	**Position**	**Tissue**	**13**	**21**	**28**
Symptom-free individual and pooled samples	**node**	**Sample**	**8(8)**	**13(14)**	**2(2)**
		Bark	8(8)	13(14)	2(2)
		Wood	0(8)	1(14)	0(2)
	internode	**Sample**	0(8)	0(14)	0(2)
	15 mm from inoculated leaf scar	**Sample**	0(8)	0(4)	0(2)
Cankered individual and pooled samples	**node**	**Sample**	**1(1)**	**10(10)**	**3(3)**
		Bark	1(1)	9(9)	3(3)
		Wood	0(1)	9(10)	3(3)
	internode	**Sample**	**1(1)**	**4(11)**	0(3)
		Bark	0(1)	2(11)	0(3)
		Wood (sub-sample)	1(1)	2(11)	0(3)
	15 mm from canker	**Total (sample)**	-	-	0(1)

At every time point, *N. ditissima* was detected in pooled samples from canker lesions at nodes, in all bark subsamples and in 12 out of 14 wood subsamples assessed. *N. ditissima* was also detected in 23 out of 24 pooled samples from asymptomatic nodes at every time point, in 23 out of 24 bark subsamples and only in one wood subsample. The pathogen was never detected at internodes adjacent to asymptomatic nodes. Conversely, five out of 15 internode samples adjacent to cankered nodes were positive, of which one was at 13 and four were at 21 *wai*. The pathogen was not detected in samples collected at 15 mm away

from either individual asymptomatic leaf scars or individual canker lesions at any of the timepoints, hence no further assessments were done at increasing distance.

Experiment 3. The proportion of macroconidia in the inoculum was 76%. Germination of conidia after 24 h was 96%. The average daily temperature ranged between -0.2 and 9.2°C, with a relative humidity comprised between 67 and 92%, over a period of 14 days starting from the date of inoculation. Cumulative incidence of inoculated pruning wounds with canker symptoms is shown in Figure 3.2.2. Disease expression was increasing at 12 *wai*, whereas no new symptoms were developing at 16 *wai*. Of the 160 DNA samples extracted from woody tissues, 159 yielded an amplicon band and were therefore included in the downstream analysis.

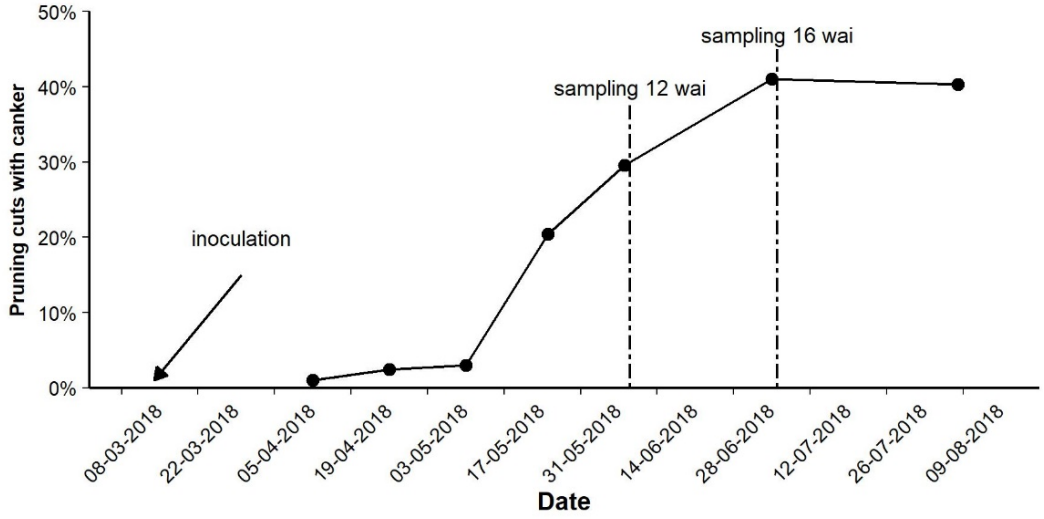

Figure 3.2.2 - Cumulative incidence of canker symptoms on apple trees inoculated via pruning cut on 14th March 2018; data are pooled for 'Royal Gala' and 'Queen Cox'.

The results of *N. ditissima*-specific real time qPCR assays on total DNA samples extracted from samples of pruning cuts inoculated with *N. ditissima* are summarised in Table 3.2.4. *N. ditissima* was always detected in bark and wood at the leading edge of canker at both 12 and 16 *wai*. All other samples collected from non-symptomatic areas neighbouring the cankers were negative, with the only exception of one sample at 10 mm from the leading edge of canker, for which the wood subsample tested positive and the bark negative. *N. ditissima* was present only in two samples from asymptomatic shoots, at 10 mm from the inoculated surface, and it was detected once in bark and once in wood. No other segment from asymptomatic shoots tested positive in the qPCR.

Table 3.2.4 – *N. ditissima* asymptomatic colonisation of orchard tree shoots inoculated via pruning wound: number of samples positive to real time qPCR assay against *N. ditissima*; positive samples are highlighted in bold and the respective data are also shown separately for bark and wood subsamples; total number of replicates is shown in brackets; data for 'Royal Gala' and 'Queen Cox' cultivars are pooled.

Weeks after the inoculation:		**12**		**16**	
Symptoms (Yes/No):		**No**	**Yes**	**No**	**Yes**
Section [a]	**Tissue**				
-2 to 3 mm (leading edge of canker)	**Sample**	-	**4(4)**[b]	-	**5(5)**
	Bark	-	4(4)[b]	-	5(5)
	Wood	-	4(4)[b]	-	4(5)
7 to 12 mm	**Sample**	0(5)	0(5)	**2(5)**	**1(5)**
	Bark	0(5)	0(5)	1(5)	0(5)
	Wood	0(5)	0(5)	1(5)	1(5)
17 to 22 mm	**Sample**	0(5)	0(5)	0(5)	0(5)
27 to 32 mm	**Sample**	0(5)	0(5)	0(5)	0(5)
37 to 42 mm	**Sample**	0(5)	-	0(5)	-

a: length of transverse section measured from the pruning cut surface (asymptomatic samples) or from the leading edge of canker (symptomatic samples);
b: 1 missing replicate in the set.

3.3 - Meta-barcoding study of fungal and bacterial endophytes across apple cultivars differing in *N. ditissima* susceptibility

Sequence quality and generation of OTUs. After quality and length filtering, 6,332,704 bacterial 16S reads were recovered from the total 128 samples. Of these, 84,227 unique sequences were clustered into 115 OTUs, including chloroplast and mitochondrial DNA sequences. Unfiltered 16S reads were aligned to OTUs, ranging from over 25,000 to over 130,000 per sample. After removal of chloroplast and mitochondrial DNA sequences, a total of 32,719 aligned reads remained (c. 0.4% of total unfiltered reads), ranging from 13 to 2,701 per sample. Sequence depth was checked after removal of chloroplast and mitochondrial OTUs and 23 samples were excluded from downstream analysis. Overall, post removal of chloroplast and mitochondrial sequences and of samples with insufficient sequence depth, 79.6% of bacterial reads, were captured by the 10% most abundant 16S OTUs.

After quality and length filtering, 2,545,933 fungal ITS reads were obtained from the samples. Of these, 50,470 unique sequences were clustered into 706 OTUs. The number of unfiltered reads which were aligned to OTUs per sample ranged from fewer than 10,000 to over 50,000. In all samples collected from the two orchards, a few OTUs had a very high read count and the majority were characterised by a low read count. Overall, 99.0% of fungal reads were captured by the 10% most abundant ITS OTUs.

Exploratory analysis. The PCAs on VST-transformed bacterial and fungal OTU counts are shown in Figure 3.3.1 and the results of ANOVA on PC1 through PC4 are shown in Table 3.3.1 for both bacteria and fungi.

For bacterial OTU data, the first four principal components accounted for 62.0% of the total variance (45.5%, 7.4%, 5.1% and 4.0% for PC1 through PC4, respectively). Unexplained variability in PC1 through PC4 was 33.2%, 37.2%, 60.4% and 71.3%, respectively. PC1 and PC2 scores were mainly influenced by orchard and scion genotype within susceptibility ($p < 0.001$). The orchard had a greater effect on PC1 (29.4%) than on PC2 (20.5%), whereas the effect of the scion genotype within susceptibility was similar on PC1 and PC2 (c. 24%). The susceptibility level had an effect on both PC1 and PC2. PC1 was also affected by block within orchard and rootstock genotype (7.8%, $p < 0.01$ and 2.8%, $p < 0.05$, respectively). The scion : rootstock interaction was not significant on the PC scores.

For fungal OTU data, the first four principal components accounted for 37.2% of the total variance (21.8%, 9.5%, 3.4% and 2.5% for PC1 through PC4, respectively). Following ANOVA (Table 4.1), unexplained variability in PC1 through PC4 was 6.6%, 38.8%, 50.3% and 57.2%, respectively. PC1 was mainly influenced by the orchard (85.9%, $p < 0.001$), whereas PC2 was mainly influenced by susceptibility level (27.3%, $p < 0.001$) and scion genotype within susceptibility (19.5% variability, $p < 0.001$). The block within orchard had a greater influence on PC2 (6.5%, $p = 0.011$) than on PC1 (1.3%, $p = 0.004$). The rootstock and the scion : rootstock interaction did not significantly affect any of the first four fungal PCs.

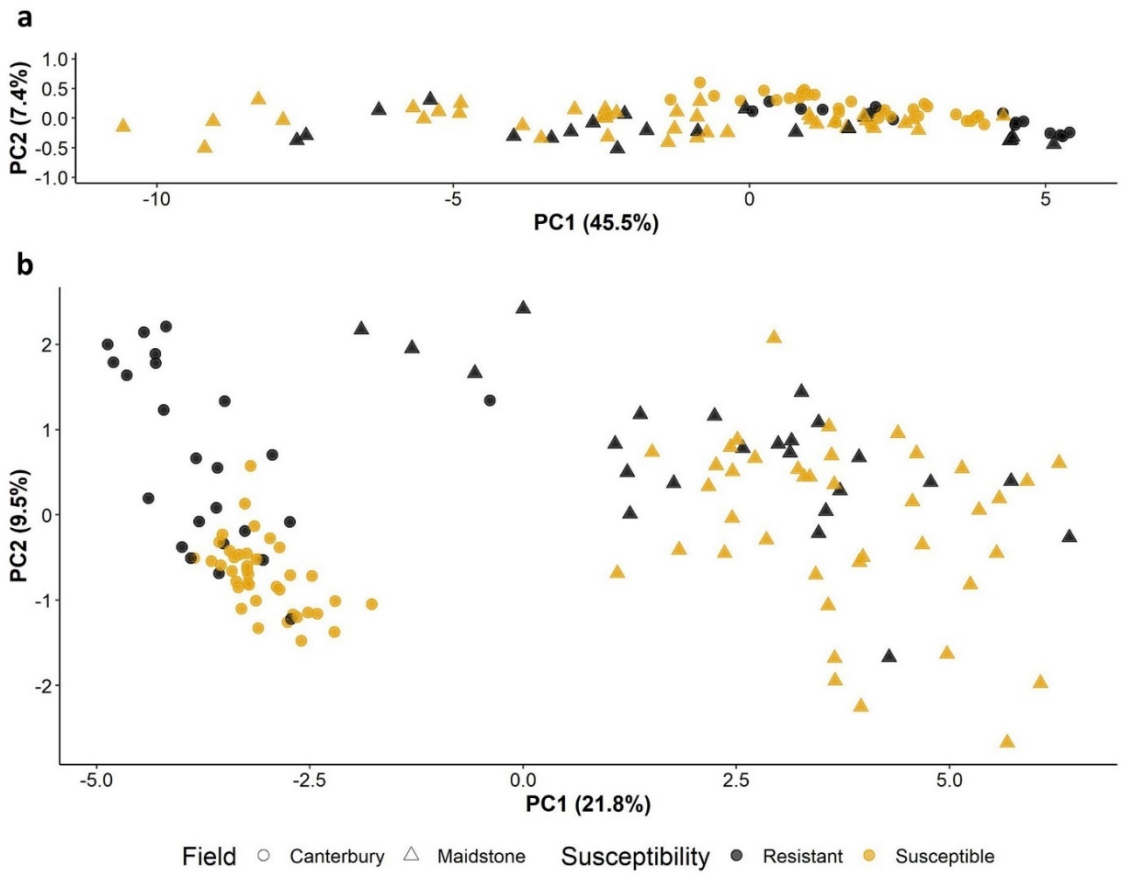

Figure 3.3.1 - Principal component analysis of bacterial (**a**) and fungal (**b**) OTUs; PC1 vs PC2 are shown in both plots and the percentage of variance explained by each PC is shown in brackets. PC axes were re-scaled by multiplying PC scores by the percentage of variance explained by the respective PC to reflect equal distances between the two direction for ease of interpretation. Points are biological replicates, point shape represents the orchard (Field) and point colour represents the susceptibility level of the cultivar.

Table 3.3.1 - Results of ANOVA on bacterial and fungal principal components (PC) 1 through 4, showing the percentage of variability (%var) in PC scores accounted for by design factors and the respective significance level (*p*); significant effects ($p < 0.05$) are highlighted in bold; the effect of the interaction Scion x Rootstock interaction was never significant and therefore is not reported.

PC	**Orchard**		**Block within orchard**		**Susceptibility**		**Scion within susceptibility**		**Rootstock**		**Residuals**
	% var	***p***	**% var**	***P***	**% var**	***p***	**% var**	***p***	**% var**	***p***	**% var**
Bacteria											
PC1	**29.4**	**<0.001**	**7.8**	**0.007**	**2**	**0.029**	**23.4**	**<0.001**	**2.8**	**0.011**	33.2
PC2	**20.5**	**<0.001**	2.7	0.438	**11.7**	**<0.001**	**24.3**	**<0.001**	0.2	0.523	37.2
PC3	**6.6**	**0.004**	**16.5**	**0.002**	0.7	0.346	**13.4**	**0.01**	0.2	0.624	60.4
PC4	1.3	0.234	2.6	0.801	**4.7**	**0.023**	**15.2**	**0.013**	0.2	0.668	71.3
Fungi											
PC1	**85.9**	**<0.001**	**1.3**	**0.004**	**1.7**	**<0.001**	**4**	**<0.001**	<0.1	0.468	6.6
PC2	**3.9**	**0.002**	**6.5**	**0.011**	**27.3**	**<0.001**	**19.5**	**<0.001**	1	0.096	38.8
PC3	0.9	0.163	**15.9**	**<0.001**	**5**	**0.002**	**25**	**<0.001**	0.4	0.362	50.3
PC4	0.8	0.232	**12.2**	**0.002**	<0.1	0.696	**26.4**	**<0.001**	7	0.258	57.2

Diversity indices. Alpha (α) diversity indices for bacterial and fungal normalised OTU data are shown in Figure 3.3.2.

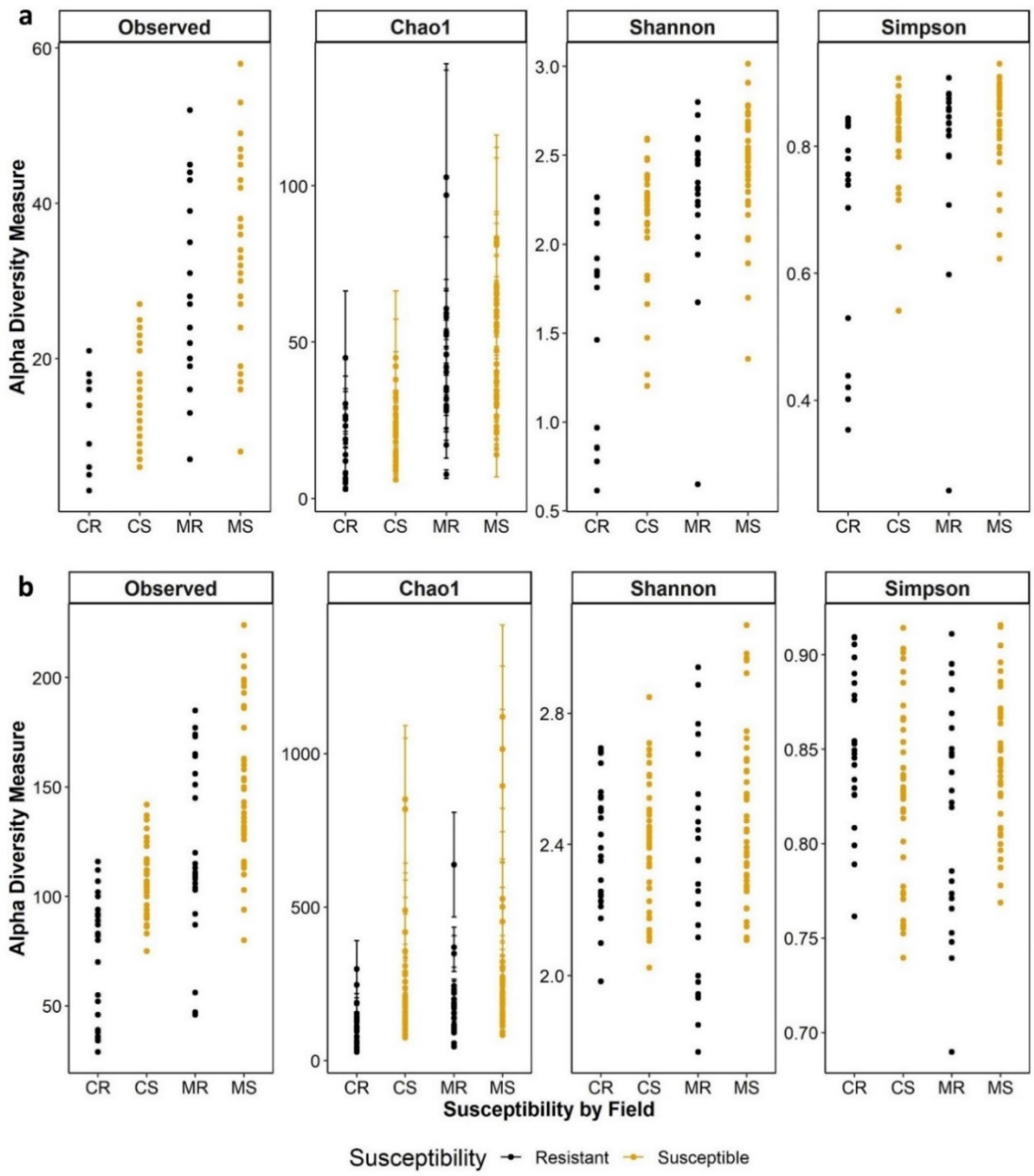

Figure 3.3.2 - Alpha (α) diversity indices (observed species, Chao1, Simpson and Shannon) for bacterial (**a**) and fungal (**b**) normalised OTU counts from resistant and susceptible cultivars at the two sites of planting (Field). **CR**: Canterbury orchard, resistant cultivars; **CS**: Canterbury orchard, susceptible cultivars; **MR**: Maidstone orchard, resistant cultivars; **MS**: Maidstone orchard, susceptible cultivars. Shannon index was calculated using the natural logarithm. Error bars of Chao1 index represent standard error. Points are biological replicates, colour refers to susceptibility level.

Following analysis by permutational ANOVA (Table 3.3.2), the estimator whose variability was most explained by the model was the observed species number, with 30.8% and 19.1% unexplained variability for bacterial and fungal OTU data, respectively. The second-best estimator was Shannon index for bacterial OTU data (37.4% unexplained variability) and Simpson for fungal OTU data (62.6% unexplained variability).

Observed bacterial species number was primarily influenced by orchard, followed by scion genotype within susceptibility level, block within orchard, rootstock and finally susceptibility level (resistant level = 21.63, susceptible level = 24.77). Observed fungal species number was primarily influenced by orchard, followed by block within orchard, scion within susceptibility level, susceptibility level (resistant = 97.63, susceptible = 128.65) and finally scion : rootstock interaction. Susceptibility level effects were higher on fungal species number (14.4%, $p < 0.001$) than on the bacterial species number (1.5%, $p = 0.042$). Shannon and Simpson indices for bacteria were influenced by the same experimental factors except for rootstock, which had no effect on either. Similarly, the scion : rootstock interaction was not significant on either of the two indices. Shannon and Simpson indices for fungi were primarily affected by scion genotype within susceptibility, followed by block within orchard. Orchard, susceptibility level, rootstock and scion : rootstock interaction had very little or no effects on both indices, with non-significant p-value.

Non-metric multidimensional scaling (NMDS) ordination (Figure 3.3.3) was used to visualise global bacterial and fungal beta diversity. For bacterial 16S data, no obvious clustering was observed in terms of orchard or susceptibility level, however the scion genotype Robusta 5 clustered away from all other samples. Fungal ITS data was clustered primarily based on the orchard. As observed with bacterial OTU data, scion Robusta 5 clustered away from the other samples. Adonis analysis (Table 3.3.2) showed that bacterial community structure, as measured by Bray-Curtis indices, was mainly affected by scion genotype within the susceptibility level, followed by orchard, block within the orchard and susceptibility level ($p < 0.001$). Fungal community composition was primarily affected by orchard, followed by scion genotype within the susceptibility level, susceptibility level and finally block within orchard ($p < 0.001$). Rootstock had little effect on bacterial beta (β) diversity (1.3% explained variability, $p = 0.045$), whereas the scion : rootstock interaction was not significant. Conversely, rootstock was not significant to fungal beta (β) diversity, and the scion : rootstock interaction had little effect (3.5% explained variability, $p = 0.039$).

Table 3.3.2 – Percentage of variability (%var) in alpha (Shannon, Simpson, observed species and Chao1) and beta (Bray-Curtis) diversity indices accounted for by design factors and interactions and the respective significance level (*p*); significant effects ($p < 0.05$) are highlighted in bold.

Measure [a]	**Orchard**		**Block**		**Susceptibility**		**Scion within susceptibility**		**Rootstock**		**Scion : Rootstock**		**Residuals**
	%var	***p***	**%var**	***p***	**%var**	***p***	**%var**	***p***	**%var**	***p***	**%var**	***p***	**%var**
Bacteria													
Observed	**38.0**	**<0.001**	**7.6**	**0.004**	**1.5**	**0.046**	**18.2**	**< 0.001**	**1.9**	**0.033**	1.9	0.584	30.8
Chao1	**35.5**	**<0.001**	6.4	0.067	0.4	0.228	**16.0**	**0.001**	0.0	0.619	3.3	0.484	38.5
Shannon	**20.3**	**<0.001**	**6.6**	**0.013**	**9.1**	**< 0.001**	**23.2**	**< 0.001**	0.1	0.745	3.3	0.490	37.4
Simpson	**6.5**	**<0.001**	**7.6**	**0.038**	**10.8**	**< 0.001**	**31.3**	**< 0.001**	0.0	0.726	4.0	0.330	39.9
Bray-Curtis	**12.3**	**<0.001**	**7.5**	**<0.001**	**3.4**	**< 0.001**	**20.6**	**< 0.001**	1.3	0.045	4.2	0.510	50.7
Fungi													
Observed	**29.7**	**<0.001**	**17.8**	**<0.001**	**14.4**	**< 0.001**	**16.0**	**<0.001**	0.4	0.092	**2.6**	**0.045**	19.1
Chao1	**2.8**	**0.031**	3.8	0.494	**7.9**	**< 0.001**	**9.3**	**0.024**	0.0	1.000	7.4	0.115	68.8
Shannon	0.3	0.474	**10.4**	**0.012**	3.0	0.062	**17.8**	**<0.001**	0.4	1.000	2.3	0.775	65.8
Simpson	0.0	0.843	**15.3**	**<0.001**	0.0	0.922	**19.5**	**<0.001**	0.3	0.396	2.3	0.878	62.6
Bray-Curtis	**36.6**	**<0.001**	**5.2**	**<0.001**	**6.2**	**<0.001**	**14.1**	**<0.001**	0.4	0.278	**3.5**	**0.039**	34.0

a: Results from permutational ANOVA of alpha diversity indices or PERMANOVA of Bray-Curtis dissimilarity matrix.

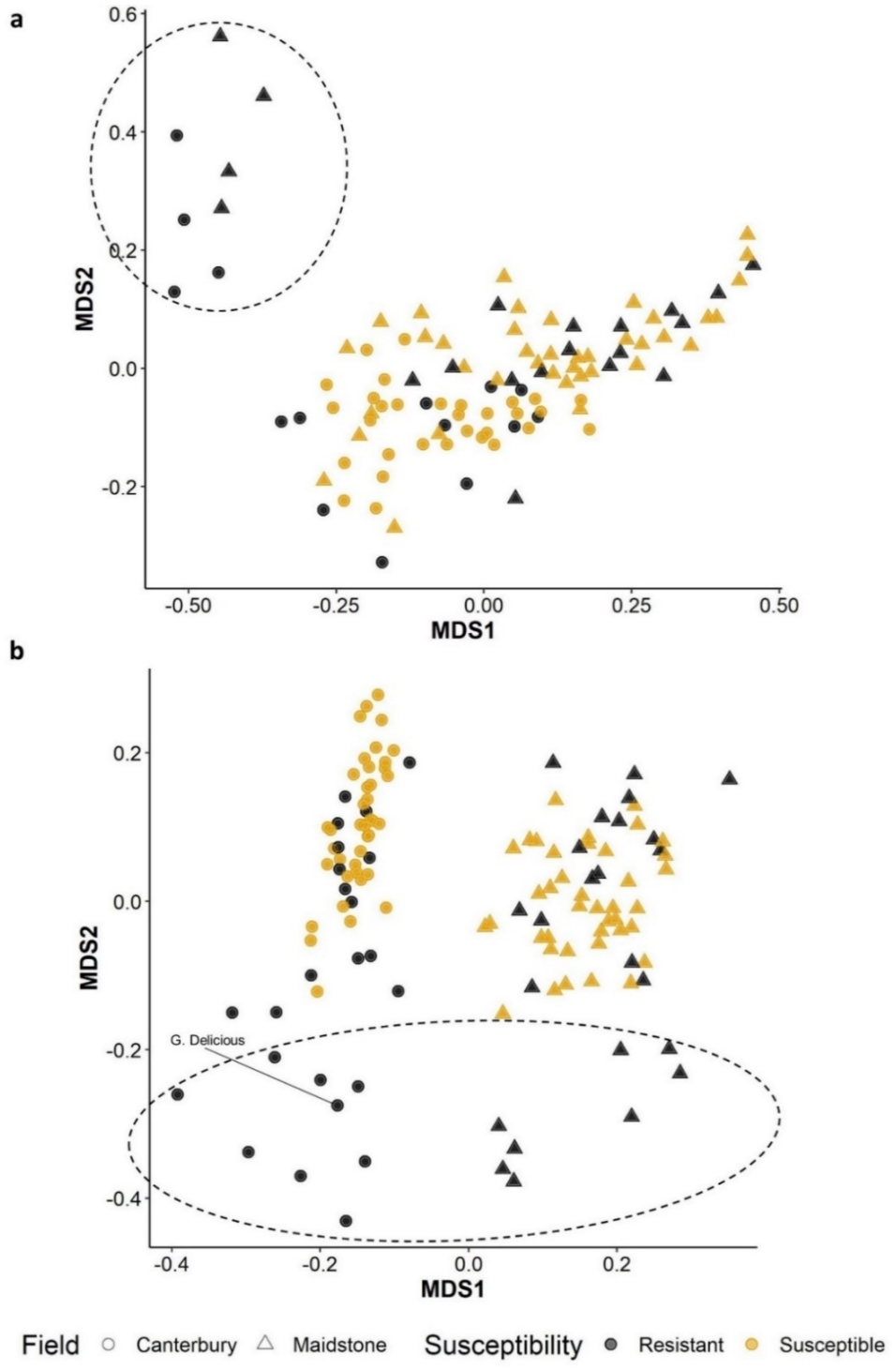

Figure 3.3.3 - Structure of microbial communities: non-metric multidimensional scaling (NMDS) ordination of bacterial (**a**) and fungal (**b**) Bray-Curtis dissimilarity matrices. Stress: 0.123 (**a**), 0.134 (**b**). Points are biological replicates, point shape represents orchard (Field) and point colour represents canker susceptibility level of the cultivar. Points enclosed in dashed line are Robusta 5 replicates, except for the point labelled as Golden Delicious in (**b**).

Differential analysis of OTU relative abundance. DESeq2 analysis identified 19 bacterial OTUs with differential relative abundance in canker-resistant genotypes ('Robusta 5', 'Golden Delicious' and 'Grenadier') compared to canker-susceptible ones ('Braeburn', 'Gala', 'Jazz', 'Kanzi' and 'Rubens'), listed in Table 3.3.3. Conversely, 32 fungal OTUs had differential relative abundance, listed in Table 3.3.4.

All 19 bacterial OTUs had lower relative abundance in canker resistant cultivars. For 18 of these, the taxon could be assigned to the genus level, including *Sphingomonas*, *Massilia*, *Hymenobacter*, *Frondihabitans*, *Curtobacterium*, *Methylobacterium, Pseudomonas* and *Rathayibacter*.

Of the 32 fungal taxa with differential abundance, eight had higher relative abundance and 24 lower relative abundance in resistant cultivars. Of the fungal taxa with higher abundance in resistant cultivars, four OTUs belonged to Ascomycota, three to Basidiomycota and one could not be identified at the phylum level. Seven OTUs were identified to the genus level. These included the yeast genera *Rhodotorula* and *Kalmanozyma* , as well as the filamentous fungi genera *Aureobasidium*, *Stemphylium* and *Dissoconium*.

Of the 23 OTUs with lower relative abundance in resistance cultivars compared to susceptible cultivars, 20 belonged to the Basidiomycota, one to the Ascomycota and two could not be identified at the phylum level. Overall, 13 taxa could be identified at the genus level. Most taxa were found in the class Tremellomycetes, orders Tremellales and Filobasidiales. The Tremellales included members in the family Bulleribasidiaceae (genera *Vishniacozyma* and *Dioszegia*), Tremellaceae (*Bulleromyces*), Phaeotremellaceae (*Gelidatrema*), Bulleraceae (*Genolevuria*) and Rhynchogastremataceae (*Papiliotrema*). The Filobasidiaceae included the genus *Filobasidium*.

Table 3.3.3 - Bacterial OTUs with differential relative abundance in resistant apple genotypes compared to susceptible genotypes, and putative lifestyle and ecology. No OTUs were found with higher relative abundance in resistant genotypes. Negative Log_2FC correspond to higher relative abundance in susceptible genotypes. Taxonomic annotations were done with UPARSE using the UNITE database and checked with BLASTn on NCBI's non-redundant nucleotide collection (nr/nt). P-value was corrected for the false discovery rate with the Benjamini-Hochberg (BH) method and statistical significance was determined at the 5% level. FC = fold change; SE = standard error; P-value (BH) = Benjamini-Hochberg corrected p-value.

UNITE taxon	OTU ID	Mean normalised count [a]	$Log_2FC \pm SE$	P-value (BH)
Sphingomonas sp. (Sphingomonadaceae)	OTU3	80.41	-5.17 ±0.74	< 0.001
Sphingomonas sp.	OTU8	56.43	-4.01 ±0.65	< 0.001
Curtobacterium sp. (Microbacteriaceae)	OTU4	26.22	-3.83 ±0.72	< 0.001
Massilia sp. (Oxalobacteriaceae)	OTU7	16.64	-3.89 ±0.67	< 0.001
Pseudomonas sp. (Pseudomonadaceae)	OTU5	16.08	-1.45 ±0.52	0.012
Pseudomonas sp.	OTU45	13.63	-3.41 ±0.59	< 0.001
Hymenobacter sp. (Flavobacteriaceae)	OTU13	13.49	-14.12 ±1.06	< 0.001
Hymenobacter sp.	OTU17	8.48	-3.34 ±1.10	0.007
Hymenobacter sp.	OTU18	7.94	-4.33 ±1.09	< 0.001
Frondihabitans sp. (Microbacteriaceae)	OTU6	7.13	-2.33 ±0.81	0.010
Methylobacterium sp. (Methylobacteriaceae)	OTU9	6.98	-1.84 ±0.84	0.049
Hymenobacter sp.	OTU33	6.41	-2.32 ±1.07	0.049
Rhizobiales	OTU16	5.82	-2.73 ±0.92	0.008
Massilia sp.	OTU11	5.01	-3.78 ±1.23	0.007
Massilia sp.	OTU27	3.93	-2.09 ±0.90	0.038
Methylobacterium sp.	OTU10	3.88	-11.37 ±1.19	< 0.001
Hymenobacter sp.	OTU29	3.22	-2.66 ±1.23	0.049
Rathayibacter sp. (Microbacteriaceae)	OTU40	1.36	-2.51 ±1.08	0.038
Hymenobacter sp.	OTU103	1.08	-13.83 ±2.04	< 0.001

[a]: mean normalised count across resistant and susceptible cultivars.

Table 3.3.4 - Fungal OTUs with differential relative abundance in resistant apple genotypes compared to susceptible genotypes, and putative lifestyle and ecology. Positive Log_2FC correspond to higher relative abundance in resistant genotypes, negative values correspond to higher relative abundance in susceptible genotypes. Taxonomic annotations were done with UPARSE using the UNITE database and BLASTn on NCBI's non-redundant nucleotide collection (nr/nt). P-value was corrected for the false discovery rate with the Benjamini-Hochberg (BH) method and statistical significance was determined at the 5% level. FC = fold change; SE = standard error; P-value (BH) = Benjamini-Hochberg corrected p-value.

Taxon [a]	**OTU ID**	**Mean normalised count** [b]	**Log_2FC ±SE**	**P-value (BH)**
Higher relative abundance				
Aureobasidium sp. (Aureobasidiaceae)	4	1785.02	1.51 ±0.38	0.001
Rhodotorula sp. (Sporidiobolaceae)	8	850.58	3.19 ±0.45	< 0.001
Fungi	6	726.54	1.18 ±0.26	< 0.001
Stemphylium sp. (Pleosporaceae)	16	214.19	1.63 ±0.30	< 0.001
Kalmanozyma sp. (Ustilaginaceae, possibly *K. fusiformata*)	26	57.42	3.18 ±1.15	0.040
Dothideales	362	14.27	1.60 ±0.51	0.015
Entylomatales	52	8.03	3.49 ±1.11	0.015
Dissoconium sp. (Dissoconiaceae, possibly *D. eucalypti*)	114	3.27	3.52 ±1.07	0.001
Lower relative abundance				
Vishniacozyma sp. (Bulleribasidiaceae)	5	1185.27	-3.20 ±0.66	< 0.001
Filobasidium sp. (Filobasidiaceae)	9	506.17	-1.67 ±0.45	0.003
Filobasidium sp. (possibly *F. wieringae*)	11	460.93	-2.45 ±0.50	< 0.001
Dioszegia sp. (Bulleribasidiaceae, possibly *D.* h*ungarica*)	10	416.16	-1.59 ±0.56	0.031
Filobasidium sp. (possibly *F. floriforme*)	14	394.16	-1.29 ±0.42	0.017
Vishniacozyma sp. (possibly *V. carnescens*)	61	221.74	-2.68 ±0.66	0.001
Gelidatrema sp. (Phaeotremellaceae, possibly *G. spencermartinsiae*)	19	155.09	-3.66 ±0.68	< 0.001
Basidiomycota	22	149.67	-1.69 ±0.57	0.023
Filobasidium sp. (possibly *F. chernovii)*	20	137.28	-2.47 ±0.48	< 0.001

Table 3.3.4 - continued

Taxon [a]	OTU ID	Mean normalised count [b]	Log_2FC ±SE	P-value (BH)
Genolevuria sp. (Bulleraceae)	27	79.86	-3.58 ±0.71	< 0.001
Fungi	24	45.96	-2.52 ±0.82	0.017
Tremellomycetes	644	41.46	-4.60 ±0.79	< 0.001
Bulleromyces sp. (Tremellaceae, possibly *B. albus*)	29	34.17	-2.08 ±0.60	0.005
Diosgezia sp.	32	22.62	-3.30 ±0.91	0.003
Fungi	31	17.54	-2.80 ±0.79	0.004
Filobasidium sp.	36	14.68	-3.36 ±0.82	0.001
Papiliotrema sp. (Rhynchogastremataceae, possibly *P. frias*)	42	11.07	-3.59 ±0.84	< 0.001
Basidiomycota	44	9.35	-5.04 ±1.02	< 0.001
Entylomatales	66	5.46	-7.36 ±2.35	0.002
Tremellales	71	4.03	-5.32 ±1.09	< 0.001
Tremellales	82	2.70	-3.59 ±1.34	0.049
Hypocreales	103	2.20	-11.87 ±2.03	< 0.001
Basidiomycota	596	0.80	-18.02 ±5.93	0.018

[a]: species-level taxa correspond to UNITE species hypotheses (SH).
[b]: mean normalised count across resistant and susceptible cultivars.

4. Discussion

4.1 - Comparison of high-throughput methods for *N. ditissima* infection

In the ELISA validation experiment, fungal antigens were detected at the leading edge of cankers on all three shoots (5/5 samples assessed), and in the asymptomatic tissue at 10 mm from the lesion on shoot C (2/2 samples assessed). These results confirm the findings in Weber & Hahn (2013), where internal spread of the pathogen was observed at distance from the canker. However, isolation of the pathogen was possible only from two out of the seven samples that were positive to the ELISA. We used a PDA-based selective medium devised to facilitate growth of the pathogen (McCracken *et al.*, 2003). Nevertheless, isolation from cankers was mostly unsuccessful. The presence in plant tissues of fungal species that can outcompete *N. ditissima* might be the main reason why the culture-dependent approach was less sensitive than the ELISA. Since isolation was laborious and time consuming (requiring over two weeks per sample), it was unsuitable for a large-scale study of *N. ditissima* colonisation of plant tissues. ELISA and qPCR, which are high-throughput tools (requiring two days and three to four days per sample, respectively), were therefore compared to select the most reliable detection method.

Overall, the ELISA was faster and more straightforward than the qPCR. The former only required sample preparation and dilution prior to the assay, whereas the latter required DNA extraction and end point PCR and agarose gel to check that DNA was amplifiable by PCR to avoid false negatives, before carrying out the assay. A partial agreement (Table 3.1.1.) was found between the ELISA and the qPCR, however the ELISA detected more positives compared to the qPCR. Several reasons may account for this discrepancy. Firstly, the subsample tested in the ELISA (4 mg) represented a greater fraction of the original sample compared to the subsample tested than in the qPCR (average 0.58 mg). This fraction was, on average, seven times greater in the ELISA sample, resulting in a seven-fold greater likelihood of detecting the fungus by ELISA compared to qPCR. In addition the DNA samples had to be diluted for use in the qPCR, therefore the concentration of target DNA template might have been reduced below the threshold for detection. Moreover, qPCR can be affected by inhibitor compounds that hinder successful amplification of DNA, and plant samples are especially rich in PCR inhibitors. On the other hand, the specific interaction between an antibody and its target antigen is considered strong enough to prevail over non-specific interactions, even in presence of very low amounts of target. However, the standard curve was more reliable for the qPCR, as shown by the R^2 values. Moreover, in the ELISA standard curve, large deviation of the standards from the curve was observed at low values of fungal biomass, resulting in low accuracy of the prediction at low levels. Most samples positive to ELISA but negative to qPCR (seven of nine) were in the lower range of the standard curve,

therefore identification of positives could not be done confidently for these samples. Hence, the qPCR was chosen as detection tool to pursue the anatomy study of *N. ditissima* asymptomatic infection.

4.2 - *Neonectria ditissima* internal colonisation of apple shoots inoculated via leaf scar and pruning wound

Asymptomatic infections were detected in artificially inoculated pruning wounds and leaf scars, but no evidence was found of asymptomatic colonisation of internal tissues at 10 mm from the infection site before any visual symptoms occurred. On the other hand, after symptoms had appeared, we detected internal spread of the pathogen in the asymptomatic tissues at 10-15 mm from canker lesions.

In previous studies, *N. ditissima* was isolated from artificially infected rasp wounds on the main stem up to 7 months after the inoculation (Walter *et al.*, 2015) or form artificially infected buds 18 months after the inoculation (Amponsah *et al.*, 2015). In our experiments, we re-isolated the pathogen from asymptomatic inoculated pruning wounds up to four months after the inoculation when the experiment was terminated. Additionally, using qPCR the fungus was found in artificially inoculated and asymptomatic leaf scars and pruning wounds, up to four and seven months after the inoculation, respectively.

Samples from trees inoculated via leaf scar in autumn were mainly collected when disease incidence was increasing (i.e. at 21 weeks after the inoculation), which indicated the presence of viable asymptomatic infections. Later on (i.e. at 28 weeks after the inoculation), incidence had reached a plateau, possibly indicating that all latent infections had become symptomatic by then. Conversely, incidence on trees inoculated via pruning cut in spring was increasing from 8 to 14 *wai* and had reached a plateau at 16 *wai*. However, as there were not enough symptomatic shoots before 12 *wai*, to ensure that comparable numbers of symptomatic and asymptomatic samples were assessed, shoots were collected at 12 and 16 *wai*. Differences in the disease expression rate over time between leaf scar-inoculated and pruning wound-inoculated trees might have been caused by the prevailing environmental conditions at the time of inoculation, which took place in autumn and spring, respectively (data not shown).

During the asymptomatic stage of the infection, the pathogen was never detected at 10 mm from the inoculated wound in any of our experiments, suggesting absence of internal colonisation beyond the entry site. Moreover, in the asymptomatic leaf scars, the pathogen was almost exclusively found in the bark tissues, which also included the vascular tissues bridging the stem and the leaf petiole. Conversely, in cankered leaf scars *N. ditissima* was found in both bark and wood. This further suggests that the asymptomatic infection is highly localised in the outmost layers of the leaf scar.

Prior to symptom expression, *N. ditissima* may reside in the infected wound as incubating conidia, or alternatively spores may germinate and then mycelium may grow at a slow rate or remain latent. In an early microscopy study on the leaf scar infection, Crowdy (1952) found that germination of conidia and local mycelial development in the vascular tissues preceded canker symptoms. When the plant managed to mechanically restrain the fungus to the leaf scar with physical barriers, 'confined lesions' were observed where the mycelium could remain viable for up to five months. More recently, Walter *et al.* (2016) hypothesised that disease expression occurs once the mycelium developing in the infected wound reaches a biomass threshold. Based on currently available data, whether *N. ditissima* resides at the infection site in the form of incubating spores or latent mycelium remains a matter of speculation. To test the hypothesis that mycelial growth occurs prior to visual symptoms, a study to quantify fungal biomass from the inoculation throughout the incubation phase and until disease expression could be carried out. The real time qPCR developed by Ghasemkhani *et al.*, (2016a), which was utilised in this present work to assess the presence of *N. ditissima* in asymptomatic plant material, was originally devised to quantify the pathogen biomass in symptomatic plant material. The assay could be further optimised to quantify fungal biomass prior to symptom expression. Alternatively, imaging tools, such as immunofluorescence or reporter genes, may help understand whether the pathogen undergoes germination followed by mycelial development at the infection point, during the latent phase of the infection.

In this study we confirmed, for the first time with a DNA-based approach, that *N. ditissima* can colonise the asymptomatic plant tissues adjacent to cankers. Dewey *et al.* (1995) and Weber & Hahn (2013) previously demonstrated the same using immunolocalization with fluorescent antibodies and isolation techniques, respectively. Dewey *et al.* (1995) did not specify the distance from the canker at which the pathogen was detected, whereas appeared Weber & Hahn (2013) tracked the fungus in the xylem up to 20 cm away from lesions on the trunk of young trees cv. 'Nicoter' ('Kanzi'), five months after symptoms occurred. In our experiments, *N. ditissima* was not frequently detected in asymptomatic tissues at 10-15 mm from canker lesions. However, we detected the fungus in both wood and bark tissues at 10-15 mm from the leading edge of the canker lesion. Spread of the pathogen beyond the leading edge of a canker lesion has a great relevance to disease management by means of pruning, as already highlighted by Crowdy (1949) and Weber (2014).

Interestingly, the researchers who developed the qPCR assay that was employed in this study could not detect the pathogen in asymptomatic plant tissues, either in bud scar wounds (Ghasemkhani *et al.*, 2016a) or in leaf scars (Ghasemkhani *et al.*, 2016b), one month after the inoculation. One possible reason for this is that the sample preparation and DNA

extraction utilised in this present work were more efficient than in Ghasemkhani *et al.* (2016a,b). In this work, we freeze-dried samples prior to flash-freezing, which allowed better grinding of plant material compared to flash-freezing alone (data not shown). Additionally, Ghasemkhani *et al.* (2016a,b) used a genomic DNA purification kit manufactured by Fermentas (now ThermoFisher Scientific), and we used instead used a QIAGEN's plant DNA extraction kit. This might have contributed to achieve higher yield and quality of total DNA extracted from plant tissues, thus enhancing the sensitivity of our method. However, an alternative explanation might be that, despite similar inoculum doses were used in Ghasemkhani *et al.* (2016a,b) (*c.* 1,000 conidia/wound) and in this study (*c.* 600 conidia/wound for the low dose applied to leaf scars), we collected asymptomatic samples for qPCR testing starting from 13 *wai*, whilst the authors assessed asymptomatic plant material earlier, namely 4 *wai*. Therefore, in our study the fungus was given a longer time to develop, which may have led to the accumulation of a larger, and hence more detectable biomass.

Longitudinal browning of xylem vessels is often found extending from canker lesions. Some authors have hypothesised that such staining might be caused by a toxin produced by the fungus in the lesion and then diffusing along the stem via xylem (Swinburne, 1975; Saville & Olivieri, 2019). It was also proposed that the staining might indicate xylem colonisation by *N. ditissima* (Kennel, 1963). Fungal hyphae were observed in association with xylem staining at sites remotely situated from the lesion (Dewey *et al.*, 1995). However, in another study (Weber & Zabel, 2010; Weber & Hahn, 2013) *N. ditissima* could not be consistently isolated from stained wood. Despite the limited evidence, the currently prevailing opinion supports a direct link between wood staining and fungal colonisation (Weber, 2014; Børve *et al.*, 2019). In our experiments, xylem staining was not found in asymptomatic shoots, whereas it was found in four symptomatic 'Royal Gala' shoots collected from polytunnel trees inoculated via pruning wounds, extending up to 70 mm from the inoculated cut surface in all shoots. Additionally, staining was observed in one symptomatic 'Royal Gala' shoot collected from an orchard tree inoculated via pruning wound, extending up to 30 mm from the inoculated cut surface. In all cases, the pathogen was only detected in the canker, but never in the stained wood, either by means of isolation or qPCR. Altogether, based on the current evidence neither of the two hypotheses should be refuted. Further studies should be undertaken to understand this aspect of disease anatomy.

4.3 - Meta-barcoding study of fungal and bacterial endophytes across apple cultivars differing in *N. ditissima* susceptibility

Global diversity of both bacterial and fungal communities in apple woody tissues was primarily shaped by the orchard location, followed by the scion genotype. Additionally, an important

proportion of the scion effect could be attributed to the differences between the two susceptibility levels to apple canker, for both bacteria and fungi. Rootstock effects were very small (bacteria) or negligible (fungi).

The effect of site is known to influence fungal endophyte community structure in woody and herbaceous plant species, as shown for example by Arnold *et al.* (2003) in *Theobroma cacao* and by Gange *et al.* (2007) in *Cirsium arvense*. In this present work, the location of the orchard (Canterbury or Maidstone, Kent, UK) was the main driver of bacterial and fungal endophyte diversity. Site effects on the apple microbiome were also found by Arrigoni *et al.* (2020) and Liu *et al.* (2020). Weather conditions were comparable across the two sites, with daily mean temperature ranging from 10.3°C to 23.1°C in Maidstone and from 10.2°C to 23.4°C in Canterbury, and daily mean relative humidity between 61% and 93% in Maidstone and between 53% and 93% in Canterbury. Overall, our results suggest an important role of the environment, possibly the nature of the surrounding vegetation (potential source of inoculum) or the characteristics and cultivation history of the orchards, in shaping the apple tree endophyte community. The two orchards used in this study were very different in terms of plant species in windbreaks and the vegetation surrounding apple trees (Figure 2.3.1). In particular, woodland areas hosting various tree species were found around the orchard in Maidstone, whilst the surrounding fields of the orchard in Canterbury was dominated by arable fields with only hedgerow trees in the vicinity of apple rows. Moreover, at the time of sampling Maidstone orchard had a high diversity of weed species at the time of sampling, whereas no weeds were observed in the Canterbury field (data not shown).

Among the different genotypes, Robusta 5 showed greater diversity of bacterial and fungal OTUs, as shown by the PCA and the NMDS plots. The genotype *Malus* x *robusta* is the most distinct among those assessed in this present study, as all the others are *M.* x *domestica* accessions. Forsline *et al.*, (2003) described Robusta 5 as a hybrid between *M. baccata* and *M. prunifolia*. This cultivar has been reported as highly canker-resistant (Bus *et al.*, 2019). The difference observed between the endophyte community structure in Robusta 5 and all other scion cultivars may reflect the genotypic difference between this genotype and all other cultivars assessed in the present work.

In this present study, 8 fungal taxa showed significantly higher relative abundance in resistant genotypes compared to the susceptible ones, whereas 19 bacterial and 23 fungal taxa showed lower relative abundance. It is possible that the amplification of non-target chloroplast and mitochondrial DNA by the 16S primers we used in this present study negatively affected the recovery of bacterial 16S sequences (Beckers *et al.*, 2016). This is suggested by the overall low proportion of bacterial reads after removal of the organelle reads. This might have caused a considerable amount of diversity not to be detected. However, the cumulative

number of reads per OTU showed that sequence depth was satisfactory for most samples, and we considered bacterial OTU data reliable, although potentially not complete.

All bacterial genera that were differentially abundant in this study have been previously isolated from apple stems (Arrigoni *et al.*, 2018, 2020; Liu *et al.*, 2018) and leaves (Yashiro *et al.*, 2011). *Pseudomonas* sp. includes species that are pathogenic on apple (Kennelly *et al.*, 2007) as well as species with biological control activity against plant pathogens (Ligon *et al.*, 2000; Weller, 2007), such as those implemented in the commercial products Bio-Save® 10LP (*P. syringae*), Bio-Save® 11LP (*P. syringae*) and BlightBan® A507 (*P. fuorescens*). *Sphingomonas* sp. and *Methylobacterium* sp. both include species with reported beneficial effects on plants, including antagonism against plant pathogens (Wachowska *et al.*, 2013; Grossi *et al.*, 2020). *Curtobacterium* sp. and *Rathayibacter* sp. include species that are pathogens on herbaceous hosts (Chase *et al.*, 2016; Murray *et al.*, 2017).

Eight fungal OTUs had higher relative abundance in the canker-resistant class. These were found across all resistant genotypes ('Robusta 5', 'Golden Delicious' and 'Grenadier'). The genera *Aureobasidium*¸ *Rhodotorula* and *Kalmanozyma* include species with known biocontrol potential against plant pathogenic fungi. For example, *Aureobasidium pullulans* controlled *Botrytis cinerea* (grey mould), *Colletotrichum acutatum* (bitter rot) or *Penicillium expansum* (blue mould) on apple post-harvest (Mari *et al.*, 2012); *R. mucilaginosa* had antagonistic activity against the necrotrophic pathogens *B. cinerea* and *P. expansum* on stored apple fruits (Li *et al.*, 2011); *K. fusiformata* (syn. *Pseudozyma fusiformata*) produces ustilagic acid, a metabolite with fungicidal activity (Golubev *et al.*, 2001), which could inhibit the growth of the phytopathogenic fungi *Phomopsis helianti* and *Sclerotinia sclerotiorum* (Kulakovskaya *et al.*, 2007). The genus *Stemphylium* includes several plant pathogenic species, including apple pathogens such as *S. botryosum* and *S. herbarum* (Behr, 1960; Jijakli & Lepoivre, 2004) and S. *vesicarium* (Weber & Dralle, 2013). However, *Stemphylium* sp. was also reported as a hyperparasite on Erysiphales fungi (Sucharzewska *et al.*, 2012). Some species of *Dissoconium* are have been isolated from sooty blotch and flyspeck of apples (Li *et al.*, 2012). However, *D. aciculare* displayed antagonistic activity against the powdery mildew causal agent *Erysiphe* spp. (Kiss, 2003).

Interestingly, most fungal taxa with lower relative abundance in the resistant cultivars compared to the susceptible ones were Basidiomycetous yeasts in the class Tremellomycetes. The genera *Vishniacozyma*, *Dioszegia*, *Gelidatrema*, *Genolevuria*, *Bulleromyces*, *Papiliotrema* (Tremellales) and *Filobasidium* (Filobasidiales) have been reported from a wide range of substrates, including plant tissues (Glushakova & Kachalkin, 2017) and decaying wood (Behnke-Borowczyk *et al.*, 2018). Species in these genera are known, or speculated, to have antifungal activity based on their morphology and physiology

(Boekhout *et al.*, 2011; Begerow *et al.*, 2017). For example, *Bulleromyces albus* (anamorph: *Bullera alba*) was shown to have antifungal activity against different species of Ascomycota and Basidiomycota (Golubev *et al.*, 1997), and haustoria have been observed in *Dioszegia* spp., suggesting a mycoparasitic lifestyle (Connell *et al.*, 2010). However, the ecological role of these Tremellales and Filobasidiales genera is still largely unexplored.

Fungal endophytes with higher relative abundance in resistant genotypes and reported antifungal activity, might directly antagonise *N. ditissima*, alone or in synergy with other microbial species. Some of these are also known as plant pathogens. However, ecological functions of endophytes are context-dependent (Newton *et al.*, 2010; Busby *et al.*, 2016). The same microorganism might behave as a pathogen, remain neutral or act as a beneficial mutualist, based on factors determined by the host, the abiotic or the biotic environment.

It is possible that some of the bacterial genera, that in this study displayed lower relative abundance in resistant genotypes, represent pathogen facilitators for *N. ditissima*. It is difficult to understand their possible ecological role in the establishment of the infection and in development of canker symptoms. *Pseudomonas* sp. and *Sphingomonas* sp., as well as many of the fungal genera, include species with the ability to parasitize other fungal organisms. Hence, they might antagonize fungi that would otherwise have an antagonistic effect against *N. ditissima*.

To understand the ecological role of the differentially abundant endophytes identified in this present study, as well as their potential as biocontrol agents against *N. ditissima*, different approaches are possible. Targeted isolation of specific endophytes could be attempted, followed by tests *in vitro,* or *in planta*, for antagonism against *N. ditissima*. The abiotic and biotic environment have a significant influence on endophyte interactions. In particular, the ecological function of an individual endophyte is likely to depend on the presence of other endophytes in the plant host. In fact, the order of arrival of different inocula on the plant has been reported to influence the nature of interactions between endophytes, the host and its pathogen (Adame-Álvarez *et al.*, 2014). Therefore, manipulation experiments employing microbial communities instead of individual microorganisms may help achieve more reliable and robust results. The relative abundance of endophytes across the different apple genotypes employed in this present work could be utilised to design and assemble complex inocula for experiments on different apple cultivars.

Several bacterial and fungal genera were found with differential relative abundance in canker-resistant apple genotypes compared to susceptible ones. Most fungal genera included members with known or predicted antifungal activity, and some have been already successfully tested as biological control agents against a variety of plant-pathogenic fungi. It

is not possible to exactly infer the ecological role of such genera in the pathosystem, and only speculations can be made. However, these results could be used to inform targeted approaches to further the research in *N. ditissima* biological control. Manipulation studies, employing either individual species inocula or complex microbial communities, could be carried out to evaluate apple tree endophytes as biocontrol agents against *N. ditissima*.

Conclusions

This project compared different methods for the detection of symptomatic and asymptomatic *N. ditissima* in woody plant samples. These were a microbiological method, consisting in the isolation of the pathogen on selective medium, a serological method (ELISA) and a DNA-dependent method (qPCR) developed by (Ghasemkhani *et al.*, 2016a). Subsequently, the asymptomatic colonisation of the apple tree by *Neonectria ditissima* was studied prior to and after visual symptoms occurred, by artificially infecting plants and then detecting the fungus utilising either re-isolation or qPCR. Finally, the diversity of bacterial and fungal endophytes associated with apple cultivars differing in European apple canker susceptibility was studied with a Next Generation Sequencing approach (meta-barcoding). The relative contribution of orchard site, scion genotype and rootstock genotype to the overall endophyte diversity was assessed, and specific endophytes associated with canker-resistant and susceptible cultivars were identified.

The main findings of the project are summarised below:

- The DNA-based assay (qPCR) developed by Ghasemkhani *et al.* (2016a) was the most reliable tool for detecting *N. ditissima* asymptomatic infection in apple trees;
- *N. ditissima* can remain asymptomatic inside the host tissues for several months before visual symptoms appear; asymptomatic *N. ditissima* infections were detected in artificially inoculated pruning wounds and leaf scars up to four and seven months after the inoculation, respectively;
- In the absence of visual symptoms, *N. ditissima* appears to be restricted to the outmost tissues of the infected leaf scar or pruning wound; however, once symptoms develop, internal colonisation may occur and the fungus can be tracked in the internal tissues at 10-15 mm from the edge of the canker lesion; internal colonisation during the symptomatic phase occurred at a slow rate, with 27% internode samples at 10-15 mm from cankers testing positive to the qPCR up to seven months after the inoculation;
- The orchard of planting had the greatest influence on global diversity of both bacterial and fungal endophytes, followed by the scion genotype, whereas the rootstock influence was small; a significant and biologically important portion of the variability explained by the scion genotype was associated with the canker susceptibility level;
- Several bacterial and fungal genera with significantly different relative abundance across the two groups of cultivars were found, and their possible ecological functions and interactions with *N. ditissima* were discussed.

The outcomes of this present project help identify short-term actions to improve European apple canker management, as well as long-term, high-priority goals for *N. ditissima* research.

The results of the study on *N. ditissima* internal colonisation of apple tissues strongly suggest that the pathogen is not a systemic endophyte. Nevertheless, it cannot be excluded that, following infection, the fungus locally develops as an endophyte as far as the disease remains in its asymptomatic phase.

The results of the study on apple endophyte diversity highlighted similarities across cultivar-specific endophyte profiles which may partially explain the different levels of *N. ditissima* resistance observed across different apple cultivars. Additionally, they suggest that horizontal colonisation by microbial inoculum from the local environment plays a major role in shaping the endophyte profile. Several of the fungal species which displayed different relative abundance between canker resistant and susceptible cultivars can antagonise fungi or promote plant growth, suggesting that they might modify disease expression (Busby *et al.*, 2016). However, due to the complexity of the apple associated microbiome and of all its potential interactions, it is difficult to dissect the individual effects on *N. ditissima*

Based on the outcomes of this present project, internal inoculum is unlikely to represent an important source of infection, either in nurseries or young orchards, both prior to and after visual symptoms have occurred. By contrast, perithecia could be observed on cankers as early as eight months after the inoculation of trees during propagation (Børve *et al.*, 2019). Overall, this combined evidence suggests that splash-dispersed or airborne conidia and spores produced on active cankers, rather than internally spread inoculum, might account for most infections in nurseries and for multiple cankers on the same tree in young orchards. Therefore, an effective strategy for European apple canker management should be based on:

- frequent monitoring of trees in the nursery or in the orchard, and prompt removal of cankers as potential sources of inoculum, as also suggested by Børve *et al.* (2018, 2019); neighbouring apple and pear orchards should be also monitored as potential sources of inoculum;
- as pruning out of cankers creates new wounds, spray treatments or wound painting with antifungal or protectant products are also recommended;
- limiting wounding during propagation could also help prevent *N. ditissima* infection (Saville & Olivieri, 2019);
- treatments targeted at key times for infection (bud-break, petal fall and leaf fall) are also recommended to efficiently reduce disease incidence (Saville & Olivieri, 2019);

Latent nursery infections caused by several different fungal pathogens are emerging as a common problem in the apple industry, potentially posing a severe risk for young orchards (Havenga *et al.*, 2019; Meng *et al.*, 2019). As such preventing latent infections during the propagation phase and/or detecting them prior to the production phase represents the first line of defence against *N. ditissima*.

N. ditissima control, either in the nursery or in the orchard, could also be achieved with the application of biocontrol agents (BCAs) as an additional control measure in alternative to synthetic fungicides or as a part of integrated control programs (Saville & Olivieri, 2019). Overall, the results of the internal colonisation study and of the apple tree endophyte survey have important consequences for the feasibility of apple endophytes manipulation studies. The environment had a greater impact on leaf scar endophyte diversity compared to the cultivar genotype. Hence, much of the endophyte diversity appeared to be non-specific to the scion cultivar. This suggests that significant manipulation of the apple tree microbiota might be possible. The fungal and bacterial community associated with leaf scars could be manipulated to antagonise *N. ditissima*, by augmenting its members or adding new ones. Moreover, since *N. ditissima* is only found at the entry site until it remains asymptomatic, it would be possible to directly target the pathogen with the BCA treatment and ensure their co-localisation. Altogether, this evidence encourages further exploration of the biocontrol potential of apple tree endophytes against *N. ditissima*.

Knowledge and Technology Transfer

- Presentation at the AHDB Horticulture Studentship Conference 2016, held at the Stratford Manor Hotel, Warwickshire, UK, 16th November 2016; title: "*Understanding endophytes to improve tree health*", by Leone Olivieri
- Presentation at the RHUL Postgraduate Symposium 2017, held at the Royal Holloway University of London, Egham, Surrey, UK, 26th April, 2017; title: "*Behind the curtains of the European apple canker pathosystem: unravelling lifestyle and interactions of apple tree endophytes*", by Leone Olivieri
- Presentation at the AHDB Fruit Agronomists' Day, held at NIAB EMR, East Malling, Kent, UK, 13th September 2017; title: "*Endophytes and Tree Health*", by Leone Olivieri.
- Presentation and poster presentation at the British Tomato Conference 2017, held at the Chesford Grange Hotel and Conference Centre, Kenilworth, Warwickshire, UK, 21st September 2017; presentation and poster title: "*Understanding Endophytes to improve Tree Health*", by Leone Olivieri
- Presentation at the Third International Workshop on Apple Canker and Replant Disease, held at NIAB EMR, East Malling, Kent, UK, 1st November 2017; title: "*Culture-dependent and immune-based approaches to understand* Neonectria ditissima *asymptomatic infection*", by Leone Olivieri
- Poster presentation at the AHDB Crops Studentship Conference 2017, held at the Stratford Manor Hotel, Warwickshire, UK, 6th November 2017; title: "*Understanding endophytes to improve tree health*", by Leone Olivieri
- Presentation at the NIAB outreach event: 'Your Plant Science – maximising the potential of Crop Researchers', held at NIAB Park Farm, Cambridge, UK, 20th November 2017; title: "*New insight into European apple canker pathosystem: providing alternative management strategies*", by Leone Olivieri
- Poster presentation at the RHUL Postgraduate Symposium 2018, held at Royal Holloway University of London, Egham, Surrey, UK, 24th April 2018; title: "*Understanding* Neonectria ditissima *latent infection*", by Leone Olivieri
- Participation into the AHDB Crops Studentship Industry Visit 2018; Lancaster University and agri-food companies in North West England, Lancashire, 3rd-4th July 2018.
- Orchard visit, academic exchange meeting and presentation at the Sino-British Joint Symposium on Fruit Tree Disease, held at the North West Agriculture and Forestry

University (NWAFU), Yangling, Shaanxi, China, 21st-23rd October 2018; presentation title: "*Culture-dependent, molecular, and serological tools to understand* Neonectria ditissima *asymptomatic infection*", by Leone Olivieri

- Participation into the 'Practical Course in Plant Pathogen Diagnostics', held at NIAB EMR, Kent, UK, 6th November 2018; the workshop covered theoretical and practical demonstrations of core plant pathogen diagnostics skills; diseases on annual and perennial crops; molecular detection of pathogens; high throughput detection and imaging tools for large scale environmental monitoring.
- Presentation at the AHDB Crops Studentship Conference 2018, held at the St Johns Hotel, Solihull, Warwickshire, UK, 26th November 2018; title: "*New insight into European apple canker pathosystem: providing alternative management strategies*", by Leone Olivieri
- Presentation at the EMRA/AHDB Horticulture Tree Fruit Day 2019, held at NIAB EMR, East Malling, Kent, UK, 28th February 2019; title: "*Can the apple canker fungus spread unseen in the tree before visual symptoms?*", by Leone Olivieri
- Presentation at the MBPP 2019, held at the John Innes Centre, Norwich, UK, 18th March 2019; title: "*Is* Neonectria ditissima *endophytic? Detecting the asymptomatic colonisation of plant tissue by a fungal tree pathogen*", by Leone Olivieri
- Presentation of the PhD project at the RHUL Postgraduate Symposium 2019, held at the Royal Holloway University of London, Egham, Surrey, UK, 1st May 2019 ; title: "*The European apple canker fungus,* Neonectria ditissima*: new insights into disease anatomy and pathoecology*" by Leone Olivieri
- Participation into the AHDB Crops Studentship Industry Visit 2019: James Hutton Institute and agri-food companies in Scotland; location: Dundee, Scotland; 8th-9th July 2019.
- Presentation of the PhD project at 'Plant Health 2019' (American Phytopathological Society annual meeting), held in Cleveland, Ohio, USA, 5th August 2019; title: "*Endophytes and tree health: disease anatomy and microbial ecology of the* Neonectria ditissima *pathosystem (European apple canker)*" by Leone Olivieri
- Poster presentation at the international symposium 'miCROPe 2019' ('Microbe-assisted crop production, opportunities challenges and needs'), held in Vienna, Austria, 4th December 2019; title: "*Microbial ecology of the European apple canker pathosystem (*N. ditissima*)*", by Leone Olivieri

- Presentation at NIAB EMR seminar, held at NIAB EMR, East Malling, Kent, UK, 4th February 2020; title: "*The anatomy of the asymptomatic infection of* Neonectria ditissima *(European apple canker)*", by Leone Olivieri.
- Presentation at the Fourth International Workshop on European fruit tree canker, online event, 5th November 2020, title: "Contributions of scion and rootstock genotypes, and orchard location to apple endophyte community structure, with specific reference to the cultivar canker susceptibility", by Leone Olivieri

References

Adame-Álvarez RM, Mendiola-Soto J, Heil M, 2014. Order of arrival shifts endophyte-pathogen interactions in bean from resistance induction to disease facilitation. *FEMS Microbiology Letters* **355**, 100–107.

Amponsah NT, Walter M, Beresford RM, Scheper RWA, 2015. Seasonal wound presence and susceptibility to *Neonectria ditissima* infection in New Zealand apple trees. *New Zealand Plant Protection* **68**, 250–256.

Anders S, Huber W, 2010. Differential expression analysis for sequence count data. *Genome Biology* **11**, R106.

Arnold A, Mejìa L, Kyllo D, Rojas E, Maynard Z, Herre E, 2003. Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences* **100**, 15649–15654.

Arrigoni E, Albanese D, Olivieira Longa, Maria C, Angeli D, Donati C, Ioriatti C, Pertot I, Perazzolli M, 2020. Tissue age, orchard location and disease management influence the composition of fungal and bacterial communities present on the bark of apple trees. *Environmental Microbiology* **22**, 2080–2093.

Arrigoni E, Antonielli L, Pindo M, Pertot I, Perazzolli M, 2018. Tissue age and plant genotype affect the microbiota of apple and pear bark. *Microbiological Research* **211**, 57–68.

Beckers B, Op De Beeck M, Thijs S, Truyens S, Weyens N, Boerjan W, Vangronsveld J, 2016. Performance of 16s rDNA primer pairs in the study of rhizosphere and endosphere bacterial microbiomes in metabarcoding studies. *Frontiers in Microbiology* **7**, 650.

Begerow D, Kemler M, Feige A, Yurkov A, 2017. Parasitism in Yeasts. In: Buzzini P, Lachance MA, Yurkov A, eds. *Yeasts in Natural Ecosystems: Ecology*. Cham: Springer, 179–210.

Behnke-Borowczyk J, Kwaśna H, Kokot K, Hałuszczak M, Łakomy P, 2018. Abundance and diversity of fungi in oak wood. *Dendrobiology* **80**, 143–160.

Behr L, 1960. Pleospora herbarum as the cause of a storage rot in Apples in Germany. *Phytopathologische Zeitschrift* **37**, 245–251.

Benjamini Y, Hochberg Y, 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B 57, 289–300. *Journal of the Royal Statistical Society, Series B* **57**, 289–300.

Berg G, Hallmann J, 2006. Control of plant pathogenic fungi with bacterial endophytes. In: Schulz B, Boyle C, Sieber T, eds. *Microbial Root Endophytes*. Berlin: Springer-Verlag,

53–69.

Berrie A, 2016. Evaluation of products for control of *Nectria galligena* on apples. *AHDB Horticulture*.

Boekhout T, Fonseca A, Sampaio JP, Bandoni RJ, Fell JW, Kwon-Chung KJ, 2011. Discussion of Teleomorphic and Anamorphic Basidiomycetous Yeasts. In: *The Yeasts (Fifth Edition)*. Elsevier B.V., 1339–1372.

Børve J, Dalen M, Stensvand A, 2019. Development of *Neonectria ditissima* infections initiated at grafting of apple trees. *European Journal of Plant Pathology* **155**, 1225–1239.

Børve J, Kolltveit SA, Talgø V, Stensvand A, 2018. Apple rootstocks may become infected by *Neonectria ditissima* during propagation. *Acta Agriculturae Scandinavica Section B: Soil and Plant Science* **68**, 16–25.

Burns M, Valdivia H, 2008. Modelling the limit of detection in real-time quantitative PCR. *European Food Research and Technology* **226**, 1513–1524.

Bus VGM, Scheper RWA, Walter M, Campbell RE, Kitson B, Turner L, Fisher BM, Johnston SL, Wu C, Deng CH, Singla G, Bowatte D, Jesson LK, Hedderley DI, Volz RK, Chagné D, Gardiner SE, 2019. Genetic mapping of the European canker (*Neonectria ditissima*) resistance locus Rnd1 from *Malus* 'Robusta 5.' *Tree Genetics and Genomes* **15**.

Busby PE, Ridout M, Newcombe G, 2016. Fungal endophytes: modifiers of plant disease. *Plant molecular biology* **90**, 645–55.

Cenis JL, 1992. Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Research* **20**, 2380.

Chase AB, Arevalo P, Polz MF, Berlemont R, Martiny JBH, 2016. Evidence for ecological flexibility in the cosmopolitan genus *Curtobacterium*. *Frontiers in Microbiology* **7**, 1–11.

Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM, 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research* **42**, D633–D642.

Connell LB, Redman R, Rodriguez R, Barrett A, Iszard M, Fonseca Á, 2010. *Dioszegia antarctica* sp. nov. and *Dioszegia cryoxerica* sp. nov., psychrophilic basidiomycetous yeasts from polar desert soils in Antarctica. *International Journal of Systematic and Evolutionary Microbiology* **60**, 1466–1472.

Crowdy SH, 1949. Observations on apple canker. III The anatomy of the stem canker. *Annals of applied biology* **36**, 483–495.

Crowdy SH, 1952. Observations on apple canker IV. The infection of leaf scars. *Annals of*

applied biology **39**, 569–580.

Deakin G, Tilston EL, Bennett J, Passey T, Harrison N, Fernández-Fernández F, Xu X, 2018. Spatial structuring of soil microbial communities in commercial apple orchards. *Applied Soil Ecology* **130**, 1–12.

Dewey FM, Li R, Swinburne T, 1995. A monoclonal antibody immunoassay for the detection of Nectria galligena in apple fruit and woody tissue. *EPPO Bulletin* **25**, 65–73.

Dubin HJ, English H, 1974. Factors Affecting Apple Leaf Scar Infection by *Nectria galligena* Conidia. *Phytopathology* **64**, 1201–1203.

Edgar RC, 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature Methods* **10**, 996–998.

Edgar RC, 2016. SINTAX: a simple non-Bayesian taxonomy classifier for 16S and ITS sequences. *bioRxiv*, 074161.

Edgar RC, Flyvbjerg H, 2015. Error filtering, pair assembly and error correction for next-generation sequencing reads. *Bioinformatics* **31**, 3476–3482.

Forsline PL, Aldwinckle HS, Dickson EE, Luby JJ, Hokanson SC, 2003. Collection, Maintenance, Characterization, and Utilization of Wild Apples of Central Asia. In: Janick J, ed. *Horticultural Reviews: Wild Apple and Fruit Trees of Central Asia*. Oxford, UK: John Wiley & Sons, Inc, 1–62.

Freeman S, Rodriguez RJ, 1993. Genetic conversion of a fungal plant pathogen to a nonpathogenic, endophytic mutualist. *Science* **260**, 75–78.

Gange AC, Dey S, Currie AF, Sutton BC, 2007. Site- and species-specific differences in endophyte occurrence in two herbaceous plants. *Journal of Ecology* **95**, 614–622.

Gardes M, Bruns TD, 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**, 113–118.

Garkava-Gustavsson L, Ghasemkhani M, Zborowska A, Englund JE, Lateur M, Van De Weg E, 2016. Approaches for evaluation of resistance to European canker (*Neonectria ditissima*) in apple. *Acta Horticulturae* **1127**, 75–81.

Ghasemkhani M, Holefors A, Marttila S, Dalman K, Zborowska A, Rur M, Rees-George J, Nybom H, Everett KR, Scheper RWA, Garkava-Gustavsson L, 2016a. Real-time PCR for detection and quantification, and histological characterization of *Neonectria ditissima* in apple trees. *Trees* **30**, 1111–1125.

Ghasemkhani M, Sehic J, Ahmadi-Afzadi M, Nybom H, Garkava-Gustavsson L, 2015. Screening for Partial Resistance to Fruit Tree Canker in Apple Cultivars. *Acta*

Horticulturae **1099**, 687–690.

Ghasemkhani M, Zborowska A, Garkava-Gustavsson L, Holefors A, Scheper R, Everett K, Nybom H, 2016b. Development of a qPCR detection procedure for fruit tree canker caused by *Neonectria ditissima*. *Acta Horticulturae* **1127**, 259–264.

Glushakova AM, Kachalkin A V., 2017. Endophytic yeasts in leaf galls. *Microbiology (Russian Federation)* **86**, 250–256.

Golubev W, Ikeda R, Shinoda T, Nakase T, 1997. Antifungal activity of *Bullera alba* (Hanna) Derx. *Mycoscience* **38**, 25–29.

Golubev WI, Kulakovskaya T V., Golubeva EW, 2001. The Yeast *Pseudozyma fusiformata* VKM Y-2821 Producing an Antifungal Glycolipid. *Microbiology* **70**, 553–556.

Gomez-Cortecero A, Saville RJ, Scheper RWA, Bowen JK, de Medeiros HA, Kingsnorth J, Xu X, Harrison RJ, 2016. Variation in host and pathogen in the *Neonectria/Malus* interaction; towards an understanding of the genetic basis of resistance to European canker. *Frontiers in Plant Science* **7**, 1365.

Grossi CEM, Fantino E, Serral F, Zawoznik MS, Fernandez Do Porto DA, Ulloa RM, 2020. Methylobacterium sp. 2A Is a Plant Growth-Promoting Rhizobacteria That Has the Potential to Improve Potato Crop Yield Under Adverse Conditions. *Frontiers in Plant Science* **11**.

Havenga M, Gatsi GM, Halleen F, Spies CFJ, van der Merwe R, Mostert L, 2019. Canker and Wood Rot Pathogens Present in Young Apple Trees and Propagation Material in the Western Cape of South Africa. *Plant disease* **103**, 3129–3141.

Herlemann DPR, Labrenz M, Jürgens K, Bertilsson S, Waniek JJ, Andersson AF, 2011. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME Journal* **5**, 1571–1579.

Illumina, 2019. 16S Metagenomic Sequencing Library Preparation.

Jijakli MH, Lepoivre P, 2004. State of the Art and Challenges of Post-harvest Disease Management in Apples. In: Mukerji KG, ed. *Fruit and Vegetable Diseases. Disease Management of Fruits and Vegetables.* Dordrecht: Springer, 59–94.

Kennel W, 1963. Zur Pathogenese des Obstbaumkrebses (Nectria galligena Bres.) am Apfel. *Die Gartenbauwissenschaft* **28**, 29–64.

Kennelly M, Cazorla F, de Vicente A, Ramos C, Sundin G, 2007. Pseudonomas syringae Diseaes of Fruit Trees. Progress Toward Understanding and Control. *Plant Disease* **91**, 4–17.

Kiss L, 2003. A review of fungal antagonists of powdery mildews and their potential as biocontrol agents. *Pest Management Science* **59**, 475–483.

Kulakovskaya E V., Kulakovskaya T V., Golubev VI, Shashkov AS, Grachev AA, Nifantiev NE, 2007. Fungicidal activity of cellobiose lipids from culture broth of yeast *Cryptococcus humicola* and *Pseudozyma fusiformata*. *Russian Journal of Bioorganic Chemistry* **33**, 156–160.

Landis JR, Koch GG, 1977. The measurement of observer agreement for categorical data. *Biometrics* **33**, 159–74.

Li HY, Sun GY, Zhai XR, Batzer JC, Mayfield DA, Crous PW, Groenewald JZ, Gleason ML, 2012. Dissoconiaceae associated with sooty blotch and flyspeck on fruits in China and the United States. *Persoonia: Molecular Phylogeny and Evolution of Fungi* **28**, 113–125.

Li R, Zhang H, Liu W, Zheng X, 2011. Biocontrol of postharvest gray and blue mold decay of apples with *Rhodotorula mucilaginosa* and possible mechanisms of action. *International Journal of Food Microbiology* **146**, 151–156.

Ligon JM, Hill DS, Hammer PE, Torkewitz NR, Hofmann D, Kempf HJ, Van Pée KH, 2000. Natural products with antifungal activity from *Pseudomonas* biocontrol bacteria. *Pest Management Science* **56**, 688–695.

Liu J, Abdelfattah A, Norelli J, Burchard E, Schena L, Droby S, Wisniewski M, 2018. Apple endophytic microbiota of different rootstock/scion combinations suggests a genotype-specific influence. *Microbiome* **6**, 1–11.

Liu J, Ridgway HJ, Jones EE, 2020. Apple endophyte community is shaped by tissue type, cultivar and site and has members with biocontrol potential against Neonectria ditissima. *Journal of Applied Microbiology* **128**, 1735–1753.

Love MI, Huber W, Anders S, 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15**, 550.

Mari M, Martini C, Spadoni A, Rouissi W, Bertolini P, 2012. Biocontrol of apple postharvest decay by *Aureobasidium pullulans*. *Postharvest Biology and Technology* **73**, 56–62.

McCracken AR, Berrie A, Barbara DJ, Locke T, Cooke LR, Phelps K, Swinburne TR, Brown AE, Ellerker B, Langrell SRH, 2003. Relative significance of nursery infections and orchard inoculum in the development and spread of apple canker (*Nectria galligena*) in young orchards. *Plant Pathology* **52**, 553–566.

McMurdie PJ, Holmes S, 2013. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* **8**, e61217.

McMurdie PJ, Holmes S, 2014. Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible (AC McHardy, Ed,). *PLoS Computational Biology* **10**, e1003531.

de Mendiburu F, 2020. agricolae: Statistical Procedures for Agricultural Research.

Meng X, Qi X, Han Z, Guo Y, Wang Y, Hu T, Wang L, Cao K, Wang S, 2019. Latent Infection of Valsa mali in the Seeds, Seedlings and Twigs of Crabapple and Apple Trees is a Potential Inoculum Source of Valsa Canker. *Scientific Reports* **9**, 7738.

Murray TD, Schroeder BK, Schneider WL, Luster DG, Sechler A, Rogers EE, Subbotin SA, 2017. *Rathayibacter toxicus*, other *Rathayibacter* species inducing bacterial head blight of grasses, and the potential for livestock poisonings. *Phytopathology* **107**, 804–815.

Newton AC, Fitt BDL, Atkins SD, Walters DR, Daniell TJ, 2010. Pathogenesis, parasitism and mutualism in the trophic space of microbe-plant interactions. *Trends in Microbiology* **18**, 365–373.

Nilsson RH, Larsson KH, Taylor AFS, Bengtsson-Palme J, Jeppesen TS, Schigel D, Kennedy P, Picard K, Glöckner FO, Tedersoo L, Saar I, Kõljalg U, Abarenkov K, 2018. The UNITE database for molecular identification of fungi: Handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Research* **47**, D259–D264.

Nirenberg H, 1976. Untersuchungen über die morphologische und biologische Differenzierung in der *Fusarium*-Section Liseola. *Mitteilungen der Biologischen Bundesanstalt für Land- und Forstwirtschaft* **169**, 1–117.

Oksanen J, Blanchet GB, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, Wagner H, 2019. vegan: Community Ecology Package.

Prince AM, Andrus L, 1992. PCR: how to kill unwanted DNA. *BioTechniques* **12**, 358–360.

R Core Team, 2019. *R: A Language and Environment for Statistical Computing* (R Foundation for Statistical Computing, Ed,). Vienna, Austria.

Saville RJ, 2014. A review of our current knowledge of *Neonectria ditissima* and identification of future areas of research. *AHDB Report*.

Saville RJ, Olivieri L, 2019. Fungal diseases of fruit: apple cankers in Europe. In: Xu X,, Fountain M, eds. *Integrated management of diseases and insect pests of tree fruit*. Cambridge: Burleigh Dodds Science Publishing, 59–83.

Schulz B, Wanke U, Draeger S, Aust HJ, 1993. Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. *Mycological Research* **97**, 1447–1450.

Sequerra J, Marmeisse R, Valla G, Normand P, Capellano A, Moiroud A, 1997. Taxonomic position and intraspecific variability of the nodule forming *Penicillium nodositatum* inferred from RFLP analysis of the ribosomal intergenic spacer and Random Amplified Polymorphic DNA. *Mycological Research* **101**, 465–472.

Sturz A V., Christie BR, Nowak J, 2000. Bacterial endophytes: potential role in developing sustainable systems of crop production. *Critical Reviews in Plant Sciences* **19**, 1–30.

Sucharzewska E, Dynowska M, Ejdys E, Biedunkiewicz A, Kubiak D, 2012. Hyperparasites of Erysiphales fungi in the urban environment. *Polish Journal of Natural Sciences* **27**, 289–299.

Swinburne TR, 1975. European Canker of Apple. *Review of Plant Pathology* **54**, 787–799.

Tilston EL, Deakin G, Bennett J, Passey T, Harrison N, O'Brien F, Fernández-Fernández F, Xu X, 2018. Candidate Causal Organisms for Apple Replant Disease in the United Kingdom. *Phytobiomes Journal* **2**, 261–274.

UNITE Community, 2019. UNITE USEARCH/UTAX release for Fungi. Version 18.11.2018.

Viera AJ, Garrett JM, 2005. Understanding interobserver agreement: the kappa statistic. *Family Medicine* **37**, 360–3.

Wachowska U, Irzykowski W, Jędryczka M, Stasiulewicz-Paluch AD, Głowacka K, 2013. Biological control of winter wheat pathogens with the use of antagonistic *Sphingomonas* bacteria under greenhouse conditions. *Biocontrol Science and Technology* **23**, 1110–1122.

Walter M, Glaister MK, Clarke NR, Von Lutz H, Eld Z, Amponsah NT, Shaw NF, 2015. Are shelter belts potential inoculum sources for *Neonectria ditissima* apple tree infections? *New Zealand Plant Protection* **68**, 227–240.

Walter M, Roy S, Fisher BM, Mackle L, Amponsah NT, Curnow T, Campbell RE, Braun P, Reineke A, Scheper RWA, 2016. How many conidia are required for wound infection of apple plants by *Neonectria ditissima*? *New Zealand Plant Protection* **69**, 238–245.

Weber RWS, 2014. Biology and control of the apple canker fungus *Neonectria ditissima* (syn. *N. galligena*) from a Northwestern European perspective. *Erwerbs-Obstbau* **56**, 95–107.

Weber RWS, Dralle N, 2013. Fungi associated with blossom-end rot of apples in Germany. *European Journal of Horticultural Science* **78**, 97–105.

Weber RWS, Hahn A, 2013. Obstbaumkrebs (*Neonectria galligena*) und die Apfelsorte "Nicoter" (Kanzi ®) an der Niederelbe. *Mitteilungen des Obstbauversuchsringes des Alten Landes* **68**, 247–256.

Weber RWS, Zabel D, 2010. Winterfrost als Ursache des aktuellen Triebsterbens an jungen Apfelbäumen. *Mitteilungen des Obstbauversuchsringes des Alten Landes* **65**, 198–204.

Webster A, Cross J, Berrie A, Johnston D, Biddelcombe C, Pennell D, 2001. *The Best Practice Guide for UK Apple Production*. London.

Weller DM, 2007. *Pseudomonas* biocontrol agents of soilborne pathogens: Looking back over 30 years. *Phytopathology* **97**, 250–256.

Wheeler B, Torchiano M, 2016. lmPerm: Permutation Tests for Linear Models.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innes MA, Gelfand DH, Sninski JJ, White TJ, eds. *PCR Protocols. A guide to methods and applications*. San Diego: Academic Press, 315–322.

Yashiro E, Spear RN, Mcmanus PS, 2011. Culture-dependent and culture-independent assessment of bacteria in the apple phyllosphere. *Journal of Applied Microbiology* **110**, 1284–1296.

Zhang Z, Schwartz S, Wagner L, Miller W, 2000. A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology* **7**, 203–14.

