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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

- Early work has paved the way for the development of novel procedures for diagnostics and control.

Background and expected deliverables

European apple canker is caused by the fungus *Neonectria ditissima* and affects apple trees in the orchard and fruits post-harvest. In the UK, the increased planting of susceptible cultivars is causing disease to become more and more important. Effective control measures are limited, due to the lack of products registered for use in apple orchards. Therefore, alternative control methods are strongly needed. Additionally, the pathogen can infect the plant during the propagation phase in nurseries and then enter a phase of latency, with the first canker lesions appearing up to three years after the trees are transplanted in the orchard. No diagnostic procedure is currently available for the early detection of this latent, asymptomatic infection.

Despite the great deal of information available on the disease epidemiology, there are substantial gaps in our knowledge of the basic biology of the pathogen which need to be addressed. An immunoassay, able to detect *N. ditissima* in infected plant material, is currently available and may be further developed for a use in diagnostic tests. However, its application requires a greater understanding of the anatomy and the temporal dynamics of the latent infection - that is where and when the fungus can be localised in plant tissues following successful infection. It is currently believed, but yet to be demonstrated, that the fungus can grow and develop asymptomatically within the plant, prior to symptom expression. This microbial lifestyle is known as endophytism.

Fungal and bacterial endophytes colonise internal plant tissues (contrarily to epiphytic microorganism, which are found on the plant surface) and cause inconspicuous and, at least transiently, asymptomatic infections. Some plant pathogens display this peculiar life-style during the early stages of infection, before switching to a pathogenic phase. Besides, several studies showed that some endophytes, acting alone or together with other endophytic species in the plant microbial ecosystem, can facilitate or antagonize plant pathogens. Therefore, they can modulate, together with the host genetics, disease expression. The study of the interactions between *N. ditissima* and the other apple tree endophytes may help to understand field resistance and provide access to untapped resources for the development of novel biocontrol strategies.

This project aims to localise the pathogen *in planta* during the asymptomatic phase of infection, with the aim of establishing an effective sampling and diagnostic procedure for the latent infection. I will also investigate the interactions between *N. ditissima* and apple tree endophytes, exploring the biocontrol potential of the apple tree endophytic microflora.

Summary of the project and main conclusions

It is currently unknown if the latent infection caused by *N. ditissima* consists in an incubation period with no colonisation of plant tissues or represents instead an endophytic phase of the pathogen life-cycle. If demonstrated, the ability of *N. ditissima* to develop in the tree as an endophyte, and to spread within plant tissues at distance from the infection site, would be highly relevant to disease management, currently based on pruning and removal of the infection from the orchard. More importantly, information on the localisation and spread of the fungus within the different plant tissues is required for the development of a sampling and diagnostic procedure, currently unavailable, to detect latent infections.

We tracked the spread of *N. ditissima* in apple trees by performing artificial inoculation of pruning wounds and re-isolation of the pathogen on an artificial growth medium. A high dose of inoculum was applied to fresh pruning cuts performed on trees of four different cultivars (Gala, Discovery, Cox and Golden Delicious). The presence of the pathogen, the distance at which it could be found from the entry wound and the tissues infected (bark or underlying wood) were assessed over time by means of traditional microbiological methods. *N. ditissima* was generally re-isolated, both prior to and after the first canker lesions appeared, from the apparently healthy woody tissue underneath the cambium, at a distance between 10 mm and 40 mm from the pruning wound. It was already known that the fungus can spread longitudinally within the stem of young apple trees, without producing any visible lesion, after the establishment of the first symptoms. Our result additionally showed that, when the entry point is represented by pruning wounds, the pathogen is localised in the internal woody tissues of the branch, at least within the first two months from the onset of infection. These findings represent the first step towards the development of a sampling strategy for the early diagnoses of latent infections, but they need to be complemented by additional information on the spread of the fungus within the plant via different types of wound and during longer periods.

The Enzyme-Linked Immunosorbent Assay (ELISA) technique was successfully used in the past for the detection of *N. ditissima* in symptomatic wooden tissues of apple tree. The ELISA is a quick and straightforward detection test based on the ability of antibodies to detect and bind to a specific target (antigen) in a liquid sample, and on the subsequent visualisation of this interaction by means of an enzymatic reaction which produces a colour change in the

sample. A different, but closely related, technique is that of Lateral Flow Devices (LFD), which are available as diagnostic tools for a number of fungal, bacterial and viral plant diseases. Antibodies are available which can recognise cellular components of *N. ditissima*. We worked on the optimisation of an ELISA assay, which once developed, would provide the cornerstone for the development of a LFD for the European apple canker. Our ELISA was able to detect the pathogen in woody apple tissues (bark and sapwood) even when the microbiological techniques were not successful (**Figure 1**). Results are promising, but further experiments to determine the limit of detection of the technique are necessary to develop a reliable tool.

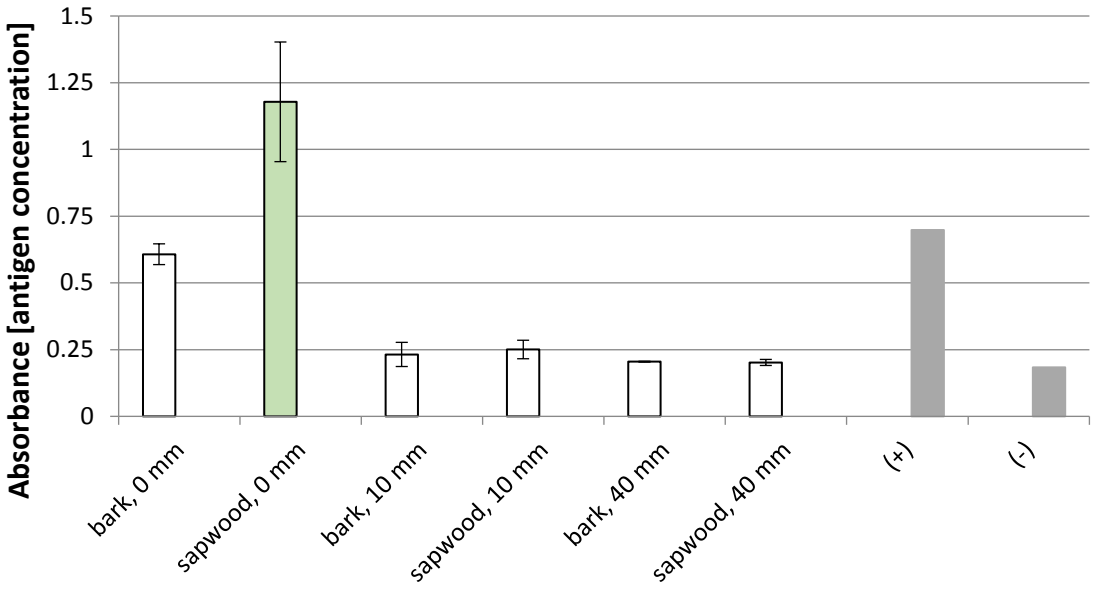

Figure 1 – ELISA on plant material from a natural infected apple branch. The measure of the concentration of *N. ditissima* antigens [absorbance] in bark and sapwood sections at different distances from the canker lesion (0, 10 and 40 mm) is reported. Grey bars correspond to positive (+) and negative (-) antigen. *N. ditissima* could be isolated only from the sapwood at the leading edge of lesion (green bar), but was detected by the ELISA also in the bark of the same branch section.

With the same protocol used to re-isolate *N. ditissima* from inoculated trees, we also collected fungal endophytes from four apple cultivars (Gala, Discovery, Cox and golden Delicious). Endophytes were classified in morphotypes, i.e. in groups based on their morphology on the artificial culture medium. A few of these fungal isolates were identified and their morphotype could be linked to reference species. Among the species identified, there were ubiquitous saprophytes previously found in association with apple leaves (such as *Alternaria* spp., *Epicoccum* spp. and *Cladosporium* spp.). The classification system based on morphotypes showed that the different apple cultivars were characterised by different endophyte profiles.

The next step of this study will involve the analysis of the correlation between the endophyte profiles characterising apple cultivars, showing different field susceptibility to European apple canker, and disease expression.

Financial benefits

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

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

Action points for growers

The current guidelines published in the Apple Best Practice Guide provide the best advice to achieve effective disease management by means of pruning, chemical control and cultural control. This current project is at an early stage but, based on our preliminary results, some suggestion can be given for the management of infected pruning wounds during the vegetative season:

- As soon as the first canker symptoms appear, branches should be immediately pruned to ensure complete removal of the infection; the minimum recommended distance from the canker lesion at which pruning should be performed is 5 cm
- Paring back of canker lesions should be avoided, and pruning should be performed instead; since the fungus is preferably localised in the sapwood, the removal of the canker lesion in the bark does not ensure removal of the infection.

SCIENCE SECTION

Introduction

Nectria canker (also known as European apple canker), caused by the fungus *Neonectria ditissima* (Tul. & C. Tul) Samuels & Rossman, is a major disease of apple tree and fruit. The disease is widespread across most apple-growing temperate regions in the world. Apple cultivars show different degrees of susceptibility to the canker, with most of both the established and the newly introduced varieties (such as Jazz, Braeburn, Rubens, Cameo, Kanzi and Zari) being particularly susceptible (Saville, 2014). The pathogen enters the plant via natural and artificial wounds, the main entry points include autumn leaf scars, fruit picking wounds and pruning cuts in the orchards, as well as wounds generated during the propagation process in the nursery. Evidence suggests that the fungus can also enter young trees during the propagation phase and remain latent until these are transplanted in the orchard, where the expression of disease symptoms can be promoted by abiotic stresses up to 3 years after transplant (McCracken et al., 2003). Losses occur at every stage of production and therefore are difficult to quantify. In the fields, *N. ditissima* causes cankers and die back of shoots, leading to loss of fruiting wood and increased pruning costs. Cankers on trunks in young orchards can be especially serious because they can girdle the stem and kill the whole plant, causing up to 10% of trees to be lost annually. Infections occurring at flowering time can result in fruit rots which either develop in orchards as eye rot (blossom-end rot) or, more commonly, remain asymptomatic and only develop post-harvest. Post-harvest losses can be up to 10% of stored fruit (Saville, 2014). In the UK, disease management almost entirely relies on pruning. Chemical control options are essentially limited to apple scab fungicides (e.g. Captan), which can be applied throughout the vegetative season and provide some protection against the apple canker. Copper fungicides, which provided good long-term protection during the dormant season (Saville, 2014; Weber, 2014), are no longer allowed since 2015. As a result of the lack of registered products for disease control, and due to the increasing planting of susceptible cultivars in the UK, apple canker incidence is increasing and is becoming more and more important (Saville, 2014).

Taxonomy and host range

Neonectria ditissima (Tul. & C. Tul) Samuels & Rossman was formerly known as *Nectria galligena* Bres. and *Neonectria galligena* (Bres.) Rossman & Samuels. Its anamorph is *Cylindrocarpon heteronema* (Berk. & Broome).

The host range of *N. ditissima* is wide: the first comprehensive list of host plants was provided by Flack and Swinburne (1977) and included more than 60 tree species from Europe and North America. Recently, Walter et al. (2015) searched the available literature and databases

and reported a total of 122 host species affected by the pathogen globally. Besides apple tree (*Malus* spp.), the pathogen can also infect pear tree (*Pyrus* spp.), but pear canker is much less severe than apple canker (Weber, 2014). It has been shown that the same isolates of *N. ditissima* can infect both apple and pear trees from neighbouring orchards (Defra Project Report OC9518, 2001). Many other broad-leaved trees and shrubs can be infected, some of which are commonly found in windbreaks surrounding apple orchards or in woodlands in their vicinity, and field observations suggest their epidemiological role as inoculum reservoirs. Walter *et al.* (2015) demonstrated that isolates from apple trees can produce conidia following infection of non-apple hosts, and these can in turn re-infect apple trees. Therefore, even though no conclusive results have been published on the actual risk of windbreaks acting as inoculum sources so far, appropriate windbreak management should not be neglected in apple canker control programs (Saville, 2014; Walter et al., 2015).

Cross-inoculation experiments performed by Flack and Swinburne (1977) with Northern-Irish isolates of the pathogen, which they referred to as *N. galligena*, suggested that *formae speciales* may also exist. However, no further studies have been pursued since then on *Neonectria* host specialisation. If confirmed, this would be a feature of practical relevance for the choice of the appropriate plant species hedgerows (Weber, 2014).

Geographic distribution

Apple canker disease occurs worldwide, having been reported from apple growing regions in Europe, North America, Chile, Australia, New Zealand, Japan and South Africa (Beresford & Kim, 2011; CAB International, 1985; Walter et al., 2015). Wet and temperate climates are especially conducive to the disease and areas above 52° latitude with frequent summer rainfall have been pointed out as the most prone to European canker, including the fruit rot phase of the disease (Beresford & Kim, 2011). Northern Germany and the Southern part of the UK, which are respectively above and slightly below such latitude, can be considered high-risk areas (Saville, 2014; Weber, 2014). Indeed, in the UK the development stages of the host characterized by high susceptibility, i.e. blossom period, preharvest and leaf fall, often coincide with climatic conditions favourable for the production of the inoculum and the onset of infection (Saville, 2014).

Life cycle and spore morphology

The disease cycle of *N. ditissima* on apple trees and fruits is shown in **Figure 2**. The overwintering source of inoculum in orchards is represented by mycelium or perithecia on cankers. Spores are produced on cankered tissues under moist conditions. Conidia (asexual spores) are formed in white-cream coloured sporodochia. Red perithecia, bearing the ascospores (sexual spores), only appear on older cankers (minimum 2 years old). Conidia

are produced first, from early spring until early autumn, and they are dispersed by rain within short distances (on the same tree or onto adjacent trees). Ascospores can be either oozed and splash dispersed locally, or ejected from the perithecium and wind-dispersed, thus can spread across long distances. Both sexual and asexual spores can infect any wound on woody tissues and fruits (Saville, 2014; Weber, 2014).

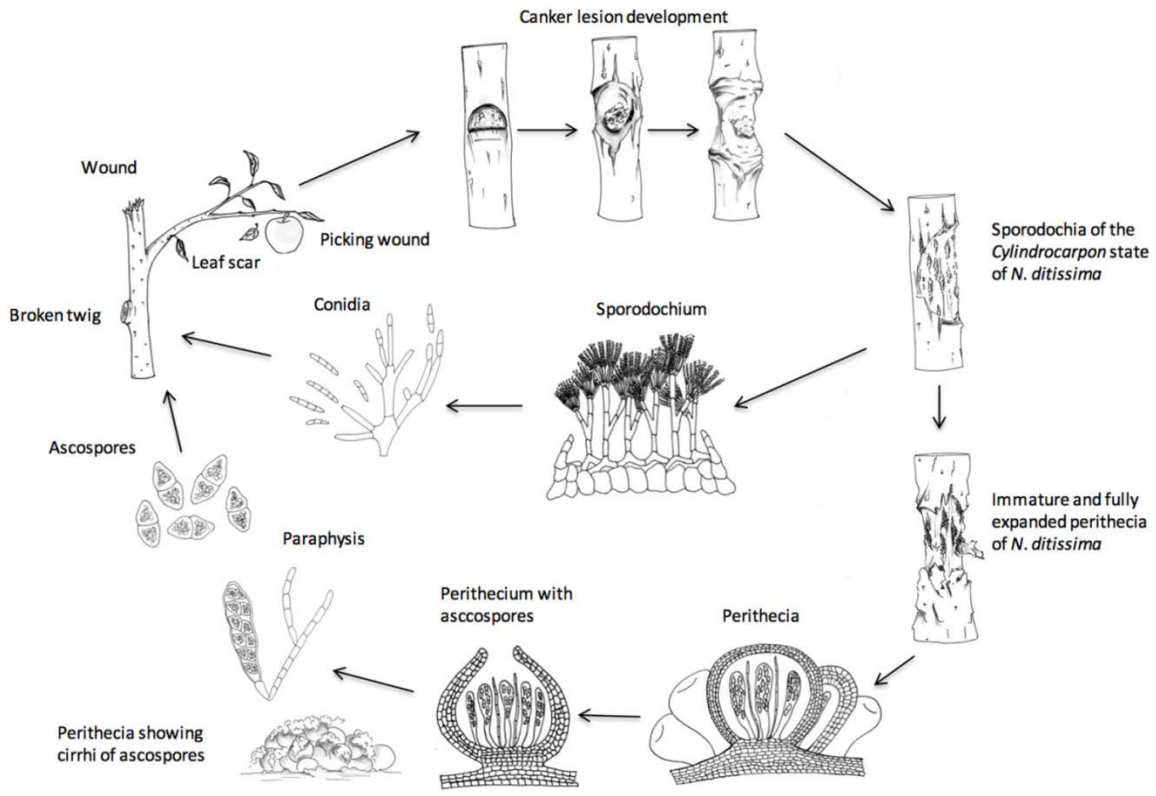

Figure 2 – Disease cycle of apple canker and fruit rot caused by *N. ditissima* (Gomez-Cortecero *et al.*, 2016; after Agrios, 1997).

Symptoms and anatomy of canker

"Canker" is a general term used to describe a typical lesion on woody plants, consisting in an open wound surrounded by swollen bark and caused by several plant pathogens and pests (Saville, 2014). The infection process of European apple canker is initiated upon entry of *N. ditissima* into the host tissue through wounds, which represent the only access point for the fungus (Crowdy, 1949). Once infection has been established, discoloration of the bark is initially observed as the epidermis turns pale brown and flakes off. At the same time, the underlying bark tissue becomes necrotic, blackened and sunken in appearance, as the cortex and the phloem gradually dry out and separate from the wood, eventually falling off and exposing the xylem (Crowdy, 1949; Weber, 2014). Usually, such symptoms are seen during spring, even though they can manifest any time of year at temperatures above freezing

(Weber, 2014). A few weeks after the manifestation of these symptoms, white or pale yellow sporodochia develop on the dead bark tissue. On cankers older than 2 years, bright red perithecia appear (Saville, 2014; Weber, 2014). From the initial entry point on stems, the fungus spreads more rapidly longitudinally than transversally, while the host tissues produces successive phellogen layers which are presumed to mechanically restrain the pathogen (Crowdy, 1949; Swinburne, 1975). As a result, old cankers on main stems (>2 years old) are elliptical, displaying a swollen, oyster shell like appearance, with radial layers of host produced phellogen radiating from the initial infection point in the centre of the lesion (Saville, 2014). In time, the canker can girdle the stem and kill it (Crowdy, 1949). Some observations have led to the speculation, not yet verified to date, that the fungus produces a diffusible toxin which spreads from the infection site to adjoining plant tissues: these consist in the death of host cells in advance of the hyphae (Crowdy, 1949; Wiltshire, 1922; Zeller, 1926), the death of distal leaves before girdling has occurred and the staining of internal wood tissue away from the lesion (Saville, 2014).

Crowdy (1949) provided the first detailed description of the anatomy of the European canker on naturally infected apple trees. *N. ditissima* colonises the cortex and the phloem at first, then enters the xylem through medullary rays (Goethe, 1877; Zeller, 1926) and reaches the lumen of vessels and tracheids via cell pits. The fungus is apparently unable to penetrate the lignified cell walls and no sign of wood disintegration is observed. More recently, Ghasemkhani et al. (2016) confirmed these early observations in a light-microscopy histopathology study on artificially inoculated young apple trees. In their study, extracellular and intracellular mycelium could be observed in all cell types (cortex, phloem, xylem, medullary rays and pith) in the canker lesions. Cortex parenchyma cells appeared deformed and collapsed, whereas vascular tissue in the wood never showed any sign of disintegration and cells retained their shape and structure even when infected.

Growth of *N. ditissima* in the xylem beyond the leading edge of the canker lesion was demonstrated with microbiological methods (Weber & Hahn, 2013) and serological methods (Dewey, Li, & Swinburne, 1995). Weber & Hahn (2013) showed that *N. ditissima* can spread vertically in the xylem of young apple trees of the highly susceptible cultivar Kanzi by up to 20 cm beyond the edges of the canker lesion, within 6 months of the first symptoms becoming visible on bark.

Epidemiology

Production and release of both conidia and ascospores is favoured by rainfall and therefore varies according to the climate. Conidia are usually produced whenever moisture is sufficient and temperature is above freezing (Weber, 2014). In Northern Germany and the UK they are

usually found from spring until autumn (Saville, 2014; Weber, 2014). In the same region, perithecia appear starting from late summer or autumn, under wet weather and cool temperatures, and their development may continue throughout winter into spring as long as the conditions remain mild (Weber, 2014). Considerable disagreement can be found in literature regarding the main period of ascospore production and discharge. According to Weber (2014), in Northern Germany, the major peak occurs in autumn, whereas other authors (Munson, 1939; Swinburne, 1971) identified spring as the season of peak discharge. In general, except for dry periods in summer or freezing conditions in winter, *N. ditissima* inoculum is available all year round in Northwestern Europe.

Many types of wound are available for infection throughout the year (Saville, 2014; Weber, 2014). Autumnal leaf scars are widely considered one of the main entry points for the pathogen (Graf, 1976; Saure, 1961b; Swinburne, 1975). In the UK, entry points caused by bud break (in early spring) and detachment of rosette leaves (in late spring) are also considered highly relevant (Swinburne, Cartwright, Flack, & Brown, 1975). In new Zealand, wounds produced by fruit thinning and picking are currently regarded as the most important entry sites (Amponsah, Walter, Beresford, & Scheper, 2015). Other entry points for the pathogen include pruning wounds, bark cracks caused by frost in winter or by growth in summer and wounds caused by insects or hail in summer (Weber, 2014).

Wounds on woody hosts become more and more resistant to infections as they heal over time, and wound age has been demonstrated to affect the susceptibility to European apple canker. The incidence of canker lesions on leaf scars (Wilson, 1966) and pruning cuts (Xu, Butt, & Ridout, 1998) was shown to be greater on fresh wounds than on older ones. This factor is highly relevant for disease management in the orchard (Saville, 2014).

The European apple canker is especially destructive when it occurs on the trunk of newly planted trees, since these need to be uprooted, resulting in serious economic losses (Weber, 2014). In an extensive epidemiological study conducted in the UK, McCracken et al. (2003), using genetic fingerprinting techniques, showed that pathogen isolates from trunk cankers on trees in newly established orchards were genetically similar, suggesting a common origin (i.e. the nursery). By contrast, isolates from peripheral cankers were shown to belong to different populations, therefore pointing at a local origin of these infections. Their work also showed that infections originated in nurseries can remain latent for up to 3 years following propagation before the first symptoms appear.

Diagnostics

The symptoms of European canker may vary depending on a number of conditions: the stage of disease development, the age of the infected trees and the cultivar affected (Gustavsson,

2012). Cankers caused by *N. ditissima* on twigs can be confused with those caused by *Neofabraea malicorticis* (syn. *Cryptosporiopsis curvispora*), thus being microscopic observation of spores is necessary for diagnostics purposes (Braun, 1997; Ghasemkhani, Holefors, et al., 2016). Diagnosis by direct isolation from infected wood can be difficult. In fact, even though some selective media which enable the isolation of the pathogen from apple tissues have been described (Amponsah et al., 2014; McCracken et al., 2003), the presence of other, faster growing fungal pathogens and endophytes such as *Fusarium lateritium*, *Botrytis cinerea* and *Penicillium expansum* still represents an issue (Langrell, 2000; Langrell, 2002; Li, 1995). Besides, consistent evidence has been produced that young apple trees can be asymptomatically infected during the propagation phase in nurseries (Brown et al.,1994; Langrell, 2000; Li, 1995; Lovelidge, 1995; McCracken et al., 2003). Recently, Wenneker *et al.* (2017) developed a methodology to detect asymptomatic infections in nursery trees by transferring them into a climate chamber with high temperature and relative humidity before they are transferred in the orchard. However, their method generally required a considerable amount of time, around 8 weeks, before the trees developed symptoms.

A sensitive and reliable diagnostic method is highly needed for fast and accurate identification of the pathogen and to assess its frequency and occurrence in propagation material (Langrell, 2002). Molecular diagnostics is widely used in plant pathology, being an effective tool for both epidemiological studies and diagnostic applications in the fields (Saville, 2014). Recently, Ghasemkhani and collaborators (2016) developed a highly specific PCR-based assay, which was able to detect *N. ditissima* and to distinguish it from closely related fungal species. They could quantify pathogen biomass in artificially inoculated apple bark and wood with visible necrosis symptoms, however the assay did not detect the fungus in symptomless, inoculated tissue or in apparently healthy tissue surrounding the infected area. Serological methods were also made available. Dewey and colleagues (1995) developed a monoclonal antibody which allowed the detection of *N. ditissima* in woody tissue by means of Enzyme-Linked Immunosorbent Assay (ELISA), which might be promising for the development of a field-based diagnostic device, such as a lateral-flow device (LFD; Saville 2014). They were also able to visualize the fungus in the cork and wood tissues in immunofluorescence assays. According to Saville (2014), two are the main challenges in the development of molecular tools for the diagnosis of *N. ditissima*: the difficulty in purifying the DNA extracted from plant woody tissues, which contain PCR inhibitor compounds, and the need for sampling strategies which can ensure representative results. The latter especially represents a major issue to be tackled. In fact, even though molecular tools allow the detection of asymptomatic infections and could therefore be used to test propagation material in nursery certification schemes, sampling tissues is destructive and cannot be applied to every individual plant. Moreover, the

pathogen can infect different types of tissue, thus making the selection of the tissues to sample critical for the development of a reliable diagnostic procedure.

Control

The control of the apple canker is implemented through a combination of cultural methods and application of protectant fungicides (Saville, 2014; Weber, 2014). Nevertheless, the current bans on several chemical products which have been previously used against the pathogen, the difficulties in implementing an efficient cultural control and the lack of alternative strategies are likely to lead to an increase in the severity of the disease in the following years.

Cultural control consists of the removal of inoculum from the orchard, nowadays representing the cornerstone of disease control. The procedures include pruning out of new cankers and paring back of lesions on scaffold branches, as well as thorough removal of infected material from the orchard. As Weber (2014) pointed out, the proper timing for pruning remains controversial: in winter it is more practical because the cankers are easily spotted on the trees (Palm, 1975), but this produces wounds that heal slower and remain susceptible for longer periods (De Jong & Van der Steeg, 2012; Van der Scheer, 1974). It has been suggested that under Northwestern Europe climatic conditions the most important period for pruning begins at flowering, when the new cankers become visible on trees (Kennel, 1963; Saure, 1974), and Weber & Hahn (2013) pointed out that susceptible varieties should be pruned several times during the growing season. As an additional measure, covering of pruning wounds with wound paints is recommended, since it can provide some protection during the time required by the plant to produce a callus layer (Saville, 2014; Weber, 2014). Paints can either provide a physical barrier against further infections, or have fungicidal/biological activity, or both. The procedure must be performed immediately after pruning, but being costly and time consuming it is seldom practiced. Additionally, some of the few fungicidal wound dressings which were available for use on apple orchards in the UK are no longer permitted (Bordeaux paste) or no longer marketed (Bezel, containing tebuconazole). Saville (2014) highlighted the possibility of testing wound sealing products from the viticulture industry, such as Vitiseal and Blocade. Since pruning entails loss of fruiting wood, a general reluctance of the growers towards this measure has been observed when the plant portion to be removed is substantial (Walter, personal communication). Adjusted nitrogen fertilisation and water programmes as well as the use of growth regulators could be used to control the growth rate and consequently reduce the incidence of infections resulting from growth cracks. However, further investigations are required in this area (Saville, 2014). The limitation of wounding during the propagation phase in nurseries may help reduce the asymptomatic infections, which are responsible of severe losses in young orchards. This can be achieved by adopting a suitable propagation method.

It has been reported that different rootstocks can also influence the susceptibility to the disease: M9 is considered to be relatively susceptible compared to M1 and M12 (Moore, 1960).

Apple scab (*Venturia inaequalis)* fungicides (during the growing season) and copper fungicides (during autumn and winter) represented the most widely used products for the control of the apple canker in Northwestern Europe (Saville, 2014; Weber, 2014). However, the new legislation prevents the use of copper for fungicidal use in the UK. Fungicides used against apple scab during the vegetation period, such as Captan (Palm, 2009), Dithianon (Cooke, 1999; Swinburne, Cartwright, et al., 1975) and Dodine (Cooke, Watters, & Brown, 1993; Saure, 1961a; Swinburne, Cartwright, et al., 1975) are generally recognised to provide some degree of protection against the apple canker both in the UK (Saville, 2014) and in Germany. In the UK, the limited availability of products which can be used against the pathogen during the growing season has encouraged research on new chemicals (within the SDHI group) and alternative treatments, e.g. foliar fertilizers containing low doses of copper (Saville, 2014).

Biocontrol agents (BCA) against *N. ditissima* have been known since the 1970's, when an isolate of *Bacillus subtilis* from apple leaf scars was found to be an effective antagonist of the pathogen *in vitro* (Swinburne, 1973; Swinburne, Barr, & Brown, 1975; Swinburne & Brown, 1976). However, it was never used in orchard applications because of the general difficulties in achieving good field performance of epiphytic biocontrol agents, which is highly dependent on the environmental conditions. More recently, interest has grown in the possibility of developing endophytic BCAs (Saville, 2014), which are less dependent on climatic conditions for their success.

There is little knowledge about the host-pathogen interaction and the race-structure of *N. ditissima* (i.e. its pathotypes). Apple cultivars show differences in their susceptibility to European apple canker. However, the genetic bases of the host resistance, as well as its expression in different tissues, are currently unknown. Even though several groups around the world are committed to such investigations, the outcomes of are not expected in the short term.

Fungal and bacterial endophytes of plants

Plant microbiome consists in a complex ecosystem of neutral, beneficial and pathogenic microorganisms which closely interact with the plant itself (Cátia Pinto & Gomes, 2016). These plant-associated microorganisms influence plant nutrition and resistance to biotic and abiotic stresses (Vandenkoornhuyse et al, 2015) and therefore its growth and health status. Several factors affect their dynamics and distribution in the plant: the host species, genotype,

age and health status as well as environmental factors, including farming and crop protection management methods (Berendsen et al., 2003; Whipps et al., 2008). Plant-associated microorganisms can be found either on plant surfaces or within inner tissues, being the former referred as “epiphytes” and the latter as “endophytes”. Among the several technical definitions of endophytism given over the years, according to Busby and colleagues (2016) the following can be adopted as a working definition: “…infections [caused by endophytes] are inconspicuous, the infected host tissues are at least transiently symptomless and the microbial colonization can be demonstrated to be internal” (Stone et al., 2000). This refers to the usual method for isolating endophytes from asymptomatic, surface-sterilised tissues. However, as pointed out by Pinto and Gomes (2016), cultivation-dependent approaches only allow for the isolation of subpopulations of culturable microorganisms from samples. Thanks to cultivation-independent approaches, such as those based on next generation sequencing, total microbial biodiversity, as well as the complex interactions of the plant microbiome, can be investigated in the natural environment (Müller & Ruppel, 2014; Pinto et al., 2014). Usually, the study of prokaryotes is based on the analysis of 16S rRNA and that of eukaryotes on the 18S rRNA or the ITS region (Cátia Pinto & Gomes, 2016).

Plant rely on genetic defences upon invasion by pathogens (Jones & Dangl, 2006). According to the disease triangle model (Stevens, 1960), the severity of symptoms which appear in the host following disease development is determined by host susceptibility (i.e. host genotype), pathogen virulence and abiotic environmental conditions. However, also plant endophytes have been shown to influence the severity of disease symptoms (Arnold et al., 2003; Freeman & Rodriguez, 1993), either decreasing (pathogen antagonism) or increasing it (pathogen facilitation) in functional assays with susceptible pants, virulent pathogens and environment conducive to disease development (Busby et al., 2016). Literature dealing with disease modification by plant endophytes has been reviewed by different authors in the last years: Compant et al. (2005), Raaijmakers et al. (2002) and Weller (1988) reviewed bacterial endophytes and Busby et al. (2016) reviewed fungal endophytes.

Compant and colleagues (2005) reviewed the literature on plant growth-promoting bacteria (PGPB; Bashan & Holguin 1998), which are associated with nearly all plant species in many different environments. The most studied group of PGPB is that of plant growth-promoting rhizobacteria (PGPR; Kloepper & Schroth 1978), which are found on root surfaces and in the rhizosphere (Kloepper et al., 1999; Kloepper & Schroth, 1978). Some of them are able to enter the root and endophytically colonise the inner tissues (Gray & Smith, 2005; Kloepper et al., 1999). Endophytic bacterial communities mainly colonize the plant via the roots (McInroy & Klopper, 1995; Sturz et al., 2000; Van Peer et al.,1990; Welbaum et al., 2004), but they can also enter via leaves, flowers and seeds (Hallman et al., 1997). PGPB stimulate plant growth

and protect plants against biotic (Bloemberg et al., 2000; Haas et al., 2002; Lugtenberg et al., 2001) and abiotic stresses (Mayak et al., 2004; Nowak & Shulaev, 2003) and their potential as biocontrol agents (BCAs) is well known. Nevertheless, their performance in field tests has been mostly inconsistent so far (Thomashow, 1996). The mechanisms of biocontrol mediated by PGPB are competition for ecological niches or specific substrates, synthesis of allelochemicals (Glick, 1995; Sturz & Christie, 2003) and induction of systemic resistance (ISR; Bloemberg & Lugtenberg, 2001; Glick, 1995; Haas et al., 2000, 2002; Lugtenberg et al., 2001).

Busby and collaborators (2016) reviewed fungal endophytes as modifiers of plant disease. Fungal endophytes have been demonstrated to establish a full range of interactions, from pathogen antagonism, to neutral interaction, to facilitation. The mechanisms by which plant endophytes modify disease severity are often not fully understood. They might act by induction of host resistance, triggering systemic acquired resistance (SAR) or induced system resistance (ISR), or direct interaction may take place between the endophyte and the pathogen. In the case of pathogen antagonism, this interaction may consist in hyperparasitism, competition for space and nutrients or antibiosis. Studies *in vitro* have demonstrated these inhibitory effects (Martín et al., 2015), however Busby and colleagues (2016) point out that more direct mechanisms are likely to exist than those known to date. Conversely, direct interactions underlying pathogen facilitation are not as well studied.

Disease modification is a widely-shared characteristic from a phylogenetical point of view. Fungal and yeast endophytic disease modifiers belong to several different taxonomic groups, with the most commonly reported antagonistic genera being: *Trichoderma*, *Aureobasidium*, *Fusarium*, *Penicillium*, *Chaetomium*, *Bionectria*, *Pichia*, *Candida*. Some genera, such as *Trichoderma*, include well known antagonistic species (Harman et al., 2004) and yeast endophytes are becoming increasingly recognized as postharvest BCAs (Spadaro & Gullino, 2004).

Materials and methods

1. Temporal and spatial dynamics of *Neonectria ditissima* infection of woody tissues in artificially inoculated apple trees and preliminary analysis of the apple endophytes associated to the infected tissues

We studied the spread of the fungus within apple tree tissues following inoculation of fresh pruning cuts. Our goal was to assess the spatial and temporal dynamics of the infection, as well as to determine the relevant sampling points to analyse the endophytic profile of apple and its correlation with disease expression.

The experiment was performed at NIAB EMR, East Malling, UK. 3-years-old potted apple trees of the cultivars Discovery, Cox (on M26 rootstock), Gala and Golden Delicious (on M9 rootstock), were transferred to a polytunnel with side vent nettings about one month before the start of experiment. Plants were constantly checked for pests and diseases and spray treatments were applied for powdery mildew and woolly aphid. Pure cultures of *N. ditissima* strain HG199 (Gomez-Cortecero et al., 2016) were grown on Sugar Nutrient Agar and Yeast (SNAY) medium for around 6 weeks. On the day of inoculation, conidia were washed from the plates with sterile distilled water and filtered through sterile muslin, the final concentration was adjusted to $4.72x10^5$ conidia/ml and the suspension was inoculated within 4 hours. Only macroconidia (2 or more septa) were counted. The germination test showed that 97% of macroconidia had successfully germinated after 6 hours. Pruning wounds were made on lateral branches and twigs of apple trees by performing a slanting cut 5 to 10 mm after a bud, and two 10 µl drops of inoculum (4,720 conidia each) were immediately applied to the cut surface at the interface between cambium and sapwood, allowing for complete absorption by the plant tissues. Three replicates per cultivar were used, four twigs per replicate were inoculated. Two 10 µl drops of sterile distilled water were applied as negative control (mock inoculum) on fresh pruning wounds. Four twigs per cultivar were inoculated with the negative control. Sampling was destructive and entailed the removal of the entire inoculated branch. Inoculation was performed on April 7th and sampling was carried out at 2 weeks (April 21st), 4 weeks (May 5th), 8 weeks (May 31st) and 16 weeks (July 25th) after inoculation (wai) immediately followed by isolation. Three inoculated and one mock inoculated twig were collected at each time point by removing around 10 cm of branch from the pruning cut to the base. Immediately after sampling, branches were processed as follows. 3.5 to 4.4 mm thick transverse sections of the branch were excised at 10, 40 and 70 mm from the pruning wound by means of a backsaw and a mitre box, and processed for the isolation of *N. ditissima* as detailed below. The isolation protocol described by Weber & Hahn (2013) was used, with some modifications. Immediately after the excision of the branch transverse sections, bark and sapwood were separated by means of a scalpel and a dissection needle and stored on ice until further processed. All tools were thoroughly cleaned and sterilised before processing each sample. Samples were surface sterilised to remove epiphytes using a modified technique based on the method no. 3 described in Schulz et al. (1993). Briefly, they were immersed in 70% ethanol for 30 s, followed by sterile deionised water for 30 s, 5% sodium hypochlorite for 60 s, 70% ethanol for 30 s and finally sterile deionised water for 120 s. Samples were pat-dried on sterile blotting paper, plated on PDA amended with iprodione (Rovral ® WG, BASF plc, Cheadle, UK) 40 ppm and rifamycin 20 ppm (McCracken et al., 2003) and incubated at room temperature until fungal growth was observed. Isolates displaying different morphology were subcultured and maintained on Potato Carrot Agar

(PCA) amended with streptomycin 80 ppm and penicillin G 60 ppm to induce sporulation and aid identification. All plates were incubated at room temperature. Isolates identified as *N. ditissima* based on their morphology were subcultured on PDA as well, and incubated for three weeks to allow mycelial growth for subsequent DNA extraction and molecular analysis.

Mycelium of isolates grown for three weeks on PDA was gently scraped off the medium by means of a micropipette tip and stored at -20ºC. Genomic DNA extraction was performed according to the method described by Cenis (1992). To assess the identity of the isolates recovered, the *N. ditissima*-specific primer set developed by Ghasemkhani et al. (2016) was used to amplify a region in the β-tubulin gene of fungal DNA. 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: 95ºC for 5 min, followed by 35 cycles of 95ºC for 15 sec, 60ºC for 30 sec and 72ºC for 15 sec, with a final extension of 72ºC for 5 min. PCR products were analysed on a 1.5% agarose gel.

All data were analysed with Microsoft Excel.

2. Optimisation of an ELISA assay for the detection of the asymptomatic *Neonectria ditissima* infection in woody tissues of apple trees

We carried out four different experiments to optimise a reliable and sensitive ELISA assay for the detection of *N. ditissima* symptomatic and asymptomatic infection. In order to validate our method, we performed ELISA assays and isolations in parallel on naturally infected and artificially inoculated apple tree woody material. A standard curve was produced for the pure *N. ditissima* antigen and for the same antigen in the presence of apple tree woody material, to assess the effects of the plant matrix on the sensitivity of the assay. Preliminary work was carried out at NIAB EMR to select an antiserum highly specific to *N. ditissima.* Supernatants from different hybridoma cell lines were screened for their specificity against antigens of *N. ditissima* and other common plant-associated fungi. The hybridoma cell line 1B1O showed high affinity for *N. ditissima* and low cross-reactivity with antigens of other fungi and was therefore selected to carry out the present work.

In **Experiment 1**, the specificity of the 1B1O hybridoma supernatant was assessed on different fungal antigens at the concentration of 10 µg/ml. Antigens were tested in duplicate and to check for any non-specific interaction a background control was included for each antigen. All steps of the assay were carried out on the background control, but no incubation with the hybridoma supernatant was performed. Normalisation was performed subtracting the

background absorbance value from the mean of the antigen readings. ELISA was carried out as described further below.

In **Experiment 2**, ELISA assays and isolations were carried out in parallel on naturally infected apple tree branches (Royal Gala on M9 rootstock, collected at NIAB EMR, East Malling, UK). Plant material was 2-3 years old and displayed well-developed canker lesions. 2 branches (thereinafter referred to as *branch A* and *branch B*) were processed as detailed below. Two consecutive transverse sections were excised at 0 (leading edge), 10 and 40 mm from the canker lesion by means of a backsaw and a mitre box, then bark and sapwood tissues of each section were separated by means of a scalpel blade and a dissection needle. Bark and sapwood from the section closer to the lesions were separately processed as follows. Samples were broken down into small fragments with an approximate width of 0.5-1 mm, the fragments were pooled, immediately placed into the ELISA plate wells with 100 µl PBS and incubated overnight at 4°C. All tools were thoroughly cleaned and sterilised before processing each sample. *N. ditissima* and *Fusarium lateritium* antigens (Ag1 and Ag4, see **Table 3** in **Results**) were included as positive and negative control respectively. All samples were assayed in duplicates and a background control (as described for **Experiment 1**) was included for each duplicate. ELISA was carried out as described further below. Bark and sapwood of the section farther away from the lesion were separated and surface sterilised to remove epiphytes. A modified technique based on the method no. 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% sodium hypochlorite for 60 s, 70% ethanol for 30 s and finally sterile deionised water for 120 s. Samples were pat-dried on sterile blotting paper, plated on PDA amended with iprodione (Rovral ® WG, BASF plc, Cheadle, UK) 40 ppm and rifamycin 20 ppm (McCracken et al., 2003) and incubated at room temperature and without parafilm until *N. ditissima* growth was observed.

In **Experiment 3**, ELISA assays and isolations were carried out in parallel on artificially infected apple tree branches (Royal Gala on MM106 rootstock). Trees were inoculated via leaf wound (produced with a scalpel blade) with a spore suspension of *N. ditissima* strain Hg199 at the concentration of $2.2x10^4$ conidia/ml in October 2016. The experiment was carried out on 1-year-old, symptomatic plant material 8.5 months after the inoculation. 1 branch (thereinafter referred to as *branch C*) was processed following the same procedure detailed for **Experiment 2**. consecutive transverse sections were excised from both leading edges of the canker lesion (±0 mm), at 10, 40 and 70 mm from the canker in the basal portion of branch (-10, -40, -70 mm) and at 40 mm (+40 mm) from the canker lesion in the distal portion of branch, corresponding to dead plant tissue. *N. ditissima* and *N. cinnabarina* antigens (Ag1 and Ag7, see **Table 3** in **Results**) were included as positive and negative

control respectively in the ELISA assay. All samples were assayed in duplicates and a background control (as described for **Experiment 1**) was included for each duplicate. ELISA was carried out as described further below.

In **Experiment 4**, an ELISA assay was carried out to calculate a standard curve and the limit of detection for the positive antigen Ag1 (*N. ditissima*) and to assess the effects of bark and sapwood matrix on the sensitivity of the method. 1B1O hybridoma supernatant was tested against four different concentrations of the antigens Ag1 and Ag7 (*N. cinnabarina*), namely 0.01, 0.1, 1 and 10 µg/ml, as well as against the same concentration of Ag1 supplemented with sapwood or bark shavings from a disease-free, 1-year-old apple tree twig (Royal Gala on M9 rootstock, collected at NIAB EMR, East Malling, UK). 100 µl antigens were incubated in wells overnight at 4°C, either alone or supplemented with sapwood or bark fragment of approximately 0.5-1 mm width. A background control (as described for **Experiment 1**; antigen concentration:10 µg/ml) and a blank (PBS alone incubated overnight at 4°C) were included for each antigen or antigen + matrix combination. 3 replicates were carried out for each measure. ELISA was carried out as detailed below.

All **ELISAs** were performed according to the method described by Dewey et al. (1995), with some modifications. Assays were carried out in 96 well MicroWell™ MaxiSorp™ flat bottom plates (Sigma-Aldrich, USA). All working volumes were 100 µl per well and wells were washed four times between incubations with 200 µl Phosphate Buffered Saline (PBS, Sigma-Aldrich) added with 0.5% Tween 20 (PBST) for 1 min. Depending on the experiment, wells were incubated overnight at 4°C with either fungal antigens 10 µg/ml (all experiments), fungal antigen 10 µg/ml and bark/wood shavings (Experiment 4), PBS and bark/wood shavings (Experiments 2, 3 and 4) or PBS (Experiment 4). Subsequently, they were washed with PBST and incubated with hybridoma supernatant (1B1O) diluted 1:10, followed by a 1:1000 dilution of goat anti-mouse IgG conjugated to biotin(Sigma-Aldrich), and then by a 1:4000 dilution of streptavidin conjugated to 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 H2SO4 1M and the absorbance at 450 nm was measured by means of a spectrophotometer.

All data were analysed with Microsoft Excel.

Results

1. Temporal and spatial dynamics of Neonectria ditissima infection of woody tissues in artificially inoculated apple trees and preliminary analysis of the apple endophytes associated to the infected tissues

The results of the inoculation and re-isolation of *N. ditissima* from apple tree woody tissues are summarised in **Table 1**. Sampling was destructive, entailing the removal of the entire branch for subsequent processing and re-isolation. Therefore, the replicates reported per each cultivar in **Table 1** represent different branches at each time point. Overall, *N. ditissima* was successfully isolated from the sapwood of nearly all transverse sections excised at 10 mm from the inoculated pruning cut, in all cultivars, at time points 1-3. The success rate of re-isolation from sapwood sections at 10mm from the entry point was 100% at 2 wai, 83% at 4 wai, 91% at 8 wai, but dropped to 25% at 16 wai. However, the isolation medium used for the fourth isolation (16 wai) showed heavy contamination by yeast species and therefore these results should not be considered reliable. *N. ditissima* could not be re-isolated from any section of the mock inoculated control branches, and it was found in the bark at 10mm from the pruning wound only in one instance (16 wai).

The earliest symptoms of apple canker were observed on Gala trees starting from 4 wai and on Discovery trees starting from 8 wai. Cox and Golden Delicious trees showed symptoms as well, as of 4 and 8 wai respectively, however these were less severe and affected a lower number of replicates. Starting from 4 wai, brown staining was detected in the xylem of replicates of all cultivars. Gala was the most affected cultivar, with the browning extending up to the sections at 40 and 70 mm from the pruning wound at both 4 and 8 wai, and Golden Delicious was the least affected cultivar, with only one replicate showing xylem browning at 4 wai.

PCR with the β-tubulin primers was carried out on isolates collected 2 and 4 wai, whose morphology on the isolation medium, PDA and PCA all corresponded to the expected morphology for *N. ditissima*. The PCR produced an amplicon band of the expected molecular weight (150 bp) for all isolates, as shown by analysis of the fragment size by agarose gel electrophoresis (data not shown).

Table 1 – Results of the re-isolation of *N. ditissima* from artificially inoculated apple tree branches. For each of the three replicates of the tested cultivars (Gala, Discovery, Cox, Golden Delicious), the transverse section (10, 40 or 70 mm) and tissue (sapwood or bark) from which the fungus was isolated, are reported. Sampling was destructive and the three replicates shown per each cultivar represent different branches at each time point. a: sw=sapwood; bk=bark. b: possible values are 10, 40 or 70 (mm). Data for mock inoculated branches not shown. c: Ga = Gala; Ds = Discovery; Cx = Cox; Gd = Golden Delicious. *: sapwood at 10 mm lost during isolation.

	tissue [a], distance [b] (mm) *N. ditissima* detected			
Cultivar (replicate)	**2 weeks after inoculation**	**4 weeks after inoculation**	**8 weeks after inoculation**	**16 weeks after inoculation**
Ga (1) [c]	sw, 10	sw, 10	sw, 10	-
Ga (2)	sw, 10	sw, 10	sw, 10	sw, 10; bk, 10
Ga (3)	sw, 10	sw, 10	sw, 10	sw, 10
Ds (1) [c]	sw, 10	sw, 10	sw, 10	-
Ds (2)	sw, 10	sw, 10	sw, 10	-
Ds (3)	sw, 10	sw, 10	sw, 10	-
Cx (1) [c]	sw, 10	sw, 10	-	-
Cx (2)	sw, 10	-	sw, 10	sw, 10
Cx (3)	sw, 10	sw, 10	sw, 10	-
Gd (1) [c]	sw, 10	-	sw, 10	-*
Gd (2)	sw, 10	sw, 10	sw, 10	sw, 10
Gd (3)	sw, 10	sw, 10	sw, 10	-

All apple tree endophytes showing different morphology on the isolation medium were sub-cultured. Overall, 20% of sapwood transverse sections resulted sterile (i.e. no microorganism was successfully isolated), whereas 7% of bark sections did. A morphotype classification method, implemented during pilot isolation experiments (data not shown), was used to group the apple endophytes based on their morphology on PCA. A few isolates were submitted to Prof. Brian Sutton for identification. Endophytes collected during isolations at 2, 4 and 8 wai were classified according to the morphotypes. Results are reported in **Table 2** and shown in **Figure 3**. Some differences could be observed in the relative amount of isolates belonging to different morphotypes across cultivars.

Table 2 – Classification of morphotypes of apple tree endophytes on PCA. Relative amount (%) of isolates of each morphotype found in each cultivar is reported. Category U includes isolates which could not be classified. Category Z refers to morphotype on PDA. a: Ga = Gala; Ds = Discovery; Cx = Cox; Gd = Golden Delicious.

ID	Morphology	Isolates identified	Ga [a] (%)	Ds [a] (%)	Cx [a] (%)	Gd [a] (%)
a	. whitish-hyaline, flat . pigment observed in the centre . white tufts may be observed	*Fusarium equiseti* (Corda) Sacc.	0.00	0.00	1.45	0.00
b	. hyaline, flat or nearly flat . can be dark-shaded . black patches or masses are observed	*Thielavia* sp. *Epicoccum purpurascens* *Stemphylium botryosum* (state of *Pleospora herbarum*)	0.00	6.67	11.59	1.37
c	. hyaline, flat . green patches or masses are observed	-	0.00	0.00	2.90	0.00
f	. grey, fluffy or slightly fluffy, can display zoning . sparse black dots are observed	-	26.00	2.22	1.45	2.74
g	. dark grey or black with hyaline areas, flat or nearly flat . very small black dots are observed	*Alternaria alternata*	*0.00*	*2.22*	*2.90*	*2.74*
h	. dark background (black, dark olive green or dark grey) with grey and/or white fluffy overlay	-	12.00	22.22	10.14	12.33
l	. dark olive green, dark . circular growth pattern, can display zoning . dusty, green spores or fluffy and sandy grey cushions of mycelium are observed	*Cladosporium cladosporioides*	12.00	22.22	21.74	2.74
m	. green or light green, flat or nearly flat . irregular edge	-	0.00	0.00	5.80	0.00
o	. white, flat, with occasional woolly tufts . no zoning observed	-	8.00	4.44	5.80	24.66

	. different structures can be observed (red-orange to yellow-brownish masses embedded in agar, pink cushions of mycelium, orange creamy masses arranged in circular pattern)					
p	. white, flat . zoning (circular growth pattern) . dark shading observed sometimes	-	4.00	0.00	14.49	2.74
q	. white, fluffy . zoning can be observed	-	2.00	15.56	1.45	21.92
s	. pigment in agar (dark to pale yellow, brown, dark red) . various mycelial morphology	-	18.00	13.33	5.80	2.74
u	. unclassified	-	12.00	5.80	0.00	23.29
z (on PDA)	. yeast-like . oil patch-like growth or irregular margins	--	6.00	11.11	8.70	2.74

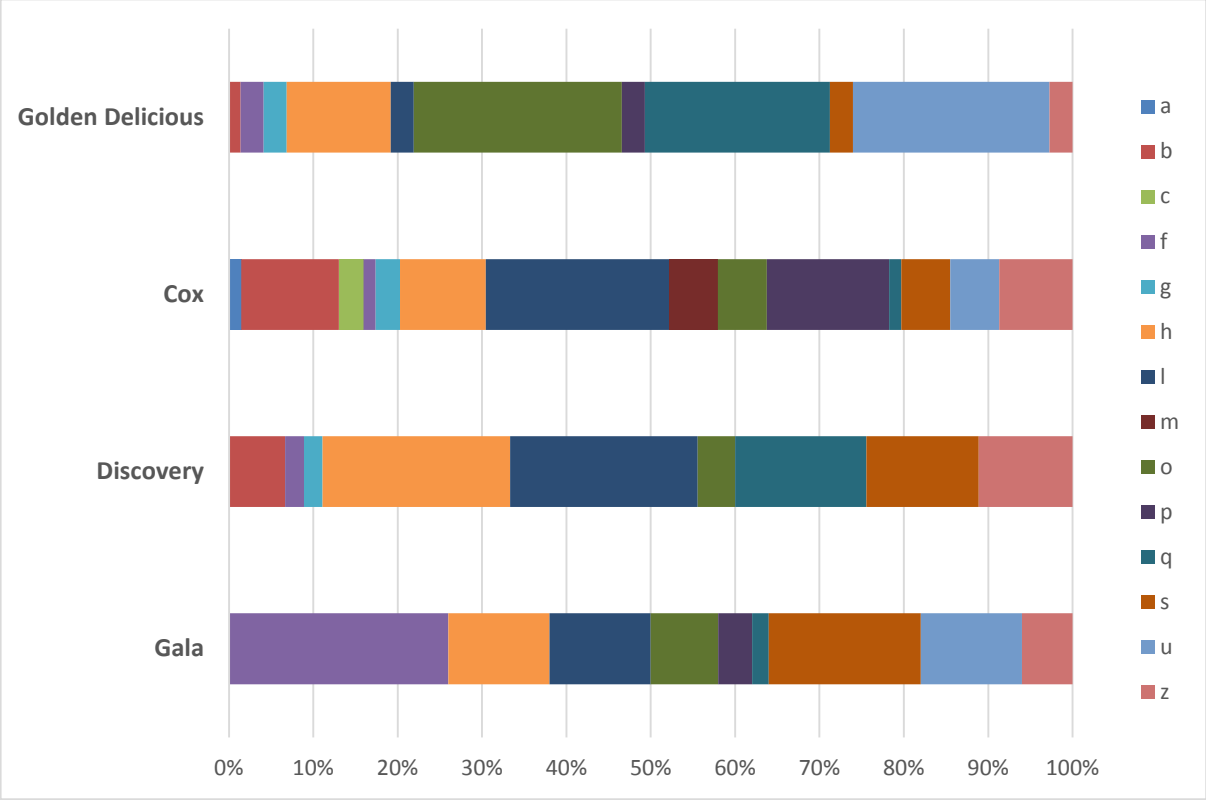

Figure 3 – Cumulative isolation frequency of the different morphotypes from the apple cultivar assessed. Legend shows the different morphotypes, listed in **Table 2**.

Most groups were found in at least three or four cultivars and they were generally isolated with different relative frequencies. This is the case, for example, of groups *F* (25% in Gala, less than 3% in other varieties) or *L* (above 20% in Cox and Discovery, 12% in Gala and less than 3% in Golden Delicious), within which *C. cladosporioides* was identified. Only one of these frequently occurring groups were found in similar amounts across the cultivars, i.e. group *G* (corresponding to 2-3% of isolates from Discovery, Cox and Golden Delicious), which *A alternata* was found to belong to. 3 morphotypes were isolated only from Cox and in low frequency: these were *A (1.45%)*, *C* (2.90%) and *M (5.80%)*.

2. Optimisation of an ELISA assay for the detection of the asymptomatic *Neonectria ditissima* infection in woody tissues of apple trees

The different fungal antigens on which the hybridoma cell line 1B1O was tested in **Experiment 1** are reported in **Table 3**. The results of the ELISA are shown in **Figure 4**. According to the normalised absorbance values, that the hybridoma cell line 1B1O showed high specificity for *N. ditissima* antigens (Ag1 and Ag3), with readings for Antigens 4-9 always below 0.2 OD_{450nm}. A high background was produced by Ag1 (*N. ditissima*) and Ag6 (*B. obtusa*).

Table 3 – Test fungal antigens. a: isolate number refers to the collection at NIAB EMR, East Malling, UK.

Antigen	Species	Isolate [a]
Ag1	*N. ditissima*	R09/05
Ag3	*N. ditissima*	R28/15
Ag4	*Fusarium lateritium*	R23/15_2
Ag5	*Monilinia laxa*	R22/15_5
Ag6	*Botryosphaeria obtusa*	PC23/15
Ag7	*Nectria cinnabarina*	R22/15_4
Ag8	*Colletotrichum acutatum*	R22/15_8
Ag9	*Phomopsis* spp. – *Diaporthe viticola*	PC19/15_PF10

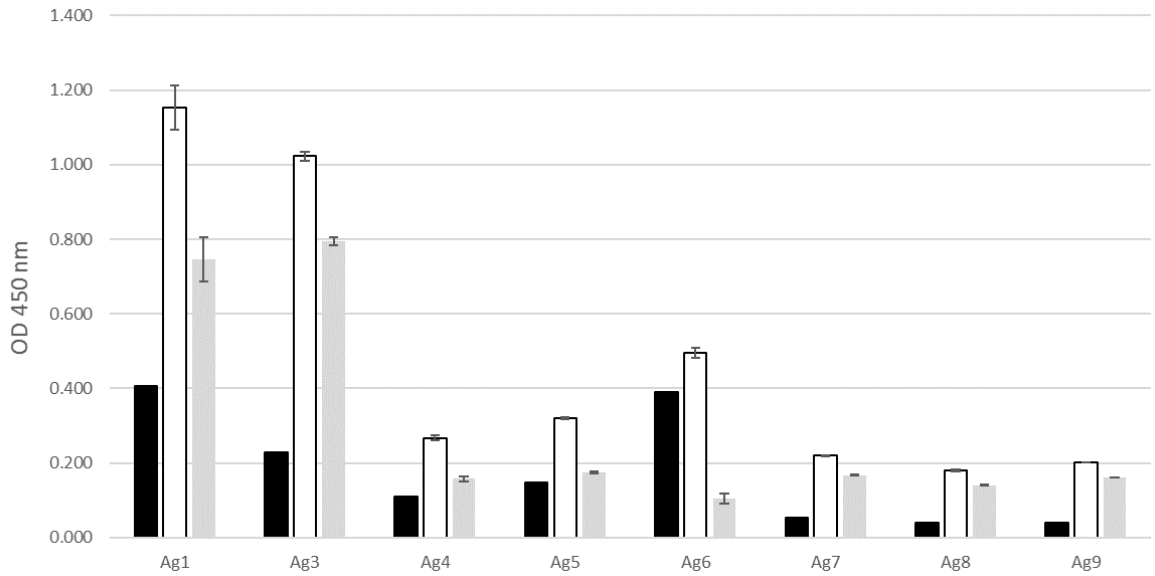

Figure 4 – **Experiment 1**: hybridoma supernatant 1B1O tested against fungal antigens (10 µg/ml). Background (black bars), raw (white bars) and normalised (grey bars) absorbance readings (OD_{450nm}) are shown. Error bars represent standard deviation.

The results of ELISA in **Experiment 2** are shown in **Figure 5**. Pictures of branch A and branch B are shown in Figure **6** and **7**. Absorbance readings were substantially higher compared to the negative control in the bark and sapwood from the leading edge of lesion on branch A and in the wood from the leading edge on branch B. The negative absorbance value recorded for the bark from the leading edge on branch B was due to the background reading being much higher than the reading from the two sample replicates, therefore this result should be not taken into account. *N. ditissima* was only isolated from sapwood and bark from the leading edge of the lesion (0 mm) on branch A.

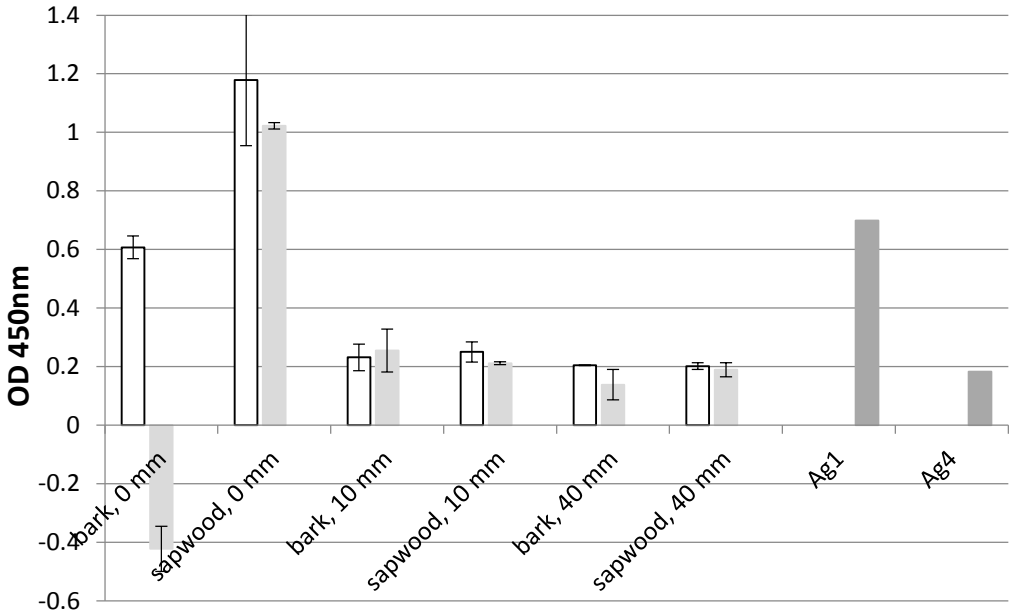

Figure 5 - **Experiment 2**: hybridoma supernatant 1B1O tested against bark and wood shavings from naturally infected, cankered apple tree branches. Absorbance readings (OD_{450nm}) normalised to the background for branch A (white bars), branch B (light grey bars) and fungal antigens Ag1 (positive control) and Ag4 (negative control) at the concentration of 10 µg/ml (dark grey bars) are shown. Data represent the average of 2 measures. Error bars represent standard deviation.

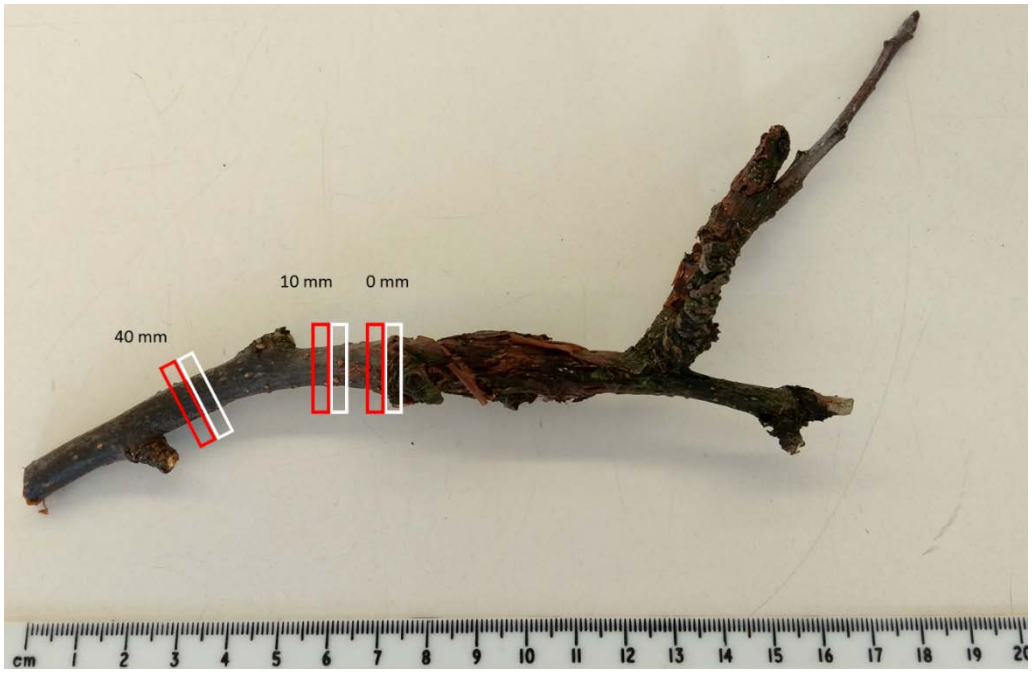

Figure 6 – Branch A (**Experiment 2**). Transverse sections excised for isolation (red rectangles) or ELISA (white rectangles) are shown.

Figure 7 – Branch B (**Experiment 2**). Transverse sections excised for isolation (red rectangles) or ELISA (white rectangles) are shown.

The results of ELISA in **Experiment 3** are shown in **Figure 8**. Pictures of branch C are shown in **Figure 9**. Absorbance readings were substantially higher compared to the negative control for bark and sapwood at both leading edges of the canker lesion (±0 mm), with much higher values on the distal (apparently healthy) part of branch (-0 mm) and for sapwood at -10 mm. Absorbance was slightly higher than the negative control for bark samples at -10 mm and +40 mm. It was not possible to isolate *N. ditissima* from any of the assessed transverse sections.

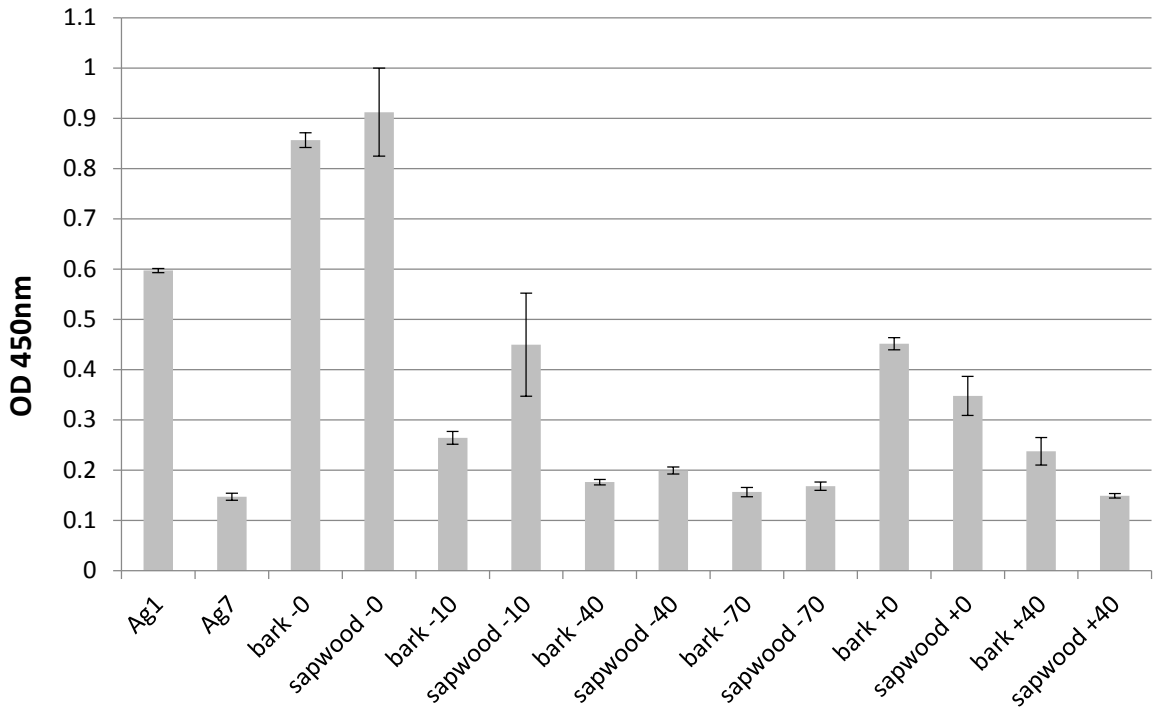

Figure 8 - **Experiment 3**: hybridoma supernatant 1B1O tested against bark and wood shavings from artificially inoculated, cankered apple tree branch. Absorbance readings (OD_{450nm}) normalised to the background for branch C and fungal antigens Ag1 (positive control) and Ag7 (negative control) at the concentration of 10 µg/ml are shown. Data represent the average of 2 measures. Error bars represent standard deviation.

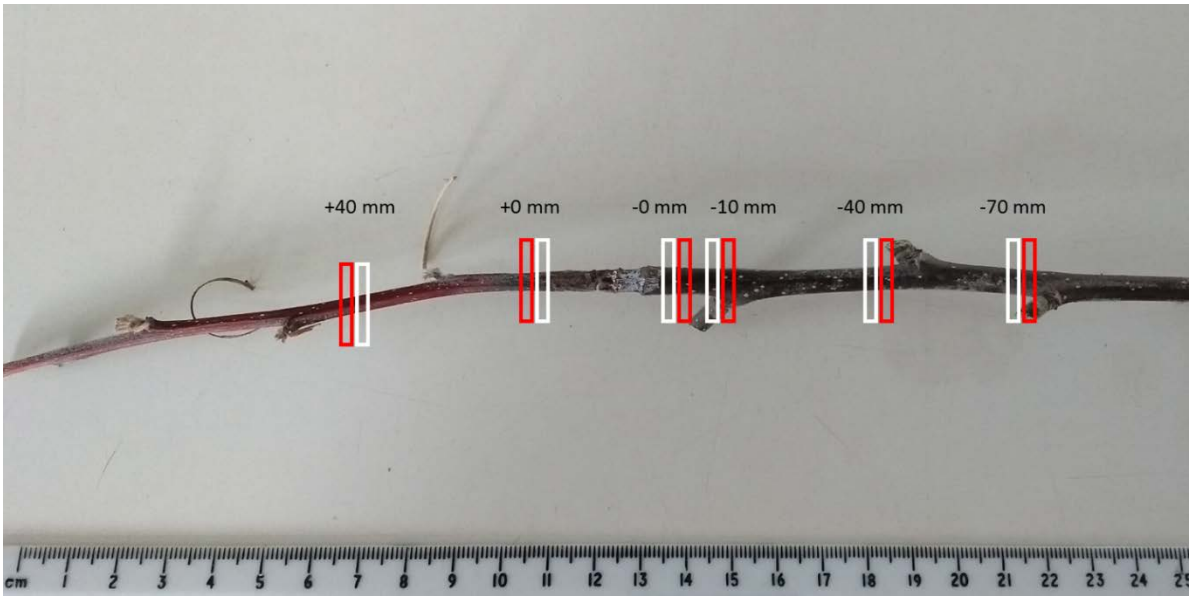

Figure 9 – Branch C (**Experiment 3**). Transverse sections excised for isolation (red rectangles) or ELISA (white rectangles) are shown.

The results of ELISA in **Experiment 4** are shown in **Figure 10** and **Figure 11**. All comparisons are based on the standard deviation. Background absorbance was different for the different tested antigens and antigens + matrix combinations, being maximum for Ag1 and decreasing through Ag1 + bark, Ag1 + sapwood and Ag7. Background absorbance for Ag1 was higher than blank absorbance, and background absorbance for Ag1 and Ag1 + bark was comparable to the respective blank values. Background for Ag7 was lower than for the antigen tested at any concentration. Absorbance was comparable for all assayed concentrations of Ag7 (10-0 µg/ml). Conversely, absorbance decreased from 10 µg/ml to 0 µg/ml (blank) for Ag1 and the Ag1 + bark/sapwood combinations. Values at 0.01 µg/ml were comparable to the blank for Ag1 and its combinations with matrices, whereas at 0.1 µg/ml only readings for Ag1 + bark/sapwood were comparable to the blank. Between 10 and 0.1 µg/ml, absorbance values were always higher for the pure Ag1 compared with its combination with bark and sapwood matrices, whereas these were always comparable to each other.

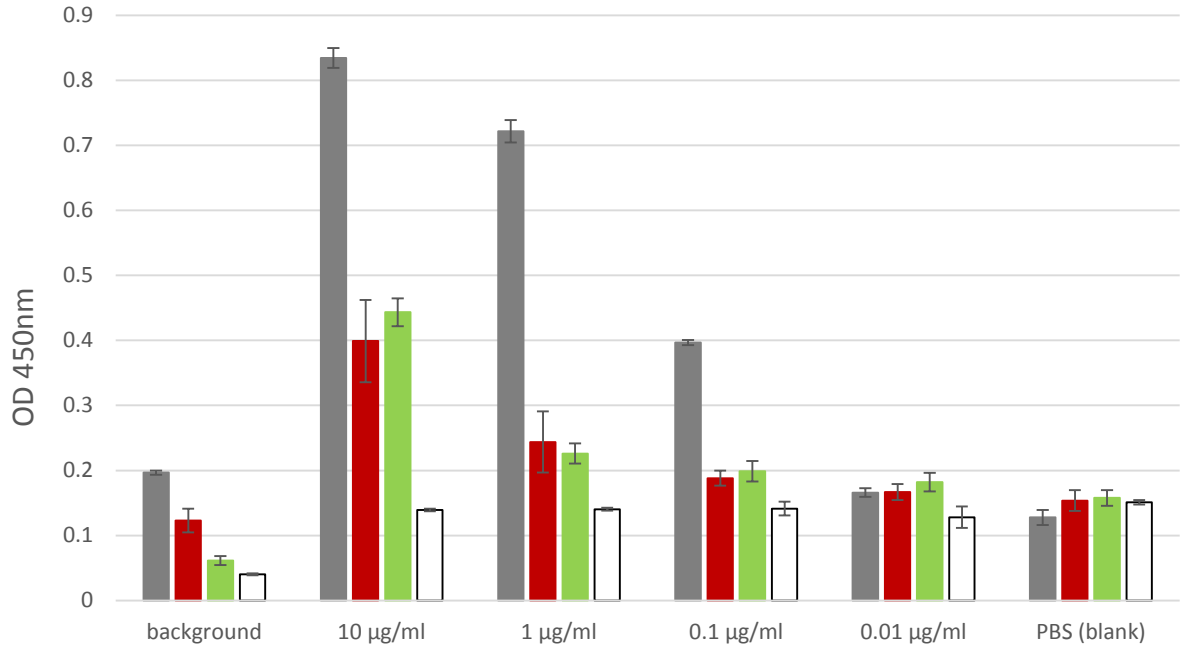

Figure 10 - ***Experiment 4****: non-normalised data. Hybridoma supernatant 1B1O tested against Ag1 (N. ditissima*, grey bars), Ag1 incubated with bark shavings (red bars), Ag1 incubated with sapwood shavings (green bars) and Ag7 (*N. cinnabarina*, white bars). *Data represent the average of 3 measures. Error bars represent standard deviation.*

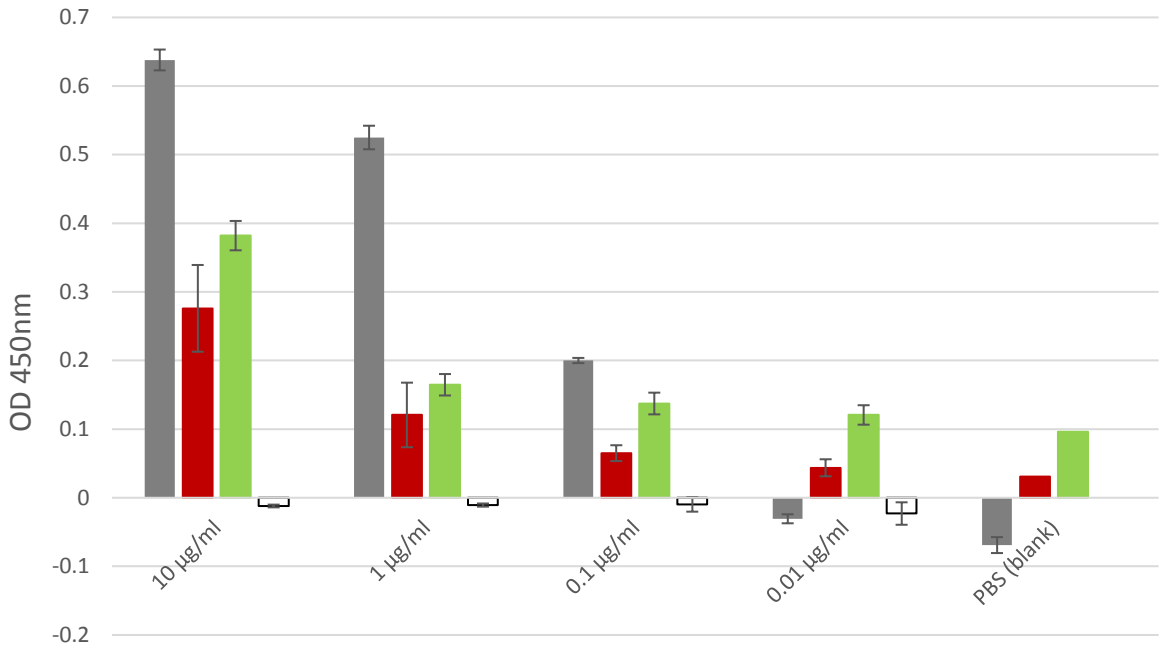

Figure 11 - **Experiment 4**: normalised data. Hybridoma supernatant 1B1O tested against Ag1 (N. ditissima, grey bars), Ag1 incubated with bark shavings (red bars), Ag1 incubated with sapwood shavings (green bars) and Ag7 (N. cinnabarina, white bars). Data represent the average of 3 measures. Error bars represent standard deviation.

Discussion

1. Temporal and spatial dynamics of Neonectria ditissima infection of woody tissues in artificially inoculated apple trees and preliminary analysis of the apple endophytes associated to the infected tissues

In the present study, we wanted to assess whether *N. ditissima* can asymptomatically colonise plant woody tissues following successful establishment of infection. Pruning cuts represent an entry point for the pathogen in commercial orchards (Saville, 2014; Weber, 2014) and display the highest susceptibility and lesion incidence following artificial inoculation compared to other types of wounds (Amponsah et al., 2015). The incidence of canker development has been shown to range from 81 to 94% or from 90 to 99% when fresh pruning wounds are inoculated at bud break or at the stage of summer elongation respectively (Xu et al., 1998). As low inoculum dose and inoculation of older wounds have been demonstrated to result in low lesion incidence (Xu et al., 1998), we applied conidia in a high dose (around 10,000 conidia per wound) to fresh pruning cuts (i.e. within two minutes from the cut). Surface moisture was shown not to influence lesion incidence in pruning wounds (Xu et al., 1998), therefore plants were stored in a vented polytunnel throughout the whole duration of the experiment to closely simulate environmental conditions in the orchard. Two highly

susceptible apple cultivars (Gala and Discovery) as well as two moderately susceptible ones (Cox and Golden Delicious), according to the classification provided by the Apple Best Practice Guide (Webster et al., 2001), were selected for the experiment.

Overall, and with the exception of the last isolation (16 wai), whose results should be not considered reliable due to the high level of yeast contamination in the isolation medium, *N. ditissima* could be re-isolated from trees of all cultivars both prior to and after the first canker lesions appeared, i.e. during both the initial incubation stage and the pathogenic stage of infection. Time elapsed before symptoms appearance varied between different cultivars. All trees were asymptomatic at 2 wai. Gala trees (highly susceptible) showed lesions greater than or equals to 10 mm already at 4 wai, followed by Discovery at 8 wai, and eventually by Cox and Golden Delicious (moderately susceptible) at 16 wai. However, tree-to-tree variation was observed in symptoms appearance, and therefore length of incubation period, within each cultivar confirming what already observed by Xu et al. (1998). Regardless of disease expression, *N. ditissima* was isolated with similar modality from each cultivar analysed. The pathogen was found in the sapwood at 10 mm from the inoculated pruning wound, it was detected growing out of the sample starting from 12-18 days after the isolation, and no other endophyte was contextually isolated. The fungus was also isolated, together with other endophytes, from a cankered Gala bark section, 10 mm away from the pruning cut, at 16 wai.

Results so far are not conclusive, but they provide some further insight on *N. ditissima* infection. Weber & Hahn (2013) previously showed that the fungus can spread longitudinally within the stem of young apple trees, without producing any visible lesion, after the establishment of the first symptoms. However, they isolated the pathogen from the entire transverse section of the stem. We showed that following the inoculation of fresh pruning wounds, the fungus is generally found in the sapwood at 10 mm from the entry point, the only exception being represented by a Gala bark section, 10 mm away from the entry point, which was entirely contained within a canker lesion. Isolation was never successful at 40 mm from the inoculated wound, therefore *N. ditissima* may reside latent or colonise the first mm of section adjacent to the wound. The apparent exclusion of the pathogen from the bark prior to disease expression may be due to mechanical plant defences or to the activity of the endophytes residing in the tissues. Indeed, 20% of sapwood transverse sections resulted sterile when isolation was attempted, against only 7% of bark sections, suggesting the presence of larger endophytic populations in the latter. However, since we made use of a selective medium amended with a fungicide and a wide-spectrum antibiotic, this observation need to be validated by repeating the experiment with non-selective media.

We carried out a preliminary screening of a subpopulation of the culturable fungal endophytic species in apple tree branches subjected to pruning cuts. Isolates were classified in

morphotypes and a few were identified in order to associate their group to known species. Some of these morphotypes include ubiquitous saprophytes, such as *Alternaria* spp. (group G), *Epicoccum* spp. (group B) or *Cladosporium* spp. (group L; Hodgson et al. 2014), and isolates belonging to these groups were frequently found across the tested cultivars. *A. alternata* and *E. nigrum* (synonym of *E. purpurascens*) were previously isolated from apple leaf scars (Swinburne, 1973) and all the 3 genera have been previously isolated from apple leaves (Camatti-Sartori et al., 2005). The classifications in morphotypes represents a useful tool to ease the screening and identification of culturable endophytes and may allow faster identification of infrequent species.

2. Optimisation of an ELISA assay for the detection of the asymptomatic *Neonectria ditissima* infection in woody tissues of apple trees

Our results so far are not conclusive, but promising the implementation of a serological method for the detection of *N. ditissima*. Data from **Experiment 1** and **4** showed that when the hybridoma supernatant incubation step was skipped, non-negligible non-specific interaction were generated by Ag1, Ag3 (*N. ditissima*) and Ag6 (*B. obtusa*), and to a lesser extent also by Ag4 (*F. lateritium*) and Ag5 (*M. laxa*), resulting in a high background reading. For Ag1, this interaction resulted in an absorbance value even higher than the blank (for which PBS was incubated overnight instead of the antigen). This should not be overlooked, as these interactions may alter the outcome of the assay. This problem could be tackled improving the washing steps (e.g., adding more detergent to the washing buffer or soaking wells for a longer time in between washes) or by using blockers (such as BSA or non-fat dry milk).

In **Experiment 4**, the hybridoma supernatant 1B1O was tested against serial dilutions of the positive antigen Ag1. Results showed that at a concentration of 0.01 µg/ml, absorbance was comparable to both the background noise and the blank. 0.1 µg/ml thus represents the lower concentration of Ag1 we could reliably detect with our current method. The sensitivity of the assay decreased in the presence of plant material (either sapwood or bark). Interestingly, the background noise was reduced as well. The absorbance reduction effect generated by bark and sapwood shavings was similar, even though overall bark produced a slightly stronger decrease.

In **Experiment 2**, we can confidently assume that *N. ditissima* was detected at the leading edge of the lesion on branch A. Interestingly, even though the absorbance of the sapwood sample from the leading edge on branch B was nearly as high that of the corresponding sample on branch A, and higher that the positive control, the isolation was not successful. If we discount procedural mistakes, this suggest that our ELISA assay is potentially more reliable than our isolation method.

The observations on the effect of plant matrix (**Experiment 4**) allow to draw some conclusions on the results of **Experiment 3**. Unfortunately, isolation of N. ditissima was never successful from branch C. A yellow, yeast-like microorganism was found in all isolation plates and a possible explanation for the failed isolation could be that it outcompeted the pathogen, even on the selective medium used. However, the ELISA assay apparently could detect the pathogen in the plant tissues. Some of the samples, and namely those with OD_{450nm} > 0.400 (more than double the value for the negative antigen), apparently leave no doubt on the presence of the fungus. However, sapwood at the leading edge on the distal part of branch (+0 mm), bark at + 40 mm on the same portion and sapwood at -10 mm on the basal part of branch produced an absorbance comparable to that of Ag1 10 µg/ml in the presence of plant material (see **Figure 8** and **Figure 11** for a comparison). This may suggest successful detection of N. ditissima in the samples. The determination of the limit of detection of the ELISA is required in order to validate all these speculations.

Conclusions

- Following artificial inoculation of pruning cuts, *N. ditissima* was re-isolated from the internal woody tissues of branches (sapwood and hardwood) at 10 mm from the entry wound, up to two months after the inoculation; the pathogen could not generally be re-isolated from the bark and it was never found in any type of tissue at 40 mm from the entry wound.
- Further experiments are required to determine the temporal and spatial dynamics of the infection via different types of commercially-relevant wound (pruning cuts and leaf scars) over a longer period.
- The ELISA, we developed, showed high specificity for *N. ditissima* and greater sensitivity compared to isolation methods for the detection of the pathogen. The plant matrix was shown to reduce the sensitivity of the assay.
- Further experiments are required to fully validate the ELISA. In partcular, the limit of detection in the presence of plant material (bark or sapwood) must be determined and the background noise should be minimised.

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