Project title:	Systemic infection and symptom expression of *Neonectria ditissima* in relation to endophytes conditioned by environmental stresses
Project number:	TF226
Project leader:	Prof Xiangming Xu NIAB EMR
Report:	Annual report, 2019 (received February 2020)
Previous report:	Annual report, October 2018
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Location of project:	East Malling, and several farms in Kent
Industry Representative:	Peter Checkley
Date project commenced:	1 October 2017
Date project completed (or expected completion date):	30 September 2021

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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

- Trees held for long periods in cold-storage led to increased canker incidence post-planting in orchards.

Background and expected deliverables

European Canker, caused by *Neonectria ditissima*, has become the most damaging disease of apple in recent years across all major apple growing regions worldwide. Modern cultivars lack effective resistance to this pathogen and in Europe, the most efficacious methods of chemical control are no longer available. Cultivars differ in their susceptibility but there is no absolute resistance. Previous work, conducted at NIAB EMR, has demonstrated that asymptomatic infection in nursery trees is a significant source of the disease in production orchards. The most economically important damage occurs when the nursery-borne latent infection becomes active and develops into canker on the main trunk during orchard establishment (within three years of planting) – leading to tree death. Ample empirical evidence suggests that stresses following planting can promote symptom expression of those nursery-borne latent infections.

An endophyte is a microbe that lives within a plant for at least part of its life cycle without causing apparent disease. Endophytes have been found in all species of plants studied to date although the endophyte/plant relationships are not well understood. Certain microbial endophytes can help plants to tolerate biotic stress, such as attacks by plant pathogens and herbivory, or abiotic stresses, including salt, drought or heat stresses. It has been shown in numerous host species that recruitment of specific microbes into the rhizosphere is partially under host genetic control and there is increasing evidence that host genetics influence the microbes occupying the endophytic niche. Endophyte composition can also be influenced by pathogen presence and crop management practices. Current research focuses on how we could exploit endophytes to produce crops that grow faster and are more resistant and hardier than crops lacking particular endophytes.

We have recently obtained preliminary data showing a link between antagonist fungal endophytes with cultivar tolerance to *N. ditissima*. One fungal endophyte group, identified as belonging to the genus *Epicoccum* (most likely as *E. purpurascens*, previously known as *E. nigrum*), is much more abundant in two canker-tolerant cultivars than in two canker susceptible cultivars. *Epicoccum purpurascens* is a known antagonist against *Monilinia laxa* (causing stone fruit brown rot) and is being commercially exploited for control of brown rot on stone fruit. It is natural, therefore, to speculate whether the abundance of *E. purpurascens* is related to

tolerance to canker development and, if so, whether we could exploit *E. purpurascens* for canker management.

In this BBSRC LINK project, we aim to build on the preliminary data to investigate whether cultivar differences in tolerance to *N. ditissima* are associated with specific endophytes and, if so, identify the organism(s) and conduct further *in vitro* and *in vivo* biocontrol assays to assess specific endophytes against *N. ditissima*. As well as the direct effect against the canker pathogen we shall study whether these specific endophytes could reduce canker development via inducing host defence systems against the pathogen. To improve breeding for canker resistance, we shall determine to what extent the recruitment of specific endophytes is genetically controlled by hosts by mapping QTLs (quantitative trait loci) and to determine the extent of overlaps of these QTLs with those mapped for canker resistance. We are conducting experiments to assess (1) to what extent recruitment of endophytes is influenced by soil characteristics and host genotypes, and (2) whether canker symptom expression is related to planting times and the abundance of specific endophytes across a number of orchards. Finally, to assist in canker management, we are investigating the extent to which endophyte profiles of a specific apple genotype can be influenced by management practices (irrigation and soil amendment).

Summary of the project and main conclusions in Year 2

We have successfully initiated all experimental studies on time.

(1) We have profiled endophytes at leaf scars of eight cultivars with differing tolerance/resistance against apple canker; the data are currently being analysed.

(2) A number of *Eppicocum* endophytes were obtained from apple and shown to have antagonistic effects against apple canker under *in vitro* tests.

(3) Drenching stool-beds with *Eppicocum* can increase the concentration of *Eppicocum* endophytes in rootstock plants. However, applying *Eppicocum* as a foliar spray to orchard trees did not result in significant increases in endophytic *Eppicocum*.

(4) Inoculation of plants with PGPR or AMF at planting time appeared to have resulted in increased tree development.

(5) Longer duration of trees in cold-storage led to increased canker incidence post-planting.

Financial benefits

These results are from the second year only of a four-year project so it is too early to quantify financial benefits to growers. However, the result that impacts commercial apple production most is the effect of storage duration on canker development.

Action points for growers

- At this stage of the project, there is only one action to recommend to growers: plant trees as soon as possible after lifting.

SCIENCE SECTION

Background

European apple canker, caused by *N. ditissima*, is a destructive disease of apple trees and current methods of control, based on protective fungicides, are only partially effective, non-sustainable, and to date reliant on copper-based fungicides which are no longer permitted. *Neonectria ditissima* has a complex lifecycle with all year-round potential of producing ascospores and conidia, which infect wounds (e.g. leaf scars & pruning cuts). The pathogen also infects fruit, leading to losses in store as a post-harvest rot. The most damaging phase of the disease is the canker on the main trunk of a young tree in newly established orchards. Most of these cankers result from infection in nurseries but remain latent until post-planting in orchards. Modern nurseries are high input operations with fungicide, nutrients and water added to encourage vigorous growth in the first two years. A nursery tree is made up of two components; a rootstock, harvested from a stool bed, and a scion, harvested from a 'mother tree', both sources can harbour latent infection which is masked by the high inputs through the nursery phase but then expressed during the establishment stage in the orchard where the tree experiences abiotic and biotic stress. This is exacerbated in modern intensive fruit wall orchard systems (c. 3000 trees/ha) where the trees are much smaller than in traditional orchards coupled with the varieties (e.g. Gala, Rubens, Jazz and Kanzi) being much more susceptible, resulting in a high incidence of tree death from trunk cankers during orchard establishment. Tree death due to canker of over 10% is common during orchard establishment for susceptible varieties (Saville, unpublished). Experience has shown that canker symptom expression in newly established orchards is related to particular sites.

Neonectria ditissima is a wound pathogen and accordingly absolute host resistance has not been observed. However, quantitative differences have been determined between genotypes in their response to this pathogen. There is currently a concerted effort in Europe and New Zealand to determine the underlying mechanisms of this resistance/susceptibility in order to breed for increased tolerance/resistance to the pathogen. Breeding apple cultivars requires a minimum of 15-20 years. Yet there is an urgent need to understand the biology of this disease to develop better management strategies in the medium term.

Endophytes associated with specific apple genotypes may be an important component affecting latent canker development, thereby contributing to field resistance. Recent evidence suggests that endophytes may induce plant defence responses, produce secondary metabolites that inhibit pathogens, directly compete with invading pathogens or a combination thereof. Resistance to Dutch elm disease (*Ophiostoma novo-ulmi*) is associated with reduced diversity in fungal endophytes in the host. Endophytes of woody angiosperms were shown to play an important role in host defence. The endophytic fungus *Muscodor albus*, originally

isolated from *Cinnamomum zeylanicum*, produces a mixture of volatile organic compounds in culture that have a wide spectrum of antimicrobial activity. Endophytes can also help plants tolerate abiotic stresses, e.g. salt and heat tolerance. Recently, it has been demonstrated that a fungal endophyte (*Piriformspora indica*) enhanced its host plant's (rice) tolerance to root herbivory through changes in gibberellin and jasmonate signalling.

It has been shown in numerous host species that recruitment of specific microbes into the rhizosphere is partially under host genetic control and there is increasing evidence that host genetics influence the microbes occupying the endophytic niche. Endophyte composition can also be influenced by pathogen presence, production system, and arbuscular mycorrhizal fungi (AMF) colonisation.

Preliminary data we obtained prior to the current study suggests that specific endophytes may be associated with cultivar differences in their susceptibility to *N. ditissima*. Orchard-specific factors (abiotic – soil type, soil water deficit, nutrient supply; and biotic – soil microbial population, including AMF and Plant growth promoting rhizobacteria (PGPR)) may indirectly influence canker symptom expression via their effects on the endophytic profile (identity or abundance) or via induction of host defence responses. Plants respond to multiple stresses differently from how they do to individual abiotic and biotic stresses, activating a specific programme of gene expression relating to the exact environmental conditions encountered. AMF and PGPR can induce specific plant defence responses. Plant hormones are major components of those pathways and regulate differential defence responses to specific types of attackers. Broadly, jasmonic acid (JA) and ethylene (ET) are responsible for elicitation of defences against necrotrophic pathogens, whereas salicylic acid (SA) is predominantly involved in defence against biotrophic pathogens. The SA- and JA-pathways can exhibit negative crosstalk - *N. ditissima* is classified as a necrotrophic pathogen; hence increased defence signalling (SA) against biotrophic pathogens (induced by external factors) may be at the expense of reduced defence against colonisation by latent infections of *N. ditissima*. Simultaneous occurrence of biotic and abiotic stresses can cause either a positive or negative plant defence response to a would-be-pathogen. This interaction between biotic and abiotic stresses is orchestrated by hormone signalling pathways, in particular abscisic acid (ABA). We hypothesise that the negative crosstalk in plant hormone signalling in response to external factors (e.g. soil water deficit, AMF, PGPR) leads to accelerated development of *N. ditissima* latent infection.

Overall objectives

The overall objective is to assess the role of endophytes in conferring resistance to *N. ditissima*, and to assess how the abundance of the specific endophytes is influenced by other biotic/abiotic factors in relation to plant defence responses and canker development. Project

outcomes will underpin the development of practical measures to reduce canker development, particularly in the early stage of orchard establishment. This will not only reduce tree death in the early establishment phase but also result in reduced secondary infection of branches and fruit due to a reduction in inoculum.

Specific objectives include: (1) confirming the association of specific endophytes with cultivar tolerance to *N. ditissima*; (2) quantifying biocontrol potential of specific endophytes that showed differential abundance between susceptible and resistant cultivars; (3) investigating whether specific endophytes induce host defence responses that may contribute to reduced canker development, (4) mapping QTLs that control recruitment of specific endophytes; (5) determining the extent to which the abundance of specific endophytes can be influenced by single or combined biotic/abiotic factors, (6) conducting a 'common garden experiment' to determine the extent of association of canker symptom development with endophytes and other abiotic/biotic factors.

In addition to funding from BBSRC and AHDB, the following industry partners also provide in-kind support: **Adrian Scripps Limited**, **Avalon Produce Limited, ENZA** (T&G global subsidiary), **Frank P Matthews Limited,** and **Worldwide Fruit Limited.**

The entire project is divided into six work packages, each dealing with specific topics. In this report, in order to have a better flow of information, we report work package by work package.

WP1: Endophytes in relation to cultivar resistance

Objectives

1. To determine whether there are differences between endophyte populations in leaf scars on 1-year-old shoots among a number of varieties and whether such differences are associated with the cultivar susceptibility to European apple canker
2. To determine whether rootstocks and environmental conditions can affect endophyte populations over time.

Material and methods

Eight scion cultivars were grafted onto two rootstocks, and the trees were planted in two sites in spring 2018. Of the eight cultivars, three were resistant and the others susceptible to canker. M116 rootstock is believed to show resistance against canker whereas M9 is susceptible. For each scion/rootstock combination, there were 15 trees at each site; trees were planted in a randomised block design of eight blocks: 7 blocks of pairs, and 1 block of single.

Figure 1a: Sampling leaf scars at Friday St Farm, 4th Oct 2018.

We chose to profile endophytes in the leaf scars because leaf scars are one of main natural entry points for canker infection under field conditions. Leaf scars were sampled at Friday St Farm and Perry Farm in Oct 2018 and June 2019 for microbiome metabarcoding analysis (Figure 1a). There were five biological replicates for each scion/rootstock combination at each site, giving 80 samples per site. One- year-old wood (shoots) with leaves still attached were (cut) collected from the leader and up to four feathers from each tree and taken to the lab.

In the lab, leaves were removed in a laminar flow hood to expose the leaf scar tissue. We did not remove epiphytic cells on the bark surface because:

1. Once the leaf has been removed, surface sterilisation also affects internal tissues of the leaf scar and change endophytes
2. Epiphytic microorganisms at the leaf scar can also affect infection and disease expression.

Instead, leaf scar tissue with minimal amount of bark was dissected with a sterile scalpel. A total of 12 leaf scars (ca. 0.3 g) per tree were dissected from 3 – 5 shoot sections, pooled and stored at -80 °C until DNA extraction, constituting one biological sample. DNA was extracted following standard protocols.

DNA preparation for amplicon sequencing, sequencing in Illumina MiSeq, subsequent sequence processing and statistical analysis followed the established protocols as NIAB EMR (Tilston et al. 2018; Deakin et al. 2018)

Results

We have completed metabarcoding on the Oct 2018 sampling time and are currently analysing the results. Preliminary results showed that:

(1) Endophyte composition differed largely between two sites, particularly for fungi (Figure 1b), indicating that the endophytes observed at the end of the first season probably entered post planting, rather than in the nursery.

(2) There are significant differences between cultivars in the overall endophyte composition, particularly between Robusta 5 (resistant) and the other seven cultivars.

(3) The overall endophyte composition did not differ significantly between canker susceptible and resistant cultivars. However, there are a number (20-30) of specific microbial groups that differ between the resistant and susceptible cultivars. Currently, we are searching literature and conducting phylogenetic analysis to infer possible functions of these specific microbial groups.

Because the results from the first batch of samples showed large differences in relative abundance of specific microbial groups, we are now proceeding with extracting DNA from the second batch of samples (collected June 2019).

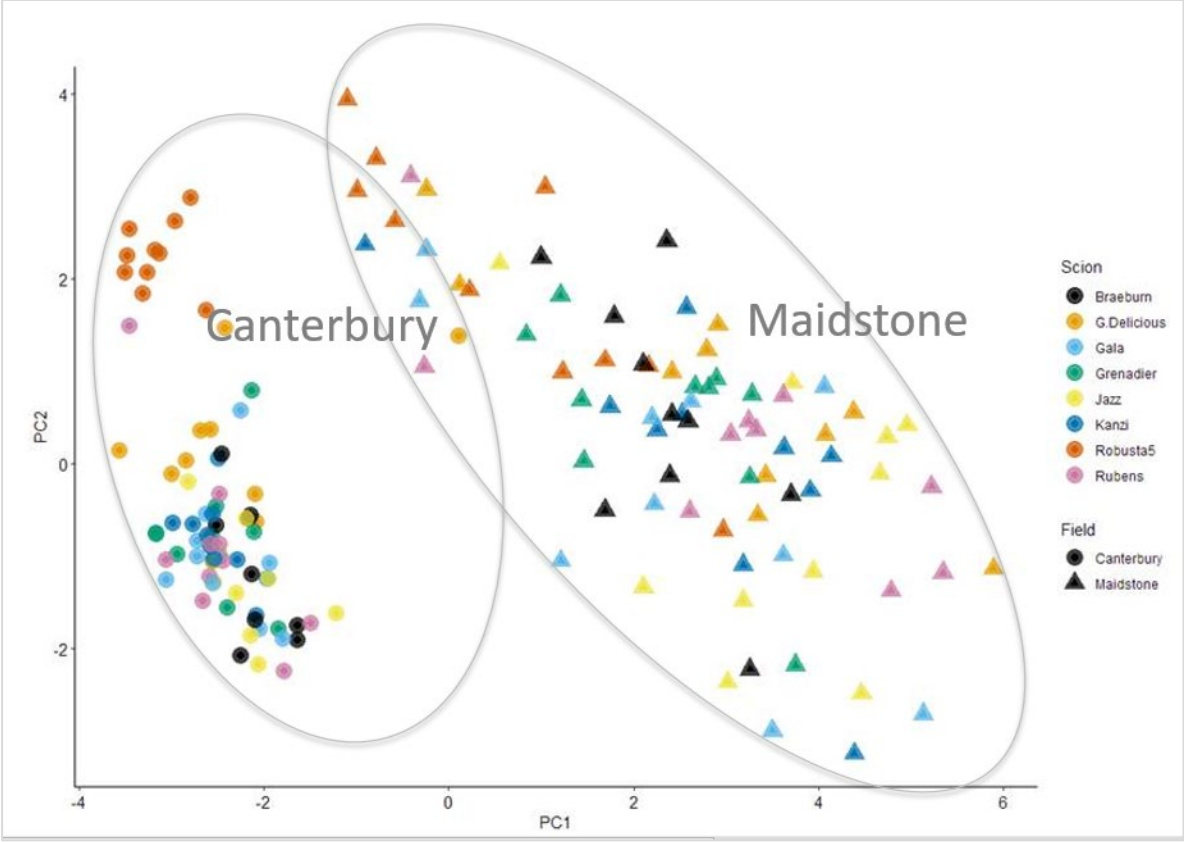


Figure 1b. Principal component analysis plot of fungal endophytes sampled from two field sites in Kent (Canterbury and Maidstone). The eight scion varieties tested and site locations are listed in the legend.

WP2: Endophyte biocontrol efficacy

In a preliminary meta-barcoding study, we identified several fungal endophytes that are significantly more abundant in canker resistant apple cultivars than in susceptible cultivars. One of them, *Epicoccum purpurascens* (previously known as *Epicoccum nigrum*), has been previously shown to have biocontrol properties against several *Fusarium spp.* (Ogórek and Plaskowska 2011), *Pythium* damping-off in cotton (Hashem and Ali 2004) and *Monilinia spp.* brown rot in peaches (Larena, Cal, and Melgarejo 2004; Cal et al. 2009).

Objectives

1. To assess whether *E. purpurascens* could control *N. ditissima in vitro* and *in vivo*
2. To determine whether there are other apples endophytes with biocontrol potential present in local apple trees

Material and methods

In vitro challenge assay: We continue to carry out *in vitro* tests to screen the collection of apple endophytic fungi for biocontrol activity against apple canker. We have tested four *Epicoccum* isolates and four *Aureobasidium* isolates, each isolated against three *N. ditissima* strains. Thus, there are 24 assay combinations, each with three replicate plates. We used the same methodology as described in the Year 1 report.

6 mm agar plugs of *N. ditissima* and *E. purpurascens* were placed at opposite ends of the 6 cm line on the plate with mycelium side down. Plates were then incubated the right way up in the dark at 20°C. Once fungal growth started, the plates were turned upside down to reduce the risk of condensation causing contamination. *Neonectria ditissima* colony size across the line on the plate was recorded twice a week for several weeks.

In a separate project on biocontrol of ash dieback we identified more than 20 fungal strains (*Epicoccum* and others) that showed good biocontrol potential against ash dieback pathogen. The *Epicoccum* strains identified here for good biocontrol of apple canker were tested against ash dieback pathogen and they all showed biocontrol potential. We are now testing if the reverse is true i.e., if 20 plus strains identified against ash dieback can a) control apple canker *in vitro* and b) if they sporulate on artificial media to facilitate inoculation and efficacy testing on trees.

Assessing biocontrol efficacy of *E. purpurascens* in planta: Four *Epicoccum* isolates that showed biocontrol potential on the plates were grown on the solid sporulation media to obtain sufficient amount of spores for inoculating trees or rootstocks. Sporulation of all four new *Epicoccum* isolates was very poor. Only one isolate sporulated at all, the rest showed no spore formation after 8 weeks of growth on lentil meal media that was previously used with good success. All tree inoculations were therefore done with the same *Epicoccum* strain (B14-1) as in season 2018/2019.

Endophyte augmentation and *In planta* biocontrol efficacy of *Epicoccum* (B14-1 strain) against apple canker: We carried out studies to determine (i) whether endophyte augmentation in the field conditions could be achieved, (ii) if so, to compare augmentation methods, and (iii) whether application of *E. purpurascens* leads to reduced canker development.

This study was carried out in field-grown M9 rootstocks with detailed methodology described in the year 1 report. In July 2018, M9 rootstock were augmented with a single UK *Epicoccum purpurascens* strain (B14-1) via either spraying onto the leaves, drenching on the roots, or both spraying and drenching of spore suspensions. At leaf fall leaves were stripped from all rootstock shoots and the leaf scars were spray inoculated with a spore suspension (10e4 spores/ml) of *N. ditissima* using a hand-held sprayer. Immediately prior to inoculation (October 2018), 3 shoots per plot were sampled and leaves and leaf scars were tested for presence of *E. purpurascens* by (1) plating surface sterilized leaf and leaf scar tissue in media, and (2) quantitative of *Epicoccum purpurascens* by qPCR.

The remaining rootstocks were harvested in mid-December 2018, size graded and stored at +4 ℃ until planting in March 2019. In summer 2019 the planted rootstocks were assessed for canker expression. In autumn 2019 the same rootstocks were sampled again to quantify the presence of Epicoccum at leaf fall more than a year after inoculation.

Results

In vitro challenge assays

Two *Epicoccum* strains (C15, C29) and one *Aureobasidium* strain (C32(1)) showed substantial levels of biocontrol ability (Figures 2 and 3), reducing the growth of all three *N. ditissima* stains 12-15 days post treatment and completely stopping the growth of *N. ditissima* by 20 days. All tested strains showed some biocontrol potential by 20 days post-treatment. *In vitro* testing of 20 fungal endophytes (*Epicoccum* and others) from ash trees are currently ongoing.

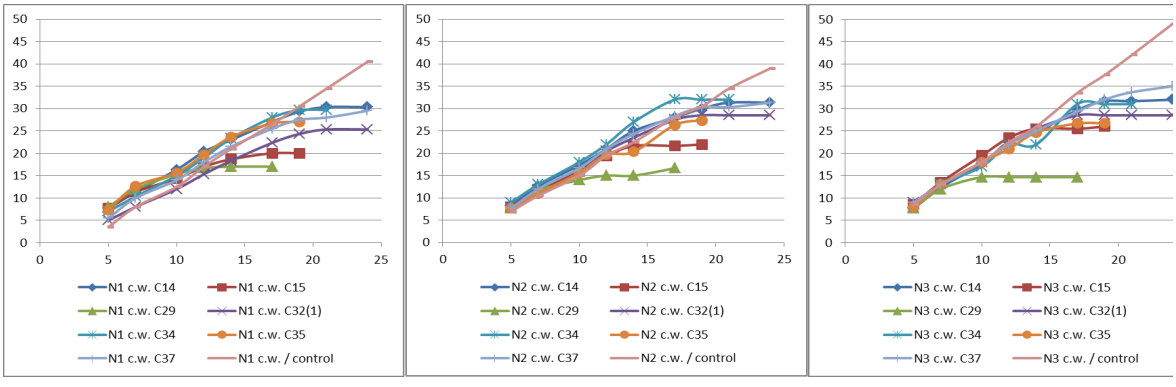


Figure 2. Growth (mm) of three *N. ditissima* strains (N1, N2 and N3) (Y-axis) over time (days) (X-axis) *N. ditissima* was either challenged with endophyte strains or without endophyte challenge (control).

Translating *in vitro* plate assay to *in vivo* assay on the trees.

Inoculated trees will be assessed for canker occurrence in the spring 2020.

Endophyte augmentation

Epicoccum purpurascens was successfully re-isolated from the inside of the leaves and leaf scars of M9 rootstocks after application of *E. purpurascens* spore suspensions, particularly with drenching, and both spraying and drenching application methods (Table 1). Spraying alone appears to be less successful. Importantly, *E. purpurascens* was not isolated from water controls. The plating results are consistent with qPCR analysis of leaf scars: the augmented samples had significantly higher amount of *E. purpurascens* DNA than water control (Figure 4).

This result indicates that when *Epicoccum* was inoculated in summer either as a spray or as a drench, it can colonise and persist until leaf fall on leaves and more importantly in and around leaf scars. Analysis of the samples collected in autumn 2019, 1 year after inoculation is ongoing and the results are expected by summer.

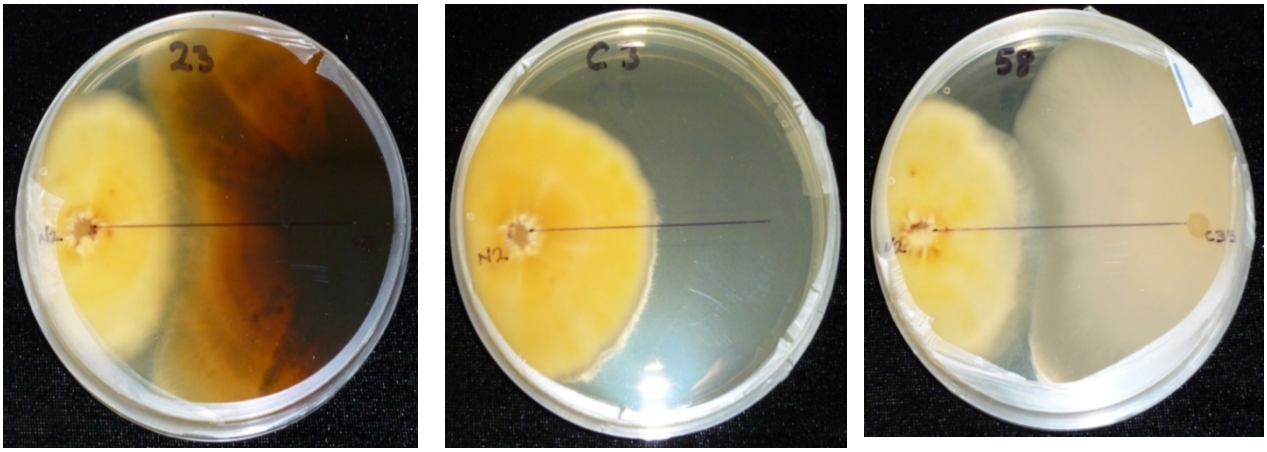


Figure 3: Challenge assay example (17 days after plating) with one of the *Epicoccum* isolates (C29) left, *Aureobasidium* (C35) right and unchallenged control (Neonectria ditissima HG199) in the centre. In both left and right examples we observed endophytes restricting the growth of *N. ditissima*.

Table 1: Number of M9 shoots from which of *E. purpurascens* was successfully isolated 3 months after treatment with spore solution of a single UK *E. purpurascens* strain (B-14). Four leaves and four leaf scars were sampled from 12 independent shoots per treatment (3 per block). Numbers below indicate the number of shoots with at least one isolate from different tissue identified as *Epicoccum spp*. by colony morphology and confirmed with ITS sequences.

Tissue type	Sprayed	Drenched	Sprayed + Drenched	Untreated control
Leaf scar	0	1	3	
Leaf	1	4	1	0

In planta biocontrol efficacy of *Epicoccum* (B14-1 strain) against apple canker

Colonisation with an endophyte may impose a growth penalty because the fungus requires a nutrient source which would be derived from the host plant. Size grading of inoculated rootstocks indicated that Epicoccum inoculation did not significantly affect rootstock growth/quality (Figure 5). This is most likely due to very small increase in concentration of *Epicoccum* in the shoots in comparison to control 0 (Figure 4). Canker expression levels were not different between treatments (Figure 6). Canker incidence however was extremely low, which could explain the lack of differences.

We repeated this experiment in 2019, trying to increase inoculation efficiency and hence obtain better data. Another 4 blocks of M9 rootstocks were inoculated with *Epicoccum* in Sep 2019 and infected with *N. ditissima* at leaf fall. Moreover, augmented rootstock planted this year will be inoculated again and observed for canker expression for at least another season. DNA from the leaf scar samples taken more than a year (Nov 2019) after initial augmentation (July 2018) are being extracted to measure the levels of *Epicoccum* at leaf fall the second season and estimate longevity of augmentation.

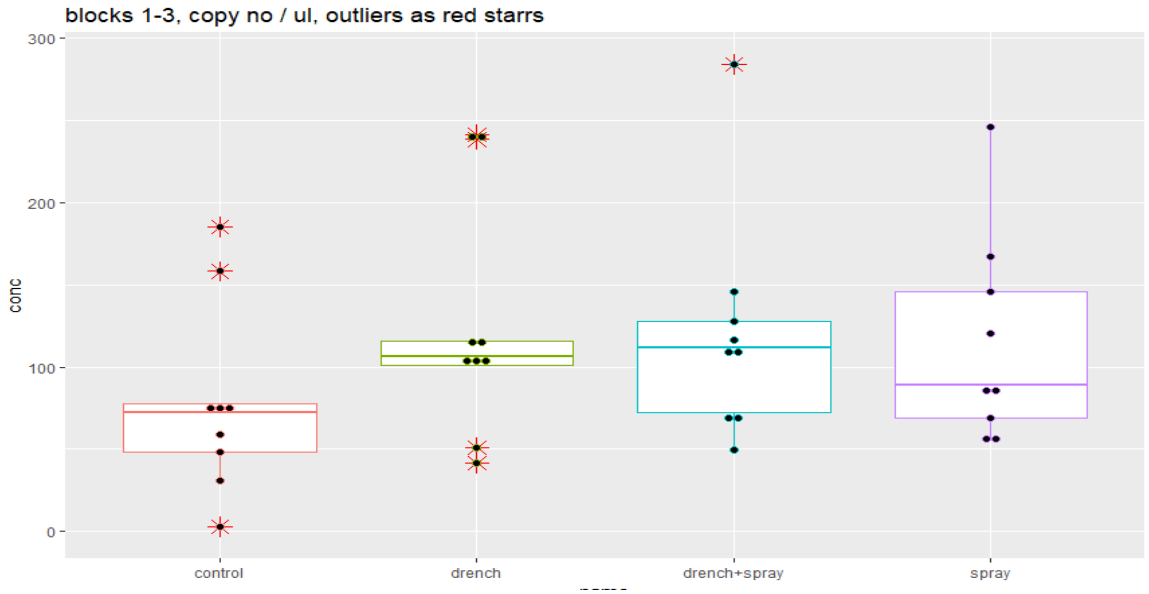
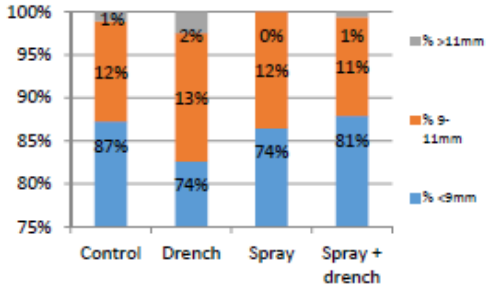
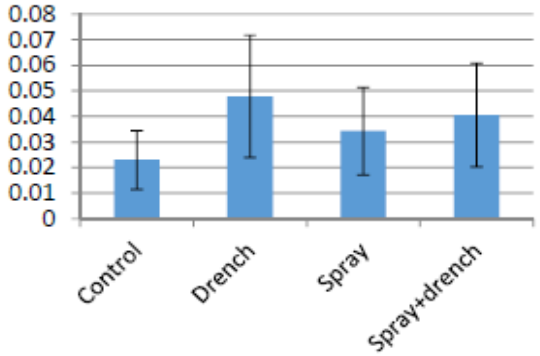


Figure 4: Box plot of *E. purpurescens* DNA concentration (copy number) in leaf scars of water treated (control) M9 rootstock and augmented with *Epicoccum purpurascens* spore spray, drench or both.



	No. of rootstocks per size		
Treatment	<9mm	9-11mm	>11mm
Control	157	21	2
Drench	133	24	4
Spray	134	21	0
Spray + drench	145	19	1

Figure 5: Size grading of *Epicoccum* inoculated M9 rootstocks. Percentage of different size classifications (left) and absolute numbers in each size classification (right).



Epicoccum treatment	No. of inoculated stems	No. of stems with canker
Control	141	3
Drench	126	6
Spray	144	5
Spray+drench	125	5

Figure 6: Percent of M9 rootstock with visible canker in summer 2019 (left) and absolute numbers of rootstocks and cankers observed (right).

WP3: Induction of plant defence response by endophytes

Objectives

1. To determine how apple trees respond to endophytes, specifically whether endophytes induce plant defence responses

Material and methods

Trees of cvs. Kanzi (susceptible) and Golden Delicious (resistant) were subjected to one of the four treatments in the nursery: inoculation with *N. ditissima* (yes, no) x inoculation with *E. purpurascens* (yes, no). Trees were planted at Hononton Farm (Scripps) in the early winter 2018/19 (Table 2). Trees were sampled summer 2019 to ascertain i) if *Epicoccum* augmentation in the nursery results in higher levels of *Epicoccum* in leaf scars in summer after planting, and ii) if higher levels of *Epicoccum* result in induced plant defences.

Table 2: Number of trees for each treatment planted at the Hononton Farm for WP3 work

Rootstock	Scion	Epicoccum inoculation	Nectria inoculation	No of trees
M9	Kanzi	+	+	30
M9	Kanzi	-	+	30
M9	Kanzi	+	-	30
M9	Kanzi	-	-	30
M9	Golden delicious	+	+	30
M9	Golden delicious	-	+	30
M9	Golden delicious	+	-	30
M9	Golden delicious	-	-	30

Two out of five blocks were sampled in summer 2019. Three different one-year-old shoots from around the tree (top, mid and bottom) were sampled from five trees per treatment per block. About 12-15 leaf scars have been sub-sampled from 3 out of 5 samples per treatment per block. Leaf scars were freeze dried and crushed. DNA was extracted and qPCR was used to quantify the amount of *Epicoccum* DNA which was normalized by the amount of plant DNA (elongation factor EF1a) to normalise for different amounts of starting material.

Results

There was a significant scion effect on the quantity of *Epicoccum* DNA in the plant tissue (Figure 7). In line with preliminary meta-barcoding data the amount of *Epicoccum* in relatively canker resistant Golden Delicious was significantly higher than in relatively susceptible Kanzi. *Epicoccum* and/or *N. ditissima* inoculation (Table 2) did not have significant effect on levels of *Epicoccum* within the scion cultivar (Figure 8). There was slight indication of elevated levels of *Epicoccum* in Kanzi, which is an encouraging indication, that augmentation with *Epicoccum*

inoculum at the nursery stage could be a valid strategy. We will extract and analyse the rest of the samples to see if we can get clearer indication.

The data overall suggests that scion genotype is stronger and more stable driver of *Epicoccum* presence than nursery augmentation. The process of uprooting, storage, and planting could also contribute to low augmentation observed in both cultivars. Augmentation of planted trees in production orchard (*Epicoccum* and *N. ditissima*) would be a better system and will be attempted in 2020 with permission from the grower (Mark Holden, Scripps).

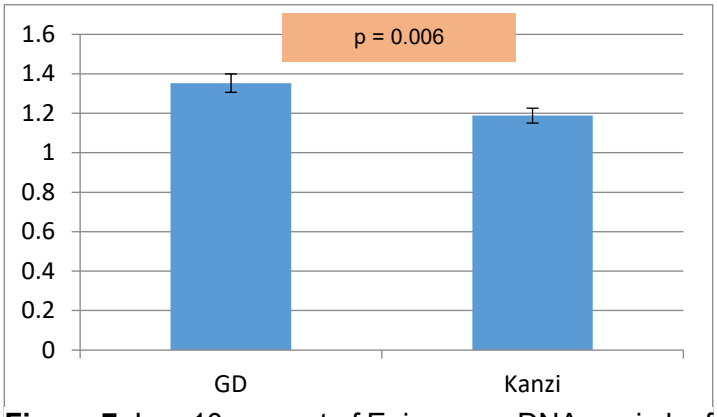


Figure 7: Log 10 amount of Epicoccum DNA per in leaf scar +/- SEM of Golden Delicious (GD) and Kanzi scions on M9 rootstock. Shoots from treatments from Table 2 are combined in a single data point.

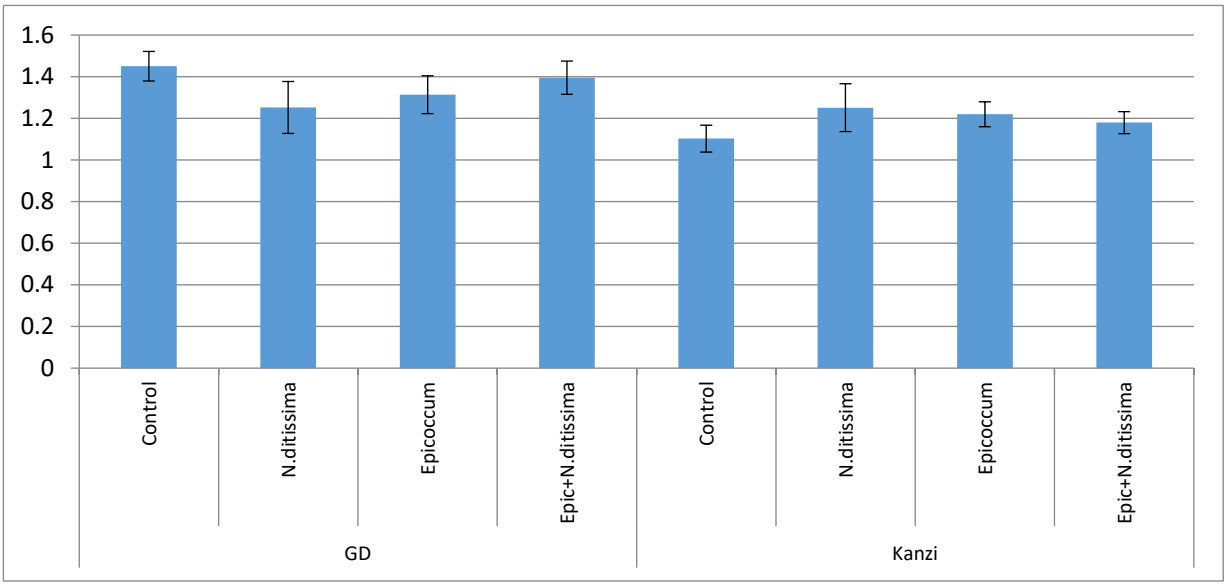


Figure 8: Log 10 amount of Epicoccum per leaf scar +/- SEM. Golden Delicious (GD) and Kanzi shoots treated with Epicoccum (July 2018, FPM), Neonectria (Nov 2018, FPM) or both are compared to water treated control.

Due to the low augmentation efficacy, we did not attempt to extract RNA and/ or metabolites to investigate whether *Epicoccum* inoculation increases plant defences. This will be done either from freshly augmented trees at NIAB EMR or at Hononton Farm (Scripps) in 2020.

At the same time as trees were sampled we also assessed them for canker expression. We recorded extremely low canker levels with a maximum of one peripheral canker per treatment in each cultivar and thus no significant differences between treatments were observed. We will continue to assess trees for canker until the end of 2020.

WP4: Mapping QTLs responsible for recruiting endophytes

Objectives

1. To map QTLs responsible for recruiting specific endophytes that have biocontrol potential against the European canker
2. To assess the overlap of these QTLs with those mapped for canker resistance in the same mapping progeny

Materials and methods

In order to choose one mapping family for the mapping study, we have profiled endophyte profiles at leaf scars for the ten parents of the five mapping populations, which have been used in another BBSRC LINK project to study canker resistance. Each parent had three biological replicates for characterizing fungal and bacterial endophytes. Detailed methodology can be found in WP1.

Results

There were no clear differences in bacterial communities between the two parents for each mapping population. Principle component analysis showed that two parents for two mapping populations (’54’ and ‘60’) differed largely in their fungal endophytes community (Figure 9).

Following discussions with breeders/geneticists and based on field canker development of the mapping populations, the ‘54’ mapping population is chosen for endophyte characterization. This population has a total 61 individuals, but only 54 genotypes were confirmed to true hybrids of the two parents.

In November 2019, leaf scars of the 54 genotypes were sampled for fungal endophyte characterization; each genotype was replicated four times. Currently, DNA are being extracted from these samples.

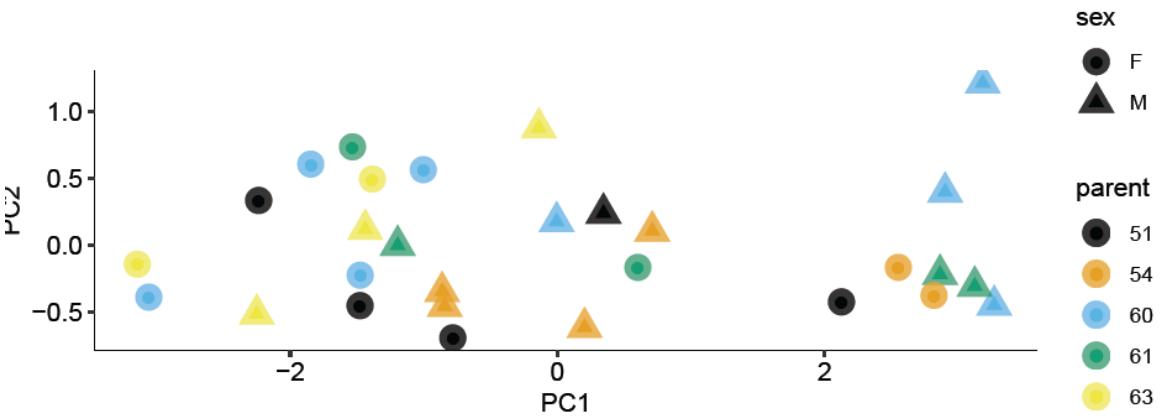


Figure 9: PCA scores of fungal endophytes for those parents (male (M) and female (F)) of the five mapping populations (51, 54, 60, 61 and 63) for canker resistance.

WP5: Effects of specific factors on endophytes

Endophytes associated with specific apple genotypes may be an important component affecting latent canker development, thereby contributing to field resistance. Recent evidence suggests that endophytes may induce plant defence responses, produce secondary metabolites that inhibit pathogens, directly compete with invading pathogens or a combination thereof. Endophytes can also help plants tolerate abiotic stresses, e.g. salt and heat tolerance. Endophyte composition can also be influenced by pathogen presence, production system, and AMF colonisation.

Objectives

1. To evaluate biological soil amendments (arbuscular mycorrhizal fungi (AMF) and plant growth promoting rhizobacteria (PGPR)) for their effects on tree health and canker expression.
2. To investigate the effects of deficit irrigation and AMF/PGPR on endophyte profiles and canker expression.

Material and method

The establishment and design of this experiment was described in the Year 1 report. Trees of cv. Gala on M9 were planted in 10 litre pots in April 2018 and grown in a polytunnel. At planting, biological soil amendment treatments were applied as follows: Control (non-inoculated), AMF (six species mix), PGPR, or a combined treatment of AMF and PGPR. Trees were grown under either 100% or 65% of watering to capacity. Half of the tress were harvested in the autumn 2018; currently we are quantifying root colonisation by AMF, plant hormones, rhizosphere and root and tissue endophyte.

For the other half of these trees, a number of leaf scars were inoculated with *N. ditissima* on 18th October 2018. Two shoots of each tree were selected to give approximately 15 leaf scars per tree. The top two or three leaves on the shoot were left and the rest of the leaves were removed manually to create leaf scars, with the top/bottom leaf scars marked with paint. The shoots were sprayed to run off with 1 x 10^4 *N. ditissima* macrospores (germination rate test = 95%). To increase humidity, large clear plastic bags were sprayed with a little water and placed over the inoculated shoot and attached with wire around wetted cotton wool inside the opening of the bag (Figure 10). The bags were removed after 24 hours. Canker

Figure 10: Plastic bags were used to maintain high humidity to promote canker infection following inoculation of leaf scars.

development was assessed in the spring 2019 and a further assessment of canker expression was carried out in October 2019. At the end of the summer 2019, samples were taken from these trees for assessment of root-associated characters.

Results

The canker data assessed in April 2019 and in October 2019. Both assessments suggest a possible reduction in canker expression with the PGPR and AMF treatments only in the well-watered trees (Figures 11 and 12). Further statistical analyses will be applied to the data.

Figure 11: Canker expression was recorded on the leaf scars pre-inoculated with *N. ditissima* macrospores

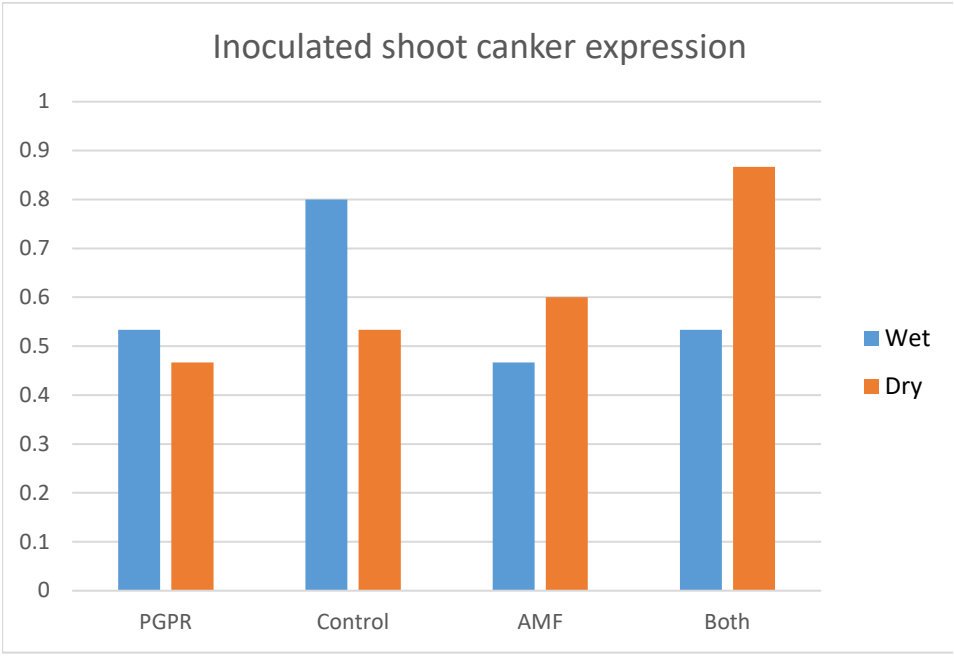


Figure 12: Average number of cankers per shoot for each treatment when assessed in October 2019.

Total height and girth measurements (trunk diameter, measured with digital callipers in two directions at 5 cm above the graft union) were also recorded for the trees grown beyond the 2018 season. Trees treated with AMF or the combined treatment grew more under reduced irrigations conditions; however the PGPR treated and combined treated trees grew more under well-watered conditions (Figure 13). Girth assessments suggest a possible increase in trunk size with AMF under well-watered conditions.

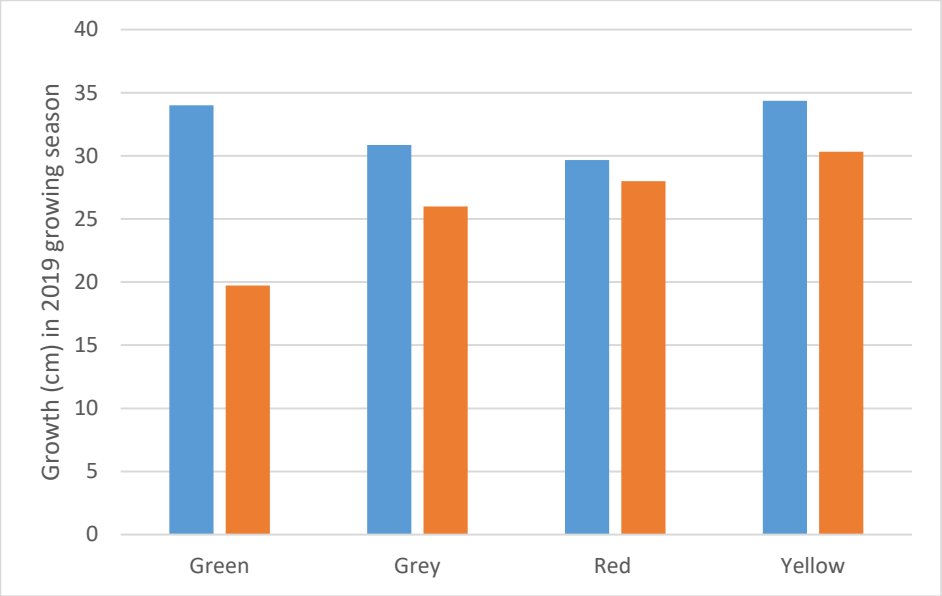


Figure 13: Growth (cm) of trees in the 2019 growing season (April-October).

We are currently quantifying AMF colonisation, rhizosphere microbial composition, plant hormones and endophytes.

WP6: Common garden experiment

Although there is ample evidence suggesting that there are specific sites that are particularly prone to canker expression, to date no research has been conducted to identify which specific factor(s) that could be responsible for promoting canker symptom development. Most importantly, empirical evidence suggests that lengthening storage time of trees between lifting and planting would worsen canker development in orchards. We propose to conduct a 'common garden experiment' to obtain a large dataset for establishing statistical association between canker expression, soil chemical and microbial properties and endophyte profiles. This large dataset could be used to formulate hypotheses for future testing.

Objectives

1. To obtain information regarding the effect of tree planting date (early winter vs early spring) on canker incidence.
2. To investigate whether canker is associated with certain soil physio-chemical characteristics, bulk soil microbiota, endophytes, and plant hormones.
3. To elucidate endophytes for apple genotypes across sites, and whether canker development is associated with particular endophyte communities.

Material and methods

Following the discussion in the Year 1 consortium meeting, we decided to focus this common garden experiment on studying the effect of cold storage on subsequent canker development in orchards (objective 1). Five cultivars (Golden Delicious, Grenadier: resistant; Gala, Braeburn Jazz, Kanzi, and Rubens: susceptible) were grafted to M9 (337) rootstocks.

In November 2018 (at leaf fall) whilst still growing at the nursery, all trees were sprayed with a moderate level of *N. ditissima* conidial suspension. This inoculation was used to ensure presence of a certain level of latent canker on all sites, increasing the usefulness of the dataset to be collected.

Trees were planted at Avalon (Friday St Farm), Scripps (Hononton Farm) and World Wide Fruit (Sheerland Farm) associated sites in early winter (December) 2018 (within a week of lifting the trees in the nursery), or refrigerated and then planted in early spring (March) 2019. Trees were assessed for canker in autumn 2019.

Results

Braeburn (susceptible) and Grenadier (resistant) had low incidence for both planting dates.

The total number of cankers summed across all three sites was 2.6 times higher with the spring planted trees than with the early winter planted trees (Table 3), showing that keeping the trees refrigerated over winter before planting increased canker incidence. The cultivar Kanzi had the highest canker incidence from early spring planted trees, followed by Jazz, Rubens and Gala. Kanzi also had the highest incidence for early winter planted trees, followed by Gala and

Rubens. These results will help inform growers to better tailor planting times to reduce canker, particularly of specific cultivars like Kanzi, Jazz, Rubens and Golden Delicious.

Trees will be further assessed for canker in spring and autumn 2020 and data analysed in the autumn. If there are still significant/consistent differences between cultivars, or sites, or planting time, we will sample and profile rhizosphere and endophyte communities and try to associate a relationship of canker incidence with specific microbial groups.

Table 3. Canker incidence (measured as canker number) of seven apple cultivars planted either in early winter (December 2018) or refrigerated and planted in early spring (March 2019). Listed from highest to lowest total canker number from all sites.

Planting time	**Cultivar**	**Scripps**	**World Wide Fruit**	**Avalon**	**All sites**
December 2018	Kanzi	10	11	3	24
	Gala	0	12	1	13
	Rubens	0	5	2	7
	Jazz	0	1	1	2
	Golden Delicious	0	0	1	1
	Grenadier	0	0	1	1
	Braeburn	0	0	0	0
	All varieties	10	29	9	48
April 2019	Kanzi	19	13	14	46
	Jazz	18	6	5	29
	Rubens	8	11	6	25
	Gala	7	5	0	12
	Golden Delicious	5	1	2	8
	Braeburn	1	1	1	3
	Grenadier	0	1	1	2
	All varieties	58	38	29	125

Research activities in the coming seasons

We have now shown canker-resistant cultivars differ in the relative abundance of a number of microbial groups from canker-susceptible cultivars. However, we need to understand (1) what are these specific microbial OTUs, and what are their potential functions, and (2) whether these specific endophytic differences persist over time. These are the two key tasks to be completed within the next 12 months. Answers to the two questions will help us assess the potential of manipulating specific apple endophytes for canker management.

We further demonstrated that a number of fungal endophyte strains (*Eppicocum*) from apple have antagonistic effects against apple canker under *in vitro* tests. We are now conducting *in vivo* tests for their biocontrol effect against the canker pathogen. If confirmed, we may be able to develop these strains into biocontrol products for use in commercial production, e.g. as a pruning wound paint. Moreover, we demonstrated that specific apple endophytes could be augmented via drenching stoolbeds but not by foliar spray treatments in orchards. We are now studying whether such an increase in a specific endophyte could persist over time, which will guide the development of future application methodology.

Most importantly, we have shown that longer duration of trees in cold-storage led to increased canker incidence post-planting. However, we now to determine whether early planting actually reduces the number of cankers or simply delays the onset of canker symptom development over time. This will be assessed in the next 12 months. Inoculation of plants with PGPR or AMF at planting time appeared to have resulted in increased tree development. Thus, combing early planting with AMF/PGPR treatments at planting may help tree establishment and reduce development of cankers, originating from nurseries and orchards.

Knowledge and Technology Transfer

(1) We presented an oral presentation on canker epidemiology and potential role of endophytes in canker development at Plant Health 2019 (APS annual meeting), Cleveland, August 3rd to 7th 2019.

(2) We presented a poster on the "Microbial ecology of the European apple canker pathosystem (*N. ditissima*)") at the microbiome conference miCROPe 2019 (Microbe-assisted crop production – opportunities, challenges and needs), Vienna, December 2nd to 5th 2019

(3) We presented a poster on "The use of beneficial microbes in commercial horticulture" at the microbiome conference miCROPe 2019 (Microbe-assisted crop production – opportunities, challenges and needs), Vienna, December 2nd to 5th 2019

(4) We gave a seminar to Agrovista growers on 'the use of beneficial microbes in commercial crop production, with specific reference to apple canker' on 29 Jan 2019. Around 100 growers/agronomists attended the meeting. -

(5) We attended a British council HEP workshop in Feb and May 2019, BKK Thailand, and presented the current work at NIAB EMR, including the work on the BBSRC canker research.

(6) We gave a seminar to Agrovista growers on 'the use of beneficial microbes in commercial crop production, with specific reference to apple canker' in Jan 2020. Around 50 growers/agronomists attended the meeting.

(7) We briefly introduced apple canker research (including the BBSRC LINK project) at NIAB EMR at the 32nd BIFGA Technical Day on 23rd Jan 2020.

(8) We gave a talk on 'Harnessing endophytes to aid apple canker control' at the AHDB Tree Fruit 2020 at NIAB EMR on 27th Feb 2020.

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