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| 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<br>NIAB EMR |
| Report: | Annual, 10/2020 |
| Previous report: | Annual, October 2019 |
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| Location of project: | East Malling, and several farms in Kent |
| Industry Representative: | Peter Checkley |
| Date project commenced: | 1 October 2017 |
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# Contents

# GROWER SUMMARY

## Headline

- One fungal endophyte showed good biocontrol potential against infection of leaf scars by the canker pathogen.

## 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 specific endophytes.

Recently, we have obtained preliminary data showing a link between antagonist fungal endophytes and 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 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 by 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 or the abundance of specific endophytes across several 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 3

We have successfully initiated all experimental studies on time; however, much of the lab molecular work has been delayed by at least six months because of COVID-19. We have applied for 6 month no-cost extension and are waiting for response from BBSRC.

(1) We have profiled endophytes at leaf scars of eight cultivars with differing tolerance/resistance to apple canker:
   a. Endophyte diversity was primarily affected by orchard location, followed by the scion, whereas the effect of rootstock was small.
   b. Several fungal and bacterial groups had differential relative abundance between canker resistant (tolerant) and susceptible cultivars. The specific fungal groups included fungal antagonists as well as plant pathogens.

(2) One *Eppicocum* endophyte from apple has been shown to have good antagonistic effects against the apple canker pathogen in field tests:
   a. Co-inoculation of both Eppicocum and canker inoculum at leaf scars can reduce the canker incidence at leaf scar by 50%.
   b. For pruning cuts, there is very limited effect of *Eppicocum* probably because of greater susceptibility of fresh pruning cuts combined with a high dose of pathogen inoculum applied.

(3) We conducted experiments to investigate how quickly and how far *Epicoccum* can colonise apple shoots through inoculated leaf scars. The results will be obtained by early 2021 (COVID-19 permits).

(4) Inoculation of plants with Plant Growth Promoting Rhizobacteria or Arbuscular Mycorrhizal Fungi at the planting time appeared to result in increased tree development but have negligible effects on canker development.

(5) Longer duration of trees in cold storage initially led to increased canker incidence post-planting but two years after planting canker incidences did not differ much between the two planting (storage) times.

## Financial benefits

The results are from only the second year of a four-year project and hence it is too early to quantify 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 site characteristics.

*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 to breed for increased tolerance/resistance to the pathogen. Breeding apple cultivars currently 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 and production system.

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, 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 several 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 (Robusta5, Grenadier, Golden delicious, Gala, Kanzi, Jazz, Braeburn, Rubens) were grafted onto two rootstocks (M116 and M9), and the trees were planted in two sites (Friday Street Farm- Avalon, and Perry Farm – WorldWideFruit) in spring 2018. Of the eight cultivars, three were considered resistant (Robusta5, Grenadier, Golden delicious) 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.

We chose to profile endophytes in the leaf scars because leaf scars are one of main natural entry points for canker infection under UK field conditions. Leaf scars for microbiome metabarcoding analysis were sampled at Friday St Farm and Perry Farm in Oct 2018, June 2019, and November 2019 (Figure 1a).

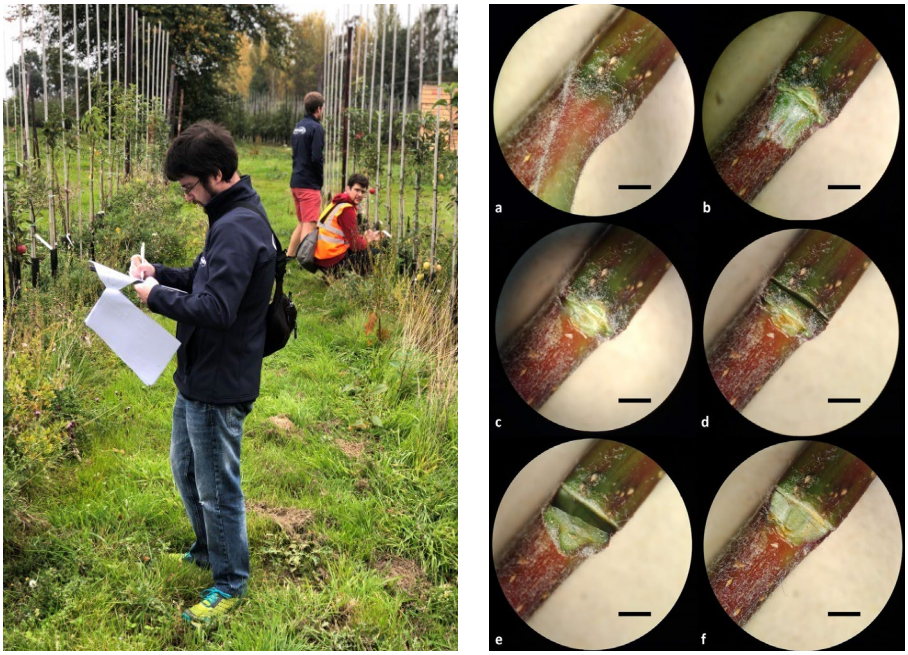


**Figure 1.** Sampling leaf scars at Friday St Farm, 4th Oct 2018 (left). Dissecting leaf scars from the shoots (right)

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 (Figure 1a). 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 using Qiagen DNAeasy plant kit.

Samples from Oct 2018 sampling time were analysed in house using Illumina MiSeq instrument. Small ribosomal subunit (16S) sequence was used to assess bacterial communities and Internal transcribed spacer (ITS) sequence was used to assess fungal communities. The result of 2018 sampling is presented below. It is worth pointing out that in the analysis of samples from Oct 2018 the total bacterial/fungal community size have not been considered. The results therefore represent only relative abundance of different species/genera to the population and do not accurately reflect absolute differences in abundance. To address this, we have used quantitative PCR to measure bacterial and fungal community sizes and better estimate the differences between samples.

Due to price of the in house sequencing we have decided to outsource the sequencing service of the subsequent time points to Novogene. We have also re-sequenced the samples from Oct 2018 sampling time to ensure consistent methodology across the time points. A small subset of samples did not pass quality control threshold used by Novogene and were not sequenced. In total we sequenced 4 replicate samples per rootstock/scion combination per site from sampling in Oct 2018 and 2019 and 3 replicate samples per rootstock/scion combination per site from sampling in June 2019.

Preliminary data analysis from in house sequenced Oct 2018 samples showed that the selected 16S primers were not specific to bacterial 16S and most sequencing reads were attributed to host (apple) mitochondrial and chloroplast 16S sequence. Consulting Novogene sequencing experts we decided to use host DNA blocking PNA primers that specifically bind to mitochondrial/plastid 16S DNA and block its amplification in the metabarcoding library preparation step. A further subset of about 20 samples did not amplify to sufficient level using blocking primers and were therefore not sequenced.

At present (1/12/20), we have received the 16S and ITS data for all samples that have passed library preparation stage; between 30 and 50 thousand high quality reads were generated per sample per amplicon. We have also measured total bacterial and fungal community sizes with qPCR. Read clustering, operational taxonomic unit (OUT) assignment and analysis of diversity is currently being analysed following the established protocols at NIAB EMR (Tilston et al. 2018; Deakin et al. 2018). The results are expected by the end of April 2021.

**Results, Season 1 (October 2018 sampling, community size not accounted for)**

(1) The diversity of bacterial leaf scar endophytes was most affected by the field or site where the trees were planted followed by the scion cultivar (Table 1). The bacterial communities of Robusta were significantly different from the other 7 cultivars indicating strong genetic control. Susceptibility to canker had smaller but nonetheless significant effect on bacterial endophyte diversity while rootstock had marginal effect (Figure 2, Table 1).

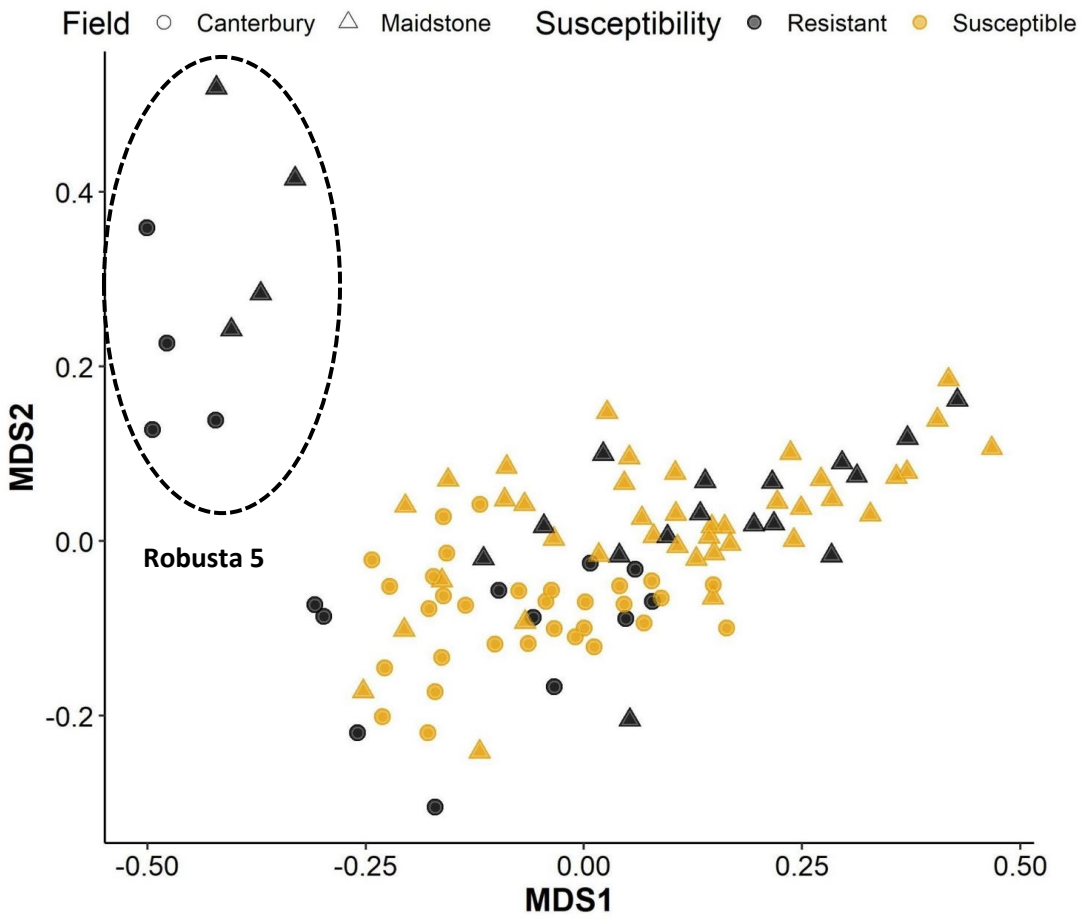


**Figure 2.** Multi-dimensional scaling plot (Bray-Curtis) of bacterial communities. Each point represents one sample. Robusta 5 samples are encircled.

**Table 1.** Proportion of variability (% var) in bacterial diversity indices explained by different factors. Measured diversity indexes are i) observed species richness, ii) Shannon entropy (frequency and distribution of species), iii) Simpson evenness index, and iv) Bray-Curtis beta diversity. *p* indicates statistical significance associated with each term.

| Indices | Field | | Block within field | | Susceptibility | | Scion within susceptibility | | Rootstock | | Scion x rootstock | | Res |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | %Var | p | %Var | p | %Var | p | %Var | p | %Var | p | %Var | p | |
| Observed | 38.0 | <0.001 | 7.6 | <0.001 | 1.5 | 0.05 | 18.2 | <0.001 | 1.9 | 0.03 | 1.9 | 0.58 | 30.8 |
| Shannon | 20.3 | <0.001 | 6.6 | 0.01 | 9.1 | <0.001 | 23.2 | <0.001 | 0.1 | 0.75 | 3.3 | 0.49 | 37.4 |
| Simpson | 6.5 | <0.001 | 7.6 | 0.04 | 10.8 | <0.001 | 31.3 | <0.001 | <0.1 | 0.73 | 4 | 0.33 | 39.9 |
| Bray-Curtis | 12.3 | <0.001 | 7.5 | <0.001 | 3.4 | <0.001 | 20.6 | <0.001 | 1.3 | 0.05 | 4.2 | 0.51 | 50.7 |

(2) Fungal leaf scar endophyte composition differed largely between two sites (Figure 2, Table 2), indicating that the endophytes observed at the end of the first season probably entered post planting, rather than in the nursery. The scion cultivar had considerable effect on the fungal communities, but the canker susceptibility seems to have smaller effect. Robusta5 fungal communities were again significantly different from the other 7 cultivars indicating strong genetic control. The rootstock had no effect on fungal endophytes.

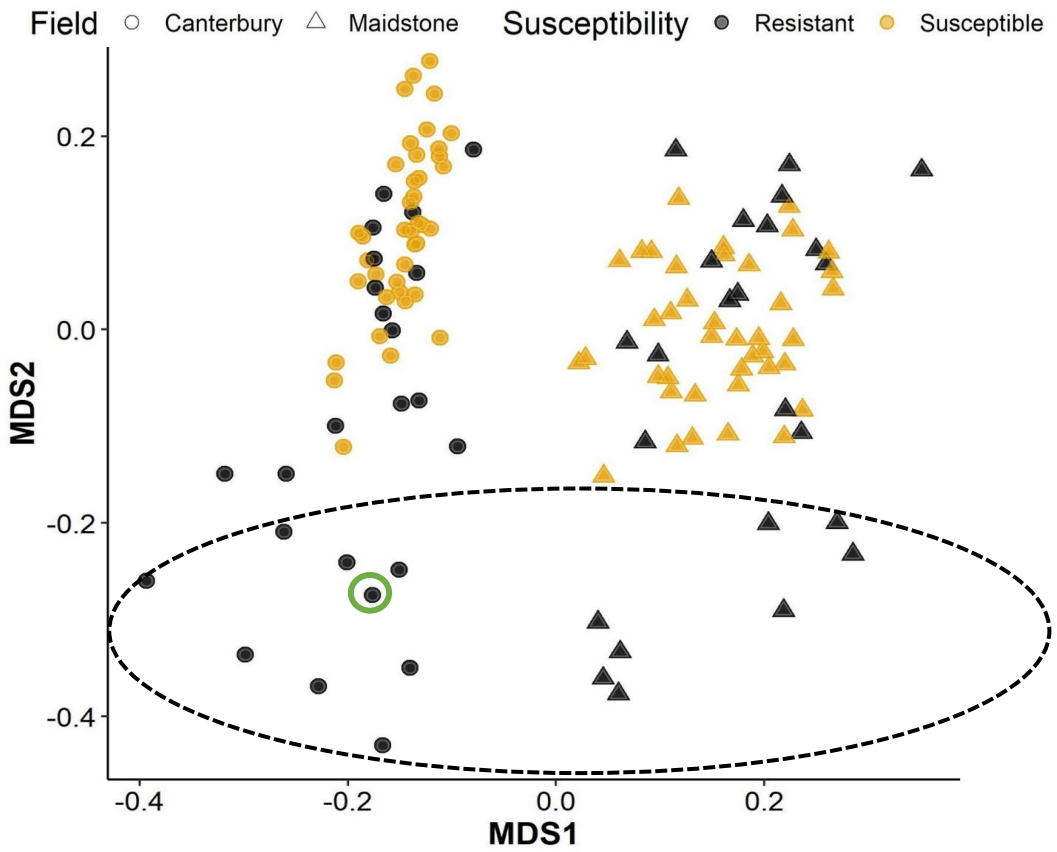


**Figure 3.** Multi-dimensional scaling plot (Bray-Curtis) of fungal communities. Each point represents one sample. Robusta 5 samples are encircled in black and one Golden delicious sample among Robust5 is encircled in green.

**Table 2.** Proportion of variability (% var) in fungal diversity indices explained by different factors. Measured diversity indexes are i) observed species richness, ii) Shannon entropy (frequency and distribution of species), iii) Simpson evenness index, and iv) Bray-Curtis beta diversity. *p* indicates statistical significance associated with each term.

| Indices | Field | | Block within field | | Susceptibility | | Scion within susceptibility | | Rootstock | | Scion x rootstock | | Res |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | %Var | p | %Var | p | %Var | p | %Var | p | %Var | p | %Var | p | |
| Observed | 29.7 | <0.001 | 17.8 | <0.001 | 14.4 | <0.001 | 16.0 | <0.001 | 0.4 | 0.09 | 2.6 | 0.05 | 19.1 |
| Shannon | 0.3 | 0.47 | 10.4 | 0.01 | 3.0 | 0.06 | 17.8 | <0.001 | 0.4 | 1.0 | 2.3 | 0.78 | 65.8 |
| Simpson | <0.1 | 0.84 | 15.3 | <0.001 | <0.1 | 0.92 | 19.5 | <0.001 | 0.3 | 0.40 | 2.3 | 0.88 | 62.6 |
| Bray-Curtis | 36.6 | <0.001 | 5.2 | <0.001 | 6.2 | <0.001 | 14.1 | <0.001 | 0.4 | 0.28 | 3.5 | 0.04 | 34.0 |

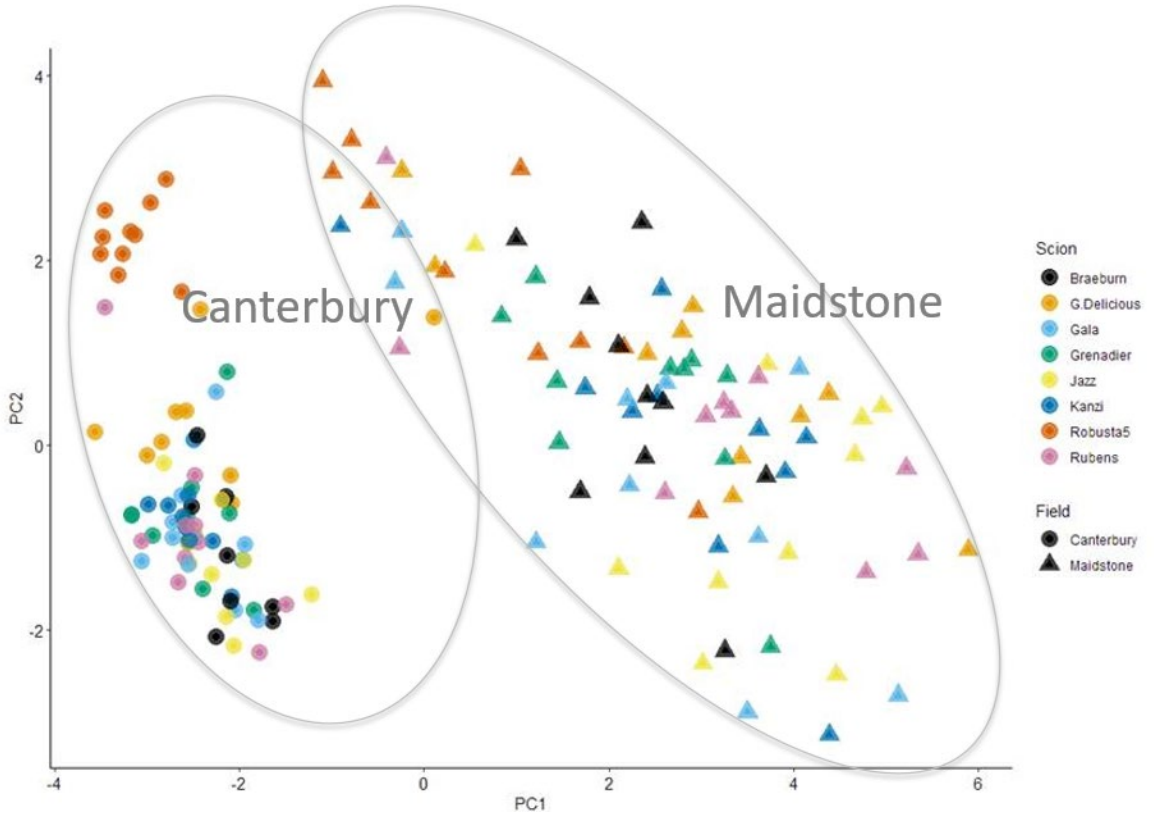


**Figure 4.** Principal component analysis plot of fungal endophytes sampled from two field sites in Kent (Canterbury and Maidstone). The eight scion varieties tested and site locations are listed in the legend.

(3) There are a number (20-30) of specific microbial taxa that significantly differ in relative abundance between the resistant and susceptible cultivars. The results in Tables 3-5 will be updated when we have qPCR data on total bacterial and fungal biomass available.

**Table 3.** Bacteria with significantly lower relative abundance in resistant scions. N, number of operational taxonomic units assigned to a particular taxon. *, low abundance taxon (mean normalised count < 10).

| Taxon | N | Putative lifestyle and ecology |
|---|---|---|
| Sphingomonas sp. | 2 | Antagonist of plant-pathogenic fungi, plant growth promoter |
| Curtobacterium sp. | 1 | Plant growth promoter |
| Massilia sp. | 3 | |
| Pseudomonas sp. | 2 | Pathogenic on apple, antifungal activity |
| Hymenobacter sp. | 6 | |
| * Frondihabitans sp. | 1 | |
| * Methylobacterium sp. | 2 | Inducer of plant defences |
| * Rathayibacter sp. | 1 | Pathogenic on herbaceous host |

**Table 4.** Fungi with significantly higher relative abundance in resistant scions. N, number of operational taxonomic units assigned to a particular taxon. *, low abundance taxon (mean normalised count < 10).

| Taxon | N | Putative lifestyle and ecology |
|---|---|---|
| Aureobasidium sp. | 1 | BCA, plant growth promoter |
| Rhodotorula sp. | 1 | BCA, plant growth promoter |
| Stemphylium sp. | 1 | Pathogen on apple (S. vesicarium; S. botryosum and S. herbarum) |
| Kalmanozyma sp. | 1 | Produces antifungal glycolipid ('killer yeast') |
| * Dissoconium sp. | 1 | Plant pathogen, mycoparasite |

**Table 5.** Fungi with significantly lower relative abundance in resistant scions. N, number of operational taxonomic units assigned to a particular taxon.

| Taxon | N | Putative lifestyle and ecology |
|---|---|---|
| Vishniacozyma sp. | 2 | Antagonist of plant-pathogenic fungi |
| Filobasidium sp. | 5 | NA |
| Dioszegia sp. | 2 | Hypothesised mycoparasitic lifestyle |
| Gelidatrema sp. | 1 | NA |
| Genolevuria sp. | 1 | NA |
| Bulleromyces sp. | 1 | Antifungal activity |
| Papiliotrema sp. | 1 | Antagonist of plant-pathogenic fungi |

We found several potential pathogen antagonistic fungal taxa that were more abundant in resistant cultivars and could therefore contribute to reduced susceptibility. Resistant scion cultivars however had also higher abundance of putative apple pathogens. The actual function of these taxa in the trees is unclear without further investigation. Moreover, their ecological function is most likely dependant on cultivar, environmental conditions, microbial community composition and other factors.

## 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.

Endophyte augmentation and *in planta* biocontrol efficacy

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 *E. 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 leaf scars spray inoculated with a spore suspension ($10^4$ spores/ml) of *N. ditissima* using a hand-held sprayer. Immediately prior inoculation (Oct 2018), 3 shoots per plot were samples 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 *E. purpurascens* by qPCR.

The remaining rootstocks were harvested in mid Dec 2018, size graded and stored at 4°C 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.

The same experiment was repeated in 2019/2020. New previously untreated block of rootstocks was amended in July 2019, *Epicoccum* concentration in the shoots assessed in October 2019 and leaf scars inoculated with Neonectria spray solution immediately after Epicoccum assessment. Rootstocks were harvested, graded, and planted out in winter 2019/20. In July 2020 canker incidence was assessed.

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 number 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.

Leaf scar protection with *Epicoccum*

In addition to *Epicoccum* inoculation, we have conducted additional experiment where leaf scar potential of *Epicoccum* was tested. At ca 50% leaf fall (Nov 2019) we selected 48 Gala trees in 12 blocks. Eight leaf scars on each tree were selected on 4 different shoots and inoculated wither with 5uL of Neonectria conidia solution ($5x10^4$ conidia/mL) or co-inoculated with *Epicoccum* spore solution (5 ul, $5x10^5$) and *Neonectria* spore solution. Canker incidence was assessed in Summer 2020.

**Results**

*In vitro* challenge assays

Two *Epicoccum* strains (C15, C29) and one *Aureobasidium* strain (C32(1)) showed substantial levels of biocontrol ability (Figures 5 and 6), 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.

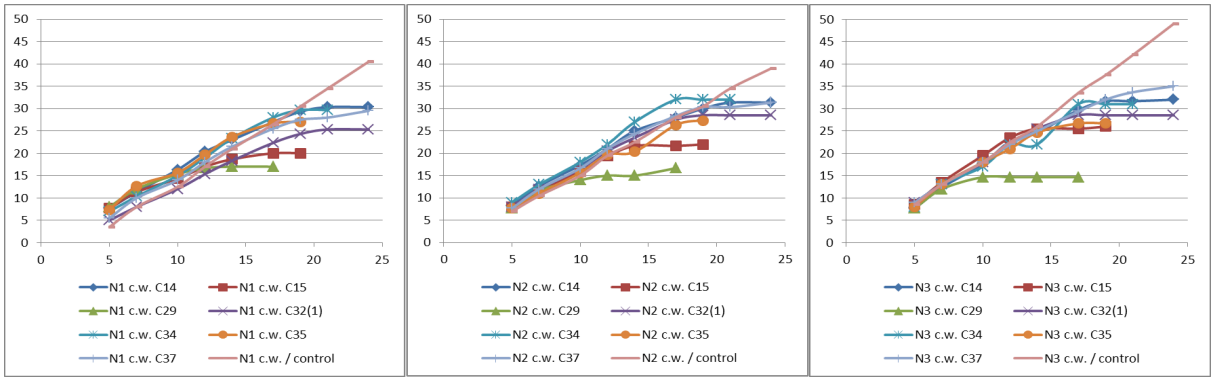


**Figure 5.** Growth (mm) of three *N. ditissima* strains (N1, N2 and N3) (Y-axis) over time (days) (X-axis) *N. ditissima* was either challenged with endophyte strains or without endophyte challenge (control).

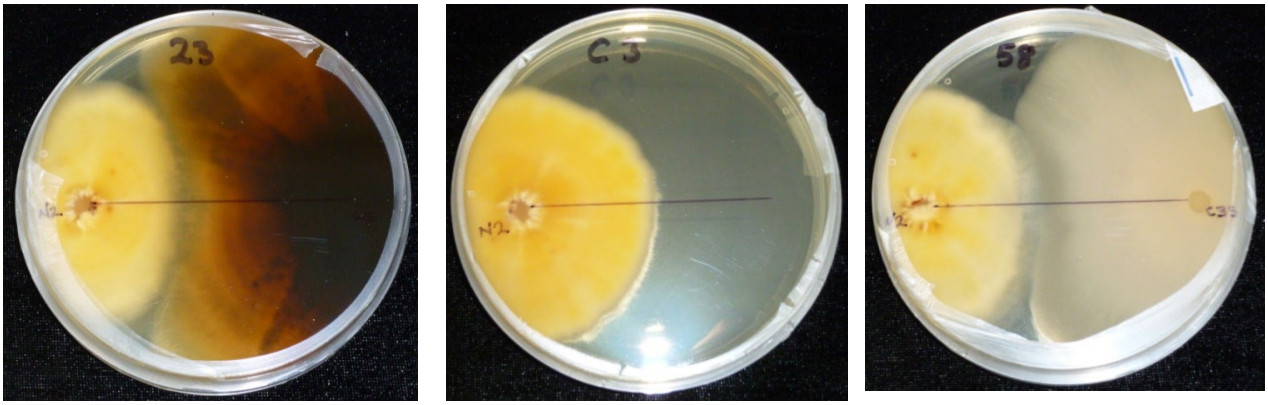


**Figure 6.** Challenge assay example (17 days after plating) with one of the *Epicoccum* isolates (C29) left, *Aureobasidium* (C35) right and unchallenged control (Neonectria ditissima HG199) in the centre. In both left and right examples we observed endophytes restricting the growth of *N. ditissima*.

Endophyte augmentation and *in planta* biocontrol efficacy

*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 6). 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 7).

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.

**Table 6.** 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 8). This is most likely due to very small increase in concentration of *Epicoccum* in the shoots in comparison to control (Figure 7). Canker expression levels were not different between treatments (Table 7). 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.

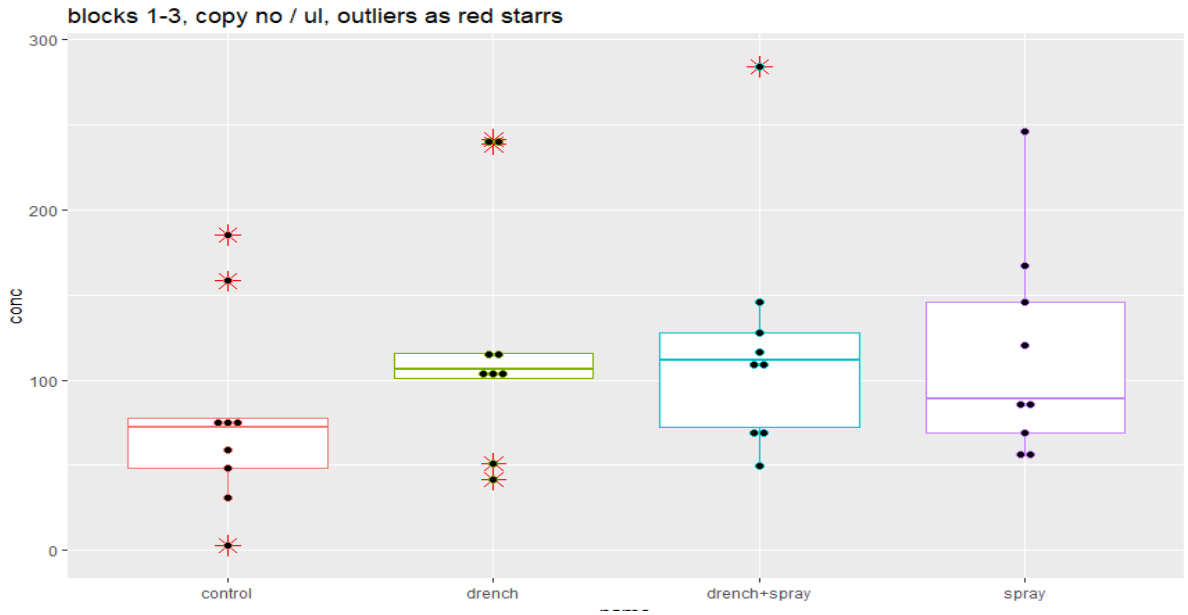


**Figure 7.** Box plot of *E. purpurascens* DNA concentration (copy number) in leaf scars of water treated (control) M9 rootstock and augmented with *Epicoccum purpurascens* spore spray, drenching or both.

**Table 7.** Number of cankers observed on *E. purpurascens* amended M9 rootstocks in summer 2019 (inoculated in 2018) and 2020 (inoculated in 2019)

| Inoculation year | Treatment | Total | Infected |
|---|---|---|---|
| 2018 | Control | 141 | 3 |
| | Drenching | 126 | 6 |
| | Spray | 144 | 5 |
| | Spray + drenching | 125 | 5 |
| 2019 | Control | 144 | 2 |
| | Drenching | 125 | 1 |
| | Spray | 124 | 6 |
| | Spray + drenching | 120 | 3 |

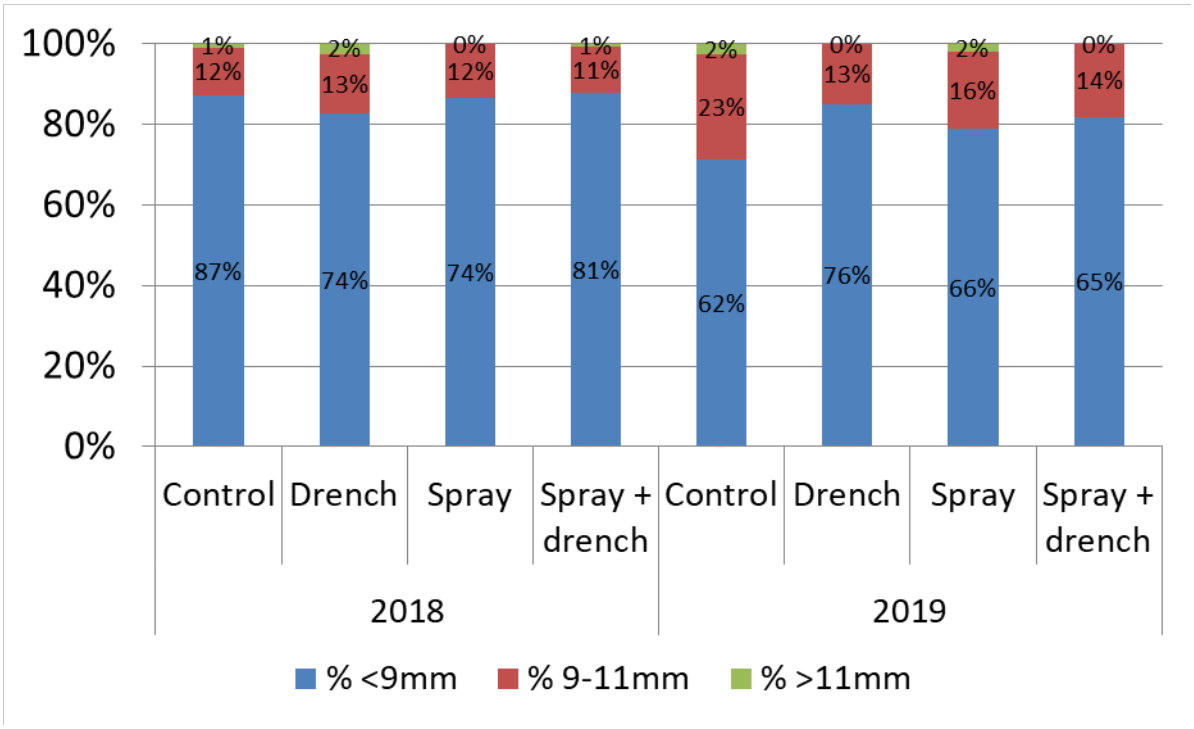


**Figure 8**: Size grading of Epicoccum inoculated M9 rootstocks. Mean percentage of different diameter grades.

Leaf scar protection

When *N. ditissima* was co-inoculated with *E. purpurascens* the canker incidence was decreased by appx 50%, from 60% incidence to just over 30%. This indicates that *E. purpurascens* could be used throughout harvest and leaf fall to protect picking and leaf scar wounds.

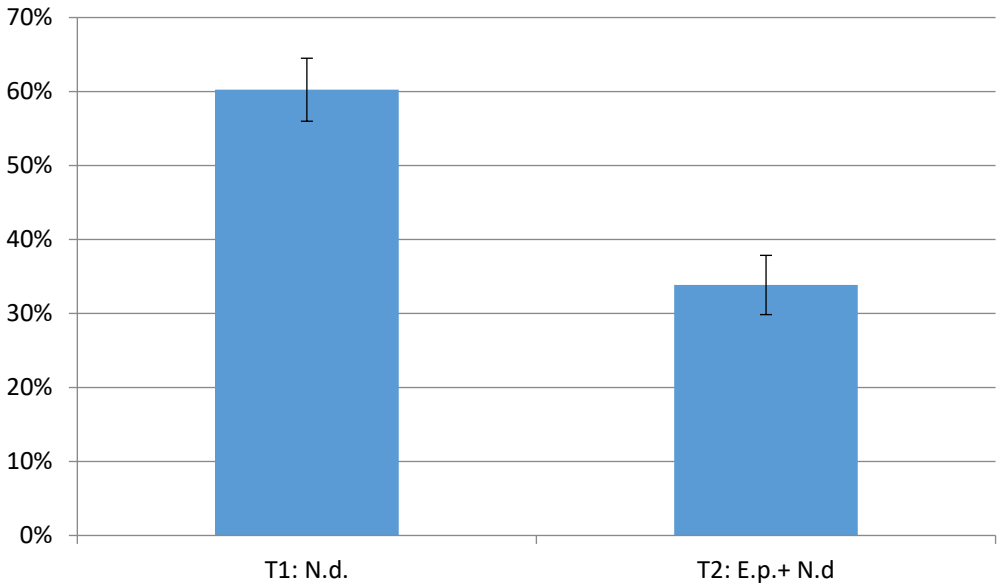


**Figure 9.** Mean frequency of canker symptom expression upon inoculation with *Neonectria ditissima* (T1:N.d.) or co-inoculation with *E. purpurascens* and *Neonectria* (T2:E.p.+N.d.). The difference between the two treatments was statistically significant (based on the Fisher LSD).

Combined efficacy of *Epicoccu*m as endophyte and leaf scar protectant

We have used a commercial orchard (Kanzi and Golden Delicious) planted at Hononton Farm in winter 2019 (Scripps) to (a) amend via spraying a subset of trees with *Epicoccum* in summer to increase its endophytic population, and (b) additionally protect leaf scars on amended trees with *Epicoccum* spore suspension sprays through leaf fall period. Trees will be assessed for canker incidence in the early summer 2021.

## 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
2. Since the canker incidence and *E. purpurascens* abundance were both very low in 2019 we decided to refocus this

### 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 8). 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 8**. Number of trees for each treatment planted at the Hononton Farm for WP3 work

| Rootstock | Scion | *Epicoccum* inoculation | *Neonectria* 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).

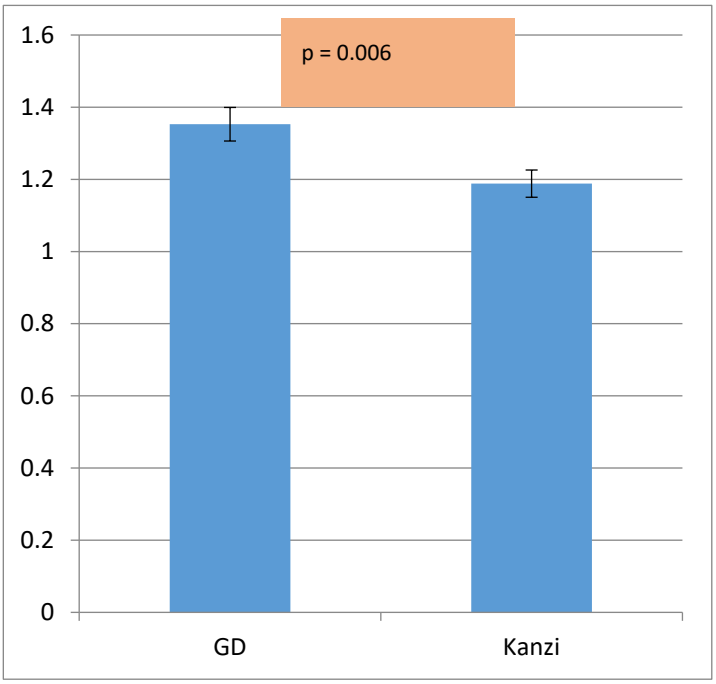


**Figure 10.** Log 10 amount of *Epicoccum* DNA per in leaf scar +/- SEM of Golden Delicious (GD) and Kanzi scions on M9 rootstock. Shoots from treatments from Table 2 are combined in a single data point.

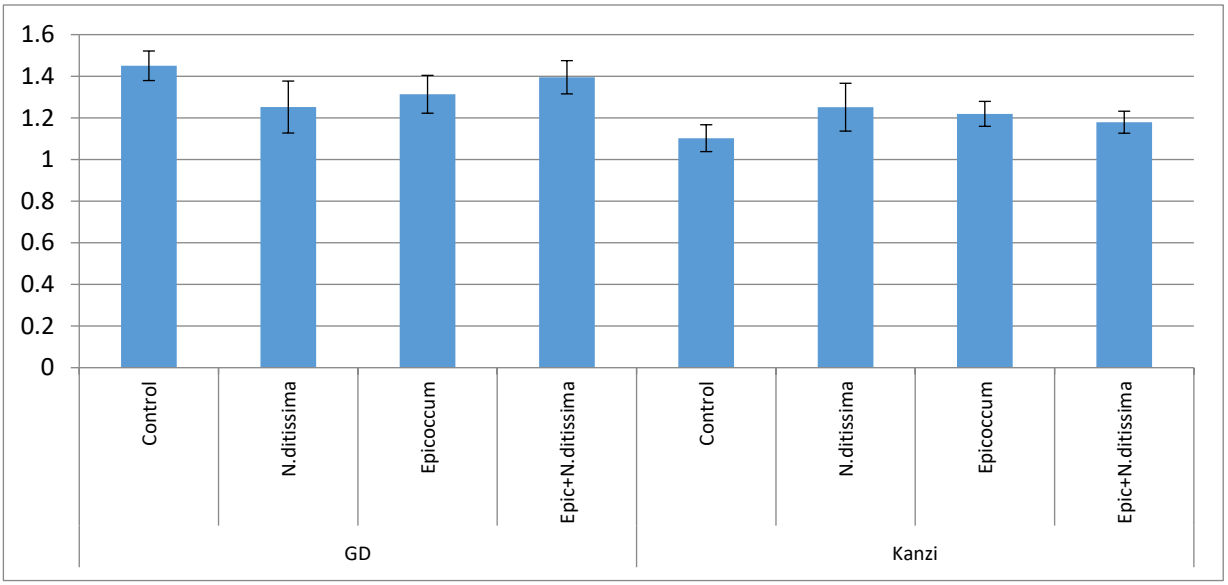


**Figure 11.** Log 10 amount of *Epicoccum* per leaf scar +/- SEM. Golden Delicious (GD) and Kanzi shoots treated with Epicoccum (July 2018, FPM), *Neonectria* (Nov 2018, FPM) or both are compared to water treated control.

Due to the low augmentation efficacy, we did not attempt to extract RNA and/ or metabolites to investigate whether *Epicoccum* inoculation increases plant defences.

At the same time as trees were sampled, we also assessed them for canker expression. We recorded extremely low canker levels with max 1 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 and results will be reported in 2021.

In 2020 we have assessed the challenges with generating meaningful data on plant defence gene expression and/or defence metabolite changes due to *Epicoccum* amendments. The main challenges were:

1. Low relative increase in *Epicoccum* abundance (based on WP2 and WP3 data) would generate at best very low signal
2. Medium to low probability that the published qPCR assays of known apple defence genes would reliably confirm or reject defence induction.
3. Considerable difficulty obtaining enough plant sap for metabolomic analysis.
4. To detect defence induction, which is most likely to be transient, the knowledge on spatial/temporal dynamics of *Epicoccum* colonisation of apple tissue is prerequisite for sampling of most relevant tissue and time points.

We have therefore re-focused this work package to address the spatial/temporal dynamics of *Epicoccum* colonisation. We decided to first investigate how quickly and how far can *Epicoccum* colonise apple shoots through inoculated leaf scars.

In September 2019 and 2020, we inoculated leaf scars of 18 Gala trees with *Epicoccum* spore solution and 18 with water control. One-year post 2019 inoculation and 10, 20 days post 2020 inoculation the shoots with inoculated leaf scars were collected, washed under tap water to remove any non-germinated spores, and sectioned into three sample types:

1. leaf scar sample
2. 3 mm thick section of bark and wood 0.5 cm above or below the leaf scar
3. 3 mm thick section of bark and wood 1 cm above or below the leaf scar

We pooled each sample type from 6 shoots collected from 3 different trees into one final sample. DNA will be extracted from all samples, *Epicoccum* abundance in inoculated shoots analysed with qPCR and compared to control shoots to ascertain how far along the shoot can *Epicoccum* spread after 10, 20 days how well can it persist in the wood over different seasons and what kind of population density it can achieve at different times post inoculation. We expect the results from this experiment by 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

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 endophyte community (Figure 12).

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 will be sampled for fungal endophyte characterization; each genotype was replicated four times.

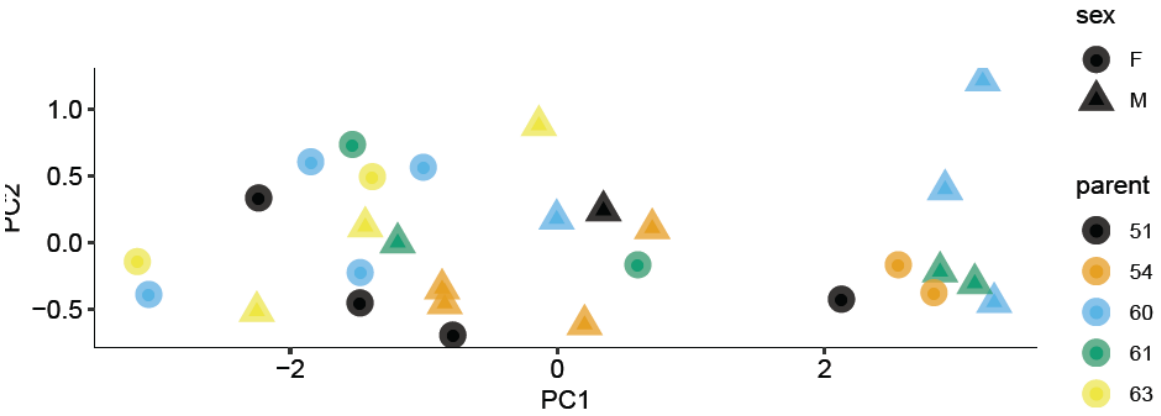


**Figure 12.** PCA scores of fungal endophytes for those parents of the five mapping populations for canker resistance.

In 2020 we have successfully sampled four replicate trees from 54 different genotypes in 'No54' mapping population for total of 216 samples. The samples were freeze dried, homogenated, DNA extracted with DNeasy kit (QIAGEN) and DNA quality checked. ITS and 16S amplicon has been sequenced by Novogene using the same mitochondrial/plastid 16S blocking primers as in WP1. All 216 samples are being sequenced and expected to generate between 30-50 K reads per amplicon each.

We have also started to quantify the total bacterial (16S) and fungal (ITS) communities in all 216 samples using qPCR. Combining total size of communities with proportional abundance

from metabarcoding we will be able to better estimate the differences in microbial abundancies between parental and different progeny lines.

These data will be used to assess whether endophyte composition is partially genetically controlled and whether specific endophytes are correlated with QTLs related to 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, several leaf scars were inoculated with *N. ditissima* on 18$^{th}$ 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 13). The bags were removed after 24 hours. Canker 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.

**Figure 13.** Plastic bags were used to maintain high humidity to promote canker infection following inoculation of leaf scars.

**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 14 and 15). Further statistical analyses will be applied to the data.

**Figure 14**. Canker expression was recorded on the leaf scars pre-inoculated with *N. ditissima* macrospores.

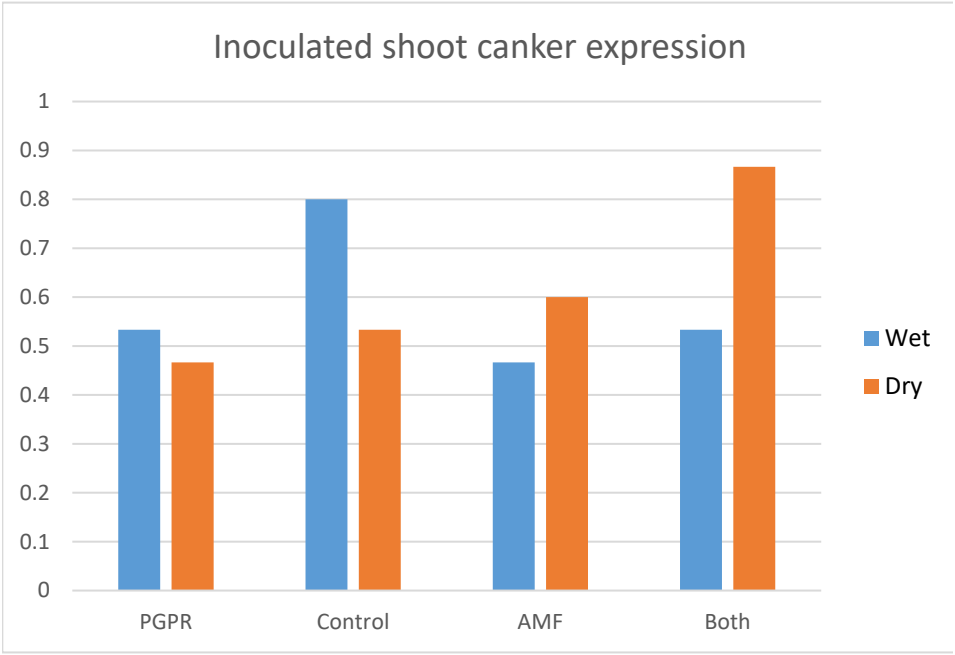


**Figure 15.** Average number of cankers per shoot for each treatment when assessed in October 2019.

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. Girth assessments suggest a possible increase in trunk size with AMF under well-watered conditions (Figure 16).

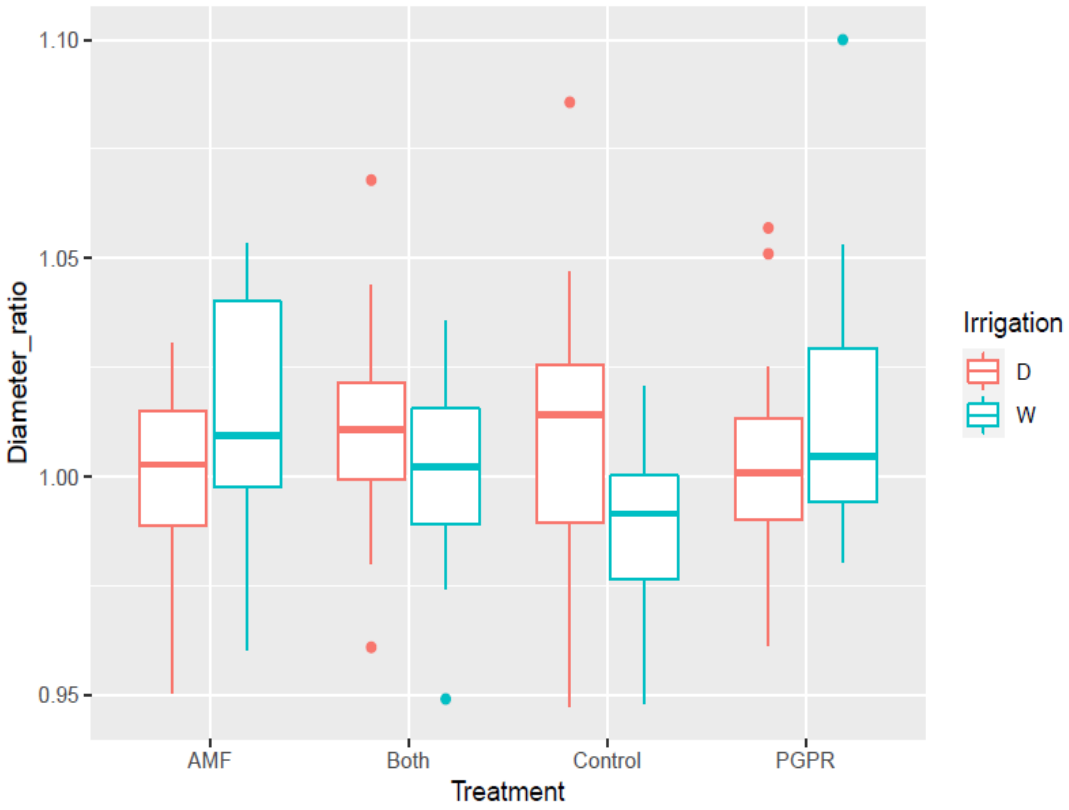


**Figure 16.** Girth increase in the 2019 growing season (April-October).

We are currently quantifying AMF colonisation, rhizosphere microbial composition 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.

### Materials 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). Seven 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, and in spring/summer 2020.

### Results

In the spring/summer 2020 assessments, there were strong varietal and site differences observed in canker number at both planting dates. World Wide Fruit was the site with the highest mean canker number (December 2018 planted: 6.8, March 2019 planted: 5.8), the while Avalon had the lowest mean canker number (December 2018 planted: 0.4, March 2019 planted: 0.5) (Table 9). Regarding varieties, Kanzi and Jazz had the highest mean canker number for both planting dates across the three sites, while Grenadier and Golden Delicious had the lowest (Table 9). Kanzi and Jazz also had higher canker number in winter (December) planted than refrigeration and planting the following spring (March). Rubens, Braeburn and Gala all had moderate canker number in both planting dates. The general trend from the 2019 canker assessments of refrigerated and then spring planted trees having higher canker appears to be changing. This may be due to the expression of latent cankers over time and increasing numbers of field-based infections. It may also be due to differences in orchard management between the sites, for example, ground cover type (mowed often or long grass under trees).

Trees will be assessed for canker again in spring 2021. In future reports, canker data analysis will be separated into A+B (rootstock +scion central leader cankers) and C+D+E (peripheral

cankers). This is due to A+B cankers mainly occurring from the nursery and peripheral cankers occurring in the field after planting.

If there are ongoing differences between cultivars and sites, we will sample and profile rhizosphere and endophyte communities and see if there is a relationship of canker incidence with specific microbial groups or orchard management.

**Table 9.** Mean canker number (mainstem + peripheral) per cultivar per tree of the seven apple cultivars planted either in early winter (December 2018) or refrigerated and planted in early spring (March 2019). Cultivars have been sorted from highest mean canker for all sites to lowest, within each planting date. Data in this table is from assessments made in late May/early June 2020.

| Planting | Cultivar | Scripps | World Wide Fruit | Avalon | Overall mean |
|---|---|---|---|---|---|
| **December 2018** | Kanzi | 5.7 | 11.5 | 1.1 | 6.1 |
| | Jazz | 2.4 | 14.2 | 0.5 | 5.7 |
| | Braeburn | 1.6 | 9.9 | 0.5 | 4.0 |
| | Rubens | 1.8 | 6.8 | 0.4 | 3.0 |
| | Gala | 1.8 | 4.1 | 0.4 | 2.1 |
| | Golden Delicious | 0.4 | 0.6 | 0.1 | 0.3 |
| | Grenadier | 0.0 | 0.2 | 0.0 | 0.1 |
| | ***Site mean*** | **2.0** | **6.8** | **0.4** | **3.0** |
| **March 2019** | Kanzi | 3.9 | 11.0 | 1.9 | 5.6 |
| | Jazz | 2.7 | 9.5 | 0.4 | 4.2 |
| | Rubens | 2.9 | 7.3 | 0.4 | 3.5 |
| | Braeburn | 1.2 | 7.9 | 0.2 | 3.1 |
| | Gala | 1.7 | 3.5 | 0.2 | 1.8 |
| | Golden Delicious | 0.2 | 1.2 | 0.1 | 0.5 |
| | Grenadier | 0.0 | 0.1 | 0.0 | 0.1 |
| | ***Site mean*** | **1.8** | **5.8** | **0.5** | **2.7** |

## Research activities in the coming seasons

We have now showed canker resistant cultivars differed in the relative abundance of several 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. Genetic control of endophytes will also be determined and interpreted in relation to QTLs for canker resistance.

We further demonstrated that several fungal endophyte strains (*Epicoccum*) from apple have antagonistic effects against apple canker under *in vitro* tests. We are now carrying *in vivo* test 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 specific endophyte could persist over time, which will guide the development of future application methodology.

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. A subset of rhizosphere samples will be sequenced to study the effects of soil amendment on rhizosphere and endophytes in relation to tree growth.

The effects of cold storage of trees, leading to increased canker incidence post-planting appears to be changing over time. We need to clarify if early winter planting (without cold storage) simply delays the onset of canker expression or reduce the number of cankers over time. This will be assessed again in spring 2021. To help data analysis and interpretation, we will divide cankers into the main stem and peripheral types. In addition, rhizosphere soils will be sampled from a subset of trees to study the site and cultivar effects on microbial recruitment, particularly in terms of canker susceptibility.

## Knowledge and Technology Transfer

(1) We presented two posters at the microbiome conference miCROPe 2019 (Microbe-assisted crop production – opportunities, challenges and needs), Vienna, December 2nd to 5th 2019

   i. "Microbial ecology of the European apple canker pathosystem (N. ditissima)"

   ii. "The use of beneficial microbes in commercial horticulture"

(2) 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.

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

(4) 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.

(5) We gave the following presentations at the fourth international canker workshop (November 2020, New Zealand):

   i. Practical value of disease forecasting for canker management

   ii. Use of endophytes for biocontrol of apple canker

   iii. Apple endophytes in relation to location, cultivar, canker susceptibility and rootstock genotypes

   iv. Apple canker management

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