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
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Location of project:	East Malling, and several farms in Kent
Industry Representative:	Peter Checkley
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Most of 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.

Contents

GROWER SUMMARY

Headline

- One fungal endophyte showed good biocontrol potential against infection of leaf scars and pruning wounds by the canker pathogen.
- Cultivar responses to the canker pathogen is consistent across three sites, although the overall canker severity differed greatly among locations
- Between-season dynamics of endophytes in the leaf scars of new extension shoots suggest that exploitation of bacterial biocontrol agents could be more advantageous over the fungal ones since bacteria appeared to be more persistent between seasons
- Specific endophytes are associated with cultivar tolerance/resistance against the canker pathogen, but not the overall endophyte community
- The abundance of some endophytes is partially genetically controlled by the apple hosts; some genetic factors controlling endophytes are co-located or close to those controlling resistance against the canker pathogen
- Post-planting drought led to reduced tree development but did not affect canker development.
- Amendment with beneficial microbes at planting did not result in significant reduction in canker development.
- Planting soon after lifting led to reduced peripheral canker development, albeit a very limited reduction.

Background and expected deliverables

European canker

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 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.

Endophytes

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.

Antagonist fungal endophytes and tolerance to N.ditissima

Recently, we have obtained preliminary data showing a link of 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 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*. In addition, we aim to understand the dynamics of an specific biocontrol endophyte colonising leaf scar tissues, to optimise their application strategy. 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 site-specific 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 key project conclusions

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.

- We have profiled endophytes at leaf scars of eight cultivars with differing tolerance/resistance against apple canker:
  a. Endophyte diversity was primarily affected by sampling time, orchard location, followed by location within an orchard and 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.

c. Bacterial endophytes at the leaf scar tissues appear to be more consistent over two seasons, suggesting that exploitation of bacterial biocontrol agents could be advantageous over the fungal ones.

- 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 effects of *Eppicocum* probably because of greater susceptibility of fresh pruning cuts combined with a high dose of pathogen inoculum applied.
  c. Consistent with the endophyte dynamics, there is only a limited survival of this *Eppicocum* strain in the leaf scars over the winter
- Studying endophytes in a F_1 mapping population indicated
  a. Abundance of several endophytes is correlated with canker development.
  b. Abundance of specific endophytes is partially genetically controlled by the hosts.
  c. QTL mapping showed that a few QTL mapped for controlling endophytes are close to or co-locate with QTLs for canker resistance.
- Amending soils with PGPR (plant growth-promoting rhizobacteria) or AMF at the planting time has negligible effects on canker development.
- Post-planting drought led to reduced tree development but did not affect canker development.
- Testing seven scion cultivars at three orchards showed that:
  a. Longer duration of trees in cold storage initially led to increased canker incidence post-planting but two years after planting such an effect of cold-storage time on canker severity is negligible.
  b. Planting soon after lifting led to reduced peripheral canker development, albeit a very limited reduction.
  c. Cultivar susceptibility to the canker pathogen is consistent across the three orchards studied.
  d. There is some indication that symptom development of main stem cankers is affected by site specific factors; these cankers result most likely from nursery infections.

Financial benefits

This project is primarily a strategic research study. The only result that directly impacts commercial apple production most is the effect of storage duration on canker development, and also the effect of drought on tree development.

Action points for growers

- Plant trees as soon as possible after lifting.
- Ensure good uniform tree-establishment is important to reduce symptom development of main stem cankers, particularly those highly susceptible cultivars
- Avoid drought following planting

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. *N. 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.

N. 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 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 related work packages, each dealing with specific topics. In this report, to have a better flow of information, we report research activities, grouped into five research themes.

Endophytes in relation to cultivar resistance, site and season

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 to what extent rootstocks, location and sampling time affect the composition of endophytes at leaf scars

Material and methods

Trial design

Eight scion cultivars (Robusta, Grenadier, Golden delicious, Gala, Kanzi, Jazz, Braebourn, Rubens) were grafted onto two rootstocks (M116 and M9), and the trees were planted in two sites (Friday Street Farm- Avalon, and Perry Farm – World Wide Fruit) 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.

Sampling leaf scar tissues and DNA extraction

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 (Fig. 1a).

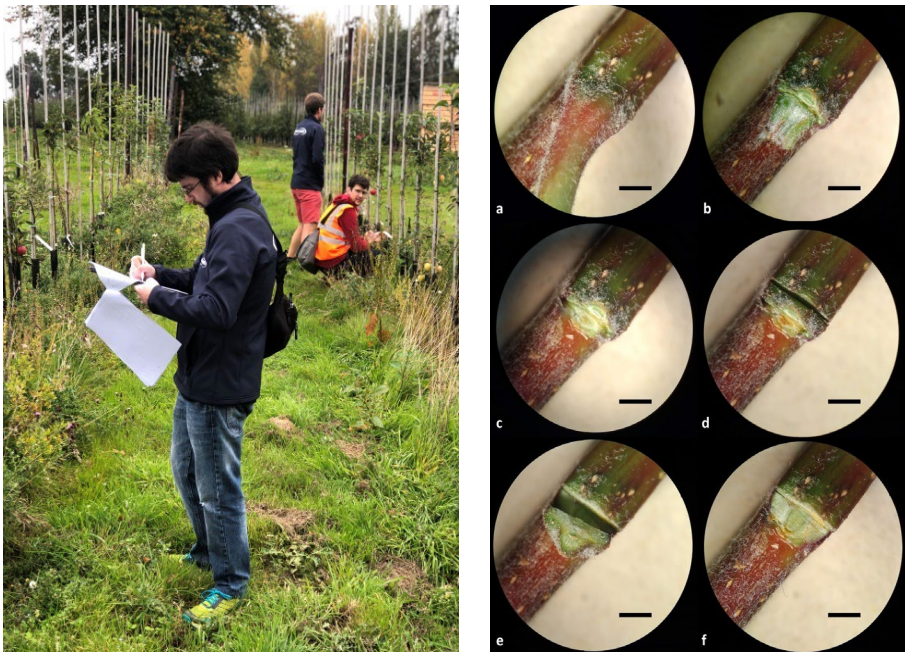

Figure 1. Sampling leaf scars at Friday St Farm, 4th Oct 2018 (left). Dissecting leaf scars from the shoots (right)

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 (Fig. 1). 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.

Amplicon sequencing

Samples were sequenced by Novogene (Cambridge). 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 the majority of 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 were did not amplify to sufficient level using blocking primers and were therefore not sequenced. We have also measured total bacterial and fungal community sizes with qPCR.

Bioinformatics and statistics

Read clustering, operational taxonomic unit (OTU) assignment and analysis of diversity followed the established protocols at NIAB EMR (Tilston et al. 2018; Deakin et al. 2018). In all statistical analysis of endophyte composition data, experimental factors included site, blocks within a site, scion genotype, rootstock genotype, sampling time and all interaction terms involving scion, rootstock and time. The three sampling time points were divided into only two classes: spring and autumn. Scion genotypes were further divided into two canker susceptibility levels based on the literature and empirical evidence: susceptible - ‘Gala’, ‘Braeburn’, ‘Kanzi’, ‘Rubens’ (Weber, 2014) and ‘Jazz’ (Berrie, 2016); and resistant - ‘Grenadier’, ‘Golden Delicious’, ‘Robusta 5’ (Gomez-Cortecero et al., 2016). Therefore, the difference among scions consisted of ‘between the two susceptibility groups’ and ‘among scions within a susceptibility group’. Only the most abundant OTUs that accounted for 99.9% of the total counts were retained for statistical data analysis. All statistical analyses were carried out with R 4.1.0 (R Core Team, 2019).

Results

qPCR data

ANOVA summary is given in Table 1. There were significant differences in the qPCR estimated population size for bacteria between sites ($P < 0.001$), between blocks within site ($P < 0.001$) and sampling times ($P < 0.05$). The differences between canker susceptibility classes was not significant but $P = 0.097$. Within site variability (between blocks) accounted for most variability (17.1%); most variability (69.6%) was, however, not due to any experimental factors.

For fungi, more experimental terms were statistically significant (Table 1) – blocks within site ($P < 0.001$), sampling time ($P < 0.001$), scion cultivars within each susceptibility class ($P < 0.001$), and the interaction between time and canker susceptibility class ($P < 0.001$). The differences between two rootstocks were close to statistical significance ($P = 0.056$). Differences between time points accounted for the most variability (59.4%) (Fig. 1) whereas only 24.3% of the variability was unaccounted for.

For both bacteria and fungi, the estimated population size was smaller for the spring samples, particularly for fungi (Fig. 2). Fungal community sizes were about two magnitudes smaller for the spring samples than the autumn samples. The interaction between time and canker susceptibility was primarily due to the lower qPCR values in the spring for the susceptible scions than the resistance ones (Fig. 2b).

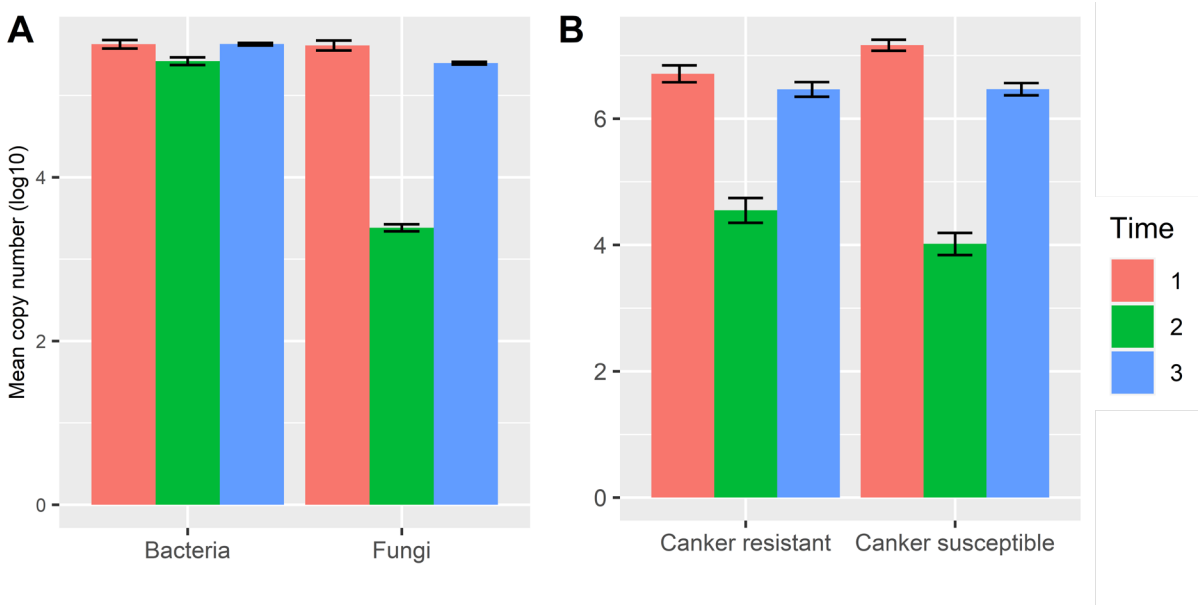

Figure 2. qPCR results of generic 16S and ITS primers for respective bacteria and fungi in samples of apple leaf scar tissues, sampled from eight scion cultivars over three time points (1 & 3 – autumn; 2 – spring) at two sites in Kent, UK; and fungal qPCR in relation to canker ressitance and sampling points.

Sequence quality and generation of OTUs. Sequence depth for all samples was sufficient for both 16S and ITS regions. Of all the samples, 279 had sufficient number of good quality 16S sequence reads. There were a total 6179 bacterial OTUs, of which the most abundant 2755 accounted for 99.9% of the total reads. Of the top 2755 bacterial OTUs, 2435, 2106, 1709, 1363 and 867 could be assigned to the rank of phylum, class, order, family and genus, respectively, at a confidence level of 80%. Proteobacteria accounted for 73.6% of the total reads; Cyanobacteria and Actinobacteria accounted for respective of 11.8% and 11.0% of the total reads; both Bacteroidetes and Firmicutes accounted for about 1.2% of the total reads. The total number of reads assigned to OTUs ranged from 3770 to 135114 (median of 41632) per sample; the total number of reads per OTU ranged from 12 to 3027318 (median of 48). The ten most abundant OTUs accounted for 71.5% of the total reads; the most abundant (*Pseudomonas* sp.) OTU accounted for 21.0% of the total reads. Of the other nine most abundant OTUs, two were from *Sphingomonas* (16.3%) and two from Streptophyta (12.8%).

In total, 315 samples had sufficient number of good quality ITS sequence reads. There were a total of 9190 fungal OTUs, of which the most abundant 3370 accounted for 99.9% of the total

reads. Of the top 3370 fungal OTUs, 1271, 1043, 896, 689, 527 and 208 could be assigned to the rank of phylum, class, order, family, genus and species, respectively, at a confidence level of 80%. Ascomycota, Basidiomycota and the unknown group accounted for 29.9%, 34.0% and 36.18% of the total reads, respectively. The total number of reads assigned to OTUs for each sample ranged from 11594 to 135642 with a median of 89988; the number of reads in individual OTUs ranged from 13 to 5939271 with a median of 42. The ten most abundant OTUs accounted for 75.5% of the total reads. The most abundant OTU was of Dothideomycetes, accounting for 22.4% of the total reads. Of the remaining nine most abundant OTUs, four could be assigned to the genus taxonomic rank (two *Vishniacozyma*, one *Rhodotorula*, and one *Alternaria*), one at the phylum rank, and other four only at the fungi kingdom rank.

Diversity indices

Table 1 gives the contribution of experimental factors to the observed variability in the bacterial and fungal alpha diversity indices, as estimated from ANOVA. For bacteria, the estimated number of OTUs (Chao1) was primarily influenced by time ($P < 0.001$) and among blocks within site ($P < 0.01$), accounting for the respective 22.8% and 7.0% of the total variability. For the Shannon and Simpson indices, the time effect was even more pronounced, accounting for 58.1% and 54.9% of the total variability, respectively; samples had much lower indices in the spring than in the autumn, consistent over the two sites (Fig. 3). Very little difference was due to scion or rootstock genotypes (Table 1).

For fungi, for the estimated number of OTUs per sample (Chao1), most (74.9%) of the observed variability is unaccounted for, only time ($P < 0.001$), site ($P< 0.01$), and within site among blocks ($P < 0.01$) were statistically significant, accounting for respective 4.8%, 2.8% and 8.4% of the total variation. For both Simpson and Shannon diversity indices, time effect was most important ($P < 0.001$), accounting for 15.0% and 22.0% of the total variability, respectively. However, unlike bacteria there is no clear pattern in the alpha-diversity indices with respect to the sites and three time points (Fig. 4).

Fig. 5 shows the number of bacterial and fungal OTUs at each sampling site or at each individual sampling point. There were many unique OTUs within each site or sampling time for both bacteria and fungi. One striking feature is that there were far more unique fungal OTUs in the spring samples (ca. 40% of the OTUs) than the bacteria (ca. 10% of the OTUs). For Shannon and Simpson indices, there were significant interactions between time and scion cultivars (time:canker and/or time:canker:scion) for both bacteria and fungi. For bacteria, such an interaction resulted mainly from the lower values in the spring samples of resistant cultivars than in the samples of susceptible cultivars; the opposite was true for fungi (Fig. 6).

NMDS ordination was used to visualise bacterial and fungal β diversities (Bray-Curtis) (Fig. 7). As for alpha-diversity, beta-diversity indices were mainly affected by time and location (site and blocks within site) (Table 1). Differences among time points accounted for 20.6% and 24.5% of the total variability in bacterial and fungal communities, respectively. Corresponding values for locations (site + blocks within site) were 22.9% and 24.1%. In the first two dimensions, spring samples appeared to be more different from the autumn samples for bacteria, and there was clear separation between the two sites for fungi (Fig 7).

ANOVA of PCA scores

The percent variance accounted for by individual PCs decreased steeply initially (Fig. S3); beyond the top 10 PCs, each of the remaining PCs accounted for very small proportion of the observed variability. Table 2 gives the ANOVA summary of the scores of the first four PCs (PC1-PC4). For bacteria, the first four PCs accounted for 30.6% of the total variance (12.4%, 8.9%,

Table 1 Percent variance in qPCR and alpha diversity indices accounted for by each experimental term based on ANOVA of qPCR and alpha diversity indices, and Adonis analysis of beta diversity indices.

Terms	qPCR		Alpha diversity						Beta diversity	
			Bacteria			Fungi				
	Bacteria	Fungi	Chao1	Shannon	Simpson	Chao1	Shannon	Simpson	Bacteria	Fungi
Site	3.6***[a]	0.3	0.1	10.1***	3.2***	2.8**	2.9***	0.9*	9.8***	17.2***
Among blocks within site	17.1***	4.2***	7.0**	4.5***	4.7***	8.4**	15.4***	9.9***	13.1***	7.3***
Time [1]	2.5*	59.4***	22.8***	58.1***	54.9***	4.8***	21.9***	15.0***	20.6***	24.5***
Scion [2]										
Canker [3]	0.3	0.4	0.1	0.0	0.0	0.1	1.2**	0.5	0.3	0.4*
Scion in canker class [4]	0.6	0.1	4.2*	1.2	1.8*	1.4	1.1	0.5	3.7***	2.4***
Rootstock [5]	0.8	0.0	0.9	0.0	0.0	0.1	0.0	0.0	0.4*	0.3
Time x Scion										
[1] x [3]	0.7	7.0***	0.2	1.2**	1.4**	0.0	3.8***	4.7***	0.6	1.2***
[1] x [4]	2.3	1.7	5.9*	1.0	1.7	3.0	8.9***	10.0***	4.7***	5.4***
Time x Rootstock	0.2	1.7***	0.2	0.1	0.5	0.3	0.5	0.2	0.3	0.3
Scion x Rootstock										
[5] x [3]	0.4	0.1	0.0	0.0	0.0	0.0	0.2	0.0	0.2	0.4
[5] x [4]	0.2	0.2	0.9	0.9	1.1	1.9	0.4	0.4	1.1	0.8
Time x Scion x Rootstock										
[1] x [5] x [3]	0.9	0.1	0.4	0.3	0.5	0.3	1.3*	0.7	0.3	0.3
[1] x [5] x [4]	1.0	0.4	2.3	1.0	2.0	2.2	1.4	1.4	1.9	1.5
Residuals	69.6	24.3	55.0	21.5	28.1	74.9	41.0	55.7	42.9	38.0

[a]: *, **, and *** indicate P < 0.05, P < 0.01, and P < 0.001, respectively.

Table 2 Percent variance in the first four principal components (PC) accounted for by each experimental term.

	Bacteria				Fungi			
ANOVA Terms	**PC1**	**PC2**	**PC3**	**PC4**	**PC1**	**PC2**	**PC3**	**PC4**
Site	27.4***[a]	0.5	0.6	2.2*	7.4***	1.4*	4.0***	13.7***
Among blocks within site	12.3***	2.9	5.4	7.5*	10.4***	29.8***	24.0***	24.1***
Time [1]	32.1***	16.0***	25.8***	2.9*	67.2***	1.5*	5.9***	8.1***
Scion [2]								
Canker [3]	0.2	0.6	0.0	0.4	0.0	0.0	0.2	0.0
Scion in canker class [4]	2.2***	2.7	0.8	2.3	0.3	0.6	0.9	2.2*
Rootstock [5]	0.4*	0.1	0.0	0.3	0.1	0.1	0.9	0.0
Time x Scion								
[1] x [3]	0.7*	0.7	0.7	0.1	0.3	0.0	0.1	0.1
[1] x [4]	3.4***	2.9	2.9	3.7	0.7	2.0	2.1	5.5**
Time x Rootstock	0.1	0.5	0.2	0.4	0.1	1.2	0.3	0.3
Scion x Rootstock								
[5] x [3]	0.1	0.5	0.3	0.0	0.0	0.0	0.4	0.2
[5] x [4]	0.5	1.0	1.7	1.9	0.1	1.5	1.5	2.2*
Time x Scion x Rootstock								
[1] x [5] x [3]	0.1	1.0	0.1	0.9	0.2	0.7	0.2	0.2
[1] x [5] x [4]	1.6	1.9	2.5	4.6	0.6	4.9*	1.9	3.0
Residuals	18.8	68.8	58.9	72.7	12.7	56.2	57.9	40.1

[a]: *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

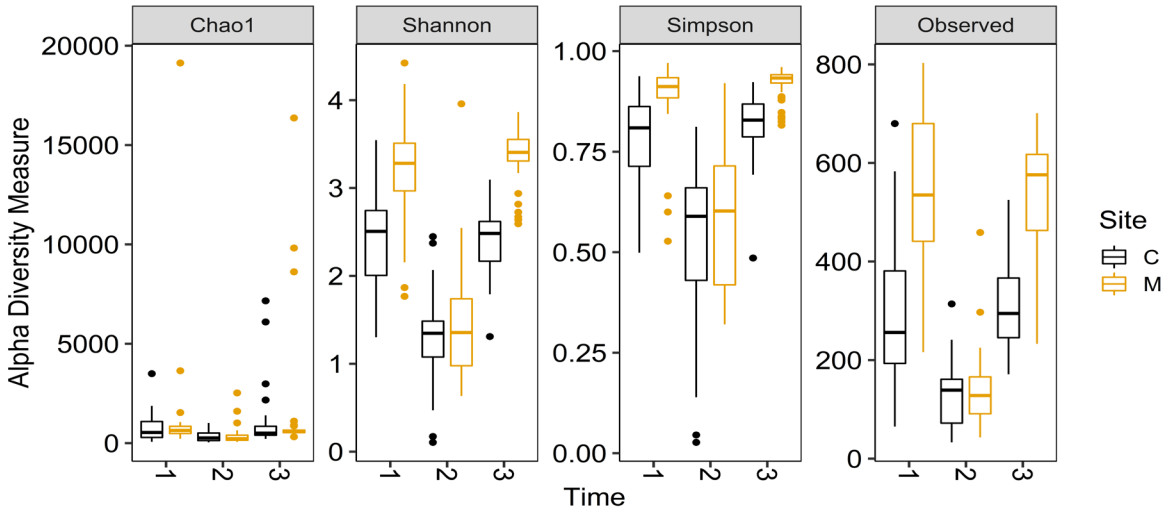

Figure 3. Within-sample (alpha) diversity indices of bacterial endophyte communities in the apple lear scar tissues, sampled from eight scion cultivars over three time points (1 & 3 – autumn; 2 – spring) at two sites (C – near Canterbury, M – near Maidstone) in Kent, UK.

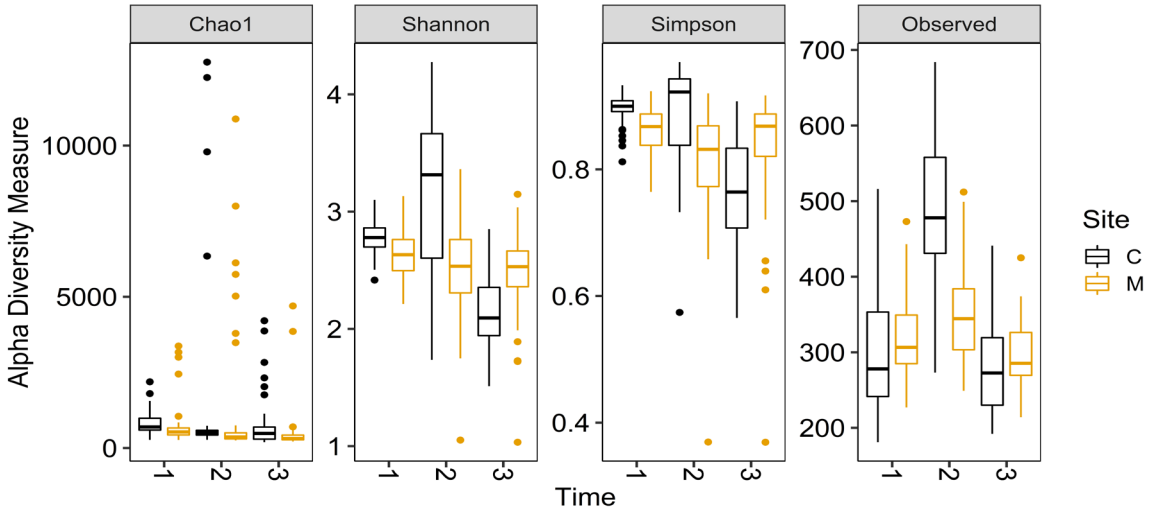

Figure 4. Within-sample (alpha) diversity indices of fungal endophyte communities in the apple lear scar tissues, sampled from eight scion cultivars over three time points (1 & 3 – autumn; 2 – spring) at two sites (C – near Canterbury, M – near Maidstone) in Kent, UK.

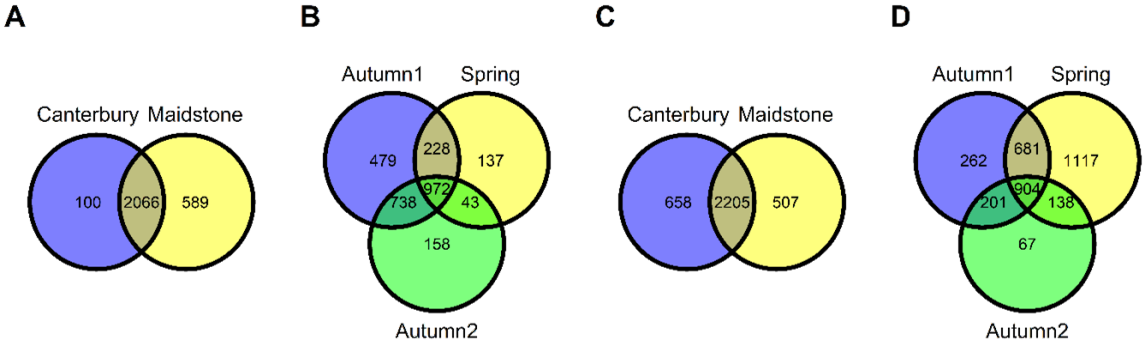

Figure 5. Vendiagrams of unique and common bacterial (A & B) and fungal (C & D) OTUs within a single site (A & C) or time point (B & D). If there was only a single sequence read for a given OTU for a time point or site, this OTU was considered absent for the site or time point.

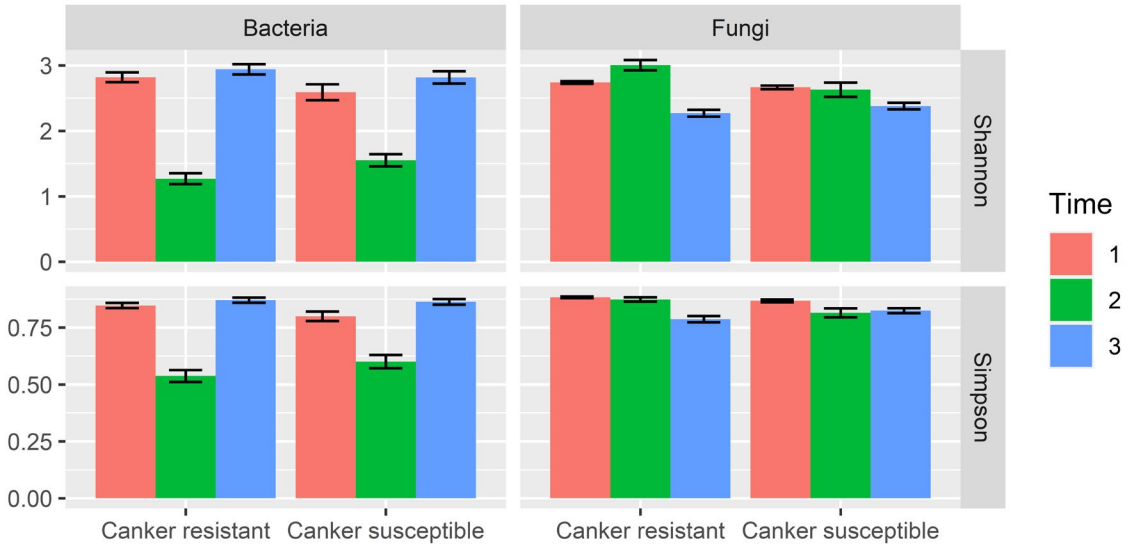

Figure 6. Within-sample (alpha) diversity indices of bacterial and fungal endophyte communities in the apple lear scar tissues in relative to three time points (1 & 3 – autumn; 2 – spring) and resistance of scion cultivars again the European apple canker disease.

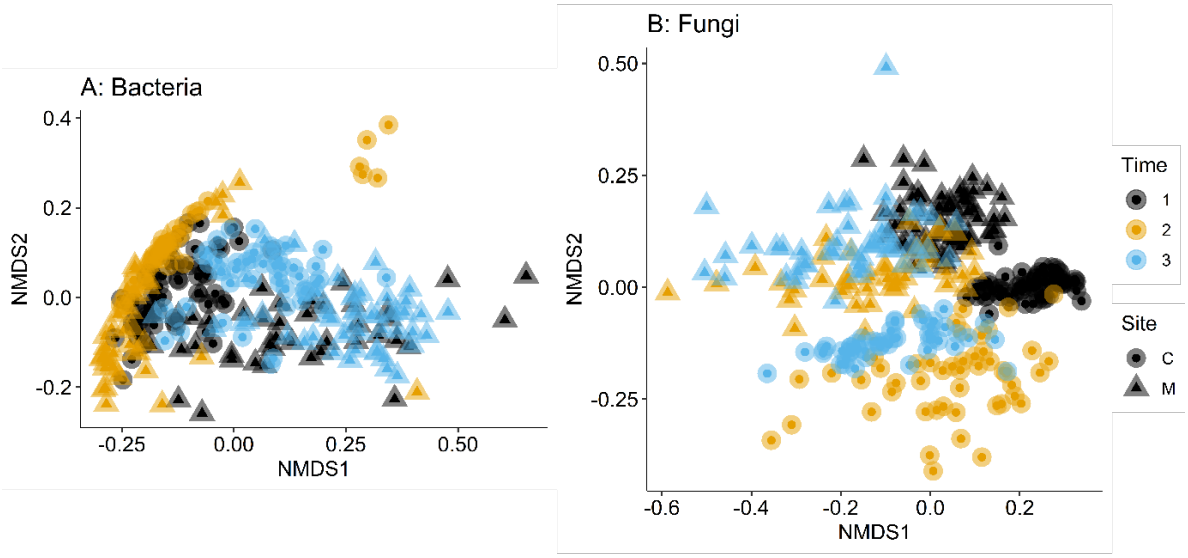

Figure 7. The first two dimesnions of NDMS analsyis of Bray-Curtis indices (beta diversity) of the bacterial and fungal endophyte communities in the apple lear scar tissues, sampled from eight scion cultivars over three time points (1 & 3 – autumn; 2 – spring) at two sites (C – near Canterbury, M – near Maidstone) in Kent, UK.

4.7% and 4.6% for PC1-PC4, respectively). Time, site and blocks within site accounted for 32.1%, 27.4% and 12.3% of the total variability in PC1, respectively. For PC2 and PC3, time was the only significant factor, accounting for 16.0% and 25.8% of the total variability, respectively. PC4 was least affected by experimental factors: nearly 73% of the total variability was unaccounted for; only the location and time effects were statistically significant. For the first two PCs, there was far less variability in the spring samples than the autumn samples, particularly at the Maidstone site (Fig. 8).

For fungi, the first four PCs accounted for only 15.5% of the total variance (7.4%, 3.2%, 2.5% and 2.4% for PC1-PC4, respectively). PC1-PC4 were all primarily affected by time and location

(Table 2). For instance, time accounted for 67.2% of the total variability in PC1; within-site differences accounted for 29.8%, 24.0% and 24.1% of the total variability in PC2-4, respectively. In contrast to bacteria, the spring samples were much more variable than the autumn samples for the first two PCs (Fig. 8).

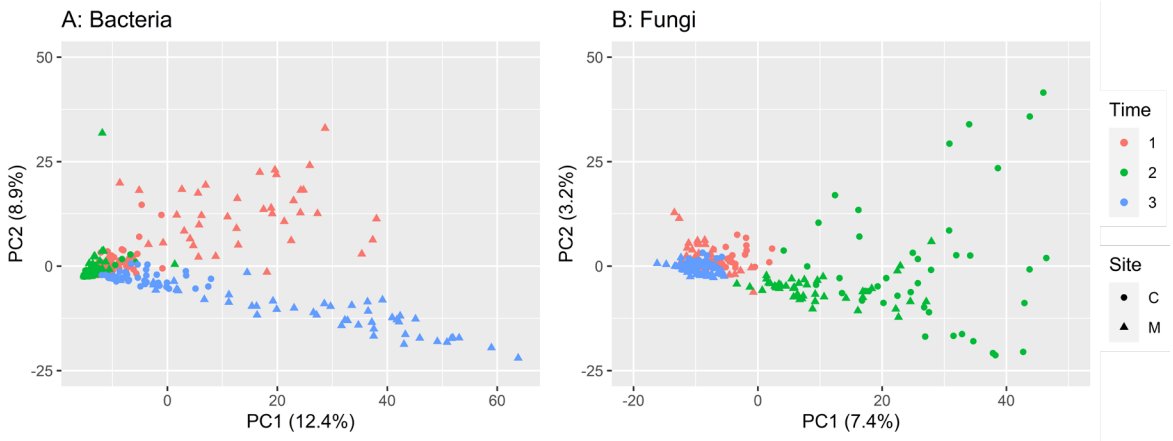

Figure 8. The first two principal compment scores for bacterial and fungal endophyte communities in the apple lear scar tissues, sampled from eight scion cultivars over three time points (1 & 3 – autumn; 2 – spring) at two sites (C – near Canterbury, M – near Maidstone) in Kent, UK.

Differential analysis of OTU relative abundance

In total, 2755 bacteria OTUs and 3370 fungal OTUs passed initial DESeq2 filtering and were subjected to differential abundance analysis for the four comparisons.

In total, 144 bacterial OTUs differed in their relative abundance between spring and autumn, of which 143 had lower relative abundance in the spring than in the autumn (Fig. 9). Log2FoldChange (LFC) values ranged from -14.8 to 0.64 (Fig. 9). Of these 143 OTUs, 101, 30, 7 and 2 OTUs were from the Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes phyla respectively; one OTU was from Cyanobacteria, and the remaining two OTUs were not assigned to the Phylum rank with at least 80% confidence (i.e., the 'unknown' group). There were 83 fungal OTUs that differed in their relative abundance between spring and autumn, with the LFC value ranging from -7.2 to 11.5. Of these 83 OTUs, 44 had lower relative abundance in the spring than in the autumn (Fig. 10). Of the 44 OTUs, 19 and 10 were from Basidiomycota and Ascomycota, respectively; the other five OTUs were from the unknown group. For those 39 OTUs with higher relative abundance in the spring, three and eight were from Basidiomycota and Ascomycota, respectively; the others were from the unknown group.

In total, 40 bacterial and 73 fungal OTUs differed in their relative abundance between the two sites. Of the 40 bacterial OTUs, 37 had higher relative abundance at the Canterbury site (Fig. 9), including 27, 8 and 2 OTUs from Proteobacteria, Actinobacteria and Firmicutes, respectively. Among the 37 OTUs was the most abundant *Pseudomonas* OTU. For 52 of the 73 fungal OTUs with differential relative abundance between the two sites, samples from the Maidstone site had greater relative abundance (Fig. 10). Most (43) of these fungal OTUs had very low number of reads (< 220); one fungal OTU (Dothideomycetes) had a very high number of reads (24184), nearly three times of the next highest one (*Vishniacozyma*), was likely to come from Aureobasidiaceae (LFC=1.42). Six of the eight *Vishniacozyma* OTUs had much higher relative abundance at the Maidstone site (including the second highest reads number – 9534) than the Canterbury site.

None of bacterial or fugal OTUs differed in its relative abundance between canker resistant and susceptible cultivars, whether over all three time points or just the two autumn sample points. Similarly the two rootstocks did not differ in the relative abundance for any bacterial OTUs and only differed in one fungal OTU, which was most likely of *Periconia* sp. - this fungal OTU had very low mean reads count (8.3).

Figure 9. Results of differential abunance analysis of individual bacterial endophyte groups in the apple leaf scar tissues for three comparisons: (A) spring vs. autumn, (B) canker resistant vs. susceptible, and (C) the two samplign sites. Analysis was carried out via the DESeq2 algothrim with the P-values adjusted using the Benjamini-Hochberg (BH) method (Benjamini & Hochberg, 1995).

Fig. 10. Results of differential abunance analysis of individual fungal endophyte groups in the apple leaf scar tissues for three comparisons: (A) spring vs. autumn, (B) canker resistant vs. susceptible, and (C) the two samplign sites. Analysis was carried out via the DESeq2 algothrim with the P-values adjusted using the Benjamini-Hochberg (BH) method (Benjamini & Hochberg, 1995).

Discussion

We quantified the relative effects of both large and small spatial scales, sampling time, scion and rootstock on bacterial and fungal endophyte communities around apple leaf scar tissues of current-season extension shoots. The results showed that endophyte composition was primarily affected by location (both local and large scales) and sampling time. There was a crash in the microbial population size in the spring, compared to the autumn, particularly for fungal endophytes. The effects of scion genotypes were limited, albeit statistically significantly, primarily through its interaction with sampling time. Rootstock genotypes in general did not significantly affect leaf scar endophyte composition.

Location is known to affect endophyte community structure in woody and herbaceous plant species on a large scales (i.e. distance of several kilometres), for example, leaf fungal endophytes in *Theobroma cacao* (Arnold et al., 2003) and *Cirsium arvense* (Gange et al., 2007), and bacterial endophytes in wild poplar (Firrincieli et al., 2020). Location effects were also demonstrated on the apple microbiome in bark (Arrigoni et al., 2020) and woody (Liu et al., 2020) tissues. Such site effects may largely be attributable to the differences in airborne microbial inoculum composition, which can be a significant source of phyllosphere endophytes (Hardoim et al. 2015; Compant, Clément and Sessitsch 2010; Bringel and Couée 2015; Hartley et al. 2015). Aerial inoculum composition can be shaped by local landscape as well as climatic conditions (Giauque and Hawkes 2013; Gomes et al. 2018; Firrincieli et al. 2020). However, different shoot parts of the Furmint grapevines were shown to harbour a common core group of fungal community in different vineyards (Knapp et al., 2021).

There was a large small-scale within-site effect, namely between blocks within an orchard, on both bacterial and fungal communities, consistent with a previous study (Vokou et al. 2012). This could be partially attributed to site-specific factors (soil pH, soil carbon, and C:N ratio) (Pacifico et al., 2019) as well as variable inoculum availability at a very local scale (Ricks & Koide, 2019). In addition, variation in microclimate may also impact the chance of successful establishment. Similarity microbial communities associated with different tomato organs appeared to correlate with the distance of a given plant organ from the soil (Ottesen et al., 2009). Other studies also suggested the effects of plant organs on endophytes (Gomes et al. 2018; Guevara-Araya et al. 2020; Wearn et al. 2012) . In the present study, only endophytes in leaf scar tissues were investigated as the research focussed on endophytes in relation to European canker development.

Both bacterial and fungal endophytes were greatly affected by sampling times, spanning 12 months. Influences of sampling time on endophyte composition have been demonstrated for other plant species, such as leaf bacterial endophytes of *Asclepias viridis*, *Ambrosia psilostachya*, *Sorghastrum nutans*, *Panicum virgatum*, and *Ruellia humilis* (Ding et al., 2013), and leaf fungal endophytes of *Cirsium arvense*, *Plantago lanceolata* and *Rumex acetosa* (Wearn et al., 2012). The present findings suggested that endophytes in apple leaf-scar tissues of current season extension shoots are primarily originated from external sources, particularly for fungi. This finding is consistent with previous findings that endophytes in woody hosts are considered predominantly non-systemic (Moricca & Ragazzi, 2008; Saikkonen et al., 2004). Although leaf microbial endophytes have been shown to be more likely result from migration of root endophytic microorganisms within the plant than from colonization of bacteria initially present on the surface of the plant species leaf grown in what conditions (Bringel & Couée, 2015), this does not preclude foliar entrance of endophytic microorganisms, as has been shown experimentally (Hartley et al., 2015). There are several strands of evidence from the present study supporting this origin inference. Firstly, there was large reduction in population sizes in terms of 16S or ITS copy numbers (as showed by qPCR) in the spring samples, compared to the autumn samples, especially for fungi. For bacteria endophytes, although population size reduction was smaller, there were significant reductions in alpha diversity indices in the spring. Secondly, there were many OTUs that were present only at individual sampling time points, indicating significant species turnover over time. This is particularly true for fungi: nearly 40% of OTUs were only present in the spring samples. Thus, although many OTUs were established in the new leaf scar areas in the spring, these OTUs were at very low abundance. These early OTUs of low abundance may have been more likely competed out by other groups during the growing season when sampled in autumn, resulting in more unique OTUs for the sampling. Finally, the overall endophyte composition in the leaf scar tissues was

little affected by either scion or rootstock genotypes, whereas the differences in rhizosphere microbiome due to rootstock genotypes were much greater (Deakin et al., 2019), suggesting movement of root microbes to leaf-scar tissues during the growing season is unlikely to be the major avenue of endophyte recruitment around leaf scars of current-season extension shoots. Of course, differences in root endophytes among host genotypes are more relevant here. Further studies are needed to confirm these speculations where endophytes in leaf scars and surrounding tissues of both previous season and current-season extension shoots are determined over time, relative to other tissues such as roots and main stems.

It should also be noted that there are clear differences in the seasonal dynamics between bacterial and fungal endophyte composition. The reduction in population size in terms of 16S or ITS copy numbers, as showed by qPCR, was far greater (at least one magnitude) in fungi than in bacteria. Furthermore, there was a much higher proportion (ca. 40%) of fungal OTUs that were only present in the spring, compared to ca. 10% for bacteria. Thus, a larger proportion of endophytic bacteria than fungi might have originated from other pre-existing apple tissues and/or these early bacterial colonisers (OTUs) in the spring were able to compete with (if any) newcomers in the growing season. This dynamics of endophytes is critically important as it will have implication for practical disease management through augmented application of beneficial microbes.

Limited effects of genotypes, compared to previous studies (Arrigoni et al., 2018, 2020; Liu et al., 2020), may simply illustrate the importance of sampling time, suggesting the importance of natural turnover events of endophytes independent of cultivars. This is reflected in the relative differences among scions for the first sampling point (Olivieri et al., 2021) in contrast with all three sampling points presented here. Recruitment of specific endophytes appears to be less genetically controlled but rather more influenced by local availability of inocula as well as other factors that are variable at a finer scale, e.g. surrounding species and microclimate. Varying degree of the interaction between time and scion genotype indicated that recruitment of specific microbial groups by genotypes is conditioned on the local microbial reservoir, indicating the dynamic nature of endophyte recruitment on new tissues over time. Such a phenomenon was also observed for apple rhizosphere microbiome over time (Deakin et al., 2019).

A number of apple endophytes in the leaf-scars tissues for the first sampling time (autumn) were associated with the susceptibility to *N. ditissima* (Olivieri et al., 2021). Specific endophytes from apple showed *in-vitro* antagonistic effects against *N. ditissima* (Liu et al., 2020). Thus, manipulating apple endophyte communities is considered as another viable approach to manage the canker disease. Apple endophyte composition could be altered through specific agronomic practices or augmented application of specific endophyte strains with biocontrol ability to improve host resistance/tolerance against *N. ditissima*. However, when analysed over all three time points, no single microbial group was significantly associated with canker resistance. However, there was some indication that canker resistance may be affected by specific microbial composition features as well as increased fungal population size in the spring. Taking all the results together, it suggests that specific endophytes introduced artificially may have difficulties to persist in high abundance over seasons, even within season from spring to Autumn.

In conclusion, we found that endophyte composition was primarily affected by location (both local and large scales) and sampling time. In addition to considerable turnover in microbial groups over time, both bacterial and fungal population suffered from crushes in their size in the spring, compared to the autumn, particularly for fungal endophytes. The present results

suggest that annual augmentation of specific endophytes around leaf-fall time may be necessary to protect leaf scars from infection in the autumn time.

Biocontrol potential of a specific fungal endophyte

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 *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.

Agar plugs (6 mm) 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. *N. ditissima* colony size across the line on the plate was recorded twice a week for several weeks.

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* spore suspension immediately after

Epicoccum assessment. Rootstocks were harvested, graded, and planted out in winter 2019/20. In July 2020 canker incidence was assessed.

Pruning and 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 (November 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. Protection of pruning wounds was also tested at the same time, on the same 48 Royal Gala trees using a separate set of eight random shoots per tree.

Colonisation and survival of E. nigrum in apple shoots

Two experiments were conducted to assess the colonisation and survival of E. nigrum (strain B14-1) in the apple tissues. Leaf scars were inoculated with *E. nigrum* B14-1 conidia at leaf fall; *E. nigrum* abundance was then measured via qPCR 10 days, 20 days and 1 year after inoculation in (i) the leaf scar tissue, (ii) the bark and sapwood 3-5 mm above and below the leaf scar, and (iii) in the bark and sapwood 8-10 mm above and below the inoculated leaf scars.

The short term colonisation experiment (10, 20 days) was conducted in October 2020 in the Middle park orchard of Royal Gala at NIAB EMR. Experiment consisted of 5 blocks each with 2 treatment plots (B14-1, control), 3 trees per plot and four test shoots per tree. Fresh leaf scars in B14-1 plots were inoculated with 10ul of B14-1 suspension at 10^5 conidia per ml. Two shoots per tree were sampled at 10 and 20 days post inoculation. Potential inoculum residues and surface growing fungi were washed from the shoots. Clean shoots were dried and dissected in the laminar flow hood to obtain 2 mm wide and 3 mm deep sections of wood and bark from each sampled shoot. Segment 1 consisted of leaf scar tissue, segment 2 consisted of bark and sapwood 3-5 mm above and below the leaf scar, segment 3 consisted of in the bark and sapwood 8-10 mm above and below the inoculated leaf scars tissue.

To investigate the survival of *E. nigrum* B14-1 in apple tissues over a period of one year, in October 2020 we sampled leaf scars that were inoculated in biocontrol trial in 2019. Each sample constituted of twelve sections belonging to the same tissue inoculation type (B14-1 or water control) and segment type (leaf scar, 3-5 mm, 8-10 mm) pooled into a single sample from four neighbouring Royal Gala trees. Sections belonging to the same segment and plot of the same experiment were pooled together, freeze dried, homogenised, and DNA extracted as described above.

Results

In vitro challenge assays

Two *Epicoccum* strains (C15, C29) and one *Aureobasidium* strain (C32(1)) showed substantial levels of biocontrol ability (Fig. 11 and 12), 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.

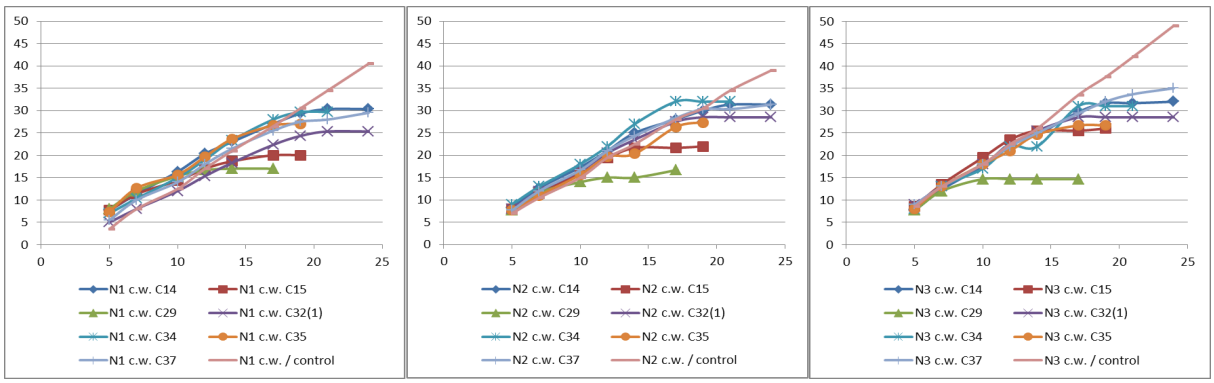

Figure 11. Growth (mm) of three *N. ditissima* strains (N1, N2 and N3) (Y-axis) over time (days) (X-axis) *N. ditissima* was either challenged with endophyte strains or without endophyte challenge (control).

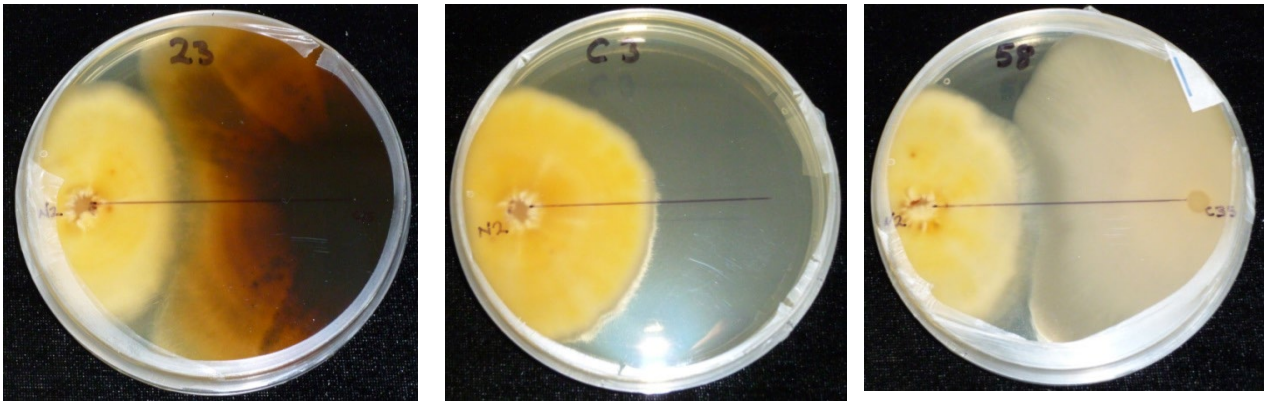

Figure 12. Challenge assay example (17 days after plating) with one of the *Epicoccum* isolates (C29) left, *Aureobasidium* (C35) right and unchallenged control in the centre. In both left and right examples we observed endophytes restricting the growth of *N. ditissima*.

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 3). 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 (Fig. 13).

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. Canker expression levels were not different between treatments. However, canker incidence however was extremely low, ranging from 1% to 5% leaf scars with canker, making statistical testing less powerful.

Similar results in term of endophyte abundance, canker development and shoot development were obtained from the 2019 experiment.

Table 3. Number of M9 shoots from which of *E. purpurascens* was successfully isolated 3 months after treatment with a single UK *E. purpurascens* strain (B14-1). 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	0
Leaf	1	4	1	0

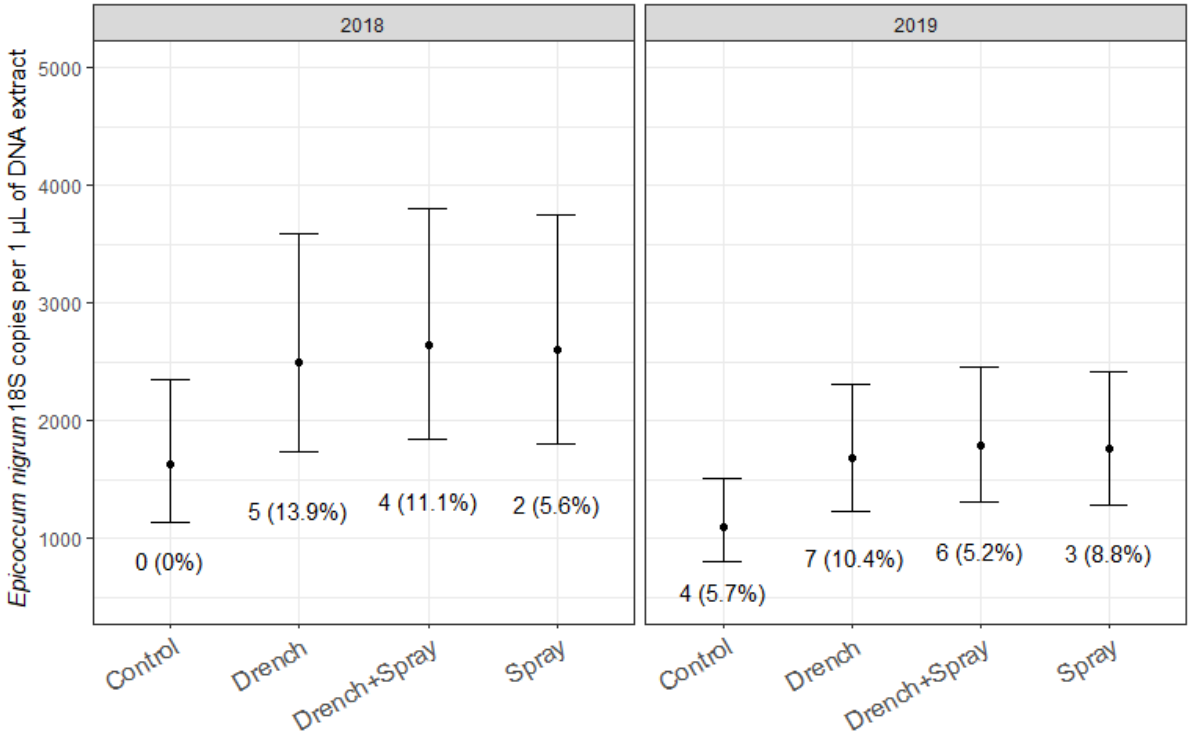

Figure 13. Mean and 95% confidence interval of *E. nigrum* 18S DNA abundance in leaf scars of M9 rootstocks as predicted from a fitted linear model. Plants in stool-beds were subjected to foliar and/or drenching treatment of *E. nigrum.*

E. nigrum B14-1 protected leaf scars and pruning wounds from N. ditissima

We tested the biocontrol potential of B14-1 against *N. ditissima* on leaf scars and pruning wounds of mature apple cv. Gala trees in the field conditions in the autumn.

The incidence of *N. ditissima* lesions was reduced by 46.4% when leaf scars were co-inoculated with *E. nigrum* B14-1 (32.5% canker incidence) in comparison to *N. ditissima* alone (60.6% canker incidence) (Fig. 14A). Co-inoculation of B14-1 with *N. ditissima* resulted in ca. 50% increase in the incidence of healthy leaf scars (52.6%) in comparison to *N. ditissima* inoculated alone (28.0%) (Fig. 14c). The reduced canker lesion incidence most likely resulted from B14-1 preventing *N. ditissima* entry rather than slowing down its colonisation progress which could result in asymptomatic infection i.e. dead bud with no visible lesion (Fig. 14B). The incidence of *N. ditissima* lesions resulting from natural infections was on 2.8 % and 8.6% for water and *E. nigrum* B14-1 only control, respectively. Increased canker lesion incidence in leaf scars inoculated only with *E. nigrum* B14-1 over water control was close to 95% significance (P = 0.08). Lesion size was the highest in *N. ditissima* inoculated leaf scars (26.6 mm), followed by *N. ditissima* + *E. nigrum* B14-1 co-inoculated leaf scars (25.5 mm), *E. nigrum* B14-1 (22.2 mm)

and water control (20.0 mm) (Fig. 14D). There were however no significant differences in mean *N. ditissima* canker lesion size between the treatments

Incidence of pruning wounds with canker reduced from 94.3% in the *N. ditissima* inoculated wounds to 87.7% in the *E. nigrum* B14-1 + *N. ditissima* co-inoculated wounds (Fig. 14E). This small reduction (6.6%) was nevertheless statistically significant (P = 0.03).

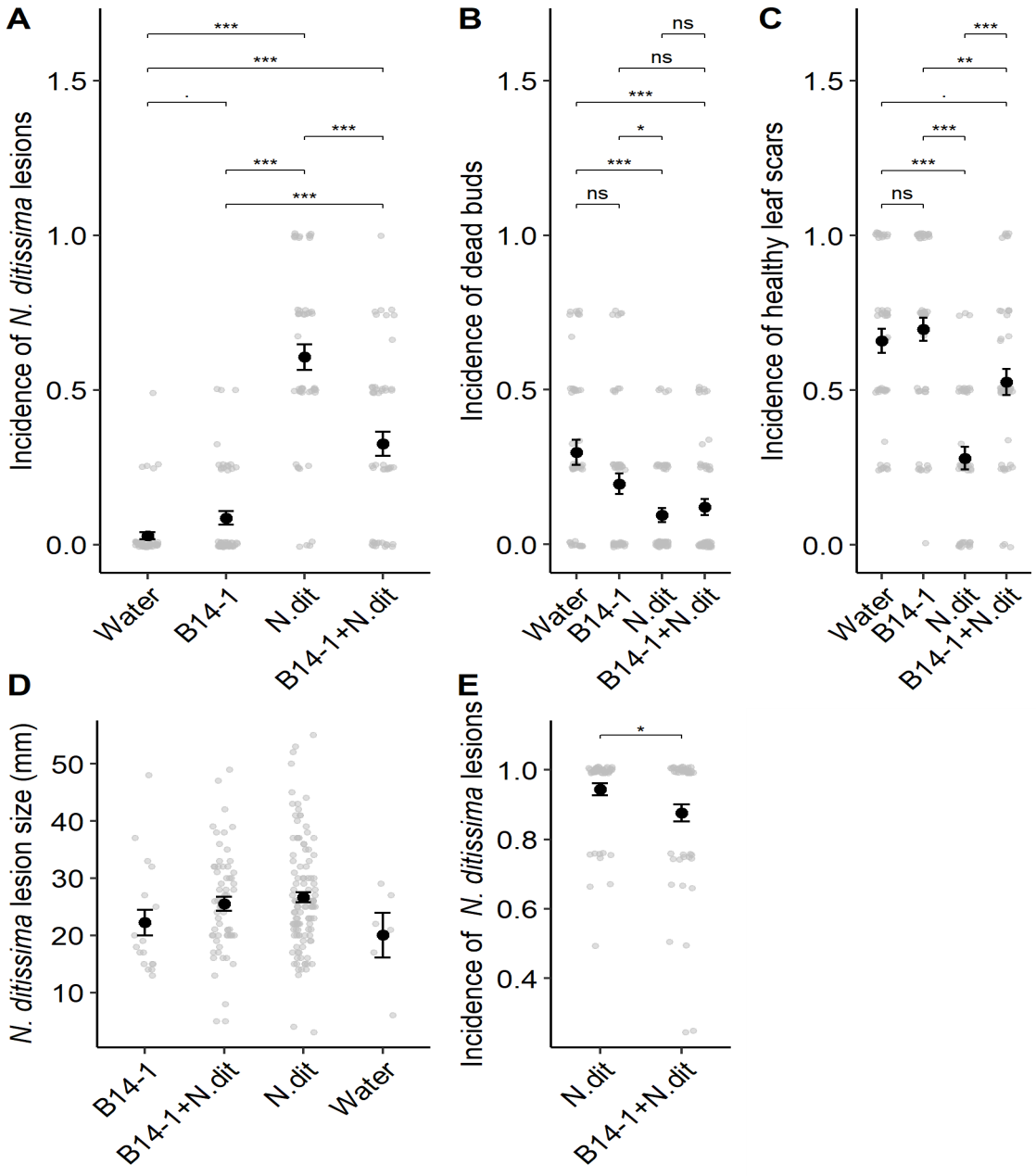

Figure 14. Incidence of cankered leaf scars (A, B, C) and pruning wounds (E), and canker lesion size (D) in leaf scars. Water control inoculated leaf scars are shown alongside with leaf scars inoculated with *E. nigrum* B14-1 (B14-1), *N. ditissima* (N.dit) alone or in combination (B14-1+N.dit). Mean and its standard error are also showed with the raw data in grey. Significant differences between the treatments are presented as: “ns” (not significant) , ”. “ (0.05 < P < 0.1), “*” (0.05 < P < 0.01), “**” (0.01 < P < 0.001) and “***” (P < 0.001).

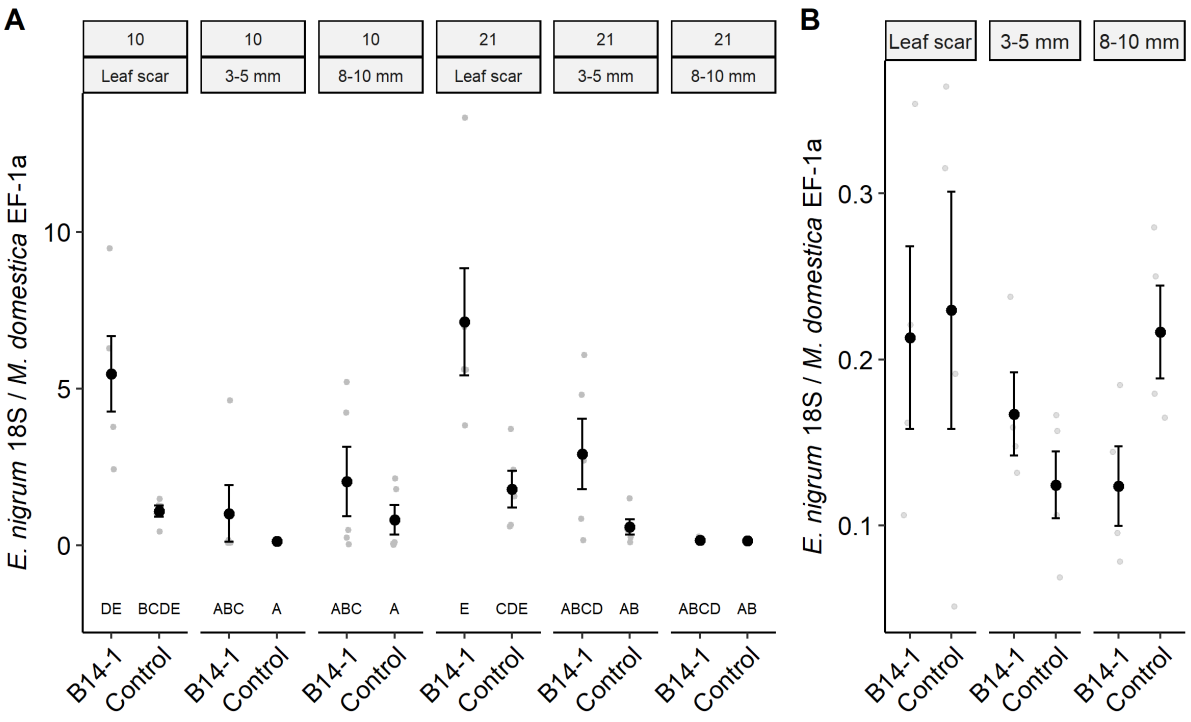

Figure 15. Concentration of *E. nigrum* DNA in leaf scar tissue, 3-5 mm above and below and 8-10 mm above and below the leaf scar at 10 days, 20 days (A) and 1 year (B) after leaf scar inoculation with live *E. nigrum* B14-1 spores (B14-1), inactivated *E. nigrum* B14-1 spores (Control in A) or water (Control in B). The *E. nigrum* DNA copy number in each sample was divided by host (*M. domestica*) EF-1a theoretical copy number to account for differences in sample size. Mean and its standard error are shown together with raw data points (in grey). Each data point represents a pool of 12 leaf scars from three separate trees. Significant differences between groups are indicated with letters, groups sharing the same letter are not significantly different.

Discussion

E. nigrum B14-1 joins a list of biocontrol Epicoccum strains

At least five species of *Epicoccum* were previously found to act as biological control agents against various plant pathogens (Taguiam et al. 2021). Most relevant examples include control of Esca disease complex of grapevine by *E. layuense* isolate E24 (Del Frari et al. 2019), control of ash dieback (*Hymenoscyphus fraxineus*) (Kosawang et al. 2018), apple proliferation phytoplasma (*Candidatus phytoplasma mali*) (Musetti et al. 2011), peach canker (*Cytospora cincta*) (Royse 1978) and brown rot of stone fruit / Peach twig blight (*M. laxa*) (Larena et al. 2005; De Cal et al. 2009) by various strains of *E. nigrum.* Moreover, endophytic *E. nigrum* strains have been shown to produce antifungal compounds and improve plant growth in sugarcane (Fávaro et al. 2012). Recently, strains of *Epicoccum* genus with *in-vitro* biocontrol potential against *N. ditissima* have also been isolated from apple trees in New Zealand (Liu et al. 2020) which shows the global presence of *Epicoccum* in apple trees.

All three strains *Epicoccum* strains isolated in this study belonged to *E. nigrum* species and demonstrated biocontrol potential in dual coculture assay. The main mechanisms were competition for nutrients and secretion of antimicrobial compounds in the media. Our observations were in line with known antagonistic mechanisms of *Epicoccum* species such as malformation and lysis of pathogen hyphae on contact (Lahlali and Hijri 2010; Bian et al. 2021) and secretion of antifungal secondary metabolites including flavipin and epicolactone,

epicoccolide and epicorazine (reviewed in Taguiam et al. 2021). The observed antifungal activity of water and organic extract of B14-1 is in line with reports of *E. nigrum* strain P16 (Fávaro et al. 2012).

To evaluate the biocontrol potential in field conditions, we need sufficient amount of *Epicoccum* inoculum. Using a suite of agar based media (data not shown) and peat/vermiculite/lentil meal solid media that was optimised for production of *E. nigrum* Link isolate 282 spores (Larena et al. 2004), we were able to produce sufficient amount of spores with B14-1, but not C15 and C29 strains. B14-1 was therefore the only strain included in the field experiments for assessment of its biocontrol efficacy against *N. ditissima.*

E. nigrum *B14-1 controlled N. ditissima in the orchard conditions*

Requirements for commercially successful endophytic biocontrol are ease of application and establishment, one or more mechanisms of action, and no harm to host under any circumstances (Card et al. 2016). Ideal endophytic biocontrol agent would rapidly and stably colonise the host tissues in commercial environment and cause no detrimental effects to the host at any stage. Research and development of biological control agents not a straightforward process. Many biocontrol studies are carried out under controlled conditions, circumventing complications associated with environmental influences, large-scale production and formulation (Latz et al. 2018). This study have shown that *E. nigrum* B14-1 can control *N. ditissima in vitro* and *in vivo* when applied directly to leaf scars and pruning wounds in commercial orchard conditions. This is the first report of successful biocontrol of *N. ditissima* in field conditions using a fungal isolate. *Bacillus subtills* isolate have been reported to control *N. ditissima* infections on leaf scars (Swinburne et al. 1975), but none of the commercial biocontrol products tested so far have been effective against *N. ditissima* in field trials (Walter et al. 2017, Papp-Rupar, unpublished). If there is additional *in vitro* antimicrobial effect of B14-1 against other apple pathogens such as *Phythopthora cactorum*, *Venturia ineaqualis* and *M. fructigena*. the B14-1strain could provide a powerful tool for apple canker control in organic and conventional apple production. The compatibility of B14-1 with commercial fungicide programs will need to be established. If necessary, fungicide resistance could be engineered into the biocontrol strain to increase its competitiveness and applicability (Zhou and Reeleder 1990).

E. nigrum B14-1 can transiently colonise apple endophytic environment

Colonisation strategy and establishment in the host is an important consideration for fungal endophyte biocontrol and has been largely overlooked by researchers so far (Latz et al. 2018). This is to our knowledge the first report where colonisation success of an endophytic biocontrol fungus was assessed on woody perennial host in field conditions. When B14-1 spores were applied on foliage in summer we detected very low, borderline significant increase in Epicoccum endophytic population in the leaf scars in the autumn. This can be due to poor establishment and leaf penetration of B14-1 in summer or due to preferential epiphytic colonisation as shown *E. nigrum* P16 on sugarcane leaves (Fávaro et al. 2012). Interestingly, endophytic colonisation success of *E. nigrum* B14-1 applied on M9 rootstocks as foliage spray and root drench were the same. This was either due to E. nigrum B14-1 locally propagating between soil and plat tissues with spores or mycelial fragments as suggested for sugarcane - *E. nigrum* P16 interaction by Favaro et al. (2012) or due to wood colonisation as observed in *E. layuense* E24 in grapevine. Inoculation method had a similarly small effect on endophytic colonisation of entomopathogenic *Beauveria bassiana* in sorghum (Tefera and Vidal 2009). Present results from B14-1 colonisation of Gala leaf scars in the autumn suggest

that B14-1 can colonise apple leaf scars and woody tissue above and below the leaf scar but not very extensively, suggesting a more towards local propagation hypothesis.

Leaf scar and wood colonisation was also not persistent across seasons. Several applications of *B. nigrum* B14-1 spores at times critical for *N. ditissima* infection as well as for *E. nigrum* establishment may be necessary to improve its colonisation and biocontrol success. It has been observed that addition of 1% KCl, 25% sodium alginate and silica gel together with higher application number significantly increased survival and establishment od *E. nigrum* on peach and nectarine surfaces (De Cal et al. 2009). More research is required to determine the tissue preference, optimal application timing and conditions, host genotype effect, and seasonal population dynamics of B14-1 in / on apple tissues and the wider orchard environment.

Mapping QTLs responsible for recruiting endophytes

We conducted an experiment to investigate the extent to which the relative abundance of endophytes in the leaf scar tissues is genetically controlled by apple hosts and, if so, to map QTLs that are responsible for specific 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

Field design and layout

To choose one mapping family for the mapping study, we 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. Following discussions with breeders/geneticists and based on field canker development of the mapping populations as well as preliminary endophyte profiling results, the '54' mapping population was chosen for endophyte characterization.

The '54' mapping population was a cross between cv. Aroma and cv. Golden Delicious made in 2015. Canker resistance is not well understood yet, but it is quantitative in nature. The two parents were selected based on empirical evidences as well as limited lab artificial inoculation data (Gomez-Cortecero et al. 2016), which classified the two parents as moderately susceptible and highly resistant/tolerant to *N. ditissima*. All 70 F1 genotypes in the family, including the parents, were grafted onto M9 EMLA rootstocks in January 2017 at East Malling, UK. The plants were maintained in pots in polytunnels until October 2017, when they were planted in a randomised block design in an orchard. There were four blocks, one tree per genotype in each block.

Inoculation with N. ditissima, sampling leaf scars, DNA extraction, and sequencing

The trees were artificially inoculated with a single isolate of *N. ditissima* (Hg199) at leaf scars in November 2018 in order to eliminate the issue of inoculum heterogeneity in the orchard. Since the research objective was to compare resistance/susceptibility of genotypes to *N. ditissima*, we decided to eliminate the issue of field inoculum heterogeneity via artificial inoculation from complicating data interpretation. The inoculum was prepared according to a published protocol (Gomez-Cortecero et al. 2016). Five artificial leaf scars were inoculated per tree, and each leaf scar (pseudo-replicate) was positioned on a separate branch. A droplet of 6 µl spore suspension (10^5 macroconidia/ml) was pipetted onto each artificial leaf scar wound and then covered with petroleum jelly (Vaseline) immediately after absorption. The petroleum jelly was removed two weeks after inoculation.

Each inoculated leaf scar was marked to allow repeated measurements over time. Canker lesion development was measured with a digital calliper at three time-points: five-, eight- and eleven-months post inoculation (mpi). A final assessment was conducted at 20 mpi on the percent branch area with foliage, percent branches with cankers and the number of cankers. Average number of cankers per branch was then calculated for each tree.

Many trees died of canker before leaf scars were sampled in the autumn 2019 before leaf-fall. Only those genotypes with a minimum of three surviving trees were sampled. In total we sampled 216 trees, including the two parents and 54 F_1 genotypes. Sampling leaf scars and extracting DNA from leaf scar samples followed a published protocol (Olivieri et al. 2021a), which was also described in the previous endophyte dynamics section. Fungal and bacterial community sizes we estimated with qPCR. DAN samples were sent to Novogene (Cambridge) for amplicon-sequencing.

Bioinformatics and statistical analysis

All subsequent bioinformatics and statistical analysis of sequence data also followed the previous endophyte dynamics section. All statistical analyses were carried out in R 4.0.3 (R Core Development Team 2019). Sequence counts data were either normalised by qPCR or the median ratio (MR) method.

The main objective of the present study was to determine whether there is significant genetic variability among F_1 progeny genotypes. This was achieved by a random effect model in which the total variability among F_1 genotypes was partitioned into environmental (V_E = between blocks + residual) and genetic (V_G = between F_1 genotypes) variability. The significance of genetic variability (component) was statistically tested by comparisons of two nest models with a Chi-square test with one degree of freedom: one with the genotypic component included and the other without. The broad sense of heritability was then estimated as: $V_G/(V_E + V_G)$. These variance components were estimated with the lmer function in the lme4 package (Bates et al. 2015). For each normalised set of counts data, two types of data were used for estimation of the genetic components: PC scores, representing the overall microbial composition, and the normalised counts data of those OTUs with highest count values (Table 4). Before PC analysis (PCA), the normalised counts data were logarithm transformed on the natural base and then standardised. Similarly, normalised OTU counts values logarithm transformed on the natural base before analysis. The random effect model was fitted to the data with the R lme4 package (Bates et al. 2015). Within the analysis of each data type (PC or OTU), the Benjamini-Hochberg (BH) adjustment (1995) was applied to correct for the false discovery rate associated with multiple testing. Statistical significance was determined at the 5% level (BH adjusted). In addition to the estimation of broad sense heritability, correlation (both Pearson and Spearman) of canker-related variables with PC scores and normalised OTU counts were calculated.

Table 4. Number of the Principal Components (PC) and OTUs with the highest counts for inclusion in estimation of differences between F_1 genotypes and correlation with canker variables

	qPCR normalised		Median of ratio normalised	
	PCs	**OTUs**	**PCs**	**OTUs**
Fungi (ITS)	30	100	80	100
Bacteria (16S)	30	200	50	200

Genotypic data for biparental population and QTL analysis

DNA from the biparental population and the two parental cultivars were extracted**.** The population was genotyped on the Illumina Infinium® 20k SNP array Genotype assignment was performed in GenomeStudio Genotyping Module 2.0 (Illumina). SNP filtering was conducted in ASSisT, leaving 8,000 SNPs to be used for further analysis.

Al those fungal and bacterial OTUs and PCs that had significant genetic components were subjected to QTL analysis. The relationship between segregations of single markers and traits were analysed with Kruskal-Wallis test (K-W) with the function kruskal.test in R. The Benjamini-Hochberg method was used to adjust p-values for the number of markers used for K-W (Benjamini and Hochberg 1995).

Verification of the QTL mapping was performed through Composite Interval Mapping (CIM) in the FullsibQTL package in R. A maximum of 10 cofactors were stipulated to locate QTLs. A random permutation test ($\alpha = 0.05$; n = 1000 replicates) available in FullsibQTL was used to determine critical LOD score for declaring the presence of true QTL. The linkage map used for the CIM analysis was produced in R-package 'OneMap'. To reduce the computational burden, markers with a pairwise recombination fraction of zero were collapsed into bins represented by the marker with the lower amount of missing data among those in the bin. A LOD Score of 3 and recombination fraction of 0.50 was considered to determine linkage between markers. Markers were ordered according to the consensus linkage map in Di Pierro et al. 2016) whereas genetic distances between markers were calculated by 'OneMap' using the Kosambi mapping population.

QTL positions of significant KW markers were determined as their position on the linkage map produced in 'Onemap'. The position of markers which had been removed in the binning process were given by a marker from within the same bin. The effect size of significant QTL was estimated in 'FullsibQTL'. All significant QTL positions were included in the calculation of QTL effects for each phenotype.

Results

Overall sequencing, OTU generating and qPCR results

Fungi. In total, there were 4,268 fungal OTUs, most of which had few reads. The top 6 and 34 most abundant OTUs accounted for over 50% and 90% of the total reads, respectively. The top 206 OTUs accounted for 99.0% of total reads and were included in subsequent analysis.

The number of reads assigned to OTUs in each sample ranged from 51,780 to 134300 with a median of 117676; the number of reads for each OTU ranged from 1,706 to 4,289,841 with a median of 7,532. The top two were both identified as *Filobasidium* spp.: *F. wieringae*, and *F. chernovii*, accounting for 18.0% and 12.0 % of the total reads, respectively. The OTUs with the third and fourth most reads were both identified as *Vishniacozyma* spp., jointly accounting 13% of the total reads. Of the 206 fungal OTUs, only 137, 118, 104, 79, 60 and 34 could be assigned to the taxonomic rank of phylum, class, order, family, genus and species with > 80% confidence, respectively. Basidiomycota and Ascomycota accounted for 59.3% and 15.8% of the total reads, whereas 24.9% of the reads could not be assigned to a phylum with > 80% confidence and were hence designated as unknown.

For 13 of the 216 samples (54 F_1 genotypes and the two parents), we failed to obtain reliable qPCR ITS values. Of the remaining samples, there were large variabilities in the ITS qPCR values among replicates within a given genotype (Fig. 16A); genotypes did not differ significantly in the ITS qPCR values.

Bacteria. One sample failed to generate sequences. In total, there were 3,639 bacterial OTUs; the top 51 and 583 most abundant OTUs accounted for > 50% and 90% of the total reads, respectively. Only the top 1,694 OTUs were included in subsequent analysis as all others jointly accounted for < 1.0% of the total reads.

Of the 1,694 OTUs the number of reads for each OTU ranged from 490 to 1,034,106 with a median of 2,654. The number of reads assigned to OTUs in each sample ranged from 14,180

to 127,718 with a median of 110,064. The top two OTUs by abundance were *Sphingomonas* sp. (4.6%) and *Methylobacterium* sp. (4.2%). Of the 1,694 bacterial OTUs, only 1,341, 1,091, 809, 605 and 388 could be assigned to the taxonomic rank of phylum, class, order, family, genus, and species with > 80% confidence, respectively. Proteobacteria and Actinobacteria accounted for 54.9% and 24.5% of the total reads, whereas 9.0% of the reads could not be assigned to a phylum.

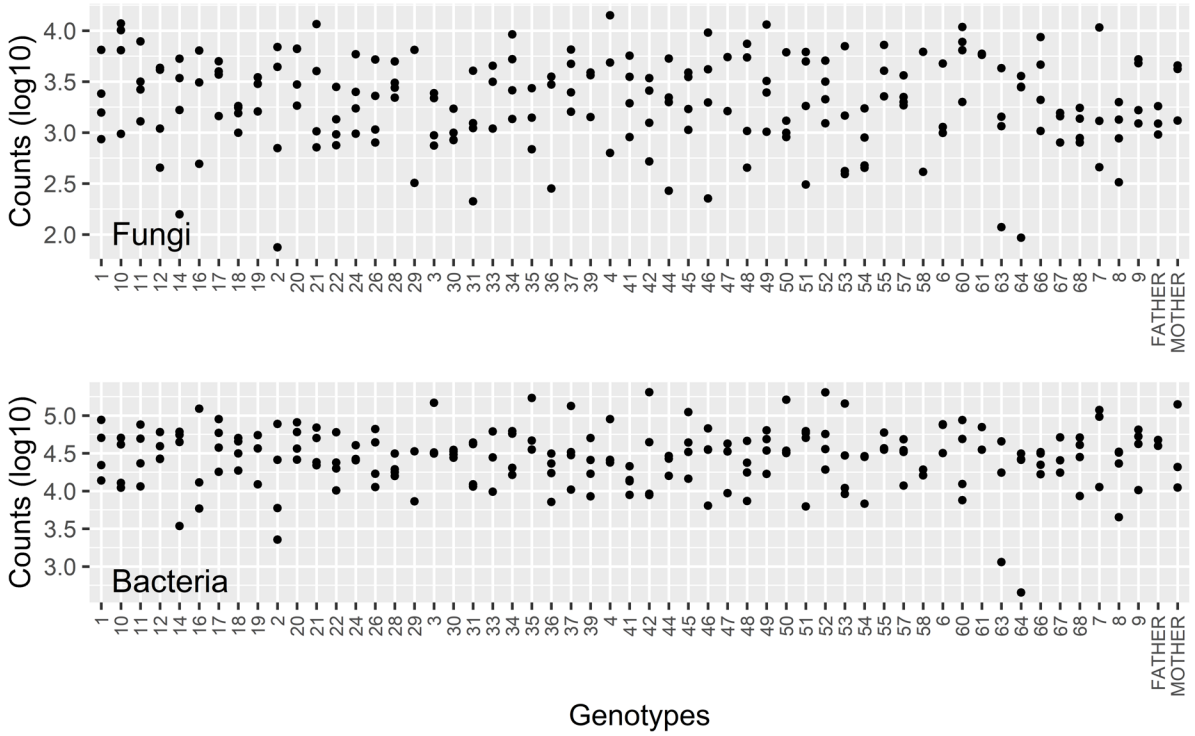

Figure 16. qPCR results of both ITS and 16S of endophytes in apple leaf scar tissues of individual trees of F_1 and parental genotypes. The cross was between cultivars Aroma and Golden Delicious.

Of the 216 samples, we failed to obtain reliable qPCR 16S values for 14 samples. For eight samples, qPCR data for both fungi and bacteria were not available. As for fungi, there were large variabilities in the 16S qPCR values among replicates within a given genotype (Fig. 16B), and genotypes did not differ significantly in the 16S qPCR values. However, there were significant ($P < 0.001$) differences in the 16S qPCR values among the four blocks.

Microbial diversity indices

Fungi. For both the qPCR- and MR-normalised data, there were no significant differences in both Simpson and Shannon indices among the 54 F_1 genotypes. But the blocks differed significantly ($P < 0.001$) in the two indices. For the qPCR-normalised data, F_1 genotypes did not differ in Bray-Curtis indices, but differed ($P < 0.001$) in the indices for the MR-normalised data, accounting for about 25% of the total variability in the indices. The two parents did not differ in all indices for either normalised data set.

Bacteria. For both the qPCR- and MR-normalised data, 54 F_1 genotypes did not differ significantly in both Simpson and Shannon indices, and the blocks differed ($P < 0.001$) in the two indices. For the qPCR-normalised data, neither F_1 genotypes nor the two parents differed in Bray-Curtis indices. In contrast, F_1 genotypes ($P < 0.001$) as well as the two parents ($P < 0.05$) differed in Bray-Curtis indices for the MR-normalised data.

Genetic components of principal component scores

Table 5 shows the summary of results.

Fungi. For the qPCR-normalised data, the first two PCs explained about 50.9% and 4.0% of the total variability. F_1 genotypes differed ($P < 0.001$) for PC5, with the corresponding estimated heritability value of 27.5%. For the MR-normalised data, the first two PCs explained only about 9.3% and 7.4% of the total variability (Fig. S1B). F_1 genotypes differed for PC1, PC4, P9, PC12, P17 and P44, with the corresponding heritability estimates of 5.5%, 33.7%, 20.8%, 23.4%, 19.4% and 21.9%.

Bacteria. For the qPCR-normalised data, the first two PCs explained about 45.3% and 3.8% of the total variability. Only for the two (PC3 and PC8) of the top 30 PCs did the 54 F_1 genotypes differ significantly ($P < 0.05$), with the respective estimated heritability of 29.5% and 30.0%. For the MR-normalised data, the first two PCs explained only about 9.2% and 8.0% of the total variability (Fig. S2B). For seven (PC2, PC7, PC8, PC16, PC17 and PC26) of the top 50 PCs, F_1 genotypes differed significantly ($P < 0.05$), with the heritability estimates ranging from 14.5% (PC13) to 33.2% (PC7).

Table 5. Number of cases where there was significant genetic variability in the PCs and top fungal/bacterial OTUs or corelation of endophytes with canker variables

	qPCR normalised				**Median of ratio normalised**			
	Fungi		**Bacteria**		**Fungi**		**Bacteria**	
	PCs	**OTUs**	**PCs**	**OTUs**	**PCs**	**OTUs**	**PCs**	**OTUs**
Genetic variance	**1**	**0**	**2**	**1**	**6**	**24**	**7**	**47**
Correlation								
Individual based								
Canker size	0 (0)*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)
Canopy	1 (1)	2 (0)	1 (1)	0 (0)	1 (1)	7 (7)	1 (1)	6 (0)
% Shoots cankered	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Family based								
Canker size	1 (1)	0 (2)	0 (0)	2 (2)	1 (1)	2 (2)	2 (2)	0 (0)
Canopy	1 (0)	5 (0)	0 (0)	0 (0)	1 (0)	8 (6)	1 (0)	4 (10)
% Shoots cankered	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)

*: **x** (**y**) indicates the **x** and **y** number of significant Pearson and Spearman correlations, respectively.

Genetic components of individual OTUs.

Table 5 shows the summary of results.

Fungi. For the qPCR-normalised data, F_1 genotypes did not differ significantly in any of the top 100 OTUs. For the MR-normalised data, F_1 genotypes differed ($P < 0.05$) for 24 out of the top 100 OTUs (Fig. 17A, Table 6). Only seven of the 24 OTUs could be assigned to the species level; two of these OTUs were both identified as *Tilletiopsis washingtonensis.* The estimate of broad sense heritability ranged from 10.2% to 34.7% with an average of 18.5%.

Bacteria. For the qPCR-normalised data, F_1 genotypes differed significantly in only one of the top 200 OTUs (OTU89 – *Roseomonas sp.*), with the estimated broad sense heritability of 27.9%. For the MR-normalised data, F_1 genotypes differed significantly for 47 of the top 200 OTUs (Fig. 17B, Table 7); The estimate of broad sense heritability ranged from 8.1% to 37.3% with an average of 18.5%. These 47 OTUs also included OTU89, indeed there were OTUs from *Roseomonas*.

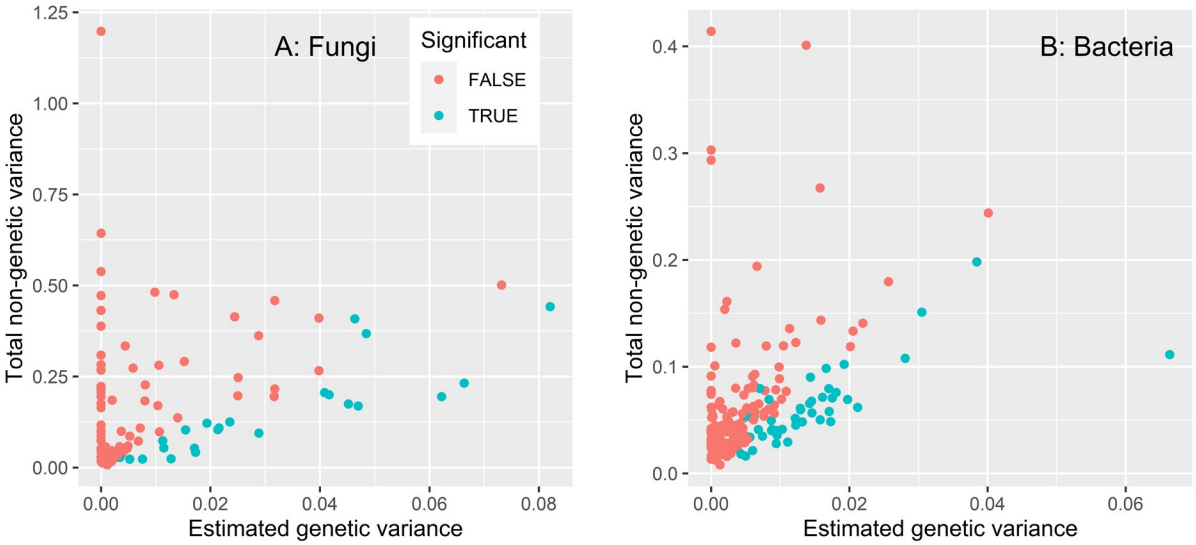

Figure 17. Estimated genetic variance plotted against the total environmental variance for the top 100 fungal and 200 bacterial OTUs based the median of ratio normalised counts data. The point colour indicates whether the genetic variance is statistically significant or not.

Table 6. Summary of those fungal OTUs within the top 100 highest counts in the median of ratio normalised counts for which the genetic differences between F_1 genotypes were statistically significant.

Taxonomy (> 80% confidence)		% Total reads	Heritability (%)
OTU29	Ascomycota	0.37	17.57
OTU3360	Basidiomycota	1.30	11.00
OTU143	Dothideomycetes	0.05	21.76
OTU35	*Entyloma*	0.19	17.26
OTU50	*Entyloma calendulae*	0.07	24.21
OTU12	Fungi	1.97	24.43
OTU1226	Fungi	0.04	11.64
OTU147	Fungi	0.07	15.67
OTU19	Fungi	0.94	24.31
OTU40	Fungi	0.65	10.20
OTU73	*Leptospora*	0.03	17.04
OTU17	*Neosetophoma*	1.51	15.79
OTU20	Phaeosphaeriaceae	0.83	23.39
OTU62	Phaeosphaeriaceae	0.05	13.31
OTU76	Pleosporales	0.04	16.57
OTU57	*Pseudoophiobolus rosae*	0.08	22.26
OTU21	*Sporobolomyces roseus*	0.62	18.66
OTU26	*Subplenodomus iridicola*	0.49	20.57
OTU24	*Symmetrospora*	0.71	10.20
OTU310	*Taphrina*	0.04	29.12
OTU71	*Taphrina*	0.05	13.02
OTU16	*Taphrina carpini*	2.04	34.73
OTU15	*Tilletiopsis washingtonensis*	1.40	16.54
OTU4179	*Tilletiopsis washingtonensis*	0.05	13.68

Table 7. Summary of those bacterial OTUs within the 200 highest counts (MR normalised) for which the genetic differences between F1 genotypes were statistically significant.

Taxonomy (> 80% confidence)		% Total reads	Heritability (%)
OTU68	Acetobacteraceae	0.18	17.87
OTU16	Actinobacteria	1.21	8.61
OTU644	Actinobacteria	0.19	26.27
OTU383	Alphaproteobacteria	0.16	23.45
OTU105	*Arthrobacter*	0.10	21.01
OTU27	Aurantimonadaceae	0.67	10.49
OTU38	*Aureimonas*	0.48	21.04
OTU128	Bacteria	0.10	14.91
OTU59	Bacteria	0.22	19.06
OTU813	Bacteria	0.11	17.52
OTU950	Bacteria	0.21	18.97
OTU52	Comamonadaceae	0.26	13.76
OTU135	*Deinococcus*	0.08	25.53
OTU18	*Deinococcus*	1.15	19.88
OTU124	Deltaproteobacteria	0.08	16.79
OTU170	*Dyadobacter*	0.10	17.86
OTU57	*Enterococcus*	0.20	17.64
OTU46	Erythrobacteraceae	0.28	12.13
OTU2048	Firmicutes	0.07	19.93
OTU5	*Kineococcus*	2.74	14.17
OTU53	*Marmoricola*	0.23	14.46
OTU13	Methylobacteriaceae	1.28	12.11
OTU3	Methylobacterium	4.27	9.34
OTU462	Microbacteriaceae	0.09	17.65
OTU504	Microbacteriaceae	0.13	14.23
OTU37	Nakamurellales	0.54	17.83
OTU123	*Nocardioides*	0.09	18.42
OTU42	*Nocardioides*	0.35	10.78
OTU7	*Novosphingobium*	1.86	22.73
OTU2625	Proteobacteria	0.09	21.81
OTU73	Proteobacteria	0.19	21.27
OTU922	Proteobacteria	0.14	25.14
OTU85	*Pseudokineococcus*	0.14	20.44
OTU100	Rhodobacteraceae	0.10	17.66
OTU30	*Rhodococcus*	0.70	8.12
OTU3169	*Rhodospirillales*	0.08	23.89
OTU126	*Roseomonas*	0.08	22.19
OTU47	*Roseomonas*	0.32	21.44
OTU84	*Roseomonas*	0.14	17.45
OTU89	*Roseomonas*	0.13	37.34
OTU20	Sphingomonadaceae	0.93	20.67
OTU114	*Sphingomonas*	0.08	15.86
OTU149	*Sphingomonas*	0.08	19.25
OTU8	*Sphingomonas*	1.76	18.97
OTU49	*Spirosoma*	0.32	27.32
OTU99	*Terracoccus*	0.10	16.25
OTU60	*Williamsia*	0.24	19.93

Canker development

Average canker size over the first three measurements (month 5, 8 and 11) ranged from 4.6 to 23.5 cm with an average of 11.1 cm (Fig. 18A). In month 20, 35 trees had cankers in all shoots (Fig. 18C). The two parents did not differ in any of the three canker measurements. In contrast, F_1 genotypes differed significantly in average canker size ($P < 0.05$), and canopy coverage (Fig. 18B) and percent shoots with canker in month 20 ($P < 0.001$). The broad sense heritability was 13.1%, 23.8% and 31.9% for average canker size, percent canopy coverage and percent shoots with canker 20 months post inoculation, respectively.

Correlation of microbial variables with canker variables. Table 5 shows the summary of results with the detailed correlation results given in Table 8 and 9.

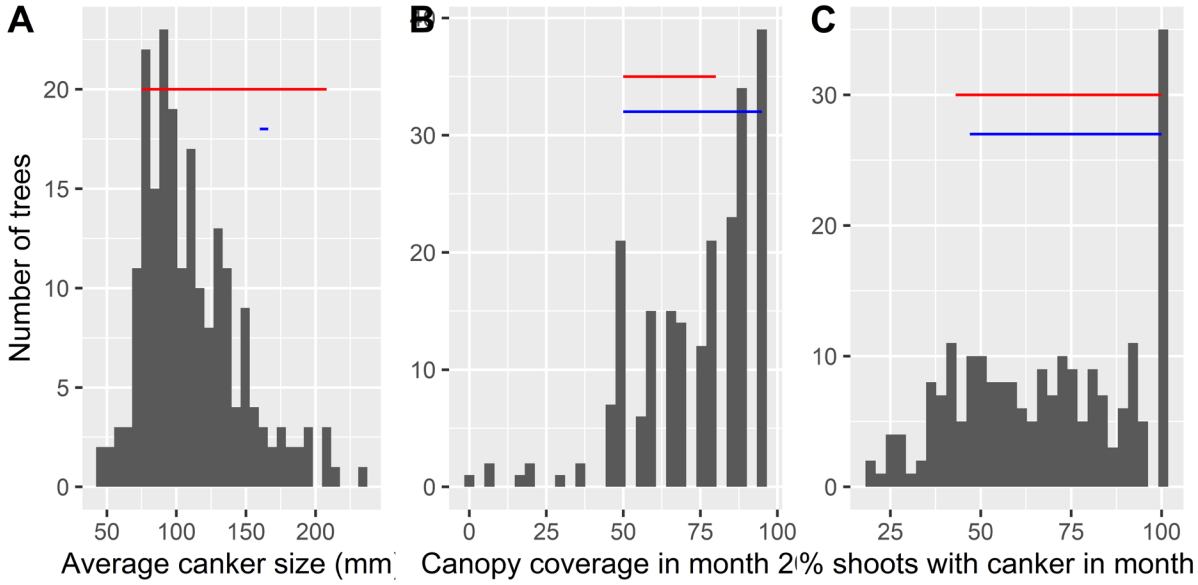

Figure 18. Histogram of three canker variables assessed on individual trees: (A) average canker sized measured five-, eight- and 11-months post inoculation, and (B) % canopy cover and (C) % shoots with cankers assessed 20 months post inoculation. The blue and red lines indicate the range of values for the female and male parents, respectively.

Fungi: Among the top 30 fungal PCs of the qPCR-normalised data, only PC5 scores were correlated with percent canopy coverage in month 20 based on the individual tree data. At the F_1 genotypic level, PC5 scores were correlated with percent canopy coverage in month 20, PC7 with canker size, and PC23 with percent shoots cankered. For the MR-normalised data, of the top 80 PCs, only PC4 scores were correlated with percent canopy coverage based on individual trees; whereas based on F_1 genotypic means, PC3 and P9 were correlated with percent canopy coverage and canker size, respectively.

In total there were 42 significant correlations of canker variables with 16 fungal OTUs; 33 of these 42 cases were for the MR-normalised data. In 26 of the 42 cases, correlation was at the level of F_1 genotypic means. In 35, 6 and 1 cases, it involved percent canopy coverage, average canker size, and percent shoots with cankers, respectively. Among these 42 significant correlations, 25 and 17 were of the Pearson and Spearman type, respectively. Correlation coefficient ranged from –0.56 to 0.51. The magnitude of correlation was greater for genotypic means (from -0.56 to 0.51) than for individual trees (from -0.30 to 0.26) (Table 8). For several OTUs, the observed correlation was inconsistent, namely different signs, between qPCR- and MR-normalised data (e.g., OTU69 in Table 5), or between individual and genotypic levels (e.g., OTU49 in Table 5), or between Pearson and Spearman correlations (e.g., OTU1896, Table 8).

Table 8. Significant Pearson and Spearman (in bracket) correlation coefficients of canker size, canopy cover and % shoots with canker 20 months post inoculation with fungal OTUs with the highest counts based on individual trees or F_1 genotypic means.

OTU ID	Taxonomy (> 80% confidence)	qPCR normalised data			Median of ratio normalised data			
		Individual	Genotypic		Individual	Genotypic		
		Canopy	Canker size	Canopy	Canopy	Canker size	Canopy	% Shoots
OTU143	Dothideomycetes			-0.43				
OTU35	*Entyloma*		(-0.47)			-0.50 (-0.55)		
OTU59	Exobasidiomycetes						-0.45 (-0.42)	
OTU36	Fungi	-0.25			-0.29 (-0.27)			
OTU43	Fungi		(-0.52)			-0.53 (-0.56)		
OTU6	Fungi	-0.28			-0.24 (-0.22)			
OTU69	Fungi			-0.50			0.47	
OTU17	*Neosetophoma*				0.24 (0.23)			
OTU20	Phaeosphaeriaceae			-0.41			-0.40	
OTU247	Phaeosphaeriaceae				-0.25			
OTU49	Phaeosphaeriaceae			-0.41	-0.27 (-0.25)		0.43 (0.45)	
OTU57	*Pseudoophiobolus rosae*			-0.45			-0.48 (-0.47)	
OTU21	*Sporobolomyces roseus*				-0.21 (0.26)		0.51 (0.50)	
OTU1896	*Vishniacozyma*				(-0.28)		-0.41 (0.44)	-0.48
OTU3	*Vishniacozyma*						0.40 (0.42)	
OTU18	*Vishniacozyma carnescens*				0.26 (0.21)			

Table 9. Significant Pearson and Spearman (in bracket) correlation coefficients of canker size, canopy cover and % shoots with canker 20 months post inoculation with most abundant bacterial OTUs based on individual trees or F_1 genotypic means.

		qPCR normalised data	Median of ratio normalised data	
OTU ID	**Taxonomy**	**Genotypic**	**Individual**	**Genotypic**
		Canker size	**Canopy**	**Canopy**
OTU58	Abditibacteriota			(0.43)
OTU93	Actinobacteria			0.47 (0.41)
OTU165	*Actinomycetospora*		0.25	
OTU67	*Allobranchiibius*			(0.43)
OTU107	*Armatimonadetes_gp5*			0.45 (0.43)
OTU108	Bacteria			(0.47)
OTU1181	Bacteria		0.22	
OTU170	*Dyadobacter*			0.50 (0.42)
OTU154	Methylobacteriaceae			(0.50)
OTU70	*Methylobacterium*			(0.42)
OTU106	Propionibacteriales	-0.49 (-0.53)		
OTU85	*Pseudokineococcus*	-0.50 (-0.54)	0.22	
OTU94	*Pseudomonas*		0.23	(0.41)
OTU72	*Sphingomonas*			0.46 (0.51)
OTU87	*Spirosoma*		0.23	
OTU545	*Tepidimonas*		0.23	

Nevertheless, several OTUs had consistent correlation patterns, including a group from *Entyloma sp.* (OTU35) and *Pseudoophiobolus rosae* (OTU57) (Table 8).

Bacteria: Among the top 30 PCs of the qPCR-normalised data, both Pearson and Spearman correlations of PC11 with percent canopy coverage were significant based on individual trees (r = -0.24, -0.32). For the MR-normalised data, PC7 was correlated with percent canopy coverage on individual trees (Pearson: -0.24, and Spearman: -0.26) (Fig. 19), whereas PC29 with average canker size (Pearson: 0.27). At the F_1 genotypic level, both PC6 and PC10 were correlated with average canker size (r = -0.50, 0.47), and PC7 with percent canopy coverage (Pearson: -0.44) (Fig. 19).

In total, there were 24 significant correlations of canker variables with 16 fungal OTUs; 20 of these 24 cases were for the MR-normalised data (Table 9), all with percent canopy cover. All four correlations of qPCR normalised data were with canker size. In 18 of these 24 cases, correlation was at the F_1 genotypic level. Half of the correlation was of the Pearson type. Correlation coefficient ranged from –0.54 to 0.51, and was greater for genotypic means (from -0.54 to 0.51) than for individual trees (from 0.22 to 0.25) (Table 9). For the qPCR-normalised data, only two OTUs (*Pseudokineococcus* sp. and Propionibacteriales) were significantly correlated with average canker size at the genotypic level (Table 6). For the MR-normalised data, several OTUs had multiple high and consistent correlations with canopy size, including Actinobacteria (OTU93), *Armatimonadetes_gp5* (OTU107), *Sphingomonas* (OTU72) and *Dyadobacter* (OTU170) (Table 6).

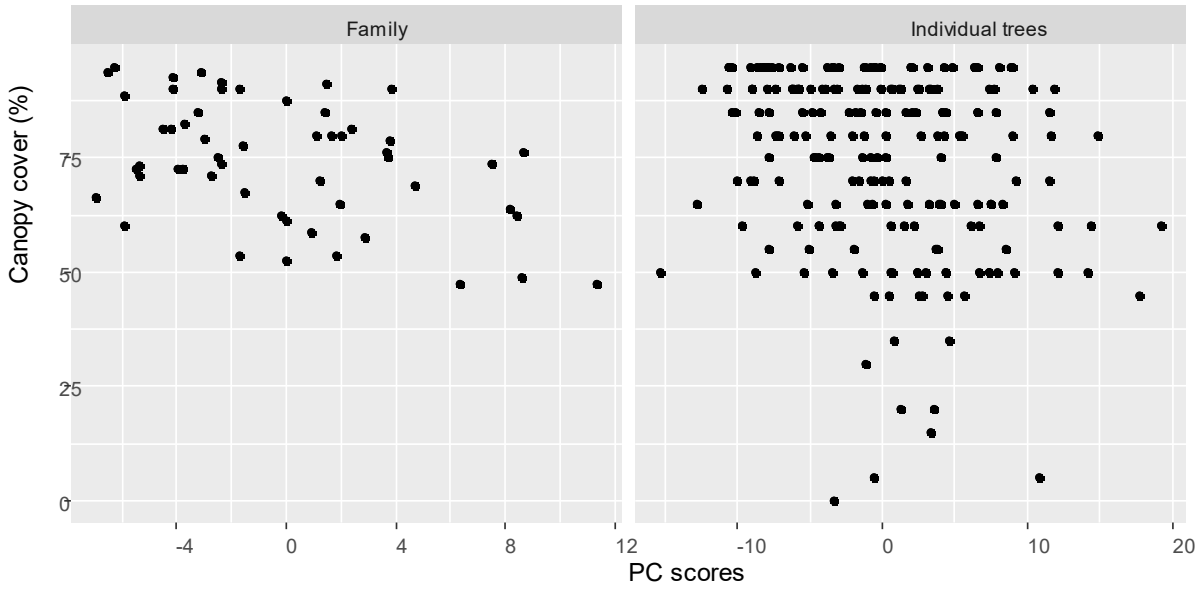

Figure 19. PC7 scores of the median of ratio normalised bacterial OTU data plotted against canopy coverage 20 months post inoculation.

QTL analysis

Bacterial endophytes. There were significant QTL associated with 21 of the 47 OTUs representing bacterial taxonomic groups (Table 10 and 11). The significant QTL hits were distributed over 15 linkage groups (LGs), however the QTL position on seven LGs (LG1, 4, 5, 10, 12, 14, 15) were associated with multiple bacterial groups. The seven PCs representing the taxonomic composition of bacterial OTUs were associated with QTL hits on five LG. Four of these were present at positions supported by multiple phenotypes. The CIM analysis resulted

in 37 significant hits across all phenotypes and LGs, whereas 17 significant marker-trait associations were identified with the K-W test (Table 10 and 11). The QTL positions on LG 4, 5, 10, 12, 14 and 15 were supported by both CIM and K-W test.

A QTL on LG4 was associated with the abundance of five bacterial OTUs and PC17. The taxonomic assignment of the associated OTUs were Proteobacteria (OTU922), Microbacteriaceae (OTU462), *Kineococcus* (OTU5), *Nocardioides* (OTU42) and *Deinococcus* (OTU135). The QTL position on LG4 of OTU135 was supported in both the CIM analysis and by K-W test. The QTL effects for LG4, as estimated from the CIM analysis, ranged between 4.5-32% depending on phenotype with a mean effect of 23%.

A QTL region on LG5 was associated with three OTUs and one PC2. The OTUs with significant QTL on LG5 were from Rhodobacteraceae (OTU100), Deltaproteobacteria (OTU124) and Rhodospirillales (OTU3169). The QTL position of OTU124 was supported by both CIM and by the K-W test. The estimated effect of the QTL on LG5 ranged between 24-31%.

A genetic region on the top of LG10 was associated with five OTU, which were from *Sphingomonas* (OTU8), *Aureimonas* (OTU38) and *Deinococcus* (OTU135). OTU59 and OTU128 could not be taxonomically classified. The QTL position was supported by K-W test for four OTU phenotypes (Fig. 20**).** A second QTL region, positioned at the bottom of LG10, was associated with the abundance of two bacterial OTU: *Deinococcus* (OTU18) and Microbacteriaceae (OTU462). The effect of the QTL on LG10 had a range of 6-50%, with a mean effect of 24%.

Significant QTL hits were identified on LG12 for three OTU and PC7. The QTL location was supported by both CIM analysis and K-W test, however only OTU84 had a significant QTL on the LG in both types of analysis. The QTL effects estimated for OTU52 and OTU84 from the CIM analysis accounted for 27 and 34% of the total phenotypic variation, respectively. The OTUs with a significant QTL on LG12 were assigned to *Aureimonas (*OTU38), Comamonadaceae (OTU52) and *Roseomonas* (OTU84).

OTU60 (*Williamsia*), OTU38 (*Aureimonas*) and PC7 were associated with QTL positioned on LG14. The QTL on LG12 was identified in both CIM and K-W analysis for the phenotypes OTU38 and PC7. The estimated QTL effects for this LG accounted for between 22-36% of the phenotypic variation.

A QTL position on LG15 was identified in abundance phenotypes of four OTUs and PC2. The taxonomic assignments of the OTUs were *Deinococcus* (OTU18), Sphingomonadaceae (OTU20), *Marmoricola* (OTU53) and Rhodospirillales (OTU3169) . The QTL position of OTU53 was confirmed by both CIM and K-W testing. The QTL on LG15 accounted for between 25-37% of the variation in bacterial phenotypes.

Fungal endophytes. There were significant QTL associated with 13 of the 22 OTUs representing fungal taxonomic groups (table 1 and 2). Four of the selected fungal PCs were associated with significant QTL. QTL associated with fungal abundance phenotypes were identified on LG1, 3, 4, 5, 6, 8, 10, 12, 14, 15, 16 and 17. The majority of QTL hits were identified with the CIM analysis, whereas only one significant marker-trait association was identified with the K-W test at LG12 (OTU12 on LG12).

A significant QTL associated with OTU26 (taxonomically assigned to *Subplenodomus iridicola*) mwas identified on LG1. The same QTL position was also associated with two bacterial OTUs (Fig. 2). The QTL on LG1 explained 19% of the variation in abundance of OTU26.

Two QTL were identified on LG3 from OTU15 (*Tilletiopsis washingtonensis*) and OTU35 (*Entyloma*). The estimated QTL effect accounted for 30 and 44% for OTU15 and OTU35, respectively. Three fungal OTUs had significant QTL hits on LG15. The QTL were estimated to account for 3-40% of the variation depending on phenotype. The fungal OTUs associated with this LG were OTU19 (no taxonomic assignment), OTU62 (Phaeosphaeriaceae) and OTU143 (Dothideomycetes).

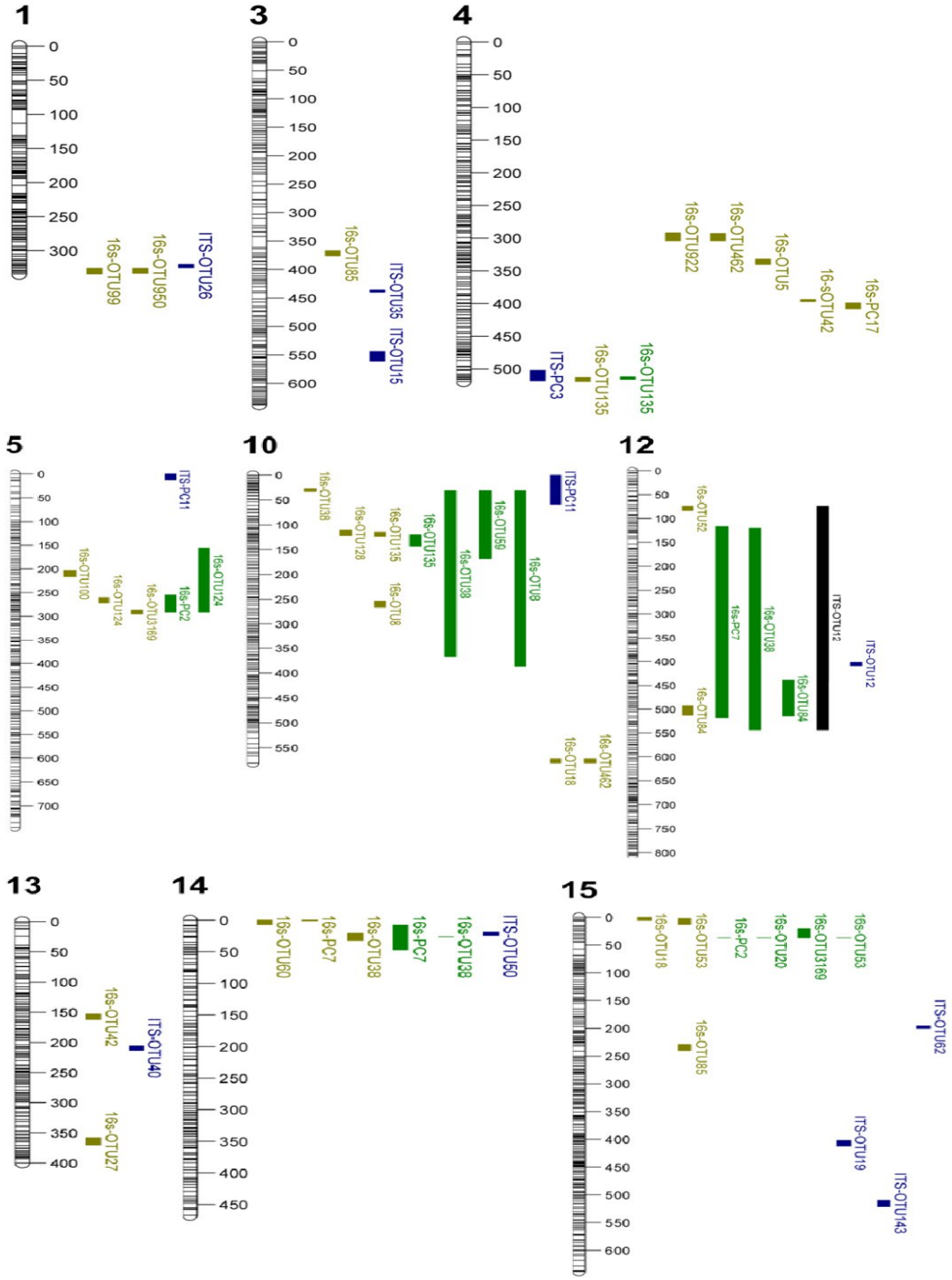

Figure 20. Linkage groups with QTL positions from the composite interval mapping (CIM) and Kruskal-Wallis (K-W) test. Phenotype colour indicates which analysis the QTL is associated with, **yellow:** bacterial CIM analysis, **blue:** fungal CIM analysis, **green:** bacterial K-W test, **black:** fungal K-W test.

Table 10. Quantitative trait loci (QTL) composite interval mapping for abundance data of bacterial (16s) and fungal (ITS) endophytes

OTUs	Taxonomy	LG	Start position	Finish position	Position of maximum	Maximum LOD-	R2
Bacteria							
OTU5	Kineococcus	4	332	340	340	9	4
OTU8	Sphingomonas	10	253	266	262	16	50
OTU18	Deinococcus	10	573	581	581	10	6
OTU18	Deinococcus	15	0	5	5	10	25
OTU27	Aurantimonadaceae	13	358	370	365	12	27
OTU38	Aureimonas	10	28	33	32	10	26
OTU38	Aureimonas	14	21	32	26	11	22
OTU42	Nocardioides	4	394	397	399	9	26
OTU42	Nocardioides	6	179	186	179	11	23
OTU42	Nocardioides	13	153	162	156	10	16
OTU52	Comamonadaceae	2	102	114	110	14	36
OTU52	Comamonadaceae	7	129	132	74	10	12
OTU52	Comamonadaceae	12	74	84	81	10	27
OTU53	Marmoricola	15	2	13	7	11	37
OTU60	Williamsia	14	0	7	0	13	35
OTU84	Roseomonas	12	493	512	499	13	34
OTU85	Pseudokineococcus	3	367	376	374	11	13
OTU85	Pseudokineococcus	6	421	432	429	11	26
OTU85	Pseudokineococcus	15	229	240	232	11	27
OTU99	Terracoccus	1	326	334	332	14	36
OTU99	Terracoccus	16	86	100	93	12	20
OTU100	Rhodobacteraceae	5	203	216	213	12	29
OTU124	Deltaproteobacteria	5	261	271	267	14	31
OTU124	Deltaproteobacteria	8	505	508	508	10	6
OTU128	Bacteria	10	111	122	120	13	36
OTU135	Deinococcus	4	513	519	516	11	29
OTU135	Deinococcus	10	115	124	120	11	22
OTU462	Microbacteriaceae	4	293	304	299	12	27
OTU462	Microbacteriaceae	10	573	581	579	11	6
OTU644	Actinobacteria	16	439	442	442	11	1
OTU922	Proteobacteria	4	292	304	304	15	32
OTU950	Bacteria	1	326	334	332	14	34
OTU950	Bacteria	17	57	63	62	12	19
OTU3169	Rhodospirillales	5	286	295	290	9	24
PC16		8	63	75	71	13	35
PC17		4	399	408	404	10	23
PC7		14	0	1	0	10	36
Fungi							
OTU12	Fungi	12	402	409	405	10	42
OTU15	Tilletiopsis	3	544	561	549	11	30
OTU19	Fungi	6	531	546	536	13	27
OTU19	Fungi	15	402	412	402	12	15
OTU26	Subplenodomus	1	321	324	322	13	19
OTU35	Entyloma	3	436	440	438	11	44
OTU40	Fungi	13	206	213	210	10	23
OTU50	Entyloma	14	19	25	25	12	35
OTU62	Phaeosphaeriaceae	15	197	200	199	10	3
OTU71	Taphrina	8	141	152	141	11	17
OTU71	Taphrina	17	312	315	314	11	34

OTU143	Dothideomycetes	15	511	521	511	11	40
OTU310	Taphrina	16	15	22	20	12	25
PC3		4	502	518	508	11	22
PC3		6	442	445	444	12	30
PC11		5	0	13	4	10	7
PC11		10	0	59	9	17	20

Table 11. Quantitative trait loci (QTL) identified from Kruskal-Wallist test for abundance data of bacterial (16s) and fungal (ITS) endophytes

OTUs	Taxonomy	Chromosome	Start position	Finish position
Bacteria				
OTU124	Deltaproteobacteria	5	156	291
OTU135	Deinococcus	4	512	516
OTU135	Deinococcus	10	120	143
OTU20	Sphingomonadaceae	15	37	37
OTU3169	Rhodospirillales	15	20	37
OTU38	Aureimonas	6	3	57
OTU38	Aureimonas	10	32	366
OTU38	Aureimonas	12	120	544
OTU38	Aureimonas	14	26	26
OTU53	Marmoricola	15	37	37
OTU59	Bacteria	10	32	169
OTU8	Sphingomonas	10	32	386
OTU84	Roseomonas	12	440	514
PC2		5	255	291
PC2		15	37	37
PC7		12	116	518
PC7		14	8	47
Fungi				
OTU12	Fungi	12	74	544

Association between QTL phenotypes and severity of European canker

The QTL region positioned in the top of LG10 was only identified in bacterial phenotypes which had a statistically significant correlation to the canker disease phenotypes. Similarly, with one exception the QTL region on LG12 and LG14 was supported by bacterial phenotypes significantly correlated to canker disease spread.

In contrast, the QTL region on LG 15 was only associated with bacterial phenotypes for which there was no significant correlation to canker development.

Discussion

The present study showed that specific components of apple endophytes as well as some individual fungal/bacterial groups in tissues around leaf scars in autumn are partially controlled genetically by host genotypes in an F_1 segregating population. Furthermore, there are specific microbial groups that are significantly correlated with canker-related plant traits. However, such corelations are often inconsistent for a given microbial group in terms of whether it is based on individual trees or genotypes or whether it is based on the Pearson or Spearman correlation.

Sequence data were normalised by two methods, qPCR data of generic 16S/ITS primers and median of ratio. In general, genotypic differences were less profound for the qPCR-normalised data than for the MR-normalised data. Moreover, there were far more cases of significant correlations of endophytes with canker-related variables for the MR-normalised data than for the qPCR-normalised data. In theory, qPCR-normalised data should provide more informative data than the MR-normalised data. Although there were often a couple of magnitude differences in qPCR values between trees, F_1 genotypes did not differ significantly in the qPCR values. We also observed a large variability in PCR efficiencies between samples (data not shown) and hence repeated qPCR was necessary for many samples. Our experience suggested that conducting qPCR analysis of fungal and bacterial DNA extracted from woody tissues with generic 16S and ITS primers is more problematic than other types of samples, such as rhizosphere soils. Nevertheless, qPCR is valuable for pathogen diagnosis, such as confirming latent infection of *N. ditissima* in leaf scar tissues (Olivieri et al. 2021a). Further studies are needed to improve quantification of overall microbial biomass in plant (particularly woody) tissues. As an alternative to the qPCR method, each sample may be spiked with a known amount of a synthetic DNA fragment to estimate absolute abundance (Tkacz et al. 2018).

The present study showed that the overall plant genetic component was similar for bacterial and fungal endophytes. For many microbial groups, albeit still a small proportion of the entire microbiome, the variability among F_1 offspring is greater than random variability, indicating existence of host genetic control. There is a significant host genetic component in several PCs, which are jointly determined by many microbial groups. There have been many reports demonstrating that plant genotypes differ in their phytobiomes associated with rhizosphere, endosphere and phyllosphere (Liu et al. 2020; Olivieri et al. 2021a; Peiffer et al. 2013; Wagner et al. 2016, 2020; Wei et al. 2019). We found significant host genetic components affecting specific endophyte components of apple leaf scars, represented by PCs, often with a moderate level of genetic variance relative to the environmental variance. However, these microbial components are only a minor proportion of the entire endophytes in the leaf scar tissues, which can be seen from the small number of PCs or OTUs with significant host genetic components. Similarly, maize inbred lines differed significantly in their rhizosphere microbiome, but the heritability level was low and the genetic relationship among the inbred lines was not correlated with the diversity characteristics of the rhizosphere microbiome (Peiffer et al. 2013). The present research is based on a F_1 population from a specific cross, hence representing genetic variance between the two specific parental genotypes only. The overall low heritability of phytobiome may be due to strong environmental effects (Clouse and Wagner 2021), including spatial and temporal variation. For instance, we recently demonstrated that although cultivars differed in their endophytes in leaf scars, orchard locations accounted for much greater variability in endophyte composition (Olivieri et al. 2021a). Plants are only able to recruit those microbial organisms present at a specific site with the recruitment outcome likely depending on frequencies of available taxa. This potential large difference in aerosol microbiome over distance may also partially explain the block effects on endophytes observed in the present study. We mapped a number of QTLs in many linkage groups for many bacterial and fungal OTUs as well as a few PCs.

Only a few specific microbial groups were significantly correlated with canker size and/or percent canopy cover. Interestingly, all significant correlations with bacterial groups indicated that higher relative abundance of these groups is associated with less canker development, mostly with better canopy coverage and in a few instances negatively with canker size. In contrast, there is no such a consistency in the correlation of fungal groups with canker development: both positive and negative corelation with canker development were observed.

Moreover, the inconsistency exists even for the same fungal group across different canker variables or parametric/rank correlations, or at the tree/genotypic levels. These differences were further confirmed with QTL mapping results – only a few bacteria-related QTLs are close to or co-located with QTLs for canker phenotypes. Reasons for these observed differences are not obvious. As discussed above, recruitment of endophytes may be considerably influenced by local aerosol microbiome, not necessarily affected by tree genotypes. Thus, individual trees may differ greatly in relative abundance for some endophytes not because of genetics but variabilities in aerosol microbiome. If these microbes have direct or indirect effects on *N. ditissima*, we may expect inconsistent correlations at the individual and genotypic levels. This may be further complicated by possible differences in the stability/persistence of individual endophytes in the leaf scar tissues at a given endophyte background. For example, fungal endophyte composition might be more easily influenced by differences in aerosol microbiome and/or external conditions than bacteria. Communities of endophytic fungi assembling in several plant species depend significantly on proximity to inoculum source as well as the identity of the plant species (Ricks and Koide 2019). It is not known what fraction of the endophytic bacterial microbiome is dispersed via the atmosphere or originated from the atmosphere (Frank et al. 2017). Further research is needed to understand relative importance of sources for plant endophytes and the effects of biotic and abiotic factors on the stability of endophyte microbiome over time before we can amend phytobiome with a reasonable level of predictability to improve crop performance.

Most of those bacterial groups with significant correlations with canker development cannot be classified into genus. *Dyadobacter* isolates are one of the major cohorts of bacteria in plant phyllosphere (Delmotte et al. 2009; Reisberg et al. 2012) but it is currently unknown what role *Dyadobacter* species plays in these communities. The relative abundance of a *Pseudomonas* OTU (OTU94) and *Sphingomonas* OTU (OTU72)is positively correlated with canopy coverage in the present study. A *Pseudomonas* strain isolated form apple endophytes was shown to be antagonistic towards *N. ditissima in vitro* tests (Liu et al. 2020). A seed-endophytic strain *S. melonis* ZJ26 confers rice with disease resistance against a bacterial pathogen and is vertically transmitted among plant generations via their seeds (Matsumoto et al. 2021). A couple of *Sphingomonas* groups were, however, more abundant in canker susceptible cultivars than in resistant cultivars (Olivieri et al. 2021a), but that particular study did not consider the actual canker severity. *Methylobacterium* normally resides in soil and water but has also been identified as a contaminant of DNA extraction kit reagents, which may lead to its erroneous appearance in microbiota or metagenomic datasets (Salter et al. 2014). Given these bacterial groups are mostly correlated with canopy cover but not with canker size, we may speculate that they may not be effective as direct competitors of *N. ditissima* but rather improve plant tolerance against consequences on plant development due to canker.

Only three of the 16 fungal groups with significant correlations with canker development can be classified into species: *Vishniacozyma carnescens* (syn= *Cryptococcus carnescens*), *Pseudoophiobolus rosae* and *Sporobolomyces roseus*. Of all the 16 groups, only *V. carnescens* is associated with reduced canker damages (but only at the individual tree level); unfortunately, there is no published information on its ecology in relation to plants. An *Entyloma* sp. (OTU35) is positively correlated with canker tolerance; but *Entyloma* is a genus of plant-pathogenic smut fungi. These fungal groups have so far not been associated with direct antagonistic effect against *N. ditissima* (Liu et al. 2020) or associated with cultivars with differential susceptibility to *N. ditissima* (Olivieri et al. 2021a). A couple of groups appear to facilitate canker development, including *P. rosae,*) and Exobasidiomycetes (OTU59). Exobasidiomycetes are a class of fungi sometimes associated with galls of plant tissues.

Further metagenome sequencing and isolation combined with challenging assays against *N. ditissima* are needed to make further progress in this area.

In summary, some components of apple endophyte microbiome as well as individual microbial groups around leaf scar tissues are partially controlled genetically by apple genotypes. Several microbial groups had significant correlation with canker development. Bacterial groups appear to be positively associated with canker tolerance. On the other hand, a few fungal groups may facilitate canker development whereas a few others may compete with the canker pathogen. The present results may be used to inform targeted approaches to further research in biocontrol of *N. ditissima* with specific microbes and breeding for resistance.

Effects of water stress, AMF and PGPR amendment on canker and 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

Treatments and experimental design

We conducted a potted-tree experiment to investigate the effect of soil amendment and water stress on apple rhizosphere and rhizoplane (including endophytes) microbiome. There were four amendments (AMF, PGPR, PGPR+AMF and Control) and two irrigation regimes (well-watered (WW) and 65% RDI (regulated deficit irrigation), giving a total of eight treatments. Trees were potted in April 2018, inoculated with *N. ditissima* in October 2018, assessed for tree and canker development in 2019, and sampled for determination of root-associated microbiome via amplicon sequencing. Trees were not re-potted during the 2018-19 winter. A commercial AMF product was used, containing six species: *Diversispora* sp., *Funeliformis mosseae*, *F. geosporus*, *Rhisophagus irregularis*, *Claroideglomus claroideum* and *Glomus microagregatum*. Similarly a mixed PGPR commercial product was used, containing *Bacillus amyloliquefaciens*, *Pseudomonas putida*, *Pseudomonas flourescens*, and *Azospirillum brassilense*.

Trees were grown in a polytunnel at NIAB EMR for four weeks prior to the irrigation treatment being applied; this was necessary to have uniform tree establishment. A total of 120 trees were arranged in a randomised block design with 8 blocks. Within each block, a split plot design was used; there were two plots, one of them randomly assigned to a water treatment; within each plot, there were four subplots, each of them randomly assigned to one of amendment treatments. For six of the eight blocks, there were two replicate trees within each subplot, hence a total of 16 trees. In the remaining two blocks, there were only 12 trees; for one block, there were two replicate trees for the WW treatment and one for the RDI treatment; the opposite was true for the other block.

Applying treatments

Trees of cv. Gala on M9 feathered maidens were potted into 10 L pots with Halstone Topsoil (Travis Perkins) on 24th April 2018; topsoil was used as it contains natural-soil-occurring microbes. Pots were placed on saucers to prevent cross contamination of AMF/PGPR and for easy assessment of whether excessive irrigation was applied. At planting, appropriate soil amendment (one of the four amendment treatments: AMF, PGPR, PGPR+AMF and Control) was applied.

To ensure the WW trees were watered to the capacity, tree water use was estimated weekly:

1. Two WW trees from each of the four amendments were chosen throughout the polytunnel and weighed on two consecutive days.
2. Tree and pot weights were recorded directly after an irrigation event on day 1, and the dripper removed from the pot.
3. Exactly 24 hours later, the tree was re-weighed, and water loss calculated to calculate water usage over a 24-hour period.
4. The estimated water use was then used to schedule irrigation the following week.

In 2018, RDI trees received 65% of water given to WW trees. In 2019, deficit irrigation was started in mid-May 2019, about six weeks after budbreak. Initially, RDI trees received 50% of water given to WW trees for 4 weeks, then and increased to 65% mid-Aug increased and again to 75% as RDI-treated trees were struggling too much in hot weather. Soil matrix potential was also measured using a soil moisture probe weekly.

Inoculation of leaf scars with N. ditissima

Inoculation of leaf scars followed a previously published protocol (Xu et al., 2021). Briefly, two shoots of each tree were selected to give approximately 20 leaf scars per tree. The top 2 or 3 leaves on each selected shoot were left and the rest of the leaves were manually removed by hands to create fresh leave leaf scars, with the position of the bottom and top leaf scars marked with a paint pen. Each selected shoot were sprayed to run off with a suspension of N. ditissima suspension, containing 1 x 10^4 macrospores per ml (germination rate of ca. 95% at the post-inoculation in vitro test). To increase humidity in the leaf scar tissue for inducing fungal infection, a large clear plastic bag was sprayed with some water, placed over the inoculated shoot and attached with wire around wetted cotton wool inside the opening of the bag. The bags were removed after 24 hours. Inoculation was conducted on 18th October 2018.

Tree growth and canker assessment, and root sampling

Tree girth (5 cm above the graft union) was measured on 10th June and 14th October 2019; similarly tree height was measured on the same dates. The measurement on the first date mostly reflected the tree growth in the first year (2018), whereas the second measurement reflected the accumulated tree growth over the two seasons. Two canker assessments were made in April and October 2019. Fine roots were sampled from 32 trees: one tree from each of the eight treatments in four blocks

DNA extraction, amplicon sequencing and sequence processing

DNA extraction followed a previously published protocol (Deakin et al., 2019), and also described in previous sections. DNA quality and quantity were checked spectrophotometrically (Nanodrop™ 1000, ThermoFisher Scientific, USA. Samples were re-extracted for DNA if their DNA concentration did not exceed 10 ng per ml. PCR amplification, library prep and metabarcoding sequencing were all carried out by Novogene UK (Cambridge, UK). For amplicon-sequencing, the target regions were ITS1-1F (ITS1-1F-F: 5'-CTTGGTCATTTAGAGGAAGTAA-3', ITS1-1F-R: 5'-GCTGCGTTCTTCATCGATGC-'3) and 16S V5-V7 (799F: 5'-AACMGGATTAGATACCCKG-3', 1193R: 5'-ACGTCATCCCCACCTTCC-3'). Samples were sequenced on the Illumina NovaSeq platform in the 250 nt paired end mode. Sequence processing was performed with the usearch v11 (https://www.drive5.com/usearch/) methods/pipelines described previously (Papp-Rupar et al., 2022).

Statistical analysis

A split-plot analysis of variance (ANOVA) was applied to the tree girth and height data to assess the effects of experimental factors. In addition to the raw girth and height data, the relative expansion rate in girth and height was also calculated and subjected to ANOVA. For canker

data, the canker incidence data were first logit-transformed and then subjected to a split-plot ANOVA. Both tree and canker data were pooled over the two replicate trees before statistical analysis.

Three different sets of OTU counts data were analysed: (1) all rhizosphere and rhizoplane data, (2) rhizosphere data only, and (3) rhizoplane only. For comparing between rhizosphere and rhizoplane data, a paired randomised block design was used – thus the comparison was based on the basis of individual trees. To assess the effect of water stress, AMF and PGPR effects, a split plot design was used where water stress was assessed at the plot level whereas AMF and PGPR at the subplot level. For all data sets, the interaction of sample type (rhizosphere or rhizoplane) with other factors was also assessed at the appropriate experimental unit. Given the nature of this split plot design, assessing water stress effect was not as powerful as assessing AMF and PGPR effects. In all analyses, a block factor was included as well. These model structures were used in all statistical analysis of alpha and beta diversity indices, Principal Component (PC) scores, and differential abundance analysis of individual OTUs. All analyses, including *alpha and beta diversity, NDMS, PC scores* and *Differential OTU abundance*, were already described in previous sections. Differentia; abundance analysis was used to compare relative abundance of individual OTUs between: (1) rhizosphere and rhizoplane [only for the all data set], (2) RDI and WW, (3) with and without AMF amendment, and (4) with and without PGPR amendment two rootstocks. All statistical analyses were carried out with R 4.1.0 (R Core Team, 2019).

Results

Tree growth data

The effect of water stress on plant height was not significant for tree height measured in June 2019, but close to statistical significance (P = 0.068) for the height measured in October 2019 (Fig. 21A) as well as the interaction between AMF and PGPR (P = 0.085). Average tree height in October 2019 was 163.2 and 174.3 cm for the water stress and full water treatment, respectively. Interaction between AMF and PGPR was due to the fact that applying for either AMF (162.6 cm) or PGPR (168.8 cm) led to a reduced height, compared to both AMF and PGPR amendment (172.0 cm) or the unamended (171.4 cm).

Irrigation significantly reduced tree girth measured in June 2019 (P < 0.01) and in October 2019 (P < 0.001) (Fig. 21B). Average tree girth in June 2019 was 10.8 mm and 11.9 mm for the RDI and WW treatments, respectively; the corresponding values for October 2019 were 11.8 mm and 13.1 mm. Once the girth in June 2019 was included in the ANOVA as a covariate, the effects of water stress was only close to statistical significance (P = 0.082). Neither AMF or PGPR amendment affected tree girth.

The incidence of canker on those inoculated leaf scars was low, about 4.2% overall (Fig. 21C). Only the interaction between water stress and AMF may have influenced canker development (P = 0.088). Canker incidence was about 5.8% for AMF amendment under the drought treatment whereas it ranged from 3.4% to 4.0% for the other three treatment combinations.

General sequencing results

Bacteria: Overall, there were 5723 bacterial OTUs across both rhizosphere and root communities, of which the top 3905 accounted for 99% of sequence reads. The top 3003 and 2943 OTUs accounted for 99% of sequence reads for the rhizosphere and root samples, respectively. Sequencing depth is sufficient for all samples as revealed by rarefaction curves.

Of the 3905 bacterial OTUs, 3250, 2771, 2061, 1351 and 788 can be assigned with confidence greater than 80% to the rank of phylum, class, order, family and genus, respectively. More

than 42% of the sequences were from Proteobacteria, compared to nearly 18% for Actinobacteria and 13% for Bacteroidetes. The number of sequences aligned to the top 3905 OTUs ranged from 54089 to 101071 per sample; the number of reads per OTU ranged from 53 to 198026. Only four of the top 10 OTUs could be assigned to the genus rank: two from *Streptomyces* (accounting for ca. 4.6% of total reads), one *Agrobacterium* (1.5%), and one *Bacillus* (0.9%). The OTU with the highest relative abundance is of *Streptomyces*, accounting for nearly 3.8% of the total reads.

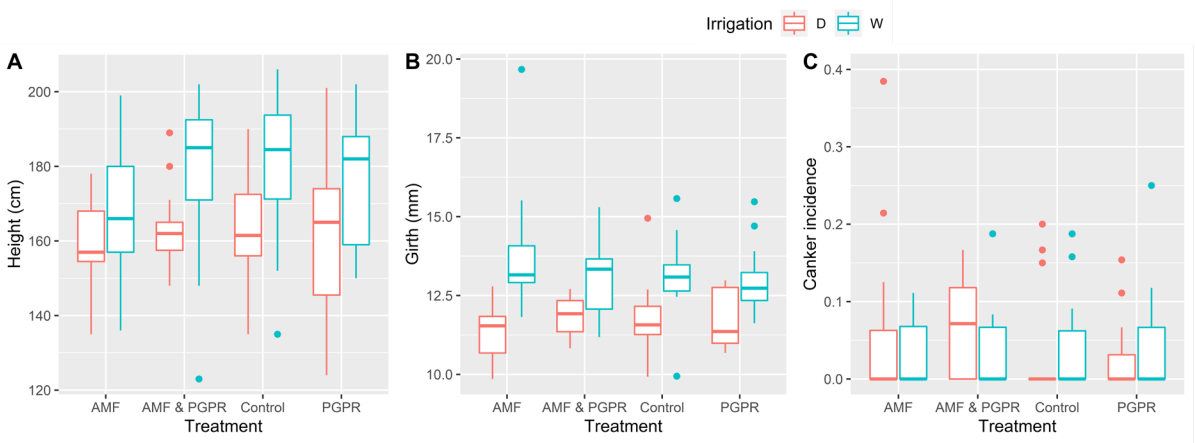

Figure 21. Boxplots of tree height (A) and girth (B), and incidence of cankers on the inoculated leaf scars (C) for each combination of water stress, AMF and PGPR treatment.

Fungi: There were 2154 fungal OTUs with the top 780 OTUs accounting for 99% of sequence reads in combined rhizosphere and rhizoplane. The top 501 and 413 OTUs accounted for 99% of the total reads for rhizosphere and rhizoplane communities, respectively. The sequencing depth is sufficient for all samples. Of the 780 fungal OTUs, 355, 267, 238, 180, 119 and 45 can be assigned with confidence greater than 80% to the rank of phylum, class, order, family, genus, and species, respectively. About 46.4% of reads cannot be identified to the phylum rank with confidence; 39.7% of sequences were from Ascomycota, compared to nearly 10% for Basidiomycota (Fig. 2B). The number of sequences aligned to those 780 OTUs ranged from 24221 to 136218 per sample (median of 109857); the number of reads per OTU ranged from 176 to 827581 (median of 888). The top 10 OTUs accounted for about 41.4% of the total reads; however, only four of the top ten OTUs could be identified to the rank below the phylum: *Penicillium* (1st), Hypocreales (5th), *Coprinellus sclerocystidiosus* (6th) and Sordariomycetes (9th).

Alpha diversity

Bacteria: Alpha diversity indices varied largely among samples, particularly for the rhizoplane (Fig. 22). For the three indices, the differences between root and rhizosphere samples were highly significant ($P < 0.001$). Rhizosphere samples were estimated to have 3352 OTUs, compared to 3136 for the root samples. Water stress only significantly reduced the Simpson indices ($P < 0.05$). Neither AMF nor PGPR initial amendment affected the overall bacterial within-sample diversities. None of treatment factors affected the alpha diversity when separate analysis was carried out for rhizosphere and rhizosphere data.

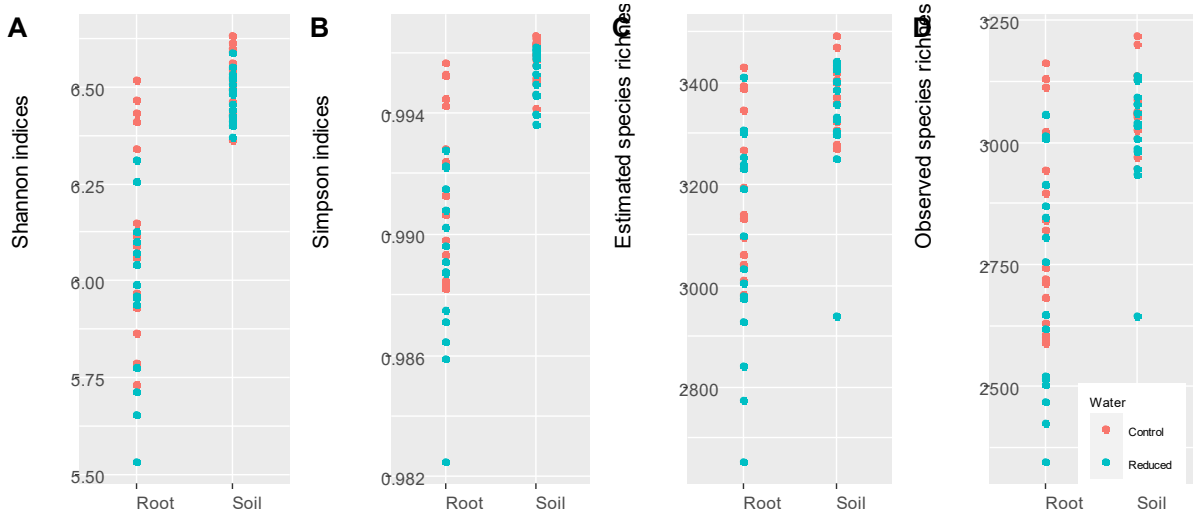

Figure 22. Bacterial alpha diversity indices (Shannon, Simpson and Chao1) together with the observed number of OTUs for each sample plotted against the sample type and water stress.

Fungi: The three within-sample diversity indices varied largely among samples (Fig. 23). The differences between rhizoplane and rhizosphere samples were significant for Shannon, Chao1 indices ($P < 0.001$), and Simpson ($P < 0.05$). Rhizosphere was estimated to have about 90 more OTUs than rhizoplane. Water stress did not significantly affect the three indices. Only species richness (chao1) was affected ($P < 0.05$) by PGPR, and the interactions between water stress and AMF and between PGPR, water stress and sample type. PGPR amendment led to an overall reduction in the number of OTUs (536 vs. 562). However, this PGPR effect was dependent on the sample type and water stress: number of fungal OTUs only reduced in the rhizoplane with PGPR amendment under water stress (Fig. 24). Number of fungal OTUs was greater for AMF treatment than the unamended under the reduced water (562 vs 539); but the opposite was true under the control condition (532 vs 563).

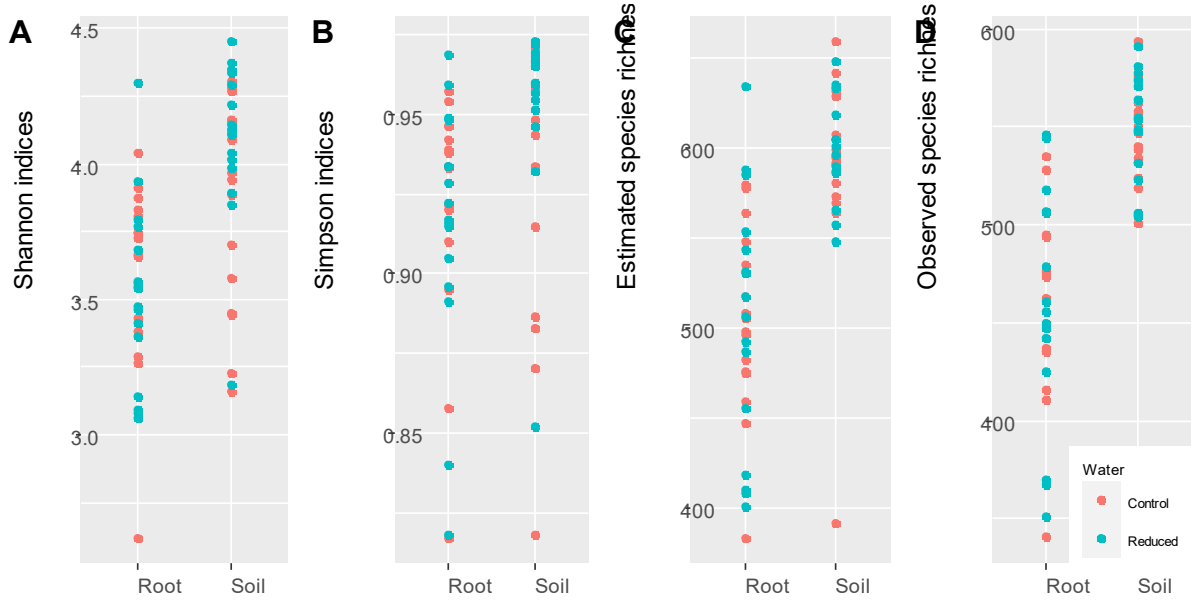

Figure 23. Fungal alpha diversity indices (Shannon, Simpson and Chao1) together with the number of observed OTUs for each sample plotted against the sample type and water stress.

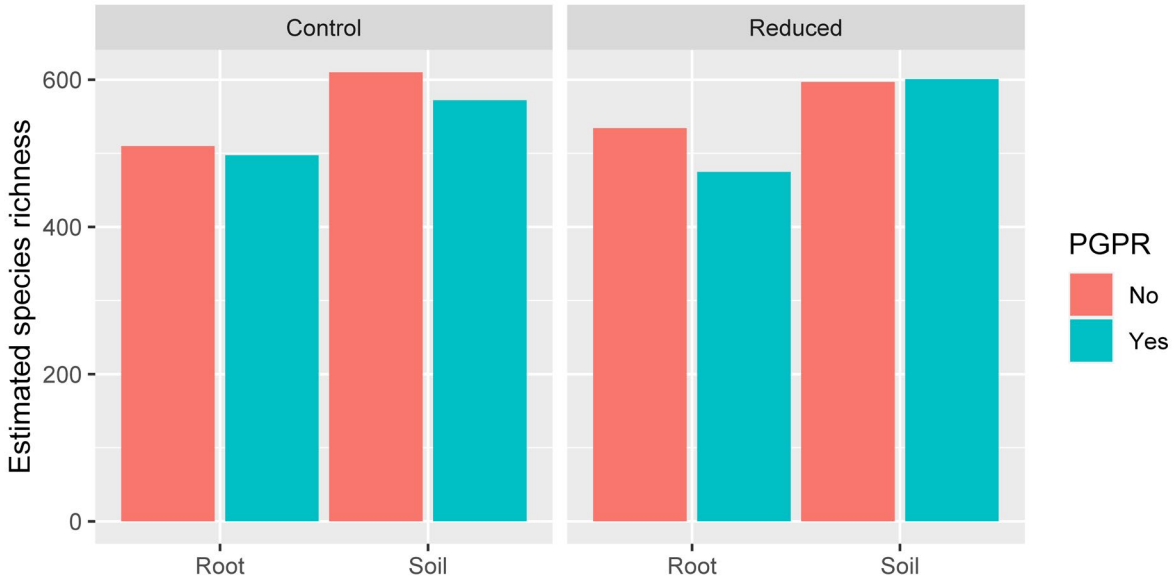

Figure 24. Estimated number of fungal OTUs for each combination of sample type (rhizoplane & rhizosphere), water stress and PGPR amendment.

Separate analysis of rhizosphere data indicated that none of treatment factors affected the three indices. For the rhizoplane communities, only the species richness was affected ($P < 0.05$) by PGPR amendment, and the interaction between water stress and AMF amendment. PGPR amendment led to reduced fungal richness: 486 vs 522. Under water stress, AMF amendment led to more fungal OTUs (529 vs 480); the opposite was true under the control condition (484 vs 523).

Beta diversity and Principal Component Analysis

Bacteria: The beta diversity indices (Bray-Curtis) were mostly affected only by sample type ($P < 0.001$) (Fig. 25). In the separate analysis of rhizosphere data, water stress and the interaction between AMF and PGPR was significant ($P < 0.05$). For rhizoplane, none of the treatment factors significantly affected the beta diversity indices.

The first six PCs accounted for 23.0%, 8.9%, 5.7%, 4.7%, 3.1% and 2.7% of the total variability in bacterial communities, respectively. The first four PCs were only affected by sample type; water stress only influenced PC6. AMF amendment affected PC5 and PC6 ($P < 0.05$), its interaction with PGPR was also significant ($P < 0.01$) for PC6. There were also significant interactions of sample type with AMF (PC5, $P <0.05$), and with water stress and AMF (PC4, $P < 0.05$). When principal analysis was applied to the roots and soil samples separately, most differences between samples were not due to experimental treatments. For rhizosphere samples, water-stress affected ($P < 0.05$) PC5; AMF affected ($P < 0.001$) PC4, and its interactions with PGPR influenced ($P < 0.05$) PC3 and PC4. For root bacterial communities, water stress affected ($P < 0.05$) PC5, and PGPR affected ($P < 0.05$) PC5.

Fungi: Fungal beta diversity indices differed between rhizoplane and rhizosphere samples ($P < 0.001$), accounting for about 21% of the total variability (Fig. 25). The effects of AMF amendment were also significant ($P < 0.05$). In the separate analysis of rhizosphere data, only AMF amendment affected ($P < 0.01$) the fungal beta diversity indices. Rhizosphere beta diversity indices were not significantly affected by treatment factors.

The first six PCs accounted for 19.1%, 7.0%, 5.3%, 3.9%, 3.8% and 3.7% of the total variability in bacterial communities, respectively. Sample type mainly affected the first two PCs ($P < 0$

.001). The first two PCs were only affected by sample type; water stress only influenced PC6 ($P < 0.05$). The interaction of AMF with water stress affected PC1 ($P < 0.01$) and with PGPR influenced PC5 ($P < 0.05$). When principal analysis was applied to the roots and soil samples separately, most differences between samples were not due to treatment factors. For rhizosphere data, water stress affected PC6 ($P < 0.05$) PC6; AMF affected PC4 ($P < 0.05$) and PC6 ($P < 0.01$), and its interactions with PGPR influenced PC4 ($P < 0.01$). For rhizoplane communities, water stress affected PC1, PC2 ($P < 0.05$), and PC5 ($P < 0.01$) PC5; AMF and PGPR amendment did not affect root fungal communities.

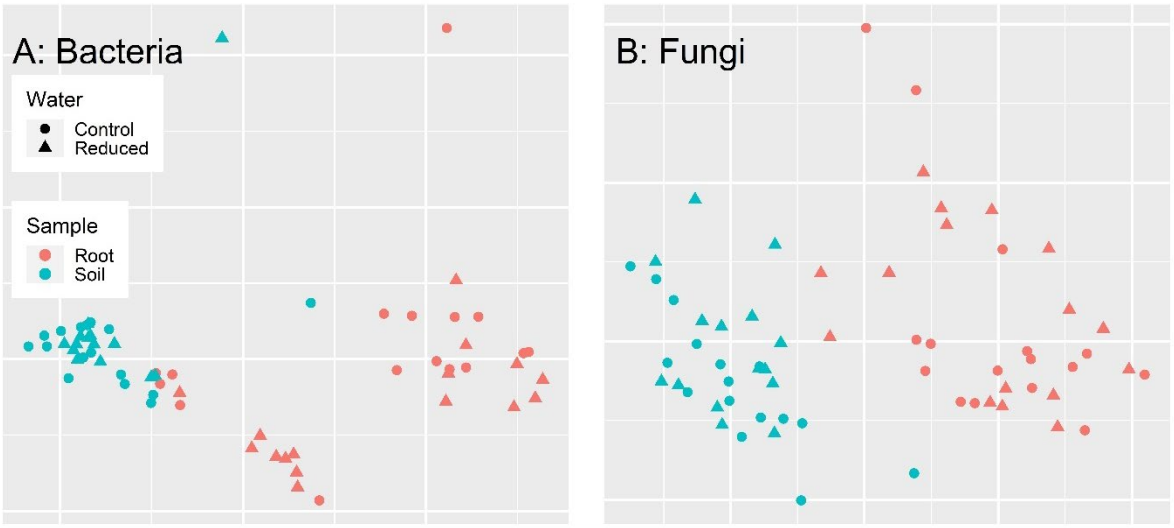

Figure 25. Non-dimensional scaling plot of the first two dimensions of beta diversity (Bray-Curtis) indices for bacterial (A) and fungal (B) communities of apple rhizosphere and roots.

DESeq2 comparison of individual OTUs

Bacteria: When both rhizosphere and root samples were analysed together, 699 of the 2925 OTUs that passed the default DESeq2 filtering criteria differed significantly in the relative abundance between root and rhizosphere communities. Of the 699 OTUs, 394 had greater relative abundance in the rhizosphere than in the roots (Fig. 26A). Water stress and amendment with either PGPR or AMF did not significantly affect the relative abundance of any of 3911 bacterial OTUs.

When the rhizosphere samples were analysed separately, none of the treatment factors significantly affected the relative abundance of any of the 3007 bacterial OTUs. For the root samples, water stress only led to increased relative abundance for one OTU (Flavobacteriaceae). Neither AMF or PGPR amendment led to significant changes in the relative abundance of bacterial OTUs.

Fungi for joint rhizosphere and root data: Of 719 fungal OTUs that passed the DESeq2 default filtering criteria 256 OTUs differed significantly in the relative abundance between root and rhizosphere communities. Of the 256 OTUs, 133 were not assigned to the phylum rank with confidence. Of these OTUs, 143 had greater relative abundance in the rhizosphere than in the roots (Fig. 26B). The large differences between root and rhizosphere communities spread across many taxonomy ranks. Of 780 fungal OTUs that passed the DESeq2 default filtering criteria seven OTUs had reduced relative abundance in the reduced water input than the control, including one OTU likely to be the AMF species (*Rhizophagus irregularis*) used in the

experiment (Table 1). Only one OTU increased its relative abundance in the reduced water input. PGPR amendment only led to decreased relative abundance for two OTUs (Table 1).

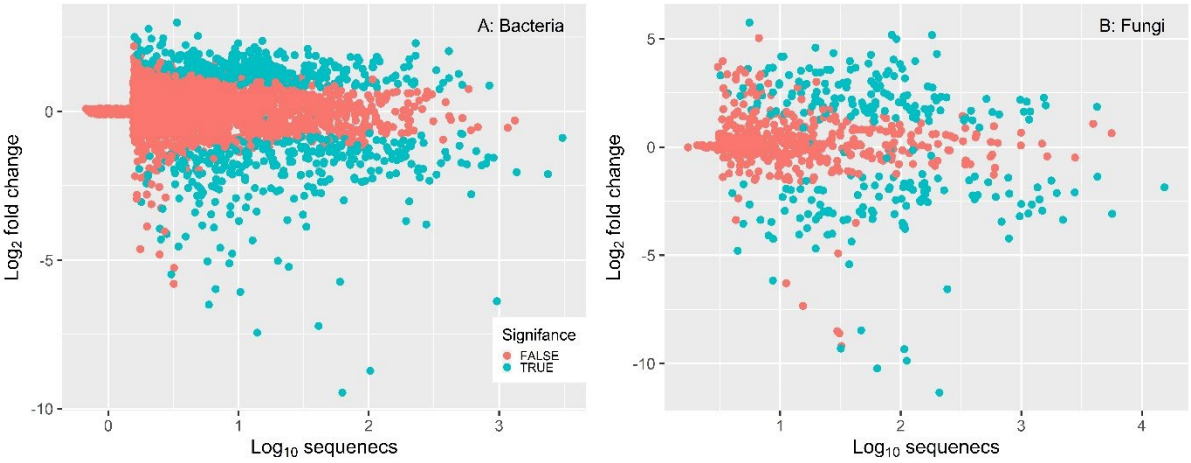

Figure 26. The log2 fold change of the relative abundance of individual bacterial (A) and fungal (B) OTUs between the rhizosphere and root communities; the cyan and red colours indicate the BH adjusted p values were less or greater than 0.05, respectively. Results were based on DESeq2 analysis.

Fungi for rhizosphere data only: Water stress affected relative abundance of 61 fungal OTUs: increased relative abundance only in 20 cases. Among these 61 OTUs, 29 cannot be assigned to the rank below phylum with confidence. These 35 OTUs included eight Glomeromycetes OTUs, all of which had the relative abundance reduced when water stress was imposed.

PGPR amendment decreased and increased abundance for one (*Acremonium pinkertoniae*) and two OTUs (including for one Glomeromycetes OTU), respectively. AMF amendment only led to significant decreases ($P < 0.05$) in the relative abundance for two fungal OTUs (*A. pinkertoniae*, and one Mortierellomycota OTU). AMF and PGPR interactions affected relative abundance of 16 OTUs: seven of which had increased relative abundance when both AMF and PGPR were applied, including *A. pinkertoniae*.

Fungi for rhizoplane and endophytes data only: Water stress affected relative abundance of 17 fungal OTUs: increased relative abundance for one OTU only. PGPR amendment led to increased abundance for one OTU (*Conocybe* sp.) only. AMF amendment did not lead to significant changes in the relative abundance of any OTUs. Joint AMF and PGPR application led to increased and decreased relative abundance of two and two OTUs, respectively.

Discussion

Rhizosphere communities, as expected, are more diverse than root communities. Such differences are not restricted to specific groups but spread evenly across all taxonomic ranks. Thus, roots may act likely a general (rather a selective) barrier. This agree with previous findings, such as *Arabidopsis thaliana* (Durán et al., 2018) and rice (Edwards et al., 2015). Within sample microbial diversity is generally not affected by water stress, AMF and PGPR amendment. Relative abundance of individual bacterial taxa, whether in the rhizosphere or in rhizoplane (including endophytes), is hardly affected by water stress, and amendment with AMF and PGPR at planting time. In contrast, relative abundance of many taxa was greatly affected by water stress (particularly in the rhizosphere) and, to a much lesser extent, by AMF and PGPR amendment. Similarly, the water regime had a dominant effect on the structure of

the microbial communities in *Festuca pratensis* and *Dactylis glomerata*, suggesting a direct effect of water on microbes (Changey et al., 2019). In contrast to the current results, drought has been shown to have drastic effects on bacterial communities (Naylor & Coleman-Derr, 2018), and far greater than on fungi (Wang et al., 2017).

Interestingly, water stress significantly reduced relative abundance of several AMF taxa in the rhizosphere. Joint application of AMF and PGPR affected relative abundance of more individual fungal taxa than individual applications, particularly in the rhizosphere. Effects of AMF inoculation on microbial abundance and diversity varied with the plant species they are associated with (Hashem et al., 2016), and environmental conditions (Hao et al., 2021).

Number of fungal OTUs in rhizoplane (including endophytes) was greater for AMF treatment than the control under the reduced water input; but the opposite was true under the control condition. Thus under water AMF led to increased fungal richness in rhizoplane (endophytes), which coincide with the observation that incidence of canker development appeared to be higher with AMF amendment under water stress. Further research is needed to assess the effects of fungal root endophytes richness on canker development.

The present study showed minimal effects of AMF and PGPR amendment at planting on tree growth and canker development following artificial inoculation. It should be noted that because of the extreme hot weather in the summer 2018 tress subjected to the well-watered treatment still visibly suffered from water stress. Thus, the trees allocated to the water stress treatment displayed more-than-mild water stress symptoms. Thus, under such a severe water stress condition, the benefit from amending soils with AMF and PGPR can be expected to be minimal. Furthermore, in order to follow commercial tree planting as much as possible, we used top soils from agricultural land, which may have already contained sufficient AMF and/or other beneficial microbes. Indeed, microscopic assessment of stained showed a high level (> 30%) of AMF root colonisation for all treatments (data not shown), including the treatment without AMF amendment. This may also explain the lack of effects associated with AMF and/or PGPR amendment.

Effects of planting dates and sites on canker development

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. Initially, we prosed 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. However, 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.

Objectives

1. To obtain information regarding the effect of tree planting date (early winter vs early spring) on canker incidence.
2. To investigate whether cultivar susceptibility/resistance is consistent across sites.

Materials and methods

Treatments and experimental design

Seven scion apple cultivars, grafted to M9 rootstocks, with varying susceptibility to *N. ditissima*, were planted at three sites in Kent, UK. Seven scion varieties were Royal Gala, Braeburn, Jazz, Kanzi, Rubens, Grenadier, and Golden Delicious. Jazz, Kanzi and Rubens are regarded as highly susceptible to *N. ditissima* whereas Grenadier and Golden Delicious tolerant/resistant against *N. ditissima*. Half of the trees were planted in December 2018 immediately after trees were lifted from the FPM nursery, Hereford, UK. The other half of trees were stored in a misted cold store at +2°C until March 2019 when they were planted out at the same sites.

At each site, there were 504 trees, half of which planted out in December 2018 and the other in March 2019. There were 72 trees per scion per site. A split-plot design was used for the experiment at each site. Within each site, there were six blocks; within each block there were seven plots, each with two subplots. Within each block, each plot was randomly assigned to one of seven cultivars; within each plot, each subplot was randomly assigned to one of the two planting times. There were six trees per subplot. For some blocks, there were insufficient number of trees for specific cultivars due to tree mortality; these were then gapped with other scion varieties. The experiment was terminated in August 2021.

Plants and management

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 (10^{-4} macro conidia per ml). This inoculation was used to ensure a certain level of latent infections of leaf scars by *N. ditissima*, increasing the chance of useful data to be collected. Trees were planted at three orchards in Pluckley (WWF), Brenchley (Scripps) and East Sutton (Avalon), Kent UK. The soil for the two orchards (Scripps and Avalon) was of the clay loam type, the remaining one (WWF) was of the sandy clay loam. The Scripps orchard had much higher copper (EDTA extractable) concentration (23.1 mg per litre) than the other two orchards (5.5 and 8.2 mg per litre).

The experiment plantings were managed following commercial practices except two specific aspects. Firstly, no canker specific products were applied, such as treatment at leaf-fall.

Secondly, canker lesions were not cut out or dead trees with cankers removed from the trial sites during the experimental duration. Canker lesions were only cut out (i.e., removed) at the time of each disease assessment; this was done in order to minimise the canker inoculum to neighbouring commercial orchards whilst not compromising the present research objective.

Canker assessment

Canker development was carried out four times: October 2019, June 2020, November 2020, and May 2021. On each occasion, all parts of every tree were assessed for visible canker lesions, with the number and location of canker lesions on the tree classified into five categories (A – rootstock and graft union; b – Main trunk above graft union; C – first branch from trunk; D – shoot from C; E – shoot from D) (McCracken et al., 2003).

Statistical analysis

All variables were summarised per sublot basis as number of canker lesions on the main stems and peripheral branches per tree, number of dead trees due to cankers, and number of trees in the category of healthy, nearly dead, and dead status on the last assessment date (May 2021). Linear mixed models were applied to the data set, in which assessment time, planting time and cultivar were treated as fixed effect factors whereas site, block within site, plot (split plot design) and subplot (experimental unit for repeated measurements) were treated as random effect factors. Number of lesions per tree was first logarithmically transformed before analysis. For tree health status on the last assessme4nt date, data were pooled across all blocks at a given site in order to increase statistical power. Logit transformation was applied to the incidence of dead or healthy trees. Logit-transformed data were then subjected to linear mixed model analysis with the site treated as a random effect factor, and planting time and cultivar as fixed effect factors.

Linear mixed models were fitted with the lme4 (Bates et al., 2015) package in R 4.1.2. Testing for individual model terms was through the lmerTest package (Kuznetsova et al., 2017): the Kenward-Roger test was used for testing fixed effects whereas the approximate Chi-square test of nest models was used for testing random effects.

Results

Main-stem cankers

Main stem Canker severity increased with time. Average number of canker lesions on the main stem was 0.11, 0.72, 1.48 and 4.43 per tree for assessment 1, 2, 3 and 4, respectively. The overall incidence of trees with main stem lesions increased from 6.7% on the first assessment to 64.0% on the last assessment. Canker development varied greatly among the three sites (Table 12). Average number of canker lesions on the main trunk was 0.53, 1.71 and 2.82 per tree for the Avalon, Scripps and WWF orchards, respectively. The overall incidence of trees with main trunk lesions 19.8%, 41.8% and 56.7% for the Avalon, Scripps and WWF orchards, respectively. The overall differences in the canker development between the two planting times were small (Table 12).

Seven scion differed in the canker development (Table 12, Fig. 27). Overall, Golden Delicious and Grenadier had much less canker development than the other five cultivars. Kanzi had much a higher incidence of canker lesions on main trunks (ca. 79.5%) than Jazz, Rubens, Gala and Rubens (between 40 and 40%), even though the incidence of peripheral cankers was similar for these five scion cultivars (Table 12).

Variance estimates and their statistical significance are given in Table 13; it should be noted that statistical significance was not necessarily correlated with the magnitude of the variance estimate. Thus, although the variance estimate for the site factor was the largest, it was only

close to statistical significance (Table 13). On the other hand, variance estimates for both plot and block terms were very small but statistically significant (Table 13). Variance estimates for the site and its interaction with time were greater than the residual variance (Table 13). Fig. 28 shows the nature of the interaction between site and time. Canker increased over time was greatest for the WWF site and least for the Avalon site, irrespective of cultivar and planting time. The interactions of site with cultivar and with both site and time were statistically significant (Table 13). Cultivar differences over time were greatest for the WWF site and least for the Avalon site (Fig. 28). Variance estimates for any random-effect terms involving the planting time was both small and statistically not significant. Variance estimates for block, plot and subplot were all small although they might be statistically significant.

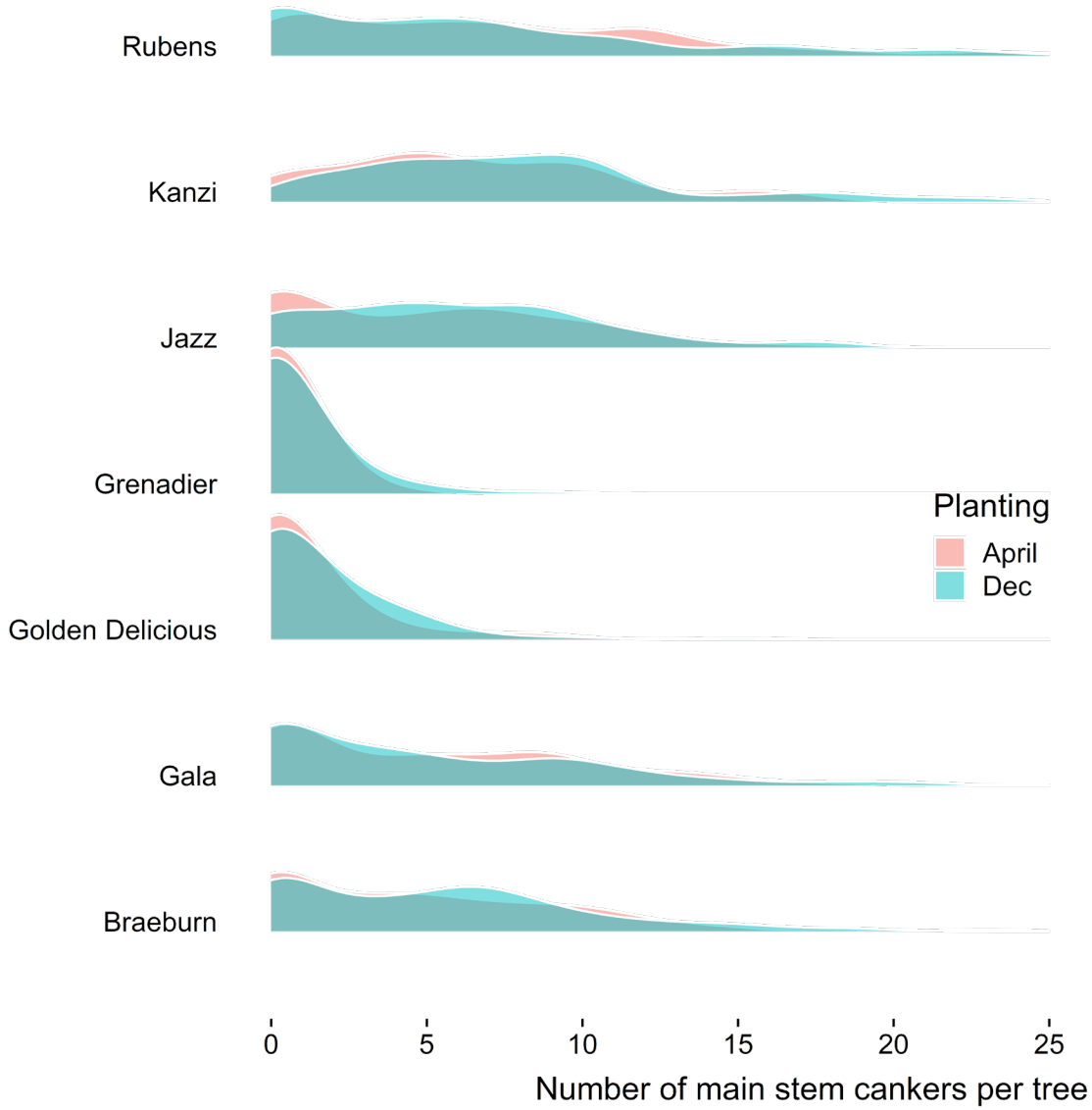

Figure 27. Density plot of number of canker lesions, when assessed in Spring 2021, on the main stem of individual trees of seven apple scion cultivars planted at three sites where trees were planted either in early winter (December 2018) or refrigerated and planted in early spring (April 2019).

Table 12. Canker development on individual scion cultivars summarised over individual plots across three sites where trees were planted either in early winter (December 2018) or refrigerated and planted in early spring (April 2019).

Factors	Number of lesions per tree		Incidence of trees with lesions	
	Main stem	Peripheral	Main stem	Peripheral
Site				
Avalon	0.53±0.11*	1.06±0.31	19.1±3.03	22.0±3.60
Scripps	1.71±0.34	6.77±1.54	41.8±4.41	55.2±5.23
WWF	2.82±0.43	7.42±1.26	56.7±5.23	61.2±5.69
Cultivars				
Braeburn	1.89±0.53	5.95±1.88	42.4±7.74	51.0±8.65
Gala	1.70±0.52	5.69±2.02	41.2±6.82	52.4±8.17
Golden Delicious	0.46±0.15	1.31±0.49	19.8±4.65	31.1±6.91
Grenadier	0.24±0.07	0.48±0.14	13.8±3.20	18.9±5.27
Jazz	2.24±0.58	7.46±2.28	49.5±7.78	56.4±8.76
Kanzi	2.89±0.60	7.35±1.77	79.5±5.68	53.9±8.07
Rubens	2.38±0.69	7.38±2.58	49.5±7.41	55.2±8.13
Planting				
December	1.64±0.28	4.78±0.94	37.9±3.88	45.0±4.38
April	1.73±0.29	5.39±1.05	40.0±3.89	46.6±4.45

*: the number after '±' is the standard error (based on individual replicate plots).

Table 13. Estimates of variances for all random effect factors in the linear mixed model analysis of canker data on individual scion cultivars summarised over individual plots across three sites where trees were planted either in early winter (December 2018) or refrigerated and planted in early spring (April 2019).

Term	Main stem	Peripheral	Main stem + Peripheral
Site	0.0936 (0.083)*	0.2319 (0.095)	0.2693 (0.072)
Site : Time	0.0632 (<0.001)	0.1760 (<0.001)	0.1742 (<0.001)
Site : Block	0.0047 (<0.001)	0.0079 (<0.001)	0.0096 (<0.001)
Site : Planting	0.0008 (0.553)	-$	-
Site : Cultivar	0.0150 (0.015)	0.0215 (0.113)	0.0241 (0.073)
Site: Time : Planting	0.0001 (0.900)	-	-
Site : Time : Cultivar	0.0213 (<0.001)	0.0643 (<0.001)	0.0584 (<0.001)
Site : Planting : Cultivar	0.0017 (0.260)	0.0030 (0.174)	0.0035 (0.108)
Plot	0.0033 (0.135)	0.0094 (0.001)	0.0108 (<0.001)
Subplot	0.0038 (0.010)	-	-
Residual	0.0530	0.0967	0.1030

*: the number in the brackets is the P value from the approximate Chi-square test via nested models as implemented in the lmerTest package (Kuznetsova et al., 2017)

$: the term was not included in the model as its inclusion would lead to non-convergence (its variance estimate was close to zero)

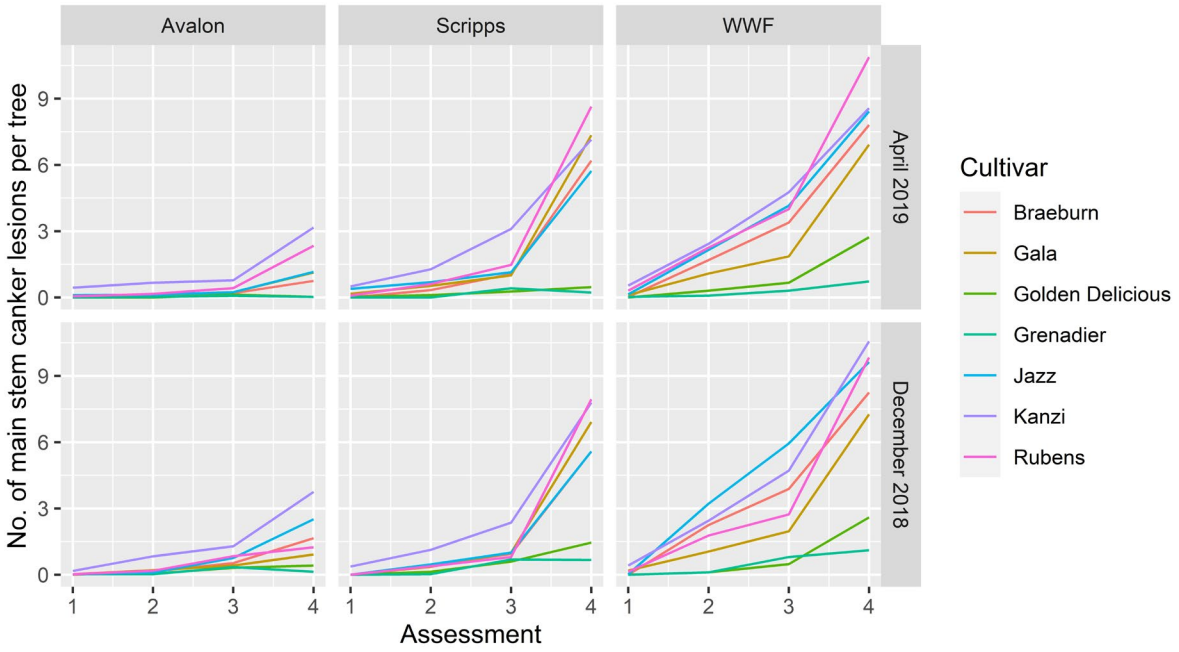

Figure 28. Number of canker lesions on the main stem on individual trees of seven apple scion cultivars at three sites where trees were planted either in early winter (December 2018) or refrigerated and planted in early spring (April 2019). Four assessments were made in Autumn 2019, Spring and Autumn 2020, and Spring 2021.

For the fixed effect factors, both time and cultivar, as well as their interaction, were highly significant (Table 14, Fig. 29A). Cultivar differences increased with increasingly later assessment. This is particularly noticeable for the differences in Grenadier and Golden Delicious with the other five cultivars on the last assessment date. The interaction of assessment time with planting time was close to significance (P = 0.066); the number of main stem lesions for the December planting increased from 0.070 (1st assessment) to 4.564 (4th assessment) whereas the corresponding values for the April planting were 0.141 and 4.304.

The overall patterns of the incidence of trees with main stem lesions in relation to site, cultivar, planting date and assessment time were similar to those observed for the number of main stem lesions except that incidence values were higher and more variable.

Table 14. Sum of squares of the fixed effect factors in the linear mixed model analysis of canker data on individual scion cultivars summarised over individual plots across three sites where trees were planted either in early winter (December 2018) or refrigerated and planted in early spring (April 2019).

Term	DF	Trunk	Peripheral	Trunk+ Peripheral
Time	3	2.061 (0.005)*	3.612 (0.005)	5.540 (0.004)
Planting	1	0.062 (0.392)	0.568 (0.030)	0.669 (0.024)
Cultivar	6	4.184 (<0.001)	8.246 (<0.001)	11.120 (<0.001)
Time : Planting	3	0.662 (0.066)	1.402 (0.002)	2.333 (<0.001)
Time : Cultivar	18	6.116 (<0.001)	9.998 (<0.001)	11.236 (<0.001)
Planting : Cultivar	6	0.676 (0.127)	0.758 (0.317)	1.043 (0.197)
Time : Planting : Cultivar	18	1.131 (0.268)	0.716 (0.986)	1.269 (0.828)

*: the number in the brackets is the P value from the Kenward-Roger test as implemented in the lmerTest package (Kuznetsova et al., 2017)

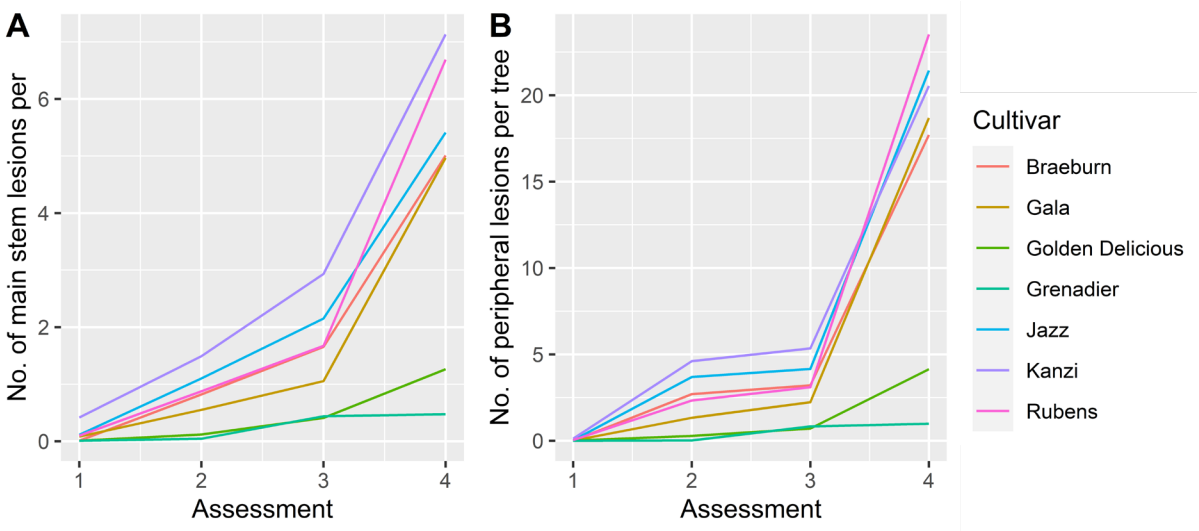

Figure 29. Number of main stem and peripheral canker lesions on individual trees of seven apple scion cultivars across three sites over two planting times. Four assessments were made in Autumn 2019, Spring and Autumn 2020, and Spring 2021.

Peripheral cankers

Peripheral canker severity increased with time. Average number of peripheral canker lesions was 0.03, 2.14, 2.81 and 15.37 per tree for assessment 1, 2, 3 and 4, respectively. The overall incidence of trees with peripheral lesions increased from 2.2% on the first assessment to 76.0% on the last assessment. Canker development varied greatly among the three sites (Table 12). Canker development varied greatly among the three sites. Average number of peripheral canker lesions was 1.06, 6.77 and 7,42 per tree for the Avalon, Scripps and WWF orchards, respectively. The overall incidence of trees with peripheral lesions was 22.0%, 55.2% and 61.2% for the Avalon, Scripps and WWF orchards, respectively. The overall differences in the canker development between the two planting times were small (Table 1). Seven scion differed in the canker development (Table 12, Fig. 30). Overall, Grenadier and Golden Delicious had much less canker development than the other five cultivars , which had similar incidences between 51% and 57% (Table 12).

Variance estimates and their statistical significance are given in Table 13. As for the main stem canker, although the variance estimate for the site factor was the largest, it was only close to statistical significance (Table 13). On the other hand, variance estimates for both plot and block terms were very small but statistically significant (Table 13). Variance estimates for the site and its interaction with time were greater than the residual variance (Table 13). Fig. 31 shows the nature of the interaction between site and time. Canker increased over time was much greater for the WWF and Scripps sites, irrespective of cultivar and planting time. In contrast to the main stem canker, the interactions of site with cultivar was not significant (Table 13). However, the three-factor interaction (site, cultivar and time) was significant; cultivar differences over time were greater for the WWF and Scripps sites (Fig. 31). Variance estimates for any random-effect terms involving the planting time was both small and not significant.

For the fixed effect factors, both time and cultivar, as well as their interaction, were highly significant (Table 14, Fig. 29B). Cultivar differences increased with increasingly later

assessment. This is particularly noticeable for the differences in Grenadier and Golden Delicious with the other five cultivars on the last assessment date. Planting date also had significant effects, albeit small: planting in December led to slightly fewer canker lesions. The interaction of assessment time with planting time was also highly significant (P = 0.002); the number of peripheral lesions for the December planting increased from 0.005 (1st assessment) to 16.3 (4th assessment) whereas the corresponding values for the April planting were 0.06 and 14.4.

The overall patterns of the incidence of trees with peripheral lesions in relation to site, cultivar, planting date and assessment time were similar to those observed for the number of peripheral lesions except that incidence values were higher and more variable.

When total number of canker lesions per tree (including both main stem and peripheral cankers) was analysed jointly, the results followed closely those for peripheral canker (Table 13 and 14).

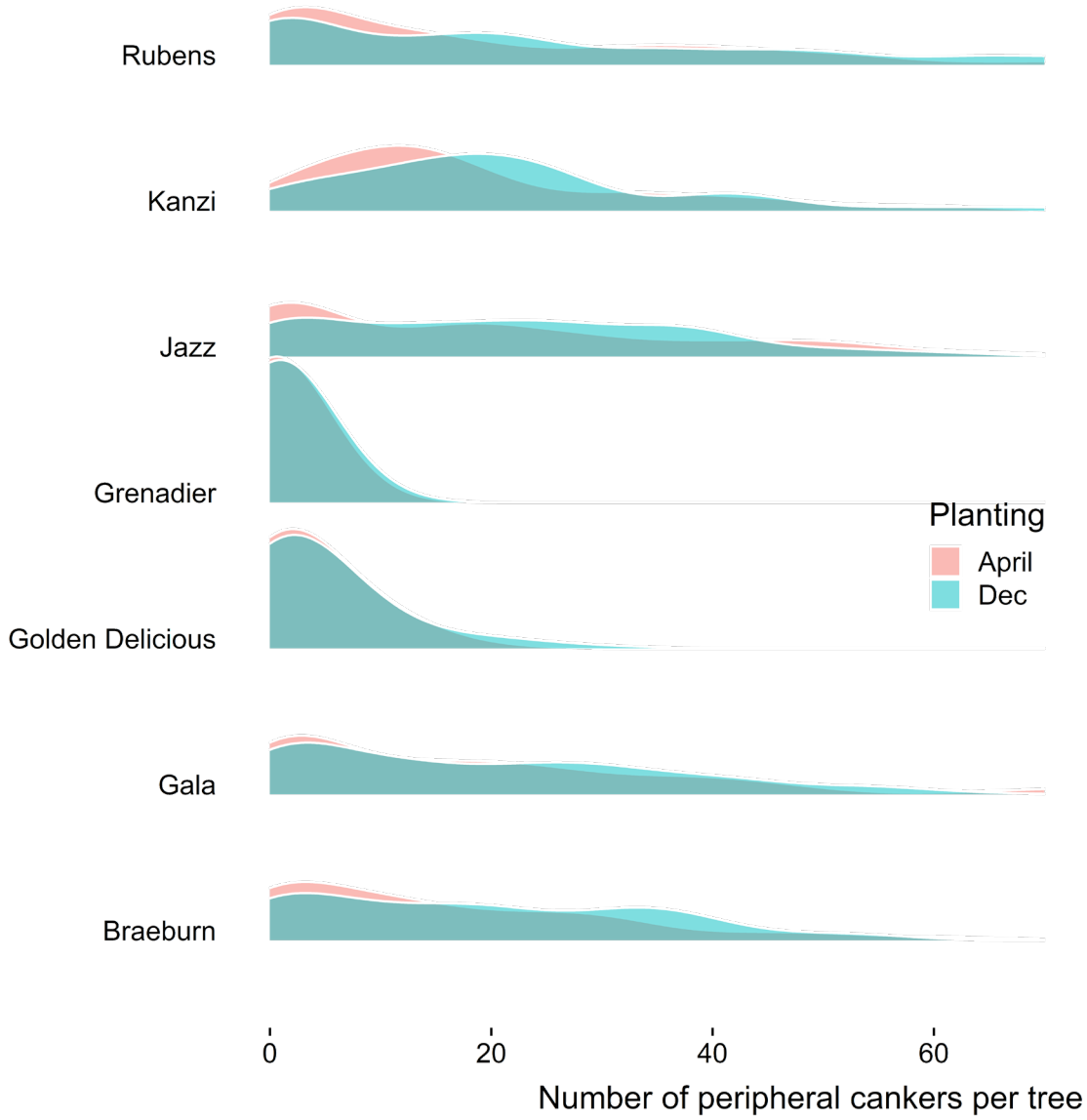

Figure 30. Density plot of number of peripheral canker lesions, when assessed in Spring 2021, of individual trees of seven apple scion cultivars planted at three sites where trees were planted either in early winter (December 2018) or refrigerated and planted in early spring (April 2019).

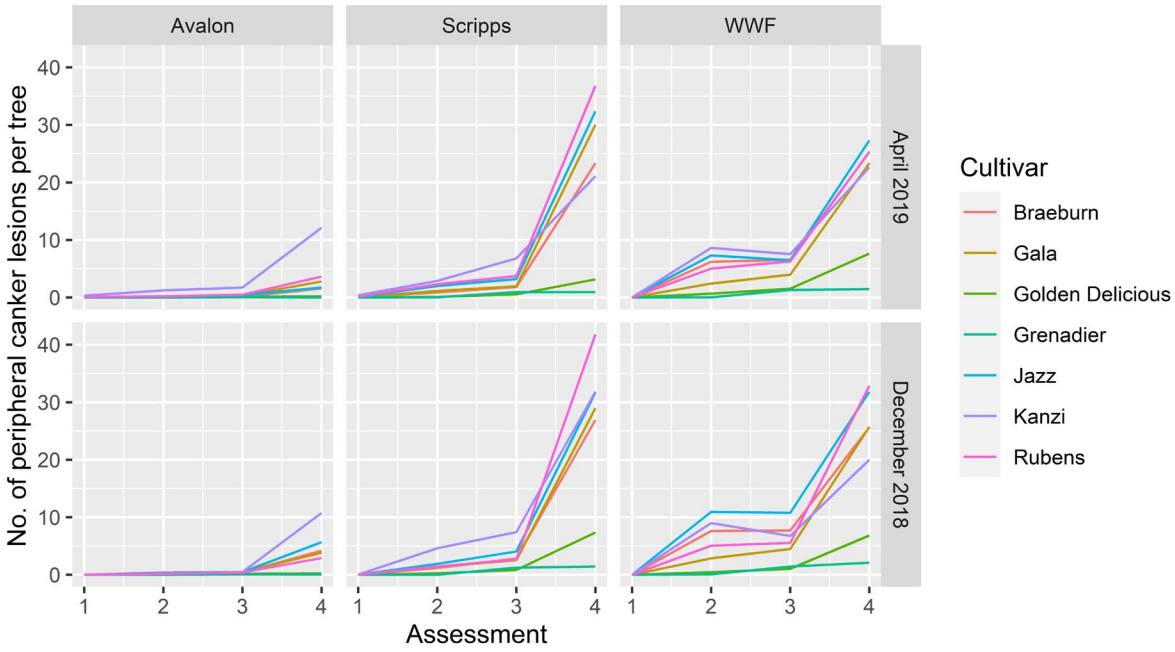

Figure 31. Number of peripheral canker lesions on individual trees of seven apple scion cultivars at three sites where trees were planted either in early winter (December 2018) or refrigerated and planted in early spring (April 2019). Four assessments were made in Autumn 2019, Spring and Autumn 2020, and Spring 2021.

Tree health

At the final assessment, 254 trees died of cankers, 308 trees were dying, and 883 trees were visually healthy. In the linear mixed model analysis of healthy trees on the last assessment date, the random-effect terms of site and its interaction with cultivar statistically significant, with the respective p value of 0.016 and 0.001); the corresponding values of their variance estimates were 3.583 and 0.937, compared the residual variance of 0.290. The nature of the interaction is shown in Fig. 32: both Golden Delicious and Grenadier had very high incidence of healthy trees on all sites whereas the incidence varied greatly with sites for the other five cultivars. The interaction between site and planting date (variance estimate = 0.058) was not significant. Of the three first-effect terms (planting, cultivar, and their interaction), only cultivar effect was significant (P = 0.001, Fig. 33). Similar results were obtained when the incidence of dead trees were analysed.

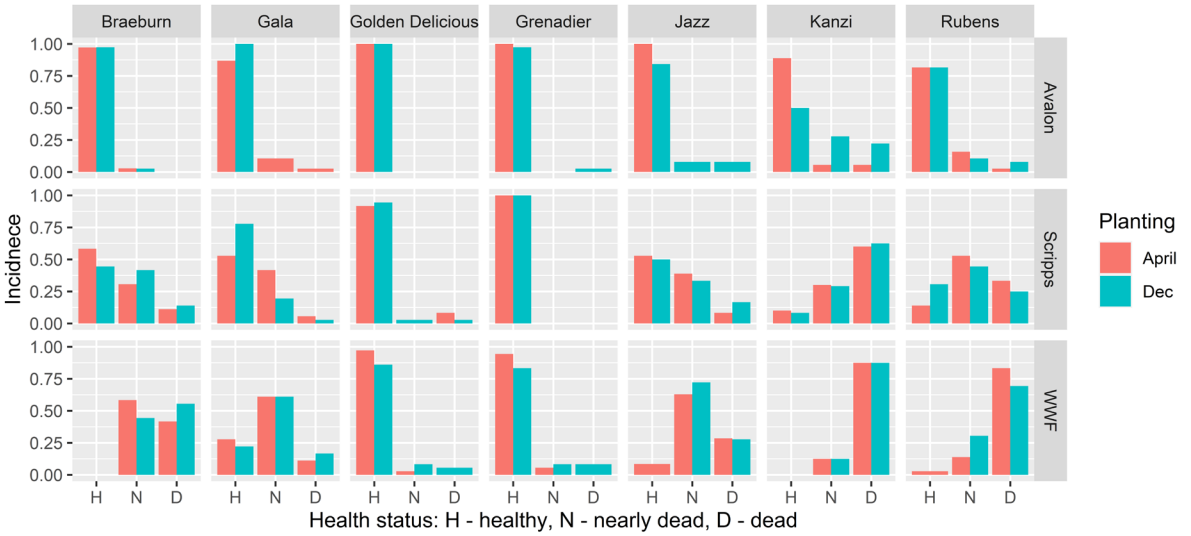

Figure 32. Incidence of trees in three health categories (healthy, nearly dead, and dead), when assessed in Spring 2021, of seven apple scion cultivars planted at three sites where trees were planted either in early winter (December 2018) or refrigerated and planted in early spring (April 2019).

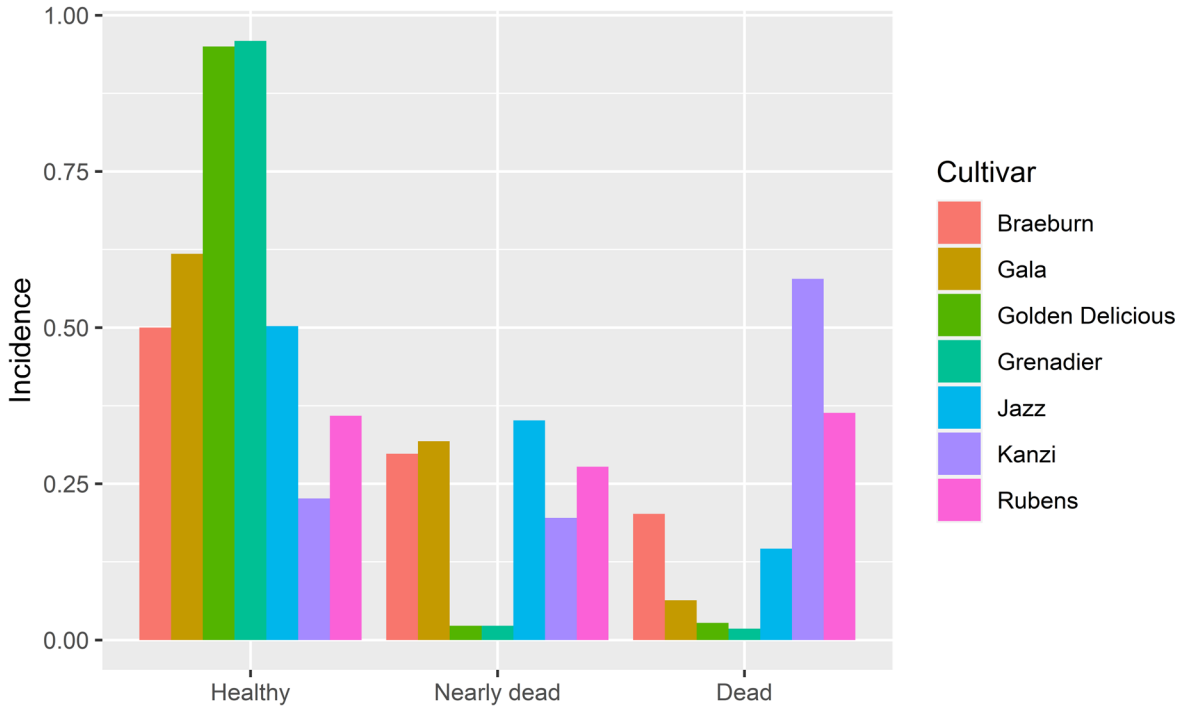

Figure 33. Incidence of trees in three health categories (healthy, nearly dead, and dead), when assessed in Spring 2021, of seven apple scion cultivars across all three sites and two planting dates.

Discussion

Canker development differed greatly among the three sites for both mainstem and peripheral cankers. Although when site was treated as a random-effect factor, its random effect was not statistically significant, probably due to a fewer sites, but its variance component was far greater than other variance components, including the residual. Indeed, when the site was treated as a fixed-effect factor, its effect was highly significant. However, in analysing the data,

we treated the site as random-effect factor in order to assess cultivar performances correctly (i.e., over a range of sites instead of just these three specific sites). Site differences may result from differences in general climatic conditions, inoculum density, and possibly soil conditions. Significant effects among local positions (blocks within a site), albeit much less than among sites, may suggest that soil conditions may also affect canker expression indirectly through influencing tree development. There were significant interactions between sites and time in canker development, primarily due to the faster canker development at the WWF site between the first and third assessments. Given the direct effects of climatic conditions on canker infection is well understood (Saville & Olivieri, 2019), we need to focus future research studies on the effects of soil properties on the expression of latent infection and on predisposing trees to canker infection; these could include soil pH, water matrix potential and soil type.

It is reassuring that cultivar differences are generally consistent, in terms of number of canker lesions and trees with lesions, for both main stem and peripheral cankers, across three sites and over two planting dates. This suggests that genetic factors responsible for reduced canker development in Golden Delicious and Grenadier are stable and effective across varying environment. Thus, exploiting these resistance factors in breeding could be rewarding. Nevertheless, similar studies need to be carried to assess the stability of other main resistance resources used in breeding for durable canker resistance in apple.

Nevertheless, the interaction between cultivar and site was statistically significant for the mainstem canker only. Given the mainstem cankers are more likely to result from latent infections in nurseries, the present result suggests that some site specific factors may interact with cultivars to affect differential expression of latent infections post-planting. that could differentially influence symptom development from latent infection. On the other hand, it appears for the new infections occurred post-planting, cultivar response was generally consisted across three sites. This interaction appears to due mostly to the relatively more mainstem cankers on Kanzi at the Avalon and Scripps sites in the early assessments. It is interesting to note that the soil in the former orchards is of the clay loam type, the WWF orchard is of the sandy clay loam. Further research is necessary to understand potential effects of main soil properties on tree establishment and canker and symptom expression of latent infections.

Planting date had only significant (albeit minimal effects) on the peripheral canker. This is surprising as we would expect that symptom development of latent infection may be more likely to be affected by cold storage. Under high humidity, cold storage may accelerate symptom development of latent infection by *N. ditissima* (Wenneker et al., 2017). However, given the effect of planting date is so small relative to other effects, management efforts should be prioritised in other areas.

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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 a talk on 'Understanding dynamics of endophytes for biocontrol of canker control' at the AAB Biocontrol and IPM conference, November 2021.

(6) 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

(7) We gave a talk on 'Using endophytes for biocontrol of canker control' at the East Malling Tree Fruit 2021 at NIAB EMR on 24th Feb 2022.

(8) We gave a talk on 'Dynamics of endophytes at leaf scars' at the East Malling Tree Fruit 2021 at NIAB EMR on 24th Feb 2022.

(9) At 12th International IOBC/WPRS Workshop on Pome Fruit Diseases, 13-16 June 2022, Plovdiv, Bulgaria, we will present

   i. Root endophytes inrelaiton to canker development at three sites

   ii. Biocontrol of the European canker with Eppicoccum

(10) We will present the genetic control of endophytes in the 18th International Scociety of Microbial Ecology Congress, August 2022, Lausanne, Switzerland

