CRANFIELD UNIVERSITY

Christopher Cook

Understanding the resilience of soil beneficials to combat Apple Replant Disease (ARD)

Environment and Agrifood Theme
Centre for Soil, Agrifood and Biosciences

PhD
Academic Year: 2018 – 2022

Supervisors: Prof. Naresh Magan, Dr Louisa Robinson-Boyer, Prof. Xiangming Xu
Associate Supervisor: Prof. Angel Medina Vaya
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ABSTRACT

Apple Replant Disease (ARD), caused by an accumulation of soil-borne fungal and oomycete pathogens in the soil, is an economically important disease of apples. Current management relies on the use of broad-spectrum chemical fumigation. Banning of chemical products has seen increased research into biological management strategies for ARD. This study aimed to test the efficacy of a number of commercially available biological soil amendments to improve the establishment of young apple trees in an attempt to minimise the detrimental effects of ARD. It also looked at changes in planting position and rootstock selection at planting to manage ARD and how climate change abiotic factors affect bulk apple soil microbiome communities.

The efficacy of single species amendments was variety specific in the field. There was a increased rate of tree girth expansion in Gala trees amended pre-planting with *Pseudomonas fluorescens* but the same effect was not observed with Braeburn trees. The single species amendments did not increase the establishment of young apple trees either individually or in a consortium of amendments in semi-field conditions. In both field and semi-field conditions pre-plant amendment significantly altered the abundance of individual operational taxonomic units (OTUs) associated with both beneficial and pathogenic taxa of plants.

The planting position in the inter-row alleyways and the genetic relationship of the new rootstock to the previously planted rootstock on the site were both identified to minimise ARD severity. In addition, a significant effect of CO_2 concentration and temperature increase on bulk soil microbiome communities was observed but this was not as significant as site management (organic vs conventional) effect.

This research will aid with the development of management strategies for ARD by integrating single species biological amendments and cultural approaches with other management practices.

Keywords:

Apple, Horticulture, Soil, Rhizosphere, Ecology, Microbiome, Climate Change

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LIST OF ABBREVIATIONS

ADONIS	Permutational Multivariate Analysis of Variance
AMF	Arbuscular Mycorrhizal Fungi
ANOVA	Analysis of Variance
ARD	Apple Replant Disease
BSM	Brassica Seed Meal
K	Potassium
LFC	Log2 Fold Change
Mg	Magnesium
NMDS	Non-Metric Multi-Dimensional Scaling
OTU	Operational Taxonomic Unit
P	Phosphorous
pA	Peak Area
PCA	Principal Component Analysis
PGPR	Plant-Growth-Promoting Rhizobacteria
RLC	Root-Length Colonisation
Sp.	Species
Spp.	Plural of Species

1 Literature Review

1.1 The Problem

Apple (*Malus domestica)* has become an increasingly important crop for protection, with 89.3 million tonnes of apples being produced worldwide in 2016. Modern systems of apple growing require high investment to induce earlier production and increased yield of the fruit (Hoestra, 1968). However, apple replant disease (ARD) has become a major hurdle to maintaining yields for growers. ARD is of concern in nurseries where the number of generations planted consecutively is higher than that in commercial orchards. In a commercial scenario, there is more chance for the trees to recover from ARD or for crops to be rotated. ARD has become a focus for research in recent years with the number of studies published rapidly increased in the last 20 years. This has been linked to the increasing impact of ARD due to the banning of broad spectrum chemical applications.

ARD is a soil-borne disease that leads to a number of negative impacts on replanted apple trees. Replanting is where trees are planted in previous orchards without rotation, land rest or cover cropping. Symptoms of the disease include stunted growth, discolouration of the apple skin, reduced yield, reduced fruit size/weight, altered fruit aroma and crop death and can result in removal of the affected tree by the grower (Mazzola & Manici, 2012; Zhu et al., 2014; Liu et al., 2014). Profitability of an orchard can be reduced by up to 50% during the orchards lifetime if ARD is present (van Schoor, Denman and Cook, 2009). The symptoms of ARD can either be extreme with characteristic symptoms, or very mild in the early stage, when effects can be easily considered as lack of nutrients/water as stunting is not drastic. However, when comparing development on fumigated soils it becomes apparent that initial ARD is present (Jackson, 1979; Jaffee, 1982a). Additional indications to the symptoms occurring above ground, below ground symptoms include discoloured roots, root tip necrosis and reduced root biomass (Mazzola and Manici, 2012). The smaller root systems therefore take up less nutrients than plants in soils without ARD (Mai, Merwin and Abawi, 1994; Somera and Mazzola, 2022). Young apple trees, particularly in the nursery, are

of particular concern, as the symptoms of ARD can occur as early as 1 year after establishment. If these young trees do not die, then characteristic ARD symptoms can emerge (Figure 1.1).

Figure 1.1 Apple Replant Disease (ARD) affected tree (left) vs a healthy tree (right). Both trees were planted in the same season.

1.2 ARD Aetiology

It is known that ARD is biotic in nature as sterilisation and pasteurisation of soils has been shown to alleviate symptoms of ARD (Somera and Mazzola, 2022). Basic soil properties are not significantly altered in ARD-affected trees reinforcing the basis of ARD as biotic (Simon et al., 2020). The current consensus is that

changes in the soil microbiota are the basis for ARD (Mazzola and Manici, 2012), including the interaction of a number of specific pathogenic microorganisms with the plant and other beneficial microbes in the soil. However, specific pathogens and their population sizes can vary greatly between samples and soils (Tesfai Tewoldemedhin et al., 2011). Additionally, the non-specific interaction of multiple pathogenic microorganisms in a pathogen complex with each other and the host may partially be responsible for varying ARD severity. Often, the specific causal agents in the rhizosphere of ARD affected soils may be an increased abundance of a subset of the pathogens associated with ARD, rather than all resulting in symptomatic trees (Nicola et al., 2018). The presence of a pathogen using DNA-based techniques alone is not however sufficient to determine its function as an ARD pathogen alone and quantitative studies of root tissues have been suggested to be a better determinant of the causal agents of ARD (Somera and Mazzola, 2022). Understanding the dynamics and composition of the soil microbiome is critical to discerning both pathogenic and beneficial components of the rhizosphere for a better interpretation of the causes and subsequent development of ARD.

Previous studies have shown differences in the dominance of various genera in separate ARD sites. For example, both Cylindrocarpon-like species and Pythium genera have been found to be present in increasing densities in untreated roots, but significantly lower in fumigation-treated soils (Mazzola and Manici, 2012). Rhizoctonia solani was also observed to be suppressed in non-cultivated orchard soils, whereas soils planted with apple seedlings for 2 years or more resulted in increased development of Rhizoctonia root rot (Mazzola, 1999), indicating increased inoculum of this pathogen may play a role in the onset of ARD. There have also been temporal changes in the dominant Phytophthora species in diseased soils. For example, in apple orchards in Washington, USA, P. cactorum was found to be the dominant pathogen. However, 12 years later P. cambivora became the major contributor to ARD (Mazzola, 1998; Mazzola and Brown, 2010). Another potentially important pathogen appears to be the nematode Pratylenchus penetrans, as increased addition of P. penetrans to soil caused a reduced dry weight of apple seedlings (Jaffee, 1982b). However, applications of

nematicides in another study were unable to increase apple tree growth (Mazzola, 1998), indicating nematodes are not a direct causal agent but can exacerbate ARD symptoms. The main causative ARD agents (Pythium spp., Phytophthora spp., Pratylenchus spp. and 'Cylindrocarpon'-like spp.) have also been identified in both nursery roots, with up to 95% of trees containing the potential causal agent P. irregulare, and in irrigation water with up to 76% of water samples collected also containing P. irregulare (Figure 1.2; (Moein et al., 2019).

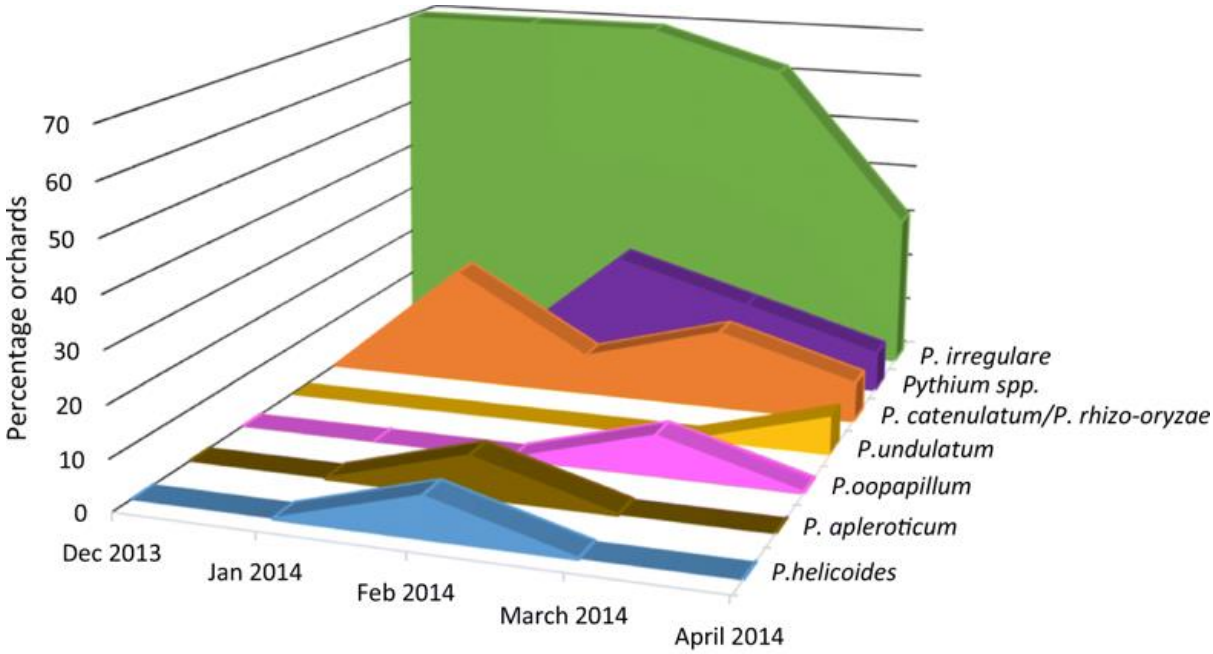

Figure 1.2 Percentage of apple orchard irrigation water containing suspected ARD causal agents over a 5 month period. A total of 13 orchards were analysed in each month. Presence/ absence was obtained by using a combination of qPCR analysis and traditional isolations (Moein et al., 2019).

There is a general acceptance that the above pathogens are the main causal agents of ARD but their relative importance and impact is yet to be understood fully. This uncertainty may explain why various organisms have been miss-labelled as important causal agents of ARD. There has been no definitive evidence of viral or bacterial causal agents of ARD. Actinomycetes were described as responsible for ARD due to their infection of roots in ARD affected soil and alleviation of symptoms achieved by steam treatment of the soil

(Westcott III, 1986). Microscopic examination of the “Actinomycete-like organism” showed that it was associated with the roots and it was concluded that it was a pathogen causing ARD (Westcott III, 1987). However, no attempt was made to confirm its pathogenicity by isolation and carrying out pathogenicity tests. Actinomycetes such as Streptomyces have been shown to supress R. solani infection, and do not appear to reduce apple growth in separate orchard sites either when applied alone or in combination with the pathogens P. irregulare or C. macrodidymum (Cohen and Mazzola, 2006; Tewoldemedhin et al., 2011a). Fusarium spp. are frequently isolated from ARD roots of trees and have been identified as an ARD causal pathogen in China using relative abundance from sequence data (WANG et al., 2018). F. solani has also been shown to reduce growth of M9 rootstocks comparatively to uninfected controls in China, but isolates of F. solani in South Africa and Washington State, USA have both been shown as non-pathogenic to apple roots (Somera and Mazzola, 2022). Other pathogens usually not associated with being root pathogens have also been reported as causal agents including Bacillus subtilis, Penicillium, and Mortierella species. This highlights the importance of fidelity when stating the relative importance of a candidate causal agent as there have been many occasions of misinterpreting the role of different microorganisms in ARD aetiology.

1.3 Role of ARD Causal Agents

When causal agents are found to be associated with ARD it is important to understand their function to avoid miss-identifying the role of non-pathogenic organisms and help to better understand their impact on apple tree growth (summarised in Table 1.1). Cylindrocarpon species have been frequently associated with ARD (Mazzola, 1998) and have also been shown to have pathogenicity towards apple seedlings. For example, Cylindrocarpon destructans have been shown to cause root rot and reduce vegetative growth (Tewoldemedhin et al., 2011b). Additionally, other species of this genus such as *C. lucidium* causes black lesions on seedling roots and *C. heteronema (=Neonectria ditissima)* causes perennial cankers of dead callus (Swinburne et

al., 1975; Tewoldemedhin et al., 2011b). *C. lucidium* has also been shown to cause the most damage to roots when acting in concert with *Pythium irregulare* (Braun, 1995). This reinforces the view that a pathogen complex or consortium rather than a single pathogen is responsible for ARD. *Cylindrocarpon*-like fungi such as *Dactylonectria torresensis,* the main component of black root rot in strawberry and raspberry, also reduces the growth of apple rootstock, making it a candidate causal agent of ARD (Manici et al., 2018a).

Table 1.1 Summary of key causal agents of ARD with their pathogenic function and related publications.

ARD Causal Agent	Function of pathogen	Publication
Cylindrocarpon destructans	Root rot/vegetative growth reduction	Tewoldemedhin *et al.*, 2011b
Cylindrocarpon lucidium	Black root lesions	Swinburne, Cartwright, Flack, & Brown, 1975
Cylindrocarpon heteronema	Perennial cankers	Tewoldemedhin, Mazzola, Mostert, *et al.*, 2011
Cylindrocarpon-like: *Dactylonectria torresensis*	Black root lesion on apple rootstock	Manici, *et al.*, 2018
Pythium ultimum	Stunted growth	Mazzola *et al.*, 2002
Pythium irregulare	Stunted growth	Tesfai Tewoldemedhin *et al.*, 2011
Pythium sylvaticum	Stunted growth	Sewell, 1981
Phytophthora cactorum	Twig necrosis, crown rot, yield decline, dominant pathogen in ARD soils	Jeffers, 1982; Tesfai Tewoldemedhin *et al.*, 2011; Mazzola, 1998
Rhizoctonia solani	Stunted growth, root rot, exacerbate *Cylindrocarpon* species linked symptoms	Burr *et al.*, 1978; Mazzola, 1997; Mazzola, 1998; Manici *et al.*, 2018b
Pratylenchus penetrans	Exacerbate ARD	Jaffee, Abawi, 1982; Mai, 1978; Johnson, 1982; Mazzola, 1998; van Schoor *et al.*, 2009

The oomycete genus *Pythium* has been widely accepted to contain a number of pathogenic species that can infect apple trees. Mazzola (2002) described consistent stunting of growth and reduced biomass of apple seedlings when infected with *P. heterothallicum, P. intermedium, P. irregulare and P. ultimum* with varying degrees of symptom severity. *P. ultimum* displayed the highest level of stunting and *P. irregulare* infection showed a persistent negative correlation with growth and exacerbated infection in the presence of brassica seed meal amendments (Mazzola et al., 2002a; Tesfai Tewoldemedhin et al., 2011). *P. sylvaticum* pathogenicity has been confirmed as all tested isolates were able to reduce the growth of apple seedlings, but without other noticeable symptoms except stunting (Sewell, 1981). Some asymptomatic *Pythium* species are able to reduce infection by pathogenic *Pythium* spp. (Mazzola et al., 2009). This is probably due to niche exclusion and competition, suggesting that some *Pythium* species may act as biocontrol agents against ARD pathogens in the rhizosphere.

A second oomycete genus associated with ARD is *Phytophthora*, a common soil-borne pathogen of apple. As well as being pathogenic towards apple, *Phytophthora* spp. cause significant economic loss in crops such as potato and alfalfa (Hansen, 2015). *P. cactorum* is more virulent than its relatives, *P. syringae* and *P. megasperma* in apple. However, all are pathogenic and able to increase twig necrosis associated with crown rot (Jeffers, 1982). *P. cactorum* also has the widest geographical range against apple in the *Phytophthora* genus (Smith, 1990). *Phytophthora* spp. been previously linked to ARD in South Africa, where *P. cactorum* was shown to infect both apple seedlings and mature trees (Tesfai Tewoldemedhin et al., 2011). Similarly, in the USA, *P. cactorum* was the dominant pathogen in a number of ARD soils (Mazzola, 1998). Cases of crown and root rot in apple due to *Phytophthora* are more common in flooded or poorly drained soils and optimum moisture content levels for growth of the plant are close to optimum levels for *Phytophthora* causing these symptoms (Merwin, 1992). It has been observed that ARD is linked to wetter years when more frequent infections by pathogens, such as *Phytophthora* spp., can occur leading to characteristic ARD symptoms.

The fungal pathogen *Rhizoctonia* is capable of attacking a wide range of host plants via root infections, leading to diseases such as stem canker, root rots, and fruit decays. It has been suggested as a possible ARD causal agent. In 1978, *R. solani* was observed as a causative agent of root rot in New York nurseries (T.J. et al., 1978), a common location for recurrent ARD. *R. solani* was later isolated from the roots of ARD-affected trees but was absent in healthy trees. This species was subsequently confirmed as pathogenic to tree roots (Mazzola, 1997). Additionally, *R. solani* isolates can cause significant reductions in apple tree biomass (Mazzola, 1998). *Cylindrocarpon*-like species were observed to work in unison with *R. solani* as the colonisation of apple roots was exacerbated when both *Cylindrocarpon*-like species and *R. solani* were present (Manici et al., 2018b). Understanding such pathogen interactions is one of the keys to understanding ARD.

Pathogenic nematodes such as *P. penetrans* had been associated with ARD since the 1980s (Jaffee, Abawi, 1982). *P. penetrans* has also been previously shown to be pathogenic towards fruit trees in both natural and greenhouse conditions and are particularly high in the soils of apple trees (Mai, 1978). *P. penetrans* can limit the growth of apple trees independently but also create lesions in the roots that provide an access point for other endophytic pathogens associated with ARD (Somera and Mazzola, 2022a). The pathogenicity of *P. penetrans* was confirmed by the application of the nematicides aldicarb and oxamyl at the time of planting, which resulted in an increase in tree growth (Johnson, 1982). In South Africa, *Pratylenchus* spp. were only present in damaging levels in one of six ARD soils tested (van Schoor, Denman and Cook, 2009), indicating it may not be the primary cause of ARD but works synergistically with other components of the ARD pathogenic complex. Similarly, other studies in the USA also identified low levels of *P. penetrans* in most soils and roots of apple orchards (Mazzola, 1998). Thus the available evidence so far points to nematodes not being primarily responsible for ARD but can exacerbate the symptoms observed.

1.4 Current ARD Management Methods

The primary tool to prevent ARD has been the use of pre-planting fumigation of the soil. Soil pasteurisation via fumigation involves the application of a volatile chemical compound to the soil which is then covered to allow effective penetration and eliminate pathogenic microorganisms and pests in the soil. Fumigation has been widely reported as successful in reducing ARD, with semi-selective chemicals (e.g., fenamiphos, matalaxyl, imidacloprid, and phosphonates) used in conjunction with fumigant formulations (e.g., chloropicirin and 1,3-dichloropropene). This approach has been effective at increasing tree growth over a 3-4 year period (Covey and Benson, 1979; Mai and Abawi, 1981; Nicola et al., 2017; Nyoni et al., 2019). However, fumigation is a sterilisation technique so it may also remove beneficial microorganisms in the soil and thus has sometimes resulted in no improvement in conserving or increasing apple tree growth and yield (Yao et al., 2006). Additionally, another consideration has to be the economics of fumigation. Often they are difficult to apply, expensive, and can be hazardous to human health. In addition, they may potentially lead to the loss of important soil micronutrients which could be important in maintaining crop health (Mazzola and Manici, 2012). The most effective broad-spectrum fumigants have now been banned in most developed countries (e.g., Methyl bromide) and the fumigants that remain are not as effective (Xu and Berrie, 2018)

Composts have been examined as a soil amendment to reduce ARD. Compost amendments have been shown to improve apple growth and yield, and increased numbers of beneficial bacteria present such as *Pseudomonas* and *Bacillus* (Liang et al., 2018). This beneficial effect was also demonstrated in many other trials (Forge, Neilsen and Neilsen, 2016; van Schoor, Denman and Cook, 2009). However, there is a lack of studies which have been focused on the effect of the individual component beneficials and their role in enhancing efficacy against ARD. In addition, it may be difficult to develop a standardised compost amendment as they vary significantly in different parts of the world. By understanding the processes of how composts stimulate plant growth in various soils would provide information about optimising soil physiochemical properties for plant growth. Some studies, however, failed to demonstrate benefits of

compost amendments in terms of ARD development, tree growth and yields (Rumberger et al., 2004a; Yao et al., 2006).

Brassica seed meal (BSM) is a pre-plant bio-fumigation tool used to prevent the onset of ARD. As an alternative to green manures, BSM has been shown to control various pathogens implicated in ARD, including *Rhizoctonia* and *Pratylenchus* (Mazzola and Mullinix, 2005). BSM also has a long term effect of reducing the recovery of specific pathogens such as *P. penetrans* (Mazzola and Manici, 2012) and *Pythium* spp. (Mazzola et al., 2009). *Brassica juncea-Sinapis alba* seed meal has been demonstrated to increase tree growth, over a period of 3 years, to levels higher than fumigated soils and altered the bacterial and fungal communities in soil (Mazzola, Hewavitharana and Strauss, 2015a). BSM is now generally considered to be one of the standard practices in treating ARD affected trees, particularly in replant soils.

Biochar was shown to increase growth of perennial ryegrass and soil microbial activity by acting as a source of carbon available to soil microbes (Gregory et al., 2014). Biochar can also change the ratios of the major soil biological components and increase soil enzyme functionality, leading to an altered soil microbiome as well as increased soil microbial activity (Zhu et al., 2017). Recent studies have shown that biochar amendment can lead to increased growth of *Malus hupehensis* seedlings: increased seedling height, dry weight, root respiration, and seedling establishment (Wang et al., 2019). The same study also noted altered fungal communities in soils and decreased abundance of *R. solani*.

1.5 Use of Specific Biological Soil Amendments

The use of soil biological soil amendments to combat soil-borne diseases is becoming more common in trying to alleviate soil-borne pathogens. Assessing the effectiveness of soil amendments is often difficult as a proper comparison to virgin healthy soil is not easy due to a lack of land space available for such trials and the soil chemical and physical properties. Microbiomes of soils can differ significantly among locations situated relatively geographically close to each

other with the additional impact of vegetation also affecting the resident soil microbiomes (Deakin et al., 2018a; Winkelmann et al., 2018b).

The use of molecular approaches has allowed comparative analysis of microbiomes between fumigated and untreated soils in term of the overall microbial composition as well as specific genera being involved in ARD. Nicola *et al.* (2017) showed a positive correlation of the relative abundance of *Bacillus, Streptomyces, Pseudomonas* and *Chaetomium* species with tree growth. Previously, plant growth promoting rhizobacteria (PGPRs) isolates have been shown to significantly increase plant height, root length and dry weight in a number of agricultural crops, such as potato, tomato, maize and wheat (Bhattacharyya and Jha, 2012). Potential mechanisms of action of PGPRs include: ACC deaminase genes (purine metabolism), siderophores (Iron transport), phosphates, phytophormones (chemical messengers in cell), cytokinins (promotes cell division), disease suppression (Bhattacharyya and Jha, 2012). These all are considered to improve plant growth.

Arbuscular mycorrhizal fungi (AMF) are geographically ubiquitous and are symbionts of most land plants by transfer of nitrogen, phosphorous and other micro-minerals to the plant in exchange for photosynthetically fixed carbon, to encouraging growth (Bever et al., 2001; Govindarajulu et al., 2005). The beneficial effects of AMF on the plant include increased root area and root dry weight and larger leaf area which has been observed in model shrubs (Kyllo, Velez and Tyree, 2003a). AMF was able to control pathogens such as a *Phytophthora* sp. as well as stimulating growth of the host plant (Herre, 2007). The available evidence generally supports the consensus that AMF is a viable amendment to improve plant establishment and subsequent growth. Additionally, recent diagnostic tools developed to track and monitor AMF inoculum in the field provide an accurate quantitative assessment of the effectiveness of AMF colonisations for decision making (Robinson-Boyer, Grzyb and Jeffries, 2009).

AMF inoculants have had limited success as an amendment to alleviate ARD, in apple and other horticultural crops. There are limited studies claiming its success as a pre-plant amendment (Forge et al., 2001; Fortuna et al., 1996; Lü, 2018;

Mehta and Bharat, 2013; Ridgway, Kandula and Stewart, 2018; Winkelmann et al., 2018b). AMF has not yet been accepted as standard practice commercially due to inconsistent effects on the growth of seedling, especially in commercial and nursery trials (personal communication, November, 2018). Application of AMF on a field-scale would also be both challenging and difficult to integrate one or a number of beneficial AMF species to the diverse mycorrhizal community within an orchard (Somera and Mazzola, 2022). To allow for larger scale use of AMF commercially, a viable formulation of AMF with sufficient shelf-life and supply must be available (Winkelmann et al., 2018b).

However, in some cases the use of AMF could have some drawbacks. Borowicz (2010) showed a lack of an increase in root and shoot fresh weights in drought conditions despite observing successful AMF colonisation (Borowicz, 2010). Similarly, beneficial effects in other studies have been limited to terms of colour parameters and phenolic compound availability in strawberry, but no changes in fresh weight, diameter or length (Castellanos-Morales et al., 2010). In apple studies, inoculation with AMF was able to increase growth of micro-propagated apple rootstock to levels similar to phosphate amendments, with the effect varying with specific AMF species (Fortuna et al., 1996). Thus, *Glomus intraradices* was most effective between 2-4 moths and *G. mossae* inoculated plants showed greatest growth response after 6 months. AMF inoculations have also been shown to both reduce levels of the pathogenic nematode *P. penetrans* in ARD conducive soils and stimulate growth, but not alleviate symptoms of ARD (Forge et al., 2001; Ridgway, Kandula and Stewart, 2018). It is also worth noting that AMF inoculations appear to affect the soil microbiome differently in organic and conventional orchards, with the former having a more similar ecology to that of virgin soils with an elevated natural AMF richness when compared to conventional soils (Purin, Filho and Stürmer, 2006).

Bacillus subtilis and *B. amyloliquefaciens* are commonly used commercially to increase plant growth by indirect inhibition of pathogens or providing access to some plant growth factors (Kloepper, Ryu and Zhang, 2004; Whipps, 2001). *Bacillus* spp. have also been shown to produce anti-microbial compounds

effective against soil microbiome components including gram-positive bacteria and fungi (Owen et al., 2015). This study also showed that a single inoculation of *B. subtilis* gave better beneficial effects than when applied in a consortium with other PGPRs. Studies have also shown that *B. subtilis* is responsible for both direct and indirect effects on plant health. Bhattacharyya and Jha (2012) found that *B. subtilis* induced resistance, promoted growth and yield in the host plant whilst simultaneously showing antibiosis and outcompeting resident pathogens (Bhattacharyya and Jha, 2012).

B. amyloliquefaciens is another important commercial amendment which has been shown to be beneficial for plant growth by increasing the availability of phytate phosphorous and producing antibiotics which exhibit anti-pathogenic effects (Borriss et al., 2002; Chen et al., 2009). Additional studies with this species showed the ability to produce anti-fungal β-1,3-glucanase which inhibited mycelial growth of the fungal pathogen *Colletotrichum langenarium.* Thus, it is considered to be a good potential biocontrol agent and antagonist of fungal pathogens (Kim and Chung, 2004). In apple orchards a study has also shown the capabilities of *B. amyloliquefaciens* to supress *Erwinia amylovora*, the major causal agent of fire blight disease (Chen et al., 2009), as well as being able to inhibit *R. solani in vitro* which has a role in ARD (Yu et al., 2002).

Pseudomonas spp. are ubiquitous bacteria in agricultural soils. Fluorescent Pseudomonads have been viewed as the most effective strains of *Pseudomonas* PGPR and have been shown to improve the growth and yield in potato, sugar beet and radish resulting in up to 44% increase in yield in the field (Sivasakthi, Usharani and Saranraj, 2014). *P. fluorescens* possesses anti-microbial properties, producing several polyketides that can target pathogens such as *Phythium* spp. to protect various crops (Girlanda et al., 2001). *P. fluorescens* protected wheat from *P. ultimum* root rot; however, its shorter shelf-life compared to *Bacillus* spore forming biocontrol strains can cause reduced viability after a number of weeks, whereas *Bacillus* spp. are more generally commercialised due to better shelf-life and similar beneficial effects on growth and yield (Haas and Défago, 2005).

There has been significant debate about the function of *P. fluorescens* as a PGPR, because it has been described as having little or no effect on pathogenic fungal communities in cucumber rhizosphere soil, despite being hypothesised to have anti-fungal effects as a soil amendment (Girlanda et al., 2001). Additionally, it was once theorised as a causal agent of ARD but this is likely a mislabelling of the species due to interference from other microbes present in the samples (Mazzola, 1998). In contrast, other studies suggest that this bacterium is capable of producing a significant reduction of blue mould of apples caused by *Penicillium* spp. as well as fire-blight caused by *Erwinia amylovora* (Etebarian et al., 2005; Pujol et al., 2006). In relation to ARD, limited studies have been conducted in the use of *Pseudomonas* spp. as a treatment for ARD. Pre-cropped orchard soils with wheat cultivars caused increased growth of apple trees in association with elevated populations of antagonistic *P. fluorescens* in the soil (Gu and Mazzola, 2003). *In vitro* studies of fluorescent *Pseudomonads* amendments resulted in increased growth of apple seedlings (Biró et al., 1998). There is a lack of knowledge of the effect of *P. fluorescens* amendments in the long-term on ARD development under field conditions.

Another potential microbial amendment includes actinomycetes, especially *Streptomyces* spp., which are usually ubiquitous across soils types and can represent nearly 40% of resident soil bacteria (Bubici, 2018). *Streptomyces* species effectively colonise rhizosphere soil around plant roots, probably reflecting the availability of root exudates. Once in the rhizosphere, *Streptomyces* species can produce a number of compounds affecting plant growth, including auxin/IAA, phosphate solubilisation, siderophore action, and various enzymes used for cycling complex nutrients into simpler forms for assimilation (Vurukonda et al., 2018). They have been shown to have plant growth-promoting effects and the capability of producing antibiotics and chitinolytic enzymes to control soilborne *Fusarium* diseases (Bubici, 2018). They have also been demonstrated to be effective plant growth promotors in a wide variety of plants including *Pinus taeda,* wheat, eucalyptus and tomato (Palaniyandi et al., 2014; Sadeghi et al., 2012; Salla et al., 2014; de Vasconcellos and Cardoso, 2009). Some *Streptomyces* spp. have also been shown to aid colonisation by mycorrhiza fungi

as an additional plant growth promoter, exhibiting a potential synergistic effect of being a PGPR and acting as a biocontrol agent (Franco-Correa et al., 2010). For example, *Streptomyces goshikiensis* produced anti-fungal organic compounds and caused a reduction in pathogenic fungi as well as promoting growth of melon (Faheem et al., 2015). A *S. corchorusii* strain was shown to produce cell wall degrading hydroplytic enzymes such as chitinase, lipase and protease, as well as stimulating rice growth under field conditions (Tamreihao et al., 2016). Because of these characteristics, there are commercial products based on these species such as Mycostop® (*Streptomyces griseovirdis*) which are marketed for the control of pathogens such as *Fusarium, Phytophthora, Alternaria* and *Pythium* (Bhattacharyya and Jha, 2012). Many of these target pathogens correspond to those considered to be involved in ARD.

1.6 Temperature, Drought and Soil Microbiomes

There is very little information on how abiotic factors may impact the prevalence of ARD. The IPCC report suggests an increase in temperature of between 2-4 ℃ by 2050. Atmospheric CO_2 exposure is expected to increase by at least twice the current levels (400 vs 800-1000 ppm CO_2) (Intergovernmental Panel on Climate Change, 2014). The IPCC report also attributed extreme daily temperature and extreme precipitation events to be “very likely” under climate change scenarios. In soil, the temperature changes may be considerably buffered but significantly higher levels of CO_2 may be occur than the present levels.

Elevated temperatures can lead to increased microbiome activity and respiration of carbon in soils influencing the rhizosphere microbial populations and their interactions with plant roots (Figure 1.3; Zogg et al., 1997). Soil organic matter degradation is under environmental constraints, influencing rates of soil organic matter degradation, which may increase under climate change stress (Davidson and Janssens, 2006).

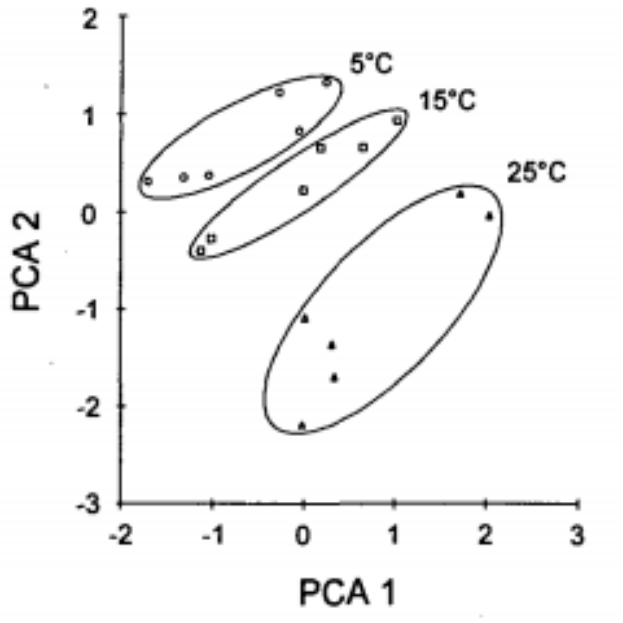

Figure 1.3 Principal component analysis (PCA) of the mole percentage distribution of 30 phospholipid fatty acids (PLFAs) after 16 week incubation at 5, 15 or 25ºC (Zogg et al., 1997).

Increased temperature also has an impact on the relative ratio of bacterial-to-fungal activity, showing a progression towards increased bacterial colonisation compared to fungal activity in agricultural soils (PietikÃ¥inen, Pettersson and BÃ¥Ã¥th, 2005). This shift in dominant organisms could change the microbial functionality and health of soils. Thus, research needs to be focused on the impact of microbiome shifts and how soil amendments will survive and function in increased soil temperatures. The initial temperature has a profound impact on microbial activity and this reaches an optimum at around 30^{o}C and then declines at >30-35^{o}C quite rapidly (Figure 1.4; Barcenas-Moreno et al., 2009), however soil temperatures, particularly deeper soils, will be unlikely to reach these values in temperate climates.

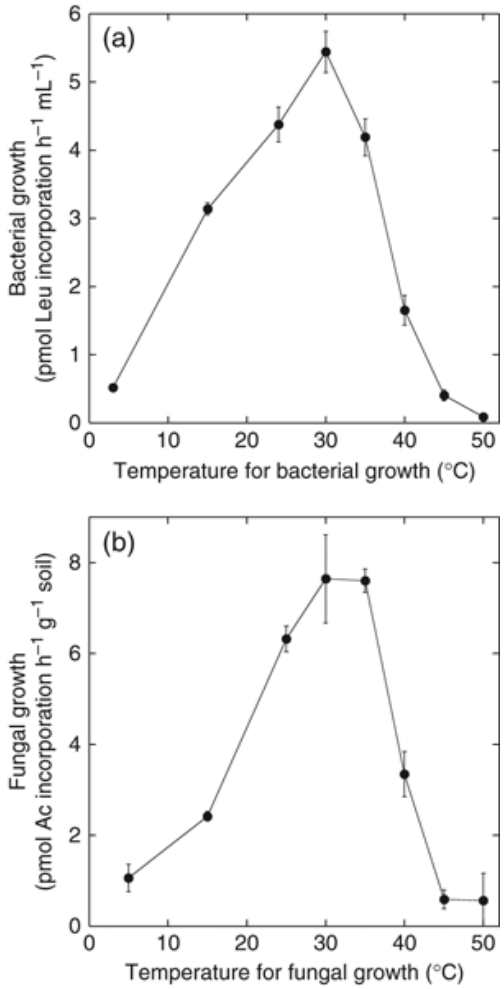

Figure 1.4 Initial temperature dependence of the soil microbial community. (a) Bacterial growth at different temperatures, estimated using leucine incorporation. (b) Fungal growth at different temperatures estimated using acetate-in-ergosterol incorporation (Barcenas-Moreno et al., 2009).

Increased temperature will also lead to increased drought stress if increased water is unavailable and both these factors together will have the highest impact on the rhizosphere microbiome. In grassland, the number of species in the microbiome was more affected than the actual community structure (Sheik et al., 2011). *Actinobacteria* were shown to recover slower than other essential carbon decomposers (*Acidobacteria*) in grassland soils, highlighting a potentially higher demand on resources such as water and associated nutrients for some bacterial phyla compared to others under increased temperatures. This study only

focussed on the top 15cm of the soil, which may not be applicable to deep-rooted trees such as apple.

Drought conditions also have an impact on the functionality and resilience of beneficial organisms both native and those amended to the soil. Root length colonisation (RLC) by AMF is increased in drought conditions, exhibiting a shift in the reliance on the mutualistic fungus in dry conditions. This reliance is due to the hyphal spanning of air gaps between shrinking roots and soil, increasing water absorption of the target plant (Augé, 2004; Robinson-Boyer, Grzyb and Jeffries, 2009). Understanding how this mutualistic relationship is increased under these conditions and the associated benefits on yield could prove important to exploit beneficial microorganisms in conventional cropping systems under abiotic stresses. Similarly, a benefit/cost analysis of the use of AMF in wheat is higher in water-stressed crops than in well-watered conditions (Al-Karaki, 1998).

Other beneficial microorganism such as PGPR have similar potential to alleviate the impact of water stress on plants. Rhizobacteria inoculations were effective in improving both growth and anti-oxidant properties and reducing build-up of toxic reactive oxygen species in basil crops (Heidari and Golpayegani, 2012). The benefits of both PGPR and AMF suggests that the use of such consortia of mixed beneficial microbes to combat water stress may provide significant benefits to plants.

1.7 Soil Microbiomes and Elevated CO_2 Exposure

Under climate change scenarios, crop plants and soils will be exposed to higher concentrations of CO_2, affecting crop production directly and/or indirectly through soil functions via soil microbial populations as well as increasingly acidic soils because of the increased level of carbonic acid. Currently, there is limited information on how increased CO_2 will impact on apple production.

In conditions of elevated atmospheric CO_2, soil organic carbon has been shown to stay the same, highlighting the inability of soils to naturally stabilise or reduce atmospheric CO_2 (Carney et al., 2007). Although elevated atmospheric CO_2

caused increased soil bacterial diversity initially, bacterial populations decreased exponentially as CO_2 concentrations increased from < 5000 to > 10,000 ppm (Ma et al., 2017). Increase in CO_2 concentration (20% above the ambient) of grassland soil cores showed an increase in the greenhouse gas N_2O released from the soil, but no effect on the nitrogen cycling communities (Brenzinger et al., 2017). Increased atmospheric CO_2 may therefore not directly affect specific beneficial microbial communities associated with plants, but may increase the rate of greenhouse gases released from soils contributing to further climate change and increasing atmospheric CO_2. However, these studies did not address the specialised nature of soils due to impact of vegetation through exudation that recruit microbes to the rhizosphere surrounding the root.

1.8 Microbiome Communities at Different Soil Depths

Both CO_2 and temperature vary with the soil depth. Increasing soil depth leads to more anaerobic conditions. At depths >2 meters, the bacterial diversity becomes more similar to that in flood plains (Steger et al., 2019). Roots at this depth will interact with a less diverse microbiome and may lack specific beneficials or facilitate the survival of pathogenic complexes. Depth differences are similar to those observed between separate biomes and locations. In deeper soils, *Archaea* are more abundant whereas *Bacteroidetes, α, β and γ-Proteobacteria* all decline exponentially as the soil depth increases; on the other hand *Acidobacteria* are relatively unaffected (Eilers et al., 2012).

Approx. 92% of root biomass in dwarfing rootstock (M9) was observed at approximately 20cm depth when compared to 40cm and 60cm where only 6% and 2% respectively were present (Ma et al., 2019). Approximately 50% of the fine root mass was scattered within the top 0-20cm of the soil depth. More vigorous rootstocks such as “Baleng Crab” have 50% of their fine root mass scattered between 100 and 150cm soil depth. Fine roots are associated with water and nutrient uptake in the plant so their spatial distribution is key to understanding the vicinity of root-associated microbiome. Fine root numbers are lower in dwarfing rootstocks than in more vigorous rootstocks (An et al., 2017).

M9 dwarfing rootstocks are unlikely to interact with microbes below 30-40cm whereas vigorous rootstocks will be utilising microbial activity and nutrients in deeper soil. It is important for growers to understand the type of rootstock they are planting to amend soils at the correct depth to avoid wastage of products, particularly in no till-systems, reducing the cost of production for the grower.

1.9 Soil Microbiomes in Organic and Conventional Production Systems

Conventional apple production systems require intensive inputs of both fertilizers and crop protection products (particularly synthetic pesticides). Consumers are now demanding more food production systems that are more environmental-friendly and sustainable (Tilman et al., 2002); (Gomiero et al., 2011). Organic farming was shown to have 30% higher species richness and 50% higher density as well as better control of a number of pests than conventional production systems (Tscharntke et al., 2012). A recent meta-analysis study showed an overall suppression of herbivores, increased natural enemies, and reduced crop damage but also reduced yield in organic systems compared to conventional agriculture (Letourneau et al., 2011).

As ARD appears to be a soil-driven condition, a key factor is soil health and nutrition after successive generations which may lead to the introduction of a pathogen or complex of pathogens. Therefore, organic farming techniques to combat ARD must focus on understanding their soil microbiota populations which provide a large impact on plant growth and ultimately yield. Despite studies showing that intensification of conventional farming has caused soils with reduced microbial diversity in comparison to organic soils, the specific changes to the microbiota are not known, suggesting that pathogenic microbes linked to ARD may be more prevalent in organic soils (Postma-Blaauw et al., 2010). Analysis of the organic soil microbiome suggests that the major determinant of microbial diversity in soils is the type of organic fertilizer added, highlighting the importance of improving and optimizing the amendments added to the soil to improve crop productivity. Potential biocontrol agents in the order *Hypocreales*

were observed at higher levels in organic systems than in conventional systems, likely due to excessive fungicide use (Hartmann et al., 2015). It should be noted that increased diversity in soils may also allow the introduction of non-native microbes that could be detrimental to tree health.

1.10 Rootstock Genetic Tolerance

In the production of apples the grafting process is essential to provide optimal growth, water and nutrient uptake and transport of minerals from roots (with help from associated rhizosphere microorganisms) (Martínez-Ballesta et al., 2010). The choice of which rootstock genotype to be used for what scion requires consideration of multiple factors. ARD susceptible rootstocks include M7 and M26 when compared to the more tolerant CG30, CG210 and CG 6210 (St. Laurent et al., 2010; Rumberger et al., 2004a). Dwarfing rootstock such as M9 are preferred in orchard spaces due to their small size and ability to produce high yields in less space. In recent years, problems associated with M9, including ARD, have led to nursery growers opting for more vigorous rootstocks such as MM106 (Frank P Matthews, personal communication, November, 2018). Different rootstocks may differ in their tolerance to ARD depending on the dominant causative pathogen(s) at specific locations (Mazzola and Manici, 2012).

Previous studies of the effects of different rootstock on ARD concluded that the rootstock genotype and the position within the orchard (grassy alleyways vs previous rows), influenced the onset of ARD (Rumberger et al., 2004a). Other studies have shown the ability of different rootstocks to select for different beneficial microbes in the rhizosphere, which may be associated with differing levels of tolerance to ARD (Isutsa and Merwin, 2000). Recent studies have focused on the elucidation of the molecular defence responses of tolerant rootstocks to the introduction of suspected ARD causal agents (Zhu, Fazio and Mazzola, 2014). The current understanding focuses on breeding of “tolerant” rootstocks which have a natural genetic tolerance to ARD. A major determinant of ARD is the genetic relationship between newly planted rootstocks and those previously planted, with those closely related to the previously planted genotype

more susceptible to ARD despite being described as genetically tolerant rootstocks (Deakin et al., 2019). The use of both tolerant rootstocks, genetically dissimilar to the previous rootstocks, and planted in the alleyways rather than the previous tree stations could provide a integrated management system to minimise ARD severity using the genetic resources of the rootstock alone.

1.11 Knowledge Gaps

To date, there is very limited knowledge on non-chemical management strategies for ARD in the UK and limited studies on the efficacy of commercially available biological soil amendments as a mechanism to increase the establishment of young replanted trees. This project has examined the implementation of a number of beneficial bio-fumigants, biocontrol agents, and plant-growth-promoting rhizobacteria in field and semi-field conditions to assess the efficacy of the amendments to increase the establishment of young apple trees as a metric of ARD severity. In addition, the the impact of cultural practices including rootstock genotype selection and planting position within replanted orchards was investigated as a strategy to minimise the detrimental effects of ARD. A hybrid system of growth and health analysis concurrently with rhizosphere microbiome community analysis was made to identify candidate beneficials and causal agents of ARD to provide a comprehensive overview of the efficacy of the biological soil amendments, within the context of climate change abiotic stresses. This project has thus addressed gaps in the current knowledge of ARD management in the UK to understand whether an integrated management strategy of cultural practices combined with pre-plant soil amendments could be an effective strategy to minimise ARD stress in replanted apple orchards.

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2 Aims and Objectives

2.1 Project Aims

The aim of the project was to identify the efficacy of beneficial microorganisms for pre-plant amendment and alternative non-chemical management strategies to improve soil health and hence reduce/eliminate ARD. The overall hypothesis of the research was that beneficial soil amendments will provide similar control of ARD as other non-chemical management strategies as an alternative to chemical fumigation.

The following hypotheses were tested:

(i) application of biological soil amendments will positively impact the host and via differences in fungal and bacterial rhizosphere microbiome communities eg. plant health and growth,

(ii) understand if biological amendments applied in a consortium are more beneficial to apple tree establishment and if they alter soil microbiome communities and soil function,

(ii) applying biological soil amendments together in a consortia will be more beneficial to the host than individual amendments by altering soil microbiome communities and soil function,

(iii) tolerant rootstocks, planting position (alleyway vs tree station), and rootstock genotype genetic relationship to the previously planted rootstock will all benefit positively impact against ARD,

(iv) temperature and CO_2 increase will alter populations of potentially beneficial and pathogenic fungi and bacteria in bulk orchard soil with different management practices.

2.2 Studies Conducted

The following studies have been carried out to meet the objectives:

(a) Biological soil amendment effect on tree establishment in field conditions

- Assess the efficacy of four biological soil amendments Rootgrow™ AMF consortium mix, *Bacillus amyloliquefasciens*, *Pseudomonas fluorescens*, and *Brassica carinata* seed meal pellets (BSM) on the establishment of apple trees.
    - Difference in response between two dessert varieties, Braeburn and Gala.
    - Effect on rhizosphere microbiome communities.
    - Identify candidate beneficial or detrimental populations in the rhizosphere.

(b) Establishment of apple rootstocks when a consortium of biological soil amendments are applied

- Impact of the application of *Diversispora* sp., *Trichoderma harzianum*, *Bacillus amyloliquefasciens*, and *Pseudomonas fluorescens* on establishment of M9 rootstocks in a potted trial.
    - Benefit associated with applying multiple amendments in consortium pre-planting.
    - Effect on rhizosphere microbial communities
    - Differences in individual populations in the soil due to applying each of the amendments

(c) Inter-Row Cropping and Rootstock Genotype Selection

- Consequence on ARD severity by rotating rootstock genotype planted in both the previous tree station and the corresponding alleyway
    - Observe if tree growth in the alleyways was greater than the previous tree station (hence ARD).
    - ARD severity differences between the different rootstock genotypes with different reported tolerances to ARD.

- Identify if rootstocks with most severe ARD were genetically related to the previously planted rootstock on the site.
- Identify both candidate beneficial populations associated with non-ARD tree rhizosphere and candidate causal agents associated with ARD trees rhizosphere.
-

(d) **Resilience of microbial communities in bulk apple orchard soil when exposed to climate change abiotic factors**

- Elucidate shifts in bulk soil microbiome communities due to increased temperature and elevated CO_2.
  - Consequence on bacterial and fungal communities due to the climatic abiotic factors.
  - Compare the response of microbiome community to climate abiotic factors in organically managed soils and conventionally (chemical) managed soils.

2.3 Connection of the Chapters

The two studies on biological soil amendments both individually and in consortium inform one another on the efficacy of the amendments in field and semi-field conditions. The rootstock genotype experiment informs both the amendment experiments as differing responses from different genotypes may facilitate a different microbiome and thus be more/less tolerant to ARD. The climate change experiment attempts to put the results from the other three chapters into context temporally, as the communities we identify currently in soils may be impacted by the abiotic stresses of climate change in future years and could therefore affect tree health and ARD. Taking the results of each of the chapters together should create a comprehensive overview of a number of non-chemical management strategies for ARD to suggest an integrated management strategy to minimise ARD severity for growers to utilise. A schematic representation of the interconnectivity of the studies is shown in Figure 2.1.

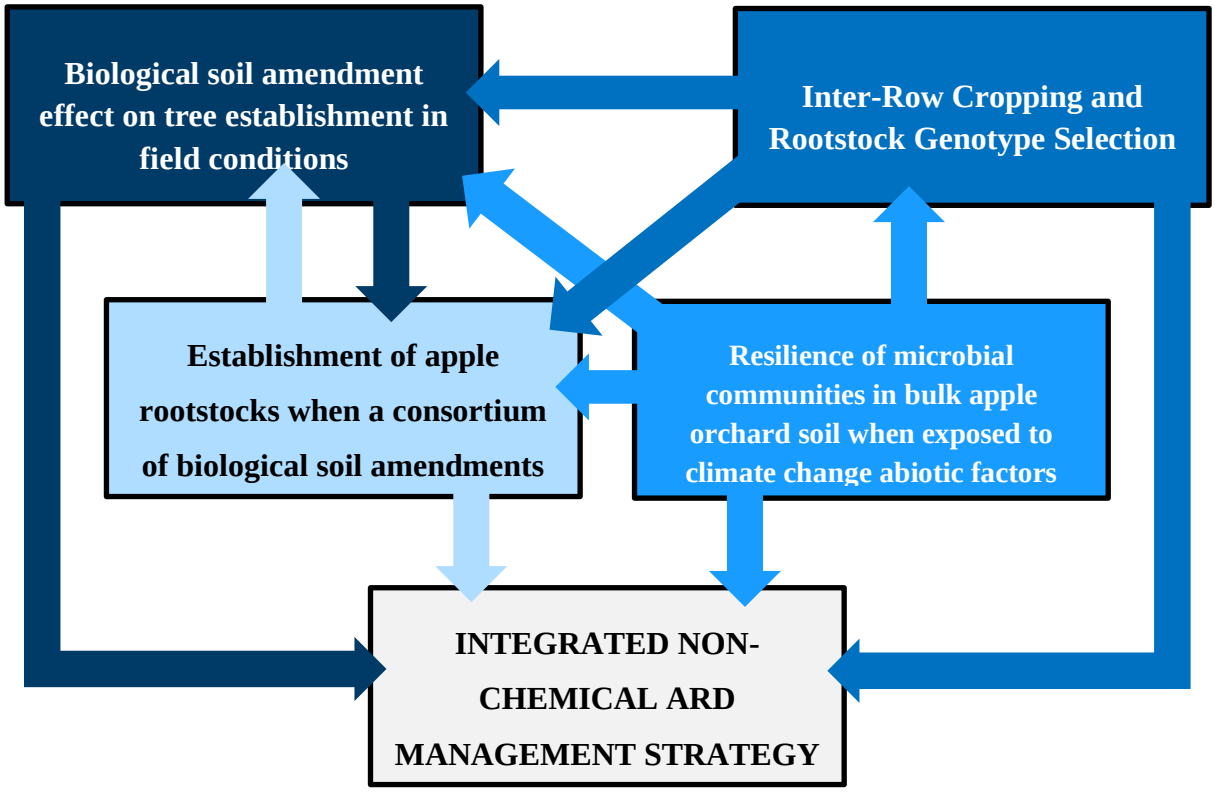

Figure 2.1 Schematic representation of the experimental work conducted and the connection of each chapter to one another and to an overall non-chemical management strategy for ARD.

2.4 List of Submitted Work

- **“Effect on microbial communities in apple orchard soil when exposed short-term to climate change abiotic factors and different orchard management practices”**
  C. Cook, N. Magan, L. Robinson-Boyer, X. Xu
  Journal of Applied Microbiology
  Submitted on the 26th August 2021. In final Review.

- **"Inter-Row Cropping and Rootstock Genotype Selection as a Management Strategy for Apple Replant Disease in a UK Cider Orchard"**
  C. Cook, N. Magan, X. Xu
  Plant and Soil
  Submitted on the 01st August 2022. In Review.

- **“The Effect of Biologically Amending Soils on Early Establishment of M9 Rootstocks and Rhizosphere Microbial Communities”**
  C. Cook, N. Magan, L. Robinson-Boyer, X. Xu
  In Preparation.

3 Early Establishment of Apple Trees with Biologically Amended Soils and the Effect on Rhizosphere Microbial Community

3.1 Abstract

3.1.1 Purpose

Apple replant disease (ARD) is a disorder where previously bountiful apple orchards produce unsatisfactory growth and yields when the same or closely related species is planted on the same site. ARD has been previously treated using broad-spectrum chemical fumigation. The use of single-species biological soil amendments to promote growth of the young trees could provide a non-chemical strategy to lower or eliminate the use of synthetic chemical fumigants.

3.1.2 Methods

Prior to planting, soils were amended with either arbuscular mycorrhizal fungi (AMF) species mix, *Bacillus amyloliquefasciens*, *Pseudomonas fluorescens*, or *Brassica carinata* seed meal pellets. Above-ground establishment of the trees was assessed for the first three growing seasons between 2019-2021 for two varieties of apple, Gala and Braeburn, on M9 rootstocks. High-throughput sequencing of fungal (ITS) and bacterial (16S) communities was used to compare differences due to the effect of treatment and variety in the rhizosphere. Individual operational taxonomic units (OTUs) were used to identify the effect on candidate beneficial or pathogenic population abundance in the soils.

3.1.3 Results

The effect of treatment was cultivar specific for Braeburn and Gala. *Pseudomonas fluorescens* was positively correlated with increased girth of Gala trees but had no effect on Braeburn trees. AMF application led to increased fruit yields of Braeburn. No differences in between-sample community diversity were detected due to different amendments but fungal communities were close to

significantly different in the rhizosphere of the two varieties (P = 0.09). A number of genera associated with plant pathogens or containing species reported as beneficial to plant growth were also identified amongst the OTUs with the greatest log2 fold change due to both treatment and variety effect.

3.1.4 Conclusions

The study suggests the effect of individual amendments is cultivar specific and strategies for biological amendment will be genotypic as well as species specific to maximise benefit. Although community-level differences were not observed due to treatment, the operational taxonomic units (OTUs) with differential abundance provide candidate beneficials for pre-planting application but suggest a synergistic effect in applying amendments such as AMF that subsequently aid the colonisation of additional beneficial microorganisms that may aid tree establishment as a strategy to minimise ARD severity.

3.2 Introduction

Apple Replant Disease (ARD), previously termed “replant problem”, is a disorder where previously high-yielding perennial fruit orchards show unsatisfactory growth and yield in replanted trees (Mai and Abawi, 1981). ARD has become increasingly difficult to control as finding virgin land to establish new orchards becomes increasingly difficult and banning of broad-spectrum chemical fumigants and formulations that remain have been reported as less effective compared to their predecessors (Somera and Mazzola, 2022b; Xu and Berrie, 2018). Apple (*Malus domestica*) can be severely affected by ARD in newly planted orchards, particularly in nursery orchards where tree turnover and successive replanting of trees are far more frequent than in fruit production orchards where older well-established trees may have a chance to recover from ARD. Modern systems of apple growing require much higher investment to induce higher yields and earlier fruit production (Hoestra, 1968), increasing the number of cases of ARD.

ARD causes a host of negative impacts on the replanted apple trees, such as stunted growth, discolouration of apple skin, reduced yield, reduced fruit size/weight, altered fruit aroma, and tree death (LIU et al., 2014; Mazzola and Manici, 2012; Zhu, Fazio and Mazzola, 2014). These changes through ARD symptoms may decrease profitability by 50% during the orchards life (van Schoor, Denman and Cook, 2009). The symptoms of ARD can be easily missed as stunting is often subtle and early stage ARD can only be detected when fumigated and unfumigated soils are compared (Jackson, 1979; Jaffee, 1982). Young apple trees, particularly in nurseries, are of particular concern as the symptoms of ARD can occur as early as 1 year after planting in the orchard. If death of these young trees does not occur, then characteristic ARD symptoms emerge. Additional to the above-ground effects described above, discoloured roots, root tip necrosis, and reduction in root biomass are all evident below the surface (Mazzola and Manici, 2012).

It is generally accepted that the cause of ARD is biotic due to basic soil properties remaining unaffected in ARD affected tree soils (Simon et al., 2020). The most

accepted hypothesis is that changes in the soil microbiome are the basis for the onset of ARD (Mazzola and Manici, 2012). The non-specific interaction of multiple pathogenic microorganisms including the oomycetes *Pythium* and *Phytophthora* and the fungi *Cylindrocarpon*, *Rhizoctonia,* and *Fusarium* with each other is likely the trigger for the onset of ARD (Winkelmann et al., 2018b). ARD is also exacerbated by the root-lesion nematode *Pratylenchus penetrans* which allows lesions in the roots as an entry point for the pathogens in the rhizosphere (Jaffee, Abawi, 1982).

The benchmark for ARD control used pre-planting fumigation of the soils by applying volatile chemical compounds to sterilise the soils of potentially pathogenic microorganisms and pests. However these broad spectrum chemicals are either currently or soon will be banned due to their negative effect on the environment (Nicola et al., 2017), making these treatments unsustainable. Non-chemical treatments include brassica seed meal (BSM), a biofumigant, that provides anti-fungal and anti-nematode action . BSM has been extensively tested as an effective alternative to chemical treatments providing a non-chemical approach amidst the uncertainty over future chemical use (Mazzola and Brown, 2010; Mazzola and Cohen, 2005; Wang and Mazzola, 2019; Weerakoon et al., 2012).

The use of single-species biological soil amendments could provide a biological alternative to broad-spectrum chemical fumigation as a renewable beneficial to stimulate increased growth of apple trees. Arbuscular mycorrhizal fungi (AMF) inoculated soils have been shown to support fewer numbers of the suspected causal agent *P. penetrans* in ARD soils as well as being a well-documented symbiont increasing growth of host plant (Forge et al., 2001; Kyllo, Velez and Tyree, 2003b). Inoculation with AMF has previously been shown to produce better establishment of apple seedlings. Inoculation with *Glomus* spp. has been shown to increase the growth of apple seedlings with a more significant impact on seedling growth seen in ARD soils (Čatská, 1994a; Mehta and Bharat, 2013). Other AMF species such as *Scutellospora calospora* have also been shown to increase seedling growth in ARD soils compared to healthy soils (Ridgway,

Kandula and Stewart, 2008). In strawberries, a mixture of *Funneliformis mosseae*, *Funneliformis geosporus* has been shown to increase plant growth in drought-stressed plants (Boyer et al., 2015) and likely could have a similar function in inoculated apple trees.

Recent studies have shown that plant-growth-promoting rhizobacteria (PGPR) can be used to increase the establishment of apple seedlings in soil. One candidate is *Bacillus* sp. such as *B. vallismortis* and *B. amyloliquefasciens* that have been shown to increase apple seedling growth in ARD soils and alter the microbiome communities present in the soils, but better establishment was seen in soils treated with methyl bromide fumigation (Duan et al., 2022c, 2022a). *Bacillus subtilis* has also been shown to promote tree growth and increase yields via biocontrol of soil-borne pathogens (Utkhede and Smith, 1992; Winkelmann et al., 2018a). Other PGPR candidates include fluorescent *Pseudomonas* species which have previously been identified as prime candidates to control plant root disease (O'Sullivan and O'Gara, 1992). Increased populations of fluorescent *Pseudomonas* have been correlated with replant disease in wheat by control of *Rhizoctonia* (Mazzola et al., 2002b). Fluorescent *Pseudomonas* species have also been shown as effective root colonisers in apple, and exhibit control of deleterious fungal and bacteria through indirect plant growth promotion such as production of antifungal antibiotics and siderophores (Sharma et al., 2017).

In this study, the efficacy of three biological soil amendments, rootgrow™ AMF consortium mix, *Bacillus amyloliquefasciens*, and *Pseudomonas fluorescens* were tested as a pre-planting amendment for two dessert orchard varieties, Braeburn and Gala, in a replanted orchard to improve the establishment and yield of the trees over a 3 year period. The efficacy of BSM as a biofumigant was also tested compared to the biological soil amendments. The study aimed to test the hypothesis that pre-plant soil amendment will positively affect the host tree establishment and yield in two different dessert apple scion varieties.

3.3 Materials and Methods

3.3.1 Orchard General Characteristics

The study was conducted on a dessert apple orchard at NIAB, East Malling in Kent (latitude 51.286170, longitude 0.450902). The soil on the site was classified in 2021 as sandy silt loam in texture with a soil pH of 6.9. The available P, K, and Mg were 31.4 mg/l, 151 mg/l, and 252 mg/l, respectively (NRM Laboratories, Winklefield Row, Berkshire, UK). The orchard has previously been a long-term planting of Royal Gala and Queen Cox on M9 rootstock.

3.3.2 Orchard Design, Rootstock, and Scion Selection

The study consisted of four non-chemical pre-planting amendments applied to two-year-old trees of Gala (Brookfield) and Braeburn (Mariri Red) on M9 rootstocks, two important apple varieties in the UK, and un-grafted 2-year-old M9 rootstocks. A split plot design of four blocks was used for the study. There were two subplots within each block randomly assigned to one of the two varieties. Each subplot consisted of five tree stations. Each of the five tree stations within the subplot was randomly assigned to one of the five treatments of the same variety. The un-grafted rootstocks arrived as pre-cut bare rooted trees and therefore were not a good comparison to the grafted trees and were removed from the study shortly after planting.

3.3.3 Pre-Planting Treatments and Planting

The trees were planted in January 2019. Pre-planting amendments were selected based on their efficacy from the literature used previously in apples or other important horticultural crops. *Brassica carinata* seed meal pellets (BSM) (Biofence®, Tozer Seeds, UK) were used as a biofumigant amendment. Holes were dug 7 days before planting and BSM was mixed with the dug soil at the rate of 300 g per tree station over a 1 m^2 area surrounding where the trunk of the tree would be planted to allow for the pellets to pick up water, break down and de-gas. Holes were then re-filled and left for 7 days to prevent waterlogging in the dug holes. Rootgrow™ AMF mixture (Plantworks®, UK) was applied as a 25 ml

scoop of the granular formulation around the tree's root ball and at the bottom of the planting hole to encourage contact of the mycorrhizae with the root system to aid colonization of the root by AMF. Two plant-growth-promoting rhizobacteria (PGPR) were selected as a single species amendment: *B. amyloliquefasciens* (Plantworks®, UK) and *P. fluorescens* (Plantworks®, UK). 500 ml of each diluted PGPR at a concentration of 3.57 x 10^6 cfu/ml was watered around the trunk of the tree to soak into the soil one week after the trees were planted. Trees were managed using commercial practice but did not have supplementary irrigation.

3.3.4 Tree Establishment and Yield Analysis

Trees were assessed for their height in cm from the graft union to the end of the leader branch using a meter rule (not including leaves). The girth of the tree trunk was measured as the circumference of the tree trunk 5 cm above the graft union. Trees were measured in the early-season (March), mid-season (July), and late-season (October/November) for three growing seasons. T1, T4, and T7 were the early season measurements in 2019, 2020 and 2021 respectively. T2 and T5 were the mid-season measurements in 2019 and 2020 (no mid-season measurement was taken in 2021). T3, T6, and T8 were the late-season measurements for 2019, 2020, and 2021. To compare the establishment of the trees due to treatment and variety difference, a repeat measurement linear mixed model was used to predict height and girth values as a measurement of establishment in the lme4 package v 1.1-28 (Bates et al., 2015). The variety and treatment effects were fixed in the model and the tree station position was treated as a random effect. The plot effect (variety position within blocks) had variance of 0 so was not included in the final model. The car R package (Fox and Weisberg, 2019) was used to calculate p-values using Wald chi-square test for the fixed effects in the model.

Yield was assessed for each tree using both the number of fruit on each tree and the weight of the fruit in kg. Fruit size was also assessed as a measure of class I. Fruit was measured on a scale between <50 mm fruit and >70 mm fruit. In 2019 only the number of fruit were measured on the trees due to the low number of fruit on each tree in the first season. In 2020 fruit quality was only measured up

to >65 mm fruit but due to an increasing number of larger fruit in 2021 the >70 mm category was added. A Poisson model was used to test paired comparisons of treatments (two treatments set to be the identical in the model) to the full model to identify statistical differences in yield between the pair of treatments.

3.3.5 Soil and Root Sampling

Rhizosphere soil was collected in November 2021 non-destructively from the orchard. Apple roots were traced from the tree and fine root was obtained from between 10 cm – 30 cm below the surface to avoid organic material from grasses and weeds present in the top 10cm of the soil. The soil aggregated to the root after light hand shaking was classified as rhizosphere soil and collected into a sterile polythene sample bag. The trowel was sterilised with 70% ethanol and fresh sterile gloves were applied between each tree to prevent cross contamination of soils. The fine root was collected into a separate polythene bag. Roots were dug from three locations symmetrically surrounding the trunk of the tree and bulked into the same sample bag. Bulk soil surrounding the root was collected into a separate polythene bag for functional analysis of the soil. 0.5 g of fine root were transferred to histology cassettes and stored in dH_2O for 24 hours until root staining to test for mycorrhizae colonisation. 0.25 g of rhizosphere soil was transferred to 1 ml Eppendorf tubes and stored at -20 ℃ prior to DNA extraction. Bulk soil was stored at -4 ℃ until required for functional tests.

3.3.6 Assessment for AMF Colonisation

To observe the structures of AMF within the root to confirm colonisation of the root, a trypan blue stain was used. Trees treated with AMF and control trees with no pre-planting amendment were the only samples stained to identify if AMF amended soils had a higher percentage of root length colonised by AMF than the control based on a standard protocol (https://invam.wvu.edu/methods/mycorrhizae/staining-roots). The cassettes containing 0.5 g of fine root were cleared in 20 % (w/v) KOH for 1 hour at 90 ℃ in a glass jar placed in a bench top water bath. Samples were then rinsed three times with dH2O to remove any KOH from the roots. Cassettes were then

covered with 2 % HCL for 1 hour at 20 ℃ and immediately covered with 0.05 % trypan blue (w/v) in lactoglycerol (5 : 1 : 1 lactic acid, glycerol, water) and incubated in the 90 ℃ water bath for 1 hour. Cassettes were briefly rinsed in dH_2O and covered and stored in 50 % (v/v) glycerol to de-stain the roots.

To quantify the percentage of root length colonised by AMF, a grid-line intersect method was used (Giovannetti and Mosse, 1980). The method involved random dispersion of roots in a 9 cm diameter petri dish with grid lines. The petri dish was then observed under a dissecting microscope and each intersection between root and grid line scored as positive or negative for the presence of stained AMF structures. The number of positive intersections with AMF structures was converted to a percentage of the total intersects observed and each sample was assigned a % root-length-colonisation (RLC) score. A minimum of 100 intersections were used for each sample. A logistic regression model was used to analyse the % RLC data.

3.3.7 Soil Extract and API®ZYM Enzyme Assay Analysis

The API®ZYM (Biomereux, France) semi-quantitative assessment of bacterial enzymatic activities system was used to identify differences in enzyme activity in the soil extract samples. Fresh soil of 5 g was oven dried at 105 ℃ for 24 hours. The soil at 0 % water holding capacity was referred to as dry soil. The dry soil was wetted to saturation with sterile dH_2O through a funnel with 11 um pore filter paper to ensure all water not saturated in the soil could run through the funnel without soil particle loss. The wet soil was then also weighed as 100 % water holding capacity. Water holding capacity of the fresh soil was then calculated per gram of soil. 10 g of the collected bulk soil surrounding the root from each sample was oven dried at 105 ℃ for 24 hours and subsequently adjusted to 60 % water holding capacity in 50 ml falcon tubes (approximately 0.28 ml sterile dH_2O/g soil) and stored in dark static conditions for 5 days. After the incubation period sterile dH_2O was added to each tube at a 1:1.5 ratio of soil to water. Tubes were thoroughly mixed on a horizontal shaker at 200 rpm for 1 hour. Soil extract was obtained by centrifugation of the tubes at 4,500 xg for 30 mins and the resulting

supernatant transferred to a fresh 50 ml falcon tube. The soil extract supernatant was stored at 4 ℃ until required for API®ZYM analysis.

API®ZYM trays were humidified with 5 ml of dH_2O before adding the enzyme strips into the trays. 65 µl of soil extract was added to each of the 20 enzyme cupules for each sample and covered. Strips were incubated at 37 ℃ for 4 hours in dark static conditions. After incubation one drop of ZYM A buffer followed by one drop of ZYM B buffer were added to each cupule and colour change allowed to develop for 5 minutes. The colour change of each cupule was scored according to the positive or negative colour change result listed in the API®ZYM manufacturer's protocol from a score of 0-5 (0/1 = negative or negligible colour change, 2/3 = intermediate colour change, 4/5 = positive result). The 20 enzymes used in the assay are listed in Table A.1.

3.3.8 Amplicon-sequencing of the Rhizosphere Soils for Bacterial and Fungal Communities

3.3.8.1 Rhizosphere DNA Extraction

The methods used for amplicon sequencing and sequencing processing to produce operational taxonomic unit (OTU) frequency tables has previously been described (Deakin et al., 2018b). DNA was extracted from 0.25 g of rhizosphere soil samples using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, USA) using the manufacturers protocol, with the additional use of a benchtop beating homogeniser to disrupt samples (Fastprep FP120, Qbiogene, USA). Both a spectrophotometer (Nanodrop 1,000, Thermo Fisher Scientific, UK) and a fluorometer (Quibit 2.0, Thermo Fisher Scientific, UK) were used to determine concentration and purity of the DNA samples. DNA was stored at -20 ℃. 20 µl of DNA sample was sent to Novogene (Cambridge, UK) for library preparation and amplicon sequencing.

3.3.8.2 Amplicon Sequencing

The quality of each DNA sample checked at Novogene prior to library preparation. PCR amplification was conducted of the bacterial 16S V4 region with the primer pair Bakt_341F/Bakt_805R (Herlemann et al., 2011) and the fungal

ITS1 and ITS2 regions with the primer pair EkITS1F/Ek28R(≡ 3126T) (Gardes and Bruns, 1993; Sequerra et al., 1997). The PCR product was then purified before sequencing using the Illumina NovaSeq 6000 platform.

3.3.8.3 Amplicon Sequencing

Sequence data was submitted to the NCBI database (Project PRJEB54727). Ambiguous reads that did not correlate to the forward or reverse primers for 16S and ITS were removed. USEARCH V11.0 (Edgar, 2013a) was used for all analyses unless otherwise stated.

The ITS fungal reads and 16S bacterial reads were processed separately. The ITS forward and reverse primers were aligned with a 10 % threshold of maximum difference in overlap an 16S reads were aligned with a 5 % threshold. Both the forward and reverse primers were removed from sequences based on their representative sequences from both fungal and bacterial reads. Merged reads with adaptor contamination or a total length fewer than 150 nucleotides for ITS reads and 300 nucleotides for 16S reads were removed. The mean number of errors per sequence was set at a threshold of 0.1 for ITS reads and 0.5 for 16 reads as quality filtering.

3.3.8.4 OTU Generation

Unique sequences were identified and any sequence with less than 4 reads were discarded from OTU generation. The sequences were sorted by decreasing read frequency and OTUs generated by clustering unique sequences with 97 % sequence similarity into an OTU. A representative sequence for each OTU was also generated. Sequence reads were mapped against each representative OTU sequence to generate an OTU frequency count table for each sample for fungal and bacterial reads separately. Taxonomy of each representative OTU sequence was predicted using a SINTAX algorithm (https://www.drive5.com/usearch/manual/sintax_algo.html) by aligning ITS OTU representative sequences to the reference database “UNITE v8.3” (Nilsson et al., 2019) combined with ITS sequences from plants endophytes are commonly sequenced from and 16S OTU representative sequences to the RDP training set v18 (Cole *et al.,* 2014).

3.3.9 Statistical Analysis of Sequence Data

All statistical analysis of OTU count data was conducted in R version 4.1.2 (R Core Development Team 2008). Singleton OTUs (count of 1) across all samples were removed. The vegan R package v2.6.2 (Dixon, 2003) was used to normalise the count data by rarefaction to the minimum number of reads (43088) before further analysis.

Alpha and beta diversity were used to compare community differences in fungi and bacteria in the rhizosphere due to variety and treatment effects. Alpha (α) diversity indices (Observed, Chao1, Shannon, and Simpson) were calculated in the phyloseq R package v1.38 (McMurdie and Holmes, 2013). The LmPerm R package v2.1 (Wheeler, 2016) was used to for permutation split-plot ANOVA analysis to detect the contribution of treatment and variety on each of the alpha diversity indices. The vegan package was used to calculate beta (β) diversity using Bray-Curtis dissimilarity. Samples were visualised using non-metric multidimensional scaling (NMDS) plots to identify dissimilarity in fungal and bacterial communities due to treatment and scion variety effect. Permutations of multivariate analysis of variance using F-tests based on the sequential sum of squares (ADONIS) based on 1,000 permutations was used to assess the effects of pre-planting treatment and variety on fungal and bacterial communities. ADONIS could not copy the split-plot design so a simple model of treatment and varietal effects was used.

The DESeq2 package v1.34 (Love, Huber and Anders, 2014) was used to identify OTUs that differed significantly in their relative abundance between the two varieties and between each treatment and the control. The top five OTUs with the highest (positive) or lowest (negative) log2 fold change and p-value ≤ 0.05 were investigated to identify taxonomy using the reference databases “UNITE v8.3” (Nilsson et al., 2019) for fungi and “RDP training set v18” (Cole *et al.,* 2014) for bacteria.

3.4 Results

3.4.1 Above-Ground Tree Establishment

The variety effect was significant for both girth (P = 1.9e-4) and height (P = 6.9e-3) (Table 3.1). Treatment effect was not significant for girth (P = 0.19) but was significant for height establishment (P = 0.01). The interaction between variety and treatment was not significant for either girth or height. Gala trees treated with *P. fluorescens* showed an increased rate of establishment compared to the other treatments and the control (Figure 3.1). Height is not a good indicator of tree establishment as mechanical damage and overbearing of fruit on the leader branches led to breakage and subsequent pruning out of many of the leaders in autumn 2020, thus no height measurements were taken in 2021. By the end-season measurement in 2020, Braeburn trees treated with AMF had damage to the leader branches causing a reduction in their height, explaining the negative trend observed in Figure 3.1. All other treatments had a similar slope trend for the Braeburn trees. Gala trees treated with *P. fluorescens*, BSM, or the control trees all showed similar slopes for height (Figure 3.1). *B. amyloliquefasciens* showed a slower rate of height establishment followed by *P. fluorescens* with the lowest rate of height increase of all the treatments.

Table 3.1 Wald Chi-squared ANOVA of repeat measurement linear mixed model for Girth and Height.

	Df	P-Value
Girth		
Cultivar	1	1.9e-4
Treatment	4	0.19
Cultivar:Treatment	4	0.70
Height		
Cultivar	1	6.9e-3
Treatment	4	0.01
Cultivar:Treatment	4	0.99

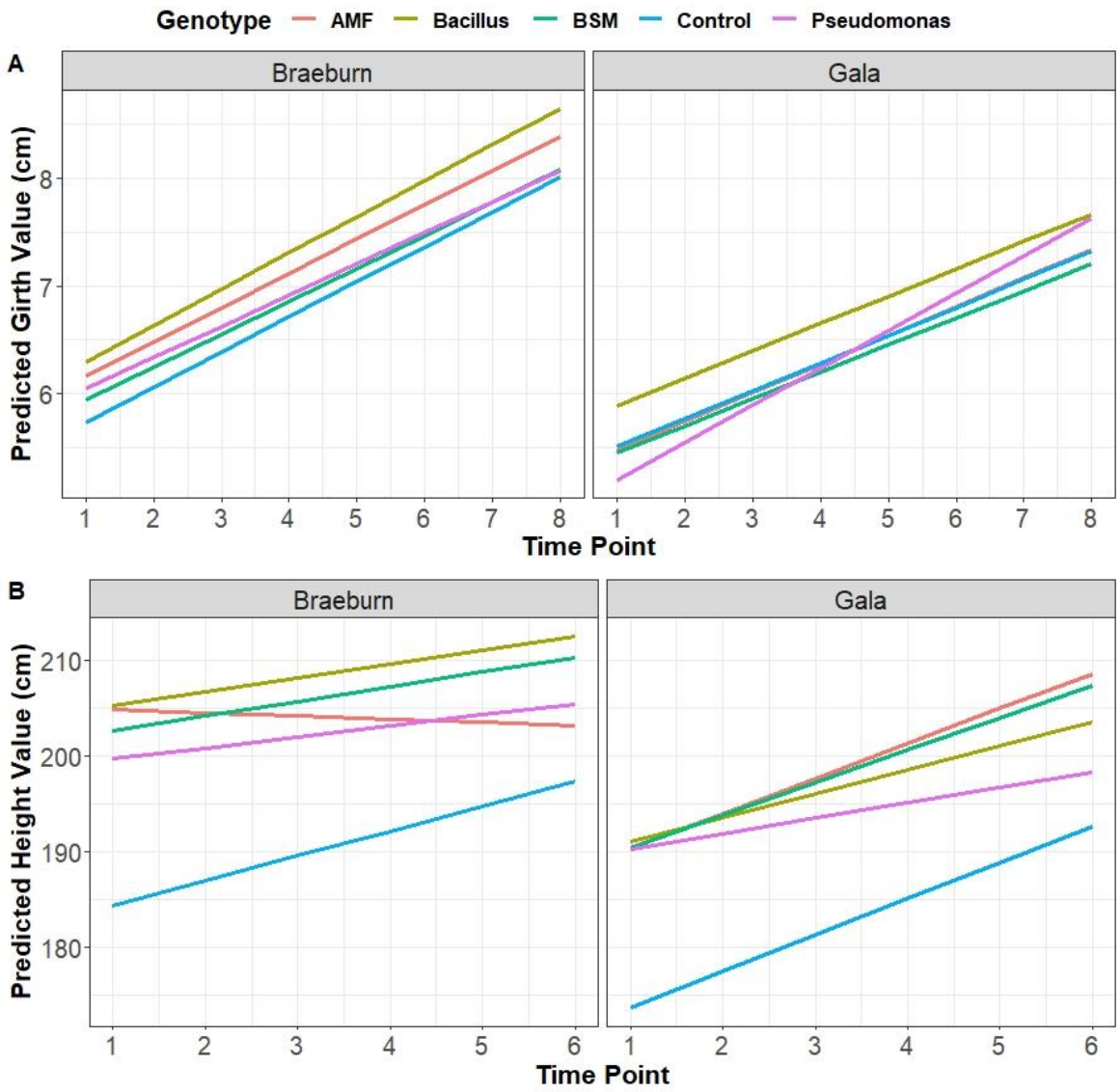

Figure 3.1 Predicted values for Girth (A) and Height (B) establishment from a repeat measurement linear mixed due to treatment effect. Cultivar and treatment effects were treated as fixed in the model and the tree position was treated as a random effect.

3.4.2 Fruit Yield

There was no significant difference between the fruit number between cultivars but there was a significant difference in the fresh weight of the fruit, with Braeburn producing a higher weight and therefore larger fruit compared to Gala (Table 3.2). Treatment effect overall was not significant across all treatments for both fruit number and fresh weight. The Braeburn trees had similar yields between treatments for both fruit number and fresh weight, other than BSM which had both a lower number of fruit and lower fruit weight than both the other treatments and the control trees (Figure 3.2). Gala trees showed lower yields in 2021 in both fruit number and yield for the BSM, *P. fluorescens* and *B. amyloliquefasciens* treatments, with BSM trees with particularly low yields of Gala fruit. AMF was identified with a significantly higher fruit number than the BSM treatment, but the paired comparison models did not identify any significant differences between treatments for fruit number (Table 3.2). The control Gala trees also had a higher fruit number than the BSM, *P. fluorescens* and *B. amyloliquefasciens* treatments but this difference was not significant between any of the treatments. Class I fruit size is shown in Figure A.1.

Table 3.2 Quasi-Poisson model of fruit number and Gaussian model of fresh weight, for paired treatments (set to be the same in model and compared to full model) to identify differences in yield between individual treatments.

Paired Model	*P-Value
Fruit Number	
Treatment	0.663
Cultivar	0.178
AMF vs Bacillus	0.550
AMF vs BSM	0.150
AMF vs Control	0.788
AMF vs Pseudomonas	0.418
Bacillus vs BSM	0.399
Bacillus vs Control	0.747
Bacillus vs Pseudomonas	0.832
BSM vs Control	0.247
BSM vs Pseudomonas	0.528
Pseudomonas vs Control	0.594
Fresh Weight (kg)	
Treatment	0.195
Cultivar	2.02e-5
AMF vs Bacillus	0.585
AMF vs BSM	0.022
AMF vs Control	0.647
AMF vs Pseudomonas	0.314
Bacillus vs BSM	0.083
Bacillus vs Control	0.937
Bacillus vs Pseudomonas	0.645
BSM vs Control	0.074
BSM vs Pseudomonas	0.202
Pseudomonas vs Control	0.595

**P-Value based on ANOVA analysis of model of paired comparisons compared to the full yield model.*

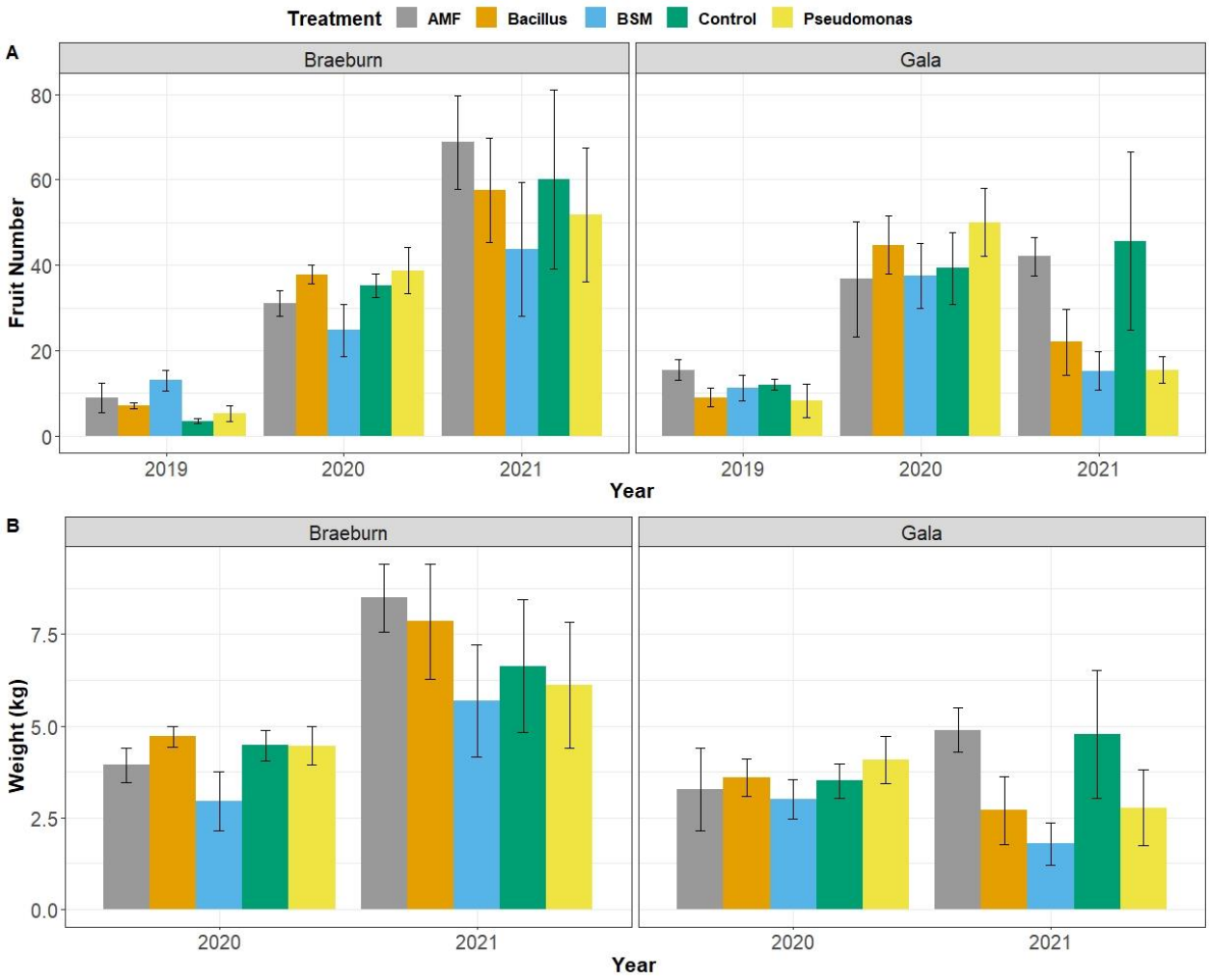

Figure 3.2 Yield data for fruit number (A) and weight (B) of the fruit. Fruit number was taken as the number of fruit on the tree irrespective of size. Weight measurements was for class I fruit only (≥ 50 mm circumference).

3.4.3 Root Length Colonisation

There was a significantly higher percentage of RLC in the AMF-treated trees than in the untreated control trees ($p = 0.007$). There was also a close to significant ($p = 0.058$) difference between Braeburn and Gala indicating some level of scion feedback on the recruitment and colonisation of mycorrhizae to the root system. Overall RLC % was low in both the AMF treated and untreated roots with ≥ 10 % of the root showing AMF structures in AMF treated roots and ≥ 5 % in the control roots (Figure A.2).

3.4.4 Soil Enzyme Functionality

The API®ZYM enzyme activity assay was used to compare enzyme functionality in soils with different pre-planting treatments. Most of the treatments showed similar mean scores for enzyme activity compared to the control soils (Table 3.3). Some notable differences in enzyme activity were the high enzyme activity of leucine arylamidase in the control Braeburn soils compared to intermediate activity in treated Braeburn soil samples and low activity in the control Gala soils. There was also intermediate activity of valine arylamidase in the Braeburn control soils with all other treatments in both Braeburn and Gala showing low or no valine arylamidase activity. β-glucosidase activity was low in all the Gala soils other than the *P. fluorescens* treated soils with intermediate activity. β-glucosidase had intermediate activity in all treated Braeburn soils and high activity in the control Braeburn soils. Finally, activity of N-acetyl-β-glucosaminidase was intermediate in the Braeburn control and Gala soils amended with *B. amyloliquefasciens* but had low activity in all other soils.

Table 3.3 Mean score data of enzyme activity for each treatment. Red colour indicates negative/low-intensity enzyme activity (0-1), orange indicates moderate enzyme activity (2-3), and green is high enzyme activity (4-5).

Enzyme	Braeburn					Gala				
	AMF	Bacillus	BSM	Control	Pseudomonas	AMF	Bacillus	BSM	Control	Pseudomonas
Alkaline phosphatase	4	3	3	5	4	3	3	4	3	4
Esterase (C 4)	5	3	4	5	5	4	3	4	3	4
Esterase Lipase (C 8)	4	3	3	4	4	3	3	4	3	4
Lipase (C 14)	1	0	0	0	0	0	0	0	0	0
Leucine arylamidase	3	2	2	4	2	2	3	2	1	2
Valine arylamidase	1	0	0	2	1	1	1	0	0	0
Cystine arylamidase	0	0	0	0	0	0	1	0	0	0
Trypsin	1	1	0	0	0	0	0	0	0	1
α-chymotrypsin	0	0	0	0	0	0	0	0	0	0
Acid phosphatase	4	4	3	4	4	3	4	3	4	3
Naphthol-AS-B1-phosphohydrolase	5	5	5	5	5	5	5	5	5	5
α-galactosidase	0	0	0	0	0	0	0	0	0	0
β-galactosidase	0	0	0	0	0	0	0	0	0	0
β-glucuronidase	0	0	0	0	0	0	0	0	0	0
α-glucosidase	0	1	0	0	0	0	1	0	0	0
β-glucosidase	2	2	2	4	2	1	1	1	0	2
N-acetyl-β-glucosaminidase	1	1	1	2	0	0	2	1	0	1
α-mannosidase	1	1	0	1	0	0	1	0	0	0
α-fucosidase	0	0	0	1	0	1	1	0	0	1

3.4.5 Summary of Amplicon-Sequencing Data

There were a total of 3,484,612 fungal reads and 2,215,773 bacterial reads across rhizosphere soil samples sequenced. 3,573 fungal OTUs were generated from the reads and 28,768 bacterial OTUs. The range of OTU counts per sample ranged from 75,198 to 109,609 for fungal OTUs with a mean number of counts of 87,115. Bacterial OTU counts per sample ranged from 43,088 to 72,465 counts and had mean number of counts of 55,394. The number of reads per OTU ranged from 2 to 31,185 reads for fungal OTUs and 2 to 2,624 reads for bacterial OTUs.

3.4.6 Diversity Indices

Alpha indices were calculated to measure within sample diversity (Observed, Chao1, Shannon, and Simpson). Alpha diversity was higher in bacterial communities than fungal communities. Block was the only significant ($P \leq 0.05$) factor affecting each of the alpha diversity indices (Table 3.4). The difference in alpha diversity between varieties contributed to 14 % of the variation in Shannon fungal alpha diversity and 14.8 % of the variation in Simpson fungal alpha diversity. Alpha diversity was slightly lower for fungal Shannon and Simpson values in the Gala variety trees (Figure 3.3B). Pre-planting treatment did not significantly affect alpha diversity (Figure 3.3A/C). The interaction between apple variety and the treatment applied contributed to a higher percentage of the variability in fungal alpha diversity (Chao1 = 9.9 %, Shannon = 17.3 %, Simpson = 17.8 %) compared to bacterial alpha diversity (Chao1 = 6.7 %, Shannon = 2.9 %, Simpson = 2.0%). The interaction between variety and treatment however was not significant.

Table 3.4 Percentage of the variability in alpha diversity indices accounted for by block, variety, soil amendment (treatment), and the interaction between treatment and variety.

Measure	Block		Variety		Treatment		Variety:Treatment	
	P value	*%*	*P value*	*%*	*P Value*	*%*	*P Value*	*%*
Fungi								
Observed	0.34	37.5	0.69	2.0	0.89	5.1	0.60	9.9
Chao1	0.34	37.5	0.69	2.0	0.77	5.1	0.63	9.9
Shannon	0.01	70.0	0.12	14.0	0.96	2.8	0.24	17.3
Simpson	0.03	72.9	0.15	14.8	1.0	2.4	0.32	17.8
Bacteria								
Observed	2e-4	86.1	0.73	1.1	0.35	15.6	0.89	6.7
Chao1	2e-4	86.1	0.73	1.1	0.34	15.6	0.88	6.7
Shannon	0.02	68.0	0.89	0.1	0.96	2.2	1.0	2.9
Simpson	0.04	64.2	0.62	1.8	0.61	7.5	1.0	2.0

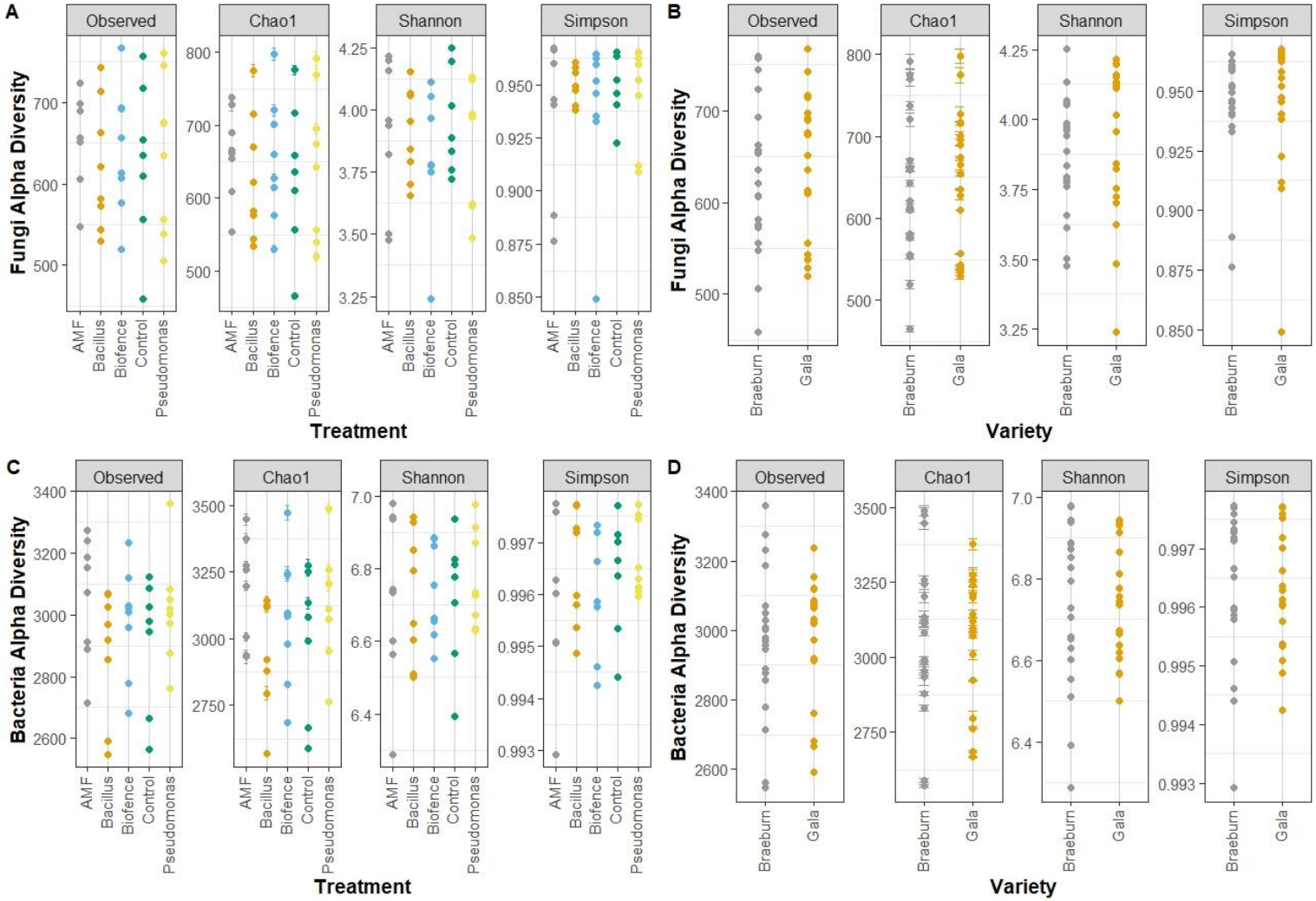

Figure 3.3 Alpha (α) diversity measures, Observed, Chao1, Shannon, and Simpson for fungi and bacteria. A and C indicate the alpha diversity values due to treatment effect, C and D indicate alpha diversity values due to variety effect.

Bray-Curtis beta between sample diversity was used to identify the differences between fungal and bacterial communities in the rhizosphere. There was no clear dissimilarity in beta diversity for either fungal (Figure 3.4A) or bacterial (Figure 3.4C) communities between treatments, which was confirmed by ADONIS analysis (Table 3.5). There were more varietal differences in the fungal communities (Figure 3.4B) than in bacteria communities (Figure 3.4D) and the variation in fungal beta diversity due to the variety effect was close to significant (P = 0.09) but only contributed to 3.5 % of the variation in fungal beta diversity. Block effect was significant for both fungal and bacterial beta diversity contributing to 12.7 % and 23.9 % of the variation in beta diversity respectively.

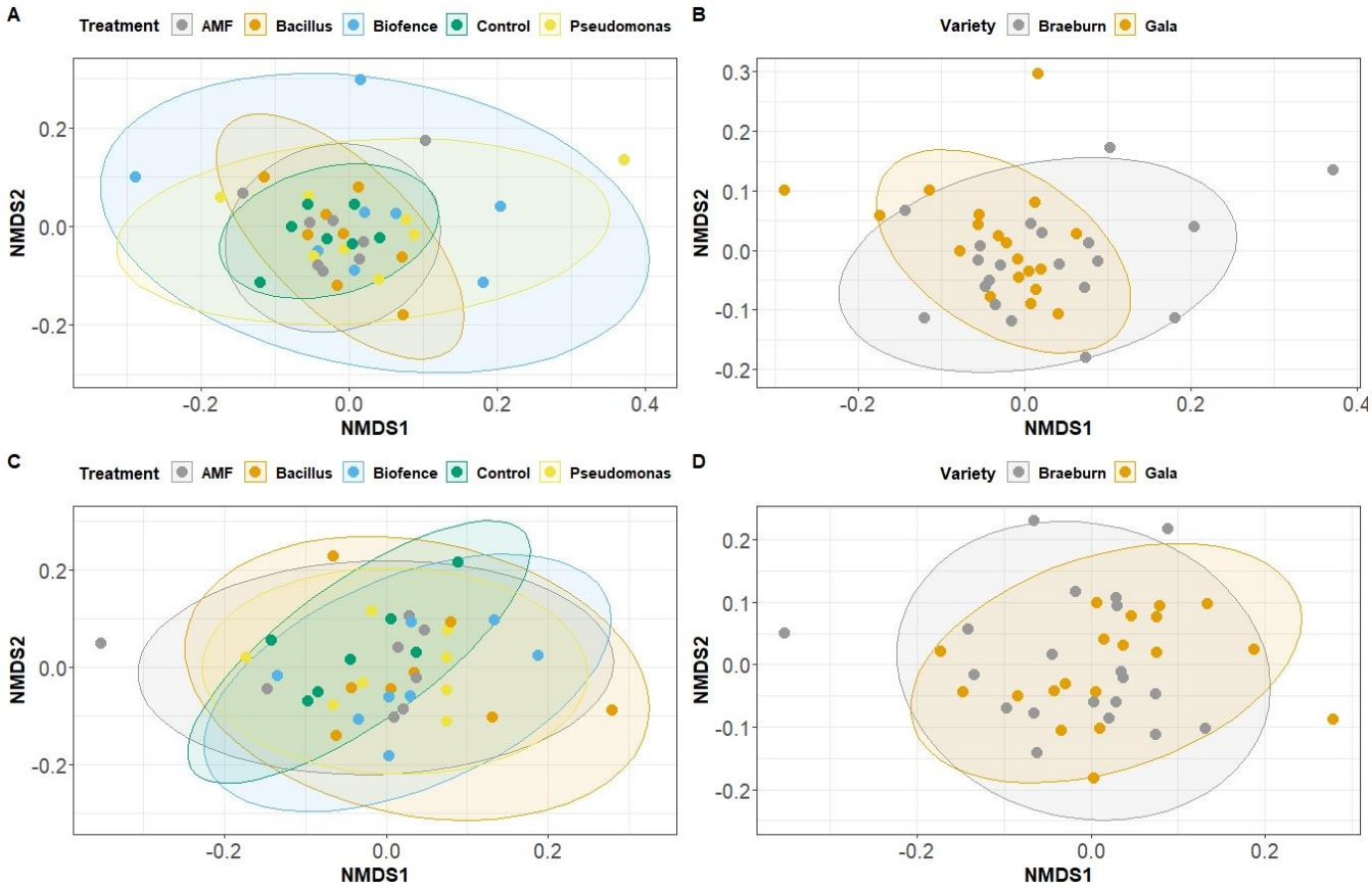

Figure 3.4 The first two dimensions of NMDS analysis of the Beta (β) diversity indices (Bray-Curtis dissimilarity) for (A) fungi coloured by treatment, (B) fungi coloured by variety, (C) bacteria coloured by treatment and (D) bacteria coloured by variety. Closer the distance between the points indicates more similarity in microbial communities between the samples.

Table 3.5 Permutational Multivariate Analysis of Variance (ADONIS) based on 1000 permutations of Bray-Cutis beta diversity accounted for by block, variety, soil amendment (treatment) factors and the interaction between treatment and variety.

	df	Sum sq	%	P-value
Fungi				
Block	3	0.41	12.7	0.003
Variety	1	0.12	3.5	0.09
Treatment	4	0.29	9.0	0.73
Treatment:Variety	4	0.30	9.1	0.72
Residuals	26	2.13		
Bacteria				
Block	3	0.73	23.9	0.001
Variety	1	0.07	2.3	0.74
Treatment	4	0.24	7.8	0.35
Treatment:Variety	4	0.25	8.1	0.69
Residuals	26	1.77		

3.4.7 Differential Abundance of OTUs

The relative abundance of OTUs was compared between the two varieties (Gala and Braeburn) and each of the treatments compared to the control. 67% of the fungal OTUs remained after DESeq2 independent filtering (mean of the normalised counts) and 55% of bacterial OTUs. 128 fungal OTUs (5.3%) and 447 bacterial OTUs (2.8%) had a significant different abundance in Gala trees (Table 3.6). 59 fungal OTUs (2.5%) and 338 bacterial OTUs (2.1%) had significant abundance in the Braeburn trees. The differences in the ten fungal or bacterial OTUs with largest log2 fold change (LFC) (five increased and five decreased) are shown in Table 3.7. Genus associated with plant pathogens *Alternaria* and *Fusarium* had increased abundance in Gala and Braeburn trees respectively. An OTU associated with *Aspergillus* spp. also had increased abundance in the Gala rhizosphere. The bacterial genus *Aquicella* and *Bdellovibrio* were more abundant in Gala, whereas a *Buchnera* spp. was identified to be more abundant in Braeburn rhizosphere.

Table 3.6 DESeq2 results summary for all differential OTUs. No. OTUs indicate the number of OTUs after DESeq2 filtering (compared between a specific pair of treatments); Log2 Fold Change (LFC) > 0 indicates a higher abundance in the first term in the comparison and a negative LFC indicates higher abundance in the second comparison term.

Model	No. OTUs	LFC > 0 (Higher)	LFC < 0 (Lower)	Low Counts
Fungi				
Gala vs Braeburn	2404	128 (5.3%)	59 (2.5%)	182 (7.6%)
AMF vs Control	2404	57 (2.4%)	93 (3.9%)	126 (5.2%)
BSM vs Control	2404	99 (4.1%)	104 (4.3%)	466 (19%)
Bacillus vs Control	2404	85 (3.5%)	106 (4.4%)	32 (1.3%)
Pseudomonas vs Control	2404	80 (3.3%)	98 (4.1%)	326 (14%)
Bacteria				
Gala vs Braeburn	15933	447 (2.8%)	338 (2.1%)	3328 (21%)
AMF vs Control	15933	311 (2%)	295 (1.9%)	6432 (37%)
BSM vs Control	15933	262 (1.6%)	318 (2.0%)	4553 (29%)
Bacillus vs Control	15933	266 (1.7%)	269 (1.7%)	5246 (33%)
Pseudomonas vs Control	15933	337 (2.1%)	311 (2.0%)	5853 (37%)

Table 3.7 OTUs with the highest log2 fold change (top five OTUs with increased or decreased abundance) identified by DESeq2 analysis. Taxonomy was predicted by comparing OTU representative sequences to the UNITE v8.3 database (along with some plant ITS sequences) for fungi and the RDP training set v18 for bacteria. 90% identity confidence threshold was used to assign taxonomy. A positive log2 fold change (LFC) indicates increased abundance in the first term in the comparison and a negative LFC indicates greater abundance in the second term. OTUs that had an unknown taxonomy are omitted from the table.

Comparison	OTU	LFC	Taxonomy
Fungi			
Gala vs Braeburn	OTU1789	29.97	*Trichosporonaceae* (f)
	OTU1375	18.07	*Alternaria* (g)
	OTU1414	-20.35	*Aspergillus* (g)
	OTU722	-19.61	*Fusarium* (g)
AMF vs Control	OTU773	23.89	*Orbiliaceae* (f)
	OTU783	21.93	*Zoopagomycetes* (c)
	OTU1789	-28.00	*Trichosporonaceae* (f)
BSM vs Control	OTU480	24.80	*Volutella* (g)
	OTU1805	22.69	*Mortierellales* (o)
	OTU1002	21.88	*Pleosporales* (o)
	OTU1789	-27.35	*Trichosporonaceae* (f)
Bacillus vs Control	OTU1337	20.47	*Pleosporales* (o)
	OTU3441	20.38	*Corpinellus* (g)
	OTU1408	20.09	*Xylaria* (g)
	OTU912	-25.02	*Issatchenkia* (g)
Pseudomonas vs Control	OTU480	21.10	*Volutella* (g)
	OTU1045	20.83	*Pyxidiophorales* (o)
	OTU1214	-27.76	*Sordariom* (c)
	OTU1789	-27.27	*Trichosporonaceae* (f)
Bacteria			
Gala vs Braeburn	OTU3918	29.80	*Aquicella* (g)
	OTU12847	28.16	*Spirosoma* (g)
	OTU6140	18.39	*Chitinophagaceae* (f)
	OTU6499	18.25	*Bdellovibrio* (g)
	OTU4008	-23.57	*Buchnera* (g)
AMF vs Control	OTU21076	21.55	*Streptomyces* (g)
	OTU2250	21.19	*Lactobacillus* (g)
	OTU3459	21.15	*Alphaproteobacteria* (c)
	OTU5114	-30.00	*Alphaproteobacteria* (c)
	OTU1836	-29.99	*Ruminococcaceae* (f)
	OTU12847	-28.96	*Spirosoma* (g)
	OTU5652	-27.96	*Haemophilus* (g)

BSM vs Control	OTU11604	21.65	*Cytophagales* (o)
	OTU5484	21.09	*Mediterranea* (g)
	OTU3918	-29.94	*Aquicella* (g)
	OTU1727	-29.46	*Phocaeicola* (g)
	OTU4443	-29.26	*Clostridiales* (o)
	OTU5114	-29.22	*Alphaproteobacteria* (c)
	OTU1173	-28.33	*Alcaligenaceae* (f)
Bacillus vs Control	OTU3626	20.37	*Myxococcales* (o)
	OTU3918	-29.82	*Aquicella* (g)
	OTU5114	-29.04	*Alphaproteobacteria* (c)
	OTU3422	-28.48	*Nocardioides* (g)
	OTU1173	-28.40	*Alcaligenaceae* (f)
Pseudomonas vs Control	OTU21987	21.00	*Legionellales* (o)
	OTU3918	-30.00	*Aquicella* (g)
	OTU2913	-30.00	*Alloprevotella* (g)
	OTU2282	-29.98	*Prevotella* (g)
	OTU2641	-29.96	*Bacterioidales* (o)
	OTU2015	-29.82	*Deltaproteobacteria* (c)

Due to the application (or absence) of the treatments, OTU abundance of a number of OTUs was significantly altered. For fungi, between 2.4% and 4.4% of OTUs had a significantly differential abundance due to the treatment, and similarly between 1.6% and 2.1% for bacterial OTUs (Table 3.6). Of the 10 OTUs with highest LFC in the AMF vs Control model (Table 3.7), none of the fungal OTUs could be assigned to a taxonomy below family. Two bacterial OTUs associated with the genus *Streptomyces* and *Lactobacillus* had increased abundance in the rhizosphere of trees treated with AMF and an OTU associated with a *Spirosoma* species was more abundant in the rhizosphere of control trees than AMF trees.

In BSM treated trees, an OTU associated with the fungal genus *Volutella* had increased abundance in the rhizosphere than in the control trees. The bacterial genus *Mediterranea* had increased abundance when BSM was applied, whereas *Aquicella* and *Phocaeicola* both were more abundant in the rhizosphere of the control trees than in the rhizosphere of BSM treated trees (Table 3.7).

Amendment with *Bacillus amyloliquefasciens* lead to the increased abundance of fungal OTUs associated with the genera *Corpinellus* and *Xylaria* but also had a reduced abundance of *Issatchenkia* compared to the control. *Aquicella* and *Nocardioides* genera were associated with bacterial OTUs more abundant in the control than the *Bacillus amyloliquefasciens* treatment (Table 3.7). Of the investigated OTUs associated with increased abundance in the *Bacillus amyloliquefasciens* treatment, they were either unknown or could not be assigned to genus level or below.

Pseudomonas fluorescens treatment was also associated with increased abundance of the genus *Volutella* than the control, similar to the BSM treatment. *Aquicella, Alloprevotella*, and *Prevotella* were all bacterial genus associated with OTUs with a higher abundance in the control than in the *P. fluorescens* soils (Table 3.7). *Aquicella* had increased abundance in the control soils than in BSM, *Bacillus amyloliquefasciens*, and *Pseudomonas fluorescens* treated trees rhizosphere. *Aquicella* was also more abundant in Gala trees than Braeburn, suggesting an increased abundance of *Aquicella* in the control Gala trees.

3.5 Discussion

This study showed the effect of pre-planting treatments varied based on the variety of apple (Braeburn or Gala). *Pseudomonas fluorescens* was identified to be beneficial for girth expansion of Gala trees but this was not reflected in the Braeburn trees. *P. fluorescens* was also not identified as beneficial for tree height or yield for either of the two varieties tested. Differences in rhizosphere local (α) and community (β) diversity for fungi and bacteria were not identified due to pre-plant treatment. There was a marked difference in fungal communities due to the scion variety, although not significant, which may suggest a scion-rootstock link that changes root exudation and thus recruitment of different communities of plant-associated fungi in the rhizosphere. *Allternaria, Aquicella* and *Bdellovibrio* were found to be more abundant in Gala rhizosphere and *Aspergillus, Fusarium*, and *Buchnera* were more abundant in Braeburn rhizosphere. *Streptomyces*, and *Lactobacillus* had a higher abundance in AMF treated rhizosphere than in the control soils. *Volultella* was also found to be more abundant in both BSM amended and *P. fluorescens* amended rhizosphere than in the control. Finally, an OTU associated *Nocardioides* was found to be more abundant in control soils than in those amended with *Bacillus amyloliquefasciens*.

It is accepted that ARD is a biological phenomenon stemming from the soil due to the success of fumigation, pasteurisation or soil sterilisation alleviating the detrimental effects of ARD (Somera and Mazzola, 2022b). Better establishment in the presence of a single strain amendment would either be attributed to a direct benefit conferred to the tree or by the strain working antagonistically against one or multiple components of the ARD disease complex of pathogens. We found *P. fluorescens* treatment to increase the girth growth rate of Gala trees but did not observe the same effect in Braeburn trees where all girth growth rates were similar irrespective of treatment. *Pseudomonads* have been well identified as beneficial rhizobacterium for crop growth in both wheat and apple (Mazzola et al., 2002b; Sharma et al., 2017) but it may be apple variety specific as to the effectiveness of the use of the amendment as we did not see the same benefit in Braeburn. We also identified elevated β-galactosidase in those rhizosphere soils,

which may link glucose production in soil to girth increase of the Gala trees. There also did not seem to be a correlation between girth establishment and height establishment for either variety. In fact, it could be well argued that each of the amendments were detrimental to Gala tree establishment by looking solely at the heights of the trees, with the control growing the best. Height however was not a good metric of establishment in the study due to mechanical damage, overbearing of leader branches by fruit, or wind damage impacting height measurements independent of the experimental variables. Girth and yield are therefore proposed as the best indicator of tree fitness and establishment in the present study.

Braeburn yields were better for both the number of fruit and the weight of the fruit in 2021 compared to Gala. Yields in Braeburn trees amended with AMF were higher, perhaps indicating by the third growing season the symbiosis with AMF aided nutrient transfer available for fruit production but was not linked to vegetative growth (girth establishment was not significantly affected). It has been previously shown AMF inoculation in apple can cause increased fruit biomass, height, root biomass, but not increased tree diameter (Berdeni et al., 2018). This aligns closely with the present results, although we could not identify the below-ground effect on root biomass as the trial was non-destructive under field conditions. In Gala trees, all the amendments but AMF appeared to produce lower yields than the unamended trees. AMF has been identified as a key beneficial in the presence of drought conditions in crops like strawberry (Boyer et al., 2015), there may have been water limitations during the 2021 season that the native (control) or AMF amended roots may have been better equipped for than the soils adapted with the other amendments for better nutrient exchange required for fruit development.

At the community level we did not identify any significant differences in community associated with the amendment applied. This implies any changes in growth and yield are attributed to a limited number of individual taxa within the rhizosphere rather than community-wide changes. Additionally, due to the application of the amendments during winter while the tree is dormant, there is

the question of the survivability of the amendments for the period of time until the tree breaks dormancy. It has been shown that the recruitment process in crops such as wheat is rapid over days rather than weeks (Buchan, Crombie and Alexandre, 2010), therefore future studies may need to investigate the temporal nature of recruitment in apple roots through exudation and timing application of biological amendments within this window. Fungal communities in Gala and Braeburn rhizospheres were also markedly different, suggesting a scion-rootstock interaction in the recruitment of microbes to the rhizosphere, which has also been previously identified with bacterial recruitment in the apple rhizosphere (Chai et al., 2022).

Although differences at a community level were not detected, changes in the abundance of individual OTUs could be linked to a pathogenic or beneficial effect on the plant in the rhizosphere. We identified many OTUs, between 57-128 OTUs with significantly different abundance in DESeq2 for fungi and 262-447 OTUs for bacteria. The large differences in abundance could be linked to both the scion effect between varieties and the effect of the amendments applied at pre-planting. Care must be taken when interpreting the function of predicted pathogens or beneficial rhizomicrobiota as the presence of the organism alone may not necessarily lead to the predicted effect (Somera and Mazzola, 2022b). Additionally, sequencing only allows for taxonomic predictions down to genus level so there may be species/strain functional differences within the soil. For these reasons the interpretations of the differential OTUs is speculative in nature as to the predicted function as a beneficial or pathogen within the soil.

Due to the large number of significantly different OTUs a subset of the top five with the highest positive or negative log2 fold change were analysed to identify their taxonomy. Two OTUs were associated with genus containing plant pathogens *Alternaria* and *Fusarium* found to be more abundant in Gala rhizosphere and Braeburn rhizosphere respectively. This suggests despite being grafted on the same rootstock (M9) the scion may confer different levels of disease tolerance to the soil through root exudation that different diseases could exploit and potentially lead to soil-borne disease. Within the *Alternaria* genus, *A.*

infectoria has been linked to leaf blotch and fruit spot in apple and in Australia apple production it is predicted to cause between 15-25 % fruit loss to the grower (Harteveld, Akinsanmi and Drenth, 2013; Harteveld et al., 2014). There is no evidence in the literature however that *Alternaria* spp. can infect roots causing disease when soilborne.

Fusarium has been extensively linked to ARD in China (Wang et al., 2018) but has also been shown to be weakly/non-pathogenic against apple roots (Somera and Mazzola, 2022). It is therefore unlikely the higher abundance would lead to a higher susceptibility of Braeburn trees to ARD. *Aspergillus* was also identified to be more abundant in Braeburn rhizosphere and has both been implicated as an airborne post-harvest pathogen of apple but also as a biocontrol agent and growth promoter against apple dieback disease in Tunisia (ben M'henni et al., 2022; Smiri et al., 2021). Further investigation/isolation of the *Aspergillus* sp. is required to accurately predict the function of *Aspergillus* in the Braeburn soils. Additionally, *Bdellovibrio,* a deltaproteobacteria that invades and can kill larger bacteria (Bratanis et al., 2020), were also more abundant in Gala soils. Although the basis of ARD is not bacterial, there could be a valuable biocontrol potential of using the bacterium as a biocontrol against soilborne bacterial diseases of apple.

In AMF amended roots, there was an increase in RLC % by AMF compared to the native AMF from either nursery or bulk soil in the control trees. AMF has been associated with mitigation of the detrimental effects of ARD but field-scale application will prove challenging as a widespread ARD management strategy (Cavael et al., 2021; Somera and Mazzola, 2022b). Alongside the colonisation of AMF itself, we also found *Lactobacillus* spp., a group of plant 'probiotics' and plant-growth-promoters (Santos, Kandasamy and Rigobelo, 2020), as significantly more abundant when AMF was applied. *Streptomyces* was also identified to be more abundant when AMF was added and although it has been associated with plant growth promotion and suppressive of the ARD pathogen *Rhizoctonia* (Faheem et al., 2015; Mazzola and Cohen, 2005; Suárez-Moreno et al., 2019; Tamreihao et al., 2016; Vurukonda et al., 2018), some *Streptomyces* strains are also well established as pathogenic in plants (Li et al., 2019). This

highlights the limitations of shotgun metabarcoding when you have species and strain variability that can be both detrimental and beneficial to the plant.

An OTU associated with the genus *Volutella* was found to be more abundant in rhizosphere of trees amended with BSM and *P. fluorescens*. *Volutella* has been linked to disease in Japanese pachysandra, alfalfa, and boxwood but has not been reported as a soilborne pathogen of apple (Chilton, 2018; Šafránková, 2007; Shi and Hsiang, 2014). *B. amyloliquefasciens* treatment appeared to be antagonistic to an OTU resembling the genus *Nocardioides* which was more abundant in control rhizosphere. Resistant cultivars of strawberry to *Fusarium* have been associated with increased abundance of *Nocardioides* spp. *B. amyloliquefasciens* amendment could therefore leave the roots more susceptible to *Fusarium* infection but *Fusarium* spp. have also been shown to not be pathogenic to apple roots (Somera and Mazzola, 2022b). An OTU associated with *Aquicella* was found to be less abundant in rhizospheres treated with BSM, *B. amyloliquefasciens*, and *P. fluorescens* but was also more significantly more abundant in Gala soils than Braeburn soils. *Aquicella* therefore appears to be associated with the control Gala trees. OTUs resembling *Aquicella siphonis* have been previously been identified as closely associated with rhizosphere communities of resistant potato genotypes against common potato scab (Kobayashi et al., 2015). It is unclear from the literature of the function of *Aquicella* spp. in apple soil.

In summary, we identified pre-planting amendment of trees in field had a mixed effect in terms of benefit. The effects were often cultivar specific highlighting the importance of scion-rootstock interaction in the recruitment process of microbes in the rhizosphere. Despite seeing benefits of amendments on the growth of the tree (*P. fluorescens* on girth) we identified AMF to increase the mean fruit number and fresh weight of fruit in Braeburn trees (although not significant) compared to the untreated trees. We did not identify any effect on community diversity of either bacteria or fungi due to the treatments applied but did notice there may a varietal effect on fungal rhizosphere diversity. A number of OTUs associated with genera containing both pathogenic and beneficial groups had the highest LFC in

abundance for bacterial and fungal OTUs due to pre-plant amendment. This highlights the limited capacity for pre-plant amendments to change the rhizosphere microbiome on a community scale but that they can be used to control the abundance of individual populations in the soil. This integrated relationship between amendment and native beneficials could provide the framework for a management strategy for ARD or other soilborne apple disease. We do however stress the need for future studies to use techniques like metatranscriptomics to identify the function soil communities and link increased abundance of particular taxa with a predicted function that may be beneficial for the tree to minimise ARD severity.

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4 The Effect of Biologically Amending Soils on Early Establishment of M9 Rootstocks and Rhizosphere Microbial Communities

4.1 Abstract

4.1.1 Purpose

Microorganisms in the rhizosphere of newly planted apple trees play a critical role in nutrient cycling, soil fertility, and carbon sequestration providing a direct effect on plant establishment. Supplementation of soils with strains of beneficial biological amendments applied individually or in a consortium may minimise the severity of Apple Replant Disease (ARD) and improve establishment of the young tree.

4.1.2 Methods

Soils were amended with one or a combinations of *Diversispora* sp., *Trichoderma harzianum*, *Bacillus amyloliquefasciens,* and *Pseudomonas fluorescens* prior to planting 1-year-old M9 rootstocks in a semi-field potted trial. High-throughput sequencing of the ITS fungal and 16S bacterial regions were used to compare community differences due to the pre-plant amendments and correlated with the rate of establishment of the rootstock as a measure of ARD severity.

4.1.3 Results

This study did not identify a beneficial effect of *Diversispora* sp., *Trichoderma harzianum, Bacillus amyloliquefasciens,* and *Pseudomonas fluorescens* as a pre-plant amendment to improve the establishment of M9 rootstocks in the first season of growth either individually or in a consortium. Each of the biological soil amendments caused changes in the abundance of a number of OTUs, which were subsequently correlated taxonomically to be associated with plant pathogenic (eg. *Verticillium*), or plant-growth-promoting (eg. *Sebacina vermifera*, *Pedobacter,* and *Bacillus*) populations.

4.1.4 Conclusions

Our results suggest that application of single species biological amendments either alone or in a consortium prior to planting does not significantly increase the establishment of rootstocks in the first season. The survivability of the amendments and functionality changes induced by the amendments themselves or by the altered taxa in the rhizosphere should also be assessed in further studies.

4.2 Introduction

The soil microbiome is a highly variable ecosystem driven by both environmental and spatio-temporal factors determining its population composition (Serna-Chavez, Fierer and van Bodegom, 2013). Microorganisms in the soil microbiome play a critical role in nutrient cycling, soil fertility, and carbon sequestration providing a direct effect on the plants and indirectly on animal species within the food chain (Fierer, 2017a). Plant-microbe interactions within the rhizosphere have long been the target of research to identify the components of the soil microbiome responsible for soil-borne disease, plant growth promotion, and the mechanisms of root exudation to recruit microbes from a highly heterogeneous environment to a specialist community in the rhizosphere. Modern advances in sequencing strategies have allowed studies to look at the microbes present in the rhizosphere and understand the individuals and biology that underpins high-intensity crop production.

To maximise the efficiency of intensive fruit production of crops such as apple (*Malus domestica*) it is important to minimise the number of pathogenic microbes present in the rhizosphere. The rhizosphere is a specialised community partially controlled by recruitment from the plant by root exudation (Haichar et al., 2008). However, pathogens in the rhizosphere can lead to soil-borne disease in apples. Apple Replant Disease (ARD) is a long-documented disease in apple production. ARD is defined as the unsatisfactory establishment and performance of a young replanted apple tree without land rest or crop rotation. ARD causes stunted growth, reduced yields, reduction in fruit quality and root biomass, and a decline in root health with symptoms consistently observed 1-3 months after planting (Mazzola and Manici, 2012). ARD can frequently cause the death of the affected trees within the first year or are removed by the grower due to unsatisfactory establishment.

The presence and interaction of pathogenic microbes in the rhizosphere have been associated with ARD. The pathogenic fungi *Rhizoctonia*, *Cylindrocarpon*, and *Fusarium* and the oomycetes *Pythium* and *Phytophthora* are all described as causal agents for ARD when their abundance is increased both alone and in a

consortium (Tewoldemedhin et al., 2011a). Lesions in the roots of the tree caused by the root-lesion nematode *Pratylenchus penetrans* probably provide an entry point for the pathogens, exacerbating the severity of ARD. Due to the biotic nature of ARD it has been previously treated using synthetic chemical fumigation of the soil to remove the pathogens present before replanting (Mai and Abawi, 1981). Alternatives to chemical fumigation are essential as government legislation continues to limit the number of chemicals available for use and those that remain are less effective than their predecessors (Xu and Berrie, 2018). Non-chemical strategies to manage ARD include planting trees in the alleyways between the previous tree stations which have distinctly different soil microbiomes (Deakin et al., 2018b; Rumberger et al., 2004b). Crop rotation can prevent the accumulation of pathogenic microorganisms and reduce ARD pressure on the apple generation following the rotation (Hewavitharana, Mazzola and DuPont, 2019). Rootstock genotypes exhibit differences in tolerance/resistance to ARD (Fazio et al., 2012; Leinfelder and Merwin, 2006; Rumberger et al., 2004b). Planting a rootstock which is tolerant to ARD, but also with a more distant genetic relationship can be an effective strategy to minimise the severity of ARD (Deakin et al., 2019; Shuttleworth, 2021; Xu and Berrie, 2018).

The use of soil amendments is another non-chemical management strategy to try and minimise the severity of ARD. Anaerobic soil disinfestation involves application of a carbon source amendment added to pre-plant soil and covered with plastic, which after a number of weeks increases the abundance of beneficial bacteria in the soils (Strauss and Kluepfel, 2015). Biochar addition has been shown to alleviate the decline in plant height, chlorophyll content, and net photosynthetic rate but did not improve fruit yield or fruit quality in a separate study (Safaei Khorram et al., 2019; Wang et al., 2014). Brassica seed meal application has also been shown to control fungal pathogens such as *Rhizoctonia*, *Cylindrocarpon*, and *Fusarium* and reduce the recovery rate of the root-lesion nematode *Pratylenchus penetrans* (Mazzola and Manici, 2012; Mazzola and Mullinix, 2005; Shuttleworth, 2021). Brassica seed meal has also been shown to alter the bacterial and fungal populations in soils and increase the growth of apple seedlings over a three-year period comparatively to fumigated

soils (Mazzola, Hewavitharana and Strauss, 2015b). Pre-plant supplementation of the soil microbiome with beneficial or biocontrol amendments is another strategy to minimise the severity of ARD on replanted trees. Increased abundance of beneficial microbiota in the rhizosphere has previously been correlated with increased tree growth (Nicola et al., 2017).

Plant growth-promoting rhizobacteria (PGPRs) are a group of organisms that improve plant growth through a wide variety of mechanisms including phosphate solubilization, siderophore production, nitrogen fixation, rhizosphere engineering and encouragement of plant-microbe symbiosis, and biocontrol action against soil-borne pathogens (Bhattacharyya and Jha, 2012). *Bacillus subtilis* has been shown to both induce plant growth and increase yields but also antibiosis and niche exclusion of soil-borne pathogens (Bhattacharyya and Jha, 2012). *Bacillus amyloliquefaciens* strains have also been shown to exhibit both plant-growth-promoting action through the action of phytases making phosphorus available for the plant and by anti-fungal activity (Chen et al., 2009; Idriss et al., 2002; Kim and Chung, 2004). *Pseudomonas* spp. are ubiquitous microorganisms in agricultural soil. *Pseudomonas fluorescens* exhibits antagonistic action against both fungal pathogens (*Penicillium* and *Erwinia*) as well as the oomycete *Pythium* (Etebarian et al., 2005; Girlanda et al., 2001; Haas and Défago, 2005; Pujol et al., 2006). *Pseudomonas* inoculation and also previously been shown to increase growth of apple trees following crop rotation with a non-woody crop. This has also been correlated with increased abundance of *Pseudomonas fluorescens* (Biró et al., 1998; Gu and Mazzola, 2003).

Beneficial fungi in the rhizosphere also act to promote plant growth both directly and indirectly. *Trichoderma* species receive nutrients from the plant through root exudates and in return improve plant nutrient uptake thus provide both biotic and abiotic stress relief (Lorito et al., 2010). *Trichoderma* strains also act as biocontrol agents through competition with other soil microorganisms for nutrients, antibiosis and production of lytic enzymes to inhibit fungi, and trigger induced systemic resistance in the host plant (Segarra et al., 2009). *Trichoderma harzianum* is one example and has been widely recognised as an effective

biocontrol agent against several soil-borne pathogens (Jensen and Wolffhechel, 1995; Papavizas, 2003). Arbuscular mycorrhizal fungi (AMF) are mutualistic filamentous fungi and the most widely distributed fungal group on Earth that improve crop growth (Jansa et al., 2002). AMF penetrates the roots and provides nutrients such as phosphorus, nitrogen, and water to the plant via their extensive hyphal network in the soil and receive organic compounds in return (Boyer et al., 2016). They also protect the plant host from pathogens and environmental stress, particularly drought (Boyer et al., 2015). Studies have also shown that the combined application of *Trichoderma harzianum* and AMF causes better root colonisation by both amendments and better productivity of brassica plants (Poveda et al., 2019).

In this study we report the results of 1-year-old apple M9 rootstock growth and associated rhizosphere microbiome when soils were amended before planting with single species biological amendments applied both individually and in a consortium. We compare the impact of the amendments on establishment of the tree compared to untreated or sterilised soils (to simulate chemical fumigation). The hypothesis of the study was that applying biological soil amendments together in a consortia will be more beneficial to the host (plant health and growth) than individual amendments by altering soil microbiome communities and soil function.

4.3 Materials and Methods

4.3.1 Soil Collection and General Characteristics

The study was conducted using soil from a recently grubbed apple orchard site at NIAB at East Malling in Kent, UK (latitude 51.286170, longitude 0.450902). The orchard was previously planted on M9 rootstocks and used for long-term apple production. Soil was mechanically dug from the top 50cm of soil across the length of the orchard into two separate 120cm x 100cm x 76cm heavy-duty plastic pallet boxes (2,000 L of soil) in March 2021. To remove large stones, debris, and macrofauna the soil was sieved through a 2.2cm pore rocking sieve and mixed in a separate empty plastic pallet box. A sub-sample of soil was sent for soil nutrient analysis at NRM Laboratories, Winklefield Row, Berkshire, UK. The soil was classified as sandy silt loam in texture with a soil pH of 6.9. The available P, K, and Mg were 31.4 mg/l, 151 mg/l, and 252 mg/l respectively.

4.3.2 Study Design

One-year-old M9 rootstocks were used as the tree host in the trial. Trees were planted in March 2021. The rootstocks were selected as those with the largest root system on the rootstock, indicating good nursery establishment; 10 L pots were used to provide adequate space for root proliferation. Rootstocks were also weighed prior to planting. 10 days before planting, approximately 5 L of soil and 30 g of Osmocote® slow-release fertiliser pellets were added to all pots.

Experiments were conducted in pots in a polytunnel using the dug bulk soil. The effect of pre-plant soil amendment on above-ground tree growth (girth, height, and above-ground weight), below-ground growth (fresh root weight and health), and rhizosphere microbiome were all evaluated. A 2x2x2x2 factorial design was used to assess the main effects of four beneficial biological soil amendments. A single species NIAB AMF, *Diversispora* sp. (NIAB / Plantworks®), Trianum (BCA), *Trichoderma harzianum* T-22 (Koppert®), Serenade, *Bacillus amyloliquefaciens* (formerly *subtilis*) strain QST 713 (Bayer®), and PGPR, *Pseudomonas fluorescens* (Plantworks®) were used as the four main treatments in the factorial design. The main effects of the treatments and their interactions

were assessed by applying treatments individually, in pairs, in threes, and all together. Three additional treatments (sterile control, Biofence®) pelleted brassica seed meal, and Rhea_AMF consortium of mycorrhizae (INOCULUMPlus®) were also applied to see their effects on rootstock establishment. The concentration of the PGPR was 1.46 x 10^7 cfu/ml, Serenade was 4.38 x 10^5 cfu/ml, and Trianum was 1 x 10^8 spore/g. Biofence was added 7 days before planting to allow time to degas and prevent toxicity to the plant. A 500ml aliquot of water was also added 7 days before planting to prevent drying out of the soil before planting. Treatments are summarised in Table 4.1.

Table 4.1 Soil amendment details, components, supplier, and volume added per sample.

Treatment	Name	Treatment Component (Supplier)	Volume/amount per tree
1	NIAB_AMF (A)	*Diversispora* sp. (NIAB / Plantworks®)	400 ml water + 25 ml scoop rootgrow™ professional granules
2	Trianum (B)	*Trichoderma harzianum T-22 (Koppert®)*	400 ml water + 0.1 g Trianum
3	Serenade (C)	*Bacillus amyloliquefaciens* (formerly *subtilis*) strain QST 713 (Bayer®)	400 ml water + 1 ml Serenade
4	PGPR (D)	*Pseudomonas fluorescens* – (Plantworks®*)*	400 ml diluted PGPR
5	A + B		400 ml water + 25 ml scoop rootgrow™ professional granules + 0.1 g Trianum
6	C + D		1 ml Serenade + 400 ml diluted PGPR
7	A + C		400 ml water + 25 ml scoop rootgrow™ professional granules + 1 ml Serenade
8	B + D		0.1 g Trianum + 400 ml diluted PGPR

9	A + D		25 ml scoop Diversispora + 400 ml diluted PGPR
10	B + C		400 ml water + 0.1 g Trianum + 1 ml Serenade
11	A + B + C		400 ml water + 25 ml scoop rootgrow™ professional granules + 0.1 g Trianum + 1 ml Serenade
12	A + B + D		25 ml scoop rootgrow™ professional granules + 0.1 g Trianum + 400 ml diluted PGPR
13	B + C + D		0.1 g Trianum + 1 ml Serenade + 400 ml diluted PGPR
14	A + C + D		25 ml scoop rootgrow™ professional granules + 1 ml Serenade + 400 ml diluted PGPR
15	A + B + C + D		25 ml scoop rootgrow™ professional granules + 0.1 g Trianum + 1 ml Serenade + 400 ml diluted PGPR
16	Control (nothing)		400 ml water
17	Biofence	*Brassica carinata* seed meal pellets (Biofence®)	300 g (1 week before planting) + 400 ml water
18	Rhea_AMF	“Rhea” five species AMF mix (INOCULUMPlus®)	25 ml scoop of powdered inoculum + 400 ml water
19	Sterilised Soil	Autoclaved (126 ℃ for 1 hour)	400 ml water

The study thus consisted of 19 treatments arranged in a random block design within the tunnel. There were six blocks with every treatment replicated once within each block (38 trees per block). Blocks 1-3 would be harvested in autumn 2021 (reported here) for analysis and blocks 4-6 would be harvested in autumn 2022. Each treatment was applied to all trees with that treatment across blocks (12 trees in 6 blocks) before the trees were randomly assigned to a position within one of the blocks. NIAB AMF and Rhea_AMF were both applied by sprinkling the inoculum on the root ball of the tree before planting and backfilling with soil. All

other amendment treatments were applied as a root dip treatment followed by watering with the required liquid amendment around the truck of the tree. The control trees were planted straight into the soil and watered with 400 ml water. Soil for sterilised treatment was autoclaved at 126 ℃ for 1 hour and stored in the polytunnel until required for planting. Sterile treatment rootstocks were planted in the autoclaved soil followed by the addition of 400 ml water.

4.3.3 Tree Management and Growth Measurements

A drip irrigation system was used for all the potted trees. Blocks 1-3, harvested in 2021 and blocks 4-6, due to be harvested in 2022, were placed on two different irrigation lines with the same timing for both. The timing for irrigation was initially set to 3 minutes once per day but was changed to 2 minutes twice a day (morning and afternoon) during the summer when the polytunnel temperature increased. Soil moisture was monitored using a W.E.T sensor probe (Delta-T Devices®) to prevent over-drying or too much moisture in the soil and irrigation was adjusted accordingly. Weeds were removed by hand when they appeared so no competition was present with the roots of the apple trees. Trees were otherwise managed using commercial practice but without fungicide application.

Initial girth measurements were made using callipers as the diameter of the trunk running both parallel and perpendicular to the length of the tunnel and using the mean of the two measurements as the girth measurement. The girth measurements were taken 5cm above the soil line and marked with non-toxic paint so the same area of the trunk was measured in November just before the destructive harvest of the trees. The height of the trees from the marked girth point was taken at planting and compared from the same point in November.

4.3.4 AMF Colonisation Assessment and Soil Sampling

Rootstocks from blocks 1-3 were destructively harvested in November, 2021. The trees were cut at the marked girth measurement point and the top above-ground portion weighed. The remaining root ball was knocked and shaken to remove loose bulk soil and rhizosphere soil was rubbed from the roots into a labelled clear polythene sample bag. Fine roots were plucked from the root ball and put

into a separate labelled sample bag for root-length-colonization (RLC; McGonigle et al., 1990) for the mycorrhizal analysis. The root ball was then shaken vigorously to remove as much of the remaining soil as possible and weighed. A 2 ml Eppendorf tube was filled with an equal volume of rhizosphere soil (1 ml soil each) from replicate treatment samples within in each block and stored at -20 ℃ for DNA extraction and sequencing. The remaining soil was stored at 4 ℃ in a cold store to maintain microbial function ready for soil function assessment. Root samples were stored at -20 ℃ prior to RLC staining.

To observe structures of AMF within the root, roots were stained with trypan blue. The method used was as described on the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi website (https://invam.wvu.edu/methods/mycorrhizae/staining-roots). This involved taking 0.5 g of fine root from each sample that was placed into histology cassettes and cleared in 20 % (w/v) KOH for 1 hour at 90 ℃ in a water bath. The cassettes were rinsed three times with water on a fine sieve to remove all KOH from root samples. Cassettes are then incubated in 2 % HCl at 20 ℃ for 1 hour, transferred to trypan blue 0.05 % (w/v) in lactoglycerol (5 : 1 : 1 lactic acid, glycerol, water) and incubated in a 90 ℃ water bath for 1 hour. To visualise roots they were destained by rinsing in 50 % (v/v) glycerol.

To quantify the total length of roots colonised by AMF, a grid-line intersect method was used (Giovannetti and Mosse, 1980). The method involves random dispersion of roots in a 9 cm diameter Petri dish with grid lines. The roots are then analysed under a dissecting microscope and each intersection between root and grid line is scored as positive or negative for AMF structures. This number was then converted to a % colonisation for each sample. A minimum of 100 intersections were used for increased accuracy. A logistic regression model was used to predict the contribution of each treatment to the percentage of root colonised by AMF.

4.3.5 API®ZYM Enzyme Assay Analysis

A further 5 g of bulk soil was dried in an oven 105 ℃ for 24 hours. The soil was then weighed to calculate dry weight of the soil. The dry soil was then wetted to saturation in a funnel with Whatman 11 um pore filter paper. This fully saturated soil was then weighed. Water holding capacity of the soil was then calculated per gram of soil. Dried soil for all samples was then adjusted to 60% water holding capacity in falcon tubes (approximately 0.3 ml dH2O/g soil) and stored in dark static conditions for 5 days. After incubation sterile dH_2O was added to each soil sample at a 1:1.5 ratio of soil to water. Tubes were shaken at 200 rpm on a horizontal shaker for 1 hour. Tubes were then centrifuged at 4,500 xg for 30 mins and the resulting supernatant was transferred to a fresh 50ml falcon tube. The supernatant was stored at 4 ℃ until required for enzymatic functional assessment.

API®ZYM strips (Biomereux) (Martínez et al., 2016) were used as a semi-quantitative assessment of 19 different enzymatic activities in the soil extract samples. API®ZYM trays were humidified with 5 ml of dH_2O before adding the enzyme strip into the tray. A 65 µl aliquot of soil extract was added to all 20 (19 + 1 control) cupules of the strip for each sample and covered with the provided lid. The strips were then incubated at 37 ℃ for 4 hours in dark conditions. After incubation one drop of ZYM A buffer and one drop of ZYM B buffer were added to each cupule and the colour change allowed to develop for 5 minutes. Colour change of the cupule was then scored according to the positive or negative colour change result listed in the API®ZYM manufacturer's protocol from a score of 0-5 (0/1 = negative or negligible colour change, 2/3 = intermediate colour change, 4/5 = positive result). The 20 enzymes including control used in the assay are listed in Table A.2.

4.3.6 Amplicon-sequencing of the Rhizosphere Soils for Bacterial and Fungal communities

4.3.6.1 Rhizosphere DNA Extraction

The method of amplicon sequencing and sequence processing follows the methods previously described (Deakin et al., 2018b). Genomic DNA was

extracted from the rhizosphere soil samples using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, USA), with the additional use of a benchtop beating homogeniser (Fastprep FP120, Qbiogene, USA). Concentration of extracted DNA and DNA quality were determined using both a spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific, UK) and a fluorometer (Qubit 2.0, Thermo Fisher Scientific, UK). 20 µl of DNA sample was sent to Novogene (Cambridge, UK) for library preparation and amplicon sequencing.

4.3.6.2 Amplicon sequencing

The quality of each DNA sample was also checked at Novogene. PCR amplification was conducted of the bacterial 16S V4 region with the primer pair Bakt_341F/Bakt_805R (Herlemann et al., 2011) and the fungal ITS1 and ITS2 regions with the primer pair EkITS1F/Ek28R(≡ 3126T) (Gardes and Bruns, 1993; Sequerra et al., 1997). The PCR product was then purified before sequencing using the Illumina NovaSeq 6000 platform.

4.3.6.3 Sequence Read Processing

Sequence data was submitted to the NCBI database (Project PRJEB54726). Any ambiguous reads that did not match either the forward or reverse primers for 16S and ITS were removed. USEARCH V11.0 (Edgar, 2013a) was used for all analyses unless otherwise stated.

Fungal and bacterial reads were processed separately. 16S reads were aligned with a maximum difference in overlap threshold of 5 %. The forward and reverse primer sequences were removed from both bacterial and fungal reads at this stage. Merged reads with adaptor contamination or a total length that is fewer than 150 or 300 nucleotides for ITS reads and 16S reads respectively were removed. Quality filtering was conducted using a mean number of errors per sequence threshold of 0.1 for ITS reads and 0.5 for 16S reads.

4.3.6.4 OTU Generation

Unique sequences were identified and any unique sequence that had less than 4 reads was discarded from the generation of OTUs. Sequences were initially sorted by decreasing read frequency and OTUs were generated by clustering the

unique sequences at 97 % sequence similarity and a representative sequence for each OTU sequence was also generated. All sequence reads were mapped against each representative OTU sequence to generate OTU count tables for fungal and bacterial OTUs. Taxonomy of each representative OTU sequence and confidence level of assignment at each taxonomic rank was predicted using a SINTAX algorithm (https://www.drive5.com/usearch/manual/sintax_algo.html) by aligning ITS OTU representative sequences to the reference database “UNITE V8.3” (Nilsson et al., 2019) and 16S OTU representative sequences to the RDP training set V18 (Cole *et al.,* 2014).

4.3.7 Statistical Analysis

4.3.7.1 Morphological Data

The difference in height and girth was calculated as the difference between the measurements taken in autumn from those when they were initially planted. For the height and girth measurements an ANOVA with the 2x2x2x2 factorial design was used to identify differences in the establishment of the trees due to the effect of applying each of the treatments NIAB_AMF, Trianum, Serenade, and PGPR. A Shapiro-Wilk normality test was used to test data met the prerequisites for parametric testing. Girth data followed a normal distribution, however height data was negatively skewed so was reflected and normalised using a square root transformation. Similarly, a factorial design ANOVA for each of the above treatments was used for the fresh weight of both the above and below ground weights of the rootstocks after Shapiro-Wilk normality test.

4.3.7.2 Sequence Data Analysis

The vegan R package V2.6.2 (Dixon, 2003) was used to normalise the OTU counts by rarefaction before further analysis. Two diversity indices, alpha and beta, were used to compare differences in fungal and bacterial communities due to pre-plant treatment. Alpha (α) diversity indices (Chao1, Shannon, and Simpson) were calculated in the phyloseq R package v1.38 (McMurdie and Holmes, 2013). The LmPerm R package V2.1 (Wheeler, 2016) was used for permutation ANOVA analysis to detect significance of treatment effect on each of the three diversity indices. Beta (β) diversity was calculated in the vegan

package using Bray-Curtis dissimilarity. Samples were visualised using non-metric multidimensional scaling (NMDS) plots to identify dissimilarity in fungal and bacterial communities due to treatments. Permutations of multivariate analysis of variance using F-tests based on the sequential sum of squares (ADONIS) based on 1,000 permutations was used to assess the effects of pre-plant treatments on beta diversity.

The DESeq2 package V1.34 (Love, Huber and Anders, 2014) was used to identify OTUs with significantly differential relative abundance due to the application of the pre-plant amendments. Only OTUs with p-value ≤ 0.05 were included in DESeq2 analysis. Of the differential OTUs identified, the five OTUs that had either the greatest (more abundant) or lowest (less abundant) log2 fold change had their OTU representative sequences submitted to the NCBI Nucleotide BLAST search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify predicted taxonomy of each differential OTU. Taxonomy was only assigned to OTUs with a percent identity ≥ 95 %.

4.4 Results

4.4.1 Above-Ground Tree Establishment

Biofence application caused detrimental changes in the soil structure, with these treatment pots not holding water as well as the others, causing either death or delayed break in the dormancy of the host rootstock. It is likely that Biofence was applied in too high a concentration for the pot size. Biofence was thus excluded from subsequent analysis of the above ground establishment. Establishment of the remaining rootstocks was good in the first eight months of growth from April-November. There were no clear visible differences in branching or leaf area between treatments.

Figure 4.1 shows there were no statistical differences in height or girth difference between any of the treatments (excluding Biofence) applied either individually or in a consortium at the eight month point. The main effect of each of the single species treatments on girth difference had p-values of 0.09, 0.32, 0.70, and 0.66 for each of the treatments, NIAB AMF, Trianum, Serenade, and the PGPR, respectively. P-values for NIAB AMF, Trianum, Serenade, and PGPR main effect for height change were 0.09, 0.69, 0.25, and 0.51. NIAB AMF had a marked effect (but not significant) for both height and girth change and appeared to link to trees with a lower girth and height difference (Figure 4.1). There were no significant differences in the interactions of treatments on height or girth change.

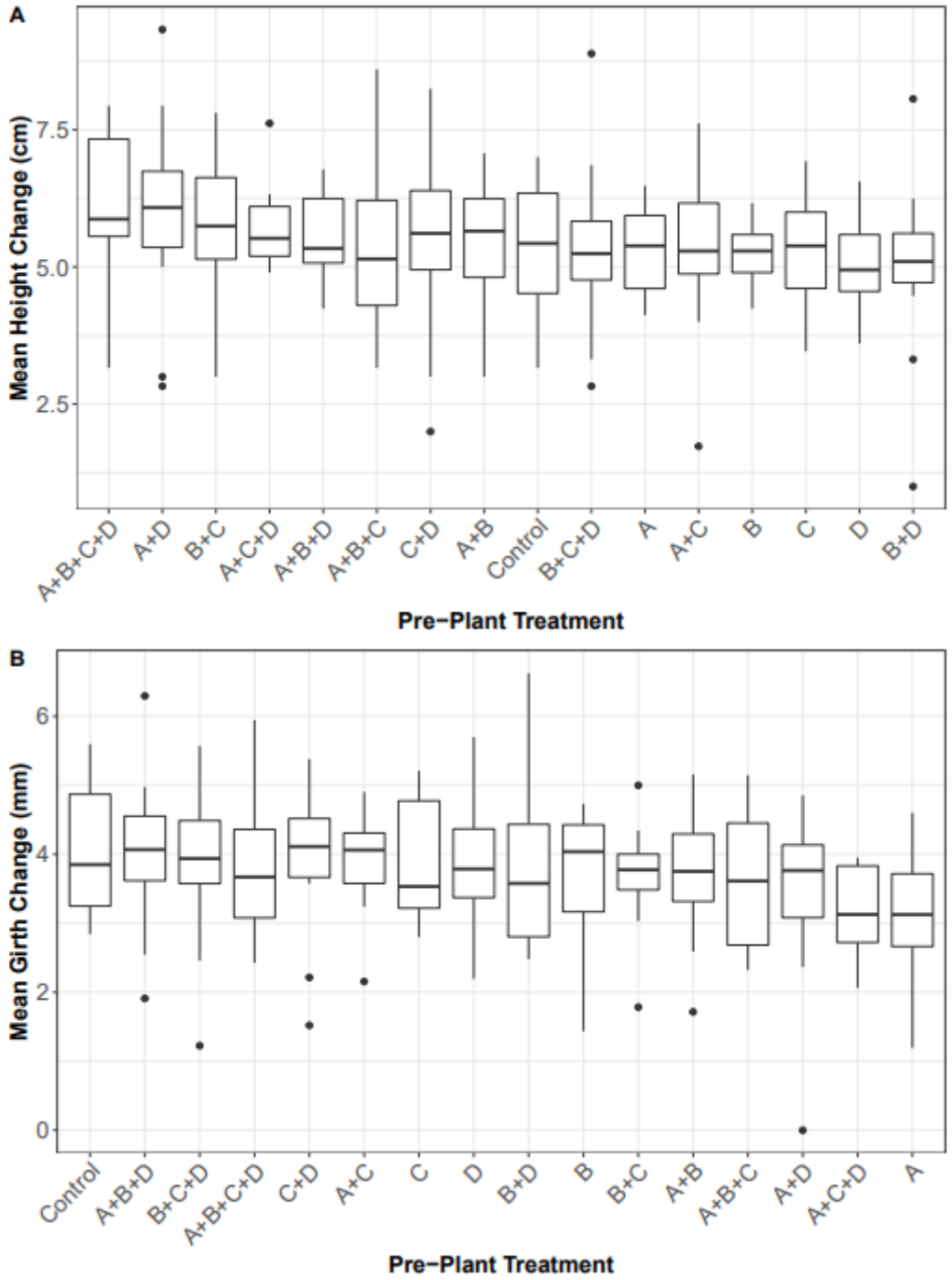

Figure 4.1 Height (A) and girth (B) change of the rootstocks in the first eight months of establishment. Treatments are labelled as described in Table 4.1. There were no statistical differences between any of the treatments for height or girth change. A = NIAB_AMF, B = Trianum, C = Serenade, and D = PGPR.

The main effect of treatments NIAB AMF, Trianum, Serenade, and PGPR on above-ground weight had P-values of 0.69, 0.13, 0.37, and 0.99 respectively. Similarly to the height and girth differences, there were also no significant differences in the interactions between any of the treatments. Figure 4.2 shows the similarities in above-ground weight between all treatments applied both individually and in a consortium.

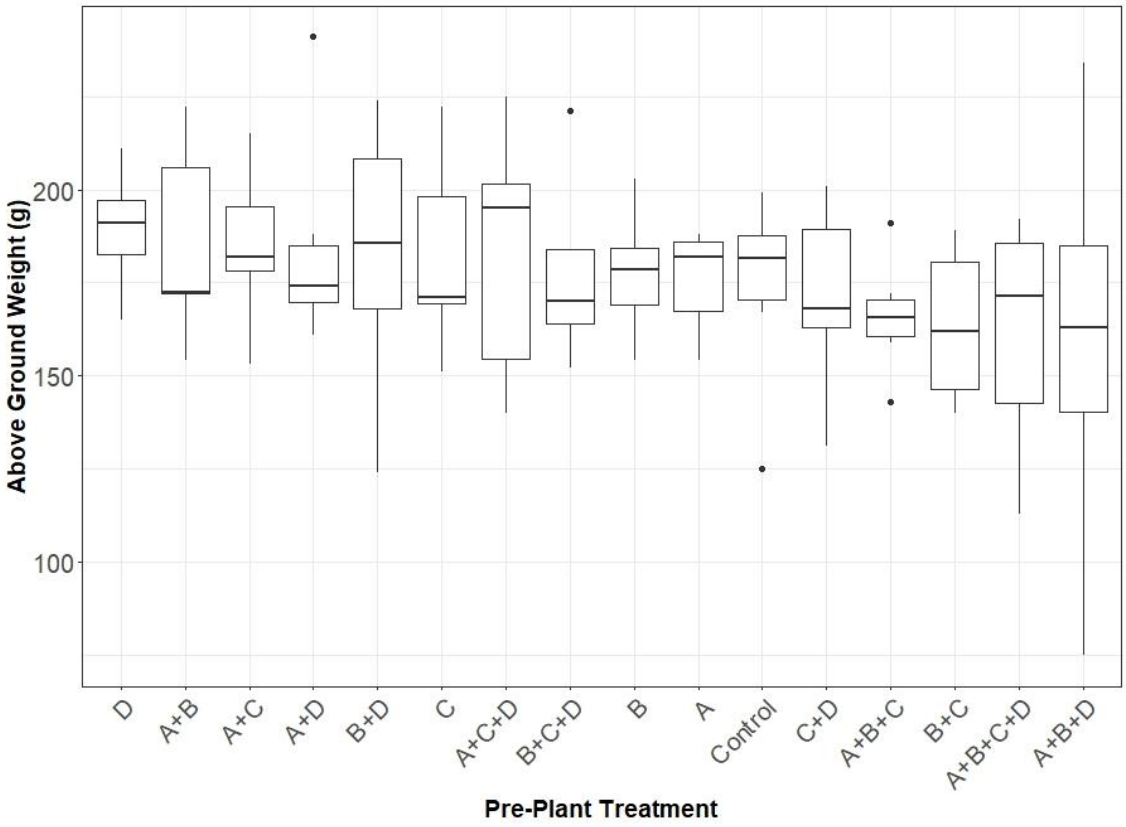

Figure 4.2 Above-ground weight of differently pre-plant amended potted rootstocks. Treatments are labelled as described in Table 4.1. There were no statistical differences between the above ground weight between treatments. A = NIAB_AMF, B = Trianum, C = Serenade, and D = PGPR.

4.4.2 Below-Ground Tree Establishment

The general health of the roots was also similar between all samples. No root tip necrosis or blackening of roots was seen in any samples other than those with the Biofence treatment. There was some differences in root ball size between samples but this did not correlate with a reduction in above-ground growth for any sample or directly with any of the treatments. The Biofence-treated root systems often did not show any growth from when they were planted and had blackening and necrosis on the root systems. The root systems that had grown when treated with Biofence were significantly smaller than the other treatments.

4.4.3 Root-Length-Colonisation of Rootstock Roots

Colonisation of roots by AMF is required for beneficial nutrient exchange between the plant host and AMF. All rootstocks had root length colonisation > 4 % in all samples (Figure 4.3). Additionally, the autoclaved sterilised soil had mean root length colonisation of between 8-9 %. Only three of the eighteen treatments had a lower mean root length colonisation (although not statistically significant) than the control pots, two of which had been treated with a consortium of the beneficials including *Diversispora* sp. (NIAB_AMF). The standard error of root length colonisation was high for many treatments which suggests the samples may not be representative of the population. Logistic regression of the root length colonisation highlighted the contribution of treatment was not significant (Table 4.2). There was also no statistical significant effect of the individual 4 treatments (NIAB_AMF, Trianum, Serenade, and PGPR) on root length colonisation. Trianum, had a p value of 0.11 and appeared to be associated with roots with lower RLC % (see Figure 4.3, Table 4.2).

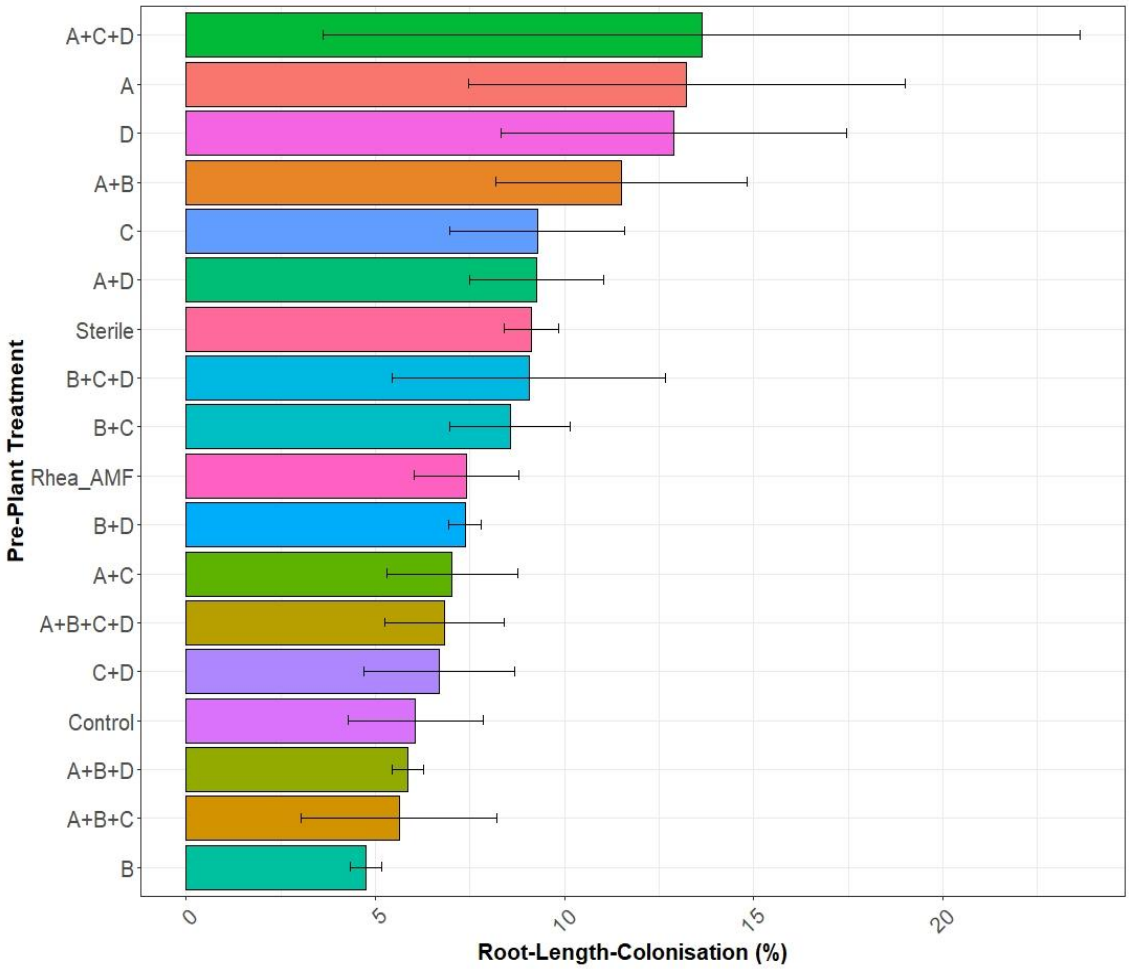

Figure 4.3 Percentage of root length colonised by AMF for each treatment. A = NIAB_AMF, B = Trianum, C = Serenade, and D = PGPR.

Table 4.2 Analysis of deviance table for root length colonisation due to treatment and block effect. The main effect of the amendments in the 2x2x2x2 factorial design were investigated by samples with the treatment vs all other samples without the amendment (including the control, not including Biofence, Rhea_AMF, and Sterile). The models were quasibinomial with logit link.

Variable	df	Deviance	Residual df	Residual Deviance	P-Value
NULL			54	1.86	
Treatment	18	0.54	36	1.31	0.35
Block	2	0.37	34	0.94	0.001
NULL			47	1.75	
Block	2	0.46	45	1.29	7.07 e-4
NIAB_AMF	1	0.02	44	1.27	0.47
Trianum	1	0.08	43	1.19	0.11
Serenade	1	0.004	42	1.18	0.71
PGPR	1	0.007	41	1.17	0.63

4.4.4 Soil Function

To compare functionality of soils with different treatments, an API®ZYM enzyme activity assay was conducted. The pattern of enzyme activity in the soils of each treatment was fairly similar across all samples (Table 4.3). There were a number of notable differences in enzyme activity for some of the treatments compared to the pattern in the control soils. NIAB_AMF (only *Diversispora* sp.) showed higher activity of C4 esterase and leucine arylamidase compared to low activity in control samples. NIAB_AMF also had medium activity for alkaline phosphatase and valine arylamidase. Treatments B+D (Trianum and PGPR) and A+B+C (NIAB_AMF, Trianum, and Serenade) both had medium activity for C4 esterase. Biofence was the only treatment with medium activity of α-fucosidase with low activity in all other samples. Rhea_AMF showed quite a different pattern from the

control samples with seven enzymes (leucine arylamidase, cytine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, and α/β-glucosidase) with medium activity compared to low activity in the control, while acid phosphatase enzyme activity was lower than the control samples

Table 4.3 Mean score data of enzyme activity for each treatment. Red colour indicates negative/low-intensity enzyme activity (0-1), orange indicates moderate enzyme activity (2-3), and green is high enzyme activity (4-5). A = NIAB_AMF, B = Trianum, C = Serenade, and D = PGPR.

Enzyme	A	B	C	D	A+B	C+D	A+C	B+D	A+D	B+C	A+B+C	A+B+D	B+C+D	A+C+D	A+B+C+D	Biofence	Rhea_AMF	Sterile	Control
Alkaline phosphatase	3	2	2	1	1	1	2	2	2	2	2	2	2	2	2	1	2	2	1
Esterase (C 4)	4	2	1	1	1	1	2	3	2	2	3	2	1	2	1	1	2	2	1
Esterase Lipase (C 8)	3	2	2	1	1	2	2	2	2	1	3	2	1	2	2	2	2	2	2
Lipase (C 14)	2	0	1	1	0	0	0	1	1	1	1	0	0	0	1	1	1	0	0
Leucine arylamidase	4	1	1	1	1	1	2	1	1	1	2	2	1	1	1	1	3	2	1
Valine arylamidase	3	1	1	1	0	1	2	1	1	1	1	2	1	1	1	1	1	1	1
Cystine arylamidase	1	1	1	0	0	1	0	0	0	1	1	1	1	1	0	0	2	1	1
Trypsin	1	0	1	0	0	0	0	1	2	1	2	1	1	0	0	0	2	0	1
α-chymotrypsin	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	2	0	1
Acid phosphatase	4	4	2	3	4	3	3	2	3	2	2	4	4	3	4	3	2	3	4
Naphthol-AS-B1-phosphohydrolase	5	5	5	5	5	5	5	4	5	5	5	5	5	5	5	5	5	5	5
α-galactosidase	0	0	0	0	1	0	0	0	0	1	0	0	1	0	1	0	2	0	1
β-galactosidase	0	0	0	1	1	0	0	0	1	1	1	1	1	0	1	0	0	1	1
β-glucuronidase	1	0	0	1	1	0	0	0	1	1	1	0	1	0	0	0	1	1	1
α-glucosidase	1	0	0	1	1	1	0	1	0	0	1	0	0	0	0	0	2	0	0
β-glucosidase	1	1	1	0	0	0	1	1	1	1	1	1	1	0	1	0	3	0	1
N-acetyl-β-glucosaminidase	1	1	1	0	0	0	1	1	0	1	1	1	1	0	0	0	1	1	1
α-mannosidase	1	0	0	0	0	1	0	0	0	0	1	1	0	0	1	0	1	0	0
α-fucosidase	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	2	1	0	0

4.4.5 Summary of Amplicon-Sequencing Data

There were a total of 5,008,880 fungal reads and 3,497,250 bacterial reads across the 57 rhizosphere samples sequenced. The number of OTUs generated was 3,571 for fungal reads and 28,768 for bacterial reads. The number of reads per sample ranged from 30,884 to 110,774 reads for fungi and ranged from 45,216 to 78,430 reads for bacteria. The range of reads per OTU ranged from 2 to 55,708 (median = 4) reads for fungal OTUs and 2 to 23,863 (median = 3) reads for bacterial OTUs.

4.4.6 Diversity Indices

Three alpha diversity indices were calculated (Chao1, Shannon, and Simpson). Alpha diversity values were higher in bacterial community than for fungal populations across all samples. The pre-plant amendment applied (treatment) was associated with 23.2 %, 23.3 %, and 25.6 % of the total variability in fungal alpha diversity for Chao1, Shannon, and Simpson respectively but were not significant for any index (Table 4.4). More of the total variability in bacterial alpha diversity was explained by treatment compared to fungi with 34.8 %, 40.2 %, and 35.3 % of the total variability for Chao1, Shannon, and Simpson. Most of the variation in alpha diversity was not explained by experimental factors for either bacteria or fungi. A separate analysis of the main effect of NIAB_AMF, Trianum, Serenade, and PGPR in the 2x2x2x2 factorial design (Table 4.5), highlighted that the contribution of the main effects of each of the treatments was small (≤ 4.8%) to the variation in alpha diversity for both bacteria and fungi and was not statistically significant. Biofence, Sterile soil, and Rhea_AMF were not included in the factorial model so the higher contribution of these treatments in the full model was associated with these treatments. Figure 4.4 highlights the similarity in alpha diversity between the consortium treatments but also shows the lower alpha diversity in the Sterile, Biofence and Rhea_AMF treated samples, agreeing with the model results.

Table 4.4 Percentage of the variability in alpha diversity indices accounted for by block and soil amendment (treatment).

Measure	Block		Treatment		Residual
	P value	*%*	*P value*	*%*	*%*
Fungi					
Chao1	0.48	4.2	0.93	23.2	72.6
Shannon	0.75	0.7	0.91	23.3	76.0
Simpson	0.98	0.1	0.84	25.6	74.3
Bacteria					
Chao1	0.78	0.6	0.43	34.8	64.6
Shannon	0.46	2.7	0.18	40.2	57.1
Simpson	0.48	2.9	0.36	35.3	61.8

Table 4.5 Percentage of the variability in alpha diversity indices accounted for by block and main treatment effect of amendments A (NIAB AMF), B (Trianum), C (Serenade), and D (PGPR). The Sterile, Biofence and Rhea_AMF treatments were not included in the model.

Measure	Block		NIAB_AMF		Trianum		Serenade		PGPR		Residual
	P Value	*%*	*P Value*	*%*	*P Value*	*%*	*P Value*	*%*	*P Value*	*%*	*%*
Fungi											
Chao1	0.14	7.9	0.22	2.2	0.47	0.4	0.27	2.4	0.66	0.1	87.0
Shannon	1.0	0.2	0.69	0.8	0.52	0.6	0.39	4.1	0.22	1.8	92.5
Simpson	0.94	0.2	0.62	1.4	0.49	0.5	0.22	3.0	0.35	2.9	92.0
Bacteria											
Chao1	0.80	0.8	0.31	3.4	0.29	1.8	0.10	4.8	0.34	1.4	87.8
Shannon	0.81	2.2	1.0	0.02	0.54	0.2	0.90	0.1	0.29	2.4	95.1
Simpson	0.45	3.7	0.51	0.5	0.80	0.5	0.59	0.4	0.78	1.4	93.6

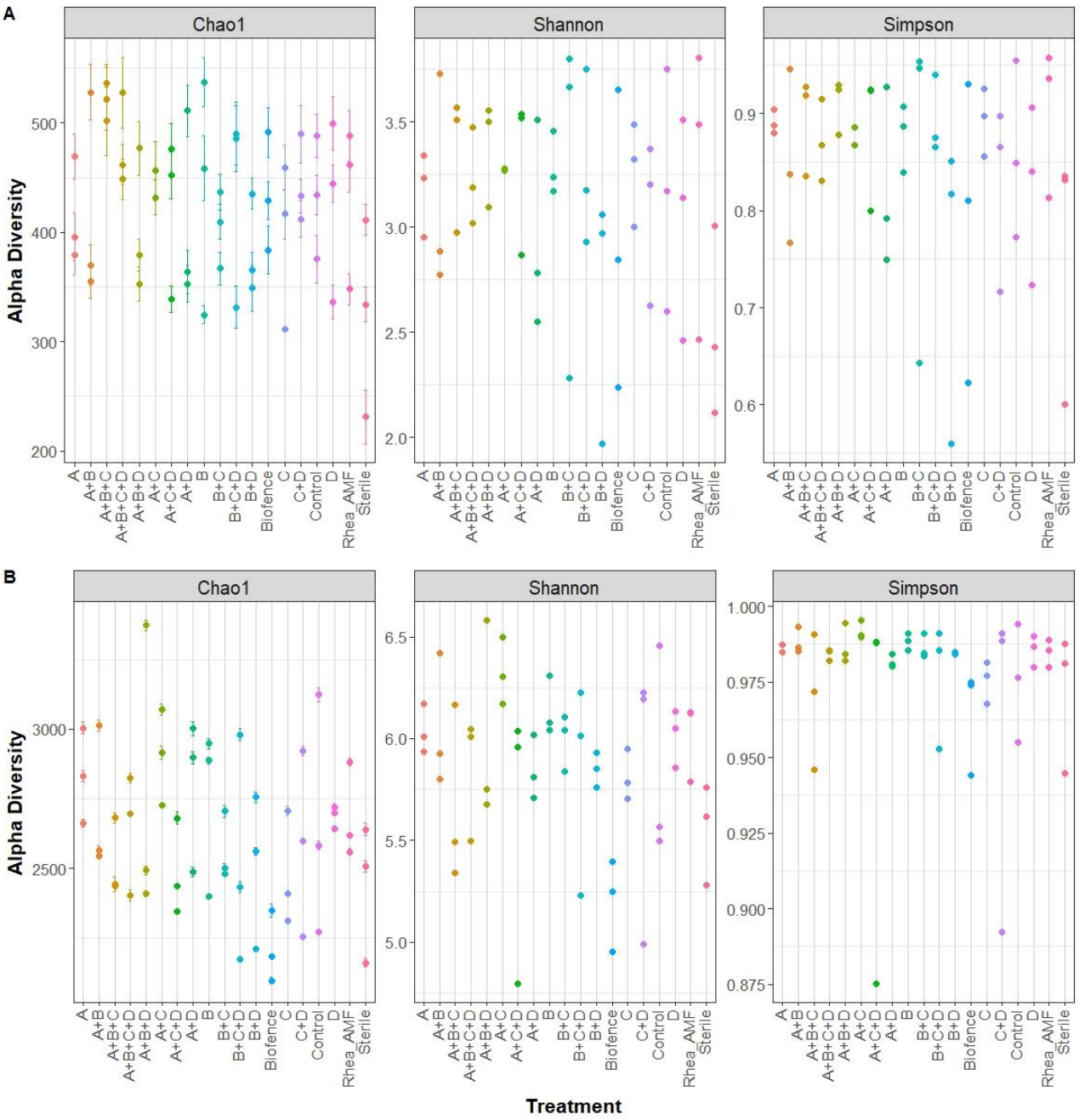

Figure 4.4 Alpha (α) diversity measures, Chao1, Shannon, and Simpson for (A) fungi and (B) bacteria. The x-axis indicates the treatment applied pre-planting of the rootstocks from Table 4.1. A = NIAB_AMF, B = Trianum, C = Serenade, and D = PGPR.

Differences in microbial communities were visualised using Bray-Curtis beta diversity. The communities of bacteria and fungi were highly similar across treatments (Figure 4.5). The Biofence treated samples and to a lesser extent the sterile soil samples were dissimilar to the other samples for both bacterial and fungal communities. ADONIS analysis highlighted both the difference in beta diversity between block and treatment effects were statistically significant for both fungal and bacterial communities (Table 4.6). The difference between blocks only accounted for 5.8 % of the variance in fungal beta diversity and 7.0 % in bacterial beta diversity. The difference in communities due to treatment accounted for 37.5 % and 39.7 % of the total variation for fungi and bacteria respectively. Approximately 50 % of the total variation was not accounted for by the block or treatment effects (56.7 % for fungi and 53.3 % for bacteria). In the factorial analysis (Table 4.7) of the NIAB AMF, Trianum, Serenade, and PGPR treatments, NIAB AMF ($p = 0.09$) and Serenade ($p = 0.07$) showed marked differences for beta diversity in fungal communities accounting for 3.5 % and 3.6 % of the total variation respectively. Serenade significantly affected beta diversity of bacterial communities ($p = 0.05$) accounting for 3.1 % of the total variation and Trianum also was almost significant with a p value of 0.11, accounting for 2.7 % of the variation. A total of 82.1 % of the variation of fungal beta diversity and 81.1 % of bacterial diversity was unexplained in the model by Block or main effects of NIAB AMF, Trianum, Serenade, and PGPR treatments.

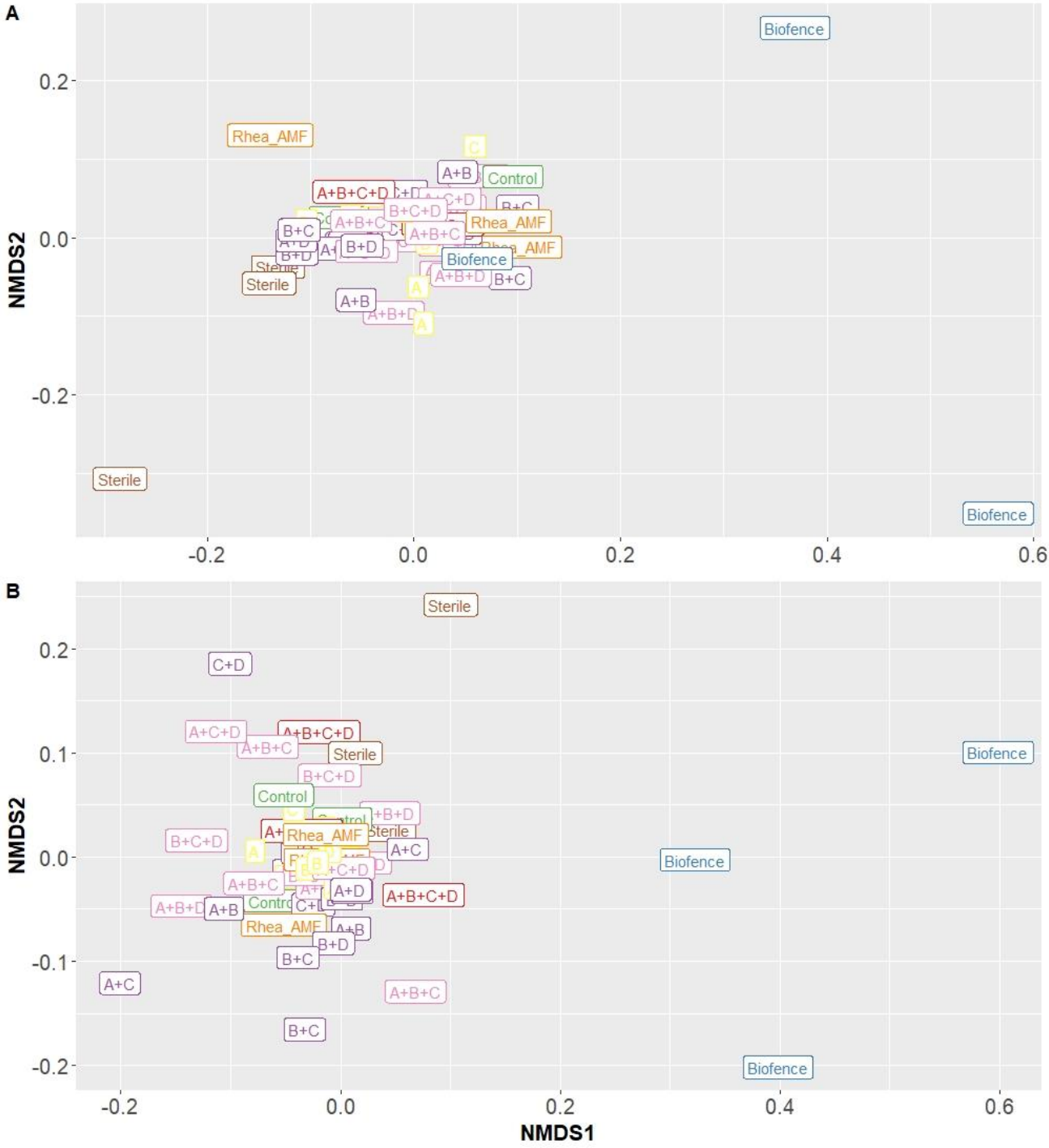

Figure 4.5 The first two dimensions of NMDS analysis of the Beta (β) diversity indices (Bray-Curtis dissimilarity) for (A) fungi and (B) bacteria. Closer the distance between the points indicates more similarity in microbial communities between the samples. A = NIAB_AMF, B = Trianum, C = Serenade, and D = PGPR.

Table 4.6 Permutational Multivariate Analysis of Variance (ADONIS) based on 1000 permutations of Bray-Cutis beta diversity accounted for by block and soil amendment (treatment) factors.

	df	Sum sq	%	P-value
Fungi				
Block	2	0.31	5.8	0.01
Treatment	18	1.98	37.5	0.01
Residuals	35	2.99	56.7	
Bacteria				
Block	2	0.37	7.0	0.001
Treatment	18	2.12	39.7	0.001
Residuals	36	2.85	53.3	

Table 4.7 2x2x2x2 factorial design Permutational Multivariate Analysis of Variance (ADONIS) based on 1000 permutations of Bray-Cutis beta diversity accounted for by block and the main effects of NIAB AMF, Trianum, Serenade, and PGPR.

	df	Sum sq	%	P-value
Fungi				
Block	2	0.26	7.9	0.02
NIAB_AMF	1	0.11	3.5	0.09
Trianum	1	0.03	0.9	0.95
Serenade	1	0.12	3.6	0.07
PGPR	1	0.06	2.0	0.42
Residuals	40	2.69	82.1	
Bacteria				
Block	2	0.33	9.1	9.99 e-4
NIAB_AMF	1	0.08	2.2	0.31
Trianum	1	0.10	2.7	0.11
Serenade	1	0.11	3.1	0.05
PGPR	1	0.06	1.8	0.60
Residuals	41	2.95	81.1	

4.4.7 Differential OTUs

Differential OTU abundance was compared between Biofence, Sterile, and Rhea_AMF trees and control trees. Additionally, the main effect of applying each of the amendments NIAB AMF, Trianum, Serenade, and PGPR treatments were compared to those that did not receive the amendment to see main differences of the amendment on OTU abundance across all samples (Table 4.8). Of the total representative OTUs, 63 % of the fungal OTUs and 66 % of the bacterial OTUs remained after DESeq2 filtering for comparison. Only a small number of OTUs, >3 % of OTUs passing DESeq2 filtering, had a significant (Benjamini and Hochberg adjusted $p \leq 0.05$) differential abundance in any of the models. Fungal OTUs were more different in the control compared to the Biofence, sterile soil or Rhea_AMF treatments compared to bacterial OTUs. There was a larger number of bacterial OTUs significantly more or less abundant due to the applying NIAB_AMF, Trianum, Serenade, or PGPR (Table 8). The number of bacterial OTUs were more abundant due to the main effect of applying the treatments ranging from 65 – 238 OTUs and OTUs less abundant ranged from 116 – 163 OTUs. Fungal OTUs ranged from 15 – 56 OTUs with significantly increased abundance and 20 – 37 OTUs with significantly lower abundance.

Table 4.8 DESeq2 results summary for all differential OTUs. No. OTUs indicate the number of OTUs after DESeq2 filtering (compared between factors); Log2 Fold Change (LFC) > 0 indicates a higher abundance in the first term in the model and a negative LFC indicates higher abundance in the second term. The NIAB_AMF, Trianum, Serenade, and PGPR describe the main effect of applying each of the treatments.

Model	No. OTUs	LFC > 0 (Higher)	LFC < 0 (Lower)	Low Counts
Fungi				
Control vs Biofence	2267	0	2	0
Control vs Sterile	2267	12	29	1802
Control vs Rhea_AMF	2267	3	2	0
NIAB_AMF	2172	24	31	0
Trianum	2172	19	27	0
Serenade	2172	56	20	370
PGPR	2172	15	37	0
Bacteria				
Control vs Biofence	19047	0	0	0
Control vs Sterile	19047	0	1	0
Control vs Rhea_AMF	19047	0	0	0
NIAB_AMF	17698	137	131	1590
Trianum	17698	219	122	1350
Serenade	17698	238	116	5769
PGPR	17698	65	163	0

Of the top five OTUs with significant differential abundance due to the application of each of NIAB_AMF, Trianum, Serenade, or PGPR only 30 % of the OTUs were assigned taxonomically at 95 % sequence identity (Table 9). Many of the taxonomies assigned to the OTUs were either saprophytic or free-living or had an unknown function in agricultural soils. *Verticillium* was identified to be more abundant due to the application of Trianum and is associated with *Verticillium* wilt in many crops. *Pencillium* (+ PGPR / - Serenade), *Curvularia* (+ PGPR), and *Sebacina* (= *Serendipita*) *vermifera* (- PGPR) were identified as candidate fungal beneficial plant-growth-promoters, although strains of *Penicillium* and *Curvularia* can also be pathogenic to plants. Application of Trianum was also positively correlated with the abundance of the beneficial bacteria *Pedobacter*, but negatively correlated with the abundance of *Bacillus* and *Azospira restricta* both of which also display plant-growth promoting action in the rhizosphere.

Table 4.9 OTUs with the highest log2 fold change (top five with increased or decreased abundance) with a predicted taxonomy from NCBI BLAST searches identified by DESeq2 analysis. 95% identity confidence threshold was used to assign taxonomy. OTUs without an ambiguous taxonomy from the NCBI BLAST search are not included in the table.

Comparison*	OTU	BLAST Taxonomy
Fungi		
+ NIAB_AMF	OTU690	*Tricholomataceae*
- NIAB_AMF	OTU1532	*Vargamyces*
	OTU496	*Agaricales*
	OTU496	*Rhizophydium*
+ Trianum	OTU1505	*Verticillium tricorpus*
- Trianum	OTU2619	*Apiothrichum porosum*
+ Serenade	OTU208	*Pyronemataceae*
	OTU1153	*Chaetomium*
- Serenade	OTU1748	*Penicillium*
	OTU690	*Tricholomataceae*
+ PGPR	OTU898	*Penicillium*
	OTU1596	*Tarzetta*
	OTU3204	*Penicillium*
	OTU1524	*Curvalaria*
- PGPR	OTU793	*Sebacina vermifera*
Bacteria		
- NIAB_AMF	OTU4283	*Megamonas funiformis*
+ Trianum	OTU3117	*Pedobacter*
	OTU5540	*Pedobacter*
- Trianum	OTU9625	*Bacillus*
	OTU6281	*Azospira restricta*
+ Serenade	OTU996	*Adlercreutzia*
	OTU223	*Bifidobacterium longum*
- Serenade	OTU25758	*Tabrizicola piscis*
+ PGPR	OTU4570	*Acinetobacter bereziniae*
- PGPR	OTU5607	Chloroplast

* (+) indicates presence, (-) indicates absence (ie. A treatment vs all other treatments and control)

4.5 Discussion

In this study over the first 9 month of establishment of the rootstocks, we did not identify any changes in the height or girth with the different treatment. Biofence was the only treatment to display slower establishment but this reduction was primarily due to death and/or delay in growth of the rootstock due to the application of too high a concentration of the biofumigant. The first 1-3 months after replanting are the considered the most susceptible stage of the tree to ARD symptoms (Mazzola and Manici, 2012). However, in this study we did not observe any above ground ARD symptoms in any of the rootstocks. It has been recently suggested that ARD symptoms only become apparent above ground between 15-24 months after replanting, and it is primarily the below ground root system that is affected during this period (Deakin et al., 2019; Tilston et al., 2020). The present study similarly did not detect an above-ground effects. In addition, root health and establishment were also similar across treatments potentially implying the possible absence of replant causal agents in the trial soils or that below ground symptoms may only emerge in the second growing season. AMF application can cause a reduction in early establishment of crops due to the shared exchange of nutrients between the fungi and roots and this could partially explain the marked reduction in above ground establishment in rootstocks that received the *Diversispora* sp. amendment. Fresh root weight was not a good indicator of root establishment due to moisture and excess soil incorporated with the root system. It is probably best to use dry weight or a visual assessment of root health as the primary identification of below-ground ARD symptoms in future studies.

It is well-established that rootstocks are able to shape the rhizosphere through the production of root exudates (Burns et al., 2015; Leisso, Rudell and Mazzola, 2017). The temporal nature of this recruitment is a rapid process with wheat species showing up to a 40 % reduction in the similarity of chemotaxic microbial communities between rhizosphere and bulk soil samples in a 3 day period (Buchan, Crombie and Alexandre, 2010). It is unclear if this process is as rapid in apple rootstocks as in cereals. The recruitment process is primarily driven by

rootstock genotype, which might explain the similarities in both establishment and microbial diversity between the treatments in the trial. Sterile soils and Biofence soils showed dissimilarity in both bacterial and microbial communities to the control and amended soil samples. Both these treatments likely had microbial diversity lowered either by biofumigation or by soil sterilisation through autoclaving. The composition of the rhizosphere microbiome was found to be influenced by the presence or absence of *Diversispora* spp. for fungal communities, *Bacillus amyloliquefasciens* for both fungal and bacterial communities and *Trichoderma harzianum* for bacterial communities. This suggests that the application of a single species can alter the rhizosphere microbiome although whether this could potentially influence the early establishment of rootstocks is not known. Analysis in future years should elucidate if these effects can lead to above or below ground effects in tree establishment. It is highly likely that single species amendments applied either individually or in a consortium have a significantly lower influence on the overall microbiome population than rootstock genotype recruitment influence on soil microbiota.

It is difficult to assess the efficacy and survivability of the amendments applied to the soil using differential abundance of general fungal and bacterial primers. Within the OTUs with the highest log2 fold change only a small number (30 %) were able to be identified to genus level or below with the majority assigned as unknown fungi or bacteria. This makes functional predictions of those differential OTUs on apple rootstock establishment challenging. OTUs associated with pathogens of crops such as *Verticillium* were associated with soils that had *Trichoderma harzianum* applied. *Verticillium* contains a number of strains associated with *Verticillium* wilt in a number of crops and has been associated with ARD conducive soils and found to be pathogenic to some rootstocks (Jiang et al., 2017; Karajeh and Owais, 2012; Xing et al., 2017). The study suggests that *Trichoderma harzianum* does not control *Verticillium* and may be associated with an increase of the pathogen, although it is unclear what the virulence of strain might be in terms of pathogenicity to apple without further testing. OTUs associated with bacterial plant-growth promoters were also associated with the

application or absence of *Trichoderma harzianum*. *Pedobacter* had an increased abundance when *Trichoderma harzianum* was applied and has been shown to have a beneficial effect on plant growth in strawberry (Morais et al., 2019). However, soils that did not have have the inoculum of *Trichoderma harzianum* had increased abundance of OTUs associated with *Bacillus,* a well known plant-growth-promoting rhizobacteria (Saxena et al., 2020), and *Azospira restricta* that has nitrogen fixing capabilities but is not well described in agricultural soils (Bae et al., 2007). Further studies therefore are required to identify how the changes due to pre-plant soil amendments such as with *Trichoderma harzianum* affect the functionality of the rhizosphere and how they might correlate with establishment of the apple rootstock as application affects both beneficial and pathogenic microbiota.

Problems also arise when taxonomy associated with OTUs contains genera that are both pathogens and beneficials in soils. Two examples were *Penicillium* associated with the absence of Serenade and the application of PGPR and *Curvularia* associated with the application of PGPR. *Penicillium* has been described as both a post-harvest pathogen of apple but also a plant-growth-promoting fungus in *Arabidopsis* (Habib et al., 2021; Hossain et al., 2008). Similarly, *Curvularia* species have P solubization function in *Parthenium hysterophorus.* Indeed, *C. mirospora* was found to be associated with leaf spot disease of *Hippeastrum striatum* in China but have yet to be reported as pathogenic to apple. It is therefore not clear for these genera if the application of PGPR would be associated with a beneficial or detrimental effect from *Penicillium* or *Curvularia*, again highlighting the limitations of solely using a single time frame for sequencing to identity function of differential OTUs.

In the case of the mycorrhizae amendments, root length colonisation was not significantly increased when the inoculum was applied to the root ball. This suggests that if any beneficial effect was to be observed in trees with this amendment is it not possible to attribute the effects directly to mycorrhizal colonisation. It is possible the AMF product may have had a low number of viable propagules. It has been shown that AMF inoculants are able to survive on

strawberry roots during cold storage between the nursery and planting (Langendorf, 2017). Thus colonisation may be due to latent AMF present from nursery fields rather than due to the amendment applied at planting. *Sebacina* (= *Serendipita*) *vermifera* was identified to have an increased abundance in the absence of PGPR and has been previously shown as a dominant endophyte in apple orchards, that enhances plant growth in switchgrass (van Horn, Somera and Mazzola, 2021; Ray and Craven, 2016). It therefore could be *S. vermifera* among other native AMFs that effectively colonise the root as opposed to the applied AMF amendments. In the study AMF was applied as a granular or powder formulation to the root system. Advances in the commercialisation of mycorrhizae amendments have suggested improved application can be made with formulations that include additives like hydrogels that are highly concentrated, free from contamination, and keep spores in close proximity to the root system for better colonisation (Pal et al., 2016). Different methods of mycorrhizae application to rootstock roots should be used to see evaluate which one may have better root length colonisation after 1-2 years.

The functionality of microbial enzymes also appeared to be relatively unchanged by the application of the majority of pre-plant amendments used in the study. Two amendments, *Diversispora* sp. and Rhea_AMF did show a different enzyme activity pattern in the soils to the other amendments, particularly in relation to esterase and the aminopeptidase leucine arylamidase. Previous studies have shown that strong competition exists between AMF and bacteria in the rhizosphere, particularly where N becomes a limited resource (Leigh, Fitter and Hodge, 2011; Nuccio et al., 2013). Aminopeptidases have also been shown to become more active in N-limited environments (Müller, Müller and Behrendt, 2004) implying that there may be competition between the AMF with bacteria in these soils. There was however, no differences in the root length colonisation and establishment of the rootstock between treatments.

The standard practice for ARD treatment has previously focussed on chemical fumigation to try and completely remove pathogen complexes from the soil. There were a large number of low counts in the sterile model implying that the

sterilisation process had been successful and either airborne contaminants or surviving communities were able to survive and colonise the rhizosphere. As diversity is lower in the sterile soils the absolute abundance was lower than the control soils. Thus, the relative abundance of communities in the sterile soil rhizosphere will be higher than in the control soils. Care must therefore be taken as differently abundant OTUs may have similar or less absolute abundance to the control soils and therefore may not have the predicted biological effect in the sterile soils as they would with the same abundance in control soils. The present study showed that each of the single species amendments was able to alter the relative abundance of fungal and bacterial OTUs across the treatments. Although there was no translation to the establishment of the tree we have identified the capacity for singular species biological amendment to alter a number of OTUs in the rhizosphere. The communication of plant and microbe in the soil is complex, with a multiple signals from both plant and associated organisms interacting to optimise nutrient exchange with the plant (Faure, Vereecke and Leveau, 2008). This study has shown that a single species can alter those signals and also change the recruitment of a number of microbes into the rhizosphere. It is not yet clear if the differential OTUs had a direct effect on the tree but it is likely they were not directly linked to establishment in the early years. Further work is required to identify the function of the OTUs affected by single species amendments and whether this can be exploited to target specific taxa in the rhizosphere as a means of biocontrol.

This study has highlighted the limited capacity of *Diversispora* sp., *Trichoderma harzianum, Bacillus amyloliquefasciens,* and *Pseudomonas fluorescens* as single pre-plant amendment to beneficially influence the establishment of M9 rootstocks in the first season of growth either individually or in consortium. We identified marked reduction in the establishment of trees amended with *Diversispora* sp. but there were no significant increases in RLC %. Thus, it is unclear if this reduction was due to the amendment with *Diversispora* spp.. In sterile soil and that amended with biofumigants, both lower fungal and bacterial diversity in the soils as well as alterations in the composition of the communities. However, care must be taken with the use of Biofence when applied to potted soil to prevent the

death of young trees. Each of the amendments were also able to alter the relative abundance of a large number of OTUs in the rhizosphere. The predicted function of the majority of the OTUs did not correlate to plant health but a number were correlated with reported plant-growth-promoting and pathogenic taxa of plants. It is unclear from the sequencing data if their altered abundance would lead to changes in establishment and yield. Future work should use technologies such as metatranscriptomics to identify both community and functional changes within the rhizosphere. Studies are also needed to track the survivability of the amendments to better understand rhizosphere colonisation by the beneficial microorganisms and aim to better understand how they interact with the rhizosphere community when introduced.

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5 Inter-Row Cropping and Rootstock Genotype Selection as a Management Strategy for Apple Replant Disease in a UK Cider Orchard.

5.1 Abstract

5.1.1 Purpose

Apple rootstock genotypes confer different levels of tolerance to apple replant disease (ARD) and vigour to a newly replanted apple tree. A hybrid management system of rotating the rootstock genotype planted between successive generations and inter-row planting in the alleyways of orchards may minimise the severity of ARD symptoms by altering the communities in the rhizosphere microbiome.

5.1.2 Methods

High-throughput sequencing of the fungal ITS and bacterial 16S regions was used to investigate the diversity, and differential taxa present in soils displaying symptoms of ARD. Candidate pathogens and beneficial microorganisms were correlated with the above-ground establishment of each rootstock genotype in a U.K. cider orchard.

5.1.3 Results

Our results suggest rootstocks that are more closely genetically related to the previous rootstock had more severe ARD. Planting in the alleyway appeared an effective strategy to minimise the severity of symptoms irrespective of rootstock genotype. The planting location effect had a higher contribution to the variation in rhizosphere microbiome than the rootstock genotype contribution. No causal agents for ARD could be identified to a taxonomic level to predict their function but two taxa associated with mycorrhizae, *Pteridiospora spinosispora* and *Paraglomus laccatum* were identified to be beneficial for the plant to minimise ARD severity.

5.1.4 Conclusions

Our findings suggest a hybrid management approach of rotating rootstock genotype to a rootstock dissimilar to those previously planted, planting rootstocks in the alleyway, and potentially using biological amendment with beneficial microorganisms could be an effective strategy to minimise severity of ARD.

5.2 Introduction

Successive planting of apple trees in the same location can cause initially high-yielding orchard trees to have reduced establishment and unsatisfactory yields. This can ultimately result in loss or removal of these trees (Mazzola and Manici, 2012). This disorder has been termed Apple Replant Disease (ARD). ARD causes stunted growth, poor fruit appearance, root tip necrosis, reduction in root biomass, and a delay in initial fruit cropping by 2-3 years (Mazzola and Manici, 2012; Liu et al., 2014; Zhu et al., 2014). The causal agents of ARD are considered to include a mixture of fungal pathogens *Cylindrocarpon* spp., *Rhizoctonia* spp., and *Fusarium* spp. and the oomycetes *Pythium* spp. and *Phytophthora* spp. (Braun, 1995; Manici et al., 2013; Mazzola and Manici, 2012; Tewoldemedhin et al., 2011a). In addition, the presence of root lesion nematodes such as *Pratylenchus penetrans* can exacerbate ARD by creating entry points for infection by these fungal pathogens via the root lesions (Mai and Abawi, 1981; Mazzola and Manici, 2012).

ARD has usually been managed by chemical fumigation of the soils to remove any pathogenic causal agents present prior to replanting. Soil fumigants such as methyl bromide and chloropicrin were commonly used as they were effective in minimising ARD. However, these have since been banned due to their damaging environmental side-effects and because they are broad-spectrum fumigants, many have become less effective in controlling ARD (Xu and Berrie, 2018). Thus, multiple non-chemical management strategies have been examined to manage ARD including the use of anaerobic soil disinfestation by either addition of plant-based-products followed by covering with plastic, or application of biofumigants such as brassica seed meal to suppress ARD through anti-fungal and anti-nematode action (Xu and Berrie 2018; Wang and Mazzola 2019). Applications of beneficial microbes such as plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) have been shown to increase yield, growth, and disease suppression but may not be as effective at controlling the oomycete pathogens implicated in ARD (Shuttleworth, 2021; Xu and Berrie, 2018).

Orchard management practices are also important to minimise ARD onset. Crop rotation for 5 years with a non-woody cover crop can reduce ARD pressure and short-term rotation with *Allium fistulosum* mixed with a *Trichoderma* spp. soil amendment was found to increase *Malus hupehensis* seedling growth compared to ARD soils but was not as effective as sterile soil (Pan et al., 2017). Rotation with a different crop such as wheat can alleviate ARD but financial restrictions and land availability make the use of a break crop an obstacle for many growers (Mazzola and Gu, 2007; Winkelmann et al., 2018b). Inter-row cropping, planting trees in the old alleyways between the previous tree rows, are alternative strategies previously shown to reduce the severity of ARD (Kelderer et al., 2012). This has been suggested to be mainly due to the presence of distinctly different rhizosphere microbiome communities between the tree rows and grass alleyways (Rumberger et al., 2004; Leinfelder and Merwin, 2006; Deakin et al., 2018). Weed management is also important and must be included when replanting in alleyways due to the detrimental effect of weed competition on the establishment of young trees which could potentially be even more severe than ARD in some cases (Deakin et al., 2019; Xu and Berrie, 2018).

Rootstock selection is a critical factor as they have differences in tolerance/resistance to ARD (Fazio et al., 2012; Leinfelder and Merwin, 2006; Rumberger et al., 2004a). Cider orchards tend to use semi-vigorous rootstocks, since generally these more vigorous rootstocks/varieties are less likely to be affected by ARD. These include MM106, which is more susceptible to ARD, and M116 which is more tolerant to ARD (Auvil et al., 2011; Deakin et al., 2019; Wang and Mazzola, 2018; Xu and Berrie, 2018). Some historically important dwarfing dessert orchard rootstocks can be very susceptible to ARD (Auvil et al., 2011). Geneva rootstocks (G16, G30, G41, and G210) have been shown to be more tolerant to ARD in some affected soils, although not all, compared to Malling rootstocks (M7, M9, M26, and MM106) and have different bacterial rhizosphere species compositions (Leinfelder and Merwin, 2006; Rumberger et al., 2004a; Wang and Mazzola, 2019). Replanting an orchard with a rootstock different to the previous rootstock genotype could be effective in reducing ARD but the genetics of ARD resistance in the rotated rootstock and its genetic relationship to the

previous rootstock need to be taken into account when deciding which rootstock to choose for rotation (Deakin et al., 2019; Shuttleworth, 2021; Xu and Berrie, 2018).

In this study, we present a continuation of the work from Deakin *et al* (2019) and report the results of tree growth and rhizosphere microbiomes in relation to rotating rootstock genotypes planted in both the previous tree station and the corresponding alleyway. The study aimed to assess (i) whether tree growth in the first five years after replanting is greater in the alleyway than in the corresponding tree station (hence ARD), (ii) whether the extent of ARD varies with rootstock genotypes, and (iii) could the differentiation in rhizosphere microbiomes between the trees in the alleyway and the corresponding original tree station contribute to relative effects of ARD. The study hypothesis was that tolerant rootstocks, planting position (alleyway vs tree station), and rootstock genotype genetic relationship to the previously planted rootstock will all benefit positively impact against ARD.

5.3 Materials and Methods

Experimental details on the orchard history, crop management, soil characteristics and experimental design for the study were provided previously (Deakin et al., 2018a, 2018b). Here we provide a brief description of relevant experimental details.

5.3.1 Orchard Design

The study was conducted on a cider orchard in the West Midlands of England in Worcestershire (latitude 52.251020, longitude -2.301711). Both rotating rootstock genotypes and planting position (alleyway vs tree station) were investigated. The study consisted of eight rootstock genotypes planted in pairs: in the previous tree station and the corresponding middle alleyway position approximately 2 m away from the tree station. Three randomised blocks were used. Each pair location within each block was randomly assigned to one of the eight rootstock genotypes. Before grubbing in 2014, the orchard had been 'Katy' apples on MM106 for blocks 1 and 2 and MM111 for block 3.

5.3.2 Rootstock and Scion Selection

Eight rootstocks were selected for the study based on their tolerance to ARD, vigour and importance to the industry. The rootstocks used were M9 (unknown pedigree), M26 (M16 × M9), M27 (M13 × M9), MM106 (Northern Spy × M1), M116 (MM106 × M27), G11 (M26 × *M. robusta* 5), G41 (M27 × *M. robusta* 5), and M200 (*M. robusta* 5 × Ottawa 3) from the East Malling breeding programme. M27, G41, G11, M9, and M200 are all dwarfing rootstocks. M26 is a semi-dwarfing rootstock and M116 and MM106 are semi-vigorous rootstocks. M27, G41 and M116 are reported to be tolerant to ARD. M9 and M26 are believed to be the most susceptible to ARD out of the eight genotypes. The root ball of each rootstock was washed before grafting to the cultivar 'Worcester Pearmain' in 2015. The trees were potted in a peat and sand mix and grown for 7 months. The land was sub soiled and rotavated before planting to prevent compaction. Pairs of trees with similar girth/height of the same rootstock were planted for a given position pair: the original tree station and corresponding alleyway, in October 2015. Trees

were managed the same as the rest of the cider trees on the site during the trial period.

5.3.3 Growth Measurements and Statistical Analysis

Initial measurements of height (from ground level) and girth (the circumference of the tree trunk 5 cm above the graft union) were taken for each tree. Each winter during dormancy (between January and March) from 2017 to 2021 trees were assessed for height and girth. Trees were marked at the point they were measured for consistency in girth measurements between time points. Height was measured from ground level to the end of the leader of the tree (not including any leaf height added to the branch at the leader's tip). The yield was the number of fruit per tree due to an abnormally low number of fruit per tree (≥ 24 individual fruit, many with 0 fruit).

All statistical analyses were conducted in R V4.0.2 (R Core Development Team 2008). In the case where one of the pairs of trees had died (either alley or tree station) the corresponding healthy tree in the pair would be removed from statistical analysis. The difference (D) in both height and girth was calculated between the alleyway and tree station for each tree pair and used in subsequent statistical analysis.

A linear mixed model with a common intercept was used to model the treatment effect on the D over time using the lme4 package v 1.1-28 (Bates et al., 2015). The genotype variable is fixed in the model whereas the location of the tree pair D is calculated from is treated as a random variable. The package ggeffects v1.1.1 (Lüdecke, 2018) was used to calculate predicted best linear unbiased predictions (BLUP) for the slopes in the model and visualised using ggplot2 package v3.3.5 (Wickham, 2011). The slope estimate represented the extent of ARD; positive slope = ARD (trees in the alleyway grew faster than in the original tree station), and ≤ 0 = no ARD. Based on the slope estimates, an ARD score was assigned to each rootstock genotype: 0 (no ARD), 1 (intermediate ARD), and 2 (severe ARD). Fruit yield was calculated as the mean number of fruit per tree was calculated and visualised in ggplot2.

5.3.4 Sampling Rhizosphere Soil

Rhizosphere soil was collected from each tree by using a sterile trowel or fork to dig under the tree to an approximately 10cm depth and detach the roots from the tree. Soil that was attached to the root after light shaking was classed as rhizosphere soil. This soil was removed from the root into a polythene sample bag and immediately cooled in an electric cool box. There were a total of 48 samples collected on the site. Between samples, tools and gloves were cleaned with 70% ethanol to prevent mixing of samples. Samples were transported to NIAB, East Malling, Kent, U.K. at 4^{o}C and subsequently stored at 4^{o}C for 24 hours until molecular processing.

5.3.5 Amplicon-sequencing of the Rhizosphere Soils for bacterial and fungal communities

5.3.5.1 Soil DNA Extraction

Amplicon sequencing and sequence processing followed the method described previously (Deakin et al., 2018). In summary, genomic DNA was extracted from 0.25 g subsample of the rhizosphere soils using the DNeasy PowerSoil Kit (Qiagen, Carlsbad, USA) along with a bead-beating benchtop homogenizer (Fastprep FP120, Qbiogene, Carlsbad, USA). DNA concentration and quality were determined using a spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific, Cambridge, UK) and a fluorometer (Qubit 2.0, Thermo Fisher Scientific, Cambridge, UK). Twenty µl of DNA sample was sent to Novogene (Cambridge, Cambridgeshire, UK) for library preparation and amplicon sequencing.

5.3.5.2 Amplicon Sequencing

The quality of DNA samples was first checked. PCR amplification was then performed for bacteria using the 16S V4 region amplified with the primer pair Bakt_341F/Bakt_805R (Herlemann et al., 2011). For fungi, the ITS1 and ITS2 regions were amplified with the primer pair EkITS1F/Ek28R(≡ 3126T) (Gardes and Bruns, 1993; Sequerra et al., 1997). PCR product was purified before library preparation. Sequencing was performed using the Illumina NovaSeq 6000 platform. Reads were paired-end 150 bp in length.

5.3.5.3 Sequence Read Processing

Sequence data was submitted to the NCBI database (Project PRJEB52534). FASTQ reads were demultiplexed. Ambiguous reads that did not match the forward and reverse read primers for 16S and ITS were removed before further processing. All analyses were conducted using USEARCH 11.0 (Edgar, 2013b) unless otherwise specified.

Bacterial and fungal reads were processed separately to create separate representative operational taxonomic units (OTUs) for bacteria and fungi. ITS forward and reverse reads were aligned with a 10% threshold of maximum difference in overlap and 16S reads were aligned with a 5% threshold. Primer sequences for forward and reverse reads were removed at this stage. Merged reads with adaptor contamination or total length fewer than 150 nucleotides for ITS reads or fewer than 300 nucleotides for 16S reads were removed. Quality filtering of the merged reads was conducted using a maximum expected error threshold of 0.5.

5.3.5.4 OTU Generation

Unique sequences were identified and any unique sequence with fewer than 4 reads were discarded for OTU generation. Sequences were sorted by order of decreasing read numbers; OTUs were generated by clustering the unique sequences at 97% sequence similarity and a representative sequence for each OTU was also produced. Then all sequence reads that passed initial quality filtering were mapped against the OTU representative sequences to generate the OTU counts tables for ITS and 16S. To predict the taxonomy of the OTUs, a SINTAX algorithm (https://www.drive5.com/usearch/manual/sintax_algo.html) was used by aligning ITS OTU representative sequences to the reference database “UNITE v8.3” (Nilsson et al., 2019) and 16S OTU representative sequences to the RDP training set v18 (Cole *et al.,* 2014).

5.3.6 Amplicon-sequencing of the Rhizosphere Soils for bacterial and fungal communities

Rarefaction curves were produced in the vegan R package v2.5.7 (Dixon, 2003) to identify if samples were adequately sequenced. OTUs with the total number of

reads fewer than 3 were discarded from further analysis. The vegan package was used to normalise the OTU counts data by rarefaction before further analysis.

Both alpha and beta diversity indices were calculated for fungi and bacteria. Alpha (α) diversity indices (Chao1, Shannon, Simpson and invSimpson) were calculated within the phyloseq package v1.34 (McMurdie and Holmes, 2013) from the rarefied counts. Permutation ANOVA analysis in the LmPerm package v2.1 (Wheeler, 2016) was used to assess the effects of planting location (alleyway vs tree station) and rootstock genotypes on alpha diversity. The rootstock genotypes were further divided into three groups based on the ARD score. The R package ggplot2 was used to visualise the alpha indices produced by phyloseq. Beta (β) diversity index (Bray-Curtis) was calculated in the vegan package from the rarefied counts data. To visualise dissimilarity between samples, non-metric multidimensional scaling (NMDS) plots were used to visualise the differences in the beta diversity. Permutations of multivariate analysis of variance using F-tests based on the sequential sum of squares (ADONIS) was used to assess the effects of planting location and rootstock genotypes on beta diversity indices. Statistical significance was based on 1000 permutations.

The DESeq2 package v1.30.1 (Love, Huber and Anders, 2014) was used to identify OTUs with significant differences in the relative abundance between rootstock genotypes with the most severe ARD score and no ARD. Log2 fold change (LFC) was shrunk within DESeq2 when extracting results from the model with a P-value threshold of 0.1 (Zhu, Ibrahim and Love, 2019). The following specific comparison was used in DESeq2 analysis to identify candidate causal agents of ARD and beneficial microorganisms:

(ARD trees at Tree Station + Non ARD tree in Alleyway) vs (Non ARD Tree Station + ARD trees in Alleyway)

More abundant OTUs in this comparison were candidate causal agents for ARD and less abundant OTUs are candidate beneficial microorganisms associated with reduced ARD. Those OTUs identified with differential abundance identified by DESeq2 had their representative sequences run through NCBI's Nucleotide

BLAST search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify possible taxonomy since many OTUs cannot be identified to the level of genus or species with sufficient confidence via the SINTAX algorithm.

5.4 Results

5.4.1 Above ground effect of rotating rootstock and planting in the alleyway

Trees in the orchard established well in the early years of growth. All six trees grafted to G11 failed to establish, probably because of incompatibility with the Worcester Pearmain scion, or a latent viral infection within the scion (Deakin et al., 2019; Robinson et al., 2003). Some tree pairs had a much healthier and more vigorous tree above ground in the alleyway compared to the corresponding tree station (Figure 5.1). The more vigorous trees displayed increased branching, spread, height, and leaf area.

Figure 5.1 Above ground effect on MM106 rootstock genotype grafted to Worcester Pearmain scion. Both trees were planted in 2016 with the tree on the right planted in the previous tree station row and the tree on the left planted in the corresponding alleyway between the previous rows.

The difference (D) in trunk girth 5 cm above the graft union between trees in the alleyway and the tree station was negligible in 2016 (Table 5.1). In 2021, five of the seven rootstocks (M116, MM106, M200, M26, and M9) had a positive D value for the girth but only three (M116, MM106, and M200) of the five also had a positive D value for the height in 2021. The mean girth value for each rootstock genotype correlated well to the known vigour conferred by the rootstock to the scion (Figure 5.2). The slope estimate from the linear random mixed model was positive for the same five rootstocks estimate (Figure 5.3): the slope estimates were 1.36 (M116), 1.14 (MM106), 0.97 (M200), 0.52 (M26), and 0.37 (M9). The slope estimate for M27 and G41 were both less than zero: -0.27 and -0.34, respectively. From these results, an ARD score was assigned to each rootstock genotype: 0 for G41 and M27 (no ARD), 1 for M9 and M26 (intermediate ARD), and 2 for M116, MM106 and M200 (severe ARD). Similar analysis of the tree height (Figure A.3) showed four of the seven rootstocks with a positive slope estimate: 22.13 (MM106), 18.41 (M116), 8.85 (M200), and 2.16 (M26). The remaining rootstocks had negative slope estimates: -2.54 (M27), -2.58 (G41), and -10.10 (M9).

Table 5.1 Average difference in girth and height between the alleyway and the previous tree station trees in the first year after planting (2016) and at the end of the trial (2021).

Genotype	Mean Girth difference (cm)		Mean Height difference (cm)	
	2016	2021	2016	2021
M116	-0.2 ± 0.16	5.0 ± 0.32	-14.8 ± 7.55	24.0 ± 3.27
MM106	0.03 ± 0.37	3.7 ± 1.19	2.8 ± 1.09	47.0 ± 16.82
M200	0.13 ± 0.24	2.0 ± 0.48	2.3 ± 10.04	11.0 ± 6.03
M26	0.2 ± 0.12	0.7 ± 0.46	12.0 ± 9.24	-6.0 ± 9.24
M9	0.1 ± 0.23	0.5 ± 1.2	8.5 ± 7.97	-23.7 ± 25.62
M27	-0.07 ± 0.12	-0.9 ± 1.79	4.0 ± 6.06	-26.0 ± 39.68
G41	0.1 ± 0.08	-1.7 ± 0.73	14.0 ± 4.90	-1.0 ± 23.68

* Number after ± is the standard error value.

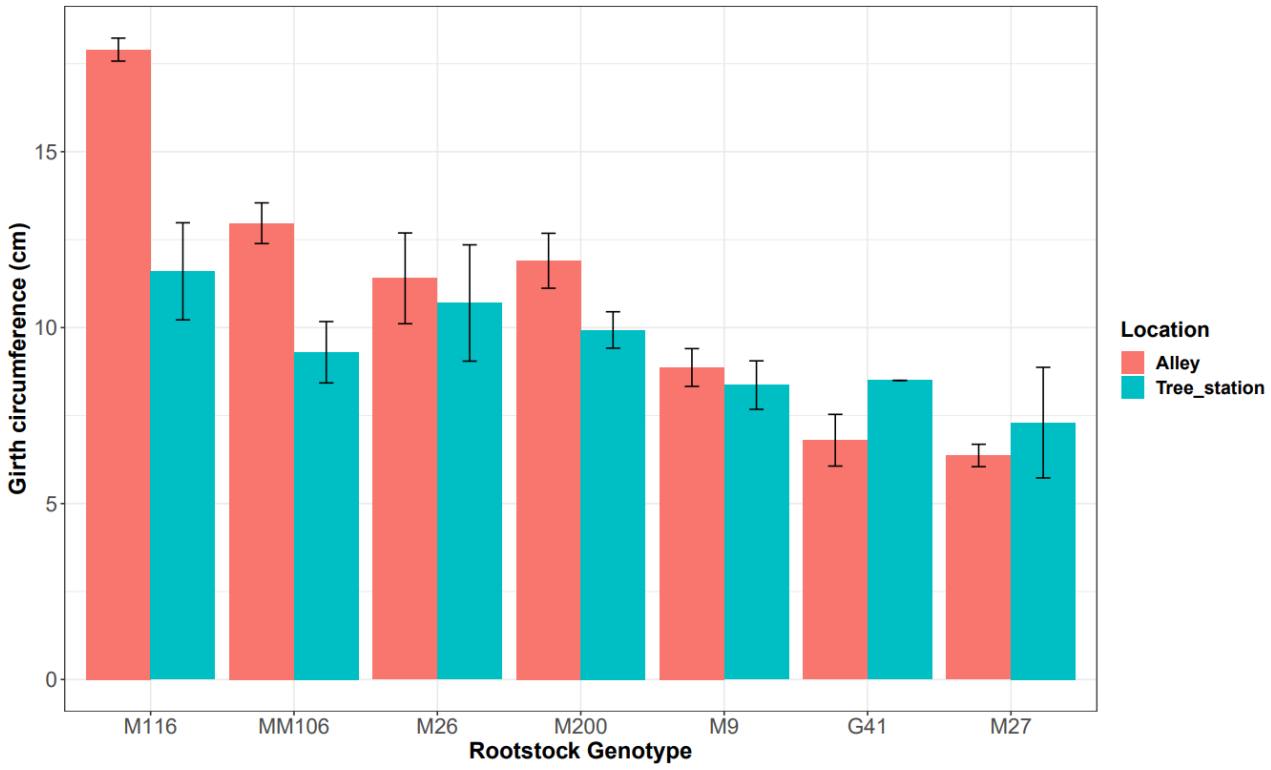

Figure 5.2 Mean girth circumference of tree trunks 5cm above the graft union in 2021. The colour of the bar indicates the location in which the tree was planted. Red = Alleyway between previous rows, Blue = Previous tree station.

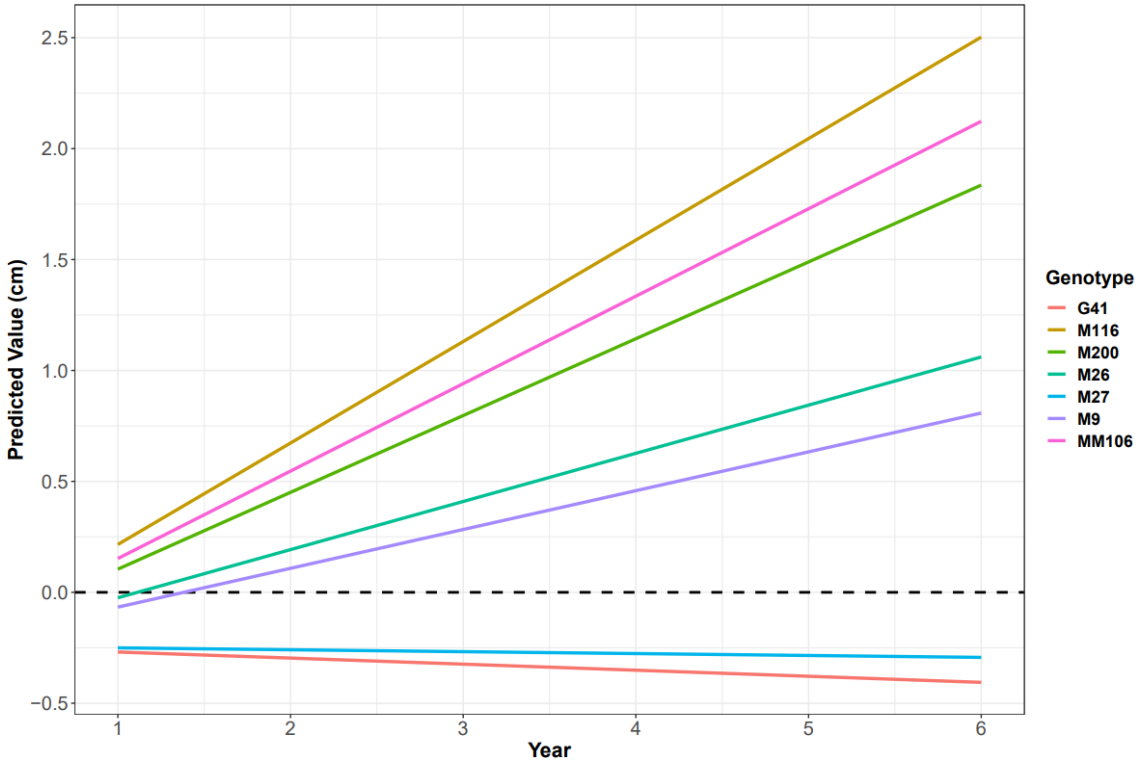

Figure 5.3 Predicted values from a randomly mixed model with a common intercept of temporal girth difference between the alleyway and the previous tree station. Genotype effect was fixed in the model and tree pair location treated as random. Slope trend was calculated from best linear unbiased prediction (BLUP) values. A value above zero indicates a replant effect on the tree planted in the tree station and a value equal to or below zero indicates no replant effect on that genotype.

The number of fruit on the trees in 2021 was low for all trees in the trial with the maximum number of fruit on one tree being 24 apples. In 17 of the 40 trees, there was no fruit when assessed in July 2021 before harvest. M116, M26, MM106, M200, and M27 all had higher mean fruit numbers in the alleyway than in the tree station; however the standard error was large, and these differences hence were not statistically significant (Figure A.4). M9 had a similar number of fruit in the alleyway and the tree station, whereas G41 had a higher number of fruit in the tree station.

5.4.2 Summary of Microbiome Data

There was a total of 1,549,832 bacterial reads and 4,730,414 fungal reads across the 40 samples. The total number of OTUs generated was 10,883 for bacteria and 4,802 for fungi. The number of reads per sample ranged from 25,305 to 45,970 for bacteria and from 2,801 to 924,636 for fungi.

5.4.3 Diversity Indices

Four alpha diversity measures (Chao1, Shannon, Simpson and InvSimpson) were calculated. Alpha diversity was generally higher for bacteria than for fungi. Bacterial alpha diversity was primarily influenced by the planting location with 15.5% and 26.5% of the variability in Shannon and Simpson/InvSimpson explained by the planting location, respectively (Table 2). Alpha diversity was higher in the original tree station than in the alleyway for bacteria (Fig S3a). Rootstock genotype effect only accounted for 5.8% of the variation in Shannon indices, 2.3% for Simpson/InvSimpson, and 15.6% for Chao1. The interaction between genotype and location accounted for between 9% and 22.3% of the total variability but was not statistically significant. Most of the variability in the bacterial alpha diversity was unexplained by the experimental factors: 57.7% (Shannon, Simpson, and InvSimpson) and 59.8% (Chao1).

Table 5.2 Percentage of the variability in alpha diversity indices accounted for by block effect, planting location (alley vs tree station) and rootstock genotype and the interaction of planting location and rootstock genotype.

Measure	Block		Location		Genotype		Location : Genotype		Residual
	P value	*%*	*P value*	*%*	*P value*	*%*	*P value*	*%*	*%*
Bacteria									
Chao1	0.92	0.9	0.32	1.4	0.49	15.6	0.31	22.3	59.8
Shannon	0.49	5.0	0.02	15.5	0.84	5.8	0.44	16.0	57.7
Simpson	0.36	5.4	0.002	25.6	1.0	2.3	0.63	9.0	57.7
InvSimpson	0.41	5.4	0.003	25.6	1.0	2.3	0.70	9.0	57.7
Fungi									
Chao1	0.002	28.2	0.04	9.8	0.39	9.5	0.40	11.6	41.0
Shannon	<2e-16	46.5	0.06	6.7	0.83	4.1	0.40	8.8	34.0
Simpson	<2e-16	43.5	0.06	6.1	0.89	4.5	0.49	9.3	36.6
InvSimpson	<2e-16	43.5	0.07	6.1	0.79	4.5	0.46	9.3	36.6

Alpha diversity for fungi was similar between the tree station and the alleyway, with the location accounting for between 6.1% and 9.8% of the total variability (Figure A.5b, Table 5.2). Both the genotype and the interaction between genotype and location effect were not statistically significant. There were large differences between blocks, contributing to 43.5% (Simpson/InvSimpson), 46.5% (Shannon), and 28.2% (Chao1) of the total variability. Fungal alpha diversity was lower for trees planted in the alleyway compared to those planted in the tree station within each of the ARD groups. Alpha diversity was similar between the three ARD groups for both bacteria and fungi (Figure A.6).

Bray-Curtis beta diversity indices were used to represent differences in microbial communities in the rhizosphere among samples. The difference between the alleyway and previous tree station samples was most pronounced in the bacterial community than in the fungal community (Figure 5.4). There were also differences in bacterial and fungal communities between ARD groups. ADONIS analysis highlighted the difference in beta diversity between blocks and between planting locations for both bacterial and fungal communities (Table 5.3). The difference between ARD severity groups was statistically significant for the fungal

community only. There were significant interactions between the planting location and rootstock genotypes in the beta diversity indices for both bacterial and fungal communities, accounting for between 13.1% and 14.8% of the total variability (Table 5.4). The magnitude of the genotype and interaction effects did not change among the three sampling times, whilst the location effect slightly increased between T2 and T4 (Table 5.4).

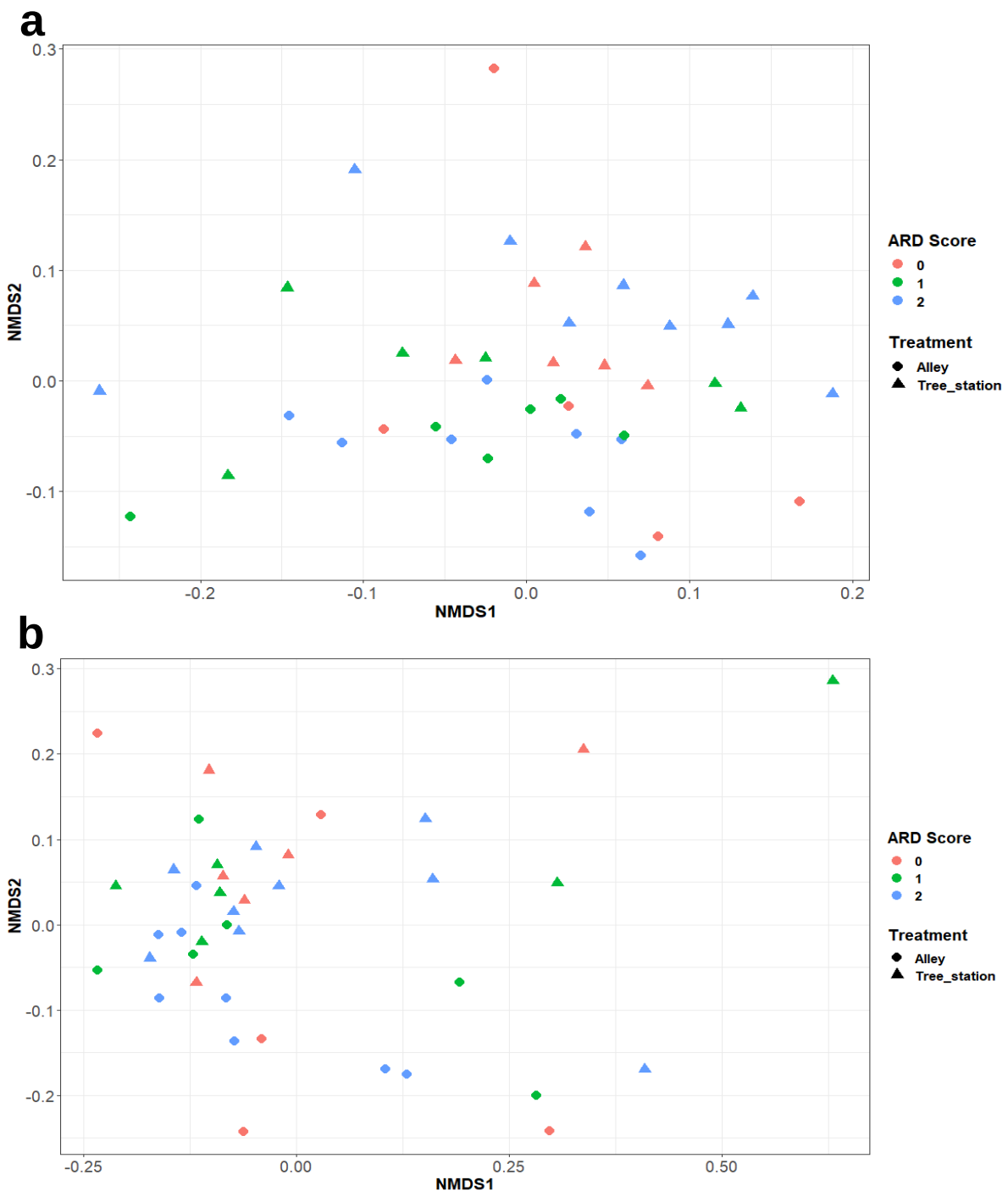

Figure 5.4 The first two dimensions of NMDS analysis of the Beta (β) diversity indices (Bray-Curtis dissimilarity) for (a) bacteria and (b) fungi. Closer the distance between the points indicates more similarity in microbial communities between the samples. The Colour of the point indicates the ARD score associated with those rootstock Genotypes. The shape of the point indicates the planting position in the alleyway (●) or the previous tree station (▲).

Table 5.3 Permutational Multivariate Analysis of Variance (ADONIS) based on 1000 permutations of Bray-Curtis beta diversity accounted for by Block, Location (Alleyway vs Tree Station), rootstock Genotype, ARD severity score and interaction terms.

		df	Sum sq	P value
Bacteria				
Block		2	0.21	9.9e-4
Location		1	0.13	9.9e-4
Genotype		6	0.25	0.56
	[1] ARD	2	0.10	0.17
	[2] Between genotypes within ARD class	4	0.15	
Location : Genotype		6	0.21	0.91
	Location : [1]	2	0.08	0.60
	Location : [2]	4	0.13	
Fungi				
Block		2	1.81	9.9e-4
Location		1	0.48	8e-3
Genotype		6	1.29	0.64
	[1] ARD	2	0.62	0.03
	[2] Between genotypes within ARD class	4	0.67	
Location : Genotype		6	1.37	0.48
	Location : [1]	2	0.46	0.35
	Location : [2]	4	0.91	

Table 5.4 Percentage of variation of microbiome data accounted for by rootstock genotypes and within-orchard location (aisle or tree stations), and their interactions, as determined by analysis of variance of all principal component scores. T = Time (Year).

Measure	T0	T1	T2	T4
Bacteria				
Location	5.64	2.72	2.80	4.92
Genotypes		14.39	14.77	14.11
Location : Genotype		14.41	13.96	13.11
Fungi				
Location	9.35	2.82	2.99	4.87
Genotypes		14.43	14.65	14.15
Location : Genotype		14.38	14.33	13.07

5.4.4 Differential OTUs between ARD and non-ARD trees

OTUs were compared between the trees with the most severe ARD (score 2) and trees that did not show ARD (score 0). After DESeq2 filtering, approximately 80% of the representative OTUs for both bacteria and fungi were retained for comparison. There were no bacterial OTUs identified to have a differential abundance between the two ARD groups. Only a small number of fungal OTUs (ca. 0.3%) significantly differed ($P < 0.1$) in their relative abundance (Table 5.5). Table 5.6 shows fungal OTUs with differential abundance from DESeq2 analysis (Table 5.5) and their associated SINTAX taxonomy predictions (at a confidence threshold of 0.65) and BLAST taxonomy. One of the OTUs predicted as a causal agent was identified as *Russula praetervisa*, a saprophyte. The remaining OTUs predicted as causal agents were not able to be correlated to a taxonomic rank low enough to predict ecology. Similarly, four of the eight OTUs identified as beneficial fungi associated with non-ARD trees were unable to be assigned with a taxonomy at a level sufficient to identify their potential function. The saprophytic genus *Podospora* was identified as more abundant in non-ARD trees. *Arthrinium arundinis* was more abundant in non-ARD trees and has been described as a plant pathogen of barley causing kernel blight but has also been described as a saprophyte and endophyte (Cano, 1992). It is unlikely to be pathogenic in this study as it was associated with the healthier non-ARD trees. Two of the putative beneficial OTUs were associated with mycorrhizae *Pteridiospora spinosispora* and *Paraglomus laccatum* (Filer and Toole, 1966; Renker, Błaszkowski and Buscot, 2007).

Table 5.5 DESeq2 results summary for all differential OTUs. No. OTUs indicate the number of OTUs after DESeq2 filtering (compared between factors); Log2 Fold Change (LFC) > 0 indicates a potential OTU candidate causal agent; LFC < 0 indicates OTU that may be a potential beneficial microorganism. LFC was shrunk using DESeq2. ARD = ARD trees at Tree Station + Non ARD trees in Alleyway, Non-ARD = Non-ARD trees at Tree Station + ARD trees in Alleyway.

DESeq2 Model	No. OTUs	LFC > 0 (higher)	LFC < 0 (lower)	Low Counts
Bacteria				
ARD vs None-ARD	8625	0, (0%)	0, (0.0%)	0, (0%)
Fungi				
ARD vs None-ARD	3821	3, (0.079%)	8, (0.21%)	3315, (87%)

Table 5.6 Differential OTUs from DESeq2 analysis to identify candidate causal agents for ARD and beneficial microorganisms working against ARD. Positive Log2 Fold Change (LFC) indicates higher OTU abundance in the first condition/treatment and vice versa for negative values. P values are Benjamini and Hochberg corrected. ARD = ARD trees at Tree Station + Non ARD trees in Alleyway, Non-ARD = Non-ARD trees at Tree Station + ARD trees in Alleyway.

DESeq2 Model	SINTAX Species/Taxa*	BLAST taxonomy	Ecology	Base Mean	LFC	P Value
Causal Agents (Fungi)						
ARD vs None-ARD	*Russmula* (g)	R*ussula praetervisa*	Saprophyte	2012.9	0.033	0.026
	Sebacinales (o)	Uncultured S*ebacina* isolate	Unknown	121.1	1.669	0.033
	Unknown Fungi (k)	Unknown Fungi	Unknown	43.9	0.041	0.065
Beneficial (Fungi)						
ARD vs None-ARD	*Pleosporales* (o)	*Pteridiospora spinosispora*	Isolated from mycorrhizae of sweetgum	4997.6	-0.129	0.026
	Agaricomycetes (c)	Uncultured *Agaricaceae*	Unknown	1581.6	-0.069	0.094
	Unknown Fungi (k)	Unknown Fungi	Unknown	48.7	-1.492	0.018
	Unknown Fungi (k)	Unknown Fungi	Unknown	67.0	-0.114	0.018
	Paraglomerales (o)	*Paraglomus laccatum*	Mycorrhizae	33.9	-0.073	0.065
	Ascomycota (p)	Uncultured *Podospora*	Saprophyte	51.2	-0.098	0.094
	Apiosporaceae (f)	*Arthrinium arundinis*	Saprophyte / Endophyte / Plant pathogen of barley	122.6	-1.502	0.026
	Hypocreales (o)	Uncultured *Hypocreales*	Unknown	87.3	-0.126	0.086

* The lowest assignable taxonomic rank with a SINTAX confidence ≥0.65.

5.5 Discussion

This study has shown that planting rootstocks closely related to the previously planted apple rootstock leads to more severe ARD. Better tree establishment was observed for those rootstocks suffering from ARD in the alleyway than in the previous tree station. Both alleyway and original tree stations had distinct bacterial and fungal communities in the rhizosphere; such a differences appear to become stable within the first year of replanting. All fungal OTUs predicted as candidate ARD causal agents could not be effectively assigned to taxonomic ranks which were sufficiently low enough to predict pathogenic effects in apple, or those that were previously reported as saprophytic. Of the eight fungal OTUs identified as potential beneficial microorganisms, two were reported from mycorrhizae and another *Arthrinium arundinis* has been shown to have antifungal and cytotoxic compounds (Zhang et al., 2018) making it an amendment candidate for a role in biocontrol.

Each rootstock confers different levels of vigour to the scion, so it sometimes becomes difficult to assess the extent of ARD between rootstocks. For instance, a vigorous rootstock can still confer better growth under ARD conditions to scions than dwarfing rootstocks that do not suffer from ARD. In this study, we measured the severity of ARD as the relative difference in tree establishment between pairs of the same scion grafted to the same rootstock genotype planted in the previous tree station and the corresponding alleyway. The present results suggested that five of the seven tested rootstocks showed a varying degree of ARD, with MM106, M116, and M200 experiencing the most severe ARD. MM106, M116 and M200 are not known for their susceptibility to ARD as they are all vigorous rootstocks. Thus even when suffering from severe ARD (planted in the original tree station), these rootstocks still conferred better tree growth than M9, G41 and M27. Only by comparing tree development between the alleyway and the previous tree stations does the ARD effect become apparent for these vigorous rootstock genotypes. This finding also suggests another possible way of combating ARD – planting more vigorous rootstocks in previous tree stations if no other methods of managing ARD are economically viable.

The above-ground effect on the most severely affected tree was consistent with the symptoms of ARD, showing stunted growth, reduced vigour and discolouration of leaves (Mazzola and Manici, 2012). The present result suggested that ARD is not limited to the first few years after planting but can persist beyond five years after planting – the difference in the tree development between the alleyway and tree station increased with time at a constant rate as indicated by the constant slope. The previously planted rootstock in the orchard was MM106; M116 is derived from the cross between MM106 and M27, implying a genetic link between the newly planted rootstock with the previous rootstock in terms of the susceptibility to ARD. The present results support that planting a genetically different rootstock from the previous rootstock can be effective to reduce ARD development. Interestingly, M27 did not exhibit any ARD effects in the late years despite being identified as susceptible to ARD in year 2 (Deakin *et al.*, 2019), highlighting the unpredictable nature of ARD during the establishment of young apple trees.

Girth differences are a better measure of the effect of ARD than height differences in this particular study. Many external factors including mechanical damage of leader branches, pruning of leader branches to remove canker lesions or high wind damage could all impact the tree height, independent of experimental factors. However, these factors would not be expected to affect tree girth directly. Similarly, yield data was highly variable and low across all rootstocks in the trial. In general, the more vigorous trees in the pairs would have a higher fruit number due to more branches able to bear fruit. The low fruit number could be due to the early removal of fruit by external influences or issues during the blossom period.

Temporally, the differences in rhizosphere microbial communities between the two locations (the previous tree station and the corresponding grassy alleyway) would be expected to be reduced due to the recruitment of similar microbes from the bulk soil by the same rootstocks (Deakin *et al.*, 2019). However, this was not supported by the present results. The contribution of the planting location to the total variation in microbial communities remained significant and had not changed much after five years of establishment. One possible explanation for this

observation could be the functional redundancies in the bulk soil microbial communities. Different rootstock genotypes may recruit microbes to the rhizosphere with similar functions, but the exact composition of microbes with specific functions may be different in the alleyway and the previous tree station due to long-term effects of herbicide applications, previous vegetation and soil compaction.

Additionally, the interaction between the rootstock genotype and the location was a significant contributor to the overall variation in bacterial alpha diversity. These results suggest that although the plant host plays an important role in shaping the rhizosphere associated with their roots through root exudation (Burns et al., 2015; Guyonnet et al., 2018; Haichar et al., 2008; Leisso, Rudell and Mazzola, 2017), the differences between the tree station and the alleyway bacterial communities could lead to functional redundancy and may partially explain the disparity between bacterial populations between the two locations after seven months (Deakin et al., 2019). The effect of genotype remained stable throughout the study suggesting the microbial recruitment from bulk soils by rootstock genotypes is relatively rapid. This highlights the importance of the early application of soil amendments especially of beneficial microorganisms to maximise recruitment into the rhizosphere of the young tree and improve the early tree establishment.

The blocking effect significantly influenced the fungal diversity in this trial implying the position within the orchard was more influential than the planting position or the genotype planted. The trial was carried out at the bottom of an orchard on a slope leading to a small stream at the base. The proximity of some blocks to the stream or water movement downwards along the slope through the blocks may have altered the soil moisture content across the orchard. This in turn would have influenced both the dominant microbiota more suited to higher moisture conditions and the soil physio-chemical properties such as pH and soil texture that all highly influence the soil microbiome (Fierer, 2017b). The differences between ARD groups were only significant for fungi, suggesting that this group are more important for ARD onset in the study area. This is consistent with the

role of ARD pathogens which are predominantly fungal or oomycetes (Somera and Mazzola, 2022b).

Using the beta diversity analyses for the communities of bacteria and fungi present in two both the tree station and the alleyway showed consistent difference in the communities present between the two locations for both microbial groups. This was similar to what was observed in the alpha diversity analyses. The difference in communities was probably mainly due to factors such as herbicide application, microbial recruitment through root exudation of the tree roots and the alleyway having previous vegetation combined with compaction via the use of heavy machinery. There was also a difference in the communities for both bacteria and fungi between rootstocks planted in the two areas within each ARD score group. In particular, the rootstocks with severe ARD had clear differences in the communities in the rhizosphere of trees in the alley and the tree station. Functional redundancy again could be a factor contributing to this effect, as the overall difference in the community was different between the alleyway and tree station with the trees perhaps recruiting different microbes, but with similar functions. These results, therefore, suggests differences at a community level alone are not directly correlated with ARD severity.

The study identified three fungal OTUs as potential causal agents of ARD and eight fungal reads as potential beneficials. However, no bacterial OTUs were found to be associated with ARD. No bacterial genera were identified as causal agents or beneficial in this study. This is similar to what was found previously that bacteria were not the causal agent of ARD (Mazzola and Manici, 2012). In addition, it is interesting to note that beneficial bacteria as biocontrol agents or as PGPRs did not increase in abundance and appeared to have little effect on ARD. This is in contrast to studies that have shown beneficial effects of a number of bacterial strains on ARD including *Bacillus* spp. and *Pseudomonas* spp. (Duan et al., 2022b; Sharma et al., 2017; Utkhede and Smith, 1992). Of the three fungal OTUs predicted as causal agents, only one OTU was identified to the species level as *Russula praetervisa*, a saprophyte in soil with no known pathogenic implications in apple. This highlights the difficulties in identifying causal agents

for ARD by the use of sequencing. Although there may be relative differences in the abundance of OTUs, it does not guarantee that the OTU will be causing disease. Similarly, pathogenicity is rarely as simple as increased abundance equates to disease symptoms. However, a small increase in pathogens or functional changes in pathogens may be sufficient enough to result in phenotypic symptoms of ARD to become evident. Members of the oomycetes and nematodes are also important causal or exacerbating agents of ARD (Tewoldemedhin et al., 2011a) that were not specifically profiled in the present study. Oomycetes can be detected using the ITS primers but were not of a high enough quality to accurately identify oomycete pathogens linked to ARD. No pathogenic oomycetes were detected with the selected primers, so future studies may need to focus on bacterial, fungal, oomycete and nematode specific primers for sequencing alongside a quantitative assessment of known ARD pathogens with specific primers to identify candidate causal agents from each community.

Two of the fungal OTUs identified as beneficial against ARD were likely to be *Pteridiospora spinosispora* and *Paraglomus laccatum*, two species of mycorrhizae (Filer and Toole, 1966; Renker, Błaszkowski and Buscot, 2007). Mycorrhizal inoculations of apple seedlings have been shown to suppress ARD symptoms and aid the establishment of the trees but not effective against oomycete pathogens (Čatská, 1994b; Xu and Berrie, 2018). Increased abundance in non-ARD trees may suggest a reduced abundance of mycorrhizae in the tree station available for root colonisation. Amendments with mycorrhizae identified in this study, or similar commercially available species, could be a viable strategy for future studies to reduce/minimise or prevent ARD in replanted trees.

One fungal OTU identified as beneficial was the species *Arthrinium arundinis*. Previously, *A. arundinis* was described as a pathogen causing kernel blight in barley and leaf edge spot of peach, despite being correlated with non-ARD trees in the present study (Cano, 1992; Ji et al., 2020). *A. arundinis* has also been shown to produce antifungal and cytotoxic compounds when isolated from the leaves of tobacco (Zhang et al., 2018). Thus, it is possible that the production of such compounds could function as components of the biocontrol strategy of this

species which contributes to competition with the causal fungal agents of ARD. This study suggests further investigation of the culturing and application of *A. arundinis* as a biocontrol agent for the control of ARD would be useful.

In summary, rotating rootstock genotypes to more genetically distantly related rootstocks between successive generations of apple orchards appears to be an effective measure to reduce ARD development and increase the above-ground establishment of replanted trees. Planting in the alleyway between previous rows is also an effective measure to mitigate ARD without rootstock rotation. The effect of initial differences in the bulk soil microbial communities of the rhizosphere microbiome of the same rootstock can be long-lasting. Despite identifying three fungal candidate causal agents of ARD, it was not possible to ascertain the ecology of these taxa. Two mycorrhizal species, *Pteridiospora spinosispora* and *Paraglomus laccatum*, were less abundant in the trees with severe ARD. This indicates that perhaps soil amendments with mycorrhizae at planting could be a viable method to minimise ARD. *Arthrinium arundinis* is reported to produce antifungal and cytotoxic compounds that may explain its association with less severe ARD. It is thus possible that a hybrid management approach of rotating rootstock genotype to a rootstock dissimilar to those previously planted, planting rootstocks in the alleyway when the orchard is replanted, and biological amendment with mycorrhizae and biocontrol inoculants could be an effective strategy to reduce/minimise the effects of ARD in replanted orchards.

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6 Effect on microbial communities in apple orchard soil when exposed short-term to climate change abiotic factors and different orchard management practices

6.1 Abstract

6.1.1 Aim

We assessed the effect of exposing apple orchard soil to different temperatures and CO_2 levels on the resident microbiome of soils from a conventionally managed and an organically managed apple orchard. The key difference between these two orchards was that synthetic fertilizers and pesticides are routinely used in the former one.

6.1.2 Methods and Results

To investigate the effect of CO_2 and temperature soil samples from each site at two depths were exposed to elevated temperature (29^{o}C) at either 5,000 or 10,000 ppm for 5 weeks or control conditions (25^{o}C + 400 ppm). Both bacterial and fungal communities were profiled with amplicon-sequencing. The differences between the two orchards were the most significant factor affecting bacterial and fungal communities contributing to 53.7% and 14.0% of Bray-Curtis β diversity variance respectively. Elevated CO_2 concentration and increased temperature affected organic orchard microbial diversity more than the conventionally managed orchard. A number of candidate beneficial and pathogenic microorganisms had differential abundance when temperature and CO_2 were elevated, but their effect on the plant is unclear.

6.1.3 Conclusions

This study has highlighted that microbial communities in bulk soils are most significantly influenced by crop management practice compared to the climate conditions used in the study. The studied climate conditions had a limited effect on microbial communities in conventionally managed soils than in organically managed soils.

6.1.4 Significance and Impact of Study

Climate change scenarios can have a significant impact on the microbiome of soils, with agronomic factors being particularly important. This study highlights the buffering capacity of conventionally managed soils compared to organically managed soils to elevated temperature and CO_2. It also identifies beneficial microorganisms sensitive to climate conditions as candidates for soil amendment and potential emerging pathogens. This study provides the basis for further work on the relative impact of changes in climatic conditions and plant development influences on the soil microbiome.

6.2 Introduction

To maximise the efficiency of high-intensity fruit production, reducing or minimising disease on fruit and trees is essential. In apple orchards, soilborne pathogens are of particular concern as due to the perennial nature of the trees, soilborne disease are difficult to prevent and treat. Apple replant disease (ARD) is an example of an important soilborne disease in apple production. ARD is a disease caused by a complex of pathogens in soil that arises in orchards when young trees are planted in an established orchard without crop rotation or land rest. Symptoms of ARD include stunted growth, reduced yields, reduction in fruit quality, and a reduction in root biomass and root health (Mazzola and Manici, 2012).

The soil microbiome is important in affecting disease development in apple. One example is a number of pathogenic microbes present in the rhizosphere of ARD trees associated with the disease. The fungal pathogens *Rhizoctonia*, *Cylindrocarpon* and *Fusarium* along with the oomycetes *Pythium* and *Phytophthora* have all been associated with ARD when their abundance is increased particularly in synergy (Tewoldemedhin et al., 2011a). *Pratylenchus Penetrans*, a root-lesion nematode, can exacerbate ARD by creating root lesions for pathogen entry into roots (Mai and Abawi, 1981; Mazzola and Manici, 2012). Similarly, beneficial microorganisms play a critical role in promoting plant growth by nutrient exchange and biocontrol action against pathogens in the rhizosphere. Plants recruit these microbes to the rhizosphere through root exudation (Haichar et al., 2008). Recent sequence data have demonstrated a positive correlation between plant growth-promoting rhizobacteria (PGPR) species and apple tree development, such as increased plant height, root length, and dry weight, and in many other important crops such as tomato, cucumber maize, and wheat (Bhattacharyya and Jha, 2012; Nicola et al., 2017). Arbuscular mycorrhizal fungi (AMF) inoculation can also increase the biomass and P content of the plant (Treseder, 2013). There is a debate as to the beneficial effect of AMF on initial plant development with some studies reporting a negative effect on growth due to AMF inoculation at early growth stages but still exhibiting beneficial effects

such as increased drought tolerance despite the initial growth suppression (Smith et al., 2010).

Large scale differences in soil microbiome are primarily down to soil-physiochemical properties. Bacterial communities are strongly defined by the pH of the soil in that particular location (Rousk et al., 2010). Smaller scale differences are attributed to the management practices applied to the soils, with organic systems tending to have higher microbial richness and exhibiting both pathogen suppression and increased abundance of beneficial microbes (Peltoniemi et al., 2021; Suyal et al., 2021). Crop disease, storage and production are all tightly controlled by climate conditions and the uncertainty around the effect of the changing climate makes development of mitigation strategies essential for global food security (Chakraborty and Newton, 2011). The geographic distribution of plant pathogens is expected to be altered as the dispersal of climatic regions change with the overall temperature increase, leading to emergence or increased severity of plant pathogens (Shaw and Osborne, 2011). Apple microbiome ecological studies in the face of climate change are needed to understand the aetiology of soilborne diseases. There is attention on the potential impact of extreme climate episodes and how this might influence microbial communities of apple orchard soils, influencing both apple tree growth and soilborne diseases

It is difficult to pinpoint the effect on soil microbiome due to changing climate factors separately as elevated CO_2 is often coupled with warming and subsequently drying of soils (Jansson and Hofmockel, 2020). Elevated atmospheric CO_2 removes the C-limitations on the microbiome as more carbon is available for growth whereas increased temperatures can increase the efficiency of enzymatic activity. Elevated CO_2 and temperature should thus be beneficial, however, studies have shown increases, decreases, or no effect on microbial biomass and activity (Drigo, Kowalchuk and Van Veen, 2008). Increase in plant biomass in elevated CO_2 conditions has also been positively correlated with increased pathogen biomass such as *Fusarium pseudograminearum* (Eastburn, McElrone and Bilgin, 2011).

Previous studies suggest that, in elevated atmospheric CO_2, soil organic carbon may remain the same, indicative of the inability of soils to naturally stabilize or reduce atmospheric CO_2 (Carney et al., 2007). Elevated atmospheric CO_2 initially increased soil bacterial diversity but decreased the diversity exponentially as concentrations exceeded 10,000 ppm (Ma et al., 2017). Exposure of soil cores collected from grassland to an elevated CO_2 concentration (20% increase above ambient) showed no effect on the nitrogen cycling communities but N_2O fluxes were doubled indicating that there may be a transient increase in N-cycling microbial community functionality rather than population shifts (Brenzinger et al., 2017). In addition, elevated CO_2 had little or no direct effect on the fungal community diversity in soil but increased total fungal biomass, although does not appear to affect fungal activity (Kandeler et al., 1998; Klamer et al., 2002; Phillips et al., 2002). However, rhizosphere populations of PGPR and fungi, particularly AMF, can play an important role in nitrogen fixation and cycling as N availability is reduced under elevated CO_2 (Drigo, Kowalchuk and Van Veen, 2008). The potential sensitivity of fungal communities to elevated CO_2 is important as they have a lower demand on N than bacteria and thus the fungal composition could be more important for nutrient exchange with roots in elevated CO_2 conditions which may be N-limiting for beneficial bacteria (Hu et al., 2001).

Changes in microbial communities could be further influenced by an increase in the release of root exudates correlated with increasing CO_2 and photosynthesis (Phillips, Fox, and Six, 2006), providing additional carbon sources which can stimulate the activity of specific components of microbial communities, especially in the rhizosphere (Kapoor and Mukerji, 2006). Many plants rely on beneficial microorganisms such as AMF and nitrogen-fixing PGPRs (Mekala and Polepongu, 2019), thus the increased abundance of these organisms would likely translate into increased plant growth. To date, however, there is little knowledge on what effects/impacts elevated CO_2 concentration and increased temperature may have on soil microbial communities and the functionality of these populations.

Soil warming generally causes shifts in both bacterial and fungal populations in soils (Hayden et al., 2012; Xue et al., 2016), with warming soils showing a positive correlation with *Fusarium* wilt control in strawberries (Hewavitharana et al., 2021). Studies have, however, also shown there is no effect of warming on soil microbiome structure or function with a +3 ^{o}C increase (Macdonald et al., 2021). As soils warm, water availability changes leading to more drought conditions, which along with the root exudation effect of the plant may explain how microbiome composition and activity are controlled by the interaction between plant, drought, and warming (Jansson and Hofmockel, 2020). Recent studies have suggested that a 30% reduction in water holding capacity is sufficient to cause a shift in the dominant fungal community members (Mekala and Polepongu, 2019). During drought conditions, AMF colonization is generally reduced but most plants benefit from mycorrhizal symbiosis during drought stress (Boyer et al., 2015; Mekala and Polepongu, 2019). (Boyer et al., 2015)(Heidari and Golpayegani, 2012)

The objective of this study was to assay the short-term effect of exposing soils to extreme increases in both temperature (25 and 29 ^{o}C) and CO_2 concentrations (400 ppm, 5,000 ppm, and 10,000 ppm) to initially understand if extreme increases above what will be seen in nature will first have an effect before selecting more representative increases in future works. The direct effect of the increased temperature and CO_2 increases would provide information on the resilience of the community structure of the resident microbiota (pathogenic, beneficial or neither) in bulk soils available for recruitment by a replanted apple tree in both a conventional and organically managed orchard. The study hypothesis was that temperature and CO_2 increase will alter populations of potentially beneficial and pathogenic fungi and bacteria in bulk orchard soil with different management practices.

(Hamada and Tanaka, 2001).

6.3 Materials and Methods

6.3.1 Soil Nutrient Analysis

Soil from an organic plot and a conventionally managed (chemical application) plot at NIAB East Malling, Kent, UK (51°17'9.5"N 0°27'12"E) were sent for nutrient analysis in January 2020 and July 2021 respectively to NRM Laboratories, Winklefield Row, Berkshire, UK. The soil type, pH, soil organic matter, P, K, Mg, and Cu content were measured for a sub-sample of soil from each site.

6.3.2 Soil Core Collection

Soil cores were collected from two orchards: one conventionally managed using industry-standard chemical applications of fertilizers, pesticides, and herbicides, and the other organically managed without the input of these synthetic fertilizers, pesticides, and herbicides at NIAB East Malling in March 2019. The conventionally managed orchard usually receives annually between 10-15 fungicide sprays for disease control that the organic orchard did not receive. The organic orchard complied with the organic farming standards in the UK and had no organic or chemical fungicides, insecticides, foliar nutrients or fertilisers applied. Apple trees that had been planted in 2009 were grubbed in October 2018; soil cores were collected from the original tree stations where the previous trees had been. A 15 cm depth soil corer (diameter 3 cm) was used to take the samples. The top 10 cm of soil was discarded and two soil cores at different depths (10 to 17.5 cm; 17.5 to 25 cm) were then collected from the same core (core was divided into two using a sterilised knife). A core from each depth was collected from 12 separate tree stations on each site. There was a total of 24 samples per site, 48 samples total. Each sample was placed into a separate polythene bag and immediately stored at 4ºC until further processing.

6.3.3 Growth Incubator CO_2 Calibration and Setup

Soil samples were transported to Cranfield University, Cranfield, Bedfordshire, UK. Growth incubators were used to create climate change conditions. Incubators were set to two conditions simulating climate changing scenarios: 29˚C (+4 ˚C above ambient) at either 5000 or 10,000 ppm CO_2 concentration

using a CO_2 gas cylinder. A tray of water was placed at the bottom of the incubator and refreshed once per week during the experiment to prevent CO_2 from drying air in the incubator. Pipes were placed at the base of the incubator to prevent CO_2 accumulation and increase the resident CO_2 concentration. Gas chromatography was used to test the peak area (pA) of CO_2 in each of the incubators converted to target ppm values. A standard curve was produced for CO_2 in the range of 0.10% and 5% and then used to check whether the target % CO_2 level was achieved with air extracted from the closed incubation chamber using a syringe. In chamber 1, 0.3% CO_2 (pA = 0.52) equalled 5,000 ppm and in chamber 2, 0.5% CO_2 (pA = 0.98) equalled 10,000 ppm. As a control treatment, soil samples were placed in a climate-controlled growth room set at 25 ^oC and ambient atmospheric CO_2 conditions (approximately 400 ppm).

6.3.4 Soil exposure to interacting climate change-related abiotic stresses of temperature x CO_2 concentration

For each site and depth combination, four of the 12 samples were randomly assigned to one of the three conditions, including the control. The two layers from the same core were exposed to the same climate condition. Soil cores were placed in surface-sterilized glass jars which were closed with lids containing a microporous layer allowing air and water vapor exchange.

The jars were placed in a random 4x4 lattice design in the two climate change incubators, 5000 ppm CO_2 + 29 ^oC (Condition A) or 10,000 ppm CO_2 + 29 ^oC (Condition B). The remaining control soils were distributed randomly in a 4x4 lattice in the climate-controlled growth room set at 25 ^oC and ambient CO_2 concentration (Control). Beakers of water were placed in the control growth room to maintain a high humidity throughout the experiment. The soil samples were all incubated for 5 weeks.

6.3.5 Next-generation sequencing of bacterial and fungal communities

After 5 weeks of exposure, soils were removed from each glass jar and the outer edges scraped off carefully with a spatula from the centre of the sample along the whole length of the core. A 2 ml Eppendorf was filled fully with the soil sample

to ensure sufficient soil was collected for each test. After taking each sample, the spatula was washed in 70% ethanol and dried. The soil samples were transported to NIAB EMR, East Malling, Kent, UK at 4^{o}C and subsequently stored at -20 ^{o}C for two weeks until used for DNA extraction.

6.3.5.1 Soil DNA Extraction and PCR Amplification

Amplicon sequencing and sequence processing followed the method used previously (Deakin et al., 2018d). In summary, genomic DNA was extracted from a sub-sample of 0.25g soil sample using the DNeasy PowerSoil Kit (Qiagen, Carlsbad, USA) in conjunction with a bead-beating benchtop homogenizer (Fastprep FP120, Qbiogene, Carlsbad, USA). DNA concentration and quality were determined using a spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific, Cambridge, UK). DNA was diluted to 2 ng/µl for PCR amplification.

The regions and primer pairs used for PCR amplification used were: for bacteria the 16S V4 region was amplified with the primer pair Bakt_341F/Bakt_805R (Herlemann et al., 2011); for fungi, the ITS1 and ITS2 regions were amplified with the primer pair EkITS1F/Ek28R(≡ 3126T) (Gardes and Bruns, 1993; Sequerra et al., 1997). PCR conditions for ITS and 16S reactions are described in Table S1. Nextera transposase adapters (Illumina, San Diego, USA) were appended to each amplicon. All PCR reactions were performed in 25 µL reaction mixtures comprising 1×PCR buffer containing 50 mM $MgCl_2$ and 1 U Platinum *Taq* DNA Polymerase (Invitrogen, Life Technologies, Carlsbad, USA), 5 mM dNTP (Fisher Bioreagents, Thermo Fisher Scientific, Pittsburgh, USA), 2 µM forward and reverse primers each (Integrated DNA Technologies, Coralville, USA), 2 ng/µl template DNA and molecular water (Sigma, St Louis, USA). PCR reaction success was checked by gel electrophoresis.

6.3.5.2 DNA Clean-Up 1

The DNA was library prepared following Illumina MiSeq manufacturer's protocols. PCR plates for both ITS and 16S amplified regions were centrifuged for 30 seconds. 50 ml of PCR product was combined with 32 µl of solid-phase reversible immobilization beads (Agencourt XP Ampure beads; Beckman Coulter, Brea, USA) and were gently mixed using a pipette and incubated at room temperature

for 5 min. Tubes were placed on a magnet to attract the beads. The supernatant, cleared of beads, was then removed; 200 µl of 80% ethanol was added to each tube and incubated for 30 seconds and the clear supernatant was removed. The 80% ethanol step and supernatant removal were repeated taking care to completely remove any excess ethanol in the tubes. Beads were air-dried until beads cracked (10+ min) and then removed from the magnet. A volume of 52.5 µl 10mM Tris pH 8.5 was added to the beads and gently pipette mixed and incubated for 2 min. The supernatant was then cleared from the beads again using the magnet and 50µl of the supernatant was collected in a fresh PCR strip and stored at -20 °C.

6.3.5.3 Index PCR and DNA Clean-Up 2

Nextera XT DNA Library Preparation Kit (Illumina) was used to barcode amplicon libraries. Nextera index 1 primer corresponded to columns 1-12 and Nextera index 2 primers corresponded to rows A-H with 5 µl of each added to each well creating a unique primer pair barcode for each sample. 35 µl KAPA HiFi Hotstart Ready Mix (Roche Sequencing Solutions Inc, Pleasanton, CA, USA) was added to each sample together with 5 µl of corresponding clean-up 1 DNA sample. Samples were gently mixed and centrifuged at 1000 xg for 1 minute. The index PCR reaction was then performed using conditions described in Table S2. Samples were then immediately cleaned up a second time as described using the Clean-up 1 procedure with 56 µl of beads initially, and 27.5 µl 10 mM tris pH 8.5 added to the dried beads to make a final volume of 25 µl of cleaned DNA was transferred to fresh tubes and then stored at -20 °C.

6.3.5.4 Library Quantification, Normalisation, and Pooling

DNA quality and concentration were checked using a spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific, Cambridge, UK) and a fluorometer (Qubit 2.0, Life Technologies, Carlsbad, USA). DNA sample of 5 µl was from each extraction and then pooled into a 1.5 ml Eppendorf tube. The pooled DNA was stored at -20 °C.

6.3.5.5 Library – PhiX Contorl denaturation and MiSeq Loading

5 µl of the pooled library (4 nM) was added to 5 µl of 0.2 nM NaOH and mixed briefly by vortexing. A volume of 990 µl of HT1 solution (MiSeq Reagent Kit v3, Illumina) was added to the tube resulting in a concentration of 20 pM. In a separate tube, the pooled library was diluted to a final concentration of 10 pM. PhiX control (Illumina) was denatured and diluted before use to the same concentration as the denatured library (10 pM). PhiX control is spiked in at 20% with a 10 pM denatured library. 120 µl 10 pM PhiX control was combined with 480 µl 10 pM denatured library and stored on ice. Immediately before loading onto the MiSeq, heat denaturation of combined PhiX-Library was incubated on a hot block at 96°C for 2 min and then mixed by inverting the tube twice. The tube was then incubated in an ice water bath for 5 min and immediately loaded onto the MiSeq cartridge and sequenced using the manufacturer's protocol. Raw reads were used for sequence read processing.

6.3.5.6 Sequence Read Processing

Sequence data was submitted to the NCBI database (Project PRJEB52533). Sequence processing followed previously reported methods/pipelines (Deakin et al., 2018d). FASTQ reads were demultiplexed into 16S and ITS fungal read datasets based on their primer sequences. Any ambiguous reads that did not match the forward and reverse read primers for 16S and ITS were removed before sequences were processed further.

Bacterial and fungal reads were processed separately to create separate representative OTUs for bacterial and fungal reads. ITS forward and reverse reads were aligned with a maximum difference in overlap of 10% threshold and 16S reads aligned similarly with a threshold of 5%. Forward and reverse primers were removed from both 16S and ITS reads. Merged reads with adaptor contamination or fewer than 150 nucleotides for ITS reads or fewer than 300 nucleotides for 16S reads were removed. Merged reads were then quality filtered using a maximum expected error threshold of 0.5.

6.3.5.7 OTU Generation

All analyses were performed using UPARSE 11.0 (Edgar, 2013b) unless otherwise specified. Sequences were dereplicated to find unique sequences, with any unique sequence with less than 4 reads being discarded from generating OTUs. Unique sequences were then sorted by decreasing frequency and clustered at 97% similarity into OTUs with a representative sequence for each OTU generated. Then an OTU table was generated by mapping all the merged reads against the representative OTU sequences. Taxonomy was predicted for generated OTUs using the SINTAX algorithm (https://www.drive5.com/usearch/manual/sintax_algo.html) by aligning ITS OTU representative sequences to the reference database "UNITE v7.1" (Nilsson et al., 2019) and 16S OTU representative sequences to the RDP training set v16 (Cole *et al.,* 2014).

6.3.6 Statistical Analysis

All statistical analyses were conducted in R V4.0.2 (R Core Development Team 2008). The vegan package V2.5.7 (Dixon, 2003) was used to produce rarefaction curves to identify outliers. Metacoder package V0.3.4 (Foster, Sharpton and Grünwald, 2017) was used to indicate the differences in microbial groups at different taxa levels between treatments. Counts with 1 count were zeroed and OTUs with no reads were subsequently removed from further analysis. Count data were normalised by rarefaction to the minimum number of reads calculated in the Vegan package for Metacoder analysis.

Alpha diversity (α) indices (Chao1, Shannon, Simpson, and invSimpson) were calculated using the Phyloseq package V1.34 (McMurdie and Holmes, 2013) from the rarefied counts. Alpha diversity indices were subject to ANOVA analysis and significance determined by permutation testing using the package LmPerm package V2.1 (Wheeler, 2016) to assess the effect of orchard management, soil core depth, climate condition (5,000 ppm +4 ^{o}C, 10,000 ppm +4 ^{o}C, and control) and the interaction between site management (conventional vs organic), depth and condition. Alpha indices were visualised in the ggplot2 package v3.3.2 (Wickham, 2011).

Beta diversity (β) index (Bray-Curtis) was calculated in the Vegan package from the rarefied OTU count data and plotted using a non-metric multidimensional scaling (NMDS) plot to visualise sample locations concerning the study factors. A permutation MANOVA (multivariate analysis of variance) using F-tests based on sequential sums of squares (ADONIS) from 1000 permutations was used to determine statistical significance. Principal components (PC) were calculated in the DESeq2 package V1.30.1 (Love, Huber and Anders, 2014). ANOVA analysis was used to determine the relative contribution of each experimental factor to the total variability in the top six PCs.

Differential OTUs were determined by the DESeq2 package to see the effect of each experimental factor on induvial taxa. Log fold change (LFC) was shrunk within DESeq2 when extracting results from the model (Zhu, Ibrahim and Love, 2019). DESeq2 adjusted P-value threshold was set to 0.1 for the significantly higher or lower abundance of OTUs. Taxonomy was assigned to each OTU with significantly different abundance with a SINTAX confidence score ≥ 0.65 at the lowest assignable taxonomic rank. For large differences in abundance, the Metacoder package was used to display a phylogeny of bacterial and fungal taxonomy, coloured by different abundance due to experimental factors. Differences in Metacoder are calculated using the Wilcoxon Rank Sum test corrected for multiple comparisons with only significant differences in taxonomy displayed on the phylogenetic tree. The Fungi tree was only labelled with taxonomy with a Wilcox p-value < 0.05. The Bacteria tree was labelled up to rank Phylum due to the complexity of the tree.

6.4 Results

6.4.1 Soil Nutrient Analysis

Soil from the conventional site was classified as sandy silt loam and the soil from the organic site classified as sandy loam. pH on both sites was 6.9. Soil organic matter content was also similar between the conventional orchard (2.5% loss on ignition) and the organic orchard (3.4% loss on ignition). P content was similar on both sites with 31.4 mg/l available on the conventional site and 31.8 mg/l available on the organic site. K levels both fell within the target index at 151 mg/l available on the conventional site and 272 mg/l available on the organic site. Excessive levels of Mg were available on the organic site at 252 mg/l compared to 63 mg/l on the organic site. Inversely Cu levels were excessive on the organic site at 18.4 mg/l and were at target levels on the conventional site at 9.6 mg/l.

6.4.2 Summary of Sequencing Data

Total bacterial counts were higher than fungal counts across all samples with 2828947 and 338920 reads, respectively. Overall OTU data for both bacteria and fungi are summarised in Table 6.1. The mean number of reads per sample was 58936 for bacteria and 7061 for fungi. Sample 30 (lower depth organic soil core exposed to 5000ppm and 29℃) had the lowest number of reads (5552 for bacteria and 808 for fungi) and so was removed from subsequent statistical analysis both bacterial and fungal analysis. Samples 22 (lower depth conventional soil core incubated at 5000 ppm and 29℃), 41 (higher depth organic soil core exposed to 10000ppm and 29℃), and 42 (lower depth organic soil core exposed to 10000ppm and 29℃) all had low reads per sample for the fungal reads (538, 31, and 67 reads respectively) so were removed from the further fungal analysis.

Table 6.1 Summary of the bacterial and fungal representative operational taxonomic units (OTUs) after quality filtering and removal of low counts. OTUs were generated using 97% sequence similarity.

	Total counts	Total OTUs	Number of OTU per sample		Number of reads per sample		Number of reads per OTU	
			Min	Max	Min	Max	Min	Max
Bacteria	2828947	11722	61	3397	5552	197191	2	14392
Fungi	338920	990	11	237	31	27039	1	8407

6.4.3 Diversity Indices

Four alpha diversity measures (Chao1, Shannon, Simpson, and InvSimpson) were calculated. Bacterial diversity was higher than for fungi across all samples. Each of the indices showed that fungal diversity was predominantly unaffected by site management, depth, or climate condition with approximately 80% of the total variability unexplained for the Shannon, Simpson, and InvSimpson indices and 96% for the Chao1 index (Table 6.2). Shannon, Simpson and InvSimpson indices for bacteria were significantly greater in the conventional orchard than in the organic orchard ($p < 2x10^{-16}$) (Figure 6.1). All bacterial alpha diversity indices were lower in the samples subjected to the two climate condition treatment samples than in the control samples (Table 6.2, Figure 6.1).

Table 6.2 Percentage of the variability in alpha diversity indices accounted for by site (organic or conventionally managed), depth of the soil core (10 to 17 cm; 18 to 25 cm), the simulated climate condition the core was exposed to (Condition A – 5000 ppm CO_2 + 29ºC; Condition B – 10000 ppm CO_2 + 29ºC; Control – 400 ppm CO_2 + 25ºC), and the interaction between management, depth and climate condition.

Measure	**Site**		**Depth**		**Condition**		**Interaction**		**Residual**
	P value	*%*	*P value*	*%*	*P value*	*%*	*P value*	*%*	*%*
Bacteria									
Chao1	0.27	1.39	0.001	13.02	0.003	17.82	0.233	3.71	64.06
Shannon	$<2x10^{-16}$	23.92	0.003	6.95	$<2x10^{-16}$	18.70	0.335	2.85	47.58
Simpson	$<2x10^{-16}$	49.75	0.179	1.14	0.045	6.48	0.0486	2.08	40.55
InvSimpson	$<2x10^{-16}$	49.75	0.375	1.14	0.031	6.48	0.402	2.08	40.55
Fungi									
Chao1	0.495	0.57	0.922	0.01	0.845	0.49	0.568	2.75	96.18
Shannon	0.548	0.65	0.232	3.39	0.554	3.87	0.182	7.43	84.66
Simpson	0.623	0.91	0.136	3.34	0.357	5.40	0.116	10.05	80.30
InvSimpson	0.505	0.88	0.231	3.41	0.239	5.47	0.108	10.11	80.13

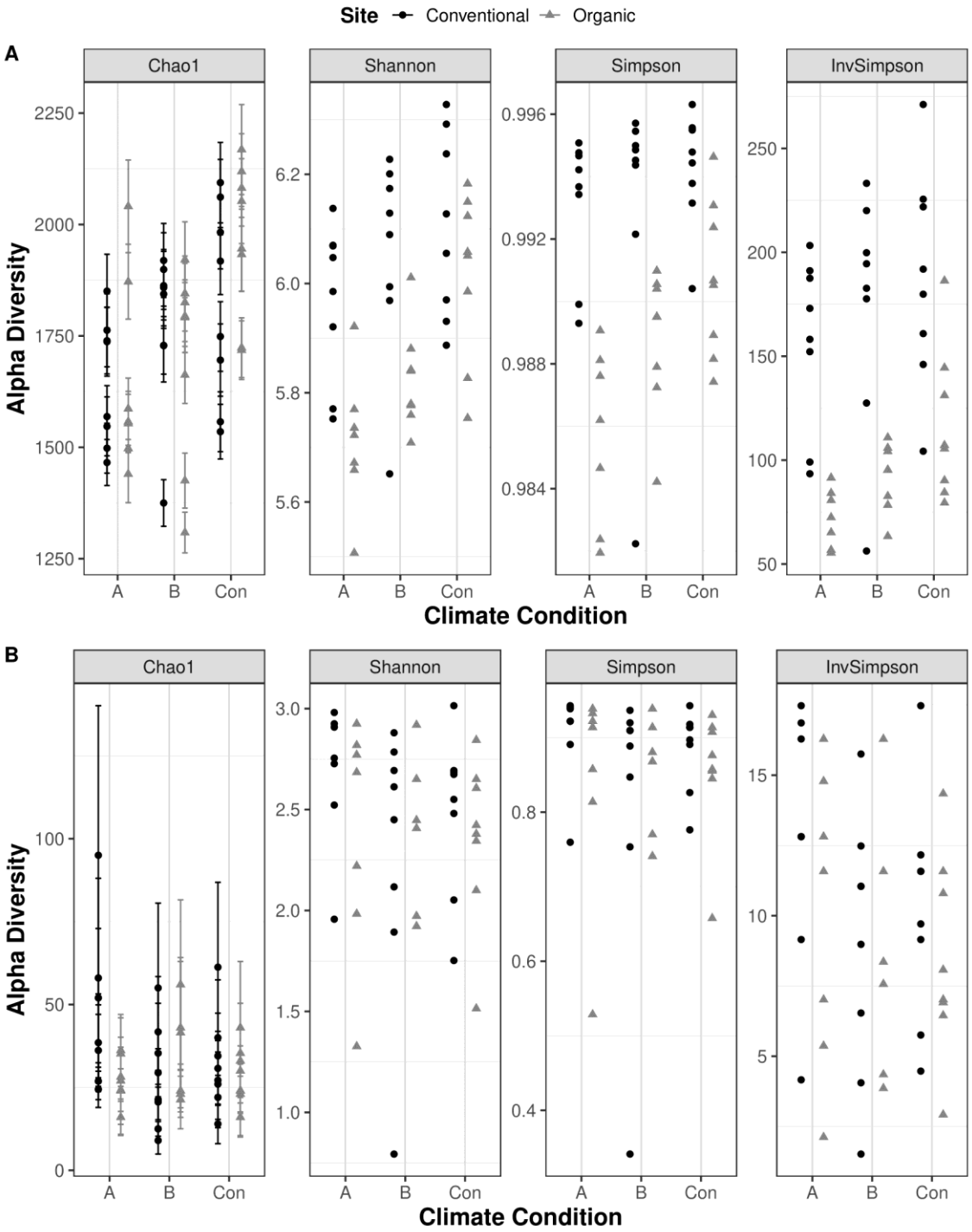

Figure 6.1 Alpha (α) diversity measures, Chao1, Shannon, Simpson and InvSimpson for (A) bacteria and (B) fungi. The x-axis indicated the simulated climate condition the cores were exposed to: A – 5000 ppm CO_2 + 29 ^oC, B – 10000 ppm CO_2 + 29 ^oC, Con – 400 ppm CO_2 + 29 ^oC. Shape of the point indicates site management, organically (▲) and conventionally (●) managed.

Bray-Curtis indices were used to represent differences in microbial communities between samples. ADONIS analysis highlighted Bray-Curtis values were significantly influenced by the site management, contributing 53.7% and 14.0% of the variability for bacteria and fungi, respectively (Table 6.3, Figure 6.2). In the conventional orchard alone, the soil depth the core was taken from contributed to 14.9% of the total variability in bacterial communities and 5.6% in fungal communities. The effect of depth on variability was similar in the organic orchard, contributing to 13.3% for bacteria and 5.2% for fungi. The depth effect was only statistically significant for bacterial populations. The condition the soils were exposed to had a statistically significant effect on both bacterial and fungal populations in the organic orchard but this was not observed in the conventional orchard soils (Table 6.3, Figure 6.3A/C). The climate condition effect contributed to 12.0% and 13.9% of the total variability in bacterial and fungal communities from the organic orchard, whereas for communities in the soil from the conventional orchard the climate condition contributed 9.2% and 8.7% for bacterial and fungal groups respectively. Bacterial communities for the control soils from the organic orchard were dissimilar to bacterial communities for both the climate-treated soils but the communities did not appear to clearly differentiate between the two climate conditions (Figure 6.3B). Fungal communities from the organic orchard appeared to differ between the control condition and the higher 10,000 ppm CO_2 condition, whereas the lower 5,000 ppm treatment was more similar to the control than the higher CO_2 condition but was not clearly different from either condition (Figure 6.3D). Interaction between the depth and climate condition was not significant in the ADONIS analysis and contributed to between 6.7% and 9.5% of the total variability in bacterial and fungal communities across soils from both sites (Table 6.3). The relative contribution of each experimental factor was quantified for the top six principal components (PC) scores. The percentage contribution of each experimental factor was similar to those from the ADONIS analysis (Table 6.3).

Table 6.3 Percentage of the variability in Bray-Curtis dissimilarity and the scores from principal component analysis (PCA) accounted for by site (organic or conventionally managed), depth of the soil core (10 to 17 cm; 18 to 25 cm), the climate condition the core was exposed to (Condition A – 5000 ppm CO_2 + 29ºC; Condition B – 10000 ppm CO_2 + 29ºC; Control – 400 ppm CO_2 + 25ºC), and the interaction between management, depth and climate condition. The depth, climate condition, and interaction are also scored for each site management separately.

Measure	**Site**		**Depth**		**Condition**		**Interaction**		**Residual**
	P value	*%*	*P value*	*%*	*P value*	*%*	*P value*	*%*	*%*
Bacteria									
	0.0001	53.7	0.008	4.2	0.155	2.7	0.371	1.9	37.6
		35.4		3.9		3.6		2.7	46.4
Organic									
Bray-Curtis			9×10^{-4}	13.3	0.019	12.0	0.123	9.5	65.2
PCA*				9.7		11.0		9.6	69.8
Conventional									
Bray-Curtis			9×10^{-4}	14.9	0.174	9.2	0.686	6.7	69.2
PCA*				11.1		8.9		7.1	72.9
Fungi									
Bray-Curtis	0.0001	14.0	0.005	4.0	0.324	4.3	0.790	3.3	74.4
PCA*		12.0		3.0		3.8		3.77	67.3
Organic									
Bray-Curtis			0.308	5.2	0.033	13.9	0.432	9.5	71.4
PCA*				4.4		14.0		10.1	71.4
Conventional									
Bray-Curtis			0.141	5.6	0.561	8.7	0.479	9.0	76.7
PCA*				5.8		7.9		8.0	78.3

*Based on the top six principal components

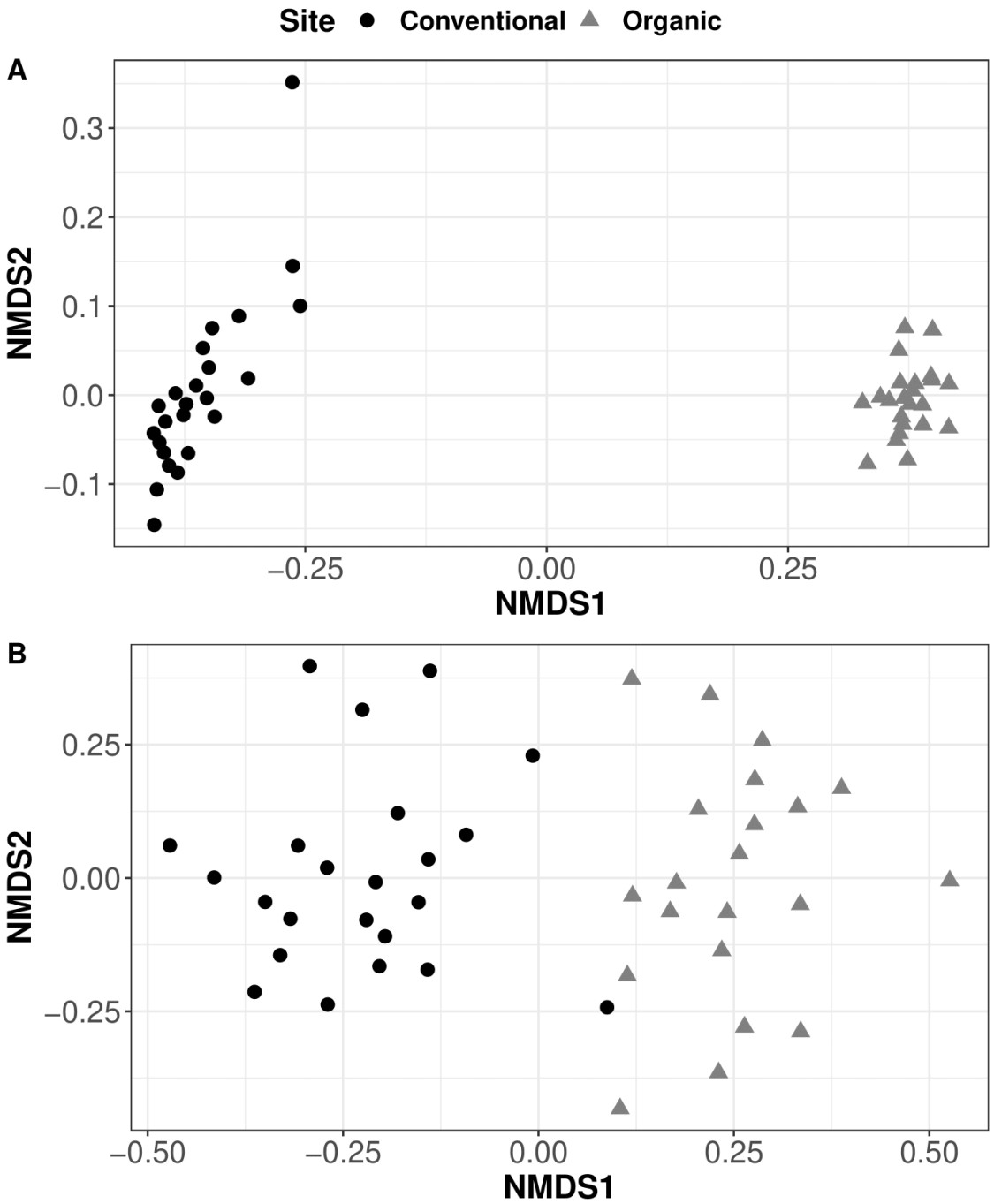

Figure 6.2 The first two dimensions of NMDS analysis of the Beta (β) diversity indices (Bray-Curtis dissimilarity) for (A) bacteria and (B) fungi. Closer the distance between the points indicates more similarity in microbial communities between the samples. The shape of the point indicates site management, organically (▲) and conventionally (●) managed.

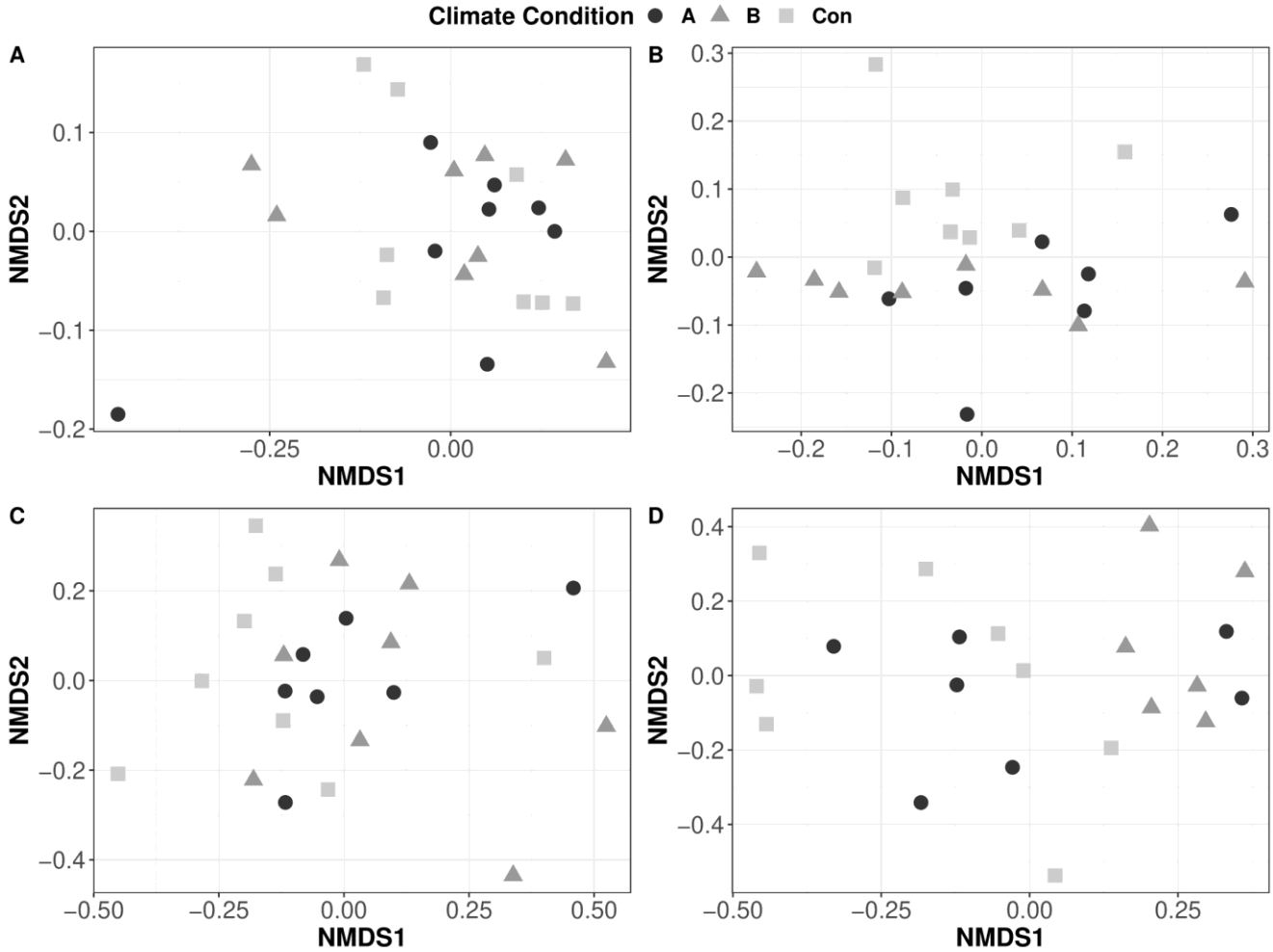

Figure 6.3 The first two dimensions of NMDS analysis of the Beta (β) diversity indices (Bray-Curtis dissimilarity) for bacterial communities in the conventionally managed (A) and organically managed (B) sites, and for fungi in the conventional (C) and organic (D) sites. Closer the distance between the points indicates more similarity in microbial communities between the samples. Shape of the point indicates climate condition treatment of the soils, A = 5,000 ppm CO_2 + 29 ℃ (●) , B = 10,000 ppm CO_2 + 29 ℃ (▲), and Con = Control, 400 ppm + 25 ℃ (■).

6.4.4 Differential OTUs

To see the effect of the climate conditions on induvial taxa, DESeq2 was used to identify OTUs with differential relative abundance between climate conditions. The effect was limited for both bacterial and fungal taxa with less than 1% of OTUs with significant differences in the relative abundances (expressed as log2 fold change - LFC) (Table 6.4). In the organic soils, bacteria relative OTU abundance was more influenced by the two climate conditions than fungal OTU relative abundance. For bacteria, 42 OTUs were identified with different abundance between condition A (5,000 ppm CO_2 + 29 ℃) and the control in organic soils; and 39 OTUs between condition B (10,000 ppm CO_2 + 29 ℃) and the control. No bacterial OTUs were different between the two climate conditions in the organic or conventional soil samples. Only four OTUs were different between either climate condition and the control for fungal OTUs in organic soils and one OTU was more abundant in condition B than in condition A. In the conventional soils, only 12 bacterial OTUs differed between either climate condition and the control (11 of which differed between condition A and the control). Only three fungal OTUs had a differential abundance between the climate conditions and control and one OTU differed between both conditions.

Table 6.4 DESeq2 results summary for all differential OTUs. No. OTUs indicates the number of OTUs after DESeq2 filtering (compared between factors); Log2 Fold Change (LFC) > 0 indicates OTUs higher in the first condition/treatment than the second; LFC < 0 indicates OTUs higher in the second condition/treatment than the first. LFC was shrunk using DESeq2. Condition A = 5000 ppm CO_2 + 29 ^oC; Condition B = 10000 ppm CO_2 + 29 ^oC; Control = 400 ppm CO_2 + 25 ^oC. Organic vs Conventional refers to the site crop management of the orchard from which the samples were obtained.

DESeq2 Model	No. OTUs	LFC > 0 (higher)	LFC < 0 (lower)	Low Counts
Bacteria				
Organic vs Conventional	11698	1386, (12%)	1357, (12%)	7545, (64%)
Organic				
Condition A vs Control	7300	18, (0.25%)	24, (0.33%)	5627, (77%)
Condition B vs Control	7300	13, (0.18%)	26, (0.36%)	5490, (75%)
Condition B vs Condition A	7300	0, (0%)	0, (0%)	15, (0.21%)
Conventional				
Condition A vs Control	8149	3, (0.037%)	8, (0.098%)	6517, (80%)
Condition B vs Control	8149	0, (0%)	1, (0.012%)	41, (0.5%)
Condition B vs Condition A	8149	0, (0%)	0, (0%)	41, (0.5%)
Fungi				
Organic vs Conventional	898	83, (9.2%)	47, (5.2%)	678, (76%)
Organic				
Condition A vs Control	589	0, (0%)	1, (0.17%)	13, (2.2%)
Condition B vs Control	589	0, (0%)	3, (0.51%)	13, (2.2%)
Condition B vs Condition A	589	1, (0.17%)	0, (0%)	13, (2.2%)
Conventional				
Condition A vs Control	658	1, (0.15%)	1, (0.15%)	63, (9.6%)
Condition B vs Control	658	1, (0.15%)	0, (0%)	63, (9.6%)
Condition B vs Condition A	658	1, (0.15%)	0, (0%)	63, (9.6%)

In contrast, 24% of bacterial OTUs differed significantly in their relative abundance between the two orchards: half of these OTUs had decreased abundance and the other half increased in the organic orchard, compared to the conventionally managed orchard. About 14.4% of fungal OTUs differed in their relative abundance between the two orchards with a higher number of OTUs more abundant in the organic orchard (9.2%) than those more abundant in the conventional orchard (5.2%).

Table 5 shows bacterial and fungal OTUs with differential abundance from DESeq2 analysis (Table 4) and their associated SINTAX taxonomy predictions. Only bacterial OTUs at rank genus and below at a SINTAX confidence threshold of 0.65 were used for increased accuracy of taxonomy assignments. All of the bacterial OTUs were identified to the rank genus. The fungus *Trichoderma evansii* was identified as more abundant after treatment with condition A or condition B than in the control soils. Two *Trichosporon* OTUs, *T. porosum,* and *T. loubieri*, were more abundant in condition B for both taxa; *T. porosum* was also more abundant in condition A than in the control.

Table 6.5 Differential OTUs from DESeq2 analysis. Positive Log2 Fold Change (LFC) indicates higher OTU abundance in the first condition/treatment and vice versa for negative values. P values are Benjamini and Hochberg corrected. Condition A = 5000 ppm CO_2 + 29 ^oC; Condition B = 10000 ppm CO_2 + 29 ^oC; Control = 400 ppm CO_2 + 25 ^oC.

DESeq2 Model	Species/Taxa*	Base Mean	LFC	P-Value
Bacteria				
Conventional				
Condition A vs Control	*Pseudonocardia* (g)	4.64	2.49×10^{-6}	0.04
	Acidobacteria – Gp7 (g)	54.92	3.25	0.04
	Pseudoxanthomonas (g)	6.73	-2.33×10^{-6}	0.04
	Janthinobacterium (g)	8.34	-4.63×10^{-6}	0.04
	Flavobacterium (g)	4.22	-3.14×10^{-6}	0.04
	Nocardioides (g)	26.16	-9.19×10^{-6}	0.09
Condition B vs Control	*Flavobacterium* (g)	35.96	-5.67×10^{-6}	0.02
Organic				
Condition A vs Control	*Sphingomonas* (g)	1132.97	0.98	0.05
	Acidobacteria – Gp3 (g)	151.13	0.67	1.23×10^{-3}
	Pseudonocardia (g)	7.93	4.96	5.44×10^{-4}
	Pseudomonas (g)	304.60	-5.28×10^{-6}	0.10
	Variovorax (g)	35.84	-6.18×10^{-6}	0.05
	Flavobacterium (g)	8.70	-3.86×10^{-6}	5.44×10^{-4}
	Chryseolinea (g)	46.12	-2.34×10^{-5}	0.02
	Acidobacteria – Gp6 (g)	37.55	-1.04×10^{-5}	0.10
	Massilia (g)	14.22	-7.36×10^{-6}	0.01
	Streptomyces (g)	9.34	-5.78×10^{-6}	0.05
	Nonomuraea (g)	3.97	-1.94×10^{-6}	0.10
	Janthinobacterium (g)	14.55	-2.87	5.44×10^{-4}
	Aeromicrobium (g)	11.43	-4.60×10^{-6}	0.01
	Pseudonocardia (g)	6.60	-4.60×10^{-6}	0.02
	Methylotenera (g)	10.15	-2.22×10^{-6}	0.06
Condition B vs Control	Acidobacteria – Gp6 (g)	17.60	1.36	1.89×10^{-3}
	Burkholderia (g)	51.13	2.77	0.04
	Acidobacteria – Gp1 (g)	28.31	1.52	0.06
	Chryseolinea (g)	23.78	1.93	7.29×10^{-4}
	Pseudonocardia (g)	7.93	5.25	1.18×10^{-4}
	Variovorax (g)	35.84	-7.15×10^{-6}	0.17
	Flavobacterium (g)	8.70	-4.66×10^{-6}	7.41×10^{-4}
	Chryseolinea (g)	46.12	-1.94	7.85×10^{-4}
	Variovorax (g)	29.69	-1.77	0.04
	Massilia (g)	14.22	-2.75	1.05×10^{-5}
	Janthinobacterium (g)	14.55	-3.52	9.62×10^{-6}
	Mucilaginibacter (g)	6.51	-2.94×10^{-6}	0.01
	Pseudonocardia (g)	6.60	-4.96×10^{-6}	6.91×10^{-3}
	Methylotenera (g)	10.15	-2.73×10^{-6}	2.29×10^{-3}

Fungi				
Conventional				
Condition A vs Control	*Trichoderma evansii*	7.47	3.45×10^{-7}	2.86×10^{-9}
Condition B vs Control	*Trichoderma evansii*	7.47	2.32×10^{-7}	3.01×10^{-4}
Organic				
Condition A vs Control	*Trichosporon porosum*	44.71	-6.50×10^{-7}	4.80×10^{-2}
Condition B vs Control	*Trichosporon loubieri*	759.96	-6.97×10^{-7}	0.05
	Trichosporon porosum	44.71	-5.58×10^{-7}	0.03
	Conocybe (g)	41.70	-2.34×10^{-7}	2.50×10^{-6}
Condition B vs Condition A	Conocybe (g)	41.70	-2.14 x 10-7	4.15×10^{-10}

* The lowest assignable taxonomic rank at genus or below with a SINTAX confidence ≥0.65.

Figure A.7 shows the differences in the relative OTU abundance between the two sites to the rank order for bacteria and fungi. Differences in bacteria were distributed across the taxa with a higher abundance in most taxa in the conventional soils compared to the organic soils. Node labels in Figure A.7 are labelled for taxa with a differential abundance with Wilcox P-value < 0.05. Differences in taxa were restricted more to specific taxa, namely higher relative abundance for *Saccharomycetales* and *Leotiomycetes* in the conventional orchard and the opposite was true for *Glomeromycetes* and *Agaricomycetes*. The order *Paraglomerales* within the class *Glomeromycetes* was also shown to be significantly more abundant in the organic orchard soil.

6.5 Discussion

Next-generation sequencing allowed for in-depth analysis of differences between microbial communities in apple orchard bulk soil and highlighted that site management (i.e., organic vs conventional) was the most significant factor affecting the microbiome in apple orchard soils. A high percentage of the variance in the microbial diversity was attributed to the site differences. Microbiomes differ between conventionally and organically managed soils with the latter possessing more heterogeneous microbial communities (Lupatini et al., 2017). This study has shown that soils in an orchard managed with conventional strategies (including the use of synthetic chemical products) had a higher alpha diversity for bacteria than an orchard managed organically (without using synthetic chemical products). Previous studies have shown that bacterial species richness increases in organic farming systems compared to conventional farming systems (Acharya et al., 2021). We however found the inverse, the reasoning for which is yet unclear and requires further studies.

Soil type, pH, P, and K availability were similar across the two sites so were unlikely to effect the soil microbiome diversity. Excessive Mg was available on the organic site, but increased concentrations of Mg more strongly affect the functionality of the soil microbiome than the taxonomic structure, which is more driven by the availability of organic nutrients such as C and N rather than inorganic nutrients (Nicolitch et al., 2019). Cu concentration was also excessive in the organic soils and increased Cu availability has been shown to be negatively correlated with bacterial local diversity in soils (Nunes et al., 2016), but the concentrations required for a significant reduction in diversity (~100-500 mg/l) is much higher than the concentration observed in this study (18.4 mg/l). It is unlikely nutrient differences between the two orchards lead to the differences in community structure observed between the two sites, but it could be a possibility that individual populations could respond to the difference in nutrients leading to changes in abundance in individual OTUs.

Fungi had similar alpha diversity between sites. The differences in the microbial communities were spread evenly across bacterial taxa but were more restricted

to specific fungal taxa. The order *Paraglomerales* within the class *Glomeromycetes*, a group of beneficial AMF species in soil, is an example of one such taxon of significance with higher abundance in the organic orchard (Schüßler, Schwarzott, and Walker, 2001). This highlights that there may be more natural symbiosis with mycorrhizae in this particular organic orchard and perhaps indicates the potential for the use of AMF inoculants in conventionally managed orchards. A recent review has discussed AMF such as *Glomeromycetes* as an amendment to mitigate ARD through benefits such as greater root system architecture, increased nutrient exchange, and regulation of root endophytes and rhizosphere ARD pathogens (Lü and Wu, 2018). We have shown that the microbiome of organic and conventional orchards are different, which may impact the onset and subsequent development of ARD in these two systems. Some other studies have also shown differences in microbiomes in organic and conventionally managed orchards (Hartmann et al., 2015) but not with elevated CO_2 concentration and increased soil temperature.

Both the two climate conditions used in the study significantly reduced bacterial alpha diversity. Bacterial diversity has been previously reported to increase with elevated CO_2 up to 10,000 ppm (Ma et al., 2017). The elevated temperature was predicted to contribute to this increasing diversity. The present study showed opposite results and suggested that other environmental factors and/or the nature of specific soils (including the history of crop production) (Powell et al., 2015) may have caused the reduction in alpha diversity in conjunction with climatic conditions, which would require further investigation in future work. Bacterial and fungal microbial communities appeared more sensitive to the two climate change conditions in organic soils than in the conventionally managed soils. The pressure of chemical application, in particular inorganic fertilisers, herbicides and pesticides, can alter both bacterial and fungal communities (Meena et al., 2020), which may lead to soils more tolerant of environmental conditions. Short-term increases in CO_2 concentration and temperature are therefore unlikely to significantly affect the diversity of fungal or bacterial communities, but the impact on the functionality of microorganisms in soils would require further investigation to identify their impact on host plant establishment.

Bacterial communities in organic soils that were not exposed to the climate conditions had different communities to both the climate conditions. It is well understood that host plants play a major role in the recruitment of microorganisms to the rhizosphere through root exudation (Burns et al., 2015; Guyonnet et al., 2018; Haichar et al., 2008). The shift in bacteria communities was rapid (5 weeks) which could alter the recruitment of bacteria to the rhizosphere if bacteria become unavailable, which may lead to the sub-optimum establishment of plant species (Hayat et al., 2010). Of course, the effect of such microbial recruitment on plant development will depend on functional redundancies in soil microbiome. Fungal populations were more resilient to the climate conditions but communities were different when CO_2 conditions reached 10,000 ppm. The lack of effect on fungal diversity at the lower concentration agrees with previous studies that found long-term CO_2 elevation did not alter fungal diversity (Klamer et al., 2002). We suggest a value between 5,000 ppm and 10,000 ppm CO_2 is a critical threshold for the resilience of fungal populations in organic soils. What the critical value for CO_2 concentration is for fungal resilience and whether soils can reach such a value for a short period of 5 weeks would require further investigation in future works.

Only a small number of individual taxa were affected by the two climate change conditions. In conventional soils a number of OTUs that are believed to have important biological function were less abundant in the elevated CO_2 concentration and increased temperature conditions compared to control soils. *Pseudoxanthomonas* was one example and is described as a biocontrol exhibiting nematostatic function but is also closely related to *Xylella* containing known pathogens, particularly *X. fastidiosa* causing disease in citrus, grape, and olives (Bansal et al., 2021; Hu et al., 2019). *Janthinocacterium* and *Nocardioides* also had lower relative abundance in the climate condition treated soils than the control and both contain species that produce anti-fungal suppression against pathogenic *Fusarium* spp. *Trichoderma evansii* had higher abundance in both the climate conditions than in the control and whilst *T. evansii* is not well described in soil; *Trichoderma* species are well-known biocontrol of plant pathogens in soil (Mukherjee et al., 2012).

In the organic soils, a number of bacterial genera associated with beneficial PGPRs had a higher relative abundance in the control soils than soils exposed to elevated CO_2 and temperature. These genera included *Pseudomonas*, *Variovorax*, *Massilia*, *Streptomyces*, and *Mucilaginibacter*. *Pseudomonas*, well established as containing PGPR species of numerous crops but also some important plant pathogens such as *P. syringae* (Sivasakthi, Usharani and Saranraj, 2014; Xin, Kvitko and He, 2018), was significantly more abundant in control soils than the 5,000 ppm condition. Similarly, *Streptomyces* species were more abundant in the control soils than in the 5,000 ppm condition only and have been described as both beneficial PGPRs, biocontrols, and pathogenic in crops such as potatoes (Li et al., 2019; Suárez-Moreno et al., 2019). As *Streptomyces* and *Pseudomonas* genera were not detected in DESeq2 analysis for the 10,000 ppm vs control model, this suggests they are tolerant to the temperature change but the relative effect of CO_2 concentration on their abundance is unclear and would require further investigation.

Burkholderia genera similarly have been described as both beneficial bacteria as a N-fixing PGPR and as an opportunistic pathogen (Angus et al., 2014) and were more abundant in the 10,000 ppm CO_2 condition than in the control soils. *Burkholderia* could therefore become an emerging pathogen causing disease in organic orchards if conditions become sufficient or inversely a new candidate soil amendment if the strain is beneficial. Identification to species rank would be required to identify whether *Pseudomonas*, *Streptomyces*, and *Burkholderia* are beneficial or pathogenic in the organic soils making them either emerging plant pathogens if climate conditions become sufficient in the case of *Burkholderia* or candidate soil amendments if the species are beneficial to plant growth. This highlights the need for identification to species level where possible when interpreting the cited function of OTUs with different abundance.

Variovorax genera are known PGPRs and often used as a model organism for plant-microbe interactions, with strains such as *V. paradoxus* shown to aid stress tolerance, and disease resistance and improve nutrient availability to the plant for growth (Han et al., 2011; Sun et al., 2018). The genus *Massilia* similarly has been

reported to show *in vitro* attributes of plant growth promotion and successfully colonises cucumber roots and seeds (Ofek, Hadar and Minz, 2012). Both *Variovorax* and *Massilia* had lower relative abundance in the organic soil samples subjected to both climate change conditions than in the control soils. *Mucilaginibacter* similarly exhibits plant growth-promoting traits when isolated from the rhizosphere of cotton (Madhaiyan et al., 2010), and was more abundant in control soils than in the 10,000 ppm condition but not differentially abundant comparing the 5,000 ppm condition to the control. *Mucilaginibacter* is likely more sensitive to the CO_2 concentration than the temperature increase, whereas both *Variovorax* and *Massilia* were sensitive to both in organic soils. This highlights the limited resilience of these beneficial bacterial groups in organic soils to climate change, which needs to be taken into consideration should these microbial groups be considered for use in commercial agriculture as a soil amendment.

These results indicated both pathogenic and beneficial microbial populations may change in bulk soils of both conventional and organic soils when extreme climate change conditions occur. The implications for the host plant are unclear. Climate change conditions (such as elevated CO_2 concentration) may likely occur irregularly and unpredictably, thus attention needs to be paid to both high and low extreme climate events. Future studies should focus on the effect of fluctuating climate perturbations in the natural environment or expose soils to both constant and varying climate conditions for longer periods known to affect microbiome populations (Guo et al., 2018) to better understand which microbial components are affected by climate changes.

This study focused solely on native microorganisms in bulk soils. Soilborne diseases such as ARD can be caused by the build-up of pathogens in the rhizosphere of roots (Mazzola and Manici, 2012). This work examined the effect of just two specific climate change scenarios on native microbial communities in bulk soils from recently grubbed apple orchards, and thus did not focus on the specific aspect of the rhizosphere where pathogens will interact with the roots. Studies have shown that increases in root exudation may be linked to increasing CO_2 (Phillips, Fox, and Six, 2006), thus the effect of changing climate conditions

may be more pronounced in the rhizosphere microbiome. Microbial communities are also influenced by water availability in both soils and plants. A 30% reduction in the soil water holding capacity is sufficient to shift the dominant fungal communities (Mekala and Polepongu, 2019). In this study, an attempt was made to maintain high moisture content but over time there was a slight drying of the soils which may have influenced microbial communities. Future work should aim to maintain moisture levels comparable to those in field conditions or include water availability as a study factor.

In summary, the present results highlighted that microbial communities in bulk soils are most influenced by the crop/soil management system, probably mostly due to the differences in the use of synthetic chemical products in organic and conventional systems. Moreover, the management practice appears to influence bacteria more than fungi. We also noted a weaker but still significant impact of the two simulated climate conditions on bacterial and fungal diversity in organic soils although the levels used are unlikely in nature. Conventionally managed soils seemed to have more similar bacterial and fungal communities when comparing the communities in the two simulated climate conditions with untreated soils. Chemical management seems to have applied selection pressure on the bulk soil microbiome leading to more tolerance to climate effects than organic soil microbiome communities. Several potential beneficial, as well as plant pathogens, may be influenced by climate change factors in both organic and conventional soils. Further studies need to examine the influence of fluctuation in temperature, CO_2 concentration, and water availability to quantify the temporal changes in the soil and rhizosphere microbiome in apple production systems.

6.6 References

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7 General Discussion and Conclusions

Due to the biotic nature of ARD, alternative non-chemical management strategies are important to provide growers with sustainable and effective methods to minimise the severity and economic pressure of ARD. With the loss of volatile chemical products for fumigation of soil due to government legislation, biological products such as brassica seed meal as a biofumigant or anaerobic soil disinfestation have been proposed as effective biological management strategies to prevent/minimise ARD (Mazzola and Manici, 2012; Strauss and Kluepfel, 2015). This work however aimed to identify the efficacy of beneficial single species microorganisms as a pre-plant amendment to improve soil health and therefore minimise the detrimental effects of ARD, which has not previously been studied in detail.

To identify the efficacy of the biological amendments, they were applied in both field and semi-field conditions to identify benefit, if any, on replanted apple tree establishment and on the communities of fungi and bacteria in the rhizosphere. Single species amendments were also compared to applying multiple amendments to one tree in consortium. Additionally, this work looks at the influence of the rootstock genetic relationship to the previously planted rootstock and inter-row planting in the grassy alleyways as a cultural management strategy for ARD. The effect of climate change stress on bulk apple soil communities in the microbiome was also investigated to decipher if changes in CO_2 concentration and temperature increase can alter the microbiome, hence changing the pool of microbes available for recruitment to the rhizosphere. Together the results of these studies aim to provide an integrated non-chemical ARD management strategy that can ultimately be utilized by growers.

Under field conditions, *Pseudomonas fluorescens* amendment was positively correlated with an increase in the rate of girth expansion of Gala trees but not for Braeburn trees. AMF application also led to a marked difference in Braeburn yields. This suggests the efficacy of the amendments in soil is also dependent on the scion variety of the apple and treatments to soil should therefore be tailored for each different variety within orchards. Although at a community level there

was no significant effect of the amendments, each of AMF, brassica seed meal, *Bacillus amyloliquefasciens*, and *Pseudomonas fluorescens* caused a significant change in the abundance of a large number of OTUs. Whereas fumigation and biofumigation treatments look at community-level changes to “sterilize” pathogens from the soil (Mazzola and Manici, 2012), we suggest biological soil amendments act to alter the abundance of individual populations within the overall community. In this way their true benefit cannot be identified using community diversity analysis and a deeper analysis of individual OTU abundance and functional gene differences in soils would provide a better idea of the benefit of the amendments in future work.

The rootstocks potted with a varying consortia of amendments (*Diversispora* sp., *Trichoderma harzianum*, *Bacillus amyloliquefasciens,* and *Pseudomonas fluorescens*) did not have a significant influence on the above-ground establishment of the rootstock. There also did not appear to be any benefit to applying multiple amendments in consortium despite a strong case for the benefit of consortia over individual amendments in the literature (Carneiro et al., 2023). Similarly to the field trial, a number of OTUs had different abundance due to each of the amendments applied. Putting both of these results together it appears biological amendments have the potential to be used as a tool to control the populations within the rhizosphere but not change the diversity of the overall community of bacteria or fungi. However, this effect does not appear to clearly correlate to the above-ground establishment and yield of the tree which in the simplest of practical conclusions would imply that field-scale application of the biological amendments would not be cost-effective for a grower to improve the establishment of their trees.

Planting rootstocks in the inter-row between previous tree stations has been shown as beneficial to replanted apple tree health (Kelderer et al., 2012). Our study confirmed this in a UK cider orchard for a number of important rootstocks in the apple industry. Rootstocks with the most severe ARD symptoms identified in the tree station were those genetically related to the rootstock previously planted in the orchard, despite being the most vigorous rootstocks. Therefore,

this suggests that growers should be planting in the inter-rows for successive generations of apple plantings to obtain better establishment from the trees in the early seasons after replanting. They should also aim to plant rootstocks genetically dissimilar to the previous rootstock planted between generations based on the results of this work. Rhizosphere diversity of bacteria and fungi was also significantly different between the trees planted in the previous tree station and alleyway. It would be interesting for future studies to test the efficacy of the biological amendments used in the field or semi-field studies to see if they affect similar OTUs in diversely different environments and if they would work differently in the previous tree-station and the inter-row.

Short-term exposure to climate change conditions was most influenced by the management system (organic vs conventional) but it was noted that there was a marked (but not significant) impact of the two climate conditions investigated on bacterial and fungal diversity. Elevated CO_2 and temperature have been shown to increase, decrease, or have no effect on microbial biomass and activity (Drigo, Kowalchuk and Van Veen, 2008). It is therefore unsurprising that soil management was found to be a more significant factor affecting microbial community diversity than the climate conditions. The effect on bulk soil was also examined, although in a field system, the influence of the plant will also contribute to the community differences seen. C-limitations would also be alleviated on the plant so more activity from the plant would lead to an increased rate of root exudation which are known to shape rhizosphere communities (Haichar et al., 2008). It is therefore unlikely from the results of the work that substantial shifts in the rhizosphere community would occur due to short-term exposure to elevated CO_2 and temperature alone (for example, during a hot summer in the UK).

The limitations of this work include the survivability of the amendments as the populations were not tracked in real time within the soil. It is therefore not clear as to the longevity of the treatments and therefore if they can benefit the tree. Additionally, in the field study the amendments were applied during January, so the cold conditions may have played a part in the survivability and efficacy of the amendments during that cold period although no evidence of reduced

survivability when applied in winter could be found in the literature so would require further investigation. Optimizing timing and application of the amendments will be critical for the acceptance of single species amendments as a pre-plant amendment for growers. The single species strains in this study were commercially available strains of each species. A better strategy may be to take a step back and research to focus on isolating beneficial microorganisms directly from apple rhizosphere and commercialize and formulate the products with a known and proven beneficial effect.

The studies also only looked at bacterial and fungal communities in the rhizosphere due to time and financial limitations of the project, but as a number of the main pathogenic complex of ARD are oomycetes (Winkelmann et al., 2018), future studies also utilizing oomycete communities would give a more accurate interpretation of ARD within the soils. Analysis of nematode numbers and species would also be beneficial to the research to give the full picture of micro and macrofauna responsible for ARD (Tilston et al., 2020). I would also suggest that future studies look at a number of different compartments additional to the rhizosphere. Analyzing communities and functionality of microorganisms in the bulk soil, rhizosphere, rhizoplane, and endophytes would better highlight the microbes recruited to the rhizosphere and rhizoplane which will interact more closely with the roots. Identification of pathogens within the root as endophytes for example would confirm their ability to infect and colonize roots which would give more credibility to their assignment as an ARD causal agent (Somera and Mazzola, 2022).

From this research, it was observed that planting successive generations of apple trees in the inter-row alleyways alongside planting genetically different rootstocks from the previously planted rootstock can both be effective to increase the establishment of the tree. ARD causal agents have been shown to have low mobility (Balbín-Suárez et al., 2021) so planting in the alleyway provides an easy-to-implement management strategy for ARD without concern over ARD pathogens migrating into the alleyways. This also allows for fruit production to continue while the previous tree stations are treated with a biofumigant or planted

with grass or cover crops to reduce the abundance of ARD pathogens. Although the benefit of single species biological soil amendments appeared limited in the two studies in pot and field, the efficacy of the amendments following biofumigation or in tandem with the inter-row cropping strategy should be tested as these could further increase the establishment of the tree. We, therefore, suggest growers put more emphasis on what they are planting and where it is being planted rather than what they apply to the soil.

The use of biological soil amendments to treat ARD is a challenging prospect, both in the scale of treatment required for multiple hectares of orchards, but also timing application of treatments for optimum survivability and efficacy. Based on the results of these studies, much more work is required before biological soil amendments can be seen as a viable alternative management strategy alone compared to wide-spectrum fumigant chemical sprays. These amendments should be seen as not an alternative, but as one tool that with optimization could be used alongside other cultural and pre-plant treatments to combat ARD.

7.1 References

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8 Suggestions for Future Work

- This study looked at the consequence of applying the amendments on the establishment of the trees on a season to season timescale. However, much of the biology likely occurs in the first days and weeks after application of the amendments. Future studies looking at the survivability and incorporation of the amendments in the rhizosphere within these early days after amendment would give important information on the biological processes in the soil. Survivability and formulation optimisation of the amendments could then be addressed.

- More detailed studies are required to identify the effect of the single species amendments on the OTUs with differential abundance in the soil. Identifying the genetic response in various compartments (bulk soil, rhizosphere, rhizoplane, endophytes, and root) to the application of the amendments could elude to the function of both the amendments in the soil, but also the function of the populations associated with the OTUs with different abundance, to better understand the efficacy of the amendments.

- Planting rootstocks in the alleyway and genetically dissimilar rootstocks have been shown to be effective strategies to minimise ARD severity. It would be interesting to also identify if pre-plant amendment with AMF, plant-growth promoters or biocontrol agents alongside these can further improve the establishment of the trees and improve yields.

- There is limited knowledge on the effect of climate change abiotic stresses (CO_2 concentration, extreme temperatures, and water stress) on apple trees in orchards. Growers would benefit from further studies on the response of the rhizosphere microbiome and tree response to these

stresses in climate change simulations to better inform the decisions and strategies they deploy for ARD management and other soilborne diseases of apple.

APPENDICES

Appendix A – Supplementary Material

A.1 Early Establishment of Apple Trees with Biologically Amended Soils and the Effect on Rhizosphere Microbial Community

Table A.1 List of enzymes and substrates in the API®ZYM strips and positive and negative result expected colour change

No	ENZYMES	SUBSTRATE	pH	RESULT	
				POSITIVE	NEGATIVE
1.	Control			Colourless or colour of the sample if it has an intense coloration	
2.	Alkaline phosphatase	2-naphthyl phosphate	8.5	Violet	Colourless or very pale yellow
3.	Esterase (C 4)	2-naphthyl butyrate	6.5	Violet	
4.	Esterase Lipase (C 8)	2-naphthyl caprylate	7.5	Violet	
5.	Lipase (C 14)	2-naphthyl myristate	7.5	Violet	
6.	Leucine arylamidase	L-leucyl-2-naphthylamide	7.5	Orange	
7.	Valine arylamidase	L-valyl-2-naphthylamide	7.5	Orange	
8.	Cystine arylamidase	L-cystyl-2-naphthylamide	7.5	Orange	
9.	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	8.5	Orange	
10	α-chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide	7.5	Orange	
11.	Acid phosphatase	2-naphthyl phosphate	5.4	Violet	
12.	Naphthol-AS-B1-phosphohydrolase	Naphthol-AS-B1-phosphate	5.4	Blue	
13.	α-galactosidase	6-Br-2-naphthyl-αD-galactopyranoside	5.4	Violet	
14.	β-galactosidase	2-naphthyl-βD-galactopyranoside	5.4	Violet	
15.	β-glucuronidase	Naphthol-AS-B1-βD-glucuronide	5.4	Blue	
16.	α-glucosidase	2-naphthyl-αD-glucopyranoside	5.4	Violet	

17.	β-glucosidase	6-Br-2-naphthyl-βD-glucopyranoside	5.4	Violet
18.	N-acetyl-β-glucosaminidase	1-naphthyl-N-acetyl-βD-glucosaminide	5.4	Brown
19.	α-mannosidase	6-Br-2-naphthyl-αD-mannopyranoside	5.4	Violet
20.	α-fucosidase	2-naphthyl-αL-fucopyranoside	5.4	Violet

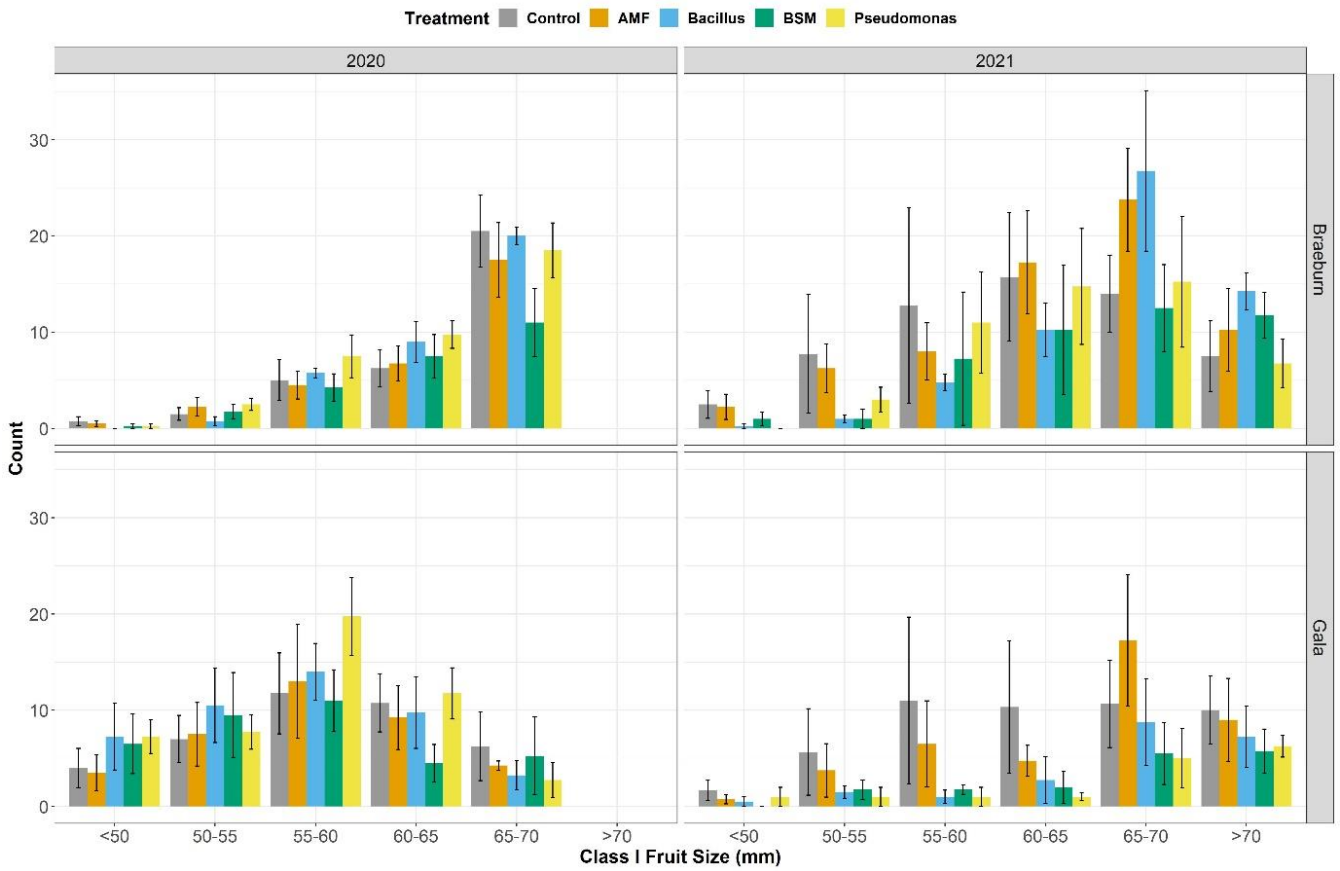

Figure A.1 Size and count of the class I fruit collected from the orchard in the 2020 and 2021 seasons. > 70 measurement was not used in 2020, so 65-70 for 2020 was classed as > 65.

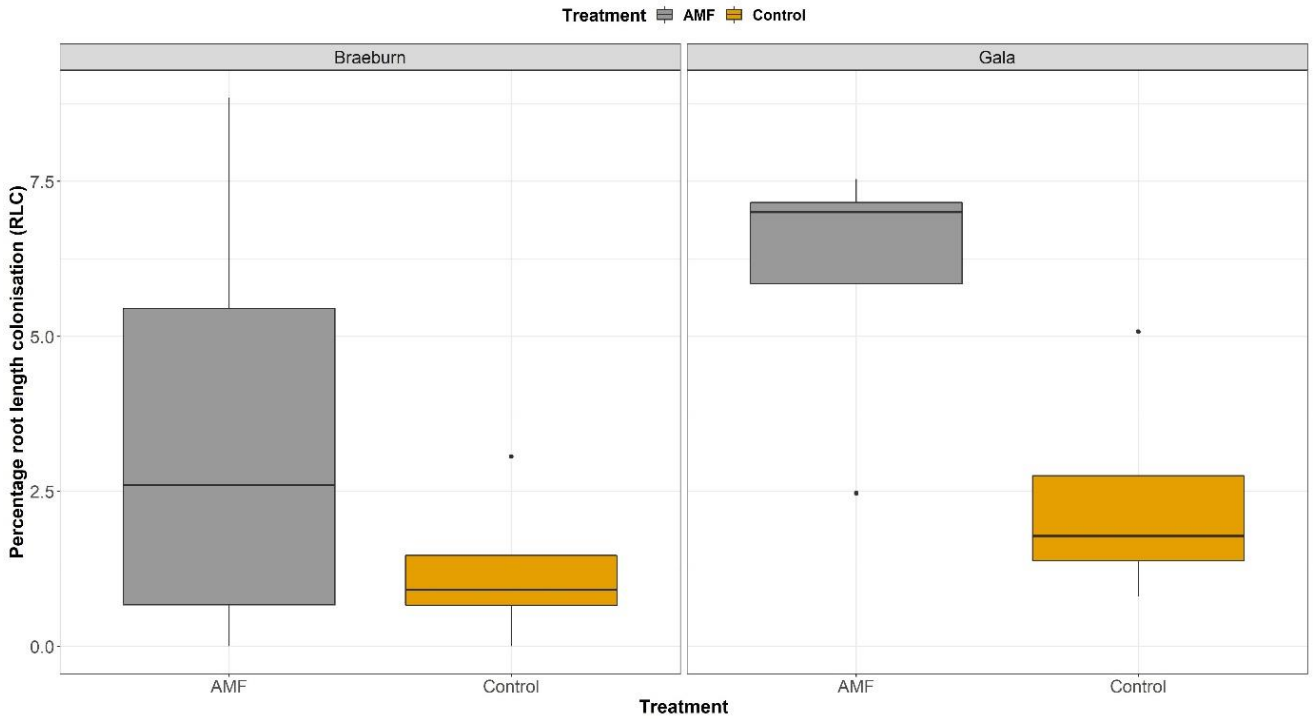

Figure A.2 Root length colonisation of roots inoculated with rootgrow™ AMF consortium mix or unamended control. Logistic regression model significance – Treatment: p = 0.007, Apple Variety: p = 0.058.

A.2 The Effect of Biologically Amending Soils on Early Establishment of M9 Rootstocks and Rhizosphere Microbial Community

Table A.2 List of enzymes and substrates in the API®ZYM strips and positive and negative result expected colour change.

No	ENZYMES	SUBSTRATE	pH	RESULT	
				POSITIVE	NEGATIVE
1.	Control			Colourless or colour of the sample if it has an intense coloration	
2.	Alkaline phosphatase	2-naphthyl phosphate	8.5	Violet	Colourless or very pale yellow
3.	Esterase (C 4)	2-naphthyl butyrate	6.5	Violet	
4.	Esterase Lipase (C 8)	2-naphthyl caprylate	7.5	Violet	
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6.	Leucine arylamidase	L-leucyl-2-naphthylamide	7.5	Orange	
7.	Valine arylamidase	L-valyl-2-naphthylamide	7.5	Orange	
8.	Cystine arylamidase	L-cystyl-2-naphthylamide	7.5	Orange	
9.	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	8.5	Orange	
10	α-chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide	7.5	Orange	
11.	Acid phosphatase	2-naphthyl phosphate	5.4	Violet	
12.	Naphthol-AS-B1-phosphohydrolase	Naphthol-AS-B1-phosphate	5.4	Blue	
13.	α-galactosidase	6-Br-2-naphthyl-αD-galactopyranoside	5.4	Violet	
14.	β-galactosidase	2-naphthyl-βD-galactopyranoside	5.4	Violet	
15.	β-glucuronidase	Naphthol-AS-B1-βD-glucuronide	5.4	Blue	
16.	α-glucosidase	2-naphthyl-αD-glucopyranoside	5.4	Violet	
17.	β-glucosidase	6-Br-2-naphthyl-βD-glucopyranoside	5.4	Violet	
18.	N-acetyl-β-glucosaminidase	1-naphthyl-N-acetyl-βD-glucosaminide	5.4	Brown	
19.	α-mannosidase	6-Br-2-naphthyl-αD-mannopyranoside	5.4	Violet	
20.	α-fucosidase	2-naphthyl-αL-fucopyranoside	5.4	Violet	

A.3 Inter-Row Cropping and Rootstock Genotype Selection as a Management Strategy for Apple Replant Disease in a UK Cider Orchard.

Table A.3 Summary of the bacterial and fungal representative operational taxonomic units (OTUs) after quality filtering and removal of low counts. OTUs were generated using 97% sequence similarity.

	Total counts	**Total OTUs**	**Number of OTU per sample**		**Number of reads per sample**		**Number of reads per OTU**	
			Min	Max	Min	Max	Min	Max
Bacteria	1549832	10883	1807	2445	25305	45970	2	2176
Fungi	4730414	4802	106	4474	2801	924636	2	49624

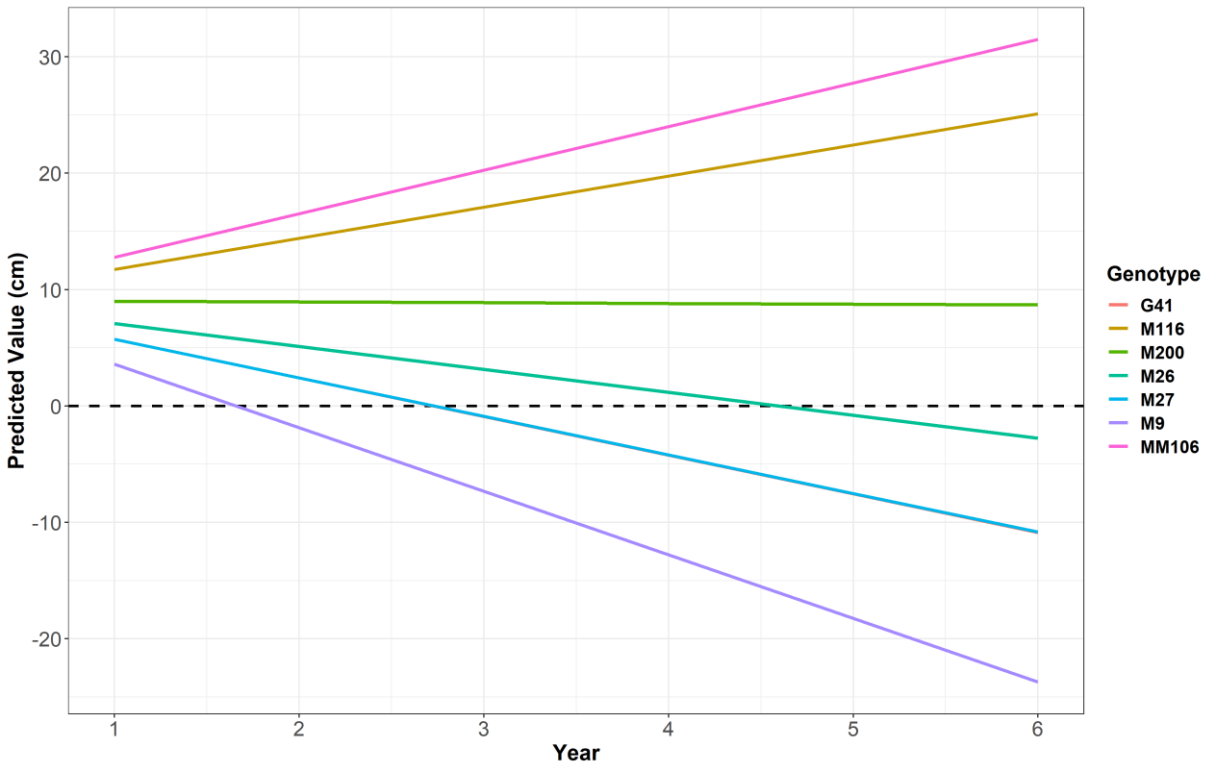

Figure A.3 Predicted values from a randomly mixed model with a common intercept of temporal height difference between the alleyway and the previous tree station. Genotype effect was fixed in the model and tree pair location treated as random. Slope trend was calculated from best linear unbiased prediction (BLUP) values. A value above zero indicates a replant effect on the tree planted in the tree station and a value equal to or below zero indicates no replant effect on that genotype.

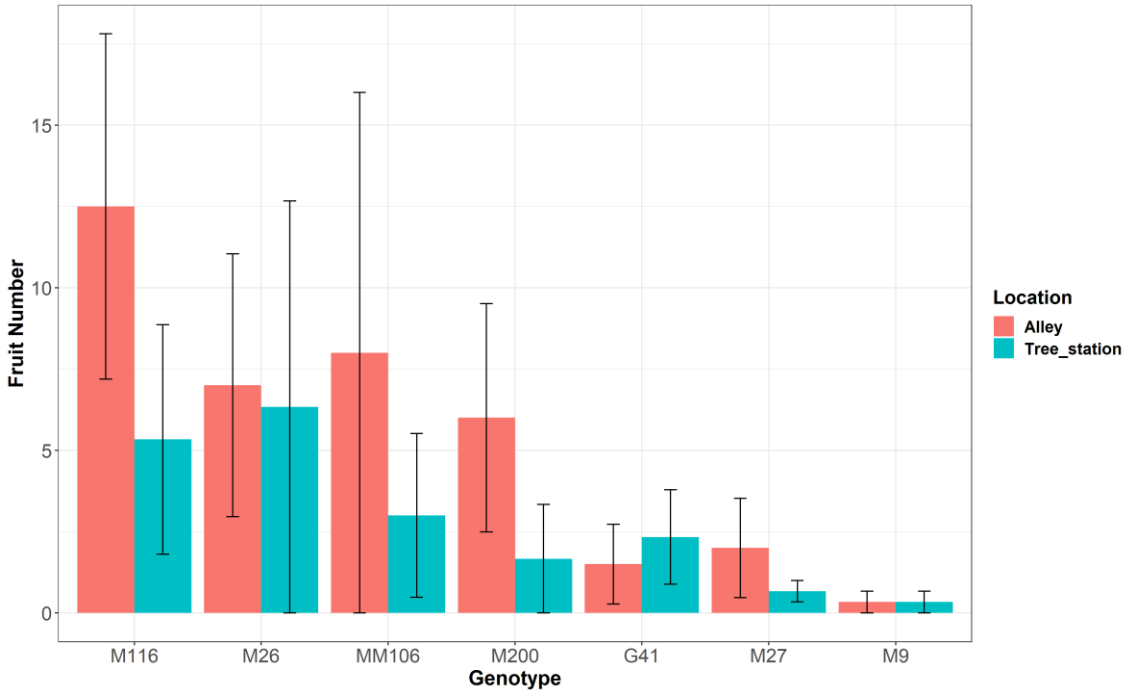

Figure A.4 Mean number of fruit counted on each tree in 2021. The colour of the bar indicates the location in which the tree was planted. Red = Alleyway between previous rows, Blue = Previous tree station.

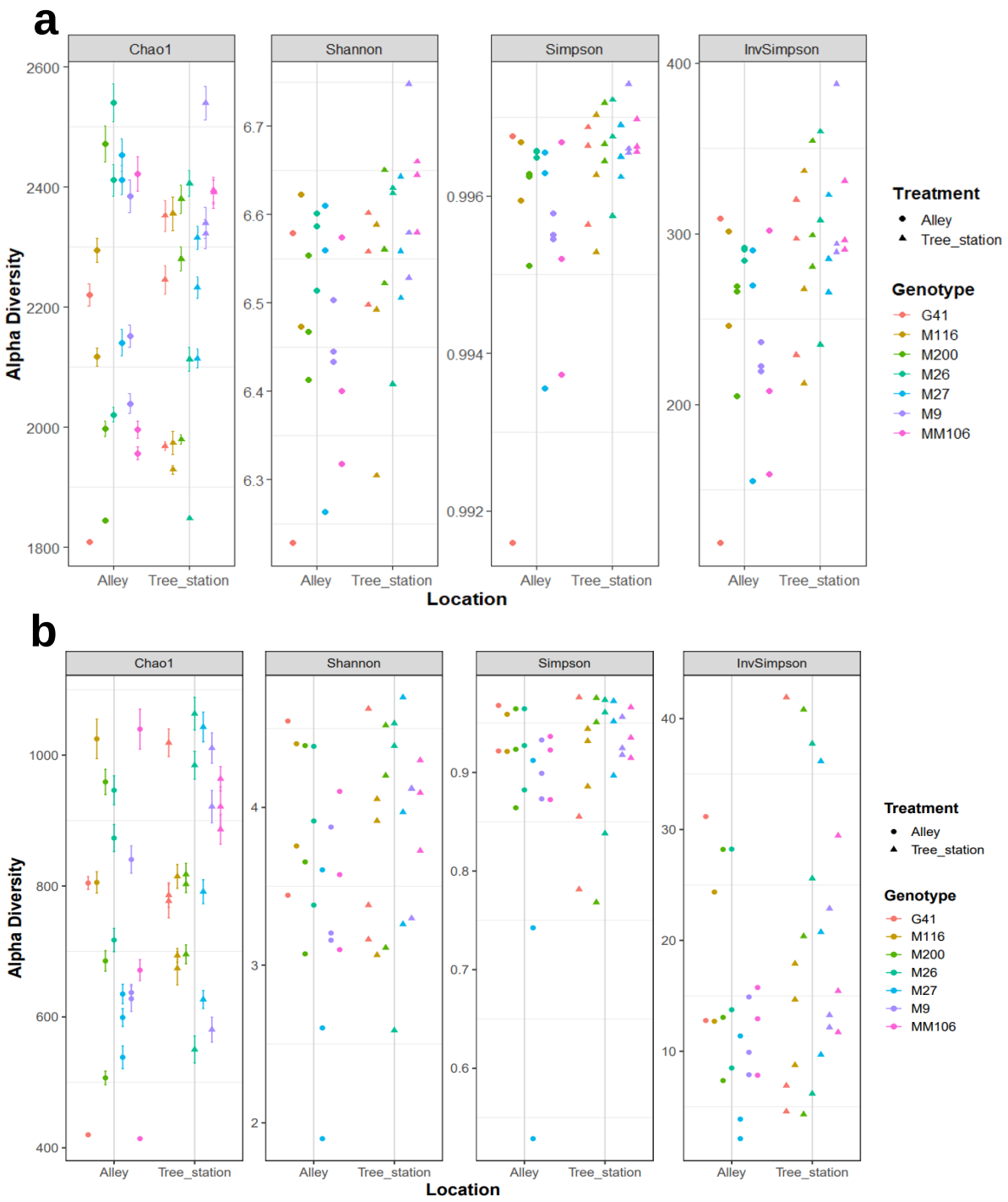

Figure A.5 Alpha (α) diversity measures, Chao1, Shannon, Simpson and InvSimpson for (a) bacteria and (b) fungi. The x-axis indicated the location the trees were replanted in, the previous tree station (▲) or the corresponding alleyway position between the rows (●). The colour indicates the rootstock genotype identity.

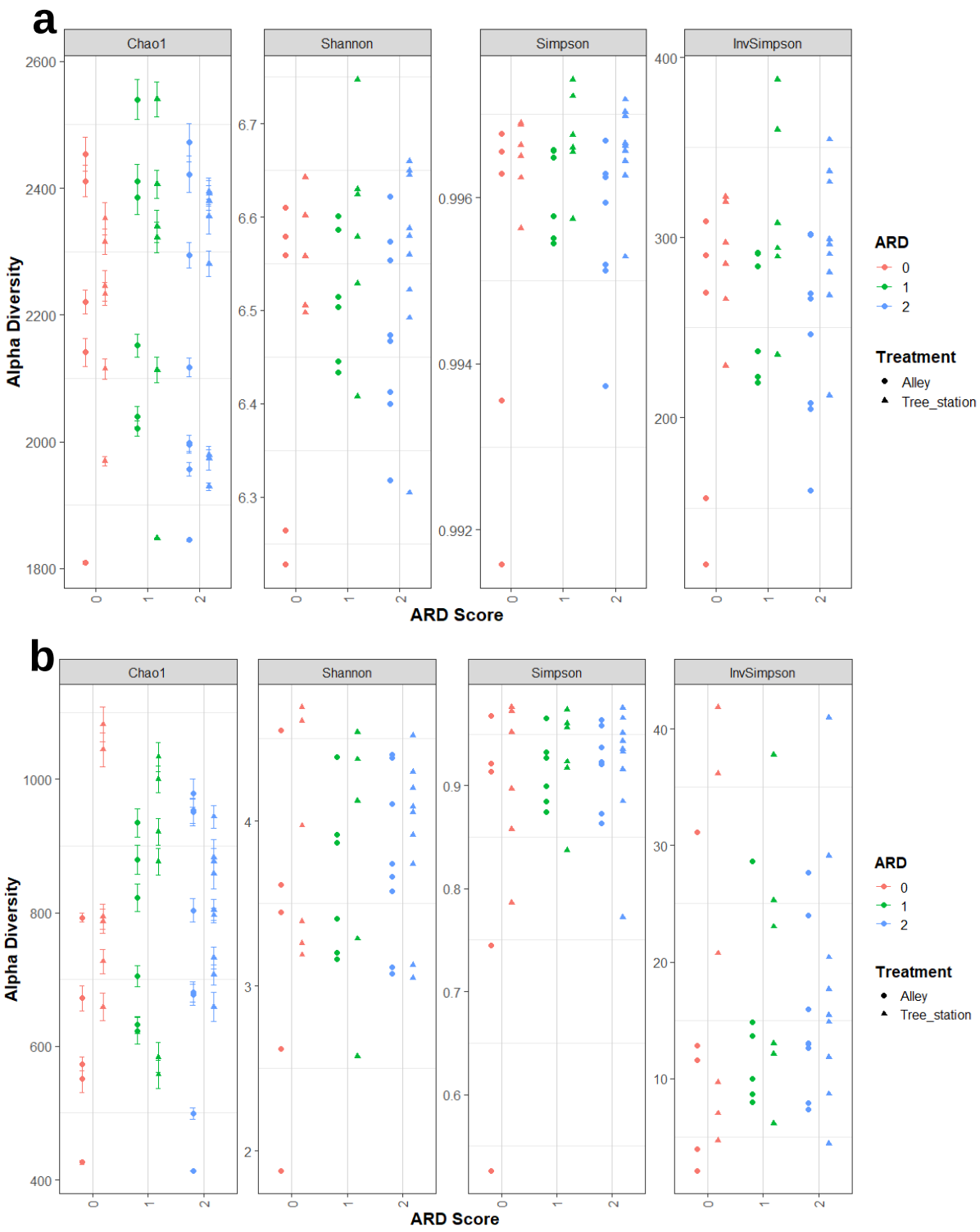

Figure A.6 Alpha (α) diversity measures, Chao1, Shannon, Simpson and InvSimpson for (a) bacteria and (b) fungi. The x-axis indicates the ARD score associated with each genotype. The shape indicates the planting location of the tree, the previous tree station (▲) or the corresponding alleyway position between the rows (●).

A.4 Effect on microbial communities in apple orchard soil when exposed short-term to climate change abiotic factors and different orchard management practices

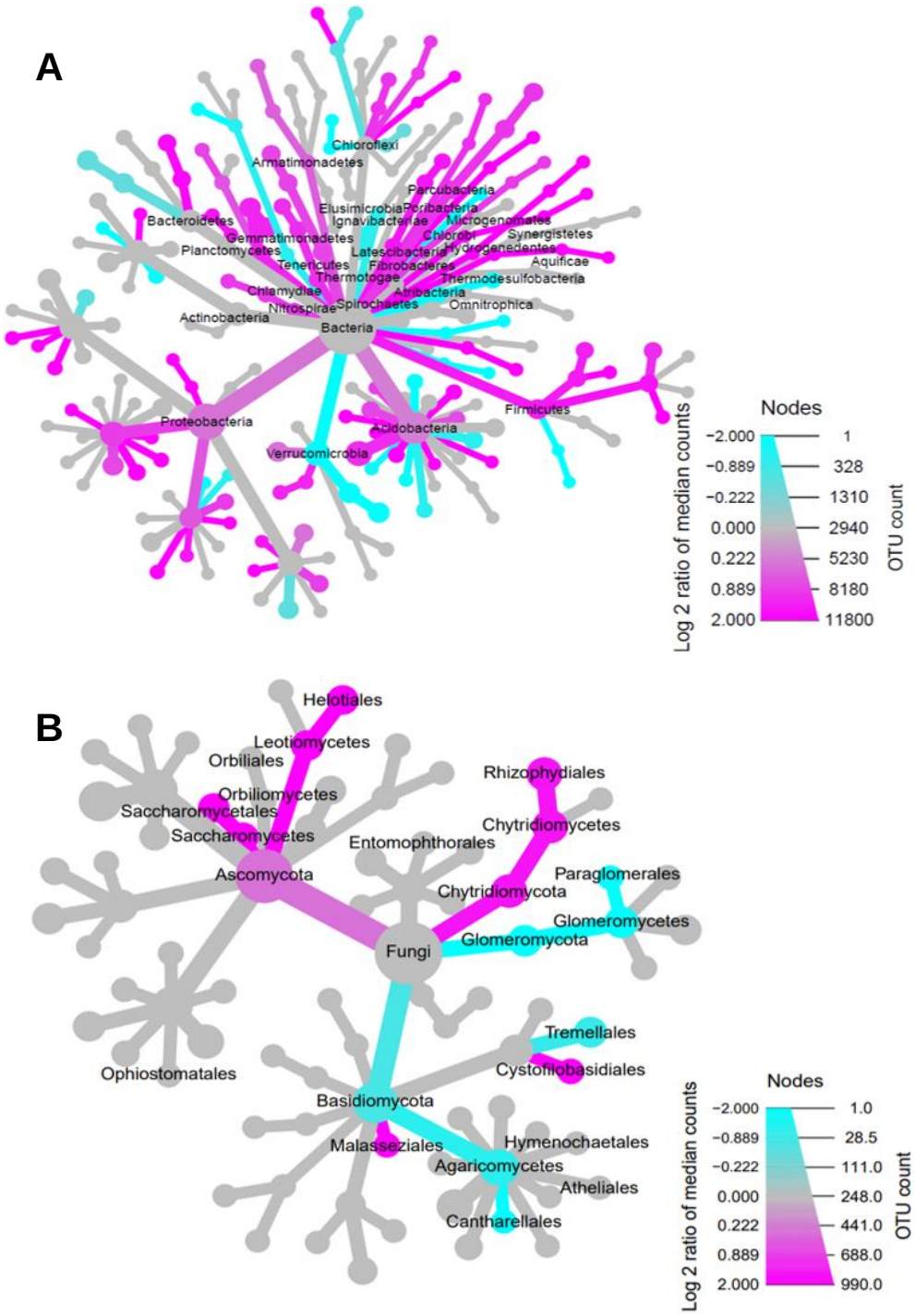

Figure A.7 Bacterial (A) and fungal (B) taxon with differential relative abundance between conventionally and organically managed orchards filtered to the rank order. Bacterial tree nodes are labelled to the rank phylum. Fungal tree nodes are labelled based on taxa with a differential abundance at Wilcox P value < 0.05. Increased abundance in the conventionally managed orchard is coloured in magenta and the organic orchard is coloured in cyan. The size of the node at each taxon rank indicates the relative OTU counts associated with that rank.

Appendix B – Poster Awards

“The Use of Soil Amendments to Improve Apple Tree Health”

C. Cook, L. Robinson-Boyer, N. Magan, X. Xu

AHDB Soft Fruit Technical Day

NIAB, East Malling, Kent, 2019

Best Poster Prize – Runner Up

“The Use of Soil Amendment to Improve Apple Tree Health”

C. Cook, L. Robinson-Boyer, N. Magan, X. Xu

AHDB Crops PhD Student Conference

Online, 2021

Best Poster Prize – Winner

The Use of Soil Amendments to Improve Apple Tree Health

Chris J. Cook[1], Dr Louisa Robinson-Boyer[1], Prof Naresh Magan[2], Prof Xiangming Xu[1]

[1]*NIAB-East Malling Research, East Malling, Kent, ME19 6BJ, U.K.* [2]*Cranfield University, Cranfield, Bedford MK43 0AL, U.K.* Email: Chris.Cook@emr.ac.uk

Figure 1: ARD Tree Symptoms

Apple Replant Disease - Problem

The phenomenon of previously bountiful perennial fruit orchards producing unsatisfactory growth and yield. "Replant disease", has become an increasing problem as it becomes more difficult to find virgin land to establish new orchard[1].

Apple Replant Disease (ARD) symptoms **(Fig. 1)** include: stunted growth, discolouration of apple skin, reduced yield, reduced fruit size/weight, reduction in root vigour and size and potentially tree death[2].

Symptoms can occur as early as 1 year after replant. Profitability of an orchard showing ARD symptoms can be reduced by up to 50%[3].

Suspect Causal Agents

- *Rhizoctonia*
- *Phytophthora*
- *Cylindrocarpon*
- *Pythium*
- *Pratylenchus penetrans*

Long Term Soil Amendment

Background

- Chemical fumigation is conventional ARD treatment but is expensive and harmful to the environment.
- Brassica Seed Meal is standard non-chemical treatment causing anaerobic disinfestation of soil.
- Candidate beneficial microorganisms may be able to promote growth and overcome ARD onset.

Methods

- Inoculate plot with soil amendment (non-chemical) at time of planting.
- Measure changes in growth (height, girth and yield).
- Compare statistical differences between treatments.

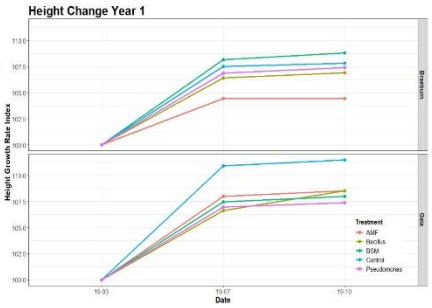

Figure 2: Height change timeseries after year 1

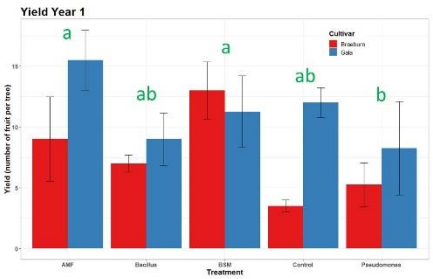

Figure 3: Yield in year 1. Significant lettering compares linear models of treatments. Same letter indicates no statistical difference

Results

- There was no statistical differences in height or girth change in year 1.
- AMF has slower height growth in the braeburn cultivar and control is outperforming all inoculations in the Gala cultivar (Figure 2).
- The yield for the Pseudomonas amendment is significantly lower than the AMF and Brassica Seed Meal (BSM) treatments (Figure 3).
- No treatment was statistically different to the control when modelling yield.

Chemical vs Biological Cost

Chemical Fumigation: **~£8,000 + VAT per hectare**

Biological Treatment: **£1,200-£2,500 per hectare**

£5,000-£7,000 saving per hectare!

Future Work

- Continue long term growth trial for two more growing seasons.
- Compare microbiome population and functionality of amended soils.
- Test resilience of a consortium of beneficial microorganisms applied to soil.
- Study soil microbial dynamics under climate change scenarios: combinations of elevated CO_2, temperature increase, and water potential stress.

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3. van Schoor, L., Denman, S., & Cook, N. C. (2009). Characterisation of apple replant disease under South African conditions and potential biological management strategies. Scientia Horticulturae.

The Use of Soil Amendments to Improve Apple Tree Health

Chris J. Cook[1], Dr Louisa Robinson-Boyer[1], Prof Naresh Magan[2], Prof Xiangming Xu[1]

[1]*NIAB-East Malling Research, East Malling, Kent, ME19 6BJ, U.K.* [2]*Cranfield University, Cranfield, Bedford MK43 0AL, U.K.* Email: chris.cook@niab.com

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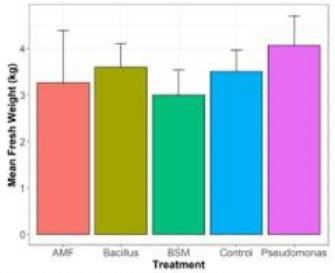

Figure 2: Gala fresh weight in second year. BSM = Brassica seed meal, AMF = Arbuscular Mycorrhizal Fungi, Control = No amendment

Suspect Causal Agents

Rhizoctonia

Phytophthora

Cylindrocarpon

Pythium

Pratylenchus penetrans

Results

- *Pseudomonas* sp. amendment had the highest mean gala fruit number and mean gala fresh weight per tree **(Fig. 2 & 3)**.
- *Bacillus* sp. was the only other amendment with higher mean fresh weight and fruit number.
- Arbuscular mycorrhizal fungi (AMF) and brassica seed meal (BSM) amendments showed lower fruit number and fresh weight than the control trees.
- AMF had the highest mean fruit number in year 1 but lowest in year 2 **(Fig. 3)**.

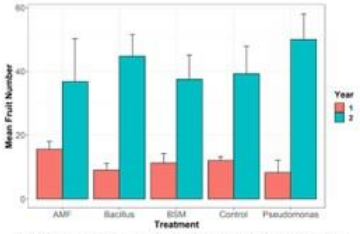

Figure 3: Mean number of marketable gala fruit >50mm per tree. . BSM = Brassica seed meal, AMF = Arbuscular Mycorrhizal Fungi, Control = No amendment

Future Work

- Continue long term growth trial for one final growing season for Gala and Braeburn cultivars.
- Compare microbiome population and functionality of amended soils.
- Test resilience of a consortium of beneficial microorganisms applied to soil.
- Study soil microbial dynamics under climate change scenarios: combinations of elevated CO_2, temperature increase, and water potential stress.

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