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Exploiting mycorrhizal selection of beneficial rhizosphere bacteria from the soil microbiome

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ABSTRACT

Soil health is dependent on its diverse communities of microbes. Many of these microorganisms enhance plant growth and enrich the soil. However, the interactions between communities of beneficial microbes remain unclear. Arbuscular mycorrhizal fungi (AMF) are responsible for the most prolific beneficial plant-fungal interaction. However, their influence on the diverse range of plant growth promoting rhizobacteria (PGPR) that also associate with plant roots is yet to be fully elucidated. This research investigates the tripartite interactions between host plant-AMF-PGPR using next-generation sequencing and culture-dependent methodology to define the effect of AMF inoculation on the taxonomic and functional characteristics of the bacterial assemblage of the root microbiome of white clover (*Trifolium repens*). Soil from two land use types (grassland and bare fallow) amended with fertiliser and/or AMF inoculants are used to describe the effect of these management components on the function of beneficial microbes in cropping systems.

The AMF *Funneliformis geosporum* affected the taxonomic composition of bacteria in the rhizosphere but not the rhizoplane. However, soil type and fertiliser were more influential determinants of bacterial taxa and function. Using split-root microcosm experiments with root exclusion meshes, the dispersal of bacteria was observed in the absence of AMF hyphae. The approaches were combined to show that root microbiome establishment is independent of AMF hyphal facilitation or selection of beneficial bacterial traits or taxa.

In vitro predictive measures were used to design a putative Phosphorus solubilising consortium comprised of synergistic P-solubilising rhizobacteria and AMF. Plant health parameters were influenced by the addition of Ca_3PO_4 but were unaffected by any microbial combination. The performance of a putative bioinoculant is dependent on many external factors which can negatively impact the intended function.

This work is an important indicator of the complexity of the soil microbiome and demonstrates the profound influence of agronomic inputs on microbial function.

Keywords:

Plant-growth promoting rhizobacteria, arbuscular mycorrhizal fungi, *Trifolium repens*, amplicon sequencing, *in vitro* culture, microbial interactions, microbial bioinoculants, plant nutrient content, Phosphorus solubilising microorganisms, sustainable agriculture.

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AMF	Arbuscular mycorrhizal fungi
ASV	Amplicon sequence variant
CAP	Canonical analysis of principal coordinates
CFU	Colony forming units
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organisation of the United Nations
GFP	Green fluorescent protein
GLM	Generalised linear model
HDTMA	Hexadecyltrimethylammonium bromide
NGS	Next-generation sequencing
NPK	Nitrogen, phosphorus, potassium (fertiliser)
OD	Optical density
OTU	Operational taxonomic unit
PCoA	Principal coordinates analysis
(q)PCR	(quantitative) Polymerase chain reaction
PGP(R)	Plant growth promoting (rhizobacteria)
PPM	Parts per million
PSB	Phosphorus solubilising bacteria
PSM	Phosphorus solubilising microorganisms
T-RFLP	Terminal restriction fragment length polymorphism

1 INTRODUCTION

1.1 General introduction

This review will introduce and examine the interactions between arbuscular mycorrhizal fungi (AMF) and plant growth promoting rhizobacteria (PGPR) in agricultural systems. The purpose of this review is to present current knowledge of these interactions and discuss the methods available for their study. The aim is to highlight the research gaps to which this project will contribute, in the context of sustainable agriculture and improved crop and soil health.

1.1.1 Global population and agriculture

More food must now be produced than ever before to satisfy the needs of a rapidly growing world population. However, with the global goal of reducing the impact of agriculture to mitigate climate change, this must be done as sustainably as possible – producing more food in the same amount of space with less environmentally damaging inputs. Food production has increased exponentially since the green revolution, which improved agricultural yield through creation of genetically advantageous crop varieties and the input of synthetic fertilisers and other agrochemicals (Khush 2001). Crop production increased three-fold, yet land use only increased by a third (Wik, Pingali et al. 2008). However, population has continued to grow at such a rate that further increases in food production are required.

In the next 30 years, there will be 9.6 billion people on earth (Gerland, Raftery et al. 2014). With an expanding population comes increased urbanisation and land use change for anthropologic purposes, such as housing and industry. Population growth is positively correlated with agricultural greenhouse gas emissions (van Beek, Meerburg et al. 2010). The FAO estimates an increase in demand for agricultural products of 60% by 2050, meaning modern farming requires a sustainable green revolution to more than double its current output (FAO 2017).

These production systems are complex and are intimately connected with the surrounding environments. To ensure the continued and sustained levels of output from arable systems, practices must be managed with long term goals in

mind. This includes, but is not limited to: reducing harmful inputs, such as chemical fertiliser; reducing the impact on farmland ecosystems, such as the damage caused by the blanket use of pesticides; maintaining soil health and conserving water usage (Papendick and Parr 1992, Khush 2001, Wezel, Casagrande et al. 2014). Shifts in consumer attitudes must also be cultivated, for example, encouraging transition towards a majority plant-based diet, and reduction in food wastage (Porter and Reay 2016). The transport of plant-based protein is more efficient both in terms of greenhouse gas emissions and total energy expenditure than delivering the same amount of animal protein (Snyder, Bruulsema et al. 2009, González, Frostell et al. 2011).

1.1.2 Agriculture contributes to climate change

Agriculture is the highest contributor of greenhouse gases in the entire food production system. Food production accounts for almost a third of global greenhouse gas production, and 80% of the greenhouse gases emitted during all food production come from direct and indirect agricultural practice (Vermeulen, Campbell et al. 2012). Freshwater inputs into farming account for 75% of the global total usage (Wallace 2000), and agriculture causes devastating pollution of fresh-water systems (Moss 2008, Wen, Schoups et al. 2017). Nitrogen fertiliser use has increased atmospheric nitrous oxide by 20% (Park, Croteau et al. 2012) and its formulation produces 100 times more methane than originally claimed: 29 Gg CH_4/yr (Zhou, Passow et al. 2019). The total cost of N fertiliser loss to the environment at each stage of production to usage in the US is over $5 billion (Good and Beatty 2011).

With an increase in food production needed, more land is required for agronomic pursuits. Land clearing to make room for agriculture releases stores of CO_2 into the atmosphere from soil and plant life (Tinker, Ingram et al. 1996). Deforestation leads to changes in rainfall, water availability and an increase in global temperatures and climate variation (Lawrence and Vandecar 2015). As such, current agricultural practices must change dramatically in order to be sustainable.

1.1.3 Climate change will affect agriculture

Agriculture and climate change are inextricably linked. As agriculture augments the rate of climate change, the effects of a rising global temperature and extreme weather events will bring many challenges to crop growth and food production. Environmental perturbations will become more frequent. For example, changes to hydrological processes mean that flooding and drought will occur more often, and these can have devastating impacts on crops and sometimes destroy them completely (Porter and Semenov 2005). Increases in temperature will affect yields, with more frequent heat waves damaging crops and inhibiting growth and also widen regions in which pests can operate and damage crops (Porter, Xie et al. 2014, Harrison, Cullen et al. 2016). Temperature shifts may also render some regions unsuitable for farming, and rising sea levels could destroy farmable land in some areas. Some staple crops are only produced in specific regions so extreme weather events here are likely to have far reaching implications for global food production (Bailey, West Jr et al. 2015). Higher CO_2 levels will have varied effects in different regions and depend significantly on crop type. Other greenhouse gases and air pollutants negatively affect crop growth; both tropospheric and global ozone have been predicted to significantly reduce yields (Booker, Prior et al. 2005, IPOC 2007).

1.2 Overview of soil microbes

Soil systems are vital to provide optimum nourishment and a suitable growth substrate for our crops, and to perform the crucial ecosystem service of sequestering carbon (Haygarth and Ritz 2009). An integral part of functioning soil systems are their diverse microbial communities, which make up a large proportion of the soil biomass (Singh, Trivedi et al. 2020). Their intensive activity involves cycling nitrogen, sequestering carbon, solubilising phosphorus and filtering heavy metal contaminants. All of these are essential services for efficient agriculture, and the microorganisms responsible have a direct impact on crop productivity (Dilnashin, Birla et al. 2020).

The soil microbiome describes all the microbial constituents and their respective activities and relationships within the soil. This includes fungi, bacteria, archaea,

protists, viruses, and nematodes. More recently it has been updated to include mobile genetic elements, plasmids and dead matter, so called “Relic DNA”, and expanded to include microbiome functions and interactions within the soil (Berg, Rybakova et al. 2020, Jansson and Hofmockel 2020). Soil microbes are adapted to live in the heterogenous habit that soil provides: pockets of air, water, roots, dense patches of nutrients and resource scarce expanses. New agricultural practices and climate change have affected the microbiome and consequently the potential benefits it can offer to plants (Jansson and Hofmockel 2020). However, with recent advances in next-generation sequencing technology, more of these losses can be identified, and their potential roles in sustainable agriculture and augmented crop growth can be described (Barea 2014). The essential services provided by the soil microbiome are vital to ecosystem function (Barea, Werner et al. 2005).

Plant roots are themselves a dynamic microcosm with a bespoke microbial community, influenced by root exudates and carbon (Brimecombe, De Leij et al. 2000). Often, these intimate relationships have evolved to be mutually beneficial and are tightly regulated (Chaparro, Sheflin et al. 2012). This happens within the rhizosphere – the interface between plant root (including the root endosphere) and soil (Hiltner 1904, Brink 2016), and more directly in the rhizoplane, which is defined as the area of soil immediately proximate to the root surface (McNear Jr 2013). Root microbial community composition is strongly influenced by plant host: root structure, plant development stage and cultivar (Cavaglieri, Orlando et al. 2009, Szoboszlay, Lambers et al. 2015). Rhizosphere activity is influenced by edaphic factors such as pH, soil carbon, temperature, aeration and water availability (Brimecombe, De Leij et al. 2000, Jones, Nguyen et al. 2009).

Microbial interactions with the plant can be beneficial, pathogenic, or benign (Bulgarelli, Schlaeppi et al. 2013). Key beneficial plant-microbe relationships include the legume-*Rhizobium* symbiosis and the relationships between roots and mycorrhizal fungi (Oldroyd 2013). These relationships have been deemed as intrinsic for modern food production and have the potential to be exploited to allow agriculture to become more sustainable (Barea, Werner et al. 2005). The

importance of the plant-associated microbial community cannot be understated, and is pivotal for optimal plant (and soil) health (Bulgarelli, Schlaeppi et al. 2013). The plant holobiont refers to the host plant and all its symbiotic microbial associates, and importantly their genetic information that contributes to evolutionary success (Vandenkoornhuyse, Quaiser et al. 2015, Lyu, Zajonc et al. 2021).

1.2.1 Fungi

Fungal community structure on plant roots is affected by factors such as cropping regime, habitat, root architecture, lifestyle (generalist or specialist) and growth stage of the host plant (Gomes, Fagbola et al. 2003, Qin, Yeboah et al. 2017, Hugoni, Luis et al. 2018, Zhang, Wang et al. 2020). These are highly diverse and dynamic communities with varied roles within the soil. These roles may be plant beneficial (Shoresh, Harman et al. 2010, Almario, Jeena et al. 2017, Ghorbanpour, Omidvari et al. 2018) or provide ecosystem services such as nutrient cycling (Burke, Weintraub et al. 2011), protection against nutrient leaching (de Vries, Thébault et al. 2013), metal decontamination (Deng, Cao et al. 2011) and improving water holding capacity (Kumar, Choudhary et al. 2016). These fungal constituents can also be pathogenic, such as the take-all causing fungus *Gaeumannomyces tritici* and *Fusarium oxysporum*. The soil-borne root pathogen *G. tritici* has been shown to build up in the soil following continuous wheat rotations. This build up is diminished when the cultivar is rotated, demonstrating the delicate balance of host plant genotype and fungal community regulation (McMillan, Canning et al. 2018).

1.2.1.1 Mycorrhizal fungi

Many valuable ecological services in the soil are carried out by mycorrhizal fungi, with mycorrhizal root colonisation conferring fitness advantages and increases chances of survival by priming a plant to be better adapted to its environment (Remy, Taylor et al. 1994). The hyphae allow fungi to adapt to and exploit the heterogeneous nature of soil and its aggregates. The formation of mycorrhizal mutualistic interactions occurs across a range of fungal taxa; 6000 species form mycorrhizal associations, making it the most common mutualism in the world

(Bonfante 2003). This partnership appears to have evolved alongside plants adapting to a terrestrial lifestyle; fossil evidence shows mycorrhizal structures in the Early Devonian period (Remy, Taylor et al. 1994). Mycorrhizal fungi exist mainly as spores and filamentous hyphae which can connect many plant root systems and explore vast volumes of soil through mycelial networks (Tiwari and Adholeya 2002).

Mycorrhizal fungi can be classified into two main groups: ectomycorrhizal and endomycorrhizal (Figure 1.1). Ectomycorrhizae form extracellular hyphal relationships with the plant root, with typical hosts being trees and shrubs. They have been shown to induce changes in root morphogenesis, with only subtle changes to the root cellular components (Bonfante 2001). Endomycorrhizae characteristically penetrate the interior of the plant root and symbiosis is formed intracellularly. The endomycorrhizae include arbuscular mycorrhizal fungi, some fine endophytes, ericoid mycorrhiza and orchid mycorrhiza (Bonfante and Anca 2009). Both endo and ectomycorrhizae share similarities in their morphology in the soil, existing as spores, hyphae and sometimes rhizomorphs.

Arbuscular mycorrhizal fungi (AMF) are purported to be a key contributor to sustainably enhancing crop production (Lone, Shuab et al. 2017) with 80 – 90% land plant species are capable of forming a mutualism with AMF (Bonfante and Genre 2010). Notably however, plants of the family *Brassicaceae* are not colonised by AMF, along with *Amaranthaceae*, *Caryophyllaceae*, *Chenopodiaceae*, *Cyperaceae*, *Juncaceae*, and *Urticaceae* (Posta and Duc 2020). The phylum Glomeromycota has coevolved with plants for 400 million years or more (Bonfante and Genre 2008). AMF lack carbon synthesis genes and therefore rely on their plant host to provide sugars from photosynthesis, and lipids. In return they supply nutrients, primarily P and K, water, and disease resistance (Luginbuehl, Menard et al. 2017). The defining feature of AMF is the arbuscules, which are the site of exchange, formed inside the cells of the plant root cortex and are “tree-like”; highly branched structures with large surface areas (Luginbuehl and Oldroyd 2017).

1.2.2 Plant growth promoting rhizobacteria

The soil is inhabited by an enormous diversity of bacteria. Plant-growth promoting rhizobacteria (PGPR) are bacteria with potential plant-beneficial activity that inhabit the rhizosphere, rhizoplane or the root. In exchange, they receive fixed carbon from the plant. PGPR perform many functions that improve plant health

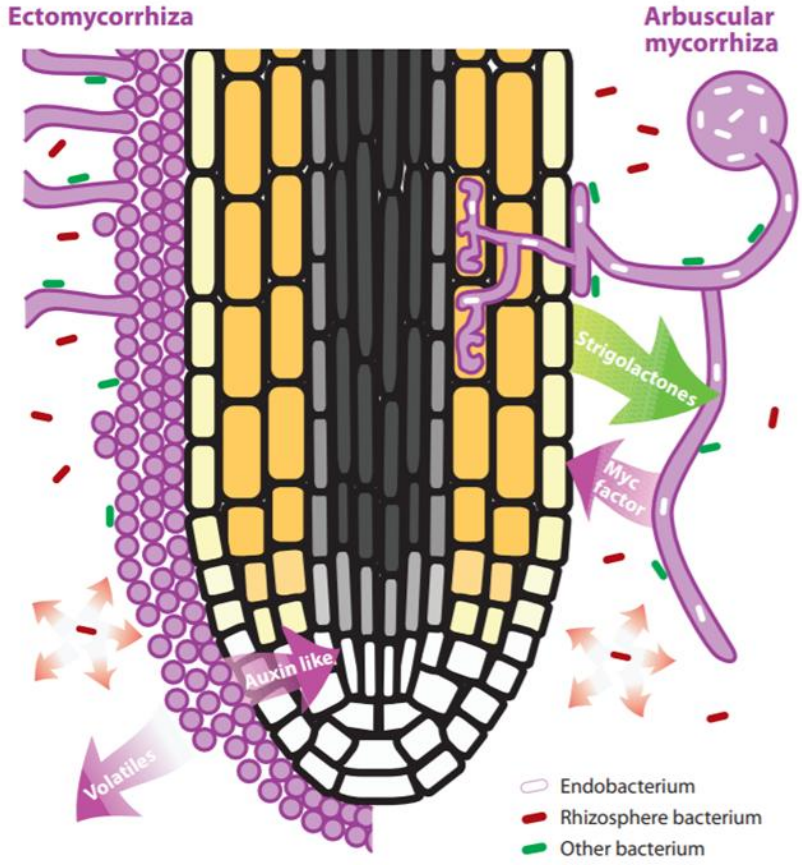


Figure 1.1. Schematic diagram of AMF and ectomycorrhizal colonisation of a plant root, including locations of endobacteria, rhizosphere bacteria and other bacteria colonising the mycorrhizal hyphae. Mechanisms of recruitment and initialising colonisation are included; plants requiring AMF partners secrete strigolactones, which stimulate Myc factor release from AMF and subsequent colonisation. Volatiles and Auxin-like factors are released from ectomycorrhizae and are perceived by the respective partners. From Bonfante and Anca (2009), copyright and permission notice in Appendix (A1).

and mitigate biotic and abiotic stresses (Hayat, Ahmed et al. 2012). Some of the factors that influence rhizosphere community composition are host plant species, root exudates, soil type and plant nutritional status (Marschner, Crowley et al. 2004, Li, Rui et al. 2014) with communities being root-zone specific (Marschner, Crowley et al. 2001). The best-known beneficial plant-bacterial interaction is the legume-*Rhizobium* symbiosis; rhizobia colonise the host plant and form

specialised organs called nodules, where they fix atmospheric nitrogen in exchange for carbon from photosynthesis (Oldroyd, Murray et al. 2011). Current work is aiming to engineer the ability to form this symbiotic partnership into non-legumes such as cereal crops as a solution to excessive nitrogen fertiliser use (Charpentier and Oldroyd 2010).

Enhanced plant nutrient status is accomplished by PGPR action via the following processes: biological atmospheric nitrogen fixation, directly increasing nutrient availability, inducing changes in root architecture that increase area available for nutrient uptake, ameliorating other beneficial host-microbe mutualisms, or a combination of the aforementioned (Vessey 2003). The legume-*Rhizobium* symbiosis provides host plants with fixed nitrogen in the form of ammonia in exchange for carbon from photosynthesis, taking place in a specialised organ, the nodule, stimulated to form by bacterial colonisation (Lindström and Mousavi 2020). However, it is not just rhizobia that perform this function for plants. Associative bacteria, although they do not form nodules, provide fixed nitrogen for a host (Baset, Shamsuddin et al. 2010). Soil bacteria with nutrient liberating activity have been reported for many of the essential nutrients required for plant growth: nitrogen, phosphorus (Masters-Clark, Shone et al. 2020), potassium (Khanghahi, Pirdashti et al. 2018), iron (Sharma, Shankhdhar et al. 2013) and zinc (Goteti, Emmanuel et al. 2013).

Abiotic stresses, such as drought, salt, heavy metal contamination and extreme temperatures, cause widespread crop damage and loss. PGPR have been reported to show promise in mitigating the damage of these detrimental edaphic factors on plant health. PGPR can regulate plant responses to abiotic stresses by regulating phytohormones and antioxidants, or by the production of volatile organic compounds (Kang, Khan et al. 2014, Liu and Zhang 2015). For example, potatoes exposed to drought, salt and heavy metal stresses were positively influenced by the inoculation of two *Bacillus* species, with plants increasing their photosynthetic capacity, tuber proline content and high levels of reactive oxygen species scavenging enzymes in response to PGPR inoculation (Gururani, Upadhyaya et al. 2013).

Disease suppression is another advantageous function of some PGPR. Mechanisms of disease suppression have been shown to include the induction of plant protective hormones, enzymes and chemicals (Ganeshamoorthi, Anand et al. 2008), physical suppression and degradation of pathogens by the PGPR inoculant (Minaxi and Saxena 2010) and secretion of antimicrobials and other protective compounds (Prasannakumar, Gowtham et al. 2015). For example, *Bacillus subtilis* 21-1 promoted plant growth and reduced incidence of different diseases in cabbage, lettuce and tomato (Lee, Lee et al. 2014).

1.2.3 Agricultural practices influence the microbiome

Intensive agriculture has profound impacts on ecosystems, the climate and microbial life. In such systems, soil is managed and manipulated for the sole purpose of maximising crop yield, often involving heavy machinery, mechanical disruption, chemical additions, and continuous cropping. These practices influence soil microbial community composition, with different agricultural amendments favouring some microbes and their specialisations over others, such as organic farming (Workneh and van Bruggen 1994). Even a single tillage event changes the abundance of individual species, microbial hydrolytic activity and community structure (Kraut-Cohen, Zolti et al. 2019). For example, continuous monocropping of peanut led to an increase in fungal diversity and abundance, while bacterial abundance decreased (Li, Dai et al. 2012). Microbial biomass, dehydrogenase activity and abundance decreased with each year of continuous cucumber crops, coinciding with a loss of plant productivity (Zhou, Gao et al. 2014).

Crop rotations are known to improve soil microbial communities by supporting more beneficial functional abilities and therefore increase biological control of disease (Larkin 2008). Understanding the complex plant-soil ecology effect on microbial populations is essential to maintain the ecological function of rhizosphere communities in order to maximise the benefit to plant growth (Berendsen, Pieterse et al. 2012, Bever, Platt et al. 2012). Similarly, the maintenance of mycorrhizal biodiversity is crucial to a sustainable and fertile soil ecosystem (Jeffries, Gianinazzi et al. 2003).

Crop yields in conventional, modern agricultural systems are heavily reliant on widespread use of fertilisers, pesticides, fungicides and herbicides (Pal, Chakrabarti et al. 2010). Due to the overuse of many of these chemicals, their efficacy has become reduced and many environmental issues have resulted, including pest resistance, biodiversity loss and human health impacts (Nicolopoulou-Stamati, Maipas et al. 2016, Mandal, Sarkar et al. 2020, Singh, Singh et al. 2020). Together, the continued use of these chemicals is contributing to many undesirable secondary effects. For example, soil degradation caused by the overuse of agrochemicals is a global problem in terms of food production and soil-related ecosystem services (Jacoby, Peukert et al. 2017). Furthermore, fertiliser use significantly decreases microbial diversity (Jangid, Williams et al. 2008, Kavamura, Robinson et al. 2019). It follows that soil bacterial diversity is often positively correlated with plant biomass (Chen, Ding et al. 2020). A reduction in microbial diversity could negatively influence the ability of the soil microbiome to facilitate plants to resist stresses.

1.3 AMF interaction with PGPR

Considering the ecological impact of both AMF and PGPR, a tripartite interaction could be auspicious in the context of plant health (Kloepper, Ryu et al. 2004). The so-called common symbiotic pathway is a shared signalling mechanism used by both mycorrhizal fungi and rhizobia, to colonise the roots of host plants (Genre and Russo 2016). This indicates a convergent evolution and a similarity in terms of entry to the host plant; further suggesting that there could be significant interactions between mycorrhizas and rhizobia, and potentially other PGPRs (Oldroyd 2013).

The mycorrhizosphere, or hyphosphere, is a term used to indicate the area of the soil directly influenced by mycorrhizal hyphae. Bacterial attachment to mycorrhizal hyphae has been demonstrated and indicates a significant and potentially highly regulated interaction (Scheublin, Sanders et al. 2010). As AMF are highly prevalent and influential constituents of the rhizosphere, it is reasonable to assume that they also exert some control or influence over their

own community of bacteria. There may be some regulation over other microbial constituents of the root microbiome, however this work will focus on bacteria.

Bacterial habitation on the surface of AMF spores and mycelium has been shown, identified as *Pseudomonas* by amplicon sequencing (Bianciotto, Bandi et al. 1996). Whether these interactions are synergistic or antagonistic is unclear. This information would be of value to farmers seeking to enhance soil health by maintaining diversity, and to encourage optimum combinations of microbes in order to provide maximum benefit to plant growth. There is mounting evidence alluding to profound interactions and regulation between AMF and PGPR at the community, individual and genetic level, with encouraging prospects for their use in agriculture, which is presented in the following.

1.3.1 Interactions at the community level

Studies including entire microbial communities are scarce as they come with the inherent difficulties that studying a tripartite, obligated symbiotic interaction can be expected to pose. However, understanding the relationships in this ecosystem will be a vital piece of information in order to safeguard soil health and ensure its optimisation for crop productivity. Mycorrhizas strongly influence bacterial presence, enzymatic function, and community structure (Nurmiaho-Lassila, Timonen et al. 1997, Vázquez, César et al. 2000, Roesti, Ineichen et al. 2005). AMF are the most influential determinant of bacterial community assemblage on grass roots, but the bacterial community does not similarly affect AMF (Singh, Nunan et al. 2008). However, a multipartite interaction of AMF and PGPR communities increases nitrogen uptake of *Brachypodium distachyon* by ten times more than plants with no soil microbes (Hestrin, Hammer et al. 2019).

1.3.2 One-to-one interaction studies

There are profound interactions between AMF and rhizosphere bacteria at the individual level. Many bio-inoculation experiments use a combination of AMF and a single strain PGPR, resulting in greater plant benefits than either microbe alone (Meyer and Linderman 1986, Gamalero, Trotta et al. 2004, Tavasolee, Aliasgharzad et al. 2011, Liu, Dai et al. 2012, Nanjundappa, Bagyaraj et al. 2019).

The main agriculturally significant benefit of AMF to plants is the supply of P (Smith and Read 2008). Due to their limited suite of exo-enzymes, it is predicted that AMF are likely to be somewhat ineffective at nutrient mobilisation from organic sources (Tisserant, Malbreil et al. 2013, Jansa, Forczek et al. 2019). Thus, recruiting or relying on microbial partners that possess such abilities is predicted (Jansa, Bukovská et al. 2013, Jansa, Forczek et al. 2019). For example, AMF have been shown to acquire the services of *Rahnella aquatilis* to mineralise soil phytate (Zhang, Shi et al. 2018).

Knowledge of communication between AMF and PGPR is lacking, however there is some evidence of microbe-microbe or microbe-host interactions being mediated by volatiles or quorum sensing (Brader, Compant et al. 2017). It may be that AMF and their respective endosymbiotic bacteria interact at the level of carbon metabolism, as endobacteria affect the metabolic profile of some AMF (Lumini, Bianciotto et al. 2007). AMF associated endobacteria have been shown to use genes for nutrient uptake, suggesting their colonisation of mycorrhizas is mutually beneficial (Bonfante 2003). Endobacteria have been shown to prime AMF immune response and improve fitness (Salvioli, Ghignone et al. 2016). An extensive survey across 28 phylogenetically distant species of AMF found that all contained bacteria-like objects and that these are diverse, vertically inherited and distinct between isolates (Bonfante and Anca 2009, Naumann, Schüßler et al. 2010). Furthermore, a unique endocellular microbiome exists in the cytoplasm of the AMF *Gigaspora margarita* (Desirò, Salvioli et al. 2014).

Mycorrhizal helper bacteria is a term that refers to bacteria shown to have a role within the proper and essential functioning of the mycorrhizal fungi and their establishment (Garbaye 1994, Bonfante and Anca 2009). These are purported to include aiding spore viability, pre-symbiotic growth of mycelium, moderating the response of potential host plant roots to fungal signalling molecules and the recognition of host and fungi and physicochemical moderation (Deveau and Labbé 2016). *G. margarita* can perceive an absence of endobacteria and expresses stress-responsive proteins (Salvioli, Chiapello et al. 2010). When its endobacteria were removed, the spore physiology and pre-symbiotic growth of

G. margarita were altered (Lumini, Bianciotto et al. 2007). Endobacteria may be both horizontally and vertically transferred during AMF reproduction, implying some obligatory symbiosis (Bianciotto, Lumini et al. 2003).

Mycorrhizal associated bacteria can colonise intracellularly, but a great deal of AMF-PGPR interaction occurs on the fungal hyphal surface in the rhizosphere of the host plant, and predominantly consist of the genera *Pseudomonas*, *Burkholderia* and *Bacillus* (de Boer, Folman et al. 2005). Importantly, the AMF species is considered more important for shaping PGPR community structure than the host plant species (Roesti, Ineichen et al. 2005, Singh, Nunan et al. 2008). However, the nature of these interactions and associations between rhizosphere bacteria and AMF still remain unclear.

1.3.3 Applications for agriculture

Soil microbes benefit crop plants in many ways, including improving nutritional status and suppressing disease, but understanding the interactions between microbial partners remains elusive. Characterising and manipulating the interactions between AMF and PGPR would be a useful step to inform sustainable agricultural practice (Johansson, Paul et al. 2004). This is the major research aim of this project. It is projected that the use of microbial bioinoculants to enhance phosphorus uptake in crops could reduce the use of P fertiliser by 50% whilst maintaining yields (Yazdani, Bahmanyar et al. 2009, Oteino, Lally et al. 2015). AMF benefit crop plants by extending the root system to provide water and nutrients, and PGPR are known to help AMF to perform these functions efficiently (Nadeem, Ahmad et al. 2014). However, antagonistic interactions may occur and so it is important to define the influence of these microbes on each other in the context of soil health and crop benefits (Trivedi, Pandey et al. 2012).

Drought was remedied in maize when AMF and PGPR were inoculated in combination but not when microbes were used separately (Ghorchiani, Etesami et al. 2018). The microbial inoculants increased P uptake, but the interactions between the constituent partners were dependent on the solubility of the P. This is advantageous as it suggests AMF and PGPR will preferentially access and supply easily available P, such as applied fertiliser, to host plants, over historical

soil stocks of Pi, such as rock phosphate (Ghorchiani, Etesami et al. 2018). Dual inoculation of AMF and a PGPR improved the efficacy of a soybean and maize intercropping system by increasing fixation and facilitating nitrogen transfer (Meng, Zhang et al. 2015).

Fungal hyphae have also been shown to be a method of bacterial translocation. For example, pollutant degrading bacteria have been shown to travel along the water films of fungal hyphae (Kohlmeier, Smits et al. 2005). There is clearly a wide and diverse scope for the interactions between these microbial partners to be exploited to benefit agriculture, whether that be through direct plant contact, maintaining soil health, reducing the need for artificial inputs such as pesticide or fertiliser, or mobilisation. These interactions must be characterised in order for them to be applied to agriculture efficiently.

The plant host is an important factor in these tripartite interactions (Kavamura, Mendes et al. 2021)*. Trifolium repens*, white clover, is a perennial legume species in the family *Fabaceae*. They are a common constituent of grassland soil, and are a popular choice for intercropping and cover cropping (Xie, Sorensen et al. 2018, Hill, Levi et al. 2021). This is due to the fact that they readily form symbiotic associations with beneficial microbes which can ameliorate both plant and soil health, by fixing nitrogen or sequestering carbon (Caradus, Woodfield et al. 1995).

1.4 Methods for the study of rhizosphere microbial communities

1.4.1.1 Culture-dependent methods

The high diversity and abundance of bacteria in soil present a problem for those attempting to study them. As an example, 33000 bacterial and archaeal taxa were identified in a single soil sample (Mendes, Kruijt et al. 2011). The difficulties for culture-dependent methodology occur primarily because isolation of bacteria from soil is laborious and only a small fraction of bacteria found in soil can be cultured *in vitro*, although in recent years culture techniques have dramatically improved (Bai, Cui et al. 2015). Bulk soil dwelling bacteria may be more

recalcitrant and resistant to culture than for example, plant associated bacteria, so the fraction or habitat also has an important a role (Stewart 2012). Libraries and collections of bacteria can be isolated, tested, and stored for repetition purposes and long-term preservation or distribution.

Isolation of microbes from soil generally involves using *in vitro* methods to culture microbes on nutrient media, with different media permitting the growth of different bacterial colonies due to factors such as nutrient content, incubation time and inoculum size (Janssen, Yates et al. 2002). Methods tend to be labour intensive and time consuming, but media are versatile and provide a wide range of testing platforms.

In vitro methods also allow physical tests of microbes to be carried out. Functional assays can quickly and efficiently reveal bacterial abilities to access nutrients, act as a biocontrol, survive stresses or produce valuable compounds, as well as synergism or antagonism between isolates. Plants recruit their microbiota to be specifically functional, which is conserved across environments, instead of a consistent taxonomic microbial profile, thus rendering the definition of the function of the community instead of its taxonomic composition essential (Burke, Steinberg et al. 2011, Bulgarelli, Garrido-Oter et al. 2015, Louca, Jacques et al. 2016, Lemanceau, Blouin et al. 2017).

Bacterial movement along fungal hyphae has been simulated and tested, and the mechanism of bacterial motility was found to be mediated by fungal hyphae hydrophobicity and influenced by the properties of bacterial adhesion (Kohlmeier, Smits et al. 2005). These types of tests can be complemented with culture independent methodologies, assessing the presence of bacterial motility genes for example. However, this type of approach can be subjective when based on best predictions. Therefore, for the most comprehensive and complete picture of community taxonomic structure, function, and abundance, both culture-dependent and -independent methods should be used in combination.

1.4.1.2 Culture independent methods

Soil microbial communities adapt and respond to changes in soil conditions and therefore monitoring their status can be indicative of soil health. Culture-independent methodologies allow soil ecology to be characterised in terms of its taxonomic diversity and biological activity, and includes techniques based on nucleic acids, enzymatic screening and biochemical analyses (Rincon-Florez, Carvalhais et al. 2013).

Using nucleic acid-based methods circumvents the requirement to grow organisms in the laboratory, and thus the limitations that culture dependent methods present (Hirsch, Mauchline et al. 2010). Recently, next-generation sequencing techniques have allowed the complex root microbiome to be better described (Mendes, Kruijt et al. 2011, Vik, Logares et al. 2013, Mendes, Kuramae et al. 2014, Luo, Gu et al. 2015, Mauchline and Malone 2017). Genomics, metagenomics and transcriptomics provide correlative information to complement the functional culture-dependent techniques previously discussed (Jansson and Baker 2016). Genomics reveals the collection of genes and proteins that exist statically in a sample. Other examples include metabolomics and transcriptomics, which can provide information about the dynamic changes to metabolites or transcribed genes respectively; describing function over time (Jansson and Baker 2016, White III, Rivas-Ubach et al. 2017).

One of the most common tools of molecular microbiological research is the use of amplicon sequencing of the genes for ribosomal RNA subunits which yield information about the presence of constituent microbes of the sample. Commonly, these are the 16S rRNA gene for prokaryotes or 18S rRNA gene or the ITS spacer region for eukaryotes such as fungi. Due to the highly conserved nature of the rRNA genes, they provide a generally accepted measure of phylogenetic diversity, given as relative abundance (Hirsch, Mauchline et al. 2010). Sequences are clustered into statistically similar consensuses called operational taxonomic units (OTU), which are quick to produce but are often rudimentary in their taxonomic resolution and subject to database bias. Recently, amplicon sequence variants (ASV) have been used with greater precision for

microbiome data as they represent actual and exact sequence variants collated without dependency on comparison to a taxonomic database (Callahan, McMurdie et al. 2017).

The properties of soil are often the limiting factor to these technologies, with its heterogeneous structure, chemical inhibitors, such as humic acid, and low yields caused by DNA/RNA contamination or disruption of nucleic acids which adsorb to soil particles (Arbeli and Fuentes 2007, Rincon-Florez, Carvalhais et al. 2013). Analytical challenges are faced when processing sequencing information as data yields are often very large, so appropriate bioinformatic analyses must be available in combination with biologically relevant interpretation. However, the intricate level of detail provided by molecular techniques provides a unique insight in to the highly complex and dynamic nature of the soil microbiome. This knowledge is essential to holistically comprehend soil as an ecosystem and preserve its activity in the context of sustainable agriculture.

1.4.2 AMF methods

As obligate mutualists, AMF present many challenges to study *in situ.* Because AMF colonise both plants and soil, their lifestyle is complex and thus, their detection is even more so. Assessing their colonisation of plant roots is one of the main metrics of the AMF mutualism and is required for the vast majority of studies, and quantification to measure treatment effects on colonisation is often done in parallel with plant health (Giovannetti and Mosse 1980). Understanding AMF community diversity is also important as plant health is augmented as AMF diversity increases (Van der Heijden, Klironomos et al. 1998, Sanders and Rodriguez 2016). There is extensive concurring research on the benefits of AMF as bioinoculants however, due to lack of suitable testing procedures or techniques, comprehensive research has been hindered; research in the field, molecular studies and the ecological significance and diversity of AMF are scarcely described (Reddy, Pindi et al. 2005, Liang, Drijber et al. 2008).

1.4.2.1 AMF quantification

Staining and microscopy are the classic and most widely used methods for quantification of AMF colonising roots. AMF structures can be quantified and identified in *in vivo* roots, or as non-vital methods that require destructive sampling and stains for fungal structures. Many types of stains are available and have been reviewed extensively, including Trypan Blue, Chlorazol black E, Aniline blue and Acid fuchsin (Gange, Bower et al. 1999). These methods depend on the microscopy skills of individuals and have low resolution to identify specific AMF species or features but are quick and easy to implement (Sharma and Buyer 2015, Voříšková, Jansa et al. 2017). Assessment via microscopy is used to describe and quantify plant host tissue colonisation by the fungus (Giovannetti and Mosse 1980).

Biochemical methods are also used to quantify AMF in plant roots and describe functionality of colonising microbes, but no universally accepted biochemical marker exists (Rosier, Piotrowski et al. 2008). Fractions of the fatty acid pool can be correlated to microscopic observations, while also serving as a method to profile microbial community structure and biomass (Balser, Treseder et al. 2005, Sharma and Buyer 2015). Glomalin is a characteristic AMF-produced glycoprotein and is synonymous with its presence in soil, but tests have proved useful for presence-absence but not for accurate quantification (Rosier, Piotrowski et al. 2008). Additionally, there is evidence that many hydrophobic proteins in soil, from sources other than AMF, contribute to the apparent glomalin content (Gillespie, Farrell et al. 2011).

1.4.2.2 *In vitro* culture of AMF

In vitro culture methods use *Agrobacterium* transformed hairy carrot or tomato roots, serving as a replicate host plant for the AMF to colonise, grown on nutrient medium instead of soil (Mosse and Hepper 1975). Tissue is kept in the dark so photosynthesis does not occur; cultures are axenic, maintained long-term, and large volumes of inoculum can be produced (with reduced contamination); in essence leading to their domestication (Kokkoris and Hart 2019). This system is ideal for mass producing large volumes of single strain inocula with minimal

inputs. However, these axenic cultures are produced in the absence of the complex soil matrix and host plant diversity in which they are evolved to occupy, and therefore the microbial diversity that they would usually encounter (Gulbis, Robinson-Boyer et al. 2013). It has been reported that bacteria in the mycorrhizosphere are essential for optimal AMF function and development. As such, it is possible that these *in vitro* cultures are missing key members of the mycobiome; their use may not be suitable to accurately represent AMF interactions with plants (Lumini, Bianciotto et al. 2007).

1.4.2.3 Culture-independent methods for AMF

Molecular techniques have allowed taxonomic identification of species, historically based on spore morphology, to become a standardised, less labour-intensive process. Using spore morphology has many limitations when isolating from the field (such as parasitisation or degradation) (Rousseau, Benhamou et al. 1996). Techniques to identify AMF have been developed, mainly using the Glomalean SSU (small subunit) or LSU (large subunit) rRNA gene to enable PCR based identification methods (Helgason, Daniell et al. 1998, Gollotte, van Tuinen et al. 2004). Other methods have included T-RFLP (Mummey and Rillig 2006) and DGGE (Liang, Drijber et al. 2008).

Molecular techniques have enabled scientists to increase plant beneficial effects of AMF in rice, by generating novel genotypes through genetic exchange (Colard, Angelard et al. 2011). Some targeted molecular studies have been utilised, and the amount of AMF genes isolated is increasing (Harrier 2001). Limitations to molecular studies are the multinucleate status of AMF spores caused by huge transfer of nuclei during spore formation. This leads to striking heterogeneity in the numbers of nuclei in sister spores, coupled with the fact that AMF are coenocytic, causes substantial variation within a single isolate (Boon, Zimmerman et al. 2010, Marleau, Dalpé et al. 2011). It is even thought that in AMF, a phenotype may be caused by the presence of multiple nuclear genomes (Boon, Zimmerman et al. 2010). Therefore, the use of molecular techniques to study AMF is most efficient if used in the context of supplementing morphological studies (Chagnon and Bainard 2015).

1.4.3 Integrative methods for the study of microbial interactions

Understanding the tripartite interactions between host plants, AMF and PGPR requires a multidisciplinary approach. The available methods include culture-dependent strategies for functional descriptions of PGPR communities and creation of libraries, transcriptomics, and mutagenesis to complement *in vitro* assays and prove the function of an isolate. This is best supplemented by applying this information and demonstrating putative PGPR function with *in planta* assays. Using a combination of both classical AMF microscopy techniques and molecular methods will remove the inherent risk of bias and will give comprehensive assessment of AMF quantity and quality of colonisation. Soil microbial community studies require culture independent techniques using complimentary amplicon, metagenomic and metatranscriptomics approaches. Metabolomic approaches are also plausible for understanding signalling between microbial partners, such as via strigolactones (Lanfranco, Fiorilli et al. 2018).

The study of both bacteria and fungi presents many inherent difficulties. Differences in lifestyle, abundance, trophism, genetics and many other factors means that revealing the *in-situ* ecology of the interactions between AMF and PGPR is laden with complications. Thus, research into this area is limited. However, one study revealed that AMF select for organic P mineralisation abilities from bacterial communities, since AMF do not have this capacity themselves (Zhang, Shi et al. 2018). In this experiment, mycorrhizal presence into a patch of organic P rich soil was controlled with different sized mesh apertures which excluded or permitted AMF hyphae. AMF were shown to recruit bacterial partners with alkaline phosphatase, and communities in AMF-free soil were significantly different to communities where AMF hyphae were present (Zhang, Shi et al. 2018). A combination of biochemical and molecular technologies was used to study functional ability and community structure respectively.

Another study used a simplified substrate with a microbial filtrate inoculum to test the difference of AMF-colonised maize versus AMF-free maize on bacterial communities. This was done using 16S rRNA gene sequencing to determine changes to bacterial communities in different soil fractions caused by AMF

(Marschner, Crowley et al. 2001). Differences in bacterial communities along mycorrhizal hyphae have been shown, also using 16S rRNA gene sequencing (Scheublin, Sanders et al. 2010). Furthermore, PCR-DGGE revealed that different AMF isolates have specifically recruited discrete microbiota to their spores (Agnolucci, Battini et al. 2015).

The use of hyphal growth tubes was pioneered to visualise the transfer of bacteria along fungal hyphae (e.g. *Fusarium*) in the field, using culture-dependent methods to isolate transmitted bacteria (Simon, Bindschedler et al. 2015). This has obvious advantages in that more complex communities can be included, as it is deployed in the field, instead of reduced diversity offered by *in vitro* studies of the same kind using AMF (Kohlmeier, Smits et al. 2005, Zhang, Shi et al. 2018). Understanding AMF and their interactions with PGPR communities can only be achieved if a variety of methods are combined.

There are complex and highly dynamic relationships that govern microbiome activity in the soil. Characterising these interactions as they relate to function will allow not only expand our understanding of soil microbiome community, but also inform how they can be best harnessed to contribute to sustainable agricultural practices. It is apparent that the soil microbiome is important for AMF function, and must be considered when creating a putative inoculant. To fully comprehend and optimise the relationship between crop and soil, we must incorporate the plant holobiont.

This work will respond to the knowledge gaps surrounding the influence of the addition of an exogenous AMF inoculant, a strategy gaining popularity for sustainably enhancing crop growth. Importantly, the theory suggests that AMF might be deleterious to plant health in managed agricultural systems using chemical fertiliser. In addition, it is not known how the application of a high dosage of an AMF species will affect the bacterial root community, both in taxonomic composition and function. There is a growing body of work suggesting intimate relationships between bacteria and AMF orchestrated by the host plant; this thesis will test if AMF and PSB work synergistically to enhance the phosphorus uptake in clover. Finally, the recent concept of the bacterial mobilisation along the

fungal “highway” is beginning to be explored, but often these experiments occur in model or *in vitro* systems. This work will respond to the knowledge gap by focusing on bacterial transport along AMF hyphae specifically, in a soil environment. Furthermore, this will be investigated in relation to recruitment of bacterial functionality and taxonomy.

2 AIMS AND OBJECTIVES

2.1 Overall aim

This project will gain novel insights to contribute to the description of the AMF-PGPR-host plant mutualism by characterising the bacterial-fungal interactions in the context of both soil and plant health. Considering the ecological impact of both AMF and PGPR, a tripartite interaction could be auspicious in the context of crop health (Adesemoye and Kloepper 2009). Whether these interactions are synergistic or antagonistic is unclear. This information will be of value to farmers looking to enhance soil health by maintaining diversity, and to encourage optimum combinations of microbes in order to provide maximum benefit to plant growth.

Constituent organisms are often studied in isolation and there are few examples of holistic studies combining organisms to study their interactions or influence on plants. Co-inoculation experiments have focused on individual bacterial species interacting with AMF, and many show positive effects on plant growth (Gamalero, Trotta et al. 2004, Tavasolee, Aliasgharzad et al. 2011, Liu, Dai et al. 2012). Therefore, combining multiple synergistic plant-beneficial bacteria with AMF as a potential bioinoculant is a promising approach.

It is vital to describe the impact of AMF on soil microbial communities to better understand their effect on soil health for sustainable agriculture. Evidence is lacking in this area, but it is known that mycorrhizas influence bacterial abundance, enzymatic function and community structure (Nurmiaho-Lassila, Timonen et al. 1997, Vázquez, César et al. 2000, Roesti, Ineichen et al. 2005).

Research into community and competition dynamics between AMF and rhizobacteria is markedly absent. The presented work will contribute to this knowledge gap by revealing how the community structure of rhizobacteria is influenced by AMF; assess if the interactions between microbial partners may be synergistic or antagonistic to plant health when subjected to variations in edaphic stresses. The goal is to reveal if putative bioinoculant combinations of AMF and PGPR have an additive beneficial effect or compete against each other for host

plant access and reward. This will incorporate testing how the use of chemical fertiliser effects these communities and their potential to be plant beneficial; revealing the ecological impacts of fertiliser use vs bioinoculants could provide a framework for decision making for a sustainable farming future.

2.2 Experimental chapter aims

2.2.1 Design and development of a system for the study of microbial interactions (Section 4).

The study of a tripartite interaction presents many challenges. The creation of suitable protocols and systems is essential, though the suitability of the putative system must satisfy many requirements. Firstly, the system must be conducive to plant growth. As AMF are obligate symbionts, and PGPR are root occupying, the substrate must provide optimum conditions for root growth and subsequently, representative enough that plant health indicators can be extrapolated to more natural systems. Substrates must be complex enough to host microbial communities and simulate conditions close to those found in the field, yet simple enough to be able to manipulate one edaphic factor at a time to disentangle the effects of stresses on the community interactions and their ability to promote plant growth. Similarly, the required system must be appropriate for downstream processing of samples.

Objectives:

- The objectives of this chapter is to trial, and implement, protocols and systems for these holistic microbiome studies to be conducted.

2.2.2 Characterise the influence of AMF on the rhizobacteria community structure and function (Section 5).

As AMF are found ubiquitously throughout the soil and associate with up to 90% of all plant species, it is likely that they are an influential determinant of rhizosphere community dynamics. As both AMF and PGPR have similar functions in terms of plant benefits, notably P provision, understanding how these

interactions change when plants are in a particular biotic or abiotic stress would be auspicious.

Objectives:

- Experiments will define the influence of two different exogenously applied single species AMF inoculants on the root microbiome of clover.
- These are then expanded to incorporate two different soil types, grassland and bare fallow, to examine how community responses might change depending on soil type.
- Finally, chemical fertiliser will be tested to assess the impact of agricultural amendments on the microbiome structure and potential plant-beneficial function, to further include how AMF influence any potential impact.

2.2.3 How do interactions between AMF and P solubilising rhizobacteria affect plant P status? (Section 6).

To sustainably enhance crop growth, it is envisioned that alternatives to artificial fertiliser might take the form of microbial bioinoculants. Understanding the competition dynamics of potential microbial combinations is essential to trial their efficacy and function before adding them to the field environment. To fully comprehend the ecological impact of these inoculants, it is important to characterise the interactions in the context of soil status, and whether the constituent partners in the inoculants affect the plant synergistically to provide benefits that are greater than the sum of their parts. It is essential to understand if AMF and PGPR require the other to perform their function for improved plant growth.

Objectives:

- Design optimum combinations of phosphorus solubilising microorganisms (PSM) by selecting for synergy *in vitro*
- Apply combinations of PSB and AMF to clover plants with the aim of revealing advantageous combinations in terms of plant biomass and nutrient content

2.2.4 Bacterial transference along AMF hyphae – is mobilisation selective for specific functions or taxa? (Section 7).

The novel and central hypothesis of this project is that AMF act as a conduit for the facilitation of PGPR to access plant roots, and that this recruitment may be selective in terms of plant beneficial effects. It is proposed that AMF can bridge soil pores and heterogeneity to access a wider range of soil bacteria, and there is potential for transport along fungal hyphae. Understanding whether this transport is selective or passive, and whether AMF may be particularly conducive to improving microbiome function under certain soil conditions, is novel for the field and would be vital to inform agricultural practice.

Objectives:

- Experiments will reveal if bacterial dispersal between plants is facilitated by AMF hyphae
- This is then further investigated to understand the direction of bacterial transference when bacteria are deployed at difference locations between the neighbouring plants
- Finally, explore the role of AMF in the establishment of the root microbiome of clover is explored, using field soil and meshes of hyphae permitting/excluding aperture sizes. The objective of this experiment is to reveal if 1. AMF select specific rhizobacteria taxa or functional capabilities and 2. Facilitate their colonisation of plant roots.

3 GENERAL METHODOLOGY

3.1 Plant preparation and planting

White clover (*Trifolium repens*, cv. Aberdai) seeds were sterilised and pre-germinated as described in Robinson, Fraaije et al. (2016). Briefly, seeds were shaken in 70% EtOH at 4°C for 10 mins, immersed in 1.5% sodium hypochlorite, inverting occasionally, before washing with sterile deionised water. Seeds were imbibed in sterile water overnight at 4°C in the dark, before draining and transferring to damp sterile germination paper placed inside Petri dishes. Only seeds of similar size were selected (by eye) to pre-germinate. These were left at room temperature in the dark for three days, until germination had occurred. Seedlings were planted in triplicate, from separate Petri dishes to avoid confounding effects across dishes and then thinned to one plant per pot once seeds had established (after one week).

3.2 Substrate preparation

Sandy loam and quartz sand were mixed at a 3:1 ratio, before being sterilised at 121°C for one hour.

3.3 Soil preparation

Soil samples were taken from two sites: Rothamsted Research Barnfield site (Grassland, Harpenden, 51.808028, -0.361487), and Rothamsted Research Woburn experimental farm (Stackyard field site, Bare fallow, Husband Crawley, Bedfordshire, 52.000293N, -0.614308). The Woburn experiment sampling site has been maintained as bare fallow for 50 years. Samples were gathered from the top 50cm of the soil, the turf removed from the Barnfield plots before collection, and then kept at 4°C until use. Before setting up experiments, soil was mixed to combine sample bags and air dried in cabinets for one week prior to sieving, using a roller mill to remove large aggregates, plant material, stones and improve homogeneity.

3.4 Soil nutrient content analysis

Chemical analysis of soil samples was performed by Rothamsted Research Analytical Chemistry unit. Analysis included Aqua regia extraction for total cations, Olsen P, total N + C on Dumas combustion analyser (Leco), NO_3-N and NH_4-N using a Skalar colourimetric flow analyser and inductively coupled plasma ICP-OES analysis for major and trace elements. Soil was finely milled using an agate ball mill for all analyses except for NO_3-N and NH_4-N analysis which was done on KCl extracts. Extractions were taken from fresh soil samples. 2M KCl was added to soil at a ratio of 1:3.2, soil:KCl, shaken at 120 strokes per minute for two hours and then filtered until a clear extract was obtained, and stored at -20°C before analysis.

3.5 Plant nutrient content analysis

Plant material was dried at 80°C for 24 hours. Dry samples were milled to 2mm using a POLYMIX PX-MFC-90 D (Kinematica AG, Switzerland). These were weighed and sent for nutrient analysis (ICP-OES majors and traces, including Olsen P) at Rothamsted Research Analytical Chemistry Unit. Measurements were obtained using an Optima 7300 DV Inductively Coupled Plasma - Optical Emission Spectrometer (ICP-OES), using a nitric and perchloric acid digestion. Values were given as PPM of dry matter.

3.6 Soil sampling from pot experiments

This protocol was adapted from Reid, Kavamura et al. (2021). Rhizosphere samples were obtained by gently removing the majority of bulk soil from roots, then shaking root systems into a clean plastic bag to collect the root-associated soil. Two sub-samples were taken: the bulk of the rhizosphere for culture-independent analysis was immediately frozen in liquid nitrogen and stored at -80°C until freeze drying. Additionally, a ~2g sample of rhizosphere soil was reserved for culture work if required and stored at 4°C for immediate use.

Rhizoplane samples were collected once the rhizosphere had been dislodged, by shaking roots vigorously in 20ml sterile water for 30s to remove the adhered soil. Roots were then removed from the solution and retained for further sampling.

A 500µl sample of the liquid rhizoplane was aliquoted in 500µl 80% glycerol, agitated to ensure thoroughly homogenous, flash frozen and stored at -80°C for culture work as a glycerol stock. The main rhizoplane suspension was also frozen in liquid nitrogen immediately after collection. This was then freeze-dried for homogeneity for culture-independent work.

Subsequent use of the word rhizoplane in this thesis pertaining to experimental work is referring the soil fraction obtained by this method of sampling. However, it is not possible to precisely separate rhizoplane from rhizosphere, so the rhizoplane fraction sampled as described is likely to consist of some rhizosphere soil in addition to the rhizoplane removed by washing.

3.7 Root sampling

Root samples were collected for AMF quantification, for both microscopy and qPCR, once the rhizoplane has been removed. Two root samples were cut from the washed roots. One was stored in 70% EtOH at 4°C for microscopy, and one was flash frozen in liquid nitrogen and freeze dried for molecular work.

3.8 DNA extractions

DNA from freeze-dried rhizosphere and rhizoplane samples (0.25g) was extracted using the DNeasy Powersoil Kit (Qiagen). One amendment to the manufacturer's protocol was made, replacing the vortex adapter step (step 2) with using a FastPrep-24 5G beadbeater (MP Biomedicals) at 30s x 2 at 5.5m/s using the Quickprep adapter. DNA quality was assessed using a Nanodrop™ 2000c Spectrophotometer (Thermofisher) and quantified using a Qubit 2.0 Fluorometer (Invitrogen) using the Qubit® dsDNA HS or BR (High-Sensitivity or Broad-Range) assay kits (Thermo Fisher Scientific). Extractants were stored at -80°C until use.

Root DNA was extracted using the method described above, using 0.09g freeze dried material which had been ground in liquid nitrogen using a sterile pestle and mortar to increase homogeneity.

3.9 qPCR

Different genes were quantified using this method, but the protocol and program had previously been optimized to be run under the same conditions:

All samples were diluted to 10ng/µl in 30µl. Quantifast kit SYBR green 2x (Qiagen) was used. All qPCRs were performed using a CFX384 touch real-time machine (Bio-Rad), conditions were set at: 1. 5 mins 95°C, 2. 10 secs 95°C, 3. 30 secs 60°C, repeat step 2 and 3 x 39, 4. 5 secs 60°C, 5. 95°C.

The list of primers for AMF (*F. geosporum*) and *gfp* are given in Table 3-1.

Table 3-1. Primers for AMF (*F. geosporum*), *gfp* used in qPCR.

Gene of interest	Forward primer	Reverse primer
AMF Large Subunit	nrLSUF GGAAACGATTGAAGTCAGTCATACCAA	FgnrLSUR CGAGAAAGTACACCAAAAGWGCCCAAT
GFP	GFPf CTGCTGCCCGACAACCAC	GFPr TCACGAACTCCAGCAGGAC

Quantification of AMF using qPCR was often inconclusive, due to the inherent difficulties of using culture-independent techniques for AMF work. A detailed discussion of such challenges is provided in Section 8.5. Thus, only microscopy-determined AMF quantification is included in this work, with AMF qPCRs included only in the appendix to support the discussive material comparing the methodology (Section A2, Figures A2-4)

3.10 Media preparation for functional assays

25 x 25 cm plates were cleaned using detergent and water, sterilised using 70% EtOH, and finally exposed to UV light for 30-60 mins before media was poured. Each plate received 100ml media. Plates were stored at 4°C until use. All plates were incubated at 25°C until assessment.

3.10.1 Zinc medium

A medium was prepared for the detection of soil bacterial isolates able to solubilise zinc. The medium including its sterile insoluble particulates were well suspended before pouring to ensure homogenous distribution of Zinc. Plates

were assessed after ten days post inoculation. Isolates positive for zinc solubilisation were able to grow, and sometimes produced a zone of clearing. Isolates which could not access zinc, did not grow. Media was prepared as follows, in one litre:

10g Dextrose (glucose); 1g $(NH_4)_2SO_4$; 0.2g KCl; 0.1g K_2HPO_4; 0.2g $MgSO_4.6H_2O$; 1g ZnO; 15g BactoAgar

3.10.2 $AlPO_4$ and $FePO_4$ media

An iron- and aluminium-phosphate solubilisation assay was prepared as described by Gadagi and Sa (2002), with some amendments. The modified basal medium was used to identify bacterial isolates that could utilise $AlPO_4$ or $FePO_4$ as a source of P. $AlPO_4$ plates were assessed after 24 hours. For $FePO_4$, plates were assessed after seven days. Discolouration of bromocresol green, caused by changes in pH were observed in positive isolates: Blue to orange for $AlPO_4$, green to dark green for $FePO_4$. The media contained the following, per litre:

10g sucrose; 0.1g NaCl; 0.5g $MgS0_4.7H_20$; 0.2g yeast extract; 0.5g NH_4Cl; 0.1g $MnSO_4.H2O$; 2g $FePO_4$ or 5 g $AlPO_4$; 20g BactoAgar; 0.025g bromocresol green.

3.10.3 Ca_3PO_4 – Pikovskaya's agar

Adapted from Pikovskaya (1948), for the detection of phosphorus solubilising bacteria. The medium uses Ca_3PO_4 as a Pi source. Plates were assessed one week after inoculation, counting colonies with visible zones of clearing, or "halo" as positive for P solubilisation. Measurements of the size of the zones of clearing were obtained only when a phosphorus solubilising index was required. For the high-throughput functional assays, this was not measured, and isolates were simply scored as positive or negative. The medium was made with the following per litre:

0.5g yeast extract; 10g dextrose; 5g Ca_3PO_4; 0.5g $(NH_4)_2SO_4$; 0.2g KCl; 0.1g $MgSO_4$; 0.0001g $MnSO_4 \cdot H_2O$; 0.0001g $FeSO_4 \cdot 6H_2O$; 15g BactoAgar.

3.10.4 Siderophore production – Blue agar CAS assay

This medium was prepared as directed in Louden, Haarmann et al. (2011). This protocol was not modified and contains a detailed list of reagents. Isolates were subjected to screening for siderophore production. The medium contains ferric iron which is bound to the indicators chrome azurol S (CAS) and HDTMA. When iron is taken out of this complex by the presence of siderophores, the media turns orange. Plates were assessed after three days in an incubator at 25°C.

3.10.5 Potassium solubilisation – Modified Aleksandrow's media

Prepared for the detection of potassium solubilising bacterial isolates. Plates were assessed after one week of growth. Colonies were considered positive if a zone of clearing was visible, and negative if not. Medium was agitated before pouring and modified to the protocol by Setiawati and Mutmainnah (2016). Prepared per litre and adjusted to pH 7.5 as follows:

5g Glucose; 0.5g $MgSO_4.7H_2O$; 0.1g $CaCO_3$; 0.006g $FeCl_3$; 2g Ca_3PO_4; 3g potash feldspar; 20g BactoAgar.

3.10.6 N hydrolysation – casein/protein hydrolysation assay

This medium contains casein in its hydrolysed form (casamino acids). Microbes that hydrolyse peptide bonds are thought to be able to process organic N (Frazier and Rupp 1928, Reid, Kavamura et al. 2021). These plates were prone to overgrowth on the large plates so for the high throughput screening, which rendered the samples uncountable. Therefore, individual plates per soil sample were used to prevent contamination between samples and assessed after three days. The medium was prepared per one litre:

5g Skimmed milk powder; 0.5g pancreatic digest of casein; 0.25g yeast extract; 0.1g D-glucose; 12.5g BactoAgar.

3.10.7 Phytate – Na-IHP solubilisation assay

Phytate-specific medium detects isolates that can use Na-IHP as a P source (Unno, Okubo et al. 2005). The media is pH sensitive (bromocresol green) and turns darker green when an isolate is positive, along with colony formation. Colony formation was assessed after one week. Counts were extremely low for this assay and agar was increased from the original protocol to create a solid media. The media contained per litre:

17g BactoAgar; 10g Na-IHP; 1g $(NH_4)_2SO_4$; 1g $MgSO_4.7H_2O$; 7g KCl; 0.1g $CaCl_2.2H_2O$; 1ml 1M FeNa-EDTA; 0.01g bromocresol green; 1 ml trace element solution (per litre) containing: 15g $Na_2EDTA.2H_2O$; 0.43g $ZnSO_4.7H_2O$; 0.24g $CoCl_2.6H_2O$; 0.99g $MnCl_2.4H_2O$; 0.22g $Na_2MoO_4.2H_2O$; 0.19g $NiCl.6H_2O$; 0.08g $Na_2SeO_3.6H_2O$; 0.15g H_3BO_3.

3.11 Assessment criteria for functional assays

Figure 3.1 is an image of each of the functional assays, with an exemplar positive (green square) and negative (red square) isolate for reference. Assays were assessed blind to prevent bias and assays were all assessed by the author. Phytate and $FePO_4$ assays were carried out for all samples but very low colony counts were obtained so these were excluded from the results in the experimental chapters.

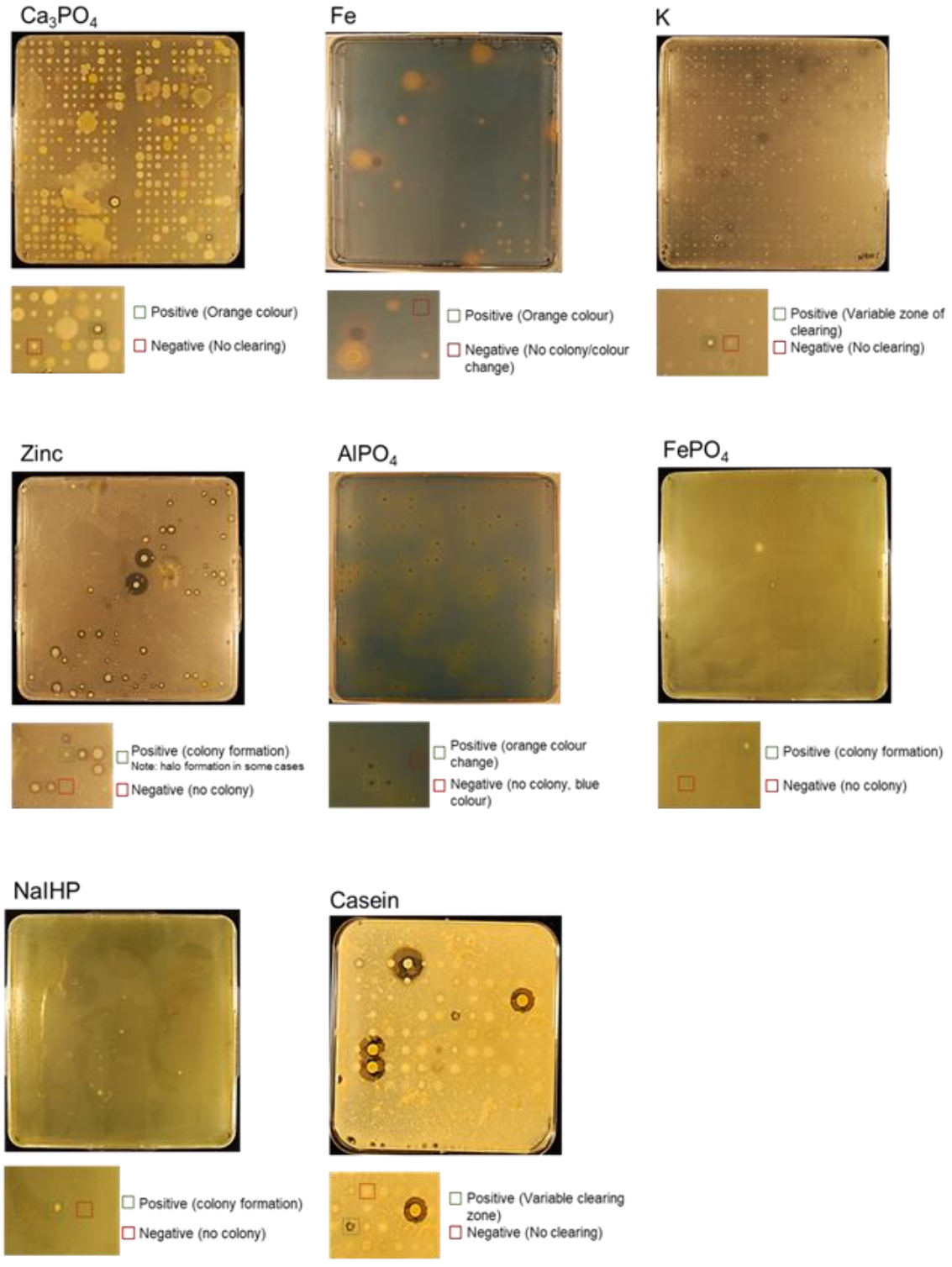


Figure 3.1. Visualisation of the assessment criteria for each *in vitro* functional assay.Green squares represent a typical inoculation point yielding a positive score for each assay, while red squares demonstrate an inoculation point with a negative score. Inoculation points (n = 94 per sample with two negative controls, n = 6 samples per plate) were given positive/negative scores only, no other measurements were taken.

3.12 Preparation and processing of amplicon dataset

Samples were prepared as described in Section 3.8. DNA was quality checked and quantified using the Qubit 2.0 Fluorometer (Invitrogen) using the Qubit® dsDNA HS or BR (High-Sensitivity or Broad-Range) assay kits (Thermo Fisher Scientific). Samples were diluted to 30ng/µl in 50µl and sent to Novogene (Cambridge, UK) for further processing and Illumina sequencing. A rhizosphere and rhizoplane extractant for each sample were sent (if these were collected). Further descriptions of the amplicon datasets can be found in their corresponding methods section.

Qiime2 was used to process the raw 16S rRNA gene amplicon sequences. Sequences were merged , denoised and de-replicated using Dada2 (Callahan, McMurdie et al. 2016). An ASV table was produced containing high-quality non-chimeric reads and a phylogenetic tree constructed using the qiime command align-to-tree-mafft-fasttree. The taxonomic database SILVA132 was used for 16S rRNA classification (Quast, Pruesse et al. 2012, Yilmaz, Parfrey et al. 2014).

R version 3.6.1 was used to analyse the Qiime2 produced taxonomy and ASV tables, which were transformed into Phyloseq (version 1.30.0) objects (McMurdie and Holmes 2013). Data was filtered according to presence (to select ASV's present in 66% of replicates) and chloroplast, mitochondrial and unassigned sequences were removed. Data were normalised using DESeq2 with a regularised logarithmic transformation except for when obtaining alpha-diversity estimates, when data were normalised by alpha rarefaction using the minimum sampling depth. For alpha diversity tests, a Kruskall-Wallis test revealed significance, and where $p < 0.05$ a Conover test was used for post-hoc comparisons.

A weighted-UniFrac distance metric with a principal coordinates analysis was conducted for all of the beta-diversity investigations after exploration of the data using three other similarity measures (unweighted UniFrac, Jaccard and Bray-Curtis). A PERMANOVA tested statistical significance. Beta-dispersion was also used to explore homogeneity between group variance using vegan::betadisper and a permutation-based test for significant differences (vegan::permutest).

A canonical analysis of principal coordinates (CAP) test was also carried out to test for interactions between the explanatory factors, constrained by treatment. This analysis also used the weighted UniFrac similarity measure. CAP outcomes were analysed using ANOVA.

Heatmaps were produced to show the top 20-25 most differentially abundant ASVs/taxa aggregated by phylum and family.

3.13 AMF root assessments – the gridline intersection method

The following assessment method was adapted from Giovannetti and Mosse (1980) as the commonly used “gridline intersection method”. Briefly, stained roots in roughly ~1 cm pieces are randomly distributed using fine forceps in a Petri dish filled with lactoglycerol (Figure 3.2). The dish has lines at 1 cm intervals horizontally and vertically. Using a dissecting microscope, at 4x magnification, follow each line horizontally and vertically. Each time the line is intersected with

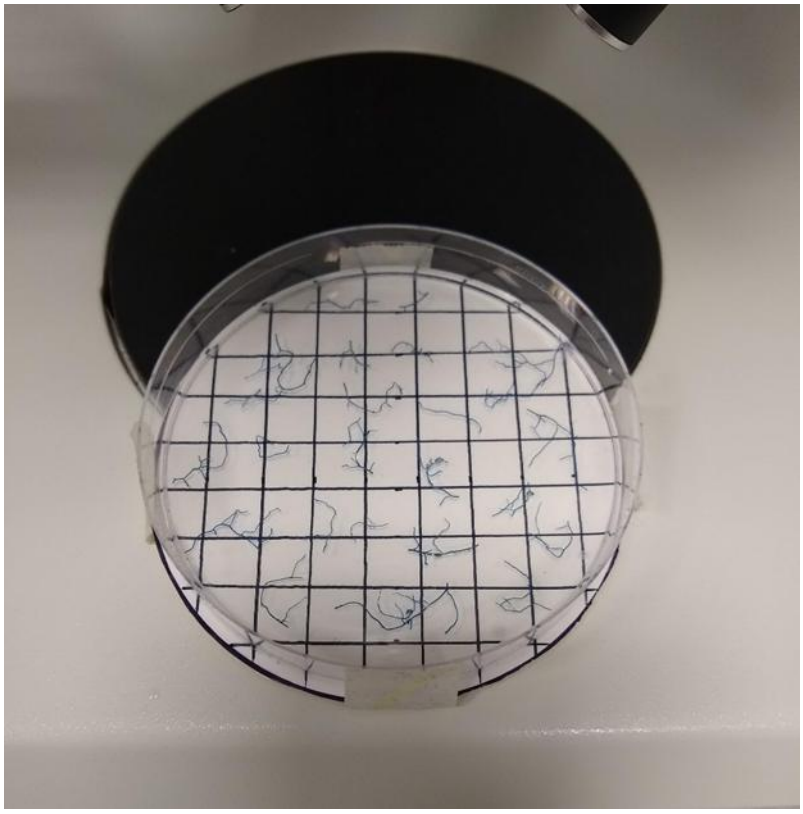


Figure 3.2. Typical set up for the gridline intersection method. Roots (~1cm) were stained with trypan blue and stored in lactoglycerol. A minimum of 50 intersections are randomly and evenly distributed in the Petri dish suspended in lactoglycerol for assessment using a dissection microscope.

a root, it is assessed to see whether it contains AMF or not. A minimum of 50 intersects are counted and the percentage of roots colonised with AMF is determined. Figure 3.3 demonstrates what was counted/discounted as an AMF colonised root.

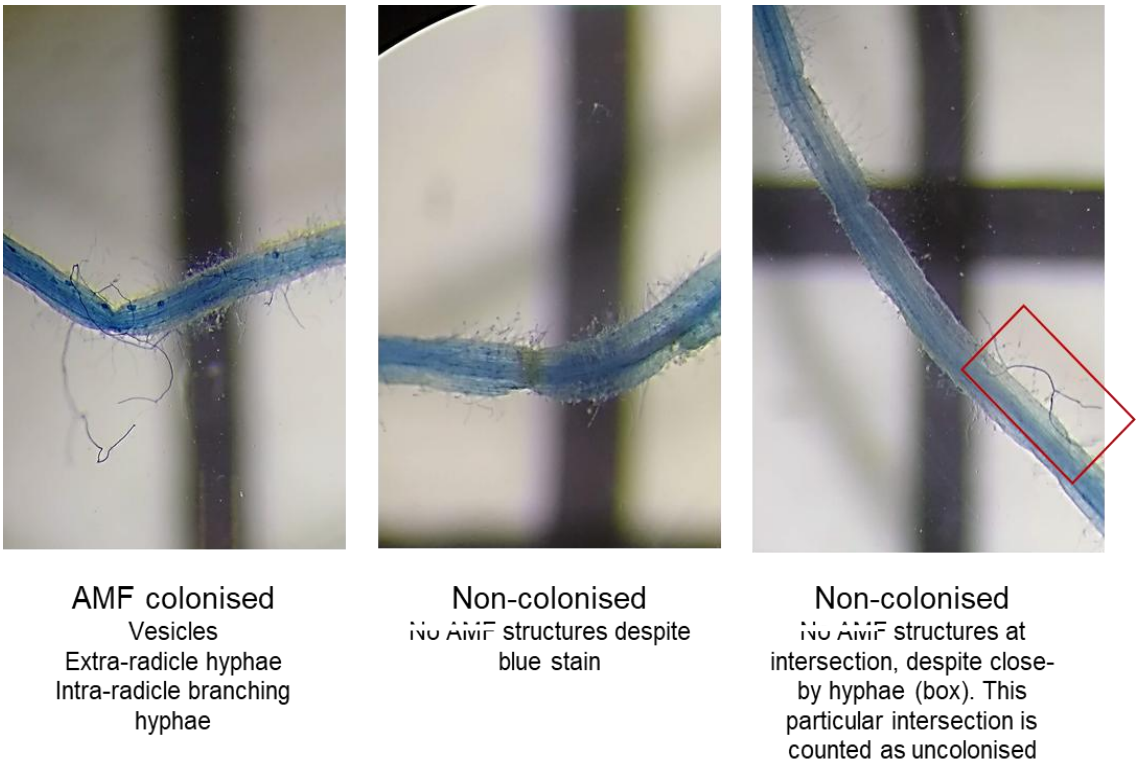


Figure 3.3. Demonstration of roots that were considered as AMF colonised and non-AMF intersections. Also shown is a root that is colonised along the root (red box) but not at the actual intersection and is therefore counted as a non-AMF colonised intersection. Magnification = 4X and scale bar = 1.5mm.

4 Designing and developing a system for the study of soil microbial interactions

4.1 Abstract

To better understand the complexities of the soil microbiome, studying microbial interactions in a suitable system that can best represent their true nature is essential. The influence of arbuscular mycorrhizal fungi (AMF) on communities of plant growth promoting rhizobacteria (PGPR) remains undefined. Studying the tripartite interaction between AMF, PGPR, and a plant host requires a system simple enough to be manipulated for scientific study, yet complex enough to host dynamic communities and to simulate the heterogeneity of soil. The development of the necessary components to pursue the proposed research are presented in this chapter. A combination of substrates was selected to suit specific experimental questions: a simplified sandy substrate in which AMF colonisation was abundant, and the use of field soils for questions pertaining to AMF interacting with bacterial communities and soil nutrient status. The use of white clover was chosen as it has a known strongly mycorrhizal phenotype, and to pursue the research questions relating to microbially-enhanced phosphorus uptake. Together these approaches were deemed the most suitable to investigate the proposed research questions.

4.2 Introduction

A 70% increase in agricultural output is required by 2050 to meet the demands of an expanding population (Hunter, Smith et al. 2017). This formidable challenge is made more onerous by the need to achieve this target without increasing the land used for arable farming, whilst reducing the amount of potentially harmful ecological inputs, such as artificial fertiliser (Tilman, Balzer et al. 2011). This ecologically conscious intensification of agriculture must look to novel alternatives to these environmentally deleterious chemicals to sustainably support the nutritional requirements of humankind.

The development of microbial bioinoculants for the supplementation of agricultural inputs intended to ameliorate plant health is a topic gaining in popularity (Santos, Nogueira et al. 2019). Soil microbes provide many essential ecosystem services and are an integral part of a functioning and healthy soil system (Jansson and Hofmockel 2020). They can improve plant health by mitigating stresses such as nutrient depletion, drought, or disease (Berendsen, Pieterse et al. 2012, Bhattacharyya and Jha 2012). Currently, the challenges faced by this approach are poor performance of these inoculants in the field compared to pot trials; the discovery of inoculants for a wider range of crops; novel solutions for areas which experience highly variable environmental stresses (Santos, Nogueira et al. 2019, Haskett, Tkacz et al. 2021). Research must focus on the discovery of high performance, host-specific, environmentally robust inoculants, and include novel deployment strategies.

In order to create these microbial supplements, research into their behaviour, community structure, and ecological impact must be comprehensive. Understanding the influence of these microbes on soil and plant health is essential to create fit for purpose inoculants that are both agriculturally enhancive and environmentally benign; they improve plant health with no adverse impacts on the surrounding environment. Studies in the field are required to reveal any ecological effects of inoculants, as are simplified pot experiments to study the detailed effects of microbial inoculants at work on plant health. With these experiments come the inherent difficulties of systems to study these complex

interactions: 1. Substrates must be suitable for plant growth throughout their lifecycle, 2. Complex enough to support microbial communities and nutrient matrices, 3. Simple enough to be able to manipulate to study one environmental variable on inoculant performance at a time, 4. Versatile enough to be able to impose multiple stresses that would affect plant growth e.g. nutrient deficiencies or drought, 5. Viable enough for repetitive and high-throughput use without unreasonable expense or consumption.

When considering a system in which to study the interactions between AMF, PGPR and a host plant, the difficulties arise both when considering the tripartite nature of these interactions and also the requirements of the individual constituents. For example, AMF are obligate mutualists, so require a plant host. Additionally, they are impacted by agricultural practices such as tillage, fertiliser use and monocropping (Jansa, Wiemken et al. 2006). The properties of soil or growth substrate also influence AMF colonisation, such as organic matter content (Albertsen, Ravnskov et al. 2006). Secondly, one must consider the requirements of PGPR. The system must be complex enough in its chemistry and physical properties to support a diverse and abundant bacterial community, and dynamic enough for these communities to carry out their functions in terms of promoting plant growth. Structure is important to host diverse communities and should not restrict movement or the ability to form networks, allowing bacteria to exist in specialised niches or microenvironments (Or, Smets et al. 2007, Juyal, Otten et al. 2021). Thirdly, a potential system must be conducive to plant growth. Optimum plant growth must be achievable within the control test medium to ensure that any treatment effects observed are from microbial additions or manipulation of stress factors and not an artefact of plant stress imposed by an unsuitable growth medium. Lastly, it is essential that a system is able to function as a testing matrix. It must be versatile and easy to manipulate, in order to impose edaphic stresses or environmental conditions that microbial inoculants can be designed to mitigate. It is more efficient to have one system that can take many forms, than to have many different systems for each potential environmental stress. The system must be well defined in order to keep its physiological properties consistent enough to

test one treatment variable at a time, while capable of supporting all the complex ecological processes that field soil provides.

Currently, systems are either too simple; such as sand, glass beads or vermiculite, which do not replicate the intricate chemistry or microbial life found in the field; or too complex, such as real soil, which has a full microbial consortia but its integrity is compromised when trying to impose stresses such as drought or sterilisation, or nutrient removal. Many putative inoculants are tested for plant growth promoting ability *in vitro*. This is useful as a high throughput measure to predict the potential for isolates to be beneficial, but it is essential that inoculants are tested *in planta* to validate their efficacy.

This chapter tests and evaluates different experimental systems in which to screen putative bioinoculants and characterise the interactions between AMF and PGPR and their effect on plant health. There must also be scope to manipulate edaphic factors within these systems in order to scrutinise the agricultural significance of the microbial partners in relation to plant health. Systems are evaluated by their ability to: support optimum plant growth, provide a matrix conducive to AMF symbiosis with a host plant, support a complex rhizosphere community of potentially beneficial microbes, and impose biotic and abiotic stresses easily. One system using sphagnum peat moss compost was particularly successful as it was extremely versatile in the manipulation of physiological properties and worked well for testing beneficial bacterial inoculants. However, this system was unsuitable for hosting AMF. The most common issue with the substrates trialled was no AMF colonisation being detected, usually due to unsuitability of the system, the protocol, or the plant host.

A large proportion of results from this chapter are published in Masters-Clark, Shone et al. (2020), “Development of a defined compost system for the study of plant-microbe interactions”, and is included in the appendix (Figure A-1).

4.3 Results and discussion

A summary of the pilot experiments carried out to find a suitable substrate for the proposed research is given in Table 4-1. Many of these substrates were deemed unsuitable, so for brevity further details of these results will not be provided. Details of the outcomes and suitability of each substrate are discussed. In summary, the most successful substrate for satisfying the requirements of the tripartite interaction was the sandy loam: sand mix, as this yielded abundant AMF colonisation, was conducive to root growth, and was simple enough to be able to conduct experimental questions that did not require complex physical structure or an indigenous microbial community. Soil sand content has been shown to be positively correlated with AMF-mediated plant growth responses and colonisation (Zaller, Frank et al. 2011). Two types of field soil were selected to complement the choice of the simplified sandy substrate, in order to provide a complex system with a full microbial community, to simulate field conditions and study agriculturally relevant questions. These came from two land use types: grassland (Barnfield) and bare fallow (Woburn). The decision flow that led to the selection of these three matrices is detailed in Figure 4.1 .

Table 4-1.Summary of pilot experiments used to test different substrates for their suitability for experimental work exploring AMF interactions with rhizobacteria. This table presents an overview of results from several pilot experiments to determine an experimental substrate to be used in the subsequent research in this thesis. Substrate details are given pertaining to composition and nutritional content, and a summary of which elements of the tripartite interaction being investigated that the substrate was suitable for.

Substrate tested	Experimental questions	Results	Substrate details	Suitable for:
Compost	Can washing compost remove soluble nutrients and can these be reinstated to recover plant growth?	Soluble nutrients are removed (notably NPK and some micronutrients) These must be reconstituted at 5X the original concentration washed out to restore plant growth. Anything above 5X gives no additional increase in plant biomass	Sphagnum peat moss, pH 5.6-6, high NPK when unwashed, low NPK when washed, Supplier: Everris, Levington F2 + sand	Nutrient manipulation Implementation of edaphic stresses (eg milling) and sterilisation Testing putative bacterial inoculants Excellent structure for plant growth Masters-Clark *et al.* (2020)
	Can nutrient deficiencies be created by the exclusion of a specific? macronutrient, and what is the optimum concentration of the reconstituted nutrients?	N and P deficiencies were created. Resulting plants has chlorotic leaves and reduced biomass. N had more of a significant effect than P deficiency		
	Do AMF affect growth of wheat or clover in different nutrient deficiencies?	No colonisation was observed in any compost experiment. Not suitable for AMF work.		
	Can P solubilising bacteria rescue growth of P deficient plants in a soluble P deficient, inorganic P supplemented medium?	Wheat had larger biomass when P solubilising microbes were present, accessing insoluble, inorganic P		
Sandy loam mix	Can washing loam soil remove the soluble nutrients?	Plants were very stressed and grew extremely poorly. Substrate was compromised by the washing process and became a dense mass unsuitable for growth	Sandy loam, no additional fertiliser	None
Sandy loam mix + sand	Is this substrate conducive to wheat, clover, barley and OSR growth and do AMF colonise in this substrate?	Efficient plant growth, clear and abundant AMF colonisation, bacterial survival and recovery	Sandy loam, no additional fertiliser, quartz sand (3:1 loam mix:sand).	Plant growth Bacterial inoculants - bacterial survival for duration of the experiment AMF colonisation

Clay + sand	Do AMF increase the growth of Medicago, Wheat or Clover in clay substrate? Are clay granules suitable for AMF colonisation?	No AMF colonisation, not conducive to plant growth and extreme variation	Low NPK, attapulgite clay granules (Profile Green Grade) and quartz sand (3:7). Washed to remove soluble nutrients (pers. comm. Kettenburg et al).	Unsuitable for AMF colonisation - no AMF structures observed on any plant species Substrate was waterlogged, anaerobic and grew moss/mould Not conducive to healthy plant growth
Woburn soil	Do AMF increase clover biomass in field soil? Is there a difference in bacterial taxonomic composition on the rhizosphere of AMF roots vs non-AMF plants?	No change in biomass *(F. geosporum*), but abundant AMF colonisation, very significant change in bacterial community when AMF are inoculated (Section 5)	Low nutrient, bare fallow soil from Rothamsted field site in Woburn. Full microbial community. Dried and sieved. Nutrient data given below.	Contains indigenous AMF at low level. AMF colonisation is abundant. Contains full bare fallow microbial community Conducive to plant growth Cannot manipulate nutrients but possible to add fertiliser to study the effects
Barnfield soil	Do AMF increase clover biomass in (low P, grassland) field soil? Is there a difference in bacterial taxonomic composition on the rhizosphere of AMF roots vs non-AMF plants?	No change in biomass *(F. geosporum*), but abundant AMF colonisation, very significant change in bacterial community when AMF are inoculated (Section 5)	Low P, grassland soil from Rothamsted site in Harpenden. Full microbial community. Dried and sieved. Nutrient data given below.	Contains indigenous AMF at low level. AMF colonisation is abundant. Contains full bare fallow microbial community Conducive to plant growth Cannot manipulate nutrients but possible to add fertiliser to study the effects

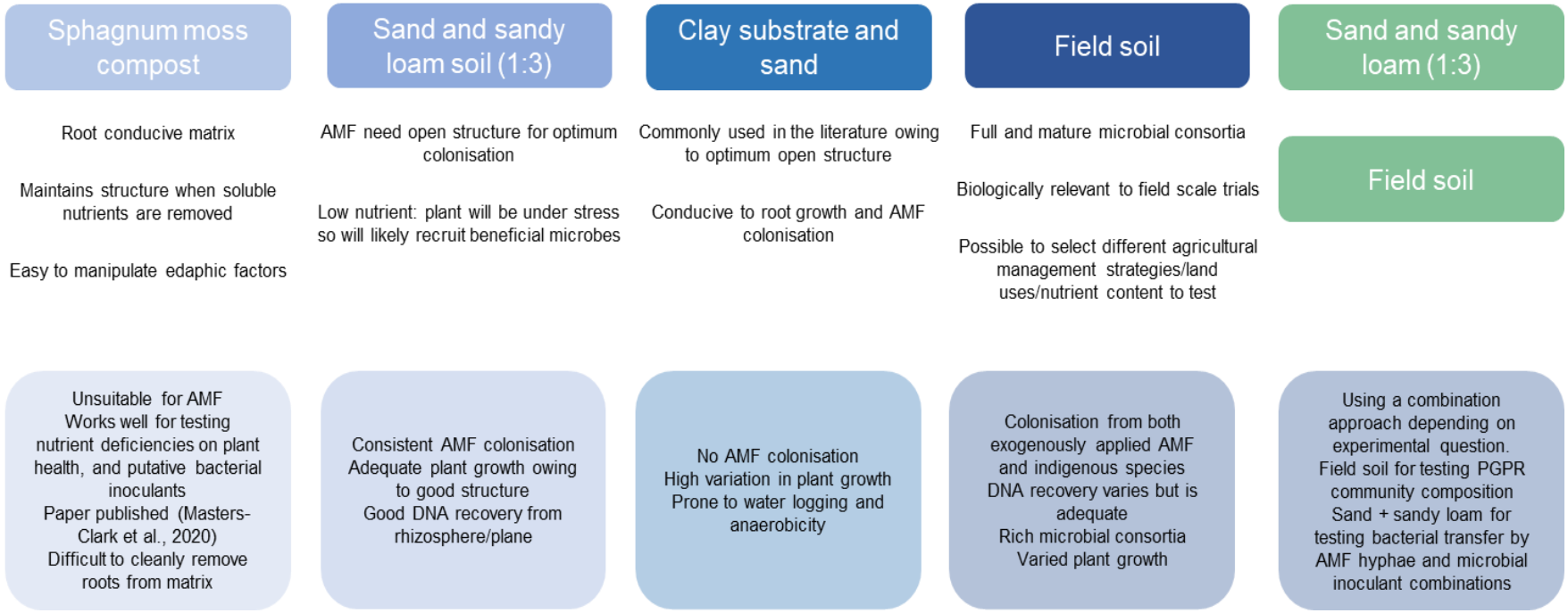


Figure 4.1. Decision flow chart depicting the trialled substrates and the rationale behind their selection. Green boxes represent the chosen substrates for the continuation of the experimental work. Outcomes from pilot experiments are given in the summary.

4.3.1 Chemical analysis of the selected substrates.

Woburn soil was analysed prior to this research and was contributed through a personal communication from a colleague (M. Abadie, with permission). All soil was analysed by the Rothamsted analytical unit. Details of sample preparation are given in Section 3.4.

Woburn soil has the lowest available P (phosphate) at 20.8 PPM. The loam substrate and Barnfield soil were comparable at 68-65 PPM respectively. Barnfield soil is maintained as a low P system, with a total P content of 673.86 PPM. Typical ranges of NH_4-N for soils are 2-10 PPM, and around 0.1-0.15% N (Marx, Hart et al. 1996, Horneck, Sullivan et al. 2011). The NH_4-N values for the measured soils are: Barnfield – 1.67 PPM, Sandy loam – 12.9, Woburn – 0.46 PPM. The NO_3-N values for the measured soils are: Barnfield – 0.38, Sandy loam – 11.62 PPM and Woburn – 0.51 PPM. Hence, Woburn can be considered to have very low available N.

Barnfield soil has the highest percentage of total carbon, which can be attributed to its cultivated nature and grassland land use type compared to the bare fallow field soil and the simplified loam and sand substrate (Hirsch, Jhurreea et al. 2017, Chen, Wang et al. 2018). The DEFRA index scale ranks for Olsen P for the tested soils are: Woburn (2), Barnfield (4) Sandy loam (4).

Table 4-2. Nutrient analyses of the three selected substrates. Values are in PPM dry soil except where % total N and C. Values are missing from Woburn as the traces analysis was not done for this soil. NO_3, NH_4 and PO_4 are converted from NO_3-N, NH_4-N and PO_4. Analysis was carried out by Rothamsted analytical unit, n = 4.

Analysis	Sandy loam:sand (3:1)	Barnfield	Woburn
Olsen P (PO_4)	68.39	65.64	20.8
NO_3	51.46	1.41	2.33
NH_4	16.61	2.14	0.6
Fe	53010.91	35732.97	
K	2033.82	3049.41	
Mg	1887.97	2672.11	
P	556.70	673.86	
Zn	60.70	82.82	
%N	0.09	0.19	0.07
%C	0.95	2.12	0.87

4.3.2 Selection of plant host.

The choice of host plant was made difficult by the requirements of AMF and its proclivity for some plants more than others, leading to varying rates of colonisation or differing levels of beneficial effects. It was important to select a host plant that is receptive to the benefits of AMF and demonstrates a responsive phenotype. The downstream processing of the host plant should be amenable to staining and DNA extraction, and able to host a diverse and dynamic rhizosphere community of microbes. Different crop species were trialled during the development stages of this project (Table 4-1), with a view to selecting a cereal or grassland crop that would benefit from AMF and PGPR addition. During the testing of the compost system, five plant species were tested, and while these showed dramatic nutrient starvation phenotypes, the unsuitability of the compost for the use of AMF became apparent after many attempts to establish AMF colonisation of host roots. Wheat was the test subject through many different

iterations of potential substrate tests, with the hope that once a suitable system was developed, its growth could be augmented by exploitation of AMF-PGPR interactions. As such an important cereal crop, and a well-defined microbiome, wheat was considered to be an exciting approach to apply the proposed bioinoculants. However, an AMF phenotype was not obtained, and colonisation was inconsistent and often absent.

White clover (*Trifolium repens*) yielded consistent colonisation and is a model legume species. Legumes require a high level of P (Hill, Simpson et al. 2006, Haling, Yang et al. 2016). Therefore, it was hoped that they would generally exhibit recruitment of AMF. Growth of clover in the sandy loam substrate with an AMF inoculant consistently increased biomass and colonisation. This biomass increase was not obtained in the field soils, but colonisation with the inoculant remained high (see: Sections 5 and 7). White clover has a fast life cycle, it is easy to grow in a variety of substrates and is a common constituent of grassland pasture (Caradus, Woodfield et al. 1995). However, its genetic variability is high which is important to note in biomass measurements (Kölliker, Jones et al. 2001).

4.3.3 Compost system.

The initial approach of a “one-size-fits-all” system using a sphagnum peat moss-based system was successful for all factors except AMF colonisation. It could be manipulated in its physical structure, chemical composition and sterilised to remove its microbial community, and was conducive to root growth and bacterial survival. However, despite many iterations, it was not suitable for AMF colonisation and therefore its use was discontinued for tripartite studies. Its uses for implementing nutrient stress on a plant or for screening putative bacterial inoculants can be found in Figure A-1 (Masters-Clark, Shone et al. 2020). It’s unsuitability for AMF colonisation were attributed to high organic matter content, humic acid interference (M. Vosatka, pers. comm.) and the inhibitory effect of peat moss on colonisation (Linderman and Davis 2003).

4.3.4 Chemical amendments.

Being able to manipulate a system's physiological, chemical, and biological properties was key to have its suitability for testing the efficacy of putative microbial bioinoculants to mitigate biotic and abiotic stress for a host plant. Another important reason for being able to control conditions such as nutrient content, structure, or water level is that AMF in particular are sensitive to changes in nutrient or water content (Ryan, Small et al. 2000, Zaller, Frank et al. 2011, Orchard, Standish et al. 2016). These can cause a decrease in colonisation, or induce a fitness cost of the mutualism to the plant if the plant is nutrient replete (Verbruggen, van der Heijden et al. 2013). Several methods of adding nutrients, either as a liquid feed or as a single starting dose were trialled and again, suitability depended on the substrate and the hypothesis.

As bacteria were screened *in vitro* for plant growth promoting ability, it was important to use the same assay components when testing the putative inoculants *in planta*. Specifically, chemicals such as tricalcium phosphate ($Ca_3(PO_4)_2$) were added as an insoluble, inorganic form of P that was, for the most part, inaccessible to plants without the help of solubilising microbes.

Additionally, to examine the taxonomic composition of the root microbiome of clover in both fertilised and non-fertilised low P field soil, an approach using slow release NPK fertiliser (osmocote) was used to be similar to that of commercial farming (Reid, Kavamura et al. 2021). This was important to emulate the impact of concentrated chemical fertiliser on the microbiome, and how the capacity of the microbiome to benefit plant growth might be affected (see: section 5).

Another method used to alter nutrient content of the trialled substrates was nutrient solutions. These were Hoagland's and modified Letcombe's solutions. The contrasting merits of these are discussed in Masters-Clark, Shone et al. (2020). These were best used to achieve a specific nutrient deficiency of one macronutrient at a time while replacing the rest of the essential nutrients needed to sustain plant growth.

4.4 Conclusion

The study of a tripartite interaction has many caveats. Satisfying the needs of both host plant and microbial partners is complicated and must be done in a way that closely replicates biologically relevant conditions in order to study the true form of these interactions. However, to investigate the fundamentals of the relationships between microbial partners, a simplistic system is required. Edaphic factors should not confound results and as many variables as possible must be controlled or standardised, to form the basis of reliable and replicable experimentation. Many substrates and systems were tested for this proposed research, and while there is no “one size fits all” solution, it is clear that some testing systems are more suitable than others for specific research. Using both field soil, simplified substrates, and different experimental set ups for each individual question allows a more versatile and dynamic approach to test these hypotheses. Defining and accepting the limitations of each system and substrate has been essential to the timely continuation of this research – development of the perfect system alone could be a lengthy preoccupation and a substantial body of research.

5 Do arbuscular mycorrhizal fungi influence rhizobacteria taxonomic structure and function?

This experimental chapter is presented in paper format.

5.1 Abstract

Arbuscular mycorrhizal fungi are the main beneficial plant-fungal interaction, and their use as a putative bioinoculant is gaining traction as a method of sustainable crop enhancement. Interactions between AMF and the root microbiome remain undefined. Understanding their influence on rhizobacteria communities is a significant step towards defining soil health in the context of sustainable agriculture. Particularly, characterising the impact of applying exogenous mycorrhizal inoculant, and examining their capacity to remain plant beneficial in managed agricultural systems. This work reveals the effect of two species of AMF inoculant on plant health and their associated rhizobacteria communities. This is further explored using soil collected from two land use types (grassland and bare fallow), with the addition of NPK fertiliser. The communities of root-associated bacteria are described using 16S rRNA gene sequencing and functional assays; related to plant biomass, nutrient content and AMF colonisation when AMF and fertiliser are included. The main driver of changes in the functional capabilities and taxonomic assemblage of rhizobacterial communities was soil type, followed by the effect of fertiliser use and root fraction (rhizosphere and rhizoplane). *F. geosporum* restructures the taxonomic composition of rhizobacteria on clover roots but does not similarly affect the functional capacity. There was an interactive effect of AMF and fertiliser use on plant health measures, becoming less advantageous when used in combination. The beneficial effect of AMF is subject to many external factors, which must be considered before its deployment as a bioinoculant.

5.2 Introduction

Climate change and concurrent population growth are the biggest challenges facing agriculture. Following the green revolution, yields have plateaued, and artificial fertilisers are becoming more expensive to produce due to rising energy and environmental costs (Savci 2012, Thirkell, Charters et al. 2017). To meet the global goals of sustainable intensification of agriculture, while mitigating the effects of climate change, alternatives to these essential agricultural additives must be explored.

Soil health is becoming an increasingly popular term in sustainability research. Soil health is defined as the continuing ability of soil to support our needs in terms of agricultural output and ecosystem services (Doran and Safley 1997, Veerman, Pinto Correia et al. 2020). In order to preserve soils and protect their function as a matrix for crop growth, we must understand the networks and processes upon which healthy soil relies. It provides essential ecosystem services, such as carbon sequestration, nutrient cycling and nitrogen fixation.

The soil hosts a complex, dynamic and ecologically significant community of microorganisms. The intricate relationships and interactions between microbes are gaining repute as a vital reservoir of ecological and agricultural function that must be conserved. Soil microbes have diverse roles within agricultural systems and are responsible for the biogeochemical cycling of both organic and inorganic nutrients (Jeffries, Gianinazzi et al. 2003). They can increase nutrient availability, enhance plant protection against pests, provide water and decontaminate heavy metals (Marschner, Crowley et al. 2011, Chitarra, Pagliarani et al. 2016, Singh, Singh et al. 2016, Barnawal, Pandey et al. 2017). Their capacity to ameliorate plant health is an exciting prospect for sustainable agriculture, potentially reducing dependency on artificial inputs such as pesticides or fertilisers.

The rhizosphere is defined as the area of soil directly affected by the root, and is affected in its chemistry by root exudates, such as protein, sugars and other phytochemicals, and sloughed plant cells, known as rhizodeposition (Hiltner 1904). Microbes consume and respond to root secretions, interacting with the plant and cycling nutrients. There are many beneficial bacteria associated with

crop plants. Bacteria such as *Pseudomonas*, *Bacillus* and *Arthrobacter* have all been purported to have roles in enhancing crop growth (Sandhya, Ali et al. 2010, Wahyudi, Astuti et al. 2011, Barnawal, Bharti et al. 2014).

Fungi also have diverse roles within the soil, but the main beneficial plant-fungal interaction is with arbuscular mycorrhizal fungi (AMF), which form associations with 80-90% land plant species (Bonfante and Genre 2010). AMF extend the host plant root system, sending out filamentous hyphae which explore the bulk soil and provide water, nutrients – primarily P and K, and resistance to biotic and abiotic stresses (Figure 5.1). In return, the host plant trades fixed carbon from photosynthesis.

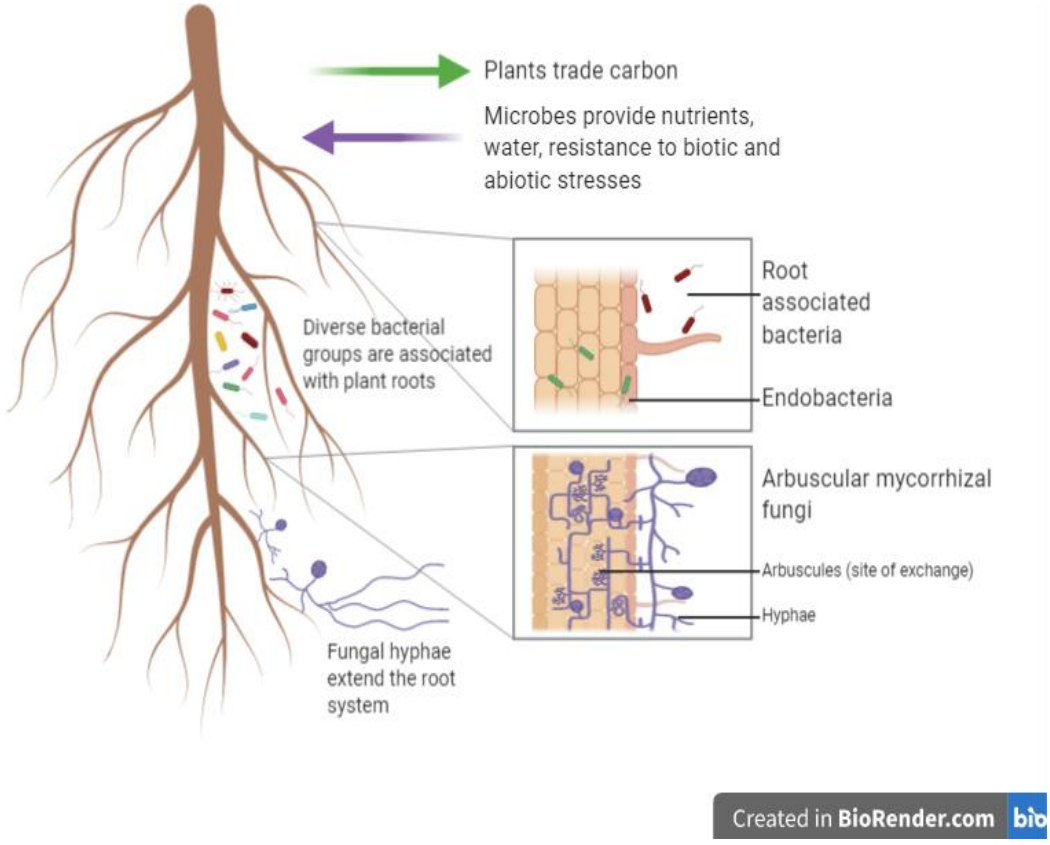


Figure 5.1. Schematic of rhizosphere bacteria and arbuscular mycorrhizal fungi interacting with the host plant root. Many different methods of plant-bacteria interaction occur. Root associated bacteria and endobacteria are shown here. Bacteria can form associations on the plant roots or within plant roots. These interactions can be beneficial or pathogenic. Plant growth promoting rhizobacteria can enhance nutrient availability and provide resistance to stresses in exchange for carbon in the form of sugars. Arbuscular mycorrhizal fungi colonise plant roots, penetrating the root cortex to form intracellular arbuscules which facilitate exchange of nutrients. The plant trades fixed carbon from photosynthesis for nutrients, primarily P and water. The fungal hyphae extend the root system into the bulk soil, dramatically increasing surface area: volume ratio and exploring a larger area of soil for nutrient patches and moisture.

As AMF and root-associated bacteria are such prominent components of the host plant rhizosphere, it is likely that the microbial constituents strongly influence and interact with each other (Adesemoye and Kloepper 2009). AMF have been shown to significantly alter bacterial taxonomic abundance and community structure (Nurmiaho-Lassila, Timonen et al. 1997, Vázquez, César et al. 2000, Roesti, Ineichen et al. 2005, Singh, Nunan et al. 2008). Additionally, bacteria appear to be essential for AMF functioning and vitality (Lumini, Bianciotto et al. 2007, Salvioli, Chiapello et al. 2010, Salvioli, Ghignone et al. 2016). Understanding these interactions in the context of plant health, and how they are influenced by edaphic factors such as soil fertility status and agronomic inputs, will help to understand the balance of processes that contribute to soil health and how best we can preserve, and harness, these interactions to enhance crop growth.

Current knowledge surrounding the interactions between the microbial constituents of the rhizosphere is lacking. However, next-generation sequencing technologies can precisely profile microbial community taxonomic composition. Recently, these techniques were used to identify significant changes in bacterial community structure when AMF were permitted or not into a patch of root-free organic P fertilised soil (Zhang, Shi et al. 2018). Bacterial communities were also shown to be strikingly different on the hyphal surface compared to the bulk soil.

5.3 Aims and objectives

This chapter aims to characterise the influence of two species of AMF, *Funelliformis geosporum* and *Rhizophagus irregularis*, exogenously applied as an inoculant, on existing bacterial communities on clover roots in field soil. This is explored further by using two soil types from different land uses (grassland and bare fallow), and with the addition of NPK fertiliser.

The following experiments aim to characterise the influence of exogenous AMF application on plant health and microbial community responses. The intention is to reveal how these communities might shift in terms of taxonomic diversity,

abundance and function when exposed to an edaphic perturbation, NPK fertiliser. Understanding how inoculants affect the microbial inhabitants of field soil, and how in turn, the use of agrochemicals affects inoculant efficacy, we can inform agricultural decision making and coerce inoculants to perform optimally.

The experimental aims are as follows:

1. Pot trials using Woburn bare fallow soil to provide a full microbial community. Clover were grown with and without exogenously applied *F. geosporum* (a common, generalist AMF species found in most soil types). The aim was to ascertain if an AMF inoculant influences the rhizobacteria taxonomic assemblage, described using amplicon sequencing.
2. Pot trials using Woburn bare fallow soil as above. Clover were grown with and without exogenously applied *R. irregularis* (a strain isolated from a salt mine, likely to have some specialist adaptations). The purpose of testing such a unique isolate was to reveal if there were species differences between AMF-mediated modification of the rhizobacteria community structure, and host compatibility within agricultural soils.
3. A third experiment was designed to further investigate the effect of *F. geosporum* on clover-associated rhizobacteria in an agricultural context. Two agronomic amendments, NPK fertiliser and *F. geosporum* inoculant, were added to clover plants separately and in combination to test the effect of these additions on bacterial taxonomic composition and functional capabilities. Two soil land use types were used – bare fallow and grassland – to reveal the effect of the agronomic inputs on different land uses. This was done with the aim of understanding how the effect of NPK and AMF inoculant use on rhizobacteria communities may differ by site, and also reveal their intended beneficial effects on plant health.

5.4 Materials and Methods

Three separate pot trials are presented in this chapter. They are designed as follows:

1. Clover plants grown in Woburn bare fallow soil, with either *F. geosporum* AMF inoculum, the AMF free carrier substrate, or no additions (n = 6)
2. Clover plants grown in Woburn bare fallow soil, with either *R. irregularis* AMF inoculum, the AMF free carrier substrate, or no additions (n = 6)
3. Clover plants grown in two soils, Barnfield (grassland) and Woburn, with either AMF inoculant (*F. geosporum*), NPK fertiliser, or AMF and NPK in combination (n = 7)

5.4.1.1 Soil preparation.

Refer to 3.2 and 3.3 for details of soil preparation. All experiments used Woburn bare fallow soil, or Woburn and Barnfield soil, which were sieved before potting.

5.4.1.2 Plant culture, harvest, and growth conditions.

Refer to 3.1 for detailed descriptions of planting and sampling.

Pots were set up in a standard glasshouse conditions (16h light, 8h dark, 21°C, 80% relative humidity) in uniform growing conditions and a complete randomised design, for all experiments, n = 6 for experiments 1 and 2, and n = 7 for experiment 3. Pots were 9x9x10cm and were filled with dry soil until ~1cm from the top.

AMF inoculum was added (species dependent on experiment) during potting: each pot was 2/3 filled, then ~5g/1 tsp inoculum was added and mixed to evenly distribute, then the final 1/3 soil added to the pot. In the case of the control treatments, the AMF-free carrier substrate of the inoculum was added, or no additions were made at all. The AMF-free substrate was included as a control to match the AMF treatment in terms of background additional taxa introduced by the substrate, and also reveal if any plant health advantage was gained from its addition. The AMF inocula consisted of the same carrier substrate with viable spores, hyphae and axenic roots from the *in vitro* growth system. The AMF species chosen were *F. geosporum* (BEG199) and *R. irregularis* (BEG145

SAMP7), provided by Symbiom as single isolates produced *in vitro*. The *F. geosporum* isolate was chosen as it is a commonly found species, present in many different terrestrial environments and constituted a suitable “generalist” species to include. The *R. irregularis* isolate is considered a “specialist”, recovered from a salt mine. It’s inclusion in these experiments was to probe if its adaptation to its specialised ecological niche would impact its ability to be beneficial in field environments and reveal if it had interesting attributes in terms of its interactions with the rhizobacteria communities. An experiment was carried out using combinations of salt tolerant bacterial isolates and *R. irregularis* which was deemed outside of the scope of this research (Figure A-6).

In experiment C, NPK fertiliser was added as osmocote (Scotts, standard NPK 15+9+11+2MgO) at 2.5g per 500g soil. Osmocote was distributed evenly over the top of the soil after one week of seedling growth.

5.4.1.3 Sampling of pot experiments.

Refer to 3.6 and 3.7 for detailed sampling methods. In experiment 1 and 2, rhizoplane samples only were sampled and sequenced whereas in experiment 3, rhizosphere and rhizoplane samples were taken.

5.4.1.4 DNA extraction and preparation of samples for sequencing

Refer to 3.8 and 3.12 for detailed methodology. In brief, DNA was extracted and quantified, before 16S rRNA gene amplicon sequencing using Illumina MiSeq. All samples were diluted to 30ng/µl using molecular grade water. For experiment 1, ITS sequencing was also carried out. This proved superfluous to the experimental question and was inconclusive for AMF quantification, so has been omitted from this work. A discussion of AMF quantification methodology is included in the general discussion (Section 8.5).

5.4.1.5 Bioinformatic processing of sequencing data.

The general protocol for bioinformatic processing of sequencing data can be found in 3.12. For experiment 3, 7 outliers identified from taxa bar plots were removed before analysis.

5.4.1.6 AMF quantification via the gridline intersection method.

Refer to 3.13. Colonisation was assessed using the gridline intersection method, with a minimum number of intersects at 50 and an average of 87, 92 and 86 for experiment A, B and C respectively (Ambler and Young 1977).

5.4.1.7 *In vitro* assessment of plant growth promotion capabilities of rhizoplane bacterial communities.

In depth protocols are given in 3.10 and 3.11. In brief, this protocol is adapted from Reid, Kavamura et al. (2021). Samples were selected in a random order and labelled blind to prevent bias. Rhizoplane glycerol stocks were diluted to 10^{-4} with sterile dH_2O. 100µl of each sample was spread onto 1/10 TSA Petri dishes with five technical replicates and plates were incubated for three days at 25°C. Single colonies were picked into 96 well plates containing 200µl 1/10 TSB, with one colony in one well, and one sample to one 96 well plate (~20 colonies per technical rep). Two wells were left blank for negative controls. These were incubated for two days at 25°C. Using a sterilised 96 prong inoculation manifold, cultures were placed onto various functional assay media, enabling the screening of six samples per 25 x 25 cm plate. These were incubated at 25°C for various lengths of time dependent on the assay:

In brief, the selected media were: Pikovskaya's agar (P solubilisation, PVK), Aleksandrovs' agar (K solubilisation, ALK), CAS agar (siderophore production/iron chelation, CAS), Casein agar (protein hydrolysis/N solubilisation), Zinc agar (zinc solubilisation), $AlPO_4$ (Aluminium phosphate solubilisation), $FePO_4$ (Iron phosphate solubilisation), and phytate media (PHY).

Colony assessments were carried out after the appropriate incubation time and were based on a positive/negative outcome. The various assays presented "positive" for the ability of interest in different ways, such as a halo or zone of clearing, colour change or simple colony growth. Details of exemplar positive and negative outcomes for each assay are given in methods 3.11. Images and colony counts were taken for each plate.

5.4.1.8 Statistical analysis.

For continuous outcome variables such as biomass and plant nutrient content, analysis of variance was used, carried out in R studio (version 4.0.2) once tests of normality, such as Shapiro-Wilk tests and exploring residual plots, had been satisfied.

To analyse the next-generation amplicon 16S rRNA gene sequencing data from all experiments, the package “Phyloseq” (McMurdie and Holmes 2013) was used with R version 4.0.2, using DeSEQ2 to normalise the data using the median sequencing depth (Love, Anders et al. 2014). Detailed statistical protocol for analysis of amplicon data is given in 3.12.

Generalised linear models were used to analyse both the percentage colonisation data and the bacterial functional assays in Genstat (21st Ed. 21.1.1). Residual plots were created for each outcome variable and showed that the underlying assumptions of the following tests were met. The analysis for each functional assay was a binomial intra-block generalised linear model, back transformed on the logit scale. When there was more than one order of fitting (for experiment 3) marginal and conditional (both including and excluding higher order terms) tests were used to screen data for consequences of the order of fitting (using the command rscreen). If there was only one order of fitting (experiment 1 and 2) terms were fitted explicitly instead of using rsceen.

The predictions are presented as the back-transformed values from the logit scale and include 95% confidence intervals. When there was evidence of overdispersion (residual of the mean deviance >1, tests were done using deviance ratios (F statistics) and the standard errors adjusted accordingly. Only significantly different predicted means for the treatments and interactions are displayed as figures. The order of fitting for experiment 3 was shown to be inconsequential for the AMF colonisation data, thus the initial model was used for all reported statistical values for percentage colonisation: AMF x NPK x Soil. For the functional assays, the order of fitting was important for two tests. For the majority, soil type was the main effect so the model Soil x AMF x NPK was fitted

and the statistical values are reported from this order of fitting unless stated otherwise.

5.5 Results

5.5.1 Experiment 1: Clover plants grown in Woburn bare fallow soil, with either *F. geosporum* AMF inoculum, the AMF free carrier substrate, or no additions

The biomass of clover inoculated with *F. geosporum* was not influenced by the addition of AMF, or the AMF-free carrier substrate (Figure 5.2, p = 0.3). The percentage of roots colonised with AMF was highest when the exogenous AMF inoculant was applied (Figure 5.3, $F_{2, 15}$ = 7.45, p = 0.006). The average percentage colonisation of the roots was 57.5%, 23.9%, and 23.6% for AMF, AMF-free substrate, and the no additions control respectively.

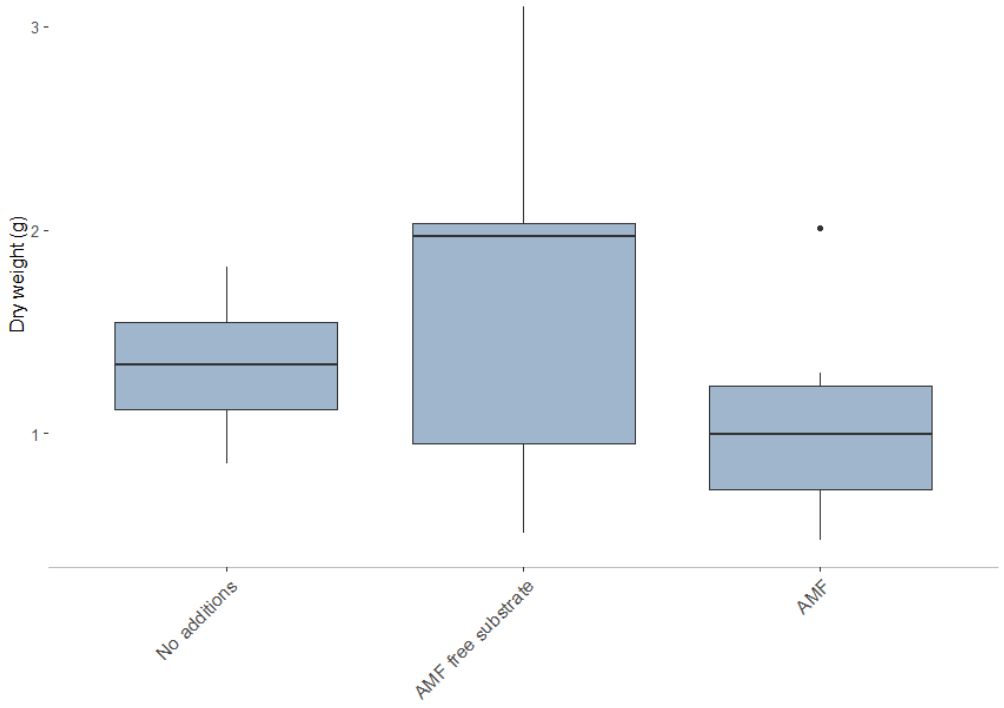


Figure 5.2. Application of *F. geosporum* does not affect the aerial biomass of clover (Woburn). Clover was grown in bare fallow soil with the following treatments: AMF – *F. geosporum* inoculum including carrier substrate, AMF-free substrate – AMF-free inoculum substrate, No additions – no inoculum or exogenous applications. Aerial biomass was weighed (g), and the mean plotted. Bars give standard error and n = 6.

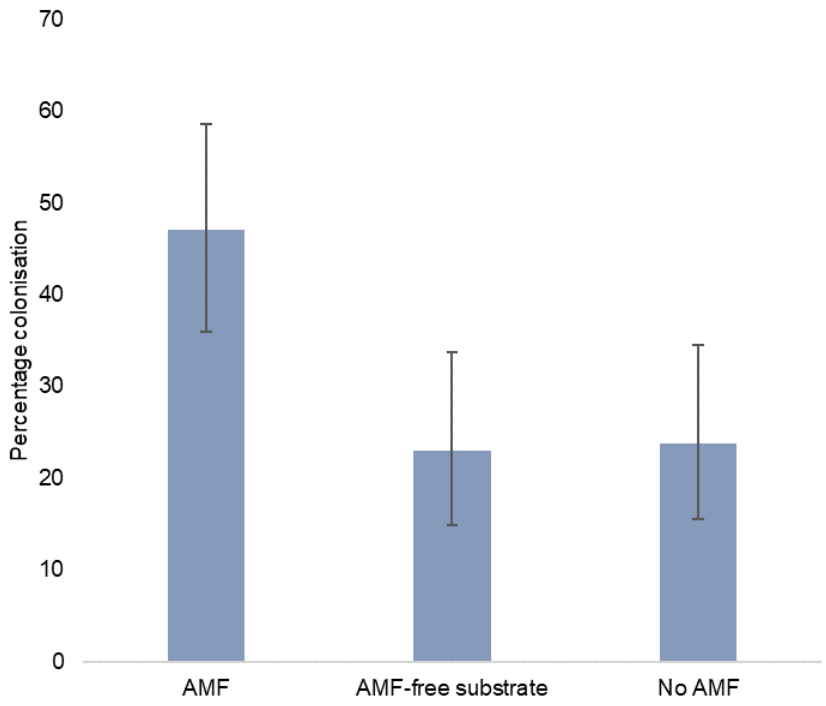


Figure 5.3. Predicted mean values for *F. geosporum* colonisation of clover roots. The mean values (proportion scale) of the percentage of roots colonised by AMF, back transformed from the logit scale, including 95% confidence intervals given by error bars.

5.5.1.1 16S rRNA gene amplicon sequencing of rhizoplane samples.

The distribution of taxa for the entire amplicon dataset is shown in Figure 5.4 displayed as the actual abundance of ASVs assigned to their corresponding phylum. In summary, the most abundant phyla are: Acidobacteria 14.3%, Actinobacteria 10.3%, Bacteroidetes 10.2%, Chloroflexi 7.0%, Firmicutes 2.9%, Gemmatimonadetes 2.8%, Proteobacteria 39% and Verrucomicrobia 3.4%.

Alpha diversity estimates showed that samples from each treatment were equally rich in taxa with a mean number of features of 748.1, Kruskall-Wallis chi-squared $p = 0.28$. Figure 5.5 shows Shannon's diversity index with a mean score of 5.93 and Simpson's evenness index with a mean score of 0.25. This included samples taken from bare fallow soil with no plant which had more observed taxa and greater diversity than rhizosphere samples.

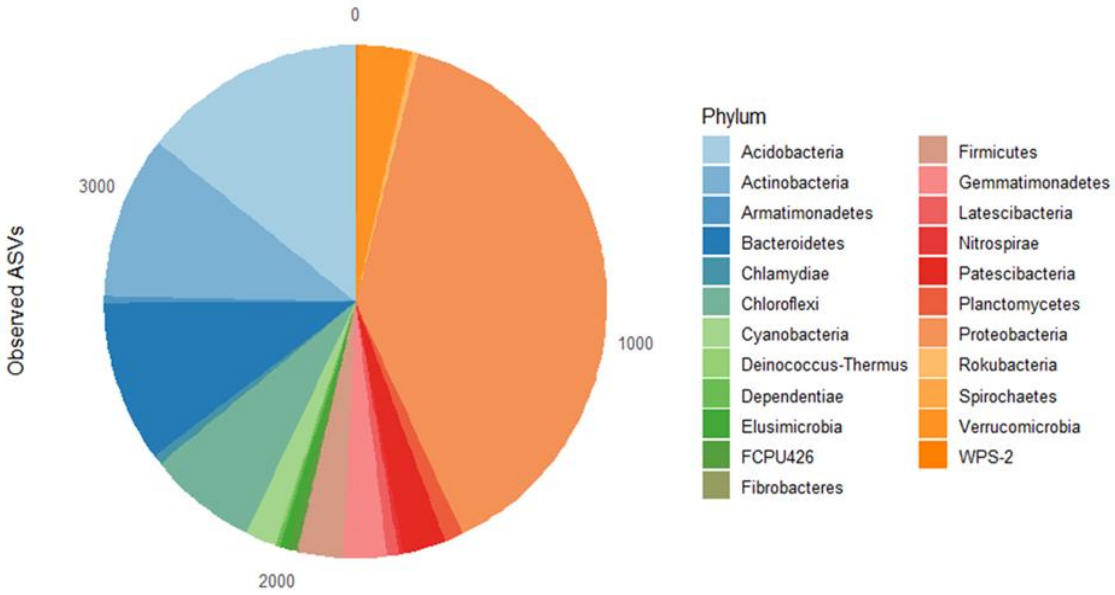


Figure 5.4. Actual abundance of ASVs aggregated by phylum for experiment 1. Counts for each unique ASV subsetted by its corresponding phylum. Pie chart shows the distribution of taxa for the entire amplicon dataset for *F. geosporum* experiment.

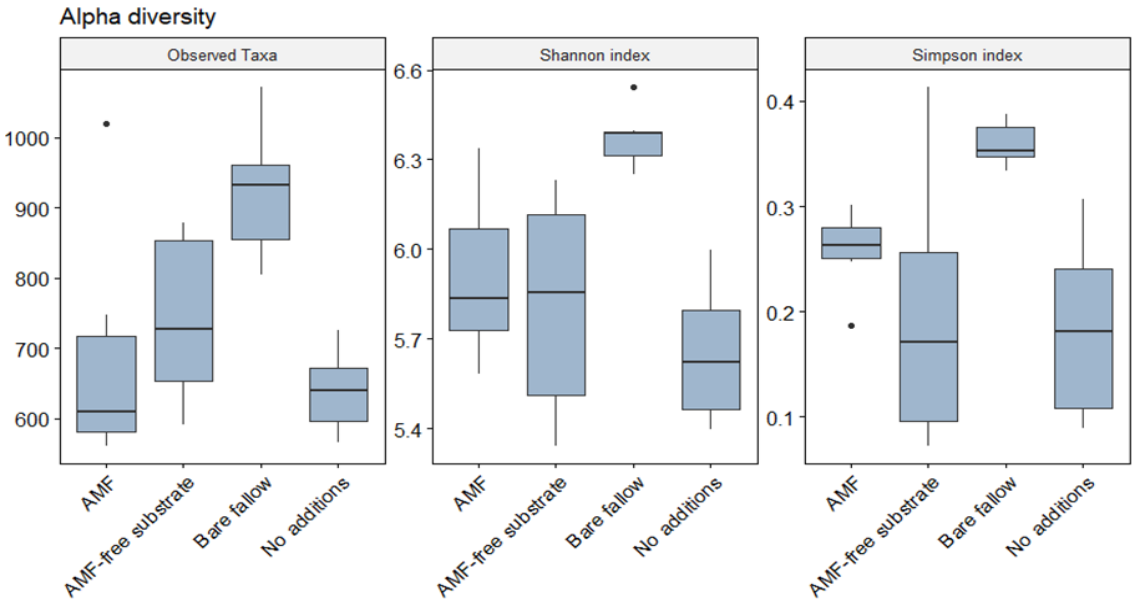


Figure 5.5. Estimates of alpha diversity in the root-associated bacterial community of clover for experiment 1. Clover was grown with either no additions, AMF inoculum (*F. geosporum*), or the AMF-free carrier substrate. Bare fallow soil samples maintained under the same conditions were also analysed. Figure shows the alpha diversity measures Shannon's diversity index and Simpson's evenness index, with number of observed taxa (unique ASVs). Bars represent standard error.

A principal coordinates analysis (PCoA) with a weighted UniFrac distance metric showed that taxonomic composition (beta diversity) is altered significantly by all three factors (AMF inoculation, AMF-carrier substrate addition and plant presence), shown in Figure 5.6 with ellipses clustering samples by treatment. PERMANOVA results are given in Table 5-1. AMF addition (R^2 = 0.06, p = 0.03) and the AMF free substrate (R^2 = 0.06, p = 0.028) affected the community composition equally, and as the AMF free substrate was included as the background of the AMF treatment, any taxa introduced as a consequence of the substrate are accounted for. Presence of a plant had the biggest influence on community structure compared to bare fallow soil (R^2 = 0.17, p = 0.001). Dispersion among groups (beta-dispersion) was homogeneous between both the AMF and AMF-free substrate treatments but significantly heterogeneous in the plant treatments (Table 5-1). The plant treatments include all the treatments except bare fallow, so within group heterogeneity is to be expected.

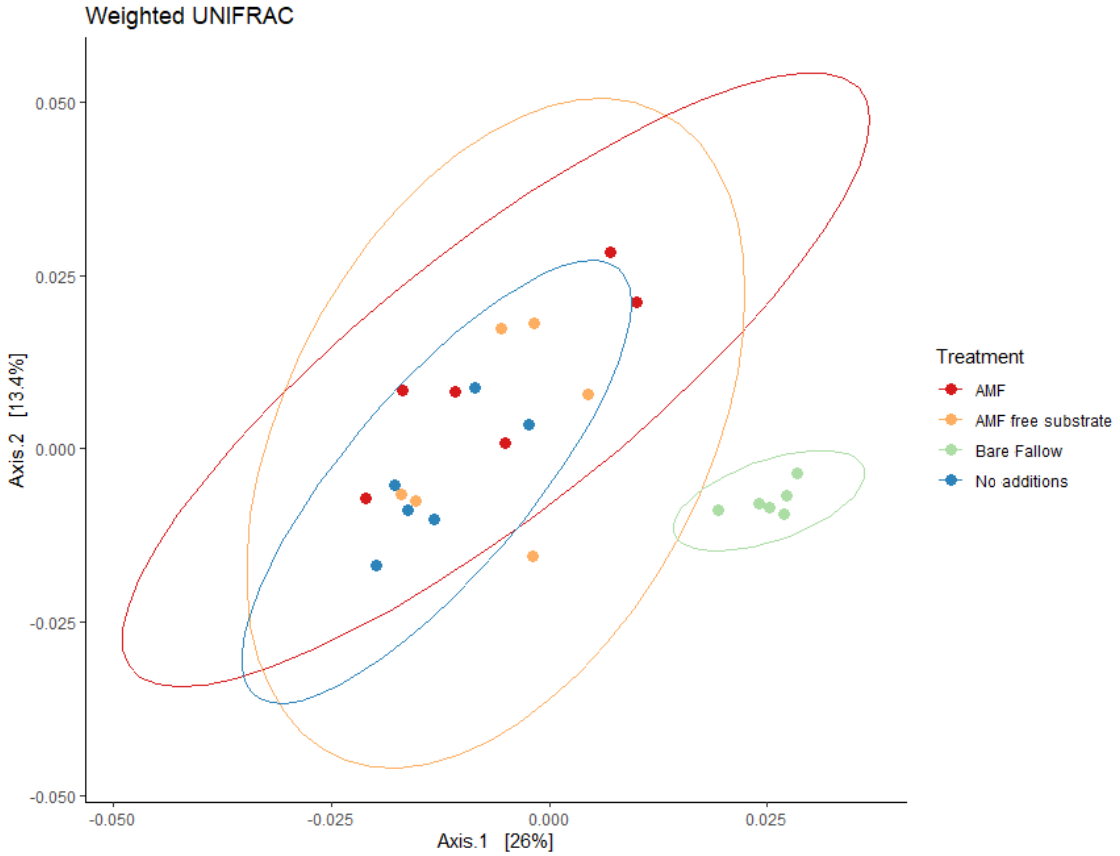


Figure 5.6. Estimates of beta diversity in the root-associated bacterial community of clover for experiment 1. Figure shows PCoA based on weighted UNIFRAC distance metrics. Plants were grown with either no additions, and AMF inoculant (*F. geosporum*), or AMF-free carrier substrate. Bare fallow soil was also maintained under the same experimental conditions for analysis.

Table 5-1. Comparisons of beta-diversity for *F. geosporum* inoculated plants and the respective controls. Bare fallow soil samples are included. Permutational analysis of variance used 999 permutations.

		adonis (permanova)			betadisper (variance of group dispersion)	
Factor	Df	F test	R^2	P	F test	P
AMF	1	1.9266	0.06605	0.034	0.4664	0.511
AMF-free substrate	1	2.0138	0.06904	0.028	0.0104	0.94
Plant	1	5.2281	0.17924	0.001	12.891	0.003
Residuals	20		0.68567			
Total	23		1			

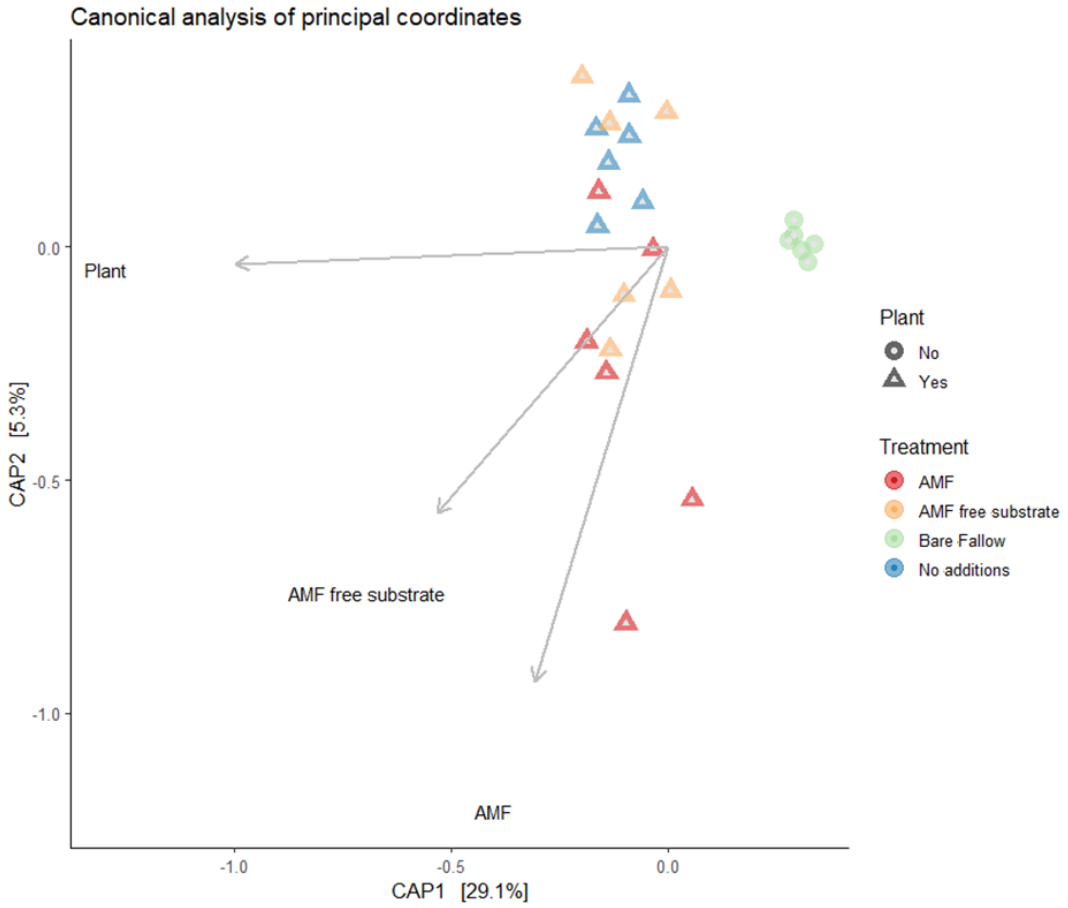


Figure 5.7. Canonical analysis of principal coordinates for ASV scores constrained by treatment for experiment 1. Scores were calculated from a weighted UNIFRAC distance measure. Colours are assigned to treatment and triangles denote samples taken from plant-containing treatments, circles represent bare fallow soil samples. The arrows point to the centre of the constrained factor. The percentage given in each axis refers to the proportion of the total variance of the data explained by each treatment factor. AMF inoculant is *F. geosporum*.

A canonical analysis of principal coordinates (CAP) was performed to be complementary to the PCoA described above, using the same distance measure (Figure 5.7). The permutational analysis of variance with 999 permutations gave a *p* value of 0.001, in agreement with the PCoA described above. This confirms the differences between groups are due to variation caused by treatment, and that none of the permuted datasets had a significantly different community assemblage than the origin dataset. The analyses are concurrent in that the plant effect is the main driver of variation in taxonomic composition, closely followed by AMF and AMF-free substrate.

The differential abundance of the top 25 most affected phyla are shown as a heatmap (Figure 5.8). Comparing the no additions treatment and the AMF-free substrate in the first instance to ascertain what bacterial groups are being applied via the carrier substrate, as these will also feature in the background of the AMF treatment. Overall the taxa are similar between the controls (AMF-free substrate and No additions), with slight differences in ASVs belonging to the family *Caulobacteraceae* (+0.6%), *Sphingobacteriaceae* (-0.3%), *Rhizobiaceae* (-0.8%), *Rhodanobacteraceae* (-1.2%), *Geobacteraceae* (+0.8%), *Roseiflexaceae* (-0.8), *Diplorickettsiaceae* (-0.8%) and *Holophagaceae* (+1.2%). Percentage change is given in relation to adding AMF free substrate to the no additions control. Thus, there are a small number of taxonomic changes caused by the control substrate.

AMF addition significantly enriches ASVs belonging to the families *Holophagaceae*, *Geobacteraceae*, *Sphingomonadaceae* and *Bulkholderiaceae* compared to the other treatments. Compared to the AMF-free substrate treatment, its closest control, AMF decreases the abundance of ASVs classified into the following families: *Deinococcaceae* (-1.5%), *Sphingobacteriaceae* (-1.6%), *Caulobacteraceae* (-1.6%) and *Burkholderiaceae* (-3.7%). AMF presence also enriches certain ASVs compared to the AMF-free substrate control, assigned to the following families: *Holophagaceae* (+1.1%), *Diplorickettsiaceae* (+1%), *Geobacteraceae* (+1.2%) and *Sphingomonadaceae* (+0.7).

Bare fallow soil has a significantly different taxonomic profile to any of the plant treatments. ASVs assigned to *Caulobacteraceae*, *Sphingobacteraceae*, *Rhizobiaceae*, *Rhodanobacteraceae*, *Geobacteraceae*, *Micrococcaceae* and *Deinococcaceae* are at a very low abundance when a plant is not affecting the system (0-0.7%). Additionally, ASVs belonging to *Bulkholderiaceae* and Sphingomonadaceae are relatively abundant, but significantly less prevalent when compared to the plant treatments. Conversely, ASVs classified in the following families are richer in bare fallow soil than plant affected soil: Acidobacteria (*Soilbacteraceae* subgroup 3 and uncultured), *Gemmatimonadaceae*, *Xanthobacteraceae*, *Bacillaceae*, Acidobacteria (*Soilbacteraceae*), Actinobacteria (uncultured), Proteobacteria (A21b) and *Nitrosomonadaceae*.

An ASV assigned to *Deinococcaceae* was the most differentially affected by plant presence, in the two controls (AMF-free substrate and no additions) which both contained a plant but no AMF, it is relatively abundant (1.9-%2.1%), but when AMF are present this is reduced to 0.4% and decreases further to 0% in bare fallow soil.

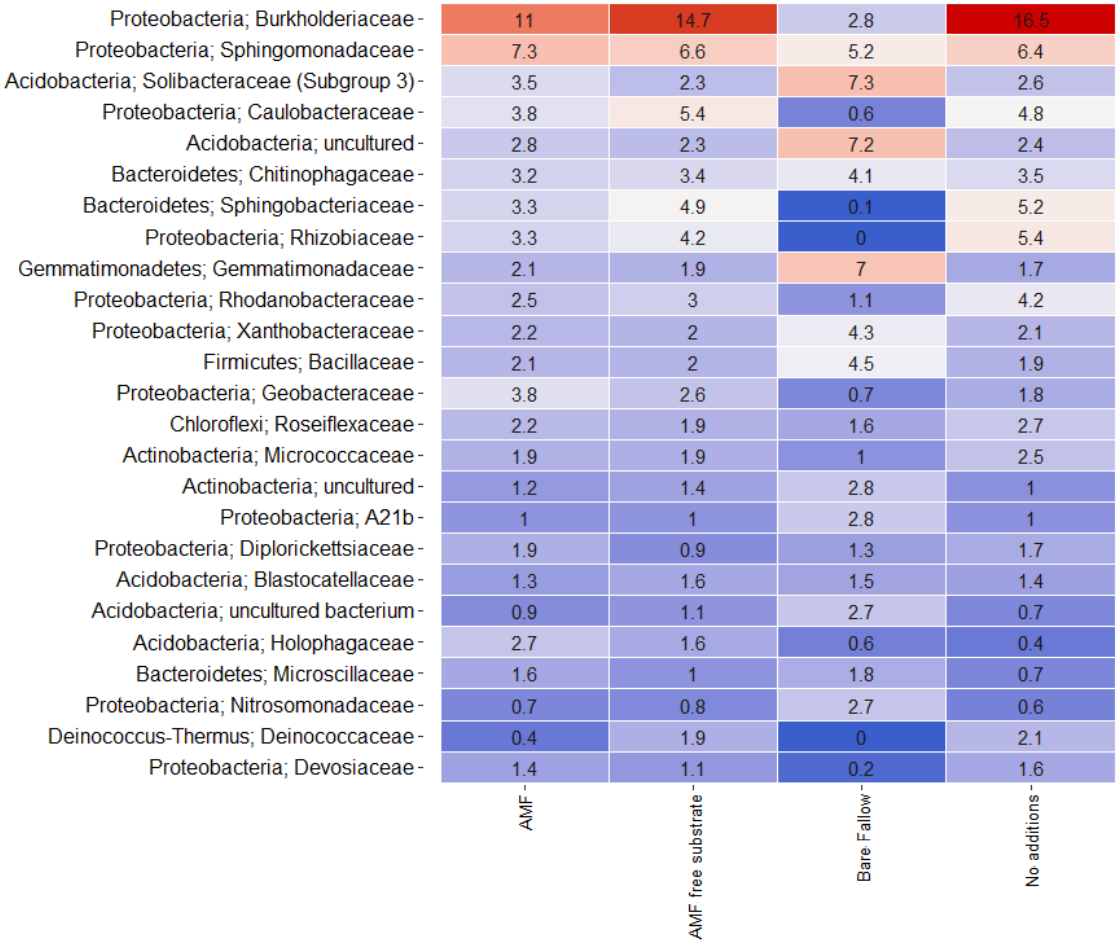


Figure 5.8. Heatmap showing mean relative abundance of the 25 most differentially abundant ASVs for experiment 1. Features are shown at phylum and family level in each treatment. Darker blue indicates a low relative abundance and red equals a higher relative abundance. AMF treatment is *F. geosporum*.

5.5.2 Experiment 2: Clover plants grown in Woburn bare fallow soil, with either *R. irregularis* AMF inoculum, the AMF free carrier substrate, or no additions.

The biomass of clover inoculated with *R. irregularis* in Woburn bare fallow soil was similar across all treatments, regardless of AMF inoculum or colonisation (p = 0.9, Figure 5.8). A generalised linear model showed that treatment made a significant difference to the percentage colonisation by AMF of clover roots ($F_{2, 15}$ = 6.68, p = 0.008, Figure 5.9). AMF treatment has around ~15% higher colonisation than either of the two non-AMF control treatments. Similar amounts of AMF colonisation were recorded in both control treatments due to indigenous AMF populations. There was no further colonisation when the AMF-free substrate

was added compared to the no additions control. An average of 36.14% of roots were colonised in the AMF treatment versus 20.3 and 21.1% in no inoculum and AMF-free substrate respectively.

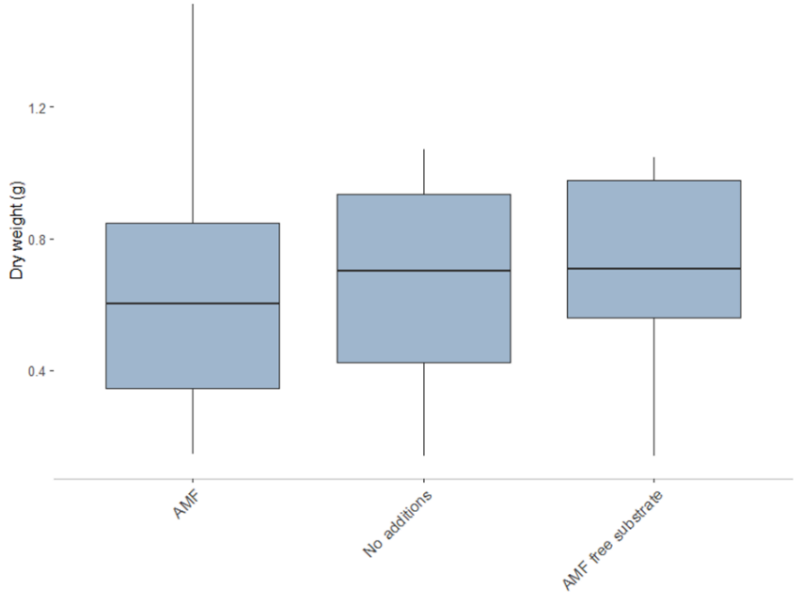


Figure 5.3. Application of *R. irregularis* does not affect the biomass of clover. Clover were grown in Woburn bare fallow soil with the following treatments: AMF – *R. irregularis* inoculum on carrier substrate, AMF free substrate – AMF-free carrier matrix (expanded clay zeolite), No additions – no inoculum or exogenous applications. Bars give standard error and n = 6.

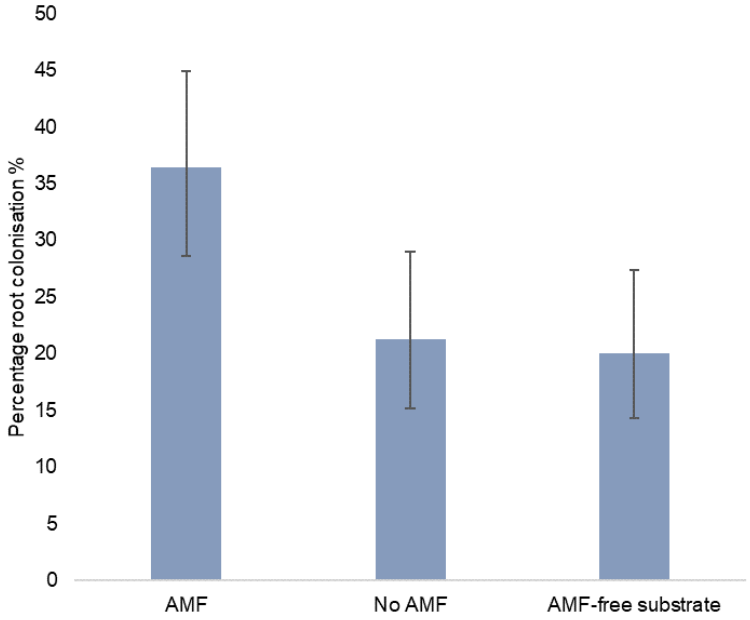


Figure 5.4. Predicted mean values for *R. irregularis* colonisation of clover roots. The mean values (proportion scale) of the percentage of roots colonised by AMF, back transformed from the logit scale, including 95% confidence intervals given by error bars.

5.5.2.1 16S rRNA gene amplicon sequencing of rhizoplane samples.

The distribution of taxa for the entire amplicon dataset is shown in Figure 5.10, displayed as the actual abundance of ASVs assigned to their corresponding phylum. In summary, the most abundant phyla are: Acidobacteria 11.1%, Actinobacteria 17.2%, Bacteroidetes 5.5%, Chloroflexi 7.8%, Firmicutes 2.8%, Gemmatimonadetes 3.5%, Planctomycetes 7.1%, Proteobacteria 32.3%, Verrucomicrobia 4.9%.

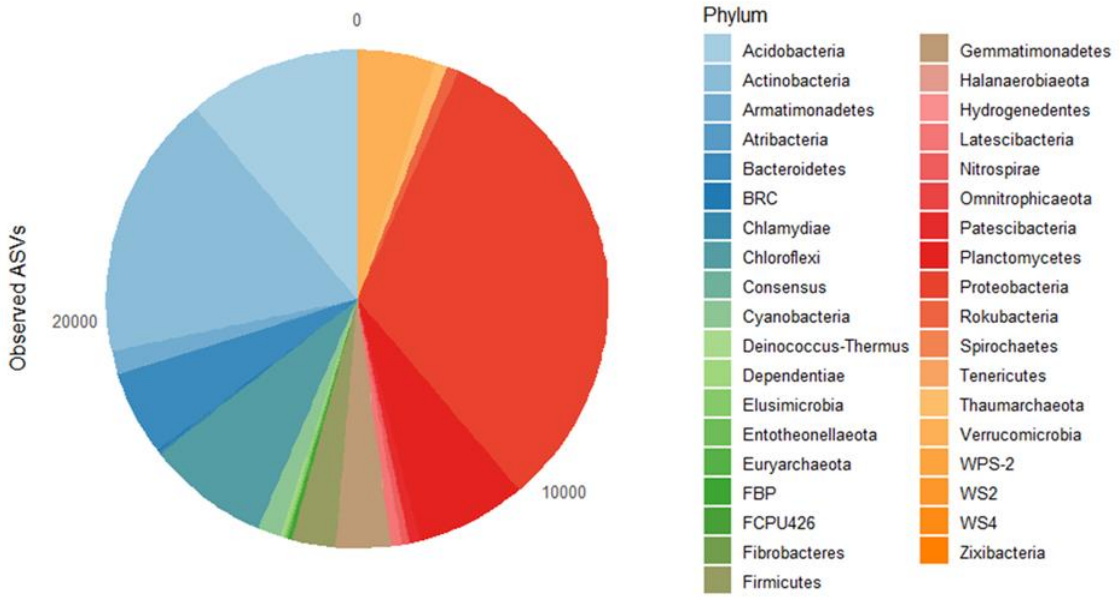


Figure 5.5. Actual abundance of ASVs aggregated by phylum for experiment 2. Counts for each unique ASV subsetted by its corresponding phylum. Pie chart shows the distribution of taxa for the entire amplicon dataset for *R. irregularis* experiment.

Amplicon sequencing of the bacterial 16S rRNA gene was used to reveal the taxonomic structure and individual abundance of the rhizoplane of clover, influenced by an AMF inoculant. A Kruskall-Wallis test showed that the within-sample evenness was comparable across treatments – samples were equally diverse in bacterial taxa. This includes bare fallow samples, without any host plant, but situated in the same experiment. The average observed taxa (number of unique ASV's) per sample was 145, with Shannon diversity index of 4.6 and a Simpson's evenness score of 0.6 (Figure 5.12). Bare fallow soil was as rich as

root samples in bacterial taxa. The AMF-free substrate did not enrich rhizosphere samples with a more diverse community, validating that this did not introduce additional bacteria and is the closest control to the AMF treatment as they have the same background inoculum substrate.

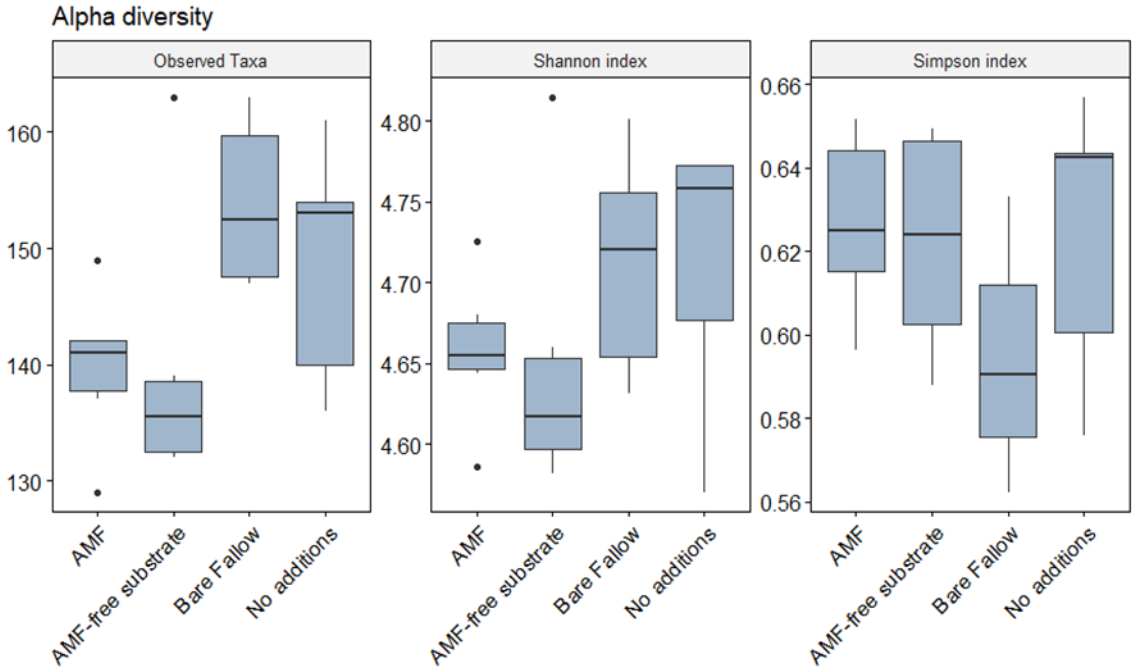


Figure 5.12. Estimates of alpha diversity in the root-associated bacterial community of clover for experiment 2. Clover was grown with either no additions, AMF inoculum (*R. irregularis*), or the AMF-free carrier substrate. Bare fallow soil samples maintained under the same conditions were also analysed. Figure shows the alpha diversity measures Shannon's diversity index and Simpson's evenness index, with number of observed taxa (unique ASVs). Bars represent standard error.

Examining beta-diversity, PCoA using a weighted UniFrac distance metric was used to show that there was a strong plant effect on the microbiome. Bare fallow soil had a significantly different taxonomic composition compared to treatments containing a plant (Table 5-2, R^2 = 0.08, p = 0.005), however it was equally diverse according to alpha diversity measures. Neither *R. irregularis* nor its carrier substrate influenced the community structure (Figure 5.13, Table 5-2).

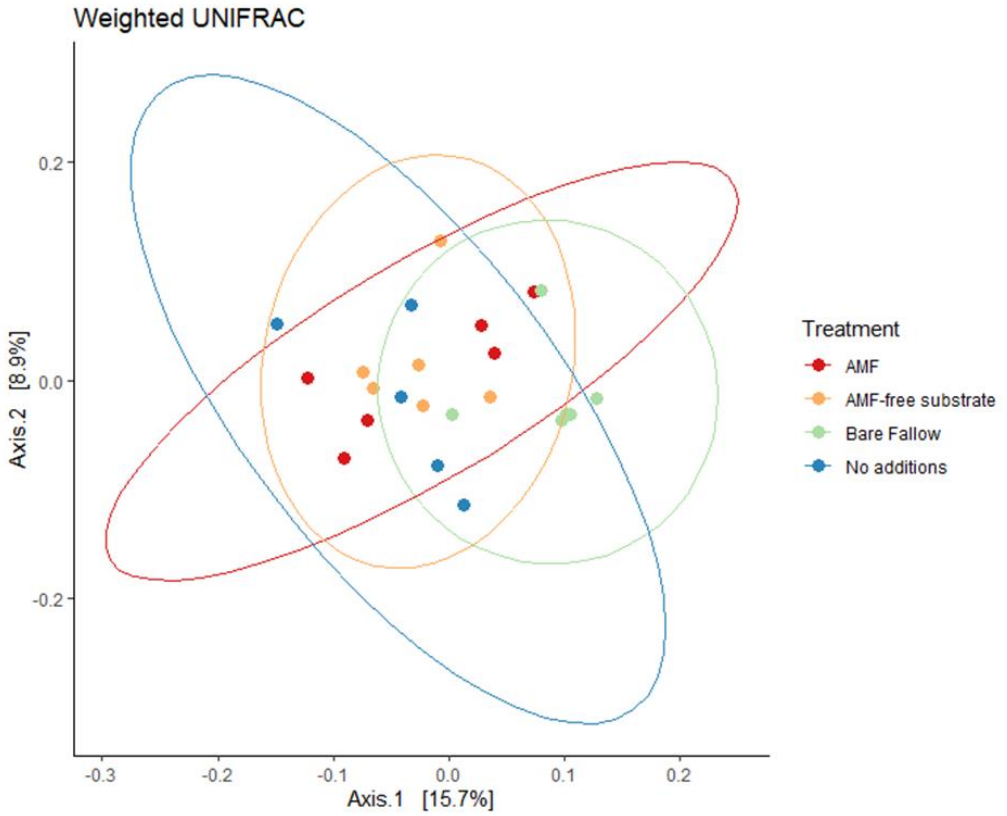


Figure 5.13. Estimates of beta diversity in the root-associated bacterial community of clover for experiment 2. Figure shows PCoA based on weighted UNIFRAC distance metrics. Plants were grown with either no additions, and AMF inoculant (*R. irregularis*), or AMF-free carrier substrate. Bare fallow soil was also maintained under the same experimental conditions for analysis.

Table 5-2. Comparisons of beta-diversity for *R. irregularis* inoculated plants and the respective controls. Bare fallow soil samples are included. Permutational analysis of variance used 999 permutations.

		adonis (permanova)			betadisper (variance of group dispersion)	
Factor	Df	F	R^2	P	F	P
AMF	1	0.98415	0.04236	0.451	2.5039	0.109
AMF free substrate	1	1.33499	0.05746	0.062	6.5419	0.012
Plant	1	1.91527	0.08243	0.005	0.5292	0.493
Residuals	19		0.81775			
Total	22		1			

A CAP was used to determine the drivers behind the beta-diversity (Figure 5.14) using the same distance metric (weighted UniFrac). The permutational analysis of variance with 999 permutations gave a *p* value of 0.009, in agreement with the PCoA described above. This confirms the differences between groups are due to variation caused by treatment, and that none of the permutated datasets had a significantly different community assemblage than the origin dataset. The analyses are concurrent in that the plant effect is an important driver of variation in taxonomic composition. However, there also appears to be clustering of the AMF-free substrate samples, separating from the AMF samples.

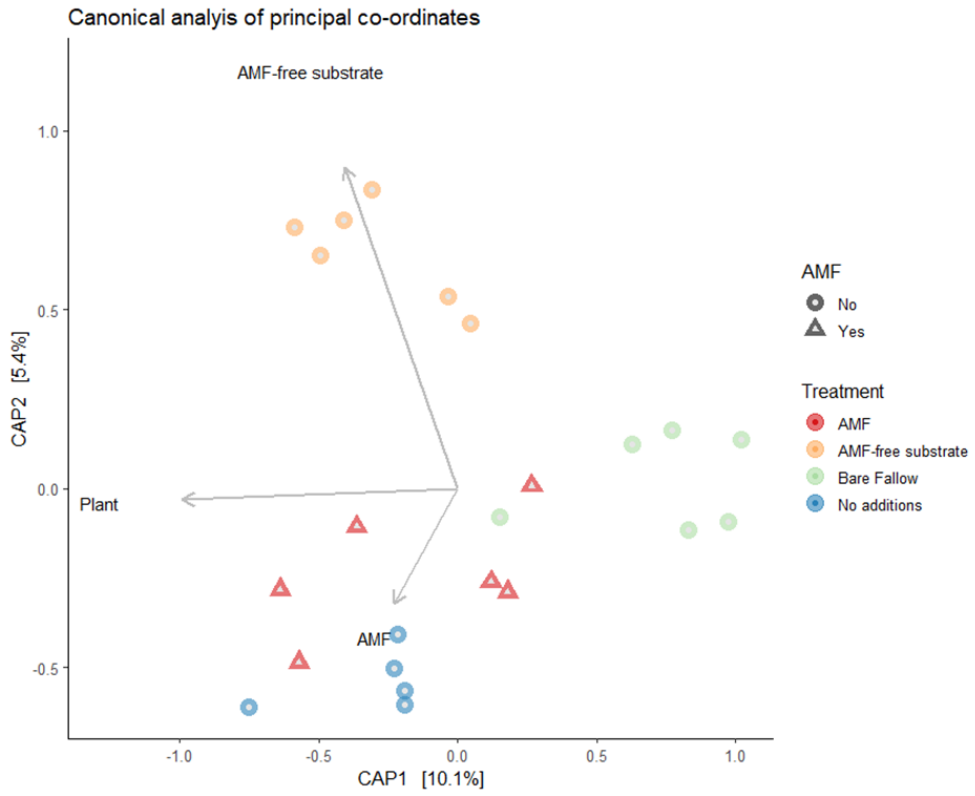


Figure 5.14. Canonical analysis of principal coordinates for ASV scores constrained by treatment for experiment 2. Scores were calculated from a weighted UNIFRAC distance measure. Colours are assigned to treatment and triangles denote samples taken from plant-containing treatments, circles represent bare fallow soil samples. The arrows point to the centre of the constrained factor. The percentage given in each axis refers to the proportion of the total variance of the data explained by each treatment factor. AMF inoculant is *R. irregularis*.

The differential abundance of the 25 most affected phyla are shown as a heatmap (Figure 5.15). Comparing the no additions treatment and the AMF-free substrate in the first instance to ascertain what bacterial groups are being applied, as these will also feature in the background of the AMF treatment – there are significantly affected ASVs. ASVs assigned to the families Actinobacteria (uncultured) (-1.9%), *Sphingomonadaceae* (-1.4%), *Chitinophagaceae* (+1%), Acidobacteria (uncultured) (+1.4%), *Xanthobacteraceae* (-2.3%), *Nitrosomonadaceae* (-0.9%), *Solirubrobacteraceae* (-1.1%), *Bacillaceae* (+1.8%), *Sphingobacteriaceae* (-1.4%), Planctomycetes WD20 (-2%), *Rhizobiaceae* (+2.4), *Isophaeraceae* (+1.5%) and Roseiflexaceae (-1.1%). Notably, many of the ASVs are declining in abundance when using the AMF-carrier substrate so it can be assumed there is some additional enrichment of taxa but predominantly changes in the structure instead of supplementary bacteria. Thus, the AMF-free substrate can serve as the closest control to the AMF treatment as they have the same microbial background addition of the carrier substrate.

Comparing AMF addition to its closest control, the AMF-free substrate treatment, there are numerous differentially abundant ASVs. *R. irregularis* inoculation increases ASVs related to families such as *Caulobacteraceae* (+2.5%), *Nocardioidaceae* (+1.5%), *Sphingomonadaceae* (+2.5%) and Actinobacteria (uncultured) (+1%). ASVs that were decreased as a result of *R. irregularis* inoculation are classified to the families: *Isosphaeraceae* (-1.3%), *Bacillaceae* (-0.8%), *Solibacteraceae* (-1%), *Pedosphaeraceae* (-0.9%) and *Chitinophagaceae* (-1.8%).

The bare fallow treatment has a significantly different taxonomic profile compared to treatments containing a plant. Compared to samples affected by a plant, bare fallow samples had lower abundance of *Chitinophagaceae* and *Burkholderiaceae*. ASVs belonging to the families *Pedosphaeraceae*, *Gemmataceae*, *Isosphaeraceae* and *Roseiflexaceae* were enriched in bare fallow samples. Actinobacteria are relatively abundant across all treatments, as are Proteobacteria and Gemmatimonadetes.

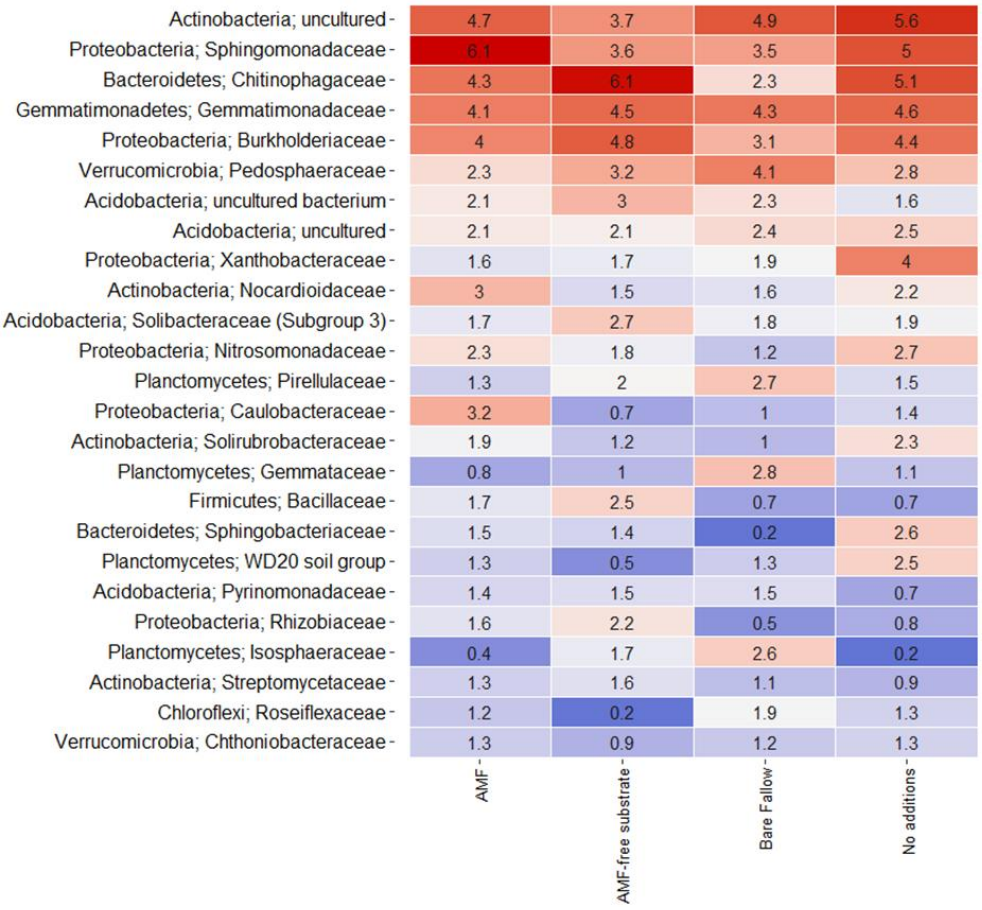


Figure 5.15. Heatmap showing mean relative abundance of the 25 most differential abundant ASVs for experiment 2. Features are shown at phylum and family level in each treatment. Darker blue indicates a low relative abundance and red equals a higher relative abundance. AMF treatment is *R. irregularis*.

5.5.3 Experiment 3: Clover plants grown in two soils, Barnfield (grassland) and Woburn, with either AMF inoculant (*F. geosporum*), NPK fertiliser, or AMF and NPK in combination.

5.5.3.1 Biomass.

NPK fertiliser increased plant biomass, in both Barnfield (p = 0.008) and Woburn soils (p = 0.001, Figure 5.16). The application of the AMF inoculant significantly increased the biomass of clover, but by the lowest relative amount compared to the other factors (p = 0.04). Soil type was also influential, with Barnfield soil yielding greater biomass than Woburn (p = 0.01). Statistical outcomes from the analysis of variance are shown in Table 5-3.

All of the treatment factors interacted, having differentially significant effects on biomass. The most significant interaction was between AMF and fertiliser ($p < 0.001$). Adding AMF and NPK in combination reduced biomass significantly in Barnfield soil, but this inhibitory effect was less clear in Woburn soil. Fertiliser and soil also interacted, increasing biomass by a greater amount in Barnfield compared to Woburn ($p = 0.001$). The effect of AMF was dependent on soil type; biomass increased more in Barnfield soil than in Woburn soil when AMF were inoculated ($p = 0.02$).

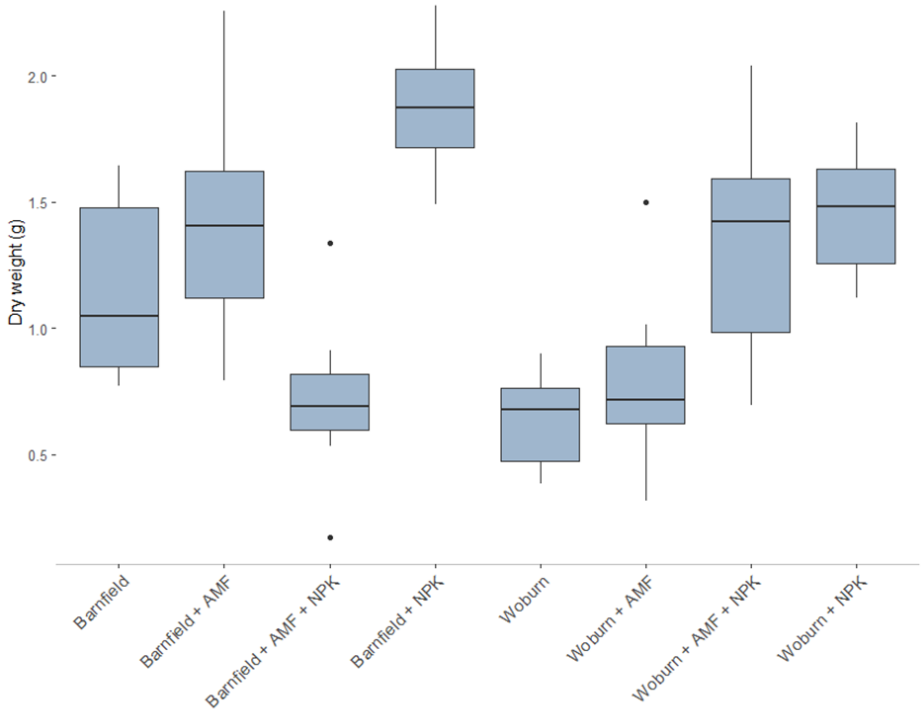


Figure 5.16. Biomass of clover grown in Barnfield and Woburn soil, with exogenously applied NPK fertiliser and AMF inoculant, separately or in combination. Clover were grown in pots with two soil types: Barnfield (low P, grassland), and Woburn (low N and P, bare fallow). Plants in the AMF treatments received *F. geosporum* inoculant (+ carrier substrate), and plants in no AMF treatments received the AMF-free carrier substrate. Plants in NPK treatments received osmocote granules onto the soil surface. Bars represent standard error, n = 7.

Table 5-3. Statistical outcomes from analysis of variance for changes in above ground biomass for experiment 3.

	ANOVA		
Factor	Df	F test	P
AMF	1	4.437	0.04054
Fertiliser	1	12.05	0.00112
Soil	1	5.968	0.01838
AMF:Fertiliser	1	19.384	0.0000613
AMF:Soil	1	5.686	0.02119
Fertiliser:Soil	1	11.952	0.00117
AMF:Fertiliser:Soil	1	8.047	0.0067
Residuals	47		

5.5.3.2 AMF colonisation.

Fertiliser application reduced AMF colonisation ($F_{1,\ 47} = 5.85$, $p < 0.02$), and soil type had a significant effect, with less colonisation in Woburn soil than Barnfield ($F_{1,\ 47} = 71.37$, $p < 0.001$), shown in Figure 5.17. The behaviour of the inoculated AMF was very significantly dependent on soil type. There was a small (but notable in the context of the experiment) effect of fertiliser interacting with soil type to affect AMF colonisation, but this was slightly above the defined 95% threshold ($F_{1,\ 47} = 3.58$, $p = 0.065$). A larger experiment could resolve this further. The addition of exogenous AMF did not affect colonisation for either soil type ($p > 0.7$ for both).

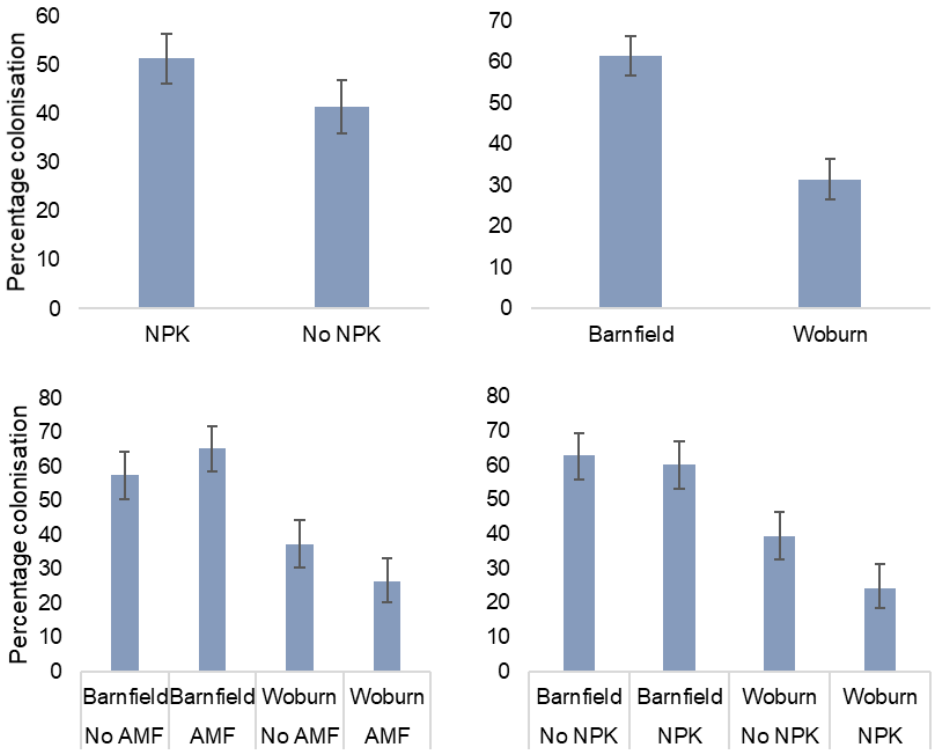


Figure 5.17. Predicted mean values for the percentage of clover roots colonised by *F. geosporum* for experiment 3. The binomial GLM predicted mean values (proportion scale) back transformed from the logit scale, including 95% confidence intervals. Only factors with significant effects on percentage colonisation are included.

5.5.3.3 Plant nutrient content.

5.5.3.3.1 Barnfield.

Results are shown in Figure 5.18.

Ca: Fertiliser did not affect plant Ca content, whereas AMF addition and the interaction between AMF and NPK both increased Ca levels ($F_{7, 47} = 5.97$, $p = 0.02$ and $F_{7, 47} = 5.13$, $p = 0.03$ respectively). Post hoc TukeyHSD tests revealed the significant difference was between Barnfield + NPK + AMF and Barnfield + NPK ($p = 0.01$).

K: Fertiliser significantly increased plant K content ($F_{1, 24} = 24.84$, $p < 0.001$) but this was not the case for AMF addition or independent of an interaction between the two factors.

Mg: Plant magnesium content was affected both by AMF and the interaction between NPK and AMF ($F_{1, 24} = 6.36$, $p = 0.02$ and $F_{1, 24} = 6.37$, $p = 0.02$

respectively). AMF and NPK in combination yielded the highest plant Mg which was significantly more than plants with NPK and no AMF (p = 0.007).

Na: Plant sodium content was not affected by any treatment.

Olsen P: Only fertiliser addition affected plant P content – AMF had no influence, nor did the factors interact (NPK: $F_{1, 24}$ = 16.4, p < 0.001). AMF and NPK in combination yielded the highest Olsen P values, significantly higher than Barnfield alone or Barnfield with AMF (p = 0.04 and 0.01 respectively). Plants grown in Barnfield with NPK alone also had greater tissue P content than with AMF alone (p = 0.03).

S: The sulphur content of plant tissue was increased by NPK only in Barnfield soil ($F_{1, 24}$ = 16.4, p < 0.001) compared to all other treatments except NPK and AMF in combination.

5.5.3.3.2 Woburn.

Results are shown in Figure 5.18.

Ca: Only fertiliser addition affected plant Ca content in Woburn soil ($F_{1, 23}$ = 8.58, p = 0.007).

K: Similarly, NPK addition affected plant K content ($F_{1, 23}$ = 57.2, p < 0.001) but AMF nor the interaction of the factors were influential. NPK and AMF in combination and NPK alone gave the highest plant K values (p = 0.96), which were significantly greater than in all other treatments without NPK.

Mg: Plant magnesium content was not affected by any of the treatments in Woburn soil

Na: Plant sodium content was not affected by any of the treatments in Woburn soil.

Olsen P: Fertiliser was the only treatment that influenced plant P content, ($F_{1, 23}$ = 39.8, p < 0.001). Post hoc comparisons revealed that every treatment containing NPK had significantly higher plant P than those treatments without fertiliser.

S: Plant tissue sulphur content was higher in plants with NPK added ($F_{1, 23} = 21.6$, $p < 0.001$).

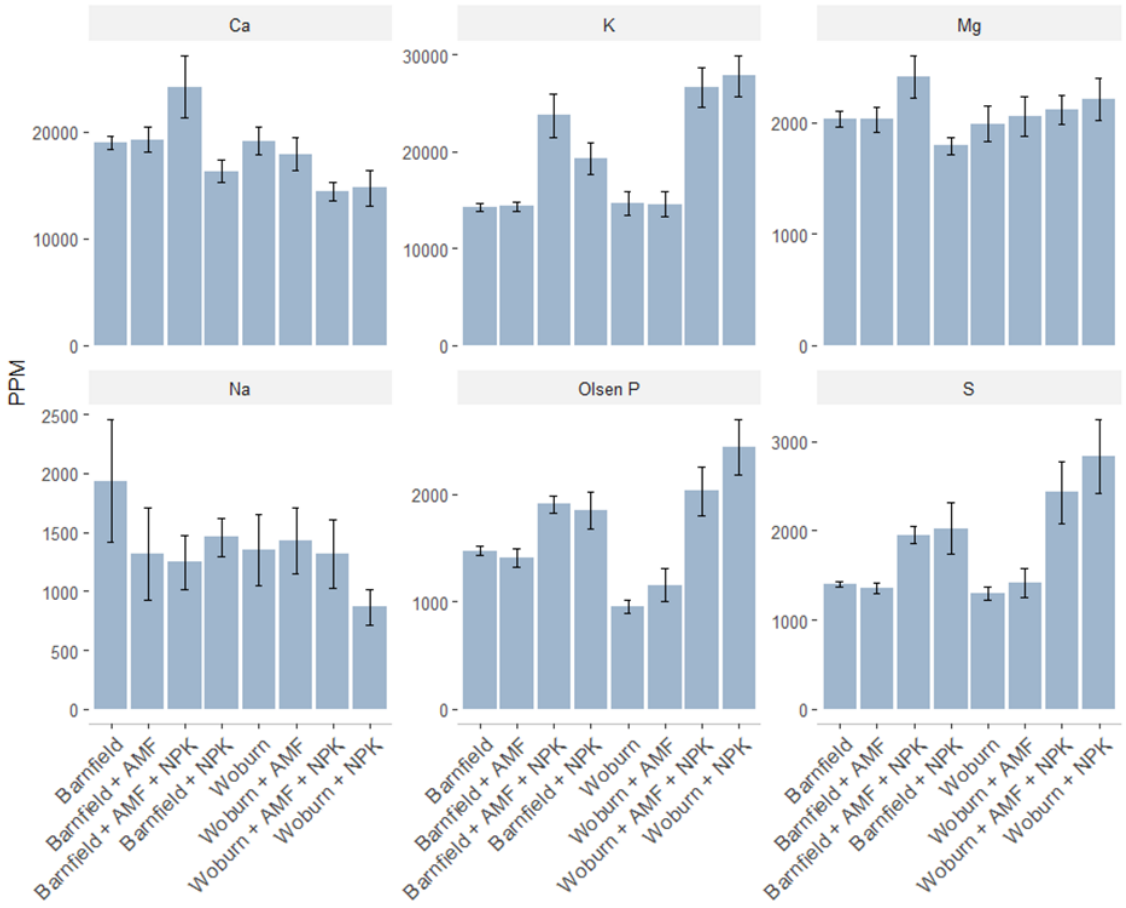


Figure 5.18. Plant aerial tissue nutrient content for experiment 3. ICP-OES analysis of some major elements including Olsen P for total plant P content. Values are given as PPM in dry matter. Bars give standard error and n = 6.

5.5.3.4 Functional assays from rhizoplane samples.

Results are shown in Figures 5.19-5.23.

$AlPO_4$: Soil type significantly affected the proportion of isolates that could solubilise $AlPO_4$ ($F_{1, 33} = 39.9$, $p < 0.001$). In Barnfield soil, 19% of isolates were positive for this function compared to 58.7% in Woburn soil. There was no AMF affect.

Casein: Both soil type and NPK had a highly significant effect on the proportion of isolates able to solubilise casein ($F_{1, 40} = 40.01$, $p < 0.001$ and $F_{1, 40} = 36.38$, $p < 0.001$ respectively). There were more positive isolates in Woburn soil than Barnfield (27.0% and 8.1% respectively). The addition of fertiliser increased the number of positive isolates regardless of soil type: 23.6% with NPK and 9.1% without NPK. There was also a notable interaction effect between soil type and NPK ($F_{1, 40} = 5.91$, $p = 0.02$) as fertiliser use increased the proportion of isolates positive for casein solubilisation in both soils, but by a greater amount in Woburn soil: A percentage increase of 3.88% compared to 34.48% for Barnfield and Woburn respectively, after NPK addition. AMF increased the proportion of positive isolates from 11.6% to 18.6% ($F_{1, 47} = 6.27$, $p = 0.016$).

K: Soil type was close to the 5% defined threshold for significance ($F_{1, 37} = 3.87$, $p = 0.057$). 2.6% of isolates were positive for K solubilisation in Barnfield soil compared with 4.1% in Woburn. The three-way interaction between soil, NPK and AMF had a highly significant effect on the proportion of isolates able to solubilise K ($F_{1, 37} = 17.95$, $p < 0.001$). In Woburn soil, AMF addition increased the proportion of K solubilising isolates following NPK addition (percentage change: 2.42%) but the proportion decreased when NPK was omitted (percentage change: -3.4%). However, in Barnfield soil the opposite was found: AMF and NPK co-application reduced the abundance of K solubilisers (percentage change: -3.67%) and when AMF were added in the absence of NPK a small relative increase in K solubilising isolates was recorded (percentage change: 2.35%).

Ca_3PO_4: There was no effect of AMF on the proportion of Ca_3PO_4 solubilising isolates. Soil type was close to the 5% defined threshold for ($F_{1, 36} = 3.83$, $p = 0.058$). Fertiliser use had a significant effect on the proportion of isolates able to liberate Ca_3PO_4 ($F_{1, 36} = 5.24$, $p = 0.03$). There were no interaction effects of the treatments. NPK addition increased the percentage of isolates positive for Ca_3PO_4 solubilisation from 3.9% to 6.1%. Barnfield had a lower percentage of Ca_3PO_4 solubilising isolates than Woburn (3.8% and 5.9% respectively).

Zn: There was no effect of AMF on the proportion of isolates capable of solubilising Zn. The interaction between soil and fertiliser affected the number of Zn solubilising isolates ($F_{1, 37}$ = 4.47, p = 0.04). NPK addition decreased the number of positive isolates in Barnfield soil (percentage change: -1.55%) but increased the proportion in Woburn soil (percentage change: 5.78%).

Fe: Soil was a strong determinant of the proportion of isolates able to chelate iron ($F_{1, 36}$ = 11.9, p = 0.001). Woburn had a greater number of positive isolates (6.99%) compared to Barnfield (2.92%). In addition, the interaction between soil and fertiliser had a significant effect, as did the three-way interaction of soil, fertiliser, and AMF ($F_{1, 36}$ = 4.93, p = 0.03, $F_{1, 36}$ = 7.51, p = 0.01 respectively). In Barnfield soil, NPK addition decreased the number of positive isolates, while in Woburn soil, NPK addition increased the proportion of positive isolates (percentage change: -2.24% and 1.75% respectively).

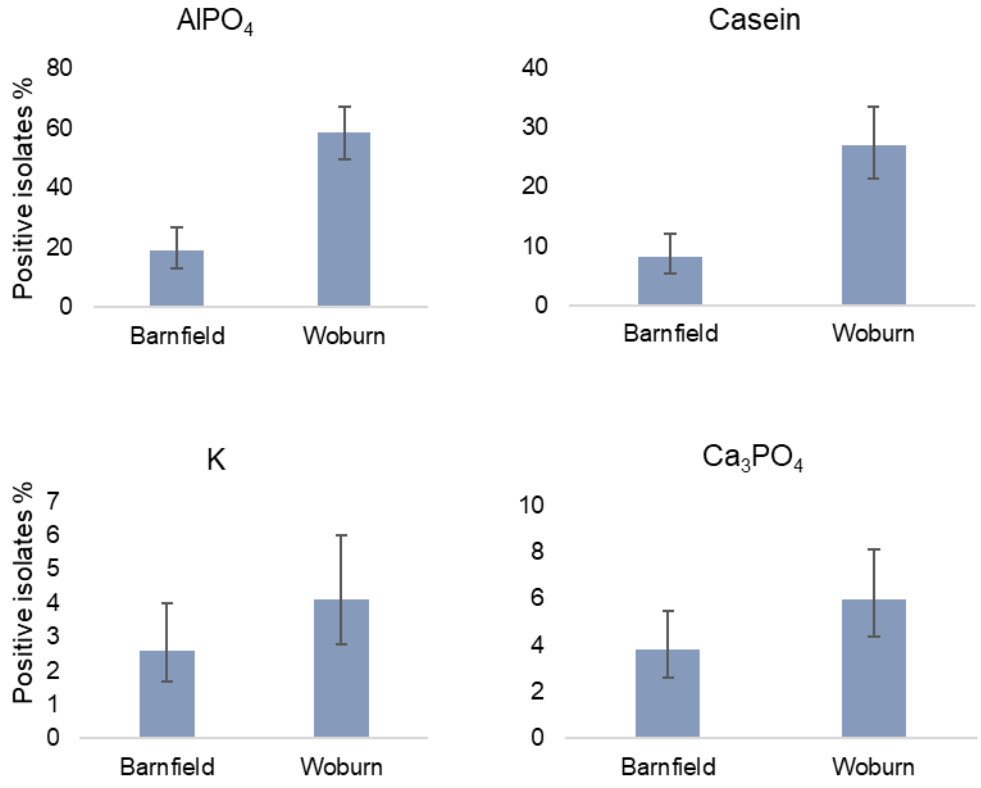


Figure 5.19. Predicted means for the functional assays for which soil type significantly affected the proportion of bacterial isolates who were positive for the respective function. Values are back transformed from the logit scale, including bars giving 95% confidence intervals.

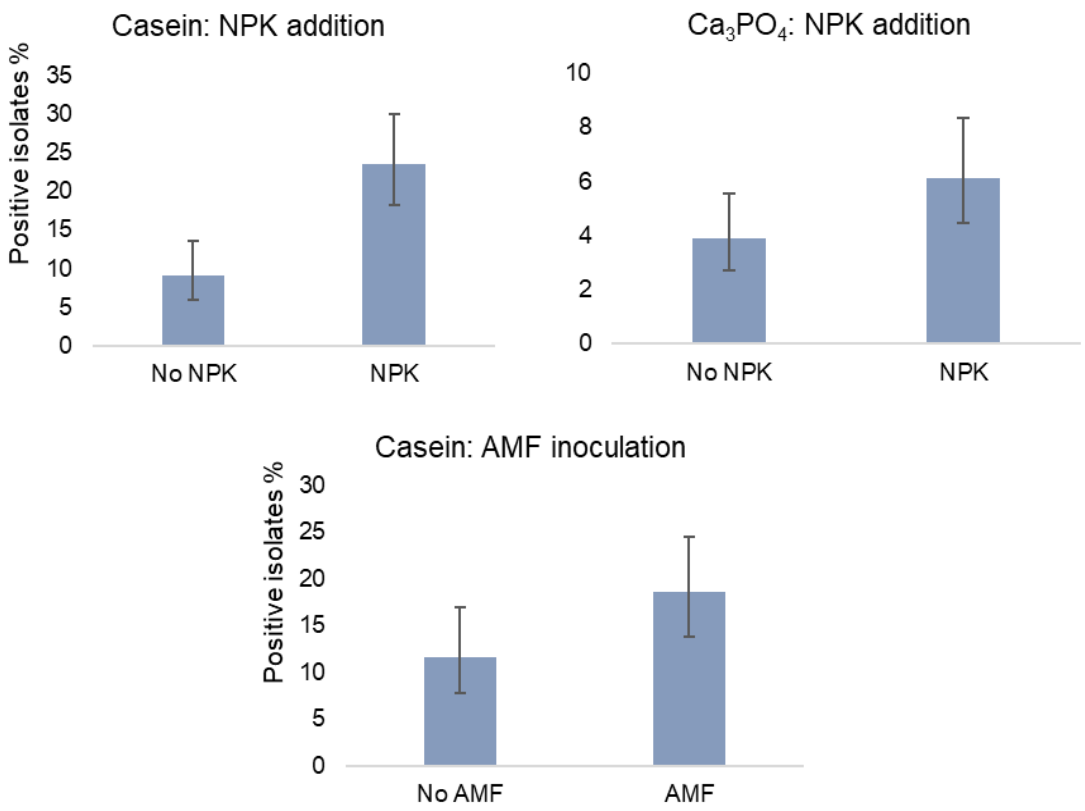


Figure 5.20. The predicted means for the functional assays significantly affected by NPK addition or AMF inoculation. Values are back transformed from the logit scale, including bars giving 95% confidence intervals.

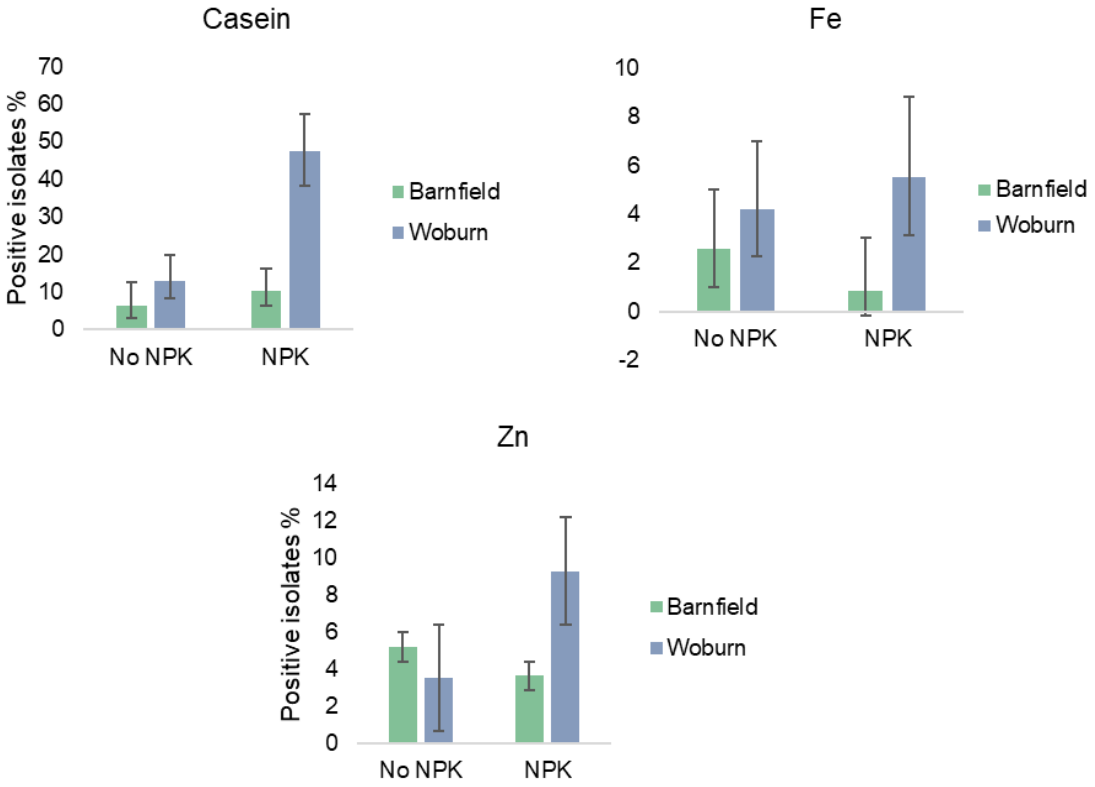


Figure 5.21. The predicted means for the functional assays significantly affected by the interaction between soil and fertiliser addition. Values are back transformed from the logit scale, including bars giving 95% confidence intervals.

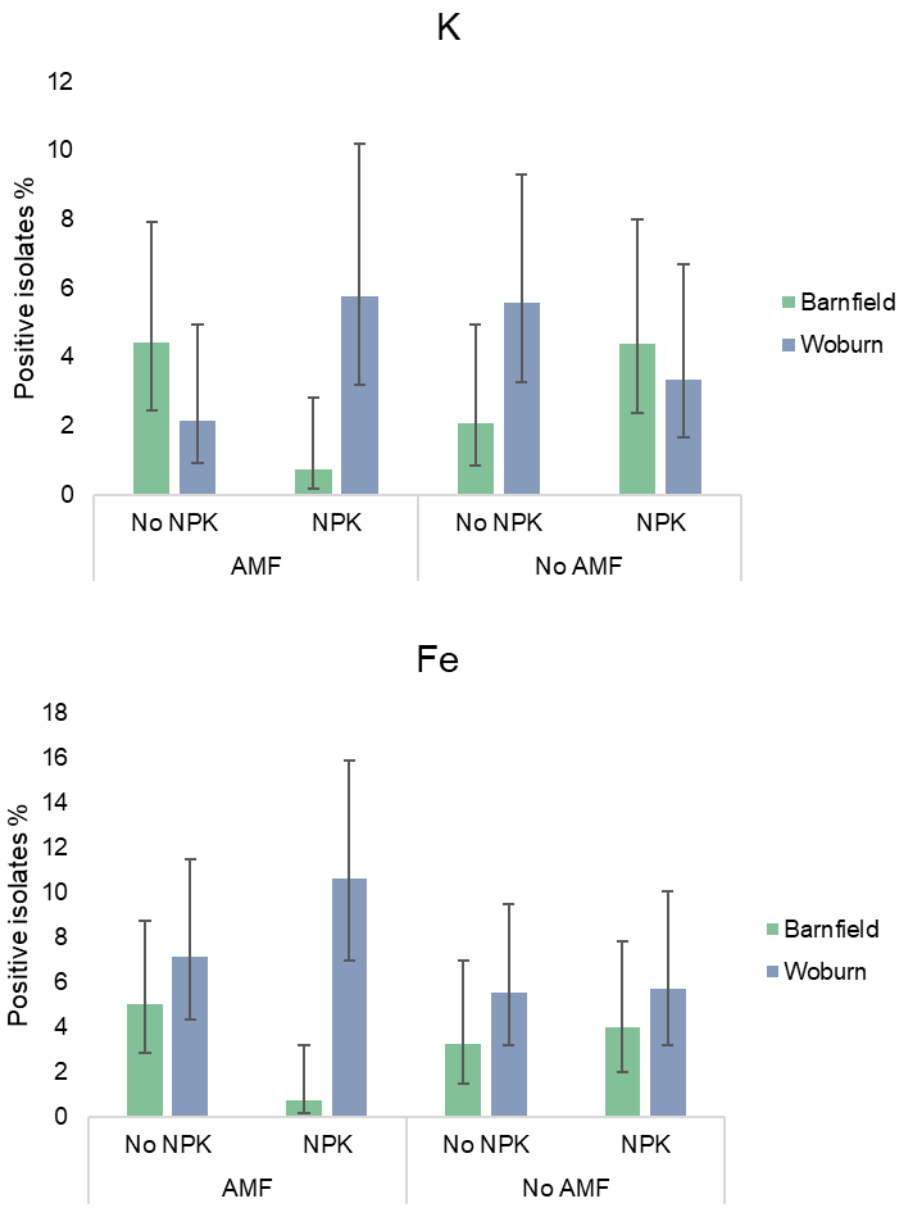


Figure 5.22. Predicted means for the functional assays in which the proportion of positive isolates are significantly affected by the three-way interaction of treatments: soil type, fertiliser and AMF addition. These factors interact to differentially affect the proportion of bacterial isolates who were positive for the respective function. Values are back transformed from the logit scale, including bars giving 95%.

5.5.3.5 16S rRNA gene amplicon sequencing of rhizosphere and rhizoplane samples.

To examine changes in taxonomic composition due to fertiliser use, land use and AMF addition, amplicon sequence variants generated from 16S rRNA gene sequencing of the rhizosphere and rhizoplane communities were analysed. The

distribution of taxa for the entire amplicon dataset is shown in Figure 5.23, displayed as the actual abundance of ASVs assigned to their corresponding phylum. In summary, the most abundant phyla are: 9% Acidobacteria, 23.4% Actinobacteria, 55.1% Bacteroidetes, 6.6% Firmicutes, 29.4% Proteobacteria, 3% Verrucomicrobia and 9% Chloroflexi. To separate soil type to avoid confounding land use type, a Venn diagram displaying the shared ASVs between Woburn and Barnfield samples is given in Figure A-7. Barnfield and Woburn had 45742 and 46803 unique ASVs respectively and 48 ASVs in common.

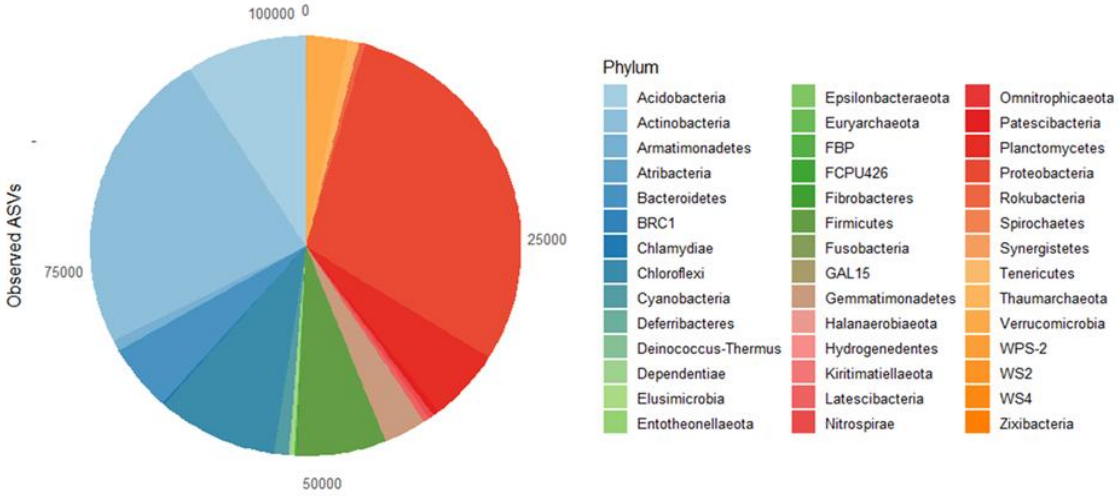


Figure 5.23. Actual abundance of ASVs aggregated by phylum for experiment 3. Counts for each unique ASV subsetted by its corresponding phylum. Pie chart shows the distribution of taxa for the entire amplicon dataset.

Indices of alpha diversity show that within-sample diversity does not differ by treatment, for either rhizosphere or rhizoplane (Kruskall-Wallis: p = 0.8 and 0.7 respectively). In the rhizosphere, there was an average richness of 978 bacterial ASV's, with an average Shannon index value of 6.5 and a Simpson's evenness score of 0.5. In the rhizoplane, there were an average of 865 bacterial ASV's, with a value of 6.4 and 0.5 for Shannon and Simpson's indices respectively (Figure 5.24). Barnfield and Woburn soils had comparatively similar species richness, with 908 and 937 observed taxa respectively, with both soils sharing a Shannon index value of 6.5.

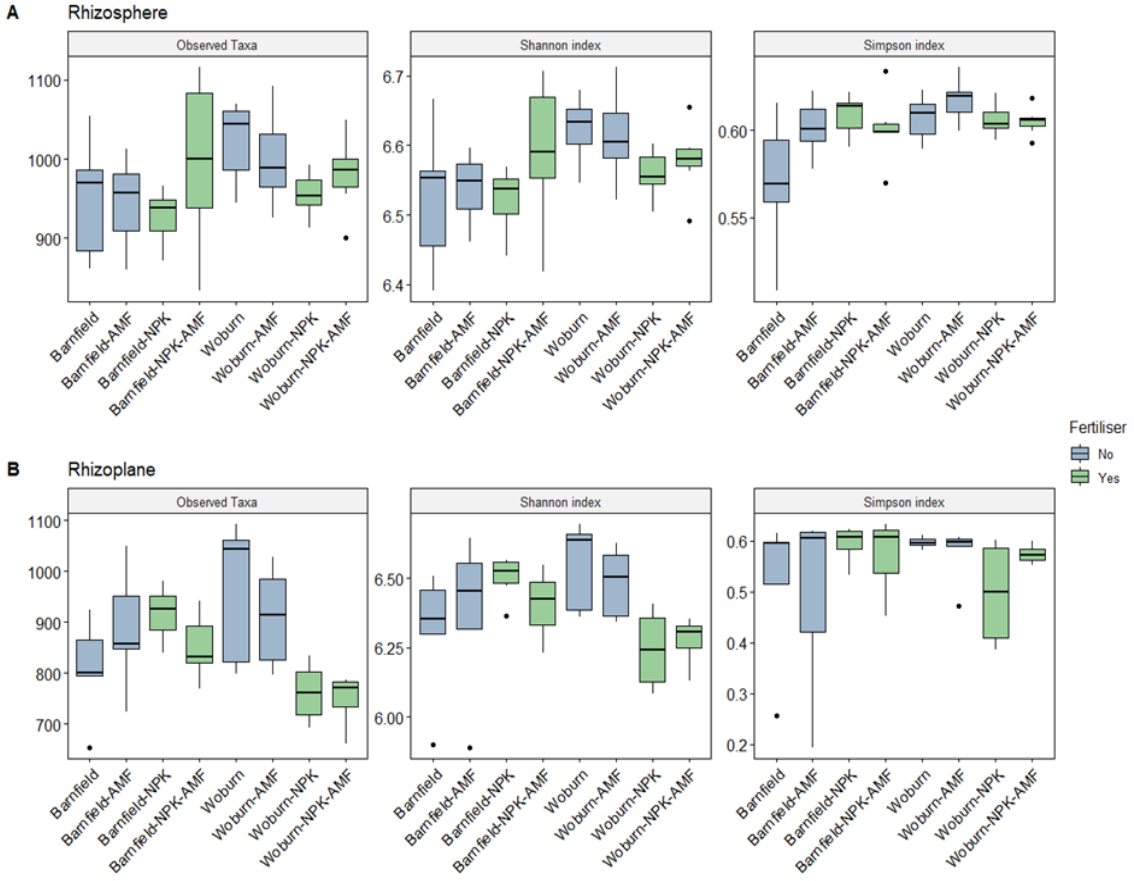


Figure 5.24. Estimates of alpha diversity in the root-associated bacterial community of clover, grown in two different soil types, with NPK fertiliser and exogenous AMF.
Figure shows the alpha diversity measures Shannon's diversity index and Simpson's evenness index with number of observed taxa (unique ASVs) subsetted by A) rhizosphere and B) rhizoplane. Colour denotes fertiliser addition for clarity, bars represent standard error.

Comparisons of beta diversity subsetted by soil type and exogenous application of both AMF and NPK fertiliser (separately and in combination) reveal that there are highly significant implications on microbial taxa (Figure 5.25). Between sample variation was calculated using a weighted UNIFRAC distance matrix to account for both phylogeny and abundance. Rhizosphere associated communities cluster most strongly according to soil type and fertiliser use (R^2 = 0.02 and p = 0.001 for both), followed by AMF inoculation (R^2 = 0.02, p = 0.006). In the rhizoplane the same clustering was observed for soil type and fertiliser (R^2 = 0.03 and 0.02 respectively, $p < 0.01$ for both), yet AMF do not have the same effect as in the rhizosphere (R^2 = 0.02, p = 0.06). In the rhizosphere, there are strong interactive effects of all test factors (Table 5-4). Conversely, there is only an interactive effect of soil type and fertiliser in the rhizoplane (Table 5-4). Beta dispersion (in-group dispersion) was tested for each treatment factor separately: AMF and NPK samples had homogenous dispersion within their respective groups for both soil fractions. Soil type had significant heterogeneous group dispersion for both rhizosphere and rhizoplane (Table 5-4).

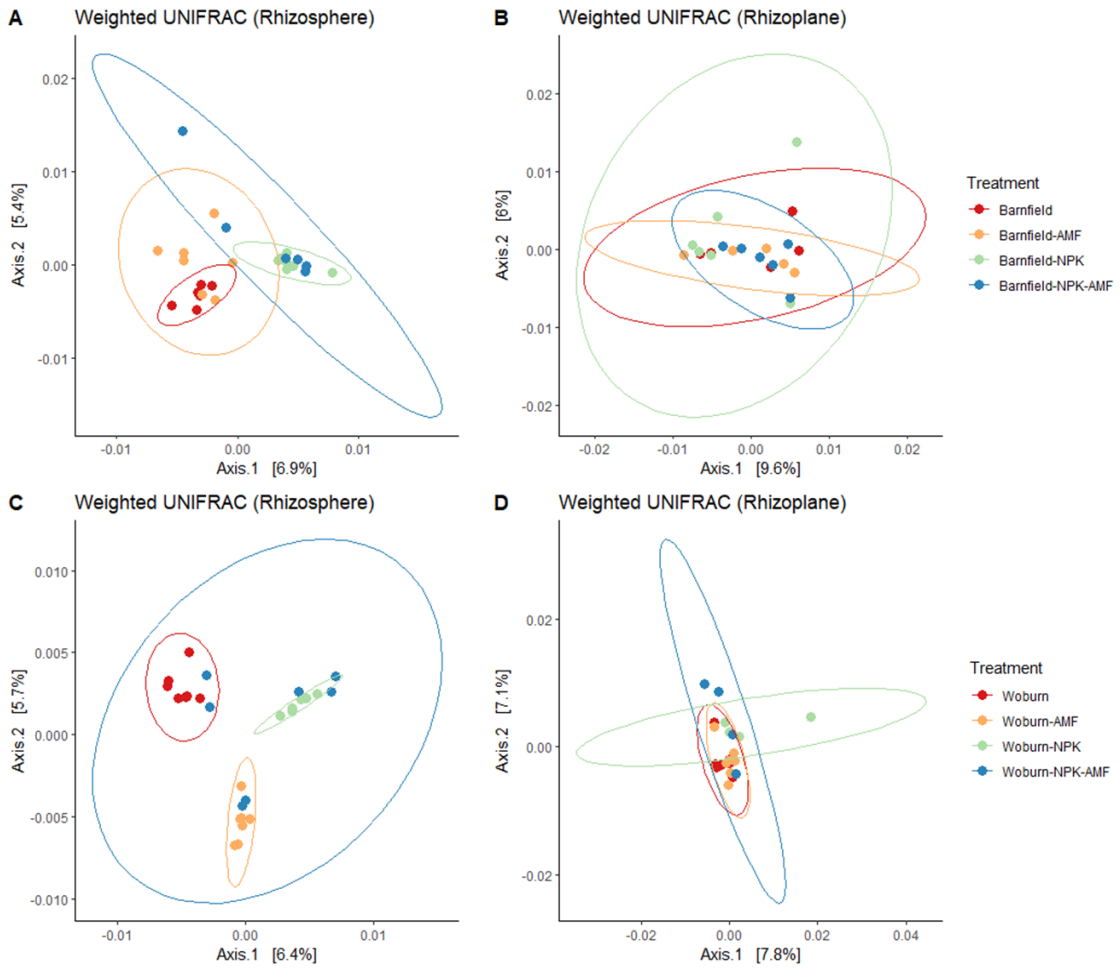


Figure 5.25. Estimates of beta diversity in the root-associated bacterial community of clover, grown in two different soil types, with NPK fertiliser and exogenous AMF. Figure shows PCoA based on weighted UNIFRAC distance metric, subset by soil type and soil fraction: A) Barnfield rhizosphere and B) Barnfield rhizoplane, C) Woburn rhizosphere, D) Woburn rhizoplane.

Table 5-4. Comparisons of beta-diversity for the treatment factors of *F. geosporum* (AMF), land use type (Soil) and NPK fertiliser addition (Fertiliser). Permutational analysis of variance used 999 permutations.

		adonis (permanova)				betadisper (variance of group dispersion)	
	Factor	Df	F test	R^2	P	F test	P
Rhizosphere	Soil	1	1.6173	0.02932	0.001	9.8396	0.004
	AMF	1	1.1222	0.02034	0.016	0.4342	0.528
	Fertiliser	1	1.4585	0.02644	0.001	0.8189	0.38
	Soil:AMF	1	1.1473	0.0208	0.002		
	Soil:Fertiliser	1	1.523	0.02761	0.001		
	AMF:Fertiliser	1	1.1278	0.02044	0.008		
	Soil:AMF:Fertiliser	1	1.1668	0.02115	0.001		
	Residuals	46		0.83389			
	Total	53		1			
Rhizoplane	Soil	1	1.3927	0.03175	0.001	5.6924	0.025
	AMF	1	1.0833	0.02469	0.063	0.0965	0.752
	Fertiliser	1	1.1734	0.02675	0.009	1.888	0.184
	Soil:AMF	1	1.059	0.02414	0.115		
	Soil:Fertiliser	1	1.1256	0.02566	0.029		
	AMF:Fertiliser	1	1.0025	0.02285	0.379		
	Soil:AMF:Fertiliser	1	1.0302	0.02348	0.245		
	Residuals	36		0.82067			
	Total	43		1			

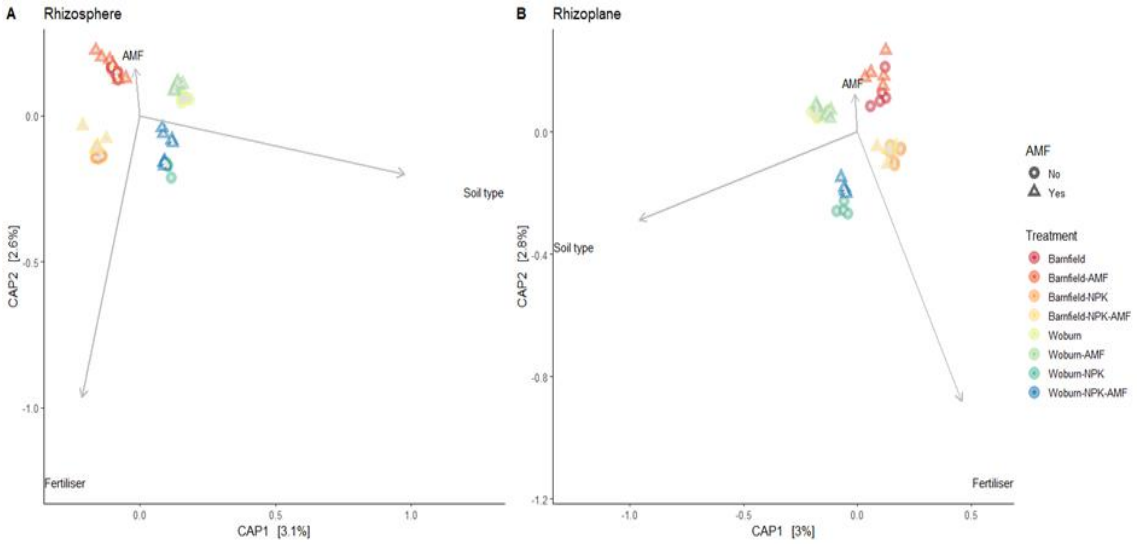


Figure 5.26. Canonical analysis of principal coordinates for ASV scores constrained by treatment, subsetted by soil fraction: rhizosphere (A) and rhizoplane (B). Scores were calculated from a weighted UNIFRAC distance measure. Colours are assigned to treatment and triangles denote AMF containing treatments and circles represent no AMF treatments. The arrows point to the centre of the constrained factor. The percentage given in each axis refers to the proportion of the total variance of the data explained by each treatment factor.

Canonical analysis of principal coordinates (CAP) was used to reveal how variations in beta diversity could be attributed to soil type, fertiliser, and AMF inoculation (Figure 5.26). A permutational analysis of variance with 999 permutations yielded a *p* value of 0.001 for both rhizosphere and rhizoplane, in agreement with the PCoA already described. This *p* value means that none of the permuted data sets has a significantly different taxonomic assemblage compared to the original data set so there are confirmed differences between the groups. Both rhizosphere and rhizoplane are clearly separated by soil type and fertiliser use, with a smaller AMF influence. This is in accordance with the results from the PCoA.

The heatmap shows the differential abundances and identities (family and phylum level) of the microbial communities of each treatment (Figure 5.27). There are some notable changes in abundance of bacterial phyla. NPK reduced Verrucomicrobia (*Pedosphaeraceae*) in both land use types. Acidobacteria were present at a very low abundance (0-0.5%) in Barnfield soil regardless of treatment, compared to 3% in Woburn soil. However, adding NPK to Woburn soil reduced two Acidobacteria families (one uncultured and *Solibacteriaceae* sub-group3) presence by almost half, from 3% to 1.8% and 2.2% to 1.4% respectively. Conversely, adding NPK to Woburn soil increased the presence of an Actinobacteria (*Nocardioidaceae*) to 2.4-2.8% from 1.8%. In Barnfield soil, NPK addition significantly reduced the abundance of Firmicutes (*Clostridaceae*) by 3.1%, but this was increased slightly by 0.5% when AMF were also included.

Actinobacteria (uncultured) were the dominant species in both Barnfield and Woburn soil, followed by two proteobacteria families and Firmicutes (*Clostridaceae*). Adding AMF to Woburn soil did not appear to significantly affect any phyla compared to Woburn alone. In Barnfield soil AMF reduced the presence of Thaumarchaeota (*Nitrososphaeraceae*) and Firmicutes (*Clostridaceae*) by 0.9% and 0.8% respectively. AMF increased the amount of Actinobacteria (uncultured family) in Barnfield soil by 0.4%).

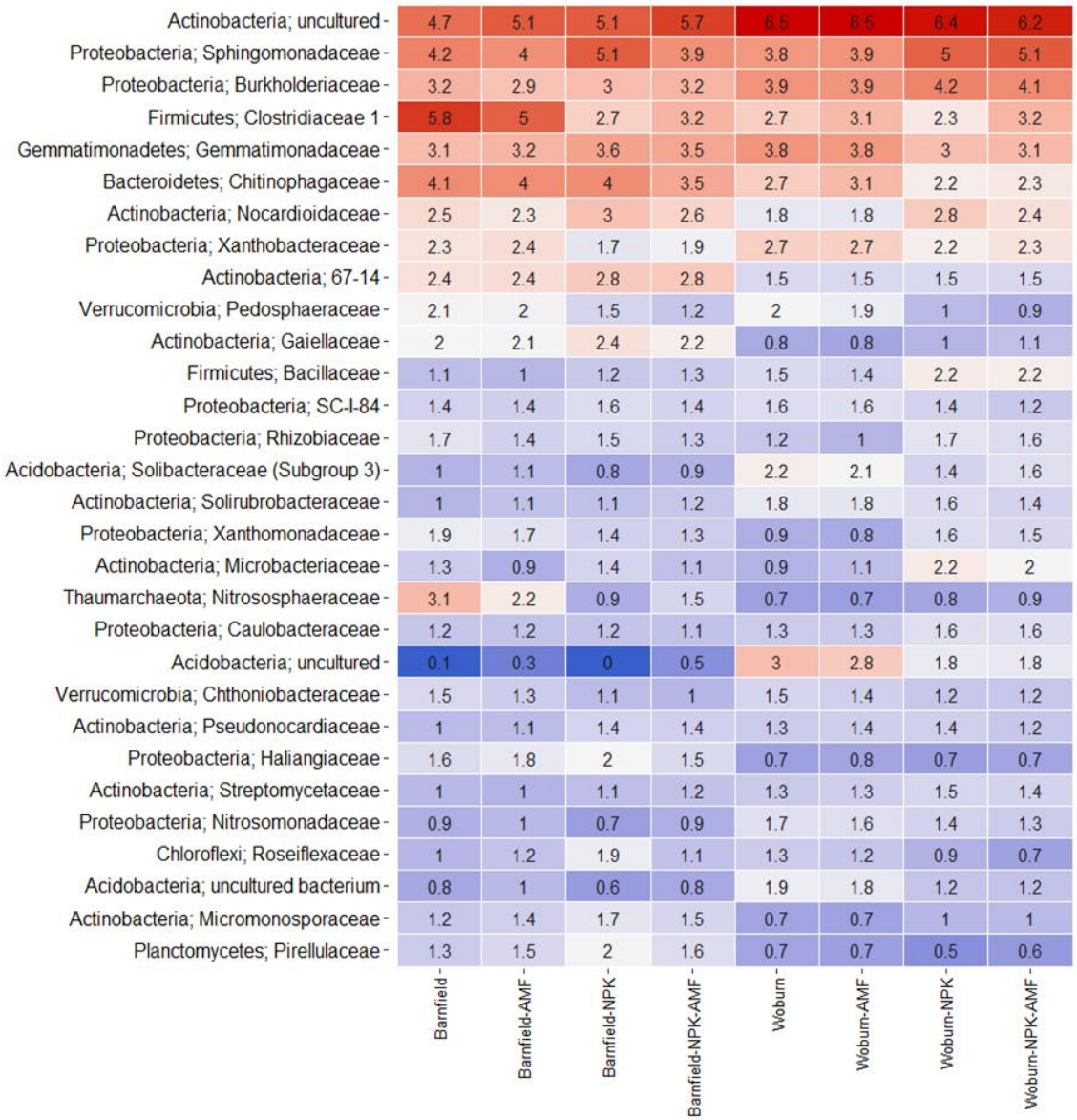


Figure 5.27. Heatmap showing mean relative abundance of the 25 most differential abundant ASVs for experiment 3. Features are shown at phylum and family level in each treatment. Darker blue indicates a low relative abundance and red equals a higher relative abundance.

5.6 Discussion

This series of experiments explored the effect of adding an exogenous AMF inoculant, a strategy for crop health augmentation which is growing in popularity, to the community of rhizobacteria in the surrounding soil. This was done to illuminate both the effect on the diversity and abundance of soil microbes and the efficacy of using such an inoculant to enhance plant growth. Relating AMF

presence to both taxonomic structure and function of the co-occurring rhizobacteria is a novel step in AMF research. It is important to note that there are many species of AMF, and clover is the chosen plant host used in this research. Community interactions will differ between soils, host plants, agricultural strategies, and many other variables, making these complex communities difficult to describe comprehensively.

The comparison of two soil types from two land uses (grassland and bare fallow) with differing nutrient contents, and the exploration of the effects of NPK fertiliser use are also novel for this field. Describing microbial communities across different agricultural landscapes and management practices will widen our understanding of the effect that anthropogenic inputs are having on the composition and function of the soil microbiome. It is important to understand the practical implications of the use of AMF inoculants for sustainable agriculture.

Points to consider are: 1. If they are as effective as claimed for plant health amelioration, 2. If the use of agrochemicals might impact or diminish the potential benefit of inoculant use, 3. If inoculants might affect the ability of *in situ* microbial communities to function in a beneficial manner.

5.6.1 *F. geosporum* (experiment 1).

Despite higher colonisation of plants in the AMF treatment, no biomass difference was observed. There are indigenous AMF in the field soil from Woburn, which colonised the non-AMF treatments equally. It would be useful for future work to identify the existing AMF community in the soil. It could be the case that the AMF beneficial effect is saturated in both treatments and exogenous application of the AMF inoculant does not increase biomass. Understanding the competition dynamics between the existing AMF constituents and an inoculated species would be advantageous for future work. A significant shift in the taxonomic composition of the rhizoplane of clover occurs when AMF were added exogenously compared to the AMF-free carrier substrate. This was used as a control so that any background additions caused by the substrate would be consistent in both AMF and non-AMF treatments. The carrier substrate did

introduce some additional taxa, comparing the plants grown with no additions and the AMF-free substrate.

The species richness of each treatment was equal in both the root-associated samples and bare fallow soil. This indicates that while the community structure might be different, the number of observed taxa is not increased. The measures of alpha diversity indicate many bacterial features and some prominent constituents with a higher relative abundance but mostly an even richness. This is consistent with results observed by Hirsch, Gilliam et al. (2009) who showed that diversity of taxa remains high in continuous bare fallow systems compared to grassland.

Both CAP and PCoA analyses were in concurrence that all three treatments affected rhizobacteria community structure. A plant in the system was the most influential determinant of taxonomic composition of the samples, with all the plant treatments having a vastly different community structure compared to bare fallow samples. This is in agreement with current knowledge that plants heavily influence their own root microbiome and the rhizosphere community is significantly different from that of the bulk soil (Smalla, Wieland et al. 2001, Kavamura, Robinson et al. 2019). Differential abundance analysis showed the effect of a plant in the system caused an increase in taxa belonging to, for example, *Sphingobacteraceae*, *Rhizobiaceae* and *Deinococcaceae*. Within the family *Rhizobiaceae,* many members are canonical root-symbiotic organisms, thus it is not surprising that ASVs relating to this family were increased in abundance due to the presence of a plant compared to bulk soil. However, nodules were not quantified in these experiments so no inference as to the number of Rhizobia can be made. The frequent plant associations of *Sphingobacteraceae* and *Deinococcaceae* are commonly reported, as is their putative plant-growth promoting or endophytic nature (Mehnaz, Weselowski et al. 2007, Marques, Pires et al. 2010, Suman, Yadav et al. 2016, Thokchom, Thakuria et al. 2017, Igiehon, Babalola et al. 2021).

Bare fallow soil had higher differential abundance of features belonging to, for example, Acidobacteria, *Bacillaceae* and *Nitrosomonadaceae*, concurrent with

results reported by Kavamura, Robinson et al. (2019). Acidobacteria and *Bacillus* species are prominent components of many ecosystems, and are often reported as dominant taxa in bulk soil, as well as being adapted to inhabit the root-soil interface (Smalla, Wieland et al. 2001, McSpadden Gardener 2004, Kalam, Basu et al. 2020). *Nitrosomonas* species have also been shown to exist abundantly in both niches, bulk soil and rhizosphere (Ruiz-Rueda, Hallin et al. 2009). Species have been identified which have significant roles in the nitrogen cycle, such as the oxidation of Ammonia (Padrão, Tortella et al. 2019).

AMF also strongly influenced the root associated bacterial community. Inoculation enriched bacteria such as *Sphingomonadaceae* and *Holophagaceae*. The most notable differentially abundant ASV belonged to the family *Burkholderiaceae*. AMF inoculation reduced its prevalence by 3.7%. *Bulkholderia sp*. have been reported to be both AMF spore-associated and AMF-endosymbiotic (Bianciotto, Bandi et al. 1996, Levy, Merritt et al. 2009), so it is possible that there is some AMF-mediated regulation altering the presence of the individuals corresponding to this ASV. It might be the case that these bacteria elect to become spore-associated if this is their preferred niche and were therefore reduced in the rhizosphere when AMF were present. AMF also markedly reduced the presence of a feature classified as *Deinococcaece*, from 2% to 0.4%. *Rhizophagus intraradices* has been shown to significantly increase relative abundance of Deinococcus-thermus in soybean, further demonstrating the complexities of species-specific AMF-bacteria interactions and AMF-host dynamics (Rodríguez-Caballero, Caravaca et al. 2017).

5.6.2 *R. irregularis* (experiment 2).

As with *F. geosporum*, *R. irregularis* inoculation caused a higher proportion of roots to be colonised with AMF. As in experiment 1, this increase was not accompanied by a change in plant biomass. Interference and competition has been shown between AMF taxa, with multi-species inocula showing no greater biomass increase than ones containing single-species AMF (Zaller, Frank et al. 2011). This could explain the lack of biomass increase when there are AMF

already present in the soil – a threshold biomass increase due to general AMF colonisation could have been reached and any further colonisation by the exogenous inocula does not confer additional growth benefit in this case. This could be explored further by using a sterilised field soil with a synthetic bacterial community, a microbial soil wash filtered for AMF spores, or mycorrhiza defective mutant host plants (Vosatka and Dodd 1998, Marsh and Schultze 2001). However, plant biomass has been shown to positively correspond to the percentage of the root that is colonised by AMF, although using this metric to predict the benefits received by the plant is still controversial (Treseder 2013). A lack of AMF-induced biomass effect was observed in both the *F. geosporum* and the *R. irregularis* experiments.

However, AMF colonisation increased by 15% following the exogenous application of *R. irregularis* compared to colonisation from indigenous species existing in the soil. *F. geosporum* inoculation caused 33% more colonisation than that from the indigenous species. This can be attributed to the generalist and more abundant lifestyle of *F. geosporum* (Labidi, Jeddi et al. 2015) in comparison with the *R. irregularis* species, which was isolated from a salt mine and is therefore likely to be more specialist.

Similar to the *F. geosporum* experiment, amplicon sequence samples were equally rich in taxa regardless of treatment. This includes samples from bare fallow soil as well as root-associated samples. Importantly, the AMF-free substrate did not introduce additional taxa, species richness was the same across all treatments. Furthermore, neither *R. irregularis* nor its carrier substrate affected the beta diversity of the samples. However, in experiment 2, only the plant was influential in determining bacterial assemblage – as bare fallow soil had different community composition compared to treatments affected by roots. As the AMF-free carrier substrate reduces relative abundance of some ASVs when comparing differentially abundant ASVs present in the no additions control – meaning it is unlikely to be introducing additional bacteria but rather it does have a slight influence on community structure. It could be possible that the carrier substrate

for *R. irregularis* contains more bacterial taxa than for *F. geosporum,* as each had a specific substrate.

R. irregularis affects the relative abundance of fewer ASVs compared to *F. geosporum*, but the percentage change of those ASVs was often greater. However, these experiments were conducted separately so direct comparisons cannot be made. ASVs belonging to *Caulobacteraceae* and *Sphingomonadaceae* both increased by 2.5%. Both of these families have putative plant-growth promoting members and have been shown to partner with AMF to enhance phosphate transfer to plants by degrading phytate (Hara and Saito 2016). *Isosphaeraceae* and *Chitinophagaceae* assigned ASVs declined by 1.3 and 1.8% respectively. Members of the *Chintinophagaceae* family have been shown to produce antifungal metabolites in some cases and be highly abundant in AMF-suppressive soils, so perhaps there is an antagonistic interaction with *R. irregularis* inoculation observed here (Loudon, Holland et al. 2014, Svenningsen, Watts-Williams et al. 2018).

The relative abundance of Proteobacteria, Gemmatimonadetes and Actinobacteria was high across all treatments. In bare fallow soil, ASVs classified as Acidobacteria and *Gematimonadaceae* were significantly more abundant than in plant-affected samples, by an increase of 4.7% and 5.1% respectively. The most significantly differentially affected ASV in bare fallow soil is assigned to *Burkholderiaceae*, which is reduced from an average of 14% in the plant treatments to 2.8% in bare fallow soil. Similarly, ASVs belonging to *Caulobacteraceae*, *Sphingobacteraceae* and *Rhizobiaceae* are reduced from an average of 4% to 0-0.6% in bare fallow soil. This suggests that there are specific taxa either adapted to, or selected by, the root niche and potentially cannot thrive without a plant host. Specifically, the examples of *Rhizobium* and *Bulkholderia,* which are known plant associated bacteria, and are not found in high abundance when there is no plant host. *Burkholderiaceae* are shown to be dominant in plant roots in P depleted soil (Gomes, Lana et al. 2018). As Woburn soil is low N and P, the results presented in this experiment support the literature that bacteria from

the family *Burkholderiaceae* may be being recruited to enhance plant nutrient status.

Future experiments studying the impact of different AMF species as inocula would be useful to understand if there is truly an AMF-species mediated difference in rhizobacteria modulation. In addition, understanding the host specific effects on colonisation and how it is affected by AMF species. Furthermore, including a mixed inoculum would be advantageous to explore this..

5.6.3 Barnfield and Woburn soils, with NPK fertiliser and *F. geosporum*.

Fertiliser was the most important determinant of clover biomass, increasing plant dry weight in both soils. AMF made some difference to biomass (p = 0.04) but this was the least influential factor. There was a highly significant interaction effect of AMF and NPK, reducing biomass significantly compared to the NPK or AMF treatments alone. This effect was most observed in Barnfield soil, and the inhibition effect of AMF and NPK was less pronounced in Woburn soil. AMF can become parasitic in some circumstances, particularly when a plant is fully nourished, but this appears to be largely dependent on host (Verbruggen and Kiers 2010, Grman 2012). Barnfield and Woburn soils also have differing nutrient content (see: section 4), which may be an explanation for the NPK:AMF interaction being dependent on soil – there could be a different nutrient repletion threshold in either soil. Fertiliser and soil also had a highly significant interactive effect (p = 0.001), biomass was increased by a greater amount in Barnfield soil than in Woburn. This could be because of the nutrient repletion threshold mentioned above – Woburn soil is very low nutrient and the addition of NPK may not increase nutrients to optimum levels compared to Barnfield. AMF and soil interacted at a relatively less significant level (p = 0.02); biomass increased in both soils, but variation was greater.

Fertiliser also was an influential factor for AMF colonisation, reducing the proportions in both soils. The addition of fertiliser is widely known to decrease

mycorrhizal colonisation (Mack and Rudgers 2008). Soil type mattered the most for the percentage of roots colonised, with less colonisation in Woburn than Barnfield. This could be because of a greater indigenous population of AMF in the grassland Barnfield soil (and therefore more roots to recruit AMF), compared with the bare fallow nature of Woburn soil. The interaction effects between AMF and soil type saw opposite effects for Barnfield and Woburn – when AMF were added to Barnfield soil, colonisation increased. When AMF were added to Woburn soil, colonisation decreased.

Plant nutrient content was differentially affected across all of the major elements measured. For most of the elements tested, fertiliser appeared to be the main determinant of plant nutrient content. Nutrient content was similar across both soils for every element except Ca (p = 0.01). For this study, soluble P was the main nutrient of interest, due to its importance in the AMF-plant mutualism. When separating the soils for comparison, NPK was the only factor that influenced plant P content, regardless of AMF inoculation. Calcium and Mg were increased in plant tissue by AMF in combination with NPK in Barnfield soil, but this was not observed with AMF alone. In addition, no difference was caused by NPK alone. No changes to Ca and Mg occurred in Woburn soil, except Ca decreasing after fertiliser addition. This might suggest that NPK addition supports the growth of microbes which may then compete with the plant for Ca. Sulphur and potassium were only influenced by NPK addition. Both S and K increased when NPK was added regardless of AMF addition for both soil types, both of which are nutrients found in the fertiliser (osmocote).

The functional assays also show a differential effect of the treatments, i.e. no one treatment caused the same trend in the proportion of isolates from rhizoplane community samples that were positive for a particular function. Each assay was modelled individually, but soil type was consistently the factor which had the most influence on the percentage of positive isolates for a given function. Examining the soil main treatment effect, Woburn soil had a higher proportion of solubilisers for $AlPO_4$, casein, K and Ca_3PO_4 than Barnfield. There were no differences between the soils for the other assays. Fertiliser affected casein and Ca_3PO_4

isolates, increasing the proportion of positive isolates when NPK was added. AMF inoculation only affected casein hydrolysis, increasing the percentage of positive isolates.

The interactions between the factors caused more complex differential effects. Woburn soil had a greater percentage of isolates than Barnfield that could solubilise casein and chelate Fe. The addition of NPK fertiliser dramatically increased the proportion of positive isolates in Woburn soil, but not Barnfield. The same trend occurred for Zn; Barnfield and Woburn had similar proportions of solubilising isolates, however the addition of NPK increased the proportion of positive in Woburn but not Barnfield.

Significant three-way factor interactions included the influence of AMF for both K and Fe. The addition of AMF resulted in increased K solubilisation positive isolate proportions in Woburn when NPK was added, but decreased proportions when NPK was not added. However, in Barnfield soil AMF inoculation significantly decreased the proportion of positive potassium solubilisation isolates in Barnfield soil when NPK was added but increased the proportion when NPK was not. In short, the proportion of solubilising isolates in response to NPK and AMF vary according to soil type. When considering siderophore producing isolates, the combination of AMF and NPK application resulted in a significant increase in abundance of Fe chelating microbes in Woburn soil. Conversely, AMF inoculation resulted in a decreased proportion Fe chelating isolates in Barnfield soil when NPK was added. However, in the absence of NPK, the addition of AMF did not significantly influence the percentage of isolates positive for iron chelation, in either soil, compared to no AMF inoculation controls.

None of the factors (soil type, fertiliser, AMF addition or soil fraction) were associated with differential bacterial richness or diversity. Both soil fractions (rhizosphere and rhizoplane) show a high overall diversity, but the number of observed taxa was slightly higher in the rhizosphere than the rhizoplane (978 and 865 unique features respectively). This means that neither fertiliser use nor AMF application affect diversity in the microbial community associated with the roots

of clover, grown in either soil. Both soil types are equally diverse despite their difference in land use; Barnfield is grassland soil, Woburn is bare fallow.

Soil type and fertiliser use were the most influential cause of changes in assemblage, in both soil fractions. This is unsurprising, due to significant differences in chemistry and physical structure of the different land use types. Both land use type and fertiliser use have been shown to significantly influence rhizobacterial community composition in Woburn soil (Kavamura, Robinson et al. 2019, Reid, Kavamura et al. 2021).

Adding fertiliser reduces the abundance of Verrucomicrobia regardless of land use type. However, the soil provenance was important for the effect of fertiliser addition in some cases, reducing the presence of Acidobacteria in Woburn soil and Firmicutes in Barnfield soil. Incidentally, Acidobacteria were highly abundant in Woburn soil compared to Barnfield. Acidobacteria are oligotrophic and better adapted to low nutrient environments such as bare fallow, bulk soil (Kalam, Basu et al. 2020). Conversely, adding NPK increased the abundance of Actinobacteria. Coincidentally, Actinobacteria were a dominant feature of both soils, along with Proteobacteria and Firmicutes. These results reflect what is known about dominant taxa in many agricultural systems (Lee, Ka et al. 2008).

5.6.4 *F. geosporum* restructures the bacterial assemblage of the rhizosphere.

The use of *F. geosporum* as an inoculant significantly alters the taxonomic composition of rhizosphere bacteria ($p = 0.01$) but not the rhizoplane ($p = 0.06$). This can be seen for both soil types as clustering of samples by treatment type in the PCoA (Figure 5.25). This is in agreement with the previous experiment, showing that in multiple experiments and soil type, *F. geosporum* remain a determining factor for rhizobacteria community assemblage. It is possible that the plant is exercising more control over its rhizoplane-associated bacteria than those determined by the AMF partner in the rhizosphere, as the explant exudate effect is less influential in the rhizosphere than clover to the root. AMF are present from

within the root and out into the bulk soil, so their influence may be less related to direct plant-root community composition and concentrated in the mycorrhizosphere, which includes the rhizosphere (Charpe 2019).

The effect of AMF inoculation on community composition is further altered by fertiliser use in the rhizosphere, but not the rhizoplane. This could be due to the specialised plant-mediated environment surrounding the root being more impactful than soil chemical composition. The effect that AMF are having on taxonomy in the rhizosphere changes depending on fertiliser use. This can be explored further by examining the functional assay data, but it is important to note that the culture-dependent work was using rhizoplane samples only.

Isolates capable of potassium solubilisation and siderophore production are differentially affected by the interaction of AMF and fertiliser. In Barnfield soil when NPK is added, there were fewer K solubilisers than when it was absent, in the presence of AMF. In the presence of AMF, NPK addition reduced the abundance of K solubilisers compared to no fertiliser (whereas without AMF, NPK addition increased K solubiliser abundance). In Woburn soil, the opposite is true: AMF treated plants have more K solubilisers when fertiliser is added. This is further evidence to illustrate the importance of validating the efficacy of potential inoculants in the intended substrate – the AMF: NPK interaction is reversed in the different soil types. NPK addition can both negatively and positively influence the AMF effect on K solubilisers, dependent on soil type. The same trend in Barnfield and Woburn soil occurs for siderophore producing isolates; NPK addition with concurrent AMF inoculation both decreases and increases the proportion of positive isolates respectively.

The interactive effect of soil and AMF is less influential over differentially abundant ASVs. In Woburn soil, there were no differentially abundant genera associated with AMF application compared to Woburn soil with no additions. *F. geosporum* only caused significant differential abundance in Barnfield soil, increasing an ASV corresponding to an uncultured Actinobacteria, and decreasing ASVs included in the families *Nitrososphaeraceae* and *Clostridaceae* which have been discussed above.

5.6.5 Conclusions.

Comparing the effect of two different AMF species as inoculants is an important step in AMF research. *Funneliformis geosporum* is a commonly found, well described species that exists in many niches (Liu, Xu et al. 2020). The *R. irregularis* isolate, despite being a well described and commonly used species in AMF research, is an isolate from a salt mine and is therefore considered a specialist, as AMF in this environment are uncommon. *F. geosporum* has been shown to be efficient at foraging P for its host plant (Liu, Xu et al. 2020) and was chosen following the first two experiments as a baseline and to serve as a representative AMF inoculant species.

The unusual provenance of the *R. irregularis* isolate was included for interest to reveal if there was a difference between species origin in terms of plant colonisation or taxonomic influence. Although neither AMF species increased biomass, *F. geosporum* did significantly restructure rhizobacterial assemblage. Comparatively, *R. irregularis* had less influence over clover root-associated bacteria. This could indeed be because of its niche adaptation and thus it is not in its ideal conditions to thrive. *R. irregularis* colonised a lower proportion of roots than *F. geosporum*.

Overall, it is important to consider species and origin when selecting or studying mycorrhizal inoculants. There are over 240 species of AMF from the phylum Glomeromycota (and potentially more as suggested by molecular studies), which all have unique traits and physiology (Lee, Eo et al. 2013). Perhaps the most auspicious route in terms of an AMF inoculant would be a mixed community, to provide the highest likelihood of rhizosphere competence from as many individuals as possible for the intended niche, and to maximise benefits and traits provided by different species. However, in this case, competition dynamics would have to be considered (Zaller, Frank et al. 2011). This work shows that there can be some fitness disadvantage to AMF colonisation in certain circumstances.

The effect of host plant structuring its own rhizosphere community is clear when comparing bacterial assemblages from plant-containing treatments and the bare fallow soil samples. Some bacteria are notably present when the system contains

a plant, such as *Rhizobiaceae*, and some are more abundant in bulk soil environment, such as some Acidobacteria, Gemmatimonadaceae and *Isosphaeraceae*. Acidobacteria are an important constituent of the crop-defined rhizosphere environment (Kalam, Basu et al. 2020). Amongst the aforementioned plant-associated ASV examples there are families representative of commonly defined root-associated bacteria, such as rhizobia and *Sphingomonas*, which contain species which have been shown to be plant beneficial (Kim, Lim et al. 2019, Luo, Wang et al. 2019). *R. irregularis* has less impact on the rhizosphere community composition compared to *F. geosporum*, but in both cases the plant effect was the most influential factor.

There were some additional bacteria added in by the addition of the mycorrhizal inoculant. This was controlled for by using AMF-free inoculant made of the carrier substrate, which consisted of the same particulate matter but without the *in vitro* AMF hyphae and spore additions. Therefore, comparing the AMF treatment with this control gave the clearest comparison of the difference that AMF presence made to the system. It has previously been shown that there are indeed bacteria associated with commercial AMF inoculants (Agnolucci, Avio et al. 2019). The inocula used in the research presented in this thesis were produced *in vitro* in a sterile environment but it was still important to control for any background additions.

Adding fertiliser increased the proportion of bacterial isolates positive for casein hydrolysation, siderophore production and zinc solubilisation in Woburn soil but not in Barnfield. Previously published work has shown a significant decrease in plant growth promoting rhizobacteria when NPK is applied in the same amounts in Woburn soil (Reid, Kavamura et al. 2021). However, despite contradicting results, perhaps host plant is the most important factor for functional recruitment. The previously mentioned work was conducted on wheat, whereas this work examined clover. As a legume it is physiologically very different and will require different nutrient ratios, particularly P for nitrogen fixation (Mitran, Meena et al. 2018). The results from this study show that there is a difference between

rhizosphere and rhizoplane for taxonomic composition, and a clear plant effect of clover.

6 How do interactions between AMF and P solubilising rhizobacteria affect plant P status?

This experimental chapter is presented in paper format

6.1 Abstract

The sustainable intensification of agriculture is required to adequately provide for an increasing population in a changing climate. Root-associated microorganisms have a wealth of plant beneficial capabilities that can improve host health and mitigate biotic and abiotic stresses. Their use as putative bioinoculants to decrease dependence on artificial fertiliser is a promising strategy, and the interactions between beneficial mycorrhizal fungi and bacteria are yet to be exploited. In the presented work, *in vitro* tests for phosphorus liberation inform selection of strategic combinatorial inoculants of an arbuscular mycorrhizal fungus (AMF) and three phosphorus solubilising bacteria (PSB). These were tested *in planta* for their ability to additively enhance the growth of clover with a recalcitrant P source. Plant health was assessed via biomass and nutrient content, particularly Olsen P. No single inoculant combination out of 14 tested conferred any greater advantage than another. Recalcitrant P status of the soil was the most influential over plant health. There was an interactive effect of AMF and supplementary P; when P and AMF were added in combination, AMF appeared to compete with the host plant for this resource, resulting in reduced plant P content. Interactive effects of bioinoculants and agrochemicals must be considered during their design. PSB performing *in vitro* functions may not reproduce this ability *in planta* as their plant-growth promoting functions are subject to external influences which may affect the efficacy as agricultural inoculants.

6.2 Introduction

Microbial bioinoculants are gaining traction as potential agricultural supplements (Santos, Nogueira et al. 2019). Microbiome assisted agriculture aims to reduce dependency on agrochemicals such as inorganic fertilisers and pesticides (Kour, Rana et al. 2020). It is essential that there is a significant reduction in use of these chemical inputs if sustainable agriculture is to prevail; increasing output yields with less harmful inputs (Khush 2001, Wezel, Casagrande et al. 2014).

The green revolution was facilitated by advanced agricultural practices and the invention of chemical fertilisers. These typically consist of the main plant macronutrients nitrogen (N), phosphorus (P) and potassium (K). These fertilisers had striking results in terms of yield augmentation (Pimentel 1996, Melillo 2012). However, in recent times this increase in yield has levelled off (Glaeser 2010, Dahlberg 2012). It is also important to note that the use of fertiliser has unintended and devastating impacts on the surrounding ecosystem, such as eutrophication and ground water contamination (Glibert, Seitzinger et al. 2005, Srivastav 2020).

Alternatives to these chemicals must be employed which are more aligned with the global goals of reducing the impacts of climate change via sustainable agriculture. However, as populations continue to grow and climate change hinders agricultural productivity, one must consider both the efficacy of any inputs, and their potential environmental impact. Thus, it is important to look at new techniques and possibilities for improving crop growth.

The soil hosts a vast diversity of microbes, an important indicator of overall soil health and functioning (Pal, Chakrabarti et al. 2010). These might be beneficial to plants, pathogenic or neutral. There are complex methods of selection, communication and exchange that occur at the root-soil interface – the rhizosphere (Oldroyd 2013). Many of the beneficial microbes form close, mutualistic associations with plant roots, especially where the environment is rich in fixed carbon from photosynthesis (Chaparro, Sheflin et al. 2012). These microbes can assist host plants to resist biotic and abiotic stresses such as nutrient liberation and fixation, tolerance to drought, salt or heavy metal

contamination and protection against disease (Goteti, Emmanuel et al. 2013, Liu and Zhang 2015, Batool, Ali et al. 2020, Masters-Clark, Shone et al. 2020, Huo, Kang et al. 2021, Reid, Kavamura et al. 2021). Harnessing the untapped potential of plant growth promotion ability amongst the soil microbial communities is key to finding successful and environmentally benign techniques for enhancing plant growth.

Many bacteria are purported to associate with plants in a beneficial manner. Well-known examples include the legume-*Rhizobium* symbiosis in which bacterial partners fix atmospheric nitrogen in specialised root organs (nodules), in return for carbon. Some species of *Pseudomonas* and *Bacillus* are known plant growth promoting rhizobacteria (PGPR), with roles including resistance to salt stress (Vives-Peris, Gómez-Cadenas et al. 2018) and induction of host systemic resistance prior to pathogen attack (Kloepper, Ryu et al. 2004). In addition to bacteria, arbuscular mycorrhizal fungi (AMF) are the most significant beneficial plant-fungal interaction, a symbiotic colonisation of plant roots exchanging nutrients (primarily Pi), water and resistance to stresses for fixed carbon. This association is formed by over 600 species of fungi and by 80-90% land plants (Bonfante and Genre 2008). As AMF and PGPR occupy a similar niche, it is likely that they interact, however, the significance of these interactions for plant health are poorly understood. It is possible that such interactions can be exploited to generate novel bioinoculants that can sustainably enhance crop growth.

Bioinoculants are exogenously applied agricultural amendments containing microorganisms with the potential to benefit plant growth (Santos, Nogueira et al. 2019). The most common uses are for N-fixation, P and K solubilisation, as well as generalist plant growth promoters (Mahdi, Hassan et al. 2010). They are a potential alternative to chemical fertilisers as they enhance soil fertility, provide renewable resources, and unlock recalcitrant soil nutrients, and are environmentally friendly when deployed (Timmusk, Behers et al. 2017).

In order to be successful, soil bioinoculants must be compatible with the host plant rhizosphere, and a full understanding of their impact on the soil microbiome and wider ecosystem is required. They must not only be non-pathogenic to plants,

but to humans and other ecologically important species. Such knowledge will lead to optimal inoculation conditions being met, which will more likely result in the desired function from the inoculant (Nosheen, Ajmal et al. 2021). Inoculants using one beneficial microbe have been tested most frequently. In terms of nutrient enhancement *Rhizobium* spp*.* are one of the most well-known and have been used for over a century to enhance N uptake (Arora, Verma et al. 2017). In addition, *Serratia* spp*.* have been shown to increase the biomass of chickpea (Zaheer, Mirza et al. 2016) and *Pseudomonas koreensis* enhanced cucumber micronutrient content when inoculated alone or with half strength NPK fertiliser (Scagliola, Valentinuzzi et al. 2021). These examples show the benefit of a single inoculant performing in controlled conditions. For a more comprehensive table of PGPR inoculants which includes functions other than enhanced nutrient acquisition see García-Fraile, Menéndez et al. (2017).

In addition to plant inoculation with a single microbial species, examples of co-inoculation, with combinations of beneficial bacteria have resulted in plant growth promotion (de Souza and de Brito Ferreira 2017, Di Salvo, Cellucci et al. 2018). There are growing numbers of examples of bioinoculants combining AMF and PGP bacteria. Pérez-de-Luque, Tille et al. (2017) demonstrated differential effects on plant growth when dual inoculating *Pseudomonas putida* with *R. irregularis*. The use of AMF and a P-solubilising *Bacillus* species increased growth of chickpeas (Saxena, Saini et al. 2015).

However, the in-field performance of putative microbial inoculants is often poor. Indeed, microbes demonstrating beneficial effects in controlled environment situations are unable to replicate these advantages *in situ* (Haskett, Tkacz et al. 2021). Factors, owing to the complexity of both the host-microbe interactions and the environments from which the inoculants are derived. Variables such as host-specificity/promiscuity, inconsistent genetic regulation of beneficial traits, persistence in the rhizosphere, competition, nutrient availability and environmental perturbations such as pH, water availability and soil temperature are attributed to inoculant failure (Tabassum, Khan et al. 2017, Haskett, Tkacz et al. 2021, Nosheen, Ajmal et al. 2021).

There is very little evidence of efficacy using a consortium of synergistic microbes for a specific PGPR function such as P solubilisation. However, Ordoñez, Fernandez et al. (2016) showed the effects of a ten-strain PSB inoculant on both AMF growth and plant P uptake. They observed wildly variable effects from previously validated P-solubilising *Pseudomonas* spp., but overall a positive interaction between AMF and PSB. To best develop and exploit potential inoculants, understanding of their provenance and composition is essential (Nosheen, Ajmal et al. 2021). Screening microbial partners with the potential for an additive effect in terms of increasing plant health is likely to yield more biologically advantageous results. This could reduce the chances of competition and increase likelihood of inoculant survival.

6.3 Aims and objectives

This chapter aims to explore the efficacy of clover inoculation with PSB either alone, in combination with other PSB isolates or with and without AMF co-inoculation. Specifically, to reveal if AMF have more benefit in terms of plant biomass and P content in the presence of P solubilising bacteria. This experiment was intended to reveal if there are synergistic or antagonistic interactions between AMF and the selected PSB to benefit plant growth.

The specific objectives are:

1. To select an optimum combination of PSB, using *in vitro* characterisation

2. To test the performance of these microbes (AMF and PSB) both separately and combined to ameliorate plant health

6.4 Materials and methods

6.4.1.1 Plant preparation and planting.

For detailed protocols, refer to section 3.1. Clover seeds were sterilised, pre-germinated and grown in a glasshouse under 16h light, 8h dark, 21°C.

6.4.1.2 Substrate preparation.

For detailed protocols, refer to section 3.2. The growth substrate (sand: sandy loam, 1: 3) was mixed and sterilised before potting.

6.4.1.3 Experimental design.

221 pots were blocked by replicate in a complete randomised block design, n = 7. Treatments included all possible combinations of three bacterial inoculants, with or without AMF co-inoculation (AMF or the AMF-free carrier substrate), and with or without Ca_3PO_4 addition, resulting in 32 treatment combinations. Blocking design is given in Figure A-8.

6.4.1.4 Microbial inoculant isolation, screening and selection.

Bacterial strains were selected from a collection isolated from the rhizosphere of many crop species. The bacteria used in this experiment were all from the faba bean rhizosphere, from Furzefield, Rothamsted Research, UK. The initial work was carried out as part of a PhD project by a lab member, Larissa Oliviera. The results of this work were used to inform selection of the plant growth promoting bacterial isolates used *in planta*. With permission, the strains were selected as part of a joint effort on the isolate collection, and joint work screening the isolates for their PGP abilities and synergy *in vitro*.

Work carried out by L. Oliviera: The compatibility of isolates was tested *in vitro* as in Sundaramoorthy, Raguchander et al. (2012). Bacteria were streaked horizontally and vertically on 1/10 TSA media, with intersections between the isolates to assess compatibility. All combinations were tested, n = 4. Each of the isolates were tested individually and in combination for plant growth promoting activity with four functional assays (described in Section 3.10) Ca_3PO_4, K, Casein and Fe. When tested as individual isolates, 1 µl culture was used; 0.5 µl for two

isolates in combination; 0.33 µl for three bacteria and 0.25 µl for four bacteria, to yield 10^8 cells in total. Again, all combinations were tested and there were four technical replicates. Plates were incubated at 25°C until assessment (section 3.10). Colony size and zone of clearing were measured and the solubilisation index was calculated (Edi-Premono, Moawad A. M. et al. 1996, Paul and Sinha 2017). Isolates were identified by 16S rRNA gene sequencing where possible (pers. comm. L. Oliviera).

The bacterial isolates selected were the most compatible *in vitro* both in terms of co-existence and P (phosphate) solubilisation:

B1: P14(9), 16S rRNA gene identification: *Pseudomonas sp.*

Solubilises: N, P, K, Fe, $AlPO_4$

B2: P14(47), 16S rRNA gene identification: Unidentified

Solubilises: P, K, Fe, $AlPO_4$

B3: P19(7), 16S rRNA gene identification: Unidentified

Solubilises: P, K, $AlPO_4$, Phytate, $FePO_4$

6.4.1.5 Microbial inoculant preparation.

Bacterial inoculants were prepared by growing overnight at 25°C at 200rpm in 50% sterile TSB. The OD was measured to ensure cultures were in the exponential growth phase (OD_{595nm} 0.6-0.8). Cultures were centrifuged at 2000 x g for 10 min to obtain a pellet, which was resuspended in Ringer's solution and OD measured again and adjusted to 0.1 to 10^8 cells (with Ringer's solution), and then inoculated on to each plant as required for each treatment. For each single isolate treatment, a total of 10^8 cells were added to plant roots. For treatments where more than one isolate was added in a mixture, the total number of cells added was also 10^8 cells, divided equally by the number of isolates in a given mixture. For example, where a mixture of 2 isolates were co-inoculated both isolates would contribute $5x10^7$ cells to the mixture. The control treatment of no microbes "none" received 1ml of Ringer's solution. Bacterial inoculation occurred after two weeks of plant growth once seedlings had established.

The commercial mycorrhizal fungal inoculant utilised contained *F. geosporum* in an expanded clay zeolite (Symbiom, Lanškroun), which is stored at 4°C. Each pot had 1tsp (~5g) AMF inoculum or AMF-free inoculum carrier added and stirred in to the top 5cm of the substrate surface.

6.4.1.6 Plant harvest and sample processing.

Refer to section 3.6 and 3.7.

To obtain root samples, soil was loosened, and the root system carefully removed from the bulk soil. The roots were gently washed by floating in water in order to best preserve AMF structures and to remove the remaining substrate. A subsample was removed and stored in 70% EtOH at 4°C prior to staining.

6.4.1.7 Root staining and AMF colonisation assessment.

Refer to section 3.7. Percentage colonisation values are presented as predicted means from a generalised linear model. Only treatments for which there were statistical differences are shown as figures.

6.4.1.8 Statistical analyses.

Analysis was carried out in R (Version 4.0.2) unless stated otherwise. A One-way analysis of variance tests was used once all the assumptions of normality had been met, followed by post-hoc TukeyHSD for pair-wise comparisons.

Percentage colonisation was analysed in Genstat (21st edition version 21.1.1) using a generalised linear model. Residual plots showed that the underlying assumptions of the following analyses were met. Marginal and conditional tests (including and excluding the higher order terms) revealed that the order of fitting the factors could be disregarded. Consequently, the model replicate + P supplementation x Bacteria 1 x Bacteria 2 x Bacteria 3 was used and the statistical values given in the results section are derived from this order of fitting. The residual of the mean deviance was 19, and all analyses for the GLM were done using the logit scale.

6.5 Results

6.5.1 Plant Biomass.

AMF did not influence plant aboveground biomass, regardless of soil P status or bacterial combination (p = 0.76). In addition, none of the microbial inoculants affected plant biomass, in either Ca_3PO_4 or non-Ca_3PO_4 supplemented treatments (p = 0.7 and 0.2 respectively, Figure 6.1 and Figure 6.2). Furthermore, when AMF was added along with the bacterial inoculants, no difference to the biomass of clover was found, regardless of P status. The addition of inorganic P increased plant biomass when compared with the no-microbe control treatments (p = 0.002). Biomass increased highly significantly in response to Ca_3PO_4 application (p < 0.001). The mean biomass was 1.09g and 2.05g for the no Ca_3PO_4 and Ca_3PO_4 treatments respectively.

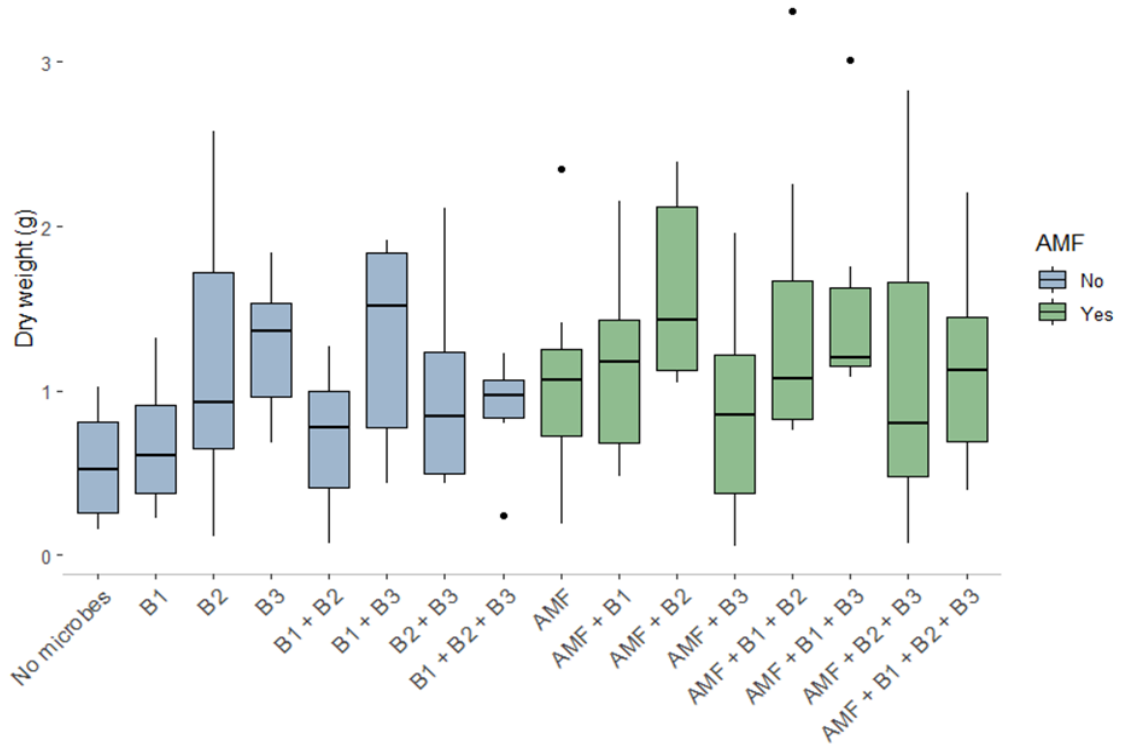


Figure 6.1. **The aerial biomass of clover with the addition of Pi solubilising microbial inoculants in the absence of Ca_3PO_4.** Treatments included combinatorial microbial inoculants from AMF (*F. geosporum*), and three Pi solubilising bacterial B1, B2, B3. This figure represents all treatments without additional Ca_3PO_4. Experiment was blocked by replicate in a complete randomised block design and n = 7.

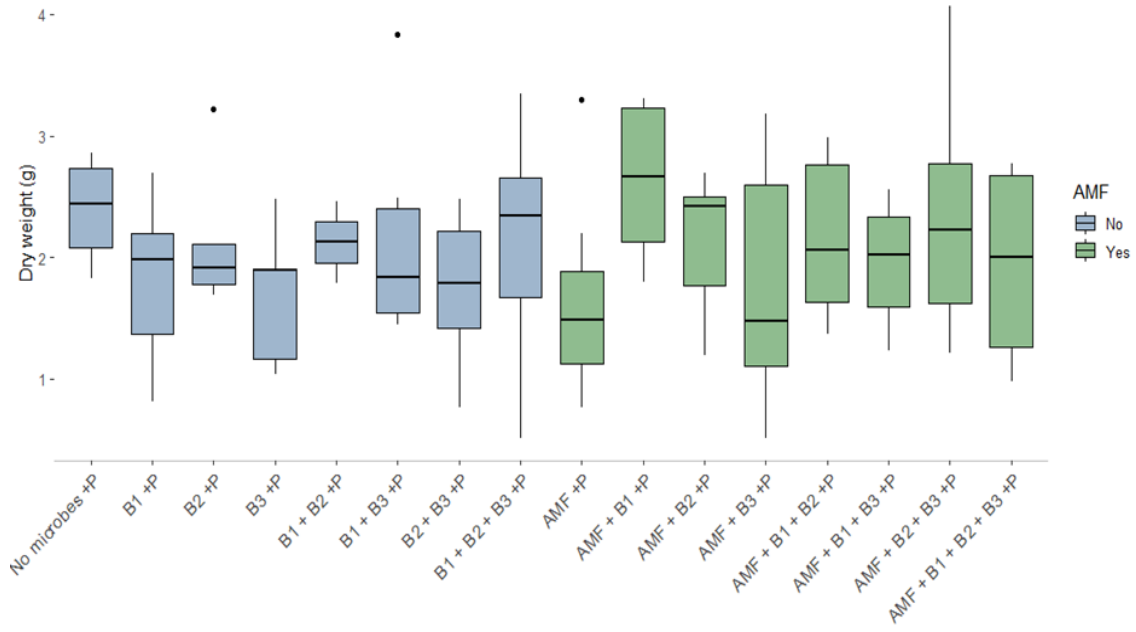


Figure 6.2. The aerial biomass of clover with the addition of Pi solubilising microbial inoculants with supplemented Ca_3PO_4. Treatments included combinatorial microbial inoculants from AMF (*F. geosporum*), and three Pi solubilising bacterial B1, B2, B3. This figure represents all treatments without additional P. Experiment was blocked by replicate in a complete randomised block design and n = 7.

6.5.2 Percentage AMF colonisation.

Only factors causing statistical differences in colonisation are presented as figures (Figure 6.3 and Figure 6.4). Marginal and conditional tests revealed the order of fitting was mostly affected by Ca_3PO_4 addition. When Ca_3PO_4 was added, AMF colonisation was reduced regardless of microbial treatment ($F_{1, 81}$ = 118.2, $p < 0.001$). Combining all microbial treatments, average colonisation was 60%, dropping to 12.3% when Ca_3PO_4 was added. There was an interaction between B1 and B2 which antagonistically affected AMF colonisation ($F_{1, 81}$ = 7.99, $p = 0.006$, Figure 6.4). There was a difference in colonisation when B2 was included, dependent on B1 presence. There was higher colonisation with B2 when B1 was not included.

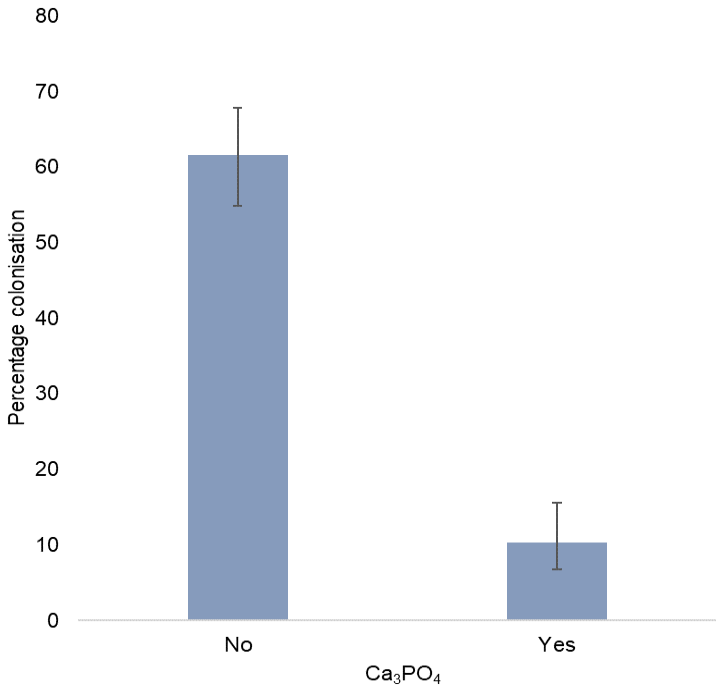


Figure 6.3. Predicted mean proportion of AMF colonised roots when Ca_3PO_4 was added. Bars show 95% confidence intervals, back transformed from logit scale, and predicted means include all combinations of inoculants.

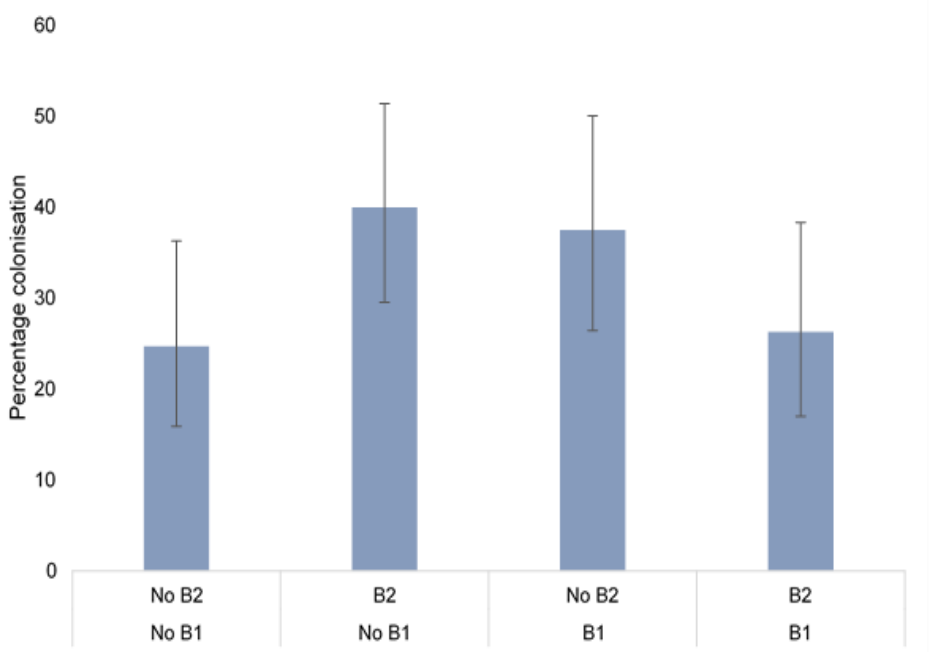


Figure 6.4. Predicted mean proportion of AMF colonised roots for Bacteria 1 and 2. Bars show 95% confidence intervals, values back transformed from logit scale.

6.5.3 Plant nutrient content.

Figure 6.5 shows the nutrient content of plants grown with different combinations of microbial inoculants. An analysis of variance was carried out on the model AMF x Bacteria 1 x Bacteria 2 x Bacteria 3. Results are presented in Table 6-1 and Table 6-2. The addition of inorganic insoluble P made significant differences (p < 0.001 for all) to the nutrient content of clover aerial tissue for all nutrients measured except sulphur. Ca_3PO_4 addition decreased Ca, K and Mg content (-5661 PPM, -5046, -641.9 mean difference in PPM, respectively) and increased plant P and Na content (1099.9 PPM and 1927.1 PPM mean difference respectively).

Olsen P. Plant P content was significantly reduced when inoculated with AMF and supplemented with Ca_3PO_4 (p <0.001). However, when Ca_3PO_4 was not included, AMF caused a significant rise in plant P content compared to plants without AMF (p < 0.001). There is an additive interaction of AMF and Bacteria 2 for Ca_3PO_4 supplemented plants, increasing plant P content when co-inoculated

(p = 0.02). When Ca_3PO_4 is not added, there is a small four-way interaction effect of all of the microbes (p = 0.05). No AMF (without additional Ca_3PO_4) had the lowest P content (mean: 1297 PPM), AMF inoculation increased this to a mean of 1716 PPM. Ca_3PO_4 addition without AMF yielded the highest plant P content (mean: 2811 PPM), which was reduced by AMF inoculation (2431 PPM) (Figure 6.6).

6.5.3.1 Bacterial effects.

There were a small number of bacterial main effects for the different nutrients surveyed. Magnesium tissue content was altered by B2 with additional Ca_3PO_4 (p = 0.02), and by B3 when Ca_3PO_4 was not supplemented. Other interaction effects between the microbial combinations are detailed in Table 6-1 and Table 6-2.

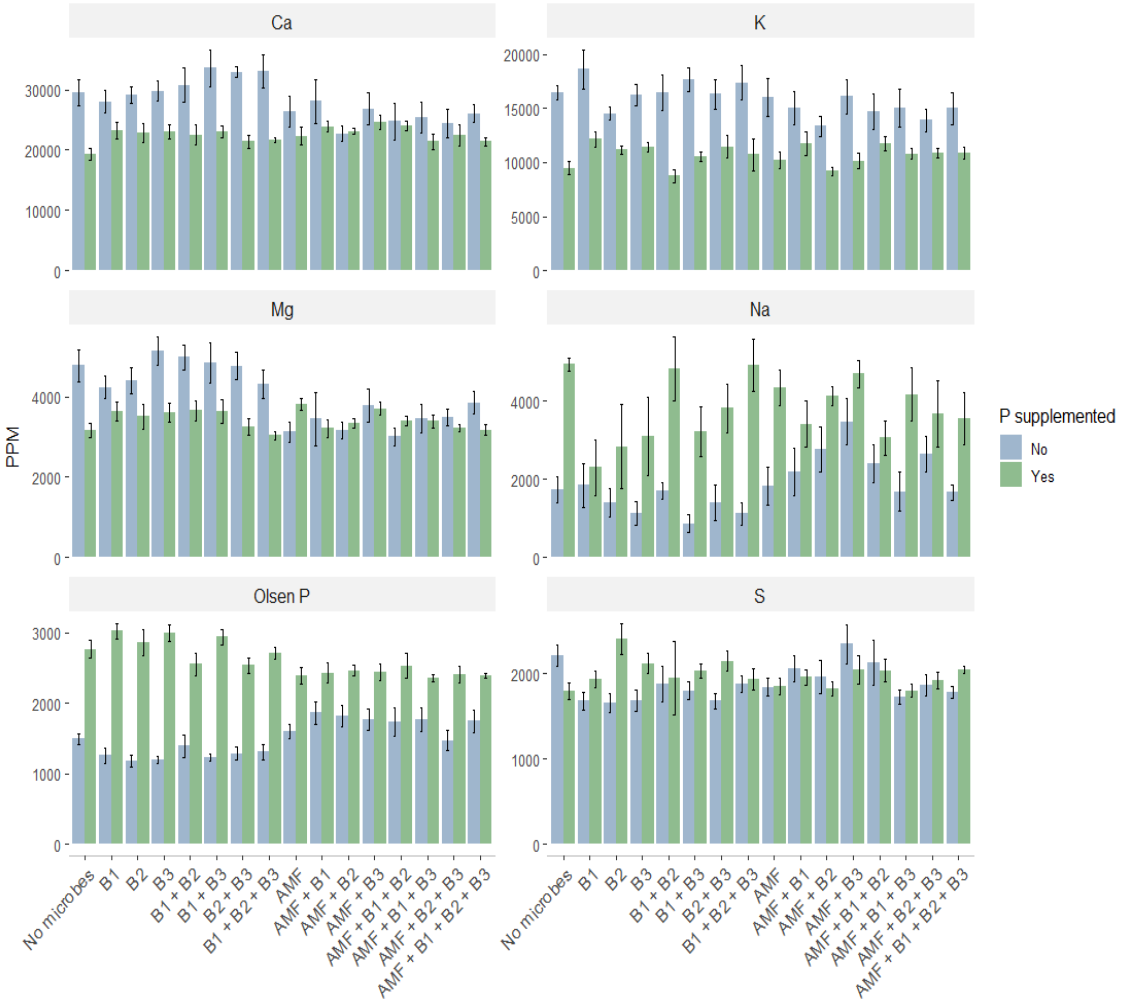


Figure 6.5. **Nutrient content of clover aerial tissue grown with different combinations of microbial inoculants**. Blue denotes plants not given supplementary Ca_3PO_4, green denotes plants with supplementary Ca_3PO_4. Values are given as PPM in dry matter. B1, B2 and B3 encode bacteria 1, 2 and 3. Bars give standard error and n = 7

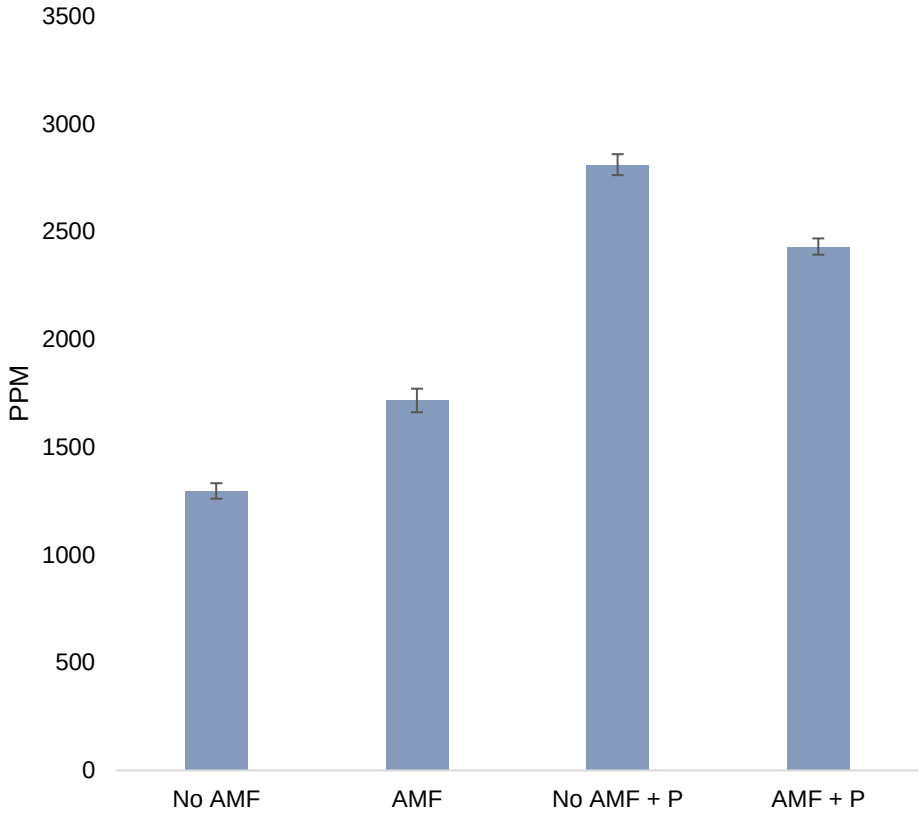


Figure 6.6. **Mean PPM Olsen P clover tissue content. Main effects of AMF and Ca_3PO_4 addition**. Means are calculated from all treatments. Bars give standard error, n = 7.

Additional P		Ca		K		Mg		Na		S		P	
	Df	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)
AMF	1	2.13	0.15	0.37	0.55	0.03	0.88	0.48	0.49	3.71	**0.06**	40.49	**0.00**
Bacteria 1	1	0.32	0.57	3.17	0.08	0.57	0.45	1.57	0.21	0.49	0.49	0.48	0.49
Bacteria 2	1	0.05	0.83	0.00	1.00	6.11	**0.02**	0.02	0.90	2.61	0.11	2.22	0.14
Bacteria 3	1	0.18	0.68	0.13	0.72	0.39	0.53	0.83	0.37	0.36	0.55	1.03	0.31
AMF:Bacteria 1	1	1.96	0.17	2.24	0.14	2.36	0.13	0.60	0.44	2.41	0.12	0.53	0.47
AMF:Bacteria 2	1	0.05	0.83	0.00	0.96	0.10	0.75	2.87	0.09	0.75	0.39	5.70	**0.02**
Bacteria 1:Bacteria 2	1	0.26	0.61	0.99	0.32	0.07	0.79	4.12	**0.05**	0.07	0.79	0.03	0.87
AMF:Bacteria 3	1	1.01	0.32	0.31	0.58	0.00	0.98	0.08	0.77	0.00	0.95	0.02	0.88
Bacteria 1:Bacteria 3	1	5.26	**0.02**	4.88	**0.03**	0.44	0.51	2.36	0.13	1.54	0.22	0.09	0.76
Bacteria 2:Bacteria 3	1	4.25	**0.04**	0.72	0.40	4.29	**0.04**	0.22	0.64	2.19	0.14	1.54	0.22
AMF:Bacteria 1:Bacteria 2	1	0.92	0.34	1.76	0.19	4.56	**0.04**	3.74	**0.06**	5.73	**0.02**	0.33	0.57
AMF:Bacteria 1:Bacteria 3	1	0.50	0.48	0.05	0.82	1.18	0.28	0.08	0.77	1.11	0.30	0.28	0.60
AMF:Bacteria 2:Bacteria 3	1	0.53	0.47	0.09	0.77	1.44	0.23	1.93	0.17	3.07	0.08	0.32	0.57
Bacteria 1:Bacteria 2:Bacteria 3	1	2.34	0.13	0.65	0.42	0.24	0.62	0.92	0.34	2.07	0.15	2.11	0.15
AMF:Bacteria 1:Bacteria 2:Bacteria 3	1	0.12	0.74	4.43	**0.04**	0.37	0.55	2.48	0.12	0.19	0.66	2.29	0.13
Residuals	77												

Table 6-1. Analysis of variance table giving F and *p* values for clover aerial tissue nutrient content with supplementary Ca_3PO_4. Significant values, or values approaching significance, are highlighted in bold, defined at $p = 0.05$.

No additional P		Ca		K		Mg		Na		S		P	
	Df	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)
AMF	1	18.67	**0.00**	6.29	**0.01**	52.28	**0.00**	18.07	**0.00**	4.43	**0.04**	39.83	**0.00**
Bacteria 1	1	0.57	0.45	1.51	0.22	0.15	0.70	2.49	0.12	0.29	0.59	0.94	0.34
Bacteria 2	1	0.17	0.68	3.18	0.08	0.29	0.59	0.02	0.89	0.87	0.35	0.24	0.62
Bacteria 3	1	1.72	0.19	0.16	0.69	3.53	**0.06**	1.05	0.31	1.07	0.30	1.04	0.31
AMF:Bacteria 1	1	0.00	0.97	1.27	0.26	0.41	0.52	2.48	0.12	0.21	0.65	0.67	0.41
AMF:Bacteria 2	1	2.11	0.15	0.02	0.89	0.01	0.91	0.03	0.86	**0.01**	0.91	0.17	0.68
Bacteria 1:Bacteria 2	1	0.13	0.72	0.48	0.49	0.79	0.38	0.03	0.87	4.99	**0.03**	0.56	0.46
AMF:Bacteria 3	1	1.27	0.26	0.01	0.91	0.77	0.38	2.20	0.14	0.09	0.77	0.03	0.87
Bacteria 1:Bacteria 3	1	0.01	0.93	0.09	0.76	0.37	0.55	4.82	0.03	0.65	0.42	0.10	0.75
Bacteria 2:Bacteria 3	1	0.32	0.57	0.71	0.40	0.30	0.59	0.38	0.54	0.14	0.71	0.02	0.90
AMF:Bacteria 1:Bacteria 2	1	0.15	0.70	0.87	0.35	0.35	0.56	0.00	1.00	0.35	0.56	0.96	0.33
AMF:Bacteria 1:Bacteria 3	1	0.56	0.46	0.05	0.82	0.27	0.61	1.21	0.27	8.49	**0.00**	0.00	0.98
AMF:Bacteria 2:Bacteria 3	1	0.26	0.61	0.31	0.58	1.52	0.22	3.02	0.09	3.18	0.08	2.09	0.15
Bacteria 1:Bacteria 2:Bacteria 3	1	0.17	0.68	0.01	0.94	0.01	0.93	0.58	0.45	**0.01**	0.91	0.07	0.80
AMF:Bacteria 1:Bacteria 2:Bacteria 3	1	1.03	0.31	0.00	0.99	3.05	0.08	1.00	0.32	4.27	0.04	4.12	**0.05**
Residuals	90												

Table 6-2. Analysis of variance table giving F and *p* values for clover aerial tissue nutrient content without Ca_3PO_4. Significant values, or values approaching significance, are highlighted in bold, defined at $p = 0.05$.

6.6 Discussion

This Chapter explores the possibility of designing optimum combinations of putative phosphorus solubilising microbial inoculants to enhance plant health and P content. The following steps were taken to ensure maximum likelihood of bioinoculant success: 1. Testing compatibility of bacterial isolates to be used within the consortia, 2. Assessing multiple combinations of the chosen microbes to mitigate risk of competition and increase the prospect of choosing a successful combination, 3. Ensuring that there is a relevant substrate source of Pi linked to the *in vitro* tests.

This protocol was established as a method of designing inoculants from an existing library to have the best chance of finding a suitable candidate that can promote plant growth and, in the case presented here, potentially increase plant P nutrition. It was also important to establish how AMF and putative Pi solubilising bacteria interact when used as a bioinoculant. Understanding the dynamics of competition or synergy is an exciting prospect in AMF research and important when considering the ecological implications of using such an inoculant in the field.

6.6.1 The effects of combinations of PSM on clover with or without supplementary P.

6.6.1.1 Biomass.

There was no significant advantage of any of the combinations of AMF and P solubilising bacteria in terms of increasing clover biomass. However, it is important to note that there was no observed disadvantage either. This implies that none of the selected isolates became inhibitory, or antagonistic, but they were not deemed beneficial. The main factor that influenced biomass was the addition of insoluble, inorganic P as Ca_3PO_4. This is purportedly inaccessible to plants and was chosen as the bacterial isolates had proven efficacy in liberating this form of Pi. Although there is little evidence of AMF mineralising Ca_3PO_4 directly, reports suggest it does interact with PSB to increase plant uptake of P when Ca_3PO_4 is present e.g. Sharma, Compant et al. (2020). The use of Ca_3PO_4 also has been shown to increase AMF colonisation of roots (Ratti, Kumar et al.

2001). Thus, we deemed it a suitable substrate for both the PSB and AMF to utilise. However, owing to the fact that the clover from the no-microbial control with supplemented Ca_3PO_4 were larger than in the non- Ca_3PO_4 treatment, it can be assumed that the increase in biomass when Ca_3PO_4 is present is not from microbial amendment. The increase in biomass due to the inclusion of Ca_3PO_4 is uniform across the supplemented treatment compared to the non-Ca_3PO_4 treatment. Therefore, it is likely to be a plant related-mechanism resulting in increased P uptake such as acidification, the release of root-exudates, carboxylation, or gaseous exchange (Hinsinger 2001, Ryan, Tibbett et al. 2012). It is also important to consider that the system was not maintained as sterile although the substrate was sterilised initially to reduce microbial background, to give inoculants the best chance to establish. It is possible that opportunistic colonisers could be having an effect. Additionally, an experiment using forms of P with different solubilities – such as orthophosphate, tricalcium phosphate and phytate – might explain this action further. Utilising transcriptomics in bioinoculant tests would reveal shifts in plant gene expression and explore inoculant-induced modifications to plant physiology.

6.6.1.2 Plant nutrient content.

Most strikingly, there is a strong differential effect of AMF on the nutrient content of clover due to inorganic, insoluble P supplementation. When there was no Ca_3PO_4 added to the system, plants colonised with AMF had altered nutrient content for every element measured. These effects were not observed with the addition of Ca_3PO_4, except in tissue Olsen P levels.

AMF also reduced plant P content compared to plants with no AMF, when Ca_3PO_4 was added to the system (Figure 6.6). Plants with no AMF had higher plant P content when supplemented with Ca_3PO_4, compared to plants with AMF. When Ca_3PO_4 was excluded, the opposite effect occurred; AMF increased plant P content compared to treatments without AMF. It is possible that when plants are P replete, competition is occurring for resources. AMF do not negate the effect of Ca_3PO_4 on plant P content entirely, but it is important to consider that plant P content is negatively affected by AMF colonisation when P is available.

The reduction in plant P content caused by AMF in Ca_3PO_4 supplemented soils remains higher than the increase caused by AMF in non- Ca_3PO_4 soil, therefore Ca_3PO_4 supplementation is still advantageous with a background of AMF. None of the PSB bacteria alone affected plant P status in any treatment. This could be due to the fact that these isolates were taken from a collection isolated from faba bean, which may mean they are not competent clover colonisers. Additionally, plant growth variability is often large and can be greater than the potential benefit seen by the use of associative microbes. High variation may mask benefits conferred by microbial inoculants; although seven replicates were used, more replication could be useful to improve observed treatment effects.

AMF colonisation and changes in root morphology are both plant-responses to low P, but these tactics compete against each other (Ma, Li et al. 2021). AMF colonisation is often negatively correlated with root hair length, particularly when P is supplemented (Orfanoudakis, Wheeler et al. 2010, Liu, Wang et al. 2018). This may lead to a trade-off in nutrient acquisition and explain the reason behind AMF decreasing plant P content when Ca_3PO_4 is added to soil.

6.6.1.3 Colonisation of clover roots by AMF.

No biomass increase was observed with the addition of the exogenous AMF inoculant *F. geosporum* in non-Ca_3PO_4 supplemented pots. Despite reasonable levels of colonisation (mean colonisation = 60%), there was no observed biomass increase that could be attributed to the use of the purported plant growth promoting AMF strain.

The proportion of roots colonised by AMF was severely inhibited by the addition of Ca_3PO_4, regardless of microbial treatment. This is contrary to Ratti, Kumar et al. (2001) who report increased levels of colonisation when soil is supplemented with Ca_3PO_4 and additional PSB are added. However, the PSB included in the aforementioned study were additionally defined as “mycorrhiza helper bacteria”. Nevertheless, taking the results from the biomass data, which shows an increase when Ca_3PO_4 is supplemented, it can be assumed that the plant is able to access the additional Pi. Therefore, if the plant is no longer in a P deficiency, it is less likely to recruit its mycorrhizal partner (Abbott, Robson et al. 1984).

There could be a reduced carrying capacity for the soil to support mycorrhizal growth, or less carbon allocation to roots by the host, or the AMF partner might confer a fitness disadvantage (e.g. carbon sink) in the absence of stress (Schroeder and Janos 2005, Smith and Read 2008, Collins and Foster 2009, Verbruggen, van der Heijden et al. 2013, Konvalinková, Püschel et al. 2017). The decrease in colonisation when P was added is to be expected – as soil P concentration increases, AMF recruitment decreases (Schroeder and Janos 2005, Ven, Verlinden et al. 2019). One observed consequence of P fertilisation is reduced total below-ground carbon allocation by the host plant, which is a strategy used for nutrient accession and microbial recruitment (Ven, Verlinden et al. 2019).

Similarly, biomass was unaffected despite the much lower levels of colonisation in the Ca_3PO_4 treatment (mean colonisation = 12.2%). The lower colonisation is likely to be because of the host plant being fully satiated for P and is therefore not actively recruiting AMF (Abbott, Robson et al. 1984). This supports reports in the literature that the mutualism is downregulated when plants have access to adequate P (Collins and Foster 2009).

AMF did not make a difference to biomass, regardless of the bacterial inoculant combination added. There appears to be no interaction between the AMF inoculant and any combination of PSB in terms of influencing plant biomass. The selection of the PSB was done using information about the bacterial isolates ability to solubilise Ca_3PO_4; their compatibility with each other *in vitro*, and their synergy in terms of Pi liberation *in vitro*. Using Ca_3PO_4 to test PSB status and including it as the source of Pi in the pot experiments provides a known substrate that could be solubilised by the inoculants (Masters-Clark, Shone et al. 2020). However, even with the pre-determining of PSB potential, *in planta* performance of inoculants was unsuccessful. This is concurrent with the experiences of many other putative bioinoculants – in field performance does not match up to predictions *in vitro* or *in silico* (Haskett, Tkacz et al. 2021). Better understanding of inoculants is required, incorporating comparative genomics including core and accessory genomes.

Checking for bacterial persistence in the rhizosphere, host competency, and quantifying bacterial recovery would be logical strategies to include in the development and selection of potential combinations of microbes. It is also important to note the many environmental and edaphic factors that can influence inoculant performance in the field.

6.6.2 Considerations and recommendations.

Microbial provenance is an important consideration for the selection of putative beneficial isolates. Different plant species have distinct microbiomes (Bouffaud, Poirier et al. 2014, Ofek-Lalzar, Sela et al. 2014). Bacteria can exhibit host-promiscuity, inhibiting the effectiveness of an in-field inoculant if there are weeds or cover crops which are not the intended beneficiary (Haskett, Tkacz et al. 2021). Conversely, bacteria can also be rigidly host specific, particularly biotrophic pathogens (Raaijmakers, Paulitz et al. 2009). Consequently, microbial inoculants will need to either be tested on a wide range of recipient species, or created for a specific host or soil type (Finkel, Salas-González et al. 2019). This is a potential constraint for the widespread use of beneficial inoculants and consideration of the need for specificity was an omission from this work and could provide an explanation for lack of improvement to plant growth. Attention could also be paid to survival in the rhizosphere, which was not done in this study.

The sterilisation of the substrate was done to remove the majority background microbiota so that the inoculated microbes could colonise in the absence of competition and have the best chance of establishing. However, it has been shown that some microbial inoculants perform better with the presence of a full microbiome as opposed to those tested in sterile, simplified substrates (Ordoñez, Fernandez et al. 2016). Additionally, there is evidence that AMF require bacteria to perform optimally and increase fitness, such as mycorrhiza helper bacteria (Bonfante and Anca 2009, Salvioli, Ghignone et al. 2016). Testing inoculants with a full microbial complement is a more realistic comparison of field/horticulture deployment, which would be the end goal. However, it can be argued that this experimental design serves as an initial screening process, evaluating host and

rhizosphere compatibility, testing in a simplified sterile substrate is a logical first step.

However, lack of observed plant growth benefits does not mean lack of interaction between AMF and bacteria. There was an antagonistic interaction of B1 and B2 on AMF colonisation of plant roots. Colonisation of roots by AMF was almost halved when B2 was combined with B1; 45% roots were colonised by AMF with B2 alone but 25% roots when B1 and B2 were together. This is contrary to the synergy between the two bacterial isolates observed *in vitro*, but it is possible that these bacteria are not beneficial to AMF. Transcriptomics, both *in vitro* and *in planta*, could explore the mechanisms further.

6.6.3 Conclusion

In line with the biomass and Olsen P results presented here, AMF have been previously observed to increase plant P content but not improve overall plant productivity (Van Der Heijden, Streitwolf-Engel et al. 2006). This highlights the importance of testing many plant health metrics to gauge the activity of putative inoculants. Despite these alterations to tissue P content, biomass was still unaltered. Further study would benefit from experiments focusing on economically advantageous metrics of plant health such as grain yield in crop plants.

Some interactive effects occurred between the bacterial inoculants, yet none yielded consistent increases in the plant health parameters tested and therefore were not termed as a successful inoculant. AMF increased plant P content when Ca_3PO_4 was not included but had the opposite effect after supplementary Ca_3PO_4. This suggested that although AMF reduce plant P content when Ca_3PO_4 is supplemented, levels remain higher than adding AMF without additional Ca_3PO_4. These results show the beneficial effects of AMF in terms of plant macronutrient status (P content in this case) might be affected when soil is chemically amended. Addition of nutrients and microbes in combination may encourage competition between the host and microbial for resources, rendering both less efficient. Conversely, the use of chemical additions might become less effective in AMF soils. Thus, it is important to find the biological and economical

balance between the benefits of adding chemical fertiliser versus exploiting the beneficial effect of microbes that fulfil the same purpose to enhance plant nutrient status.

The interactive effects of putative inoculants are undoubtedly complex. In order to test interactions between PGPR and AMF, a reliable AMF phenotype is needed. This was only obtained through plant P content and not biomass. There were some interactions between bacterial inoculants affecting AMF root colonisation but none that notably improve plant health. The pre-screening approach adopted to give the best chance of finding a successful inoculant is valid but might be best paired with a higher throughput *in planta* test to increase likelihood of success.

7 Bacterial transference along AMF hyphae – is mobilisation selective for specific functions or taxa?

This experimental chapter is presented in paper format.

7.1 Abstract

Mycorrhizal fungi are the main beneficial plant-fungal interaction and curate the bacterial assemblage in their hyphosphere. There is growing evidence of bacterial dispersal along fungal hyphae both in soil and *in vitro*, but the network of AMF hyphae is just beginning to be explored as a potential conduit for bacteria to gain preferential access to plant roots. Particularly, investigating AMF-mediated selectivity over bacterial functional capabilities is a novel approach. This work uses mesh of different aperture sizes to create compartments containing clover plants, connected through bulk soil via AMF hyphae to reveal transference of a *gfp* transformed bacterial strain (*Pseudomonas putida* KT2440::*gfp*). Additionally, it explores the taxonomic composition and functional capabilities of the root microbiome of clover plants permitted or denied access to field soil via AMF hyphae. Migration of *P. putida* occurred between plants only when AMF were not present. Indeed, AMF plants appeared to have a higher concentration of *P. putida* compared to dispersal to the neighbouring plant where AMF was not inoculated. Rhizobacteria communities did not differ in their taxonomic assemblage or functional abilities regardless of AMF access to field soil. Together these results suggest that bacterial dispersal through soil can occur passively along non-specific fungal hyphae without facilitation or selectivity by AMF. The rhizobacteria of an AMF plant might experience a greater “pull”, perhaps via a transpiration gradient created by hyphae, to concentrate bacteria towards the root surface.

7.2 Introduction

Host plants exercise stringent and dynamic regulation of their microbial partners (Smalla, Wieland et al. 2001, Söderberg, Olsson et al. 2002, Kavamura, Robinson et al. 2019). These partners can provide plant-growth promoting benefits such as nutrient provision, drought tolerance and disease resistance. The soil is a heterogeneous ecosystem, with microenvironments, chemical gradients, structural variations, and rich communities of microorganisms. How bacteria arrive at plant roots is not fully elucidated. Juyal, Otten et al. (2021) postulate that bacteria might not be distributed randomly in the soil and exist in microenvironments dependent on soil structure. Therefore, to migrate towards the exudate rich root environment, rhizobacteria may need to mobilise through the soil.

Fungi provide a network of mycelium, bridging air spaces and soil aggregates as a continuous water filmed matrix that enables bacterial dispersal (Wick, Remer et al. 2007). *In vitro* studies have confirmed that fungal hyphae do act as a conduit for bacterial mobilisation (Kohlmeier, Smits et al. 2005, Zhang, Kastman et al. 2018). However, extrapolating from *in vitro* experiments to imagine the behaviour of soil dwelling microorganisms must be done with caution. This being said, revealing the movement of bacteria in soil is difficult because of the opacity and heterogeneity of soils. Movement of bacteria along fungal hyphae has been shown to be strongly microorganism-dependent, with traits such as hydrophobicity of hyphae or bacterial motility having a great influence on mobilisation (Kohlmeier, Smits et al. 2005). There are also other edaphic factors such as soil structure and chemical composition which can affect bacterial dispersal, so experimental systems must be carefully considered (Juyal, Eickhorst et al. 2018).

AMF are a key beneficial plant-fungal interaction; thus it is likely that they are also an important determinant of root microbiome composition. Interactions between AMF hyphae and other microorganisms (in the “hyphosphere”) are substantial (Turrini, Avio et al. 2018). Bacterial community structure is conserved across

some AMF species (*Glomus versiforme* and *R. irregularis*), demonstrating their ability to selectively determine their microbiome (Emmett, Lévesque-Tremblay et al. 2021). There is mounting evidence of AMF regulating hyphosphere bacterial taxonomic composition and functional capability. AMF cannot mobilise organic phosphorus, and have been shown to recruit bacteria with alkaline phosphatase activity to mitigate this (Zhang, Shi et al. 2018). Additionally, in conjunction with the host, AMF select bacteria which are beneficial for the symbiosis and the plant (Frey-Klett, Chavatte et al. 2005).

It is known that AMF also influence rhizobacteria at a taxonomic level. AMF have been shown to modify the root microbiome of a host plant, not just those directly associated with spores or hyphae. In response to salt stress, AMF modify the bacterial community of peanut, increasing abundance of Proteobacteria and Firmicutes, and consequently trigger a higher soil urease activity level (Ci, Tang et al. 2021). AMF also modify the root microbiome of Lanthanum-spiked maize to increase the abundance of PGP bacteria, including those with heavy metal tolerant functions (Hao, Zhang et al. 2021).

Recently, bacteria have been shown to disperse along AMF hyphae. For example, mobilisation of *Rahnella aquatilis* (a known PSB) was observed *in vitro*, dependent on the influence of hyphal exudates, suggesting that facilitation is not merely a passive translocation along the physical structure (Jiang, Zhang et al. 2021). In addition, AMF hyphae were shown to facilitate the transfer of *Bradyrhizobium sp*. to the roots of soybean and enabled subsequent nodulation (de Novais, Sbrana et al. 2020).

The methods by which this recruitment and regulation occurs remain unknown. However, there is evidence of bacterial regulation by fungal-mediated quorum sensing. For example, between the human pathogenic fungus *Candida albicans* and *P. aeruginosa* to permit co-existence in biofilms (De Sordi and Mühlschlegel 2009). Additionally, quorum sensing signalling molecules from bacteria are detected, and even degraded by some fungi (Uroz and Heinonsalo 2008, Tarkka, Sarniguet et al. 2009). Alternatively, bacterial secondary metabolites could be implicated in bacterial-fungal signalling. These metabolites elicit a growth

response and gene expression changes in fungi, including mycorrhizal fungi (Schrey, Schellhammer et al. 2005, Riedlinger, Schrey et al. 2006, Tarkka, Schrey et al. 2006, Schrey and Tarkka 2008, Tarkka, Sarniguet et al. 2009).

Understanding the role that AMF play in the deliverance or recruitment of bacterial communities to plant roots will lead to a more holistic understanding of microbial community dynamics. In essence, if AMF are an important determinant that enable the microbiome to be able to function optimally for plant health, it will have impacts for soil management practices for the future. This information can be useful for sustainable agricultural practices – we can optimise, protect, or cultivate interactions in the microbiome to maximise plant beneficial effects.

7.3 Aims and objectives

The overall aim of this chapter is to track the movement of bacteria through bulk soil facilitated by AMF hyphae. It is hypothesised that bacteria travel along fungal hyphae, bridging soil pores and aggregates to attain proximity, and even preferential access, to host roots. Importantly, it aims to determine whether this transport is active or passive - are AMF actively recruiting bacteria of certain species or with particular attributes? The following experiments were designed to reveal changes in the functional capabilities of the microbiome of clover that are facilitated by AMF. In short, do AMF preferentially select or recruit bacterial species with specific functions, or from certain taxonomic groups.

Specific objectives

1. To assess the non-selective movement of *gfp* labelled *Pseudomonas putida* KT2440::*gfp* (hereafter referred to as *P. putida*) along AMF hyphae using root exclusion/hyphae permitting meshes, culture and qPCR.

2. To ascertain in which direction the conduction of bacteria is occurring along hyphae (towards the root, or away from the host with the physical growth of the hyphae). Using root excluding/hyphae permitting meshes, and *P. putida*, the direction of “pull” from the AMF will be revealed by deploying the bacteria in

different locations. Quantification of bacterial movement and recovery of *P. putida* by culture and qPCR will determine the role of AMF in the direction of travel of bacteria from the inoculation point.

3. To investigate whether AMF selectively facilitate bacterial colonisation of a host plant, and to describe the resulting rhizobacteria community both taxonomically and functionally. This will be explored using clover grown in a simplified, sterile substrate. Using root excluding, hyphae permitting/excluding meshes, AMF will have access to a full microbial community in surrounding low P field soil. This will reveal 1: If the root microbiome that forms around the clover is influenced by AMF transferring bacteria from the field soil into the simplified substrate, and 2: whether the functional capabilities of this community are selected for by AMF.

7.4 Materials and Methods

Three experiments are presented in this Chapter. A summary of their aims is provided, and more details of the specific methods and experimental design for each experiment are described subsequently.

7.4.1 Box experiment 1.

This experiment was designed to reveal if *P. putida* would disperse through the bulk soil passively, with the physical direction of growth of AMF hyphae. It was hypothesised that *P. putida* would colonise a neighbouring plant facilitated by AMF hyphae growth, a potential conduit for bacterial transport in bulk soil. One plant was grown inside a root-excluding/hyphae-permitting mesh compartment and inoculated with AMF and *P. putida*. The other plant was grown outside of the mesh. The two plants were therefore only connected by fungal hyphae, not by roots. The spread of *P. putida* was detected using culture and qPCR.

7.4.2 Box experiment 2.

Following the results from experiment 1, it was necessary to design an experiment which could demonstrate the direction of “pull” being experienced by *P. putida*. Two plants were positioned at either end of a box, both inside a

separate mesh compartment filled with and surrounded by the same substrate. One plant was inoculated with AMF. Again, the intention was that the two plants were connected via a hyphal “bridge”, without roots touching. *P. putida* was inoculated either on the AMF plant, the neighbouring plant, or in the centre of the box between the two mesh compartments. The spread of *P. putida* was detected via culture and qPCR.

7.4.3 Field soil and mesh, experiment 3.

This experiment aimed to describe the formation of the rhizobacteria community establishing on a plant grown in a sterile simplified substrate, permitted or denied access to field soil via AMF hyphae. The aim was to reveal which bacteria would colonise plant roots (described by both taxonomic abundance and function), but importantly what of this was facilitated by AMF. When mesh restricts the movement of AMF (including fine endophytes), is there a difference in the bacterial community structure? A clover plant was grown in simplified substrate, inside a mesh compartment of two different aperture sizes (20µm: AMF permitting, 1µm: AMF excluding). This compartment was surrounded by a low P grassland field soil to provide an indigenous microbial community.

7.4.4 Field soil sampling, processing, and nutrient analysis

For detailed protocols for soil and substrate preparation, refer to 3.3 and 3.4. The specific details for each experiment are given below.

Experimental design for root excluding mesh experiments

Two box experiments were carried out (1 & 2). General set-up was consistent between both experiments. A 3:1 ratio of sandy loam soil and quartz sand was sterilised at 121°C for one hour. Rectangular boxes (28 x 17.5 x 17 cm) were filled to equal volume and weight (7kg) of sterile substrate. Mesh was acquired from PlastOk associates LTD (Merseyside) of pore size 20µm or 1µm. Both sizes of mesh exclude roots but permit water and nutrient flux. However, 20µm mesh permits fungal hyphae whereas 1µm mesh excludes both roots and fungal hyphae, along with fine endophytes and potentially some bacterial species.

Experiments 1-3 were in a complete randomised design. For experiment 1: n = 5, experiment 2: n = 6 and experiment 3: n = 8.

The specific set up of each box experiment is as follows:

1. Two clover plants were set up at either end of a box filled with sterilised sand: sandy loam substrate. One plant was grown inside a root-excluding/hyphae-permitting mesh of 20µm. The other was grown in the bulk soil. Inside the mesh compartment the plant was inoculated with AMF (*F. geosporum*), and *P. putida.*

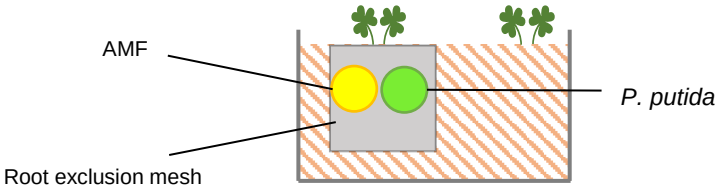


Figure 7.1. A schematic depicting the set-up of experiment 1

2. A subsequent experiment was set up similarly, with two plants both inside a mesh sock (20µm). One plant received AMF inoculum and the neighbouring plant received AMF-free carrier substrate only. In addition, *P. putida* was added in one of three locations: the AMF plant (location 1), the centre of the box in the bulk soil equidistant between the two mesh compartments (location 2), or the neighbouring, non-AMF plant (location 3).

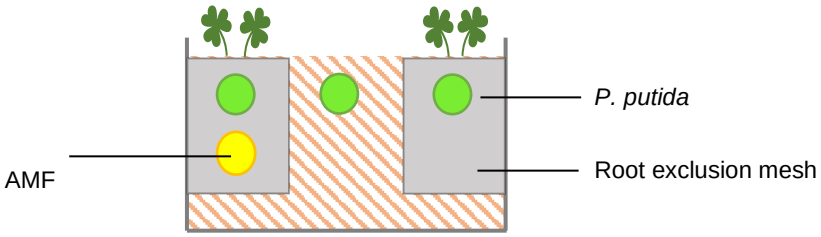


Figure 7.2. A schematic depicting the set-up of experiment 2. *P. putida* was deployed in three different locations. Location 1: On AMF plant, Location 2: in bulk soil between meshes, equidistant between plants, Location 3: On the neighbouring, non-AMF plant.

3. A final experiment using field soil was set up, experiment 3. This used a deep square pot (10x10x20cm) filled with low P grassland field soil. A mesh compartment of different aperture size (either 1 or 20µm) and filled with sterilised simple substrate was buried so that it was surrounded completely by field soil. The plant was grown inside the mesh sock, with either AMF or AMF-free carrier substrate.

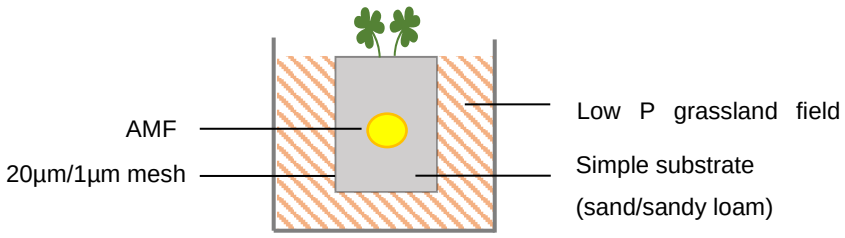


Figure 7.3. A schematic depicting the set-up of experiment 3.

7.4.5 Substrate, AMF and plant material preparation

Refer to 3.1 and 3.2 for detailed protocols for experimental set up and material preparation. *F. geosporum* was the AMF inoculant used in all experiments.

7.4.6 Bacterial inoculation

For detection of bacterial movement, *P. putida* with constitutively expressed *gfp* (KT2440::*gfp*) was used (referred to as *P. putida*). Overnight cultures were prepared in 50% TSB shaken at 200rpm at 25°C. Suspension OD was taken to ensure the cultured bacteria were in the exponential growth stage in the culture medium (OD_{595}). Cultures were centrifuged at 2000 x g for 10 minutes and resuspended in Ringer's solution. OD was taken again and then adjusted to 0.1 to obtain 10^8 cells. 1ml of suspension was applied to the appropriate position in the boxes by inserting a pipette tip ~2cm below the soil surface. Where applied to a section containing plants, culture was pipetted directly onto seedling roots.

7.4.7 Sample processing and plant harvest

Experiments were harvested at flowering. For both experimental types, aerial biomass was removed and dried at 80°C for 24 hours before weighing. Once weighed, dry samples were milled using a POLYMIX PX-MFC-90 D (Kinematica AG, Switzerland). These were weighed and sent for nutrient analysis (ICP-OES majors and traces, including Olsen P) and Rothamsted Research Analytical Chemistry Unit.

Experiment 1 used samples from the rhizoplane for qPCR work and rhizosphere for culture work. Experiment 2 and 3 sampled both the rhizosphere and rhizoplane for culture work and qPCR. Refer to 3.6 and 3.7 for sampling protocol.

7.4.8 Bacterial assessment

For the mesh experiments, culture dependent and independent methods were used to assess the transfer or persistence of *P. putida* in the rhizosphere. Soil samples were diluted with sterile water to 10^{-1} and 100µl spread onto plates of 1/10 TSA with gentamicin 20ng/ml, for which KT2440::*gfp* has resistance. Dilutions of up to 10^{-5} were trialled but 10^{-1} gave the largest catchment of the rhizosphere community, and selective agar was sufficient to reduce background colonies sufficiently to reveal GFP-expressing colonies. Plates were sealed and incubated for five days before counting GFP colonies under UV light.

The freeze-dried rhizoplane and rhizosphere samples were used for qPCR and amplicon sequencing dependant on the experimental type. DNA from the field soil experiment (experiment 3) was used for 16S rRNA gene Illumina amplicon sequencing. The mesh experiments (experiments 1 and 2) used qPCR to quantify *gfp*. For DNA extraction and quantification, refer to 3.8.

7.4.9 qPCR

Refer to general methods for qPCR set up and conditions (Section 3.9). For the AMF specific quantification, the primers used were: nrLSUF (GGAAACGATTGAAGTCAGTCATACCAA) and FgnrLSUR (CGAGAAAGTACACCAAAAGWGCCCAAT). For the *gfp* specific quantification,

the primers used were: GFPf (CTGCTGCCCGACAACCAC) and GFPr (TCACGAACTCCAGCAGGAC).

7.4.10 Functional assays

Detailed protocols given in Sections 3.10 and 3.11 for preparation of media and assessment criteria. Assays for phytate and $FePO_4$ were tested but colony counts were so low that these two assays were excluded from the results. The included assays were Ca_3PO_4, $AlPO_4$, casein hydrolysis, iron-chelation, K and Zn.

7.4.11 Amplicon sequencing

Details of sample preparation and analysis for 16S rRNA gene sequencing using Illumina MiSeq are given in general methods (Section 3.12).

7.4.12 Statistical analysis

For continuous normally distributed outcome variables such as biomass, statistical significance was calculated using analysis of variance. For percentage colonisation and functional assay data, analysis was carried out using a binomial generalised linear model. Residual plots were created for each outcome variable and showed that the underlying assumptions of the following tests were met. The analysis was a binomial intra-block generalised linear model, transformed on the logit scale. The predictions are presented as the back-transformed values from the logit scale and include 95% confidence intervals. When there was evidence of over dispersion (residual of the mean deviance >1), tests were done using deviance ratios (F statistics) and the standard errors adjusted accordingly.

For bioinformatic processing and statistical analysis of 16S rRNA gene amplicon datasets, refer to general methods (Section 3.12).

7.5 Results

7.5.1 Experiment 1.

7.5.1.1 Biomass

Clover inoculated with the AMF inside the mesh had significantly greater biomass than those uninoculated outside the mesh ($p < 0.001$, Figure 7.4). Plants grown in boxes without AMF in the system did not differ in biomass regardless of whether they were grown inside or outside of the mesh compartment. *P. putida* inoculation did not influence plant biomass. Box 1 contains a visualisation of experiment 1 set up for reference.

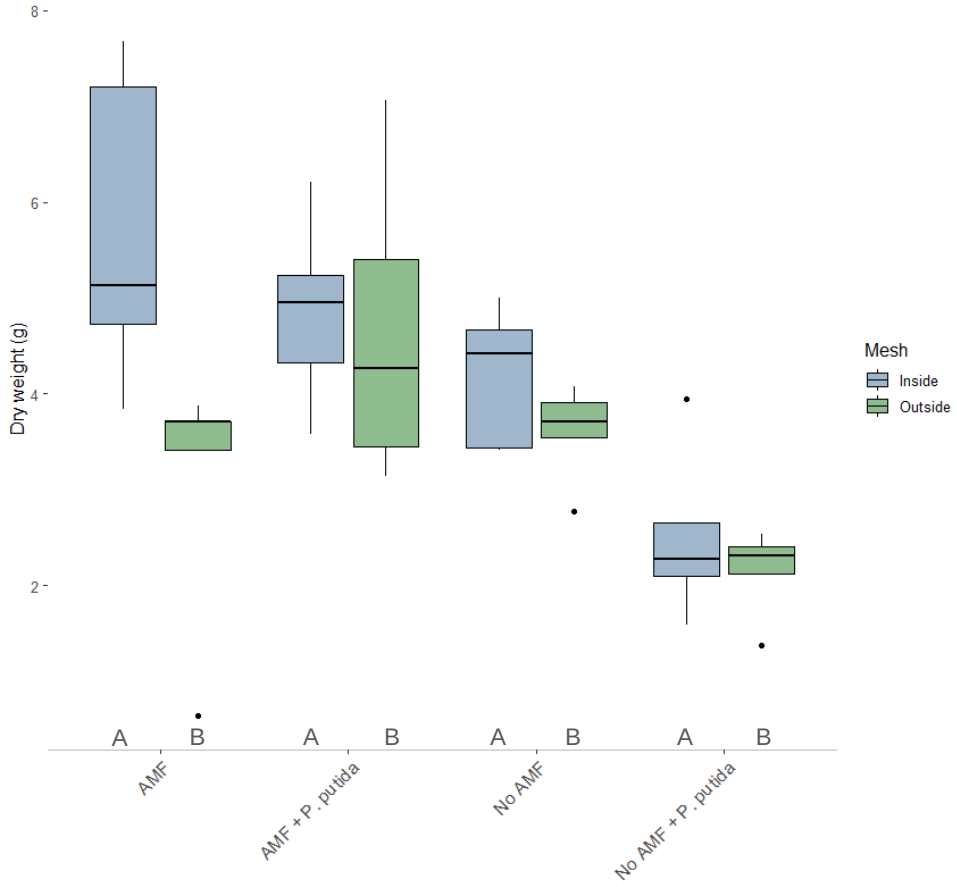
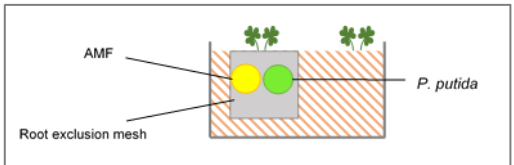


Figure 7.4. Aerial biomass of clover plants (experiment 1). AMF and *P. putida* inoculants were deployed to the plant inside the mesh (A), and plants outside the mesh were given the AMF-free carrier substrate as a control (B). Dry weight was taken at flowering, n = 5



Box 1. Visualisation of experiment 1 set up

7.5.1.2 AMF quantification

AMF colonisation was quantified via the gridline intersect method (Figure 7.5). Plants originally inoculated with AMF (inside the mesh) had greater colonisation than those outside the mesh (the neighbouring plant) ($F_{1, 15} = 14.38$, $p = 0.002$). Mean percentage colonisation of the neighbouring plants (outside mesh) was 29.6%, and 58.5% for all plants given AMF inside the mesh. While colonisation is greater at the site of AMF inoculation, there is sufficient colonisation of the neighbouring plant to assume an adequate hyphal connection between the two.

Inoculation of the *P. putida* had no effect on the AMF colonisation of plant roots ($F_{1, 15} = 4.14$, $p = 0.06$). There was no interaction effect of the two factors. There was a higher proportion of root colonisation by AMF in the AMF inoculated plant compared to the neighbouring plant outside the mesh regardless of whether the meshed plants had also received the *P. putida* inoculant. When examining the *P. putida* and AMF inoculated pots, there was 53.6% AMF colonisation in the AMF inoculated plant, compared to 20.5% in the neighbouring plant outside the mesh ($p = 0.009$). In the pots inoculated with AMF in the absence of *P. putida*, again there were higher levels of colonisation in the AMF inoculated plant inside the mesh (average 63.4%), and lower in the neighbouring plant outside the mesh (46.8%).

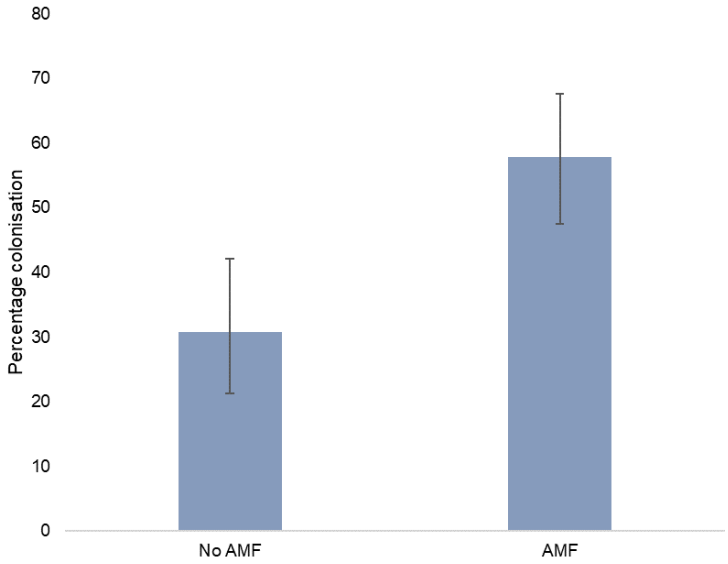


Figure 7.5. Experiment 1. Predicted means for the percentage of clover roots colonised with AMF. Values are predicted from raw data from microscope assessment of clover roots using the gridline intersection method. Values are generated from a binomial GLM, back transformed from the logit scale. The plant inside the mesh was given the inoculum, and the neighbouring plant (no AMF) outside the mesh received the AMF-free carrier substrate. Predictions include all treatments with or without AMF. Bars give back-transformed 95% confidence intervals and n = 5.

7.5.1.3 GFP movement

7.5.1.3.1 qPCR

Rhizosphere – *gfp* was detected on the site of original deployment (inside mesh) in both the AMF + *P. putida* and no AMF + *P. putida* treatments, whereas in the neighbouring plant *gfp* was only detected in the absence of AMF inoculant (Figure 7.6). The AMF treatment retained more *gfp* copy numbers than the non-AMF treatment (average log-transformed copy numbers: 4.74 and 0.74 respectively).

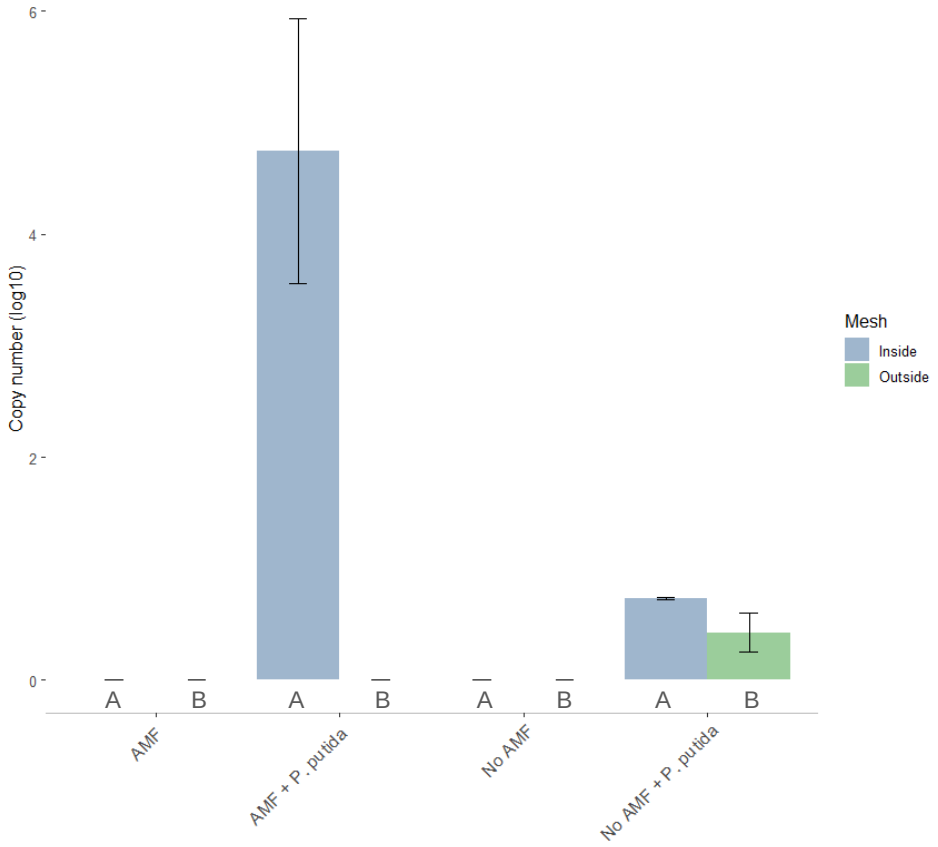


Figure 7.6. Experiment 1. *gfp* copy numbers from qPCR per gram of dried rhizosphere soil sample. Quantification of *gfp* in the rhizosphere of clover via qPCR in different configurations of mesh, AMF inoculant and *P. putida*. The plant inside the mesh was given the AMF inoculum (A), and the neighbouring plant outside the mesh (B) received the AMF-free carrier substrate. Treatments are as follows: AMF – AMF inoculum only on meshed plant, AMF + *P. putida* – AMF inoculum and *P. putida* on meshed plant. Bars give standard error, data was log transformed and n = 5.

7.5.1.3.2 Culture

Colonies expressing GFP were only observed in the treatments in which *P. putida* was deployed, so contamination of treatments did not occur. An average of 78 *P. putida* CFUs were counted in the AMF + *P. putida* treatment inside the mesh, at the site of deployment (Figure 7.7). None were observed from samples outside of the mesh. An average of *P. putida* CFUs grew from culture of the No AMF treatment inside the mesh where the *P. putida* was inoculated, and 4 *P. putida* CFUs were cultured from the neighbouring plant, outside the mesh.

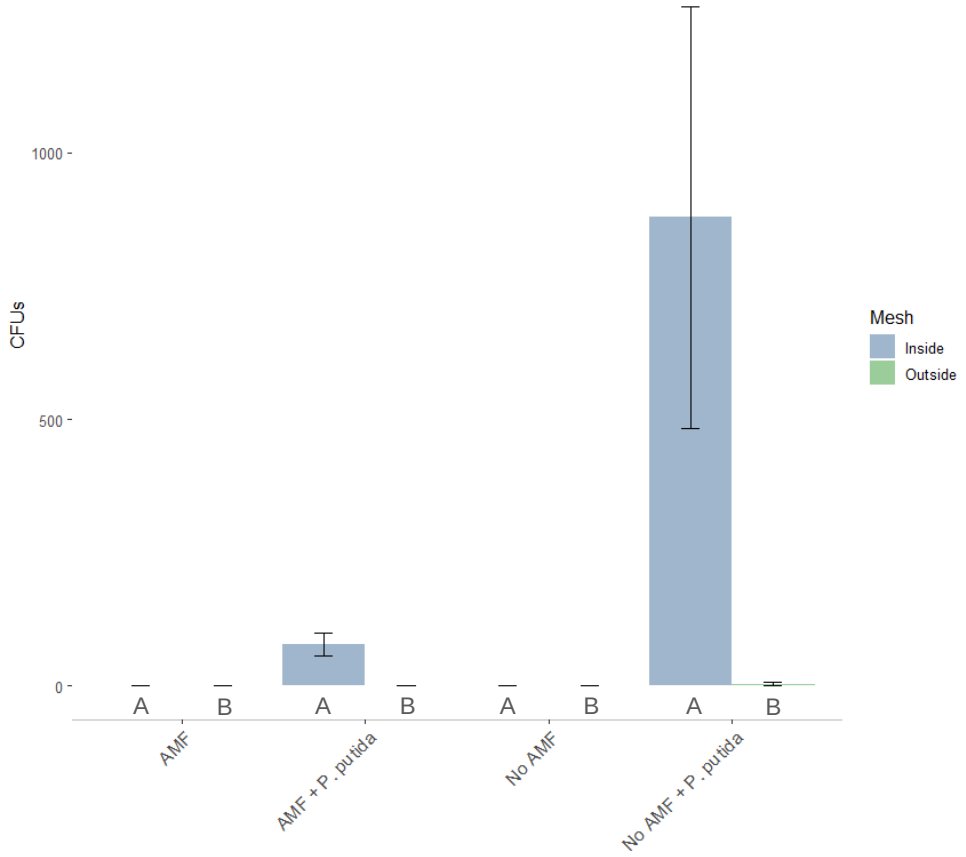
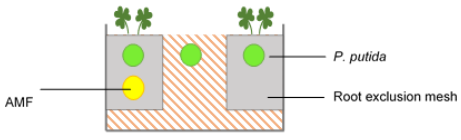


Figure 7.7. Experiment 1. *P. putida* CFU counts from the rhizosphere of clover at 10^{-1} dilution. Colony counts from *Pseudomonas* selective agar plates (with gentamycin) under UV light. *P. putida* was inoculated into "AMF + *P. putida*" and "No AMF + *P. putida*". The plant inside the mesh was given the AMF inoculum (A), and the neighbouring plant outside the mesh (B) received the AMF-free carrier substrate. Treatments are as follows: AMF – AMF inoculum only on meshed plant, AMF + *P. putida* – AMF inoculum and *P. putida* on meshed plant. Bars give standard error and data was log transformed and n = 5.

7.5.2 Experiment 2.

7.5.2.1 Biomass

Comparing the plants given AMF initially to the neighbouring plant, there was a highly significant difference in biomass ($p < 0.001$). No other factors influenced biomass except AMF inoculation (Figure 7.8). Box 2 contains a visualisation of experimental set up for reference.



Box 2. Visualisation of experiment 2 set up

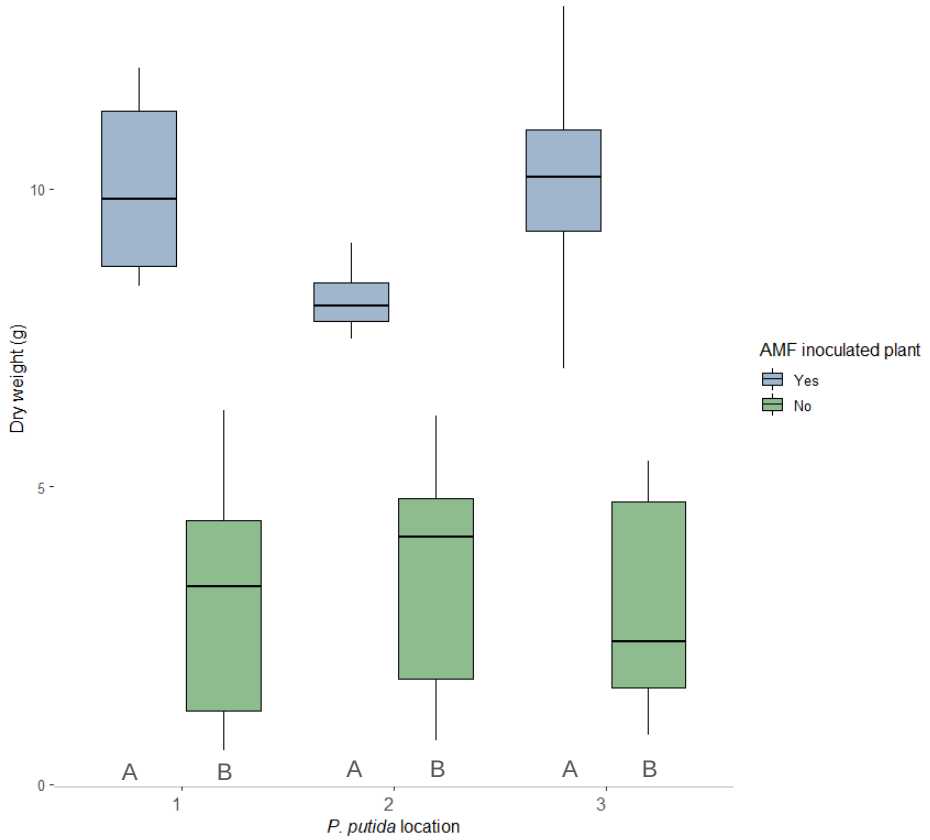


Figure 7.8. Experiment 2. Dry aerial biomass of clover. Dry aerial biomass of clover grown with or without an AMF inoculum (*F. geosporum* + carrier substrate). The plant inside the mesh was given the inoculum (A), and the neighbouring plant (B) outside the mesh received the AMF-free carrier substrate. Location refers to the deployment of *P. putida*. Location 1: The AMF plant, Location 2: The centre of the box in the bulk soil between the two mesh compartments, Location 3: the neighbouring plant without AMF inoculum. Bars give standard error and n = 6.

7.5.2.2 AMF colonisation

AMF inoculation was a significant determinant of root colonisation ($F_{1, 30} = 45.92$, $p < 0.001$), but neither *P. putida* inoculation location nor the interaction of the two factors had an impact (Figure 7.9). The average colonisation of AMF inoculated plants versus the neighbouring plants were 84.3% and 43.8% respectively.

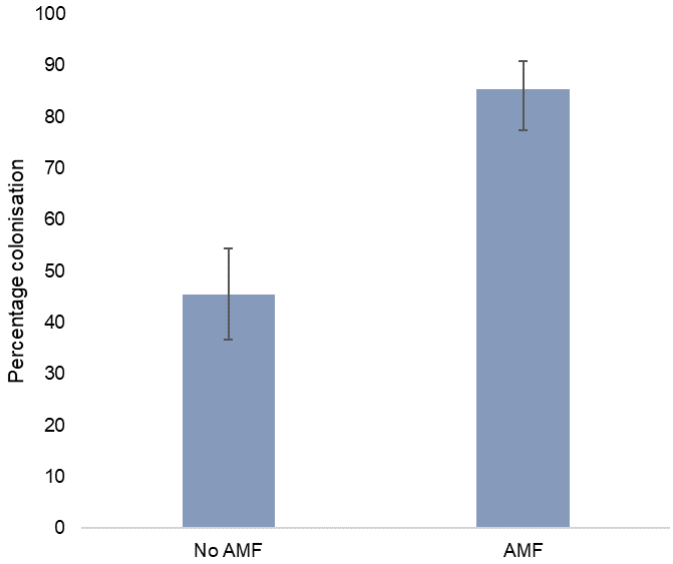


Figure 7.9. Experiment 2. Predicted means from GLM for percentage root colonisation by AMF of clover. Percentage of clover roots colonised with AMF assessed using the gridline intersect method. GLM predicted values are back transformed from the logit scale to give proportion. The AMF treatment plant (AMF) was given the AMF inoculum and carrier substrate, and the neighbouring plant (No AMF) received the AMF-free carrier substrate. Bars give confidence intervals back transformed from the logit scale and n = 6

7.5.2.3 GFP detection

7.5.2.3.1 *In vitro* culture

In the rhizosphere, when *P. putida* was deployed in location 1 (on the AMF plant), colony counts were detected on both plants (Figure 7.10). When *P. putida* was inoculated in between the two mesh compartments (location 2) and on the non-AMF plant (location 3), *P. putida* cultures were recovered only from the non-AMF plant. In the rhizoplane, the same trends were observed except for samples from location 1 treatment; *P. putida* colonies were not found on the neighbouring plant.

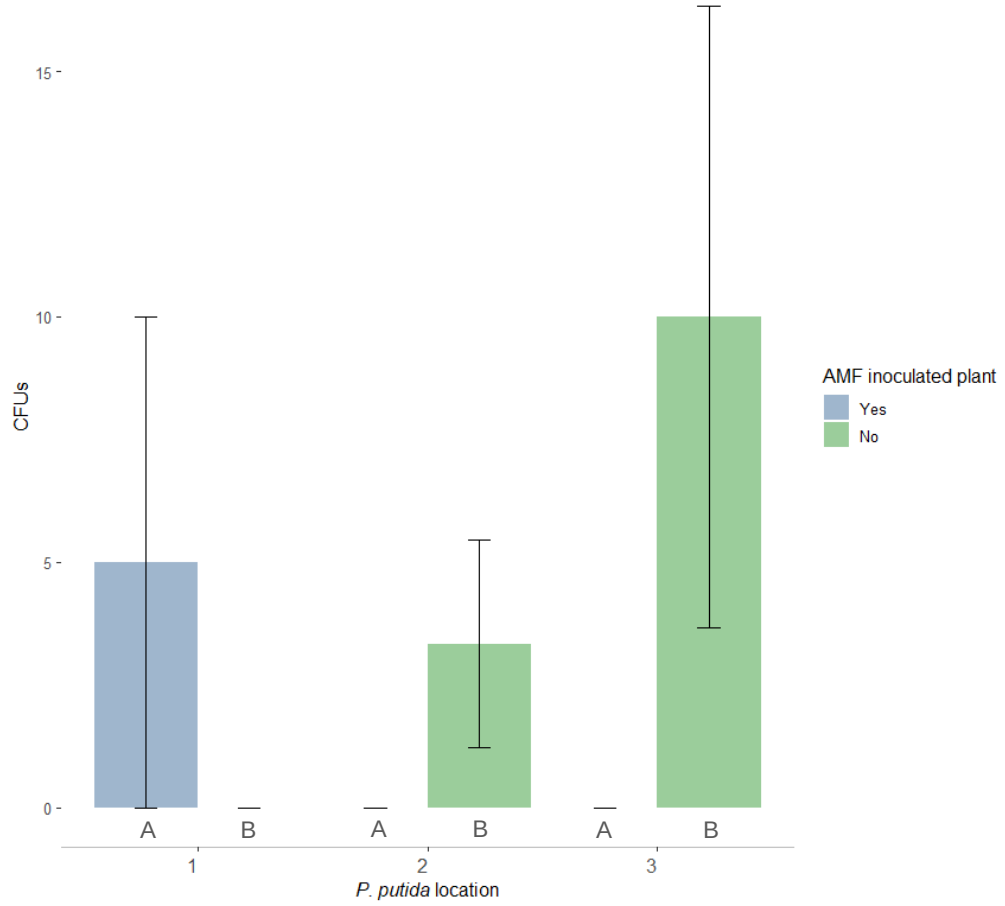


Figure 7.10. Experiment 2. *P. putida* CFU counts from the rhizosphere of clover. Samples were plated onto *Pseudomonas* selective agar with gentamycin and counted under UV light for *P. putida* colonies. The AMF treatment plant (A) was given the AMF inoculum and carrier substrate, and the neighbouring plant (B) received the AMF-free carrier substrate. Location refers to the deployment of *P. putida*. Location 1: The AMF plant, Location 2: The centre of the box in the bulk soil between the two mesh compartments, Location 3: the neighbouring plant without AMF inoculum. Bars give standard error and n =6.

7.5.2.3.2 qPCR for *gfp*

The results from qPCR for *gfp* were inconclusive as copy numbers were below detectable levels and a signal could not be distinguished from noise created by primer dimers.

7.5.3 Experiment 3.

7.5.3.1 Biomass

No difference in biomass was observed for any treatment ($F_{2,21}$ = 1.26, p = 0.30, Figure 7.11). Box 3 contains a visualisation of experimental set up for reference.

7.5.3.2 AMF colonisation

Treatment made a significant difference to AMF colonisation ($F_{2, 21}$ = 7.03, p = 0.005). The GLM predicted means of 63% for both of the AMF inoculated treatments (AMF 20µm and AMF 1µm, and 41% for the no AMF treatment (No AMF 20µm) (Figure 7.12).

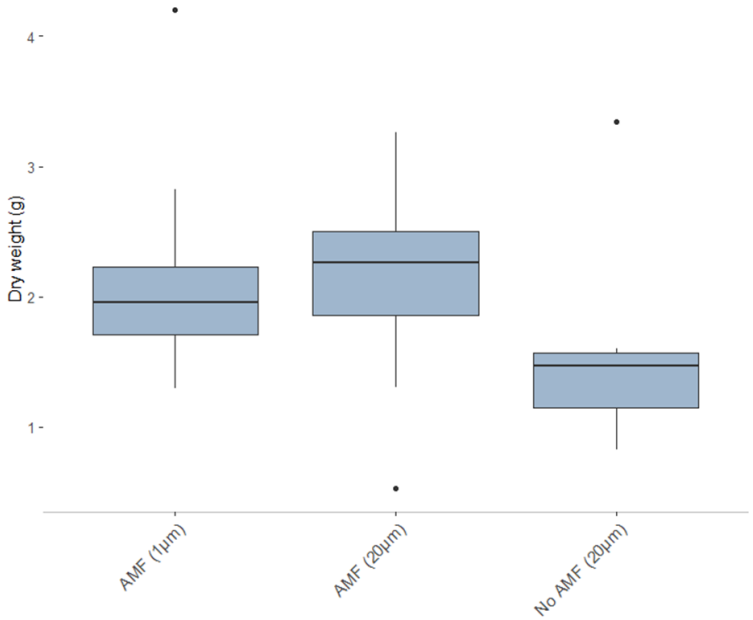
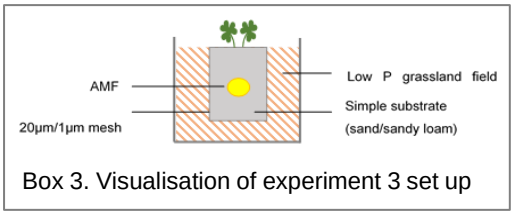


Figure 7.11. Experiment 3. Dry aerial biomass of clover. Biomass of clover grown with or without an AMF inoculum (*F. geosporum* + carrier substrate). Compartments created using two different mesh aperture sizes permitted (20) or excluded (1) AMF hyphae from accessing the surrounding field soil.



Box 3. Visualisation of experiment 3 set up

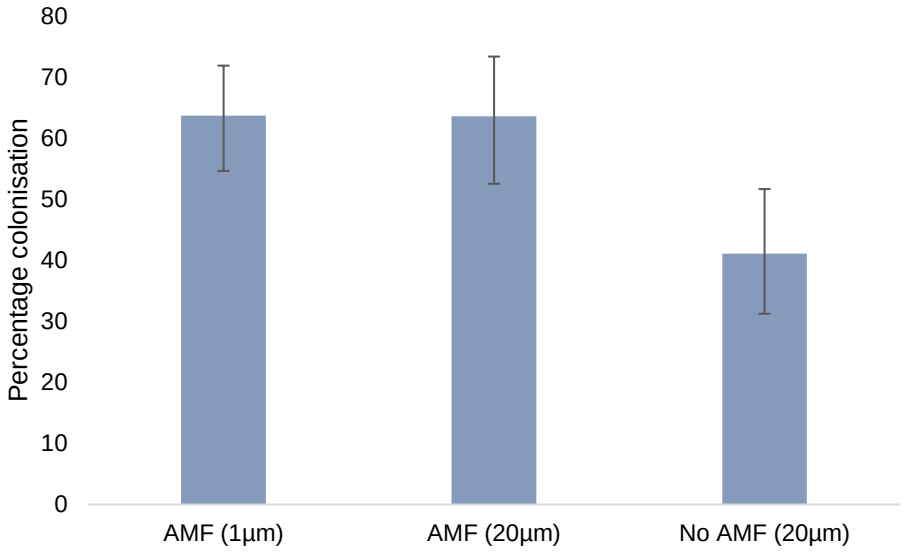


Figure 7.12. Experiment 3. Predicted mean values for percentage of clover roots colonised by AMF. GLM predicted values are back transformed from the logit scale to give proportion. Plants were grown with or without AMF inoculum, inside a mesh "sock" of different apertures, filled with a simplified substrate, surrounded by low P grassland field soil. Bars represent 95% confidence intervals back transformed from the logit scale, n = 8.

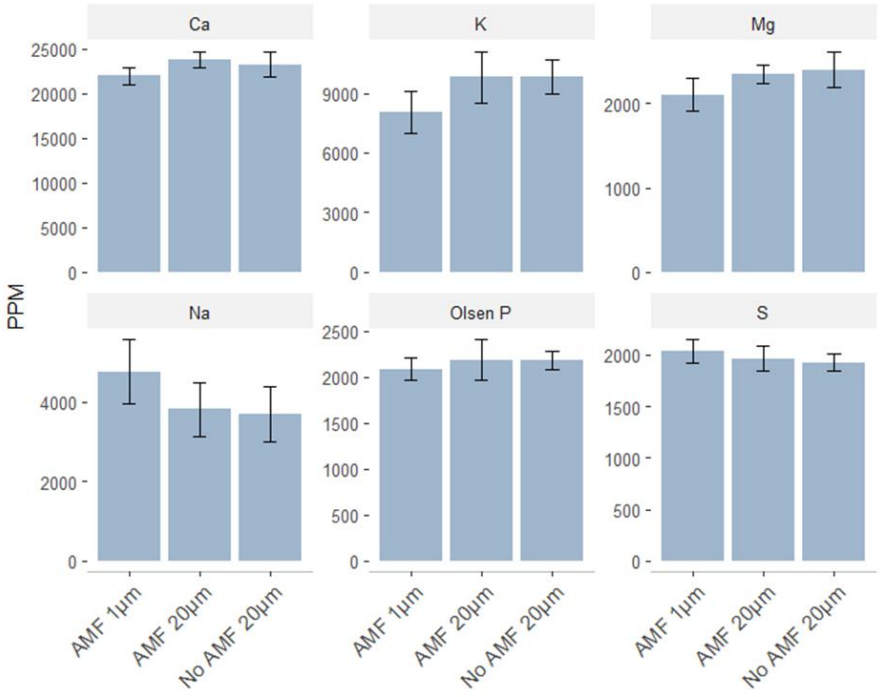


Figure 7.13. Plant aerial tissue nutrient content for experiment 3. ICP-OES analysis of some major elements including Olsen P for total plant P content. Values are given as PPM in dry matter. Bars give standard error and n = 6.

7.5.3.3 Plant nutrient content

Treatment did not influence the nutrient content of the plant material, for any of the nutrients measured, including Olsen P (Figure 7.13).

7.5.3.4 Functional assays

Treatment did not affect the proportion of plant growth promoting isolates, for any of the functional assays (Figure 7.14). The treatment of note was Zn with a probability score of 0.07, but as this is slightly above the defined 5%, it was not deemed significant. Compared to the other assays however, it could be said that there is more evidence for a treatment effect for Zn. $AlPO_4$: ($F_{2, 22}$ = 2.13, p = 0.15). AMF 20µm had the highest predicted mean value for $AlPO_4$ solubilisers (~50%), with the other two treatments at 37.0±.0.9%. Casein: ($F_{2, 9}$ = 0.36, p = 0.7). AMF 20µm had the lowest predicted mean value for casein solubilisers (~12.77%), with the other two treatments at 15.3±0.25%. K: ($F_{2, 20}$ = 1.49, p = 0.25). All treatments had comparable proportions of K solubilisers (AMF 1µm: 5.2%, AMF 20µm: 5.9%, No AMF 20µm: 8.4%). Ca_3PO_4 ($F_{2, 22}$ = 0.11, p = 0.9). All treatments had comparable proportions of Ca_3PO_4 (AMF 1µm: 10.64%, AMF 20µm: 10.64%, No AMF 20µm: 9.42%). AMF 1µm had the lowest amount of Zn solubilising isolates, with the GLM predicting a mean percentage of 11.44%. AMF 20µm was predicted to have a mean proportion of 17.69% and No AMF 20µm was predicted at 19.15%. Fe: ($F_{2, 22}$ = 1.92, p = 0.17). AMF 1µm had the lowest amount of Fe solubilising isolates, with the GLM predicting a mean percentage of 3.19%. AMF 20µm was predicted to have a mean proportion of 5.72% and No AMF 20µm was predicted at 6.54%

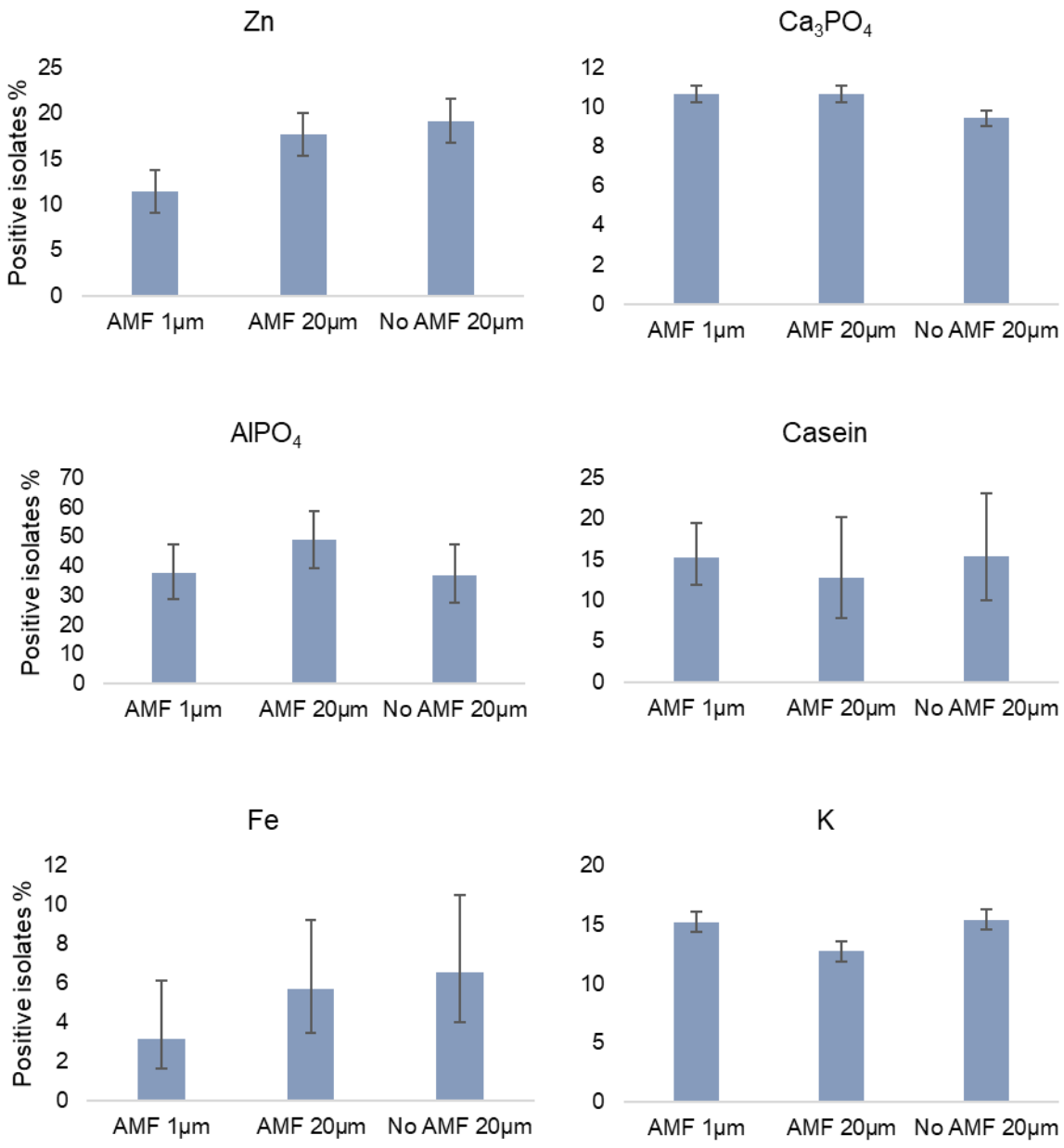


Figure 7.14. Predicted mean proportion of positive isolates for each of the functional assays. Values were back transformed to proportions from the logit scale. Mean values are given as %. Bars give back transformed 95% confidence intervals. Each sample (n = 8 per treatment) consisted of a library of n = 94 isolates from a rhizosphere culture.

7.5.3.5 16S rRNA gene amplicon sequencing

The distribution of taxa for the entire amplicon dataset is shown in Figure 7.15, displayed as the actual abundance of ASVs assigned to their corresponding phylum. In summary, the most abundant phyla are: Acidobactera 4.3%, Actinobacteria 12%, Bacteroidetes 13.5%, Firmicutes 6.1%, Proteobacteria 42.1%, Planctomycetes 5.2%, Verrucomicrobia 4%.

Samples are equally taxonomically diverse according to alpha diversity estimates (Figure 7.16). Kruskall-Wallis test showed that the alpha diversity within samples did not differ across treatments (p = 0.8 and 0.7 for rhizosphere and rhizoplane respectively). The roots of clover became colonised with an equal number of species despite the restrictive mesh or the AMF. The average number of observed taxa was 980 and 921 for the rhizosphere and rhizoplane respectively. The Shannon diversity index for each of the soil fractions was 6.6 and 6.5 respectively, with a Simpson's evenness measure of 0.6 for both fractions.

Neither AMF addition or mesh size altered taxonomic composition (beta diversity) in the rhizosphere or rhizoplane of clover, analysed using a principal coordinates analysis (PCoA) with a weighted UniFrac distance metric (p = 0.07 and 0.9 respectively, Figure 7.17). Estimates of beta-dispersion show homogeneity between groups in terms of species evenness (p > 0.3 for all factors). A CAP shown in Figure 7.18. yielded a permutational analysis of variance output of p > 0.1 for both rhizosphere and rhizoplane, further indication of no influence of either of the treatment factors on the bacterial community composition in either soil fraction.

Differentially abundant features are shown in the heatmap in Figure 7.19. Columns are similar across all three treatments. ASVs that are notably differentially abundant relative to the other treatments are mostly found within in the 1µm mesh treatment. The 20µm treatments (with and without AMF) have comparable ASV counts. ASVs belonging to the phyla *Bacteroidetes* (-0.9%), Actinobacteria (-0.65%), Firmicutes [*Clostridiaceae*] (+0.6%) and Firmicutes

[*Paenicaillaceae*] (-0.4%) are those which show the most change compared to the average of the 20µm treatments.

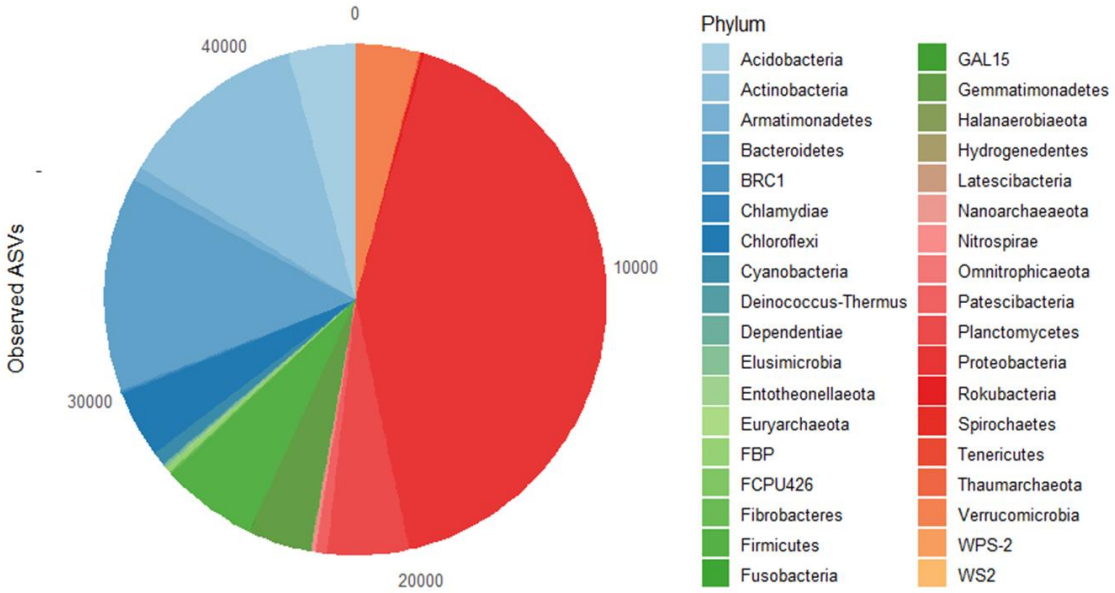


Figure 7.15. Actual abundance of ASVs aggregated by phylum. Counts for each unique ASV subsetted by its corresponding phylum. Pie chart shows the distribution of taxa for the entire amplicon dataset for experiment 3.

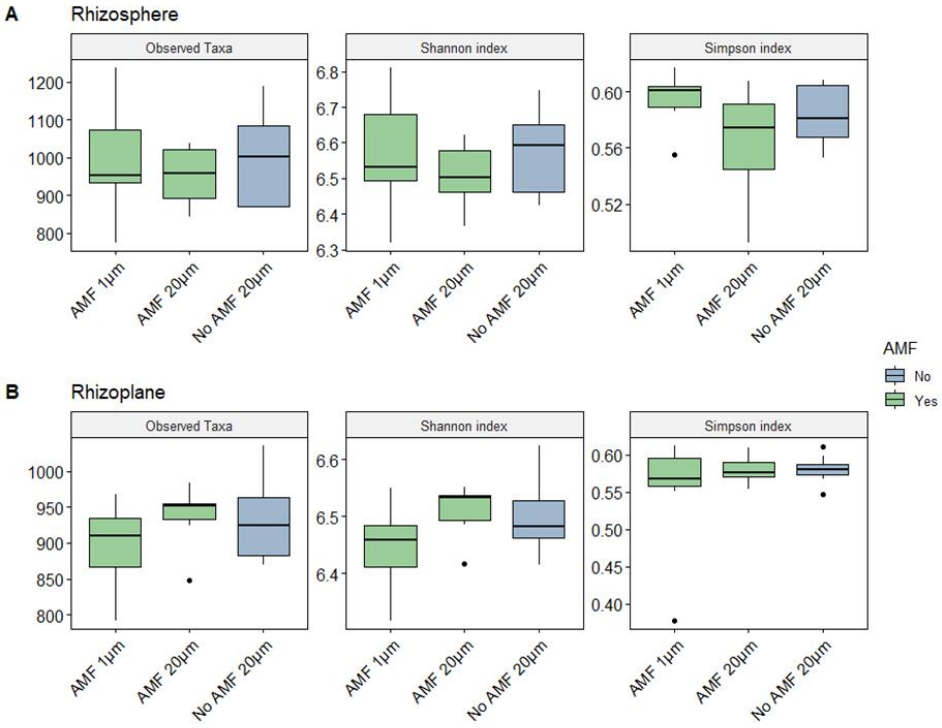


Figure 7.16. Estimates of alpha diversity in the root-associated bacterial community of clover. Clover was grown on a simplified sandy substrate with mesh that either permitted (20) or denied (1) AMF access to a surrounding bulk soil. Figure shows the alpha diversity measures Shannon's diversity index and Simpson's evenness index, with number of observed taxa (unique ASVs). Bars represent standard error.

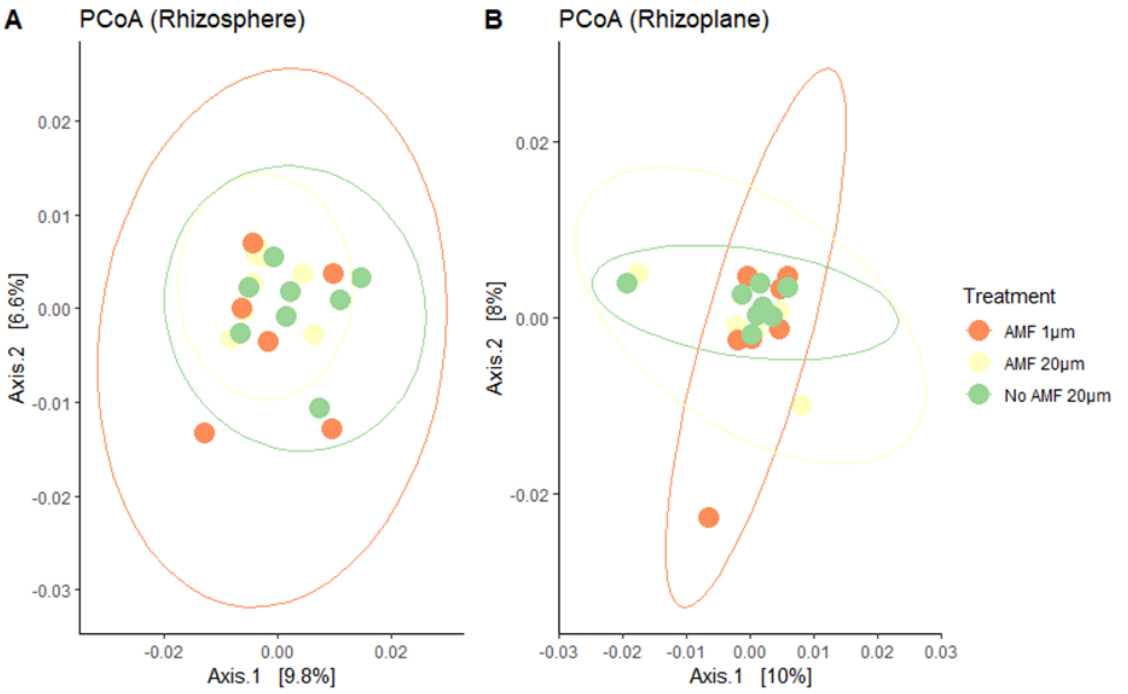


Figure 7.17. Estimates of beta diversity in the root-associated bacterial community of clover. Figure shows PCoA based on weighted UNIFRAC distance metrics, split by root fraction rhizosphere (A) and rhizoplane (B).

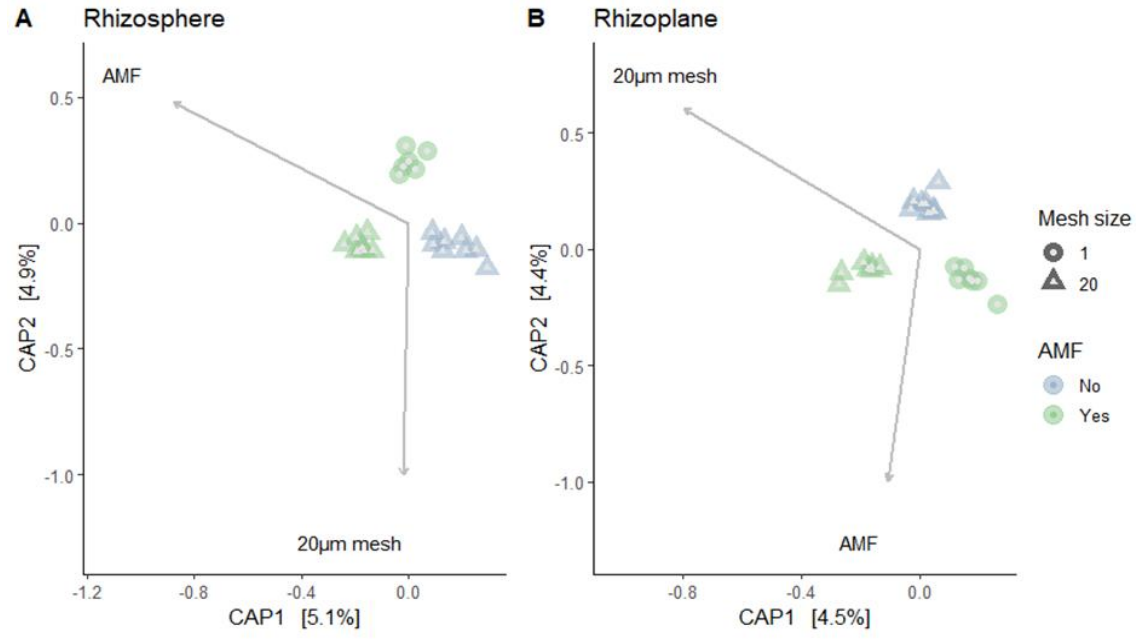


Figure 7.18. Canonical analysis of principal coordinates for ASV scores constrained by treatment. Scores were calculated from a weighted UNIFRAC distance measure. Colours are assigned to AMF inoculation and triangles denote AMF permitting mesh, circles represent AMF excluding mesh. The arrows point to the centre of the constrained factors. The percentage given in each axis refers to the proportion of the total variance of the data explained by each treatment factor. AMF inoculant is *F. geosporum*.

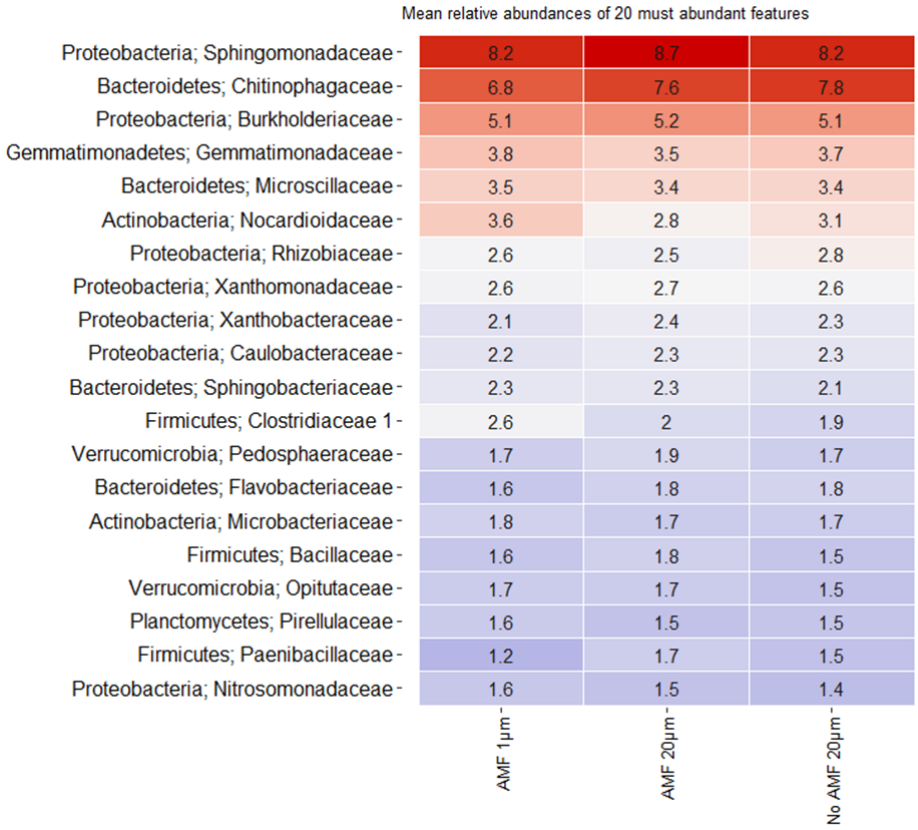


Figure 7.19. Heatmap showing mean relative abundance of the 20 most differentially abundant ASVs. Features are shown at phylum and family level in each treatment. Darker blue indicates a low relative abundance and red equals a higher relative abundance. AMF treatment is *F. geosporum*.

7.6 Discussion

7.6.1 Experiment 1.

The culture and qPCR quantification of *P. putida* show that when AMF is not present, and roots of neighbouring plants are prevented from touching, there is bacterial transference from between plants. When AMF were present, *P. putida* did not spread through the soil to colonise the neighbouring plant. This is contrary to the initial hypothesis, that bacteria might be mobilised physically with the outwards growth of the hyphae, which was towards the neighbouring plant during colonisation. As the bacteria appeared concentrated towards the AMF host plant, it could be the case that a transpiration gradient created by the water transference of the AMF is the source of this “pull”. AMF plants have been shown to have a greater transpiration rate than non-AMF plants (Hallett, Feeney et al. 2009). In the non-AMF treatments, bacteria could be dispersing via multiplication or chemotaxis. It has been shown that *Pseudomonas fluorescens* can spread through bulk soil in the absence of water movement (Juyal, Otten et al. 2021).

There was a significant statistical difference in the colonisation between the AMF inoculated plant vs the neighbouring plant. There was an average of over 29% colonisation in the neighbouring (non-AMF) plants, and 58.5% for the AMF plants. The qPCR data for the specific AMF species in the inoculum showed no difference; AMF colonisation was equal inside (the original site of inoculation) and outside the mesh (the neighbouring plant). Taken together, it was assumed that an adequate hyphal connection was created between the two plants. If there was greater colonisation in the AMF inoculated plant, *P. putida* inoculated here would experience a greater effect of transpiration and therefore be retained on the host plant. The qPCR is specific for the genus of fungi contained in the inoculum (*Funneliformis sp*.), whereas the microscopy records generic AMF structures, which could be representative of a variety of species.

Bacterial dispersal signified by detection of *P. putida* was concurrent in terms of presence/absence detection between qPCR and culture methodologies. *P. putida* was recovered from the neighbouring plant only when AMF were not present in the system, i.e. bacterial dispersal was not facilitated by AMF and did

not occur when AMF were not present. However, *P. putida* did persist in the rhizosphere of AMF-inoculated plants (inside mesh). Though, culture and qPCR methodologies disagree as to which plant retained the highest proportion; qPCR showed AMF and *P. putida* (inside mesh) had the highest relative concentration of *P. putida* whereas culture showed that the non-AMF and *P. putida* inoculated plant had the highest relative *P. putida* colony counts. Nevertheless, both methodologies yield the same trend; that *P. putida* dispersal only occurred when AMF were not inoculated. Comparing the two methods, culture-dependent work has a low detection threshold – soil was diluted to 10^{-1} after trialling dilutions up to 10^{-5}. The low dilution allowed a broader scope of the rhizosphere to be captured whilst using *Pseudomonas* specific agar with antibiotic selection cut through some of the noise to reveal mostly the colonies of interest. There are discrepancies between culture and qPCR methodologies for detecting specific bacterial species in soil samples, qPCR is more sensitive than culture for quantifying *Bulkholderia sp*. for example (Trung, Hetzer et al. 2011). However, given the inhibitory properties of soil components such as humic acid on molecular methods, a combination approach was deemed the most suitable for this research (Hoshino and Inagaki 2012).

7.6.2 Experiment 2.

The biomass of the plants initially inoculated with AMF was higher regardless of the location of the GFP labelled *P. putida.* The biomass did not differ between all plants given AMF, regardless of treatment. The same occurred for all plants in the neighbouring mesh, without AMF; biomass was similar. This is reflected by the percentage root colonisation for the plant initially given the AMF inoculant – it has greater colonisation than the neighbouring plant. However, as with experiment 1, the roots of the neighbouring plant were still colonised on average by 43.8%, so it can be assumed that a substantial hyphal connection has formed between the two plants.

The movement of inoculated bacteria was assessed via both culture and qPCR. The results from the culture-dependent methodology support the results

presented from experiment 1. When *P. putida* was deployed onto the AMF plant, it remained there and did not disperse to colonise the neighbouring non-AMF plant. This is seen most clearly in the rhizosphere, with no colonies detected on the neighbouring plant. The rhizoplane data shows some movement to the neighbouring plant, but this was an average of 1 colony. When *P. putida* was inoculated in locations 2 and 3, both of these are away from the AMF-plant and thus are likely to experience less pull from AMF hyphae, direction of travel occurs towards the neighbouring plant only; no colonies were detected from the AMF plant in either location 2 or 3 treatments for both rhizoplane and rhizosphere.

7.6.3 Experiment 3.

This experiment aimed to reveal if there was a selection by AMF for particular species or functions to colonise the roots of a clover host plant, from a full microbial complement from field soil. The use of root-excluding mesh prohibited the roots of the host clover plant to enter the field soil. AMF hyphae were permitted to enter the soil zone using 20µm mesh but prohibited by 1µm mesh. This technique was similar to an experimental set up descried by Zhang, Shi et al. (2018), although the hyphal excluding mesh used in their work was 0.45µm. A study by Albright and Martiny (2018) used mesh compartments to permit or deny bacterial dispersal. To prevent bacterial movement, they used an aperture size of 0.22µm. The work presented here used a slightly larger aperture of 1µm. It was hoped that this would be conducive for most soil bacteria to be able to access the plant, but also to prevent the interference of fine endophytes (Gannon, Manilal et al. 1991). However, some bacteria may exist at a larger size, such as *Pseudomonas aeruginosa* which can be 0.5-0.8µm in diameter but 1.5-3µm in length (Wu and Li 2015). Both mesh sizes allow diffusion of water and nutrients.

Biomass was not influenced by any treatment, even when plants were grown in 1µm mesh and AMF were not permitted to access the field soil. Even when AMF were not inoculated, there was no growth benefit from being inoculated or not. There were still high levels of AMF colonisation in the treatment without an exogenous AMF inoculant applied. This can be attributed to indigenous AMF in

the soil and thus explains why the biomass of the three treatments, regardless of AMF application or not, is the same.

AMF colonisation was identical (63%) in both AMF inoculation treatments. It is encouraging that the plants were colonised similarly, even though access was restricted to the bulk soil for one treatment. Possible explanations for this is that there are no microbes in the bulk soil being brought into the plant by the AMF (where hyphae are permitted access) that are affecting colonisation (Xavier and Germida 2003). However, alpha diversity estimates reveal equal richness in each system, regardless of mesh. Thus, it can be assumed that bacterial flow is not restricted by the mesh and can colonise equally between the two mesh types. Although it is important to note that the substrate inside the mesh was not maintained as sterile and watering could introduce additional taxa. Colonisers could occur from other routes, not just bacteria inhabiting the field soil exclusively.

Using culture-dependent methods to assess the plant-growth promoting capabilities of the rhizoplane bacterial communities, it was found that there were no differences in the functional abilities for any of the treatments. This suggests that the functional capacity of the root microbiome is not under the influence of AMF hyphae. Eight assays in total were used to give a general overview of the proportion of the bacterial community from a rhizosphere sample to have putative PGP functions. Four P-related assays were used, however two were not included because of extremely low counts (phytate and $FePO_4$). The two remaining P assays ($AlPO_4$ and Ca_3PO_4) again showed no differences when AMF could access the soil vs when they could not. This is contrary to what was shown by Zhang, Shi et al. (2018) who demonstrate that AMF can recruit bacteria with alkaline phosphatase to aid in organic P mineralisation. However, in this research differing nutrient availability was not tested, which is purported to be the stimulus for taxonomic change in the aforementioned study.

Similarly, in the amplicon sequencing data set, it appears that there are not significant differences in any of the systems, regardless of mesh size or AMF inoculation. The bacterial community does not change regardless of AMF access to field soil, and roots appear to have similar community composition in all

treatments. This may mean that the plant-mediated recruitment is more important than AMF influence for rhizobacteria community establishment. Plants are known to be highly influential determinants of their own root-associated community (Smalla, Wieland et al. 2001, Söderberg, Olsson et al. 2002, Kavamura, Robinson et al. 2019). These experiments reveal in clover, that when establishing a microbiome, AMF play no role over recruitment of specific taxa or function in this system.

7.7 Conclusion

AMF may not be an influential factor determining recruitment of a new root-associated community in terms of taxonomic composition or functional capabilities when a plant grown in a sterilised substrate becomes colonised. There is no down-regulation of putative plant-growth promoters occurring, if AMF were competing to be the main beneficiary for example. There were no differences between the treatments meaning each system was essentially the same. *P. putida* dispersal appears to occur without the help of AMF, as shown in experiment 1 and 2, and regardless of AMF treatment all of the root communities in experiment 3 culminated as being the same. Considering the experimental set up, previous work presented in section 5 using Barnfield soil (the field soil used in experiment 3) was also amplicon sequenced and returned a richness of 908 unique ASVs. The average number of bacterial features recovered in experiment 3 in this chapter was 980, so it can be assumed that clover grown in Barnfield soil directly (section 5, experiment 3), and clover grown in the sterile simple substrate surrounded by Barnfield soil were equally rich in taxa. This could be considered evidence that the bacteria colonising the plant are most likely from the soil and not from the watering or environmental colonisation.

In summary, these experiments show that there is an effect on bacterial movement due to AMF hyphae. This movement appears to be passive facilitation, perhaps along the water film of the hyphae along a transpiration gradient. There is no selective control over bacterial dispersal or functional profile on AMF hyphae. Rhizobia have been shown to use fungal hyphae as a means to access

legume roots (Zhang, Li et al. 2020), but this may be simply an exploitation of the physical conduit instead of an active recognition and selection by the fungal species. The AMF does not appear to act on behalf of the host to encourage a beneficial microbiome in terms of functions that could ameliorate plant health. However, the benefits of bacteria for the AMF themselves were not assessed here. Research into whether AMF are actively recruiting bacteria which are beneficial for themselves would further enhance our understanding of this tripartite relationship, as differences in the bacterial taxa of mycorrhizosphere have begun to be revealed (Battini, Grønlund et al. 2017).

8 GENERAL DISCUSSION

8.1 Main findings

The general discussion of this research will summarise key experimental outcomes, limitations, and intellectual contribution. It will highlight the link between the experimental chapters and explore the impact of this research for future study.

The aim of this project was to begin to describe the interactions between arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria. The purpose was to attempt to exploit these interactions to ameliorate plant health. The AMF-plant interaction is well defined, as are many plant-PGPR interactions. However, the influence of the microbial partners on each other is unexplored. This can be attributed to the difficulties in fully characterising a multifaceted and highly dynamic interaction, in an environment as heterogeneous as soil.

A tripartite interaction is undoubtedly complex and to enable the proposed research the experimental systems in which to study these interactions had to be developed (Section 4). The main difficulty was the cultivation of AMF and providing a system in which they would consistently proliferate. A combination of approaches was adopted, with experimental systems harbouring different attributes based on the research question.

Two AMF single species inoculants were deployed to investigate their effect on white clover and its root microbiome. *F. geosporum* was selected as the representative species for the remainder of the investigative work in this project, owing to its consistent colonisation phenotype, generalist nature, and influence on rhizobacteria assemblage. This was further explored by using NPK fertiliser, two soil types and the AMF inoculant, to characterise the interactions in an agriculturally relevant context (Section 5).

Putative plant growth promoting bacteria were characterised *in vitro* and the most synergistic in terms of their ability to liberate Pi were selected. These were then tested *in planta* with AMF to discover advantageous combinations that would improve plant health in regard to nutrient content and biomass (Section 6).

Bacterial transference through bulk soil along fungal hyphae has begun to be described but remains unresolved. Particularly, if AMF play a selective or passive role in dispersal. The mobilisation of *P. putida* along fungal hyphae was investigated using exclusion meshes and plants connected only via AMF hyphae (Section 7). The establishment of a root-associated community was then explored to ascertain if taxonomic composition or functional capabilities are determined or selected for by AMF.

The main findings of this work are highlighted below and discussed in the following section:

1. A combination approach of experimental systems to study tripartite interactions must be employed
2. Sphagnum peat moss compost is an ideal substrate for nutrient manipulation and testing putative bacterial inoculants
3. There is an inhibitory effect of combining NPK and AMF on the biomass of clover in grassland soil
4. Fertiliser and land use type are the most influential determinants of rhizobacteria taxonomic and functional assemblages associated with the roots of white clover
5. *F. geosporum* alters the taxonomic composition of the rhizosphere of clover in field soil, but not the rhizoplane
6. Inorganic, insoluble Ca_3PO_4 use influences the biomass of clover to a greater extent than any combination of phosphorus solubilising microbes
7. The addition AMF and Ca_3PO_4 separately increased clover plant P content, but AMF become competitive when these are added in combination
8. Bacteria become concentrated on the roots of an AMF inoculated plant instead of dispersing along the hyphae
9. Bacterial dispersal between neighbouring clover plants was observed to occur only in the absence of AMF
10. AMF do not selectively recruit for specific bacterial taxa or functions
11. The rhizobacteria community establishment of clover is independent of AMF

8.2 Design and deployment of putative microbial bioinoculants.

The use of microbial bioinoculants is, in theory, a sustainable and environmentally benign method of agricultural enhancement. Whilst there are a plethora of potentially favourable abilities residing within the microbial constituents of the soil, for an inoculant to be efficient or successful, it must be robust enough to withstand the edaphic perturbations that the agricultural landscape experiences. This project has shown that the beneficial effects of both AMF and PGPR are altered by edaphic influences, coupled with the improbability of optimal field performance of beneficial traits predicted *in vitro*. The interactions between a host plant and their microbial partners is dynamic and can become beneficial or detrimental depending on the environmental conditions or pre-existing microbes already colonised, for example (Svenningsen, Watts-Williams et al. 2018). Putative inoculants must therefore either be so well defined that their efficacy can be proven to be pervasive, or bespoke to a specific environment to operate within a defined set of parameters. An example of this might be in horticulture or controlled environment farming where conditions are maintained and stable (Aini, Dwi Yamika et al. 2019). An inoculant could then be produced to work well for this set of specific circumstances – host competence, persistence in the substrate, and consistent growth enhancement regardless of other exogenous inputs.

This project has also shown that the plant host itself appears to be the main determinant of its own root associated microbial community. AMF affected taxonomic composition in the rhizosphere, but not the rhizoplane, indicating a tighter control closer to the plant roots but also AMF influence in the wider soil environment. Additionally, it was shown that during community recruitment/establishment, AMF do not selectively facilitate colonisation of rhizobacteria of specific taxa or with a particular function. Regardless of AMF inoculation in the experimental system, the root-associated community culminated with near identical taxonomic and functional composition. Therefore, while it is important to characterise inoculants fully and use *in vitro* predictive methods, it appears that plants will curate their microbiome regardless (Söderberg, Olsson et al. 2002). With soil being such a rich ecosystem and

already containing a wealth of microbial diversity, it is likely that these beneficial organisms and their functions contained within the inoculant are already residing in the soil. Soil health is inextricably linked to its microbial diversity (Allison and Martiny 2008). Thus, it may be a more auspicious strategy to encourage the environment to be as diverse as possible, and for the plant to be able to capitalise on the existing microbial aid already available (Allison and Martiny 2008, Bardgett and Caruso 2020). This project showed that the use of Ca_3PO_4 and AMF together became inhibitory to plant P content. There must be a delicate balance between the addition of supplementary agrochemicals and allowing the soil microbiome to function at its best to improve plant growth. Perhaps this balance could be defined economically: Is it more financially advantageous to reduce fertiliser additions and use AMF inoculants instead? Or perhaps encouraging diversity and reducing fertiliser use to optimise the benefits that can be obtained from a vigorous root-microbiome. More research is needed to find the optimum trade-off between the beneficial effects of a fully equipped and uninhibited microbiome, and the use of fertiliser. For sustainable agriculture to prevail, the short-term goal of maximum yield must be entwined with the improvement and enhancement of soil health to ensure its continued capacity to function.

Despite attempts to increase the likelihood of a successful bioinoculant, by screening for synergistic Pi liberation *in vitro*, the three bacterial inoculants were not advantageous *in planta*, neither in terms of biomass nor plant nutrient content. There were no apparent interactions - antagonistic or additive – between AMF and the combinations of PSB. As there are so many factors that influence inoculant performance *in situ*, it is unsurprising that these were unsuccessful. Due to the high chance of inoculant failure, and to generate the most chance of discovering an effective combination, high-throughput testing systems must be developed to screen as many candidates as possible (as there is such a low rate of success). Despite all the predictive power that molecular and bioinformatic protocols now provide, there is still an element of luck required. The limitations of the microbial inoculant screening in this project were that only three putative bacteria could be tested due to large experiment size. Additionally, including microbial provenance and rhizosphere competence would have been

advantageous. It is not known if the selected isolates exhibited any host specificity, which would be likely to affect persistence or performance.

8.3 Are inoculated AMF still beneficial in managed agricultural systems?

The work presented in this thesis has demonstrated that AMF can be beneficial in certain systems. However, it has also shown that the mutualism is highly dynamic and is sensitive to the use of agrochemicals, supported in the literature (Toljander, Santos-González et al. 2008). Notably, separate experiments (Sections 5 and 6) using additional Ca_3PO_4 and NPK have a great influence over plant biomass and nutrient content; but the combined use of these chemicals and AMF have a competitive interaction. The use of the chemicals also reduced AMF colonisation, but in section 5 this effect was dependent on soil type (colonisation was unaffected by NPK in grassland soil but NPK decreased colonisation in bare fallow soil).

While AMF can have beneficial effects, in general, arable systems are fertilised as standard. This might mean that overall, the AMF mutualism is downregulated, or even inhibitory – as shown by results in this thesis and the literature. Typically in agricultural systems, AMF diversity is low (Verbruggen and Kiers 2010, Verbruggen, van der Heijden et al. 2013). Farmers need to maintain high yields in the short term to maximise profits, so reducing fertiliser use is unlikely to occur in the absence of an alternative that can be guaranteed to mimic the yield enhancement of fertiliser. Two approaches are considered involving the incorporation of beneficial microbes without compromising the need to produce viable yields. The first is that diversity in the soil microbiome can be encouraged as a way of building more resilient ecosystems (Bardgett and Caruso 2020). For example, AMF are not just beneficial to plants, but also improve soil structure and stability (Martin, Mooney et al. 2012). However, this would rely on changing agricultural practices such as incorporating fallow years or reducing agrochemical use that typically (but not always) inhibit or downregulate beneficial communities (Meena, Kumar et al. 2020, Reid, Kavamura et al. 2021). This work has shown there is a significantly greater proportion of plant-growth promoting traits in bare

fallow soil. This may be because the microbial inhabitants must adapt to a more generalist, harsh lifestyle without the exudate rich root niche, promoting the accumulation of a greater suite of functions (Hirsch, Gilliam et al. 2009, Goulding, Murray et al. 2010).

The second, could be the deployment of microbial inoculants, including the use of AMF, to enhance the practice of intercropping or cover crops. These strategies use non-cash crops or typically legumes, that will fix nitrogen into the soil, to give the soil a rebuild period between seasons of regular production (Dang, Gong et al. 2020). This work used white clover which is a popular species for soil health restoration. A rotation of clover, without the need for fertiliser, can be used to improve soil health in a field over a season (Hill, Levi et al. 2021). Couple this with the use of microbial inoculants, which will not only enhance plant growth but also supplement beneficial populations in the soil. This could be the niche for microbial bioinoculants to maximise the soil restoration properties of a cover crop season.

8.4 AMF effect on dispersal, community composition and functional selection of rhizobacteria

The original hypothesis of this research was that AMF may potentially exert some selection over the taxa or functions of its associated bacteria and allow them preferential access to plant roots. The mechanism of bacterial dispersal along the mycelial network in the soil is beginning to be described (de Novais, Sbrana et al. 2020), so it was proposed that the AMF-mediated recruitment of PGPR could be a method of plant access to a more diverse bacterial pool. However, the data show that AMF are not implicated in the establishment of the root-microbiome. In addition, this work reveals that AMF affect bacterial taxa of an existing community in the rhizosphere, but not the fraction most closely associated with the root which includes the rhizoplane. This is evidence of the strong plant-mediated rhizobacteria curation process which is well represented in the literature (Smalla, Wieland et al. 2001, Kavamura, Robinson et al. 2019). The experimental work shows that there are confounding effects of soil land use type and fertilisation on the microbiome, in agreement with published findings (Gomes, Lana et al. 2018).

However, mobilisation of bacteria along the physical structure of the hyphae is occurring (Kohlmeier, Smits et al. 2005, Simon, Bindschedler et al. 2015, de Novais, Sbrana et al. 2020). This has been shown to be influenced by hyphal exudates, which again evidences some level of fungal control (Jiang, Zhang et al. 2021). This work shows that this is not at the level of selecting for plant beneficial function. The AMF are not differentially regulating rhizobacteria that may compete against it to be a plant beneficiary, nor are they encouraging populations of bacteria with plant advantageous functions. There may be other traits selected for which were not measured in this study, for example conferring advantages to the AMF themselves. It is known that AMF do curate the assemblage of bacteria in their hyphosphere (Scheublin, Sanders et al. 2010, Nuccio, Hodge et al. 2013), but this may be entirely separate from the needs of the plant host. Further investigation into the functional significance of the community changes that were observed in this research would clarify this. For example, metatranscriptomics, mutagenesis studies, or probing functional groups bioinformatically.

8.5 Impact and intellectual contribution

This research has adopted multifaceted techniques in almost all aspects of its approach to attempt to define such a complex tripartite relationship. Combinations of experimental systems, using both culture dependent and independent methods, and different strategies of AMF methodology had to be used to adapt to each experimental question. To describe a microbial community fully, both functional capacity and taxonomic assemblage must be considered.

Reliable and reproducible quantification and identification of AMF is challenging. In this work, two methods of AMF quantification were used to obtain reliable data: classical microscopy and qPCR. This was done because the gridline intersection method is highly subjective and does not resolve quantification beyond presence/absence – it is not possible to distinguish between species. However, there were problems encountered with qPCR which also make this method less reliable. With AMF being multi-nucleate, tube shape structures, absolute quantification is unlikely, and qPCRs done in this work were often noisy with

primer dimers or a higher melt peak than the target. This could be from related species, as the primers were designed for *Funneliformis sp*. and not *F. geosporum* specific. There remains a large amount unknown about the genetic information of AMF species, which is perhaps why designing optimum and specific primers is challenging. This was encountered during ITS sequencing during experiment 1 (section 5.4.1.4). This data was inconclusive and deemed beyond the scope of this research. However, when investigating the phylum Glomeromycota, to which AMF belong, there were discrepancies due to the taxonomic database (UNITE, Nilsson, Larsson et al. (2018)) being unresolved and insufficient for AMF species. More research is needed to define AMF species at the molecular level to be able to use culture-independent methodologies for their quantification and identification.

The compost system can be used to screen putative PGPRs under bespoke nutrient conditions. *Pseudomonas* that could solubilise P *in vitro*, can solubilise this *in planta* to increase the biomass of wheat in available P starved (but inorganic P replete) conditions (Masters-Clark, Shone et al. 2020). This is a significant contribution to knowledge, as the system can be adapted to test many aspects of plant growth and enhancement using bacterial inoculants. However, it was not suitable for AMF work.

The significant alterations to bacterial taxonomic composition in the rhizosphere of clover when *F. geosporum* is inoculated is impactful for this research field. It has been shown that AMF similarly influenced the bacterial communities on the roots of maize, however this was using a microbial filtrate inoculum and not field soil as in this study (Marschner, Crowley et al. 2001). The knowledge that exogenous AMF application will incur a taxonomic change to rhizobacteria composition is important in the context of understanding the influence of agronomic inputs.

Ruling out AMF involvement in rhizobacteria community establishment is highly novel and has disproved the original hypothesis of this study. This work shows that while AMF have been shown to affect bacterial assemblage in the

rhizosphere of an established community, they are not influencing the rhizoplane, nor selecting which bacteria are transferred to a host plant.

The concentration of bacteria towards the AMF host plant along AMF hyphae, perhaps along a transpiration gradient, is a novel observation. Previous work involving fungal-mediated bacterial transfer has been done predominantly *in vitro*, but this has rarely been attempted in a realistic, soil-based system. The type of experiments done in the presented research (using more realistic substrates) permit more complex questions such as understanding the mechanism of mobilisation; is it selective via metrics other than those that were explored in this work. The relevance of the bacteria being transported along AMF hyphae across soil aggregates has been thus far unexplored and will significantly change our understanding of physical interactions and of movement and transport below ground. There are interesting applications in this field, such as using hyphae as a potential bacterial deployment mechanism in contaminated soils, or to bridge soil pores and aggregates to deliver beneficial inoculants.

The use of combination inoculants is gaining traction in research settings, but few combine AMF and multiple PSB. The methodology for obtaining optimum combinations of bacteria and AMF is exciting and it is likely that with a higher throughput *in planta* system, auspicious combinations could be identified. No advantages in plant biomass were found within the initial microbes selected, but this approach is pertinent in that the synergy of the microbes within the inoculant is verified pre-deployment, whereas in many commercial inoculants, beneficial microbes may be combined with no consideration to how they many impact one another.

Taken together, this research is valuable as it illustrates the importance of understanding and conserving the soil microbial consortia as an integral component of soil and plant health. Showing that fertiliser use and soil type impact the ability of the plants to recruit, and interact with, beneficial microbes will be essential to inform the shift towards protective and sustainable crop systems. It is clear from this work that there are significant interactions between AMF and PGPR, but these may not be on behalf of the plant host. Understanding the

delicate balance and complex relationships of these ubiquitous communities in the soil will enable us to develop strategies to preserve and protect them, but most importantly, how we can use them to our advantage to sustainably enhance crop growth while minimising harmful inputs.

8.6 Future work

Whilst this work did not reveal any promising combinations of PSM that could be used to supplement plant growth, the steps taken to achieve the highest chance of discovery are still relevant. These could be combined with further pre-screening *in silico*, and a higher through-put testing system to increase the numbers tested and therefore the likelihood of success. Other approaches could be to design auspicious synthetic communities of PGPR which deliver both diversity, increasing inoculant resilience, but also encompassing a greater suite of taxa and functions. Haskett, Tkacz et al. (2021) suggest engineering communities to be better adapted to rhizosphere survival or to prevent downregulation of beneficial traits, however regulations currently do not allow this. Additionally, it would be interesting to focus research on the plant transcriptomic responses to changes in microbial community composition. For example, how do different taxonomic assemblages relate to the expression of nutrient uptake genes. Additionally, approaching this from a plant-breeding perspective could allow for greater reliance or recruitment of beneficial microbes over the uptake of chemical fertiliser (Porter and Sachs 2020, Cobb, Duell et al. 2021). This strategy would help to support and increase soil microbial diversity and reduce dependency on artificial fertiliser.

A promising field within soil microbiome studies is bioprospecting for beneficial compounds, which negate the need for organism survival/dependency on environmental parameters. Characterising the AMF-PGPR interactions in respect to their communication or recruitment may reveal some promising chemical candidates for improving soil microbiomes or plant receptivity to beneficial microorganisms.

In addition, whilst describing the interactions between AMF and PGPR *in situ* in the soil is biologically relevant, it would be interesting to reveal these associations

at the micro-scale. *In vitro* culture of AMF was outside the scope of this project but recent advances in microfluidic devices, confocal microscopy and -omics technology would allow the description of specific and directed interactions between bacteria and AMF in the absence of the noise of the soil environment. This could be particularly useful for imaging the dispersal of bacteria along AMF hyphae.

This project describes the relationship between AMF and PGPR in a specific set of circumstances. Soil type and fertiliser use were shown to be major determinants of these interactions. To further our understanding of these complex dynamic communities, future work must explore more soil and land use types, different agrochemical inputs, diverse host plant species, AMF-inoculant species as just some examples. Soil is enormously complex, and it is likely that the dynamics of its microbiome will never be fully defined.

8.7 Conclusions

This thesis has provided evidence of the enigmatic complexity of the soil microbiome. It is clear that agricultural practices impact microbial communities and interfere with their potential to be beneficial. Improvements to soil health must include its microbial constituents to ensure the soil's continued capacity to function and contribute its essential ecosystem services of crop cultivation and nutrient cycling. The relationship between arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria has been shown to be subject to both extrinsic and intrinsic regulation. This must be carefully considered when designing and deploying putative microbial bioinoculants – they can be affected negatively by environmental parameters and fluctuations.

While AMF are important components of the root-associated microbiome, their beneficial properties are subject to many external factors and cannot be relied upon to consistently enhance plant growth in managed agricultural systems. Their interactions with rhizosphere bacteria are potentially more selfish than first hypothesised and this work shows that they do not curate bacterial assemblages on behalf of the plant. The exploitation of these interactions to sustainably enhance crop growth is still possible, but further work defining the soil microbiome

itself is necessary. This work highlights the importance of understanding the communities of microorganisms in the soil, but also the enormity of the task.

REFERENCES

Abbott, L. K., A. D. Robson and G. De Boer (1984). "The effect of phosphorus on the formation of hyphae in soil by the vesicular-arbuscular mycorrhizal fungus, *Glomus fasciculatum*." The New Phytologist **97**(3): 437-446.

Adesemoye, A. O. and J. W. Kloepper (2009). "Plant–microbes interactions in enhanced fertilizer-use efficiency." Applied Microbiology and Biotechnology **85**(1): 1-12.

Agnolucci, M., L. Avio, A. Pepe, A. Turrini, C. Cristani, P. Bonini, V. Cirino, F. Colosimo, M. Ruzzi and M. Giovannetti (2019). "Bacteria associated with a commercial mycorrhizal inoculum: community composition and multifunctional activity as assessed by Illumina sequencing and culture-dependent tools." Frontiers in Plant Science **9**: 1956.

Agnolucci, M., F. Battini, C. Cristani and M. Giovannetti (2015). "Diverse bacterial communities are recruited on spores of different arbuscular mycorrhizal fungal isolates." Biology and Fertility of Soils **51**(3): 379-389.

Aini, N., W. S. Dwi Yamika and R. W. Pahlevi (2019). "The effect of nutrient concentration and inoculation of PGPR and AMF on the yield and fruit quality of hydroponic cherry tomatoes (*Lycopersicon esculentum* Mill. var. cerasiforme)." Journal of Applied Horticulture **21**(2).

Albertsen, A., S. Ravnskov, H. Green, D. F. Jensen and J. Larsen (2006). "Interactions between the external mycelium of the mycorrhizal fungus *Glomus intraradices* and other soil microorganisms as affected by organic matter." Soil Biology and Biochemistry **38**(5): 1008-1014.

Albright, M. B. and J. B. Martiny (2018). "Dispersal alters bacterial diversity and composition in a natural community." The ISME journal **12**(1): 296-299.

Allison, S. D. and J. B. Martiny (2008). "Resistance, resilience, and redundancy in microbial communities." Proceedings of the National Academy of Sciences **105**(Supplement 1): 11512-11519.

Almario, J., G. Jeena, J. Wunder, G. Langen, A. Zuccaro, G. Coupland and M. Bucher (2017). "Root-associated fungal microbiota of nonmycorrhizal *Arabis alpina* and its contribution to plant phosphorus nutrition." Proceedings of the National Academy of Sciences **114**(44): E9403-E9412.

Ambler, J. R. and J. Young (1977). "Techniques for determining root length infected by vesicular-arbuscular mycorrhizae." Soil Science Society of America Journal **41**(3): 551-556.

Arbeli, Z. and C. L. Fuentes (2007). "Improved purification and PCR amplification of DNA from environmental samples." FEMS Microbiology Letters **272**(2): 269-275.

Arora, N. K., M. Verma and J. Mishra (2017). Rhizobial Bioformulations: Past, Present and Future. Rhizotrophs: Plant Growth Promotion to Bioremediation. S. Mehnaz. Singapore, Springer Singapore**:** 69-99.

Bai, L., J. Cui, W. Jie and B. Cai (2015). "Analysis of the community compositions of rhizosphere fungi in soybeans continuous cropping fields." Microbiological Research **180**: 49-56.

Bailey, R. L., K. P. West Jr and R. E. Black (2015). "The epidemiology of global micronutrient deficiencies." Annals of Nutrition and Metabolism **66**: 22-33.

Balser, T. C., K. K. Treseder and M. Ekenler (2005). "Using lipid analysis and hyphal length to quantify AM and saprotrophic fungal abundance along a soil chronosequence." Soil Biology and Biochemistry **37**(3): 601-604.

Bardgett, R. D. and T. Caruso (2020). "Soil microbial community responses to climate extremes: resistance, resilience and transitions to alternative states." Philosophical Transactions of the Royal Society B **375**(1794): 20190112.

Barea, J. (2014). "Future challenges and perspectives for applying microbial biotechnology in sustainable agriculture based on a better understanding of plant-microbiome interactions." Journal of Soil Science and Plant Nutrition **15**(2): 261-282.

Barea, J. M., D. Werner, C. Azcón-Guilar and R. Azcón (2005). Interactions of arbuscular mycorrhiza and nitrogen-fixing symbiosis in sustainable agriculture. Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment. D. Werner and W. E. Newton. Dordrecht, Springer Netherlands**:** 199-222.

Barnawal, D., N. Bharti, D. Maji, C. S. Chanotiya and A. Kalra (2014). "ACC deaminase-containing *Arthrobacter protophormiae* induces NaCl stress tolerance through reduced ACC oxidase activity and ethylene production resulting in improved nodulation and mycorrhization in *Pisum sativum*." Journal of Plant Physiology **171**(11): 884-894.

Barnawal, D., S. Pandey, N. Bharti, A. Pandey, T. Ray, S. Singh, C. Chanotiya and A. Kalra (2017). "ACC deaminase-containing plant growth-promoting rhizobacteria protect *Papaver somniferum* from downy mildew." Journal of Applied Microbiology **122**(5): 1286-1298.

Baset, M., Z. Shamsuddin, Z. Wahab and M. Marziah (2010). "Effect of plant growth promoting rhizobacterial (PGPR) inoculation on growth and nitrogen incorporation of tissue-cultured *Musa* plantlets under nitrogen-free hydroponics condition." Australian Journal of Crop Science **4**(2): 85-90.

Batool, T., S. Ali, M. F. Seleiman, N. H. Naveed, A. Ali, K. Ahmed, M. Abid, M. Rizwan, M. R. Shahid, M. Alotaibi, I. Al-Ashkar and M. Mubushar (2020). "Plant growth promoting rhizobacteria alleviates drought stress in potato in response to suppressive oxidative stress and antioxidant enzymes activities." Scientific Reports **10**(1): 16975.

Battini, F., M. Grønlund, M. Agnolucci, M. Giovannetti and I. Jakobsen (2017). "Facilitation of phosphorus uptake in maize plants by mycorrhizosphere bacteria." Scientific Reports **7**(1): 1-11.

Berendsen, R. L., C. M. Pieterse and P. A. Bakker (2012). "The rhizosphere microbiome and plant health." Trends in Plant Science **17**(8): 478-486.

Berg, G., D. Rybakova, D. Fischer, T. Cernava, M.-C. C. Vergès, T. Charles, X. Chen, L. Cocolin, K. Eversole, G. H. Corral, M. Kazou, L. Kinkel, L. Lange, N. Lima, A. Loy, J. A. Macklin, E. Maguin, T. Mauchline, R. McClure, B. Mitter, M. Ryan, I. Sarand, H. Smidt, B. Schelkle, H. Roume, G. S. Kiran, J. Selvin, R. S. C. d. Souza, L. van Overbeek, B. K. Singh, M. Wagner, A. Walsh, A. Sessitsch and M. Schloter (2020). "Microbiome definition re-visited: old concepts and new challenges." Microbiome **8**(1): 103.

Bever, J. D., T. G. Platt and E. R. Morton (2012). "Microbial population and community dynamics on plant roots and their feedbacks on plant communities." Annual Review of Microbiology **66**(1): 265-283.

Bhattacharyya, P. N. and D. K. Jha (2012). "Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture." World Journal of Microbiology Biotechnology **28**(4): 1327-1350.

Bianciotto, V., C. Bandi, D. Minerdi, M. Sironi, H. V. Tichy and P. Bonfante (1996). "An obligately endosymbiotic mycorrhizal fungus itself harbors obligately intracellular bacteria." Applied and Environmental Microbiology **62**(8): 3005-3010.

Bianciotto, V., E. Lumini, P. Bonfante and P. Vandamme (2003). "'Candidatus glomeribacter gigasporarum' gen. nov., sp. nov., an endosymbiont of arbuscular mycorrhizal fungi." Int J Syst Evol Microbiol **53**(Pt 1): 121-124.

Bonfante, P. (2001). At the interface between mycorrhizal fungi and plants: the structural organization of cell wall, plasma membrane and cytoskeleton. Fungal Associations. B. Hock. Berlin, Heidelberg, Springer Berlin Heidelberg**:** 45-61.

Bonfante, P. (2003). "Plants, mycorrhizal fungi and endobacteria: a dialog among cells and genomes." The Biological Bulletin **204**(2): 215-220.

Bonfante, P. (2003). "Plants, mycorrhizal fungi and endobacteria: a dialog among cells and genomes." Biol Bull **204**(2): 215-220.

Bonfante, P. and I. A. Anca (2009). "Plants, mycorrhizal fungi, and bacteria: a network of interactions." Annual Review of Microbiology **63**: 363-383.

Bonfante, P. and A. Genre (2008). "Plants and arbuscular mycorrhizal fungi: an evolutionary-developmental perspective." Trends in Plant Science **13**(9): 492-498.

Bonfante, P. and A. Genre (2010). "Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis." Nature Communications **1**(48): 1-11.

Booker, F. L., S. A. Prior, H. A. Torbert, E. L. Fiscus, W. A. Pursley and S. Hu (2005). "Decomposition of soybean grown under elevated concentrations of CO_2 and O_3." Global Change Biology **11**(4): 685-698.

Boon, E., E. Zimmerman, B. Lang and M. Hijri (2010). "Intra-isolate genome variation in arbuscular mycorrhizal fungi persists in the transcriptome." Journal of Evolutionary Biology **23**(7): 1519-1527.

Bouffaud, M. L., M. A. Poirier, D. Muller and Y. Moënne-Loccoz (2014). "Root microbiome relates to plant host evolution in maize and other Poaceae." Environmental Microbiology **16**(9): 2804-2814.

Brader, G., S. Compant, K. Vescio, B. Mitter, F. Trognitz, L.-J. Ma and A. Sessitsch (2017). "Ecology and genomic insights into plant-pathogenic and plant-nonpathogenic endophytes." Annual Review of Phytopathology **55**(1): 61-83.

Brimecombe, M. J., F. A. De Leij and J. M. Lynch (2000). The effect of root exudates on rhizosphere microbial populations. The rhizosphere, CRC Press**:** 111-156.

Brink, S. C. (2016). "Unlocking the secrets of the rhizosphere." Trends in plant science **21**(3): 169-170.

Bulgarelli, D., R. Garrido-Oter, P. C. Münch, A. Weiman, J. Dröge, Y. Pan, A. C. McHardy and P. Schulze-Lefert (2015). "Structure and function of the bacterial root microbiota in wild and domesticated barley." Cell Host & Microbe **17**(3): 392-403.

Bulgarelli, D., K. Schlaeppi, S. Spaepen, E. V. L. Van Themaat and P. Schulze-Lefert (2013). "Structure and functions of the bacterial microbiota of plants." Annual review of plant biology **64**: 807-838.

Burke, C., P. Steinberg, D. Rusch, S. Kjelleberg and T. Thomas (2011). "Bacterial community assembly based on functional genes rather than species." Proceedings of the National Academy of Sciences **108**(34): 14288.

Burke, D. J., M. N. Weintraub, C. R. Hewins and S. Kalisz (2011). "Relationship between soil enzyme activities, nutrient cycling and soil fungal communities in a northern hardwood forest." Soil Biology and Biochemistry **43**(4): 795-803.

Callahan, B. J., P. J. McMurdie and S. P. Holmes (2017). "Exact sequence variants should replace operational taxonomic units in marker-gene data analysis." The ISME Journal **11**(12): 2639-2643.

Callahan, B. J., P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson and S. P. Holmes (2016). "DADA2: high-resolution sample inference from Illumina amplicon data." Nature Methods **13**(7): 581-583.

Caradus, J., D. Woodfield and A. Stewart (1995). "Overview and vision for white clover." NZGA: Research and Practice Series **6**: 1-6.

Cavaglieri, L., J. Orlando and M. Etcheverry (2009). "Rhizosphere microbial community structure at different maize plant growth stages and root locations." Microbiological Research **164**(4): 391-399.

Chagnon, P.-L. and L. D. Bainard (2015). "Using molecular biology to study mycorrhizal fungal community ecology: Limits and perspectives." Plant Signaling & Behavior **10**(7): e1046668-e1046668.

Chaparro, J. M., A. M. Sheflin, D. K. Manter and J. M. Vivanco (2012). "Manipulating the soil microbiome to increase soil health and plant fertility." Biology and Fertility of Soils **48**(5): 489-499.

Charpe, A. M. (2019). Biotic and Abiotic Stress Management by AM-Mediated PGPRs. Plant Growth Promoting Rhizobacteria for Sustainable Stress Management. R. Z. Sayyed. Singapore, Springer Singapore. **Volume 2: Rhizobacteria in biotic stress management:** 325-343.

Charpentier, M. and G. Oldroyd (2010). "How close are we to nitrogen-fixing cereals?" Current Opinion in Plant Biology **13**(5): 556-564.

Chen, Q.-L., J. Ding, Y.-G. Zhu, J.-Z. He and H.-W. Hu (2020). "Soil bacterial taxonomic diversity is critical to maintaining the plant productivity." Environment International **140**: 105766.

Chen, S., W. Wang, W. Xu, Y. Wang, H. Wan, D. Chen, Z. Tang, X. Tang, G. Zhou and Z. Xie (2018). "Plant diversity enhances productivity and soil carbon storage." Proceedings of the National Academy of Sciences **115**(16): 4027-4032.

Chitarra, W., C. Pagliarani, B. Maserti, E. Lumini, I. Siciliano, P. Cascone, A. Schubert, G. Gambino, R. Balestrini and E. Guerrieri (2016). "Insights on the impact of arbuscular mycorrhizal symbiosis on tomato tolerance to water stress." Plant Physiology **171**(2): 1009-1023.

Ci, D., Z. Tang, H. Ding, L. Cui, G. Zhang, S. Li, L. Dai, F. Qin, Z. Zhang and J. Yang (2021). "The synergy effect of arbuscular mycorrhizal fungi symbiosis and exogenous calcium on bacterial community composition and growth performance of peanut (*Arachis hypogaea* L.) in saline alkali soil." Journal of Microbiology **59**(1): 51-63.

Cobb, A. B., E. B. Duell, K. B. Haase, R. M. Miller, Y. Q. Wu and G. W. Wilson (2021). "Utilizing mycorrhizal responses to guide selective breeding for agricultural sustainability." Plants, People, Planet.

Colard, A., C. Angelard and I. R. Sanders (2011). "Genetic exchange in an arbuscular mycorrhizal fungus results in increased rice growth and altered mycorrhiza-specific gene transcription." Applied and Environmental Microbiology **77**(18): 6510-6515.

Collins, C. D. and B. L. Foster (2009). "Community-level consequences of mycorrhizae depend on phosphorus availability." Ecology **90**(9): 2567-2576.

Dahlberg, K. (2012). Beyond the green revolution: The ecology and politics of global agricultural development, Springer Science & Business Media.

Dang, K., X. Gong, G. Zhao, H. Wang, A. Ivanistau and B. Feng (2020). "Intercropping alters the soil microbial diversity and community to facilitate nitrogen assimilation: a potential mechanism for increasing proso millet grain yield." Frontiers in Microbiology **11**: 2975.

de Boer, W., L. B. Folman, R. C. Summerbell and L. Boddy (2005). "Living in a fungal world: impact of fungi on soil bacterial niche development." FEMS Microbiology Reviews **29**(4): 795-811.

de Novais, C. B., C. Sbrana, E. da Conceição Jesus, L. F. M. Rouws, M. Giovannetti, L. Avio, J. O. Siqueira, O. J. Saggin Junior, E. M. R. da Silva and S. M. de Faria (2020). "Mycorrhizal networks facilitate the colonization of legume roots by a symbiotic nitrogen-fixing bacterium." Mycorrhiza **30**: 389–396.

De Sordi, L. and F. A. Mühlschlegel (2009). "Quorum sensing and fungal–bacterial interactions in *Candida albicans*: a communicative network regulating microbial coexistence and virulence." FEMS Yeast Research **9**(7): 990-999.

de Souza, J. E. B. and E. P. de Brito Ferreira (2017). "Improving sustainability of common bean production systems by co-inoculating rhizobia and azospirilla." Agriculture, Ecosystems & Environment **237**: 250-257.

de Vries, F. T., E. Thébault, M. Liiri, K. Birkhofer, M. A. Tsiafouli, L. Bjørnlund, H. Bracht Jørgensen, M. V. Brady, S. Christensen, P. C. de Ruiter, T. d'Hertefeldt, J. Frouz, K. Hedlund, L. Hemerik, W. H. G. Hol, S. Hotes, S. R. Mortimer, H. Setälä, S. P. Sgardelis, K. Uteseny, W. H. van der Putten, V. Wolters and R. D. Bardgett (2013). "Soil food web properties explain ecosystem services across European land use systems." Proceedings of the National Academy of Sciences **110**(35): 14296.

Deng, Z., L. Cao, H. Huang, X. Jiang, W. Wang, Y. Shi and R. Zhang (2011). "Characterization of Cd- and Pb-resistant fungal endophyte Mucor sp. CBRF59 isolated from rapes (*Brassica chinensis*) in a metal-contaminated soil." Journal of Hazardous Materials **185**(2): 717-724.

Desirò, A., A. Salvioli, E. L. Ngonkeu, S. J. Mondo, S. Epis, A. Faccio, A. Kaech, T. E. Pawlowska and P. Bonfante (2014). "Detection of a novel intracellular microbiome hosted in arbuscular mycorrhizal fungi." The ISME Journal **8**(2): 257-270.

Deveau, A. and J. Labbé (2016). Mycorrhiza helper bacteria. Molecular Mycorrhizal Symbiosis**:** 437-450.

Di Salvo, L. P., G. C. Cellucci, M. E. Carlino and I. E. G. de Salamone (2018). "Plant growth-promoting rhizobacteria inoculation and nitrogen fertilization increase maize (*Zea mays* L.) grain yield and modified rhizosphere microbial communities." Applied Soil Ecology **126**: 113-120.

Dilnashin, H., H. Birla, T. X. Hoat, H. B. Singh, S. P. Singh and C. Keswani (2020). Chapter 32 - Applications of agriculturally important microorganisms for sustainable crop production. Molecular Aspects of Plant Beneficial Microbes in Agriculture. V. Sharma, R. Salwan and L. K. T. Al-Ani, Academic Press**:** 403-415.

Doran, J. and M. Safley (1997). "Defining and assessing soil health and sustainable productivity." Biological Indicators of Soil Health.: 1-28.

Edi-Premono, M., Moawad A. M. and P. L. G. Vlek (1996). "Effect of phosphate solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere." Indonesian Journal of Crop Science **11**(13-23).

Emmett, B. D., V. Lévesque-Tremblay and M. J. Harrison (2021). "Conserved and reproducible bacterial communities associate with extraradical hyphae of arbuscular mycorrhizal fungi." The ISME Journal: 1-13.

FAO (2017). The future of food and agriculture – Trends and challenges. Rome.

Finkel, O. M., I. Salas-González, G. Castrillo, S. Spaepen, T. F. Law, P. J. P. L. Teixeira, C. D. Jones and J. L. Dangl (2019). "The effects of soil phosphorus content on plant microbiota are driven by the plant phosphate starvation response." PLOS Biology **17**(11): e3000534.

Frazier, W. C. and P. Rupp (1928). "Studies on the proteolytic bacteria of milk I. A medium for the direct isolation of caseolytic milk bacteria." Journal of Bacteriology **16**(1): 57-63.

Frey-Klett, P., M. Chavatte, M. L. Clausse, S. Courrier, C. L. Roux, J. Raaijmakers, M. G. Martinotti, J. C. Pierrat and J. Garbaye (2005). "Ectomycorrhizal symbiosis affects functional diversity of rhizosphere fluorescent pseudomonads." New Phytologist **165**(1): 317-328.

Gadagi, R. S. and T. Sa (2002). "New isolation method for microorganisms solulbilizing iron and aluminum phosphates using dyes." Soil Science and Plant Nutrition **48**(4): 615-618.

Gamalero, E., A. Trotta, N. Massa, A. Copetta, M. G. Martinotti and G. Berta (2004). "Impact of two fluorescent pseudomonads and an arbuscular mycorrhizal fungus on tomato plant growth, root architecture and P acquisition." Mycorrhiza **14**(3): 185-192.

Ganeshamoorthi, P., T. Anand, V. Prakasam, M. Bharani, N. Ragupathi and R. Samiyappan (2008). "Plant growth promoting rhizobacterial (PGPR) bioconsortia mediates induction of defense-related proteins against infection of root rot pathogen in mulberry plants." Journal of Plant Interactions **3**(4): 233-244.

Gange, A. C., E. Bower, P. G. Stagg, D. M. Aplin, A. E. Gillam and M. Bracken (1999). "A comparison of visualization techniques for recording arbuscular mycorrhizal colonization." New Phytologist **142**(1): 123-132.

Gannon, J., V. Manilal and M. Alexander (1991). "Relationship between cell surface properties and transport of bacteria through soil." Applied and Environmental Microbiology **57**(1): 190-193.

Garbaye, J. (1994). "Tansley Review No. 76 Helper bacteria: a new dimension to the mycorrhizal symbiosis." New Phytologist **128**(2): 197-210.

García-Fraile, P., E. Menéndez, L. Celador-Lera, A. Díez-Méndez, A. Jiménez-Gómez, M. Marcos-García, X. A. Cruz-González, P. Martínez-Hidalgo, P. F. Mateos and R. Rivas (2017). Bacterial probiotics: A truly green revolution. Probiotics and Plant Health. V. Kumar, M. Kumar, S. Sharma and R. Prasad. Singapore, Springer Singapore**:** 131-162.

Genre, A. and G. Russo (2016). "Does a common pathway transduce symbiotic signals in plant–microbe interactions?" Frontiers in Plant Science **7**: 96.

Gerland, P., A. E. Raftery, H. Ševčíková, N. Li, D. Gu, T. Spoorenberg, L. Alkema, B. K. Fosdick, J. Chunn, N. Lalic, G. Bay, T. Buettner, G. K. Heilig and J. Wilmoth (2014). "World population stabilization unlikely this century." Science **346**(6206): 234.

Ghorbanpour, M., M. Omidvari, P. Abbaszadeh-Dahaji, R. Omidvar and K. Kariman (2018). "Mechanisms underlying the protective effects of beneficial fungi against plant diseases." Biological Control **117**: 147-157.

Ghorchiani, M., H. Etesami and H. A. Alikhani (2018). "Improvement of growth and yield of maize under water stress by co-inoculating an arbuscular mycorrhizal fungus and a plant growth promoting rhizobacterium together with phosphate fertilizers." Agriculture, Ecosystems & Environment **258**: 59-70.

Gillespie, A. W., R. E. Farrell, F. L. Walley, A. R. S. Ross, P. Leinweber, K.-U. Eckhardt, T. Z. Regier and R. I. R. Blyth (2011). "Glomalin-related soil protein contains non-mycorrhizal-related heat-stable proteins, lipids and humic materials." Soil Biology and Biochemistry **43**(4): 766-777.

Giovannetti, M. and B. Mosse (1980). "An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots." New Phytologist **84**(3): 489-500.

Glaeser, B. (2010). The green revolution revisited: critique and alternatives, Taylor & Francis.

Glibert, P. M., S. Seitzinger, C. A. Heil, J. M. Burkholder, M. W. Parrow, L. A. Codispoti and V. Kelly (2005). "Eutrophication." Oceanography **18**(2): 198.

Gollotte, A., D. van Tuinen and D. Atkinson (2004). "Diversity of arbuscular mycorrhizal fungi colonising roots of the grass species *Agrostis capillaris* and *Lolium perenne* in a field experiment." Mycorrhiza **14**(2): 111-117.

Gomes, E. A., U. G. Lana, J. F. Quensen, S. M. de Sousa, C. A. Oliveira, J. Guo, L. J. Guimarães and J. M. Tiedje (2018). "Root-associated microbiome of maize genotypes with contrasting phosphorus use efficiency." Phytobiomes **2**(3): 129-137.

Gomes, N. C. M., O. Fagbola, R. Costa, N. G. Rumjanek, A. Buchner, L. Mendona-Hagler and K. Smalla (2003). "Dynamics of fungal communities in bulk and maize rhizosphere soil in the tropics." Applied and Environmental Microbiology **69**(7): 3758-3766.

González, A. D., B. Frostell and A. Carlsson-Kanyama (2011). "Protein efficiency per unit energy and per unit greenhouse gas emissions: Potential contribution of diet choices to climate change mitigation." Food Policy **36**(5): 562-570.

Good, A. G. and P. H. Beatty (2011). "Fertilizing nature: A tragedy of excess in the commons." PLOS Biology **9**(8): e1001124.

Goteti, P. K., L. D. A. Emmanuel, S. Desai and M. H. A. Shaik (2013). "Prospective zinc solubilising bacteria for enhanced nutrient uptake and growth promotion in maize (*Zea mays* L.)." International Journal of Microbiology **2013**: 869697.

Goulding, K., P. Murray, S. Sohi, L. Gilliam, J. Williams, I. Clark and P. Hirsch (2010). Soil without plants: the consequences for microorganisms and mesofauna. 19th World Congress of Soil Science, Soil Solutions for a Changing World.

Grman, E. (2012). "Plant species differ in their ability to reduce allocation to non-beneficial arbuscular mycorrhizal fungi." Ecology **93**(4): 711-718.

Gulbis, N., L. Robinson-Boyer and G. K. Robinson (2013). "Studying the microbiome of AMF cultivated *in vitro*." Aspects of Applied Biology (Positive Plant Microbial Interactions: Their role in maintaining sustainable and natural ecosystems) **120**: 71-76.

Gururani, M. A., C. P. Upadhyaya, V. Baskar, J. Venkatesh, A. Nookaraju and S. W. Park (2013). "Plant growth-promoting rhizobacteria enhance abiotic stress tolerance in *Solanum tuberosum* through inducing changes in the expression of ROS-scavenging enzymes and improved photosynthetic performance." Journal of Plant Growth Regulation **32**(2): 245-258.

Haling, R. E., Z. Yang, N. Shadwell, R. A. Culvenor, A. Stefanski, M. H. Ryan, G. A. Sandral, D. R. Kidd, H. Lambers and R. J. Simpson (2016). "Growth and root dry matter allocation by pasture legumes and a grass with contrasting external critical phosphorus requirements." Plant and Soil **407**(1): 67-79.

Hallett, P. D., D. S. Feeney, A. G. Bengough, M. C. Rillig, C. M. Scrimgeour and I. M. Young (2009). "Disentangling the impact of AM fungi versus roots on soil structure and water transport." Plant and Soil **314**(1): 183-196.

Hao, L., Z. Zhang, B. Hao, F. Diao, J. Zhang, Z. Bao and W. Guo (2021). "Arbuscular mycorrhizal fungi alter microbiome structure of rhizosphere soil to enhance maize tolerance to La." Ecotoxicology and Environmental Safety **212**: 111996.

Hara, S. and M. Saito (2016). "Isolation of inositol hexaphosphate (IHP)-degrading bacteria from arbuscular mycorrhizal fungal hyphal compartments using a modified baiting method involving alginate beads containing IHP." Microbes and Environments: ME15206.

Harrier, L. A. (2001). "The arbuscular mycorrhizal symbiosis: a molecular review of the fungal dimension." Journal of Experimental Botany **52**: 469-478.

Harrison, M. T., B. R. Cullen and R. P. Rawnsley (2016). "Modelling the sensitivity of agricultural systems to climate change and extreme climatic events." Agricultural Systems **148**: 135-148.

Haskett, T. L., A. Tkacz and P. S. Poole (2021). "Engineering rhizobacteria for sustainable agriculture." The ISME Journal **15**(4): 949-964.

Hayat, R., I. Ahmed and R. Sheirdil (2012). An overview of plant growth promoting rhizobacteria (PGPR) for sustainable agriculture. Crop Production for Agricultural Improvement. Dordrecht, Springer**:** 557-579.

Haygarth, P. M. and K. Ritz (2009). "The future of soils and land use in the UK: soil systems for the provision of land-based ecosystem services." Land use policy **26**: S187-S197.

Helgason, T., T. J. Daniell, R. Husband, A. H. Fitter and J. P. W. Young (1998). "Ploughing up the wood-wide web?" Nature **394**(6692): 431-431.

Hestrin, R., E. C. Hammer, C. W. Mueller and J. Lehmann (2019). "Synergies between mycorrhizal fungi and soil microbial communities increase plant nitrogen acquisition." Communications Biology **2**(1): 233.

Hill, J., R. Simpson, A. Moore and D. Chapman (2006). "Morphology and response of roots of pasture species to phosphorus and nitrogen nutrition." Plant and Soil **286**(1): 7-19.

Hill, N. S., M. Levi, N. Basinger, A. Thompson, M. Cabrera, J. Wallace, E. Saikawa, A. Avramov and J. Mullican (2021). "White clover living mulch enhances soil health versus annual cover crops." Agronomy Journal **113**(4): 3697-3707.

Hiltner, L. (1904). "Uber neuere Erfahrungen und Probleme auf dem Gebiet der Bodenbakteriologie und unter besonderer Berucksichtigung der Grundungung and Brache." Arbeit. Deut. Landw **98**: 59-78.

Hinsinger, P. (2001). "Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review." Plant and Soil **237**(2): 173-195.

Hirsch, P. R., L. M. Gilliam, S. P. Sohi, J. K. Williams, I. M. Clark and P. J. Murray (2009). "Starving the soil of plant inputs for 50 years reduces abundance but not diversity of soil bacterial communities." Soil Biology and Biochemistry **41**(9): 2021-2024.

Hirsch, P. R., D. Jhurreea, J. K. Williams, P. J. Murray, T. Scott, T. H. Misselbrook, K. W. Goulding and I. M. Clark (2017). "Soil resilience and recovery: rapid community responses to management changes." Plant and Soil **412**(1): 283-297.

Hirsch, P. R., T. H. Mauchline and I. M. Clark (2010). "Culture-independent molecular techniques for soil microbial ecology." Soil Biology and Biochemistry **42**(6): 878-887.

Horneck, D. A., D. M. Sullivan, J. S. Owen and J. M. Hart (2011). "Soil test interpretation guide."

Hoshino, T. and F. Inagaki (2012). "Molecular quantification of environmental DNA using microfluidics and digital PCR." Systematic and Applied Microbiology **35**(6): 390-395.

Hugoni, M., P. Luis, J. Guyonnet and F. e. Z. Haichar (2018). "Plant host habitat and root exudates shape fungal diversity." Mycorrhiza **28**(5): 451-463.

Hunter, M. C., R. G. Smith, M. E. Schipanski, L. W. Atwood and D. A. Mortensen (2017). "Agriculture in 2050: Recalibrating targets for sustainable intensification." BioScience **67**(4): 386-391.

Huo, Y., J. P. Kang, J. C. Ahn, Y. J. Kim, C. H. Piao, D. U. Yang and D. C. Yang (2021). "Siderophore-producing rhizobacteria reduce heavy metal-induced oxidative stress in *Panax ginseng* Meyer." Journal of Ginseng Research **45**(2): 218-227.

Igiehon, N. O., O. O. Babalola, X. Cheseto and B. Torto (2021). "Effects of rhizobia and arbuscular mycorrhizal fungi on yield, size distribution and fatty acid of soybean seeds grown under drought stress." Microbiological Research **242**: 126640.

IPOC (2007). Climate Change: Impacts, Adaptation and Vulnerability, Contribution of WG II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge, UK, Cambridge University Press**:** 976.

Jacoby, R., M. Peukert, A. Succurro, A. Koprivova and S. Kopriva (2017). "The role of soil microorganisms in plant mineral nutrition—current knowledge and future directions." Frontiers in Plant Science **8**: 1617.

Jangid, K., M. A. Williams, A. J. Franzluebbers, J. S. Sanderlin, J. H. Reeves, M. B. Jenkins, D. M. Endale, D. C. Coleman and W. B. Whitman (2008). "Relative impacts of land-use, management intensity and fertilization upon soil microbial community structure in agricultural systems." Soil Biology and Biochemistry **40**(11): 2843-2853.

Jansa, J., P. Bukovská and M. Gryndler (2013). "Mycorrhizal hyphae as ecological niche for highly specialized hypersymbionts–or just soil free-riders?" Frontiers in Plant Science **4**: 134.

Jansa, J., S. T. Forczek, M. Rozmoš, D. Püschel, P. Bukovská and H. Hršelová (2019). "Arbuscular mycorrhiza and soil organic nitrogen: network of players and interactions." Chemical and Biological Technologies in Agriculture **6**(1): 1-10.

Jansa, J., A. Wiemken and E. Frossard (2006). "The effects of agricultural practices on arbuscular mycorrhizal fungi." Geological Society, London, Special Publications **266**(1): 89-115.

Janssen, P. H., P. S. Yates, B. E. Grinton, P. M. Taylor and M. Sait (2002). "Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*." Applied and Environmental Microbiology **68**(5): 2391-2396.

Jansson, J. K. and E. S. Baker (2016). "A multi-omic future for microbiome studies." Nature Microbiology **1**(5): 16049.

Jansson, J. K. and K. S. Hofmockel (2020). "Soil microbiomes and climate change." Nature Reviews Microbiology **18**(1): 35-46.

Jeffries, P., S. Gianinazzi, S. Perotto, K. Turnau and J.-M. Barea (2003). "The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility." Biology and Fertility of Soils **37**(1): 1-16.

Jiang, F., L. Zhang, J. Zhou, T. S. George and G. Feng (2021). "Arbuscular mycorrhizal fungi enhance mineralisation of organic phosphorus by carrying bacteria along their extraradical hyphae." New Phytologist **230**(1): 304-315.

Johansson, J. F., L. R. Paul and R. D. Finlay (2004). "Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture." FEMS Microbiology Ecology **48**(1): 1-13.

Jones, D. L., C. Nguyen and R. D. Finlay (2009). "Carbon flow in the rhizosphere: carbon trading at the soil–root interface." Plant and Soil **321**(1): 5-33.

Juyal, A., T. Eickhorst, R. Falconer, P. C. Baveye, A. Spiers and W. Otten (2018). "Control of pore geometry in soil microcosms and its effect on the growth and spread of *Pseudomonas* and *Bacillus* sp." Frontiers in Environmental Science **6**: 73.

Juyal, A., W. Otten, P. C. Baveye and T. Eickhorst (2021). "Influence of soil structure on the spread of *Pseudomonas fluorescens* in soil at microscale." European Journal of Soil Science **72**(1): 141-153.

Kalam, S., A. Basu, I. Ahmad, R. Sayyed, H. A. El Enshasy, D. J. Dailin and N. Suriani (2020). "Recent understanding of soil *Acidobacteria* and their ecological significance: A critical review." Frontiers in Microbiology **11**: 2712.

Kang, S.-M., A. L. Khan, M. Waqas, Y.-H. You, J.-H. Kim, J.-G. Kim, M. Hamayun and I.-J. Lee (2014). "Plant growth-promoting rhizobacteria reduce adverse effects of salinity and osmotic stress by regulating phytohormones and antioxidants in *Cucumis sativus*." Journal of Plant Interactions **9**(1): 673-682.

Kavamura, V. N., R. Mendes, A. Bargaz and T. H. Mauchline (2021). "Defining the wheat microbiome: Towards microbiome-facilitated crop production." Computational and structural biotechnology journal **19**: 1200.

Kavamura, V. N., R. J. Robinson, R. Hayat, I. M. Clark, D. Hughes, M. Rossmann, P. R. Hirsch, R. Mendes and T. H. Mauchline (2019). "Land management and microbial seed load effect on rhizosphere and endosphere bacterial community assembly in wheat." Frontiers in Microbiology **10**: 2625.

Khanghahi, M. Y., H. Pirdashti, H. Rahimian, G. Nematzadeh and M. G. Sepanlou (2018). "Potassium solubilising bacteria (KSB) isolated from rice paddy soil: from isolation, identification to K use efficiency." Symbiosis **76**(1): 13-23.

Khush, G. S. (2001). "Green revolution: the way forward." Nature Reviews Genetics **2**(10): 815-822.

Kim, Y.-J., J. Lim, J. Sukweenadhi, J. W. Seok, S.-W. Lee, J. C. Park, A. Taizhanova, D. Kim and D. C. Yang (2019). "Genomic characterization of a newly isolated rhizobacteria *Sphingomonas panacis* reveals plant growth promoting effect to rice." Biotechnology and Bioprocess Engineering **24**(1): 119-125.

Kloepper, J. W., C.-M. Ryu and S. Zhang (2004). "Induced systemic resistance and promotion of plant growth by *Bacillus* spp." Phytopathology **94**(11): 1259-1266.

Kohlmeier, S., T. H. Smits, R. M. Ford, C. Keel, H. Harms and L. Y. Wick (2005). "Taking the fungal highway: mobilization of pollutant-degrading bacteria by fungi." Environmental Science & Technology **39**(12): 4640-4646.

Kokkoris, V. and M. Hart (2019). "*In vitro* propagation of arbuscular mycorrhizal fungi may drive fungal evolution." Frontiers in Microbiology **10**(2420).

Kölliker, R., E. Jones, M. Jahufer and J. Forster (2001). "Bulked AFLP analysis for the assessment of genetic diversity in white clover (*Trifolium repens* L.)." Euphytica **121**(3): 305-315.

Konvalinková, T., D. Püschel, V. Řezáčová, H. Gryndlerová and J. Jansa (2017). "Carbon flow from plant to arbuscular mycorrhizal fungi is reduced under phosphorus fertilization." Plant and Soil **419**(1): 319-333.

Kour, D., K. L. Rana, A. N. Yadav, N. Yadav, M. Kumar, V. Kumar, P. Vyas, H. S. Dhaliwal and A. K. Saxena (2020). "Microbial biofertilizers: Bioresources and eco-friendly technologies for agricultural and environmental sustainability." Biocatalysis and Agricultural Biotechnology **23**: 101487.

Kraut-Cohen, J., A. Zolti, L. Shaltiel-Harpaz, E. Argaman, R. Rabinovich, J. Stefan and D. Minz (2019). "Effects of tillage practices on soil microbiome and agricultural parameters." Science of The Total Environment **705**: 135791.

Kumar, A., A. K. Choudhary and V. K. Suri (2016). "Influence of AM fungi, inorganic phosphorus and irrigation regimes on plant water relations and soil physical properties in okra (*Abelmoschus esculentus* L.) – pea (*Pisum sativum* L.) cropping system in Himalayan acid alfisol." Journal of Plant Nutrition **39**(5): 666-682.

Labidi, S., F. B. Jeddi, B. Tisserant, M. Yousfi, M. Sanaa, Y. Dalpé and A. L.-H. Sahraoui (2015). "Field application of mycorrhizal bio-inoculants affects the mineral uptake of a forage legume (*Hedysarum coronarium* L.) on a highly calcareous soil." Mycorrhiza **25**(4): 297-309.

Lanfranco, L., V. Fiorilli, F. Venice and P. Bonfante (2018). "Strigolactones cross the kingdoms: plants, fungi, and bacteria in the arbuscular mycorrhizal symbiosis." Journal of Experimental Botany **69**(9): 2175-2188.

Larkin, R. P. (2008). "Relative effects of biological amendments and crop rotations on soil microbial communities and soilborne diseases of potato." Soil Biology and Biochemistry **40**(6): 1341-1351.

Lawrence, D. and K. Vandecar (2015). "Effects of tropical deforestation on climate and agriculture." Nature Climate Change **5**(1): 27-36.

Lee, E.-H., J.-K. Eo, K.-H. Ka and A.-H. Eom (2013). "Diversity of arbuscular mycorrhizal fungi and their roles in ecosystems." Mycobiology **41**(3): 121-125.

Lee, S.-H., J.-O. Ka and J.-C. Cho (2008). "Members of the phylum *Acidobacteria* are dominant and metabolically active in rhizosphere soil." FEMS Microbiology Letters **285**(2): 263-269.

Lee, S.-W., S.-H. Lee, K. Balaraju, K.-S. Park, K.-W. Nam, J.-W. Park and K. Park (2014). "Growth promotion and induced disease suppression of four vegetable crops by a selected plant growth-promoting rhizobacteria (PGPR) strain *Bacillus subtilis* 21-1 under two different soil conditions." Acta Physiologiae Plantarum **36**(6): 1353-1362.

Lemanceau, P., M. Blouin, D. Muller and Y. Moënne-Loccoz (2017). "Let the core microbiota be functional." Trends in Plant Science **22**.

Levy, A., A. J. Merritt, M. J. Mayo, B. J. Chang, L. K. Abbott and T. J. J. Inglis (2009). "Association between *Burkholderia* species and arbuscular mycorrhizal fungus spores in soil." Soil Biology and Biochemistry **41**(8): 1757-1759.

Li, P., C.-C. Dai, X.-x. Wang, T. Zhang and Y. Chen (2012). "Variation of soil enzyme activities and microbial community structure in peanut monocropping system in subtropical China." African Journal of Agricultural Research **7**.

Li, X., J. Rui, Y. Mao, A. Yannarell and R. Mackie (2014). "Dynamics of the bacterial community structure in the rhizosphere of a maize cultivar." Soil Biology and Biochemistry **68**: 392-401.

Liang, Z., R. A. Drijber, D. J. Lee, I. M. Dwiekat, S. D. Harris and D. A. Wedin (2008). "A DGGE-cloning method to characterize arbuscular mycorrhizal community structure in soil." Soil Biology and Biochemistry **40**(4): 956-966.

Linderman, R. and E. Davis (2003). "Soil amendment with different peatmosses affects mycorrhizae of onion." HortTechnology **13**(2): 285-289.

Lindström, K. and S. A. Mousavi (2020). "Effectiveness of nitrogen fixation in rhizobia." Microbial Biotechnology **13**(5): 1314-1335.

Liu, C.-Y., P. Wang, D.-J. Zhang, Y.-N. Zou, K. Kuča and Q.-S. Wu (2018). "Mycorrhiza-induced change in root hair growth is associated with IAA accumulation and expression of EXPs in trifoliate orange under two P levels." Scientia Horticulturae **234**: 227-235.

Liu, R., M. Dai, X. Wu, M. Li and X. Liu (2012). "Suppression of the root-knot nematode [*Meloidogyne incognita* (Kofoid & White) Chitwood] on tomato by dual inoculation with arbuscular mycorrhizal fungi and plant growth-promoting rhizobacteria." Mycorrhiza **22**(4): 289-296.

Liu, S., J. Xu, H. Huang, J. Zhu, J. Tang and X. Chen (2020). "Changes in the mycorrhizal fungal community in host roots over five host generations under low and high phosphorus conditions." Plant and Soil **456**(1): 27-41.

Liu, X.-M. and H. Zhang (2015). "The effects of bacterial volatile emissions on plant abiotic stress tolerance." Frontiers in Plant Science **6**(774).

Lone, R., R. Shuab, S. Khan, J. Ahmad and K. K. Koul (2017). Arbuscular mycorrhizal fungi for sustainable agriculture. Probiotics and Plant Health. V. Kumar, M. Kumar, S. Sharma and R. Prasad. Singapore, Springer Singapore**:** 553-577.

Louca, S., S. M. S. Jacques, A. P. F. Pires, J. S. Leal, D. S. Srivastava, L. W. Parfrey, V. F. Farjalla and M. Doebeli (2016). "High taxonomic variability despite stable functional structure across microbial communities." Nature Ecology & Evolution **1**(1): 0015.

Louden, B. C., D. Haarmann and A. M. Lynne (2011). "Use of blue agar CAS Assay for siderophore detection." Journal of Microbiology & Biology Education **12**(1): 51-53.

Loudon, A. H., J. A. Holland, T. P. Umile, E. A. Burzynski, K. P. Minbiole and R. N. Harris (2014). "Interactions between amphibians' symbiotic bacteria cause the production of emergent anti-fungal metabolites." Frontiers in Microbiology **5**: 441.

Love, M., S. Anders and W. Huber (2014). "Differential analysis of count data–the DESeq2 package." Genome Biol **15**(550): 10-1186.

Luginbuehl, L. H., G. N. Menard, S. Kurup, H. Van Erp, G. V. Radhakrishnan, A. Breakspear, G. E. D. Oldroyd and P. J. Eastmond (2017). "Fatty acids in arbuscular mycorrhizal fungi are synthesized by the host plant." Science **356**(6343): 1175.

Luginbuehl, L. H. and G. E. D. Oldroyd (2017). "Understanding the arbuscule at the heart of endomycorrhizal symbioses in plants." Current Biology **27**(17): R952-R963.

Lumini, E., V. Bianciotto, P. Jargeat, M. Novero, A. Salvioli, A. Faccio, G. Bécard and P. Bonfante (2007). "Presymbiotic growth and sporal morphology are affected in the arbuscular mycorrhizal fungus *Gigaspora margarita* cured of its endobacteria." Cellular Microbiology **9**(7): 1716-1729.

Lumini, E., V. Bianciotto, P. Jargeat, M. Novero, A. Salvioli, A. Faccio, G. Bécard and P. Bonfante (2007). "Presymbiotic growth and sporal morphology are affected in the arbuscular mycorrhizal fungus *Gigaspora margarita* cured of its endobacteria." Cellular Microbiology **9**(7): 1716-1729.

Luo, B., W. Gu, J. Zhong, Y. Wang and G. Zhang (2015). "Revealing crosstalk of plant and fungi in the symbiotic roots of sewage-cleaning *Eichhornia crassipes* using direct *de novo* metatranscriptomic analysis." Scientific Reports **5**(1): 15407.

Luo, Y., F. Wang, Y. Huang, M. Zhou, J. Gao, T. Yan, H. Sheng and L. An (2019). "*Sphingomonas* sp. Cra20 increases plant growth rate and alters rhizosphere microbial community structure of *Arabidopsis thaliana* under drought stress." Frontiers in Microbiology **10**: 1221.

Lyu, D., J. Zajonc, A. Pagé, C. A. Tanney, A. Shah, N. Monjezi, L. A. Msimbira, M. Antar, M. Nazari and R. Backer (2021). "Plant Holobiont Theory: The Phytomicrobiome Plays a Central Role in Evolution and Success." Microorganisms **9**(4): 675.

Ma, X., X. Li and U. Ludewig (2021). "Arbuscular mycorrhizal colonization outcompetes root hairs in maize under low phosphorus availability." Annals of Botany **127**(1): 155-166.

Mack, K. M. and J. A. Rudgers (2008). "Balancing multiple mutualists: asymmetric interactions among plants, arbuscular mycorrhizal fungi, and fungal endophytes." Oikos **117**(2): 310-320.

Mahdi, S. S., G. Hassan, S. Samoon, H. Rather, S. A. Dar and B. Zehra (2010). "Bio-fertilizers in organic agriculture." Journal of Phytology **2**(10): 42-54.

Mandal, A., B. Sarkar, S. Mandal, M. Vithanage, A. K. Patra and M. C. Manna (2020). Impact of agrochemicals on soil health. Agrochemicals Detection, Treatment and Remediation, Elsevier**:** 161-187.

Marleau, J., Y. Dalpé, M. St-Arnaud and M. Hijri (2011). "Spore development and nuclear inheritance in arbuscular mycorrhizal fungi." BMC Evolutionary Biology **11**(1): 51.

Marques, A. P., C. Pires, H. Moreira, A. O. Rangel and P. M. Castro (2010). "Assessment of the plant growth promotion abilities of six bacterial isolates using *Zea mays* as indicator plant." Soil Biology and Biochemistry **42**(8): 1229-1235.

Marschner, P., D. Crowley and R. Lieberei (2001). "Arbuscular mycorrhizal infection changes the bacterial 16 S rDNA community composition in the rhizosphere of maize." Mycorrhiza **11**(6): 297-302.

Marschner, P., D. Crowley and Z. Rengel (2011). "Rhizosphere interactions between microorganisms and plants govern iron and phosphorus acquisition along the root axis–model and research methods." Soil Biology and Biochemistry **43**(5): 883-894.

Marschner, P., D. Crowley and C. H. Yang (2004). "Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type." Plant and Soil **261**(1): 199-208.

Marsh, J. F. and M. Schultze (2001). "Analysis of arbuscular mycorrhizas using symbiosis-defective plant mutants." New Phytologist **150**(3): 525-532.

Martin, S., S. Mooney, M. Dickinson and H. West (2012). "The effects of simultaneous root colonisation by three Glomus species on soil pore characteristics." Soil Biology and Biochemistry **49**: 167-173.

Marx, E. S., J. M. Hart and R. G. Stevens (1996). "Soil test interpretation guide."

Masters-Clark, E., E. Shone, M. Paradelo, P. R. Hirsch, I. M. Clark, W. Otten, F. Brennan and T. H. Mauchline (2020). "Development of a defined compost system for the study of plant-microbe interactions." Scientific Reports **10**(1): 7521.

Mauchline, T. H. and J. G. Malone (2017). "Life in earth–the root microbiome to the rescue?" Current Opinion in Microbiology **37**: 23-28.

McMillan, V. E., G. Canning, J. Moughan, R. P. White, R. J. Gutteridge and K. E. Hammond-Kosack (2018). "Exploring the resilience of wheat crops grown in short rotations through minimising the build-up of an important soil-borne fungal pathogen." Scientific Reports **8**(1): 9550.

McMurdie, P. J. and S. Holmes (2013). "phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data." PloS one **8**(4): e61217.

McNear Jr, D., H., (2013). "The Rhizosphere - roots, soil and everything in between." Nature Education Knowledge **4**(3): 1.

McSpadden Gardener, B. B. (2004). "Ecology of *Bacillus* and *Paenibacillus* spp. in agricultural systems." Phytopathology **94**(11): 1252-1258.

Meena, R. S., S. Kumar, R. Datta, R. Lal, V. Vijayakumar, M. Brtnicky, M. P. Sharma, G. S. Yadav, M. K. Jhariya and C. K. Jangir (2020). "Impact of agrochemicals on soil microbiota and management: A review." Land **9**(2): 34.

Mehnaz, S., B. Weselowski and G. Lazarovits (2007). "*Sphingobacterium canadense* sp. nov., an isolate from corn roots." Systematic and Applied Microbiology **30**(7): 519-524.

Melillo, E. D. (2012). "The first green revolution: Debt peonage and the making of the nitrogen fertilizer trade, 1840–1930." The American Historical Review **117**(4): 1028-1060.

Mendes, L. W., E. E. Kuramae, A. A. Navarrete, J. A. van Veen and S. M. Tsai (2014). "Taxonomical and functional microbial community selection in soybean rhizosphere." The ISME Journal **8**(8): 1577-1587.

Mendes, R., M. Kruijt, I. de Bruijn, E. Dekkers, M. van der Voort, J. H. M. Schneider, Y. M. Piceno, T. Z. DeSantis, G. L. Andersen, P. A. H. M. Bakker and J. M. Raaijmakers (2011). "Deciphering the rhizosphere microbiome for disease-suppressive bacteria." Science **332**(6033): 1097.

Meng, L., A. Zhang, F. Wang, X. Han, D. Wang and S. Li (2015). "Arbuscular mycorrhizal fungi and rhizobium facilitate nitrogen uptake and transfer in soybean/maize intercropping system." Frontiers in Plant Science **6**(339).

Meyer, J. R. and R. G. Linderman (1986). "Response of subterranean clover to dual inoculation with vesicular-arbuscular mycorrhizal fungi and a plant growth-promoting bacterium, *Pseudomonas putida*." Soil Biology and Biochemistry **18**(2): 185-190.

Minaxi and J. Saxena (2010). "Disease suppression and crop improvement in moong beans (*Vigna radiata*) through *Pseudomonas* and *Burkholderia* strains isolated from semi arid region of rajasthan." BioControl **55**: 799-810.

Mitran, T., R. S. Meena, R. Lal, J. Layek, S. Kumar, B. Meena and R. Datta (2018). Role of soil phosphorus on legume production. Legumes for soil health and sustainable management. Singapore, Springer**:** 487-510.

Moss, B. (2008). "Water pollution by agriculture." Philosophical Transactions of the Royal Society B **363**: 659-666.

Mosse, B. and C. Hepper (1975). "Vesicular-arbuscular mycorrhizal infections in root organ cultures." Physiological Plant Pathology **5**(3): 215-223.

Mummey, D. L. and M. C. Rillig (2006). "The invasive plant species *Centaurea maculosa* alters arbuscular mycorrhizal fungal communities in the field." Plant and Soil **288**(1): 81-90.

Nadeem, S. M., M. Ahmad, Z. A. Zahir, A. Javaid and M. Ashraf (2014). "The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments." Biotechnology Advances **32**(2): 429-448.

Nanjundappa, A., D. J. Bagyaraj, A. K. Saxena, M. Kumar and H. Chakdar (2019). "Interaction between arbuscular mycorrhizal fungi and *Bacillus* spp. in soil enhancing growth of crop plants." Fungal Biology and Biotechnology **6**(1): 23.

Naumann, M., A. Schüßler and P. Bonfante (2010). "The obligate endobacteria of arbuscular mycorrhizal fungi are ancient heritable components related to the *Mollicutes*." The ISME Journal **4**(7): 862-871.

Nicolopoulou-Stamati, P., S. Maipas, C. Kotampasi, P. Stamatis and L. Hens (2016). "Chemical pesticides and human health: the urgent need for a new concept in agriculture." Frontiers in Public Health **4**: 148.

Nilsson, R. H., K.-H. Larsson, A. F S. Taylor, J. Bengtsson-Palme, T. S. Jeppesen, D. Schigel, P. Kennedy, K. Picard, F. O. Glöckner, L. Tedersoo, I. Saar, U. Kõljalg and K. Abarenkov (2018). "The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications." Nucleic Acids Research **47**(D1): D259-D264.

Nosheen, S., I. Ajmal and Y. Song (2021). "Microbes as biofertilizers, a potential approach for sustainable crop production." Sustainability **13**(4): 1868.

Nuccio, E. E., A. Hodge, J. Pett-Ridge, D. J. Herman, P. K. Weber and M. K. Firestone (2013). "An arbuscular mycorrhizal fungus significantly modifies the soil bacterial community and nitrogen cycling during litter decomposition." Environmental Microbiology **15**(6): 1870-1881.

Nurmiaho-Lassila, E. L., S. Timonen, H. K. and R. Sen (1997). "Bacterial colonization patterns of intact *Pinus sylvestris* mycorrhizospheres in dry pine forest soil: An electron microscopy study." Canadian Journal of Microbiology **43**: 1017-1035.

Ofek-Lalzar, M., N. Sela, M. Goldman-Voronov, S. J. Green, Y. Hadar and D. Minz (2014). "Niche and host-associated functional signatures of the root surface microbiome." Nature Communications **5**(1): 1-9.

Oldroyd, G. E., J. D. Murray, P. S. Poole and J. A. Downie (2011). "The rules of engagement in the legume-rhizobial symbiosis." Annual review of genetics **45**: 119-144.

Oldroyd, G. E. D. (2013). "Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants." Nature Reviews Microbiology **11**(4): 252-263.

Or, D., B. F. Smets, J. Wraith, A. Dechesne and S. Friedman (2007). "Physical constraints affecting bacterial habitats and activity in unsaturated porous media–a review." Advances in Water Resources **30**(6-7): 1505-1527.

Orchard, S., R. Standish, D. Nicol, V. Gupta and M. H. Ryan (2016). "The response of fine root endophyte (*Glomus tenue*) to waterlogging is dependent on host plant species and soil type." Plant and Soil **403**(1): 305-315.

Ordoñez, Y. M., B. R. Fernandez, L. S. Lara, A. Rodriguez, D. Uribe-Velez and I. R. Sanders (2016). "Bacteria with phosphate solubilizing capacity alter mycorrhizal fungal growth both inside and outside the root and in the presence of native microbial communities." PloS one **11**(6): e0154438.

Orfanoudakis, M., C. T. Wheeler and J. E. Hooker (2010). "Both the arbuscular mycorrhizal fungus *Gigaspora rosea* and *Frankia* increase root system branching and reduce root hair frequency in *Alnus glutinosa*." Mycorrhiza **20**(2): 117-126.

Oteino, N., R. D. Lally, S. Kiwanuka, A. Lloyd, D. Ryan, K. J. Germaine and D. N. Dowling (2015). "Plant growth promotion induced by phosphate solubilizing endophytic *Pseudomonas* isolates." Frontiers in Microbiology **6**: 745.

Padrão, J., G. Tortella, S. Cortez, N. Dias, A. Nicolau and M. Mota (2019). "Nitrifying soil bacterium Nitrosomonas europaea: operational improvement of standard culture medium." Journal of Soil Science and Plant Nutrition **19**(2): 270-276.

Pal, R., K. Chakrabarti, A. Chakraborty and A. Chowdhury (2010). "Degradation and effects of pesticides on soil microbiological parameters-a review." International Journal of Agricultural Research **5**(8): 625-643.

Papendick, R. I. and J. F. Parr (1992). "Soil quality—The key to a sustainable agriculture." American Journal of Alternative Agriculture **7**(1-2): 2-3.

Park, S., P. Croteau, K. A. Boering, D. M. Etheridge, D. Ferretti, P. J. Fraser, K. R. Kim, P. B. Krummel, R. L. Langenfelds, T. D. van Ommen, L. P. Steele and C. M. Trudinger (2012). "Trends and seasonal cycles in the isotopic composition of nitrous oxide since 1940." Nature Geoscience **5**(4): 261-265.

Paul, D. and S. N. Sinha (2017). "Isolation and characterization of phosphate solubilizing bacterium *Pseudomonas aeruginosa* KUPSB12 with antibacterial potential from river Ganga, India." Annals of Agrarian Science **15**(1): 130-136.

Pérez-de-Luque, A., S. Tille, I. Johnson, D. Pascual-Pardo, J. Ton and D. D. Cameron (2017). "The interactive effects of arbuscular mycorrhiza and plant growth-promoting rhizobacteria synergistically enhance host plant defences against pathogens." Scientific Reports **7**(1): 16409.

Pikovskaya, R. I. (1948). "Mobilization of phosphorus in soil in connection with the vital activity of some microbial species." Mikrobiologiya **17**(362-370).

Pimentel, D. (1996). "Green revolution agriculture and chemical hazards." Science of the Total Environment **188**: S86-S98.

Porter, J. R. and M. A. Semenov (2005). "Crop responses to climatic variation." Philosophical transactions of the Royal Society of London. Series B, Biological sciences **360**(1463): 2021-2035.

Porter, J. R., L. Xie, A. J. Challinor, K. Cochrane, S. M. Howden, M. M. Iqbal, D. B. Lobell and M. I. Travasso (2014). Food security and food production systems. In: Climate Change 2014: Impacts, Adaptation, and Vulnerability. . Cambridge, UK and New York, USA, Cambridge University Press. **Part A: Global and Sectoral Aspects. Contribution of working group II to the fifth assessment report of the intergovernmental panel on climate change:** 485-533.

Porter, S. D. and D. S. Reay (2016). "Addressing food supply chain and consumption inefficiencies: potential for climate change mitigation." Regional Environmental Change **16**(8): 2279-2290.

Porter, S. S. and J. L. Sachs (2020). "Agriculture and the disruption of plant–microbial symbiosis." Trends in ecology & evolution **35**(5): 426-439.

Posta, K. and N. Duc (2020). Benefits of arbuscular mycorrhizal fungi application to crop production under water scarcity. Drought-Detection and Solutions. G. Ondrasek, Books on Demand**:** 25-37.

Prasannakumar, S. P., H. G. Gowtham, P. Hariprasad, K. Shivaprasad and S. R. Niranjana (2015). "*Delftia tsuruhatensis* WGR-UOM-BT1, a novel rhizobacterium with PGPR properties from *Rauwolfia serpentina* (L.) Benth. ex Kurz also suppresses fungal phytopathogens by producing a new antibiotic-AMTM." Letters in Applied Microbiology **61**(5): 460-468.

Qin, S., S. Yeboah, X. Xu, Y. Liu and B. Yu (2017). "Analysis on fungal diversity in rhizosphere soil of continuous cropping potato subjected to different furrow-ridge mulching managements." Frontiers in Microbiology **8**: 845-845.

Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies and F. O. Glöckner (2012). "The SILVA ribosomal RNA gene database project: improved data processing and web-based tools." Nucleic Acids Research **41**(D1): D590-D596.

Raaijmakers, J. M., T. C. Paulitz, C. Steinberg, C. Alabouvette and Y. Moënne-Loccoz (2009). "The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms." Plant and Soil **321**(1): 341-361.

Ratti, N., S. Kumar, H. Verma and S. Gautam (2001). "Improvement in bioavailability of tricalcium phosphate to *Cymbopogon martinii* var. motia by rhizobacteria, AMF and *Azospirillum* inoculation." Microbiological Research **156**(2): 145-149.

Reddy, S. R., P. K. Pindi and S. M. Reddy (2005). "Molecular methods for research on arbuscular mycorrhizal fungi in India: problems and prospects." Current Science **89**(10): 1699-1709.

Reid, T. E., V. N. Kavamura, M. Abadie, A. Torres-Ballesteros, M. Pawlett, I. M. Clark, J. Harris and T. H. Mauchline (2021). "Inorganic chemical fertilizer application to wheat reduces the abundance of putative plant growth-promoting rhizobacteria." Frontiers in Microbiology **12**: 458.

Remy, W., T. N. Taylor, H. Hass and H. Kerp (1994). "Four hundred-million-year-old vesicular arbuscular mycorrhizae." Proceedings of the National Academy of Sciences of the United States of America **91**(25): 11841-11843.

Riedlinger, J., S. D. Schrey, M. T. Tarkka, R. Hampp, M. Kapur and H. P. Fiedler (2006). "Auxofuran, a novel metabolite that stimulates the growth of fly agaric, is produced by the mycorrhiza helper bacterium *Streptomyces* strain AcH 505." Applied and Environmental Microbiology **72**(5): 3550-3557.

Rincon-Florez, V., L. Carvalhais and P. Schenk (2013). "Culture-independent molecular tools for soil and rhizosphere microbiology." Diversity **5**: 581-612.

Robinson, R. J., B. A. Fraaije, I. M. Clark, R. W. Jackson, P. R. Hirsch and T. H. Mauchline (2016). "Wheat seed embryo excision enables the creation of axenic seedlings and Koch's postulates testing of putative bacterial endophytes." Scientific Reports **6**: 25581.

Rodríguez-Caballero, G., F. Caravaca, A. J. Fernández-González, M. Alguacil, M. Fernández-López and A. Roldán (2017). "Arbuscular mycorrhizal fungi inoculation mediated changes in rhizosphere bacterial community structure while promoting revegetation in a semiarid ecosystem." Science of the Total Environment **584**: 838-848.

Roesti, D., K. Ineichen, O. Braissant, D. Redecker, A. Wiemken and M. Aragno (2005). "Bacteria associated with spores of the arbuscular mycorrhizal fungi *Glomus geosporum* and *Glomus constrictum*." Applied and Environmental Microbiology **71**(11): 6673-6679.

Rosier, C., J. Piotrowski, A. Hoye and M. Rillig (2008). "Intraradical protein and glomalin as a tool for quantifying arbuscular mycorrhizal root colonization." Pedobiologia **52**: 41-50.

Rousseau, A., N. Benhamou, I. Chet and Y. Piché (1996). "Mycoparasitism of the extramatrical phase of *Glomus intraradices* by *Trichoderma harzianum*." Phytopathology **86**(5): 434-443.

Ruiz-Rueda, O., S. Hallin and L. Bañeras (2009). "Structure and function of denitrifying and nitrifying bacterial communities in relation to the plant species in a constructed wetland." FEMS Microbiology Ecology **67**(2): 308-319.

Ryan, M., D. Small and J. Ash (2000). "Phosphorus controls the level of colonisation by arbuscular mycorrhizal fungi in conventional and biodynamic irrigated dairy pastures." Australian Journal of Experimental Agriculture **40**(5): 663-670.

Ryan, M., M. Tibbett, T. Edmonds-Tibbett, L. Suriyagoda, H. Lambers, G. Cawthray and J. Pang (2012). "Carbon trading for phosphorus gain: the balance between rhizosphere carboxylates and arbuscular mycorrhizal symbiosis in plant phosphorus acquisition." Plant, Cell & Environment **35**(12): 2170-2180.

Salvioli, A., M. Chiapello, J. Fontaine, A. L. Hadj-Sahraoui, A. Grandmougin-Ferjani, L. Lanfranco and P. Bonfante (2010). "Endobacteria affect the metabolic profile of their host *Gigaspora margarita*, an arbuscular mycorrhizal fungus." Environmental Microbiology **12**(8): 2083-2095.

Salvioli, A., S. Ghignone, M. Novero, L. Navazio, F. Venice, P. Bagnaresi and P. Bonfante (2016). "Symbiosis with an endobacterium increases the fitness of a mycorrhizal fungus, raising its bioenergetic potential." The ISME Journal **10**(1): 130-144.

Sanders, I. R. and A. Rodriguez (2016). "Aligning molecular studies of mycorrhizal fungal diversity with ecologically important levels of diversity in ecosystems." The ISME Journal **10**(12): 2780-2786.

Sandhya, V., S. Z. Ali, M. Grover, G. Reddy and B. Venkateswarlu (2010). "Effect of plant growth promoting *Pseudomonas* spp. on compatible solutes, antioxidant status and plant growth of maize under drought stress." Journal of Plant Growth Regulation **62**(1): 21-30.

Santos, M. S., M. A. Nogueira and M. Hungria (2019). "Microbial inoculants: reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture." AMB Express **9**(1): 1-22.

Savci, S. (2012). "Investigation of effect of chemical fertilizers on environment." Apcbee Procedia **1**: 287-292.

Saxena, J., A. Saini, I. Ravi, S. Chandra and V. Garg (2015). "Consortium of phosphate-solubilizing bacteria and fungi for promotion of growth and yield of chickpea (*Cicer arietinum*)." Journal of Crop Improvement **29**(3): 353-369.

Scagliola, M., F. Valentinuzzi, T. Mimmo, S. Cesco, C. Crecchio and Y. Pii (2021). "Bioinoculants as promising complement of chemical fertilizers for a more sustainable agricultural practice." Frontiers in Sustainable Food Systems **4**(305).

Scheublin, T. R., I. R. Sanders, C. Keel and J. R. van der Meer (2010). "Characterisation of microbial communities colonising the hyphal surfaces of arbuscular mycorrhizal fungi." The ISME Journal **4**(6): 752-763.

Schrey, S. D., M. Schellhammer, M. Ecke, R. Hampp and M. T. Tarkka (2005). "Mycorrhiza helper bacterium *Streptomyces* AcH 505 induces differential gene expression in the ectomycorrhizal fungus *Amanita muscaria*." New Phytologist **168**(1): 205-216.

Schrey, S. D. and M. T. Tarkka (2008). "Friends and foes: streptomycetes as modulators of plant disease and symbiosis." Antonie Van Leeuwenhoek **94**(1): 11-19.

Schroeder, M. and D. Janos (2005). "Plant growth, phosphorus nutrition, and root morphological responses to arbuscular mycorrhizas, phosphorus fertilization, and intraspecific density." Mycorrhiza **15**(3): 203-216.

Setiawati, T. C. and L. Mutmainnah (2016). "Solubilization of potassium containing mineral by microorganisms from sugarcane rhizosphere." Agriculture and Agricultural Science Procedia **9**: 108-117.

Sharma, A., D. Shankhdhar and S. Shankhdhar (2013). "Enhancing grain iron content of rice by the application of plant growth promoting rhizobacteria." Plant Soil and Environment **59**: 89.

Sharma, M. P. and J. S. Buyer (2015). "Comparison of biochemical and microscopic methods for quantification of arbuscular mycorrhizal fungi in soil and roots." Applied Soil Ecology **95**: 86-89.

Sharma, S., S. Compant, M.-B. Ballhausen, S. Ruppel and P. Franken (2020). "The interaction between *Rhizoglomus irregulare* and hyphae attached phosphate solubilizing bacteria increases plant biomass of *Solanum lycopersicum*." Microbiological Research **240**: 126556.

Shoresh, M., G. E. Harman and F. Mastouri (2010). "Induced systemic resistance and plant responses to fungal biocontrol agents." Annual Review of Phytopathology **48**: 21-43.

Simon, A., S. Bindschedler, D. Job, L. Y. Wick, S. Filippidou, W. M. Kooli, E. P. Verrecchia and P. Junier (2015). "Exploiting the fungal highway: development of a novel tool for the in situ isolation of bacteria migrating along fungal mycelium." FEMS Microbiology Ecology **91**(11).

Singh, B. K., N. Nunan, K. P. Ridgway, J. McNicol, J. P. Young, T. J. Daniell, J. I. Prosser and P. Millard (2008). "Relationship between assemblages of mycorrhizal fungi and bacteria on grass roots." Environmental Microbiology **10**(2): 534-541.

Singh, B. K., P. Trivedi, E. Egidi, C. A. Macdonald and M. Delgado-Baquerizo (2020). "Crop microbiome and sustainable agriculture." Nature Reviews Microbiology **18**(11): 601-602.

Singh, B. R., A. Singh, S. Mishra, A. H. Naqvi and H. B. Singh (2016). Remediation of heavy metal-contaminated agricultural soils using microbes. Microbial Inoculants in Sustainable Agricultural Productivity, Springer**:** 115-132.

Singh, D., S. K. Singh, A. Modi, P. K. Singh, V. Y. Zhimo and A. Kumar (2020). Impacts of agrochemicals on soil microbiology and food quality. Agrochemicals Detection, Treatment and Remediation, Elsevier**:** 101-116.

Smalla, K., G. Wieland, A. Buchner, A. Zock, J. Parzy, S. Kaiser, N. Roskot, H. Heuer and G. Berg (2001). "Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed." Applied and Environmental Microbiology **67**(10): 4742-4751.

Smith, S. and D. Read (2008). Mycorrhizal Symbiosis.

Snyder, C. S., T. W. Bruulsema, T. L. Jensen and P. E. Fixen (2009). "Review of greenhouse gas emissions from crop production systems and fertilizer management effects." Agriculture, Ecosystems & Environment **133**(3): 247-266.

Söderberg, K. H., P. A. Olsson and E. Bååth (2002). "Structure and activity of the bacterial community in the rhizosphere of different plant species and the effect of arbuscular mycorrhizal colonisation." FEMS Microbiology Ecology **40**(3): 223-231.

Srivastav, A. L. (2020). Chemical fertilizers and pesticides: role in groundwater contamination. Agrochemicals Detection, Treatment and Remediation, Elsevier**:** 143-159.

Stewart, E. J. (2012). "Growing unculturable bacteria." Journal of bacteriology **194**(16): 4151-4160.

Suman, A., A. N. Yadav and P. Verma (2016). Endophytic microbes in crops: diversity and beneficial impact for sustainable agriculture. Microbial Inoculants in Sustainable Agricultural Productivity, Springer**:** 117-143.

Sundaramoorthy, S., T. Raguchander, N. Ragupathi and R. Samiyappan (2012). "Combinatorial effect of endophytic and plant growth promoting rhizobacteria against wilt disease of *Capsicum annum* L. caused by *Fusarium solani*." Biological Control **60**(1): 59-67.

Svenningsen, N. B., S. J. Watts-Williams, E. J. Joner, F. Battini, A. Efthymiou, C. Cruz-Paredes, O. Nybroe and I. Jakobsen (2018). "Suppression of the activity of arbuscular mycorrhizal fungi by the soil microbiota." The ISME Journal **12**(5): 1296-1307.

Szoboszlay, M., J. Lambers, J. Chappell, J. V. Kupper, L. A. Moe and D. H. McNear (2015). "Comparison of root system architecture and rhizosphere microbial communities of *Balsas teosinte* and domesticated corn cultivars." Soil Biology and Biochemistry **80**: 34-44.

Tabassum, B., A. Khan, M. Tariq, M. Ramzan, M. S. I. Khan, N. Shahid and K. Aaliya (2017). "Bottlenecks in commercialisation and future prospects of PGPR." Applied Soil Ecology **121**: 102-117.

Tarkka, M. T., A. Sarniguet and P. Frey-Klett (2009). "Inter-kingdom encounters: recent advances in molecular bacterium–fungus interactions." Current Genetics **55**(3): 233-243.

Tarkka, M. T., S. Schrey and U. Nehls (2006). "The α-tubulin gene AmTuba1: a marker for rapid mycelial growth in the ectomycorrhizal basidiomycete Amanita muscaria." Current Genetics **49**(5): 294-301.

Tavasolee, A., N. Aliasgharzad, G. R. Salehi, M. Mardi, A. Asgharzadeh and S. Akbarivala (2011). "Effects of co-inoculation with arbuscular mycorrhizal fungi and rhizobia on fungal occupancy in chickpea root and nodule determined by real-time PCR." Current Microbiology **63**(2): 107-114.

Thirkell, T. J., M. D. Charters, A. J. Elliott, S. M. Sait and K. J. Field (2017). "Are mycorrhizal fungi our sustainable saviours? Considerations for achieving food security." Journal of Ecology **105**(4): 921-929.

Thokchom, E., D. Thakuria, M. C. Kalita, C. K. Sharma and N. C. Talukdar (2017). "Root colonization by host-specific rhizobacteria alters indigenous root endophyte and rhizosphere soil bacterial communities and promotes the growth of mandarin orange." European Journal of Soil Biology **79**: 48-56.

Tilman, D., C. Balzer, J. Hill and B. L. Befort (2011). "Global food demand and the sustainable intensification of agriculture." Proceedings of the National Academy of Sciences **108**(50): 20260-20264.

Timmusk, S., L. Behers, J. Muthoni, A. Muraya and A.-C. Aronsson (2017). "Perspectives and challenges of microbial application for crop improvement." Frontiers in Plant Science **8**: 49.

Tinker, P. B., J. S. I. Ingram and S. Struwe (1996). "Effects of slash-and-burn agriculture and deforestation on climate change." Agriculture, Ecosystems & Environment **58**(1): 13-22.

Tisserant, E., M. Malbreil, A. Kuo, A. Kohler, A. Symeonidi, R. Balestrini, P. Charron, N. Duensing, N. F. dit Frey and V. Gianinazzi-Pearson (2013). "Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis." Proceedings of the National Academy of Sciences **110**(50): 20117-20122.

Tiwari, P. and A. Adholeya (2002). "*In vitro* co-culture of two AMF isolates *Gigaspora margarita* and *Glomus intraradices* on Ri T-DNA transformed roots." FEMS Microbiology Letters **206**(1): 39-43.

Toljander, J. F., J. C. Santos-González, A. Tehler and R. D. Finlay (2008). "Community analysis of arbuscular mycorrhizal fungi and bacteria in the maize mycorrhizosphere in a long-term fertilization trial." FEMS microbiology ecology **65**(2): 323-338.

Treseder, K. K. (2013). "The extent of mycorrhizal colonization of roots and its influence on plant growth and phosphorus content." Plant and Soil **371**(1): 1-13.

Trivedi, P., A. Pandey and L. M. S. Palni (2012). Bacterial inoculants for field applications under mountain ecosystem: present initiatives and future prospects. Bacteria in Agrobiology: Plant Probiotics. D. K. Maheshwari. Berlin, Heidelberg, Springer Berlin Heidelberg**:** 15-44.

Trung, T. T., A. Hetzer, A. Göhler, E. Topfstedt, V. Wuthiekanun, D. Limmathurotsakul, S. J. Peacock and I. Steinmetz (2011). "Highly sensitive direct detection and quantification of *Burkholderia pseudomallei* bacteria in environmental soil samples by using real-time PCR." Applied and Environmental Microbiology **77**(18): 6486-6494.

Turrini, A., L. Avio, M. Giovannetti and M. Agnolucci (2018). "Functional complementarity of arbuscular mycorrhizal fungi and associated microbiota: the challenge of translational research." Frontiers in plant science **9**: 1407.

Unno, Y., K. Okubo, J. Wasaki, T. Shinano and M. Osaki (2005). "Plant growth promotion abilities and microscale bacterial dynamics in the rhizosphere of Lupin analysed by phytate utilization ability." Environmental Microbiology **7**(3): 396-404.

Uroz, S. and J. Heinonsalo (2008). "Degradation of N-acyl homoserine lactone quorum sensing signal molecules by forest root-associated fungi." FEMS Microbiology Ecology **65**(2): 271-278.

van Beek, C. L., B. G. Meerburg, R. L. M. Schils, J. Verhagen and P. J. Kuikman (2010). "Feeding the world's increasing population while limiting climate change impacts: linking N_2O and CH_4 emissions from agriculture to population growth." Environmental Science & Policy **13**(2): 89-96.

Van der Heijden, M., J. Klironomos, M. Ursic, P. Moutoglis, R. Streitwolf-Engel, T. Boller, A. Wiemken and I. Sanders (1998). "Mycorrhizal fungal diversity determines plant diversity, ecosystem variability and productivity." Nature **396**: 69-72.

Van Der Heijden, M. G., R. Streitwolf-Engel, R. Riedl, S. Siegrist, A. Neudecker, K. Ineichen, T. Boller, A. Wiemken and I. R. Sanders (2006). "The mycorrhizal contribution to plant productivity, plant nutrition and soil structure in experimental grassland." New Phytologist **172**(4): 739-752.

Vandenkoornhuyse, P., A. Quaiser, M. Duhamel, A. Le Van and A. Dufresne (2015). "The importance of the microbiome of the plant holobiont." New Phytologist **206**(4): 1196-1206.

Vázquez, M., S. César, R. Azcón and J. M. Barea (2000). "Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum*, *Pseudomonas*, *Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants." Applied Soil Ecology **15**(3): 261-272.

Veerman, C., T. Pinto Correia, C. Bastioli, B. Biro, J. Bouma and E. Cienciela (2020). "Caring for soil is caring for life." EU Soil Health and Food Mission Board: Brussels, Belgium.

Ven, A., M. S. Verlinden, E. Verbruggen and S. Vicca (2019). "Experimental evidence that phosphorus fertilization and arbuscular mycorrhizal symbiosis can reduce the carbon cost of phosphorus uptake." Functional Ecology **33**(11): 2215-2225.

Verbruggen, E. and E. T. Kiers (2010). "Evolutionary ecology of mycorrhizal functional diversity in agricultural systems." Evolutionary Applications **3**(5-6): 547-560.

Verbruggen, E., M. G. van der Heijden, M. C. Rillig and E. T. Kiers (2013). "Mycorrhizal fungal establishment in agricultural soils: factors determining inoculation success." New Phytologist **197**(4): 1104-1109.

Vermeulen, S. J., B. M. Campbell and J. S. I. Ingram (2012). "Climate change and food systems." Annual Review of Environment and Resources **37**(1): 195-222.

Vessey, J. K. (2003). "Plant growth promoting rhizobacteria as biofertilizers." Plant and Soil **255**(2): 571-586.

Vik, U., R. Logares, R. Blaalid, R. Halvorsen, T. Carlsen, I. Bakke, A.-B. Kolstø, O. A. Økstad and H. Kauserud (2013). "Different bacterial communities in ectomycorrhizae and surrounding soil." Scientific Reports **3**(1): 3471.

Vives-Peris, V., A. Gómez-Cadenas and R. M. Pérez-Clemente (2018). "Salt stress alleviation in citrus plants by plant growth-promoting rhizobacteria *Pseudomonas putida* and *Novosphingobium* sp." Plant Cell Reports **37**(11): 1557-1569.

Voříšková, A., J. Jansa, D. Püschel, M. Krüger, T. Cajthaml, M. Vosátka and M. Janoušková (2017). "Real-time PCR quantification of arbuscular mycorrhizal fungi: does the use of nuclear or mitochondrial markers make a difference?" Mycorrhiza **27**(6): 577-585.

Vosatka, M. and J. C. Dodd (1998). "The role of different arbuscular mycorrhizal fungi in the growth of *Calamagrostis villosa* and *Deschampsia flexuosa*, in experiments with simulated acid rain." Plant and Soil **200**(2): 251-263.

Wahyudi, A. T., R. P. Astuti, A. Widyawati, A. Mery and A. A. Nawangsih (2011). "Characterization of *Bacillus* sp. strains isolated from rhizosphere of soybean plants for their use as potential plant growth for promoting rhizobacteria." Journal of Microbiology and Antimicrobials **3**(2): 34-40.

Wallace, J. S. (2000). "Increasing agricultural water use efficiency to meet future food production." Agriculture, Ecosystems & Environment **82**(1): 105-119.

Wen, Y., G. Schoups and N. van de Giesen (2017). "Organic pollution of rivers: Combined threats of urbanization, livestock farming and global climate change." Scientific Reports **7**(1): 43289.

Wezel, A., M. Casagrande, F. Celette, J.-F. Vian, A. Ferrer and J. Peigné (2014). "Agroecological practices for sustainable agriculture. A review." Agronomy for Sustainable Development **34**(1): 1-20.

White III, R. A., A. Rivas-Ubach, M. I. Borkum, M. Köberl, A. Bilbao, S. M. Colby, D. W. Hoyt, K. Bingol, Y.-M. Kim and J. P. Wendler (2017). "The state of rhizospheric science in the era of multi-omics: A practical guide to omics technologies." Rhizosphere **3**: 212-221.

Wick, L. Y., R. Remer, B. Würz, J. Reichenbach, S. Braun, F. Schäfer and H. Harms (2007). "Effect of fungal hyphae on the access of bacteria to phenanthrene in soil." Environmental Science & Technology **41**(2): 500-505.

Wik, M., P. Pingali and S. Brocai (2008). "Global agricultural performance: past trends and future prospects."

Workneh, F. and A. H. C. van Bruggen (1994). "Microbial density, composition, and diversity in organically and conventionally managed rhizosphere soil in relation to suppression of corky root of tomatoes." Applied Soil Ecology **1**(3): 219-230.

Wu, M. and X. Li (2015). Chapter 87 - *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Molecular Medical Microbiology (Second Edition). Y.-W. Tang, M. Sussman, D. Liu, I. Poxton and J. Schwartzman. Boston, Academic Press**:** 1547-1564.

Xavier, L. J. and J. J. Germida (2003). "Bacteria associated with *Glomus clarum* spores influence mycorrhizal activity." Soil Biology and Biochemistry **35**(3): 471-478.

Xie, Y., J. N. Sorensen, K. K. Petersen and H. L. Kristensen (2018). "Stepwise incorporation of white clover (Trifolium repens L.) as fertiliser increases nitrogen fixation and improves nitrogen retention when intercropped with leek (Allium porrum L.)." Plant and soil **422**(1): 541-554.

Yazdani, M., M. A. Bahmanyar, H. Pirdashti and M. A. Esmaili (2009). "Effect of phosphate solubilization microorganisms (PSM) and plant growth promoting rhizobacteria (PGPR) on yield and yield components of corn (*Zea mays* L.)." World Academy of Science, Engineering Technology **49**: 90-92.

Yilmaz, P., L. W. Parfrey, P. Yarza, J. Gerken, E. Pruesse, C. Quast, T. Schweer, J. Peplies, W. Ludwig and F. O. Glöckner (2014). "The SILVA and "all-species living tree project (LTP)" taxonomic frameworks." Nucleic Acids Research **42**(D1): D643-D648.

Zaheer, A., B. S. Mirza, J. E. Mclean, S. Yasmin, T. M. Shah, K. A. Malik and M. S. Mirza (2016). "Association of plant growth-promoting *Serratia* spp. with the root nodules of chickpea." Research in Microbiology **167**(6): 510-520.

Zaller, J. G., T. Frank and T. Drapela (2011). "Soil sand content can alter effects of different taxa of mycorrhizal fungi on plant biomass production of grassland species." European Journal of Soil Biology **47**(3): 175-181.

Zhang, L., N. Shi, J. Fan, F. Wang, T. S. George and G. Feng (2018). "Arbuscular mycorrhizal fungi stimulate organic phosphate mobilization associated with changing bacterial community structure under field conditions." Environmental microbiology **20**(7): 2639-2651.

Zhang, W., X.-G. Li, K. Sun, M.-J. Tang, F.-J. Xu, M. Zhang and C.-C. Dai (2020). "Mycelial network-mediated rhizobial dispersal enhances legume nodulation." The ISME journal **14**(4): 1015-1029.

Zhang, X., H. Wang, G. Ash, D. Yu and H. Wang (2020). The influence of rhizosphere soil fungal diversity and complex community structure to wheat root rot disease.

Zhang, Y., E. K. Kastman, J. S. Guasto and B. E. Wolfe (2018). "Fungal networks shape dynamics of bacterial dispersal and community assembly in cheese rind microbiomes." Nature Communications **9**(1): 336.

Zhou, X., D. Gao, J. Liu, P. Qiao, X. Zhou, H. Lu, X. Wu, D. Liu, X. Jin and F. Wu (2014). "Changes in rhizosphere soil microbial communities in a continuously monocropped cucumber (*Cucumis sativus* L.) system." European Journal of Soil Biology **60**: 1-8.

Zhou, X., F. H. Passow, J. Rudek, J. C. von Fisher, S. P. Hamburg and J. D. Albertson (2019). "Estimation of methane emissions from the U.S. ammonia fertilizer industry using a mobile sensing approach." Elementa: Science of the Anthropocene **7**.

APPENDICES

A.1 Figure 1.

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A.2 Development of a defined compost system for the study of plant microbe interactions

A significant amount of work pertaining to Section 4 was published in the following research paper (Figure A-1). The system was designed to be a system in which plant-microbial interactions could be studied, however its use was discontinued due to its unsuitability for AMF work.

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Development of a defined compost system for the study of plant-microbe interactions

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Plant growth promoting rhizobacteria can improve plant health by providing enhanced nutrition, disease suppression and abiotic stress resistance, and have potential to contribute to sustainable agriculture. We have developed a sphagnum peat-based compost platform for investigating plant-microbe interactions. The chemical, physical and biological status of the system can be manipulated to understand the relative importance of these factors for plant health, demonstrated using three case studies: 1. Nutrient depleted compost retained its structure, but plants grown in this medium were severely stunted in growth due to removal of essential soluble nutrients - particularly, nitrogen, phosphorus and potassium. Compost nutrient status was replenished with the addition of selected soluble nutrients, validated by plant biomass; 2. When comparing milled and unmilled compost, we found nutrient status to be more important than matrix structure for plant growth; 3. In compost deficient in soluble P, supplemented with an insoluble inorganic form of P ($Ca_3(PO_4)_2$), application of a phosphate solubilising *Pseudomonas* strain to plant roots provides a significant growth boost when compared with a *Pseudomonas* strain incapable of solubilising $Ca_3(PO_4)_2$. Our findings show that the compost system can be manipulated to impose biotic and abiotic stresses for testing how microbial inoculants influence plant growth.

Novel and optimised alternatives to artificial fertilisers, which can be deleterious to the environment, are required to achieve sustainable intensification of agriculture[1]. Plant growth promoting microbes found at the root-soil interface – part of the root microbiome – have an underexploited arsenal of capabilities that can improve plant health, growth and nutrient status[2]. The network of interactions between microbial partners and the host plant is complex and not well understood. This knowledge is essential for the production of plant growth promoting bioinoculants, which are predicted to be an important part of the solution to feeding the world's growing population[3–5].

Plant growth promoting rhizobacteria (PGPR) have been shown to increase acquisition of many nutrients and improve plant health and productivity where nutrients may be limiting. These nutrients include iron[6], phosphorus[7] and potassium[8]. The ability of PGPR to mitigate abiotic stresses such as drought[9], salt[10] or alkaline soil[11] has been demonstrated in many crop plants. Potato plants exposed to all the aforementioned stresses in combination survived when inoculated with two *Bacillus* species[12]. The mechanism of tolerance, induced by the bacteria, was shown to be an increase in reactive oxygen species scavenging enzymes and an improvement in photosynthetic performance[12]. *Pseudomonas* species are important PGPR, overcoming drought stress in maize[13] and often possessing many of the characteristic traits of plant growth promotion: production of siderophores, indole-acetic acid and ammonia[14]. Microbiome research is also focusing on the potential of beneficial microbes to improve agricultural resilience and soil health, enhancing practices such as phytoremediation[15], the creation of disease suppressive soils[16], and reducing dependency on artificial fertilisers[17]. Bacterial inocula have been shown to reduce incidence of disease across a broad range of both fungal and bacterial diseases, enhanced further when mixed inocula are used[18].

In order to assess the potential benefits of PGPR there is a need for the development of a defined testing platform. Previous work has used simplified inert substrates, such as sand, glass beads, vermiculite or perlite, but these are less conducive to root growth and are a much less intricate and realistic matrix than soil[7,19]. Conversely,

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Figure A-1 Publication by Masters-Clark et al., 2020 as part of experimental work in Section 4. .

the use of real soil is often unsuitable due to inherent difficulties in altering soil nutrients, without also affecting soil physical and biological status, making the importance of each individual component for plant health difficult to quantify. We argue that sphagnum peat moss compost, hereafter referred to as compost, provides a suitable substrate to test factors that influence plant growth. Other systems are either too simple to replicate *in vivo* conditions well enough to test inoculants, such as vermiculite, or too complex to manipulate nutrient levels, such as soil. Compost is an irregular, organic matter matrix with absorption and adsorption properties that provides chemical complexity and an excellent structure for plant growth whilst being easy to manipulate and implement stresses. Tomato plants grown in substrates containing peat showed improved growth compared with using other media such as sand and perlite[20], and growth of daisies was also improved when vermiculite was supplemented with compost and sphagnum moss peat[21]. Compost provides the optimal structure for plant growth, allowing the effect of implemented stresses or microbial inoculants to be measured.

Many experiments use *in vitro* functional screening assays to predict the ability of bacteria to promote plant growth via biotic and abiotic stress resistance. While this is the accepted method, it is important to be able to test that these abilities can be replicated in an *in planta* system[22]. For example, genomic screening of bacteria for plant growth promoting genes is a useful indicator of potential PGPR function. However, plant growth promoting genes are often not expressed *in planta*, perhaps due to an incompatible plant-microbe interaction. It is therefore necessary to test potential inoculants *in vivo* to confirm their plant growth promoting efficacy[23]. In order to develop a system to test microbial interactions *in planta*, it is essential to be able to define the physical matrix and soluble nutrient status of the system, and to understand how changing these factors affect plant growth.

In this work we describe a defined compost system and demonstrate the use of this medium with three case studies: 1) Compost nutrient status – removal and reconstitution; 2) Compost physical structure status – the effect of pore size on plant growth; 3) The deployment of microbial inoculants, with the example of a P solubilising bacterial isolate, to mitigate soluble-P deficiency.

Materials and methods

Case study 1 tested the impact of washing compost on nutrient status and the potential for this impact to be reversed with the addition of nutrients. The nutrient levels of washed and unwashed compost were obtained, and the growth of several crop plants were tested in this medium. Different methods of reconstituting the nutrient levels of the compost were tested to re-establish wheat biomass to that of the unwashed compost. Case study 2 identified the relative importance of matrix physical structure as well as nutrient status for wheat growth. This involved milling to reduce pore size and growing wheat plants in both washed and unwashed compost. Case study 3 investigated the ability of a P solubilising microbial inoculant to support wheat growth in a soluble P deficient system.

Removal of soluble nutrients and assessment of washed compost. The compost used was sphagnum peat moss (95%), with added silver sand (5%); with a 0-3 mm particle size; pH 5.3-6.0 using lime; additional fertiliser N-144, P-73 and K-239 mg/L; 0.1 kg/m^3 Micromax micronutrient fertiliser; and a wetting agent as standard (Levington F2 + sand, supplied by ICL, Ipswich, UK). Compost was washed to remove soluble nutrients by flooding one-part compost to eight-parts tap water, mixing and breaking up any aggregates, and draining through a 0.63 µm sieve. This process was repeated three times. The effects of washing the compost on the component macro- and micronutrients were obtained using three methods: combustion analysis to measure total N, (LECO TruMac combustion analyser – Dumas method), nutrient extraction (i.e. NH_4, NO_3 and Olsen P) and X-ray fluorescence. The X-ray fluorescence was carried out on a Bruker Tracer 5i portable X-ray fluorescence (pXRF) spectrometer using Bruker's EasyCal software. Milled and dried samples were loaded into xrf cups lined with Prolene films.

Bulk density was measured as mass per unit volume, calculated using the mass of oven-dry washed compost and its volume. Compost was packed to the same density in a known volume and then weighed. The pH change of the compost before and after washing was measured, using a 1:2.5 sample: water ratio. Measurements were taken using a PerpHecT ROSS Micro Combination pH electrode (Thermofisher).

Plant germination and selection of seeds. Seeds were sterilised as described by Robinson *et al*[24]., immersed in 70% ethanol for ten minutes, followed by submersion in sodium hypochlorite (1.5%) for one hour. After washing thoroughly, seeds were left overnight in sterile water at 4 °C in the dark, before choosing seeds of a similar size and placing them on damp sterile germination paper (Anchor, Minnesota 55101, USA) in a Petri dish. These were germinated in the dark at room temperature for three days. For planting, seeds were chosen with a coleoptile of length between 3 and 5 mm. Wheat (Cadenza) was used for all experiments except in supplementary figure S4, where different plant species were tested in washed and unwashed compost. These were: Wheat (Cadenza), Barley (Atlas), Clover (Merula), Oilseed rape – (OSR, Aries).

Reconstitution of nutrients in washed compost. Two methods were tested to reconstitute the nutrient content of the washed compost, with the aim of controlling nutrient levels. These enabled the testing of the importance of specific nutrients for plant growth. Firstly, plants in pots (9×9×10 cm, containing 150 g air-dried washed or unwashed compost) were watered daily with 50 ml Hoagland's solution with or without soluble phosphate[25] (Supplementary table S1). Secondly, a modified version of Letcombe's solution (Supplementary table S1) was applied. The differences between the amount of N, P and K in unwashed and washed compost was determined and used to calculate the amount of each nutrient to be added to each pot of washed compost. A single concentrated dose of the primary macronutrients, N, P and K (as required) was applied using a pipette enabling even distribution of the solution over the surface of the compost. This was followed with the addition of 300 ml of

micronutrient solution, in staggered doses until all the solution was absorbed by the compost without subjecting the plants to waterlogged conditions. Growing plants were then watered as necessary with tap water.

Preparation of soluble P deficient compost, supplemented with recalcitrant tricalcium phosphate. Washed compost was supplemented with tricalcium phosphate, $Ca_3(PO_4)_2$, (Sigma) as an insoluble inorganic P source, at a 200:1 ratio[7]. Once germinated, a single plant was transferred to each pot and plants were watered with 50 ml of a modified Hoagland's solution (without P) daily.

Milling of compost to reduce particle size. The effect of the compost particle size on wheat growth was tested. Washed and non-washed compost was air dried before milling using a hammer mill with a 0.5 mm mesh. The compost was re-wetted by submersing the bottom of the pots in water from a tray prior to transplanting seedlings to prevent hydrophobicity issues during plant growth. To ensure the amount of compost was comparable, each pot was allocated 150 g compost prior to milling.

Plant measurements and harvesting. In all plant experiments, wheat was grown until flowering, n = 7 unless stated otherwise in figure legend. Aerial biomass was harvested and weighed after drying at 80 °C for 36 hours.

***In vitro* bacterial phosphate solubilisation assay.** Microbes were isolated in August 2017 as described by Mauchline *et al.*[26] from the rhizosphere of oat, bean and wheat plants grown at Furzefield, Rothamsted Research (51.809094, -0.380412). This involved vortexing roots in sterile water to sample the rhizosphere soil, which was centrifuged to obtain a pellet. A library of bacterial rhizosphere isolates was obtained, and these were screened *in vitro* for the ability to solubilise an example of an insoluble form of phosphate, $Ca_3(PO_4)_2$. Pikovskaya medium (PVK) uses an inorganic, insoluble form of P ($Ca_3(PO_4)_2$) and turns clear when the inoculated strains liberate PO_4-. The medium was prepared as described by Pikovskaya[27]. Isolates from the library were grown for 48 hours in tryptic soy broth (TSB) (Sigma) at 28 °C in a shaker, and 1 µl of each culture was pipetted onto PVK media, with 12 isolates gridded per plate. Sterile TSB served as a negative control. The inoculated plates were incubated at 25 °C for seven days. To confirm that their ability to solubilise $Ca_3(PO_4)_2$ was persistent, P-solubilising rhizobacterial isolates were subjected to sub-culturing three times on PVK.

The ability of rhizobacterial isolates to access insoluble phosphate was described by the phosphorus solubilisation index (PSI)[28,29] as: (colony diameter + halo diameter) / colony diameter. In total, of the 578 bacterial isolations, 82 were capable of solubilising $Ca_3(PO_4)_2$, and 60 were selected for 16S rRNA gene sequencing, and an additional random selection of 32 isolates unable to solubilise $Ca_3(PO_4)_2$ were also sequenced as a comparison.

Microbial DNA isolation, PCR and 16S rRNA gene sequencing. To identify the P solubilising and non-solubilising bacteria, isolates were subjected to 16S rRNA gene sequencing. Bacterial isolates were cultured overnight in TSB at 25 °C. Bacterial DNA was released using microLYSIS-Plus (Microzone, United Kingdom). Lysates were diluted 10-fold prior to PCR.

16S rRNA gene PCR was carried out on microbial DNA isolate extracts using the primers 341f(5'CCTACGGGAGGCAGCAG)[30] and 1389r (5'ACGGGCGGTGTGTACAA)[31]. PCR reactions were carried out using the ThermoPrime 2x ReddyMix PCR Master Mix Kit (Thermo Fisher Scientific). Once prepared, the samples were placed in a thermocycler for PCR and subjected to the following conditions: 95 °C 1 min, followed by 30 cycles of 95 °C 1 min, 60 °C 1 min, 72 °C 1 min and a final extension step of 72 °C for 5 min. The PCR product was purified (Qiagen, Venlo, Netherlands) prior to sequencing.

Sequencing of 16S rRNA gene PCR products was carried out by Eurofins MWG/Operon (Germany) using a PCR product concentration of 10 ng µl^{-1} with the 341f primer. Sequences produced were tested with the BLASTN algorithm using default settings[32].

Bacterial culture, quantification, washing, inoculation. The P-solubilising pseudomonad (Psol) isolate with the highest solubilisation index and a non-P-solubilising pseudomonad (nonPsol) were chosen as they were morphologically and phylogenetically similar and were derived from the same host plant (oat). In addition, the 16S rRNA gene sequence identified them to both belong to the *Pseudomonas fluorescens* species complex. Psol recorded the highest solubilisation index (4.37). The average PSI score of all isolates positive for P solubilisation was 2.71 and the minimum score was 0.72. NonPsol did not solubilise $Ca_3(PO_4)_2$ *in vitro* returning a score of 0.

To inoculate wheat plants, cultures of each isolate were grown in 50% TSB at 28 °C in a shaker overnight. Cultures were measured spectrophotometrically to confirm that they were in the exponential growth phase (OD_{600} 0.6-0.8). When this was the case, the cultures were spun at 353 x g for ten minutes, the supernatant removed, and cells resuspended in Ringer's solution. Both cultures were diluted using Ringer's solution to OD_{600} 0.1 to ensure that a consistent 10^8 cells were applied per pot. For each plant, 1 ml of the Ringer's diluted culture was applied onto the root, ten days after planting. A control treatment using no bacterial inoculant was included in this experiment.

Statistical analysis. Significance was defined as $p < 0.05$ using a two-way ANOVA and Tukey HSD post-hoc tests for pairwise comparisons. All statistical calculations were carried out in R and all assumptions of the statistical tests were met, n = 7 unless stated otherwise.

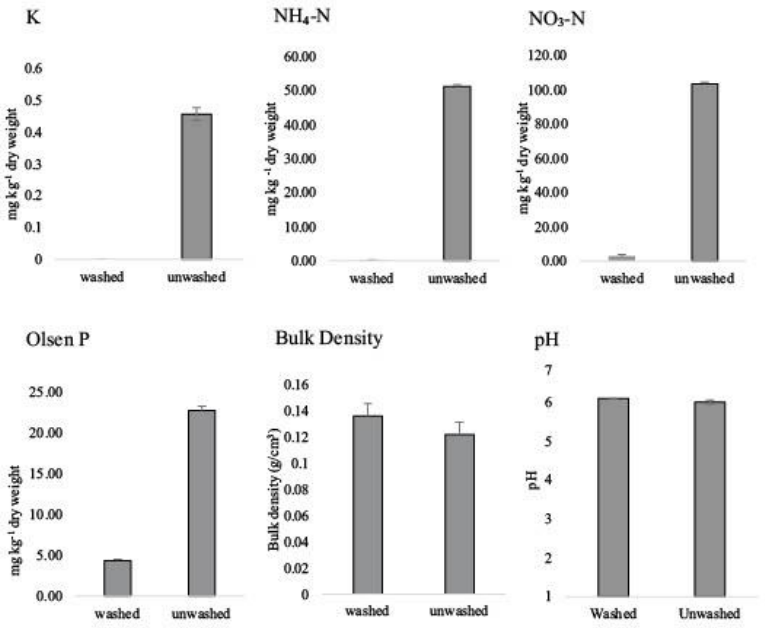

Figure 1. Nutrients, bulk density and pH in washed and unwashed compost. Extracted nutrient concentrations are expressed as mg kg^{-1} dry weight soil. Bulk density was measured as mass per unit volume, calculated using the mass of oven-dry compost (Md) and its volume (V), using the formula Db = Md/V. Bars show standard error.

Results

Effect of washing compost on soluble N, P and K and other elements. Nutrient analysis showed that, of the total nutrients, washing compost removes: 100% of K, 99.7% NH_4, 97.1% of NO_3 and 80.96% of P (Fig. 1). Other secondary macro- and micronutrients were included in the analysis; washing reduced the levels of, for example, magnesium, aluminium and sulphur (Supplementary table S2).

Effect of washing compost on bulk density and pH. Washing the compost did not change its bulk density or pH, compared to unwashed compost (Fig. 1). The pH of the compost in many of the different nutrient configurations was tested; all nutrient treatments were not significantly different from the average pH within the given range of the unwashed compost (5.3-6.0) (Supplementary figure S3).

N, P and micronutrient reconstitution. Growth of plants in washed compost was significantly reduced in the four crops (Supplementary figure S4). However, adding the predicted required amount, 1X, of each nutrient as calculated from the nutrient analysis (Supplementary table S2) to replace what had been removed by washing was insufficient to adequately recover plant growth (Fig. 2). We found that it was necessary to supply 5X the amount of soluble N and P to the washed compost (Fig. 2) in order to fully recover plant biomass to the level of plants grown in unwashed compost. Nutrients were also added at 10 and 20X but these did not result in further increases in plant biomass.

Biomass of plants in milled compost. Nutrient status of compost was more important for wheat growth than particle size; reducing particle size reduced growth when nutrients are not limiting. Milling compost reduced the plant biomass in unwashed compost ($p < 0.05$) (Fig. 3). In the case of nutrient depleted compost, the reduction of the particle size did not have a significant effect on the resulting plant biomass ($p > 0.9$). Washing the compost and removing the nutrients had a substantially greater effect on wheat biomass than reducing the particle size.

Phosphorous solubilising bacteria influence wheat biomass. The 16S rRNA gene sequenced *Pseudomonas* sp. bacteria differed in their ability to access recalcitrant P ($Ca_3(PO_4)_2$); the P-solubilising bacteria increased wheat growth (Fig. 4). The 16S rRNA gene sequencing data informed the choice for both bacterial

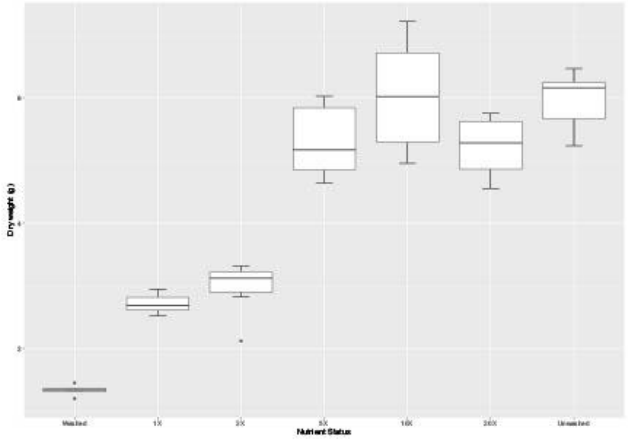

Figure 2. Biomass (as dry weight) of wheat grown in washed compost given nutrients from 1X, 2X, 5X, 10X and 20X the amounts removed by washing. Dry weight is the aerial biomass of wheat harvested at flowering. Central line represents the median, n = 6. Different letters indicate significant difference ($p < 0.05$).

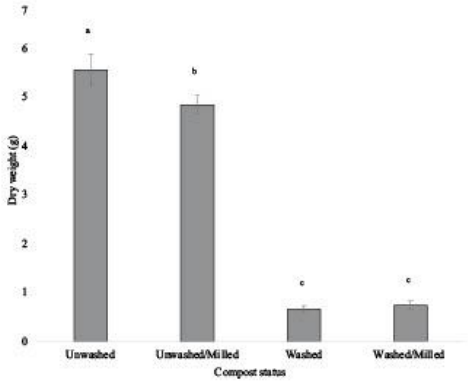

Figure 3. Dry biomass of wheat grown in milled or unmilled, washed or unwashed compost. Plants were grown in the following conditions: Control - unwashed (nutrient replete); Milled - a reduced pore size and replete nutrients; Washed - compost with soluble nutrients removed; Washed/Milled – soluble nutrient deplete and reduced pore size. Wheat above-ground dry biomass was measured at flowering. Bars show standard error, n = 5. Different letters indicate significant difference ($p < 0.05$).

isolates chosen for this experiment and identified them as *Pseudomonas* sp. The inoculation of a phosphate solubilising (Psol) bacterial isolate was compared to a non-solubilising bacterial isolate (nonPsol) from the same host, and a control treatment with no bacterial inoculant. The mean above ground biomass of the two bacterial treatments was compared using a two-tailed t-test with equal variance ($p = 0.02$) and plants in the Psol treatment had 18.01% more biomass than plants in the nonPsol treatment. NonPsol showed no difference in biomass compared to the no bacterial control ($p = 0.8$), whereas plants inoculated with Psol had a greater biomass than plants with no bacteria ($p = 0.01$).

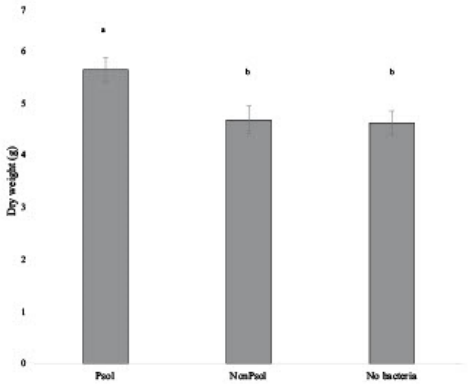

Figure 4. Wheat, with tricalcium phosphate as a source of P, grown with P solubilising or non-P solubilising *Pseudomonas*, or no bacterial inoculant. Psol is a P solubilising *Pseudomonas* species isolate, nonPsol is a non-solubilising *Pseudomonas*. Error bars represent standard error, n = 7. Different letters indicate significant difference ($p < 0.05$).

Discussion

Nutrient limited compost system. As expected, washing the compost reduces soluble nutrient availability and plants subsequently grown in this medium have a significantly reduced biomass. Neither the bulk density or pH of the compost changes significantly after washing so the reduction in above ground growth was attributed to the removal of many of the soluble nutrients, primarily N, P and K (Supplementary table S2).

In order to reconstitute the nutrient levels of the washed compost system, two different nutrient solutions were trialled to find the most efficient and successful recovery of plant growth. It is essential that nutrient levels can be easily manipulated to simulate different nutrient deficiencies for use in experiments with plant growth promoting microbes. Hoagland's solution provided highly effective and reliable recovery of plant growth when used in the phosphate solubilisation bioassay (Supplementary table S1). This method involved daily watering with a prescribed dose of Hoagland's solution, allowing nutrients to be replaced gradually. Although Hoagland's solution can easily omit P, the recipe adds many different sources of N, in combination with other micronutrients. As such, this method is not suitable for mineral N depletion experiments. Removing all sources of N would mean many other essential micronutrients that constitute Hoagland's solution would be eliminated. The second nutrient solution tested was a modified version of Letcombe's solution for wheat (Supplementary table S1). One major advantage of this approach is that the nutrients are given as one single dose at the beginning of the experiment and thereafter plants are watered with tap water, significantly reducing the workload.

Reconstituting the compost with the exact amount of N and P calculated to have been removed by washing was insufficient to fully recover plant growth. In order to support plant growth to the same biomass level as that of the unwashed compost, it was necessary to add 5X the original amount of nutrients. It is likely that this can be attributed to the loss of fine particles and their associated nutrients during the washing process. These particles have a high surface area:volume ratio and the mineralisation of organic nutrients to inorganic soluble forms will be reduced in washed compost. Two further concentrations were also tested (10X and 20X), but these had no further effect on plant growth and biomass indicating that the system was replete with nutrients.

The nutrient status of the washed compost is more important than pore size for plant biomass (Fig. 2). Washing compost removes nutrients and results in a pronounced reduction in plant biomass. However, when comparing wheat grown in unwashed compost, milling resulted in a reduced wheat biomass, whereas for washed compost milling had no effect on wheat biomass. It may be the case that under nutrient replete conditions that the reduced biomass seen with milling could be attributed to reduced oxygenation of the soil, water logging and compaction affecting root structure[33], but this is secondary to the effect of nutrient depletion.

The compost system is a more intricate, ecologically complex matrix than for example, sand or hydroponics. This will mean simulations can be closer to those found in the field – the translation of experiments from compost to field may be more realistic than, for instance, *in vitro* experiments or those using an inert mineral substrate such as vermiculite. Additionally, real soil is not suitable for experiments such as these as it is difficult to manipulate their biotic, abiotic and physical states. The development of soils depleted in particular nutrients would take many years to achieve, whereas the compost system presented here can be readily manipulated for the control of these factors. The main application of this system is in testing microbial interactions and their impact on plant health. The ability of microbes, either as singular inoculants or as synthetic communities, to enhance plant growth can be screened in a standardised, easily manipulated system that can simulate many environmental conditions: nutrient deficiencies, altered physical structure, a reduced microbial pool and abiotic stresses such as drought or flooding.

Influence of phosphate-solubilising pseudomonads on the aerial biomass of wheat in P-deficient compost. The P solubilising bacterial strain was chosen as it had the highest PSI score. The non-solubilising bacterial strain was chosen as it was not able to solubilise P but was the same species, came from the same host and was morphologically similar to the P solubilising strain. When wheat was inoculated with phosphate-solubilising bacteria (*Pseudomonas* sp.), under depleted soluble P condition but supplemented with recalcitrant P, it had increased above ground biomass relative to wheat inoculated with a non-solubilising *Pseudomonas* species, or a no bacteria control. This suggests that the P solubilising *Pseudomonas* sp. was able to liberate P from inorganic, insoluble $Ca_3(PO_4)_2$, promoting plant growth. The inclusion of a no bacteria control treatment addresses the potential issue of pH difference between agar and compost systems influencing $Ca_3(PO_4)_2$ solubilisation. The *in vitro* screen used for P solubilisation is at pH 7, whereas the compost system has a pH ~6. As the compost substrate is slightly more acidic than PVK agar, the recalcitrant source of P could be more soluble in the compost system, making PO_4 in the $Ca_3(PO_4)_2$ more readily available to the plant. However, our data shows that plants inoculated with bacteria unable to solubilise P and plants grown with no bacterial inoculant have a similar biomass ($p=0.8$), and the addition of the P solubilising bacterial inoculant increases wheat biomass relative to the other treatments ($p=0.02$). This demonstrates that it is the addition of Psol that benefits the growth of wheat, either directly or indirectly, in the compost system.

Isolates screened *in vitro* for their ability to solubilise phosphate may not replicate this *in planta*. The use of PVK media and $Ca_3(PO_4)_2$ as the recalcitrant source of phosphate is well documented[34], but soils contain many kinds of metal-phosphate sources and other organic P sources, so screening for other P solubilisation mechanisms in the future should also be considered, to replicate conditions found naturally in soil[35]. Rhizosphere competence, the chemical, biological and physical properties of the growth medium, and microbial competition are all factors that may influence the potential of an inoculant to perform the same functions *in planta* that are observed *in vitro*. Isolates screened using this technique, including *Enterobacter* and *Burkholderia* sp. - both displaying a high phosphate solubilising ability *in vitro* - yielded contrasting results *in planta*, either increasing or having no effect on the biomass of sorghum respectively[36]. Our experiment used compost supplemented with $Ca_3(PO_4)_2$, thus *in vitro* screening using the same phosphate source was appropriate. For use in natural soil systems, the use of mixed communities with a variety of PGPR functions and phosphate solubilisation abilities is likely to give more success in field situations.

The use of plant growth promoting bioinoculants is an exciting avenue for exploration of alternatives to mineral fertilisers in agriculture. Previously published work focusing on phosphate solubilising microbes has conflicting results. For example, pea plants had increased biomass when inoculated with *Pseudomonas* sp., using $Ca_3(PO_4)_2$ as the recalcitrant P source[7], and the growth of walnuts was improved by separate inoculation with *Pseudomonas chlororaphis* and *Pseudomonas fluorescens*, previously screened for phosphate solubilisation using PVK media[37]. The ability to manipulate the washed compost system means high-throughput screenings of plant growth promoters under specific conditions, such as nutrient depletion, pathogen challenge or drought, are possible in a consistent and defined matrix.

Conclusion

Washing compost significantly reduces soluble nutrient pools, including N, P and K, without affecting its structure or pH. We show that nutrient levels can be manipulated to simulate different nutrient deficiencies and study the potential for microbial inoculants to overcome these deficits. The compost provides a standardised testing system defined in some of its biological, chemical and physical attributes. This defined system creates a platform for detailed and complex plant-microbe interactions to be studied and the simple manipulation of nutritional status provides a reliable screening platform for PGPR. The system is closer to conditions found in the field than the commonly used inert mineral substrates or hydroponics, so we believe that results are more relevant for sustainable agriculture. It has great potential as a platform to provide rigorous and reproducible pre-field testing of commercial microbial inoculants to replace or complement mineral fertilizers.

Data availability

The 16S rRNA sequences used in the current study are available in the NCBI GenBank, accession: MT181117 and MT181116.

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References

1. Garnett, T. *et al.* Sustainable intensification in agriculture: premises and policies. *Science* 341(6141), 33–34 (2013).
2. Berendsen, R. L., Pieterse, C. M. & Bakker, P. A. The rhizosphere microbiome and plant health. *Trends in Plant Science* 17(8), 478–486 (2012).
3. Bhattacharyya, P. N. & Jha, D. K. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiology Biotechnology* 28(4), 1327–1350 (2012).
4. Vessey, J. K. Plant growth promoting rhizobacteria as biofertilizers. *Plant and Soil* 255(2), 571–586 (2003).
5. Vejan, P., Abdullah, R., Khadiran, T., Ismail, S. & Nasrulhaq Boyce, A. Role of plant growth promoting rhizobacteria in agricultural sustainability—a review. *Molecules.* 21(5), 573 (2016).
6. Arikan, Ş. *et al.* Effect of plant growth promoting rhizobacteria on Fe acquisition in peach (*Prunus Persica* L) under calcareous soil conditions. *Journal of Plant Nutrition* 41(17), 2141–2150 (2018).
7. Oteino, N., *et al.* Plant growth promotion induced by phosphate solubilizing endophytic Pseudomonas isolates. *Frontiers in Microbiology.* 6 (2015).
8. Basak, B. B. & Biswas, D. R. Co-inoculation of potassium solubilizing and nitrogen fixing bacteria on solubilization of waste mica and their effect on growth promotion and nutrient acquisition by a forage crop. *Biology and Fertility of Soils* 46(6), 641–648 (2010).
9. Lim, J. H. & Kim, S. D. Induction of drought stress resistance by multi-functional PGPR *Bacillus licheniformis* K11 in pepper. *The Plant Pathology Journal* 29(2), 201 (2013).

10. Yao, L., Wu, Z., Zheng, Y. & Kaleem, I. Growth promotion and protection against salt stress by *Pseudomonas putida* Rs-198 on cotton. *European Journal of Soil Biology* 46(1), 49–54 (2010).
11. Kumar, A., S. Sharma & S. Mishra. Effect of alkalinity on growth performance of *Jatropha curcas* inoculated with PGPR and AM fungi. *Journal of Phytology* 1(3) (2009).
12. Gururani, M. A. *et al.* Plant growth-promoting rhizobacteria enhance abiotic stress tolerance in *Solanum tuberosum* through inducing changes in the expression of ROS-scavenging enzymes and improved photosynthetic performance. *Journal of Plant Growth Regulation* 32(2), 245–258 (2013).
13. Sandhya, V., Ali, S. Z., Grover, M., Reddy, G. & Venkateswarlu, B. Effect of plant growth promoting *Pseudomonas* spp. on compatible solutes, antioxidant status and plant growth of maize under drought stress. *Journal of Plant Growth Regulation* 62(1), 21–30 (2010).
14. Joseph, B., Ranjan Patra, R. & Lawrence, R. Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.). *International Journal of Plant Production* 1(2), 141–152 (2012).
15. Novo, L.A., P.M. Castro, P. Alvarenga & E.F. da Silva. Plant growth–promoting rhizobacteria-assisted phytoremediation of mine soils. *Bio-Geotechnologies for Mine Site Rehabilitation*, 281–295 (2018).
16. Raaijmakers, J. M. & Weller, D. M. Natural plant protection by 2, 4-diacetylphloroglucinol-producing *Pseudomonas spp.* in take-all decline soils. *Molecular Plant-Microbe Interactions* 11(2), 144–152 (1998).
17. Yazdani, M., Bahmanyar, M. A., Pirdashti, H. & Esmaili, M. A. Effect of phosphate solubilization microorganisms (PSM) and plant growth promoting rhizobacteria (PGPR) on yield and yield components of corn (*Zea mays* L.). *World Academy of Science, Engineering Technology* 49, 90–92 (2009).
18. Liu, K., McInroy, J. A., Hu, C. H. & Kloepper, J. W. Mixtures of plant-growth-promoting rhizobacteria enhance biological control of multiple plant diseases and plant-growth promotion in the presence of pathogens. *Plant Disease*. 102(1), 67–72 (2018).
19. Simons, M., Brand, I., Wijffelman, C. & Lugtenberg, B. Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting Pseudomonas bacteria. *Molecular plant-microbe interactions* 9(7), 600–607 (1996).
20. Sahin, Ü., Ozdeniz, A., Zulkadir, A. & Refik, A. The effects of different growing media on yield, quality and growth of tomato (*Lycopersicon esculentum* Mill.) grown and irrigated by drip irrigation method under the greenhouse conditions. *Turkish Journal of Agriculture* 22(1), 71–80 (1998).
21. Vendrame, A.W., I. Maguire & K.K. Moore. Growth of selected bedding plants as affected by different compost percentages. in Proceedings of the Florida State Horticultural Society. Florida: Florida State Horticultural Society (2005).
22. Baez-Rogelio, A., Morales-García, Y. E., Quintero-Hernández, V. & Muñoz-Rojas, J. Next generation of microbial inoculants for agriculture and bioremediation. *Microbial Biotechnology* 10(1), 19–21 (2017).
23. Hassen, A.I., F Bopape & L. Sanger, Microbial inoculants as agents of growth promotion and abiotic stress tolerance in plants, in Microbial inoculants in sustainable agricultural productivity 23-36 (Springer, 2016).
24. Robinson, R. J. *et al.* Wheat seed embryo excision enables the creation of axenic seedlings and Koch's postulates testing of putative bacterial endophytes. *Scientific Reports* 6, 25581 (2016).
25. Hoagland, D.R. & D.I. Arnon. The water-culture method for growing plants without soil. *California Agricultural Experiment Station*. 347(2nd edit) (1950).
26. Mauchline, T. *et al.* An analysis of Pseudomonas genomic diversity in take-all infected wheat fields reveals the lasting impact of wheat cultivars on the soil microbiota. *Environmental Microbiology*. 17(11), 4764–4778 (2015).
27. Pikovskaya, R.I. Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. *Mikrobiologiya*. 17(362-370) (1948).
28. Edi-Premono, M., Moawad A. M. & P.L.G. Vlek, Effect of phosphate solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. *Indonesian Journal of Crop Science*. 11(13-23) (1996).
29. Paul, D. & Sinha, S. N. Isolation and characterization of phosphate solubilizing bacterium *Pseudomonas aeruginosa* KUPSB12 with antibacterial potential from river Ganga, India. *Annals of Agrarian. Science*. 15(1), 130–136 (2017).
30. Muyzer, G., De Waal, E. C. & Uitterlinden, A. G. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied Environmental Microbiology* 59(3), 695–700 (1993).
31. Marchesi, J. R. *et al.* Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Applied and Environmental Microbiology* 64(2), 795–799 (1998).
32. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. Basic local alignment search tool. *Journal of Molecular Biology* 215(3), 403–410 (1990).
33. Passioura, J. Soil structure and plant growth. *Soil Research* 29(6), 717–728 (1991).
34. Zheng, B.-X. *et al.* Identification and characterization of inorganic-phosphate-solubilizing bacteria from agricultural fields with a rapid isolation method. *AMB Express* 8(1), 47 (2018).
35. Bashan, Y., A.A. Kamnev & L.E. de-Bashan. A proposal for isolating and testing phosphate-solubilizing bacteria that enhance plant growth. *Biology and Fertility of Soils*, 1-2 (2013).
36. Collavino, M. M., Sansberro, P. A., Mroginski, L. A. & Aguilar, O. M. Comparison of *in vitro* solubilization activity of diverse phosphate-solubilizing bacteria native to acid soil and their ability to promote *Phaseolus vulgaris* growth. *Biology and Fertility of Soils* 46(7), 727–738 (2010).
37. Yu, X., Liu, X., Zhu, T. H., Liu, G. H. & Mao, C. Isolation and characterization of phosphate-solubilizing bacteria from walnut and their effect on growth and phosphorus mobilization. *Biology and Fertility of Soils* 47(4), 437–446 (2011).

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Author contributions

E.M.C. carried out the experimental work and wrote the manuscript, with supervision and revisions by T.M., P.H., I.C., E.B. and W.O. T.M. conceived the presented method. E.S. and M.P. contributed the phosphorous solubilisation screening and the milling experiments respectively.

Competing interests
The authors declare no competing interests.

Additional information
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A.3 AMF qPCR

AMF qPCR was inconclusive for many of the experiments and was deemed most suitable for presence/absence reporting, instead of absolute quantification. This was attributed to several reasons: insufficient sequence information for species specific primers, inadequate AMF taxonomic database information, the multinucleate and aseptate nature of AMF exacerbating heterogenous distribution in soil samples. The following figures contain the AMF qPCR results for the experiments for which they were carried out.

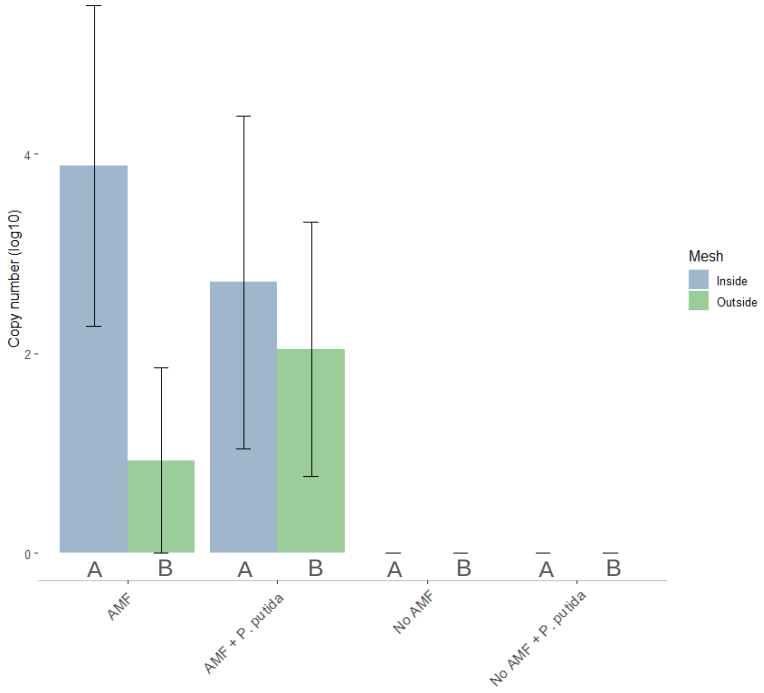

Figure A-2. AMF LSU copy number per g of dry rhizosphere (experiment 1, section 7). Mean quantitative PCR data for *Funneliformis sp*. LSU. Clover plants were grown inside (A) or outside (B) a root-excluding, hyphae permitting mesh. Colonisation of roots by AMF was measured using qPCR for a genus specific large subunit. All microbial inoculants (AMF and *P. putida*) were given to the plant inside the mesh (A). Bars represent standard error, data was transformed by log10, n = 5.

In figure A-2, there was no detection of AMF in control no AMF treatments. This confirms that the sterilisation of the substrate was sufficient to destroy any AMF present in this substrate, and that watering via the hose did not introduce any discernible levels of AMF into the system. AMF were present in the AMF treatments, on both the original site of inoculation (inside the mesh) and the neighbouring plant (outside the mesh), meaning a hyphal connection was formed

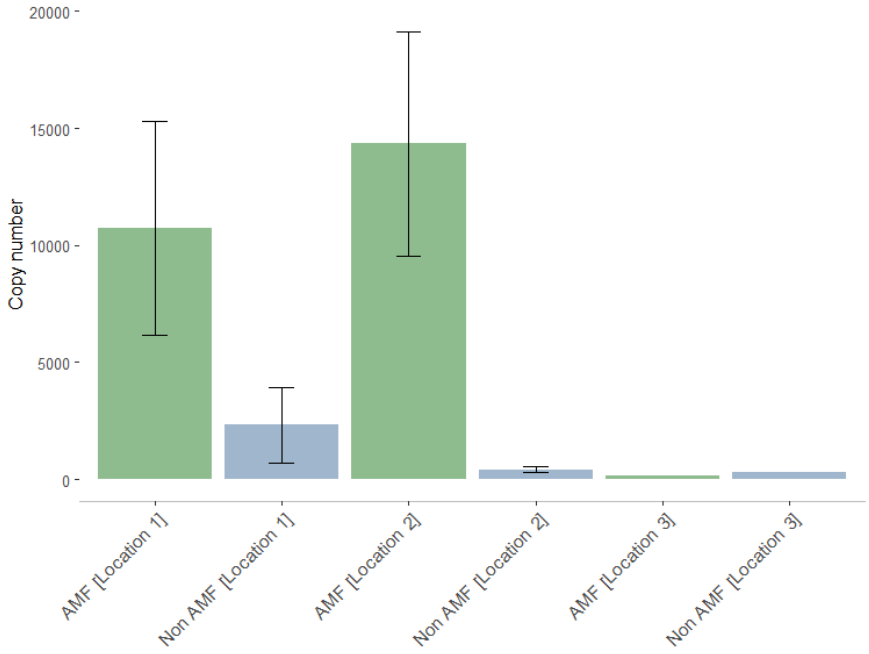

Figure A-3. AMF LSU copy number per g of dry rhizosphere (experiment 2, section 7). Mean quantitative PCR data for *Funneliformis sp*. LSU. Clover plants were grown inside a root-excluding, hyphae permitting mesh. Colonisation of roots by AMF was measured using qPCR for a genus specific large subunit. All AMF inoculant was deployed in location 1. Locations refer to inoculation point of *P. putida*. Bars represent standard error, data was transformed by log10, n = 5.

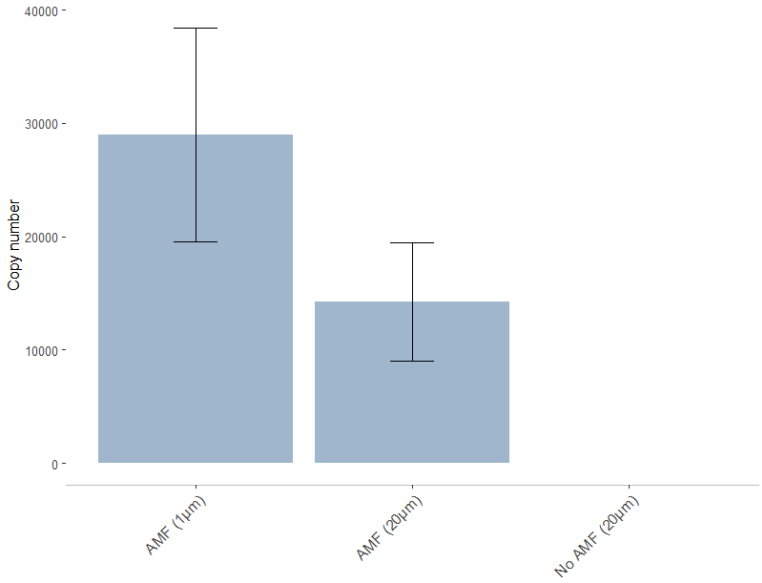

Figure A-4. AMF LSU copy number per g of dry rhizosphere (experiment 3, section 7). Mean quantitative PCR data for *Funneliformis sp*. LSU. Clover plants were grown meshes of different aperture sizes Colonisation of roots by AMF was measured using qPCR for a genus specific large subunit. All AMF inoculant was represent standard error, data was transformed by log10, n = 5.

between the two plants (Figure A-2). This occurred regardless of pseudomonad

inoculation (p = 0.1). In Figure A-3, there is highly variable data which contradicts the microscopy data. There was very low detection of AMF in the location three treatments, which is contracted by the microscopy results. The aim of this experiment was to create an AMF hyphal connection between the AMF and the neighbouring plant. Although low copy numbers, there was still AMF presence detected between the neighbouring plants, reinforcing the experimental design and the microscopy results. However, these data show that qPCR is not currently resolved enough for exact quantification and is sufficient for presence/quantification.

For experiment 3 (Section 7), *Funneliformis* sp. was detected in both of the AMF inoculated treatments. Although AMF were seen to colonise the non-inoculated treatment using microscopy, this was assumed to be indigenous AMF from the field soil compartment, and therefore may not be AMF from the *Funneliformis* genus.

A.4 Salt stress experiment.

This experiment was carried out as part of a collaboration with the Czech Academy of Sciences and Symbiom, who provided the mycorrhizal inoculants. *R. irregularis* was isolated from a salt mine and therefore was assumed to have a salt tolerant lifestyle. Two bacterial isolates from the library isolated from Furzefield (Section 6.4.1.4) were selected after screening in vitro for salt tolerance and other PGP activity. These were tested separately and in combination with the AMF, at different salt concentrations, to assess plant health under these conditions. The addition of NaCl significantly affected biomass regardless of microbial inoculant ($p < 0.001$). Microbial inoculant did not affect biomass (p = 0.06).

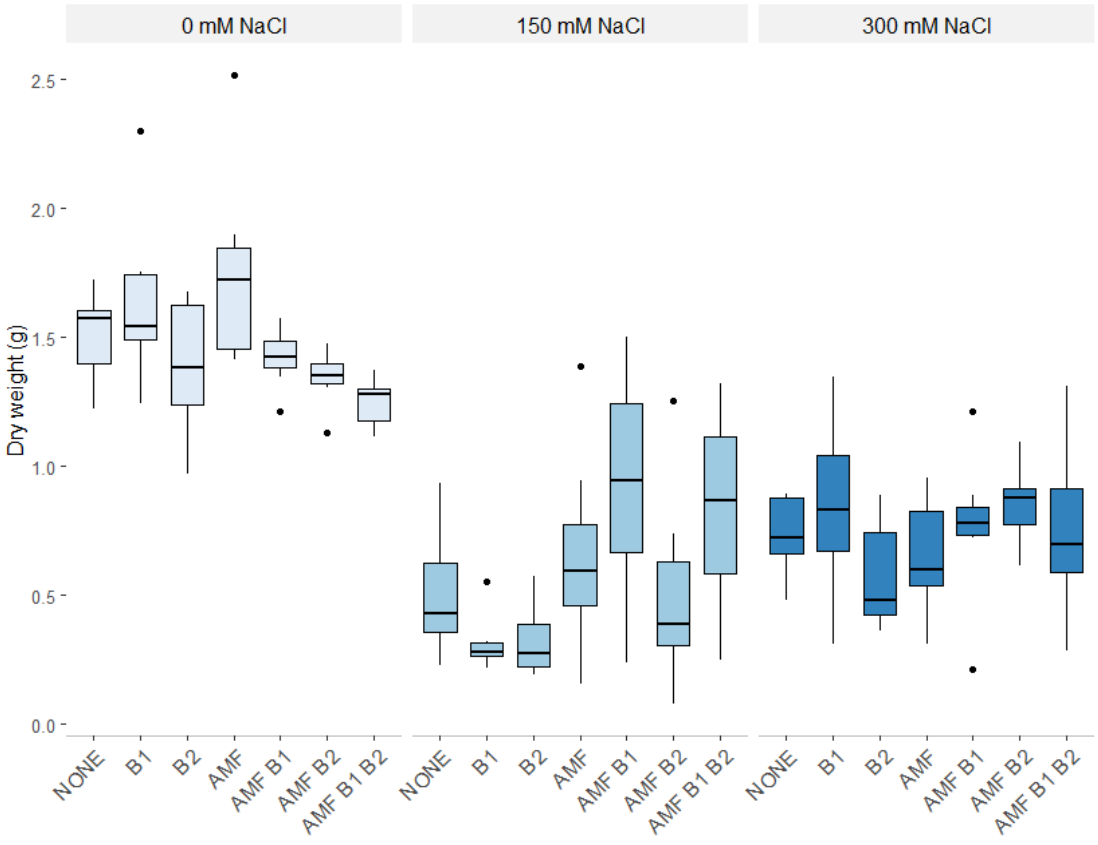

Figure A-5 Biomass of clover when subjected to three different salt concentrations with combinations of salt tolerant microbial inoculants.

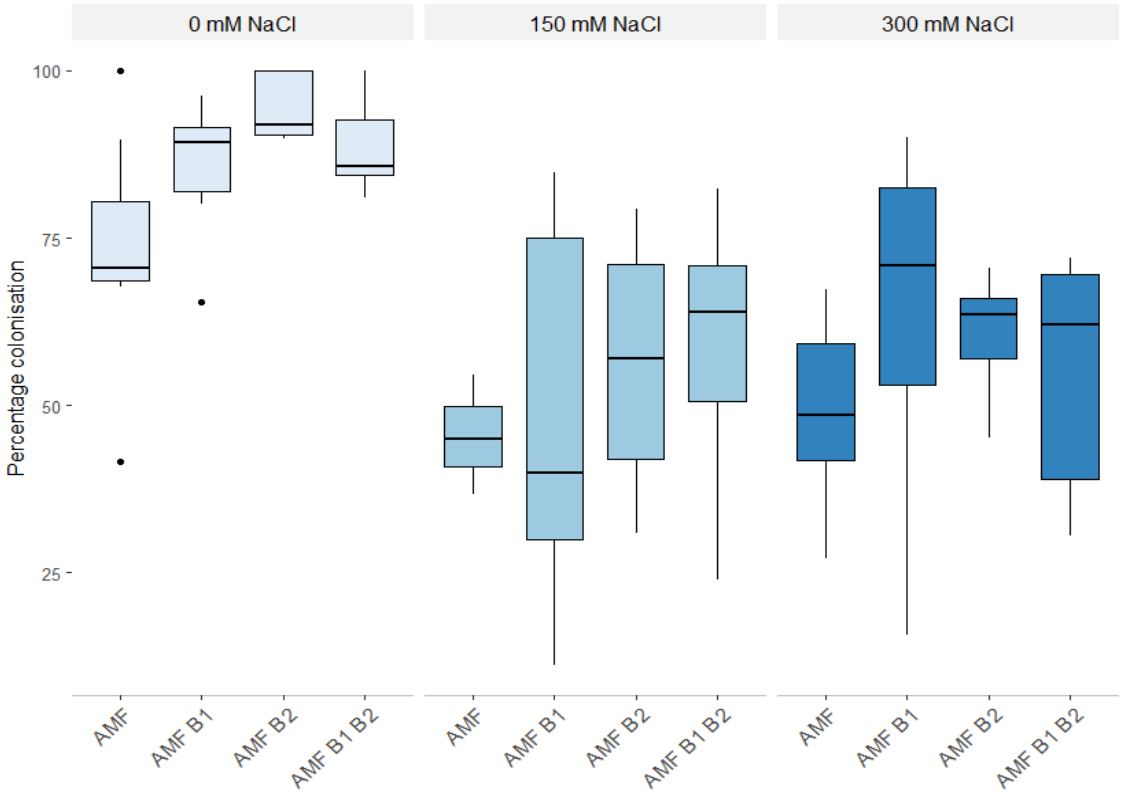

Figure A-6. Percentage of clover roots colonised by AMF (*R. irregularis*) when subjected to three different salt concentrations with combinations of salt tolerant microbial inoculants.

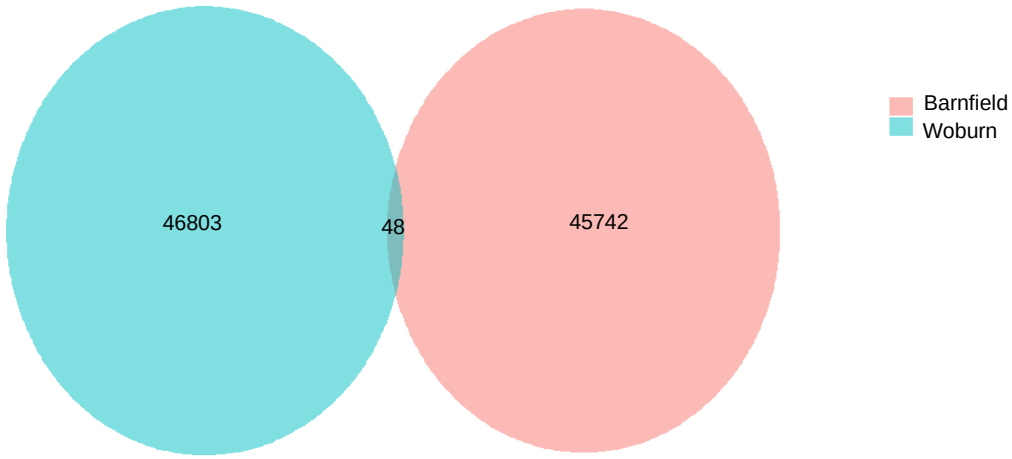

Figure A-7. Venn diagram showing the shared ASVs for both Woburn and Barnfield soils. Numbers give the number of reads unique to each soil type and those in common.

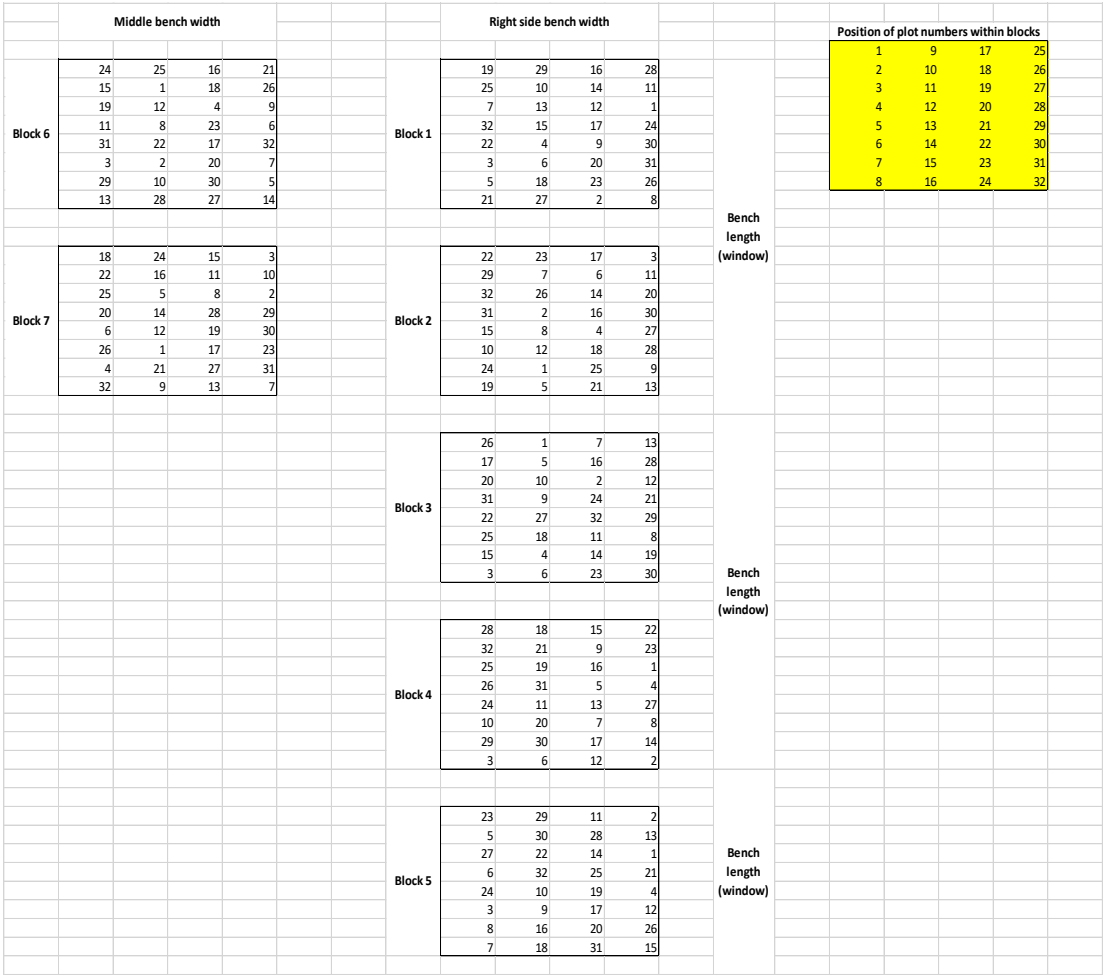

Figure A-8. Complete randomised block design, blocking for replicate effect. Microbial inoculants experiment (Section 6). Plot number = treatment type.