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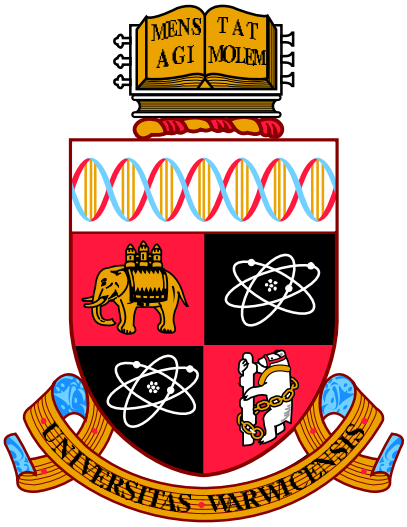

The effect of beneficial microbes on plant fitness and microbiome – an integrated approach

by

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This work is dedicated to my family, to Sophie Piquerez and to those who believed in me, supported me and taught me to follow my dreams

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Declarations

This thesis is presented in accordance with the regulations for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work in this thesis has been undertaken by myself except where otherwise stated.

Abstract

The plant root microbiota, consisting of diverse microbial communities within and around the plant roots, can significantly influence plant development and stress tolerance. The goal of this thesis was to analyse the microbe-microbe and plant-microbe interactions in the root. For this purpose, *Serendipita indica,* a mutualistic fungal root endophyte, able to improve plant performance and disease resistance in host plants, was studied. Strategies of the fungus to establish colonisation within the plant were studied by investigating the effect of putative *S. indica* effector proteins on the plant auxin signalling pathway. Single *S. indica* effectors were found to alter the expression of auxin-related genes, with consequent effects on plant growth, indicating a potential competitive advantage of *S. indica* gained through effectors against other microbes. Furthermore, by analyzing the effect of two beneficial fungal endophytes, *S. indica* and *Colletotrichum tofieldiae* on the rhizobiome composition of *Arabidopsis thaliana,* inoculated individually on plant roots, I aimed to reveal plant- and/or microbe-derived patterns that are involved in shaping the root microbiome. Using a gnotobiotic system and a synthetic bacterial community, I demonstrated the persistence of the bacterial community in the plant root, as well as the transition of *C. tofieldiae* to a potentially pathogenic lifestyle hereby affecting plants' survival. To further test these patterns in a natural environment, a bacterial taxon commonly utilized in agriculture, *Rhizobia*, was used as a mutualist root symbiont of the legume *Medicago truncatula. Rhizobia* strains were tested for their ability to stimulate plant growth and nutrient supply as well as their competitiveness in representative UK soil types and their different natural microbiomes. *Sinorhizobium meliloti* 1022 was found to enhance plant growth and nitrogen acquisition in all soil types, altering the bacterial community structure and composition of the root endosphere, while the fungal community structure and composition was driven only by the soil characteristics. Overall, this work contributes towards elucidating the stability of beneficial plant-microbe interactions in natural communities and in different environments. The long-term aim of such study is to integrate molecular and environmental factors to generate customized beneficial microbiomes that can be applied to sustainably support crop production.

Abbreviations

AMs	Arbuscular mycorrhizas
A. thaliana	*Arabidopsis thaliana*
ATS	Arabidopsis thaliana salts
C. tolfidiae	*Colletotrichum tolfidiae*
CbetaPs	beta-subclass Proteobacteria
cDNA	complementary Deoxyribo-Nucleic Acid
C_T	Cycle threshold
dai	Days after inoculation
DC_T	Delta cycle hreshold
DDC_T	Double delta cycle threshold
DEX	Dexamethasone
DMSO	dimethyl sulfoxide
DMSO	Dimethyl sulfoxide
eV	empty Vector
GC-MS	Gas Chromatography–Mass Spectrometry
GFP	Green Fluorescent Protein
GUS	Beta-glucuronidase
GUS	β-glucuronidase
IAA	Indole-3-acetic acid
ILA	Indole-3-lactate
ITS	Internal transcribed spacer
LUC	Luciferase
M. truncatula	*Medicago trancatula*
MES	2-(N-morpholino)ethanesulfonic acid
mRNA	messenger Ribo-Nucleic Acid
MS	Murashige and Skoog medium
MUG	4-Methylumbelliferyl-β-D-glucuronide
NAA	Naphthaleneacetic acid
NCBI	National Center for Biotechnology Information
OTU	Operational Taxonomic Unit
P. striata	*Pseudomonas striata*
P. syringae	*Pseudomonas syringae*
PCoA	Principle Coordinate analysis
PCR	Polymerase Chain Reaction
RA	Relative Abundance
RLU	relative light units
rpm	revolutions per minute

rRNA	Ribosomal ribonucleic acid
S. indica	*Serendipita indica*
S. medicae	*Sinorhizobium medicae*
S. meliloti	*Sinorhizobium meliloti*
SIE	*Serendipita indica* effector
Tukey's HDS	Tukey's Honest Significant Difference
TY medium	Tryptone – yeast extract medium

Chapter 1: Introduction

1.1 The importance of soil microbiome for sustainable agriculture

The increasing demand for food, in combination with the major agricultural challenges, such as pests, plant diseases caused by microbes, drought stress and soil nutrient deficiencies drive the need for sustainable solutions to improve crop protection and increase crop yield (Ericksen et al., 2009; Garnett, 2013). Plant pathogens such as *Magnaporthe oryzae* (rice blast), *Fusarium graminearum* (fusarium head blight), *Pseudomonas syringae* and *Ralstonia solanacearum*, can cause severe crop yield losses (Dean et al., 2012; Mansfield et al., 2012). On the other hand, beneficial microbes such as mycorrhizal fungi and plant growth-promoting rhizobacteria have remarkable effects on plant growth, health and tolerance to biotic and abiotic stresses (Berg, 2009; Finkel et al., 2017; Pineda et al., 2010). In recent years, a lot of attention has been directed towards the analyses of complex microbial communities associated with plants (Hacquard et al., 2015). These so-called microbiomes have been found to hold a wealth of plant beneficial microbes and as such considered to be a promising and underexploited resource for agriculture (Mendes et al., 2013). As plant-associated microbiomes have key roles in plant development and health (Berendsen et al., 2012), understanding their function in plant processes would encourage the use of microbiomes' beneficial properties as a potential sustainable solution to agricultural challenges. In turn, intensive agriculture as well as the farming methods can affect microbial communities and can lead to soil decline (Hartman et al., 2017, 2018). Understanding the effect of soil factors on microbiome parameters (e.g. community assembly, structure, composition, diversity) and the consequent effect on plant growth and health can reveal consequences of farming practices on soil ecosystem functioning and encourage the development of novel cropping practices.

The root, as principal tissue for nutrient and water acquisition, is important for plant health and development (Hodge et al., 2009). In addition, plant roots represent a very specific and rich niche for microbes and only a few of them can overcome defence mechanisms of plants and establish colonisation within the root

tissue (Bai et al., 2015; Hacquard et al., 2015). The rhizosphere, in particular, is a highly competitive environment for microbes as it is enriched in plant-derived nutrients. Microbes can form complex interactions with the plant root ranging from parasitic, in the case of soil-borne plant pathogens leading to the reduction of crop yield (Müller et al., 2016), to mutualistic interactions, as in the case of legume-rhizobia symbioses, which improve plant development and health (Garg and Geetanjali, 2009). However, the full potential of these interactions, as well as the underlying mechanisms are largely unknown. The high complexity, diversity and variation of these interactions demand for holistic approaches in order to capture the whole spectrum of microbiome's capabilities.

1.2 Plant – microbe interactions from a molecular perspective

1.2.1 The role of hormones in plant-microbe interactions

The continuous pressure of the environment on crops, leading to crop diseases and yield loss, has created the need for deeper understanding of how plants face and resist environmental cues such as biotic and abiotic stresses (Conforti, 2011). Plants bear a remarkable plasticity to cope with changing environments. Plant cells have developed highly sensitive perception and transduction systems to monitor their environment and adapt cellular behaviour in response to external changes. Plant hormones take an essential role in integrating environmental stimuli and, in combination with other signalling pathways, initiate physiological responses (Davies, 2010), including stress adaptive processes (Xu et al., 2018). So far, auxin (IAA), abscisic acid (ABA), ethylene (ET) brassinosteroids (BRs), cytokinins (CKs), gibberellins or gibberellin acid (GA), jasmonate (JA), nitric oxide, salicylic acid (SA), and strigolactones (SLs) are defined as plant hormones (Santner and Estelle, 2009). Various plant hormones and the crosstalk between them are involved in the regulation of plant - microbe interactions (Xu et al., 2018). For example, JA and SA are part of the plant defense signaling during pathogen attack (Koornneef and Pieterse, 2008; Kunkel and Brooks, 2002), while CKs and IAA can modify growth processes as part of host-microbe interaction (Gamas et al., 2017; Sukumar et al., 2013). In addition, microbes are able to produce hormones as a strategy to

influence plant metabolism in order to facilitate plant colonisation (Costacurta and Vanderleyden, 1995). Due to their ability to regulate plant response (i.e. to integrate environmental stresses) (Vurukonda et al., 2016) microbe-derived hormones are considered as a valuable source for plant-strengthening bioagents (Egamberdieva et al., 2017).

Auxin, in particular, has drawn a lot of attention regarding its function in plant microbe-interactions, due to its well-known function in plant growth and defense (Teale et al., 2006). It is synthesised in meristematic tissues and its homeostasis is essential for plant metabolism (Kazan, 2013). Auxin depletion and accumulation in cells play a crucial role in plant development (Ljung, 2013; Sorefan et al., 2009). Auxin can accumulate very locally (i.e. within one cell or in a small group of cells) or show a gradient distribution among cells to control certain pathways and responses (Sorefan et al., 2009). Auxin affects growth as it is involved in cell division, elongation and differentiation and is highly mobile within the plant by utilising a very specific transportation system (Teale et al., 2006).

Auxin is also involved in different stages of plant-microbe interactions, including pathogenic interaction and the formation of symbioses (Boivin et al., 2016). The down-regulation of auxin signalling is often part of a defence response of plants against pathogens. Therefore auxin is an important target for microbes in order to establish plant colonisation (Spaepen and Vanderleyden, 2011). The manipulation of auxin signalling pathways can be achieved through microbial effectors or microbial production of auxin molecules (Kunkel and Harper, 2018). Notably, the majority of rhizosphere-associated bacteria can produce IAA (Patten and Glick, 1996). This exogenous IAA can increase the susceptibility of plants to microbes allowing microbial proliferation within plant tissues (Spaepen and Vanderleyden, 2011). Auxin is also involved in the establishment of beneficial plant symbioses, for example, in the formation of rhizobial nodules (van Noorden et al., 2007). Due to its significance in the establishment of beneficial and pathogenic interactions, the ability of microbes to manipulate the auxin signalling pathway can outcompete other microbes and, thus, represent a competitive advantage.

1.2.2 The key role of microbial effectors in plant – microbe interactions

Plants have a sequence of defence mechanisms against microbes that attempt to colonise them. They recognise pathogen-associated molecular patterns (PAMPs), such as bacterial flagellin or fungal chitin, and trigger the activation of immunity (pattern triggered immunity) (Couto and Zipfel, 2016; Zipfel, 2014). Microbes secrete effectors, molecules that can alter host cell structure and function (Kamoun, 2006), in order to overcome plant immune response. Plants have evolved a second layer of immunity, termed effector triggered immunity (ETI), to recognise microbial effectors and initiate defence mechanisms against those microbes (Cui et al., 2014; Selin et al., 2016). For this reason, microbes keep evolving their effector repertoire in order to escape the plant immune system and maintain their ability to colonise plants for reproduction. In response, the plant keeps evolving the recognition and defence mechanisms, resulting in continuous cycles of co-evolution (Dodds and Rathjen, 2010; Jones and Dangl, 2006).

Microbial effectors can act in the apoplast or inside the host cell, where they target and modify plant proteins (Hogenhout et al., 2009). Effectors may have single or multiple host targets and they can mimic plant hormones or other plant molecules. Certain effectors can move between plant cells to establish infection sites (Djamei et al., 2011; Hogenhout et al., 2009). By influencing cellular signalling processes of the host, e.g. by suppressing host defence mechanisms, microbes essentially improve their ability to colonise and spread (Win et al., 2012). In this respect, the effector repertoire provides a competitive advantage and defines the success of microbes in colonising plants (Selin et al., 2016). In a community context, members of the microbiome may affect the virulence of other microbes, determining the outcome of the plant - microbe interaction (Xin et al., 2018). For example, certain microbes used as biocontrol agents against plant pathogens, activate defense responses as a strategy for disease protection (Pieterse et al., 2014), potentially through the secretion of effector proteins. Understanding the mechanisms of these interactions will enhance our ability to predict and manipulate their function and ensure their effectiveness in agricultural applications.

Microbial effectors have attracted a lot of attention as determinants of the establishment of pathogenic and beneficial plant-microbe interactions (Baker et al., 1997). In addition, effectors hold a great potential for agriculture, e.g. in defining disease resistance traits in breeding programmes (Vleeshouwers and Oliver, 2015). However, there are certain limitations in unravelling the biological functions of microbial effectors. For instance, the large diversity of effectors, with no specific functional signatures within their amino acid sequence, makes it challenging to assign functions to effectors. In addition, the lack of methods for genetic manipulation of microbes (e.g. for many fungi) interferes with functional effector studies (Rafiqi et al., 2012). Many approaches have been used to study the effectome, as the entirety of the microbial effector repertoire of a given microbe, mainly for pathogens. Such approaches include pathogenicity assays (Schechter et al., 2007), as well as machine learning that enables effector systematic analysis and prediction (Wang et al., 2017a). Petre et al. (2014) have highlighted the necessity of new high-throughput approaches for the identification and functional characterization of new effector proteins (effectomics). Combining comparative genomics, structural and functional studies (Anderson et al., 2015) could give a holistic understanding of the role of microbial effectors in plant microbe interactions.

1.3 Plant interactions with beneficial bacteria and fungi

1.3.1 The *Medicago truncatula* – rhizobia interaction

Bacteria are highly abundant in all plant compartments (Hacquard et al., 2015) and hold key roles in soil nutrient cycling, influencing various processes of the ecosystem (Harris, 2009). They are found as free living, closely associated with the root surface or within the root tissue. Bacteria affect plants in multiple ways: synthesizing molecules, when associated with plants (e.g. phytohormones), enabling nutrient acquisition from the soil (either by altering root architecture or by actively making certain compounds available to the plants) and providing disease tolerance and protection (Hayat et al., 2010).

Symbioses of legumes with nitrogen-fixing rhizobia represent agriculturally important plant-bacterial associations as they can enrich the soil with nitrogen (Fournier et al., 2008; Jones et al., 2007; Oldroyd et al., 2011; Rangin et al., 2008). *Medicago truncatula* is a model organism for legumes (Cook, 1999). Legumes secrete flavonoids in order to attract nitrogen fixing rhizobia, which form nodules in the roots of the plant (Bertin et al., 2003; Hassan and Mathesius, 2012; Oldroyd et al., 2011). Nodules provide low oxygen conditions, which rhizobia require for the conversion of N_2 into ammonia, using the enzymatic complex called nitrogenase (Day et al., 2001; Gage, 2004; Gibson et al., 2008). Thus, in this symbiotic relationship rhizobia provide ammonia to plants, while plants provide carbon and energy to the bacteria (Poole et al., 2018). Furthermore, nodules, have been found to enclose microbial communities, with possible beneficial effects on the plant (Hartman et al., 2017; Martínez-Hidalgo and Hirsch, 2017; Velázquez et al., 2017). However, functional studies are required for unravelling the function of the nodule microbiome.

Rhizobial-legume symbioses are regulated by genetic and environmental factors (e.g. recognition mechanisms, soil nitrogen availability). For rhizobia to enter plant roots, the plant has to recognize the bacterial-derived Nod factors for the activation of a series of processes that will enable successful nodulation (Oldroyd et al., 2011). The plant-host has control over the survival of the bacteria within the nodule. It is responsible for supporting the metabolic processes of bacteria, provide the necessary nutrients, as well as the limited oxygen conditions required for nitrogen fixation (Jones et al., 2007; Mergaert et al., 2006). Thus, the plant defense pathways can regulate the number of nodules, resulting in negative control (autoinhibition) of nodulation (van Brussel et al., 2007). Such inhibition signals are regulated by plant hormones, having a key role in plant-microbe interactions (Sun et al., 2006).

1.3.2 The *Arabidopsis thaliana* - *Serendipita indica* interaction

Fungi take important roles in the function of ecosystems and it is hypothesized that early symbioses with fungi enabled plants to colonise land (Brundrett, 2002). The most extensively studied beneficial plant-fungi symbioses

are mycorrhizas. Arbuscular mycorrhizas (AMs) establish arbuscules inside root cells of their host plants. AMs provide nitrogen and phosphorus to host plants and, in turn receive carbohydrates from their hosts. The establishment of mycorrhizas is based on complex communication signals and metabolic processes (Parniske, 2008).

In addition to mycorrhizas, plants can undergo beneficial symbioses with a diversity of other fungal species. The root fungal endophyte *Serendipita indica*, which is closely related to some mycorrhizal fungi (e.g. orchid mycorrhizas)(Weiss et al., 2004), is another well-studied microbe that forms mutualistic relationships with a broad range of plants. *S. indica* is a mutualistic fungal root endophyte of the order Sebacinales (Basidiomycota) that was isolated in the Indian Thar desert and was observed to improve plant phosphorus supply and disease resistance (Qiang et al., 2012a). Similar to other symbionts it receives carbon from host plants, essential for its growth and survival (Aslam et al., 2019). Members of the order Sebacinales are known as asymptomatic endophytes, that have been found in a large variety of plants from different environments and ecosystems around the world. These fungi are thought to enhance the ecological competitiveness of their host plants (Weiß et al., 2011) and their ubiquitous presence might indicate a superior competitiveness of sebacinoid fungi in colonizing plant roots.

Various studies have aimed at identifying the molecular nature of the beneficial interaction between the fungus and its host plants (Becker et al., 2005; Cosme et al., 2016; Draft et al., 2011; Lahrmann et al., 2013; Molitor and Kogel, 2009). *S. indica* has been found to suppress plant innate immunity and alter hormone homeostasis during infection. This involves upregulation of genes related to auxin, gibberellin, abscisic acid and down-regulation of salicylic acid-related genes (Schäfer et al., 2009). In addition, plant ethylene signalling has been found to be essential for host colonisation (Camehl et al., 2010). While these studies indicate the potential of *S. indica* in improving crop production, its application in the field was less promising (Serfling et al., 2007). It suggests, as it is for other beneficial microbes, to exploit their full beneficial potential as bioagents, we need to understand their behavior within complex microbial communities.

1.4 Plant microbe interactions at the community level

Root microbiota, as diverse microbial communities in and around plant roots, can significantly influence plant development and stress tolerance (Bulgarelli et al., 2015). Microbes have specialized to alter plant signaling and metabolism in order to create a habitat that is sufficiently supplied with plant-derived nutrients to support microbial survival and proliferation (Ortíz-Castro et al., 2009). In turn, plants can influence the composition of microbial communities in the rhizosphere through root exudates, attracting plant beneficial microbes to the root (Bulgarelli et al., 2013). It is thus important to understand the specific role of different microbes in complex ecosystem processes and the influence of nutrient availability and other environmental factors on their activity.

1.4.1 Microbial interactions within the community

Bacteria and fungi often share micro-environments and their interactions drive many processes in a given ecosystem, and can contribute to plant and animal fitness (Deveau et al., 2018). These microbial groups have evolved various strategies in order to co-exist and/or thrive in the ecosystem they share. For example, fungal hyphae can provide micro-habitats for bacteria (Frey-Klett et al., 2011) and can facilitate bacterial migration along hyphae (Warmink et al., 2011). In turn, certain fungi rely on bacterial endosymbionts for their growth and function (Bonfante and Desirò, 2017). This close relationship between bacteria and fungi relies on sophisticated ways to balance their interactions.

In soil-plant ecosystems, microbes have evolved various strategies to communicate and influence the environment around them (Tseng et al., 2009). Bacteria use quorum sensing to mediate the establishment of colonisation within the plant. Quorum sensing, as defined by Miller and Bassler (2002), is the regulation of gene expression in response to fluctuations in cell-population density. This is achieved using signal molecules such as lipid-like diffusible factors and signalling peptides, as well as N-acyl homoserine lactones. These signal molecules mediate bacterial virulence, biofilm development, production of cell-wall-degrading enzymes, bacteria adhesion and movement (Leach et al., 2017; Miller

and Bassler, 2002). Quorum sensing is also involved in interspecies communication, and quorum sensing signals can trigger competition responses in other microbes, such as antibiotic production (Dubuis et al., 2007). Another bacterial mechanism of communication is the production of volatile organic compounds, which affect virulence, biofilm formation and stress survival mechanisms in adjacent bacteria (Lee et al., 2015). Fungi can also produce volatile organic compounds, with some of them exerting antimicrobial activity (Kanchiswamy et al., 2015), regulate fungal pathogenicity (Tsitsigiannis and Keller, 2007), or activate plant defense pathways plants (Li et al., 2016a). Quorum sensing and volatile organic compounds production can also affect plant metabolism, development and defense by modifying respective plant pathways (Hartmann et al., 2014; Kanchiswamy et al., 2015).

Quorum sensing signals and volatile organic compunds play important roles in the interspecies and inter-kingdom communication. It has been shown that these signals can affect the phytobiome composition and the outcome of the interactions within the plant (Leach et al., 2017). For example, certain fungal compounds such as mycotoxins can inhibit the perception and response of some quorum sensing signals (Bacon et al., 2017; Rasmussen et al., 2005). Fungal volatiles, in turn, can affect motility of bacteria (Schmidt et al., 2016). Plants can mimic fungal volatiles to regulate fungal colonisation and pathogenicity (Gao and Kolomiets, 2009), as well as to mimic or inhibit bacterial quorum sensing and signalling (Corral-Lugo et al., 2016; Vikram et al., 2010). The complexity of communication within microbiomes and between microbes and plants makes the study of microbe-microbe and plant-microbe interactions challenging. These interactions are highly dynamic and the recruitment of different approaches is necessary in order to resolve and, thus, understand underlying mechanisms involved in the spatiotemporal organization of these activities.

1.4.2 The expansion of microbiome research

The advancement in sequencing technologies and the increased computational power has led to a large number of microbiome-based studies (Fierer, 2017; Prosser, 2015). The common approach used recently in microbiome

research, is the barcoded amplicon sequencing using specific conserved regions of microbes; usually the 16S ribosomal RNA (rRNA) for bacteria and the intergenic spacer (ITS) region for fungi. Amplicon sequencing has been an essential tool for microbiome studies as it allows simultaneous analyses of multiple samples and, by increasing the complexity of experiments, it allow us to observe general patterns in microbiome structure and composition (Bulgarelli et al., 2012; Lundberg et al., 2012a; Peiffer et al., 2013; Tkacz et al., 2015).

1.4.3 The use of synthetic microbial communities in gnotobiotic systems

Following the advancement in sequencing technologies, there are novel methods to culture previously unculturable microbes, encouraging new studies and efforts to isolate and characterize new microbial strains (Bai et al., 2015; Browne et al., 2016; Vartoukian et al., 2010). These isolates are often recruited for the construction of synthetic microbial communities to study mechanisms involved in the assembly of microbial communities by the plant as well as to uncover and explain shifts in microbiome structure and/or composition in response to specific changes in the system (Bai et al., 2015; Niu et al., 2017). These experimentally tractable, synthetic microbial communities can be used in gnotobiotic systems, allowing the targeted manipulation of various factors of a system in order to test specific hypotheses (Vorholt et al., 2017). Gnotobiotic is defined as a system in which all the organisms are known and all parameters defined (Wagner, 2008). For this reason, gnotobiotic system-based analyses often start with a sterile environment and controlled conditions, where known organisms are added aseptically (Simons et al., 1996; Wagner, 2008). This sophisticated approach enables the functional characterization of individual microbes within a community context and/or in the presence of a plant. Such approaches will increase our understanding of the interactions within the microbiome (Levy et al., 2018).

The use of synthetic microbial communities in gnotobiotic systems can assist us in characterizing the function of microbiome members within a community and in association with a plant; thus, associating genotypes with phenotypes (Vorholt et al., 2017). This approach has enabled the identification of keystone microbes, the lack of which can lead to the collapse of microbiomes (Niu et al.,

2017), or their presence in the root can "buffer" negative effects of a microbial species on the plant (Hartman et al., 2017). It suggests that even individual species can have significant effects on microbiome dynamics and functions. This experimental approach can enable us to overcome ecosystem complexity and build fundamental knowledge on the processes within (plant) microbiomes.

1.5 Bridging the lab-field gap

An ultimate aim of microbiome research is to be able to use the knowledge gained in the laboratory, to the field; e.g. to improve agricultural ecosystem functioning. However, agricultural practices have a strong impact on the microbiome structure, diversity and function. Such effects, though, cannot be studied in a laboratory experimental set up. Therefore, more agriculturally relevant experiments are necessary to advance our understanding of microbiome and its functions (Sergaki et al., 2018).

The physicochemical properties of soils, such as pH, texture, organic matter and nutrient content can vary largely. These soil properties can strongly affect microbiome structure and composition either by creating specific environmental niches, or by influencing plant root exudate availability and consequently, the microbial assembly by the plant (Lareen et al., 2016). Notably, it has been observed that soil types can have stronger effects on bacterial community structure than geographical location (Gelsomino et al., 1999) and can drive the succession and the stability of microbiomes in plant rhizospheres (Tkacz et al., 2015). Soil pH, nitrogen and phosphate availability can shift the abundance of pathogenic as well as beneficial microbes (Duffy et al., 1997; Dumbrell et al., 2010; Höper et al., 1995; Rotenberg et al., 2005). Similarly, soil microbiome structure changes with the addition of fertilizers (Toljander et al., 2008). It highlights the importance of studying the effect of soil characteristics on microbiome, gaining knowledge applicable in an agricultural setting. Studying plant-microbe interactions using approaches with increasing complexity, from the molecular level (e.g. effectome) to the ecosystem level (e.g. in a field setting), will allow the exploitation of these interactions for the improvement of crop yield and health.

1.6 Aims of this thesis

Microbe-microbe and plant-microbe interactions are highly complex systems. At the molecular level, hormones play a key role in the establishment of plant-microbe interactions and microbial effectors enable the manipulation of the host for the successful microbial establishment. The bipartite interactions between *M. truncatula – Sinorhizobium* spp and *A. thaliana – S. indica* are well-studied model systems of mutualistic plant-microbe interactions. However, microbial interactions in a community context are highly complex. The development of amplicon sequencing, along with the development of gnotobiotic system and the construction of synthetic microbial communities have assisted in improving our understanding of the factors that affect the microbiome structure and composition. Furthermore, this knowledge needs to be transferred to the field, studying agriculturally relevant factors that may affect the microbiome, and consequently plant performance, such as soil characteristics. This work aims to give an insight into these interactions using different approaches.

This thesis consists of three chapters containing experiments and data that aimed at identifying strategies of *S. indica* to establish colonisation within the root (Chapter 3), plant or/microbe derived patterns that shape the root microbiome in a defined system (Chapter 4) as well as in natural soil types, along with exploring the competitiveness of beneficial root symbionts (Chapter 5).

In more detail, in Chapter 3 potential strategies for the establishment of the *S. indica* colonisation of the plant were investigated. The effect of single putative effectors on auxin signalling was evaluated using a high-throughput protoplast screening approach followed by root growth inhibition assays of *Arabidopsis* plants expressing *S. indica* effectors. Basal expression of auxin related genes of those *Arabidopsis* plant lines were examined, in order to evaluate whether the expressed effector can regulate auxin signalling. This work aimed to identify *S. indica* putative effector proteins that affect auxin signalling as a strategy of the fungus to establish colonisation within the plant and potentially influence the plant-associated microbiome.

Chapter 4 has a focus on the role of beneficial microbes in shaping the plant-associated microbiome, improving plant yield. In this chapter, I employed a gnotobiotic system and a synthetic microbial community to study the effects of *S. indica* and *Colletotrichum tofieldiae* on *A. thaliana* growth and bacterial community structure. This approach aimed to reveal the effect of a single beneficial fungus on the core bacterial community structure and diversity of *A. thaliana*, and the consequent effect on plant growth.

Following the reductionist approach, the work presented in Chapter 5 intended to increase the system complexity investigating the plant-microbe interactions in a more agriculturally relevant experimental set up. For these experiments, I used three different natural UK soils, collected from agricultural fields, the legume *Medicago truncatula* and three *Sinorhizobium* strains. The purpose of these experiments was to investigate the competitiveness of *Sinorhizobium* strains in agricultural soils and their effect on plant growth, phytobiome, as well as the dependency of these effects on soil characteristics.

Chapter 2: Materials and Methods

2.1 Plant material and growth

For the experiments with *Arabidopsis thaliana* the ecotype Col-0 wild type (Nottingham *Arabidopsis* Stock Centre accession N60000) was used. For the protoplast screening, Col-0 plants were grown in a compost/sand/vermiculite mix (6:1:1) for 4-5 weeks, in a growth cabinet in a 22°C light/20°C dark cycle (12 hours light). For the auxin experiments, *pEG201::HA-SIE* (SIE = *Serendipita indica* effector) lines were used, along with Col-0 and *pAtUBQ10::HA-GFP* plants as controls. For these experiments, seeds were sterilized with chlorine gas, by mixing 50 ml bleach (14% v/v NaOCl in aequeous solution) and 3 ml of concentrated HCl (32% v/v) in a desiccator, for 4 hours. After adding 1 ml of 0.02% w/v sterile Phytoagar (Duchefa Biochemie) solution, seeds were sown on ½ MS agar plates; 2.151 g l^{-1} Murashige and Skoog basal salts (M5524-10L Sigma-Aldrich), 0.5 g l^{-1} 2-(N-morpholino)ethanesulfonic acid (MES) hydrate, pH adjusted to 5.7 with 1 M KOH, and 0.7% w/v Phytoagar, and kept at 4°C in the dark for 2 days before transferring them to growth cabinet in a 22°C light/20°C dark cycle (12 hours light).

For the *Arabidopsis* microbiome experiments, seeds were sterilised in a NaOCl solution. To this end, seeds were first mixed with H_2O 0.02% Tween20, which was then replaced by 70% ethanol. Seeds were then incubated for 5 min in 1 ml of a 3% v/v NaOCl solution and then washed thoroughly 7 times with sterile water. At the end of the sterilization, the seeds were left in sterile 0.2% w/v Gelrite (Duchefa Biochemie) solution. Seeds were sown on squared petri dishes containing ATS (*Arabidopsis thaliana* salts) medium (Lincoln, 1990) supplemented with 4.5 g l^{-1} Gelrite and kept at 4°C in the dark for 2 days before transferring them to a growth cabinet at 20°C and 10 hours light, 14 hours dark.

For the experiments with *Medicago truncatula*, the ecotype Jemalong A17 wild type was used. Seeds were scarified with concentrated sulfuric acid for 20-30 min and washing three times with water. This was followed by sterilisation in 7%

v/v NaOCl for 5 min and washed 8 times with sterile water. Seeds were then placed on 1.5% phytoagar plates and hydrated by the sequential addition of three droplets of water. To provide high humidity, sterile wet growth pouches were placed in the lids of the square plates. Plates were kept at 4°C in the dark for 4 days before they were transferred to a growth cabinet at 25°C in the dark for 2 more days. Seeds were then transferred to pots containing autoclaved sterile perlite (Sinclair Pro) covered with a thin layer of sterile vermiculite (Sinclair Pro) to maintain humidity, grown at 24°C light/22°C dark cycle (16 hours light).

For the experiments in perlite, *Medicago trancatula* genotype Jemalong A17 plants were inoculated 1 day after being transferred to the pots. Fresh shoot weight and the fungal colonisation were quantified 49 days after inoculation (dai) (corresponding to 52 day old plants).

For the microbiome experiments, *Medicago trancatula* genotype Jemalong A17 plants were inoculated 1 day after being transferred to the pots, and they were left in perlite for 11 days more. Watering was performed on demand using nutrient solution (0.5 mM $MgSO_4*7H_2O$, 0.7 mM KH_2PO_4, 0.8 mM $Na_2HPO_4*2H_2O$, 20 µM FeNaEDTA, 8 µM $MnSO_4*H_2O$, 4 µM $CuSO_4*5H_2O$, 7.34 µM $ZnSO_4*7H_2O$, 16 µM H_3BO_3, 4.13 µM Na_2MoO_4, 1 mM $CaCl_2$, pH to 6.5) without N. Seedlings were then transferred to the pots containing the natural soil types. 7cmx7cm (fp7) pots had been filled with the soils and bottom watered with RO (Reverse Osmosis) water. 8 technical repetitions were set up per treatment and soil type. To identify the best sampling time point, shoot dry weight was measured 46, 56, 63 and 70 dai (49, 59, 66 and 73 day old plants respectively). Quantification of fungal colonisation was done 56 dai (59 day old plants). Samples for microbiome analysis were taken 63 dai (66 day old plants).

2.2 Microbial strains, growth conditions and inoculation

Serendipita indica (formerly *Piriformospora indica*, German collection of microorganisms and cell cultures Deutsche Sammlung für Mikroorganismen und Zellkulturen, DSMZ, Braunschweig, Germany, accession number DSM 11827) was grown on Complex Medium (CM) (Pham et al., 2008) for 8-10 weeks, at 25°C

in the dark. *S. indica* spore suspension was prepared as in Banhara et al. (2015a), counted under the light microscope using a counting chamber and diluted in sterile milliQ water at the concentration of 500,000 spores/ml. For the Arabidopsis microbiome experiments, the bacterial collection from (Bai et al., 2015) was used. 200 bacteria were grown at 20°C in 96 well plates with 50% Tryptic Soy Broth (Sigma-Aldrich) and pooled together (in equal ratios), creating in this way the synthetic community (SynCom). The synthetic community was adjusted to OD_{600} 0.5 and 1 ml (approx. 10^8 cells) was added to 50 ml of ½ MS media (including vitamins).

Sinorhizobium meliloti strains WSM1022 and WSM1021 and *Sinorhizobium medicae* WSM419 were kindly donated by Jason Terpolilli (Murdoch University, Perth, Australia). They were grown in TY (Tryptone – yeast extract) medium (5 g l^{-1} tryptone, 3g/l yeast extract, 4.766g/l HEPES, pH 7 with KOH, 6 ml l^{-1} 1 M $CaCl_2$ added after autoclaving) at 28°C for 2 days. 100 mg l^{-1} streptomycin was used to select for WSM1021, and 20 mg l^{-1}chloramphenicol for WSM419 and WSM1022. For the plant inoculation, the rhizobia strains were grown overnight in liquid TY, at 28°C and 220 rpm shaking. Rhizobia were then harvested by centrifugation at 2880 x *g* for 10 min at 4°C, washed 3 times with sterile water to get rid of any nutrients and antibiotics from the media, before they were resuspended to a final OD_{600} of 0.05 in sterile milliQ water.

The co-inoculation of *Serendipita indica* with *Sinorhizobium meliloti* strains was performed using a mix of both microbes. The bacterial culture was centrifuged at 2880 x *g* for 10 min at 4°C and the supernatant was replaced with equal volume of the spore suspension. After mixing appropriately, each plant was inoculated from the top into the perlite using a pipette, with 250 µl of the suspension.

2.3 Protoplast-based luciferase reporter assay

For the isolation of protoplasts, leaves from 24 plants were sliced into 1 mm strips and transferred into 6 ml of enzyme solution (20 mM MES pH 5.7, 0.4 M mannitol, 20 mM KCl, 1.5% w/v cellulase R10 (Duchefa Biochemie), 0.4% w/v

macerozyme R10 (Duchefa Biochemie) in a 5.5 cm petri dish. The enzyme solution was vacuum infiltrated into the tissue twice at -0.8 bar for 5 minutes, before incubation at 25°C for 3 hours. After the incubation the protoplasts were filtered through a 70 µm nylon filter into a 50 ml falcon tube and rinsed with the 6 ml volume of W5 washing solution (2 mM MES, pH 5.7, 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, autoclaved). The cells were then centrifuged at 100 x *g* for 2 minutes at 4°C, the supernatant was discarded, and protoplasts resuspended in the 12 ml of cold W5 solution (4°C). Protoplasts were centrifuged again and resuspended in MMG solution (4 mM MES, pH 5.7, 0.4 M mannitol, 15 mM $MgCl_2$, autoclaved). The number of protoplasts was determined using a Fuchs-Rosenthal haemacytometer and adjusted to 330,000 protoplasts per ml in MMG solution. The DNA mix used for the transformation consisted of 1 µg each of plasmids *pGH3.3::LUC* (ABRC, CD3-913), *pAtUBQ10::GUS* (Abel and Theologis, 1994), and *pEG201::HA-SIE* (SIE construct) (Earley et al., 2006), or the empty vector *pAtUBQ10::HA-GFP* (Abel and Theologis, 1994) as control. The SIE sequences were synthesised by LifeTech Scientific and cloned into the *pEG201* using the Gateway system (Earley et al., 2006) by members of P. Schäfer group (University of Warwick). All plasmids extracted from *Escherichia coli* liquid cultures (grown overnight in Luria broth, 37°C, 100 rpm shaking) using the ZymoPURE Plasmid Midiprep Kit (Zymo Research). The DNA was mixed with 30 µl of the protoplast solution (around 10,000 cells), and 33 µl of freshly prepared transfection solution (40% w/v PEG 4000, 0.2 M mannitol, 100 mM $CaCl_2$,) in order to start the transformation. The cells were then shaken at 1000 rpm on an orbital shaker for 1 min, incubated at room temperature for 15 minutes. The transformation was stopped by adding 170 µl of W5 solution and incubating the mix at 1000 rpm for 1 minute. The cells were spun down for 1 minute at 100 x *g*, before 160 µl of the supernatant were removed and cells were resuspended in 140 µl of WI solution (4 mM MES, pH 5.7, 0.5 M mannitol, 20 mM KCl, autoclaved) upon shaking at 1000 rpm for 1 minute. The transformed cells were incubated overnight in a growth cabinet at 22°C, 12 hours light/12 hours dark.

On the next day, the protoplasts were spun down at 100 x *g* for 2 min, and 100 µl of the supernatant were removed. The protoplasts were then treated with the synthetic auxin 1-Naphthaleneacetic acid (NAA, 500 nM final concentration) or

water as control. For the luciferase reporter assay, white 96-well 0.5 ml polypropylene plates (Nunc™ #267350) were used. 10 µl of the treated protoplasts were pipetted into each well and 20 µl of the Beetle Luciferin luciferase substrate (Promega, #E1602) was added. Measurements were taken with a Photek camera and the LUC activity data were extracted from the images with the Image32 software (Photek), as relative light units (RLU). After that, 100 µl of 1x lysis buffer (Luciferase Assay System, Promega # E1500) were added, and protoplasts were shaken at room temperature at 1000 rpm for 30 sec. For the GUS (β-glucuronidase) assay, 10 µl of the lysate were pipetted into each well of a transparent flat bottom 96-well plate and 100 µl of GUS substrate (1 mM 4-methylumbelliferyl-beta-D-dglucuronide (MUG), 10 mM Tris-HCl pH 8, 2 mM $MgCl_2$) were added. The mix was incubated at 37°C for 1 hour and fluorescence measurements taken using a TECAN GENios microplate reader (Tecan Life Sciences AG) (excitation filter 360 nm, emission filter 465 nm) equipped with the Magellan software (version Standard, Tecan Life Sciences AG). The Magellan software measures the GUS activity as relative light units (RLU) for each well in the 96-well plate, which is used for further analysis.

2.4 Analysis of protoplast-based luciferase reporter assay data

For the data analysis the luminescence values of the activity of the promoter in the control treatment was compared with the auxin treatment, as well as the values of the empty vector with those of the effectors. All luminescence values were normalised using the data from the GUS assay, by dividing the LUC values by the corresponding GUS (and then multiplied with a high factor to get values in a similar range as the raw data).

For the selection of the best candidates, the ratio of the difference in the normalised LUC expression of the effector to that of the empty vector (eV) in the mock treatment was multiplied by the ratio of the difference of the normalised LUC expression of the effector to that of the empty vector (eV) under the NAA treatments:

$$\text{Ranking ratio} = \frac{\text{SIE normalised LUC expression in mock}}{\text{eV normalised LUC expression in mock}} \times \frac{\text{SIE normalised LUC expression in NAA treatment}}{\text{eV normalised LUC expression in NAA treatment}}$$

2.5 Auxin tests

Arabidopsis plants were grown on ½ MS plates as described in 2.1 for 7 days and then transferred to ½ MS plates containing 40 nM indole-3-acetic acid (IAA) in dimethyl sulfoxide (DMSO) or DMSO as control (3.2 µl in 800 ml MS). 7 days later, pictures of the whole plate were taken and the primary root length was measured using the ImageJ software (https://imagej.net).

2.6 RNA extraction, cDNA synthesis, qPCR

Frozen plant tissue samples were ground using a Tissue Lyser (QIAGEN) at 25 Hz for 1 min on each side. The frozen ground material were kept on ice and 1 mL Trizol was added to them and were vortexed thoroughly. Then, 200 µl chloroform was added and samples were vortexed for 15 sec. Samples were incubated on ice for 2-3 mins and then centrifugated for 30 mins at 13000 rpm at 4°C. The colourless supernatant (approximately 400 µl, avoiding the gDNA-containing interphase) was transferred into a 1.5 mL RNase-free microcentrifuge tube containing 500 µl isopropanol. RNA was precipitated overnight at -20°C. Precipitated RNA was pelleted by centrifugation at 13000 rpm for 50 min at 4°C. The supernatant was discarded and the pellet washed in 75% ethanol. The ethanol was removed and the pellet dried for 5 mins on ice, before being resuspended in 10-30 µl nuclease-free water. The samples were vortexed, incubated at 35°C for 10 mins and RNA concentration was measured using NanoDrop (ThermoFisher Scientific).

Genomic DNA was removed from samples using a DNase digest prior to cDNA synthesis. The reaction mix per sample for this process was: 2 µg sample RNA, 2 µl 10x reaction buffer with $MgCl_2$, 2 µl DNase I (1 U/µl, Thermo

Scientific), 0.5 µl Ribolock RNase inhibitor (40 U/µl, Thermo Scientific), made up to total volume of 20 µl with nuclease-free water. The mix was incubated at 37°C for 30 mins in a thermal cycler, before the addition of 2 µl of 50 mM EDTA (Thermo Scientific) and a further 10 min incubation at 65°C, to inactivate the DNase. RNA concentration was re-measured by Nanodrop.

cDNA synthesis was performed using Quanta Biosciences qScript™ cDNA Synthesis Kit (#95047-100). The reaction mix per sample for this process was: 1 µg DNase-treated RNA, 4 µl 5x qScript Reaction Mix, 1 µl qScript Reverse Transcriptase, made up to total volume of 20 µl with nuclease-free water. Samples were incubated in a thermal cycler for 5 mins at 25°C, 30 mins at 24°C, 4 mins at 85°C, then held at 8°C. The resulting cDNA was diluted to 5 ng/µl with H_2O_{bidest} and stored at -20°C.

For quantifying transcriptional expression of genes, qPCR was performed. The reaction mix per sample for this process was: 8 µl SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich), 0.4 µl forward primer, 0.4 µl reverse primer, 2 µl DNA template, 9.2 µl MilliQ water was used. Each sample tested with each primer pair in triplicate (technical replicates). For the quantification of *Arabidopsis thaliana UBQ5*, the primers used were 5' – ATGACTCGCCATGAAAGTCC as forward and 5' – ATGACTCGCCATGAAAGTCC as reverse (Jacobs et al., 2011). The primers for the auxin-responsive genes *GH3.3 (AT2G23170)* and *IAA5 (AT1G15580)* were 5'- GGAGATTCAACGTATTGCCA as a forward primer and 5' – GGTTGGCATCAACTTCCTTT as reverse for *GH3.3* and 5'- CGTTGAAGGAAAGTGAATGTG as a forward primer and 5' – ATCCAAGGAACATTTCCCAA as reverse for *IAA5.* The primers were designed to bind on the exons of the sequences. The PCR reaction was performed using a thermocycler Agilent Technologies Stratagene Mx3005P, with : 5 minutes at 95°C, 40 cycles of [15 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C], followed by 1 min at 95°C, 10 sec at 55°C, 10 sec at 95°C. Gene expression for the gene of interest in each sample was normalised against *UBQ5* expression by calculating the mean 2^{-DCT} across each triplicate, where DC_T is the difference between the C_T value of *UBQ5* in the sample and the C_T value of the gene of interest. Fold change from

the mock treatment was calculated using the 2^{-DDCT} method (Schmittgen and Livak, 2008).

2.7 Quantification of fungal colonisation using qPCR

For the quantification of fungal colonisation, genomic DNA was extracted from roots using the Doyle and Doyle method for rapid DNA isolation (Doyle and Doyle, 1987). This DNA was used as a template for qRT-PCR which was conducted as described above (section 2.6). The primers used for the quantification of *S. indica ITS* were 5'- CAACACATGTGCACGTCGAT as a forward primer and 5' – CCAATGTGCATTCAGAACGA as reverse (Deshmukh et al., 2006a). For the quantification of *Arabidopsis thaliana UBQ5*, the primers used were 5' – CCAAGCCGAAGAAGATCAAG as forward and 5' – ATGACTCGCCATGAAAGTCC as reverse (Jacobs et al., 2011). For the quantification of *Medicago truncatula Medtr6g084690 (PDF2)*, the primers used were 5' – GATCAAGAATTGGCGTTGGT as forward and 5' – TAGGCGACCATCTGACATTG as reverse (Jacobs et al., 2011). For the quantification of the DNA a reaction mix containing 8 µl SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich), 0.4 µl forward primer, 0.4 µl reverse primer, 2 µl DNA template, 9.2 µl MilliQ water was used. Each sample tested with each primer pair in triplicate (technical replicates). The PCR reaction was performed using a thermocycler Agilent Technologies Stratagene Mx3005P, with : 5 minutes at 95°C, 40 cycles of 15 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C, 1 min at 95°C, 10 sec at 55°C, 10 sec at 95°C. The amount of fungal DNA as the expression of *ITS* was normalised against the *UBQ5* gene quantity for *Arabidopsis* samples and *PDF2* for *Medicago* samples, was determined by the 2^{-DDCT} method (Schmittgen and Livak, 2008).

2.8 *Arabidopsis thaliana* microbiome preparation, sampling and processing

Arabidopsis plants were inoculated with *S. indica* spore suspension, *C. tolfidiae* mycelium (Hiruma et al., 2016), or sterile water 3 days after germination, and 2 days after inoculation were transferred into the sterile Flowpots system

(Kremer et al., 2018). The soil (peat)/vermiculite mix used in the system was washed with water, sterilized twice by autoclaving and was incubated at 65°C until being completely dehydrated. The Flowpots were prepared as in Durán et al., 2018, using microbial strains as reported in Bai et al., 2015. Two seedlings were placed in each flowpot and 6 flowpots in each box (24 seedlings were used for the microbe free samples and 36 seedlings for all the rest treatments). The plants were kept at 22 °C, 11 hours light/13 hours dark, and 54% humidity for 6 weeks. The seedlings were then extracted from the pot, washed in washing buffer (1x TE + 0.1% Triton X-100) and flash frozen in Lysing Matrix E and in liquid nitrogen. Bulk soil was sampled in Lysing Matrix E (MP Biomedicals, Fisher Scientific) in liquid nitrogen. The fresh weight of the plant shoot was measured.

Three samples fractions were used in the experiment. Input culture is the mix of all the microbes inoculated as the "core microbiome". As matrix is referred the bulk soil, sampled away from the roots and as root is defined the plant root endosphere and rhizosphere, after the removal of all soil particles attached to it.

2.9 *Arabidopsis thaliana* microbiome library preparation

The Lysing Matrix E tubes with the samples were homogenized at 5,600 rpm for 30 seconds using Precellys 24 tissue lyzer (Bertin Technologies, Montigny-le-Bretonneux, France), and DNA was extracted from all samples using the FastDNA SPIN Kit for soil (MP Biomedicals) according to the manufacturer's instructions. The DNA concentrations were measured using PicoGreen dsDNA Assay Kit (Life technologies) and diluted to 3.5 ng/µl. Bacterial 16S rRNA genes were amplified using V4–V7 region of bacterial 16S rRNA with the primers 799F 5' - ACGTCATCCCCACCTTCC and 1192R 5' – CAAGCAGAAGACGGCATACGAGAT (Durán et al., 2018). The Fungal ITS region was amplified with the primer pair fITS7 5' - GTGARTCATCGAATCTTTG and ITS4 5' – TCCTCCGCTTATTGATATGC (Durán et al., 2018). Each sample was amplified in duplicate by two independent PCR mixtures (plus respective no template controls). For the PCR BIORON DFS-Taq was used (including 3% BSA). For the PCR program 55°C annealing temperature was used for 30 sec and 1 min elongation time. After the PCR, the

replicates of each sample were pooled together for the cleaning procedure. Per sample, 1 µl of Antarctic phosphatase was used, 1 µl of Exo I and 2.44 µl of Antarctic phosphatase Buffer. The mix was incubated at 37°C for 30 minutes, followed by 15 minutes at 85°C to deactivate the enzymes. The samples were then centrifuged for 10 minutes at 3000 rpm and the supernatant was transferred to a new plate for the second PCR. This reaction was set up and run in the same as the first one, but with unique barcodes (as in Durán et al., 2018) for each sample attached on the forward primer. The duplicates of each sample were combined and the final product was then purified with Agencourt AMpure XP (Beckman Coulter), following the manufacturer's instructions, quantified with PicoGreen (ThermoFisher Scientific). All the samples were pooled together in a tube and quantified with Qubit. The DNA was then adjusted to 200 ng/µl and diluted to 1:2, 1:10, 1;100. The libraries were subjected to MiSeq paired-end sequencing using Nextera XT 300bp amplicon size.

2.10 *Medicago truncatula* microbiome experiment preparation, sampling and processing

For the *Medicago truncatula* microbiome experiments, the seedlings after germination on agar plates (as described at 2.1), were transferred to sterile FP9 pots with sterile perlite, covered with a 0.5 cm layer of sterile vermiculite on the top, wet with nutrient solution ($CaCl_2$ $2H_2O$ 6 mM, KH_2PO_4 1 mM, $FeC_6H_5O_7$ 75 µM, $MgSO_4$ $7H_2O$ 1 mM, K_2SO_4 0.25 mM, $MnSO_4$ H_2O 6 µM, H_3BO_3 20 µM, $ZnSO_4$ $7H_2O$ 1 µM, $CuSO_4$ $5H_2O$ 0.5 µM, $CoSO_4$ $7H_2O$ 0.05 µM, Na_2MoO_4 $2H_2O$ 0.1 µM, NH_3NO_3 0.1mM) at 24°C light/22°C dark cycle (16 hours light). One day later the seedlings were individually inoculated with 250 µl of rhizobia inoculum prepared as described at 2.2 11 days after inoculation, the seedlings were transferred into individual sterile FP9 pots with the different soil types. 63 days after inoculation, plants were harvested; above ground tissue was collected, dried at 65°C for 3 days and weighed. The below ground tissue was harvested in falcon tubes to be processed for the construction of microbiome libraries. Bulk soil samples were obtained before the start and after the end of the experiment. Roots were processed in pairs; 2 individual roots were pooled together as one microbiome sample. 4

samples per sample type were collected for every biological replicate and the experiment was performed 4 times (4 biological replicates, 4 technical replicates each, 16 samples per sample type in total). All the roots close to the surface, the bottom and the sides of the pot were removed, in order to keep only the core root tissue in the middle of the pot, which should be the least affected by the environment. The root samples were then thoroughly washed with sterile water in a sterile hood and the first wash was centrifuged at 4,000 x *g* for 15 min, in order to concentrate the rhizospheric soil. Supernatant was carefully discarded and 500 µl of the pellet was collected into a Lysing Matrix E (MP Biomedicals) tube and flash frozen in liquid nitrogen as the rhizospheric sample. The roots were further washed with 3ml of washing buffer (1x TE + 0.1% Triton X-100) and 3 times with sterile water. They were then cut in small pieces with sterile scissors and transferred into the matrix tube and flash frozen in liquid nitrogen as the endospheric sample.

For this study, 5 different sample types/fractions were used; input soil, unplanted soil, soil, rhizosphere and endosphere. The input soil sample was collected at the start of each experiment, before transferring the plants to the soil. The unplanted soil came from soil pots grown without a plant alongside with the planted pots, in order to capture the influence of the plant. The bulk soil is referred as soil, sampled away from the roots, as rhizosphere (rhizo) the microbes at the surface of the root and as root associated (endo) the inside of the root, clean from all the microbes on the surface of the root.

2.11 *Medicago truncatula* microbiome library preparation

The Lysing Matrix E tubes with the samples were homogenized at 5,600 rpm for 30 seconds using Precellys 24 tissue lyzer (Bertin Technologies, Montigny-le-Bretonneux, France), and DNA was extracted from all samples using the FastDNA SPIN Kit for soil (MP Biomedicals) according to the manufacturer's instructions. The DNA concentrations were measured using PicoGreen dsDNA Assay Kit (Life Technologies) and diluted to 5 ng/µl. The libraries were constructed using 2-step PCR using Q5® Hot Start High-Fidelity DNA Polymerase (NEB) and 10-20 ng of DNA template. The taxa primers used for the first PCR, for the amplification of bacterial 16S (341f/785r (region V3-V4)) were 341f 5' –

CCTACGGGNGGCWGCAG and 785r 5' – GACTACHVGGGTATCTAATCC (Thijs et al., 2017) and for the fungal ITS2 region, ITS4 5' – GTGAATCATCGAATCTTTGAA and ITS86F 5' – TCCTCCGCTTATTGATATGC (Op De Beeck et al., 2014). For the PCR program, 53°C annealing temperature and 25 cycles were used for bacteria, 55°C and 35 cycles for fungi and 20 sec elongation time for both. The PCR product was run in 1.2% agarose gel and cleaned up with Agencourt AMpure XP (Beckman Coulter) following the manufacturer's instructions. The second (Index) PCR was performed using the Illumina index primers as listed at Table S2.1. For the PCR program, 55°C, 15 sec elongation and 8 cycles. The PCR product was cleaned using SequalPrep Normalization Plate (96) Kit (Thermo Fisher Scientific, Catalog number A1051001). All samples were pooled together and quantified using NEBNext® Library Quant Kit for Illumina® (NEB), according to the manufacturer's manual. The libraries were subjected to MiSeq paired-end sequencing using Nextera XT 300bp amplicon size.

2.12 Analysis of microbiome sequencing data

2.12.1 Raw data analysis

The reads from the MiSeq run were processed using Trimmomatic (Bolger et al., 2014) trimming at the quality level of Q15 reads for *Arabidopsis* microbiome data and Q20 for the *Medicago* microbiome. The sequencing data were processed using Qiime1 (Caporaso et al., 2010), UPARSE (Edgar, 2013) and USEARCH (Edgar, 2010) and taxonomy was assigned using the Greengenes database (DeSantis et al., 2006; McDonald et al., 2012). The number of raw reads, filtered reads and the number of reads at the OTU table level are shown on Table S2.2. The reads at the end of the raw data analysis were rarefied (normalised all samples to the same total number of reads) at 2400 reads for the *Arabidopsis* bacterial community, 2000 for the *Arabidopsis* fungal community, 1000 for *Medicago* bacterial community and 5000 reads for the fungal community, in order to be able to compare the samples (Weiss et al., 2017). This process may lead to the loss of low abundant and rare microbiome reads, but it is suitable for analysing and understanding big changes in the microbiome, rather than focusing to the taxa that

are present in very low numbers in the samples (McMurdie and Holmes, 2014). The statistical analysis was performed using Qiime1 and Past3 (Hammer et al., 2001). The full pipeline of all the downstream analysis is presented at Figure 2.1.

2.12.2 Alpha (α) and beta (β) diversity

The OTU table with the rarefied reads was used to calculate the alpha (α) diversity as a measure of OTU richness, using Observed OTUs and Shannon Index as measure of within-sample diversity (Sepkoski, 1988). The OTU table was also used for the calculation of the beta (β) diversity, which is the between samples diversity (Sepkoski, 1988). The Bray-Curtis dissimilarity matrix was used for all the downstream analysis, as well as for the calculation of the community beta diversity. The Bray-Curtis dissimilarity matrix was used for the Principles Coordinate Analysis (PCoA). Plotting the first two axis of the PCoA revealed the between-samples diversity, which shows whether the microbiome of each sample differ from the rest (Beals, 1984).

2.12.3 Statistics - Significance tests

The significance of the differences for alpha diversity was tested using ANOVA followed by Tukey's Honest Significant Difference test (HSD), and for beta diversity. The significance of the differences was tested using analysis of similarities (ANOSIM) using the Bray-Curtis similarity index or. ANOSIM generates an R value between 0 and 1, with 0 being the null hypothesis (the samples are indifferent), and 1 representing high dissimilarity among the samples (Chapman and Underwood, 1999), along with a p value that quantifies the significance of those differences. The contribution of the different taxa to the observed microbial community differences was assessed using the rarefied OTU table along with the assigned taxonomy and the Similarity Percentages (SIMPER) Analysis (K. R. Clarke, 1993) using the Bray-Curtis similarity index. The differentially enriched OTUs were calculated using the Kruskal-Wallis test in order to reveal the taxa that are significantly enriched or depleted in the different treatments or sample types.

2.12.4 Pearsons' correlations

Pearsons' correlation was used to find correlation between soil and plant chemical characteristics, aboveground dry weight, bacterial and fungal alpha diversity (Marić et al., 2016). The R value of 1 indicates strong positive correlation, -1 strong negative correlation and 0 indicates no correlation. The p value indicates the significance of the correlation.

2.12.5 Canonical Correspondence Analysis (CCA)

Chemical soil and plant characteristics were associated with the fungal and the bacterial community structure using the Canonical Correspondence analysis (CCA) which reveals the relationship between the ordination of the OTUs matrix and the environmental variables (Gonzalez et al., 2015). The Canonical Correspondence analysis was calculated using Past3 (Hammer et al., 2001). CCA is similar to PCoA, as it uses ordination matrix but includes Pearson's correlations of the environmental variables with the clusters of the microbiome samples (Gonzalez et al., 2015). Here, the environmental variables are represented by lines, with the direction of the line indicating increase of that variable and the length representing the strength of the correlation. The position of the sample points in respect to the lines of the variables represent their association. Using this approach, it was possible to unravel the effects of *S. meliloti* 1022 on the plant microbiome and its correlation with changes of the plant aboveground nutrients.

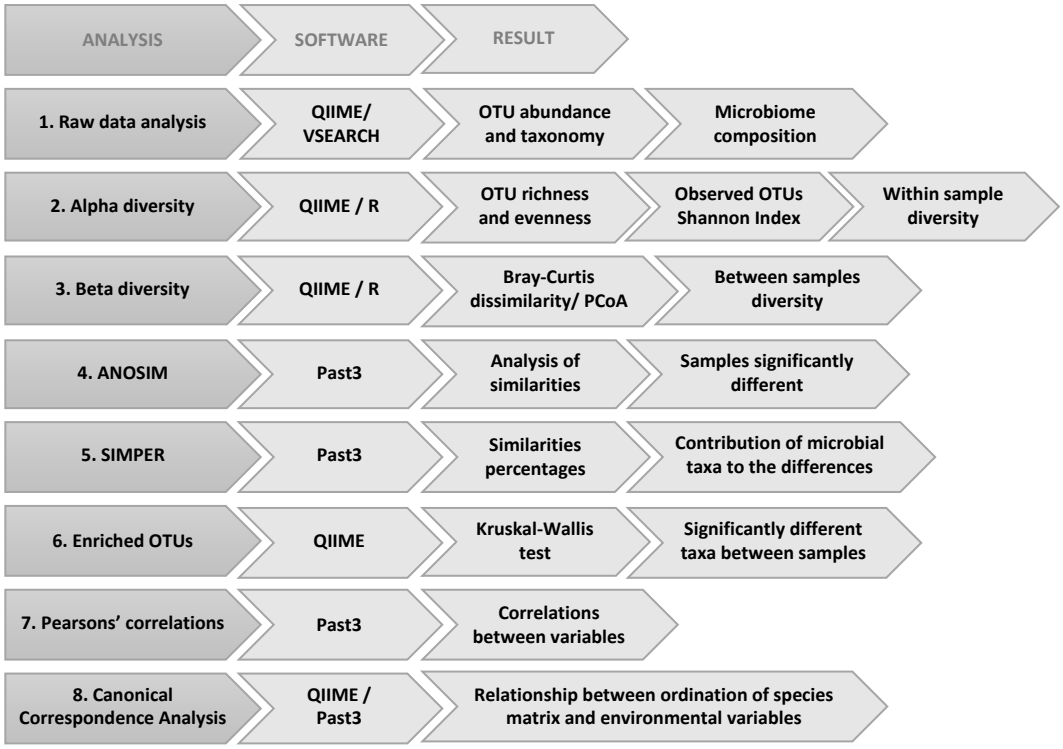


Figure 2.1: Infographic of microbiome analysis pipeline.

2.13 Soil collection and nutrient content analysis

The soils for Chapter 5 were collected from the locations given by the following GPS coordinates: high NP soil 52°50'25.2"N 0°01'48.8"W 52.840334, -0.030209, low N high P soil 52°12'22.0"N 1°36'21.7"W 52.206114, -1.606030, low NP soil 52°12'33.3"N 1°36'27.5"W 52.209239, -1.607634. For the collection of the low NP and low N high P soil the top 20 cm of soil was removed, as those hadn't been ploughed. The soil collection of the high NP was performed on ploughed soil. The soils were kept at 4˚C.

Soil and plant sample preparation and analysis was performed by the postdoctoral researcher Dr. Beatriz Lagunas, from Dr Miriam Gifford's group (University of Warwick) at the University of Nottingham. Soil and plant samples were dried previous to any analysis at 80˚C for 24h. Soil samples were sieved through a 2mm sieve and ground with mortar and pestle. Plant samples were ground

using stainless steel grinding jars (Qiagen). pH and conductivity were measured on 2mm soil samples incubated with distilled water (5 g on 12.5 ml) after shaking for 30 minutes using a combined pH electrode.

Multi elemental analysis was performed by Induced Coupled Plasma Mass Spectrometry (ICP-MS; Thermo-Fisher Scientific iCAP-Q; Thermo Fisher Scientific, Bremen, Germany; University of Nottingham). For the soil sample preparation, a full 'hydrofluoric-perchloric-nitric acid' digestion was used on 0.2g of ground soil using Certified Reference Material ® 2711a (Montana II soil) (Sigma-Aldrich) as control. For the plant sample preparation, we used a microwave-facilitated nitric acid digestion on 0.2g of ground plant tissue using Certified Reference Material ® 1573a (Tomato leaves) (Sigma-Aldrich).

The main inorganic anions (nitrate and phosphate) were analysed in soil samples using a Thermo Scientific Dionex ICS-1100 Ion Chromatography System, University of Nottingham. Soil extraction was performed using 30 ml milliQ water on 3 g of 2mm soil shaking overnight at room temperature. Samples were then centrifuged at 2000rpm for 10min. The supernatant was filtered through a 0.2 μm filter and kept at 4˚C until analysed.

Total Carbon and Nitrogen analysis was performed by total combustion using a LECO Trumac® CN/N determinator. 0.5g of ground soil or plant material were weighed into a ceramic boat and loaded into the furnace of the instrument set at 1350˚C.

Chapter 3: *Serendipita indica* effector proteins as a strategy to establish a compatible root environment

3.1 Introduction

An important part of plant-microbe interactions is the manipulation of plant hormone pathways by microbes. Changes in plant hormones, in combination with other signalling pathways, can regulate physiological responses (Davies, 2010), plant (systemic) resistance and interplant communication (Xu et al., 2018). Therefore, the ability of microbes to manipulate these signals within a plant not only allows them to colonise plants but also confers them a competitive advantage for getting access to valuable plant resources.

The phytohormone auxin (e.g. indole acetic acid, IAA) is involved in a large variety of developmental processes in plants (Vanneste and Friml, 2009), as well as in the establishment of the interaction between microbes and their host plants (Barker and Tagu, 2000; Vadassery et al., 2008). Auxin holds an instrumental role in orchestrating processes in plant development and adaptive growth (Naser and Shani, 2016), cell division and enlargement, organ development (Kopittke, 2016), as well as in stress responses and defence mechanisms (Egamberdieva et al., 2017; Kazan and Manners, 2009). The suppression of auxin signalling has been linked with antimicrobial activity (Fu et al., 2011; Navarro et al., 2006). In addition, it has been shown that growth-promoting microbes can directly or indirectly (e.g. via nitric oxide) change IAA biosynthetic and signalling pathways in plants to interfere with the plant metabolism (Creus et al., 2005; Molina-Favero et al., 2008; Spaepen et al., 2007). Overall this indicates the significance of auxin in the establishment of plant-microbe interactions and explains why a diversity of microbes would target auxin metabolism and/or signalling.

Microorganisms can produce IAA as a tool to influence the internal auxin levels of the plant and induce the plant signal accordingly, as a strategy to establish colonisation within the plant (Tudzynski and Sharon, 2013; Yang et al., 2007).

Notably, an IAA-overexpressing mutant of the ectomycorhizza *Hebeloma cylindrosporum* was found to colonise plant roots faster and deeper than the wild strain, as well as triggering faster transcriptional response by the plant (*Pinus pinaster*) (Charvet-Candela et al., 2002; Reddy et al., 2006; Tranvan et al., 2000). Hilbert et al. (2012) showed that *S. indica* gene *PiTam1* holds a key role in establishing the biotrophic interaction of the fungus with the plant, but the lack of it did not compromise the growth promotion effect, suggesting additional mechanisms involved in this plant – microbe interaction. In more detail, they demonstrated that *S. indica* can produce the phytohormones indole-3-acetic acid (IAA) and indole-3-lactate (ILA). The production of these phytohormones is involved in the successful establishment of the fungus in the roots, with the gene *PiTam1* having a key role in their production. Therefore, microbially-produced IAA is a strategy for microbes to influence plants auxin signalling pathway, as this pathway plays a key role in microbial establishment within the root.

Since auxin has a key role in the establishment of microbes in the root, microbes have developed additional strategies to affect plant auxin signalling pathway, besides the production of IAA. For example, the well-studied *Pseudomonas syringae* Type III effector *AvrRpt2* has been found to alter the plant auxin signalling pathway in order to establish its colonisation (Chen et al., 2007; Cui et al., 2013). In particular, Cui et al. (2013) found that the effector *AvrRpt2* promotes pathogenicity via the stimulation of the Aux/IAA protein turnover, important negative regulators in the plant auxin signalling pathway. Interestingly, *AvrRpt2* is not the only *P. syringae* effector that possesses this ability, but other effectors demonstrated a similar activity, indicating that this may be a general ability among pathogenic bacteria. Similar activity has been observed also from rhizosphere-associated, plant growth promoting microbes (Kunkel and Harper, 2018). Specifically, van Puyvelde et al. (2011) demonstrated the secretion of effectors by plant growth promoting *Azospirillum brasilense* in the presence of auxin, as an adaptation mechanism of the microbe to the plant root. Taken together, microbial effectors give a competitive advantage to the microbes, enabling their establishment within the plant tissue via the manipulation of the plant auxin signalling.

The role of plant hormones in the interaction with the fungus *S. indica* has been well studied and reviewed (Arma et al., 2012; Gill et al., 2016; Xu et al., 2018). These reviews highlight alterations of the plant hormone levels for IAA, cytokinin (CK) and gibberellins (GA) that are associated with increased plant growth and yield, while changes in abscisic acid (ABA), ethylene (ET) and salicylic acid (SA) upon fungal colonisation enhance plant stress tolerance. The observed growth promoting effect in various host plants by *S. indica* could be due to reprogramming of auxin signalling. Microbe-associated plant phenotypes such as auxin-induced root branching would be beneficial for plants as well as it can facilitate nutrient acquisition in soil (Lee et al., 2011; Shen et al., 2018). There has been research into possible *S. indica*-derived factors that may be responsible for the effective interaction and manipulation of host plants aiming to unravel the mechanisms of action evolved and employed by the fungus (Sirrenberg et al., 2007). These factor can be bioactive metabolites such as auxins (Sirrenberg et al., 2007) or fungal effector proteins (Rafiqi et al., 2013).

Microbial effectors are proteins that are transferred into the plant cells by the fungus and physically interact with molecules – targets of plant pathways (Boller and He, 2009; Dodds and Rathjen, 2010; Mukhtar et al., 2011). *S. indica* putative effectors were found to represent 10% of the fungal genes induced during the barley-fungus interaction (386 genes) (Zuccaro et al., 2011). Rafiqi et al. (2013) studied the secretome of *S. indica*, with special focus on the effectors, highlighting the important role they seem to hold in the *S. indica* – plant interaction. They predicted *S. indica* putative effectors with certain function, e.g. protease or chitin-binding, which are known to contribute to fungal virulence, using whole genome sequencing of the fungus along with the use of *in silico* analysis. However, these putative effectors have not been yet associated with auxin signalling. It is yet unknown how the fungus manipulates auxin signalling and changes the distribution/concentration of auxin in root cells (Xu et al., 2018).

Considering the high relevance of auxin for the establishment of plant-microbe interactions, including, *S. indica* symbioses, I hypothesized that certain effectors of *S. indica* have the ability to alter auxin signalling in plants. I specifically addressed this question as the manipulation of host auxin signalling might represent

a key advantage for *S. indica* in outcompeting other microbes and, hence, modifying root microbiomes. In addition, considering the broad host spectrum of *S. indica*, auxin might represent a universal target for *S. indica* to manipulate its different hosts. For this study, single *S. indica* effectors were analysed, with no presence of the fungus. I investigated the effect of the fungal effectors in altering *A. thaliana* growth (as auxin-regulated process) and auxin signalling using a combination of systems.

The experimental approach used in this chapter consisted of a protoplast screening and *in vivo* assessment of the function of putative *S. indica* effectors. More specifically, I used a high-throughput screening in *A. thaliana* leaf protoplasts which were transformed with individual *S. indica* putative effectors, along with an auxin-responsive gene in association with a luciferase reporter and tested using synthetic auxin. The effect of each effector in the expression of the auxin responsive gene was evaluated using the signal from the luciferase expression. The putative effectors were transformed in *A. thaliana* plants under constitutive promoter and used for root growth inhibition assays on plates and basal gene expression quantification.

3.2 Results

3.2.1 Leaf protoplast screening of effectors to identify effector function in response to auxin

A library of *S. indica* effectors was generated in the Schäfer laboratory, (University of Warwick). Dr. Frances Burton, former PhD student, identified 852 *S. indica* effector candidates using RNA sequencing of *A. thaliana* roots colonised by the fungus, 3 and 10 dai (unpublished data). These effector candidates were filtered based on their sequences and resulted to a final list of 150 effector candidates (see list with number-coded effectors in Table S3.1) (unpublished data). For this thesis, in a first step the *S. indica* effectors were screened for affecting the expression of the auxin marker gene *GH3.3* using an *A. thaliana* leaf protoplast assay as summarized in Figure 3.1. The gene *GH3.3* is commonly used as an indicator for increased auxin levels, as its expression is upregulated in response to auxin (Stringlis et al., 2018). Therefore, the promoter of the auxin-inducible *GH3.3* promoter was fused to a luciferase (*LUC*) gene. Leaf protoplasts were transformed with three plasmids simultaneously, *pGH3.3::LUC*, *pAtUBQ10::GUS*, and *S. indica* effector (SIE) constructs (*35S::SIEs*), or the empty vector *pAtUBQ10::HA-GFP* as a control. Transformed protoplasts were then treated with synthetic auxin (NAA) and luciferase activity was measured, as described in the methods section. In total, 150 effectors were analysed in this assay.

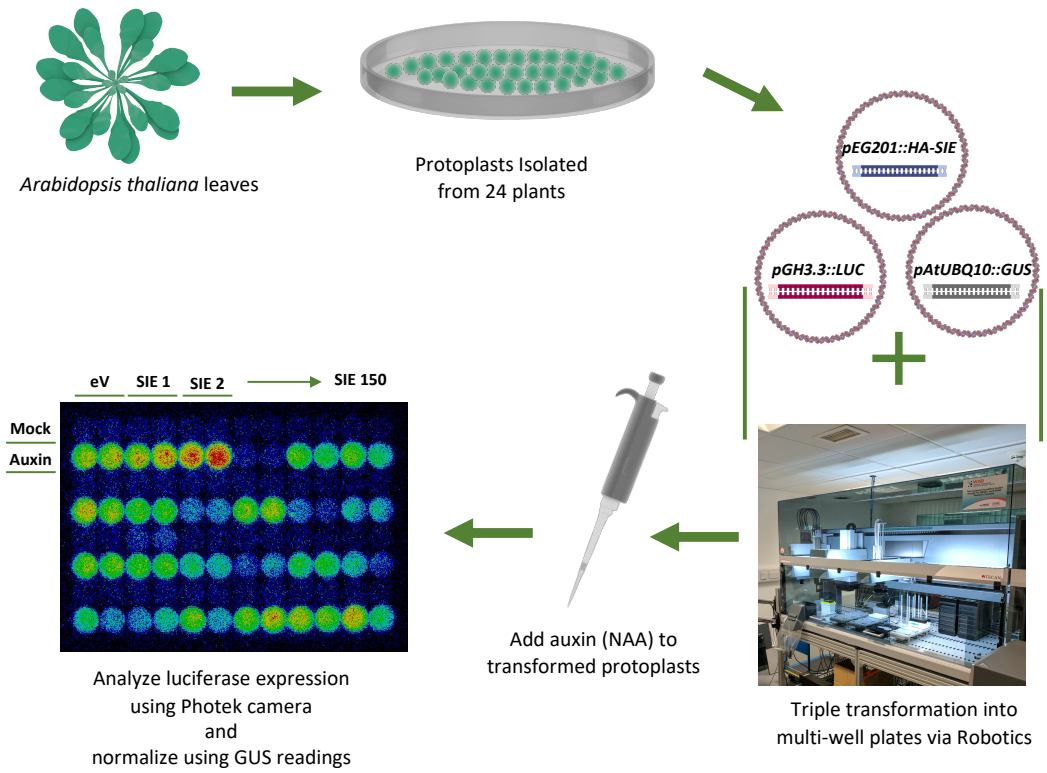


Figure 3.1: Pipeline for effector screening using the Arabidopsis protoplast assay. Protoplasts were isolated from leaves of 24 plants, co-transformed with three plasmids, SIE construct, pGH3.3::LUC and pAtUBQ10::GUS, using a robotic system. The transformed protoplasts were treated with auxin (NAA) or sterile H_2O (Mock) and subsequently luciferase expression was measured using Photek camera, and GUS readings for normalization of the luciferase expression. [Arabidopsis plant, petri dish, protoplasts, plasmids and pipette] illustrations courtesy of somersault18:24, Creative Commons license CC BY-NC-SA 4.0

From the 150 putative effectors tested, twenty effectors with the most significant effects were used for further analyses. Effector-mediated responses were ranked from the strongest induction to the strongest suppression of the expression of *GH3.3*. For this ranking, the ratio of the difference in the LUC activity of the effector to that of the empty vector (eV) in the mock treatment was multiplied by the ratio of the difference of the *LUC* expression of the effector to that of the empty vector (eV) under the NAA treatments (see equation 2.1 in methods section) (Table S3.2). Based on these calculations, the 20 effectors that more strongly suppressed or induced the expression of the auxin marker were used for further experiments;

these were the *S. indica* effectors (SIEs) 149, 87, 67, 83, 7, 16, 24, 88, 56, 148 (suppressing *GH3.3* expression) and effectors SIEs 10, 144, 64, 75, 126, 76, 69, 9, 81, 59 (inducing *GH3.3* expression) (Figure 3.2).

The effectors with the strongest response to auxin were used to generate *A. thaliana* plants that constitutively expressed the effector under the 35S promoter. Expressing the effectors in *A. thaliana* would allow us to study the effect of each effector on plant growth and its response to the addition of exogenous auxin. SIEs 12, 30, 77, 103, 106 strongly suppressed the activity of the marker gene but were not chosen for further analysis as the strongly reduced LUC activity in SIE-expressing protoplasts was further associated with low GUS expression, the marker used for normalisation, indicating cell death or a strong general interference with cellular transcription and/or translation.

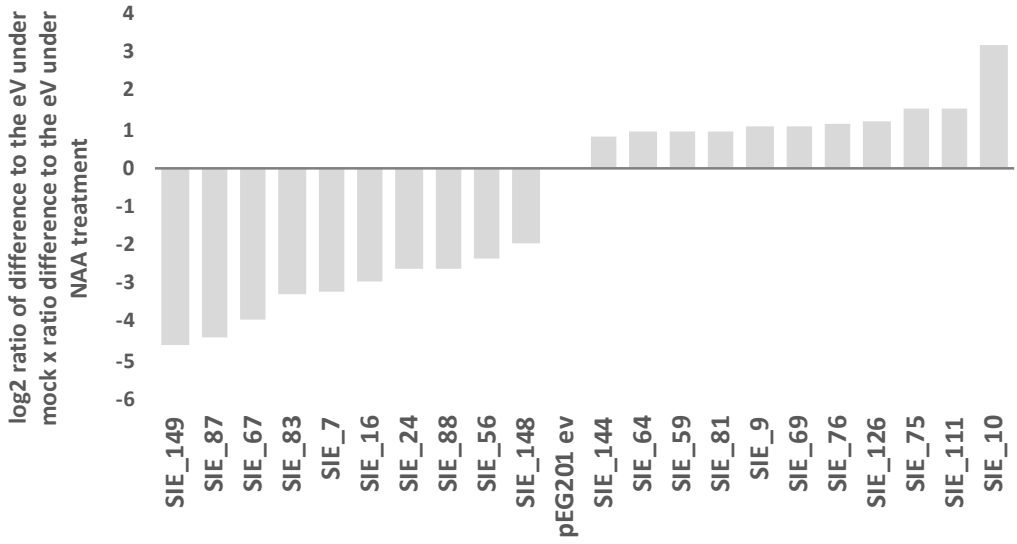


Figure 3.2: S. indica effectors that significantly affected the GH.3.3 expression in A. thaliana protoplasts. Bars represent log2 ratio of the fold changes of mock-treated samples multiplied by the fold change of auxin–treated protoplasts, of the top candidate effectors that suppress or induce GH.3.3 expression in protoplasts in comparison to the empty vector (ev) pEG201. Data are based on normalized luminescence from protoplasts transformed with an effector over protoplasts transformed with the empty vector with (ratio treatment) or without (ratio mock) treatment.

3.2.2 Plants expressing *S. indica* effectors can affect root growth

The protoplast assay based on *GH.3.3* expression was the initial screening tool to identify *S. indica* effectors with strong effect on auxin signalling. This screening was followed by root growth inhibition assays, which were performed on a subset of the initial effector pool (20 effector candidates, less than 15%). This assay was carried out to see if the effectors that caused transcriptional changes in the auxin pathway in protoplasts would also result in an altered growth when constitutively expressed at the whole plant level. This approach could provide robust evidence for the involvement of the effectors in signal transduction and growth regulation processes. For these experiments the auxin compound IAA was used, as is the auxin molecule more commonly found in nature (Woodward and Bartel, 2005).

A. thaliana Col-0 plants were stably transformed with the different chosen effectors and propagated to obtain T1 generation plants by Dr. Silke Lehmann (P. Schäfer group, University of Warwick). T2 seeds from three independent transformation lines were pooled to perform the root growth analysis in order to compensate for possible positional effects of the transgene. Such T2 pools were tested for 10 effectors that suppressed auxin responses in the protoplast screening and 10 effectors that induced the response (Figure 3.2). In order to identify the right auxin concentration for the analysis, root growth inhibition of Col-0 seedlings was measured using different concentration of IAA (Figure 3.3). Plants grown at 40 nM IAA showed 32% reduction of root length in comparison to mock and this concentration was used for subsequent root growth inhibition phenotyping in the presence of auxin.

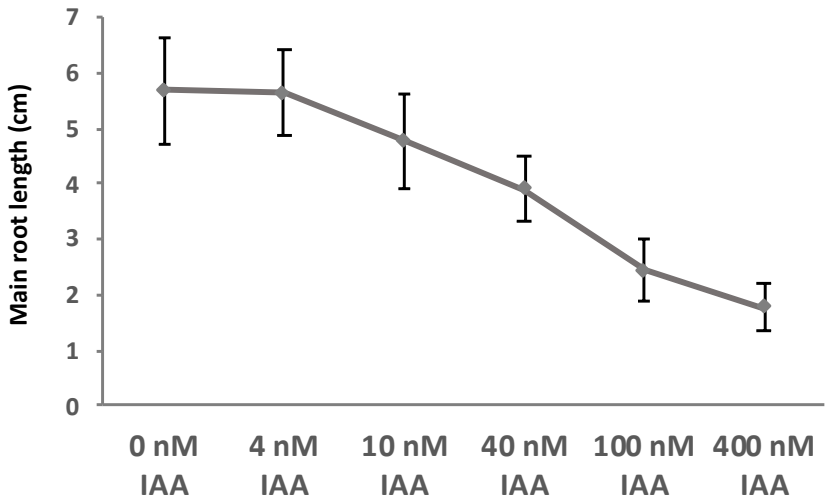


Figure 3.3: Root growth inhibition in the presence of different concentrations of auxin (IAA). Average main root length (cm) of 14 days old Col-0 seedlings grown on different IAA concentrations from 0 to 400 nM IAA. Data were collected from 126 plants per treatment (3 biological replicates, 42 plants per replicate) and mean values are shown with standard deviations.

Initially, the root growth inhibition assays were performed using the T2 pools of plants expressing the SIEs. *A. thaliana* lines expressing the 20 effectors were tested on 40 nM IAA to measure root growth on each plate. SIE88, 144, 64, 24 and 59 had significantly longer primary roots than GFP control lines on control plates. Lines expressing SIE10, 75, 76 and 81 had significantly shorter roots than GFP lines on control plates (Figure 3.4A). On the other hand, lines expressing SIE24, 144, 59, 88, 67, 87, 16, 149, 69 and 64 grow longer roots in the presence of auxin in comparison to control plants expressing GFP. *A. thaliana* lines expressing SIE75, 76 and 10 had smaller roots as compared to control plants under inhibitory auxin concentration (Figure 3.4B). Notably, all the lines that had significantly different growth on the control plates, had also significantly different growth in the presence of IAA. An exception was the line expressing SIE81, which did not show significantly different growth in the presence of IAA.

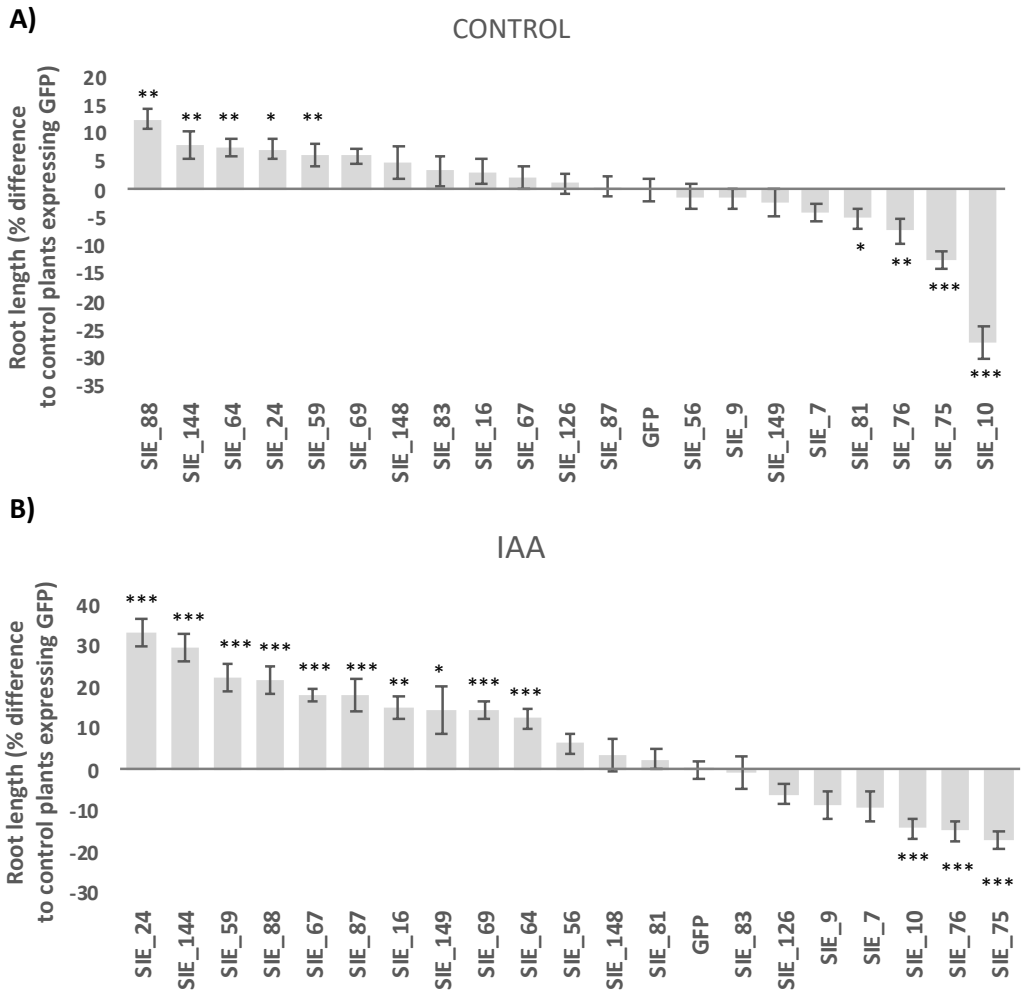


Figure 3.4: Main root length relative to the control plants expressing GFP treated with DMSO (control) or 40 nM IAA. Root length of plants presented as percentage (%) of the root length (cm) of GFP-expressing control plants grown in the same conditions; ***A)*** *control MS plates with DMSO;* ***B)*** *MS plates with 40 nM IAA. Data were collected from 42 plants per line and treatment. Mean values are shown with standard errors. Significance is based on t-test with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$*

By comparing the results from protoplast screening results with the results of the root growth inhibition assay in the presence of auxin (40 nM IAA), nine effectors, SIE149, 87, 67, 16, 24, 88, 76, 75 and 10, had similar effects when expressed in plants under a constitutive promoter and expressed in leaf protoplasts. On the other hand, there are certain contradictions between the protoplast screening method and the root growth inhibition assay. For example, SIE144 and 75 induced

the expression of *GH3.3* in protoplasts, in the presence of inhibitory auxin concentrations (Figure S3.1) but showed increased root growth in the presence of inhibitory auxin concentration (SIE144) or reduced root length (SIE75). However, 45% of the protoplast screening results were confirmed when effectors were expressed in whole plants and tested on plates containing IAA.

Plants expressing SIEs that demonstrated the strongest effect on root growth in the presence of inhibitory auxin levels were tested as independent lines and not pools, in order to exclude the possibility that the observed effects were due to positional effects of SIE insertions in the genome. *A. thaliana* lines expressing SIE75 could not be analyzed due to low seed germination rate of all lines carrying this construct. The screening of the independent lines expressing the selected SIEs revealed two effectors, SIE10 and 24, with strong effects on plant growth as well as consistency of the effect among the independent lines, although with some variation (Figure 3.5). *A. thaliana* lines 10_6 and 10_8 showed significantly decreased root length both in the presence and absence of IAA in comparison to GFP-expressing plants, and similar to the results of the pool. Line 24_1 showed significantly increased root length on control and IAA plates in comparison to GFP expressing plants, and similar to the pool of the three independent lines. Line 24_3 had a similar increase of root length to the pool on IAA plates, but not on the control plates. Line 24_2 showed no significant difference to GFP lines (Figure 3.5).

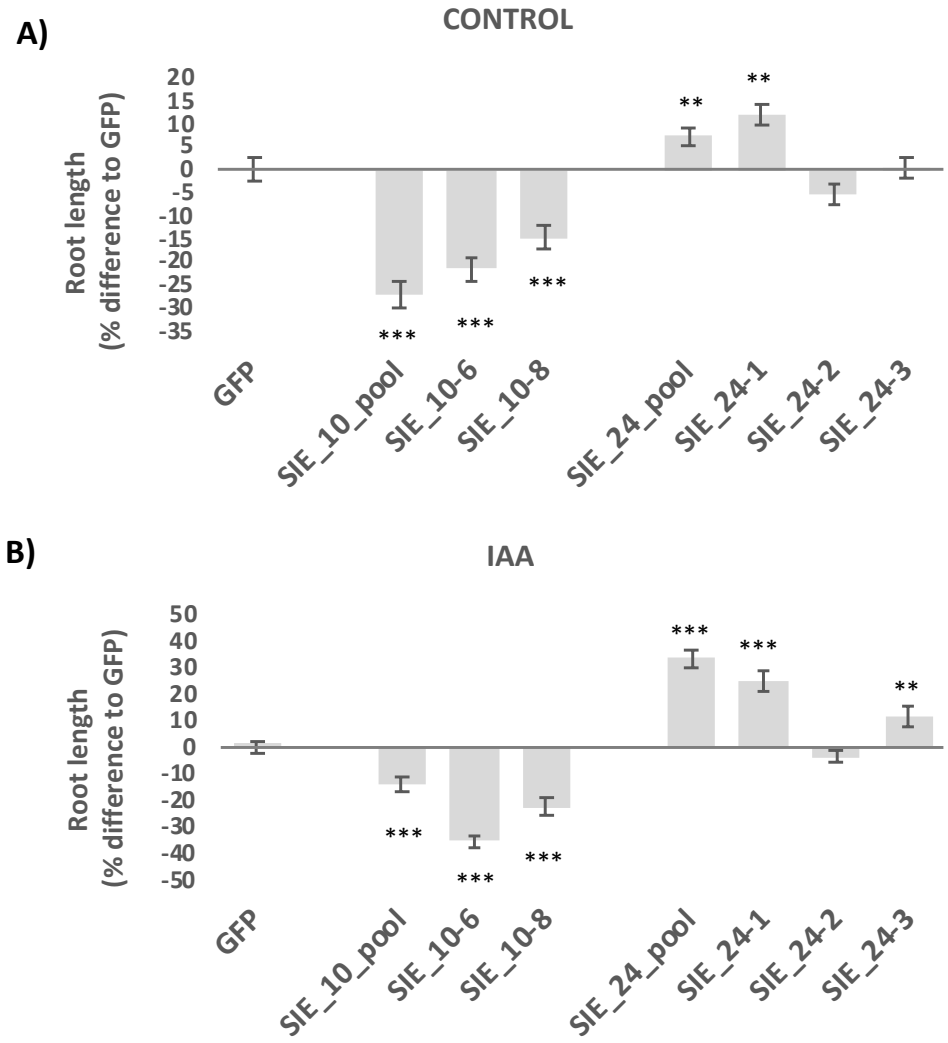


Figure 3.5: Main root length of plants expressing SIEs (as pools and as independent lines) relative to control plants expressing GFP when grown on control and 40 nM IAA plates. Root length of plants is presented as percentage (%) of the root length (cm) of control plants. ***A)*** *Control MS plates with DMSO;* ***B)*** *MS plates with 40 nM IAA. Data were collected from 42 plants per line and treatment. Mean values are shown with standard errors. Significance is based on t-test with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$*

In summary, many plants expressing SIEs were affected in root growth, in control condition but also under inhibitory auxin levels. This effect was initially investigated using pools of *A. thaliana* lines expressing the same effectors in order to eliminate any possible positional effect on the transgene. Independent lines from these SIEs showing the strongest effect as pools were selected and tested

individually. Therefore, lines 10_6 and 24_1, expressing SIE10 and SIE24, respectively, were chosen for measuring the basal expression of auxin responsive genes.

3.2.3 Basal expression of auxin responsive genes is altered in *A. thaliana* seedlings expressing *S. indica* effectors

Auxin responses are often characterised using three groups of auxin-responsive genes: *GH3s*, *IAAs* and *SAURs* (Weiste and Dröge-Laser, 2014). *GH3* genes are involved in the conjugation of amino acids to IAA and are responsible for auxin homeostasis, regulating plant growth and responses to stress (Park et al., 2007; Staswick, 2005). On the other hand, the expression of *IAA* genes is known to be depleted in the absence of auxin and rapidly induced with the addition of auxin (Hagen and Guilfoyle, 2002). The gene *IAA5* is found to have increased expression as a response to inhibitory auxin concentrations (Uchida et al., 2018). Therefore, *GH3.3* and *IAA5* are robust genes for the investigation of changes in auxin signalling mediated by *S. indica* effectors.

A. thaliana lines expressing SIE10 and 24 were used to examine whether the altered root growth under control and inhibitory auxin levels was associated with an altered plant auxin signalling. Root and leaf samples from 11 days old seedlings (growing on MS plates without IAA) expressing GFP as a control, SIE10_6 or SIE24_1 were sampled for RNA extraction. Generated cDNAs were subsequently used to quantify the expression of auxin responsive genes *GH3.3* and *IAA5* by RT-qPCR.

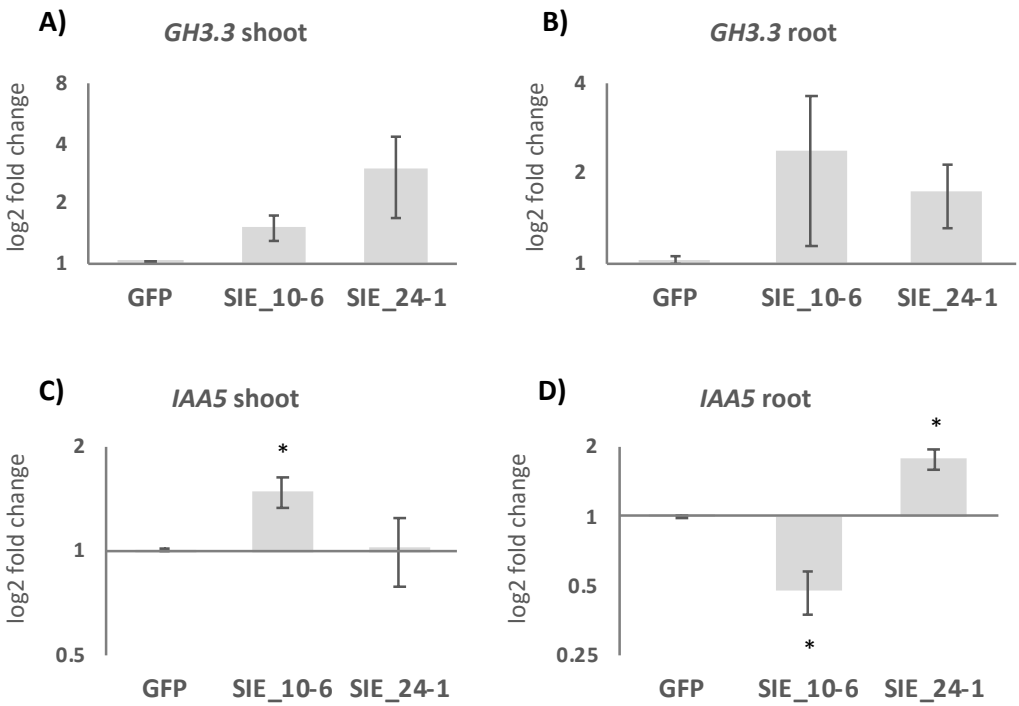


*Figure 3.6: Basal expression of the auxin-responsive genes GH3.3 and IAA5 in Arabidopsis lines expressing GFP, SIE10 or SIE24. log2 fold change of gene relative expression (ΔΔCt) of auxin reporter gene GH3.3 (A, B) and IAA5 (C, D) in shoot and roots of Arabidopsis plants expressing SIE10 or SIE24., Mean fold change from 2 biological replicates are shown with standard errors. Significance is based on t-test with * $p < 0.05$*

The basal expression of *GH3.3* gene was not significantly altered in leaves or roots of *A. thaliana* lines expressing SIEs (Figure 3.6A and B). The basal expression of the auxin responsive gene *IAA5* was significantly upregulated in the shoot of plants expressing the SIE10_6, but not SIE24_1 (Figure 3.6C). In contrast, *IAA5* expression in roots was significantly downregulated in lines expressing SIE10_6 and significantly upregulated in SIE24_1 (Figure 3.6D). The *IAA5* gene expression in the roots expressing SIE10 and SIE24 follows the root growth of these lines, with line 10_6 having decreased root growth and 24_1 increased, in comparison to the GFP line.

3.3 Discussion

One of the factors that can determine the proliferation and survival of a species is its ability to gain a competitive advantage (Lareen et al., 2016; Stubbendieck et al., 2016). Here I demonstrated that *S. indica* may gain that advantage through its effector proteins, obtaining the ability to manipulate the plant auxin pathway resulting in altered growth and response to inhibitory auxin concentrations. Manipulating the auxin pathway is a strong competitive advantage for a microbe, as auxin controls many other processes of the plant (Vanneste and Friml, 2009) and has a key role in plant-microbe interactions (Barker and Tagu, 2000; Ludwig-Müller, 2000; Vadassery et al., 2008). Recently, attention has been drawn on how microbial effector proteins can influence the microbiome and its interaction with the plant (Snelders et al., 2018). It is possible that those effector proteins enable the fungus to efficiently manipulate and colonise its host root system, but also potentially to create a favourable environment around it. Using those effectors, the fungus may be more competitive against other colonisers, or even manipulate the plant to shape a different microbiome around it (Haney and Ausubel, 2015), in order to benefit plant growth and health. Effector research may reveal which of these effector proteins can give an advantage to the entire microbiome or to specific microbiome members and how they use them to shape their environment. This study raises a lot more questions and possibilities in plant-microbe interaction research, as effectors of beneficial microbes may be the key to unravelling basic mechanisms of the complex microbe-microbe and plant-microbe interactions as well as source for innovation and subsequent applications in agriculture.

The role of *S. indica* effectors in plant auxin signalling was investigated using different methods. A high throughput screen of 150 SIEs using an *A. thaliana* leaf protoplast assay with an auxin reporter resulted in a selection of 20 effectors (Figure 3.2). This selection was done using the multiplied ratio of mock-treated effector/mock-treated control and NAA-treated effector/NAA-treated control (Figure 3.1), as an effort to picture the whole scale of the effect of SIEs and identify those SIEs with the strongest effect on auxin response. However, some SIEs that

affect such plant auxin responses and growth during *S. indica* colonisation may have not been identified in this screening step. Nevertheless, these are putative effectors identified based on certain characteristics of their sequence that are found in known effectors (Rafiqi et al., 2013), and one would need to prove the transfer of effectors into host cells and show their site and tissue of action (Schilling et al., 2014); e.g. have an effect on root cells as the tissue colonized by *S. indica* (Deshmukh et al., 2006b). Furthermore, this work focuses on only one aspect of effectors function and is using one auxin responsive gene, thus, comprehensive research would potentially reveal more effectors with strong effect on plant growth and signalling. In addition, even if an effector does not have an effect on leaf cells, it may still have an impact on plant signalling when expressed at the whole plant level or in specific tissues. The development of a high-throughput system for root protoplast screening of effectors would give a more complete picture of their activity. Root protoplasts are sensitive and have a low transformation rate (Mathur et al., 1995), while this high throughput method required a large amount of cells with high transformation and survival rate. Nevertheless, this screening with leaf protoplasts revealed *S. indica* effectors that strongly affect auxin signalling, as confirmed in experiments with whole plants. A summary of the results is presented in the Tables 3.1 and 3.2.

Table 3.1: Summary table of results from different methods examining the effect of SIEs on plant root growth and auxin signalling, indicating the selection of the candidates in each step.

	***GH3.3* expression**	**Root growth inhibition assays of pools**	
effectors	**Protoplast screening**	**Control**	**40 nM IAA**
SIE_149	**suppressor**	**no effect**	**increased**
SIE_87	**suppressor**	**no effect**	**increased**
SIE_67	**suppressor**	**no effect**	**increased**
SIE_83	**suppressor**	**no effect**	**no effect**
SIE_7	**suppressor**	**no effect**	**no effect**
SIE_16	**suppressor**	**no effect**	**increased**
SIE_24	**suppressor**	**increased**	**increased**
SIE_88	**suppressor**	**increased**	**increased**
SIE_56	**suppressor**	**no effect**	**no effect**
SIE_148	**suppressor**	**no effect**	**no effect**
control	**pEG201 eV**	**GFP**	**GFP**
SIE_144	**inducer**	**increased**	**increased**
SIE_64	**inducer**	**increased**	**increased**
SIE_59	**inducer**	**increased**	**increased**
SIE_81	**inducer**	**decreased**	**no effect**
SIE_9	**inducer**	**no effect**	**no effect**
SIE_69	**inducer**	**no effect**	**increased**
SIE_76	**inducer**	**decreased**	**decreased**
SIE_126	**inducer**	**no effect**	**no effect**
SIE_75	**inducer**	**decreased**	**decreased**
SIE_10	**inducer**	**decreased**	**decreased**

As control, pEG201 eV was used in the protoplast assay and A. thaliana lines expressing GFP for the root growth inhibition assays. "suppressor" and "inducer" refer to the GH3.3 expression in the protoplast screening, "increased" and "decreased" refer to root growth of SIE-expressing lines on plate assays and "no effect" indicates non-significant differences based on t-test ($p < 0.05$).

Table 3.2: Summary table of results from different methods examining the effect of SIE 10 and 24 on plant root growth and auxin signalling.

	GH3.3 expression	Root growth inhibition assays		Basal gene expression of *IAA5*	
Lines	**Protoplast screening**	**Control**	**40 nM IAA**	**In leaves**	**In roots**
SIE_24	suppressor	increased	increased	-	-
Line 24_1		increased	increased	no effect	increased
Line 24_2		no effect	no effect	-	-
Line 24_3		no effect	increased	-	-
control	pEG201 eV	GFP	GFP	GFP	GFP
SIE_10	inducer	decreased	decreased	-	-
Line 10_6		decreased	decreased	increased	decreased
Line 10_8		decreased	decreased	-	-

As control, pEG201 eV was used in the protoplast assay and A. thaliana lines expressing GFP for the root growth inhibition assays and for the basal gene expression assesement."suppressor" and "inducer" refer to the GH3.3 expression in the protoplast screening, "increased" and "decreased" refer to root growth of SIE-expressing lines on plate assays, or the basal IAA5 gene expression and "no effect" indicates non-significant differences based on t-test ($p < 0.05$).

3.3.1 Constitutive expression of *S. indica* effector proteins in *A. thaliana* plants affect root growth

Auxin regulates plant growth, consequently suppressors of auxin signalling enable roots to grow longer (Teale et al., 2006). Indeed, some effectors that were characterised as suppressors of auxin signalling, as observed by the suppression of *GH3.3* expression in the protoplast assay, showed increased root length in the presence of inhibitory auxin concentration (Table 3.1). On the contrary, three of these effectors that were characterised as inducers of auxin signalling showed decreased root length, both in basal conditions and inhibitory auxin concentrations (Table 3.1).

However, certain effectors showed contrasting effect between the two assays, the protoplast screening and the root growth inhibition assay (Table 3.1). This phenomenon (inducers of auxin signalling in protoplast assay and suppressors

of auxin signalling in the root growth inhibition assay) could be explained by differential levels of expression of the given effectors, due to the transient and stable expression methods. Alternatively, the observed SIE effects on *GH3.3* expression might be caused by ectopic effector expression. In addition, SIEs might have different effect depending on the plant tissue (Schilling et al., 2014), which could explain the observed phenotypes. For example, Strehmel et al. (2016) demonstrated that *S. indica* inoculation has substantially different effect in *A. thaliana* root and the leaf tissue, with many plant molecules being increased in the roots upon inoculation, such as amino-acids, but not in the leaves. In support of this, auxin signalling has been found to be differentially regulated depending on plant tissue, with different auxin pathways and genes being involved in the shoots and in the roots (Novák et al., 2012). For this reason, coupling this high-throughput leaf protoplast screening with a root (or other tissue if necessary) protoplast screening would give information of the activity of effectors in different tissues, whereas characterising the transcriptional profile of those plants could reveal a first insight into the mode of action of those effectors.

The SIE24 was found to suppress auxin signalling in leaf protoplasts, as well as inducing main root growth in the presence and absence of auxin (Table 3.2). Since immune responses are strongly associated with auxin signalling (Naseem et al., 2015), *S. indica,* may be using SIE24 to manipulate auxin signalling and subsequently suppress any immune response by the plant, in order to establish colonisation within the root. However, this could be observed with a transcriptomics study. Similarly, Chen et al. (2007) showcased the effect of a single effector on auxin signalling using *A. thaliana* lines expressing the *P. syringae* type III effector *AvrRpt2* under constitutive promoter and lacking *RPS2,* the *AvrRpt2*-responsive gene in the plant. They found that such lines had longer primary roots, like the SlE24, as well as increased number of lateral roots, and higher sensitivity to auxin. Therefore, SlE24 may play a key role in the establishment of *S. indica* in the *A. thaliana* root, similarly to the *P. syringae* effector *AvrRpt2*.

On the other hand, SIE10 was found to induce auxin signalling in leaf protoplasts and affect negatively the main root growth. The induction of auxin signalling by the fungus may be an alternative role to that of SIE24. It is suggested

that IAA can affect gene expression of certain microbes (Spaepen and Vanderleyden, 2011). For example, addition of IAA regulates the metabolic pathways of *Escherichia coli*, increasing its growth and adaptation to environmental stresses (Bianco et al., 2006a, 2006b). In addition, IAA in low concentrations can induce the growth of *Saccharomyces cerevisiae*, while in high concentrations it blocks its growth (Prusty et al., 2004). Taken together, the role of SIE10, as an inducer of auxin signalling, may have an effect in the interaction of microbes with the plant, possibly by securing its own proliferation within the root, limiting the presence of antagonistic microbes, or enabling the establishment of beneficial microbes within the root. However, when SIE10 is constituently expressed in the whole plant, the constant induction of auxin signalling may affect other mechanisms within the plant, compromising its growth.

The strong effect of *S. indica* effectors on *A. thaliana* auxin response is clear. In the protoplast screening, the activity of certain effectors led protoplasts potentially to cell death or had strong interference with cellular transcription and/or translation. Furthermore, the strong effect on plant growth, even the loss of seed viability in certain cases, indicates the detrimental effect of those effectors in plant health when expressed under constitutive promoter. Expressing microbial effectors under inducible promoter(s) as commonly used for such studies, e.g. estrogen-inducible or DEX-inducible expression of *P. syringae* effectors *HopAI1* and *AvrRpm1* respectively in plants (Mackey et al., 2003; Zhang et al., 2007), would enable the further investigation of these interactions in depth.

3.3.2 Constitutive expression of *S. indica* effector proteins in *A. thaliana* plants alter the expression of auxin responsive genes

Independent *A. thaliana* lines expressing SIE10 and SIE24 were grown (on ½ MS) and samples were collected for gene expression of auxin reporter genes. Lines 10_6 and 10_8 had similar phenotype with the pool of these, while lines 24_2 and 24_3 did not show the same phenotype as the pool, as they were not significantly different from the GFP line. These differences between independent lines might be either due to different expression levels of the effector and/or T-

DNA insertion positional effect. Therefore, lines 24_1 and 10_6 were chosen to study the expression levels of auxin responsive genes.

The genes used to study the effect of the effectors in the auxin signalling pathway were *GH3.3*, which is the auxin-responsive gene used in the protoplast screening, and another auxin marker *IAA5*. Lines 10_6 and 24_1 were found to alter the expression of gene *IAA5* in different ways. In contrast to the protoplast assay, expression of *GH3.3* was not affected in both lines, which might lie in the nature of the assay. It is important to recognise that a *pGH3.3::LUC* construct was used in the protoplast assay. Therefore, post-transcriptional regulation of *GH3.3* transcript might not operate as compared to effector-expressing plants. In addition, the physiological state of a protoplast can be quite different to that of a whole plants and pathways recruited by the effectors in single cell protoplasts are not necessarily accessible for manipulation or the manipulation is compensated (e.g. in communication with neighbouring cells). Auxin is a highly mobile hormone, and its transportation, as well as the gradient accumulation among cells play crucial role in the activity of auxin (Ljung, 2013; Sorefan et al., 2009; Teale et al., 2006), and can be observed in the whole plant context, as the protoplast system lacks such complexity. It further indicates the significance of using more than one reporter in confirming protoplast-based expression profiles.

The differential expression of *IAA5* in SIE10 or SIE24 lines might point towards an explanation of the different auxin-related phenotypes for both lines. An increase in the expression of *IAA5* is associated with an activation of auxin signalling pathway (Uchida et al., 2018) and *IAA5* is downregulated during suppression of auxin signalling (Dharmasiri et al., 2005). The expression of *IAA5* in the SIE10 line is up-regulated in the shoot and down-regulated in the root, indicating activation of auxin signalling in the shoot and suppression of auxin signalling in the root. Suppression of auxin signalling is associated with decreased susceptibility to pathogens (Navarro et al., 2006) and can have direct effect on the establishment of microbes in the plant, independently of their ability to produce auxin (Spaepen and Vanderleyden, 2011). While this ability of the fungus has been demonstrated, e.g. protection of barley from *Fusarium graminearum* (Deshmukh and Kogel, 2007), SIE10 may play a crucial role in the protection of the plant from

soil-derived pathogens. The possible induction of auxin signalling in the shoot by SIE10 could be a result of plant defence response to biotic factor (Bari et al., 2009), as the effectors are not expected to be found in leaf tissue since *S. indica* is a root endophyte. This might explain the compromised growth of the plant in the presence of the SIE10.

In contrast, *IAA5* was significantly up-regulated in roots (while expression in the shoot was unaltered) of *A. thaliana* lines overexpressing SIE24 (Uchida et al., 2018). Consistent with *IAA5* upregulation, line SIE24_1 showed increased root growth under mock conditions. Interestingly, SIE24_1 line showed improved root growth under inhibitory auxin concentrations. It has been shown that root growth inhibition caused by high concentrations of auxin can be overcome in the presence of *S. indica* and rescue plants from negative effects of the hormone (Vadassery et al., 2008). This could be a possible modulation of the pathway through a fungal effector such as SIE24. Furthermore, this activity of the effector may be a strategy to improve nutrient acquisition by affecting root architecture and length, similarly to plant growth promoting bacteria which improve plant performance through auxin-mediated enhancement of root structure, independently of their ability to synthesise IAA (Spaepen and Vanderleyden, 2011). Transcriptome analysis of this line could reveal first insights into the effect of this effector on auxin and plant signalling.

The beneficial effect of *S. indica* on host plants is likely a combination of different mechanisms. Effectors might take an essential role in manipulating plant signalling in order to stimulate those mechanisms. However, *S. indica* effectors can have very different effect in different plant tissue. Increased plant growth in the presence of the fungus has been associated with high levels of auxin in roots, whereas the auxin concentration in leaves was found unaffected (Hilbert et al., 2012; Lee et al., 2011). In my work, differential expression of auxin reporter genes in the shoot and the root samples indicate the presence of distinct mechanisms in the regulation of auxin or/and other signalling pathways in the different plant organs and tissues. Even though the fungus is present in plant roots, it may cause different responses in other plant tissues, potentially involving different effectors or/and different mechanisms. Unraveling the function of *S. indica* effectors in establishing

beneficial plant-microbe interactions and in activating beneficial activities in host plants can reveal mechanisms to improve plant growth under changing environments.

3.3.3 Conclusions and future perspectives

This work, as part of a larger project within the Patrick Schäfer group, illustrates the effect of a mutualistic fungus on plant signalling through effector proteins. It shows that single *S. indica* effectors affect plant auxin signalling and provides a high-throughput approach to study the involvement of microbial effectors on plant signalling of *A. thaliana*. Alongside, this work provided an insight to the effectiveness and the limitations of the pipeline used, as a high-throughput tool to identify effectors that influence plant signalling pathways. The alteration of the auxin-responsive genes expression was used as an indication for effector functions in to the regulation of the auxin pathway. However, my analysis was a first step, as a proof of concept, and more extensive research is required to understand the mode of actions of *S. indica* effectors on plant auxin signalling. Understanding thoroughly the involvement of microbial effectors in plant signalling could enable the development of new tools to stimulate plant growth and signalling, as well as the interactions with the microbiome.

High-throughput experiments using protoplast to test gene functions have been used before (Confraria and Baena-González, 2016; Li, 2016), often for the study of transcription factors (Wehner et al., 2017) and with the use of robotic systems (Dlugosz et al., 2016; Lenaghan and Neal Stewart, 2019). However, the method has not been used for the study of fungal effectors and their effect on plant signalling. Here, I demonstrate the usefulness of this system, established by group members of the P. Schäfer group. Some *S. indica* effectors that have been identified to have a strong effect on auxin signalling from the protoplast screening, showed also a strong effect when they were stably expressed in plants. This suggests a high efficiency of the protoplast screening to identify suitable candidates for further experiments, however, considering that their effect might change when expressed in whole plants. The results of my experiment could be supported further by

identifying host proteins interacting with effectors via methods such as Yeast-2-Hybrid or affinity tandem mass spectrometry (Mukhtar et al., 2011; Vasilescu et al., 2004; Weßling et al., 2014). This would elucidate potential mechanisms for effector functions *in planta* to support effector activities observed in the protoplast and whole plant screens.

Chapter 4: Influence of beneficial fungi on the composition of the *Arabidopsis thaliana* core root microbiome – a reductionist approach

4.1 Introduction

The progress in the development of new tools and experimental approaches has enabled us to study new aspects of interactions within microbiomes as well as microbial interactions with the host (Goodrich et al., 2017). In particular, advancements in sequencing technologies and computational power together with the reduction of sequencing costs has encouraged a large number of studies of soil and plant microbiome (Fierer, 2017; Prosser, 2015). One of the most common type of sequencing in microbiome research is the metataxonomic amplicon sequencing, amplifying a specific conserved region of the genome, usually 16S rRNA (Bacteria and Archaea) or the intergenic spacer (ITS) region (Eukaryotes). This technology has enabled researchers to determine general patterns in microbiome structure and diversity (Bulgarelli et al., 2012; Lundberg et al., 2012b; Peiffer et al., 2013; Tkacz and Poole, 2015). However, amplicon sequencing reveals only the structure of the microbiome but does not provide any information on the real function of the community or individual community members.

In recent years, there have been significant efforts to unravel the complexity of soil and plant microbiomes (Bulgarelli et al., 2012; Lundberg et al., 2012b) and to incorporate those findings into strategies for a more sustainable agriculture that has the potential to propel a next green evolution (Jez et al., 2016). In order to effectively exploit the potential of microbiomes, it is important to develop strategies that will enable us to study their functional capabilities (Bashiardes et al., 2018). Despite the extended research on microbiome structure (Schlaeppi and Bulgarelli, 2015) it is necessary to associate such structures with microbial dynamics and community functioning (Sánchez-Cañizares et al., 2017). Moreover, it is important to study and understand the whole functional range of each microbe individually and in a

community context. It has been shown that even species of the same genus can have substantially different lifestyles, from pathogenic to mutualistic, in dependency of environmental parameters (Hacquard et al., 2016; Hiruma et al., 2016), or the horizontal transfer of certain functional genes (Qiu et al., 2009). Understanding the functional diversity of microbes can enable us to develop strategies that will employ the beneficial traits within microbiomes for agriculture.

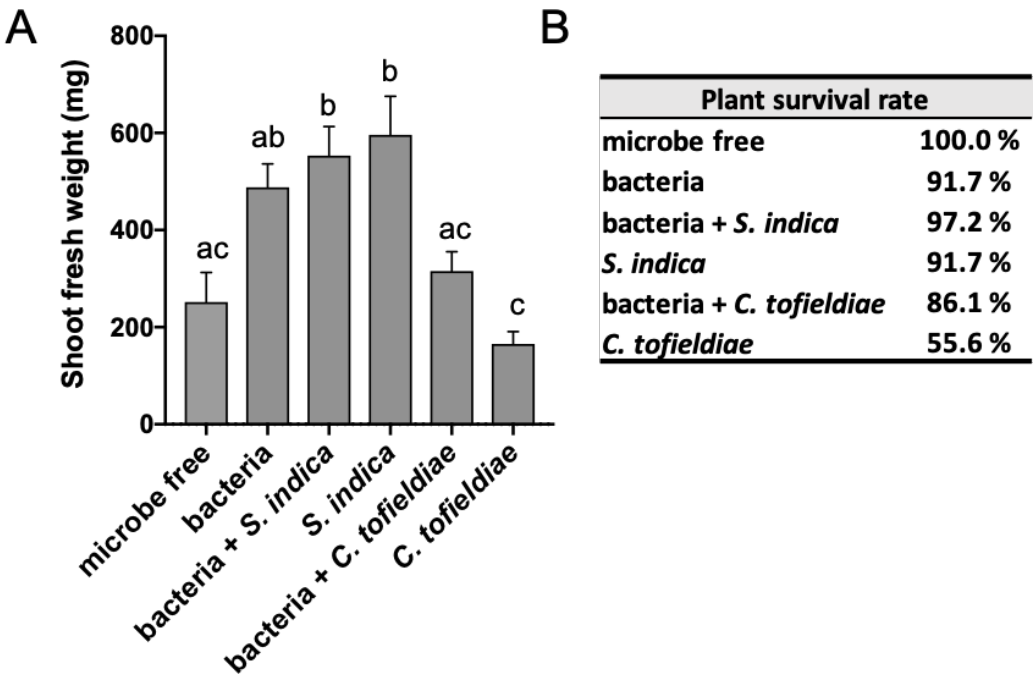
New approaches and experimental set ups have been employed in order to overcome those challenges originating from the complexity of microbiomes' functional diversity. There have been extensive efforts to isolate, cultivate and characterise microbial isolates (Bai et al., 2015; Levy et al., 2018; Mauchline et al., 2015). Those isolates can be used for the construction of synthetic microbial communities and test specific hypotheses in a very defined experimental environment (e.g. gnotobiotic systems) (Finkel et al., 2017; Lebeis et al., 2015), such as how the differences in the composition of microbial community influence the response of the plant to phosphate stress (Herrera Paredes et al., 2018). Such defined systems represent excellent tools to describe the dynamics and functional properties of individual microbes as well as microbiomes. This set up can further reveal key species that drive the community composition and function (Niu et al., 2017), recruitment patterns of the host (Bai et al., 2015), as well as microbe-microbe interactions and their effect on plant growth (Hartman et al., 2017; Herrera Paredes et al., 2018). Interestingly, Niu et al., (2017) used synthetic microbial communities on legume roots in a sterile system and showed that removing one strain from the system caused the total collapse of the microbial community. This indicates the significant role of each organism in the system and that even small changes can affect the whole structure of a microbiome. Furthermore, Hartman et al., (2017), using a similar setting with a small synthetic microbial community, showed that the negative effect of a microbe on maize plants could disappear in the presence of other microbes, even though all of them remained abundant in the plant root. These studies provide examples for interactions between microbes and the host that we probably could not uncover without the use of synthetic communities. Therefore, understanding the dynamics within the microbiome will allow us to understand and more effectively exploit it for a more sustainable crop production.

The aim of this study was to understand the complex interactions within microbial communities and to uncover their effect on plant growth. I hypothesized that the presence of a fungus in the roots might alter the bacterial microbiome, employing different bacteria. I also hypothesize that the presence of the fungus and the potential effect on the microbiome might affect plant growth. To test these hypotheses, we employed a synthetic bacterial community, initially isolated from *Arabidopsis thaliana* rhizosphere and characterized as *Arabidopsis* root core microbiome (Bai et al., 2015), and tested it in a gnotobiotic system. In collaboration with Stephane Hacquard and Prof. Paul Schulze-Lefert at the Max Planck Institute in Cologne (Germany) we investigated how 2 different fungi, the mutualist *Serendipita indica* and *Colletotrichum tofieldiae*, shape the composition of the *Arabidospis* core microbiome under controlled conditions. *S. indica* and *C. tofieldiae* are both root endophytes that assist the plant in phosphorus acquisition and improve plant growth (Hiruma et al., 2016; Wu et al., 2018). For this study, plant roots colonized by *S. indica* or with *C. tofieldiae* were exposed to the collection of about 200 bacterial species. This "core microbiome" was applied to *Arabidopsis* roots in order to monitor any changes in core microbiome composition and plant growth as a result of co-inoculation with or without *S. indica / C. tofieldiae*

4.2 Results

4.2.1 Effect of the fungi on plant growth and root core microbiome

The analysis of fresh shoot weight of plants at 6 weeks after transfer into the gnotobiotic system (Figure 4.1A) showed that the presence of the bacteria increased plant growth ($p<0.05$) from 25.2 g, (for microbe free samples; n=24) to 49.2 g average shoot weight in the presence of the bacterial microbiome (n=33). In the presence of *S. indica* as well as both *S. indica* and the bacterial microbiome, plant growth increased to an average shoot weight of 58.6 g (n=33) and 55.1 g (n=35), respectively ($p < 0.05$ and $p < 0.01$, respectively). The average fresh shoot weight of the *C. tofieldiae* treated plants was 16.53 g (n=20) and significantly lower than the bacteria treated plants ($p < 0.01$) and plants treated with *S. indica* ($p < 0.001$). In the presence of the bacterial community the average weight of the *C. tofieldiae*-treated plants was almost doubled, with an average of 31.57 g (n=31), however still significantly different from *S. indica*-treated plants ($p < 0.01$) and *S. indica*-treated plants including the bacterial community ($p < 0.05$). In addition, differential plant survival was observed in the different treatments. Notably, the survival rate of plants inoculated with *C. tofieldiae* was reduced by 44.4% as compared to microbe free plants, which was increased by 30.5% in the presence of the bacterial microbiome in the system (Table 4.1B).



Plant survival rate	
microbe free	100.0 %
bacteria	91.7 %
bacteria + *S. indica*	97.2 %
S. indica	91.7 %
bacteria + *C. tofieldiae*	86.1 %
C. tofieldiae	55.6 %

Figure 4.1: Growth and survival of Arabidopsis plants growing in flowpots system for 6 weeks. A) Fresh shoot weight. Mean values are shown with standard error and letters indicate the significance according to ANOVA followed by to Tukey's HDS ($p < 0.001$) (n=20-35, depending on survival). B) Plant survival rate shown as percentage.

By employing next generation sequencing we could reveal the Relative Abundance (RA) of *S. indica* and *C. tofieldiae* in the system (ITS amplicon) (Figure 4.2), as well as the structure and composition of the bacterial microbiome (16S amplicon) (Figures 4.3-4), as described in Materials and Methods. The analysis of the sequencing data showed the presence of fungal and plant DNA. The plant DNA was identified by blasting the OTU_1 (Table S4.1) using the NCBI BLAST platform (https://blast.ncbi.nlm.nih.gov/). As shown in Figure 4.3A, *S. indica* was significantly suppressed in the presence of the bacteria both in soil and root as compared to samples without bacteria ($p < 0.001$), based on RA. As a result, in the presence of bacteria, plant DNA was mostly amplified with ITS primers (Cheng et al., 2016). A similar effect was observed for *C. tofieldiae,* which was significantly reduced in the presence of bacteria both in the root and substrate matrix ($p < 0.001$), based on RA. In all samples, some reads (3-6%) belong to OTUs with very low abundance (~1-20 reads) and present in one or very few samples only. These OTUs are considered as

contaminants for this study, with 21% of the reads belonging to contaminants in the matrix samples from *C. tofieldiae* and bacteria-treated plants.

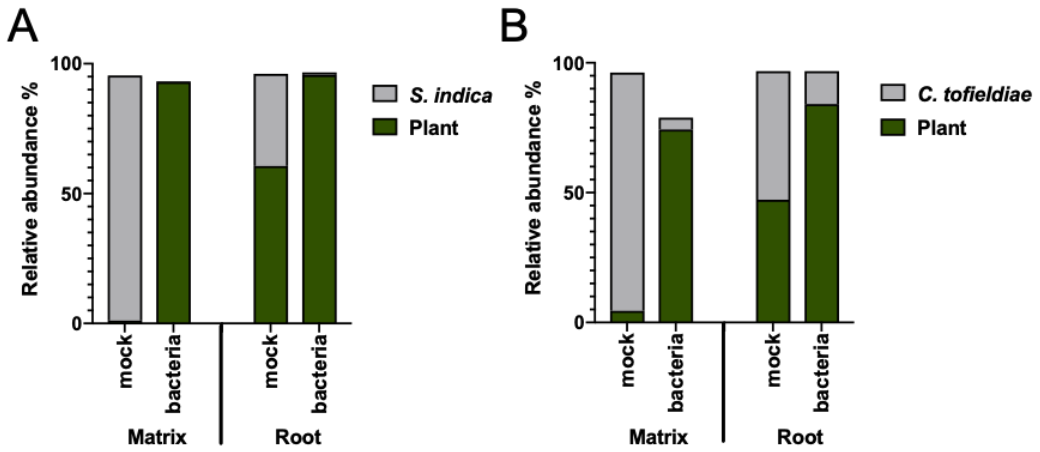


Figure 4.2: Relative abundance of fungi and plant. Relative abundance (RA) of fungi and plant (in %) in matrix and root samples, in the presence (bacteria) or absence (mock) of the bacterial community. Presented as a percentage of all reads in each sample. A) RA of S. indica and plant (%) and B) RA of C. tofieldiae and plant (%)

Focusing on the effects of fungi on the bacterial microbiome, the α-diversity was calculated using rarefied data and presented as the number of observed OTUs (richness) and Shannon Index (evenness) (Figure 4.3A, B). The input culture samples contain the most OTUs on average, however this number is not significantly higher than the number of observed OTUs in the roots under any treatment. The OTU richness in the matrix samples was the lowest on average and significantly lower than the input culture samples ($p < 0.01$ for the samples without fungi/*C. tofieldiae* and $p < 0.05$ for *S. indica*-treated samples). Regarding the evenness of the samples, calculated as Shannon Index, a similar pattern was observed. The input culture samples were the most even on average, but not significantly different than the root samples, nor from the matrix samples containing fungus-inoculated plants. The matrix samples that contain no fungus had significantly lower evenness than the input culture samples and the root samples with fungi ($p < 0.01$). In addition, root samples treated with fungi were significantly more even than the matrix samples containing fungi ($p < 0.05$). Summarising, higher sample richness and evenness was observed at the input culture

samples, followed by the root samples, while the matrix samples presented the lowest sample richness and evenness.

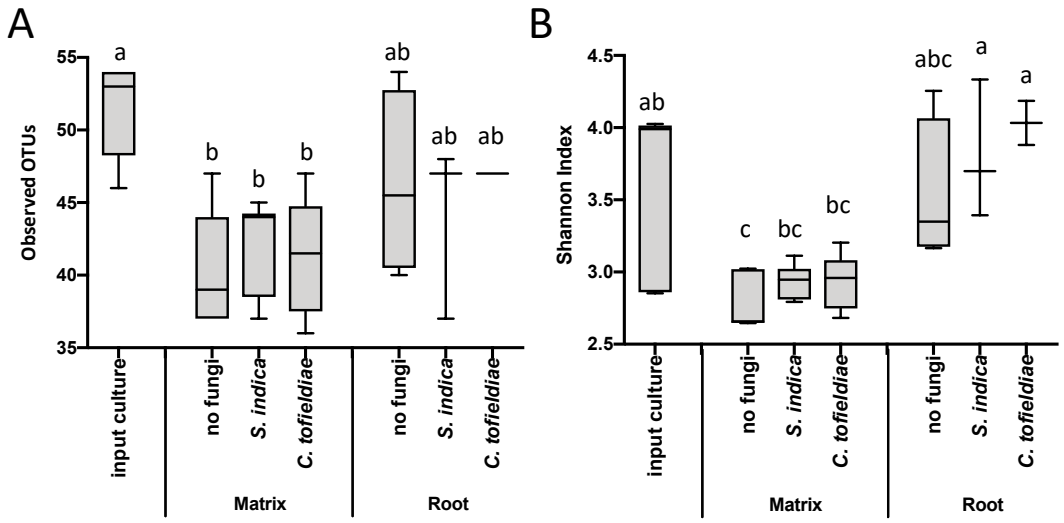


Figure 4.3: Boxplot of alpha diversity indices. Mean values are shown with standard error, whiskers represent quartile distribution, letters represent significant differences according to ANOVA followed by to Tukey's HDS ($p < 0.001$): A) Number of observed OTUs in each sample, B) Shannon Index of each sample. (n=2-6, depending on survival)

In order to detect differences between samples β-diversity was determined using Principle Coordinates Analysis (PCoA) based on Bray-Curtis distances on the rarefied OTU table. The results were visualized using the first two dimensions of PCoA (Figure 4.4). The input culture clusters independently from all samples whereas the rest of the samples have no specific cluster pattern.

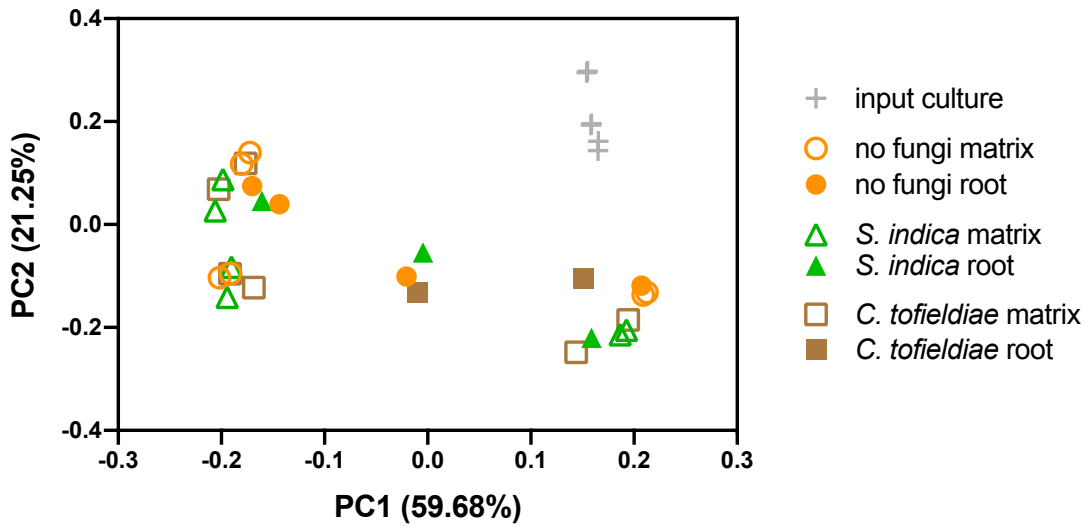


Figure 4.4: Bacterial microbiome structure was determined using principle coordinates analysis (PCoA). The first two dimensions of PCoA are plotted based on Bray-Curtis distances (rarefied reads used). The treatments are color coded and represented with different symbols, the root samples differentiate from the matrix as filled/empty respectively. (n=2-6, depending on survival)

In turn, the composition of the microbiome changes from the matrix to the root fraction (Figure 4.5). The significant changes between the compartments and the treatment was tested individually for each bacterial taxon with Analysis of Similarities (ANOSIM) (Table 4.1). Comparisons scoring R values close to 1 are highly different, whereas 0 indicate indifference; p values indicate the significance of the difference observed. The relative abundance of most of the bacterial taxa was significantly different from the input culture, with Bacilli significantly and strongly reduced in all compartments in comparison to the input (Table 4.1C). The β-Proteobacteria taxon is strongly but not significantly different in the root compartment in comparison to input (Table 4.1E).

Moving on to the differences between the matrix and the root compartment, Actinobacteria are significantly enhanced in the root compartment in comparison to the matrix (Table 4.1B). The relative abundance of Flavobacteria is significantly reduced in the roots treated with *C. tofieldiae* in comparison to all matrix samples, whereas β-Proteobacteria is significantly increased (Table 4.1A and E respectively). α-Proteobacteria have different relative abundance in the matrix an with the root, with more significant the difference of the relative abundance between the matrix

containing *S. indica* and the roots of all treatments (Table 4.1D). γ-Proteobacteria relative abundance in the matrix samples containing either *S. indica* or *C. tofieldiae* is significantly different from that in the root with no fungi and root with *S. indica* (Table 4.1F). Only the relative abundance of β-Proteobacteria tends to be different in the root between the different treatments, however this difference is not significant (Table 4.1E).

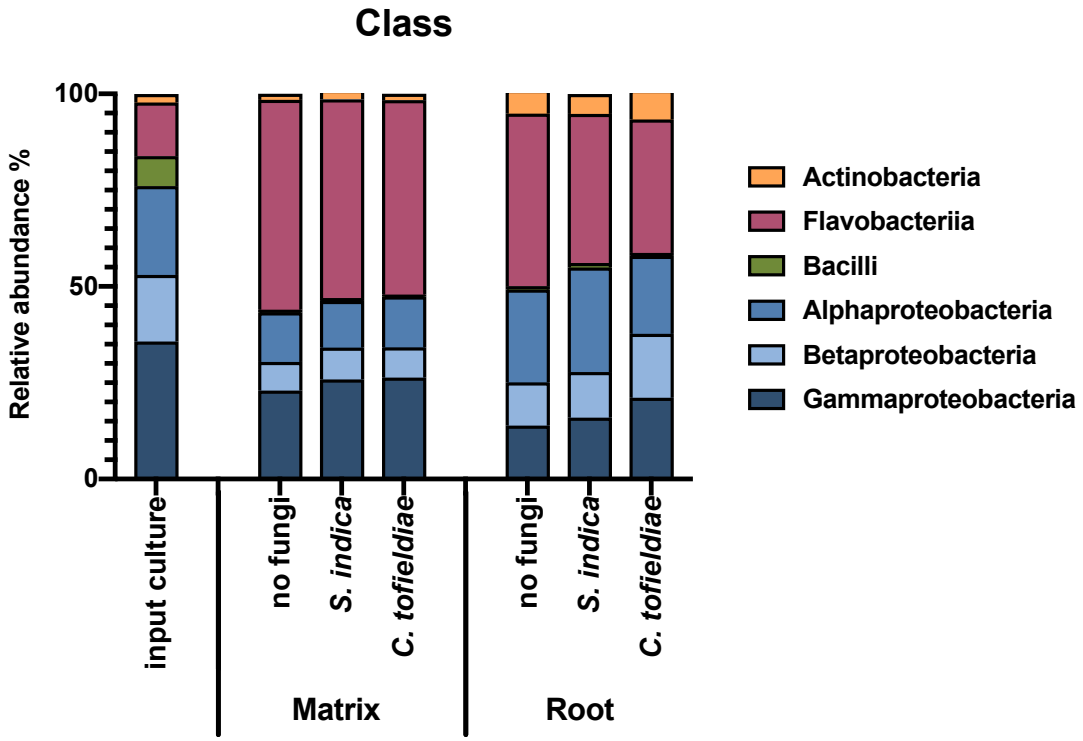


Figure 4.5: Relative abundance of each class in the matrix and root samples treated with S. indica, C. tofieldiae or no fungi. RA is represented using stacked bar plot of the calculated means of rarefied reads.

Table 4.1: Heatmap of significant changes in the microbiome composition.

A) **Flavobacteriia**

p values	input culture	no fungi soil	S. indica soil	C. tofieldiae soil	no fungi root	S. indica root	C. tofieldiae root
input culture							
no fungi soil	0.0023						
S. indica soil	0.002	0.4917					
C. tofieldiae soil	0.0027	0.7107	0.8997				
no fungi root	0.0063	0.1226	0.0477	0.0967			
S. indica root	0.0816	0.0627	0.0732	0.0817	0.8336		
C. tofieldiae root	0.0727	0.0347	0.0342	0.038	0.6697	0.5075	

R values	input culture	no fungi soil	S. indica soil	C. tofieldiae soil	no fungi root	S. indica root	C. tofieldiae root
input culture							
no fungi soil	0.837						
S. indica soil	0.8056	-0.01667					
C. tofieldiae soil	0.7963	-0.07037	-0.1074				
no fungi root	0.6111	0.1865	0.3373	0.1865			
S. indica root	0.4259	0.3951	0.3765	0.3086	-0.1481		
C. tofieldiae root	0.3333	0.9583	1	0.9583	-0.1786	0	

B) **Actinobacteria**

p values	input culture	no fungi soil	S. indica soil	C. tofieldiae soil	no fungi root	S. indica root	C. tofieldiae root
input culture							
no fungi soil	0.1469						
S. indica soil	0.0286	0.8175					
C. tofieldiae soil	0.0808	0.5234	0.5108				
no fungi root	0.0052	0.0051	0.0043	0.0111			
S. indica root	0.011	0.0127	0.0149	0.0116	1		
C. tofieldiae root	0.0354	0.0369	0.0339	0.0357	0.8015	0.7099	

R values	input culture	no fungi soil	S. indica soil	C. tofieldiae soil	no fungi root	S. indica root	C. tofieldiae root
input culture							
no fungi soil	0.1148						
S. indica soil	0.3315	-0.1037					
C. tofieldiae soil	0.2463	-0.05833	-0.0213				
no fungi root	0.5595	0.8056	0.881	0.7302			
S. indica root	0.6358	0.8395	0.9321	0.7531	-0.2963		
C. tofieldiae root	0.8646	0.9792	1	0.9479	-0.1429	-0.25	

C) **Bacilli**

p values	input culture	no fungi soil	S. indica soil	C. tofieldiae soil	no fungi root	S. indica root	C. tofieldiae root
input culture							
no fungi soil	0.0023						
S. indica soil	0.0024	0.9887					
C. tofieldiae soil	0.0022	0.4548	0.2917				
no fungi root	0.0048	0.6918	0.8846	0.204			
S. indica root	0.0123	0.1552	0.2871	0.0454	0.542		
C. tofieldiae root	0.0364	0.8985	0.8613	0.57	1	0.3908	

R values	input culture	no fungi soil	S. indica soil	C. tofieldiae soil	no fungi root	S. indica root	C. tofieldiae root
input culture							
no fungi soil	1						
S. indica soil	0.9963	-0.1444					
C. tofieldiae soil	0.9926	-0.01111	0.04259				
no fungi root	0.996	-0.1071	-0.1488	0.1032			
S. indica root	1	0.216	0.03704	0.3765	-0.08333		
C. tofieldiae root	1	-0.25	-0.2188	-0.07813	-0.3214	0.1667	

D) **Alphaproteobacteria**

p values	input culture	no fungi soil	S. indica soil	C. tofieldiae soil	no fungi root	S. indica root	C. tofieldiae root
input culture							
no fungi soil	0.0243						
S. indica soil	0.0065	0.5306					
C. tofieldiae soil	0.0112	0.5099	0.8715				
no fungi root	0.803	0.0233	0.0114	0.0161			
S. indica root	0.6136	0.0361	0.0118	0.0112	0.832		
C. tofieldiae root	0.7814	0.282	0.033	0.0684	0.3955	0.2093	

R values	input culture	no fungi soil	S. indica soil	C. tofieldiae soil	no fungi root	S. indica root	C. tofieldiae root
input culture							
no fungi soil	0.4259						
S. indica soil	0.6593	-0.0537					
C. tofieldiae soil	0.513	-0.03333	-0.09444				
no fungi root	-0.1349	0.4603	0.7341	0.5476			
S. indica root	-0.1049	0.5309	0.8765	0.7531	-0.1481		
C. tofieldiae root	-0.1667	0.09375	0.6458	0.4167	0.1429	0.25	

E) **Betaproteobacteria**

p values	input culture	no fungi soil	S. indica soil	C. tofieldiae soil	no fungi root	S. indica root	C. tofieldiae root
input culture							
no fungi soil	0.002						
S. indica soil	0.0024	0.3112					
C. tofieldiae soil	0.0024	0.7589	0.9402				
no fungi root	0.1313	0.0154	0.0542	0.0081			
S. indica root	0.2794	0.0131	0.0125	0.0119	0.741		
C. tofieldiae root	0.8935	0.0347	0.034	0.0356	0.1393	0.1019	

R values	input culture	no fungi soil	S. indica soil	C. tofieldiae soil	no fungi root	S. indica root	C. tofieldiae root
input culture							
no fungi soil	0.042						
S. indica soil	0.0504	1					
C. tofieldiae soil	0.0504	1	1				
no fungi root	1	0.3234	1	0.1701			
S. indica root	1	0.2751	0.2625	0.2499	1		
C. tofieldiae root	1	0.7287	0.714	0.7476	1	1	

F) **Gammaproteobacteria**

p values	input culture	no fungi soil	S. indica soil	C. tofieldiae soil	no fungi root	S. indica root	C. tofieldiae root
input culture							
no fungi soil	0.0947						
S. indica soil	0.3463	0.3793					
C. tofieldiae soil	0.5275	0.3169	0.8081				
no fungi root	0.0163	0.0194	0.0047	0.0054			
S. indica root	0.0974	0.1329	0.0269	0.0118	0.6864		
C. tofieldiae root	0.4618	0.8884	0.4283	0.5027	0.0679	0.1957	

R values	input culture	no fungi soil	S. indica soil	C. tofieldiae soil	no fungi root	S. indica root	C. tofieldiae root
input culture							
no fungi soil	0.1352						
S. indica soil	0.04259	-0.03148					
C. tofieldiae soil	-0.01481	0.01852	-0.1037				
no fungi root	0.4802	0.4921	0.869	0.8889			
S. indica root	0.3704	0.179	0.7037	0.7037	-0.1296		
C. tofieldiae root	-0.01042	-0.3021	-0.09375	-0.02083	0.8214	0.6667	

p value
< 0.05
< 0.01
< 0.001

R value
0
0.25
0.5
0.75
1

Heatmap of p and R values from ANOSIM test for significance between samples groups for each bacterial class observed in the microbiome. A) Flavonobacteria; B) Actinobacteria; C) Bacilli; D) Alphaproteobacteria; E) Betaproteobacteria; F) Gammaptoteobacteria.

In summary, *Arabidopsis* core microbiome had a positive effect on the plant growth in comparison to the microbe free, as well as rescuing the growth and increasing the survival of plants inoculated with *C. tofieldiae*. A significant increase

of plant yield was observed only in the presence of *S. indica*, with and without the bacterial community. However, both fungi were eliminated from the root in the presence of the bacterial community and they had no effect on bacterial community alpha (α) nor beta (β) diversity. Significant differences were observed in the alpha diversity and the bacterial composition when comparing the input with the rest of the samples and between the compartments.

4.3 Discussion

The use of gnotobiotic system and synthetic communities can give insights into microbial community dynamics and functional effects on plants. In this experiment, two plant beneficial fungi were individually tested in such a system, with the addition of a synthetic bacterial community.

4.3.1 *S. indica* and its combination with the core microbiome improves plant growth in the gnotobiotic system

The plant fresh weight results highlight the improved plant growth in the presence of the microbiome (Figure 4.1A). *S. indica* has a positive effect on plant growth as expected (Carvalhais et al., 2013), showing significantly higher aboveground plant yield in the presence and in the absence of the bacterial community, in comparison to the microbe free plants. The combination of *S. indica* inoculated plants with the bacterial community did not have a significant additive effect on the growth, either because this weight could be the maximum that the plants could reach at this stage of development or due to other complex interactions within the community.

4.3.2 *C. tofieldiae* has negative effect on plant growth and survival

C. tofieldiae did not improve plant growth, but instead, its presence led to high plant mortality (50%). In addition, the fresh shoot weight of the plants treated with *C. tofieldiae* had reduced growth in comparison to those treated with *S. indica*, with and without the presence of the bacterial community. While *C. tofieldiae* has been identified as an endemic root endophyte of *Arabidopsis thaliana* promoting plant growth in phosphate starvation conditions (Hiruma et al., 2016), in this gnotobiotic system it showed an inhibitory effect on plant survival. It is suggested that *C. tofieldiae* is able to improve growth by improving phorsporus uptake, and this ability is induced by phosphorus starvation processes (Hiruma et al., 2016). Considering that the soil substrate (common peat) used in this experiment is not

depleted in phosphorus, may explain the absence of growth promotion, however, it does not explain the low survival rate of the plants treated with *C. tofieldiae*.

Even though *C. tofieldiae* is studied for its beneficial effect on phosphorus upatake and plant growth promotion (Hiruma et al., 2016), *Colletotrichum* species are known as common plant pathogens (Perfect et al., 1999) causing diseases on crops worldwide, leading to pre- and post- harvesting losses (JEFFRIES et al., 1990). In fact, *Colletotrichum* spp. has been characterized as the eighth most important plant pathogen, among the well-known *Fusarium* and *Botrytis* species, based on its scientific and economical importance (Dean et al., 2012). However, it is known that *Colletotrichum* species can have very different repertoire of genes, as well as completely different host invention strategies and host specificity (O'Connell et al., 2012). Studying the transciptomes of *C. tofieldiae* and its pathogenic relative *C. incanum*, genomic signatures were determined to signify the lifestyle transition of this genus, from pathogenic to mutualistic (Hacquard et al., 2016). This study further suggests that *C. tofieldiae* is potentially saprotrophic, thus its pathogenicity genes are not expressed during plant infection, in contrast to its relative *Colletotrichum*. Regarding the plant, its metabolic responses were differentially affected during *C. tofieldiae* colonisation in accordance to phosphate availability, with defense responses being activated in the presence of replete phosphorus (Hacquard et al., 2016). Since the soil substrate used in our experiment was not phosphorus depleted, it is possible that plant defense mechanisms were activated in the presence of *C. tofieldiae*, resulting to plant fatality. Notably, fungi used by Durán et al. (2018) are part of the *Arabidopsis* core microbiome that, when inoculated without other microbes, exhibit a negative effect on plants. It indicates that plant fungal endophytes can exert pathogenic activities under certain conditions. This effect was observed with *C. tofieldiae* in our experiments, which is also characterized as an endemic endophyte of Arabidosis root (Hiruma et al., 2016). Studies have found that mono-associations of fungal endophytes with the plant restrict plant growth (Durán et al., 2018; Keim et al., 2014; Kia et al., 2017). This indicates that the plant alone cannot modulate fungal colonisation and its potential negative effect, but the microbial communities may play a role in balancing the plant-fungal interactions. However, such negative effect was not

observed in the case of *S. indica*, indicating its close beneficial relationship with the plant.

The negative effect of *C. tofieldiae* on the plant growth and survival, was masked by the bacterial community. In the presence of the core microbiome, the *C. tofieldiae* treated plants reached plant growth similar to that of plants without the *C. tofieldiae* treatment and rescued 30% of the plants from the detrimental effects of the fungus. The differences observed in the *C. tofieldiae* – treated root microbiome are mostly in the relative abundance of Flavobacteria and β-Proteobacteria, with Flavobacteria being decreased and the β-Proteobacteria increased. De Boer et al. (2001) showcased the mycolytic activity of members of β-Proteobacteria (CbetaPs), which are able to inhibit fungal growth followed by the proliferation of β-Proteobacteria. The decrease of Flavobacteria members may be just an effect of the proliferation of β-Proteobacteria. These observations highlight the contribution of the root core microbiome to the plant health, development and defense towards root pathogens, in agreement to previous findings (Haas and Défago, 2005; Whipps, 2001) and its potential in agriculture.

4.3.3 The presence of the bacterial community eliminates the fungal presence in the plant root and the matrix

In order to further investigate the interaction between the fungal species studies in this chapter and the core microbiome, the relative abundance of the fungi at the end of the experiment was calculated. The chosen method to determine fungal presence and colonisation was through amplicon sequencing. However, this method only gives information on the relative abundance and it is more suitable for samples of higher complexity (i.e. microbiome community samples). Nevertheless, the amplicon sequencing data gave an insight in the relative abundance of the fungi in the system, showing a significant reduction ($p < 0.001$) of the relative abundance of both fungi in the presence of the bacterial community. The reason for the unsuccessful colonisation of the root by *S. indica,* despite applying the optimal inoculation method (Ghaffari et al., 2016; Harrach et al., 2013; Pedrotti et al., 2013; Vahabi et al., 2016), could be due to competition with certain members of the

bacterial community as observed in the past between certain bacterial and fungal species (Mille-Lindblom et al., 2006). As previously mentioned, *S. indica* has been studied for its beneficial effects but also for the interactions with other organisms. It is well known that the bacteria compete with fungi for the same niche (Rousk et al., 2008), but also it has been shown that certain bacteria can feed on fungal hyphae or fungal exudates (Leveau and Preston, 2008). In more detail, the fluorescent *Pseudomonas*, *Bacillus* spp. and *Burkholderia* spp. are known as biological control agents based on their ability to produce antifungal metabolites and affect the symbiosis of fungal mutualists with the plant (Raaijmakers et al., 2009). In fact, assays on plates have shown that *Pseudomonas* strains, like *Pseudomonas fluorescens* WS5 and SS101, inhibited the growth of *S.indica* (Varma et al., 2012). Moreover, Meena et al., (2010) showed that the root colonisation of *S. indica* can be regulated by *Pseudomonas striata*. Representatives of those taxa were present in the synthetic community and they might have influenced the growth of *S. indica* and its successful colonisation through those antifungal compounds. Furthermore, Kumar Bhuyan et al., (2015) investigated the interaction of *S. indica* with the bacteria *Azotobacter chroococcum*. While one *Azotobacter* strain showed a positive interaction with the fungus, another strain of the same species had inhibitory effects, highlighting the ability of bacteria to influence the growth and physiology of the fungus. As a result, it is important to keep in mind that different interactions amongst biological kingdoms will have a direct influence on the host plants (Kumar Bhuyan et al., 2015). These examples showcase how bacteria can influence *S. indica* growth and colonisation, and it is a likely explanation of the inhibition of *S. indica* in the gnotobiotic system experiments (Figure 3.22). Similar might be the case of *C. tofieldiae*, even though the inhibition of plant pathogenic fungi by endophytic bacteria is broadly known (Berg and Hallmann, 2007; Haas and Défago, 2005; van Loon et al., 2002). An interaction experiment as presented by Vorholt et al., (2017), where interactions among all members of the microbiome are tested, could provide further details and reveal positive or negative interactions between the members of the bacterial collection and the fungi, and give some indications on synergism and antagonism of the fungus with those bacteria.

4.3.4 The robustness and the limitations of the gnotobiotic system

The alpha and beta diversity of the microbiome could provide information on the structure and the composition of the microbiome. While the beta diversity revealed no differences between the compartments and the treatments, the alpha diversity revealed significant differences in the richness and evenness of the microbiome between the input culture, the root samples and the matrix samples. The matrix samples had the lowest species richness and evenness. The differences in the microbial community observed between the matrix and the root are expected, due to competition at the root surface as well as root exudates that are produced by plants that attract or repel certain microbes (Carvalhais et al., 2011; Dennis et al., 2010). In addition, the bacterial culture used was isolated and characterised as the core rhizobiome of *Arabidopsis*, indicating the preference of those bacteria to inhabit the root rhizo/endosphere of the plant (Bai et al., 2015), which might explain the lower number of OTUs observed in the matrix samples. The fungal pre-treatment did not have an effect on the number of observed OTUs, but certain differences were observed at the Shannon Index values (species evenness). In more detail, the species evenness of the samples treated with fungi (matrix and root) was not significantly different than that of the input culture. In contrast, the Shannon Index of the matrix samples containing only bacteria where significantly lower than the input culture and the root samples containing fungi. This could indicate that the fungi may play a role in the establishment of the microbes in this habitant.

It would be challenging to further investigate the biological basis of the results of this experiment, and whether the interactions observed are due to the specific experimental set up. One first step to unravel the fundamental mechanisms of those interactions would be investigating the dynamics between the microorganisms and the plants at early stages of colonisation by harvesting samples at different time points, as research has shown that there are enormous differences in the microbiome structure and composition over time (Qiao et al., 2017; Yuan et al., 2015). Such an experiment would give an insight into the establishment of the balance in the system as well as how this is achieved and would provide further information on the robustness and reproducibility of the experimental system. Furthermore, sampling the rhizosphere and the endosphere independently would be

an additional step that could give different results. That is because rhizosphere microbiome is more similar to the soil microbiome (Hacquard et al., 2015), and therefore might be masking the differences, if the endospheric microbiome was more distinct. Nevertheless, the knowledge gained from this pioneering experiment is a promising starting point for the usage of such a simplified gnotobiotic system in revealing regulatory principles in the establishment of microbial communities.

Similar research employing gnotobiotic system and synthetic microbial communities, has confirmed the practicality and utility of such experimental set up observed in my experiments. For instance Durán et al. (2018) used the same experimental set up to study interkingdom interactions and demonstrated the necessity of the bacterial microbiome for plant growth and survival. They employed part of the bacterial collection used in this study, as well as a collection of fungi and Oomycetes. Their study demonstrated that the fungal community was detrimental for plant survival but was suppressed in the presence of the bacteria, which rescued the plant. They further showed that the Flavobacteriaceae, Pseudomonadaceae, Comamonadaceae and Rhizobiaceae are highly competitive families in this system. Those families are also the most abundant families in my study in all samples (Figure S4.1), which confirms the robustness and reproducibility of this experimental set up, as well as of the observed results. Furthermore, even though the number of the input bacteria was 148 isolates, they could only detect 65 OTUs in their MiSeq run. Similarly, in my experiment the input number of bacterial isolates was 200 but only 78 OTUs (or 54 after rarefying) were detected after sequencing. In both experiments the number of observed OTUs is similar, as well as the observed balance in the community structure (most abundant families). This is probably due to some of the strains being lost from inoculation to harvesting day or during the MiSeq library preparation, or too low in abundance to be detected in the sequencing. They also showed that the combination of bacterial and fungal community induced similar levels of plant growth as observed in plants inoculated with the bacterial community only, and the maximum growth was achieved only in the presence of all bacterial, fungal and oomycete communities. Taken together, it highlights the reproducibility of data and, hence, robustness of the experimental set up used in my work.

4.3.5 Conclusions and future perspectives

The gnotobiotic system can enable the study of the mechanisms involved in the dynamic changes within the community, as well as the lifestyle transition of microbes under certain conditions. This current work showcases that the *Arabidopsis thaliana* core microbiome establish its colonisation in the root and maintains a similar community structure irrespectively of the fungal treatments used. In addition, this core bacterial community is able to rescue plants from fatality caused by *C. tofieldiae*. The observed effect of the bacteria on fungal colonisation could have remarkable potential for agricultural applications as biocontrol agents for fungal pathogens (Haas and Défago, 2005; Whipps, 2001). Research suggests that recruitment of certain microbes by the root can enhance plant health and resistance to pathogens (Berendsen et al., 2012; Hartmann et al., 2009). Understanding these interactions could enable the manipulation of microbiomes, for example, through microbiome engineering (Orozco-Mosqueda et al., 2018), and to combat devastating plant pathogens (Sergaki et al., 2018).

The reproducibility of the system as observed when comparing our study with that of Duran et al. (2018) make this system a robust tool for unraveling the complex interactions within this small, simplified ecosystem, particularly in combination with other 'omics methods such as transcriptomics, proteomics and metabolomics. Such an approach would enable the understanding of the differential effect of two fungi known as beneficial, with *S. indica* maintaining its beneficial properties while *C. tofieldiae* had detrimental effect on the plant growth and survival. Furthermore, while it highlights the potential of the beneficial symbiont *S. indica* inoculant for field application, it is important to better study its establishment and the interactions in the presence of other competitive microbial communities in order to ensure its effectiveness when used as agricultural inoculant.

Chapter 5: Influence of rhizobia strains on the composition of the core root microbiome of *Medicago truncatula* in natural UK soils

5.1 Introduction

Lab based research has set a benchmark for studying the fundamentals of microbiome, however, the application of microbiomes in agriculture should focus on improving the most vital aspects of crop production, such as nutrient availability, soil fertility and health (Syed Ab Rahman et al., 2018). In this respect, microbiome research can provide sustainable solutions by uncovering the interactions within microbiomes and with the host under natural conditions. Bridging the lab – field gap has become a necessity in order to exploit the full potential of microbiomes in agriculture. However, it requires more short and long-term field experiments, aiming to understand the complex dynamics of the communities in nature and the effect of the environment and the farming practices on them (Parnell et al., 2016; Schlaeppi and Bulgarelli, 2015).

Nitrogen, phosphorus and potassium can be a major limitation for plants which often is compromised by the use of fertilizers. While excessive use of fertilizers is associated with environmental pollution (Good and Beatty, 2011), extensive research on soil and plant-associated microorganisms that enhance plant production has shown the enormous potential of using microbial communities as a sustainable solution to certain unsustainable farming practices (Bakker et al., 2012; Busby et al., 2017; Chaparro et al., 2012; Schlaeppi and Bulgarelli, 2015; Sergaki et al., 2018). In this respect, legumes can play a vital role. Legume plants hold major roles in agriculture and in ecological systems (e.g. enriching soil with nitrogen), making them very important for ecosystem functioning including agricultural fields (Sprent et al., 2017). This eco-beneficial potential of legumes is closely linked with their ability to establish bacterial symbioses. Certain soil bacteria support plants, e,g., plant growth-promoting rhizobacteria (PGPR), are known as natural biofertilizers and are occupiers of the same

niche in the plant as phytopathogens and microbes stimulating plant nutrient acquisition (Glick, 2015; Martínez-Viveros et al., 2010). One taxon of PGPR is rhizobia, which are known for their ability to form symbiosis with legume roots and fix atmospheric nitrogen (Zahran, 1999). These bacteria are able to take up nitrogen (N_2) from the air, a molecule not available to the plant, and transform it to plant available ammonium (NH_4^+) (Udvardi and Poole, 2013). In exchange, the plant host will give rhizobia carbon compounds such as sugars and amino acids (Oldroyd et al., 2011). Harnessing the full potential of this symbiotic relationship can establish levels of nitrogen required in agricultural crop production and decrease the use of chemical fertilizers (Howieson et al., 2000). Production of nitrogen fertilizers is dependent on the Haber-Bosch process, an energy-demanding process that uses 1-2% of world's energy resources. Moreover, for every ton of N-fertilizer manufactured and used, ~9.7 tons of greenhouse gas CO_2-equivalent are produced (Smith, 2002). Therefore, decreasing the production and use of nitrogen fertilizers would have a beneficial impact on the environment developing strategies to achieve this is therefore a main goal for sustainable agriculture.

In addition to nitrogen, soil phosphorus availability is a major limitation for plant growth and fitness, including legume plants associated with rhizobia (Vance et al., 2003). Even though phosphorus is present in many soil types across the world, it is mostly inaccessible always for plants (Akinrinde et al., 2006). While arbuscular mycorrhizas are well-studied as plant symbiotic fungi that can enhance plant phosphorus supply (Richardson, 2002), this can have negative effects on plant growth in cases where mycorrhiza compete with plants for nitrogen under low nitrogen conditions (Wang et al., 2018). *Serendipita indica* (formerly *Piriformospora indica*) has demonstrated an increased ability to improve phosphorus nutrition, along with plant growth (Johri et al., 2015). The co-inoculation of *S. indica* and a growth promoting rhizobacteria (*Pseudomonas striata*) has previously shown synergistic effects; e.g. enhancing plant growth and colonisation by the PGPR bacteria (Meena et al., 2010). Based on these findings, *S. indica* was used with a *Sinorhizobium meliloti* strain and the model legume *Medicago trancatula* to investigate their combined effect on plant yield and nutrient acquisition.

I used *Medicago truncatula* for my studies as an accepted model organism for legumes and for the association with nitrogen-fixing bacteria (Barker et al., 1990; Cook, 1999). A nitrogen-fixing bacterium often associated with this model organism is *Sinorhizobium meliloti*, which has been extensively studied (Galibert et al., 2001; Jones et al., 2007; Marx et al., 2016). The *Sinorhizobium meliloti* strain 1021 is most commonly studied with *Medicago truncatula* (Zhao et al., 2012), while additional nitrogen-fixing strains such as *Sinorhizobium meliloti* 1022 and *Sinorhizobium medicae* 419 (formerly *Sinorhizobium meliloti*) have attracted some attention in recent years (Terpolilli et al., 2008). *Sinorhizobium meliloti* 1022 was isolated from a field of a Greek island and it is characterised as a highly effective rhizobial strain (Terpolilli et al., 2013). *Sinorhizobium medicae* 419, formerly known as *Sinorhizobium meliloti WSM* 419 (Hellweg et al., 2009) was isolated from Sardinia (Howieson and Ewing, 1986) and it has been studied for its positive effect on plant growth (Larrainzar et al., 2014; Reeve et al., 2006; Tiwari et al., 1996). These three strains were chosen for this study in order to investigate how synergistic interactions with co-inoculated *S. indica* might differ when in combination with rhizobia with variation in N-fixing efficiency

Nutrient uptake can be facilitated and significantly altered by the microbiome (Carvalhais et al., 2013). Revealing the microbial dynamics that influence changes in plant growth and nutrition under different environmental conditions could encourage the exploitation of microbiomes in agriculture (Sergaki et al., 2018). Alongside with the tripartite interaction between *Medicago trancatula, Sinorhizobium* spp. and *S. indica*, microbial communities as well as plant and soil biochemical characteristics were examined, aiming to unravel the effects of the co-symbioses on the system. Microbiome composition changes in dependency of environment biochemical properties, day length, geographic position and biotic factors (Lozupone and Knight, 2007). Correlations between soil bacterial communities and environmental factors have been made before, and it is believed that bacterial abundances can be predicted by those environmental factors (Griffiths et al., 2011). Research on the microbiome is complex due to its dependency on the conditions of soil ecosystems, one of the most biodiverse ecosystems on earth (Whitman et al., 1998). Microbiome research has both shown that microbial communities with different composition have similar functional capacities (Nannipieri et al., 2003; Wertz et al., 2006) as well as that microbial community composition reflects specific function (Balser and Firestone, 2005;

Paterson et al., 2011). This striking contradiction reflects the complexity and challenges associated with microbiome studies and highlighting the necessity in assigning functions to microbiome members within ecosystem processes (Koranda et al., 2014). For this reason, the tripartite system was analysed in different UK agricultural soil types that differ in defined biochemical characteristics. The three soil types were characterised according to their phosphate (PO_4^{3-}) and nitrate (NO_3^-) content as “Low NP”, “Low N high P”, “High NP” soil.

The working hypothesis was that rhizobia are able to alter the microbiome, assisting the recruitment of beneficial taxa inside the root in order to benefit the plant. It was also expected that in the presence of rhizobia and the recruited microbiome, the plant nutrient composition would be altered, as a consequence of the altered microbiome. It was expected that microbial community composition will vary depending on the soil type leading to different results in plant growth and nutrition. The ultimate aim was to unravel key functions of microbe - microbe and plant root – microbe interactions associated with the environment and the phenotypes observed.

5.2 Results

5.2.1 *S. meliloti* spp. differ in their ability to promote growth in dependency of nutrient composition in natural soils

For the establishment of a complex tripartite system comprising a fungus, a bacterium and a plant, it is important to establish an inoculation procedure that will ensure the colonisation of the plant by all microbes and the presence of the beneficial attributes of the microbes. The co-inoculation of *S. indica* and the rhizobia strains was first tested in perlite, prior moving on to the three soil types. *M. truncatula* plants were inoculated 1 day after being transferred to the perlite pots. Fresh shoot weight and the fungal colonisation were quantified 49 dai (52 day old plants). For the experiments in the natural soil types, *M. trancatula* plants were inoculated with the microbes as mentioned above, or with sterile water (mock), in perlite pots and transferred to the pots with natural soil 11 days later (12 day old plants). The efficiency of the co-inoculation was assessed using phenotypic data and quantification of the fungal colonisation 63 dai, (66 day old plants).

The plant yield results, measured as the aboveground dry weight, showed no significant additional enhancement of plant growth in plants co-inoculated with *S. meliloti* spp. and *S. indica* (Figure 5.1). The visual assessment using qPCR of plant fungal colonisation showed that despite having a good colonisation in the presence of the different rhizobial strains in perlite, *S. indica* was significantly reduced or not detectable in natural soil types (Figure 5.2).

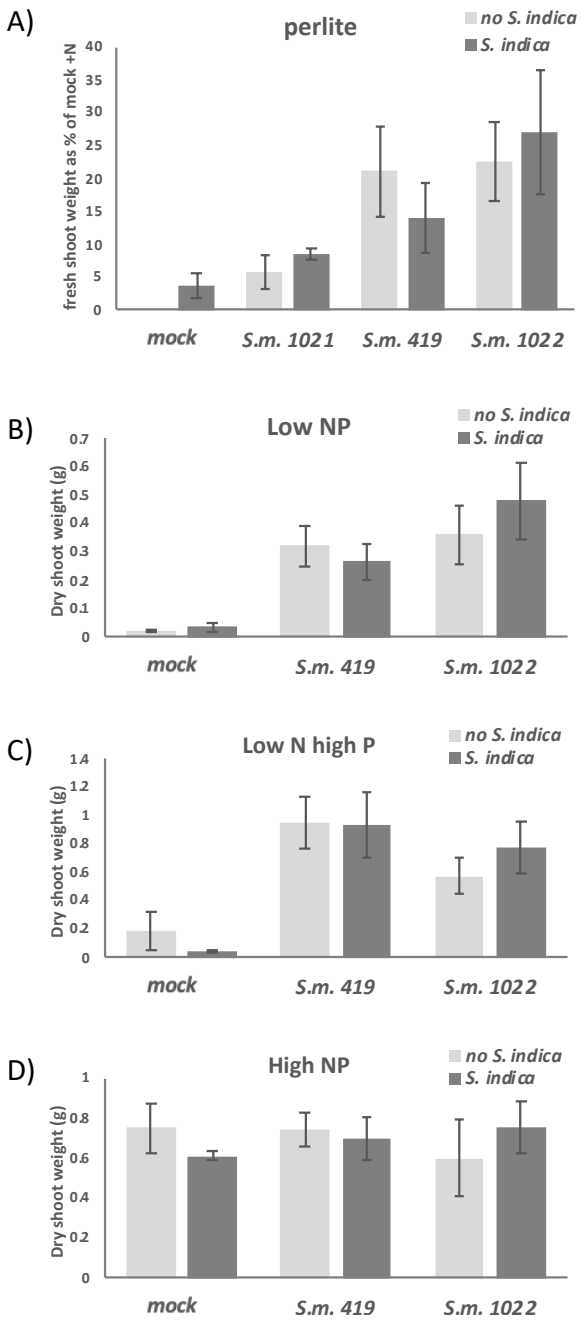


Figure 5.1: Effect of co-symbiosis on plant yield. Plant yield (g) under different conditions and substrates in the absence (light grey bars representing single treatments) or the presence (dark grey bars representing co-inoculations) of S. indica. A) Fresh shoot weight of 52 day old M. truncatula plants relative to control treated plants grown with replete nitrogen (15mM NH_4NO_3), 49dai. B) Dry shoot weight of 66 day old M. truncatula plants grown in low NP soil type, 63 dai. C) Dry shoot weight of 66 day old M. truncatula plants grown in low N high P soil type, 63 dai. D) Dry shoot weight of 66 day old M. truncatula plants grown in high NP soil type, 63 dai. Mean values are shown with standard error.

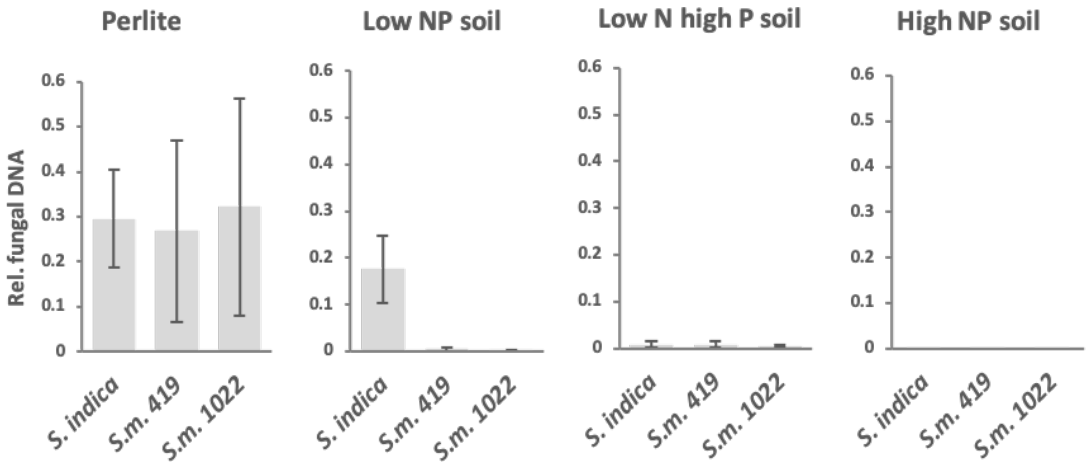


Figure 5.2: Root colonisation by S. indica. Amount of S. indica fungal genomic DNA relative to plant genomic DNA found in root samples of plants inoculated with S. indica (left) or inoculated with S. indica and the high efficiency rhizobial strains (middle and right). Results are from all substrates analysed, in perlite 49 dai (52 day old plants) and in the 3 different soil types 56 dai (59 day old plants). Mean ΔCt values are shown with standard error.

The analyses suggest that the differences in bacterial and fungal growth rates requires a well-balanced amount of fungal vs. bacterial inoculum. As it is challenging to optimise inoculum concentrations and conditions (which might further differ between soil types) to buffer such microbe-microbe growth dynamics it was decided to focus on characterising the mechanism and effects of the rhizobial strains on plant fitness.

5.2.2 Characterisation of soil types

The soils used in this experiment were chosen for their distinct characteristics and representative for around 70% of UK soil types used in agriculture. Various tests were performed in order to assess their texture, nutrient content and microbiome composition at the start of the experiment. Crop rotation data for the preceeding 4 years prior to the collection of the soils for this experiment was also obtained from the farmers or researchers that managed the land (Table 5.1).

Soil texture and the organic matter content were quantified (Figure 5.1A) and no significant differences were found using ANOVA followed by Tukey's HSD test. However, there is significantly more silt and less sand content found in the high NP soil in comparison to the other two soil types (Figure 5.2B). Based on Rhodes (2013), low NP and low N high P soil types are categorised as sandy loams, and the high NP is categorised as loam (Rhodes, 2013). The high NP soil is also characterised by significantly higher pH and conductivity (Figure 5.4A and B). The soil nitrogen (N) and phosphorus (P) available to the plant are significantly different in every soil type as indicated by the soil attributes (e.g. low, high) (Figure 5.4C, D). However, low N high P soil type has significantly higher available P than the high NP soil. Soil C:N ratio, C % and N% content is significantly higher at the high NP soil type in comparison to the low NP soil type (Figure 5.4E, F).

Table 5.1: Crop rotation for each soil type for the last 4 years before soil collection for these experiments.

Soil	2013/14	2014/15	2015/16	2016/17
Low NP	Winter wheat	Grass / weeds	Grass / weeds	Grass / Weeds
Low N high P	Winter wheat	Oilseed rape	Winter wheat	Winter Barley
High NP	Fennel	Celeriac	Beet	Wheat

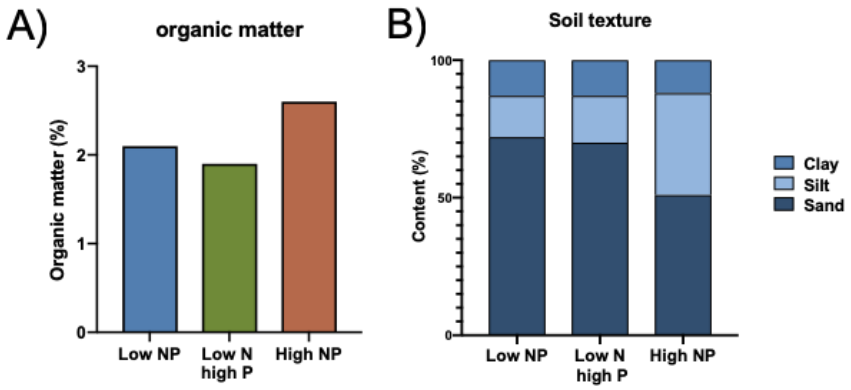


Figure 5.2: Soil organic matter and texture characteristics. A) Percentage of organic matter found in each soil type referred to total soil weight and B) soil texture presented as the percentage of clay, silt and sand content for each soil type.

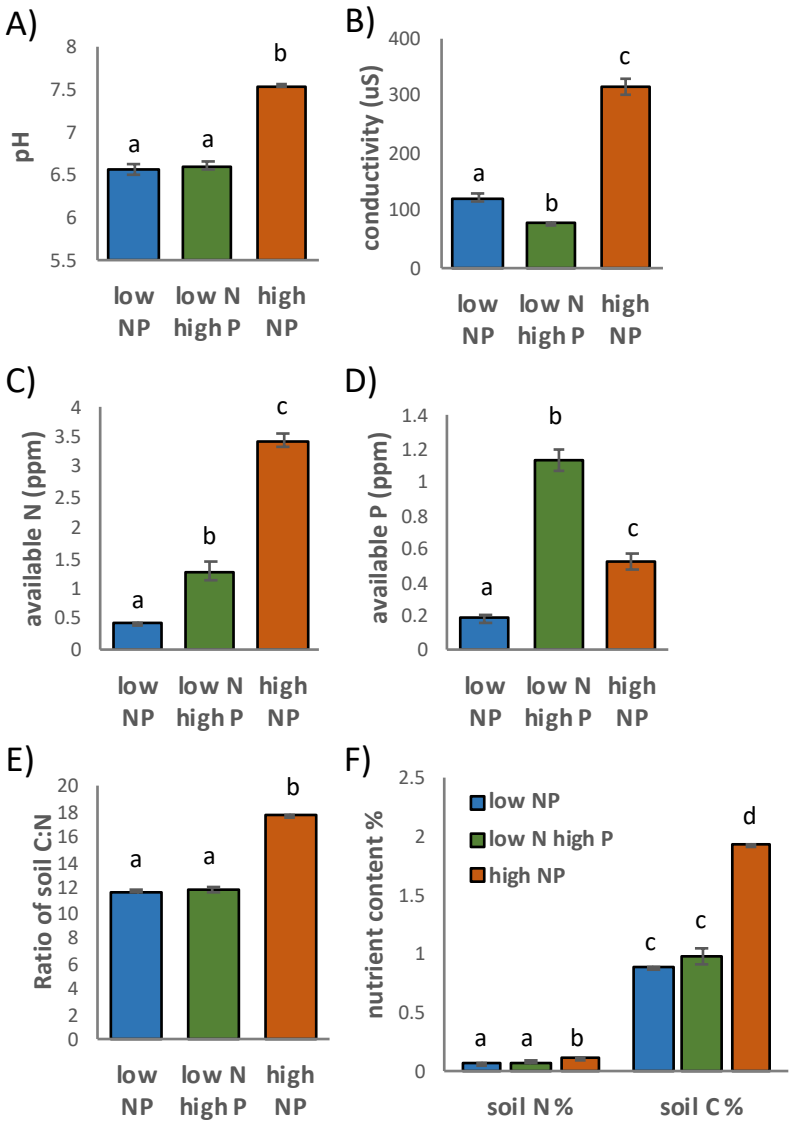


Figure 5.4: Soil chemical characteristics before the experiments were set up showing initial differences amongst soil types. A) pH; B) Conductivity; C) Available nitrogen (N); D) Available phosphorus (P); E) Ratio of soil carbon:nitrogen (C:N); F) Percentage of total soil nitrogen (soil N%) and total soil carbon (soil C%). Means are shown with standard error and letters represent significant differences according to ANOVA followed by to Tukey's HDS ($p < 0.001$).

An important soil characteristic for this experiment is the microbiome composition. Therefore, the soil microbiome was sampled before the start of the experiment and sequenced using Illumina Miseq. The microbiome alpha and beta diversity, determining the within sample and between sample diversity respectively, was analysed using rarefied reads. The number of observed OTUs in each sample was

calculated as a measure of alpha diversity. The number of observed bacterial OTUs in the High NP input soil was significantly higher than the input soil samples of low NP soil type. Moreover, the planted and unplanted (soil without plant) samples of the high NP soil type contained significantly higher numbers of OTUs than any other sample. When plotting the beta diversity in a PCoA plot, this revealed a cluster of all high NP soil type samples, while the low NP and the low N high P soil types cluster together (Figure 5.5).

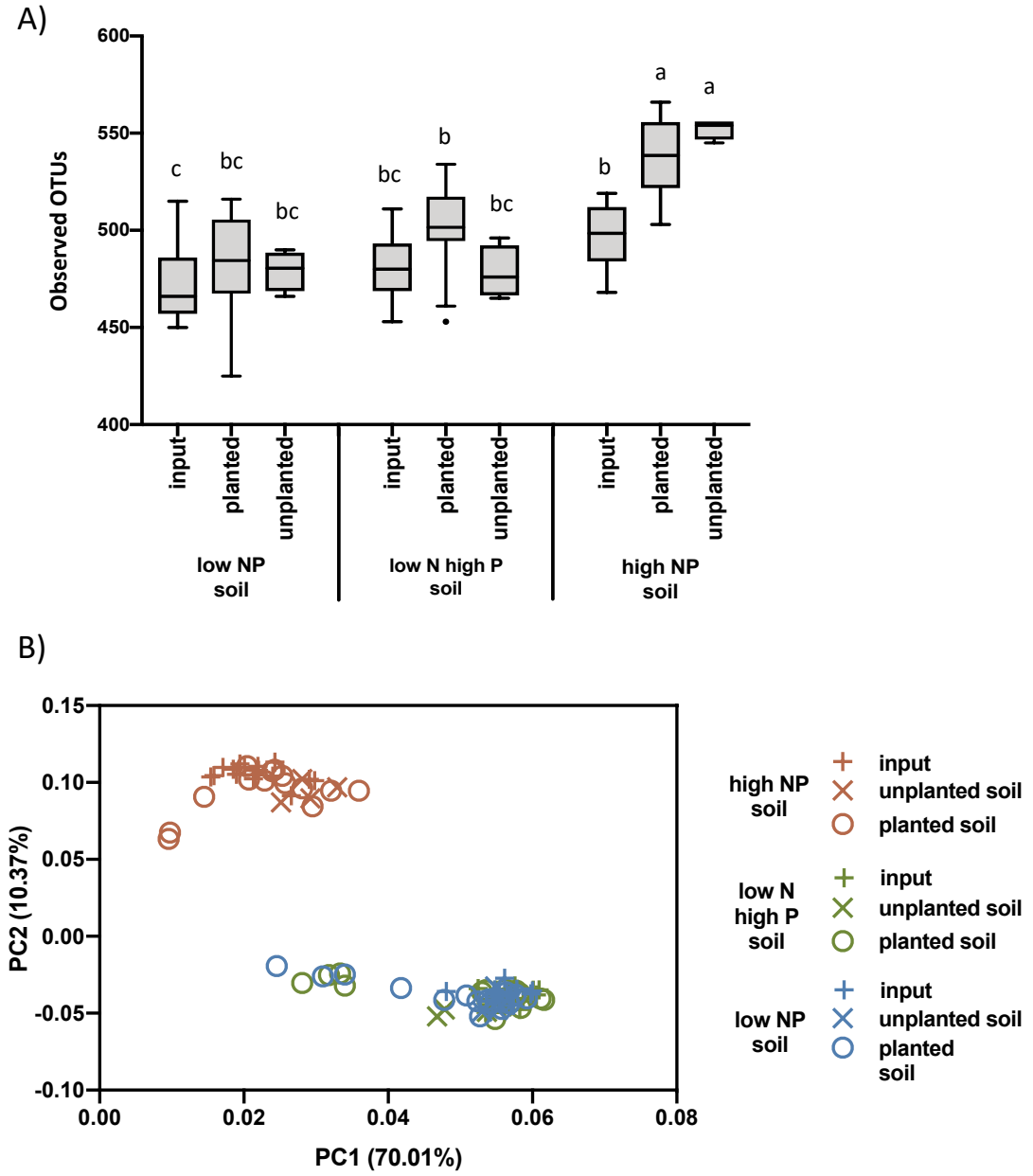


Figure 5.5: Bacterial community richness and structure. A) Bacterial community alpha diversity presented as the number of observed OTUs in each sample type: input soil, planted (mock-treated) and unplanted soil in the different soil types. Letters

represent significant differences according to ANOVA followed by to Tukey's HDS ($p < 0.001$). Boxplots represent the median value (black bar inside the box) and whiskers represent quartile distribution; outlier data points are depicted as black dots outside the boxplots. B) beta diversity presented as bacterial community structure using Principle Coordinates Analysis (PCoA). The first two dimensions of PCoA are plotted based on Bray-Curtis distances (rarefied reads used) and they explain 80.38% of the variation in the data.

In contrast to the bacterial community results, the number of fungal observed OTUs in the High NP soil was significantly lower than in the rest of the soils. Different to bacterial samples, fungal samples clustered according to the soil type in three clusters representing the beta diversity in the PCoA plot (Figure 5.6).

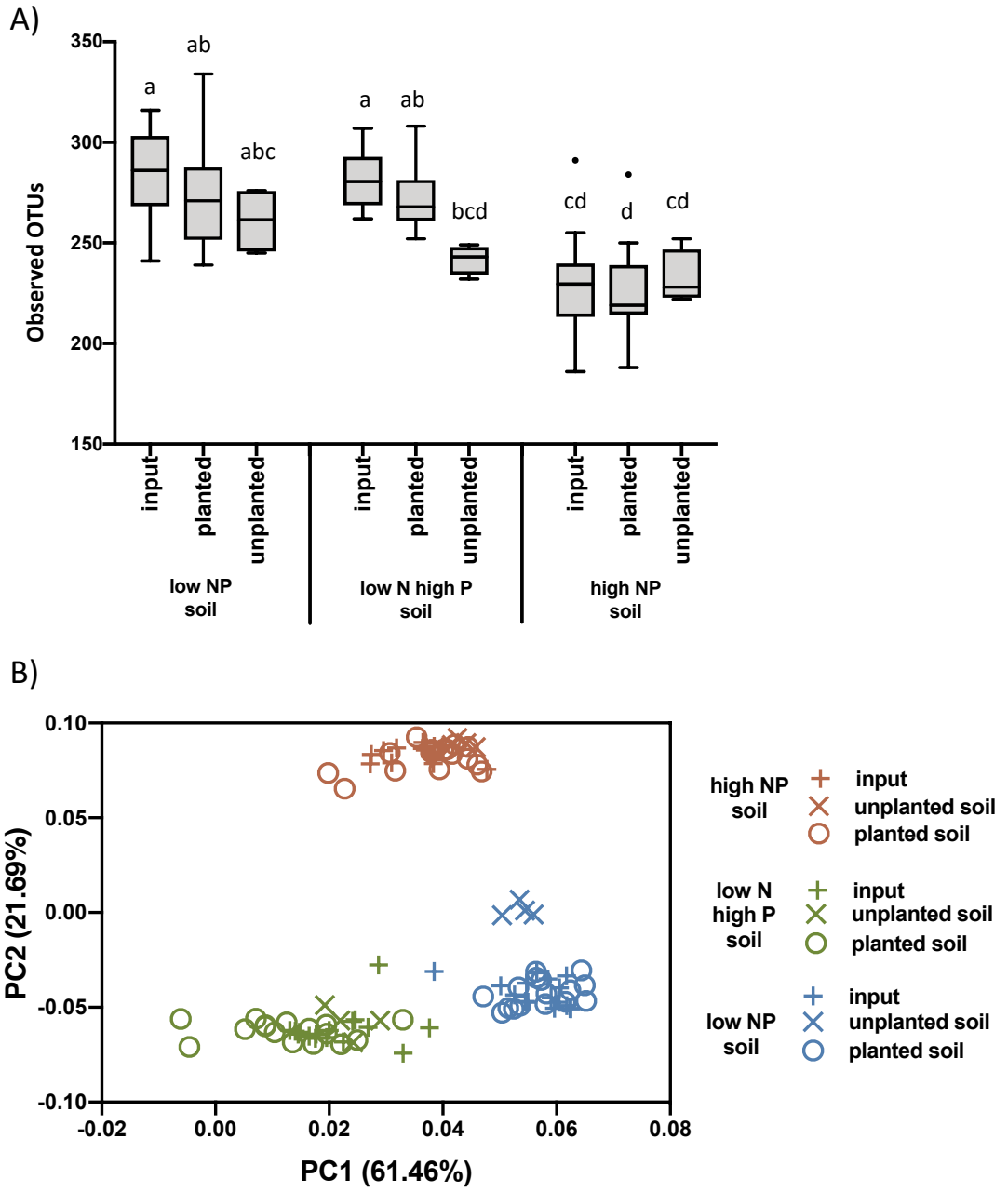


Figure 5.6: Fungal community richness and structure. A) Fungal community alpha diversity presented as the number of observed OTUs in each sample type: input soil, planted (mock-treated) and unplanted soil in the different soil types. Letters represent significant differences according to ANOVA followed by to Tukey's HDS ($p < 0.001$). Boxplots represent the median value (black bar inside the box) and whiskers represent quartile distribution, outlier data points are depicted as black dots outside the boxplots. B) beta diversity presented as bacterial community structure using Principle Coordinates Analysis (PCoA). The first two dimensions of PCoA are plotted based on Bray-Curtis distances (rarefied reads used) and they explain 83.15% of the variation in the data.

The alpha and beta diversity of the soil microbiome revealed different patterns in the bacterial and the fungal community. To further assess differences in the composition of the microbial communities, taxonomy was assigned to the OTUs and presented as relative abundance % (RA) (Figure 5.7).

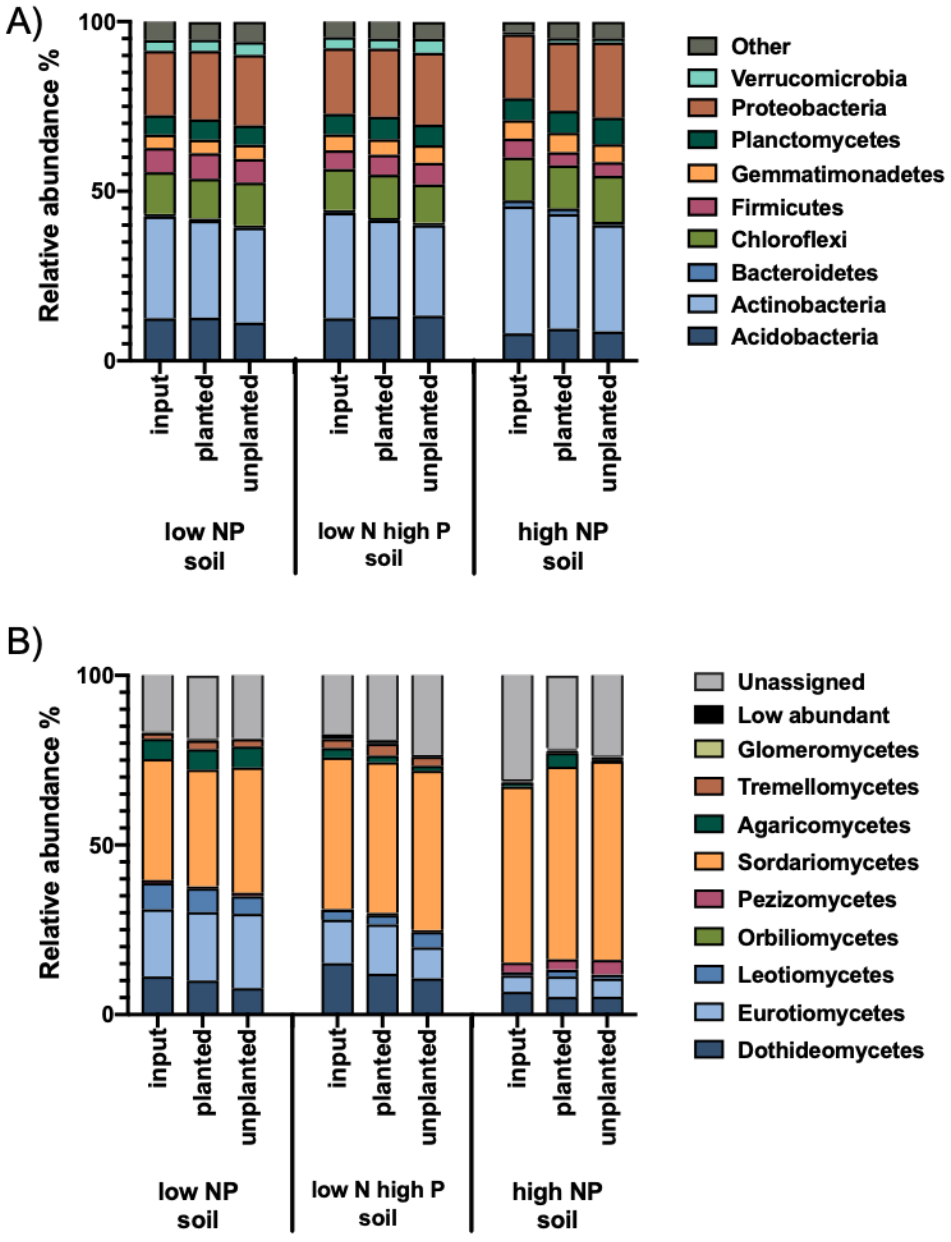


Figure 5.7: Bacterial and fungal community composition. A) Relative abundances % of bacterial taxa at the phylum level, and B) fungal taxa at the order level, shown as percentage of all reads and colour-coded, calculated using rarefied reads. "Other"

refers to unassigned and low abundant bacterial taxa, while this is split to "Unassigned" and "Low abundant" taxa for fungi.

The subsequent analysis of the bacterial community composition using SIMPER showed similarities among soil samples (~8% overall dissimilarity) with the Actinobacteria contributing 27% to the differences, followed by Acidobacteria which contribute 16% to the differences (Table 5.2). In the fungal community (~18% overall dissimilarity based on SIMPER), Sordariomycetes contributed 29% to the differences observed among soil samples, followed by the Eurotiomycetes which contribute 22% to the differences (Table 5.2).

Table 5.2: Microbial taxa contributing to the differences in community structure.

Bacteria		Mean values								
		Low NP soil			Low N high P soil			High NP soil		
Taxon	Contrib. %	input	planted	unplanted	input	planted	unplanted	input	planted	unplanted
Actinobacteria	27.33	30	28.6	28	31.1	28.4	26.8	37.5	33.8	31.4
Acidobacteria	16.5	12.6	12.8	11.3	12.6	13	13.3	8.12	9.5	8.68
Firmicutes	10.89	7.13	7.48	6.95	5.55	5.9	6.42	5.57	3.87	4.03
Verrucomicrobia	10.27	3.17	3.31	3.8	3.31	2.99	4.17	0.754	1.25	1.23
Proteobacteria	9.214	19	20.2	20.8	19.3	20.1	21.1	18.8	20.2	22.2
Other	6.222	5.42	5.31	5.97	4.55	4.96	5.03	2.98	4.84	4.9
Gemmatimonadetes	5.857	3.92	4	4.2	4.68	4.54	5.17	5.45	5.79	5.33
Chloroflexi	5.573	12.4	11.8	12.7	12.1	12.8	11.2	12.6	12.7	13.6
Planctomycetes	4.99	5.75	6.01	5.67	6.09	6.66	6.08	6.5	6.43	7.8
Bacteroidetes	3.164	0.683	0.608	0.625	0.787	0.658	0.7	1.71	1.62	0.9

Fungi		Mean values								
		Low NP soil			Low N high P soil			High NP soil		
Taxon	Contrib. %	input	planted	unplanted	input	planted	unplanted	input	planted	unplanted
Sordariomycetes	29.09	35.7	34.3	37	44.6	44.4	47	51.9	56.8	58.5
Eurotiomycetes	22.07	19.8	20.2	21.9	12.8	14.6	9.16	4.82	5.99	5.3
Unassigned	13.32	16.5	18.4	18.5	17.4	19.1	23.5	30.7	21.4	23.6
Dothideomycetes	10.84	11.2	10.1	7.79	15.2	12	10.7	6.74	5.25	5.31
Leotiomycetes	8.051	7.79	6.99	5.2	2.91	2.76	4.54	0.91	1.95	1.16
Agaricomycetes	7.213	6.01	6.07	6.16	2.85	1.91	1.4	1.21	4.05	0.71
Pezizomycetes	4.218	0.84	0.62	0.94	0.3	0.64	0.58	2.85	3.02	4.26
Tremellomycetes	3.587	1.65	2.56	2.2	2.6	3.54	2.69	0.44	0.64	0.52
Other	1.059	0.26	0.28	0.2	1.36	0.91	0.46	0.44	0.38	0.43
Glomeromycetes	0.4548	0.22	0.44	0.08	0.04	0.13	0.05	0.05	0.39	0.11

The mean values of the Relative Abundance (%) of all samples are shown. Analysis of Similarity Percentages (SIMPER) showing bacterial taxa that contributed most of the dissimilarities (≥2%) of the bacterial communities and the fungal taxa that contributed to the dissimilarities of the fungal communities.

The observed differences of microbiomes from different soil types could be explained by edaphic factors and therefore daphic factors were integrated with the bacterial and fungal microbiome using Canonical Correspondence Analysis (Figure 5.8). This analysis revealed that the bacterial and fungal community of the high NP soil type is associated with increased soil pH, conductivity, total C and available N, while the fungal community of this soil type is associated with high CN ratio. The fungal community of the low N high P soil type is associated with increased available P, whereas the fungal community of the low NP soil type is associated with the depletion of all edaphic factors and slight increases of total N in the soil. Soil pH, conductivity, CN ratio, total soil C and total N as well as soil N and P available to the plant and explain the 75.39% of the differences in the bacterial community structure and the 75.72% of the differences in the fungal community structure as shown from the CCA plots.

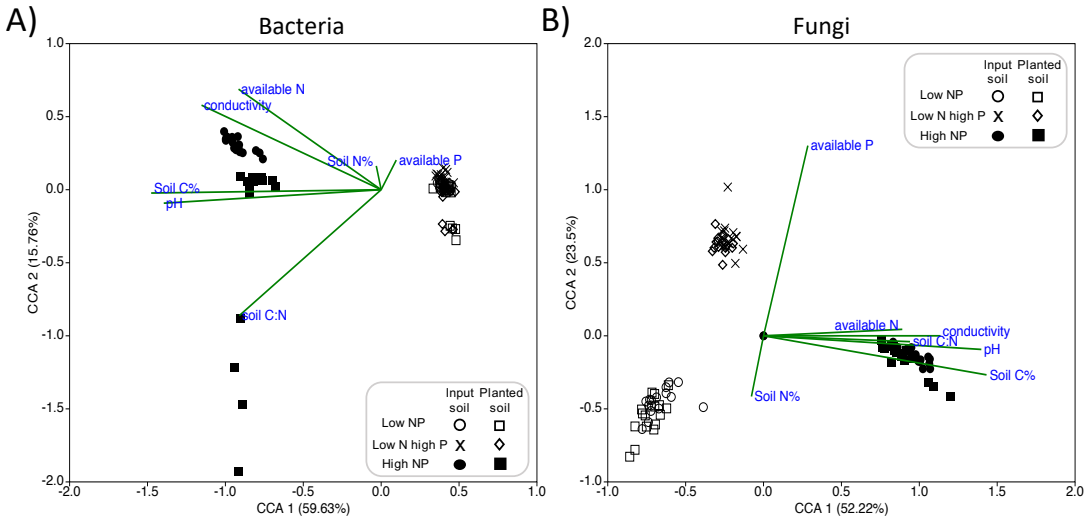


Figure 5.8: Correlation of edaphic factors with microbial community structure. Canonical Correspondence Analysis (CCA) illustrating the effect of edaphic factors on the A) soil bacterial and B) fungal community structure in the input and planted (mock-treated) soil samples. The two first axis of the CCA are plotted explaining the 75.39% and the 75.72% of the differences in the bacterial and fungal community structure respectively.

Overall, the three soil types are characterized by distinct microbial communities as well as varying biochemical characteristics. The high NP soil type has

higher conductivity, pH, available nitrate, C:N ratio, C and N content and lower fungal OTU richness than low NP and low N high P soil types, as well as distinct bacterial community structure. Fungal community structure was different between all soil types and bacterial community composition is characterised by the strong presence of proteobacteria. These differences between the chosen soil types will help unravel the different effect that soil factors can have on plant growth and nutrient acquisition, as well as the effect of rhizobial inoculants under different conditions.

5.2.3 Effect of rhizobial strains on *Medicago truncatula* growth in different soil types

The three different soil types as described above were used to investigate growth promoting properties of the three rhizobial strains on *Medicago truncatula* (Figure 5.9). *M. trancatula* plants were inoculated 1 day after being transferred to perlite pots and were moved to the pots with natural soil 11 dai (14 day old plants). The efficiency of the co-inoculation was assessed using phenotypic data and quantification of the fungal colonisation 63 dai, (66 day old plants).

Overall, rhizobial strain 419 enhanced plant growth in low NP and low N high P soil types, whereas 1022 enhanced plant growth in all soil types (Figure 5.10). The most appropriate sampling time point was determined to be 63 dai using growth curves (Figure S5.1).

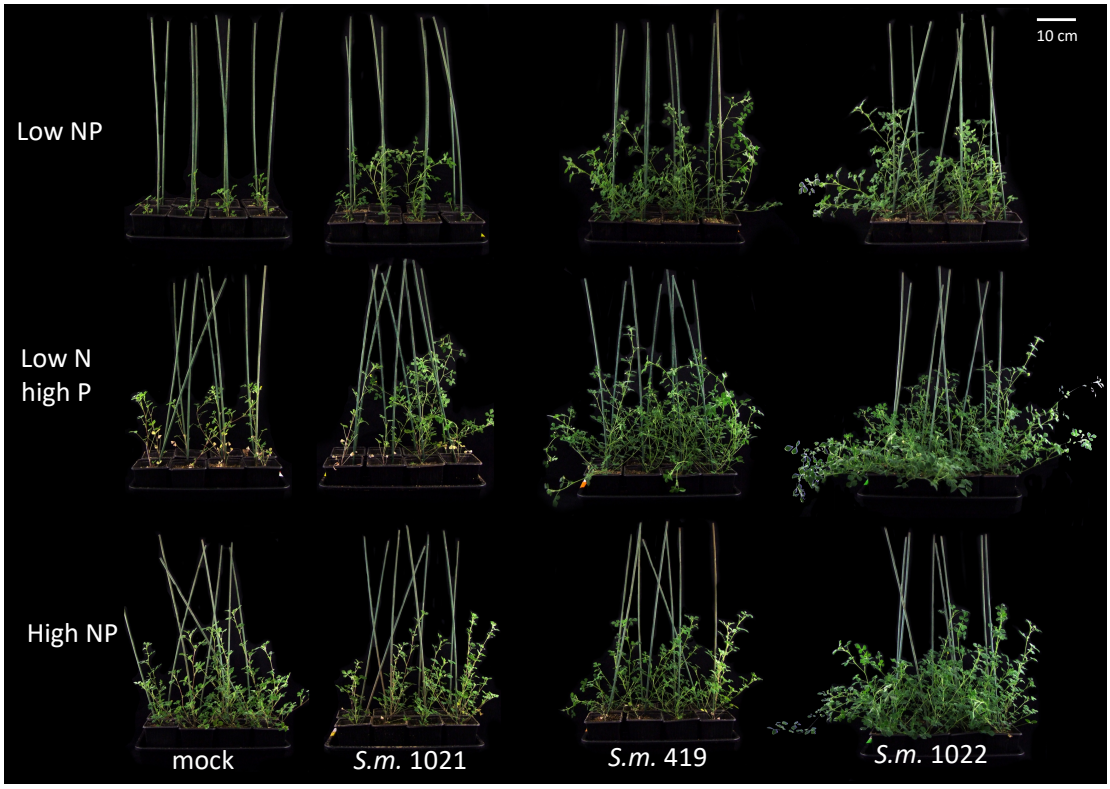


Figure 5.9: Comparison of plant growth of Medicago truncatula in different soil types and treatments. Representative picture of 4 biological repeats showing 66 day old Medicago truncatula plants grown under different treatments (mock, Sm1021, Sm419, Sm1022) and soil types (low NP, low N high P, high NP), 63 dai. Treatments represent mock (sterile water) and rhizobia inoculations with 3 different Sinorhizobium strains (S. meliloti 1021, S. medicae 419, S. meliloti 1022).

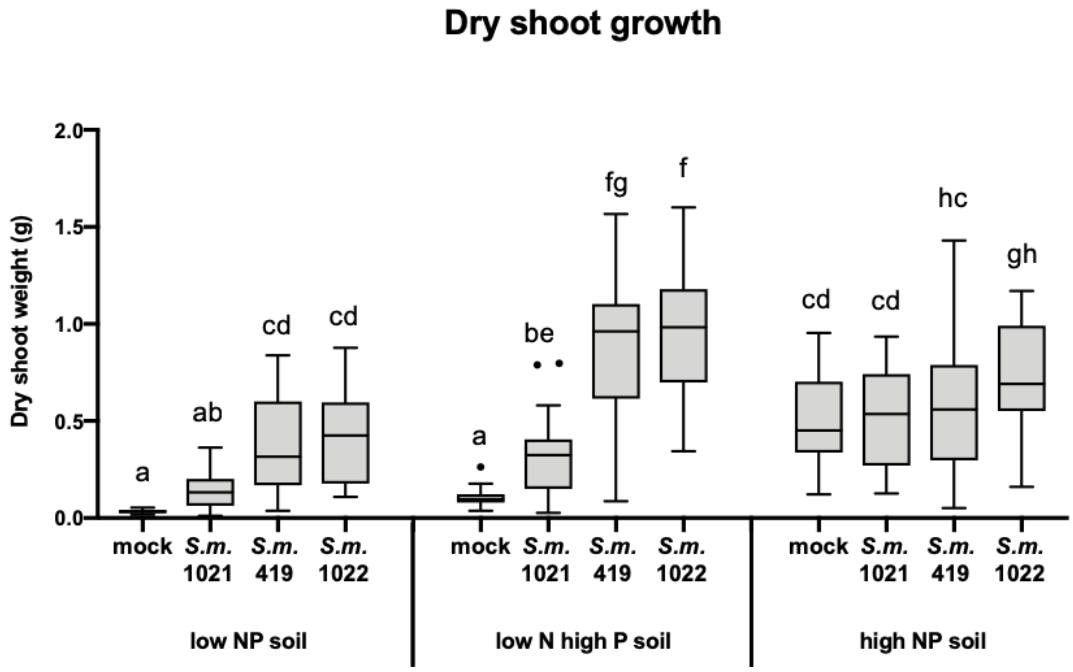


Figure 5.10: Dry shoot weight of Medicago truncatula in different soil types and treatments. Box plots indicating 66 day old Medicago truncatula plant weight under different treatments and soil types, 63 dai, based on above ground dry weight (g). Treatments represent mock (sterile water) and rhizobia inoculations with 3 different Sinorhizobium strains (S. meliloti 1021, S. medicae 419, S. meliloti 1022). Letters represent significant differences according to ANOVA followed by to Tukey's HDS ($p < 0.001$). Boxplots represent the median value (black bar inside the box) and whiskers represent quartile distribution. Outlier data points are depicted as black dots outside the boxplots.

Finally, from these results it was clear that only *S. meliloti* 1022 increased the plant yield significantly in all soil types (based on ANOVA followed by Tukey's HSD test, $p < 0.001$) (Figure 5.10). Thus, *S. meliloti* 1022 was chosen for the following experiments studying the microbiome changes in the presence and absence of the rhizobial inoculant, in different soil types and the consequent effect on plant growth and nutrient acquisition.

5.2.4 *S. meliloti* 1022 alters the bacterial but not the fungal community structure in *Medicago truncatula* roots in different soil types

Roots from mock and *S. meliloti* 1022-treated *Medicago truncatula* plants were collected and processed for sequencing as described at the methods sections 2.10 and 2.11. As soil is referred to bulk soil from pots grown with plants, sampled in distance from the roots, rhizosphere (rhizo) was defined as the microbes at the surface of the root and endosphere (endo) covered the inside of the root, clean from all the microbes on the surface of the root. Dried plant shoot tissue and soil samples were stored at room temperature for further nutrient analyses at the end of the experiment. These data enable the association of changes in microbial community structure, plant and soil nutrient content as well as the effect of the rhizobial strain.

Microbiome libraries were constructed individually for fungi and bacteria and were subjected to Next Generation Sequencing using Illumina Miseq. The sequencing data were analysed using QIIME and plotted using principle coordinates analysis (PCoA) based on Bray Curtis distance matrices (Figure 5.11). All samples were plotted together in order to observe common patterns and differences between the soil types, however, individual PCoA plots for each soil type can be found at the next section. Bacterial and fungal communities of soil and rhizosphere samples of high NP soil clustered together and separately from all the rest of the samples, whereas soil and rhizosphere samples of low NP and low N high P clustered closer, as previously observed when analysing only the input, planted (mock-treated) and unplanted soils. In more detail, bulk soil bacterial communities of low NP and low N high P soil types clustered together, whereas some rhizosphere samples tended to create a separate cluster, between soil and endosphere samples (Figure 5.11A). Bulk soil and rhizosphere microbiomes were not separated by the treatment. The endosphere samples created two clear clusters distinguished by the treatment (Figure 5.11A). The bulk soil fungal communities in low NP and low N high P soil types clustered separately, yet closer than to the high NP soil type. However, some rhizosphere samples from low NP and low N high P soil types clustered between soil and rhizosphere samples (Figure 5.11B). All endosphere samples of all soil types and treatments clustered together.

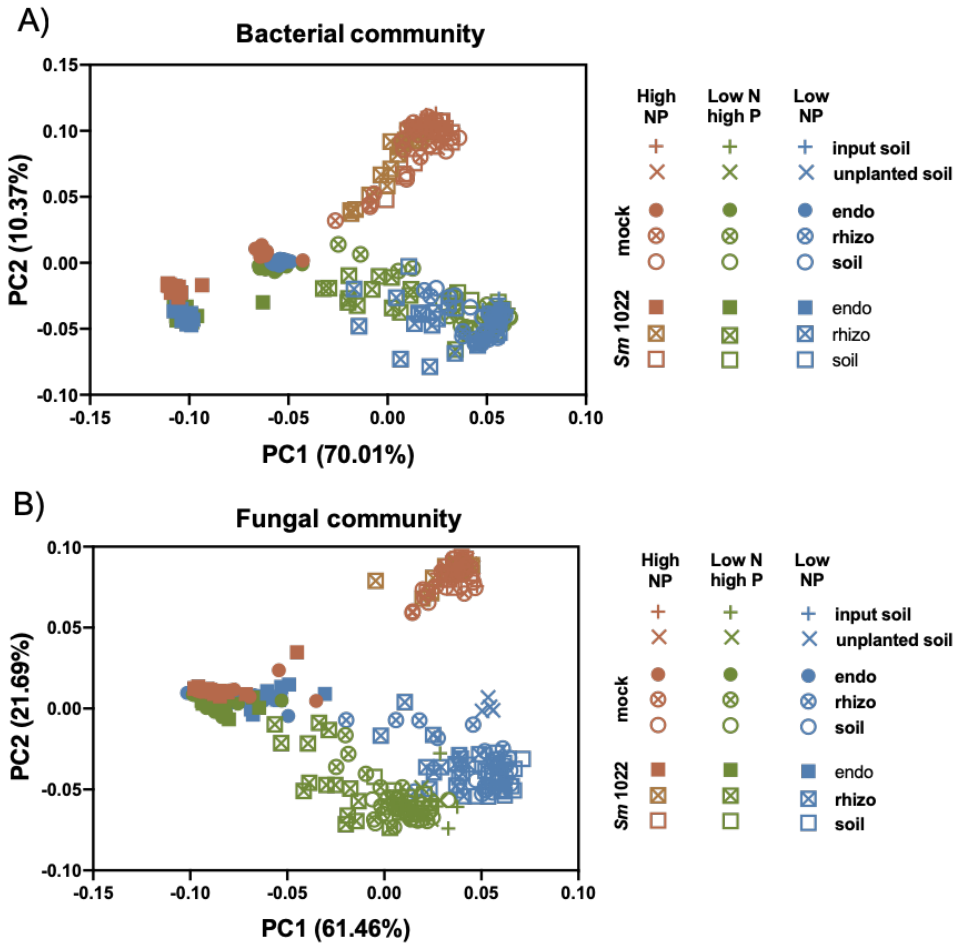


Figure 5.11: Bacterial and fungal community structure including all treatments and soil types. Bacterial community (A) and fungal community (B) beta diversity presented as microbiome structure using Principle Coordinates Analysis (PCoA). The first two dimensions of PCoA are plotted based on Bray-Curtis distances (rarefied reads used) and they explain 80.38% and 83.25% of the variation in the data respectively.

Following the determination of microbiome beta diversity using PCoA, Analysis of similarities (ANOSIM) was performed for bacterial and fungal microbiomes to determine if there were any significant changes in the community structure between the different sample types. The P and R values were calculated and presented as a heatmap (Tables 5.2 and 5.3).

The heatmap is divided by the diagonal from top left to bottom right corresponding to same sample comparisons, the ANOSIM-P values are presented on the bottom left side of that diagonal. P values <0.05 indicate significant differences

and are highlighted in blue, e.g community structure of the mock-treated endosphere in low N high P soil type is significantly different (P-value 0.0276) from the community of the mock-treated endosphere in low NP soil type. ANOSIM-R values are presented on the top right side of the diagonal. R values that are very close to 1 show highly different samples and are coloured in dark blue, e.g community structure in the mock-treated endosphere in low N high P soil type is very different (R-value 0.9745) from the community in the mock-treated endosphere in low NP soil type, and values closer to 0 indicate indifferent samples. The data on the ANOSIM tables indicate that the differences observed on the PCoA plots above (Figure 5.11) are significant. Therefore, most of the bacterial microbiome samples of the high NP soil type are highly different than the other two soil types and all endosphere samples are highly different from all other samples (Table 5.3). Similarly, most of the fungal microbiome samples of the high NP soil type are highly different than the other two soil types, but endosphere samples present higher level of similarity (Table 5.4). These tables demonstrate that the differences observed at Figure 5.11 are significant.

Table 5.3: Heatmap of significant changes in the bacterial community composition between all samples.

ANOSIM on bacteria samples

p value < 0.05	R value 0 / 0.25 / 0.5 / 0.75 / 1		High NP								Low NP								Low N high P							
				mock			Sm1022					mock			Sm1022					mock			Sm1022			
			input soil	endo	rhizo	soil	endo	rhizo	soil	unplanted soil	input soil	endo	rhizo	soil	endo	rhizo	soil	unplanted soil	input soil	endo	rhizo	soil	endo	rhizo	soil	unplanted soil
High NP		input soil		1	0.6127	0.3692	1	0.6586	0.282	0.7681	1	1	1	1	1	0.9993	0.9961	1	1	1	0.8271	1	1	1	1	1
	mock	endo	0.0276		0.9972	1	0.9996	0.9949	1	1	1	1	1	1	1	0.9999	1	1	1	0.9982	0.9904	1	1	1	1	1
		rhizo	0.0276	0.0276		0.3563	1	0.1202	0.3439	0.23	0.8882	1	0.910	0.947	1	0.9435	0.932	0.8616	0.906	1	0.6973	0.9354	1	0.9575	0.9277	0.9234
		soil	0.0276	0.0276	0.0276		1	0.485	-0.03322	-0.136	0.9879	1	0.998	0.997	1	0.9981	0.9833	0.9913	0.9902	1	0.7752	0.9947	1	1	0.9953	1
	Sm1022	endo	0.0276	0.0276	0.0276	0.0276		0.9787	1	1	1	1	1.000	1.000	0.7257	0.963	1	1	1	1	1	1	0.5558	0.9928	1	1
		rhizo	0.0276	0.0276	1	0.0276	0.0276		0.4683	0.3188	0.9885	1	0.987	0.995	0.9902	0.9664	0.9959	0.9807	0.9893	1	0.8147	0.9954	0.9833	0.9365	0.9946	0.9983
		soil	0.0276	0.0276	0.0276	1	0.0276	0.0276		-0.1441	0.9298	1	0.979	0.970	1	0.9954	0.9549	0.9193	0.9421	1	0.788	0.9595	1	0.9996	0.9605	0.9678
		unplanted soil	0.2484	0.138	1	1	0.1104	1	1		1	1	0.999	1.000	1	0.9902	0.9378	1	1	1	0.5773	1	1	1	1	1
Low NP		input soil	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.138		1	0.5839	0.02763	1	0.7929	0.1729	0.2253	0.8	1	0.4701	0.4164	1	0.9923	0.4946	0.8496
	mock	endo	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.1104	0.0276		1	1	1	0.9997	1	1	1	0.9745	0.9495	1	0.9987	1	1	1
		rhizo	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.2208	0.0276	0.0276		0.3904	1	0.4747	0.35	0.3332	0.7084	1	0.2956	0.4771	1	0.9526	0.5023	0.444
		soil	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0552	1	0.0276	0.0276		1	0.736	-0.03805	-0.107	0.5786	1	0.4284	0.2964	1	0.9803	0.3099	0.2974
	Sm1022	endo	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.1104	0.0276	0.0276	0.0276	0.0276		0.9012	1	1	1	1	0.9998	1	0.3365	0.9708	1	1
		rhizo	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.1656	0.0276	0.0276	0.0276	0.0276	0.0276		0.6254	0.5975	0.8521	0.9997	0.443	0.8081	0.901	0.523	0.8088	0.6932
		soil	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.3036	0.8004	0.0276	0.1656	1	0.0276	0.0276		0.01416	0.6807	1	0.3097	0.2965	1	0.9683	0.271	0.2906
		unplanted soil	0.1656	0.138	0.0828	0.0552	0.138	0.1104	0.0552	1	1	0.0828	1	1	0.1104	0.138	1		0.9239	1	0.1675	0.2257	1	0.9836	0.1835	0.7135
Low N high P		input soil	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0828	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.1104		1	0.4507	0.1707	1	0.9875	0.2005	0.6172
	mock	endo	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0828	0.0276		0.9287	1	0.9857	0.9993	1	1
		rhizo	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	1	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.3864	1	0.0276	0.0276		0.2806	0.9899	0.6418	0.2776	0.11
		soil	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.7176	1	0.0276	0.0276	0.0276		1	0.9725	0.01432	0.08222
	Sm1022	endo	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0552	0.0276	0.0276	0.0276	0.0276	0.1104	0.0276	0.0276	0.138	0.0276	0.0276	0.0276	0.0276		0.9043	1	1
		rhizo	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.1104	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.138	0.0276	0.0276	0.0276	0.0276	0.0276		0.979	0.9855
		soil	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.1656	0.0276	0.0276	0.0276	0.1104	0.0276	0.0276	0.552	1	0.0276	0.0276	0.0552	1	0.0276	0.0276		0.0001841
		unplanted soil	0.0828	0.138	0.1656	0.2208	0.1104	0.1656	0.138	1	0.1656	0.0828	1	1	0.138	0.1104	1	1	0.0552	0.0828	1	1	0.0552	0.0276	1	

Results of ANOSIM on the different samples using the bacterial microbiome data. The left side of the table is presented the p values of the test, color coded as light blue for the p value < 0.05 and on the right side of the table is presented the R values of the ANOSIM test, color coded as indicated, from light blue being indifferent (R=0) to dark blue indicating the strongest differences (R=1)

Table 5.4: Heatmap of significant changes in the fungal community composition between all samples

ANOSIM on fungi samples

p value < 0.05	R value 0 / 0.25 / 0.5 / 0.75 / 1		High NP								Low NP								Low N high P							
				mock			Sm1022					mock			Sm1022					mock			Sm1022			
			input soil	endo	rhizo	soil	endo	rhizo	soil	unplanted soil	input soil	endo	rhizo	soil	endo	rhizo	soil	unplanted soil	input soil	endo	rhizo	soil	endo	rhizo	soil	unplanted soil
High NP		input soil		0.9877	0.5469	0.5292	1	0.6196	0.5958	0.6427	1	1	0.9989	1	1	0.9999	1	1	1	1	0.8576	1	0.9671	0.9929	1	1
	mock	endo	0.0276		0.9752	0.9864	0.03946	0.9683	0.9867	0.9754	0.9914	0.3885	0.9693	0.9871	0.3119	0.8485	0.9906	0.9851	0.9873	0.5246	0.967	0.9871	0.5375	0.9776	0.9869	0.9804
		rhizo	0.0276	0.0276		0.08449	0.9998	0.0179	0.1223	-0.2458	1	1	0.9978	1	1	0.9993	1	1	1	1	0.8606	1	0.9511	0.9912	1	0.9993
		soil	0.0276	0.0276	1		0.9999	0.1197	-0.0009603	-0.3471	1	1	0.9977	1	1	0.9992	1	1	1	1	0.8445	1	0.9642	0.9909	1	0.9973
	Sm1022	endo	0.0276	1	0.0276	0.0276		0.9948	1	1	1	0.4797	0.9999	1	0.3744	0.9461	1	1	1	0.6221	0.9969	1	0.6306	1	1	1
		rhizo	0.0276	0.0276	1	0.1932	0.0276		0.1169	-0.1499	1	0.9927	0.9977	1	0.994	0.9922	1	1	1	0.9965	0.8457	1	0.9305	0.9901	1	1
		soil	0.0276	0.0276	0.2208	1	0.0276	0.138		-0.3857	1	1	0.9981	1	1	0.9998	1	1	1	1	0.8477	1	0.9628	0.9925	1	1
		unplanted soil	0.0276	0.0552	1	1	0.1104	1	1		1	1	0.9949	1	1	0.9998	1	1	1	1	0.667	1	0.9113	0.9823	1	1
Low NP		input soil	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0552		0.9912	0.3767	0.09308	1	0.5359	0.1239	0.3888	0.9869	1	0.8362	0.9886	0.9725	0.9846	0.9834	0.999
	mock	endo	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.138	0.0276		0.9567	0.978	0.008838	0.7604	0.9781	0.9939	1	0.4115	0.9852	1	0.4839	0.9892	1	1
		rhizo	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0828	0.0276	0.0276		0.3307	0.9941	0.2456	0.2512	0.02838	0.834	0.9977	0.7655	0.8262	0.9248	0.8577	0.8178	0.9126
		soil	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0552	1	0.0276	0.0276		0.9988	0.5138	0.005593	0.2778	0.9996	0.9999	0.865	0.9994	0.9635	0.9874	0.9989	1
	Sm1022	endo	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0828	0.0276	1	0.0276	0.0276		0.7758	0.9994	1	1	0.3336	0.9868	1	0.437	0.9958	1	1
		rhizo	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.138	0.0276	0.0276	0.0276	0.0276	0.0276		0.4878	0.2736	0.8628	0.8436	0.7774	0.849	0.6733	0.7998	0.8452	0.9369
		soil	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0828	0.276	0.0276	0.0276	1	0.0276	0.0276		0.08859	0.989	1	0.866	0.9909	0.969	0.9916	0.9876	0.9997
		unplanted soil	0.0276	0.0828	0.0828	0.1104	0.1656	0.0828	0.0552	1	1	0.0828	1	1	0.0828	1	1		1	1	0.6642	1	0.9547	0.9598	1	1
Low N high P		input soil	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0552	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.1656		0.9988	0.2446	0.1464	0.9303	0.5089	0.07196	0.9123
	mock	endo	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0828	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0552	0.0276		0.9778	0.9984	0.08899	0.9812	0.9985	1
		rhizo	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.1932	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.5244	0.0276	0.0276		0.1333	0.8492	0.05929	0.1814	0.1138
		soil	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.1104	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.1104	0.0828	0.0276	0.138		0.9278	0.4697	0.02091	0.8214
	Sm1022	endo	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.138	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0828	0.0276	1	0.0276	0.0276		0.7824	0.9226	0.8551
		rhizo	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0552	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0828	0.0276	0.0276	1	0.0276	0.0276		0.4791	0.45
		soil	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0828	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0276	0.0552	1	0.0276	0.0276	1	0.0276	0.0276		0.8745
		unplanted soil	0.0552	0.0552	0.138	0.0828	0.0552	0.0828	0.0828	1	0.0552	0.1104	0.0552	0.1104	0.138	0.0828	0.0828	1	0.0828	0.0276	1	0.0552	0.138	1	0.1104	

Results of ANOSIM on the different samples using the fungal microbiome data. The left side of the table is presented the p values of the test, color coded as light blue for the p value < 0.05 and on the right side of the table is presented the R values of the ANOSIM test, color coded as indicated, from light blue being indifferent (R=0) to dark blue indicating the strongest differences (R=1)

The alpha and beta diversity analyses revealed the broad differences in the microbiome. Using analysis of similarity percentages (SIMPER), it was possible to identify the taxa that contributed the most to the observed differences. The bacterial OTU2, assigned to Rhizobiales species, and fungal OTU3, assigned to Sordariomycetes species of the Ascomycota phylum, had the most significant contribution to the differences observed among all samples in the bacterial and the fungal community, respectively (Table 5.5). The bacterial OTU2, a Rhizobiales species, contributed 12% to the differences in the bacterial community, followed by two other Proteobacteria species, *Methylibium* and a Sphigomonaceae species, as well as a Micrococcaceae species of the Actinobacteria Phylum, contributing 1% to the differences each. The fungal OTU3, Sordariomycetes species, contributed 14% to the differences, followed by another Sordariomycetes species which contributed 6% to the differences, other Sordariomycetes species including *Fusarium* and a Eurotiomycetes species, that contribute to 1-4% to the observed diversity.

Table 5.5: Microbial taxa contributing to the differences in community structure among all samples.

Bacteria

Overall average dissimilarity	samples	Taxon	Contrib. %	Taxonomy
78.21%	all	OTU2	12.01	Proteobacteria/Alphaproteobacteria/**Rhizobiales**
		OTU4	1.867	Proteobacteria/Betaproteobacteria/Burkholderiales/Comamonadaceae/**Methylibium**
		OTU8	1.063	Actinobacteria/Actinobacteria/Actinomycetales/**Micrococcaceae**
		OTU13	1.007	Proteobacteria/Alphaproteobacteria/Sphingomonadales/**Sphingomonadaceae**

Fungi

Overall average dissimilarity	samples	Taxon	Contrib. %	Taxonomy
71.48%	all	OTU3	14.08	Ascomycota/Sordariomycetes/Hypocreales/Incertae sedis/Ilyonectria/**Ilyonectria macrodidyma**
		OTU2	6.887	Ascomycota/**Sordariomycetes sp**
		OTU4	4.666	Ascomycota/Incertae sedis/Incertae sedis/Pseudeurotiaceae/**Pseudeurotium sp**
		OTU6	2.917	Ascomycota/Sordariomycetes/**Hypocreales sp**
		OTU5	2.856	Ascomycota/Eurotiomycetes/Chaetothyriales/**Herpotrichiellaceae sp**
		OTU9	1.714	Ascomycota/Sordariomycetes/Hypocreales/Bionectriaceae/Bionectria/**Bionectria ochroleuca**
		OTU13	1.614	Ascomycota/Sordariomycetes/Hypocreales/Nectriaceae/**Fusarium sp**
		OTU44	1.561	Ascomycota/Sordariomycetes/Sordariales/Lasiosphaeriaceae/**Podospora sp**

Analysis of Similarity Percentages (SIMPER) identifies taxa that contribute to most of the dissimilarities (≥1%) between all the samples of the bacterial and fungal communities.

Due to the high complexity of the experiment, including samples from different soil types, compartments, treatments and microbial kingdoms, the bacterial and fungal communities were analysed as per soil type, in order to be able to observe the effect of the *S. meliloti* 1022 on the microbiomes in the different soil types.

As described earlier, the alpha and beta diversity of the bacterial community is presented using the number of Observed OTUs and with a PCoA based on Bray-Curtis respectively, for each soil type (Figure 5.12). Similar patterns are observed in all soil types, with the number of OTUs being significantly lower in the endosphere, independently of the soil type and treatment. This number is even lower in the presence of *S. meliloti* 1022 and the beta diversity PCoA plot shows that mainly the endosphere is clustered according to the treatment (Figure 5.12). Notably, only the thizosphere samples of low NP and low N high P soil types differ significantly to both the bulk soil and the endosphere samples in the presence of *S. meliloti* 1022, whilst this is not the case for the mock treatment.

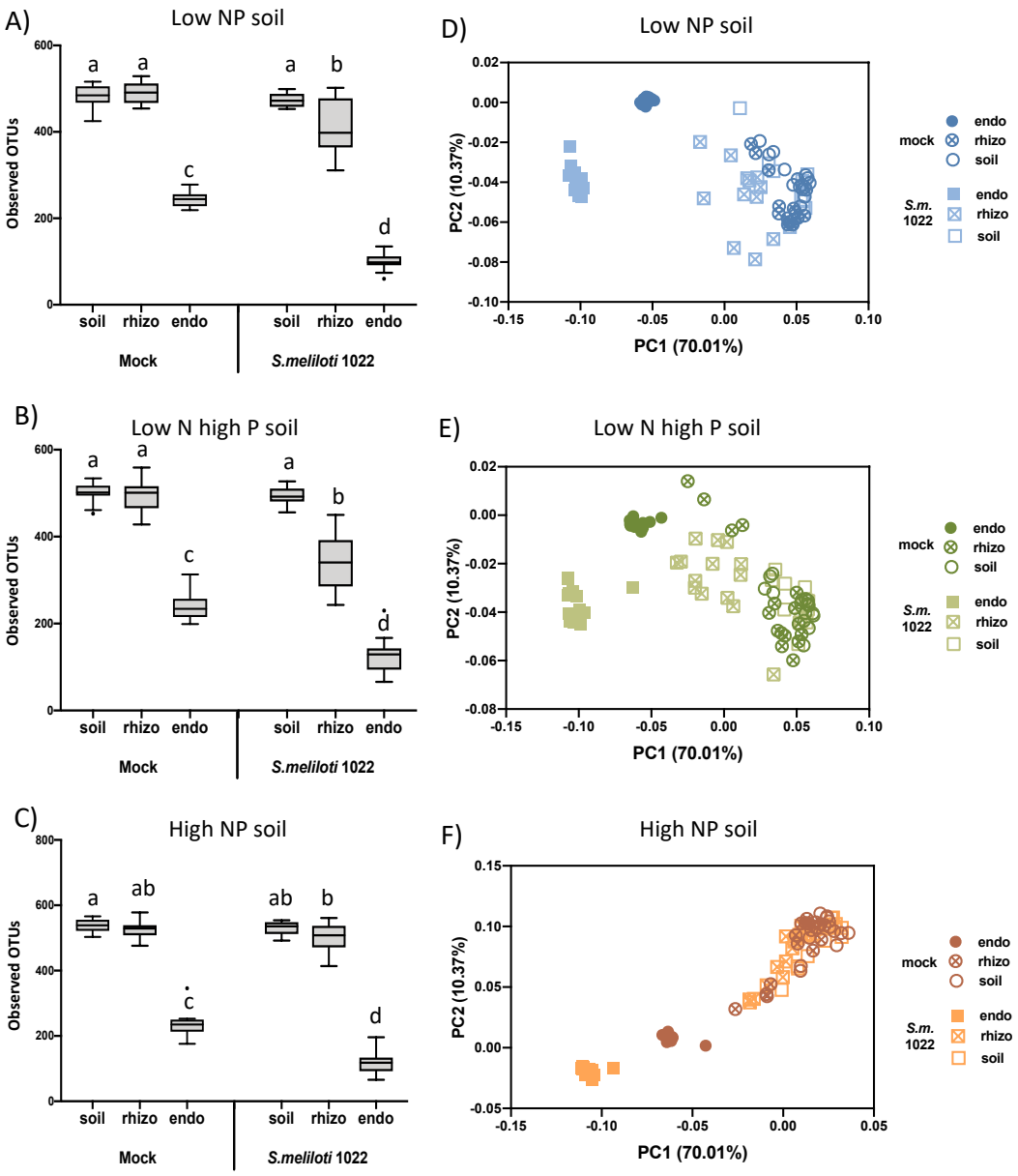


Figure 5.12: Bacterial community richness and structure in the three different soil types. A-C) Alpha diversity of bacterial community presented as the number of observed OTUs in each sample type. Letters represent significant differences according to ANOVA followed by Tukey's HDS test ($p < 0.001$). D-F) Beta diversity presented as microbiome structure using principle coordinates analysis (PCoA). The first two dimensions of PCoA are plotted based on Bray-Curtis distances (rarefied reads used). A) and D) refer to low NP soil type, B) and E) to low N high P soil type, C) and F) to high NP soil type.

The alpha and beta diversity of the fungal communities were analysed in the same way as the bacterial community. Similar patterns were observed in low NP and

low N high P soil types, with strong effects of the compartment on both alpha and beta diversity whereas the presence of the rhizobial inoculant had no effect on the fungal community (Figure 5.13A, B, D, E). In the high NP soil type the microbiome of the rhizosphere was indifferent from the soil microbiome, while endosphere microbiome clusters had less OTUs independently of the rest of the samples (Figure 5.13C and F). For all soil types, *S. meliloti* 1022 had no effect on the fungal community.

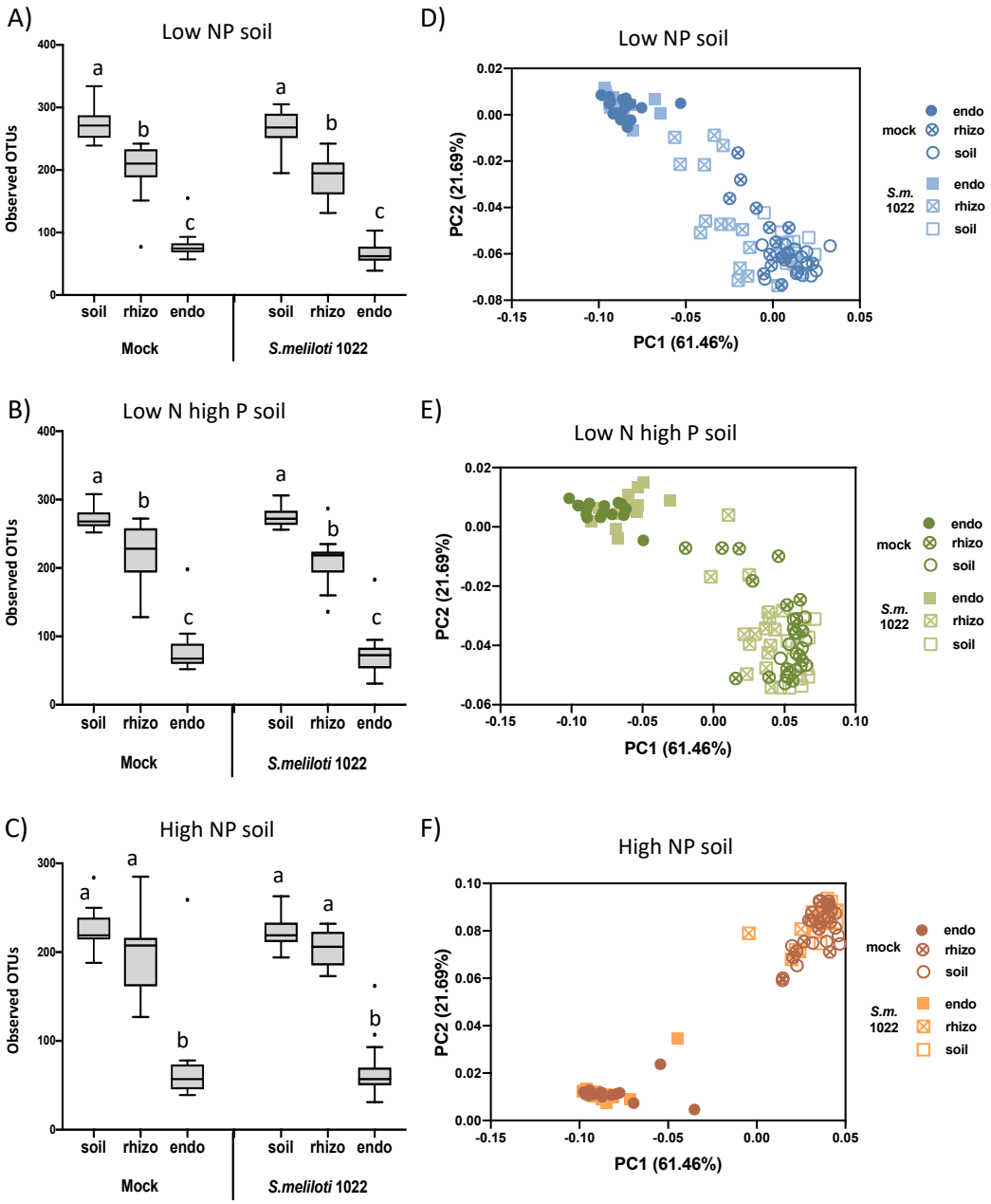


Figure 5.13: Fungal community richness and structure in the three different soil types. Fungal community A), B), C) alpha diversity presented as the number of observed OTUs in each sample type, letters represent significant differences according to

ANOVA followed by Tukey's HDS test ($p < 0.001$) and D), E), F) beta diversity presented as microbiome structure using Principle Coordinates Analysis (PCoA). The first two dimensions of PCoA are plotted based on Bray-Curtis distances (rarefied reads used). A) and D) refer to low NP soil typel, B) and E) to low N high P soil type, C) and F) refer to high NP soil type.

Overall, *S. meliloti* 1022 affected the bacterial microbiome diversity of the root endosphere in all soil types, but not the fungal microbiome diversity. Observed differences in the diversity of fungal communities were caused by edaphic factors. Major contributions to these differences have the alpha-Proteobacteria and the Order Sordariomycetes respectively.

5.2.5 *S. meliloti* 1022 alters the composition of bacterial but not fungal community in *Medicago truncatula* roots in different soil types

In order to investigate the effect of the rhizobial inoculant on the microbiome composition, the RA of bacterial and fungal taxa was analysed. This analysis revealed taxa with different abundances in the different compartments and soil types in the presence of *S. meliloti* 1022.

The RA of the bacterial taxa was calculated from rarefied reads and was analysed at the phylum level. A gradient effect of the compartment was observed, with an increase of Proteobacteria from soil to endosphere in both treatments and all soil types (Figure 5.14). Analysis of similarity percentages (SIMPER) showed that OTU2, belonging to Proteobacteria and assigned as Rhizobiales sp., had the most significant contribution to the differences observed between mock and *S. meliloti* 1022 samples in the endosphere and rhizosphere of all soil types (Table 5.6). This taxon was increased in the presence of the rhizobial inoculant in all samples. However, different species contributed to differences in the bacterial community composition between mock and *S. meliloti* 1022-treated samples in the endosphere and rhizosphere of all soil types (Table 5.6). In high NP soil type, a beta-proteobacteria species (Burkholderiales, Comamonadaceae family) contributed 11% to the differences in the endosphere and 2.5% to the rhizosphere between the treatments. In the low N high P

soil type another alpha-proteobacteria species contributed 2.8% to the differences in the endosphere and an Actinobacteria species contributed 3.3% to the differences between the mock and *S. meliloti* 1022 samples in the rhizosphere. In the low NP soil type Actinobacteria and Chloroflexi species contributed 2.9-3.9% to the differences observed in the endosphere and a different Actinobacteria species contributed 3.2% to the differences observed in the rhizosphere between the treatments (Table 5.6).

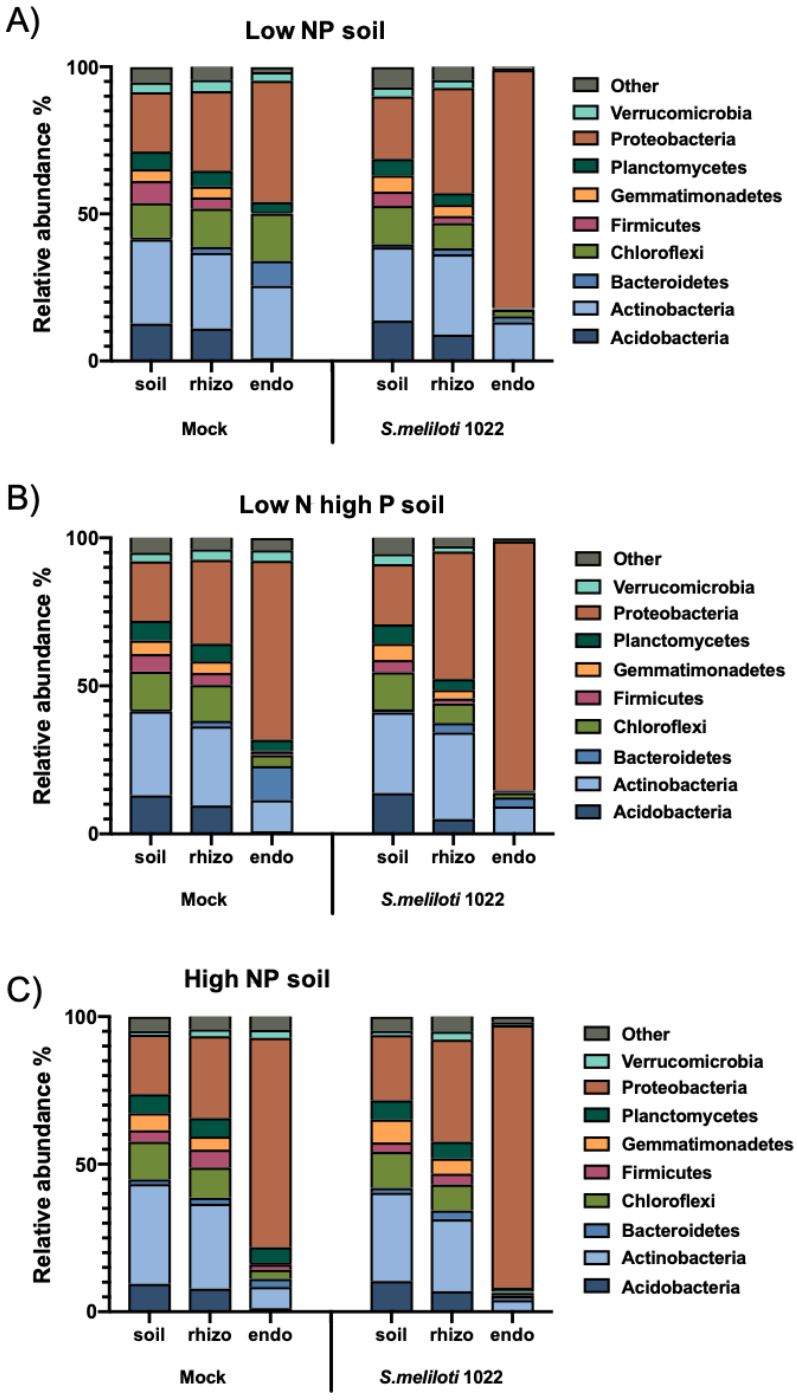


Figure 5.14: Bacterial community composition in three different soil types. Relative abundance of bacterial taxa at the phylum level in A) low NP soil type, B) low N high

P soil type and C) high NP soil type; shown as percentage of all reads calculated using rarefied reads. "Other" refers to unassigned and low abundant bacterial taxa.

Table 5.6: Microbial taxa contributing to the differences in community structure in the different soil types.

endosphere samples	Taxon	Contrib. %	mean value		Taxonomy
			mock	*S.m.* 1022	
High NP	OTU2	42.77	4.87	691	Proteobacteria/Alphaproteobacteria/**Rhizobiales sp**
	OTU4	11.31	228	46.9	Proteobacteria/Betaproteobacteria/Burkholderiales /Comamonadaceae/**Methylibium sp**
Low N high P	OTU2	41.46	1.94	682	Proteobacteria/Alphaproteobacteria/**Rhizobiales sp**
	OTU13	2.833	65.8	26.8	Proteobacteria/Alphaproteobacteria/Sphingomonadales /**Sphingomonadaceae sp**
Low NP	OTU2	41.87	0.25	694	Proteobacteria/Alphaproteobacteria/**Rhizobiales sp**
	OTU9	3.896	80.1	22.8	Actinobacteria/Actinobacteria/**Actinomycetales sp**
	OTU115	3.309	63.4	8.62	Chloroflexi/Chloroflexi/Roseiflexales/**Kouleothrixaceae sp**
	OTU24	2.948	56.7	7.85	Chloroflexi/Chloroflexi/Roseiflexales/**Kouleothrixaceae sp**

rhizosphere samples	Taxon	Contrib. %	mean value		Taxonomy
			mock	*S.m.* 1022	
High NP	OTU12	2.521	13.8	39.6	Proteobacteria/Betaproteobacteria/Burkholderiales /Comamonadaceae/**Hydrogenophaga sp**
	OTU2	2.112	4.07	25.4	Proteobacteria/Alphaproteobacteria/**Rhizobiales sp**
Low N high P	OTU2	10.47	1.25	149	Proteobacteria/Alphaproteobacteria/**Rhizobiales sp**
	OTU18	3.369	6.75	54.4	Actinobacteria/Actinobacteria/Actinomycetales /Nocardioidaceae/**Aeromicrobium sp**
Low NP	OTU2	7.549	0.267	95.1	Proteobacteria/Alphaproteobacteria/**Rhizobiales sp**
	OTU5	3.265	0.0667	41.1	Actinobacteria/Actinobacteria/Actinomycetales /Pseudonocardiaceae/**Pseudonocardia sp**

Analysis of Similarity Percentages (SIMPER) identified taxa that contributed most of the dissimilarities (≥2%) of the bacterial communities between mock samples and those inoculated with S. meliloti 1022 (S.m 1022), in the endosphere and rhizosphere respectively, in the different soil types. Mean values of the Relative Abundance are shown for the mock and the S. meliloti 1022 treatment.

In order to investigate the differences that were associated with *S. meliloti* 1022 in the endosphere and rhizosphere microbiome, changes in the relative abundances of bacterial taxa at the order level were calculated by comparing their abundances in the *S. meliloti* 1022 and mock samples. The abundance of the taxa that significantly changed as calculated using Kruskal-Wallis test with p value < 0.001, were plotted as the log2 ratio of their abundance in the *S. meliloti* 1022-treated plants in relation to mock-treated plants (only taxa with significant changes were plotted) (Figure 5.15). In

all soil types, alpha-Proteobacteria were significantly enriched in the endosphere after *S. meliloti* 1022 treatment, with beta-, gamma- and delta-Proteobacteria being depleted, along with all other taxa (Figure 5.15). In the rhizosphere of low NP soil type, only 4 taxa were significantly affected negatively in the presence of the rhizobial inoculant (Figure 5.16A), while the rhizosphere of high NP soil type was not significantly affected. The relative abundance of alpha-Proteobacteria was also significantly increased in the rhizosphere microbiome of the low N high P soil type, along with the Actinobacteria, whereas the rest of the taxa had significantly reduced relative abundances in comparison to the mock-treated rhizosphere samples (Figure 5.16B).

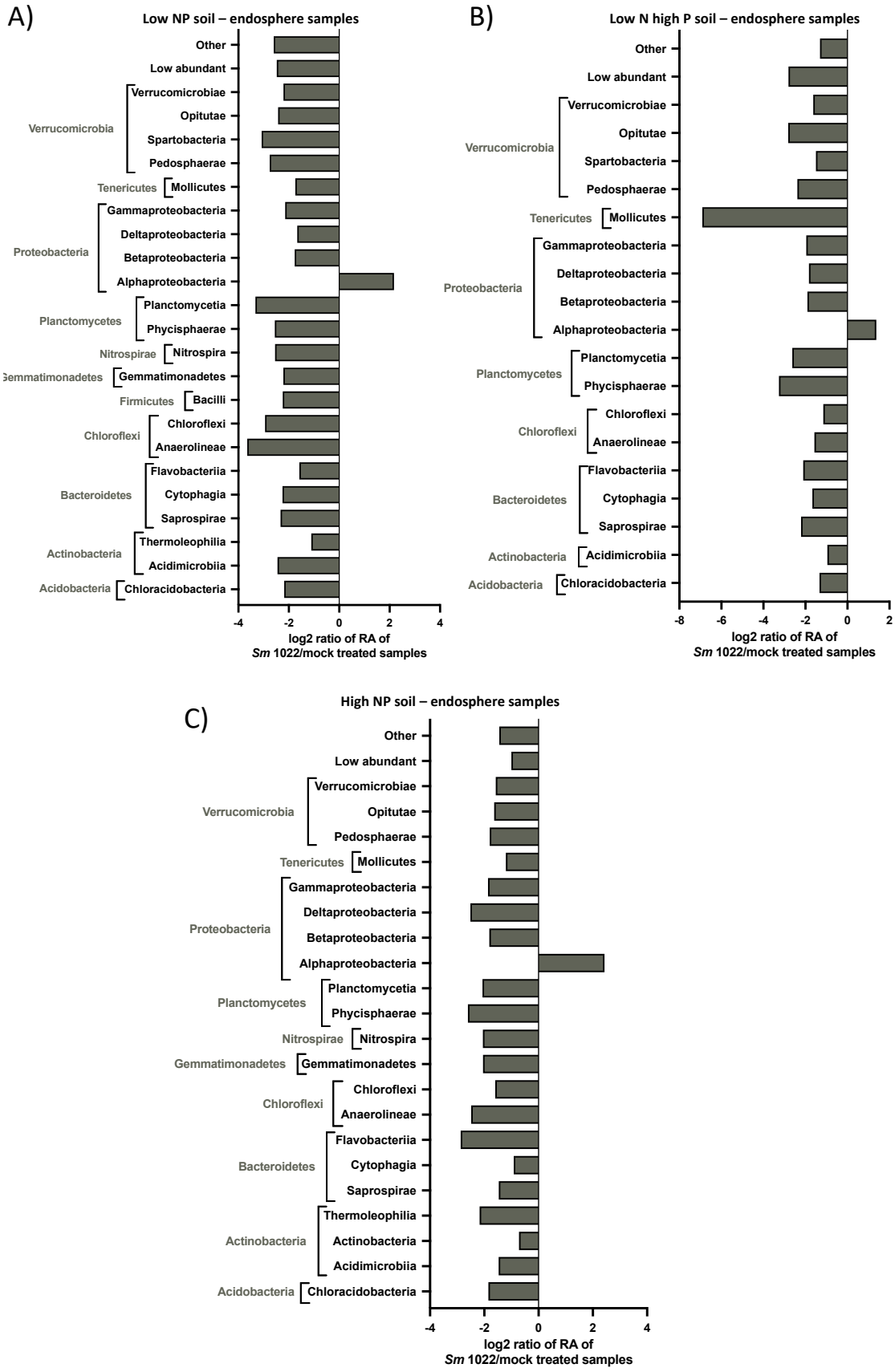


Figure 5.15: Differentially enriched bacterial taxa in the endosphere of Medicago trancatula in three different soil types. Differentially enriched bacteria in the

endosphere of plants grown at A) low NP soil type, B) low N high P soil type, and C) high NP soil type. Data presented as relative abundance in S. meliloti 1022-treated samples divided by mock-treated samples. All taxa presented are identified as significantly enriched/depleted using Kruskal Wallis test ($p < 0.001$).

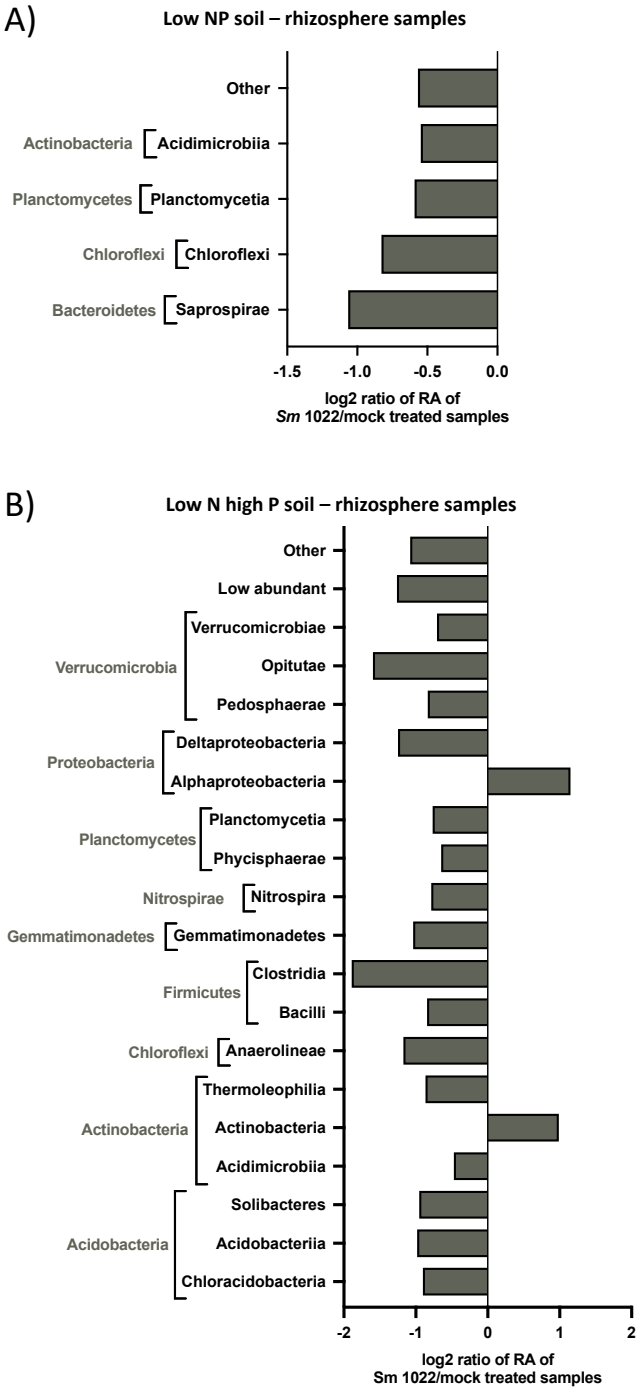


Figure 5.16: Differentially enriched bacterial taxa in the rhizosphere of Medicago trancatula in the three different soil types. Differentially enriched bacterial of plants

grown at the A) low NP soil type, B) low N high P soil type. Data presented as relative abundance in S. meliloti 1022-treated samples divided by mock-treated samples. All taxa presented are identified as significantly enriched/depleted using Kruskal Wallis test ($p < 0.001$).

As performed with the bacterial community, the fungal community composition was assessed using the calculated relative abundance % (RA). RA analysis in the fungal community reveals most significant differences in the endosphere of all soil types, with Sordariomycetes having a higher relative abundance in the endosphere as compared to other compartments. Moreover, some taxa present in soil and rhizosphere samples such as the Pezizomycetes seem to be excluded from the endosphere compartment (Figure 5.17).

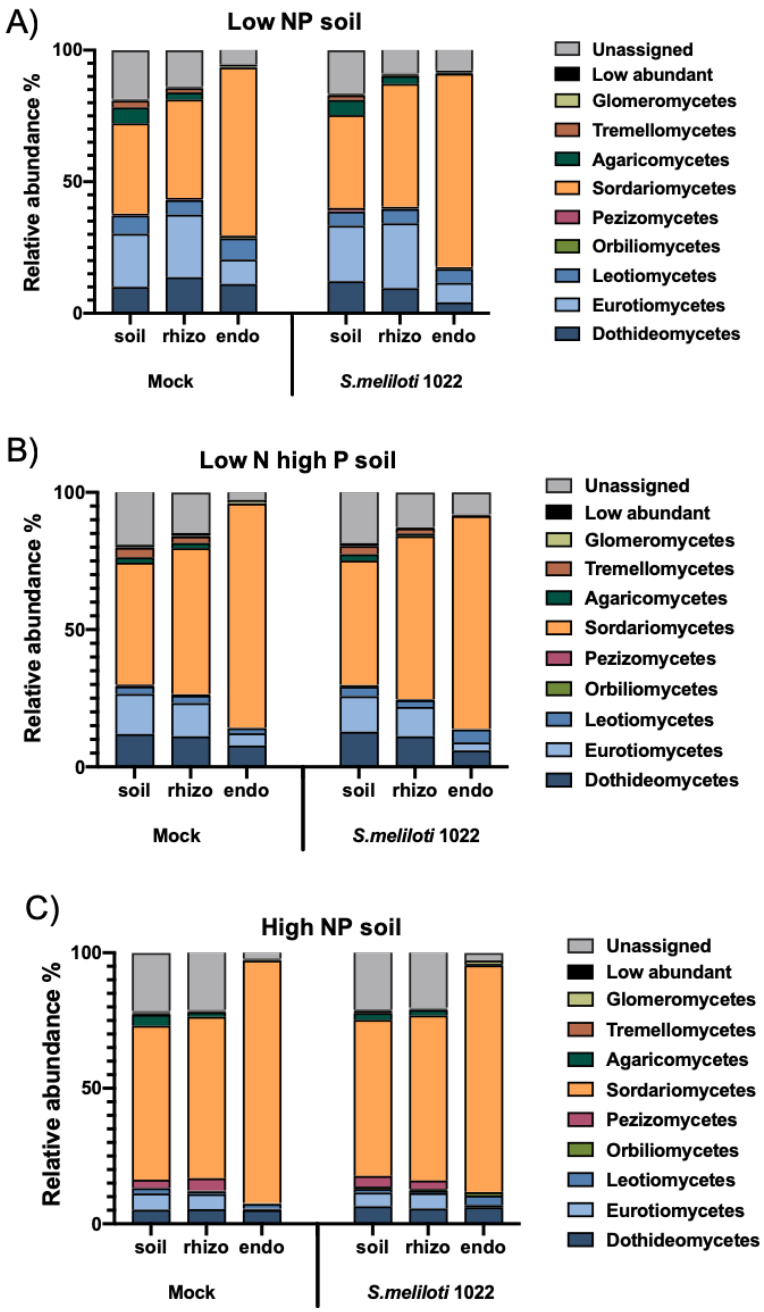


Figure 5.17: Fungal community composition in the three different soil types. Relative abundance of fungal taxa at the order level in A) low NP soil type, B) low N high P soil type and C) high NP soil type; shown as percentage of all reads and colour coded, calculated using rarefied reads.

The differences in the composition of the fungal community were analysed using similarity percentages (SIMPER). The SIMPER analysis showed that OTU3, belonging to Sordariomycetes and assigned as Hypocreales species most significantly contributed to the differences observed between mock and *S. meliloti* 1022 samples in

the endosphere of all soil types (Table 5.7) and the rhizosphere of the low NP soil type (Table 5.8). Notably, a *Fusarium* species contributed 5% to the differences observed between the treated and mock samples in endosphere of low N high P and low NP soil types (Table 5.7). In rhizosphere samples of high NP and low N high P soil types, OTU2 belonging to a Sordariomycetes species contributed most significantly to the differences observed (Table 5.8). Of all taxa that contributed to the differences in the fungal community between the mock and *S. meliloti* 1022-treated samples, more than 3% belonged to the Ascomycota phylum (species of the order Sordariomycetes). However, none of those taxa was significantly affected in the presence of *S. meliloti* 1022 as calculated using the Kruskal-Wallis test with a threshold of p value < 0.001.

Table 5.7: Fungal taxa contributing to the differences in community structure in the endosphere, in the different soil types.

endosphere samples	Taxon	Contrib. %	mean value		Taxonomy
			mock	Sm1022	
High NP	OTU3	19.68	2.22E+03	2.62E+03	Ascomycota/Sordariomycetes/Hypocreales/Incertae sedis/Ilyonectria/**Ilyonectria macrodidyma**
	OTU27	10.47	510	7.67	Ascomycota/Sordariomycetes/Microascales/Ceratocystidaceae/Thielaviopsis/**Thielaviopsis basicola**
	OTU41	7.854	246	223	Ascomycota/Sordariomycetes/Hypocreales/Hypocreaceae/**Trichoderma sp**
	OTU359	7.547	366	118	Ascomycota/Sordariomycetes/Hypocreales/**Nectriaceae sp**
	OTU55	7.263	313	243	Ascomycota/Sordariomycetes/Hypocreales/Incertae sedis/Ilyonectria/**Ilyonectria robusta**
	OTU54	4.955	196	253	Ascomycota/Dothideomycetes/**Pleosporales sp**
	OTU75	4.398	196	52.7	Ascomycota/Sordariomycetes/Hypocreales/Nectriaceae/**Cylindrocarpon sp**
Low N high P	OTU3	15.72	1.67E+03	1.10E+03	Ascomycota/Sordariomycetes/Hypocreales/Incertae sedis/Ilyonectria/**Ilyonectria macrodidyma**
	OTU6	12.77	927	700	Ascomycota/Sordariomycetes/**Hypocreales sp**
	OTU40	9.965	470	300	Ascomycota/Sordariomycetes/Hypocreales/Nectriaceae/**Nectria sp**
	OTU13	5.059	323	396	Ascomycota/Sordariomycetes/Hypocreales/Nectriaceae/**Fusarium sp**
	OTU85	4.714	105	269	Ascomycota/Sordariomycetes/Hypocreales/Incertaesedis/Ilyonectria/**Ilyonectria mors-panacis**
	OTU2	4.554	29.8	266	Ascomycota/**Sordariomycetes sp**
	OTU9	4.312	146	161	Ascomycota/Sordariomycetes/Hypocreales/Bionectriaceae/Bionectria/**Bionectria ochroleuca**
	OTU374	3.639	0	216	Ascomycota/Sordariomycetes/Hypocreales/Nectriaceae/Fusarium/**Fusarium fujikuroi**
Low NP	OTU3	19.24	2.49E+03	2.66E+03	Ascomycota/Sordariomycetes/Hypocreales/Incertae sedis/Ilyonectria/**Ilyonectria macrodidyma**
	OTU48	6.599	227	119	Ascomycota/Leotiomycetes/Helotiales/Incertae sedis/Tetracladium/**Tetracladium maxilliforme**
	OTU13	5.032	153	244	Ascomycota/Sordariomycetes/Hypocreales/Nectriaceae/**Fusarium sp**
	OTU5	4.656	288	220	Ascomycota/Eurotiomycetes/Chaetothyriales/**Herpotrichiellaceae sp**
	OTU26	4.54	236	82.1	Ascomycota/**Dothideomycetes sp**

Analysis of Similarity Percentages (SIMPER) identified taxa that contributed most of the dissimilarities (≥3%) of the fungal communities in the endosphere between mock samples and those inoculated with S. meliloti 1022 (S.m 1022), in the different soil types. Mean values of the Relative Abundance are shown for the mock and the S. meliloti 1022 treatment.

Table 5.8: Fungal taxa contributing to the differences in community structure in the rhizosphere, in the different soil types.

rhizosphere samples	Taxon	Contrib. %	mean value		Taxonomy
			mock	Sm1022	
High NP	OTU2	7.071	976	1.07E+03	Ascomycota/**Sordariomycetes sp**
Low N high P	OTU2	5.959	782	736	Ascomycota/**Sordariomycetes sp**
	OTU6	5.198	152	328	Ascomycota/Sordariomycetes/**Hypocreales sp**
	OTU3	4.831	192	223	Ascomycota/Sordariomycetes/Hypocreales/Incertae sedis/Ilyonectria/**Ilyonectria macrodidyma**
Low NP	OTU3	9.383	319	831	Ascomycota/Sordariomycetes/Hypocreales/Incertae sedis/Ilyonectria/**Ilyonectria macrodidyma**
	OTU9	4.643	188	273	Ascomycota/Sordariomycetes/Hypocreales/Bionectriaceae/Bionectria/**Bionectria ochroleuca**
	OTU69	4.022	202	49.9	Ascomycota/**Dothideomycetes sp**
	OTU1738	3.516	10.1	202	Ascomycota/Eurotiomycetes/Eurotiales/Trichocomaceae/Penicillium/**Penicillium brevicompactum**

Analysis of Similarity Percentages (SIMPER) identified taxa that contributed most of the dissimilarities (≥3%) of the fungal communities in the rhizosphere between mock samples and those inoculated with S. meliloti 1022 (S.m 1022), in the different soil types. Mean values of the Relative Abundance are shown for the mock and the S. meliloti 1022 treatment.

In summary, only bacterial communities (especially alpha-Proteobacteria) were affected in the presence of *S. meliloti* 1022 in all soil types, particularly in root endosphere. In contrast there were no significant changes in the composition of fungal communities in the presence of the rhizobium.

5.2.6 The plant bacterial microbiome composition correlates with changes in plant nutrients in all different soil types

To investigate the possible biological factors associated with the root microbiome changes described previously, environmental factors were examined to

determine if there was any correlation with these microbial abundance changes. Edaphic factors such as pH, conductivity, total soil C and N, soil available NO_3^- and PO_4^{3-} were measured at the end of the experiment and compared with the initial measurements prior to the experiments. No significant changes were observed for any of the edaphic factors in the presence of *S. meliloti* 1022 at the end of the experiments (Figures 5.18, 5.19, 5.20) based on ANOVA test followed by Tukey's HSD test ($p < 0.001$), except of soil C:N ratios in high NP soil type which is significantly reduced in the mock samples (Figure 5.20). However, some of those factors were significantly altered at the end of the experiment in comparison to the beginning (after 63 days), such as the depletion of soil available NO_3^- in low N high P and high NP soil types (Figure 5.19A). In addition, soil total elements were measured by Inducted Coupled Plasma-Mass Spectrometry (ICP-MS) and none of them were significantly changed in the presence of *S. meliloti* 1022, except soil Cu, which was significantly depleted in the presence of the rhizobial inoculant (Table 5.9).

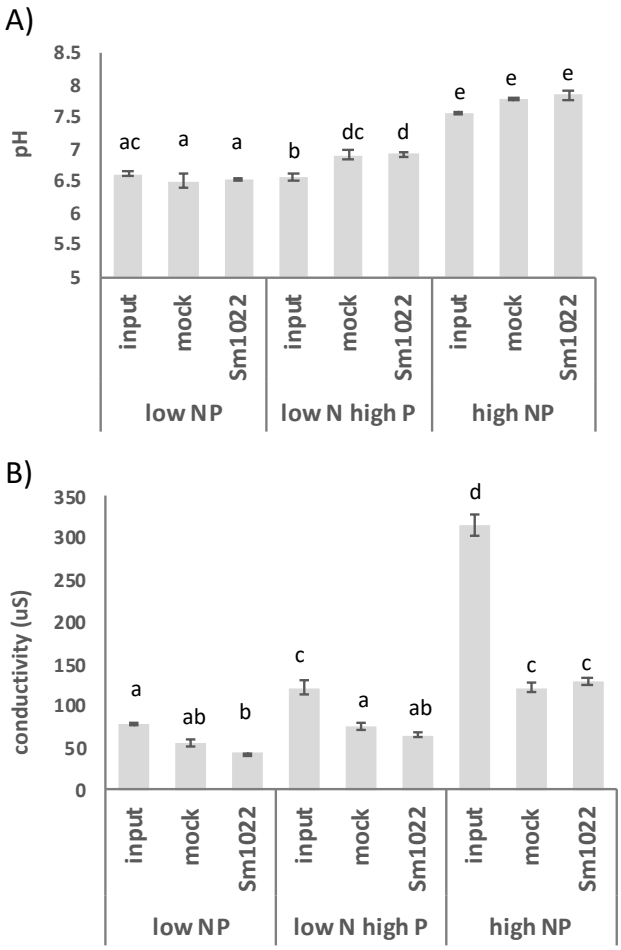


Figure 5.18: Soil pH and conductivity in soil types upon different treatments. A) pH and B) conductivity under different treatments and soil types. Letters represent significant differences according to ANOVA followed by Tukey's HDS test ($p < 0.001$).

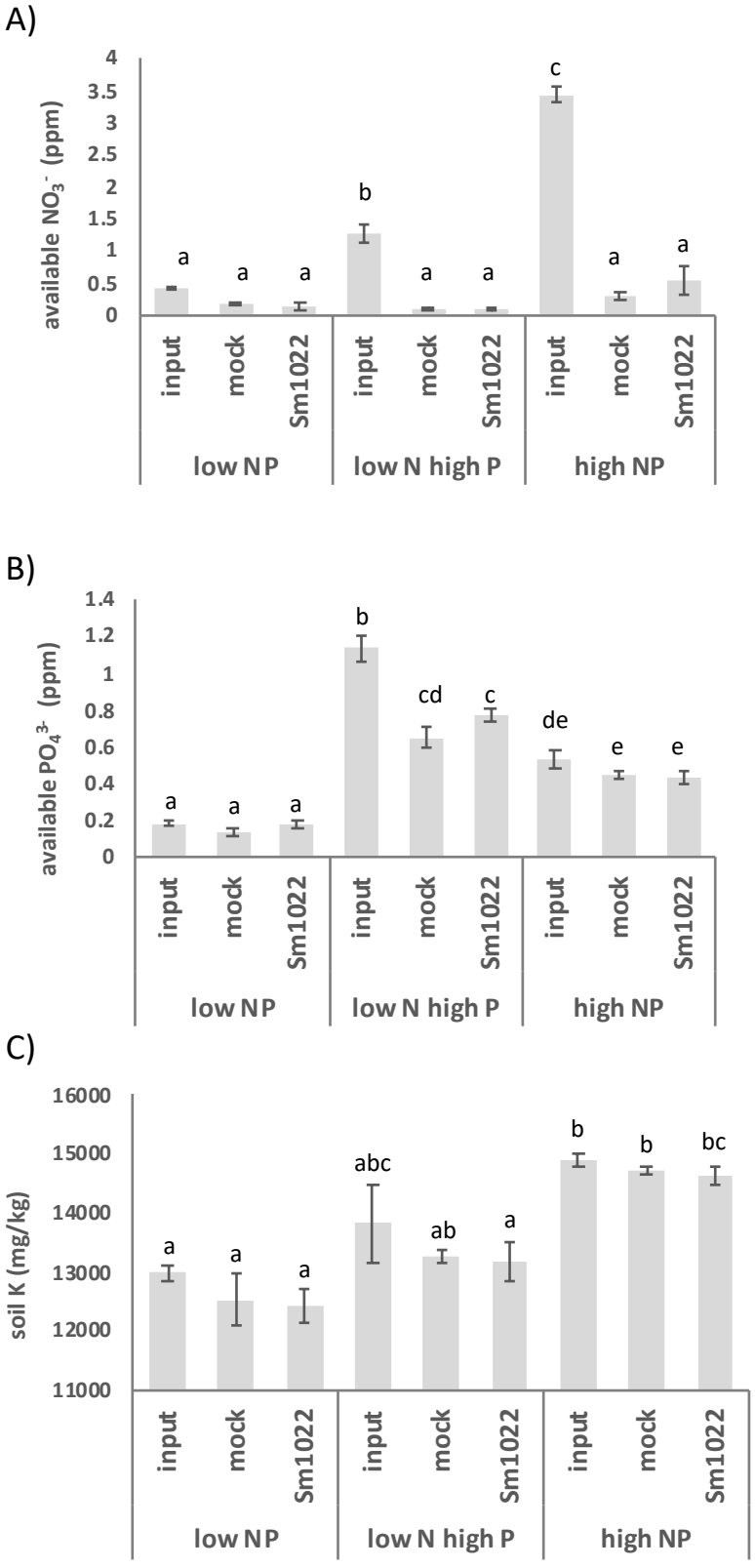


Figure 5.19: Soil available NO_3^-, PO_4^{3-} and K soil types upon different treatments. A) Soil available NO_3^-, B) soil available PO_4^{3-}, and C) soil K under different treatments and soil types. Letters represent significant differences according to ANOVA followed by Tukey's HDS test ($p < 0.001$).

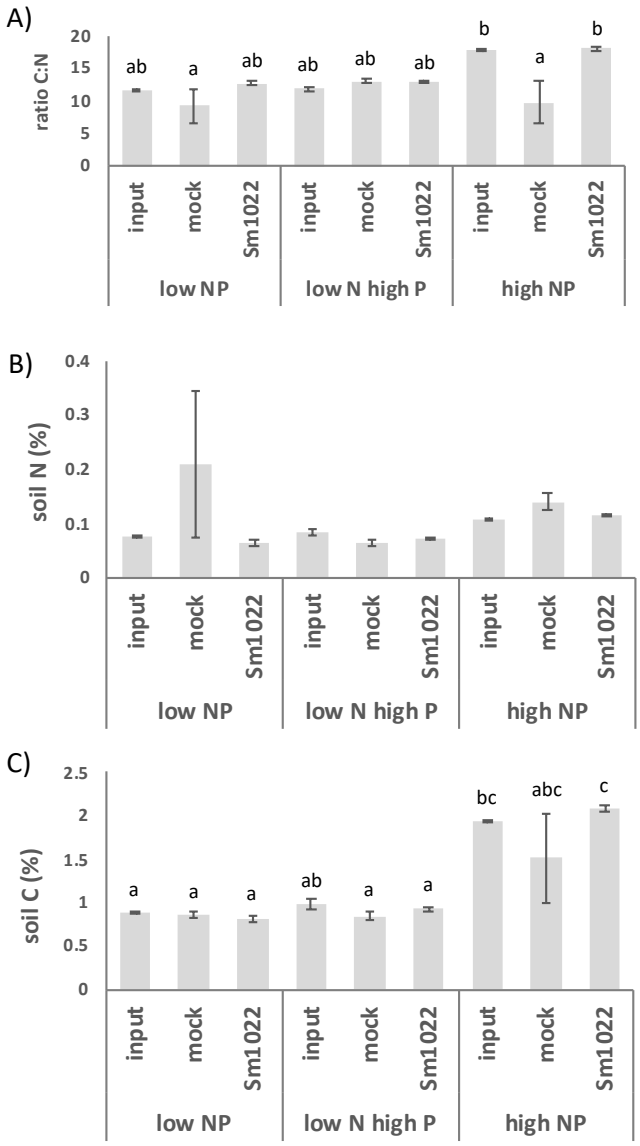


Figure 5.20: Soil CN, C% and N% soil types upon different treatments. A) Soil C:N ratio, B) soil N (%), and C) soil C (%) under different treatments and soil types. Letters represent significant differences according to ANOVA followed by Tukey's HDS test ($p < 0.001$).

Examining the changes of the aboveground plant nutrients in the presence of *S. meliloti* 1022, more distinct changes were observed in comparison to the soil types. The plant C:N ratio was significantly decreased in the presence of *S. meliloti* 1022 in all soil types (Figure 5.21A), as a result of the significant increase of the plant N in the presence of *S. meliloti* 1022 in all soil types (Figure 5.21B), as calculated using ANOVA followed by Tukey's HSD test for significance ($p < 0.001$).

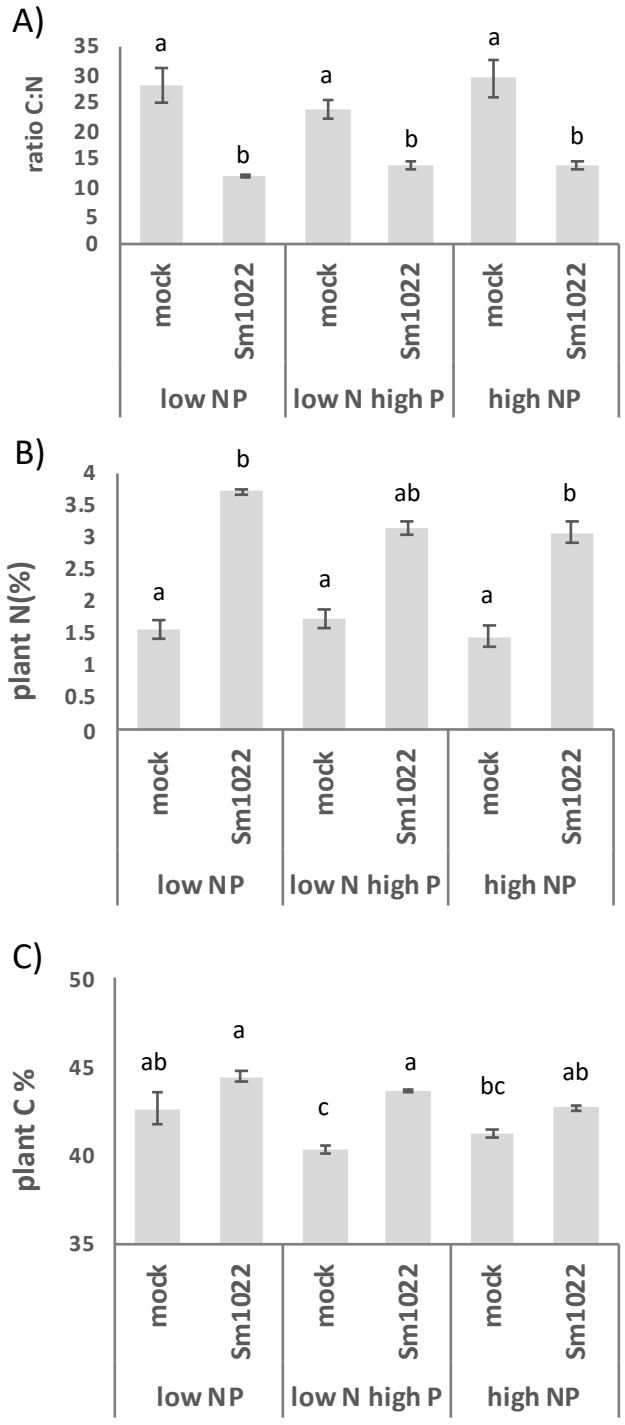


Figure 5.21: Plant C:N ratio and C and N contents soil types upon different treatments. A) Plant C:N ratio, B) plant N content (%), C) plant C content (%), under different treatments and soil types. Letters represent significant differences according to ANOVA followed by Tukey's HDS test ($p < 0.001$).

The extent of correlation was tested between the different edaphic factors, plant nutrients and microbial number of OTUs (alpha diversity), using Pearson's correlation (with Bonferroni corrected p-values). In mock samples, there was a strong positive correlation between aboveground dry weight and pH, conductivity and soil C%, and less strong correlation with soil C:N ratio and available P (Figure 5.21A). Strong positive correlations were also observed between pH and soil C%, conductivity and available N, bacterial and fungal alpha diversity (as number of observed OTUs) as well as a negative correlation between plant N% and plant CN ratio. On the other hand, in the presence of *S. meliloti* 1022, stronger correlations were found between edaphic factors and plant nutrients, while the correlations between plant dry weight and soil factors are no longer observed (Figures 5.22A, B). The strongest positive correlations were detectable between pH and conductivity, soil CN ratio, soil C%, soil N% and plant K as well as a negative correlation of those with plant C%. In addition, a negative correlation was found between plant C%, N% and plant K, while the correlation between bacterial and fungal alpha diversity (as number of observed OTUs) remained positive.

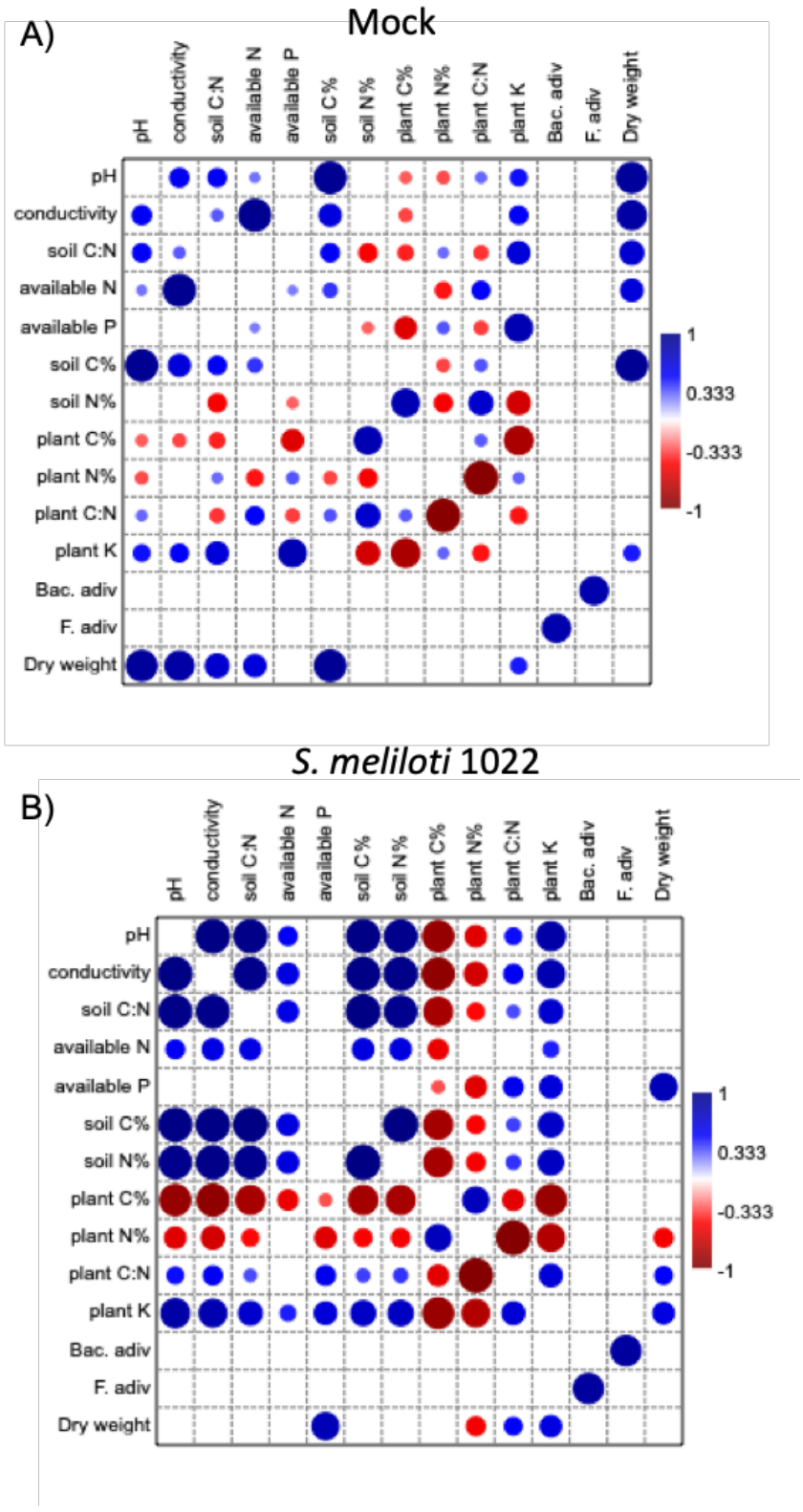


Figure 5.22: Correlation of soil and plant biochemical characteristics, microbial richness and shoot dry weight upon mock and S. meliloti 1022 treatment, in the three soil types. Pearson's correlations between microbial observed OTUs as alpha diversity measure (adiv), plant and soil factors in A) all mock samples and B) all S.

meliloti 1022 samples of all soil types. Dark blue indicating strong positive correlation (1) and dark red indicating strong negative correlation (-1). Point sizes indicates the strength of the correlation, with larger size for stronger correlation. Only the correlations with p < 0.05 (Benferroni corrected p values) are shown.

The environmental variables measured and described above were tested for correlation with the microbiome using the Canonical Correspondence Analysis (CCA). The soil pH, conductivity, soil available N, P and soil C:N explained 73.81% of the differences in the soil bacterial community structure and 77.94% of the differences in the soil fungal community in the different soil types. The soil bacterial community from high NP soil type samples correlated with increased soil pH, conductivity and total soil C, while the bacterial communities of the other two soils correlated with the decrease of those (Figure 5.23A). The soil fungal community from high NP soil type samples correlated with soil pH, conductivity, soil C%, C:N ratio and soil available N, while the other two soil fungal communities correlated with a decrease in those parameters (Figure 5.23B). In more detail the soil fungal community from low N high P soil type samples correlated with the increase of available P (PO_4^{3-}) and the low NP soil type fungal community correlated with a decrease in available P (PO_4^{3-}).

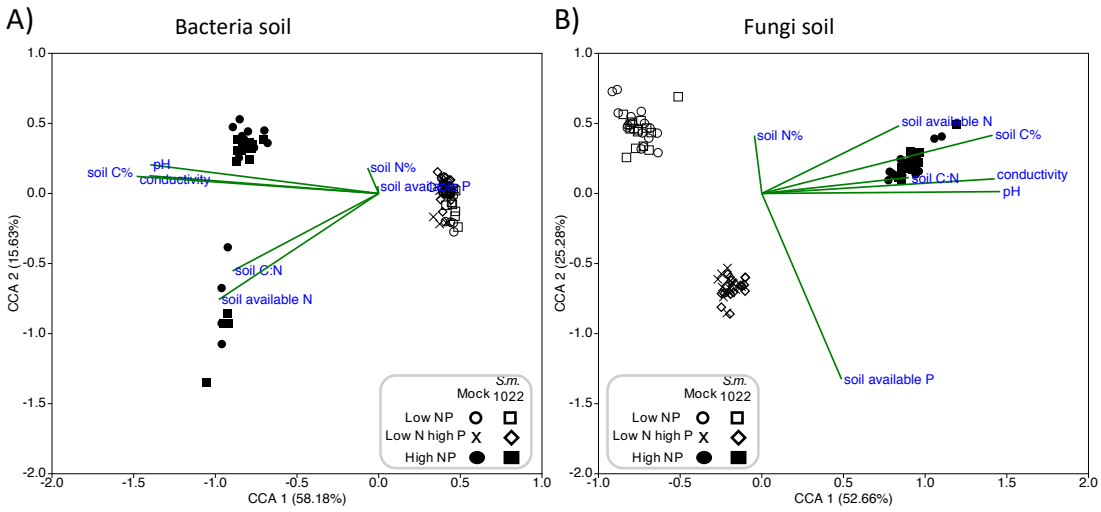


Figure 5.23: Correlation between edaphic factors and soil microbial community structure of mock and S. meliloti 1022-treated plants grown in three different soil types. Canonical Correspondence Analysis (CCA) illustrates the effect of edaphic

factors on the soil A) bacterial and B) fungal community structure. The two first plotted axes of the CCA explaining 73.81% and 77.94% of the differences in the bacterial and fungal community structure, respectively

Next, compartments were analysed that are more affected by the presence of *S. meliloti* 1022. Bacterial and fungal communities of the root rhizosphere and endosphere were found to be associated with certain soil and plant factors. The bacterial endosphere community was clustered according to soil type and treatment, each of the clusters being associated with different factors (Figure 5.24). In the endosphere, the bacterial community structure of plant treated with *S. meliloti* 1022 was strongly associated with increased C% and N% in all soil types; however, the samples of the high NP soil type were also associated with increased soil available N (NO_3^-) and P (PO_4^{3-}), soil C:N and pH. The bacterial community of the mock-treated endosphere was associated with increased plant total K and decreased plant C% in the high NP soil type. In low N high P soil type, the bacterial community of the mock-treated endosphere was correlated with increased plant C:N ratio, with decreased plant total K and all soil factors tested in the low NP soil type (Figure 5.24A). The bacterial community structure in the rhizosphere of high NP soil type was correlated with increased plant K, soil pH, CN and available N (NO_3^-) (Figure 5.24C). The bacterial community of the *S. meliloti* 1022 treated plants in low N high P soil type was correlated with high plant C% and N%, while the bacterial community of mock-treated plants was associated with decreased soil available P (PO_4^{3-}) (Figure 5.24C). Overall, the soil and plant factors tested could explain 75.87 % of the variation observed within the bacterial community in the endosphere and 59.65 % in the rhizosphere.

In contrast, the fungal community of the endosphere was clustered only according to the soil type in both soil endosphere and rhizosphere. The fungal community of plants grown in high NP soil type correlated with increased soil pH, soil and plant C:N ratio and soil available N. The fungal community of low N high P soil type was associated with high available P (PO_4^{3-}) in the soil and the fungal community of low NP soil type was associated with increased plant C% (Figure 5.24B). Overall, the soil and plant factors tested could explain 65.71 % of the variation observed within fungal community in the endosphere and 70.55 % of this variation in the rhizosphere.

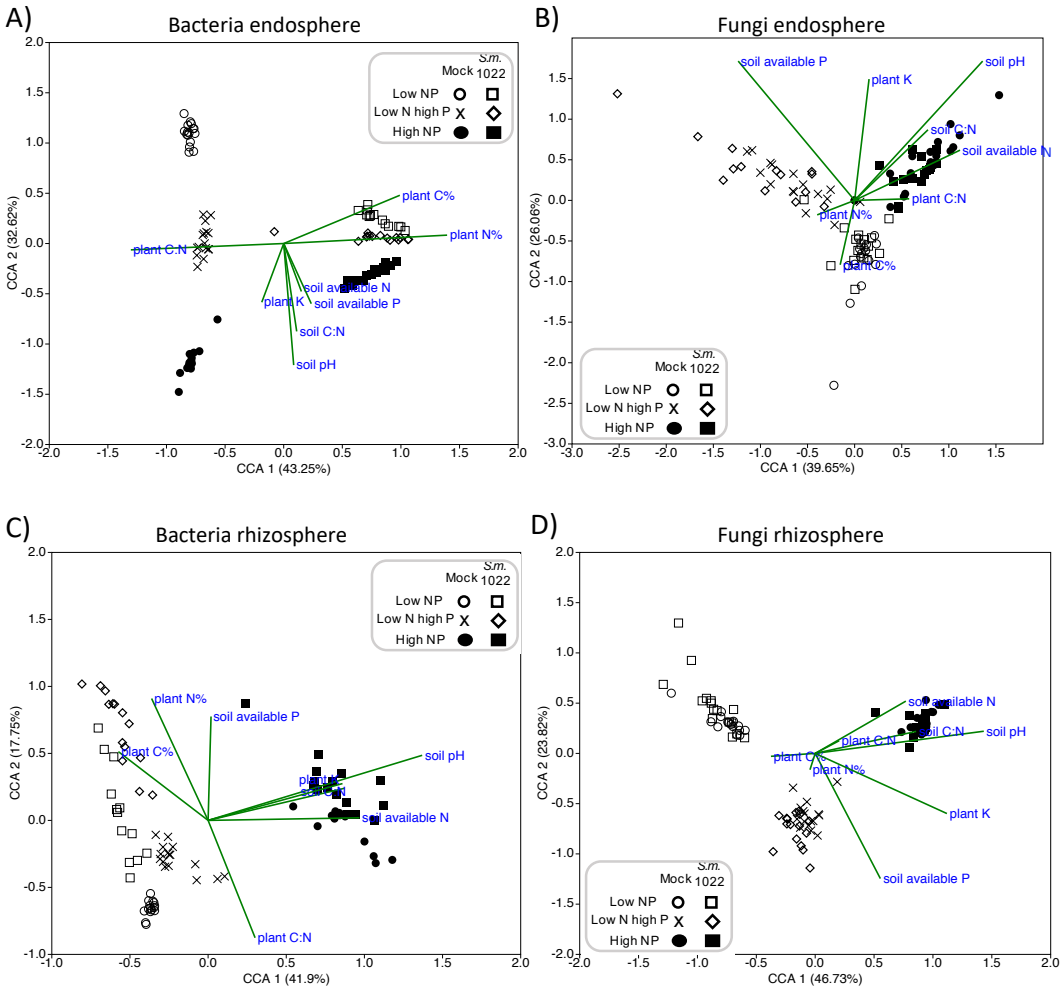


Figure 5.24: Correlation between soil and plant biochemical characteristics and the structure of rhizosphere and endosphere microbial communities of mock and S. meliloti 1022-treated plants grown in three different soil types. Canonical Correspondence Analysis (CCA) of A) bacterial endosphere, B) fungal endosphere, C) bacterial rhizosphere and D) fungal rhizosphere communities in association with plant and soil edaphic factors. CCA illustrating the effect of edaphic factors on the structure of A) bacterial endosphere, B) fungal endosphere, C) bacterial rhizosphere and D) fungal rhizosphere communities. The two first plotted axes of the CCA explain A) 75.87 %, B) 65.71 %, C) 59.65 % and D) 70.55 % of the differences.

Summing up the results from correlation analysis between all of the edaphic and microbiome parameters, the analyses found that plant N content (%) was significantly increased in the rhizobia-treated plants and that *S. meliloti* 1022 had the strongest effect on plant yield. This was in contrast with the findings for plant yield of

the mock-treated samples, which was strongly correlated with pH, conductivity and soil C%. Moreover, it was shown that changes in bacterial and fungal communities correlated differently with the environmental variables tested in this experiment, with soil pH, conductivity, available NO_3 and PO_4 play a key role in shaping microbial community structure.

5.3 Discussion

This study aimed to gain a better insight into rhizobia-plant interactions in natural soils, how those interactions alter soil and plant-associated microbiomes, and to what extent the interaction outcomes are affected by soil environmental variables. This study further aimed to improve our understanding of rhizobia-plant interactions in the context of soil and plant biochemical properties. Soil pH, salinity, temperature and farming practices typically reduce rhizobial diversity and population in soils (Slattery et al., 2001) and alter their effectiveness in root colonisation and abilities to fix nitrogen (de Castro Pires et al., 2018; Gyogluu et al., 2018; Slattery et al., 2001, 2004). Considering the biological and agricultural potential of the use of mutualistic microbes it is necessary to identify conditions that are tolerated by N-fixing rhizobia and those that maximise or limit their beneficial activities.

5.3.1 Competitiveness of fungal symbionts in natural soils

During the initial trials for these experiments the co-inoculation of the rhizobial strains with *S. indica* was not successful in natural soils, despite the successful co-inoculation in perlite. As a result, *S. indica* did not exert any beneficial effects in *M. truncatula*. Similarly, the results of this experiment align with previous research on the suppression of fungi by the natural soil microbiome (Wilson et al., 2007), and on arbuscular mycorrhiza fungi being suppressed by Acidobacteria (Svenningsen et al., 2018), a bacterial taxon abundant in the soil types used in this experiment (8-13% of all reads). In addition, *S. indica* was recently shown to depend on thiamine-producing bacteria for vegetative growth (Jiang et al., 2018). Interestingly, most fungi cannot produce thiamine indicating the need for different inoculation strategies for beneficial fungi. Experiments on bacterial and fungal establishment at the different developmental stages of *Medicago* plants has revealed that fungi establish colonisation of the endosphere at later developmental stages than bacteria (Mougel et al., 2006). All the above, along with the knowledge that bacteria dominate all plant compartments (Hacquard et al., 2015) might have contributed to the reduced competitiveness of *S. indica* in this

system. In addition, even though rhizobia are known for their growth promoting properties (Vacheron et al., 2013), the results of this experiment show that they might interact differently with the host in dependency of natural microbiome and soil types. This observation highlights the necessity of field-based experiments in order to understand the "behaviour" of these complex interactions in natural systems.

5.3.2 Efficiency of *Sinorhizobium* species varies and also differs depending on the soil type

Sinorhizobium species used to inoculate the *Medicago trancatula* are well studied (Galibert et al., 2001; Jones et al., 2007; Marx et al., 2016). While *S. meliloti* 1021 is the most common species studied in association with *Medicago* species (Zhao et al., 2012), it was recently demonstrated that strain 1021 is less efficient compared to the addition of external nitrogen, therefore might not be the most efficient nitrogen-fixing bacteria for *Medicago truncatula* (Terpolilli et al., 2008). This was confirmed by this present study, as it had the smallest plant growth promotion effect in low N high P soil type and no significant growth promotion in low NP and high NP soil type, in comparison to the mock-treated plants. This might be due to inefficient compatibility with the plant host as mentioned before (Terpolilli et al., 2008), or due to the weak competitiveness of this strain against the soil microbiome. The inoculant *S. medicae* 419 promotes growth more efficiently that the 1021 strain, however, there is no additive effect in high NP soil type, something that was observed only with the 1022 strain. According to competition experiments by Rangin et al. (2008) some *S. medicae* strains are less competitive than certain *S. meliloti* strains, which might be case for *S. medicae* 419 in high nutrient environments such as the high NP soil type. In addition, Garau et al. (2005) showed that *S. medicae* 419 is more efficient in acidic soils. Absence of significant growth promotion effect of *S. medicae* 419 was observed only in the soil with the most alkaline pH, the high NP soil type, which may explain the absence of the enhancing effect of this strain. Moreover, the success of *S. meliloti* 1022 to nodulate host plants could be due to its ability to recruit plant beneficial microbes in dependency of soil type and not necessarily the plant genotype (Leite et al., 2016).

Moreover, since colonisation success of rhizobia is dependent on soil factors (Slattery et al., 2001, 2004) it is important to characterise the efficiency of the commonly used rhizobial species in different environments.

5.3.3 Each soil type is characterised by distinct microbial communities

The three soil types studied here did not simply have different biochemical characteristics (edaphic factors), but also possess distinct soil microbiomes. Low NP and low N high P soil types had similar bacterial community structures, while high NP soil type differed from both soils. This might be explained by similar biochemical characteristics of low NP and low N high P soil types. For instance, it is known that increased N content results in an alteration to the microbial community and consequently its enzymatic activity as a whole, with increased biomass of gram negative bacteria and decrease of labile C in the soil (Cusack et al., 2011). Fungal communities differed between all soil types indicating a higher dependency or specificity of soil fungal microbiomes and its soil environment as observed previously (Smith et al., 2014). The OTU richness (alpha diversity) of the bacterial community in the high NP soil type was significantly higher at the end of the experiment (63 dai) as compared to the input soil, and all the samples from low NP and low N high P soil type, and irrespective of the presence of the plant; most likely due to a higher nutrient availability (e.g. nitrate) in this soil (Curd et al., 2018). In contrast, the number of observed fungal OTUs was lower in the high NP soil type, which might be a result of the bacterial proliferation (Mackie and Wheatley, 1999). Moreover, the suppression of fungi has been associated with the presence of Acidobacteria in natural soil (Svenningsen et al., 2018), a bacterial phylum that is present in all soils used in this experiments, and had increased relative abundance in the high NP soil type. A common characteristic among all soil types is the gradient reduction of OTU numbers towards the root endosphere. OTU number tends to reduce at locations in close proximity to the root as has been reported before (Shi et al., 2015) and reflects the required specialisation of microbes and their ability to manipulate host processes for colonisation.

5.3.4 Strong effect of *S. meliloti* 1022 on the bacterial community structure but not on the fungal community structure in the endosphere compartment

S. meliloti 1022 has a strong effect on the bacterial community mainly on the endosphere compartment and to a lesser extent in the rhizosphere, while there was no observed effect of the rhizobial inoculant in the soil microbiome. The endosphere compartment sampled in this project included the interior microbiome of the nodules. Therefore, the observed shift is highly due to the high accumulation of rhizobia in the endosphere. Studies have similarly shown that nodules consist of microbiomes that are distinct from microbiomes of the rhizosphere/endosphere (Leite et al., 2016; Xiao et al., 2017; Zgadzaj et al., 2016). The microbiome of the nodule has been associated with plant growth enhancement, as the plant can recruit plant beneficial microbes (Velázquez et al., 2017). This might also contribute to the high growth-promoting activity of *S. meliloti* 1022. The absence of any effect of the rhizobial inoculant on fungal communities in any of the analysed compartments could be due to different niche competition requirements of bacteria and fungi (Rousk et al., 2008). Nodules represent a tissue which is quite different from the main root and the distinct microbiome composition of roots vs. nodules suggests more specific changes, for example, the recruitment of beneficial microbes inside the nodule (Leite et al., 2016; Velázquez et al., 2017). In future work, nodules might be an interesting location to mine for novel microbes with plant growth promoting effects.

It was not possible to identify *S. meliloti* 1022-specific sequences directly in the samples, due to the large number of rhizobia present in soils. However, one bacterial OTU was particularly abundant in the bacterial community. This is OTU2, which contributed 12% to the differences observed among all samples (Table 5.5) and more than 40% to the differences observed between mock and *S. meliloti* 1022 samples in the endosphere (Table 5.6). When OTU2 was investigated it shows 100% similarity to *Sinorhizobium meliloti* strain (Figure S5.2), and thus, it is likely to belong to *Sinorhizobium meliloti* 1022. It is important for future work to design primers to distinguish this strain from other rhizobia, to enable quantification in the samples, relative to plant DNA. This would also make it possible to determine the efficiency of inoculation in each soil type. The design of such primers (also

applicable to natural soil samples) is challenging considering the lack of genome information but could be aided by the sequence data from OTU2 here.

5.3.5 Strong effect of *S. meliloti* 1022 on the bacterial community composition but not on the fungal community composition in the endosphere compartment

The bacterial community composition was affected in the presence of *S. meliloti* 1022 in all soil types, particularly in the plant endosphere with alpha-Proteobacteria being at the centre of those changes. This effect can be associated with the presence of *S. meliloti* 1022 in the root (Figure 5.14). However, the alpha-Proteobacteria also dominate the endosphere of mock treated plants. Taken these together, the strong effect of *S. meliloti* 1022 in the endosphere may be a result of a synergistic effect of the rhizobial inoculant with the native, soil-derived rhizobial species. According to experiments on root microbiomes at different developmental stages, Proteobacteria were identified to be very rapid colonisers, making them robust competitors uninfluenced by the environmental and edaphic factors (Yuan et al., 2015). Proteobacteria are robust colonisers, comparatively taking over from the rest of the bacteria (Shi et al., 2015), highlighting the close association of this order with the root.

S. meliloti 1022 has no obvious effect on fungal community structures and compositions in any compartments. Rhizobia are commonly studied for their association with arbuscular mycorrhiza (Wang et al., 2011; Xie et al., 2016b) mainly because their genetic requirements for symbiosis with the plant are partially common between the two taxa (Parniske, 2000). Since this fungal taxon was mainly absent from the soils used, rhizobia seem to not influence strongly the rest of the fungal taxa. In turn, soil type had the strongest effect on fungal communities in all analysed samples. Differences in the abundance of species of Sordariomycetes were most obvious, followed by differences in Dothideomycetes in the endosphere and Eurotiomycetes in the rhizosphere. All three orders belong to the phylum of Ascomycota which comprises the majority of the fungal reads and therefore highlights a well-known association with *Medicago truncatula* plants (Mahmoudi

et al., 2019). Sordariomycetes is one of the largest order of Ascomycota with members following different lifestyles ranging from plant endophytes, saprotrophs to pathogens. The nature of interaction of detected Sordariomycetes with *Medicago truncatula* is however unknown. Surprisingly, arbuscular mycorrhiza species from the order of Glomeromycetes were not overrepresented in analysed samples although they have been described as having strong associations with *Medicago truncatula* and rhizobia (Ané et al., 2004; Mougel et al., 2006; Oldroyd, 2005). The high percentage of unassigned fungal taxa has been discussed before and might reflect the high number of uncharacterised fungal species in nature (Mahmoudi et al., 2019).

5.3.6 *S. meliloti* 1022 promotes plant growth irrespectively of the soil characteristics

The significant correlations between edaphic factors, plant C, N, K and aboveground dry weight, in the presence and the absence of *S. meliloti* 1022, highlight the beneficial effect of the rhizobial inoculant (Figure 5.22). Despite the strong correlation of the plant dry weight with soil characteristics (pH, conductivity, soil CN, soil available phosphate and nitrate, soil C%) in the absence of *S. meliloti* 1022, there was no strong correlation between those in the presence of *S. meliloti* 1022. This makes *S. meliloti* 1022 a highly efficient and competitive strain in soils even if there are varying levels of nitrate and phosphate – it is beneficial in diverse soils. Notably, it is suggested that plants might be cultivated with their compatible rhizobia for highest colonisation efficiency and beneficial activities, instead of solely relying on native rhizobia (Thrall et al., 2000). These findings highlight the necessity for studies identifying beneficial microbes that are compatible with plant species under a diversity of environmental conditions, in order to be able to use PGPR strains most effectively in agriculture (Sergaki et al., 2018).

The correlations between soil and plant nutrient characteristics also changed in the presence of *S. meliloti* 1022, in comparison to mock-treated. Soil pH, soil C:N ratio, soil C% and soil N% are positively correlated as expected (Rezaei and Gilkes, 2005), and plant K is also positively correlated. This indicates a strong

correlation of the individual factors describing the soil ecosystem. However, direct links between pH and microbiome are difficult to be made as pH affects many processes in the system including soil C and N availability (Griffiths et al., 2011). The negative correlation of soil pH, soil C:N ratio, soil C% and soil N% with plant C% indicates that as plant C% increases, the soil nutrients decrease, as they are likely used towards for plant growth. Soil and root microbes have been reported to affect aboveground parts of plants by supporting nutrient supply and resistance to pathogens (Kopriva et al., 2017; Laksmanan et al., 2014; Liu et al., 2007). Therefore, the presence of *S. meliloti* 1022 enables plants to make better use of certain soil factors either directly by *S. meliloti* 1022 or indirectly as a result of *S. meliloti* 1022 altering the microbiome or root system architecture (Li et al., 2016b), irrespectively of the soil type.

5.3.7 Soil characteristics differently affect the bacterial and fungal community structure

The higher soil pH, conductivity, total C and available N (NO_3^-) of the high NP soil type are correlated with observed changes in soil bacterial and fungal community structure (Figures 5.23). The fungal community structure in low NP soil type is associated with a decrease of all edaphic factors and a slight increase of N% in the soil. The correlation of those soil factors with microbial communities have been previously observed, with soil conductivity, C, N and P in particular affecting the abundance of certain bacterial and fungal groups in the soil (Xue et al., 2018). This makes sense as they are determinants of microbial nutrition and proliferation. These correlations indicate the strong effect of the soil factors on shaping the microbiome as the nutrient abundance enables the proliferation of microbes that might be eliminated in nutrient depleted conditions due to competition (Hibbing et al., 2010; Jones et al., 2018).

High levels of available phosphate in the low N high P soil type strongly affected fungal community structures. Fabiańska et al. (2018) have observed similar shifts in fungal communities as a result of phosphorus availability in the soil, usually driven by the plant, in order to increase phosphorus acquisition.

Furthermore, they observed a role of the plant phosphate starvation response in the interaction of the fungi with the plant. Limited phosphate availability leads to a phosphate starvation response in plants, which affects the establishment of fungi in the root (Breuillin et al., 2010; Fabiańska et al., 2018; Nagy et al., 2009), and essentially may be the main driver of the observed differences in the fungal community. Further studies on the mechanistic understanding of these changes would assist in maximising the beneficial effect of soil fungal communities on plant phosphorus acquisition by the plants.

The soil available P (PO_4^{3-}) in low N high P soil type is strongly decreased at the end of the experiment in the presence and the absence of *S. meliloti* 1022. Soil available P is mobilised and acquired by the roots to be used for plant growth (Shen et al., 2011). Hence, this was not observed in the other two soil types. Similarly, the soil nitrate was found to be reduced at the end of the experiment. It is likely taken up by the plants as indicated, in particular, by higher N contents in plants inoculated with *S. meliloti* 1022. These changes highlight the agricultural importance of this microbe. As in agricultural fields, plant-fixed nitrogen would be transferred to the soil with post-harvest plant biomass that remains in the field, significantly enhancing the soil available nitrogen for the next crop generations.

Soil conductivity also plays a role in microbiome structure. Conductivity is determined by the clay and salt content, as well as the mineral content, which essentially shape the environmental conditions for the microbes in the soil, such as the availability of substrate, air and water (Xue et al., 2018). The reduction of soil conductivity in the low N high P and high NP soil types at the end of the experiment could be due to the reduction of the soil elements that plants have taken up to grow. This effect would also occur for the available soil N (NO_3^-), which is probably used to the same extent by any plant irrespective of treatments. The significantly increased levels of nitrogen in the plant inoculated with *S. meliloti* 1022, in comparison to mock-treated, might therefore indicate the utilisation of other forms of nitrogen such as atmospheric N fixed by *S. meliloti* 1022 (Stern, 1993).

Since success of rhizobial inoculants is dependent on soil factors (Slattery et al., 2001, 2004), it is important to characterise the efficiency of the commonly

used rhizobial inoculants in different environments. Our study demonstrated the limitations of commonly studied rhizobial strains based on the presented finding with different UK soil types. The robustness of *S. meliloti* 1022 in supporting *Medicago trancatula* A17 in different UK soil types with varying nutrient availability makes the strain 1022 a highly performing rhizobial inoculant with potential for use in UK agriculture. Moreover, this works raises further questions on the mechanisms underlying our observations, which could open new strategies for utilising rhizobia and soil microbiomes as tools to sustainably improve agricultural crop production and soil health.

Chapter 6: Discussion

6.1 Outlook

A major challenge ahead of us is the eco-sustainable production of food under the prospect of an increasing world population (9.5 billion people by 2050), climate change and continuous loss of arable land (Conforti, 2011). Such challenge demands innovative and sustainable solutions especially for agricultural crop production. My thesis work aimed to provide an integrated approach, from studying plant-microbe interactions at the molecular level, to a simplistic approach for studying microbiomes and finally to a large-scale study of agricultural relevance. This work provides an insight into the potential of such approaches and their limitations. Additionally, this work contributes towards strengthening our fundamental understanding of microbe-microbe and plant-microbe interactions, in anticipation of sustainably improving agricultural crop production.

6.2 The potential of (*S. indica*) effectors on the establishment of fungal colonisation and in engineering plant microbiomes

Microbes have the ability to manipulate plant processes through phytohormones (Spaepen et al., 2007) and the root-associated microbiome can cause an induction of phytohormones (Yuan et al., 2015). Understanding the mechanisms underlying the interaction might help to i) manipulate microbiomes, and ii) generate new tools for agriculture. Expressing fungal effectors in *Arabidopsis*, transiently in a protoplast system and stably *in planta*, I demonstrated the strong effect of single effectors on plant growth and auxin signalling. These results triggered new approaches for tackling the initial biological question. For instance, investigating the role of effectors in the establishment of *S. indica* in *Arabidopsis* roots in the presence of the core microbiome, or in *Medicago truncatula* roots in the presence of the natural microbiome was shown to be relevant, such that they could be developed as agricultural “vaccines” (Laere et al., 2016) that have an improved competitiveness in the field.

As shown in Chapter 3, one single *S. indica* effector can have a substantial effect on *Arabidopsis* growth, by altering the auxin signalling pathway, which is involved in many fundamental processes of plant growth, health and interaction with microbes (Barker and Tagu, 2000; Vadassery et al., 2008; Vanneste and Friml, 2009). This effect indicates that *S. indica* affects an important process of its host plant. The plant root is a dynamic environment and effectors could help microbes, like *S. indica,* to compete and establish their presence within a microbial community. Akum et al. (2015), for example, showed that an *S. indica* effector protein (PIIN_08944) is involved in the establishment of the fungus in plant roots by affecting pattern-triggered immunity and the salicylic acid signalling pathway. The suppressive activity of *S. indica* against root and leaf plant pathogens is well known and achieved by the ability of the fungus to enhance plant immunity (Kari Dolatabadi et al., 2012; Molitor et al., 2011; Molitor and Kogel, 2009; Serfling et al., 2007). By manipulating the host's signalling pathways, the fungal endophyte could also assist in the recruitment of microbes. *S. indica* can alter metabolism and signalling of phytohormones (Aslam et al., 2019) that have been found to modulate the ability of soil microbes to colonise roots (Haney and Ausubel, 2015; Lebeis et al., 2015). In this respect, it is interesting that *S. indica* supports the growth of a biotrophic oomycete, potentially through effector-driven alterations of the host signalling (Akum et al., 2015). This indicates the significance of effectors serving a beneficial fungus to get established in the competitive rhizosphere/endosphere habitat and to support another unrelated microbe. However, can such singular effector-driven activities affect root microbiomes? The assessment of the role of the whole *S. indica* effector repertoire and of other beneficial organisms can help in understanding the mechanisms and the full potential of such microbes in generating an environment that is not only beneficial for plants but for a broader spectrum of the microbial community. As a result, we might learn which plant and microbe-derived factors and responses can shape microbial communities.

Microbial effectors, thus, can be tools - not only to understand the interactions of microbes with plants – but also to have an impact on the composition of the microbial community by using the plant. A plant is able to alter the microbiome and the environment through hormonal signalling and root exudates, attracting or repulsing certain microbes (Leach et al., 2017). For example, Badri et

al. (2013a) applied *Arabidopsis* root exudates to root associated microbiome and observed that each compound induces the growth of microbes, as well as that the root exudates synergistically determine the community composition. Using a different approach, Lebeis et al. (2015) demonstrated that the signalling pathway of the hormone salicylic acid is involved in the modulation of microbial recruitment by the root, moderating the colonisation of specific microbial groups. Therefore, the ability of *S. indica* to alter plant processes can outline strategies to engineer plant microbiomes, enriched in beneficial microbes. More studies on how microbial effectors may influence the microbiome would enable the identification of key effectors with valuable activities for agricultural crop production.

My work presents a robust high-throughput approach to identify effectors that can redirect plant signalling. This screening approach should be followed up by identifying host proteins interacting with interesting effectors via methods such as Yeast-2-Hybrid or affinity tandem mass spectrometry (Mukhtar et al., 2011; Vasilescu et al., 2004; Weßling et al., 2014). Such methods that allow the study of multiple interactions between microbial effectors and plant targets simultaneously will be essential for the identification of suitable candidates for further experiments, from the plethora of effectors that exist in nature. Following the initial identification of promising effector candidates with defined activities, the complexity of the experiments needs to be increased in order to unravel all different aspects of the mode of action. Akum et al. (2015) identified the involvement of a *S. indica* effector in different processes of *Arabidopsis* plants, using a fungal mutant lacking the effector of interest and a plant line over-expressing this effector. Using this material in gnotobiotic system would be a strategy to identify the role of effectors in altering microbial communities and to study and uncover underlying mechanisms. However, the genetic manipulation of filamentous fungi has been challenging due to the low efficiencies and flexibility of current methods (Shi et al., 2017). Various novel genome editing tools have recently been developed (Wang et al., 2017b), with CRISPR/Cas being the most promising approach for editing fungal genomes. The development of CRISPR/Cas technology will assist in the exploitation of the potential of fungi (Wang et al., 2017b). With such a method, fungal effector sequences can be altered in very defined ways (e.g. knock-out, motif editing, etc.) and used to unravel their role in plant manipulation and in changing plant

microbiomes. For example, generating *S. indica* strains lacking the two effector candidates identified in Chapter 3, and testing them in a gnotobiotic system with a synthetic microbial community (as described in Chapter 4), may reveal the involvement of these effectors in *S. indica* establishment within the plant root and how that further affects other microbes or microbiomes.

The study of effectors will reveal fundamental and conserved plant processes that are affected by effectors from phylogenetically distantly related microbes (Win et al., 2012). The characterisation of *S. indica* effectors may help identifying common strategies of beneficial microbes to manipulate host plants. Besides this, microbial effectors can be a valuable tool to dissect the signalling pathways and unravel fundamental principles of plant biology and immunity (Feng and Zhou, 2012).

Another aspect in this respect is the potential effect of microbial effectors on less obvious processes, such as pest control (Casteel and Hansen, 2014). It has been shown that microbes alter plant perception of herbivores and support plant immunity against herbivores (Ceja-Navarro et al., 2015; Giron et al., 2016). Furthermore, Badri et al. (2013) showed that the root microbiome can affect plant leaf metabolomes, which influences insect feeding behaviour, and inhibition of its herbivorous activity. Microbes that live in symbioses with pests can secrete effectors into the saliva of insects to suppress plant immunity and to induce a metabolic reprograming in plants in order to create the appropriate nutritional environment for the insect (Body et al., 2013; Kaiser et al., 2010). This research highlights the role of effectors in the metabolic manipulation of the host as an ecological competitive advantage. Moreover, it outlines the importance of effectors in multi-kingdom organism interactions and their economic potential in the suppression of diseases and pests in agriculture.

In the effort to increase the complexity of effectome studies, the role of the effectors should be assessed in the context of a microbial community. Do effectors from different microbes have a synergistic effect on altering plant processes? For example, Zolla et al. (2013) showed that soil microbiome induces drought perception by the plant, as well as the induction of the drought tolerance signalling

pathway, affecting the expression of drought related marker genes. Microbial effectors have been found to affect the drought signalling pathway (Rajput et al., 2015). Therefore, effectors may be playing a key role in this induction of the drought tolerance signalling pathway by the microbiome. Since drought is a major constrain for agriculture, particularly for farmers with limited access to water (Molden, 2013), identifying microbiomes that confer drought tolerance, or tolerance to biotic and abiotic stresses, as well as their mechanism of action, would be of high economic importance, improving crop tolerance and yield in challenging conditions.

6.3 Gnotobiotic system as a tool in microbiome research

Gnobiotitic systems have been proven to be very valuable in dissecting complex processes within multi-organism interactions (such as microbiomes) and to answer highly specific questions (Durán et al., 2018; Herrera Paredes et al., 2018). Such systems, in combination with minimal or synthetic microbial communities, give the flexibility to alter multiple different factors of the ecosystem in a very targeted way, such as soil characteristics, environmental conditions, the composition and complexity of the microbial community (Vorholt et al., 2017). These characteristics make gnotobiotic systems an ideal tool for studying the effectome in a microbiome context as mentioned above, but also the complex interactions within microbial communities and between plants and microbiomes.

6.3.1 Potential and limitations of gnotobiotic systems as reproducible tools to study the microbiome and basic interactions

Microbiome research has mainly been descriptive and is often short in providing insights in to the function of members of the community (Busby et al., 2017). The use of gnotobiotic systems and synthetic communities have brought a revelation to the microbiome research, and as indicated in this work (Chapter 4), has a high rate of robustness and reproducibility when it is compared with similar studies. The system enabled me to observe the high competitiveness of the *Arabidopsis* core bacterial community against beneficial and pathogenic fungal endophytes, pre-inoculated in the root (Chapter 4). This experiment showcased that

the bacterial community comes to a similar balance after a certain period of time irrespective of the initial treatments. Similarly, using such an experimental set up, researchers were able to show altered functionality of microbes without changing their relative abundance (Hartman et al., 2017), as well as the collapse of the microbial community with the removal of a single strain from the system (Niu et al., 2017). It indicates that gnotobiotic systems in combination with synthetic communities are a powerful approach to advance our understanding of microbial community dynamics and function.

The individual components of an ecosystem have a role in its function, and if the aim is to improve plant growth and health, a clear understanding of the most crucial ecosystem members is important. Interestingly, Durán et al. (2018) concluded that the maximum plant growth is achieved in the presence of a diversity of microbes comprising bacteria, fungi and oomycetes. The experimental set up of gnotobiotic system and the use of synthetic communities, could assist in gaining a better understanding of the dynamics of microbiome and its interaction with plants. This approach would be particularly helpful for uncovering mechanisms that have been proved to be challenging in microbiome research and seen as difficult to be studied with the traditional, descriptive approaches. For example, DNA-based approaches provide information of the microbial abundances but not about their activity; e.g. more than 90% of microbial community members are inactive or dormant (Fierer, 2017). On the other hand, rare microbiome members, contributing less than 0.1% to the overall relative abundance, belong to the striking 91% of the metabolically active microbes in and around the plant root (Dawson et al., 2017), highlighting the importance of the low abundant microbiome members in the system's processes and the functionality of the community. Rare species are considered as key players in the community that drive ecosystem function (Jousset et al., 2017). In this respect, gnotobiotic systems would be excellent tools to study the role of rare microbes in the microbiome, as well as the sequence of community assembly (Fukami and Morin, 2003), which can be a major determinant of biodiversity and productivity of the system. In addition, other organisms including viruses, archaea, nematodes, and insects play important roles for the ecosystem diversity and function (Adam et al., 2017; Castillo et al., 2017; Elhady et al., 2017;

Pratama and van Elsas, 2018), suggesting the necessity for more holistic and comprehensive studies of multitrophic interactions.

Considering the above observations when studying microbiomes, one should be careful on the conclusions made from an experiment in gnotobiotic systems, and generally when making associations between microbiome and plant phenotypes. Especially considering the limitations of the system used and the influence of other factors affecting the ecosystem's balance (Vorholt et al., 2017). Vorholt et al. (2017) suggested that understanding of such fundamental principles requires reproducible experimental approaches which allow to change specific factors, such as environmental/growth conditions, composition and diversity of communities, gene modifications, in order to be able to create links between made changes vs. observed effects or phenotypes. In addition, such experimental approaches including metagenome analyses should be complemented with other methods, such as transcriptomics, proteomics, metabolomics and metaphenomics (Sergaki et al., 2018; Vorholt et al., 2017). As highlighted by my work in Chapter 3, effectors alter the auxin signalling pathway leading to substantial changes in plant growth and responses to high levels of auxin, as well as their potential effect in plant-microbe interactions. Therefore, depending on the scientific question, it might be worthwhile to include the analysis of microbial effectors (Uhse and Djamei, 2018) and hormones as a complement in microbiome research to advance our understanding of underlying ecological interactions.

6.3.2 Approaches to advance our understanding of microbial communities

In recent years, various approaches have been used to study plant microbiomes, including metagenome analyses to receive the genetic potential of microbiomes (Ofek-Lalzar et al., 2014). Efforts have been made to extract RNA from roots, aiming at identifying metabolically active microbes and their involvement in plant pathways (Chaparro et al., 2014; Turner et al., 2013; Yergeau et al., 2014). Metatranscriptomics, as a functional expression analysis, can be combined with other methods to enhance our understanding of interactions. For example, Chaparro et al. (2013) could associate the root microbiome with the metabolome of *Arabidopsis* at different developmental stages, using

pyrosequensing of the microbiome mRNA in the rhizosphere and GC-MS to identify the metabolic profile of the plant. This approach revealed the functional genes of the community that could be correlated with plant metabolites. Turner et al. (2013) used metagenomic and metatranscriptomic profiling of microbiomes, as well as plant mutants, in order to identify multi-kingdom differences in the abundance as well as their metabolic capabilities (e.g. methylotrophy, cellulose degradation, H_2 oxidation). Such studies indicate that combining metagenomics and metatranscriptomics can enable us to functionally characterise microbiomes and to unravel the role of microbes in altering defined plant processes.

Unlike RNA which has a short turnover time (Prosser, 2015), proteins have a higher level of stability in the environment (Armengaud et al., 2012). Using metaproteomics, we can capture expressed proteins and thus, metabolic signature and activities in a given sample (Heyer et al., 2015; Wilmes and Bond, 2006). Exoproteomics or exometaproteomics, in particular, focus on the ecologically more important proteins involved in microbe – microbe and plant - microbe interactions (Armengaud et al., 2012; Lidbury et al., 2016). This approach would be very interesting to use in the experiments with *Medicago truncatula* and *Sinorhizobium spp.,* and the natural soil to gain a deeper insight into the biochemical processes within the system. The downside of this approach is that it requires sufficient amounts of material (Johnson-Rollings et al., 2014) which may be difficult to obtain for certain experimental set ups in addition to analytical challenges such as the correct assignment of detected peptides based on public databases and the required computational power (Muth et al., 2013, 2016; Timmins-Schiffman et al., 2017). The size of my experiment did not allow such an approach, but it would be of high relevance to be used in future experiments in order to disprove function-based hypotheses. Moreover, meta(exo)proteomics could be an excellent tool for identifying extracellular enzymes involved in plant – microbe interactions and in phosphate mobilization within roots (Lidbury et al., 2016).

Along with the employment of a combination of methods to improve our understanding of fundamental functional principles in microbiomes, time course experiments can provide an insight into the timeline of interactions and reveal mechanisms involved in each step of an interaction. It is known that plant

development significantly affects the composition of the rhizobiome (Chaparro et al., 2014; Rasche et al., 2006). Investigating further the microbial composition at different developmental stages could provide vital information on microbiome recruitment processes and the dynamics underlying the establishment of plant-microbe/microbe-microbe interactions over time (Qiao et al., 2017; Yuan et al., 2015). Such approaches would also help to better understand the establishment of core microbiomes in *Arabidopsis* roots in experiments using gnotobiotic systems, as well as the dynamic changes of natural soil microbiomes when exposed to *Medicago truncatula* roots inoculated with *S. meliloti* 1022.

6.3.3 Challenges in microbiome analysis

Due to the fast development of microbiome research, a lot of discussion arises on the appropriate approach for the analysis of microbiome data (Buttigieg and Ramette, 2014; Paliy and Shankar, 2016; Ramette, 2007; Song et al., 2018). Uncovering the earth's biodiversity is becoming a communal effort (Alivisatos et al., 2015), however, the diversity of data collection and analysis methods remains a big challenge for microbiome research (Thompson et al., 2017). This technical variation can lead to inconclusive results when comparing different studies. Venter et al. (2016) observed that different methods for microbiome analyses showed different outcomes. In addition, a high number of biological replicates is necessary to apply statistical tools that determine significant factors in studies in the field of microbial ecology and to ensure validity and reproducibility of experiments with high complexity. The Earth Microbiome project, as a collaborative effort to capture earth's microbial diversity (Gilbert et al., 2014b), continuously critically evaluates the methods used for this purpose (Gilbert et al., 2014a).

There are different methods available for microbiome data analyses with different advantages and disadvantages (Di Bella et al., 2013). Nearing et al. (2018) did a comparative analysis of different analysis pipelines and concluded that all different approaches lead to a similar community structure. However, the number of observed species significantly differed depending on the pipeline, as well as the alpha diversity. This aspect would be important if rare microbiome members are most important for a study (Nearing et al., 2018). In my work, I was looking for

major shifts caused by the inoculant. Therefore, the pipeline used (QIIME1) was sufficient to answer hypotheses. If I was looking for certain, low abundant species, the DADA2 pipeline (Callahan et al., 2016) would be better suited as it can detect a higher number of species due to the machine learning approach it uses (Nearing et al., 2018). Nevertheless, the analytic approach should be critically evaluated for each individual experiment and in dependence of the research objective. Using standardised/universal sampling and analysis protocols would help achieving higher reproducibility and compatibility of the research (Buttó and Haller, 2017).

6.4 The necessity of a fundamental understanding of individual microbes' properties, capabilities and lifestyles - Approaches to ensure inoculant competitiveness

Microbes have a broad spectrum of capabilities and the best exploitation of the gnotobiotic system's potential could be achieved with the advancement of our fundamental understanding of microbe's lifestyle, responses to the environment, genetic and functional potential (Sergaki et al., 2018). The employment of methods that can characterize microbiomes more accurately down to the genus level and beyond are highly necessary. For example, in my work, it was not possible to identify many of the Rhizobiales species beyond the order level, due to limitations in taxonomy assignment, which makes the exploration of microbial diversity and function more difficult. Furthermore, I showed the highly variable efficiency of different strains that belonged to the same genus, which highlights the necessity for functional characterisation of individual species, genera or even strains. As Schlaeppi and Bulgarelli (2015) suggested, in order to achieve that, large-scale, high-throughput techniques are needed.

6.4.1 The case of *Serendipita indica*

The use of the synthetic communities was found to be an excellent tool to study basic microbe interactions at the community level. It assisted in finding the inefficiency of the spore inoculation method used for *S. indica*, which was further proved to be true under natural conditions. Rabiey et al. (2017) demonstrated the strong potential of *S. indica* as an agricultural inoculant for UK soils, with strong

effects on wheat growth, soil microbial community diversity and long-term survival in the soil without the plant under UK weather conditions. However, the inoculation method for their experiments was mixing fungal spores and mycelium with the soil (Rabiey et al., 2017), similarly used in other studies (Anith et al., 2018; Das et al., 2012; Kumar et al., 2009; Narayan et al., 2017). This method might ensure the establishment of *S. indica* in plants, but the feasibility of using such a technique in agriculture and in larger scale experiments, should be carefully examined, particularly for the large mycelium quantities it would require.

While *S. indica* has been studied extensively in its association with plants (Arma et al., 2012; Banhara et al., 2015b; Cosme et al., 2016; Jacobs et al., 2011; Lahrmann et al., 2013; Qiang et al., 2012b; Rabiey et al., 2017; Schäfer et al., 2009; Vadassery et al., 2008; Varma et al., 2012), there are only a few studies on its interaction with other microbes. Meena et al. (2010) showed that *S. indica* had a synergistic effect with *Pseudomonas striata*, enhancing the bacteria's proliferation and increasing chickpea biomass. A similar effect was observed by Anith et al. (2015), who showed that the co-inoculation of the fungus with *Bacillus pumilus* improved the growth of tomato seedling. In all cases, the combined effect of the fungus and the inoculant was stronger than the effect of the single inoculation, something that I did not observe in my experiments suggesting a lower competitiveness of *S. indica* in my experimental set-up. Similarly, Kumar Bhuyan et al. (2015) showed that different strains of a plant beneficial bacteria, *Azotobacter chroococcum*, had contrasting effects on the growth of the fungus in the absence of a plant. They demonstrated that under the same experimental conditions, two of the tested strains had opposite effects on fungal growth. While *Azotobacter chroococcum* strain WR5 promoted *S. indica* growth, *Azotobacter chroococcum* strain M4 inhibited its growth. These findings show the complexity of microbial interactions and the necessity for a combination of approaches to study them. Regarding *S. indica*, I observed in my experiments that its proliferation in plant roots was inhibited in the presence of the synthetic microbial community in the gnotobiotic system, as well as in the presence of the natural microbiome. In order to understand this effect, it would be important to study the interaction of *S. indica* with microbiomes. One approach could be to build an interaction network as demonstrated by Helfrich et al. (2018) based on bipartite interactions between the

fungus and members of the synthetic community. Using such a high-throughput approach, it is possible to study pairwise interactions of microbes on plates, identifying inhibitory, neutral or auxotrophic relationship between microbes. This could potentially be used in the design of experiments with synthetic communities in order to be able to decompose all the microbial interactions within the community.

A more detailed, functional characterisation of *S. indica* growth and interaction with other microbes would assist in understanding the nature of interactions between *S. indica* and other microbes under different conditions. Such an effort was made by Jiang et al. (2018), who investigated the bipartite interaction of the fungus with the beneficial microbe *Bacillus subtilis.* They demonstrated that *S. indica* is auxotrophic for thiamine, which has a role in carbon cycling, and that the presence of *Bacillus subtillis* could recapitulate the auxotrophic growth of the fungus in thiamine-free media. In addition, they found that this auxothrophic interaction was present only when microbial inoculation was separated in space and time. It highlighted how different aspects of microbial interactions and the importance of their spatial and temporal organisation within the system may determine the outcome of microbial interactions in the microbiome.

6.4.2 The case of *Colletotrichum tolfidiae*

Colletotrichum tolfidiae is characterised as plant growth and nutrition enhancing microbe (Hiruma et al., 2016) However, when used in my gnotobiotic system, it had a negative effect on *Arabidopsis* plants. Studies on *Colletotrichum* spp. have shown that plant derived signals are able to reprogram fungal gene expression and determine the interaction with plants. In addition, the pathogenicity of the microbe was also found to be linked to the effector repertoire of the fungus (O'Connell et al., 2012). Combining the gnotobiotic system approach with metatranscriptomics and effectomics would unravel the mechanisms underlying the potential transition of *C. tolfidiae* between beneficial and pathogenic lifestyle.

The lifestyle transitions of microbes between mutualism and parasitism have been a major concern. For example, fungal endophytes that are recognised as

pathogens, such as *Fusarium graminearum*, can exist inside the plant root without causing any disease symptoms (Lofgren et al., 2018). Interestingly, *Fusarium* species were found in all UK agricultural soil types used in my experiments, as well as in the roots of *Medicago truncatula*. In fact, they were found to contribute up to the 5% of the observed differences in the fungal community of the *M. truncatula* endosphere, with increased relative abundance in the presence of *S. meliloti* 1022. Considering the strong growth promotion by the rhizobial strain, *Fusarium* species may even play an important role in this beneficial effect. Furthermore, even Sebacinales, the order that *S. indica* belongs to, is considered to have evolved from a saprotrophic ancestor (Garnica et al., 2016; Weiß et al., 2016). It is suggested that fungi that are closely related to pathogens or saprotrophs maintain certain pathogenic traits. However, the underlying mechanism driving the transition between pathogenicity and mutualism is unknown (Fesel and Zuccaro, 2016). My experiments, showing the detrimental effect of beneficial *C. tolfidiae* on *Arabidopsis* plants, highlight the necessity to understand such mechanisms, as well as the need for an improved understanding of the whole range of genetic and functional capabilities of microbes. Furthermore, we need to assess the level of the expression of those capabilities when microbes are grown individually, but also in association with plants or other microbes and under different environmental conditions. This effort will enable a more accurate and efficient use of microbes in agriculture, and an engineering of microbiomes in a more predictable way.

6.4.3 The case of *Sinorhizobium spp.*

Sinorhizobium species are used in agriculture as beneficial bioagents. My experiments showed varying efficiencies of different *Sinorhizobium* strains in three representative UK soil types (Chapter 3.3). The well-studied *Sinorhizobium meliloti* strain 1021 was found to be the least efficient in improving growth of *Medicago truncatula,* irrespective of soil type and nitrate availability. These findings align with those from Terpolilli et al. (2008), who demonstrated the low efficiency of *S. meliloti* 1021, using 8 different *Medicago* species. In this study, plants treated with strain 1021 were found to have lower dry weight and shoot N content compared to the N-treated plants and formed less active nodules in comparison to *S. medicae* 419 (Terpolilli et al., 2008).

While it has been discussed that plant species should be matched with the appropriate inoculants (Thrall et al., 2000), my work shows that the soil type plays a key role in their efficiency to enhance growth. *S. medicae* 419 appeared as a highly efficient strain at low pH, but this effect was less strong at higher pH. Therefore, for higher inoculant efficiency, not only the host plant genotype should be considered, but also edaphic factors of the field site. These findings highlight the necessity for field-based or field soil-based studies, testing different plant hosts and environmental conditions in order to assess the efficiency and the suitability of an inoculant.

This work presents evidence of the complexity of microbial interactions within a given community and with the environment, which can disturb the expected performance of certain microbes. The reduced competitiveness of *S. indica*, the detrimental effect of the previously shown beneficial *C. tolfidiae* and the varying efficiency of *Sinorhizobium* species indicate current gaps in our knowledge and understanding of microbe – microbe and plant – microbe interactions. Fundamental understanding of the genomic and functional capabilities of microbes, as individuals and as part of microbial communities, will accelerate our understanding of microbial interactions, recruitment pattern by the plant and their function in a given ecosystem.

6.5 Future approaches to examine and exploit microbial bioagents for agricultural application

Microbiome research has shown that unexplored diversity could be a substantial biotechnological source (De Souza et al., 2016). De Souza et al. (2016) suggest that studies that focus on specific microbial groups will miss their collective contributions to processes such as nitrogen fixation, and that the microbial diversity within the root may play an important role in nitrogen fixation. Along these lines, *S. meliloti* 1022 was found to alter bacterial community structures and compositions, as well as to enhance plant growth and to increase N content in the aboveground plant tissue in different soil types with varying P and N content. On

the other hand, it is known that high nitrogen levels in soil can inhibit nodulation (Van Noorden et al., 2016). It might indicate that *S. meliloti* 1022 assists in the recruitment of microbes that improve growth and N acquisition from the soil. This ability of *S. meliloti* 1022 indicate the potential of microbes to improve plant growth nutrition in ways that are usually disregarded. Moreover, Thonar et al. (2017) demonstrated that the combination of three inoculants (*Trichoderma*, *Pseudomonas*, and *Bacillus* strains) in a field experiment increased maize growth and nutrient acquisition. Understanding the role of each individual microbe, their mode of action and how they perform in combination with other microbes will allow us to identify combinations of microbes under different or specific soil/environmental conditions to maximise plant health and production.

Particularly, for improving nitrogen fixation, more thorough research is needed to understand the complex interactions with plants and within nodules. Notably, Zgadzaj et al. (2016) showed that the nodule has a very distinct microbiome and that symbiosis drives the establishment of specific microbes with potential growth promoting traits. Such beneficial microbes might also support the plant growth promotion by *S. meliloti* 1022 in my experiments, particularly in the high nitrate soil, where nodulation might have been inhibited, yet the growth promotion in the presence of the inoculant was clear. Nodule microbiomes may harbour novel mechanisms to sustain plant growth and nutrition acquisition as well as supporting other microbes involved in establishing and maintaining nodulation.

As previously mentioned, the comprehensive genetic and functional characterisation of individual microbes, as well as the characterisation of their interactions are essential to advance our knowledge about the full spectrum of microbiome functions. *In vitro* assessment of plant growth promoting traits/genes is not necessarily translated into a plant phenotype. Similarly, genes found to have no function under laboratory conditions may play key roles in a community context and modulate competitive interactions or/and support the establishment of symbiosis under field conditions (Poole et al., 2018). Whole genome sequencing of microbial isolates collections and the use of synthetic microbial communities in the gnotobiotic system in combination with plant lines with genetic variation are promising strategies for the identification of such traits in strains and encourage the

discovery of new plant growth promoting genes (Finkel et al., 2017). For example, whole genome analysis of *Paenibacillus polymixa* revealed genes related to IAA production, compound important for plant manipulation (Xie et al., 2016a). In addition, studying transcriptome or proteome responses of microbes as part of complex plant microbiome and exposed to stressful conditions can provide essential information of community assembly and plant growth promoting processes (Fernández et al., 2013; Lidbury et al., 2016; Mauchline et al., 2006). For example, Lidbury et al. (2016) examined the responses of agriculturally relevant *Pseudomonas* strain in low phosphate conditions using comparative exoproteomics, revealing key proteins of the different strains involved in their growth promoting activity in low phosphate conditions.

In recent years, new tools and methods have been developed to study microbe-microbe and plant-microbe interactions. For example, with the use of bio-reporters for sugars, polyols, amino and organic acids, or flavonoids in a *Rhizobium* spp. mutants, in combination with metabolomics and transcriptomics, Pini et al. (2017) were able to describe the development of rhizobia-legume symbioses in space and time. Such approaches can help us in unravelling plant-microbe dynamics in the rhizosphere as well as in the identification of compounds (e.g. proteins, metabolites) involved in those interactions. Furthermore, microfluidic systems using plants and fluorescent microbes allow spatio-temporal analysis of microbial interactions in the root and provide information on the establishment of microbes and the competition between them (Massalha et al., 2017). Both approaches would be useful for understanding of the establishment of *S. indica* and the rhizobial strains in the presence of other microbes, as well as the recruitment of certain microbes by the inoculants. However, the experimental set up of those may have certain limitations on the hypothesis that can be tested.

6.6 Transferring knowledge from the lab to the field

Even though experiments using bipartite interactions under controlled conditions have increased our understanding of processes involved in microbial interactions, they give little information on the processes that occur under natural settings (de Boer, 2017). This also applies to plant-microbe interactions as shown

from my experiments, involving *Medicago truncatula, Serendipita indica, Sinorhizobium* and agricultural soils, where the microbes did not perform as expected. *Serendipita indica* did not promote plant growth as expected, while *Sinorhizobium* species behaved very differently depending on the soil type. In particular, the well-studied *S. meliloti* strain 1021 was found to have no growth-promoting effect in all soil types. This also holds true for arbuscular mycorrhizal fungi, which are well-known plant-growth promoting microbes. However, certain species can inhibit plant growth under conditions such as low temperature, light and even low phosphorus availability (Johnson et al., 1997; Smith and Smith, 1996). Moreover, it was recently shown that arbuscular mycorrhizae can be suppressed by the soil microbiome (Svenningsen et al., 2018). While there is an increasing interest in using microbes that can utilise soil P that is unavailable for plants, their use in the field remains limited, as the dynamics within the natural ecosystem is still poorly explored (Richardson, 2002). In addition, the observed growth promotion by microbial inoculants may not be a consequence of an attribute observed in the laboratory, such as phosphorus mobilisation (Richardson, 2002). Instead, it could be due to the recruitment of other beneficial microbes. This may be the case for the highly efficient *S. meliloti* strain 1022, which improves plant growth and N acquisition even in soil types with high N content, potentially through the recruitment of other beneficial microbes. Taken together, there is an urgent need for applying lab-gained knowledge in the field.

Transferring knowledge from the laboratory to the field, would mean that farming practices should also be taken into account. It is known that crop rotation has a positive impact on plant yield, which could be a result of changes in the microbiome composition. Hilton et al. (2018) demonstrated the significant effect of crop rotation on the composition of bacterial and fungal communities. In addition, studies have shown that crop rotation increases microbial diversity and richness as different plants alter soil characteristics in different ways (Venter et al., 2016). In my study, mainly high NP soil type had a cultivation history with different crops, resulting in a distinct microbiome and higher bacterial species richness, increased nutrient content (perhaps due to mineral fertilisation) and higher plant yield of mock-treated plants. In contrast, the field from which I obtained the low NP soil type was uncultivated while the low N high P soil type was used for winter wheat

for two consecutive years. This possibly resulted to a reduced fertilizer input, and consequently reduction of nitrogen, as well as the formation of bacterial communities with similar structure. Furthermore, Li et al., (2019) found that monocropping caused a reduction of microbial diversity, an enrichment of rare microbiomes and a loss of microbial traits associated with plant growth enhancement, e.g. underrepresentation of plant growth promoting microbes with function in nutrient metabolism and phytohormones biosynthesis. These findings highlight the impact of farming practices on microbiome – associated traits that can affect crop production.

6.7 Future perspectives

There is the need for microbiome research strategies with a clear focus on the functional characterisation of microbial communities in order to exploit the full potential of microbiome activities for agricultural crop production (Figure 4.1). In order to do this, isolation and functional characterisation of microbes is needed, as well as of all the organisms of the ecosystem, e.g. plant-related nematodes, viruses, and insects, as they may contribute to certain processes of the ecosystem (Brussard, 1997). To do this, the employment of high throughput assays that will increase the speed of the screening would be necessary, targeting different aspects of microbial fitness and parameters that affect the assembly of microbiomes. For example, cytology-based approaches, such as the microplate-reader system that enables the visualization of gene expression during microbial bipartite interactions (Hennessy et al., 2017), is a promising high-throughput screening tool to dissect microbial interactions. Such approach could also reveal components of microbial interactions that can potentially have commercial value, such as antibiotics (Tyc et al., 2014). Furthermore, studying the response of individual microbes to plant microbiome or to abiotic stresses, using combination of approaches (e.g., proteomics, transcriptomics), can reveal genes associated with plant growth promotion properties and microbial recruitment (Fernández et al., 2013; Lidbury et al., 2016; Martino et al., 2018; Mauchline et al., 2006). The identification of such genes/traits could be excellent tools in manipulating microbiomes, plant growth and health.

Characterised microbes can build the basis for the construction of synthetic communities by combining microbial strains with different activities and design communities with varying levels of complexity. Gnotobiotic system can serve as robust 'tools' to study the activities of such communities (e.g. on plant fitness), as it allows to change and test defined parameters, and is particularly informative when coupled with other approaches such as 'omics-based analyses. Gnotobiotic systems can be the first step in transferring this knowledge to the field, where other aspects, such as farming parameters including crop rotation, tillage, etc. can be tested. In addition, universal standards should be applied for microbiome research in order ensure reliability and reproducibility of results and their biological relevance.

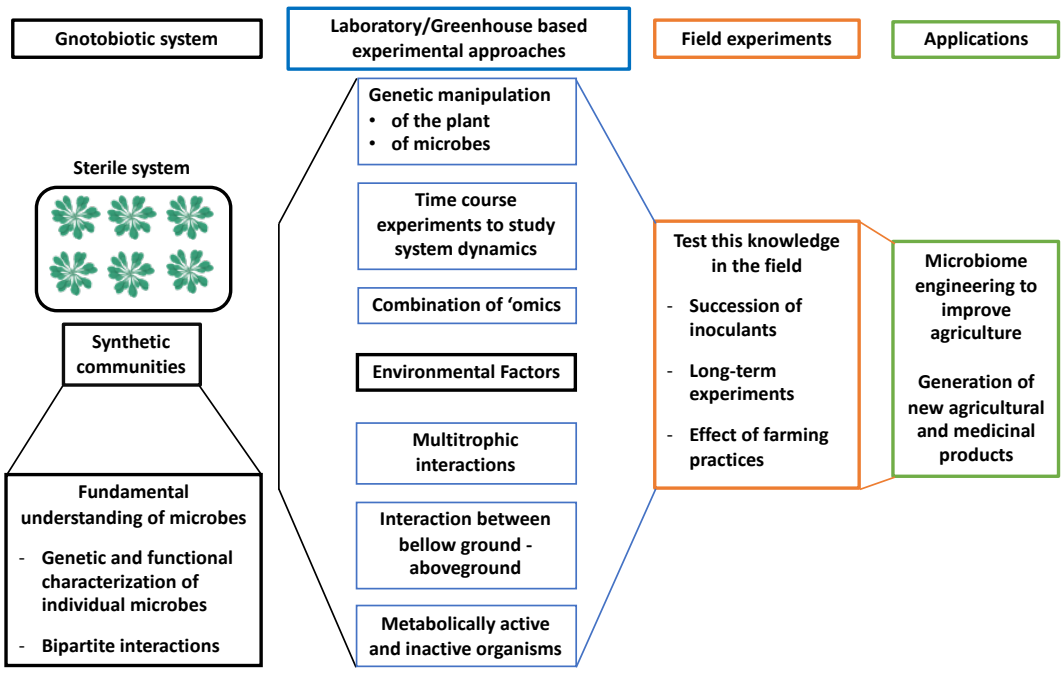


Figure 6.1: Multi-scale approaches for microbiome research. The generation of microbial isolates collections enables the increase of our fundamental understanding of microbes and their use in the gnotobiotic system as synthetic microbial communities. This system allows the use of various experimental approaches to study plant-microbe interactions and test specific hypothesis. The knowledge from these laboratory/greenhouse experimental approaches should be tested in the field and further apply it to improve agriculture. [Arabidopsis plants] illustrations courtesy of somersault18:24, Creative Commons license CC BY-NC-SA 4.0

6.8 General conclusions

The conclusions of this integrated approach to study plant-microbe interactions are as follows:

The study of *S. indica* effectors using a combinational approach, gave an insight to strategies for the establishment of fungal colonisation within the root. This study opens new potential for manipulation of plants and microbiomes that can be achieved through mechanistic understanding of the microbial effectors' activity. The key conclusions of this chapter are:

- Single *S. indica* effectors alter auxin hormonal signaling
- Effectors confer a competitive advantage to the fungus
- Potential of deploying microbial effectors to manipulate crops

The use of a synthetic microbial community and plants inoculated with fungal endophytes in a gnotobiotic system revealed competition relationships among microbes giving the following conclusions:

- The gnotobiotic system is a reproducible tool to study to deconstruct the microbiome and study basic interactions – complement with other methods
- *S. indica* promotes growth, however, when it is in a microbiome context, the method of inoculation matters.
- *C. tolfidiae* can have detrimental effect on plant survival, which is reverted by the *Arabidopsis* core bacterial community
- *Arabidopsis* core bacterial community structure is similar in all treatments within this system, indicating the robustness of the core microbiome.

Increasing the complexity of the experimental set up, the efficiency of three *Sinorhizobium strains* to increase *M. truncatula* yield and nitrogen content was evaluated using three agricultural UK soil types. Increasing the output of this experiment, the fungal and bacterial communities were examined, along with the soil and aboveground plant tissue chemical characteristics. This experimental set up gave agriculturally relevant conclusions:

- *S. meliloti* 1022 is the most efficient growth promoting rhizobia symbiont for *Medicago trancatula* A17 among *S. medicae* 419 and *S. meliloti* 1021 in three different soil types with variable nutrient availabilities.
- *S. meliloti* 1022 has an additive effect on *Medicago trancatula* A17 growth in high nitrate conditions.
- Edaphic factors affect fungal and bacterial soil community structures.
- The rhizobial inoculant influences the composition of bacterial communities in the endosphere in all soil types while it had no effect on the fungal community in any compartment (soil, rhizophere, endosphere).
- *S. meliloti* 1022 supports plant nitrogen supply.
- The presence of *S. meliloti* 1022 has greater effect on the plant growth than the edaphic factors.
- *S. meliloti* 1022 potentially enhances plant yield in a different way in the high NP soil type than in the low NP and low N high P soil type, potentially through recruitment of beneficial microbes into the root.

The work presented in this thesis gives meaningful and promising insights for the improvement of crop production. Research is being directed towards agricultural applications by characterising microbiomes and their functions in sustainably improving crop production systems. This has a huge impact, comparable to that of human probiotics. Such applications of microbiome research include the host-mediated microbiome engineering to sustainably improve agriculture, though the genetic selection or manipulation of plant to recruit beneficial or disease-suppressive microbes (Mueller and Sachs, 2015; Raaijmakers and Mazzola, 2016). Other aspects of microbiome engineering can be atomisation of beneficial microbes into the flowers in order to support the growth of the next generation of plants (Mitter et al., 2017), multi-generation selection of microbiome (Panke-Buisse et al., 2015) and direct seed, seedling, soil, endosphere or plant wound inoculation of beneficial microbiome (Orozco-Mosqueda et al., 2018) have all demonstrated excellent potential to sustainably improve agriculture. My work on *S. indica* effectors, the gnotobiotic system testing individual effectors and the evidence for the robustness of *S. meliloti* as *M. truncatula* inoculant in natural soils give an insight into the potential of these approaches in microbiome research and in agriculture as well as creating a basis for further research.

Appendix

Table S2.1: Index primers used for the Medicago microbiome libraries.

Index primers (all 5'-3')	
S502	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTC
S503	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGTC
S505	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTC
S506	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTC
S507	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGTC
S508	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTTCGTCGGCAGCGTC
S510	AATGATACGGCGACCACCGAGATCTACACCGTCTAATTCGTCGGCAGCGTC
S511	AATGATACGGCGACCACCGAGATCTACACTCTCTCCGTCGTCGGCAGCGTC
S513	AATGATACGGCGACCACCGAGATCTACACTCGACTAGTCGTCGGCAGCGTC
S515	AATGATACGGCGACCACCGAGATCTACACTTCTAGCTTCGTCGGCAGCGTC
S516	AATGATACGGCGACCACCGAGATCTACACCCTAGAGTTCGTCGGCAGCGTC
S517	AATGATACGGCGACCACCGAGATCTACACGCGTAAGATCGTCGGCAGCGTC
S518	AATGATACGGCGACCACCGAGATCTACACCTATTAAGTCGTCGGCAGCGTC
S520	AATGATACGGCGACCACCGAGATCTACACAAGGCTATTCGTCGGCAGCGTC
S521	AATGATACGGCGACCACCGAGATCTACACGAGCCTTATCGTCGGCAGCGTC
S522	AATGATACGGCGACCACCGAGATCTACACTTATGCGATCGTCGGCAGCGTC
N701	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG
N702	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGG
N703	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGG
N704	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGG
N705	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGG
N706	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGG
N707	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGG
N710	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGG
N711	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGG
N712	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGG
N714	CAAGCAGAAGACGGCATACGAGATTCATGAGCGTCTCGTGGGCTCGG
N715	CAAGCAGAAGACGGCATACGAGATCCTGAGATGTCTCGTGGGCTCGG
N716	CAAGCAGAAGACGGCATACGAGATTAGCGAGTGTCTCGTGGGCTCGG
N718	CAAGCAGAAGACGGCATACGAGATGTAGCTCCGTCTCGTGGGCTCGG
N719	CAAGCAGAAGACGGCATACGAGATTACTACGCGTCTCGTGGGCTCGG
N720	CAAGCAGAAGACGGCATACGAGATAGGCTCCGGTCTCGTGGGCTCGG
N721	CAAGCAGAAGACGGCATACGAGATGCAGCGTAGTCTCGTGGGCTCGG
N722	CAAGCAGAAGACGGCATACGAGATCTGCGCATGTCTCGTGGGCTCGG
N723	CAAGCAGAAGACGGCATACGAGATGAGCGCTAGTCTCGTGGGCTCGG
N724	CAAGCAGAAGACGGCATACGAGATCGCTCAGTGTCTCGTGGGCTCGG
N726	CAAGCAGAAGACGGCATACGAGATGTCTTAGGGTCTCGTGGGCTCGG
N727	CAAGCAGAAGACGGCATACGAGATACTGATCGGTCTCGTGGGCTCGG

Table S2.2: Number of reads before and after the analysis of the microbiome sequencing data

	M. truncatula **bacteria**	***M. truncatula*** **fungi**	***A. thaliana*** **bacteria**	***A. thaliana*** **fungi**
Number of raw reads	**23.6 million**	**27.8 million**	**18.5 million**	
Number of reads after filtering	**5.9 million**	**12.9 million**	**9.3 million**	
Number of reads in OTU table	**1.9 million**	**8.9 million**	**5.2 million**	**1.7 million**

Table S3.1: List of S. indica effectors gene names with the number used in the screening process.

# in screen	GeneName
1	PIIN_00011
2	PIIN_00073
3	PIIN_00112
4	PIIN_00113
5	PIIN_00199
6	PIIN_00200
7	PIIN_00225
8	PIIN_00455
9	PIIN_00561
10	PIIN_00602
11	PIIN_00740
12	PIIN_01005
13	PIIN_01255
14	PIIN_01377
15	PIIN_01379
16	PIIN_01577
17	PIIN_01719
18	PIIN_01768
19	PIIN_01892
20	PIIN_01931
21	PIIN_02015
22	PIIN_02096
23	PIIN_02227
24	PIIN_02492
25	PIIN_02570
26	PIIN_02571
27	PIIN_02572
28	PIIN_02598
29	PIIN_02649
30	PIIN_02839
31	PIIN_02849
32	PIIN_03075
33	PIIN_03088
34	PIIN_03188
35	PIIN_03190
36	PIIN_03222
37	PIIN_03271
38	PIIN_03283
39	PIIN_03459
40	PIIN_03461
41	PIIN_03467
42	PIIN_03691
43	PIIN_03806
44	PIIN_03863
45	PIIN_04084
46	PIIN_04114
47	PIIN_04206
48	PIIN_04207
49	PIIN_04219
50	PIIN_04220

# in screen	GeneName
51	PIIN_04270
52	PIIN_04291
53	PIIN_04301
54	PIIN_04539
55	PIIN_04661
56	PIIN_04685
57	PIIN_04931
58	PIIN_04932
59	PIIN_04941
60	PIIN_05021
61	PIIN_05094
62	PIIN_05098
63	PIIN_05217
64	PIIN_05242
65	PIIN_05338
66	PIIN_05452
67	PIIN_05670
68	PIIN_05674
69	PIIN_05848
70	PIIN_05864
71	PIIN_05865
72	PIIN_05872
73	PIIN_05875
74	PIIN_05879
75	PIIN_05932
76	PIIN_05933
77	PIIN_06047
78	PIIN_06133
79	PIIN_06305
80	PIIN_06398
81	PIIN_06410
82	PIIN_06443
83	PIIN_06535
84	PIIN_06568
85	PIIN_06796
86	PIIN_06837
87	PIIN_06901
88	PIIN_07076
89	PIIN_07104
90	PIIN_07138
91	PIIN_07145
92	PIIN_07212
93	PIIN_07327
94	PIIN_07333
95	PIIN_07381
96	PIIN_07425
97	PIIN_07657
98	PIIN_07672
99	PIIN_07915
100	PIIN_07917

# in screen	GeneName
101	PIIN_07952
102	PIIN_08029
103	PIIN_08228
104	PIIN_08307
105	PIIN_08375
106	PIIN_08513
107	PIIN_08516
108	PIIN_08524
109	PIIN_08623
110	PIIN_08672
111	PIIN_08714N
112	PIIN_08768
113	PIIN_08836
114	PIIN_08972
115	PIIN_08982
116	PIIN_09044
117	PIIN_09051
118	PIIN_09175
119	PIIN_09181
120	PIIN_09206
121	PIIN_09321
122	PIIN_09409
123	PIIN_09466
124	PIIN_09504
125	PIIN_09625
126	PIIN_09643
127	PIIN_09644
128	PIIN_09658
129	PIIN_09723
130	PIIN_09744
131	PIIN_09791
132	PIIN_09887
133	PIIN_09899
134	PIIN_09929
135	PIIN_09968
136	PIIN_10187
137	PIIN_10216
138	PIIN_10223
139	PIIN_10467
140	PIIN_10493
141	PIIN_10643
142	PIIN_10951
143	PIIN_10962
144	PIIN_10989
145	PIIN_11103
146	PIIN_11297
147	PIIN_11336
148	PIIN_11572
149	PIIN_11578
150	PIIN_11654

Table S3.2: Raw data from the protoplast screening of the top effector candidates.

	difference to LUC expression of eV under mock treatment (%)					***difference to LUC expression of eV under NAA treatment (%)***			
effector	mean	SD	SE	t-test	effector	mean	SD	SE	t-test
SIE_87	-71.35	40.52	28.65	0.10	SIE_83	-222.29	0.91	0.64	0.00
SIE_144	-46.30	75.95	53.70	0.42	SIE_7	-214.83	38.32	27.10	0.01
SIE_149	-43.41	10.13	7.16	0.02	SIE_149	-92.83	0.65	0.46	0.38
SIE_16	-33.06	13.83	9.78	0.05	SIE_87	-91.84	1.32	0.94	0.38
SIE_67	-25.38	5.41	3.82	0.02	SIE_67	-91.33	3.14	2.22	0.39
SIE_24	-23.01	13.43	9.50	0.08	SIE_16	-90.42	13.54	9.58	0.40
SIE_7	-19.88	0.10	0.07	0.00	SIE_88	-83.12	1.37	0.97	0.44
SIE_56	-18.25	2.99	2.12	0.07	SIE_148	-80.57	0.95	0.67	0.01
SIE_83	-15.56	1.91	1.35	0.15	SIE_24	-79.24	3.56	2.52	0.47
SIE_88	-2.90	15.48	10.95	0.33	SIE_56	-76.60	6.55	4.63	0.05
eV	-0.09	0.28	0.09		SIE_76	-35.52	91.19	64.48	0.54
SIE_148	32.66	0.59	0.42	0.01	SIE_59	-5.79	22.23	15.72	0.64
SIE_111	39.30	6.36	4.50	0.05	eV	0.00	0.00	0.00	
SIE_69	41.63	12.60	8.91	0.40	SIE_81	5.29	23.16	16.38	0.78
SIE_126	50.07	12.35	8.73	0.03	SIE_64	7.13	23.83	16.85	0.98
SIE_9	64.30	33.53	23.71	0.16	SIE_9	23.66	73.33	51.85	0.60
SIE_76	69.96	3.77	2.67	0.16	SIE_69	46.06	31.89	22.55	0.38
SIE_64	78.08	26.48	18.73	0.10	SIE_126	49.26	36.85	26.06	0.58
SIE_75	82.35	2.16	1.52	0.11	SIE_10	56.60	11.50	8.13	0.04
SIE_81	83.51	16.87	11.93	0.13	SIE_75	57.80	4.97	3.51	0.14
SIE_59	103.54	21.99	15.55	0.04	SIE_144	61.42	12.27	8.68	0.38
SIE_10	479.72	117.20	82.88	0.03	SIE_111	107.10	51.34	36.30	0.13

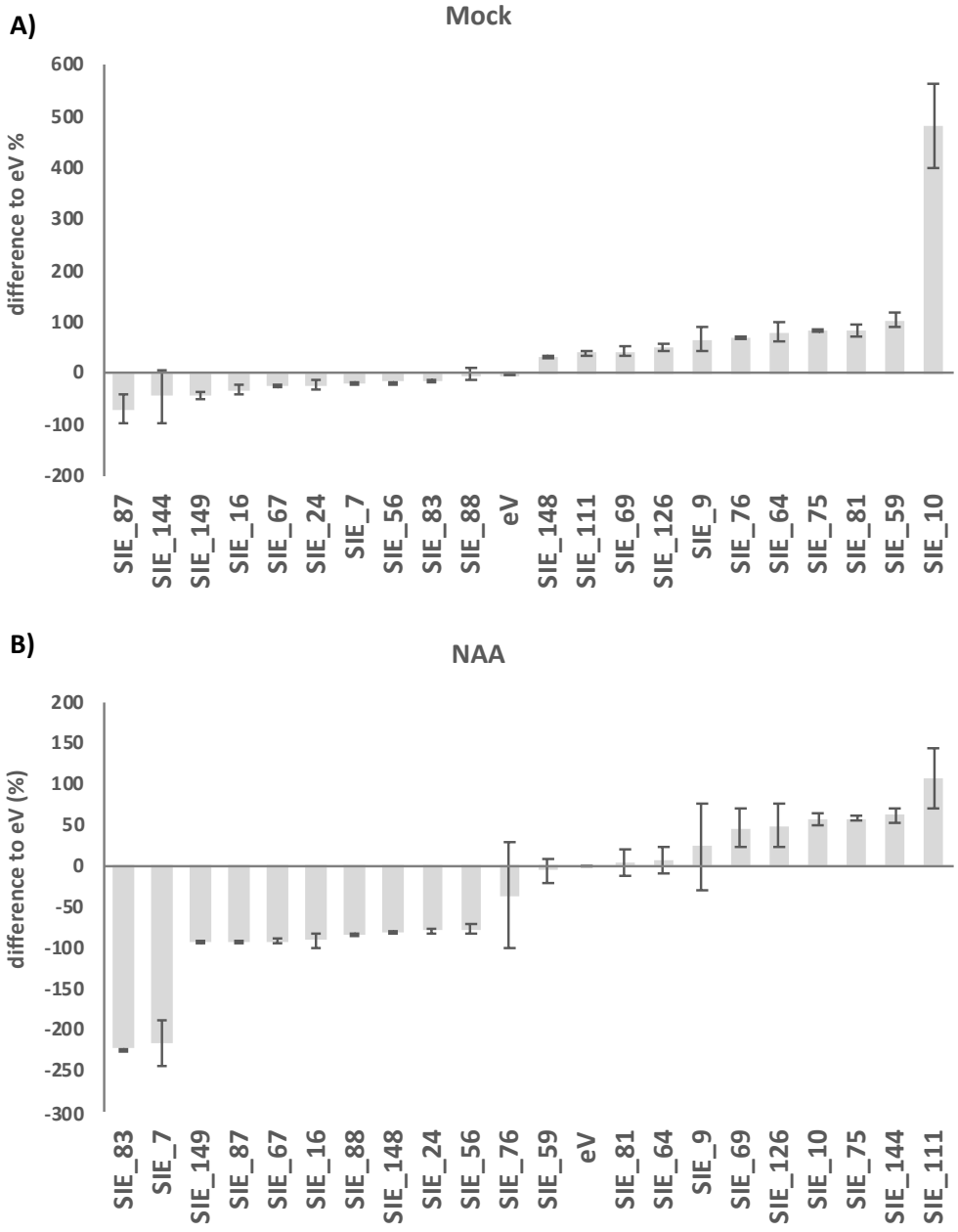


Figure S3.1: Normalized luciferase expression relative to eV GFP in control and NAA treated protoplasts. Luciferase expression is presented as percentage (%) of the luciferase expression of protoplasts transformed with the eV under the respective treatment; ***A)*** *Mock or* ***B)*** *NAA treatment. Mean values are shown with standard errors.*

Table S4.1: Sequence of OTU1 as identified by Qiime1 software

OTU1	AACGCAAGTTGCGCCCCAAGCCTTCTGGCCGAGGGCACGTCTGCCTGGGTGTCACAAATC GTCGTCCCTCACCATCCTTTGCTGATGCGGGACGGAAGCTGGTCTCCCGTGTGTTACCGCA CGCGGTTGGCCTAAATCCGAGCCAAGGACGCCTGGAGCGTACCGACATGCGGTGGTGAAC TTGATCCATTACATTTTATCGGTCGCTCTTGTCCGGAAGCTGTAGATGACCCAAAGTCCATA TAGCGACCCCAGGTCAGGCGGGATTACCCGCTGAGTTTAA

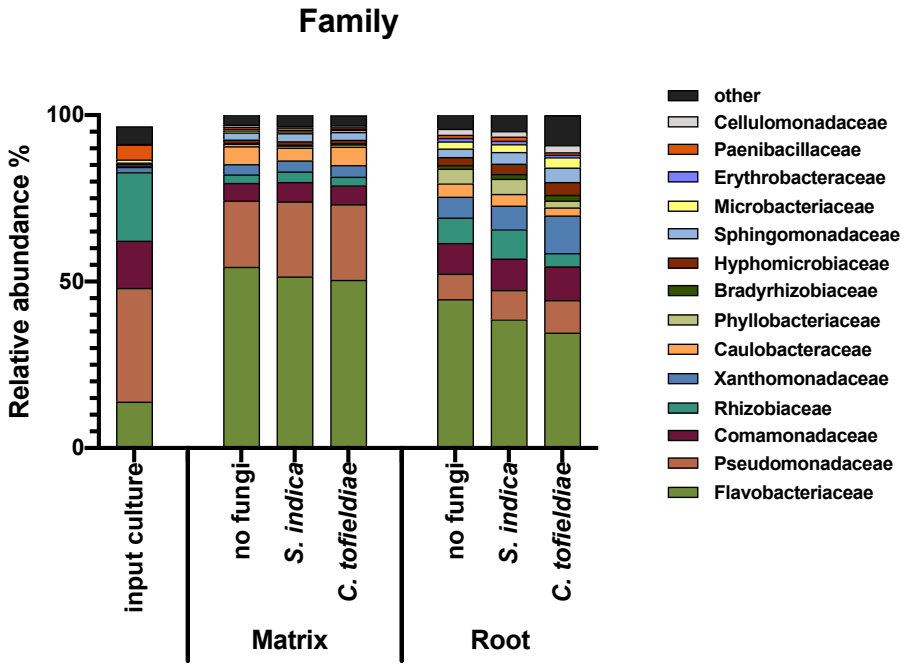


Figure S4.1: Relative abundance of each family in the matrix and root samples treated with S. indica, C. tofieldiae or no fungi.

RA is represented using stacked bar plot of the calculated means of rarefied reads.

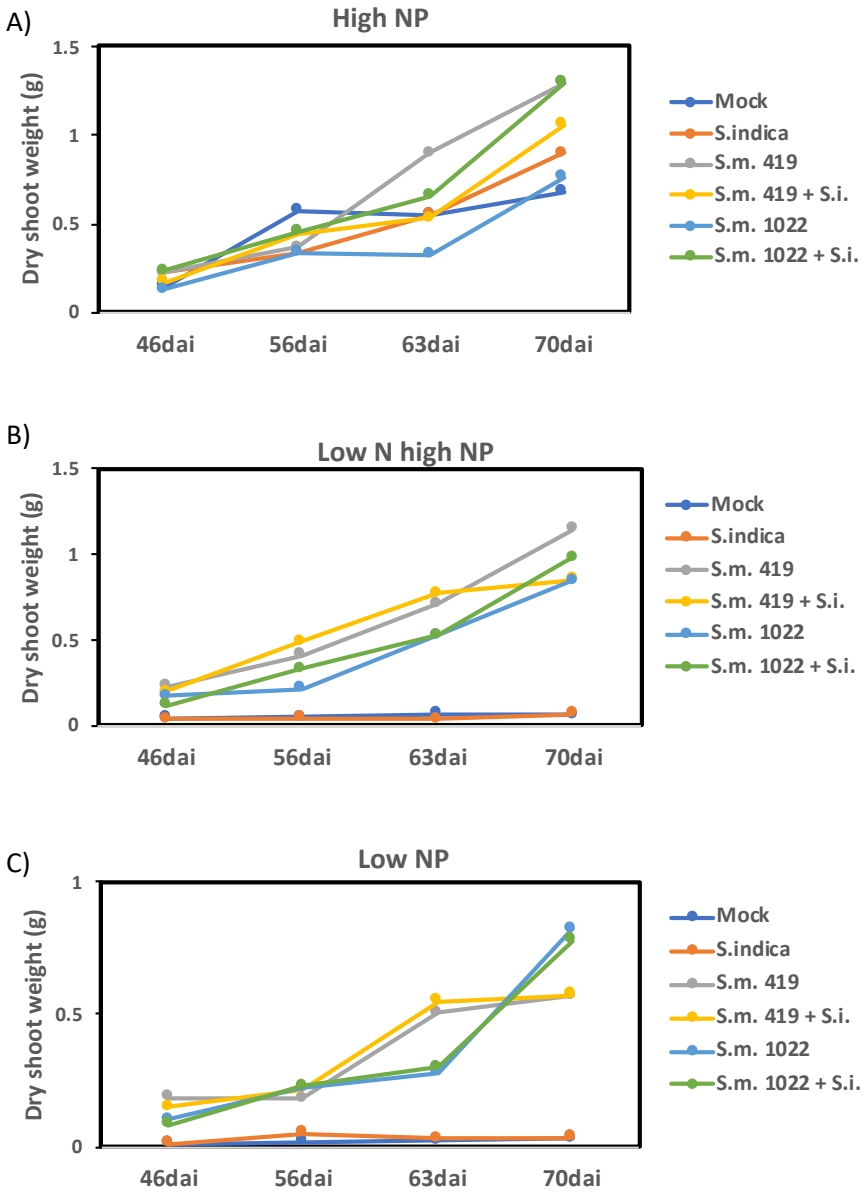
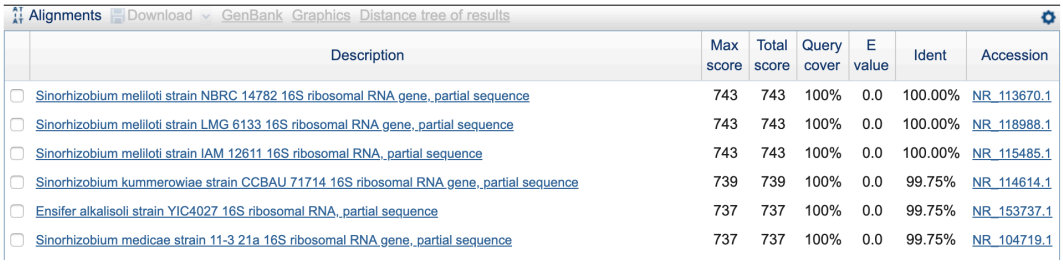


Figure S5.1: Growth curves of Medicago trancatula plants growing in different soil types, with different treatments. Mean values of dry aboveground weight (in g) are plotted for 4 time points, at 46dai, 56dai, 63dai and 70dai.



Alignments | Download | GenBank | Graphics | Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Sinorhizobium meliloti strain NBRC 14782 16S ribosomal RNA gene, partial sequence	743	743	100%	0.0	100.00%	NR_113670.1
Sinorhizobium meliloti strain LMG 6133 16S ribosomal RNA gene, partial sequence	743	743	100%	0.0	100.00%	NR_118988.1
Sinorhizobium meliloti strain IAM 12611 16S ribosomal RNA, partial sequence	743	743	100%	0.0	100.00%	NR_115485.1
Sinorhizobium kummerowiae strain CCBAU 71714 16S ribosomal RNA gene, partial sequence	739	739	100%	0.0	99.75%	NR_114614.1
Ensifer alkalisoli strain YIC4027 16S ribosomal RNA, partial sequence	737	737	100%	0.0	99.75%	NR_153737.1
Sinorhizobium medicae strain 11-3 21a 16S ribosomal RNA gene, partial sequence	737	737	100%	0.0	99.75%	NR_104719.1

Figure S5.2: Result from bacterial OTU2 alignment with available genomes on BLAST platform.

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