Plant genotype, immunity and soil composition control the rhizosphere microbiome

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Abstract

Three model plant and three crop plant species were grown for three generations in sand and compost. Pots were inoculated with 10 % soil initially, and with 10% of growth medium from the previous generation in generations 2 and 3, keeping replicates separate for all three generations. The microbiome community structure of the plant rhizosphere in each generation was characterised using ARISA DNA fingerprinting and 454 sequencing. Rhizosphere bacterial and fungal communities are different from those in bulk soil and there are also differences in the microbial community between different plant species. Plants both select and suppress specific bacteria and fungi in the rhizosphere microbiome, presumably via composition of their root exudates. Two out of three most abundant bacteria selected in the rhizosphere were isolated. These isolates proved to possess plant growth promotion properties. Plants are able to "farm" the soil in order to enrich it with plant growth promoting rhizobacteria (PGPR) species. However, in some plant species rhizospheres, invasions of opportunists and pathogens take place, mimicking events in plant monocultures.

Other experiments using this multi-replicate system allowed for statistical analysis of the influence of *Arabidopsis* and *Medicago* mutants on the rhizosphere microbiome. Three groups of *Arabidopsis* mutants were tested: plants unable to produce aliphatic glucosinolates, plants impaired in the PAMP-triggered immune response and plants unable and over-expressed in methyl halides production and one group of *Medicago* mutants which are impaired in the mycorrhization ability. All these plant genotypes, except those for methyl-halide production and one genotype involved in PAMP response, significantly altered the rhizosphere microbiome.

Abbreviations

196-11	35S::HOL (*Arabidopsis* line)
AMA	Acid minimal agar
AMS	Acid minimal salts
ARISA	Automated Ribosomal Intergenic Spacer Analysis
BLAST	Basic Local Alignment Search Tool
bp	base pair
CCC	Capacity and Capability Challenge program
cfu	Colony forming units
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
EB	Elution buffer (Qiagen)
EDTA	Ethylenediaminetetraacetic acid
gDNA	Genomic DNA
GUI	Graphical User Interface
HOL	Harmless to Ozone Layer
ISR	intergenic region (16S – 23S rRNA gene)
ITS	intergenic spacer (18S – 28S rRNA gene)
LB	Luria Bertani media
LCA	Lowest Common Ancestor
MANOVA	Multivariate ANalysis Of VAriance

MAP	Mitogen activated proteins
MDS	MultiDimensional Scaling
MEGAN	MEtaGenome ANalyzer
MS media	Murashige and Skoog media
MYB28/29	MYeloBlastosis 28/29
Myc^-	Mycorrhization mutant
NCBI	National Centre for Biotechnology Information
Nod^-	Nodulation mutant
OD	Optical density
OTU	Operational Taxonomic Unit
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEG 6000	Polyethylene glycol 6000
RPM	Revolutions per minute
RAM1	Required for Arbuscular Mycorrhization 1
RAM2	Required for Arbuscular Mycorrhization 2
ROS	Reactive oxygen species
rRNA	ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
TY media	tryptone yeast extract media
UMS	Universal minimal salts
v	volume

w	weight
WT	wild type

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Chapter 1: Introduction to plant microbe interactions

1.1 Plant growth efficiency as a result of biotic and abiotic conditions of soil

Soil is a key influence on plant growth. Plants take up most of their required nutrients via their roots, apart from CO_2, which is taken up by leaves which also capture light. Nutrient availability is a result not only of the nutrient levels in the soil, but also depends on soil structure, water regime, pH value, temperature, salt stress and mineral composition. This system is influenced by the presence of living organisms in the soil. The number of animals, fungi, protista, archaea and bacteria in soils is enormous and their influence on the soil and plant health is complicated, from the simple softening of soil in the case of moles and earthworms to nutrient cycling in case of simpler organisms. Soil invertebrates have been found at 3700 to 8200 individuals/m^2 in forest soil (Gongalsky, 2013). The number of fungi is even greater although abundance of filamentous fungi is hard to determine. However, the number of yeast, single-celled fungi, was estimated to be 60 – 115500 cfu/g of soil (Birkhofer *et al.*, 2012). Bacterial numbers present in the soil are estimated to be in around 10^6 to 10^9 cells/g of soil, depending on the sampling location (Torsvik *et al.*, 1990; Watt *et al.*, 2006). The number of different bacterial species has been estimated using metagenomic approaches. However, estimates obtained using that approach vary from less than 5000 bacterial species per gram of soil (Morales & Holben, 2009; Tringe *et al.*, 2005), through a few thousands (Roesch *et al.*, 2007; Torsvik *et al.*, 1990) up to 10^6 (Gans *et al.*, 2005), However, the last value is highly controversial due to concerns about the data analysis methods (Volkov *et al.*, 2006).

1.2 Microbial diversity in different environments

Microorganisms are believed to occupy all environments on Earth where living organisms have been found. Early research focusing on enrichment methods found that the same

groups of organisms can be found in very different environments. This led to the theory that "everything is everywhere, but the environment selects" known as the Beijerink (also known as Baas-Becking) hypothesis. This theory assumes that every environment harbours exactly the same bacterial species. However, which are most abundant is controlled by the environment. We know now that this theory is not always true as different habitats may be occupied by very distinctive microbial communities (De Wit & Bouvier, 2006). Research studies using culture-independent methods of assessing microbial structure have unravelled the community composition of human gut (The_Human_Microbiome_Project_Consortium, 2012), soils (Fierer & Jackson, 2006), hydrothermal vents (Xie *et al.*, 2011), acid mine drainage water (Denef *et al.*, 2010), air (Bowers *et al.*, 2011) and many more using greater and greater sequencing depth (Figure 1.2.1).

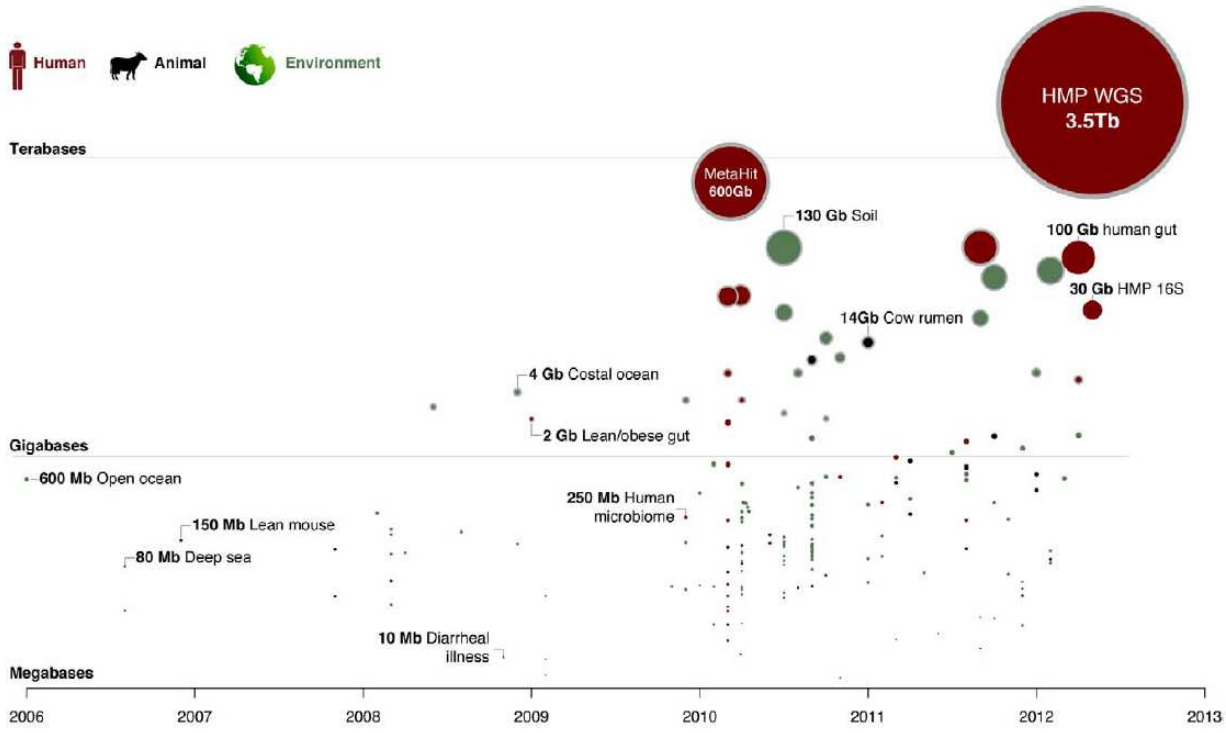


Figure 1.2.1 Progress in the sequencing efforts towards better understanding of the microbial communities of different environments. X axis – years, Y axis – sequencing depth. HMP – Human Microbiome Project. Figure taken from the review by Gevers et.al. (Gevers *et al.*, 2012)

The greatest effort has been on microbial communities of the human gut, skin, oral cavity, nasal and urogenital system, as it relates to our health and immunity. It was shown that the

bacterial community is very diverse between different niches and between different individuals (beta diversity) and within the samples (alpha diversity). Some niches like the oral cavity have a high alpha diversity, with very rich bacterial communities occupying different sites in the human mouth (saliva, tongue, gums) but relatively low beta diversity, where different individuals have a relatively similar community structure. The opposite is true for the gut habitat, which may reflect that different diets and/or human genome influences the community structure here. Some of the differences can be correlated with the ethnicity, pH value of the niches or even body mass index of the individuals. However, the correlation is not great, as it is probably disturbed by a diet, daily cycles, etc. (these variables were not tested) (The_Human_Microbiome_Project_Consortium, 2012). Moreover the microbiome changes over human life (Yatsunenko *et al.*, 2012) and it may be disturbed by antibiotic treatment (Peterfreund *et al.*, 2012) or even surgery (Graessler *et al.*, 2012) as shown by detailed sequencing studies of the human gut microbiome. Even within each niche the differences in the microbiome may be very significant as presented in the study focused on the detailed analysis of the skin microbiome (Figure 1.2.2) (Grice & Segre, 2011). This study shows that human skin is extremely diverse environment and the microbial structure is dependent on the ecology of the skin surface, which varies at different topographical location and also with individual factors (i.e. age, sex) and environmental factors (clothing, antibiotic usage, life style of the host).

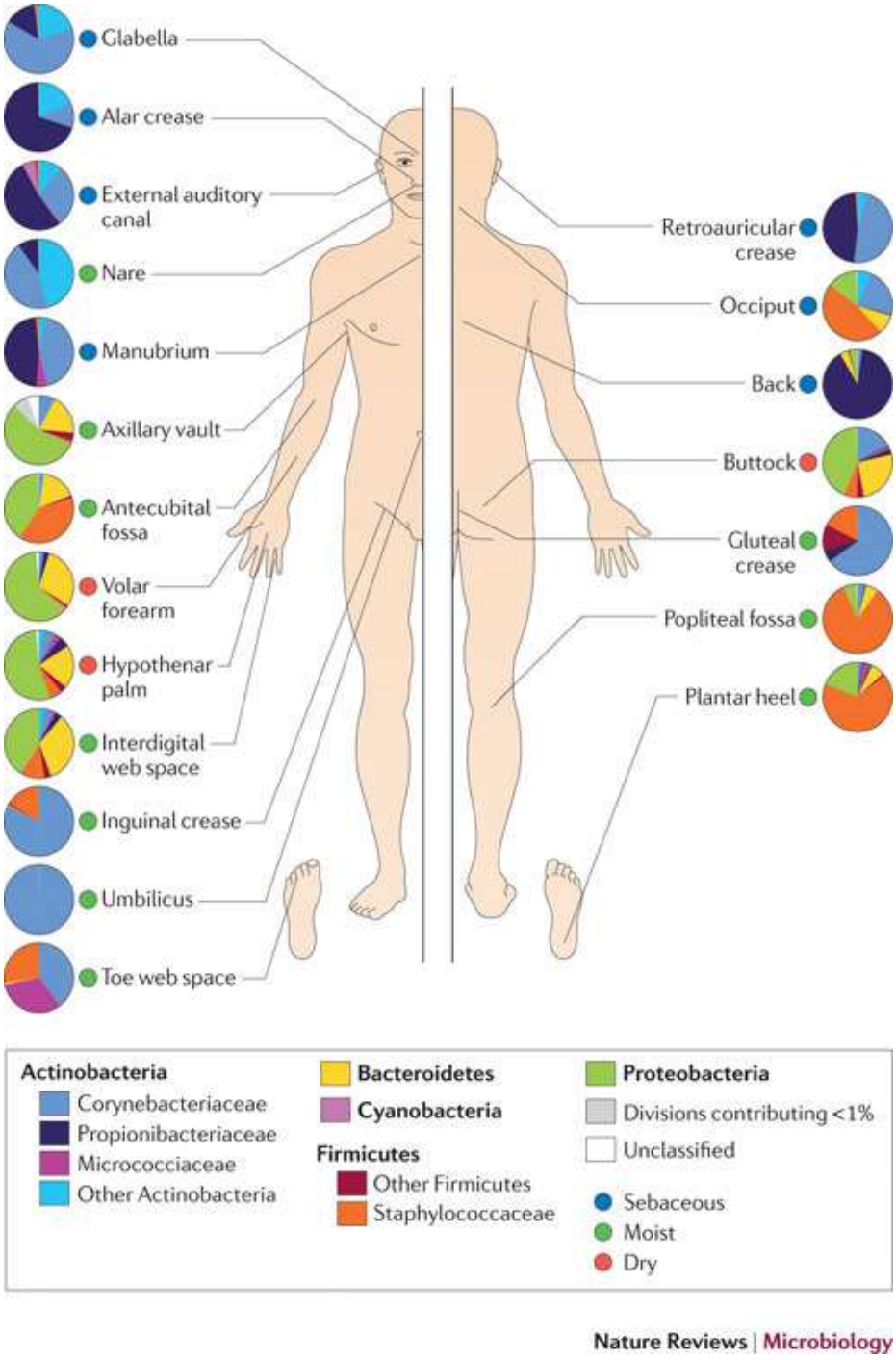


Figure 1.2.2 The skin microbiome structure analysed at various location on human body. Figure taken from the review by Grice and Segre (Grice & Segre, 2011)

1.3 Factors controlling the rhizosphere microbial community

As shown in section 1.2, the microbial composition of different environments is very diverse as the conditions controlling growth of microorganisms are very distinct. Not all bacterial groups are found in all these environments. Some environments, with very specific physical and chemical properties may harbour relatively simple microbial communities. Examples of such environments are hydrothermal vents at the bottom of the oceans (Xie *et al.*, 2011) and acid mine drainage bacterial biofilms (Denef *et al.*, 2010). However, we cannot exclude the possibility that other microbial groups were just not found because of insufficient sequencing depth. Connecting the microbial composition differences to the abiotic conditions is one of the greatest challenges in modern microbiology and geomicrobiology.

In soil communities factors like soil pH (Fierer & Jackson, 2006), land use (Osborne *et al.*, 2011), mineral composition (Carson *et al.*, 2009), soil water chemistry (Baneras *et al.*, 2012), C:N ratio (Nuccio *et al.*, 2013), soil carbon content (Xue *et al.*, 2013), soil water deficiency (Bouskill *et al.*, 2013), root exudates (Badri & Vivanco, 2009; Dennis *et al.*, 2010) and probably many more play a role in shaping the microbial world. Comparing soil communities sampled across the globe ranging from boreal to tropical forests led to the conclusion that soil pH is the dominant factor (Fierer & Jackson, 2006). Soil pH may have a direct influence on single cell organisms due to stress, or indirectly controlling other soil properties like nutrient availability, toxic compounds mobility, etc. (Delgado-Baquerizo *et al.*, 2013). Interestingly, the geographical distance between sampled environments and climate did not have a major influence on the community (Peiffer *et al.*, 2013). The other important factor controlling soil borne microorganisms is land use. Agriculture and planned forestry have a very significant impact on the environment (Knief *et al.*, 2005), leading to changes in the local, regional and global composition of water and air chemistry. The macro floral and faunal communities are highly influenced by changes in land use. An interesting question arises: does land use have an influence on soil microbial communities?

Recent studies focusing on this question revealed that indeed land use significantly alters the soil community. There are significant changes in the soil microbiome between woodland, pasture and re-vegetated land (Osborne *et al.*, 2011). However, a different study uncovered that the microbial community may reoccupy disturbed soil and its structure can

be relatively similar (in case of bacteria, but not fungi) to the non-disturbed one. This study showed that the bacterial community re-shaped to the initial structure in 18 years after soil was disturbed by bauxite mining (Banning *et al.*, 2011)

An interesting argument concerning microbial diversity in soil was posed by Carson (Carson *et al.*, 2009). Soil sampling strategies normally requires at least 1 g of soil for microbial DNA isolation (Delmont *et al.*, 2011). However, it is unclear how heterogeneous the sample is for microbial life. This question was indirectly addressed studying the influence of soil mineral composition on the bacterial community. Indeed, Carson showed that P_2O_5, K_2O, CaO, MgO, Al_2O_3, SiO_2, Fe_2O_3, Na_2O and SO_3 compounds significantly influence the community.

There are many reviews available concerning factors that influence the microbial community in the soil and especially in the rhizosphere (Badri & Vivanco, 2009; da Rocha *et al.*, 2009; Lugtenberg & Kamilova, 2009; Oldroyd *et al.*, 2005). A summary of the interactions in presented on figure 1.3.1

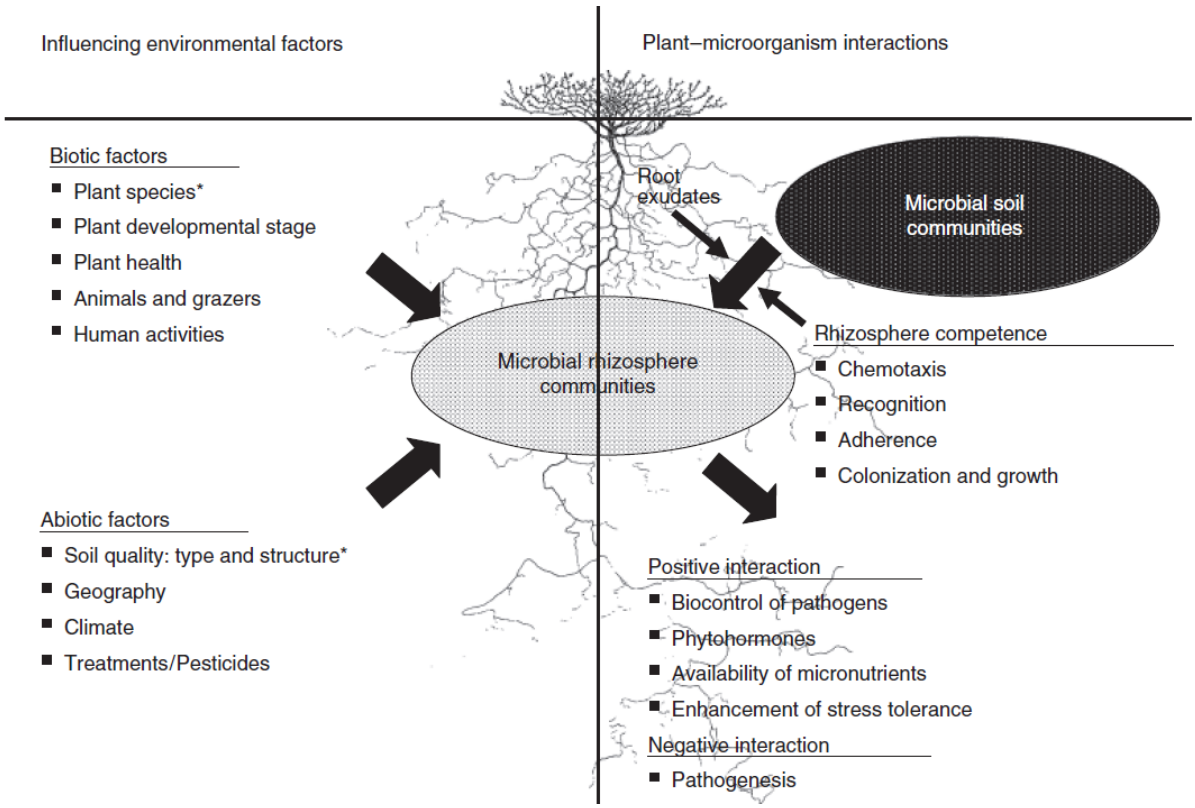


Figure 1.3.1 Factors influencing soil microbial communities. Picture taken from the review by Berg & Smalla (Berg & Smalla, 2009).

1.4 Root exudates manipulate the soil microbial community

Selected abiotic factors presented above play a significant role in shaping the community. However, the influence of biotic factors still remains to be uncovered. Even though, there are indications that soil predation (e.g. nematodes, protista, viruses) shifts the community (Yergeau *et al.*, 2010), it is assumed that plants play a decisive role. Plants influence the soil type over hundreds and thousands of years, changing its physical and chemical properties. Plant roots with the aid of mycorrhizal fungi penetrate into the ground and weather minerals – either by cracking or dissolution. Plant roots secrete a variety of compounds. Organic acids are the compounds that actively change the soils physical and chemical properties by dissolving minerals and indirectly shaping the microbial world.

However, plants may influence the microbial communities in a direct way. Between 5 and 21 % of photosynthetic assimilated carbon is secreted through roots into the rhizosphere (Lugtenberg & Kamilova, 2009; Marschner, 1995). Why such substantial amounts of carbon compounds are released to the environment in unclear. One possible explanation is the role of excreted carbon compounds in order to enhance abundance and activity of bacterial N-fixers. Except in agriculture where using N-fertilizers, nitrogen shortage constrain plant growth. Moreover it was found that some of the compounds are actively released by the plant using ABC transporter systems (Badri *et al.*, 2009). It was suggested that plants may actively farm rhizosphere microbial communities in order to enhance the population of Plant Growth Promoting Rhizobacteria (PGPR) and to obtain plant hormones, acquire defence against soil pathogens and increase nutrient uptake. Hiltner in 1904 observed that the rhizosphere, where the root secretion takes place is greatly enriched in microorganisms (Hartmann, 2008). It is very probable that the greater number of bacteria in the rhizosphere is driven by nutrient availability coming from released exudates.

Different plant races, cultivars or accessions of the plants excrete different compounds (Bakker *et al.*, 2013; Rovira, 1969). A well characterized *Arabidopsis* secretome consists of: carbohydrates, amino acids, organic acids, flavonols, lignins, coumarins, aurones, glucosinolates, anthocyanins, indole compounds, fatty acids, sterols, allomones, proteins

and enzymes (Narasimhan *et al.*, 2003) and the composition of the root exudates changes with the plant developmental stage, where exudation of sugars and sugar alcohols decreases relative to the release of phenolics and amino acids (Chaparro *et al.*, 2013). Other plants also secretes vitamins, nucleosides and inorganic ions and gaseous molecules (Dakora & Phillips, 2002). If the microbial communities can be modified by the release of carbon compounds by roots, then it can be assumed that different plants species harbour different microbial community in their rhizospheres (Costa *et al.*, 2006; Kuske *et al.*, 2002). The same is true for different accessions of the plants belonging to the same species (Micallef *et al.*, 2009; Zancarini *et al.*, 2012).

The problem with research on soil bacteria is the fact that most of them are uncharacterized and cannot be grown in the laboratory. It is estimated that 95-99 % of the soil bacteria have not yet been cultured (Nichols, 2007) although the reason why most bacteria are uncultured is unclear. Some species may require very specific compounds to grow, some may grow very slowly and are outcompeted by faster growing bacteria (da Rocha *et al.*, 2009). The term "great plate count anomaly" was coined to illustrate the difference between cell counts observed under the microscope compared to the number of colonies that can be grown on Petri plates (Staley & Konopka, 1985). Recent research showed that many soil bacteria may be cultured, however, special methods have to be used (explained later in the text). Bacteria that are r-strategists (fast growth when nutrients are available) normally outcompete K-strategists, which grow slowly. Their ability to colonize soils over short time is high, however over longer periods it is K-strategy that allows for occupation and maintaining its high abundance (Finlay, 2007). K-strategists due to their tolerance of harsh conditions of nutrient limitation normally found in soils are more likely to be found in any soils, while r-strategists are very abundant in the disturbed soils and/or with an input of nutrients. Even though r/K strategy concepts were coined to use in macro-ecology, some principals of this theory also applies in microbial world. However, as bacteria can rapidly switch they metabolism according to environmental changes this ecosystem is much more complicated.

Because of different bacterial growth rates cultivation methods had to be modified to favour slower growing bacteria. One approach is to highly dilute the inoculum and plate it on a minimal media and incubate for a long time. In this case the nutrient level is low, which prevents the R-strategists from colonizing the culture (George *et al.*, 2011). Another

approach is the addition of xylan to minimal media in order to select for bacteria able to degrade plant cell wall polysaccharides (Sait *et al.*, 2002). Research has also focused on better mimicking soil conditions using polycarbonate membranes and sterilized soil extract as a nutrient source (Ferrari *et al.*, 2005). However, in order to study the whole soil community in their natural habitat culture-independent methods have to be used.

1.5 DNA fingerprinting methods used for assessing microbial structure in the rhizosphere

There are several molecular methods used in the study of environmental microbial communities. Denaturing Gradient Gel Electrophoresis (DGGE), Terminal Restriction Length Fragment Polymorphism (T-RFLP) and Automated Ribosomal Intergenic Spacer Analysis (ARISA) are technique widely used in the microbial ecology. DGGE is a PCR dependent method where two or more samples of amplified 16S rRNA genes (DNA may come from an environmental sample) are run next to each other in an acrylamide gel with a denaturing agent (7 M urea + 40 % formamide). The more resistant fragments (higher G+C content) migrate through the gel for longer creating a band once they melt (Muyzer *et al.*, 1993). As this method is based on an amplified fragment of the 16S rRNA gene all the DNA fragments are of the same size. At the beginning of June 2013 the PubMed database contained 6179 publications, which included DGGE in its title or abstract. Another molecular biology method is T-RFLP that also relies on PCR amplified fragments of the 16S rRNA gene. One of the primers used in this method is fluorescently labelled. Other genes may also be used here, if the research focuses on a particular group of bacteria (for example *nifH* in the case of diazotrophs). The PCR product is digested with a restriction enzyme (e.g. *HhaI*) and run on a polyacrylamide gel and the fluorescent signal is read by sequencing equipment. As the DNA fragments differ in sequence their restriction sites are located at different places and the product of digestion contains DNA fragments of different sizes (Liu *et al.*, 1997). Comparing the banding patterns of samples gives an insight into the similarity of different communities. According to the PubMed database (June 2013) T-RFLP has been used 796 times (and TRFLP was used 133 times). The third method mentioned at the beginning of this subchapter is ARISA. This method allows for the best resolution in studying microbial structure and is most reproducible (Danovaro *et al.*, 2006; Fisher & Triplett, 1999). A detailed explanation of this

method is provided in the Material and Method section. In brief, ARISA is based on the PCR amplification of the 16S – 23S rRNA intergenic region, where one of the PCR primers is fluorescently labelled. DNA fragments vary in size between different bacterial species, so it is possible to obtain a whole "fingerprint" of the community based on the spacer between these two genes. PCR fragments are run on a polyacrylamide gel and their length is determined using detection of the fluorescent signal, usually with a DNA sequencing machine. ARISA was used only 134 times according to the PubMed website (and automated ribosomal intergenic spacer was used 182 times – as on June 2013). There are several reasons why it is less popular than DGGE or T-RFLP. First of all, this method is relatively new (late 1990-ies) and some laboratories perfected other methods before that time. The other reason is the need of using genotyping equipment (also needed for T-RFLP), which is relatively expensive and the reagents needed in order to run the method are also quite expensive (fluorescent standard ladder, Hi-di formamide). DGGE is the most widely used method probably because it is possible to characterize bacteria species of interest, by cutting off and sequencing gel bands that are responsible for differences between samples. There are some methods to annotate the ISR fragments obtained through ARISA method to the bacterial species level as the method developed by Grant and Ogilvie (Grant & Ogilvie, 2004). In the summary, this method involves sequencing a fragment of the 16S rRNA gene with the ISR fragment amplified during the same polymerase chain reaction. Once the ISR length is compared (excluding 16S rRNA fragment) to the previously obtained ISR fingerprint the 16S rRNA gene is annotated using available microbial databases.

1.6 Plant growth promotion

The tools described above have revolutionised our understanding of microbial community structure in the natural environment, but we have much less information on the function of uncultured microbes. In the present context, we have rather little information on whether they are beneficial (or deleterious) for plant growth.

Even small numbers of bacteria that are beneficial to the plant may play an enormous role in promoting plant growth. Bacteria that promote plant growth are called Plant-Growth-

Promoting Rhizobacteria (PGPR). These bacteria excrete metabolites that are directly or indirectly beneficial to the plant health (Lugtenberg & Kamilova, 2009).

One plant growth promoting effect of bacteria is the expression of ACC deaminase to regulate ethylene production by plants. Plants release ethylene in response to various stresses (temperature, pathogen and insect attacks, chemicals and water levels). However, elevated amounts of ethylene may be a cause of epinastic curvature, leaf abscission, rotting and inhibition of plant growth (Finlay, 2007). *Psudomonas putida,* which is the model organism for this research is able to balance the level of ethylene using ACC deaminase. This enzyme is responsible for reducing the concentration of 1-aminocyclopropane-1 carbozylate leading to lower ethylene production by plants (Glick *et al.*, 1998).

Bacteria also stimulate growth of plants by production of plant hormones. *Pseudomonas* and *Serratia* are model bacteria in studies concerning production of indoleacetic acid (auxin). Bacteria with increased production of these phytohormones can increase plant growth by 20 percent (Finlay, 2007). Other bacteria (e.g. *Azospirillium*) produce plant hormones like pyrrolquinoline quinone, gibberellins and cytokinins (Lugtenberg & Kamilova, 2009) that also stimulate plant growth.

There are also more indirect ways that bacteria promote plant growth. One of them is the ability of PGPRs to sequestrate iron using siderophores from the rhizosphere environment. PGPR chelate iron from the insoluble from of Fe^{3+}. Chelated iron is taken up by plants (although plants secrete siderophores, they have a lower affinity for binding iron than microbial siderophores). Moreover uptake of iron by plants thanks to beneficial bacteria reduces the iron availability in the rhizosphere. That in turn leads to slower growth of other microorganisms (especially fungi) that may be parasitic toward the plant (Bal *et al.*, 2013; Finlay, 2007; Shippers *et al.*, 1987; Traxler *et al.*, 2012).

1.6.1 Nodulation

Well studied plant-microbe interactions include biofertilization, where microorganisms provide plants with essential nutrients. Some bacteria belonging to the order Rhizobiales of

the Alpha-Proteobacteria subphylum form nodules on leguminous plant roots, inside which they convert atmospheric N_2 into plant-available NH_3 and in return feed on carbon compounds released by the plant (Oldroyd *et al.*, 2011). There are also other bacteria that can help plants to obtain ammonia, either as an endosymbiont (*Burkholderia* and *Cupriavidus* in case of leguminous plants of the genus *Mimosa* (Gyaneshwar *et al.*, 2011)), or as free living bacteria in the soil (*Azotobacteraceae* and Cyanobacteria). Nodulation appeared for the first time roughly 60 millions years ago (Figure 1.6.1) (Doyle, 2011).

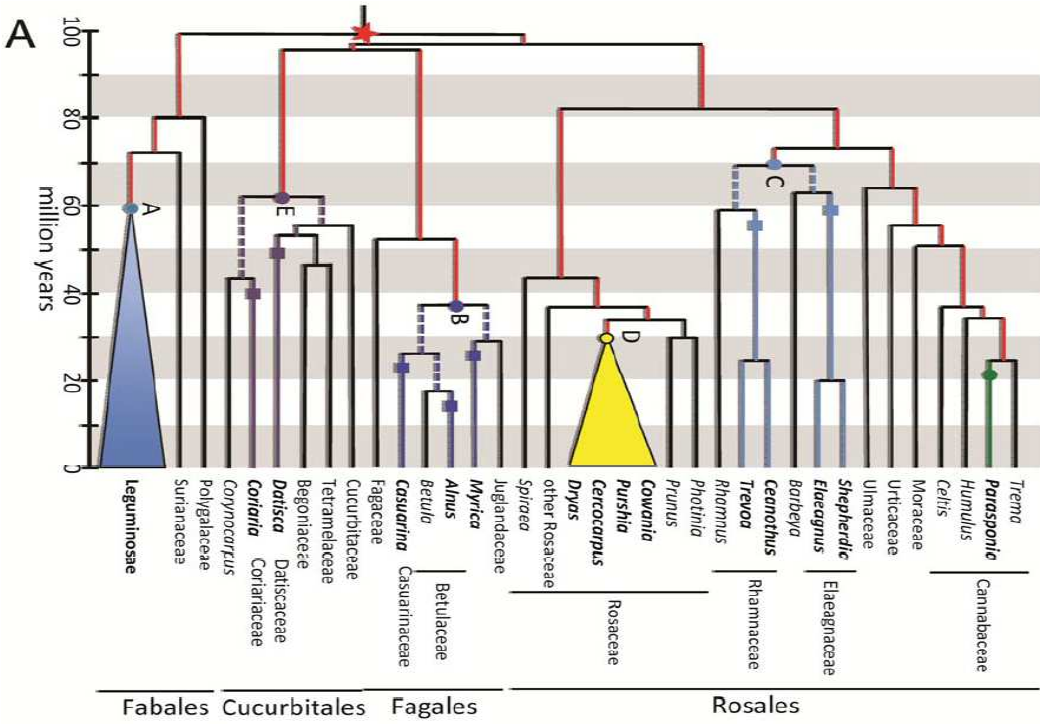


Figure 1.6.1 Chronogram showing the occurrence of nodulation across different plant groups. Nodulated groups are annotated with bold font and their lineages are colored. Red star indicates the time of predisposition for nodulation. Figure taken from (Young *et al.*, 2011).

There is also a distinct group of actinorhizal plants that can form association with N-fixing bacteria. The well known example of this relation is nodulation of Alder (*Alnus)* by symbiotic *Frankia* genus.

Nodulation will be described in more details for the legume – rhizobia symbiosis, as these associations are very well characterized and in a focus of part of this thesis. Plants secrete flavonoids in order to attract rhizobia from the surrounding soil. This secretion uses ATP

dependent system from the ABC transporter super family (Untiet *et al.*, 2013). A detailed metabolic profile of these compounds was performed for *Medicago truncatula*, indicating that they consist of chalcones, flavanones, isoflavones, and pterocarpans (Farag *et al.*, 2008). It was shown that flavonoids are responsible for attraction of *Rhizobium meliloti* towards the plant (Dharmatilake & Bauer, 1992) (Figure 1.6.2). Rhizobia are able to recognize these signals which include and production of lipochitooligosaccharide nodulation (Nod) factors (genes *nod*A, B, C and D code for the Nod factor) (Fisher & Long, 1992).

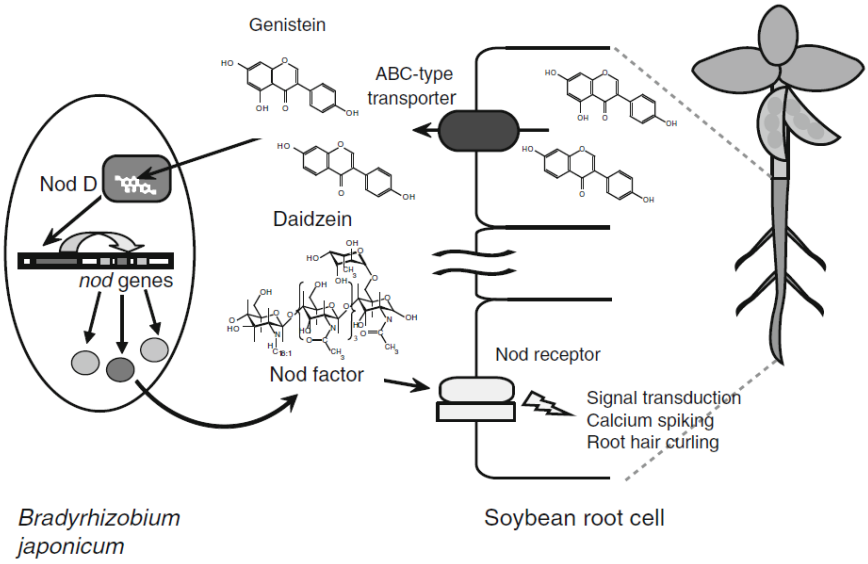


Figure 1.6.2 Cross-talk between the host plant (Soybean) and the symbiotic bacteria (Bradyrhizobium japonicum). Genistein is one of isoflavons in the flavonoids group. Figure taken from (Sugiyama, 2012).

Plant host/bacterial signalling is quite species specific as different plants produce different flavonoid compounds (Figure 1.6.3). Flavonoid compound binds to the bacterial NodD receptor and induce expression of *nod* genes, which in turn lead to production of the Nod factors (Oldroyd & Downie, 2004). *nod* A,B,C and D are ubiquitous among rhizobia, however species-specific *nod* genes modify the Nod factor structure, especially in its side chain

(Fisher & Long, 1992). Finally, Nod factor are secreted by ABC exporters such as the NodL and NodJ transporters (Downie, 1998; Spaink, 2000).

Plant	Compound
Alfalfa (*Medicago sativa*)	Luteolin
	7,4′-Dihydroxyflavone
	7,4′-Dihydroxyflavanone
	4,4′-Dihydroxy-2′-methoxychalcone
	Chrysoeriol
	Trigonelline
	Stachydrine
Barrel Medic (*Medicago truncatula*)	7,4′-Dihydroxyflavone
Black Locust (*Robinia pseudoacacia*)	7,4′-Dihydroxyflavone
	Apigenin
	Naringenin
	Chrysoeriol
	Isoliquiritigenin
Common bean (*Phaseolus vulgaris*)	Eriodictyol
	Naringenin
	Genistein 7-*O*-glycoside
	Delphinidin
	Petunidin
	Malvidin
	Myricetin
	Quercetin
	Kaempferol
Common vetch (*Vicia sativa*)	3,5,7,3′-Tetrahydroxy-4′-methoxyflavanone
	7,3′-Dihydroxy-4′-methoxyflavanone
Cowpea (*Vigna unguiculata*)	Daidzein
	Genistein
	Coumestrol
Miyakogusa (*Lotus japonicus*)	Unknown
Pea (*Pisum sativum*)	Apigenin
	Eriodictyol
Rostrate sesbania (*Sesbania rostrata*)	7,4′-Dihydroxyflavanone
Soybean (*Glycine max*)	Daidzein
	Genistein
	Coumestrol
White clover (*Trifolium repens*)	7,4′-Dihydroxyflavone
	Geraldone
White lupine (*Lupinus albus*)	Erythronic acid
	Tetronic acid

Figure 1.6.3 List of flavonoid compounds released by different leguminous plants. Table taken from the review by (Sugiyama, 2012).

Plants recognize Nod factor using Nod receptors (Figure 1.6.4) (in case of *Medicago* these are NFP and LYK3 that perceive the Nod factors - Figure 1.5.4) located in the plasma membrane. These turn induces influx of Ca^{2+} into root hairs. A protein Dmi1 is required for the plant to start Ca^{2+} spiking (oscillations in Ca^{2+} concentration) in the plant cytosol (Oldroyd & Downie, 2004). This initiates root hair curling in response to transcriptional changes controlled by NSP1, NSP2 and NIN transcription factors (Figure 1.6.4 and 1.6.5). At this point rhizobia are already attached to the surface of the root hairs using exopolysaccharides, glucomannan and cellulose fibrils (Downie, 2010; Gibson *et al.*, 2008). Root hairs curl and engulf the attached bacteria, which grow down the plant root through the infection thread. Root cells begin to divide forming nodule primordia. Plant cortical cells grow and divide to form the nodule structure. Inside the plant cell bacteria are released from the infection thread and surrounded by a plant derived symbiosome (also called peribacteroid) membrane (Sugiyama, 2012). The mature form of nitrogen-fixing bacteria are

known as bacteroids and act like plant organelles. In the process of developing into bacteroids there are dramatic changes in bacterial gene expression compared to the free-living state (Karunakaran *et al.*, 2009; Ramachandran *et al.*, 2011)

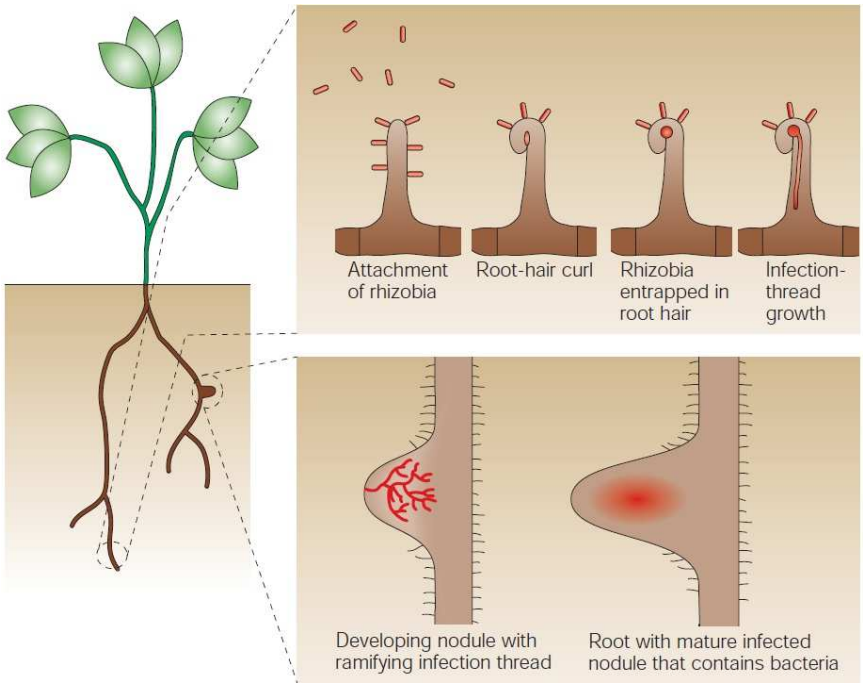


Figure 1.6.4 Steps of rhizobia infection of the leguminous plant. Figure taken from (Oldroyd & Downie, 2004)

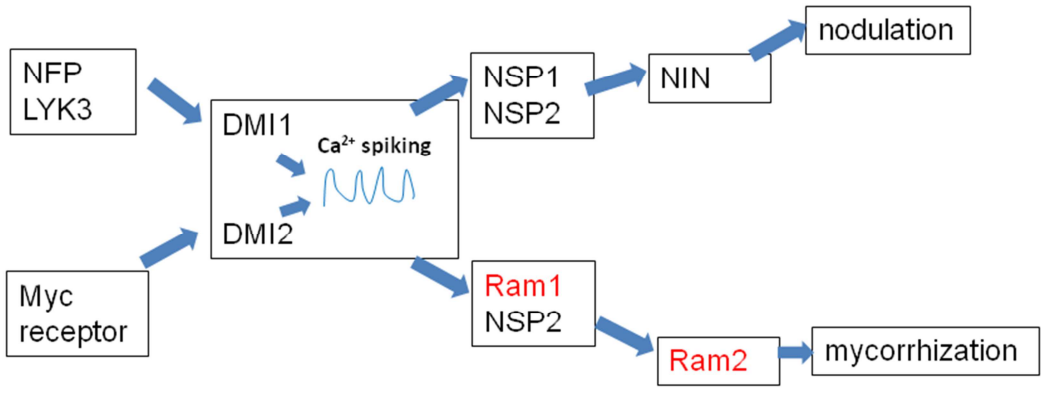


Figure 1.6.5 Nodulation and mycorrhization pathway in *Medicago truncatula*. NFP – Nod factor perception, LYK3 – ortholog of NFR-1 (serine/threonine receptor kinase gene), DMI1-2 ("does not make infection" genes), NSP1-2 (nodulation signalling pathways – GRAS domain transcription factors), NIN - GRAS domain transcription factors, RAM1 and RAM2 - (Required for Arbuscular Mycorrhization – RAM1 is a GRAS domain transcription factor of *ram2 gene,* RAM2 – encodes GPAT proteins (Gobbato *et al.*, 2012; Maillet *et al.*, 2011; Oldroyd *et al.*,

2005; Wang *et al.*, 2012). The genes of interest for the *Medicago* mycorrhiza influence on the rhizosphere microbiome are highlighted in red.

1.6.2 Mycorrhization

Another key nutrient that plants take up from the soil with the help from microorganisms is phosphorous. In acidic and alkaline soils, phosphorous is normally bound with aluminum and iron (forming strengite and varescite) or with calcium (forming apatite), respectively. Plants, however, are not able to take up bound phosphorous. They require phosphate either as $H_2PO_4^-$ or HPO_4^- (Schachtman *et al.*, 1998). Some bacteria due to release of organic acids are able to chelate the cations bound to the phosphate anion thus releasing this compound into the soil (Vassilev *et al.*, 2006). However, this process is insufficient for plants to obtain all the necessary phosphate (especially in the acidic soils). In case of plants forming relations with mycorrhizal fungi most of the available phosphorus is provided to the plants by these fungi present in the soil (Smith *et al.*, 2003). However, it is worth noting that bacteria are also able to solubilize phosphorous from the bound form and therefore provide this essential nutrient for the plants. Mycorrhiza evolved during the Early Devonian period (Pirozynski & Malloch, 1975). The early occurrence of this relationship is well documented in the fossil record, such as in the sedimentary rocks of the Rhynie Chert in Scotland (Krings *et al.*, 2007), in paleobotanical data (Berbee & Taylor, 2007) and by phylogenetic analysis based on DNA sequencing (James *et al.*, 2006a). Mycorrhizal fungi are difficult to study as they are unculturable, asexual, multinucleate and obligatory biotrophs. So far more than 5000 plant species were found to interact with arbuscular mycorrhizal (AM) fungi (Bouwmeester *et al.*, 2007). AM fungi thrive in soil as spores until they can detect the plant influence. They germinate and they release hyphae through the soil in search of a host plant root. At the contact with the plant, fungi form appressoria, through which they gain an access to the intercellular space of the root using LCO signals (sulphated and non-sulphated lipochitooligosaccharides) in response to plant strigolactone production (Akiyama *et al.*, 2005; Maillet *et al.*, 2011). The next step for the fungus is the formation of the branched hyphae (arbuscules) inside the cortical cells (Harrison, 2005). They are surrounded by the plant plasma membrane inside the plant cells. Plants supply the hyphae with the carbon source and in turn receive phosphate. Plant phosphate transporters responsible for the

phosphate uptake from the AM fungi have been characterized (Harrison *et al.*, 2002). The AM fungi – plant host cross-talk is similar to the nodulation process and some common pathway presented on the figure 1.5.5 is conserved between these two processes (Parniske, 2000). The pathway diverges after the calcium ions oscillation into nodulation (NSP1/NSP2) and mycorrhization (NSP2/RAM1) (Oldroyd *et al.*, 2005).

The role of mycorrhization in plant growth and in microbial community structure in soils has always been regarded as very important. Furthermore, *Medicago truncatula* is a model plants for nodulation and mycorrhization research that is extensively studied for the influence of these two nutritional associations.

Previous research has focused on assessing the microbial community structure influenced by *Medicago* plants with and without the ability to form mycorrhizal associations (Offre *et al.*, 2007). However, the plants used in the study (Offre *et al.*, 2007) were all impaired in the ability of nodulation, so the effects on the community changes observed may be associated with mycorrhization or nodulation. Moreover the experiments described by Offre *et al.* (2007) are not entirely convincing, due to low number of replicates (only 3 biological replicates per genotype and growth medium). In this study a detailed rhizosphere bacterial and microbial eukaryotic community structure changes due to the inability of *Medicago* plants to mycorrhizate will be discussed (RAM experiment - Chapter 5.2.7). In this study two plant mutants were assessed for their influence on the rhizosphere community:

The RAM1 (**R**equired for **A**rbuscular **M**ycorrhization) gene encodes a mycorrhizal specific GRAS-domain transcription factor (GRAS stands for **G**IBBERELLIC-ACID INSENSITIVE (GAI), **R**EPRESSOR of GAI (RGA) and **S**CARECROW (SCR)). RAM1 regulates the expression of another gene involved in mycorrhization called RAM2 (Gobbato *et al.*, 2012), which encodes a GPAT protein (**g**lycerol-3-**p**hosphate **a**cyl **t**ransferase), responsible for production of cutin monomers excreted as a wax layer on the roots. This layer is crucial for arbuscular fungi and oomycetes to attach to the roots (Wang *et al.*, 2012). Mutation in either of these genes causes the plant to be impaired in mycorrhization but does not interfere with nodulation (Figure 1.6.5).

1.6.3 Plant immunity triggered by microorganism and their influence on the plant growth

Other indirect effect of beneficial microorganisms on the plant health is stimulation of the natural plant defense against soil borne pathogens. Some PGPR elicit induced systemic resistance (ISR) in host plants which then gain resistance against particular pathogens by producing jasmonic acid and ethylene (Figure 1.6.6) (Pieterse *et al.*, 1996). Plant MAMP (Microbial Associated Microbial Patterns) is involved here. Plants can develop resistance against pathogens using salicylic acid pathway (this immunity is called acquired systemic resistance). Plant PAMP (Pathogen Associated Molecular Patter) system (recognition, gene activation and response) is involved in this process.

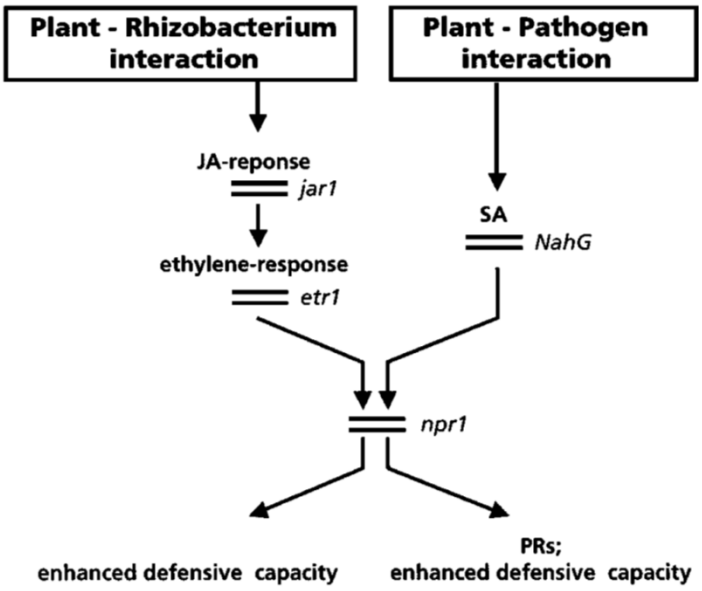


Figure 1.6.6 Signal pathways leading to induced systemic resistance (ISR) and systemic acquired resistance (SAR). Figure taken from (van Loon *et al.*, 1998)

Microorganisms in the rhizosphere are able to switch on the plant defense system. Plants detect microbe-associated molecular patterns (MAMPs) and prepare themselves against a real pathogen infection. Examples of the beneficial microbe associated induced resistance in plants are shown in the Table 1.6.1

Table1 | **Evidence for, and effectiveness of, induced resistance in plants by *Trichoderma* species**

Species and strain	Plant	Pathogens	Evidence or effects	Time after application	Efficacy
T. virens G-6, G-6-5 and G-11	Cotton	*Rhizoctonia solani*	Protection of plants; induction of fungitoxic terpenoid phytoalexins	4 days	78% reduction in disease; ability to induce phytoalexins required for maximum biocontrol activity
T. harzianum T-39	Bean	*Colletotrichum lindemuthianum*; *Botrytis cinerea*	Protection of leaves when T-39 was present only on roots	10 days	42% reduction in lesion area; number of spreading lesions reduced
T. harzianum T-39	Tomato, pepper, tobacco, lettuce, bean	*B. cinerea*	Protection of leaves when T-39 was present only on roots	7 days	25–100% reduction in grey-mould symptoms
T. asperellum T-203	Cucumber	*Pseudomonas syringae* pv. lachrymans	Protection of leaves when T-203 was present only on roots; production of antifungal compounds in leaves	5 days	Up to 80% reduction in disease on leaves; 100-fold reduction in level of pathogenic bacterial cells in leaves
T. harzianum T-22; *T. atroviride* P1	Bean	*B. cinerea* and *Xanthomonas campestris* pv. phaseoli	Protection of leaves when T-22 or P1 was present only on roots; production of antifungal compounds in leaves	7–10 days	69% reduction in grey-mould (*B. cinerea*) symptoms with T22; lower level of control with P1. 54% reduction in bacterial disease symptoms.
T. harzianum T-1 & T22; *T. virens* T3	Cucumber	Green-mottle mosaic virus	Protection of leaves when *Trichoderma* strains were present only on roots	7 days	Disease-induced reduction in growth eliminated
T. harzianum T-22	Tomato	*Alternaria solani*	Protection of leaves when T-22 was present only on roots	3 months	Up to 80% reduction in early blight symptoms from natural field infection
T. harzianum T-22	Maize	*Colletotrichum graminicola*	Protection of leaves when *Trichoderma* strains were present only on roots	14 days	44% reduction of lesion size on wounded leaves; no disease on non-wounded leaves
Trichoderma GT3-2	Cucumber	*C. orbiculare*, *P. syringae* pv. lachrymans	Protection of leaves when *Trichoderma* strains were present only on roots; induction of lignification and superoxide generation	1 day	59% and 52% protection from disease caused by *C. orbiculare* or *P. syringae*, respectively
T. harzianum	Pepper	*Phytophthora capsici*	Protection of stems when *Trichoderma* strains were present only on roots; enhanced production of the phytoalexin capsidiol	9 days	~40% reduction in lesion length
T. harzianum NF-9	Rice	*Magnaporthe grisea*; *Xanthomonas oryzae* pv. oryzae	Protection of leaves when NF-9 was present only on roots	14 days	34–50% reduction in disease

Table 1.6.1 Examples of ISR in different plant species triggered by Trichoderma species. Table taken from (Harman *et al.*, 2004)

Plants defend themselves against insects, herbivores and microorganisms using sophisticated response systems. Recently characterized immune system components reacting against bacterial signals – Microbe-Associated Molecular Patterns (MAMPs) and Pathogen-Associated Molecular Patterns (PAMPs) are illustrated on figure 1.6.7. PAMP signals - *elf18* (bacterial peptide derived from bacterial elongation factor Tu) and *flg22* (bacterial peptide derived from flagellin) (Zipfel *et al.*, 2006) are recognised by plant EFR (EF-TT RECEPTOR KINASE) and FLS2 (FLAGELLIN SENSING2) receptors, respectively. Plants can defend themselves against fungi as well, recognizing chitin oligosaccharides released from the invading fungal hyphae (de Jonge *et al.*, 2010). Activated receptors trigger plant immune systems using SERK proteins (SOMATIC-EMBRYOGENESIS RECEPTOR-LIKE KINASE). The details of the SERK pathway reactions are not fully understood. It is known, that there are five different proteins belonging to the SERK family in *Arabidopsis* (SERK 1-5). SERK 1, SERK 4, SERK 2 and SERK 3 bind to the FLS2 receptor and SERK 2 and SERK 3 can bind to the EFR receptor (Roux *et al.*, 2011). SERK proteins initiate a plant immune response of synthesis of reactive oxygen species, mitogen-activated proteins, upregulation of defence genes and callose deposition on the plant surface. The biggest effect on the plant immunity occurs in SERK3 mutants, which can be even more severe when accompanied by mutation of SERK 4. Mutations in SERK 1, SERK 2 or SERK 4 alone do not produce a phenotype, apart from SERK 4 which changes seed germination rates. SERK 5 was not mutated as this protein does not bind to any receptors (Roux *et al.*, 2011). Experiment focusing on deciphering the impact of mutations of the PAMP pathway is shown in the chapter 5 (PAMP experiment).

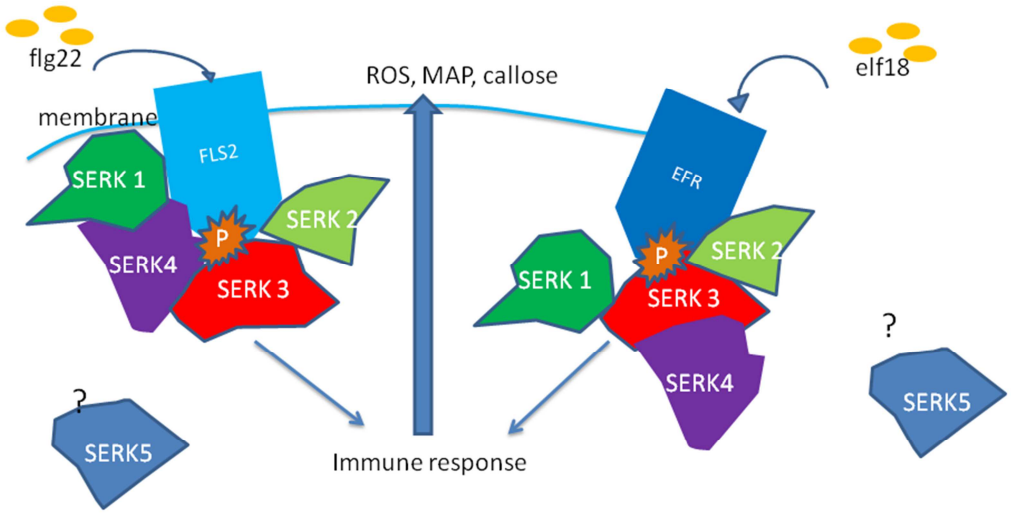


Figure 1.6.7 Plant immune system triggered by SERK-dependent PAMP signals based on (Roux *et al.*, 2011) ROS – reactive oxygen species, MAP – mitogen-activated proteins

The PAMP system is crucial for the plant resistance against pathogen in soils. In this PhD thesis the influence on the rhizosphere microbiome of three different plant mutants in the PAMP pathways are compared (Chapter 5.2.1).

Some plants are able to defend themselves against pathogens by releasing antimicrobial compounds (phytoalexins) via their roots into the rhizosphere. A well studied example of this process is glucosinolate excretion by plants belonging to the family Brassicaceae. Glucosinolates are a class of compounds derived from amino acids and glucose. Normally they are produced in response to herbivore attack. However, glucosinolates also have an antimicrobial effect and are released by plants in order to suppress bacterial attacks on roots (Ratzka *et al.*, 2002). *Arabidopsis*, belonging to the Brassicaceae family, is a model plant for studying the effect of glucosinolates on herbivores and microorganisms. Two transcription factor genes called *myb28* and *myb29* have been identified as controlling the aliphatic glucosinolates production (Hirai *et al.*, 2007; Sonderby *et al.*, 2007). *Arabidopsis* plants with impaired aliphatic glucosinolate production are more susceptible for non-host bacteria invasions (Fan *et al.*, 2011). In this PhD thesis, results of experiments focusing on understanding the influence of the glucosinolates on the rhizosphere microbiome are shown. Experiments were conducted comparing the rhizosphere community of wild type *Arabidopsis* with mutants impaired in glucosinolate production (MYB experiment).

Another possible plant defence system against pathogens is the release of methyl halides through their leaves and roots. Methyl iodide, methyl chloride and methyl bromide are a group of toxic compounds made naturally by members of the Brassicaceae family. Their biological purpose is still unknown, although it was proposed they may play a role as insect and microbe repellents (Rhew *et al.*, 2003). Indeed, methyl bromide was used as a soil fumigation agent in order to suppress soil pathogens, nematodes and fungi. This practice was abandoned due to the increasing evidence of the role of methyl halides in reducing the stratospheric ozone layer (http://ozone.unep.org/new_site/en/montreal_protocol.php) The genes responsible for methyl halides production were found to be strongly expressed in the roots of *A*. *thaliana* plants (Lars Ostergaard and Evelyn Koerner – personal communication). In this PhD thesis results of experiments focused on understanding the influence of the methyl halides on the rhizosphere microbiome are shown. Experiments were conducted comparing the rhizosphere community of *Arabidopsis* wild type against mutated plants impaired in production as well as overexpressing methyl halides production (HOL experiment).

Suppressive soils are one of the best examples of a beneficial microbe-plant association in soil. At the beginning of the last century it was observed that the yield of wheat crops drastically decreased if plants were cultivated for a few years on the same field. The disease was called take-all, due to the great losses in the crop production. However, plants grew better in the following years and produced roughly 80 % of the initial yield. In countries like Australia and central USA, where wheat was the main crop, the reduction in yield was a major issue. Farmers started to use the crop rotation or were willing to wait a few years in order to obtain what we call now “suppressive soils” (Figure 1.6.8) (Asher, 1981). Suppressive soils are soils in which pathogens are absent or highly suppressed, where they cause little or no damage to plants (Baker KC, 1974). Fungal pathogens that cause most economical damage to the crops have been identified and characterized: *Gaeumannomyces graminis* var. *tritici, Fusarium oxysporum*, *Aphanomyces euteiches*, *Heterodera avenae*, *H. schachtii*, *Meloidogyne* spp., *Criconemella xenoplax*, *Thielaviopsis basicola*, *Phytophthora cinnamomi*), *Phytophthora infestans*, *Pythium splendens*, *Pythium ultimum*, *Rhizoctonia solani*, *Streptomyces scabies*, *Plasmodiophora brassicae*, and *Ralstonia solanacearum*

(Weller *et al.*, 2002). It is not known if suppressive soil is resistance to just one or multiple fungal pathogens.

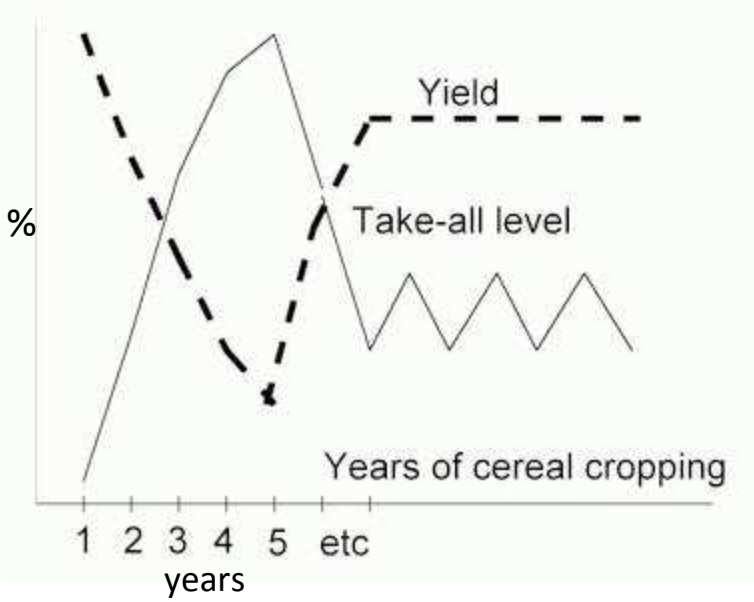


Figure 1.6.8 Take-all disease influence on the wheat crop production.

2,4-diacetylphloroglucinol produced by *Pseudomonas fluorescence spp.* was identified as having antifungal against the take-all pathogen *Gaeumannomyces graminis* var. *tritici* (Weller *et al.*, 2007). It was shown that the population of *Pseudomonas* increases in the rhizosphere over a period of several years and once it reaches 10^5 cfu per gram of root it is able to suppress the fungal pathogen.

More recently it has been shown that organisms other than *Pseudomonas spp*. contribute to take-all disease suppression. Whole arrays of bacteria are selected in suppressive soils. Many of the selected bacteria belong to Burkholderiaceae, Xanthomonadales and Actinobacteria (Mendes *et al.*, 2011).

These findings suggest that plants recruit beneficial microorganisms in the rhizosphere in order to better take up nutrients from the soil or defend themselves against pathogens. As shown above (Figure 1.6.8) soil needs a few years in order to gain pathogen-suppressive properties. To mimic these interactions between microbes and plants experiments were conducted over multiple generations of plants (Swenson *et al.*, 2000) and concluded that soil microorganisms may play a crucial role in plant growth. In this experiment *Arabidopsis* plants were grown over 15 generation. Each generation of plants was started from seeds

coming from an external source. Initially all the plants were grown in the same well mixed and autoclaved soil (before the planting autoclaved soil was mixed with a small amount of fresh soil) in separate containers. After the first generation the plants biomass was measured and the soil in which three plants with the highest biomass (high treatment) and with the lowest biomass (low treatment) were grown was used as a microbial inoculum for the “offspring” generations. Offspring generation were prepared from the autoclaved soil to which a small amount of soil (as a microbial inoculum) from the previous generation was added (either soil coming from the high or low treatment). Over 13 times out of 15 generations the high treatment plants had a higher biomass than the low treatment ones (Figure 1.6.9).

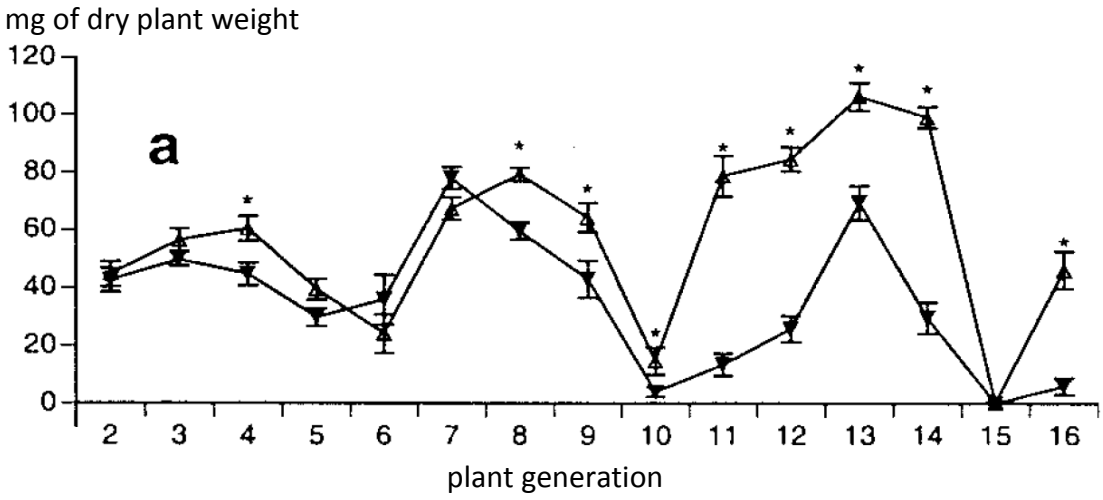


Figure 1.6.9 Two lines of *Arabidopsis thaliana* plants grown over multiple generations. Upward-pointing triangles represent "high biomass" line and downward-pointing triangles "low biomass" line of plants. Asterisks points out the generations with statistically different biomass between the two treatments ($p<0.05$). The 15th generation was overtaken by a fungus pathogen. Figure taken from (Swenson *et al.*, 2000).

Plants were grown using the same physical conditions and the only variable was the structure of offspring microbial inoculum from the previous generation. This experiment indicates that the rhizosphere community structure influences plant growth. However, the exact mechanism of this effect was not tested. In chapter 2 and 3 rhizosphere community profiles (including the rhizosphere of *Arabidopsis thaliana*) are tested over three generations of model and crop plants. Our study shows how plant selection alters the microbial community and how this microbial diversity can collapse due to either opportunistic or pathogenic invasions.

1.7 Phyllosphere microbial community

Plant leaves and stems are a microbial niche for more than 10^{26} bacterial cells worldwide and these bacteria are able to feed on compounds released by the plant (Delmotte *et al.*, 2009). The main focus of the research concerning phyllosphere has been always laid on plant pathogens, for example cereal rust and powdery mildew caused by fungi, leaf blight and leaf spot, caused by bacteria. *Pseudomonas syringae* can indirectly damage plants

during freezing condition by secretion of Ina proteins that enhance the water to form ice crystals on the leaves surface causing wounds, through which these bacteria gain entrance to plant cells (Clarke *et al.*, 2010; Lindow *et al.*, 1982).

However, most of the phyllosphere bacteria are actually non-pathogenic. The phyllosphere environment is enriched with methanol, a side product of cell-wall metabolism and many of the bacteria living there use this C1 compound as a carbon and energy source (Galbally & Kirstine, 2002). Nitrogen is a limiting nutrient in this environment, so bacteria that are able to fix atmospheric nitrogen have an advantage (Delmotte *et al.*, 2009). Moreover microorganisms are exposed to various fluctuating stresses like temperature and water availability, UV radiation and the presence of reactive oxygen species (Lindow & Brandl, 2003). Metaproteomic study confirmed that many of the proteins involved in stress resistance are being actively produced by the microorganisms in the phyllosphere (Delmotte *et al.*, 2009).

The diversity of the phyllosphere microbial community is relatively poor compared to the rhizosphere. 16S rRNA gene cloning and sequencing of the phyllosphere microbiome showed that this environment is around four times less diverse than a farm soil (Figure 1.6.1).

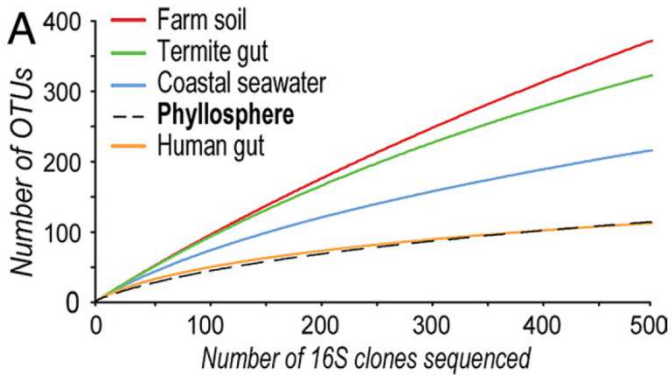


Figure 1.7.1 Rarefaction curves comparing the microbiome diversity of different environments. Figure taken from (Delmotte *et al.*, 2009)

1.8 Aims of this project

The literature is very rich in research concerning plant influences on the rhizosphere microbiome. Since the early 1990s researchers have compared the rhizosphere of different plant species using fingerprinting methods and is some cases sequencing of the clone libraries. Examples include flax and tomato (Lemanceau *et al.*, 1995), strawberry and oilseed rape (Costa *et al.*, 2006), chickpea, rape and Sudan grass (Marschner, 2001). In all cases the fingerprint of the bacterial rhizosphere community was different for different plants species. Recently, due to advances in the sequencing methods, it is possible to compare rhizosphere communities in more detail, identifying the main microbial players behind the observed differences. However, up to date only one research publication was focused on detailed comparison of the rhizosphere bacterial microbiome of two or more plant species: *Deschampsia antarctica* (Poaceae) and *Colobanthus quitensis* (Caryophyllaceae) (Teixeira *et al.*, 2010) . There are an emerging number of publications focused on the impact of different accessions or mutants of the same plant species on the rhizosphere microbiome. Different accessions of *Arabidopsis thaliana* select for a different community structure (Micallef *et al.*, 2009) and different tissue of this plant have a different bacterial community. The phyllosphere is preferably colonized by (endo- or epiphyte) Gammaproteobacteria and Alphaproteobacteria, while plant roots are dominated by (either endo or epi-phyte) Actinobacteria and Bacteroidetes (Bodenhausen *et al.*, 2013). However, other researchers that focused on the below ground community comparing root endophytic and rhizosphere bacteria claim that the former is enriched with Proteobacteria, Actinobacteria and Bacteroidetes, while the later with Acidobacteria and Planctomycetes (Bulgarelli *et al.*, 2012). This published study also indicates that the *Arabidopsis* rhizosphere is actually not much different from the bulk soil (Figure 1.8.1). Another recent publication confirmed these relationships (Lundberg *et al.*, 2012).

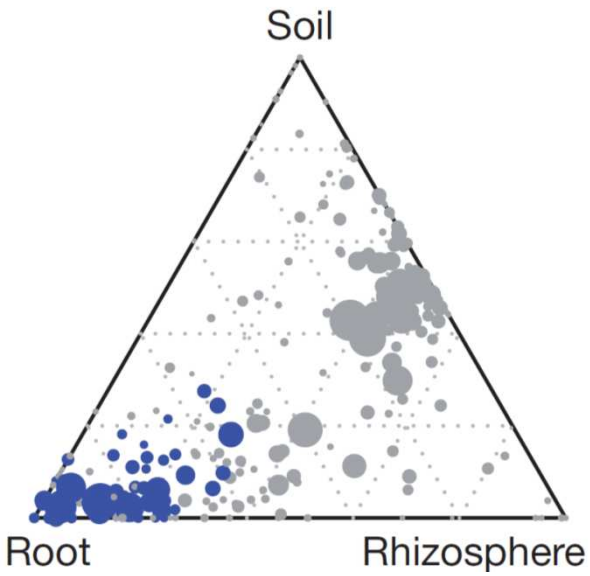


Figure 1.8.1 The differences in the bacterial community structure between bulk soil, rhizosphere and root compartment. Each dot is one bacterial OTU (explained in Chapter 2.8.1). Bacterial OTU significantly enriched in the root tissues are annotated as dark blue dots (all other OTUs are represented as grey dots). Figure taken from (Bulgarelli *et al.*, 2012).

The observation that the rhizosphere and bulk soil communities have a similar structure contradicts previous studies indicating a large shift in the community between bulk soil and rhizosphere of this model plant (Micallef *et al.*, 2009). The different conclusions may come from the fact that different methods were used in order to characterize the bacterial communities – Micallef, et.al. used T-RFLP, RISA (a semi-automated version of ARISA) and low resolution clone library construction, while the research of Bulgarelli, et.al. was based on high throughput 454 sequencing. (However the choice of primers used, favours one phylum over others. Chloroflexi in particular made up roughly 80% of the sequences in the rhizosphere and bulk soil, while CARD-FISH analysis of the samples did not confirm this). This difference between two publications reflects the advance in culture-independent methods of community structure analysis that took place in the period of just a few years. In this PhD thesis great focus is placed on the comparison between bulk soil and rhizosphere community structure.

Even though, available data indicates that plant species have an influence on the microbial community, none of them really showed it using multireplicate experiments and only a few

focused on comparison between different plant species rhizospheres: arctic plants (Teixeira *et al.*, 2010) and wheat, maize, rape, barrel medic (Haichar *et al.*, 2008). Moreover, the literature is lacking research focused on the development of community structure over time using the newest culture-independent methods.

The main focus of this PhD project was to test plant species and generation influence on the bacterial and fungal community using two molecular, culture-independent methods (ARISA and 454 pyrosequencing) and two different plant growth media. Experiments were performed using multireplicate systems, normally 20-24 biological replicates. Such an approach allows for a statistical analysis of the community structure. More than 10 years ago, it was shown that the microbial community can be manipulated by the plant, and the community influences plant growth (Swenson *et al.*, 2000). That research gave a rise to the hypothesis that plants may "farm" their rhizosphere community for their advantage and this claim was based solely on the plant physiological traits. In this PhD project we expand this issue on the question what is happening to the microbial community over plant generations. (chapter 3 and chapter 4)

Another aim of this PhD project was to focus on PGPR that are present in soil. Much research has been focused on isolation of numerous bacteria and screening them for their plant growth promoting properties (Govindasamy *et al.*, 2008). However, their real abundance in the soil is unknown. Moreover there is no evidence that isolated bacteria are plant dependent or actually plant species specific. To address these concerns a three generation experiment of model plants grown under poor soil conditions was developed. The same plant species was grown for three successive generations. Plants were grown using a smaller and smaller amount of soil (diluted with sand). Plants were sub-cultured using the rhizosphere community over generations. We focused on the answering the following question: what kind of processes of microbial selection take place in the soil? These questions could only be answered using novel molecular methods in assessing microbial structure. This aim of the experiment was to enrich the rhizosphere soil samples with organisms that are plant dependant, are abundant in their environment and may be PGPRs. Such bacteria may be used one day commercially as a natural biocontrol or growth promoting agent.

The third main aim of the project (chapter 5) was an interest in understanding the mechanisms of plant selection of the rhizosphere microbiota. Different plant species have a very different array of root compounds released into soil that may shape microbial communities. To address this problem the effect of specific mutants compared to wild type plants on the rhizosphere community was tested. Knowing that a particular plant is impaired in production of a single compound (or actually a group of compounds, that are similar to each other and their effect on the microorganisms is similar) allowed for a detailed analysis of the influence of this compound on the rhizosphere community. The effect of aliphatic glucosinolates, methyl halides and the plant immune system response in *Arabidopsis* as well as the mycorrhization ability in *Medicago* was tested on the bacterial and fungal community. There is some research done using plant mutants in order to characterize the influence of a single plant metabolite on the microbial community. However, the novelty of the research presented here is the statistical power behind each experiment and the screening of the community using two powerful molecular methods (ARISA and 454 pyrosequencing).

Chapter 2: Materials and methods

2.1 Introduction

Rhizosphere communities were analyzed and compared between different growth conditions and between different plant species as hosts. The community came from a natural soil environment at Bawburgh farm and was altered using selected factors during plant growth.

Studying rhizosphere microbial communities requires the use of isolation-independent molecular methods. In this chapter two methods are described: ARISA fingerprinting (chapter 2.7) and high-throughput sequencing (chapter 2.8) and data analysis is described in chapter 2.9. All the steps proceeding molecular methods are described in chapters 2.1–2.6, including plant growth conditions and DNA isolation. Research on the isolated strains is explained in chapters 2.10 and 2.11.

2.2 Soil collection and preparation

Soil was collected from a naturally grassed and unfertilized part of the Church Farm in Bawburgh, Norfolk (52°62’76’’N, 1°17’85’’E). Covering vegetation was stripped off and soil was collected from a depth of 10-30 cm. Soil was air-dried for 1-2 days and sieved through a 1 cm mesh in order to remove stones and roots. Soil was extensively mixed using a cement mixer. Mixing is essential in order to ensure homogeneity of the initial microbial community structure. A soil sample was sent for chemical analyses (The Macaulay Land Use Research Institute, Edinburgh). Analysis showed that Bawburgh soil is poor in nutrients (NO_3^- 3.49 mg/kg, P^{-3} 120.5 mg/kg, K^+ 168.2 mg/kg, Mg^{2+} 33.55 mg/kg) compared to agricultural soil. The pH (7.5) is neutral with a typical amount of organic matter (2.92%) for grassland soil.

2.3 Plant seeds surface sterilization and germination

Seeds were surface-sterilized using ethanol and bleach (Table 2.3.1) in order to eliminate contaminating microbial communities that were present on the seeds. Seeds were germinated on Murashige and Skoog (MS) medium containing essential macro- and micro-elements for plant growth. The medium was supplemented with sucrose to enhance plant growth as well as to test if any bacteria or fungi were not successfully killed during sterilization. MS plates that were contaminated with microorganisms were thrown away.

Plant species	Ethanol concentration	Time of exposure to ethanol	Bleach (sodium hypochloride) concentration	Time of exposure to bleach
Arabidopsis	70 % with triton X-100 (0.5 %) 95 %	3 min 1 min		
Medicago (seeds scarified)	70 %	1min	4 %	3 min
Brachypodium	70 %	30 sec	10 %	6 min
Turnip	70 % with triton X-100 (0.5 %) 95 %	3 min 1 min		
Pea	70 %	30 sec	2 %	5 min
Wheat	70 %	30 sec	5 %	3 min

Table 2.3.1 Seed surface sterilization methods

2.4 Testing the effect of nitrogen levels on plant growth and *Medicago* nodulation

In order to allow plants to grow in low level nutrient conditions, in a farm soil / sand mix (Silver sand, washed, lime free, horticultural grade, manufactured by J.Arthur Bower's, Sinclair, Lincoln), 5 ml of nutrient solution (Table 2.4.1) was added once a week and 10 mg of KNO_3 was added once to each pot (50 ml pots almost fully filled with the soil/sand mixture) one week after planting. This amount of nitrogen was chosen based on a *M.truncatula* nodulation assay.

M.truncatula was grown in sand and a different amount of nitrogen (KNO_3) was added. Some treatments were inoculated with 10^5 cfu of *S. medicae*, a typical *M.truncatula* symbiont. After 4 weeks of plant growth the number of nodules on the roots was scored. *M.truncatula* was chosen as one of the model plants in the rhizosphere microbial structure experiments and it was important to allow plants to be nodulated. Nodulation only occurs in relatively low soil nitrogen level and in the presence of *Rhizobium*. When plants are supplemented with N-fertilizer nodulation ceases (Omrane & Chiurazzi, 2009). In two conditions no nitrogen was added and plants had to form symbiosis with *Rhizobium* in order to obtain this macro element. Plants that were not inoculated with *Sinorhizobium medicae* WSM 419 could form only a few nodules (Figure 2.4.1). A low number of nodules is caused by insufficient *Sinorhizobium* number in the soil mixture (10 % soil/90 % silver sand was used in this experiment). Inoculated plants could be nodulated under different nitrogen levels added to the soil. There was a decline in the number of nodules at the level of 50 mg of KNO_3. Eventually the level of 10 mg of KNO_3 (10 μM) was chosen for all the further experiments.

Chemical	Stock concentration	Stock volumes	Volume in 50L	Final concentration
$CaCl_2.2H_20$	1M	73.51g/500ml	50ml	1mM
KCl	100mM	3.73g/500ml	50ml	100µM
$MgSO_4.7H_2O$	800mM	98.59g/500ml	50ml	800µM
Fe EDTA	10mM	1.84g/500ml	50ml	10µM
H_3BO_3	350mM	2.16g/100ml	5ml	35µM
$MnCl_2.4H_2O$	90mM	1.78g/100ml	5ml	9µM
$ZnCl_2$	8mM	0.109g/100ml	5ml	0.8µM
$Na_2MoO_4.2H_2O$	5mM	0.121g/100ml	5ml	0.5µM
$CuSO_4.5H_2O$	3mM	0.075g/100ml	5ml	0.3µM
KH_2PO_4		25g/1L	1L	
Na_2HPO_4		28.4g/1L	1L	

Table 2.4.1 Ingredients of N-free nutrient solution (Poole *et al.*, 1994)

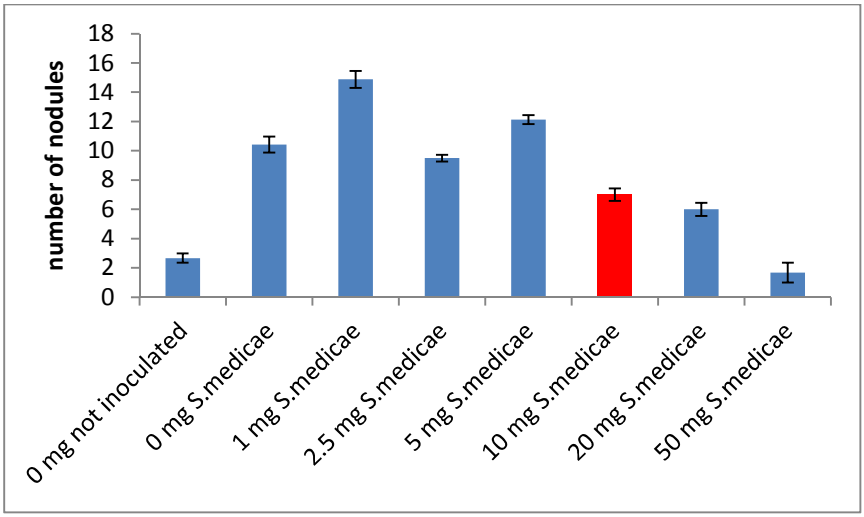


Figure 2.4.1 *Medicago* growth assay using different nitrogen level fertilizer (n=8 for each condition). Bawburgh soil (10 %) was mixed with autoclaved silver sand (90 %). Red bar represents chosen nitrogen level. Plants were grown in 50 ml non-transparent beakers fully filled with the soil/sand mixture.

2.5 Plant growth conditions

All plants were kept in a closed growth room at 12 h/12 h light/dark conditions at a temperature of 23°C. This was *Arabidopsis* specialized growth room with stable light/dark cycles and temperature. A summary of different growth conditions is presented in the Table 2.5.1. Different experiment (different plant species, soil richness, DNA or RNA isolation) required different growth conditions. At the beginning of the result chapters there is a short explanation of the condition used in the study. All plants were grown in separate, closed containers (either 50 ml or 100 ml beakers, Falcon tubes or boiling tubes). Model plants (*Arabidopsis thaliana* Col-0*, Medicago truncatula* A-17 Jemalong and *Brachypodium distachyon* Bd 21) were grown in 50 ml beakers (Figure 2.5.1) and crop plants (*Brassica rapa* R-O-18, *Pisum sativum* Avola and *Triticum aestivum* Paragon) were grown in 100 ml beakers. Only wheat and *Brachypodium* in the Capacity and Capability Challenge program (CCC) project (explained in chapter 6) were grown in Falcon tubes and boiling tubes, respectively. Closed and separate pots as a growth medium are needed to stop microorganisms from entering or escaping the plant rhizosphere. Moreover, closed conditions allow all plants

exudates to be retained in the pots. The disadvantage of such a system is its artificial character and possibility of water-logging. For all critical experiments the location of plants was randomized using an online number randomizer (http://www.randomizer.org/). The example of such randomization is presented on picture 2.5.1. Sand and compost was sterilized using “glassware run” with the temperature of 134°C for 20 min at approximately 2.1-2.2 bars.

The sterilization effect was tested by plating out sand and compost (separately). Sand and compost (1g) was diluted with 5 ml of water, mixed for 10 min using Vortex and 100 µl was spread on TY plates. No bacteria or fungi growth was observed (less then 50 cfu in 1g of soil). However, some fungal spores could survive as the compost left in the autoclave box for approximately 1 week was normally covered with a green lawn of fungus. For some reason this fungus was unable to grow on TY media, but was able to colonize Levington compost. Probably spores of this fungus were able to survive autoclaving.

Figure 2.5.1 Plant growth conditions. Different plant species were grown next to each other in separate pots. The location of trays was changed at least once a week.

Exp-eriments	Plant species	Pots / Falcons/ Boiling tubes	Soil mixture	Watering and nutrition	Growth period	Analyses done
Sand experiment – 3 generations	*Arabidopsis*, *Medicago*, *Brachypodium*, unplanted	50 ml beakers	10% Bawburgh soil/90% autoclaved silver sand (v/v)	Watered with autoclaved deionised water with 10mg KNO_3 and 4 x 5 ml of standard nutrient solution	4 weeks	ARISA, 454 pyrosequencing
Compost experiment – 3 generations	*Arabidopsis*, *Medicago*, *Brachypodium*, unplanted, *Brassica*, *Pisum, Triticum*, unplanted	Model plants – 50 ml beakers Crop plants – 100 ml beakers	10% Bawburgh soil/90% autoclaved F2 Levington compost (v/v)	Watered with autoclaved deionised water only	4 weeks	ARISA, 454 pyrosequencing, plant dry mass
Myb mutant	*Arabidopsis* WT, *myb28/29*, unplanted	50 ml beakers	10% Bawburgh soil/90% autoclaved F2 Levington compost (v/v)	Watered with autoclaved deionised water only	4 weeks	ARISA, 454 pyrosequencing, plant dry mass
Ram mutants - compost	*Medicago* WT, *ram1, ram2*, control	50 ml beakers	10% Bawburgh soil/90% autoclaved F2 Levington compost (v/v)	Watered with autoclaved deionised water only	4 weeks	ARISA, 454 pyrosequencing, plant dry mass

Table 2.5.1 Plant growth conditions for all conducted experiments.

PAMP mutants	*Arabidopsis* WT, *cerk1*, *bak1-5 bkk1-1* , *bak1-5 bkk1-1 cerk1* , unplanted	50 ml beakers	10% Bawburgh soil/90% autoclaved silver sand (v/v)	Watered with autoclaved deionised water with 10mg KNO_3 and 4x 5 ml of standard nutrient solution	4 weeks	ARISA, 454 pyrosequencing, plant dry mass
Exp-eriments	Plant species	Pots / Falcons/ Boiling tubes	Soil mixture	Watering and nutrition	Growth period	Analyses done
CCC project – *Achromobacter* and *Arthrobacter*	*Brachypodium*, *Triticum*	Triticum – 50 ml Falcon tubes, *Brachypodium* – boiling tubes	Medium vermiculate	5ml of nutrient solution with 10mg KNO_3	Triticum – 2 weeks, *Brachypodium* – 3 weeks	RNA isolation and Ilumina sequencing
HOL experiment	*Arabidopsis* WT, *hol*, 196-11, unplanted	50 ml beakers	10% Bawburgh soil/90% autoclaved F2 Levington compost (v/v)	Watered with autoclaved deionised water only	1,2 and 4 weeks	ARISA
growth medium details	sand: silver sand, washed lime free, horticultural grade, J.Arthur Bower's, Sinclair, Lincoln, UK		compost: Levington F2, Seed and Modular compost, Sphagnum moss peat with added 150 mg/l of N, 200 mg/l of P and 200 mg/l of K, Scott's, Ipswich, UK		vermiculate: medium size, Sinclair, Lincoln, UK	

Table 2.5.1 Plant growth conditions for all conducted experiments.

2.6 Sample preparation and soil DNA isolation

As the main purpose of this PhD project was to examine the microbiome in plant rhizospheres, it was necessary to develop a method that allowed separation of soil influenced by root exudates (normally up to 1 mm away from the roots) from the remaining soil in the pot. During harvest plants were gently pulled out from the soil using 95 % alcohol-washed forceps. Plants were then shaken twice in order to discard loosely adhered soil. Depending on the plant species and the size and architecture of the root structure, different amount of soil adhered to the roots was collected (from 2 to more than 5 g per plant). The shoot was cut off and the root with the rhizosphere soil was placed in a 50 ml Falcon tube. PBS solution (30 ml) was added and the tube was vortexed for 30 seconds. Roots were then removed using sterile forceps and the soil suspension was centrifuged at 4000 rpm for 5 minutes to collect any suspended bacteria in the soil pellet. The supernatant was decanted and 1 g (wet weight) of the remaining soil was placed in pre-prepared and autoclaved bead tubes. Bead tubes were prepared using 2 ml screw cap tubes (StarLab,Germany) filled (8 % of the volume) with 0.1 mm zirconia/silica beads (Thistle Scientific,UK) and 0.5 mm glass beads (8 % of the volume) (Thistle Scientific,UK) and one 2 mm glass ball (Sigma-Aldrich,UK). Samples were snap-frozen in liquid nitrogen and kept at -80 °C for further analysis.

DNA was isolated by a method adapted from Griffiths (Griffiths *et al.*, 2000). All steps (unless stated otherwise) were conducted on ice. CTAB (0.5 ml) solution (Cetyltrimethylammonium bromide (5 %) diluted in potassium phosphate buffer (120 mM) and phenol-chloroform-isoamyl alcohol 25:24:1 (0.5 ml) was added to each sample. Cells were lysed using a Fast-Prep bead beater for 30 sec at the speed of 5.5 for three times leaving samples for 3 minutes on ice between shakings. Samples were spun at 13,000 rpm for 5 min. The supernatant was transferred to a new Eppendorf tube (2 ml) and mixed with chloroform-isoamyl alcohol 24:1 (0.5 ml) (Sigma-Aldrich,UK) in order to remove remaining phenol. Samples were then shaken to form an emulsion and spun at 13,000 rpm for 5 min. The supernatant was transferred to a new Eppendorf tube (2 ml) and precipitated using 1 ml of PEG solution (Polyethylene glycol 6000 (30 %) diluted in NaCl (1.6 M)). Samples were left at room temperature for 1-2 hours and spun down at 13,000 for 10 min. DNA pellets were then washed with ethanol (70 %).

This method allows for relatively fast and cost effective DNA isolation. However, during this process humic acids present in the soil are also dissolved and may be found in the final product. Humic acids interfere with DNA polymerase causing PCR reactions to fail (LaMontagne *et al.*, 2002) (Figure2.6.1). DNA was amplified using ARISA primers (ITSF and ITSReub) – described later. Amplification using this primer pair produces a smear of DNA fragments as it amplifies bacterial ITS fragment of varying sizes between different bacteria species.

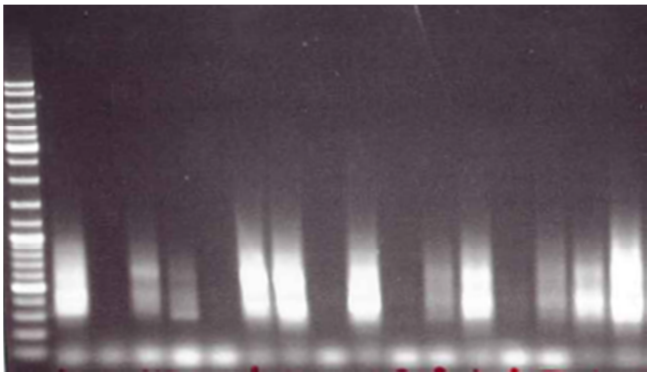


Figure 2.6.1 Agarose gel (0.8 %) with PCR product run against Generuler 1kb standard (Thermo Scientific). Not all samples have been successfully amplified.

This problem was overcome with using ZYMO research One-step™ PCR inhibitor removal kit (Epigenetics, Irvine, USA). Samples were treated according to the manufacture's protocol. Using this kit allowed successful PCR amplification of isolated DNA (Figure2.6.2)

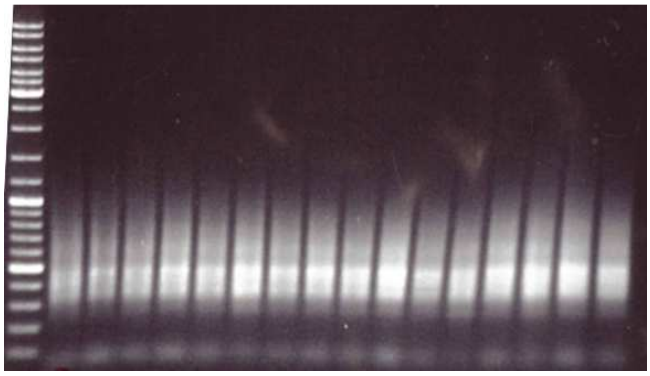


Figure 2.6.2 Agarose gel (0.8 %) with PCR product run against Generuler 1kb standard (Thermo Scientific). All samples have been successfully amplified after DNA clean-up step (PCR products shown were amplified from the same DNA samples as on Figure 2.6.1).

2.7 ARISA method

Automated Ribosomal Intergenic Spacer Analysis (ARISA) is a DNA fingerprinting method (Fisher & Triplett, 1999). It is a relatively simple and inexpensive method, allowing a great number of samples to be examined at once. ARISA can be roughly divided into 3 sections: PCR amplification of Intergenic Spacer Region (ISR), sample preparation for polyacrylamide gel and data analysis. It has been successfully used on bacteria, archaea and eukaryotic communities. ARISA is based on amplification of ISR regions of the microbial community and comparing ISR profiles against each other. The novelty of the ARISA method over RISA is the usage of a fluorescently labelled primer for PCR. Only bacterial ARISA will be described below, as this method was used with most of the rhizosphere samples. A very brief introduction to eukaryotic ARISA will be provided in the chapter 5.2.7, where this method was used to study one set of samples.

The ARISA method was developed in order to examine microbial population structure (Fisher & Triplett, 1999). It is one of a few DNA fingerprinting methods used in environmental microbiology along with Terminal Restriction Fragment Length Polymorphism (T-RFLP) and Denaturing Gradient Gel Electrophoresis (DGGE). The advantage of ARISA over other method is higher accuracy (Danovaro *et al.*, 2006). To this date, rhizosphere, root tissue, soil, composting pile, biofilm on coral reefs, lake, cow rumen, sea, sewage biofilm and many more bacterial communities have been examined using ARISA (Beman *et al.*, 2011; Biswas & Turner, 2012; Borneman & Triplett, 1997; Jami & Mizrahi, 2012; Mougel *et al.*, 2006; Sawall *et al.*, 2012; Schloss *et al.*, 2003; Shade *et al.*, 2012). ARISA has therefore has been proven to be a universal and accessible DNA fingerprinting method.

The low cost and relative time-efficiency of this method allowed screening of multiple replicates. Replication is essential in the case of microbial structure as it is a very dynamic and ever-changing system. In the following experiments (unless otherwise stated) 24 biological replicates for each plant species were tested using ARISA.

All bacteria have at least one copy of the rRNA operon on their chromosome. The rRNA operon consists of 3 genes: 5S, 16S and 23S, which code for rRNA subunits. The rRNA operon has highly conserved regions (16S rRNA and 23 rRNA) separated by highly variable

regions (Intergenic Spacer Region – ISR). The 16S and 23S rRNA genes sequences are evolutionary conserved in the bacterial kingdom. This allows the design of oligonucleotides that can bind near the 3' end of 16S gene and the beginning (5' end) of the 23S gene (Figure 2.7.1).

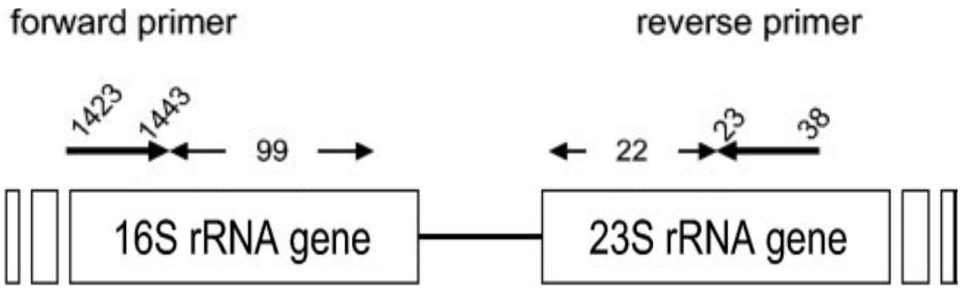


Figure 2.7.1 Position of the primers (ITSF and ITSReub) on the bacterial rRNA operon based on *E.coli* ribosomal gene (figure adapted from (Cardinale *et al.*, 2004)). Forward primer binds to 1423-1443 bp region of 16S and the reverse primer binds to 23-38bp region of 23S gene.

Some parts of the ISR code for tRNAs and some are non-coding (Anton *et al.*, 1998). ARISA is based on the comparison of the length of the ISR. Its length may vary between different bacteria species, strains or even a single bacterial chromosome may have different ISR lengths (where there are multiple copies of the rRNA genes). On the other hand different groups of bacteria may have an identical ISR length, while being evolutionary very distant. Despite these issues ARISA can be used in comparing microbial communities. In a rich bacterial population the fact that a few different species may produce the same ISR fragments should not have a major impact on the final result, assuming that the community consist of thousands of different species.

The first pair of bacterial primers to be used for ISR amplification was GAAGTCGTAACAAGG (forward) and CAAGGCATCCACCGT (reverse) (Jensen *et al.*, 1993). The forward primer matches a highly conserved region of the 16S rRNA gene and binds very well, however, the reverse primer was based on sequences from only five bacterial and four plant chloroplast 23S rRNA genes and poorly amplifies targets (Gonzalez *et al.*, 2003). Different sets of bacterial primers have been designed and used for samples taken from different

environments (Fisher & Triplett, 1999; Ranjard *et al.*, 2001). A comparison between different primers sets used for samples from different environments was also conducted (Cardinale *et al.*, 2004). The most universal environmental primers, with the widest range of spacer sizes and DNA template concentration are ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3'). The 6FAM fluorescence probe is attached to the forward primer.

DNA was isolated from soil microorganisms, purified using a Zymo research kit (described above) and quantified using a Nanodrop ND-1000 spectrophotometer. A standardized amount of DNA (1µl of 5 ng/µl concentration) was used for ARISA PCR preparation using colourless Gotaq mastermix (5 µl), autoclaved Molecular Biology Grade water (3 µl) and forward and reverse primers (1 µl of 10 pmol concentration of each). PCR conditions were as follows: 95°C for 5 min, 30 cycles of 94°C for 1min, 55°C for 1 min and 72°C for 2 min with the final extension (1 cycle) of 72°C for 10 min and cooled down to 4°C. PCR products were run on an agarose gel (0.8 %), stained in EtBr (0.08 %) for 15 min and the bands were visualized using UV transilluminator (Syngene, USA) (Figure 2.6.1 and 2.6.2).

Each PCR product (1 µl) was mixed with 10 µl of Hi-di formamide (Life technologies) and 0.4 µl of LIZ1200 size standard (GeneScan). Samples were submitted to the John Innes Centre, NRP, Norwich to be run on ABI3730 sequencer. The output is an electropherogram, which was uploaded into Peak Scanner 1.0 software (Applied Biosystems – Life technologies, Grand Island, NY, USA) (Figure 2.7.2). The orange peaks represent LIZ1200 size standard.

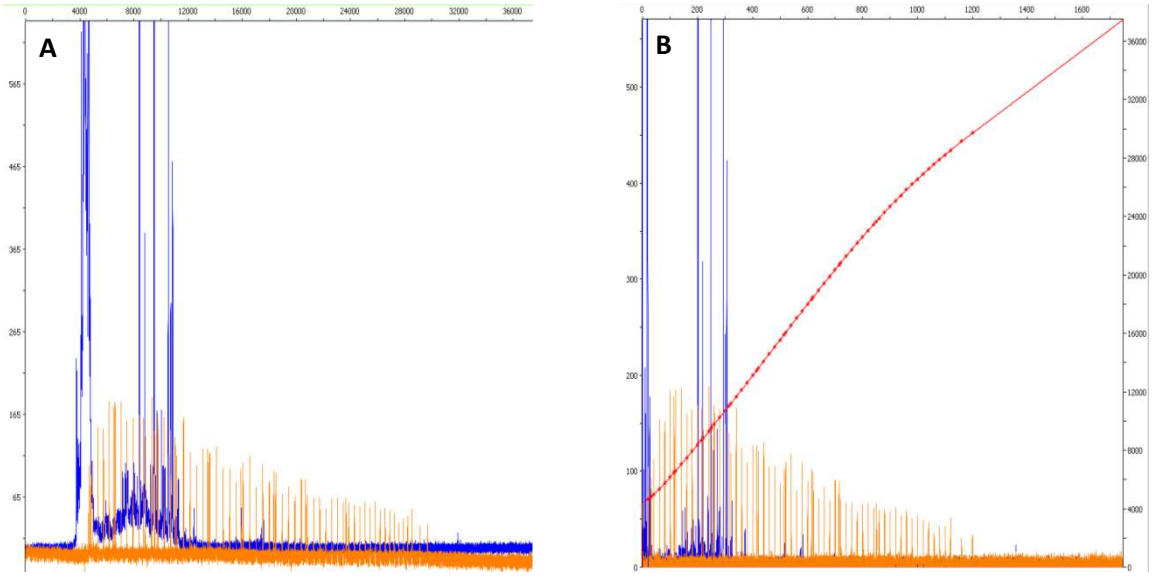


Figure 2.7.2 Electropherogram of ARISA sample: (A) raw fluorescence data - fluorescence intensity over time (B) analyzed data - normalized fluorescence units (fu) over fragment size in bp. Blue lines represent fluorescence of 6FAM dye and orange colour is LIZ1200 size standard.

Electropherogram data was changed into its tabulated numerical version (Figure 2.9.1). For further analysis 100 sizes with the highest fluorescence were chosen. All the sizes must be at least 100 bp in size and the minimum fluorescence intensity threshold was 20. Sizes with fluorescence intensity smaller than 20, were considered as noise.

2.8 454 Life Science (Roche) GS FLX pyrosequencing

454 Pyrosequencing FLX is a high-throughput sequencing method. It enables sequencing of about 400,000 DNA fragments with the average length of 300-400 bp at one run using a PicoTiter plate. 454 sequencing is based on detection of inorganic pyrophosphate (PPi), which is released at the time of nucleotide synthesis. The first step is to amplify DNA using biotinylated primers (emPCR). Amplicons are attached to the very small beads and the non-biotynylated DNA strand is denaturated. The Denaturated DNA strand and all other reagents are washed off and the sequencing primer is added.

The plate is washed with nucleotides and every time a nucleotide is attached to the newly formed DNA strand PPi is released. ATP sulfyrase converts ADP plus PPi into ATP and P_i. ATP reacts with luciferase producing oxyluciferin and light. An ultra-sensitive camera captures these flashes of light every time a new nucleotide is added. The reaction is repeated until no more light flashes are detected (http://www.channelwolf.com/lvv/sem6/index_files/Page542.htm, (Elahi & Ronaghi, 2004)).

454 pyrosequencing allows multiplex sequencing. Using specially designed primers it was possible to sequence 36 different sets of samples on each quarter of PicoTiter plate in this PhD project (a full list of primers used in 454 pyrosequencing can be found in Table S1).

Two sets of samples were submitted to the Genome Centre for high-throughput sequencing; sand experiment and compost experiment including Myb, Ram and PAMP mutants (see Table 2.4.1). In both cases separate bacterial and fungal specific primers were used. Reverse primers consist of 3 parts; primer A, which binds to PicoTiter beads, MID region used for sample recognition (bar coding) and sequence targeting 16S rRNA/28S rRNA gene. The forward primer is build from two parts: primer B as the final part of the PCR product and sequence targeting 16S rRNA/18S rRNA gene.

Bacterial communities were examined using the hyper-variable 16S rRNA fragment V1-V2 position 27-338 for *E.coli* (Figure 2.8.1) (Hamady & Knight, 2009). Amplification of this fragment allows different bacterial species to be distinguished due to the high sequence variability.

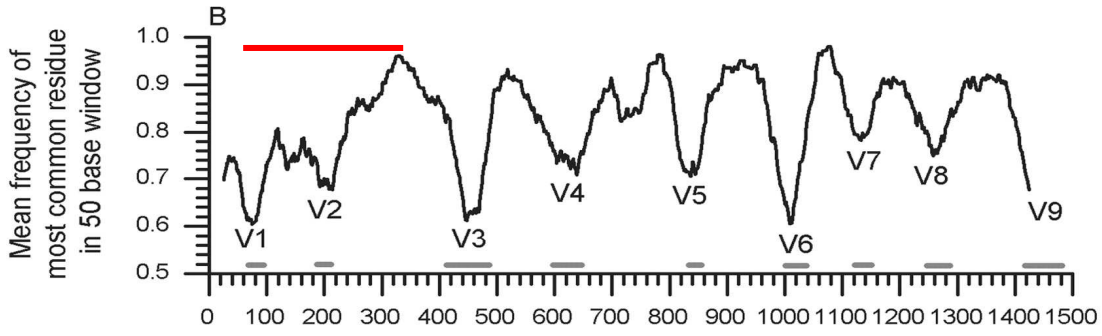


Figure 2.8.1 Hypervariable regions of 16S rRNA gene for *E.coli*. Red rod indicates the amplified region (http://www.rna.icmb.utexas.edu/)

The fungal community was examined using fungal specific primers that bind to the ITS region between 18S and 5.8S rRNA genes (fig 2.8.2 and Table S1). Primers were designed to amplify mostly Ascomycota and Basidiomycota phyla (Buee *et al.*, 2009). Fungal molecular taxonomy is based on ITS region, as fungal ribosomal small subunit (SSU) is much less variable than the bacterial one.

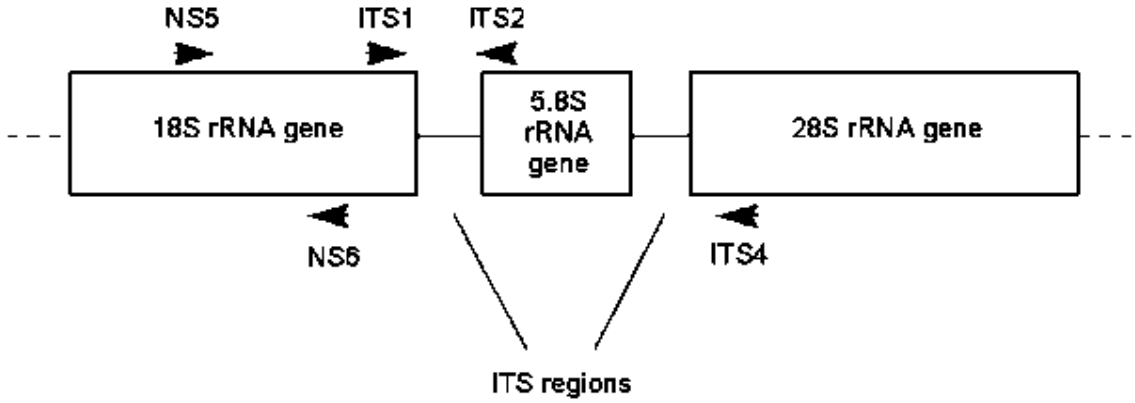


Figure 2.8.2 Fungal rRNA operon with annotated binding sites for ITS primers

PCR reactions were prepared using pooled DNA samples. From every generation for each plant species 3 pooled samples were produced (4 plants or unplanted x 3 generations x 3 replicates = 36 barcoded samples). The samples were pooled always according to the same pattern: PCR sample 1 was pooled from DNA samples 1-8, PCR sample 2 from DNA samples 9-16 and PCR sample 3 from DNA samples 17-24.

DNA samples were PCR amplified using bacterial and fungal primers using the same conditions: 94°C for 4 min, 30 cycles of 94°C for 30 sec, 50°C for 1 min and 72°C for 90 sec with the final extension of 72°C for 10 min. Samples for the sand experiment (Chapter 3.3) were processed at the Genome Analysis Centre, NRP, Norwich and sequenced using 10/16th of a 454 plate. For each of the PCR amplifications the products were purified using an equal volume of AMPure XP beads (Beckman Coulter, USA) and the concentration of the purified products quantified using the Quant-iT hsDNA Assay Kit (Life Technologies, USA). Samples were then normalized to 5 ng/ µl and equimolar pooled based on the barcodes used.
For the compost experiment (Chapter 3.7.) PCR products were purified using GeneJet PCR purification kit (Thermo Scientific, UK) and quantified using Quantifluor kit (Promega,UK).

Amplified DNA products (50 ng) from each sample was mixed together in order to produce 3 master-samples for bacteria and 3 master-samples for fungi. The bacterial and fungal samples were mixed 3:1 (by DNA amount) and sent for sequencing using 3 quarters of a 454 FLX plate.

Samples for the sand and for the compost experiment were then subjected to emPCR to generate template beads which were then sequenced on a Roche 454 GSFLX sequencing platform according to the manufacturers' instructions.

A quality check was done on raw 454 pyrosequencing data. Reads that did not contain PCR primer sequence at the 5' end were discarded. The sand experiment produced 85,010 bacterial reads (94% of the original number before filtering) and 98,613 fungal reads (75%) (Table S2) and the compost experiment produced 199,976 bacterial reads (84%) and 54,701 fungal reads (88%) (Table S3).

2.8.1 Initial analysis using 454 pyrosequencing data

The first step in analysing 454 data was sample separation according to their MID sequence and location on the 454 plate. Then bacterial reads were separated from the fungal ones according to target primer sequence (Table S2 and S3). Once the samples were separated two different data analysis approaches were used (Figure 2.8.3).

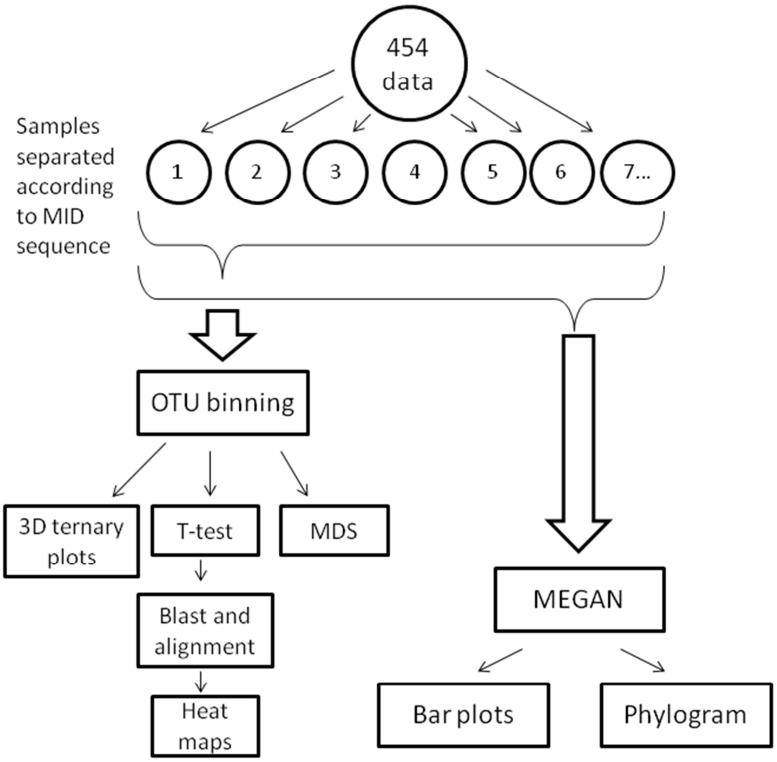


Figure 2.8.3 Pathways of analyzing 454 pyrosequencing data

The first one included binning reads into Operational Taxonomic Units (OTUs) according to the sequence similarity between the reads. USearch 6.0 software was used to produce OTUs (Edgar, 2010). Rarefaction curves were produced using MOTHUR software (www.mothur.org) (Schloss et al., 2009) and OTUs were binned at different similarity levels (Figure 2.8.4). A Similarity of 95 % was chosen as it produced a reasonable number of OTUs at the similarity level roughly corresponding to genus level (Stackebrandt, 1994).

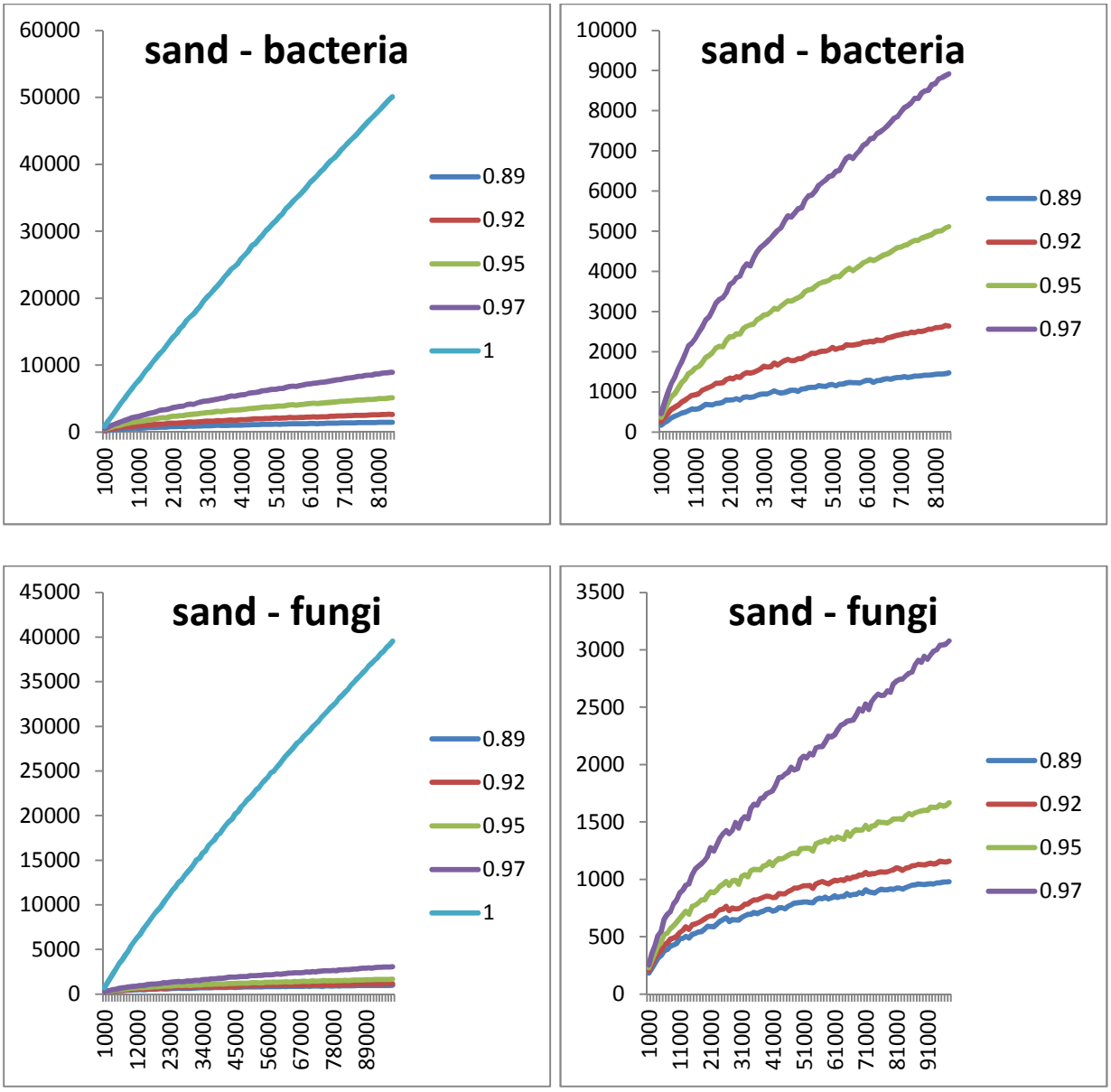


Figure 2.8.4 Rarefaction curves produced for 454 pyrosequencing reads. Horizontal axis represents number of reads taken into analysis and the vertical axis shows the number of resulting OTUs. Numbers represent the similarity value at which sequences were binned together (e.g. 0.89 means that reads were binned using 89% similarity threshold).

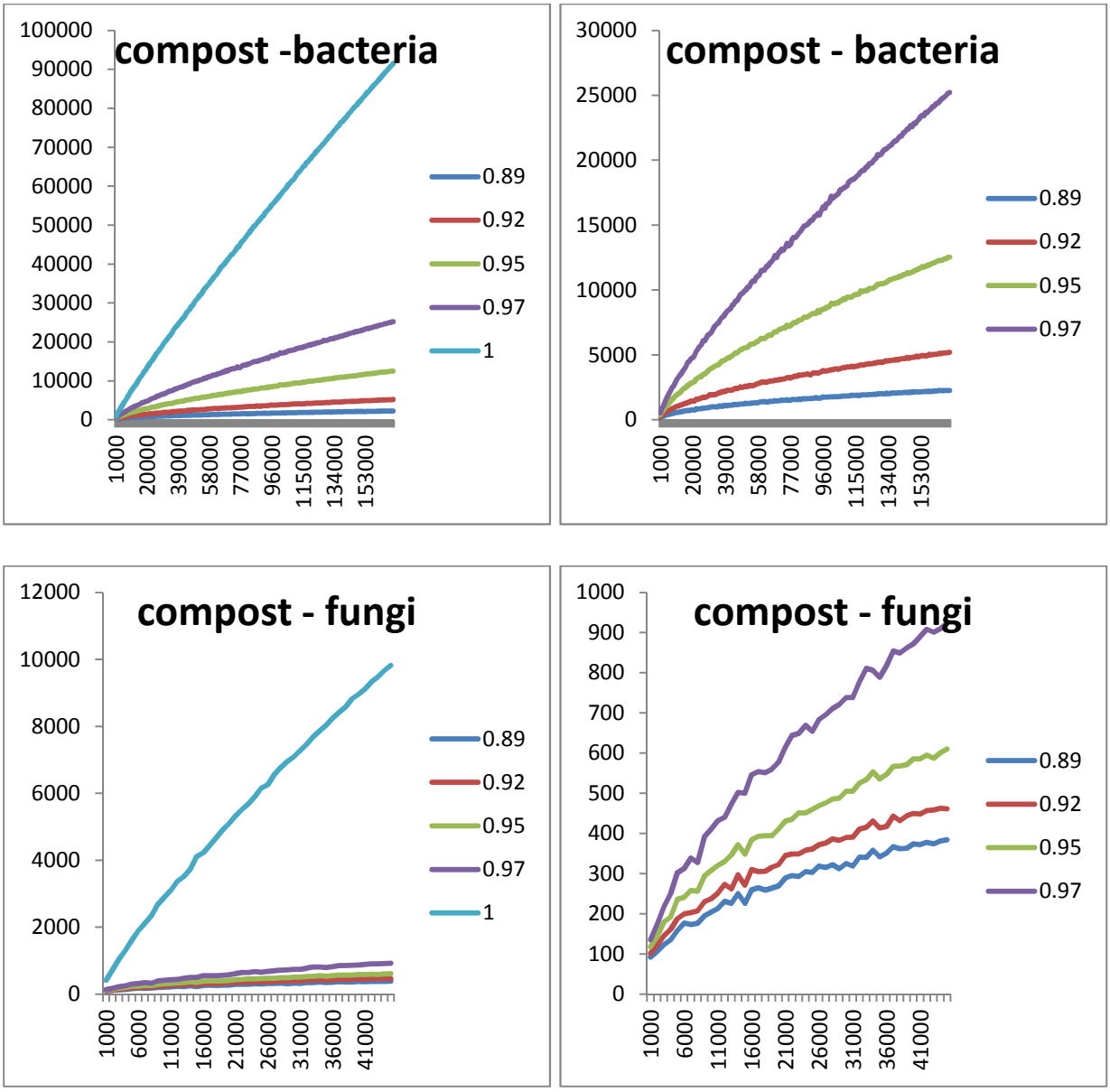


Figure 2.8.4 Rarefaction curves produced for 454 pyrosequencing reads. Horizontal axis represents number of reads taken into analysis and the vertical axis shows the number of resulting OTUs. Numbers represent the similarity value at which sequences were binned together (e.g. 0.89 means that reads were binned using 89% similarity threshold).

Heat maps display selected and depleted OTUs in rhizosphere relatively to unplanted control. T-test (p value <0.05) was used to compare abundance of each of the OTUs found in the rhizosphere against unplanted control samples. Once, the list of OTUs was assembled (either rhizosphere selected or depleted) a representative sequence from each of them was

aligned against each other using an R script. The read that starts the "seed" in the Usearch software was chosen to be a representative sequence. All other reads belonging to the same seed (OTU) are similar to the initial one in at least 95 %, however it can not be excluded that the similarity between remaining reads in an OTU is lower than 95 %. A phylogram was constructed using Dendroscope software. The representative sequence for each OTU was used in BLAST search against the SILVA database version 1.06 (Pruesse *et al.*, 2007) in the case of bacteria and NCBI GenBank nucleotide collection in case of fungi. BLAST files were uploaded into MEGAN software in order to identify the Lowest Common Ancestor (LCA) of the all reads belonging to particular OTU (Huson *et al.*, 2009). An example analysis using the MEGAN software is explained below. In most cases the LCA could be identify to the genus level as predicted from the OTU binning procedure.

The other analysis pathway in interpreting 454 data was the use of MEGAN on non-binned data. In this case, a BLAST report done on each sample (see below) was uploaded into the software and the relative abundance of bacterial phyla or fungal subphyla/classes was exported into Microsoft Excel. Excel was used to produce bar graphs representing bacterial structure at high taxonomic level.

MEGAN also produces phylogram plots. Each node is represented as a pie chart with relative abundance of this node among examined samples. An example MEGAN phylogram with pie charts is shown in figure 2.8.5. Only 2-4 samples may be presented using this method, as including more samples produces complex pie charts that are difficult to interpret.

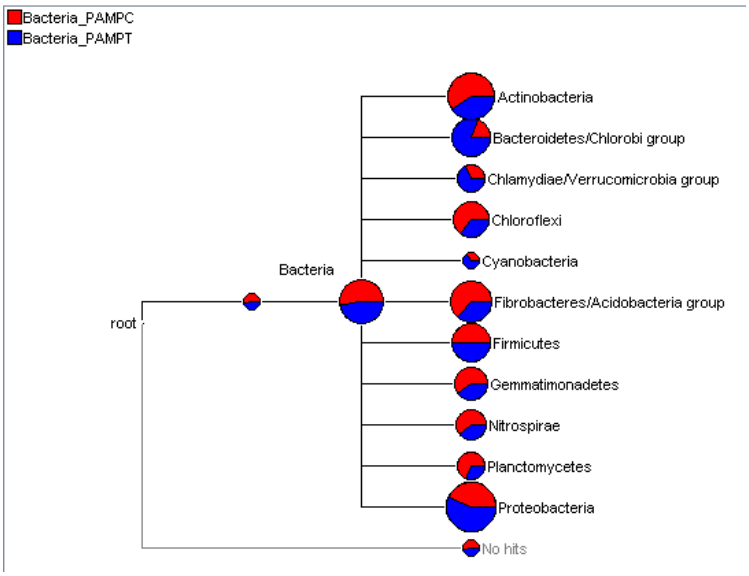


Figure 2.8.5 An example of MEGAN phylogram comparing community between two samples.

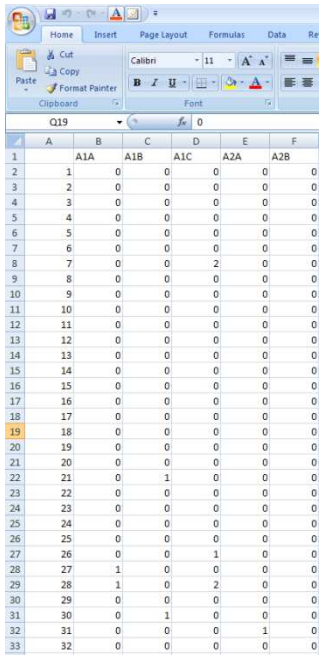
MEGAN compares the annotations of each read in a group (for example total community of *Arabidopsis* rhizosphere in the 1st generation of the sand experiment – data here is not binned into OTUs) and chooses the LCA, a taxonomic level to which all the reads belong. The very important parameter in LCA assignment is the number of reads from each sample taken into account. This number is regulated by the BIT score (one of the BLAST index) and it is calculated as a percentage of the top score. Depending on different LCA parameter different number of reads is included and the annotation of the sample may differ. The LCA parameter used in MEGAN was set to top 1% for all analysis.

2.9 Data analysis for ARISA and 454 pyrosequencing data

ARISA and 454 pyrosequencing produced data very similar to each other in structure. Both methods yield data on the abundance of OTUs in multiple samples. The ARISA data presented as fluorescence intensity against ISR fragment size and 454 data are presented as OTU number against its abundance (number of reads allocated to a particular OTU). The methods used for OTU construction from the 454 pyrosequencing data are explained in the

next subchapter. Data transformation and many of the statistical analysis were conducted in exactly the same way for both data sets and that is why there will be presented together unless stated otherwise.

	Dye Color	Dye/...	Size	Height
63		B, 63	512.9463	9034
64		B, 64	518.3261	21
65		B, 65	520.4861	25
66		B, 66	524.496	26
67		B, 67	524.7795	25
68		B, 68	529.6385	41
69		B, 69	531.8247	56
70		B, 70	533.2819	68
71		B, 71	534.3748	91
72		B, 72	536.9246	123
73		B, 73	539.9595	76
74		B, 74	542.811	25
75		B, 75	544.7686	82
76		B, 76	546.4829	86
77		B, 77	549.3841	65
78		B, 78	554.0913	25
79		B, 79	554.5421	27
80		B, 80	560.2058	114
81		B, 81	562.4698	156
82		B, 82	563.4169	322
83		B, 83	567.0422	51
84		B, 84	572.6503	3776
85		B, 85	575.0852	121
86		B, 86	579.1323	819
87		B, 87	583.6688	22
88		B, 88	585.3591	38
89		B, 89	590.1834	37



	A	A1A	A1B	A1C	A2A	A2B
1						
2	1	0	0	0	0	0
3	2	0	0	0	0	0
4	3	0	0	0	0	0
5	4	0	0	0	0	0
6	5	0	0	0	0	0
7	6	0	0	0	0	0
8	7	0	0	2	0	0
9	8	0	0	0	0	0
10	9	0	0	0	0	0
11	10	0	0	0	0	0
12	11	0	0	0	0	0
13	12	0	0	0	0	0
14	13	0	0	0	0	0
15	14	0	0	0	0	0
16	15	0	0	0	0	0
17	16	0	0	0	0	0
18	17	0	0	0	0	0
19	18	0	0	0	0	0
20	19	0	0	0	0	0
21	20	0	0	0	0	0
22	21	0	1	0	0	0
23	22	0	0	0	0	0
24	23	0	0	0	0	0
25	24	0	0	0	0	0
26	25	0	0	0	0	0
27	26	0	0	1	0	0
28	27	1	0	0	0	0
29	28	1	0	2	0	0
30	29	0	0	0	0	0
31	30	0	1	0	0	0
32	31	0	0	0	1	0
33	32	0	0	0	0	0

Figure 2.9.1 Structure of raw data for A) ARISA obtained using Peak Scanner, B) 454 pyrosequencing OTUs obtained using Usearch

ARISA data were exported to Microsoft Excel and OTUs identified using T-Align (Smith et al., 2005) (www.inismor.ucd.ie/~talign/). The binning interval was set to 0.8 bp. Such binning is necessary in order to compare ISR profiles from different samples. Binning generates a table of the frequency of each fragment size. The ARISA and OTU data was then analysed using Primer 6.0 software. Each value was standardized (value of each fluorescent peak height was divided by the total fluorescence value for each sample) and square root transformed by the PRIMER 6.0 software (Figure 2.9.2). Standardization was needed because different samples had different values of total fluorescence. Standardized data were square root transformed in order to minimize the influence of the common taxa. This software was used for constructing a similarity matrix using the Bray-Curtis similarity coefficient. The Bray-Curtis coefficient is widely used in ecological studies in order to compare multivariate data collected at multiple study sites. The result (between 0 and 1, or as implemented in PRIMER

software 0 % - 100 %) represents the relation between the observations that are different to the observations that are the same at the two corresponding study sites. A Bray-Curtis similarity coefficient of one means identical samples and 0 means completely different.

$$s_{jk} = 100\left\{1 - \frac{\sum_{i=1}^{p}|y_{ij} - y_{ik}|}{\sum_{i=1}^{p}(y_{ij} + y_{ik})}\right\}$$

Figure 2.9.2 Bray-Curtis similarity coefficient, where (i) is an ISR size or OTU number and (j) and (k) are relative fluorescence values or OTUs abundance for compared samples.

2.9.1 MANOVA test

In order to separate the samples belonging to different groups (for example to distinguish rhizosphere samples of *Arabidopsis* from *Medicago* samples) a statistical analysis was used. Multivariate analysis was performed on the data obtained by ARISA method as described in (Osborne *et al.*, 2011). MANOVA output indicates the significance level between two groups of data on the base of F-test performed on the Bray-Curtis distance matrix with 1000 permutations using ADONIS implemented in the "vegan" package of the R program. MANOVA was used to determine if the differences between groups of rhizosphere samples are statistically significant. Replicates from each group were taken at random and compare against each other. OTU data was not analysed using MANOVA tests as there are only 3 samples for each group of data. The low number of replicates was insufficient to successfully use MANOVA.

The lower number of replicates is required to separate groups the less similar these groups are. In the MANOVA tables provided in the results section the size means the number of replicates taken at random from each group. If the permutation test gives P values > 0.05 such groups are not considered to be different from each other. In order for P values to be low the β diversity (between the two groups of samples) has to be much higher than the α diversity (diversity within each group).

For simplicity, in the results chapters the MANOVA tables represent the number of rhizosphere/unplanted replicates needed in order for 950 out of 1000 permutations to reach a significance of $p < 0.05$.

2.9.2 Analysing community structure using ternary plots

MDS plots are very useful for analysing the overall similarities between samples. However, in order to look for specific ISR sizes (or OTUs) that are responsible for the observed differences other representation methods were needed. Initially bar graphs were produced in Microsoft Excel showing the 15-20 most abundant ISR sizes for each sample. However, their interpretation is very time consuming and may be misleading as not all ISR sizes can be shown. There was a need for a more graphical approach to represent the community structure. Ternary plots were chosen as they can represent the whole structure and are easy to interpret (Figure 2.9.3). Each generation is shown as a separate triangle and each ISR is shown as a dot in case of 2 dimensional graphs and ball in case of 3D graphs. The location of ISR is a result of their plant species specificity. The size represents relative abundance in each generation. The dot and ball colour is calculated according to:

$$C\ (\%) = [1 / (3 \times U / (A+M+B))] \times 100$$

where U is ISR relative abundance in unplanted control (fluorescence intensity of a particular ISR divided by total fluorescence, or OTU abundance divided by total number of 454 reads), A is *Arabidopsis*, M is *Medicago* and B is *Brachypodium*. C is a value between 0 and 100%. The formula [(3 x U / (A+M+B)) x 100] gives the soil selection, so to express results as rhizosphere selected the reciprocal is used [1 / (3 x U / (A+M+B))] x 100]. For the crop plants the same equation was used, just the plants species were exchanged. To simplify the colour code a scale of only 4 colours was used: 0-25 % blue, 25-50 % green, 50-75 % orange and more than 75 % red. It was necessary to use the formula unplanted/sum of rhizosphere as some of ISRs were missing from the unplanted control. Only ISR with a contribution of at least 1 % in at least one rhizosphere were taken into analyses (and 0.1 % in case of OTUs). Red balls located in the middle of a triangle represent ISR rhizosphere specific, but not plant species specific ISRs. If the red ball is situated in any of the corners

then this ISR is rhizosphere and plant species specific. If the ball is coloured green or blue and it is in the middle of the graph it represents ISR that was suppressed in the rhizosphere of all plants. If a blue ball is found in the corner this ISR was suppressed by all plants apart from the one in whose corner it is located. ISRs that are present in successive generations can be connected by a line. The number of lines indicates how similar the community is between generations.

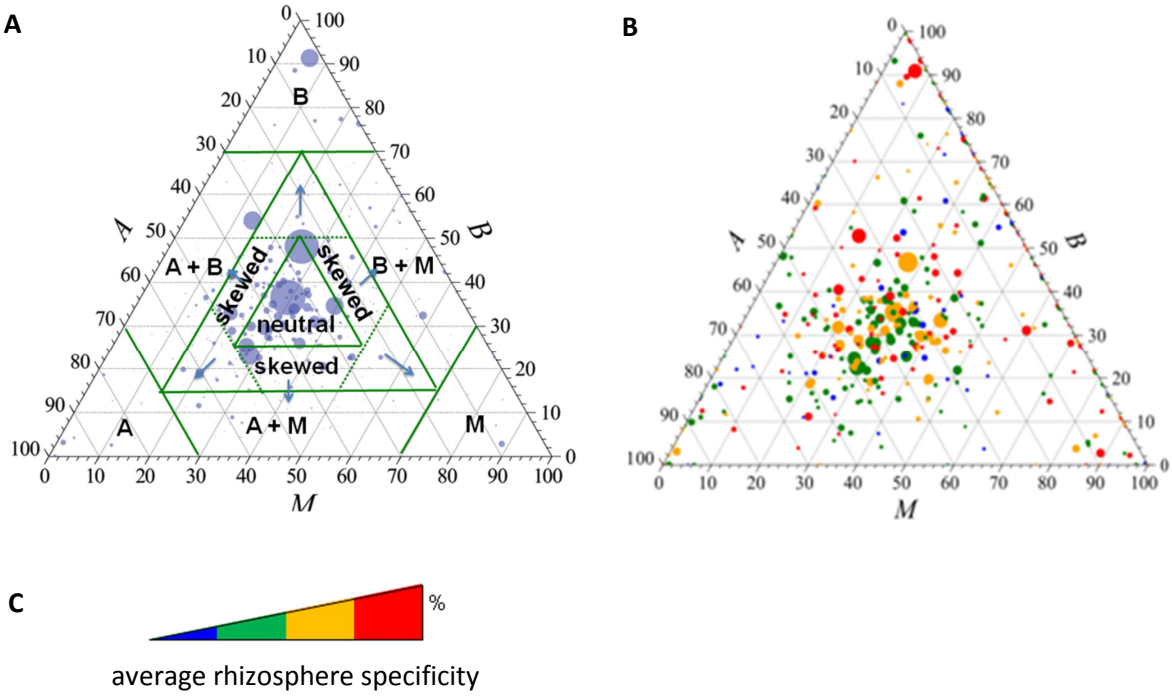


Figure 2.9.3 Two-dimensional examples of ternary plots. A is *Arabidopsis*, M is *Medicago* and B is *Brachypodium* rhizosphere. A) Ternary plot with plant species specific areas B) Ternary plot with coloured ISR/OTUs used C) Rhizosphere specificity scale.

In order to produce 3D ternary plots the initial step was to prepare 2D ternary plots for each generation. For 2D plots Veusz, a GUI scientific plotting and graphing package (http://home.gna.org/veusz) was used to get a ternary plot frame and then a custom written Python 2.7 program was used to overlay and colour code the ISR/OTU's values, and to render the annotated SVG. 3D plots were generated using a python program and Java applet Jmol (http://jmol.sourceforge.net) to view the 3D scene in Firefox browser. The best view was captured and annotated.

2.9.3 Community richness and diversity using Shannon index

Data analysis using nMDS and ternary plots suggested a great diversity loss over generations in the sand experiment. In order to present this effect in a numerical way, richness and diversity indexes were needed. Initially it was decided to use a very simple approach to examine the community. The ARISA and OTU data were standardized and sorted for each sample according to fluorescence intensity and abundance, respectively. Richness is expressed as a number of different ISRs or OTUs needed to reach 50 % of the total abundance.

The richness index does not capture the abundance of the least abundant ISRs or OTUs. In order to examine the whole community structure it was necessary to use one of the ecological diversity indexes. The Shannon index quantifies the uncertainty associated when making a prediction. In case of ARISA or 454 data it is the uncertainty of finding a new ISR or OTU when going through the data at random. It is calculated according to:

$$H' = -\sum_{i=1}^{R} p_i \log p_i$$

where p_i is the abundance of a particular ISR or OTU. The Shannon index is more powerful in showing the diversity than the richness index is. However, due to the fact that the richness index is very easy to interpret both indexes will be presented in the results chapters.

2.10 Soil bacteria isolation methods

Bacterial strains were isolated from the 3rd generation rhizosphere of *Brachypodium*, *Arabidopsis* and *Medicago* grown in the sand. Soil (1 g) was taken from each plant species rhizosphere, diluted in di-ionised water and shaken for 15 minutes. After that time samples were centrifuged at 1000 rpm for 1 minute and the supernatant was collected. The supernatant was then centrifuged at 4,000 rpm for 5 minutes and the pellet was used for plating out on agar plates after serial dilution. In case of sand experiment 3 different media were used: TY, LB, AMA with glucose (10 mM) and ammonia (10 mM). Compost samples

were plated onto TY plates only. Plants were kept at 27°C for 2-3 days. Approximately 50 colonies were chosen from each rhizosphere sample and subcultured onto new TY plate. Sub-culturing helped to obtain single, not contaminated colonies from which DNA was isolated using the alkaline PEG method (PEG 200 (60 g) diluted in KOH (2 M, 0.93 ml) and water (39 ml), pH adjusted to 13).

ARISA was performed in order to determine the size of the ISR region for each isolate. Comparing ISR sizes of isolated strains with the ISR profiles obtained during sand and compost experiment did not yield a simple correlation between peak sizes. Thus the abundance of soil isolates with an ISR size equal to the common ISR sizes found in the rhizosphere, were not confirmed by 454 pyrosequencing. However, during isolation experiments many colonies were examined for their 16S rRNA gene sequence. These sequences were later used for BLAST comparison against the 454 pyrosequencing data. Most of the isolated bacteria were absent or not abundant in the soil (at least based on the 454 data), however two of the colonies were found to match to the two common OTUs.

2.11 *Achromobacter* and *Arthrobacter* DNA and RNA isolation

Two isolated strains (*Achromobacter sp.* and *Arthrobacter sp.*) found to be among the dominant species in the soil experiment (based on sand 454 pyrosequencing data) were used for further research. Genomic DNA was isolated and sent for sequencing. At the time of writing this PhD thesis only a partial information about the results are obtained. Moreover RNA was isolated and enriched for mRNA, however as I am still waiting for the data to come this are will not be covered in the introduction or in the material and method section.

As *Achromobacter* is Gram-negative and *Arthrobacter* is Gram-positive two different methods for gDNA isolation were used. Both strains were grown overnight in 200 ml TY flasks at 27 °C. 1 ml of the bacterial cultures were spun down at 4,000 rpm for 10 min.

Achromobacter cells were lysed using lysing buffer (1 mg/ml of Proteinase K diluted in SDS (0.5 %)) for approximately 2 hours at 55 °C. After incubation samples were incubated again

at 65 °C for 20 min using NaCl (150 µl, 5 M) and CTAB solution (80 µl) (CTAB (10 g) in NaCl (0.7 M)). Then Phenol: Chloroform: Isoamyl alcohol (25:24:1) (700 µl) was added and samples were shaken using orbital shaker for 10 min. Samples were spun down at 4000 RPM and the supernatant was collected. DNA was precipitated using isopropanol (420 µl) and spun down at 4000 RPM for 10 min. 70 % ethanol was added to the pellet and centrifuged again (1000 RPM for 10 minutes). The pellet was air dried and resuspended in elution buffer (Qiagen) buffer. Contaminating RNA was removed by using Riboshredder according to the manufacturer's specification (Epicentre) (Figure 2.11.2).

Genomic DNA from *Arthrobacter* was isolated using salting out procedure (Kieser, 2000). Bacterial culture (1 ml) was resuspended with SET buffer (75 mM NaCl, 25 mM EDTA pH8, 20 mM Tris-HCl pH7.5) and lysozyme (10 µl) was added. Sample was incubated at 37 °C for 1 hour. After incubation proteinase K (140 µl) was added and mixed with SDS (10 %, 600µl). Sample was incubated for another 2 hours at 55 °C. Then NaCl (5M, 2 ml) was added and the sample was thoroughly mixed. Chloroform (5 ml) was added and the sample was mixed for 30 min using orbital shaker. Samples were spun down (4000 RPM for 10 min) and the supernatant collected. Isopropanol (0.6 sample volume) was added to the supernatant to precipitate DNA, which was spun down at 4000 RPM for 10 min. DNA was cleaned using ethanol (70 %), air dried and resuspended in elution buffer (Qiagen). Samples were depleted of RNA contamination using Riboshredder according to the manufacturer's specification (Epicentre) (Figure 2.11.2). Even though visually there was no RNA contamination (maybe apart from *Arthrobacter* gDNA – lane 3) Riboshredder and subsequent column purification cleaned DNA from smaller fragments. It is worth noting that the aim here was to check if the DNA isolation worked rather than to asses it's quality. All the quality and quantity assessments were done by TGAC.

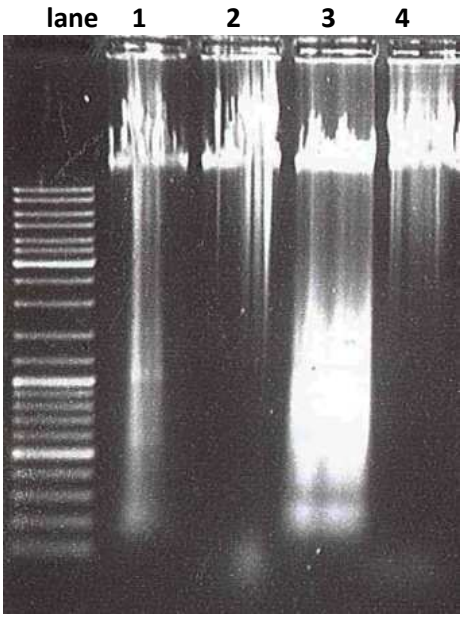


Figure 2.11.2 Electrophoresis gel with gDNA samples. Samples were run against Generuler 1 Kb ladder (Thermo scientific), ladder DNA amounts: bright bands: 99 ng, pale bands: 26-33 ng, lane 1: gDNA of *Achromobacter*, lane 2 gDNA of *Achromobacter* after RNA removal, lane 3: gDNA of *Arthrobacter*, lane 4 gDNA of *Arthrobacter* after RNA removal.

One gDNA sample from each strain was sent to the Genome Analysis Centre, Norwich for 454 sequencing using half a 454 plate for each sample.

2.12 Growth media and buffers composition

The recipes for all media and buffers used throughout this PhD project are given below.

LB and LB agar

Tryptone 10.0 g

Yeast Extract 5.0 g

NaCl 10.0 g

Add 1000 ml glass distilled water

Adjust to pH 7.0 with 1 M NaOH.

For solid medium add 10.0 g agar

MS agar 0.8%

Murashige and Skoog medium (including vitamins*)

4.41 g

Sucrose 30.0 g

Add 1000 ml glass distilled water

Adjust pH to 5.8 with 1 M NaOH

Bacto agar 8.0 g/l

M9 medium and agar

Na_2HPO_4 6.0 g

KH_2PO_4 3.0 g

NaCl 0.5 g

NH_4Cl 1.0 g

Add 1000 ml glass distilled water

Adjust to pH 7.4

Bacto agar 24 g

10 X PBS

Sodium di-hydrogen orthophosphate (NaH_2PO_4) 2.48 g

Di-sodium hydrogen orthophosphate (Na_2HPO_4) 21.36 g

Sodium Chloride (NaCl) 87.66 g

Add 1000 ml glass distilled water

pH 7.4

TY medium and agar

Tryptone 5.0 g

Yeast Extract 3.0 g

$CaCl_2$ $6H_2O$ 1.32 g

Add 1000 ml glass distilled water

For solid medium add 10.0 g agar

RNAlater

20 mM of EDTA, dihydrate.

25 mM of Sodium Citrate, trisodium salt dehydrate.

70 g of Ammonium sulphate.

Prepare stock solution of EDTA and Sodium citrate.

0.5 M EDTA pH 8.0 (dissolve 18.61 g/100ml, adjust the pH to 8.0 with NaOH while stirring) and 1 M of Sodium citrate (dissolve 29.4 g/ 100 ml).

In a beaker, take 4 ml of 0.5 M EDTA, 2.5 ml of 1 M sodium citrate, 93.5 ml of sterile water and 70 g of Ammonium sulphate. Stir the contents on low heat till the salt completely dissolves. Allow it cool, with constant stirring. Adjust the pH to 5.2 with 1N H_2SO_4. Transfer to a Screw capped bottle and store in room temperature or 4°C.

Use twice the volume of the culture.

AMA - Acid Minimal Salts medium and agar

0.5 ml	1M K_2HPO_4 (c/r)	Di-potassium hydrogen orthophosphate anhydrous (0.5 mM)
0.5 g	$MgSO_4.7H_2O$	Magnesium sulphate (2 mM)
0.2 g	NaCl	Sodium Chloride
4.19 g	MWT 209.3	MOPS (20 mM)
1000 ml	glass distilled water	
1 ml	Solution A	
2 ml	Solution B	Do not store for more than 1 week
1 ml	Solution C	Added aseptically after sterilising

UNLESS dispensing small volumes in which case add solution C before sterilising.

Rhizobium Solution A - see overleaf

Ingredients:

15 g	$EDTA\text{-}Na_2$
0.16 g	$ZnSO_4.7H_2O$ (Zinc sulphate heptahydrate)
0.2 g	$NaMoO_4$ (Sodium molybdate di-hydrate)
0.25 g	H_3BO_3 (Boric acid)
0.2 g	$MnSO_4.4H_2O$ (Manganese sulphate tetrahydrate)
0.02 g	$CuSO_4.5H_2O$ (Copper sulphate pentahydrate)
1 mg	$CoCl_2.6H_2O$ (Cobalt chloride hexahydrate)
	(dissolve 100mg in 100 ml GDW and add 1ml)

Make up to 1L in glass distilled water

Method:

Dissolve each ingredient in turn before adding the next.

Make up w/v to 1 L with glass distilled water.

Store at 4 °C.

Rhizobium Solution B

Ingredients:

1.28 g	$CaCl_2.2H_2O$ (87.1 mM) (final after addition to main medium 0.17 mM)
0.33 g	$FeSO_4.7H_2O$
100 ml	glass distilled water

Method:

Dissolve each ingredient in 50mls water then combine.

Store at 4 °C for no more than 1 week.

Rhizobium Solution C

Ingredients:

1 g	Thiamine hydrochloride
2 g	D-Pantothenic acid Ca salt
1 mg	Biotin (Dissolve 100mg in 1000ml glass distilled water and add 10 ml and store the rest of biotin at -20 °C)

Make up to 1L with glass distilled water

UMS medium and agar

Universal Minimal Salts (UMS) which is modified from previously described AMS as follows; EDTA Na_2 1 µM, $CoCl_2.6H_2O$ 4 µM, $CaCl_2.2H_2O$ 510 µM and $FeSO_4.7H_2O$ 40 µM.

***Composition of Murashige and Skoog salts and vitamins**

Component	**mg/L**	**Vitamins**	**mg/L**
Ammonium nitrate	1650.0	Glycine	2.00
Boric acid	6.2	Myo-Inositol	100.00
Calcium chloride anhydrous	332.2	Nicotinic acid	0.50
Cobalt chloride • $6H_2O$	0.025	Pyridoxine HCl	0.50
Cupric sulfate • $5H_2O$	0.025	Thiamine HCl	0.10
Na_2-EDTA	36.70		
Ferrous sulfate • $7H_2O$	27.8		
Magnesium sulfate	180. 54		
Manganese sulfate • H_2O	16.9		
Molybdic acid (sodium salt) • $2H_2O$	0.25		
Potassium iodide	0.83		
Potassium nitrate	1900.0		
Potassium phosphate monobasic	170.0		
Zinc sulfate • $7H_2O$	8.6		

2.13 454 sequencing strategy for the compost experiment and comparison of efficiency against the sequencing performed for the sand experiment

DNA samples collected during compost experiment were submitted for 454 pyrosequencing. As in the sand experiment, the bacterial and fungal communities were assessed using the same barcoded primers. However, in case of compost, bacterial and fungal PCR products were mixed in the ratio of 3:1 before submitting. It was expected that fungi will have a simpler structure and deep sequencing is not necessary for analysis of the dominant fungal species. The assumption proved to be true and the OTU rarefaction curves show that with 54701 full reads in total (including mutants rhizospheres) the number of OTUs at the similarity level of 95% was still relatively away from a plateau (Figure 2.8.4). The sequencing statistics are presented in Table 2.13.1. Even though the amount of DNA after PCR amplification was quantified and standardized for all the samples some of them were sequenced insufficiently. All the bacterial samples have 1000 reads or more and the number of reads is consistent between the samples. However some fungal samples have a very low yield in the number of reads obtained (U100 1A has only 25 reads), while the average is 521 reads per sample.

bacteria				fungi			
A1A	1968	P1A	2141	A1A	819	P1A	747
A1B	3027	P1B	2057	A1B	767	P1B	788
A1C	2940	P1C	1645	A1C	668	P1C	78
A2A	2145	P2A	1817	A2A	383	P2A	739
A2B	1899	P2B	1877	A2B	371	P2B	493
A2C	1868	P2C	1814	A2C	753	P2C	691
A3A	2242	P3A	2132	A3A	174	P3A	548
A3B	2678	P3B	2864	A3B	853	P3B	943
A3C	1839	P3C	1943	A3C	27	P3C	942
B1A	2336	U1001A	1573	B1A	317	U1001A	25
B1B	2379	U1001B	1822	B1B	735	U1001B	389
B1C	3310	U1001C	2172	B1C	936	U1001C	625
B2A	2120	U1002A	1870	B2A	462	U1002A	509
B2B	2252	U1002B	1462	B2B	476	U1002B	315
B2C	1819	U1002C	1571	B2C	527	U1002C	438
B3A	2264	U1003A	1724	B3A	677	U1003A	722
B3B	2319	U1003B	1892	B3B	658	U1003B	781
B3C	2149	U1003C	2179	B3C	581	U1003C	787
Br1A	2081	U501A	2110	Br1A	554	U501A	599
Br1B	1606	U501B	2400	Br1B	481	U501B	565
Br1C	1803	U501C	2131	Br1C	594	U501C	741
Br2A	1843	U502A	2403	Br2A	42	U502A	543
Br2B	1664	U502B	1950	Br2B	106	U502B	245
Br2C	1732	U502C	2453	Br2C	162	U502C	642
Br3A	1623	U503A	1956	Br3A	649	U503A	453
Br3B	1725	U503B	1848	Br3B	87	U503B	419
Br3C	1395	U503C	2189	Br3C	288	U503C	349
M1A	2037	W1A	1556	M1A	463	W1A	129
M1B	2268	W1B	1482	M1B	321	W1B	571
M1C	1906	W1C	1879	M1C	589	W1C	575
M2A	1169	W2A	1361	M2A	63	W2A	590
M2B	2890	W2B	1455	M2B	727	W2B	284
M2C	2370	W2C	1706	M2C	553	W2C	451
M3A	1772	W3A	2338	M3A	538	W3A	697
M3B	2291	W3B	2081	M3B	623	W3B	789
M3C	2106	W3C	2748	M3C	397	W3C	898

Table 2.13.1 Number of full reads (containing both primer sequence) for the compost experiment. A=*Arabidopsis*, M=*Medicago*, B=*Brachypodium*, U50=unplanted 50ml, Table Br=turnip, P=pea, W=wheat, U100=unplanted 100ml, 1,2,3=generation number, A,B,C=replicate number

192 samples in total were sent for 454 sequencing using 3/4 of a 454 plate for all the sequencing. Based on sequencing efficiency during the sand experiment, the aim was to obtain at least 2000 reads for every bacterial sample and 500 for every fungal sample. In most cases these conditions were met (Table 2.13.1). Some comparison of sequencing efficiency between the sand and compost experiments is provided in Figure 2.13.1. Sequencing efficiency was slightly higher in the compost experiment. Sequencing was performed using the same chemistry so the differences may only be the result of chance.

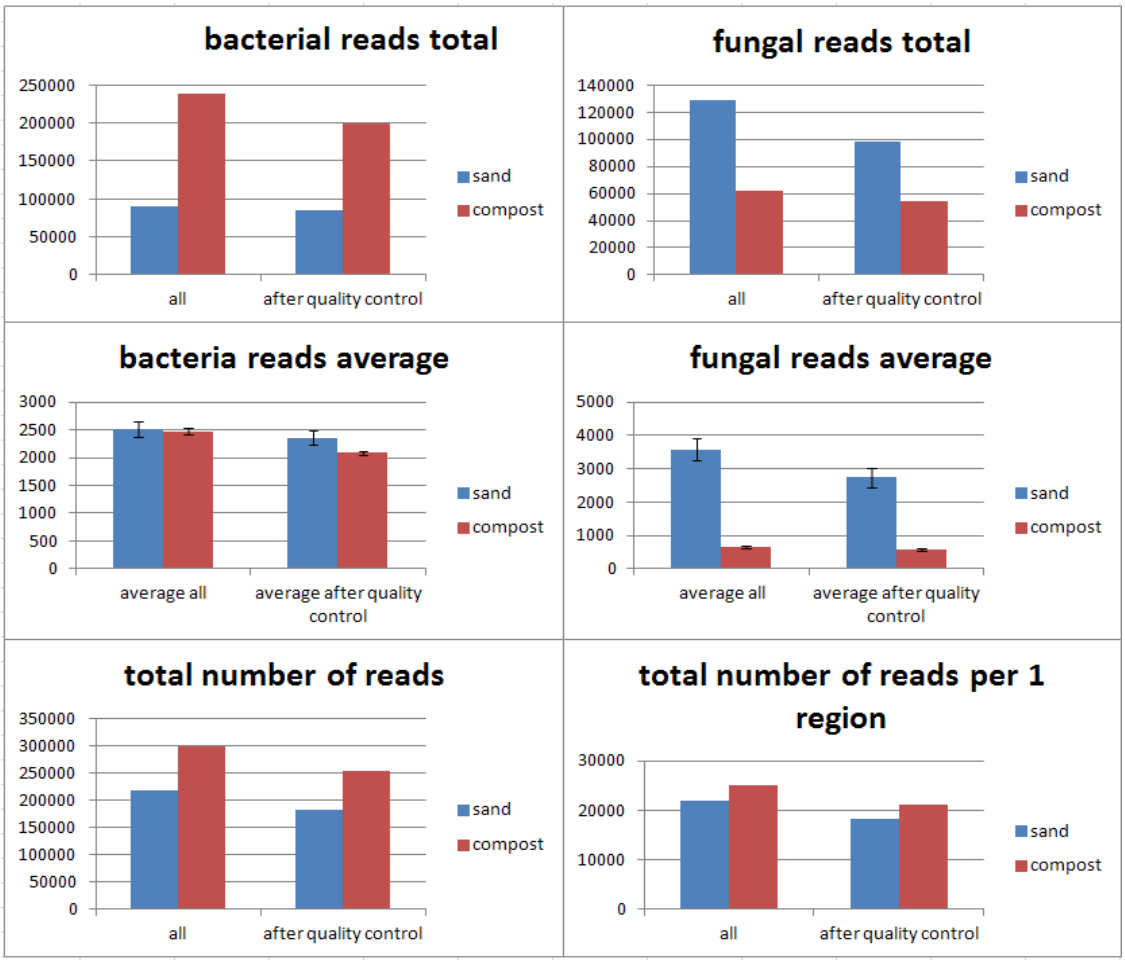


Figure 2.13.1 Comparison of sand and compost experiments 454 sequencing efficiency.

Chapter 3: Assessing bacterial and fungal rhizosphere succession for plants grown in sand using ARISA and 454 pyrosequencing

3.1 Introduction

The aim in the first experiment was to assess changes in the rhizosphere community between model plant species over generations. Three model plants were chosen. *Arabidopsis thaliana* Col-0 (thale cress) is a well-studied annual plant belonging to the Brassicaceae family. It is however unusual in that it does not form mutualistic relations with mycorrhiza. *Medicago truncatula* A17-Jemalong (barrel medic) is a model legume that can be nodulated and form mycorrhizal interactions. *B.distachyon* Bd21 (purple false brome) is a model plant for grasses. It can form mycorrhizal interactions, but cannot be nodulated. The species were chosen because the genomes of these plants have been successfully sequenced (The_Arabidopsis_Genome_Initiative, 2000; Vogel, 2010; Young *et al.*, 2011) and are widely used in plant–microbe research. Moreover, they grow easily in closed pots and have a compact physical stature. The aims of the experiment were to determine:

1. Is the rhizosphere microbial community different from the community of the bulk soil (later called unplanted control);

2. Are there differences between the rhizosphere microbiomes of different plant species,

3. Does the rhizosphere community change over successive plant generations.

The first question has already been partly answered by studying rhizosphere influence on the bacterial and fungal community. The most powerful studies describe the *Arabidopsis* and maize influence on the rhizosphere bacterial community (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012; Peiffer *et al.*, 2013). However at the time of starting this PhD experiments not much was known about the differences between rhizosphere and bulk soil. Existing studies were done either using only fingerprinting methods and/or using low number of biological replicates, which are essential in order to gain statistical power in analysing such complicated environment (Offre *et al.*, 2007).

The second question we wanted to answer was also partly answered by studying rhizosphere of strawberry and oilseed rape (Costa *et al.*, 2006) and arctic grass and perlworts (Teixeira *et al.*, 2010). The novelty of this PhD research is looking into differences between microbial communities using model plants. Model plants have advantage in studying their influence on the rhizosphere as more and more is known about their root excretome (Badri & Vivanco, 2009; Micallef *et al.*, 2009). Future research, which aims to connect plant root secretion compounds to the change in the microbial structure around the roots, will give a powerful new opportunity for plant-microbes interaction studies. Moreover, results presented in this chapter are coming from plants grown in controlled environment, which minimize the difference in pH, temperature, water content, etc., normally encountered in the real farm conditions.

Actually, the most interesting question to answer was the third one: what are the changes in the rhizosphere community over successive plant generations. The only published work on that topic was studied by (Swenson *et al.*, 2000) and (Badri *et al.*, 2009), where the authors present the changes in the rhizosphere community of a few *Arabidopsis* ABC-transport mutant over two generations.

In order to simplify the system, an initial 10 % of soil inoculum was mixed with autoclaved silver sand (v/v). Soil is an extremely rich and diverse environment in the microbiology and geochemistry aspect. As microbiome succession was at the focus of this work, it was decided to impoverish the growth system so the microbiome would have to rely more strongly on the plant derived compounds (i.e. carbon, nitrogen, vitamins, amino acids, etc.). Moreover a similar approach was used by Swenson et.al. in his work, where he examined the influence of soil microbiome on the plant growth over multiple generations (Swenson *et al.*, 2000). The main idea behind this multigeneration experiment was to test if there are any rhizosphere community changes over plant generations, as was assumed in Swenson's work. Three model plants were tested in order to support the findings, i.e. if the community change is depended on the plant species and if so, what are the differences between different plant species. After 4 weeks of plant growth, a rhizosphere sample was taken for DNA isolation and the remaining soil (25 %) was used to inoculate the successive generation.

In this case the amount of soil carbon that is not being deposited by a plant growing in the previous generation drops 4 times. It means that in the first generation there was 10 % of farm soil, but only 2.5 % in the second one (4 times less) and only 0.6 % in the third one (again 4 times less – comparing to the second generation). This mechanism should cause the microbial population to be more and more dependent on the host plant root exudates in every successive generation (Figure 3.1.1). This effect should select for fast-growing plant dependant bacterial and fungal species. This approach was designed to test for plant selection of microbial communities. It did not have the aim of explaining or mimicking the real field community changes under monoculture growth.

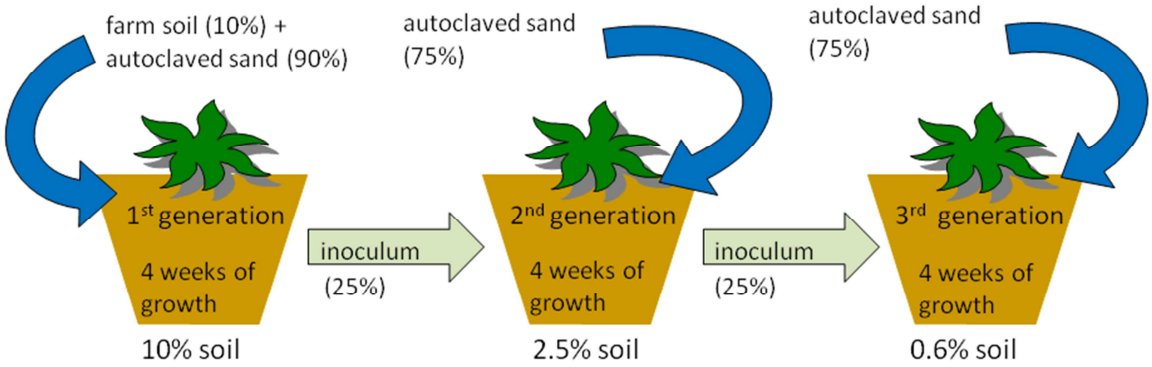


Figure 3.1.1 The sand experiment design. Plants were watered with N-free nutrient solution (5 ml) once a week. On the planting day KNO_3 (0.5 mg) and one week later additional 9.5 mg of KNO_3 was added (plants could be "burned" if all nitrogen was added to the seedlings).

The three model plants were grown for three generations. Unplanted soil/sand was used as a control and was maintained (generation preparation, watering, temperature etc.) in the same way as the rhizosphere samples. At the end of each generation, rhizosphere soil (see chapter 2 for more details about sampling procedure) was used for microbial DNA isolation. An unplanted control lines (24 lines) was started at the same time as the rhizosphere lines. DNA samples for the unplanted control were isolated from a well mixed soil/sand mix (the same concentration of soil to the sand as in the rhizosphere lines) after removal of the top 1 cm of the soil/sand. In total 288 samples were obtained ((3 plant species + unplanted) x 3 generations x 24 biological replicates). ARISA analysis was successfully carried out on 255 of these samples, as a small number of samples were discarded due to various problems.

ARISA allowed for fast and cost-efficient characterization of bacterial communities. However, it is only a fingerprinting method and does not allow annotation of the bacterial species. In order to do that, 454 Roche ("next-generation") pyrosequencing was used. This is more expensive than ARISA so it was impossible to analyse all the samples separately. The replicates (up to 24) from each plant in each generation were pooled into 3 pseudo-replicates. Throughout the experiments the same method of pooling was used: samples 1-8, 9-16 and 17-24 were pooled into 3 pseudo-replicate samples. Pooled DNA samples were amplified for the 16S rRNA gene using 27F and 338R primer pairs (based on *E.coli* 16S rRNA gene sequence) and for the fungal Intergenic Spacer Region (ITS) using ITS1 and ITS2 primer pair (Buee *et al.*, 2009). DNA samples (one for each kingdom) were amplified with 36 different MID barcoded primers (Table S2) for sand grown plants. Barcoding was essential in order to separate the reads into their corresponding samples after sequencing. Bacterial and fungal sequences were easily separated by their target primers (either 338R or ITS1 primer sequence). Sequencing statistics are presented in the Table 4.2.21.

These two methods complement each other; multi-replicate ARISA was used to gain statistical power in order to separate bacterial communities, while 454 sequencing was used to identify the organisms present in the community. ARISA data allows for community analysis at the strain level, while 454 sequencing data was used to analyse the communities at the genus (OTU data) and phylum level. Importantly, both methods were run using the same DNA samples, so the results obtained can be directly compared.

3.2 Results and Discussion

3.2.1 ARISA and pyrosequencing analysis of bacterial succession over 3 generations of plants grown in sand

In the first generation the rhizosphere communities of each of the three plants were different from unplanted bulk soil. In addition there was a clear plant species influence as data points belonging to a particular plant clustered together (Figure 3.2.1). The *Brachypodium* rhizosphere was more diverse as points are more scattered and more distant

from other samples. *Medicago* and *Arabidopsis* rhizospheres appear relatively similar to each other.

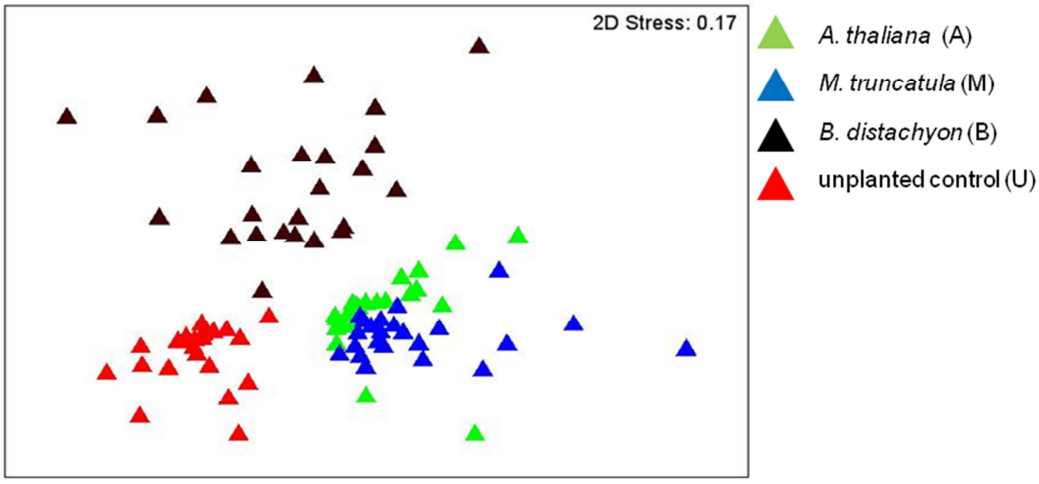


Figure 3.2.1 MDS plot of ARISA fingerprints of the bacterial community of the 1st generation of model plants. Each triangle represents the relative position of the rhizosphere community structure of a single plant.

The visual impression of the MDS plots is confirmed by MANOVA statistics (Table 3.2.1). Table 3.2.1 shows how many data points from each group are needed in order to show significant difference between these groups. For most comparisons 4 or 5 data points taken at random is enough, apart from *Arabidopsis* – *Medicago* group that needs at least 8 data points in order to be successfully differentiated. However the most important thing is that all different rhizosphere and bulk soil communities are significantly different from each other.

	A1	M1	B1	U1	A2	M2	B2	U2	A3	M3	B3	U3
A1		8	5	4	4	4	4	4	4	4	4	4
M1	8		5	5	4	4	4	4	4	4	4	4
B1	5	5		5	4	5	4	4	4	4	5	4
U1	4	5	5		4	5	4	4	4	4	4	5
A2	4	4	4	4		7	5	5	4	6	5	4
M2	4	4	5	5	7		10	6	5	7	6	5
B2	4	4	4	4	5	10		5	5	6	9	4
U2	4	4	4	4	5	6	5		10	6	5	5
A3	4	4	4	4	4	5	5	10		5	5	5
M3	4	4	4	4	6	7	6	6	5		12	8
B3	4	4	5	4	5	6	9	5	5	12		6
U3	4	4	4	5	4	5	4	5	5	8	6	

Table 3.2.1 Pairwise MANOVA analysis of the sand experiment rhizosphere communities ($p<0.05$). The MANOVA test consists of repetitive F-tests performed on two groups of samples using defined number of replicates within each sample. For each comparison the F-test was repeated 1000 times. Number indicate how many samples from each of the groups is needed for all the performed F-tests to reach significance level ($p<0.05$). Colour was used for better visualization of the results.

A- *Arabidopsis* rhizosphere community,

M- *Medicago* rhizosphere community,

B- *Brachypodium* rhizosphere community,

U - unplanted soil community,

1,2,3 – generation number

In the second generation, the bacterial communities in the unplanted control samples became more diverse, while still remaining different from the rhizospheres of plants (Figure 3.2.2 and Table 3.2.1). Plant rhizosphere samples formed tighter clusters. In the first generation *Arabidopsis* and *Medicago* rhizosphere samples were clustered closely together. However in the second generation *Brachypodium* and *Medicago* samples are located together. MANOVA analysis needs 10 data points chosen at random to separate the *Medicago* and *Brachypodium* rhizosphere communities. Despite the relative similarities between some groups of samples, all rhizosphere lines are statistically different from each other and from the unplanted control.

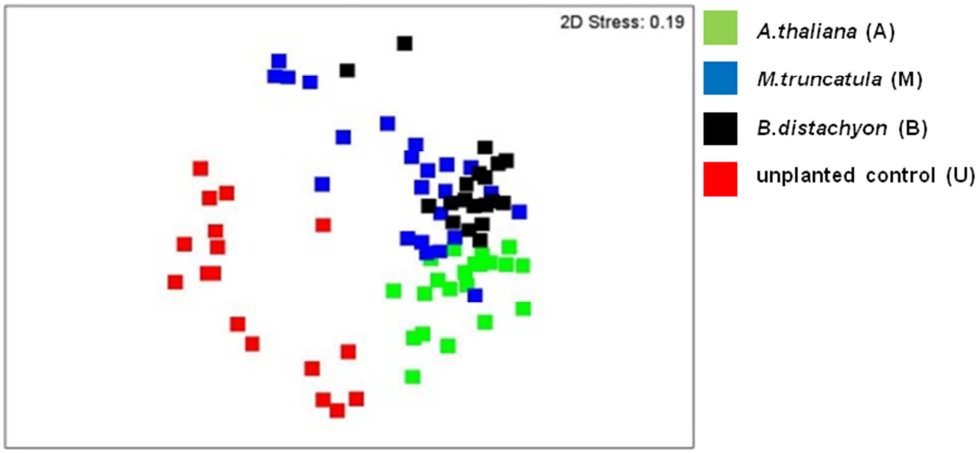


Figure 3.2.2 MDS plot of the ARISA fingerprints of the bacterial community of the 2nd generation of model plants.

In the 3rd generation communities of the unplanted controls were all very similar (Figure 3.2.3). The *Arabidopsis* community samples clustered tightly together, with the diversity between samples was smaller than for *Medicago* and *Brachypodium* samples. *Medicago* and *Brachypodium* rhizospheres continued to be more similar to each other, with MANOVA analysis revealing 12 data points are needed to separate them (Table 3.2.1). Again, as in the case of the 2nd generation, all the rhizosphere lines are statistically different from each other.

It is necessary to mention that the growth of *Arabidopsis* plants in the final generation was impaired (Figure 3.2.3 B). Plants were much smaller than in the previous generations.

Unfortunately, the plant biomass data was not collected during this experiment. As the nutrition level was similar and all the essential plant macro- and micronutrients were provided this growth difference is probably caused by underlying microbial processes in the rhizosphere. Of course, it can not be excluded that some important, but rare micronutrient that is absent from the plant mineral solution was a limiting factor for *Arabidopsis* growth (but not for *Medicago* or *Brachypodium*).

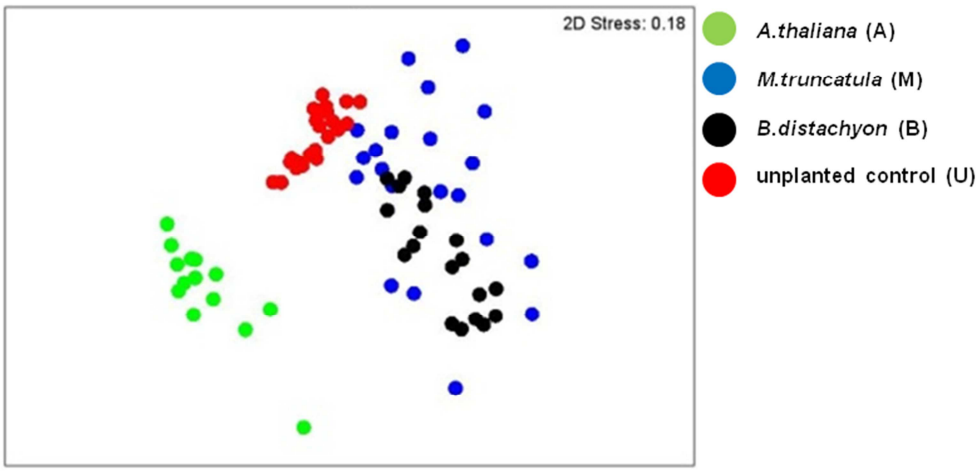


Figure 3.2.3A MDS plot of ARISA fingerprints of the bacterial community of the 3rd generation of model plants.

Figure 3.2.3 B *Arabidopsis* growth over three generations. From the left: 1st, 2nd and the 3rd generation. Pictures were taken 4 weeks after planting on the day of sampling.

In order to show the community shift over multiple generations, all data points were combined into one MDS plot (Figure 3.2.4). The large number of data points makes these

plots difficult to interpret, so the three generations have been marked using coloured ovals that enclose data from each generation. In order to simplify the graphical representation, data from 24 replicates were binned into 3 pseudo-replicates (Figure 3.2.5). The diversity for each of the subsample is shown as standard error bars (SE represent the variation on XY surface of MDS). In the case of the 1st generation and a few other pseudo-replicates the SE is so small that it is hidden beneath the plotted symbols. Most importantly binning did not change the underlying pattern or interpretation of the ARISA data.

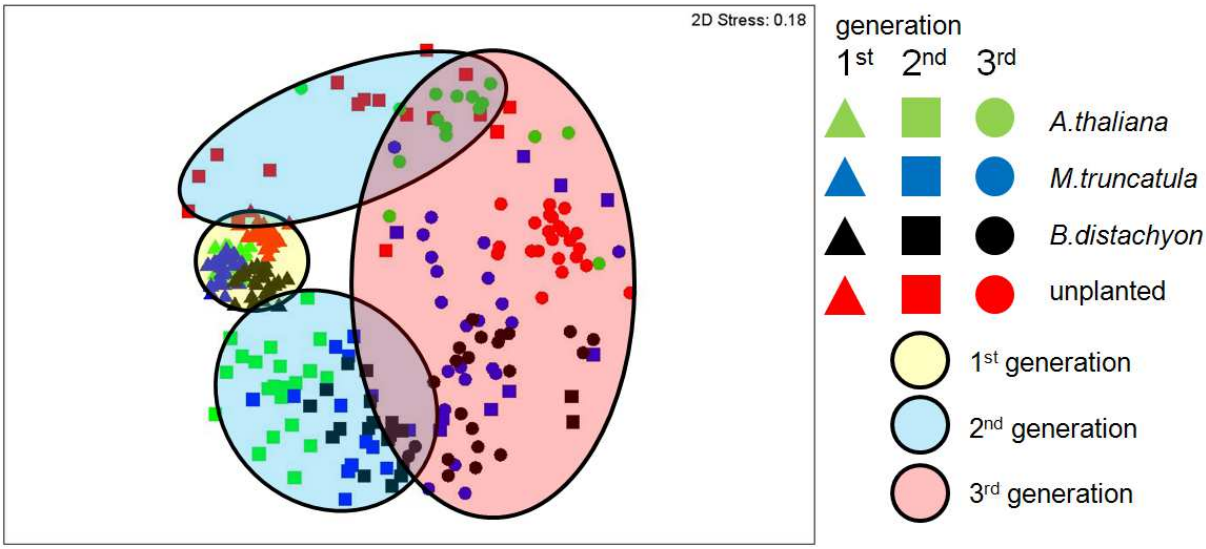


Figure 3.2.4 MDS plot of ARISA fingerprints of the bacterial community showing all 3 generation of model plants in the sand experiment using all data points.

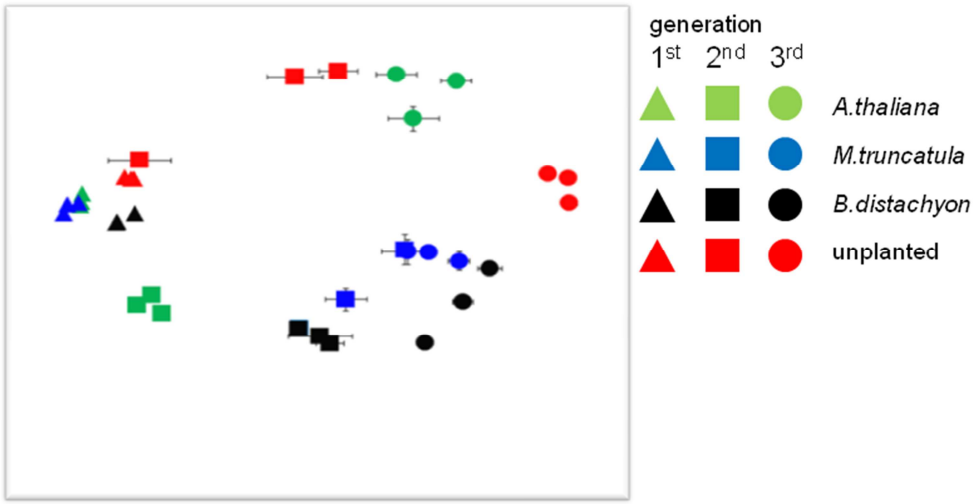


Figure 3.2.5 MDS plot of ARISA fingerprints of the bacterial community showing all 3 generation of model plants in the sand experiment using 3 pseudo-replicates of ARISA fingerprints for each plant in each generation. Standard errors are calculated for variations of location of data points on the 2D MDS surface. To simplify comparisons, the ARISA data have been combined into bins that correspond to the same groups of samples that were pooled before pyrosequencing (see later in this chapter).

The first generation communities are more similar to each other than to those in the 2nd and 3rd generations. Furthermore, the *Medicago* and *Brachypodium* rhizosphere become very different in the second generation from *Arabidopsis* and unplanted control. In the 3rd generation, *Medicago* and *Brachypodium* communities stay relatively similar to each other and to their previous generation, while *Arabidopsis* and unplanted control community change significantly compared to the second generation.

As is the case for ARISA, 454 data binned into OTUs can be used to construct MDS plots. Instead of ISR fragment size and fluorescence intensity, for 454 data there is OTU number and its abundance (number of reads classified as a particular OTU). However, as only 3 samples were analyzed for every plant in each generation, data points on MDS plots do not have standard errors. MANOVA tests were not run because of low number of replicates

The change of community structure over generations obtained using 454 sequencing (Figure 3.2.6) shows a very similar overall picture to the one based on ARISA data (Figure 3.2.5). In

the first generation all samples were clustered together. In the second generation the rhizosphere of communities of all three plants are similar to those of the first generation, while the unplanted control becomes very different. As in the case of ARISA, one replicate of unplanted control in the 2nd generation clusters relatively close to the 1st generation group. In the 3rd generation the unplanted control is still very different from the rhizosphere. The *Arabidopsis* rhizosphere community became similar to unplanted control. This observation not only confirms the overall community structure in the sand experiment using ARISA, but also validates it as an excellent method for analysing community structure.

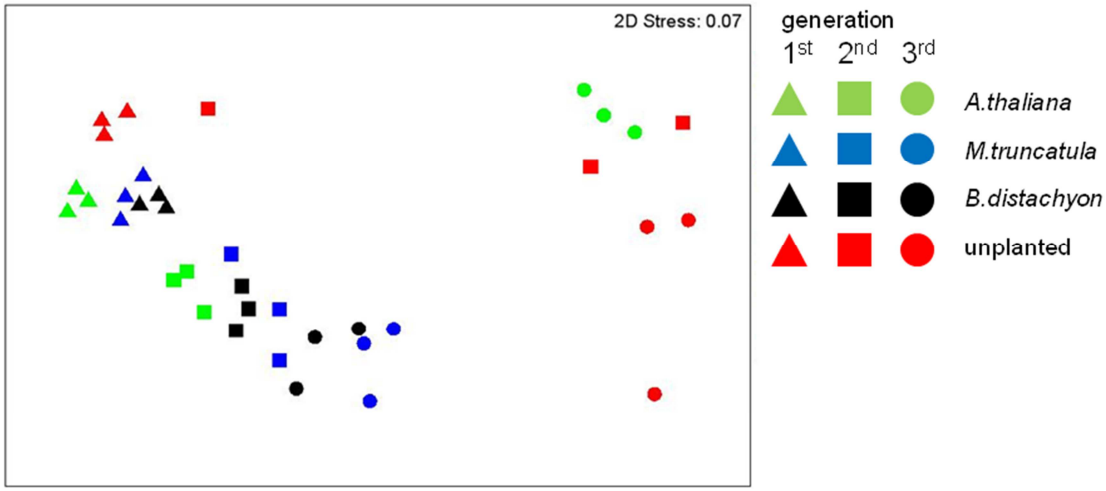
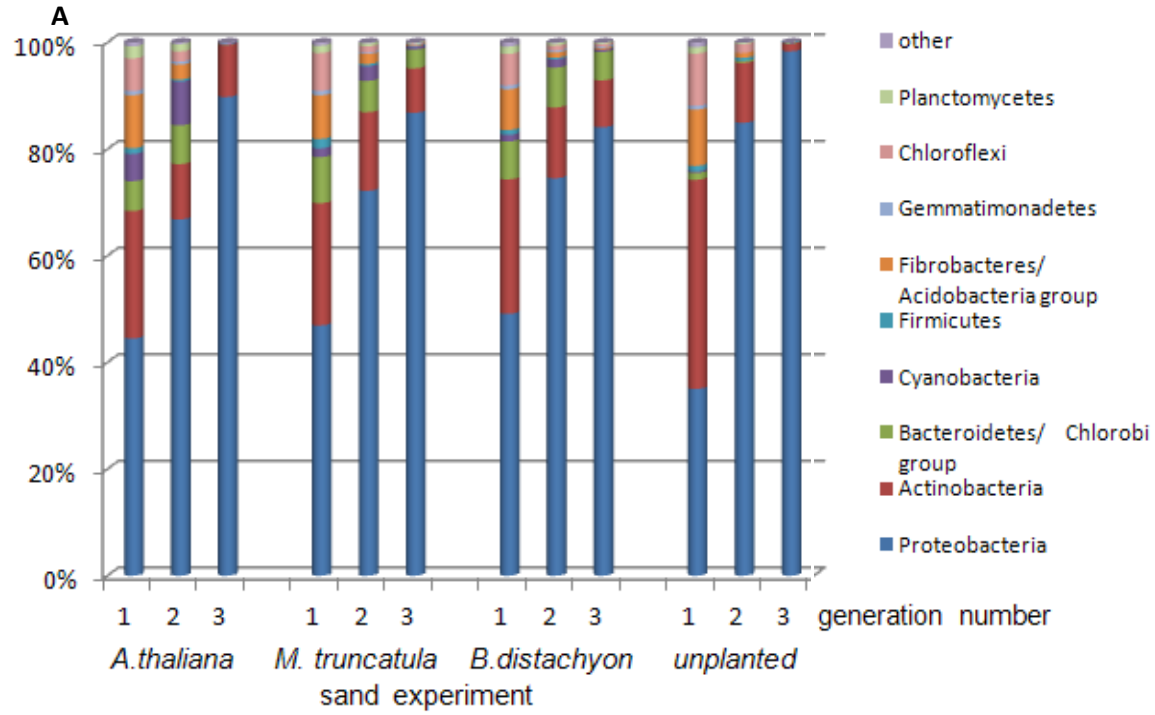


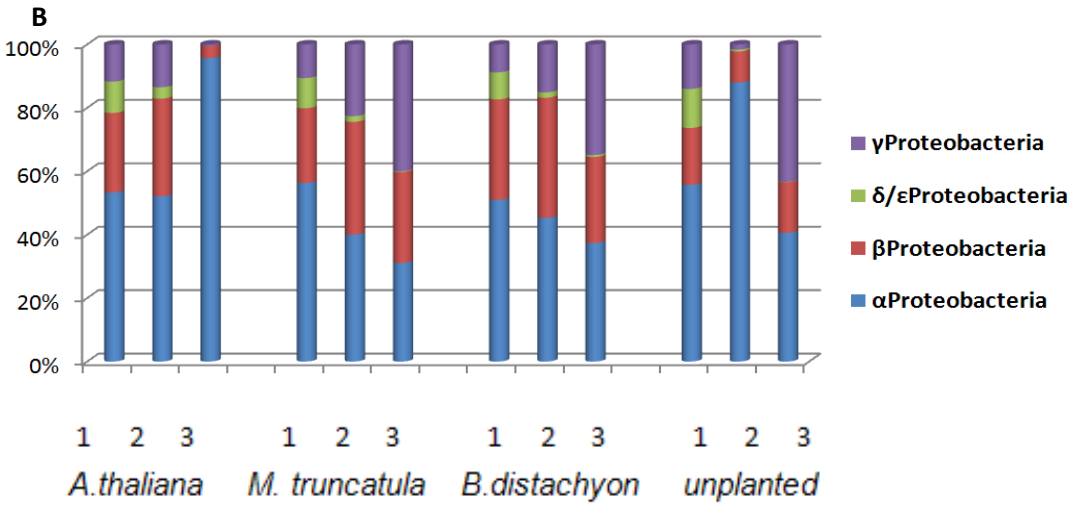
Figure 3.2.6 MDS plot of the bacterial community of plants grown in sand. Community structure is derived from bacterial OTU abundance (454 sequencing data).

Overall, growth of plants in sand showed a strong plant species selection of rhizosphere bacterial communities. Even though the dominant force shaping the communities over the generations (Table 3.2.1) is the effect of time, probably in turn driven by the dilution of soil with Silver sand, plants are able to modify that pattern and select particular bacterial species. The comparison of the power of plants in each generation to modify the community and the influence of the generations can be made using pairwise MANOVA analysis. MANOVA analysis shows that generations are the major force behind community changes as only 4 or 5 replicates from each individual plant species and unplanted control are enough to separate the community between generations (Table 3.2.1). While MANOVA

analysis performed on each individual generation between different plant species (Table 3.2.1) requires 4-12 replicates to show a significant difference.

The phylum Proteobacteria was dominant throughout the whole experiment. Its dominance increased over generation starting from ~40 % in the 1st generation and reaching ~90 % in the 3rd generation in the rhizospheres and almost 100 % in the unplanted control (Figure 3.2.7 A). The increasing dominance of Proteobacteria over generations correlates with the diversity collapse (explained below). Even though the increase in Proteobacteria is not statistically significant, the fold difference between generations is clear. The second most abundant phylum was Actinobacteria. In the first generation the abundance of this phylum reached ~20 % in the rhizospheres and more than 30% in unplanted control. As described below (Figure 3.2.12) many OTU assigned to Actinobacteria were more abundant in the unplanted control. The other dominant phyla were Bacteroidetes, Cyanobacteria and Acidobacteria. Due to the relative increase in Proteobacterial abundance all the other phyla decreased in the successive generation. The *Arabidopsis* rhizosphere in the 3rd generation and unplanted control in the 2nd and 3rd generation were totally dominated by Proteobacteria. However, the *Arabidopsis* rhizosphere was very different from unplanted control at the subphylum level (Figure 3.2.7 B). In the case of *Medicago* and *Brachypodium* rhizospheres, Gammaproteobacteria become more and more dominant.


A
100%
80%
60%
40%
20%
0%
other
Planctomycetes
Chloroflexi
Gemmatimonadetes
Fibrobacteres/ Acidobacteria group
Firmicutes
Cyanobacteria
Bacteroidetes/ Chlorobi group
Actinobacteria
Proteobacteria
1 2 3 1 2 3 1 2 3 1 2 3 generation number
A.thaliana M. truncatula B.distachyon unplanted
sand experiment


B
100%
80%
60%
40%
20%
0%
γProteobacteria
δ/εProteobacteria
βProteobacteria
αProteobacteria
1 2 3 1 2 3 1 2 3 1 2 3
A.thaliana M. truncatula B.distachyon unplanted

C

phylum	A1	M1	B1	U1	A2	M2	B2	U2	A3	M3	B3	U3
Chloroflexi	c	bc	abc	c	abc	a	a	ab	a	a	a	a
Gemmatimonadetes	d	d	cd	bcd	abcd	abcd	ab	abcd	abc	abc	a	a
Acidobacteria	c	c	bc	bc	ab	a	a	a	a	a	a	a
Firmicutes	bc	c	abc	abc	abc	abc	abc	abc	ab	abc	a	ab
Cyanobacteria	b	a	a	a	c	a	a	a	a	a	a	a
Bacteroidetes	ab	b	ab	a	b	ab	b	a	a	ab	ab	a
Actinobacteria	b	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	a
Proteobacteria	ab	ab	ab	a	ab	ab	b	ab	ab	ab	ab	ab

subphylum	A1	M1	B1	U1	A2	M2	B2	U2	A3	M3	B3	U3
αProteobacteria	ab	ab	ab	ab	ab	ab	ab	b	ab	a	ab	ab
βProteobacteria	a	a	ab	a	ab	ab	b	a	a	ab	ab	a
δ/εProteobacteria	e	de	cde	abcd	bcde	abc	abcd	ab	a	a	ab	a
γProteobacteria	ab	ab	a	ab	ab	ab	ab	a	a	ab	ab	b

Figure 3.2.7 Community structure at A) phylum level, B) subphylum level of Proteobacteria for the sand experiment obtained by 454 sequencing – values averaged from 3 replicates, C and D) One-way ANOVA with Tukey test (significance level 0.05).

The diversity of ISR fragments decreased over generations (Figure 3.2.8 A, B). The diversity collapse was stronger in the unplanted control than in the rhizospheres. Decreased numbers for richness and diversity over generations indicates that some bacterial species/strains were highly selected.

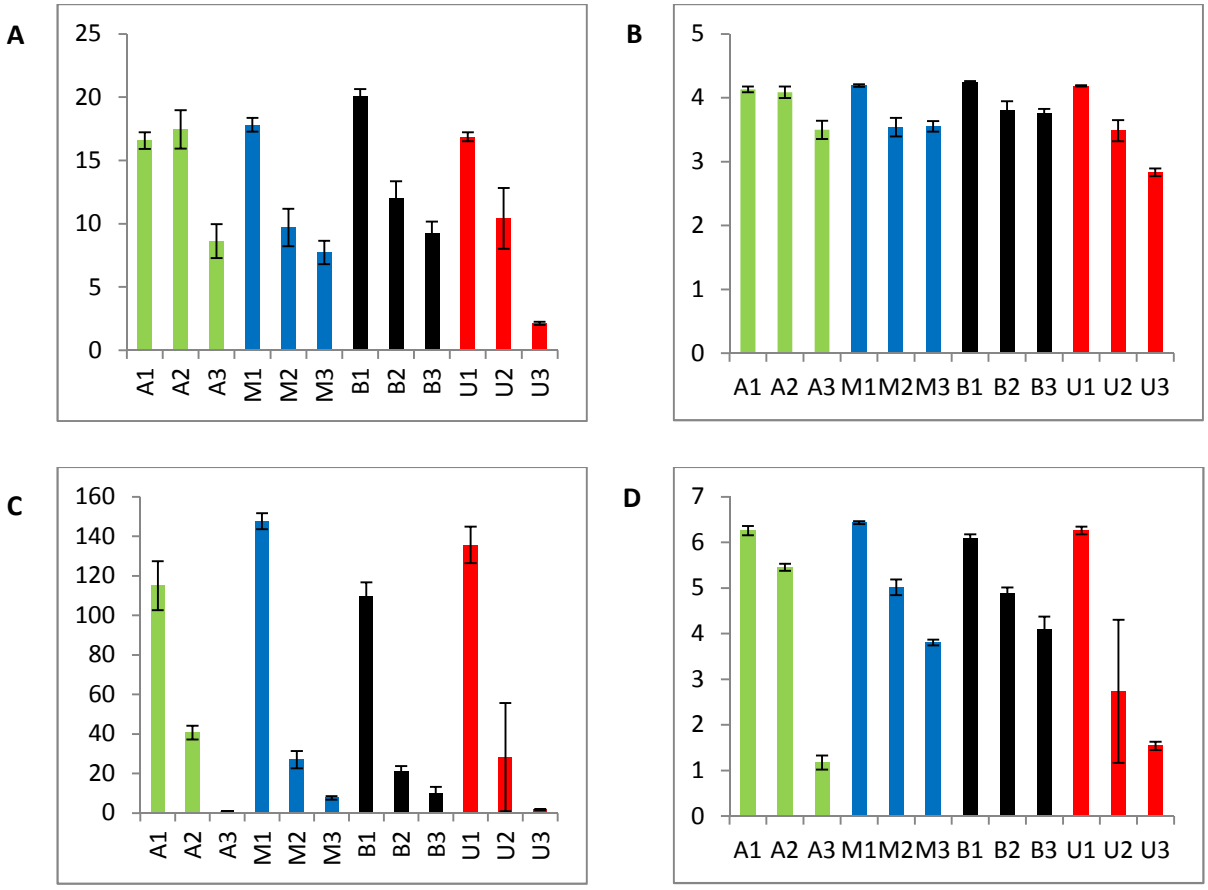


Figure 3.2.8 Richness (A, C) and Shannon diversity index (B, D) for ARISA (A and B) and 454 sequencing data (C and D). Richness is the number of different ISRs or OTUs needed to reach 50% of the total fluorescence/OTU abundance. Error bars ±SEM.

Paired test ($p<0.05$) results (X - X not significant, X -*- X significant), for ARISA n=16,24 for 454 data n=3 for each plant/unplanted in each generation.

A) A1 - A2 -*- A3, M1 -*- M2 - M3, B1 -*- B2 - B3, U1 -*- U2 -*- U3

A1 – U1	A2 -*- U2	A3 -*- U3
M1 – U1	M2 – U2	M3 -*-U3
B1 -*- U1	B2 – U2	B3 -*- U3

B) A1 - A2 -*- A3, M1 -*- M2 - M3, B1 -*- B2 -*- B3, U1 -*- U2 -*- U3

A1 – U1	A2 -*- U2	A3 -*- U3
M1 – U1	M2 – U2	M3 -*-U3
B1 - U1	B2 – U2	B3 -*- U3

C) A1 -*- A2 -*- A3, M1 -*- M2 -*- M3, B1 -*- B2 -*- B3, U1 -*- U2 - U3

A1 – U1	A2 - U2	A3 - U3
M1 – U1	M2 – U2	M3 -*-U3
B1 - U1	B2 – U2	B3 -*- U3

D) A1 -*- A2 -*- A3, M1 -*- M2 -*- M3, B1 -*- B2 -*- B3, U1 - U2 - U3

A1 – U1	A2 - U2	A3 - U3
M1 – U1	M2 – U2	M3 -*-U3
B1 - U1	B2 – U2	B3 -*- U3

454 sequencing data accords with the ARISA showing the diversity of the bacterial community reduces over generations. The rhizosphere community in the third generation was dominated by a few OTUs in *Medicago* and *Brachypodium* and a single OTU in *Arabidopsis* and the unplanted control. This indicates that *Arabidopsis* was not able to support the community in its rhizosphere or cannot suppress the dominant oligotrophs (the analysis of the dominant bacterial groups is described below), which can also be found in the unplanted control. The diversity collapse at the strain and genus level probably correlates with increased abundance of Proteobacteria in the rhizosphere and bulk soil.

An important aspect in interpreting MDS plots is to identify changes in the abundance of individual ISR sizes and OTUs that contribute most strongly. Analysis of the dominant ISR sizes and OTUs for each plant species in each generation gives the most detailed picture of community changes (Figure 3.2.9 and Figure 3.2.10). Rhizosphere ISR and OTU abundance was compared against their abundance in the unplanted control. Some of the dominant ISRs and OTUs were actively selected in the rhizosphere in the 1st generation. However, it was in the 2nd generation that the abundance of almost all rhizosphere-specific ISRs were highly upregulated. The third generation was dominated by a few ISRs that can be found in the rhizosphere and unplanted control. However, which of these ISRs were most common in the rhizosphere is plant species specific. ISR size of 495.62, 1049.2 and 1050.4 were only present in the *Arabidopsis* rhizosphere, but not *Medicago* or *Brachypodium*. However, ISRs of sizes 579.45 and 669.8 could be found in the *Medicago* and *Brachypodium* rhizosphere, but not in *Arabidopsis*. This observation indicates that plants actively suppress bacterial species/strains in their rhizospheres. *Arabidopsis* depleted bacteria with ISR size of 579.45 and 669.8, while *Medicago* and *Brachypodium* selected against ISR sizes of 495.62, 1049.2 and 1050.4. Even, though these ISR sizes represent some oligotrophic species (as they were also found in the unplanted control), plants modified their abundance and actively selected against them. 454 sequencing data in the analysis described below fully supports this observation. Some ISRs like 548.13 in case of *Brachypodium* are highly plant species specific.

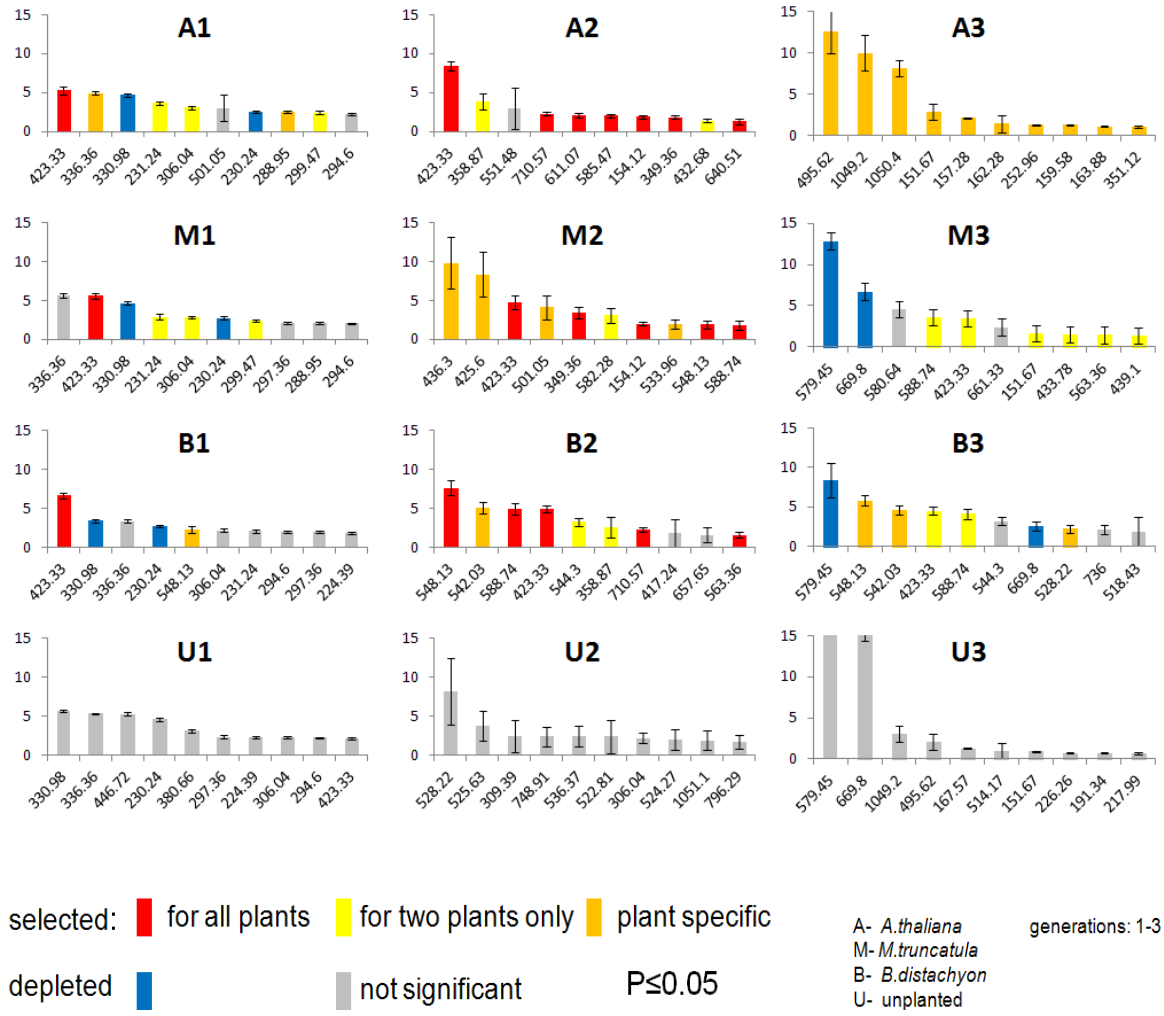
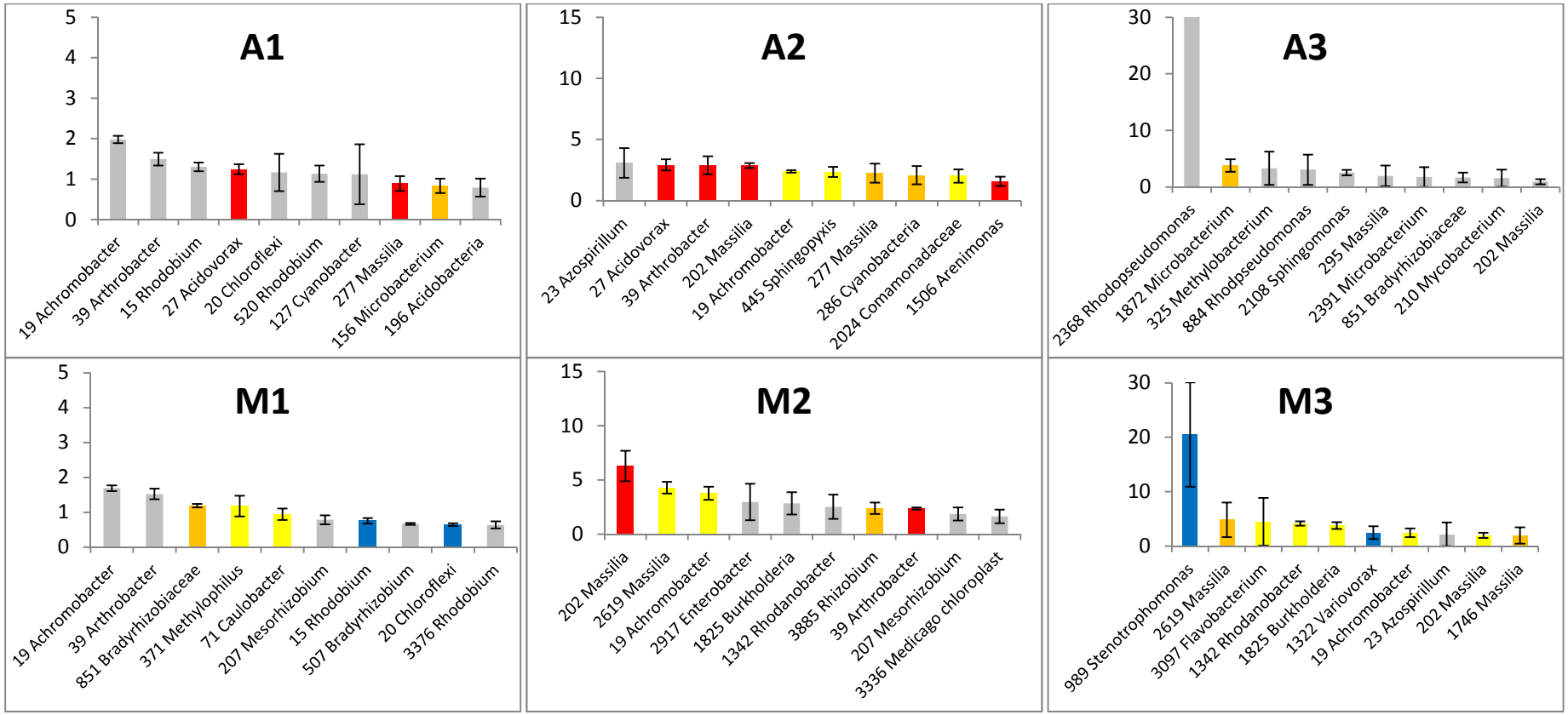


Figure 3.2.9 Bar graphs representing the 10 most abundant ISR sizes found in the rhizosphere of model plants and unplanted soil samples using ARISA method. Vertical axis represents the relative abundance in percentage of the total community. Coloured bars represent rhizosphere selected or depleted ISRs selected using t-test.

Based on the 454 sequencing data the third generation is dominated by OTU 989 *Stenotrophomonas*, 1322 *Variovorax*, 2393 *Pseudomonas* (*Medicago*, *Brachypodium* and unplanted) and 2368 *Rhodopseudomonas* (*Arabidopsis* and unplanted) (Figure 3.2.10). These 4 soil opportunists swept through the plant rhizosphere but even here there was a plant effect as *Arabidopsis* suppressed OTU 989 *Stenotrophomonas*, 1322 *Variovorax* and 2393 *Pseudomonas,* while *Medicago* and *Brachypodium* selected against OTU 2368 *Rhodopseudomonas.* Overall, plant species specific suppression of soil opportunists was observed using the ARISA method.

Some OTUs are very plant specific and were found in multiple generations: OTU 27 *Variovorax*, OTU 202 *Massilia*, OTU 39 *Arthrobacter*, OTU 19 *Achromobacter*, OTU 1342 *Rhodanobacter*. OTU 19 *Achromobacter* and OTU 39 *Arthrobacter* were isolated from the rhizosphere of *Medicago* and Brachypodium and further experiments were conducted (chapter 6).


A1
5
4
3
2
1
0
19 Achromobacter
39 Arthrobacter
15 Rhodobium
27 Acidovorax
20 Chloroflexi
520 Rhodobium
127 Cyanobacter
277 Massilia
156 Microbacterium
196 Acidobacteria
A2
15
10
5
0
23 Azospirillum
27 Acidovorax
39 Arthrobacter
202 Massilia
19 Achromobacter
445 Sphingopyxis
277 Massilia
286 Cyanobacteria
2024 Comamonadaceae
1506 Arenimonas
A3
30
20
10
0
2368 Rhodopseudomonas
1872 Microbacterium
325 Methylobacterium
884 Rhodpseudomonas
2108 Sphingomonas
295 Massilia
2391 Microbacterium
851 Bradyrhizobiaceae
210 Mycobacterium
202 Massilia
M1
5
4
3
2
1
0
19 Achromobacter
39 Arthrobacter
851 Bradyrhizobiaceae
371 Methylophilus
71 Caulobacter
207 Mesorhizobium
15 Rhodobium
507 Bradyrhizobium
20 Chloroflexi
3376 Rhodobium
M2
15
10
5
0
202 Massilia
2619 Massilia
19 Achromobacter
2917 Enterobacter
1825 Burkholderia
1342 Rhodanobacter
3885 Rhizobium
39 Arthrobacter
207 Mesorhizobium
3336 Medicago chloroplast
M3
30
20
10
0
989 Stenotrophomonas
2619 Massilia
3097 Flavobacterium
1342 Rhodanobacter
1825 Burkholderia
1322 Variovorax
19 Achromobacter
23 Azospirillum
202 Massilia
1746 Massilia

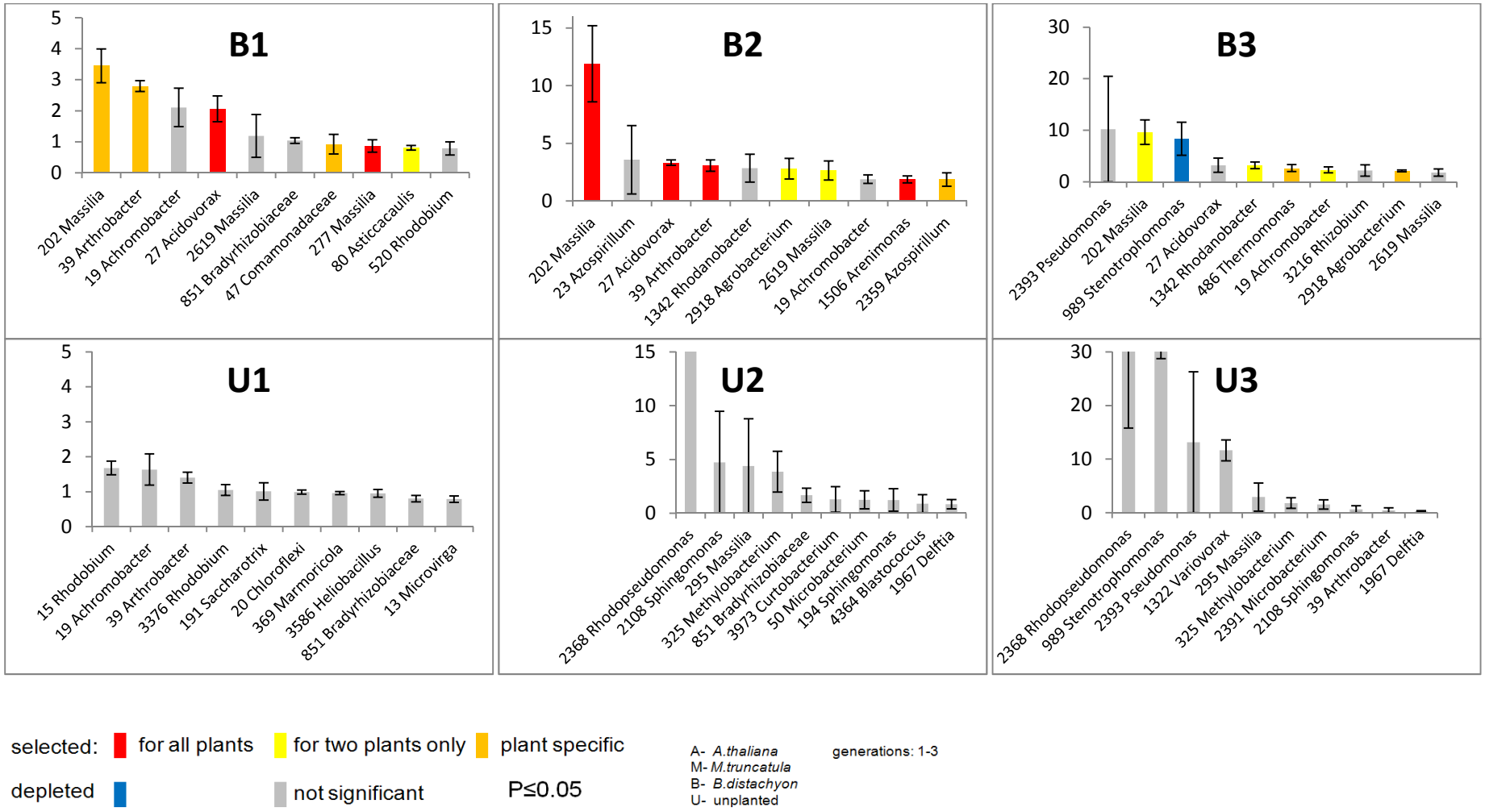


Figure 3.2.10 Bar graphs representing 10 most abundant bacterial OTUs found in the rhizosphere of model plants and unplanted soil samples using 454 sequencing. Vertical axis represents the relative abundance in percentage of the total community. Coloured bars represent rhizosphere selected or depleted OTUs selected using t-test.

ARISA data analysed using 3D ternary plots indicate that the communities were relatively similar to each other in the 1st generation, as most of the ISRs are located in the middle of the triangle and their colour is either green or orange (Figure 3.2.11 A) (for the description of 3D ternary plot please see chapter 2.9.2).

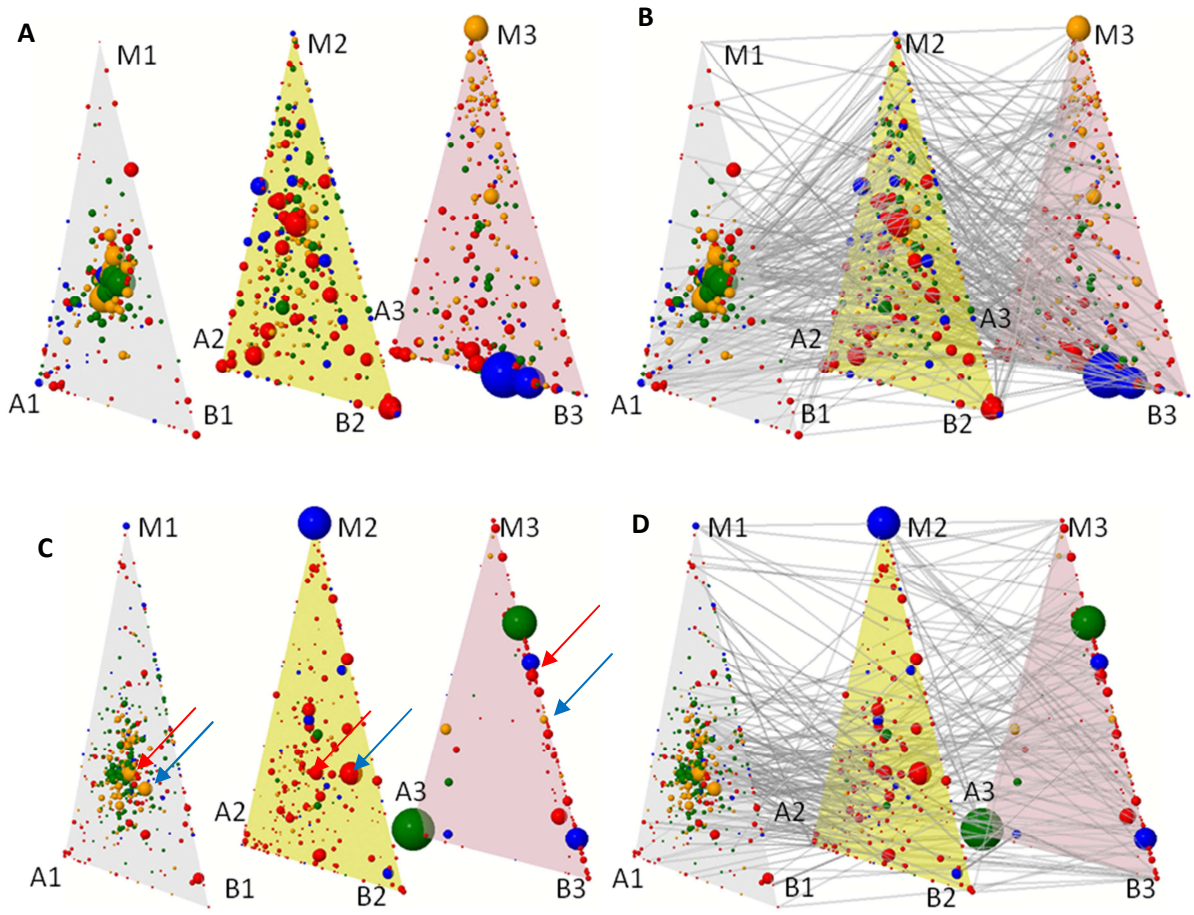


Figure 3.2.11 Three-dimensional ternary plots of the sand experiment using ARISA (A and B) and 454 sequencing (C and D). Plots A and C focus on the community structure. Plot B and D show how many ISRs and OTUs were present in successive generations. Interpretation of the ternary plots is explained in material and methods section. A1, A2, A3, M1, M2, M3 and B1, B2, B3 is *Arabidopsis*, *Medicago* and *Brachypodium* rhizosphere in successive generations, respectively. The dominant ISR or OTU is annotated as a ball of a particular volume (size 8 in Veusz software) and all other ball sizes are calculated relatively to the dominant one. C) Red and blue arrows show the location of OTU 19 *Achromobacter* and OTU 39 *Arthrobacter*, respectively.

However, some ISR are shown as red balls in corners, which are responsible for the observed differences between plant rhizospheres and unplanted control. The second generation was dominated by ISRs annotated as red balls, which are more likely to be located towards the corners of the plots. There is also an increasing number of ISRs annotated as blue balls. Such a pattern indicates that the community was modified separately by each plant species and that the rhizosphere of all plants becomes very different from the unplanted control. The third generation is divided into ISRs annotated as orange balls next to the *Arabidopsis* corner, indicating that the *Arabidopsis* community was different from *Medicago* and *Brachypodium* and relatively similar to unplanted control. There are numerous shared rhizosphere specific ISRs (red balls) on the axis between *Medicago* and *Brachypodium,* consistent with considerable overlap in their communities as shown by MDS plots (Figure 3.2.3). There were also two dominant ISRs annotated as blue balls between *Medicago* and *Brachypodium*. These ISRs were not selected by any of these plants; they were suppressed in the *Arabidopsis* rhizosphere. Most of the ISRs can be tracked from the previous to the next generation (Figure 3.2.11 B).

454 sequencing data confirms the overall bacterial community structure shown by the ARISA data. In the first generation, the rhizosphere and bulk soil shared a similar bacterial community as most of OTUs are represented as green or orange balls (Figure 3.2.11 C). A single generation was not enough to influence the majority of the community. However, there are some highly rhizosphere and plant species dependent OTUs in the corners. In the second generation the rhizosphere became very different from the unplanted control. The plant species influence was more and more pronounced. There was an opportunistic invasion (OTU 2368 *Rhodopseudomonas*) in the unplanted control and the same OTU was common in the *Medicago* rhizosphere in the second generation (big blue ball in the *Medicago* corner). However, it is unlikely that this OTU was selected by *Medicago*, rather it was suppressed in the *Arabidopsis* and *Brachypodium* rhizospheres. This highlights the power of ternary plots to identify the difference between positive selection of a microorganism as opposed to suppression. The *Arabidopsis* rhizosphere in the 3rd generation was dominated by OTU 2368 *Rhodopseudomonas*. This OTU was strongly suppressed in the *Medicago* and *Brachypodium* rhizospheres. It is the same OTU that was found in the 2nd generation of the *Medicago* rhizosphere (annotated as a blue ball). The

Medicago and *Brachypodium* rhizospheres were dominated by OTU 989 *Stenotrophomonas*, 1322 *Variovorax* and 2393 *Pseudomonas*. All these species were probably not actively selected by plants as they were also most common in the unplanted control. However plants were able to suppress different groups of these opportunists. Even though there are invasive invasions from the soil *Brachypodium* and *Medicago* still selected a substantial number of rhizosphere specific organisms (annotated as red balls on the axis between *Medicago* and *Brachypodium* corners). OTU 19 *Achromobacter* and OTU 39 *Arthrobacter* were common in the rhizosphere of *Brachypodium* and *Medicago* throughout the experiment (annotated as red and blue arrows on Figure 3.2.8 C). It indicates that these species are highly rhizosphere specific despite the invasion of soil opportunists. The lines connect OTUs that can be found in successive generations (Figure 3.2.11 D). There are more lines between the 1st and 2nd generation than there are between the 2nd and the 3rd. This indicates that the 1st and 2nd generation share more bacteria. This is probably due to the occurrence of opportunist invasions in the 3rd generation causing the remaining OTUs to become less common and so they were not detected by the sequencing (also ternary plots only show OTUs with the relative abundance of 0.1 % and more). The overall pattern of connections between OTUs found in the successive generation is very similar to the one observed in the ARISA analysis (Figure 3.2.11 B).

Heat maps allow detailed analysis of rhizosphere communities. Two graphs were constructed using 454 sequencing OTU data. T-tests were used to identify OTUs significantly selected or depleted in the rhizosphere compared with unplanted controls in each generation and are represented as heat maps (Figure 3.2.12). Colour represents the fold difference against unplanted control. Warmer colours represent stronger selection and cooler colour stronger depletion. OTUs were ordered according to their phylogeny.

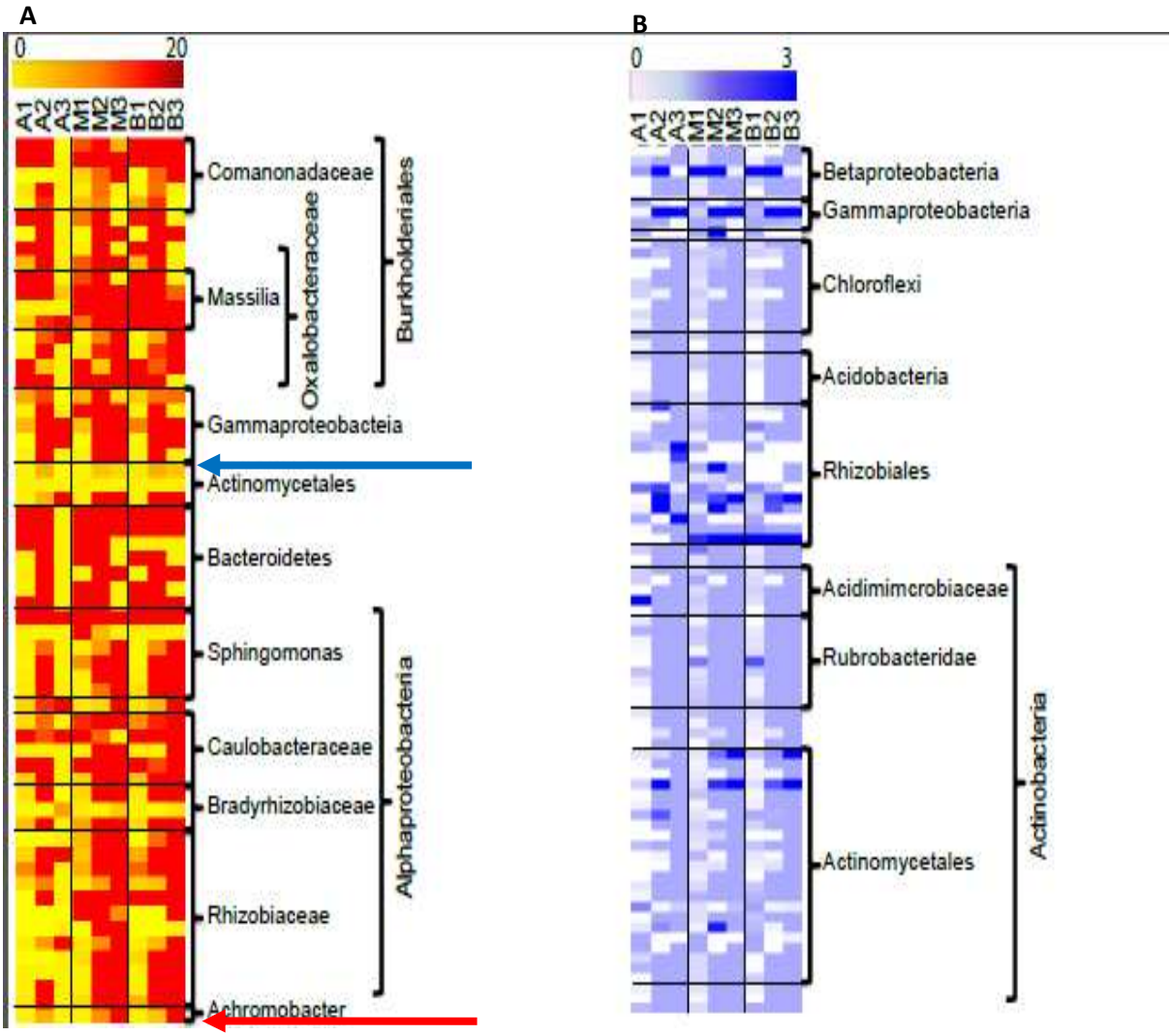


Figure 3.2.12 Heat maps showing A) Selected and B) Depleted OTUs in the rhizosphere against unplanted control. OTUs abundance was compared using t-test ($p < 0.05$) against unplanted control. Presented OTUs are selected or depleted in at least one rhizosphere in at least one generation. Colour scale represents fold difference against the unplanted control. The red and blue arrows point out *Achromobacter* and *Arthrobacter* OTU (There is more detailed discussion about these bacteria in chapter 6).

The rhizosphere environment selects for OTUs belonging to Burkholderiales and Alphaproteobacteria (Figure 3.2.12 A). It is worth noting that there were four strongly selected OTUs of *Massilia* in the *Medicago* and *Brachypodium* rhizospheres (and to a lesser extent in the *Arabidopsis* rhizosphere*)*. Rhizobiales was the dominant order of Alphaproteobacteria.

Heat maps also allow for detailed analysis of OTUs that were significantly less common in the rhizosphere compared to the unplanted control. Overall the rhizosphere suppression was less strong than the selection. Many of the OTUs were selected more than 20 times stronger in the rhizosphere than in the unplanted control, while most of the OTUs that were suppressed in the rhizosphere are in the range of the 3 fold difference against the unplanted control (this difference is represented on the heat scale on the top of the Figure 3.2.12 B).

Actinobacteria were strongly depleted in the rhizospheres of all plants. Moreover OTUs belonging to Chloroflexi and Acidobacteria were only found among the depleted list. Plants clearly favour bacteria belonging to particular phylogenetic groups (Alphaproteobacteria and Burkholderiales) and suppress other taxa (Actinobacteria, Chloroflexi and Acidobacteria).

3.2.2 Summary and discussion of the results for ARISA and 454 pyrosequencing of bacterial community of model plants in the sand experiment

The sand experiment showed how the bacterial community changed in the plant rhizospheres over generations in nutrient-poor sand conditions. Bacteria became greatly dependent on the plant influence. Species like *Massilia*, *Achromobacter* and *Arthrobacter* were actively selected in the rhizosphere. Many species belonging to Alphaproteobacteria and Burkholderiales were also highly selected. Plants suppressed Actinobacteria (apart from *Arthrobacter*), Acidobacteria and Chloroflexi. The bacterial community lost its diversity at the strain and genus level over plants generations. This was probably caused by a strong plant selection, as some bacteria species became very common in the rhizosphere. Loss of diversity was also caused by increased numbers of soil opportunists, which in the 3rd generation invasion through the rhizospheres. Proteobacteria were the dominant phyla in this study and their abundance increased over generations. The generation strongly influenced the community structure as the rhizosphere and unplanted control bacterial community changed with time (apart from being strongly modified by plants).

3.2.3 Pyrosequencing analysis of fungal succession over 3 generations of plants grown in sand

Fungal DNA samples isolated from all three generations of model plants grown in sand were submitted for 454 sequencing. DNA pooling was performed in the same way as for bacterial 454 pyrosequencing. The fungal community was less diverse than the bacterial community, with the total number of OTUs about 4 times lower, even though the total number of reads was higher (Table S3). Richness and Shannon diversity indexes were also much lower than in the case of the bacterial community. As with the bacterial community, the fungal community showed a steep decline in diversity over time (in case of *Arabidopsis* and partially *Medicago* and *Brachypodium* between successive generations and in the case of all plants between first and the third generation only). However there are no major differences between rhizosphere samples and unplanted control in the final generation (Figure 3.2.13).

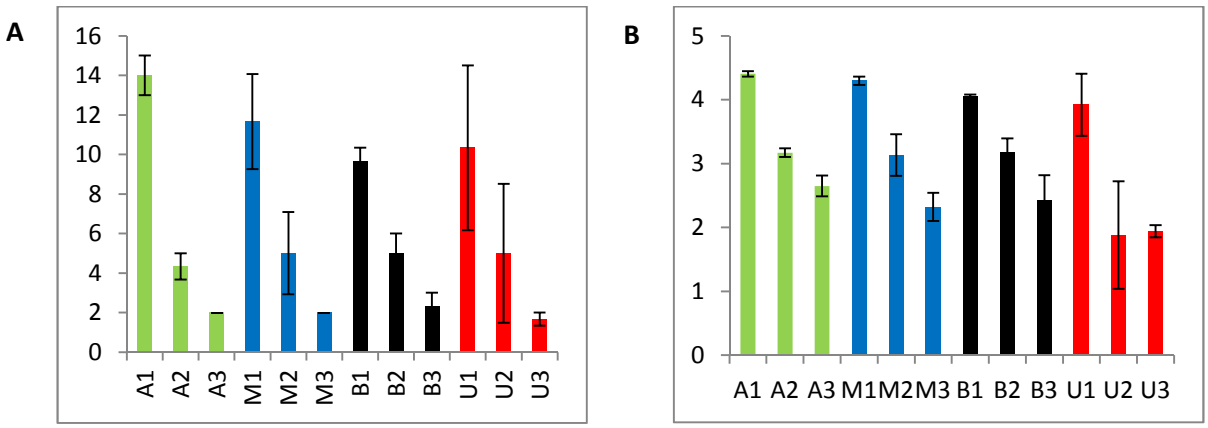


Figure 3.2.13 Richness (A) and Shannon diversity indexes (B) for fungal 454 pyrosequencing data. Richness is presented as the number of different OTUs needed to reach 50% of the total abundance. Error bars ±SEM.

Paired test ($p<0.05$) results (X - X not significant, X -*- X significant), for 454 data n=3 for each plant/unplanted in each generation.

A) A1 -*- A2 -*- A3, M1 - M2 - M3, B1 -*- B2 - B3, U1 - U2 - U3

A1 – U1	A2 - U2	A3 - U3
M1 – U1	M2 – U2	M3 -U3
B1 - U1	B2 – U2	B3 - U3

B) A1 -*- A2 -*- A3, M1 -*- M2 - M3, B1 -*- B2 - B3, U1 - U2 - U3

A1 – U1	A2 - U2	A3 - U3
M1 – U1	M2 – U2	M3 -U3
B1 - U1	B2 – U2	B3 - U3

Even though fungi had a less diverse community than bacteria the pattern of succession over generations was similar. The only major difference is that the fungal community was less heterogeneous as data points on the MDS plot are located close to each other (Figure 3.2.14). As in case of the bacterial community, the fungal community confirms that the *Arabidopsis* rhizosphere in the 3rd generation is very different from other rhizosphere samples and from unplanted control.

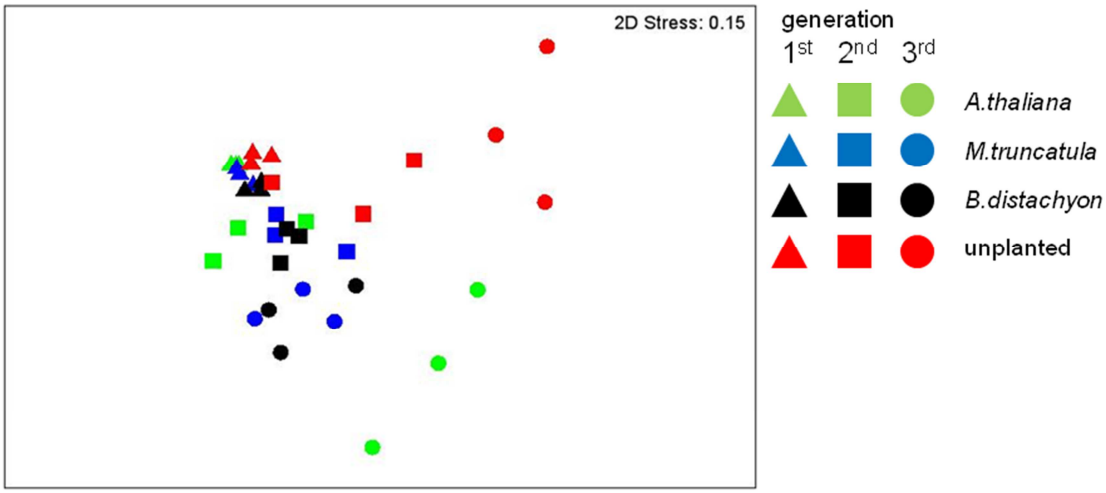
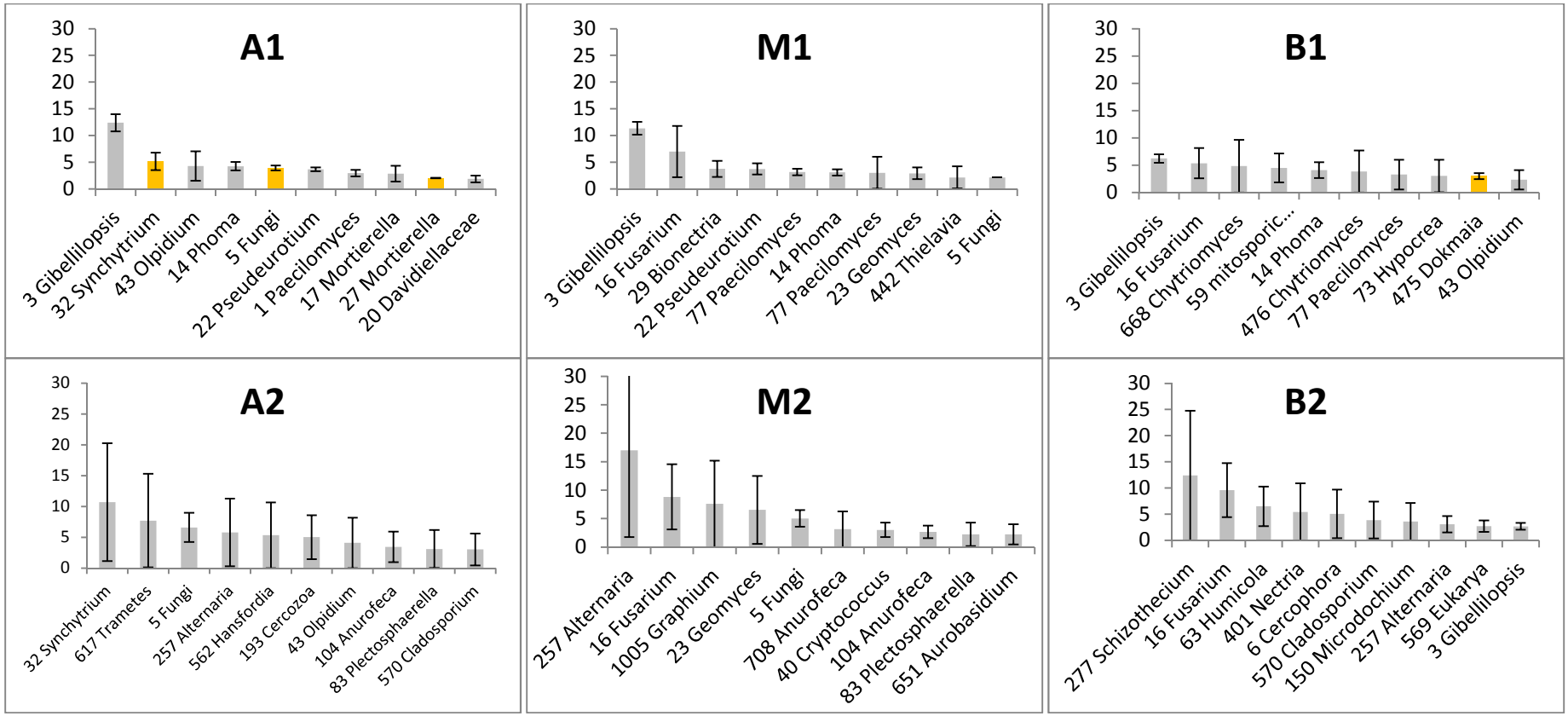


Figure 3.2.14 MDS plot of the fungal community presenting sand experiment community structure based on fungal OTUs abundance (454 sequencing data)

Dominant fungal OTUs are presented on Figure 3.2.15. Due to high diversity between replicates only a few OTUs pass a t-test. Differences between replicates became greater with successive generations.


A1
30
25
20
15
10
5
0
3 Gibellilopsis
32 Synchytrium
43 Olpidium
14 Phoma
5 Fungi
22 Pseudeurotium
1 Paecilomyces
17 Mortierella
27 Mortierella
20 Davidiellaceae
M1
3 Gibellilopsis
16 Fusarium
29 Bionectria
22 Pseudeurotium
77 Paecilomyces
14 Phoma
77 Paecilomyces
23 Geomyces
442 Thielavia
5 Fungi
B1
3 Gibellilopsis
16 Fusarium
668 Chytriomyces
59 mitosporic...
14 Phoma
476 Chytriomyces
77 Paecilomyces
73 Hypocrea
475 Dokmaia
43 Olpidium
A2
32 Synchytrium
617 Trametes
5 Fungi
257 Alternaria
562 Hansfordia
193 Cercozoa
43 Olpidium
104 Anurofeca
83 Plectosphaerella
570 Cladosporium
M2
257 Alternaria
16 Fusarium
1005 Graphium
23 Geomyces
5 Fungi
708 Anurofeca
40 Cryptococcus
104 Anurofeca
83 Plectosphaerella
651 Aurobasidium
B2
277 Schizothecium
16 Fusarium
63 Humicola
401 Nectria
6 Cercophora
570 Cladosporium
150 Microdochium
257 Alternaria
569 Eukarya
3 Gibellilopsis

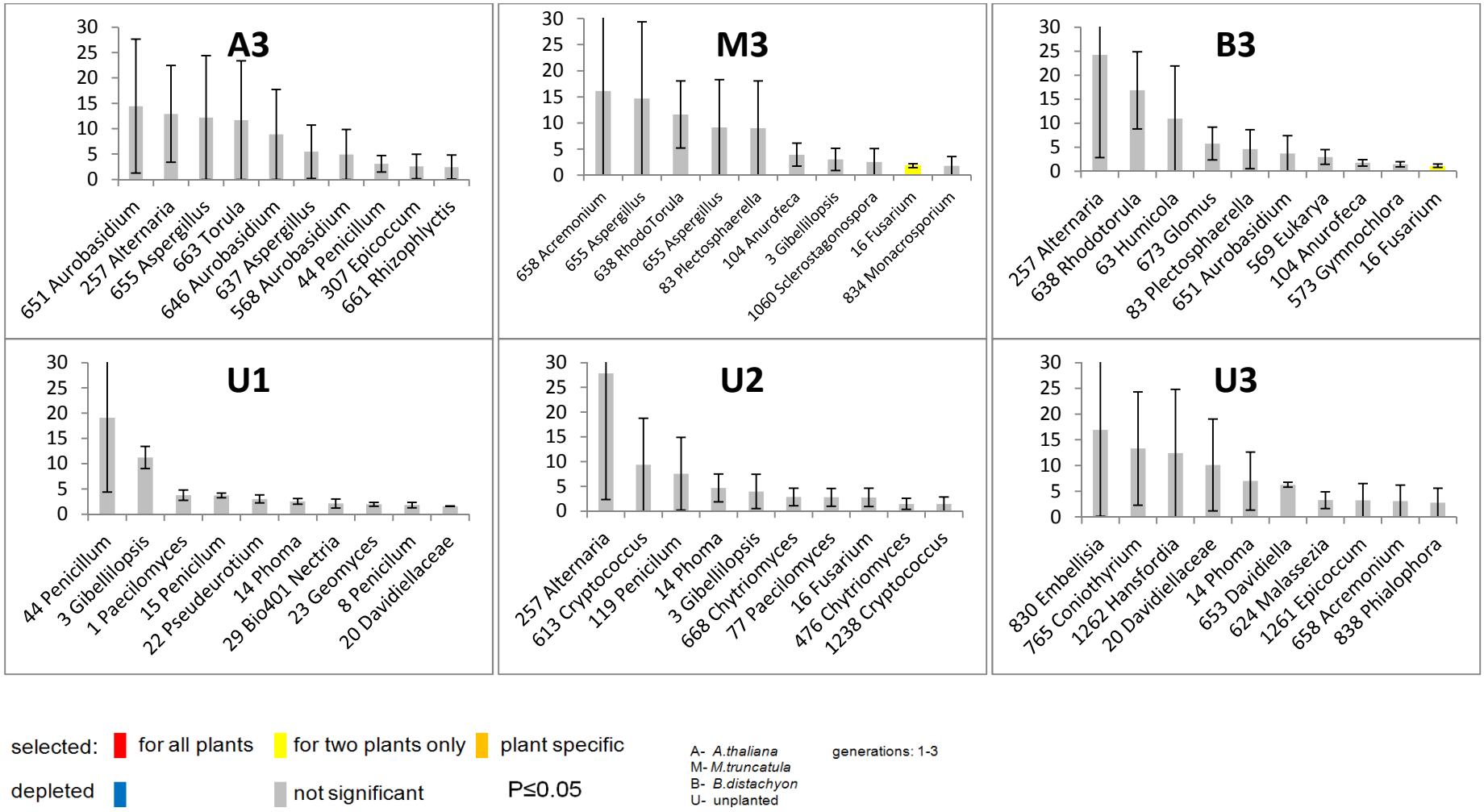
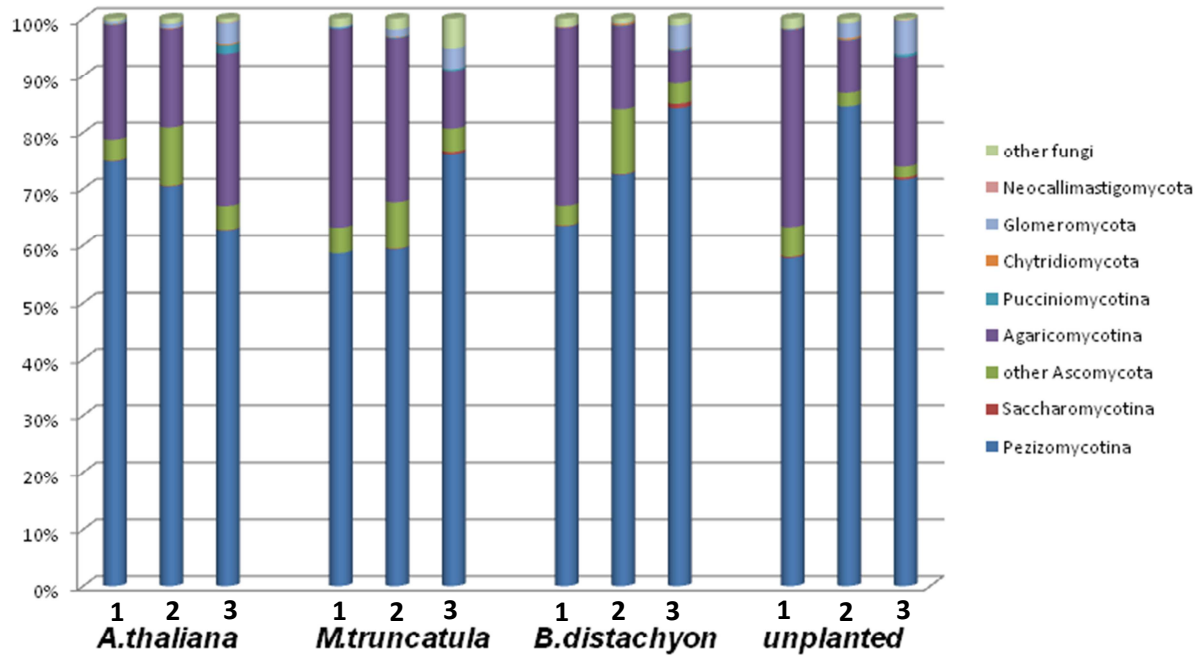


Figure 3.2.15 Bar graphs representing the 10 most abundant fungal OTUs found in the rhizosphere of model plants and unplanted soil samples using 454 sequencing. Vertical axis represents the relative abundance in percentage of the total community. Coloured bars represent rhizosphere selected or depleted OTUs annotated using t-test.

The fungal community at the phylum/division/subdivision level was relatively similar between different plant species and between successive generations (Figure 3.2.16). The Pezizomycotina were dominant with 60-70 % abundance. The second and third dominant fungi taxa were Agaricomycotina and other Ascomycota. It is difficult to propose any theory behind fungal community composition influenced by plants as the rhizospheres and unplanted control were relatively similar to each other. Moreover, there are major differences between replicates (Figure 3.2.15), which makes any interpretation difficult. A study of forest soil using the same primers for fungal ITS amplification showed that Basidiomycota were the dominant division in the community (Buee *et al.*, 2009). Farm soil used in the sand experiment was dominated by Pezizomycotina and other Ascomycota, which belong to the division Ascomycota.



One-way ANOVA with Tukey test (significance level 0.05)

taxa	A1	M1	B1	U1	A2	M2	B2	U2	A3	M3	B3	U3
Chtridiomycota	a	a	a	a	a	a	a	a	a	a	a	a
Agaricomycotina	a	a	a	a	a	a	a	a	a	a	a	a
other fungi	a	a	a	a	a	a	a	a	a	a	a	a
Glomeromycota	ab	ab	a	a	ab	a	ab	a	a	ab	b	ab
Neocallimastigomycota	a	a	a	a	a	a	a	a	a	a	a	a
Pezizomycotina	a	a	a	a	a	a	a	a	a	a	a	a
Pucciniomycotina	a	a	a	a	a	a	a	a	a	a	a	a
Saccharomycotina	a	a	a	a	a	a	a	a	a	a	a	a

Figure 3.2.16 Fungal community structure in the 3 generations of model plants grown in sand conditions. Classification based on LCA (lowest common ancestor) determined using MEGAN. ANOVA with Tukey test (0.05 significance) showed no significant differences between rhizospheres and generations.

The lower total number of OTUs for the fungal 454 sequencing data resulted in lower number of selected and depleted rhizosphere OTUs against unplanted control (Figure 3.2.17) than for bacteria. Fungal selection is not as strong as in the case of bacteria (as the maximum colour scale is halved), however, some fungal OTUs are highly depleted in the rhizosphere. Many of the selected fungal OTUs could only be annotated to the kingdom level, because of low bit scores obtained during BLAST. These OTUs probably represent fungal species that have not been taxonomically characterized. Two OTUs assigned to the genus of *Anurofeca* were strongly selected in the rhizosphere of all plants (apart from *Arabidopsis* in the 3rd generation). The literature describes isolated *Anurofeca* either as symbionts or pathogens of snails and frogs (Baker *et al.*, 1999; Figueras *et al.*, 2000; Hertel *et al.*, 2004). It is very unlikely that rhizosphere fungi are closely related to obligate animal symbionts and pathogens and probably represent other fungal species related to sequenced *Anurofeca* species. The NCBI GenBank database is under-represented for fungi and precise assignment is very difficult

Almost all depleted OTUs belong to Pezizomycotina subdivision of Ascomycota. Three OTUs were found to be highly depleted in the rhizosphere: OTU 235 belonging to *Trichocomaceae* family (*Trichocomaceae* are extensive fungal family including genera like *Aspergillus* and *Penicillium*) and other two OTUs belonging to *Hypocreales* order.

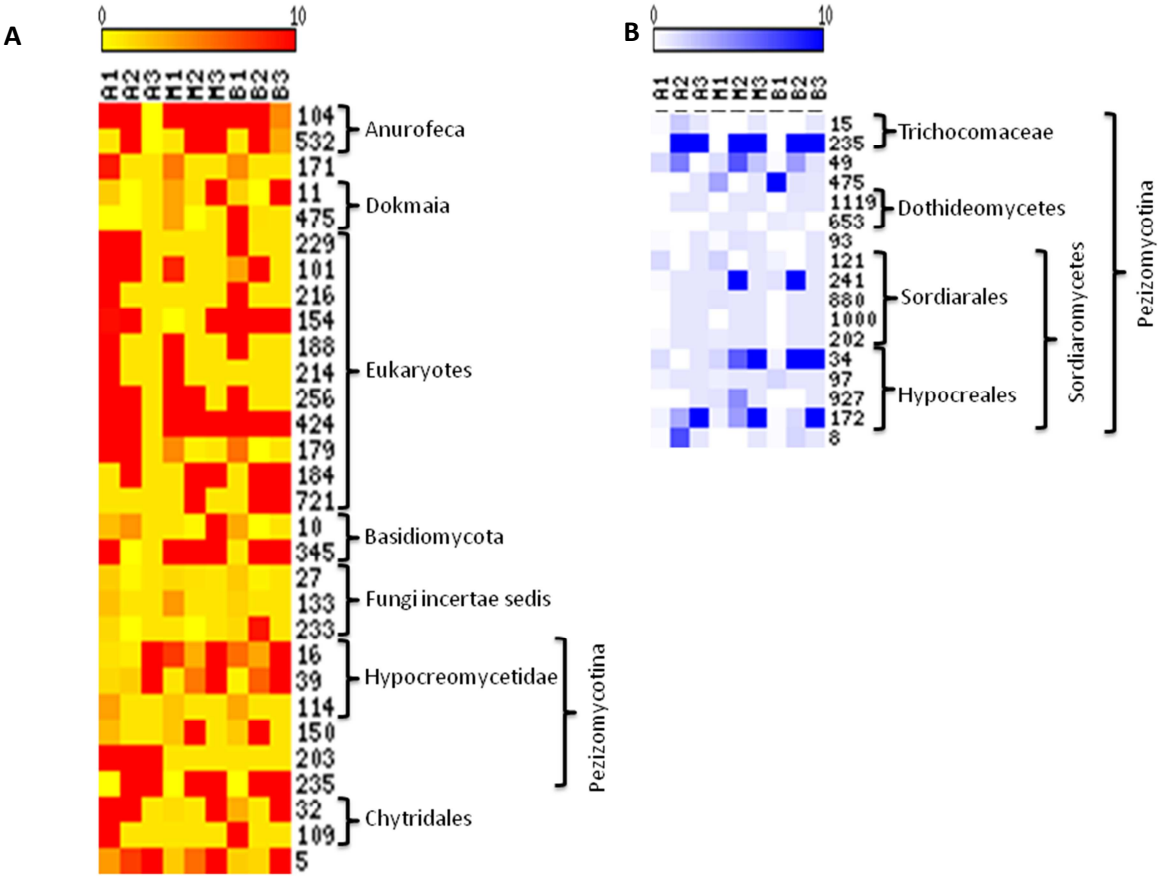


Figure 3.2.17 Heat maps showing A) selected and B) depleted fungal OTUs in the rhizosphere against unplanted control. OTU abundance was compared using t-tests against unplanted control. OTUs were selected or depleted in at least one rhizosphere in at least one generation. Colour scale represents fold difference against the unplanted control.

Ternary plots (Figure 3.2.18) indicate that fungal community in the first generation was rather similar between different plant species and between rhizosphere and unplanted control. There were a few highly selected OTUs, especially in the *Brachypodium* rhizosphere. Many OTUs move from the central area of the first triangle, representing the 1st generation move towards corners in successive triangles (Figure 3.2.18 B). This clearly indicates a high plant species dependence of fungal OTUs. Only a few centrally located OTUs can still be found in the 2nd generation. The third generation was dominated by plant species specific OTUs as almost none of them can be found in the middle of the graph. However, it is essential to interpret these findings with great caution as ternary plots are based on the average OTU abundance and do not show differences found between replicates. Analysis of

the community structure on figure 3.2.15 shows that there was a lot of variation in fungal structure.

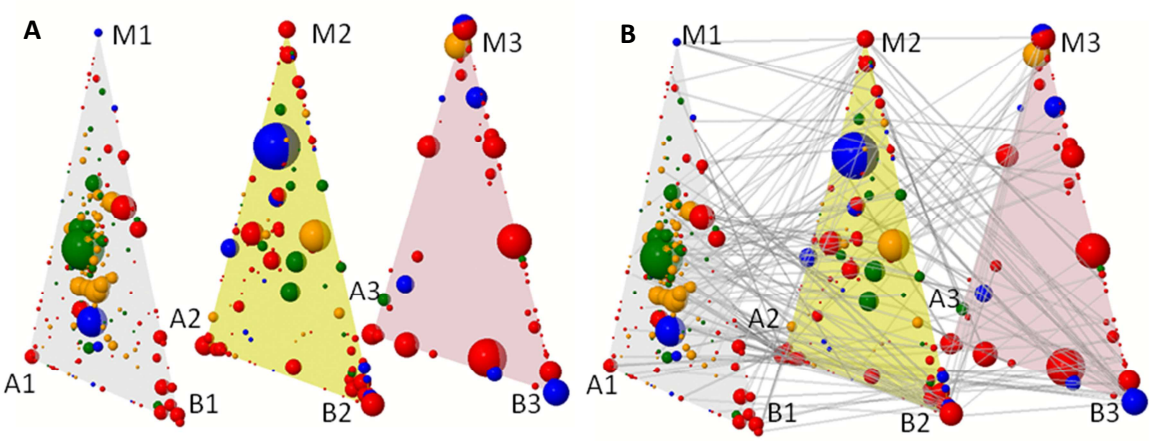


Figure 3.2.18 Three-dimensional ternary plots of the sand experiment using fungal OTUs constructed based on 454 sequencing. Plot A represents the community structure. Plot B shows how many OTUs can be found in successive generations. Interpretation of the ternary plots is explained in material and methods section. A1, A2, A3, M1, M2, M3 and B1, B2, B3 is *Arabidopsis*, *Medicago* and *Brachypodium* rhizosphere in successive generations, respectively. The dominant OTU is annotated as a ball of a particular volume (size 8 in Veusz software) and all other ball sizes are calculated relatively to the dominant one.

3.2.4 Summary and discussion of the results for 454 pyrosequencing of fungal community of model plants

The fungal community was examined using 454 pyrosequencing only. Fungal community structure changes over plant generations following the pattern observed with the bacterial community. This relationship may be either caused by plant influence or by bacterial community influence (and vice versa). The *Arabidopsis* rhizosphere and unplanted control are again much different from *Medicago* and *Brachypodium* rhizosphere. Fungal diversity was much lower than bacterial diversity. A number of fungal OTUs were selected in the rhizosphere, but many of these cannot be annotated beyond the level of phylum. However, only a few fungal OTUs were suppressed by plants and all of them belong to Pezizomycotina.

3.2.5 Discussion

The results obtained in the sand experiment show a strong plant selection of bacterial and fungal species. This observation confirms previous findings where different plant species, flax and tomato (Lemanceau *et al.*, 1995), strawberry and oilseed rape (Costa *et al.*, 2006), chickpea, rape and Sudan grass (Marschner, 2001) and three alpine plants (Becklin *et al.*, 2012) all had distinctive rhizosphere bacterial and fungal communities.

By the third generation, model plants had rhizosphere microbiomes dominated by bacterial opportunists. There was also a relative increase in Proteobacteria during second and third generations (not always statistically significant) that may reflect the disturbance caused by the selection system in sand. The increase in Proteobacteria was greater in unplanted sand in the second and third generations compared with plant rhizospheres. Initial farm soil community from Bawburgh farm was shaped by natural grassland over many years. Thus even though diversity (richness and Shannon diversity index) reduced substantially in the rhizosphere microbiomes in generations two and three, plants still exerted a significant selection relative to soil. Interestingly, Proteobacteria also increase in abundance in the human gut microbiome for the patients which are infected by *Clostridium difficile.* This infection is often a side effect of antibiotic treatment. However this effect can be reversed by faecal transplant, where infected gut microbiota is replaced by a highly diverse “healthy” microbiota often from a donor (Hamilton *et al.*, 2013). This suggests an increase in the relative abundance of Proteobacteria is often a signature of disturbance.

Moreover it was shown that the rhizosphere strongly influenced the microbiome compared to bulk soil. In the previous reports (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012) authors claim that rhizosphere is not much different from the bulk soil, which is in clear contrast to research showing a significant shift in the microbiome structure between these two environments (Micallef *et al.*, 2009). The results of the sand experiment at least partially explain this controversy. Bulgarelli, et. al. (2012) and Lundberg, et.al. (2012) used *Arabidopsis* to compare the structure of the bacterial community between bulk soil, rhizosphere and endosphere at the phylum level and OTU (97 % similarity – species level).

Micallef, et.al. (2009) also used *Arabidopsis* as the plant influencing the bacterial microbiome in the rhizosphere, but used RISA to characterize the differences between rhizosphere and bulk soil community at the strain/species level. RISA is an earlier version of ARISA, with only minor technical differences. Results of the sand experiment indicate that *Arabidopsis,* compared to other plants (*Medicago* and *Brachypodium*) had a weaker influence on the microbiome in the rhizosphere. The differences are clearly visible using ARISA, however the OTU data (constructed with 95 % similarity – genus level) and at the phylum level showed minimal differences.

Plants actively select for plant growth promoting rhizobacteria, as all rhizospheres, except *Arabidopsis* in the final generations were strongly enriched with *Achromobacter sp.* and *Arthrobacter sp*. These strains were tested for their PGPR properties (chapter 6) and positively influence the plant growth (based on plant dry weight assay). Many previous studies focused on isolation of PGPR strains from bulk or rhizosphere soil (Govindasamy *et al.*, 2008), however in my study I have first enriched the soil with plant-dependent bacteria. Thanks to that approach, it is more convincing that these strains are highly competitive for the plant exudates and have PGPR properties. Modern research focusing on PGPR strains should be able to demonstrate their soil survival abilities and not only their potential (based on genome mining studies) or actual PGPR properties (Berg *et al.*, 2006).

Results of this experiment also showed a collapse of diversity of bacterial and fungal communities over plant generations. Loss of diversity was a result of opportunist invasions that took place in the 3rd generation, but also because of plant selection. Invasions were probably possible because of poor nutritional conditions of the growth medium (more and more diluted soil mixed with sand).

Apart from plant influence on the rhizosphere microbiome this study also showed that community is drifting over time. The major component of the total community structure over three generations was a drift (to the right side of MDS plots) of the rhizosphere and bulk soil communities. There are two possible mechanisms in explaining this observation. The first one is the fact that the microbial community was adjusted to real field conditions, with the soil temperature and moisture fluctuations. Here the community was exposed to stable temperature 23°C and watering regime. Such dramatic environmental changes must

have an influence on cells metabolism and subsequent the community profile. The only possible way to overcome this problem would be to grow plants in real field conditions. However, this approach is not really repeatable (as the weather changes all the time) and many more other factors would play a role in the microbiome structure formation – macrofauna, nutrients leaching, plant stress (draught, wind, parasites, etc.). The compromise approach would be to keep plants in the growth room with conditions more similar to the outside world (fluctuations in the temperature – assuming that plants used in this study grow over summer- temperature could fluctuate between 10 and 20°C). Due to technical issues (shared growth room) I was unable to grow plants in such conditions and compare the results to the previously obtained. The other possibility is the fact that community is never in the stable state. Rasche et.al showed that over two years of sampling farm soil and analysing the bacterial and archaeal community structure, there was a time-drift and these communities were never the same over time (Rasche *et al.*, 2011). The other supporting observation was made by Lauber et. al where temporal differences in the microbial community were much greater than the spatial ones (normal farm, low-input farm and grassland) (Lauber *et al.*, 2013). Thus even though it is widely accepted that the land use, covering vegetation (Osborne *et al.*, 2011), soil and other environmental factors play a major role in shaping microbial community. Interestingly, it seems that time has a powerful impact comparable only with the soil pH in shaping the microbiome (Lauber *et al.*, 2009). In case of the three generation project where soil pH should be relatively stable (not measured, but the plant nutrient solution should buffer any changes) time was the major factor shaping the overall community. However, it is crucial to state that plants were able to sustain relation with some bacterial and fungal species (either beneficial or deleterious) over generations.

One way to study the microbial community changes caused by plants but not influenced by the diversity loss or soil opportunists would be to repeat the experiment using a different soil inoculum. That would test if different soils also are susceptible to these problems. However, such an approach means that many different starting soil inocula would have to be tested over multiple plant generation experiments. The other possibility is to change the sand for a rich nutritional medium and such an approach was pursued. The results of the three generation of plants grown in rich compost will be presented in the following chapter.

Chapter 4: Assessing bacterial and fungal rhizosphere succession for plants grown in compost using ARISA and 454 pyrosequencing

4.1 Introduction to 3 generations experiment of model plants grown in the compost

In order to study microbial diversity loss and opportunist invasions seen in Chapter 3 a new approach to study microbial succession was developed. The three generation experiment was repeated in the compost conditions using the same model plants and additional crop plants; *Brassica rapa* (turnip), *Pisum sativum* (pea) and *Triticum aestivum* (wheat). These are crop plants widely used in research. Turnip is closely related to *Arabidopsis* as they both belong to Brassicaceae family. Pea is related to *Medicago*, they both belong to Faboidea subfamily in the family of Fabaceae and both of these plants can be nodulated. Wheat and *Brachypodium* belong to Pooideae subfamily in the family Poaceae, which includes all the grasses. In the later analysis pea will be annotated on figures and tables as "P", turnip as "Br" and wheat as "W". The compost experiment was designed to enable us to study:

1. Model and crop plant rhizosphere enrichment with bacteria and fungi beneficial to the plants growth. Such enrichment has a potential application in agriculture

2 If model and crop plants belonging to the same families are able to shape the microbial community according to plant evolutionary relationships.

3. If there is an overall community drift. Such a community drift was observed in the sand experiment. However, whether it was caused by the soil dilution or by environment conditions is unclear

The first question will answer whether plants are able to enrich the rhizosphere with PGPR. The sand experiment showed that this is indeed possible (chapter 3 and chapter 6). However, is this process taking place in rich soil conditions is still unclear. Many PGPR were found to inhibit the rhizosphere (Berg *et al.*, 2006) and endosphere (Gottel *et al.*, 2011; Mao

et al., 2011). It was found that bioenergy plants (in this case: maize, switchgrass, *Miscanthus* and tallgrass prairie) increases the expression of bacterial genes responsible for N-cycling in their rhizospheres (Mao *et al.*, 2011). Here we will try to focus on the identification of the PGPR species that are common in different plant species rhizospheres and preferably in both growth conditions (sand and compost).

The second question has not been studied so far. Even though our approach will give only limited access to the problem as only two plant species from each family will be studied, it is the first step into deciphering the plant evolution in relation to the rhizosphere microbiome structure.

The third question relates to the sand experiment but also to Swenson's work (Swenson *et al.*, 2000). Swenson showed that plant growth is determined by the underlying microbial processes taking place in the soil. However, are these processes are caused by the plant influence over the rhizosphere community, or rather by a natural community drift (caused by the fact that the microbial community must adapt to the new environmental conditions).

The compost experiment design is very similar to the sand one, except that instead of sand, nutrient-rich compost was added and the rate of inoculation between generations was reduced to 10 %. (Figure 4.1.1)

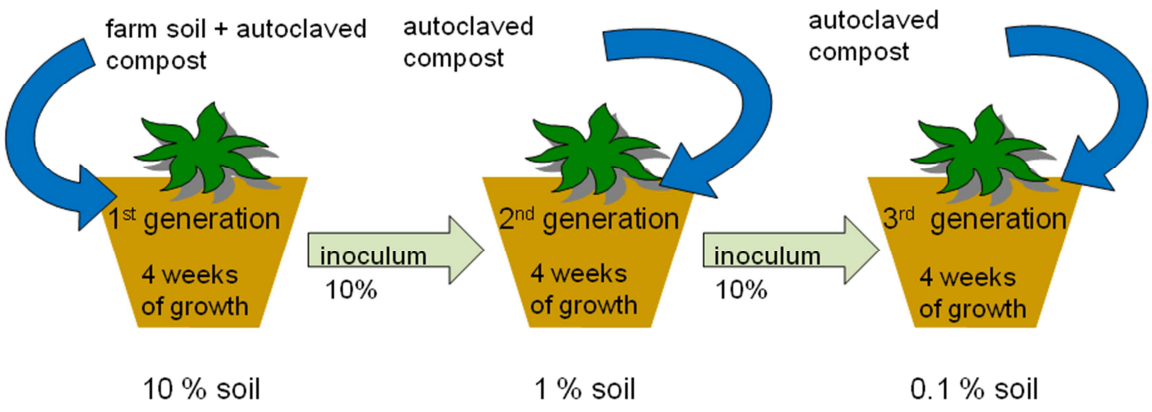


Figure 4.1.1 Diagram illustrating compost experiment design

No fertilizer was added to any of the pots as the compost was rich enough to sustain plant growth. The nutrient concentration of the compost was as follows: NO_3^- 1366 mg/kg, PO_4^{-3} 1247 mg/kg, K^+ 973 mg/kg, Mg^{2+} 3738 mg/kg) with pH 5.8 and high amount of organic matter (50 %). These values are roughly 100 times higher than the nutrient concentration values for the Bawburgh soil. Legume plants did not nodulate throughout the experiment, probably due to the high nitrogen content in the compost. In this experiment model plants were kept in 50 ml beakers as usual, however crop plants were grown in 100 ml beakers. Crop plants grow much bigger and needed bigger pots in order to expand the root system. This difference required two sets of unplanted controls using 50 ml and 100 ml volumes. This difference must be taken into account when making comparisons between model and crop plants.

4.2 Results and Discussion

4.2.1 ARISA and pyrosequencing analysis of bacterial succession over 3 generations of plants grown in compost

The bacterial communities in the rhizosphere of model plants in the first generation examined using ARISA show significant differences between plant species (Figure 4.2.1 A and Table 4.2.1 A). Crop plants exerted a stronger influence on the rhizosphere as data

points from each group cluster together (Figure 4.2.1 B). MANOVA confirms the MDS observation. In the case of crop plants not more than 9 replicates is enough to statistically differentiate all the groups from each other (Table 4.2.1 B). However, at least 14 replicates are needed to separate the influence of model plant species. Only the *Arabidopsis* rhizosphere seems to be very different from the other plants and unplanted control.

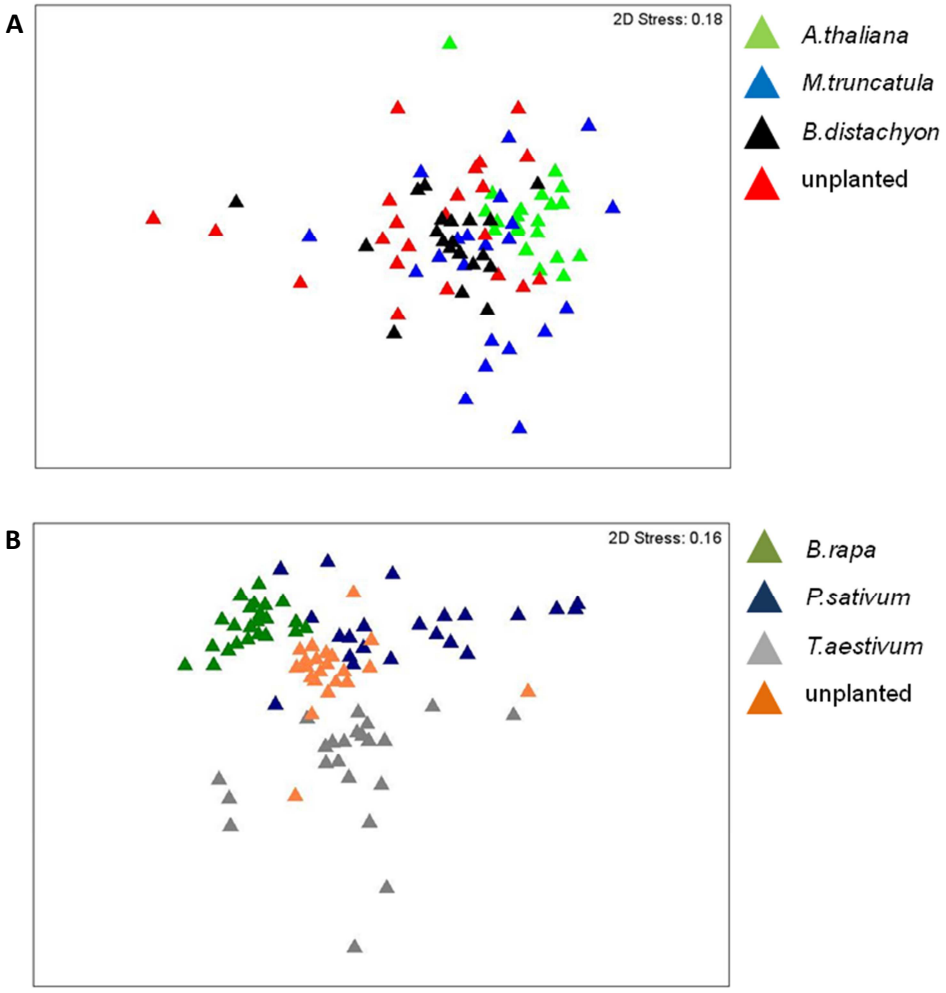


Figure 4.2.1 MDS plot of the 1st generation of A) Model plants and B) Crop plants in compost experiment

A

	A1	M1	B1	U1	A2	M2	B2	U2	A3	M3	B3	U3
A1		6	5	7	4	4	4	4	4	4	4	4
M1	6		14	9	4	5	5	4	4	4	5	4
B1	5	14		13	4	5	5	4	4	4	4	4
U1	7	9	13		4	5	6	4	5	4	4	5
A2	4	4	4	4		11	8	5	6	4	4	5
M2	4	5	5	5	11		15	5	7	5	4	8
B2	4	5	5	6	8	15		5	7	6	4	12
U2	4	4	4	4	5	5	5		6	4	4	5
A3	4	4	4	5	6	7	7	6		5	5	9
M3	4	4	4	4	4	5	6	4	5		4	5
B3	4	5	4	4	4	4	4	4	5	4		4
U3	4	4	4	5	5	8	12	5	9	5	4	

B

	Br1	P1	W1	U1	Br2	P2	W2	U2	Br3	P3	W3	U3
Br1		6	5	5	4	4	4	4	4	4	4	5
P1	6		6	9	4	5	4	5	5	5	5	5
W1	5	6		7	5	5	5	4	5	5	5	4
U1	5	9	7		4	5	5	4	4	4	4	4
Br2	4	4	5	4		9	5	4	7	5	5	4
P2	4	5	5	5	9		5	5	5	7	6	5
W2	4	4	5	5	5	5		4	6	5	7	4
U2	4	5	4	4	4	5	4		4	4	4	5
Br3	4	5	5	4	7	5	6	4		10	7	4
P3	4	5	5	4	5	7	5	4	10		6	4
W3	4	5	5	4	5	6	7	4	7	6		4
U3	5	5	4	4	4	5	4	5	4	4	4	

Table 4.2.1 Pairwise MANOVA analysis of the bacterial rhizosphere communities ($p<0.05$)

A) A- *Arabidopsis,* M- *Medicago,* B- *Brachypodium*, U - unplanted soil (50 ml pot)

B) Br- turnip, P- pea, W1- wheat, U - unplanted soil (100 ml pot)

1,2,3 – generation number

The rhizosphere community was examined at the end of the 2nd generation of plant growth. Both model and crop plant rhizosphere communities became very different from the unplanted control (Figure 4.2.2 A and B). This observation is very similar to the one made in case of the sand experiment. In sand the 2nd generation showed a clear rhizosphere effect i.e. separation of rhizosphere versus unplanted control. In the compost experiment, model plant rhizosphere communities are more similar to each other. MANOVA analysis indicates that in order to separate any rhizosphere from unplanted control 6 replicates needed to be taken from each group. However, to separate the rhizospheres of different plant species at least 15 replicates were required (Table 4.2.1). The *Medicago* and *Brachypodium* rhizospheres had a relatively similar bacterial community structure. A high similarity between *Medicago* and *Brachypodium* rhizospheres was also observed in the sand experiment. Crop plant communities are easier to separate as normally analysis on 5 replicates from each group yields significant separation. Only the turnip and pea communities need at least 15 replicates in order to be separated.

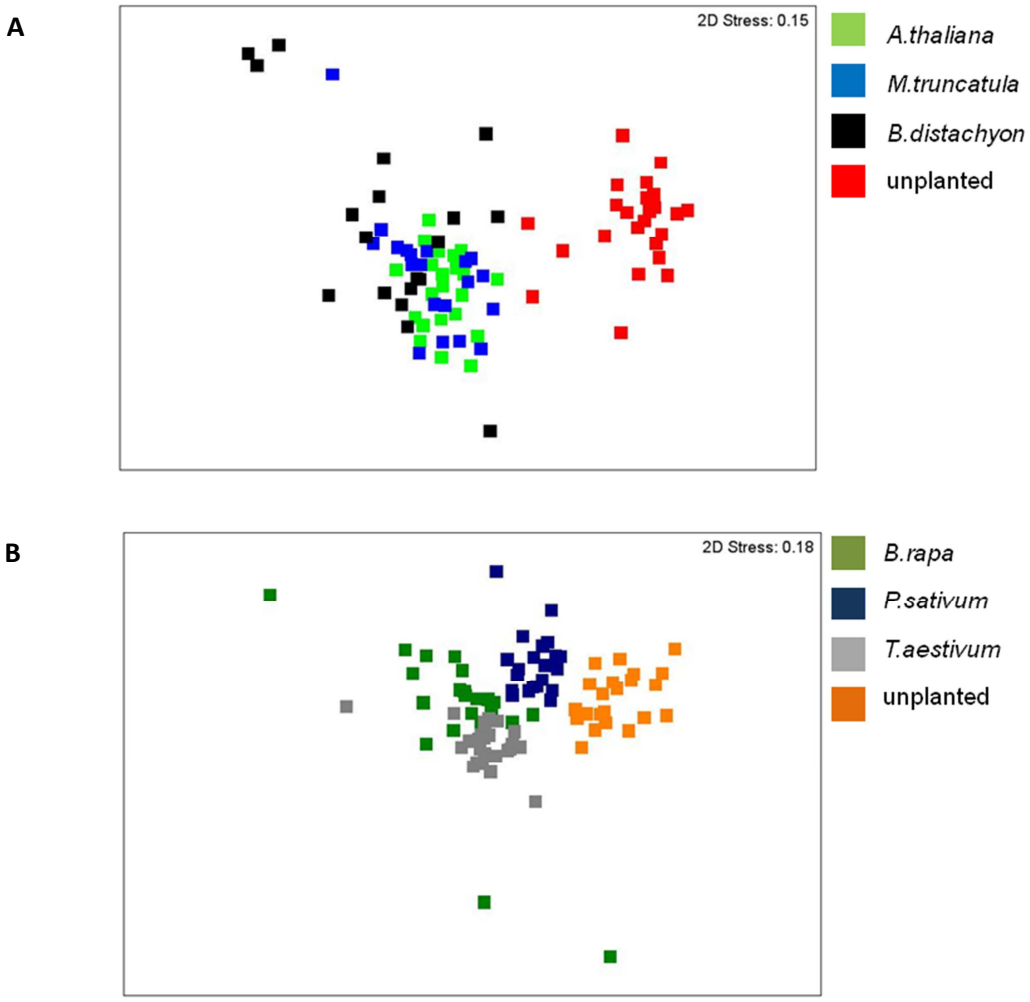


Figure 4.2.2 MDS plot of the 2nd generation of A) model plants and B) crop plants in compost. MDS plot based on ARISA data.

The community in the 3rd generation of the compost experiment showed stronger differences between plant species. In the case of the model plants *Medicago* and *Brachypodium* the communities were relatively similar to each other (Figure 4.2.3 A). However, due to lower diversity within the groups (based on MDS plot analysis) MANOVA separates them using only 4 replicates (Table 4.2.1). The unplanted control became very heterogeneous compared to the rhizosphere (there was also high heterogeneity in *Arabidopsis* samples). It can be explained by previous research indicating that bulk soil diversity is much greater than the rhizosphere (Garcia-Salamanca *et al.*, 2013). The *Arabidopsis* rhizosphere community separates into three distinct clusters. The most abundant cluster is similar to the *Brachypodium* rhizosphere and to unplanted control, while the other two form distinct groups. This problem was not studied further due to project

time constraints. Crop plants replicates form distinct clusters. As in case of the second generation the microbial communities of turnip and pea were closer together, yet still significantly different.

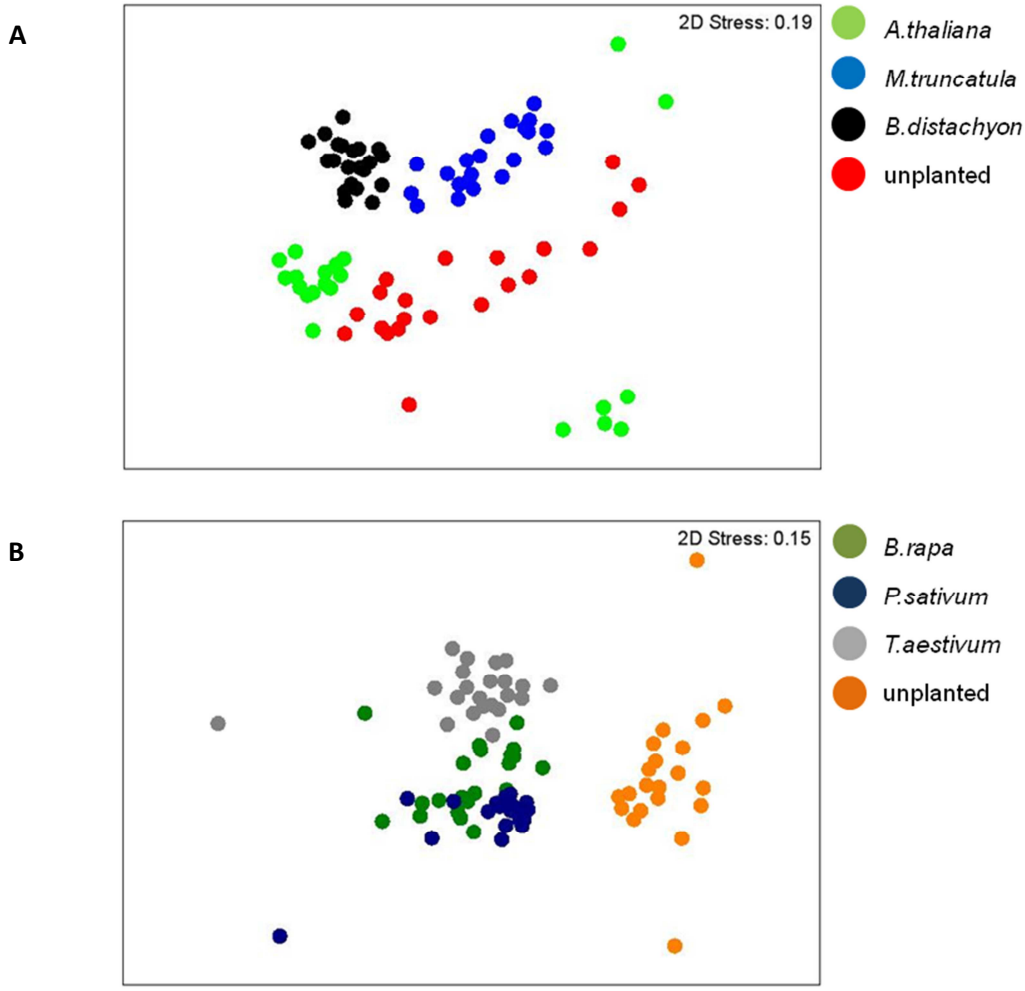


Figure 4.2.3 MDS plot of the 2nd generation of A) model plants and B) crop plants grown in compost. MDS plot based on ARISA data.

Combining all data points together produced an MDS plot that is very difficult to interpret (Figure 4.2.4 A). Particular groups of data points seem to form clusters. However, the most interesting observation is that the overall community shifted from the 1st generation onwards (Figure 4.2.4 B). A similar shift was observed in case of model plants grown in sand (Figure 3.2.4). In the case of sand experiment the shift may be explained by the dilution rates of farm soil and compounds not derived from the root exudates. However, in the compost condition, where the nutrient levels are very high, farm soil dilution can not be responsible for the observed trend. It is proposed that the overall community coming from relatively poor farm soil is adapting to the rich compost conditions (the temperature and watering regime also were much different from the outdoor environment). The generation experiment provides community structure snapshots of that process.

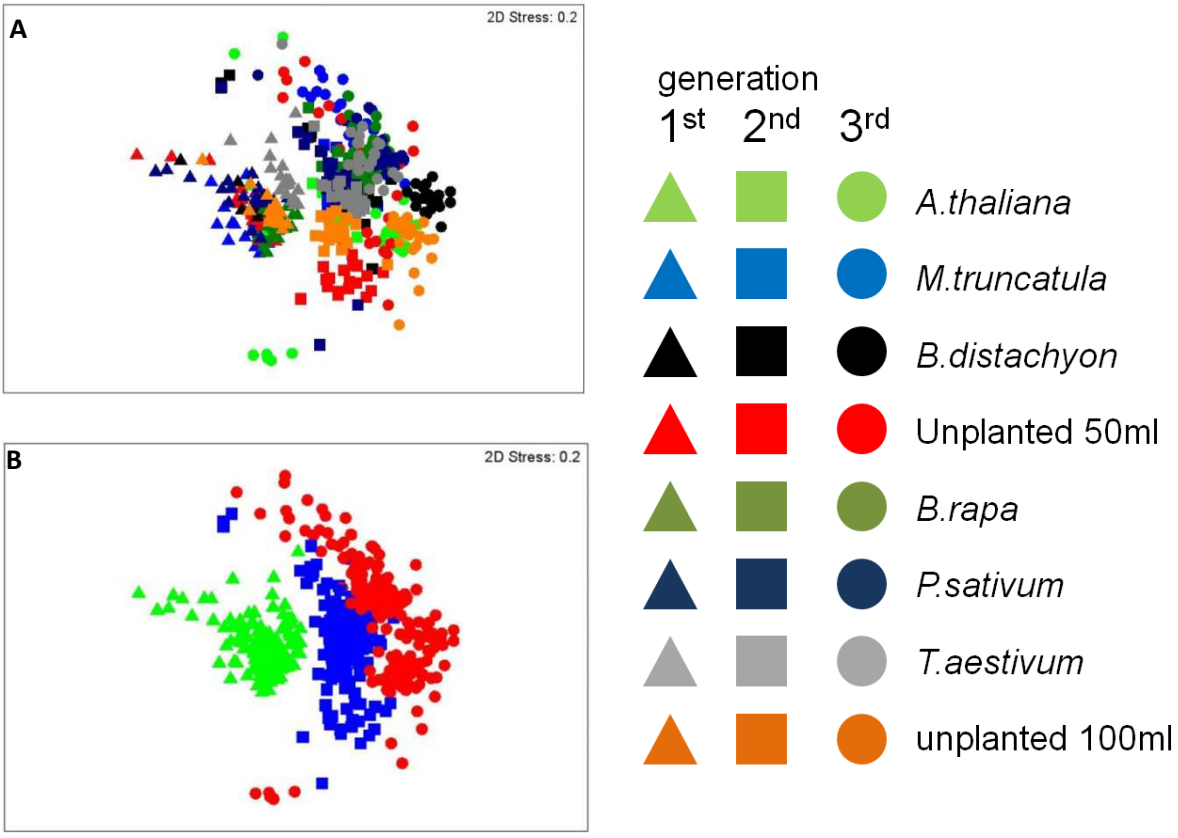


Figure 4.2.4 MDS plot showing all 3 generation of model and crop plants grown in compost using all data points. A) Plant species driven colour code (see legend), B) Generation driven colour code: green – 1st, blue – 2nd, red - 3rd generation.

In order to simplify visualisation the community structure for all three generations was pooled into 3 samples for every plant in every generation (Figure 4.2.5). The first generation is relatively homogenous with all the plant species and controls co-located, except the wheat rhizosphere. There was a significant distance between 1st and 2nd generation communities. The third generation data points are located closer to the 2nd generation. The community spread slowed down indicating that the bacterial communities were closer to their equilibrium state. The crop plant rhizospheres were relatively similar to each other and very different from the unplanted control, while model plant rhizosphere communities were much more heterogeneous. The heterogeneity may be observed between the replicates of *Arabidopsis* as well as between different plant species. Model plants are located closer to the unplanted control than crop plants suggesting that larger crop plants have a stronger influence on the rhizosphere community.

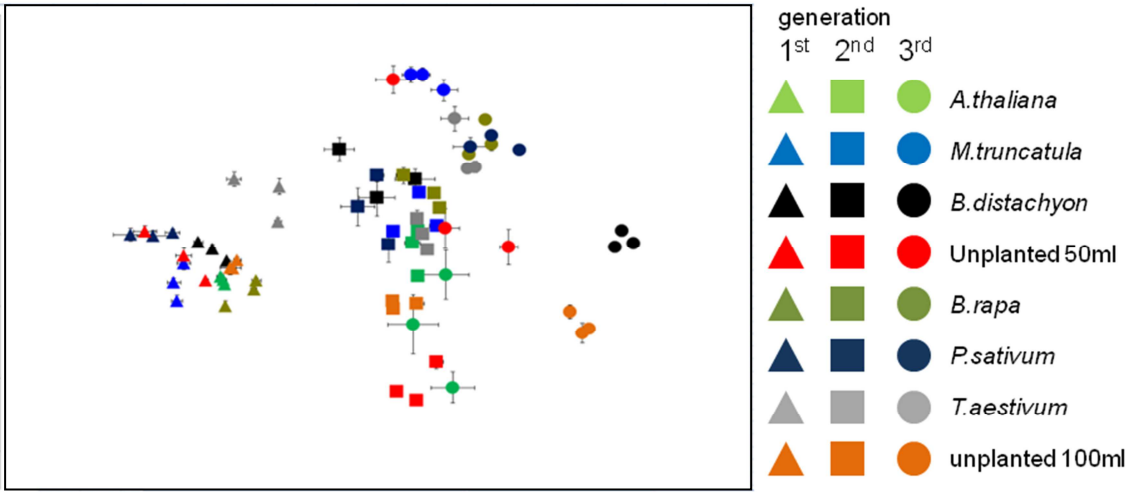


Figure 4.2.5 MDS plot showing ARISA results of the compost experiment using three pooled samples for each plant species in each generation. Error bars show the standard error of the mean.

The microbial community in the compost experiment was also studied using 454 sequencing. The detailed analysis of sequencing efficiency and comparison against the sequencing performed on the samples from the sand experiment is presented in Chapter 4.2.6.

Bacterial 454 sequencing data binned into OTUs shows a similar pattern to ARISA. There was a very strong generation effect, with a large change from generation 1 to generation 2, and smaller change in generation 3. These data also shows considerable influence of the plants with samples from the rhizosphere within a single generation being very similar to each other (Figure 4.2.6). Bacterial communities at the genus level (OTUs were binned using 95 % reads sequence similarity – Figure 2.8.4) were less heterogeneous between replicates of the same plant in the same generation. Unplanted controls (50 ml and 100 ml) were located close to each other, indicating the existence of a very similar bacterial community. The *Arabidopsis* rhizosphere community was also very similar to the unplanted control community, as samples were very close to each other. Again crop plants had a greater effect on the community with samples located far away from the unplanted controls.

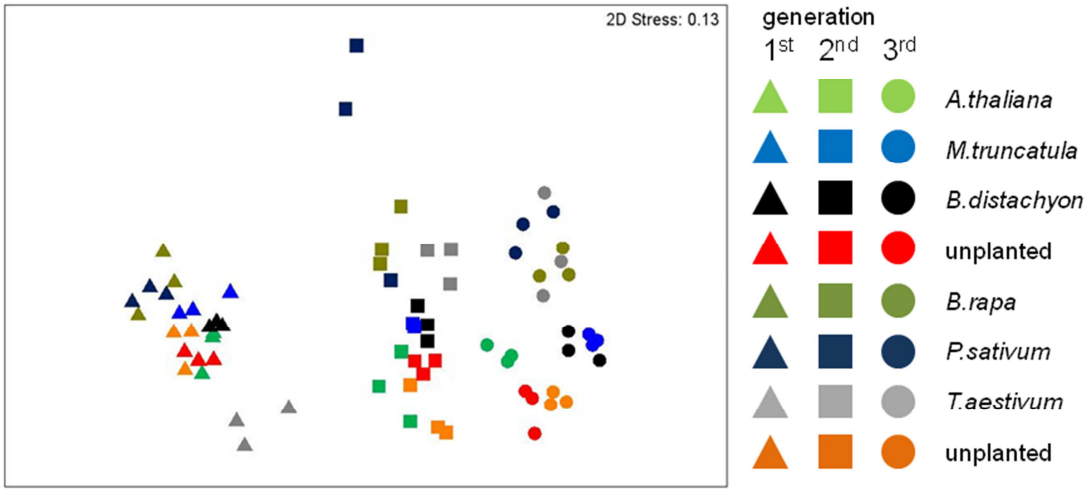


Figure 4.2.6 MDS plot of the bacterial community from the rhizosphere of model and crop plants grown in compost. Plot is based on the 454 pyrosequencing data binned into OTUs.

The bacterial community at the phylum level was relatively stable over generations. There were no major differences between different rhizospheres and bulk soil. There were some differences in abundance of Firmicutes and Actinobacteria for model plants and Proteobacteria, Acidobacteria, Gemmatimonadetes, Firmicutes and Bacteroidetes in the case of crop plants. Proteobacteria made up 60-70 % of the community, however their abundance did not increase in successive generations (as was the case in sand– Figure 3.2.7). Actinobacteria and Bacteroidetes made up around 20 % of the total community

(Figure 4.2.7 A). Within the Proteobacteria, Gammaproteobacteria became more abundant in successive generations, however only statistically in case of model plants (4.2.7 B).

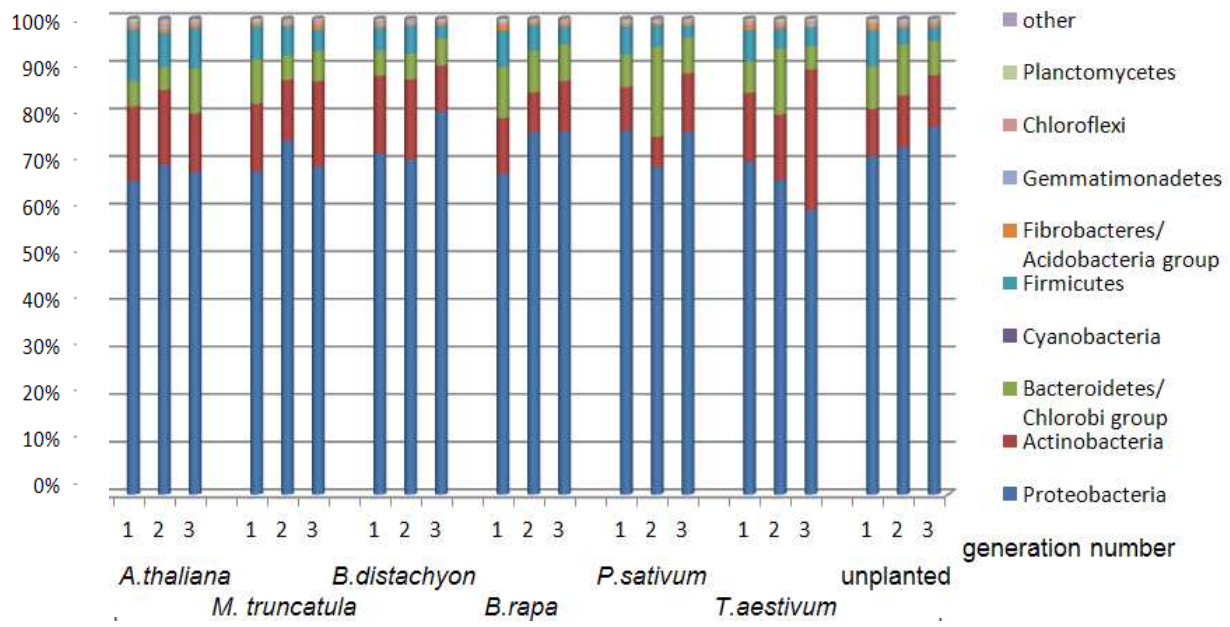


Figure 4.2.7 Bacterial community structure at A) phylum level, B) subphylum level of Proteobacteria for the model and crop plants grown in compost obtained by 454 sequencing – values averaged from 3 replicates

Statistics based on One-way ANOVA with Tukey test for multiple comparisons

A) phylum	A1	M1	B1	U1	A2	M2	B2	U2	A3	M3	B3	U3
Chloroflexi	a	a	a	a	a	a	a	a	a	a	a	a
Gemmatimonadetes	a	a	a	a	a	a	a	a	a	a	a	a

Acidobacteria	a	a	a	a	a	a	a	a	a	a	a	a
Firmicutes	c	ab	ab	ab	ab	ab	ab	ab	bc	ab	ab	a
Cyanobacteria	a	a	a	a	a	a	a	a	a	a	a	a
Bacteroidetes	a	a	a	a	a	a	a	a	a	a	a	a
Actinobacteria	ab	ab	b	ab	ab	ab	ab	ab	a	a	ab	a
Proteobacteria	a	a	a	a	a	a	a	a	a	a	a	a
Planctomycetes	a	a	a	a	a	a	a	a	a	a	a	a

A) phylum	Br1	P1	W1	U1	Br2	P2	W2	U2	Br3	P3	W3	U3
Chloroflexi	a	a	a	a	a	a	a	a	a	a	a	a
Gemmatimonadetes	b	ab	a	ab	ab	ab	ab	ab	ab	ab	ab	ab
Acidobacteria	b	ab	ab	ab	a	ab	ab	ab	ab	ab	a	ab
Firmicutes	bc	abc	abc	c	abc	abc	ab	a	a	abc	abc	a
Cyanobacteria	a	a	a	a	a	a	a	a	a	a	a	a
Bacteroidetes	b	ab	a	ab	ab	ab	ab	ab	ab	ab	ab	ab
Actinobacteria	a	a	a	a	a	a	a	a	a	a	b	a
Proteobacteria	ab	ab	ab	ab	ab	ab	a	ab	ab	ab	ab	b
Planctomycetes	a	a	a	a	a	a	a	a	a	a	a	a

B) subphylum	A1	M1	B1	U1	A2	M2	B2	U2	A3	M3	B3	U3
Alphaproteobacteria	a	a	a	a	a	a	a	a	a	a	a	a
Betaproteobacteria	a	a	a	a	a	a	a	a	a	a	a	a
Delta/epsilon-proteobacteria	ab	a	ab	ab	ab	ab	ab	b	ab	ab	ab	ab
Gammaproteobacteria	a	a	ab	abc	abc	abc	bc	abc	c	abc	d	c

B) subphylum	Br1	P1	W1	U1	Br2	P2	W2	U2	Br3	P3	W3	U3
Alphaproteobacteria	a	a	a	a	a	a	a	a	a	a	b	a
Betaproteobacteria	a	a	a	a	a	a	a	a	a	a	b	a
Delta/epsilon-proteobacteria	a	a	a	a	a	a	a	a	a	a	b	a
Gammaproteobacteria	a	a	a	a	a	a	a	a	a	a	b	a

Bacterial diversity and richness were relatively stable over generations and between different plant species at the strain/species level (Figure 4.2.8). In most plants, except wheat, there was a significant decrease in the Shannon index and richness values in the 2nd generation. The *Brachypodium* rhizosphere community was much more diverse in the 3rd generation. There was a sharp decrease in the turnip rhizosphere diversity between the 1st and 2nd generations.

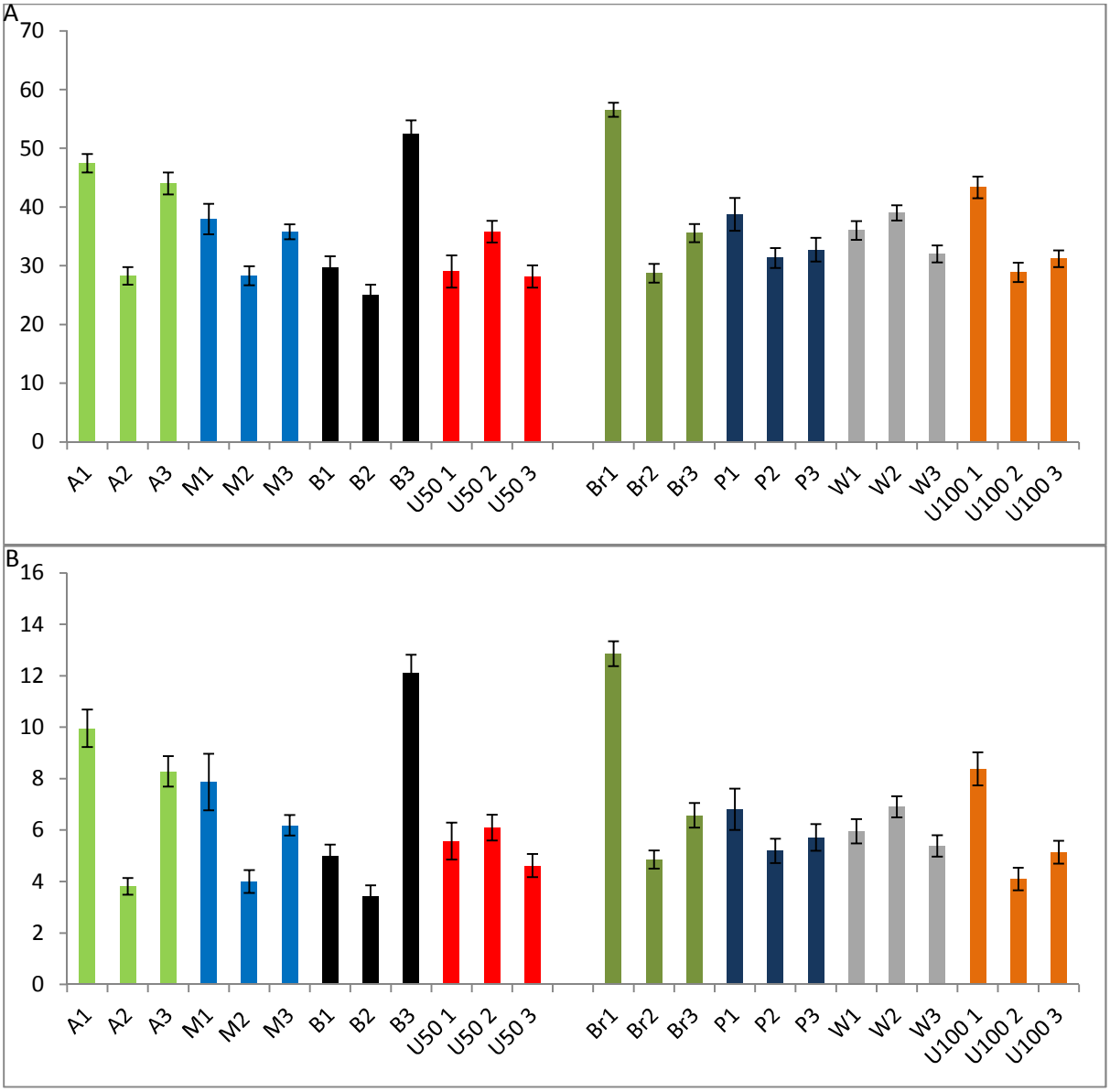


Figure 4.2.8 A) Shannon diversity index and B) richness for ARISA data of the 3 generations of model and crop plants grown in compost.

Paired test ($p<0.05$) results (X - X not significant, X -*- X significant), for ARISA n=16,24 each plant/unplanted in each generation.

A) A1 -*- A2 -*- A3, M1 -*- M2 -*- M3, B1 - B2 -*- B3, U1 - U2 - U3

A1 -*- U1	A2 -*- U2	A3 -*- U3
M1 -*- U1	M2 -*- U2	M3 -*-U3
B1 - U1	B2 -*- U2	B3 -*- U3

Br1 -*- Br2 - Br3, P1 -*- P2 - P3, W1 - W2 -*- W3, U1 - U2 - U3

Br1 -*- U1	Br2 - U2	Br3 - U3
P1 - U1	P2 - U2	P3 -U3
W1 - U1	W2 - U2	W3 - U3

B) A1 -*- A2 -*- A3, M1 -*- M2 -*- M3, B1 -*- B2 -*- B3, U1 - U2 -*- U3

A1 -*- U1	A2 -*- U2	A3 -*- U3
M1 -*- U1	M2 -*- U2	M3 -*-U3
B1 - U1	B2 -*- U2	B3 -*- U3

Br1 -*- Br2 - Br3, P1 - P2 - P3, W1 - W2 - W3, U1 -*- U2 - U3

Br1 -*- U1	Br2 - U2	Br3 -*- U3
P1 - U1	P2 - U2	P3 -U3
W1 -*- U1	W2 -*- U2	W3 - U3

In contrast to the sand experiment (chapter 3.2) there was no diversity collapse at the genus level in the compost experiment with Shannon diversity index and richness stable over generations (Figure 4.2.9) (apart from turnip and wheat between first and second generation) . Presumably, this is because compost provides higher concentrations of a wider range of carbon sources and more physical heterogeneity than sand. There was a slight dip in the bacterial diversity in the 2nd generation (not significant for most plants, but the trend is consistent for all plants). Diversity in the 3rd generation was in most cases similar to that in the 1st generation. Two independent unplanted controls (50 ml and 100 ml pots) show the same pattern of community diversity. The richness in unplanted controls drops over generations from 120 to 80. This may represent natural bacterial succession, where bacterial species better adapted for growth in rich soil become more abundant.

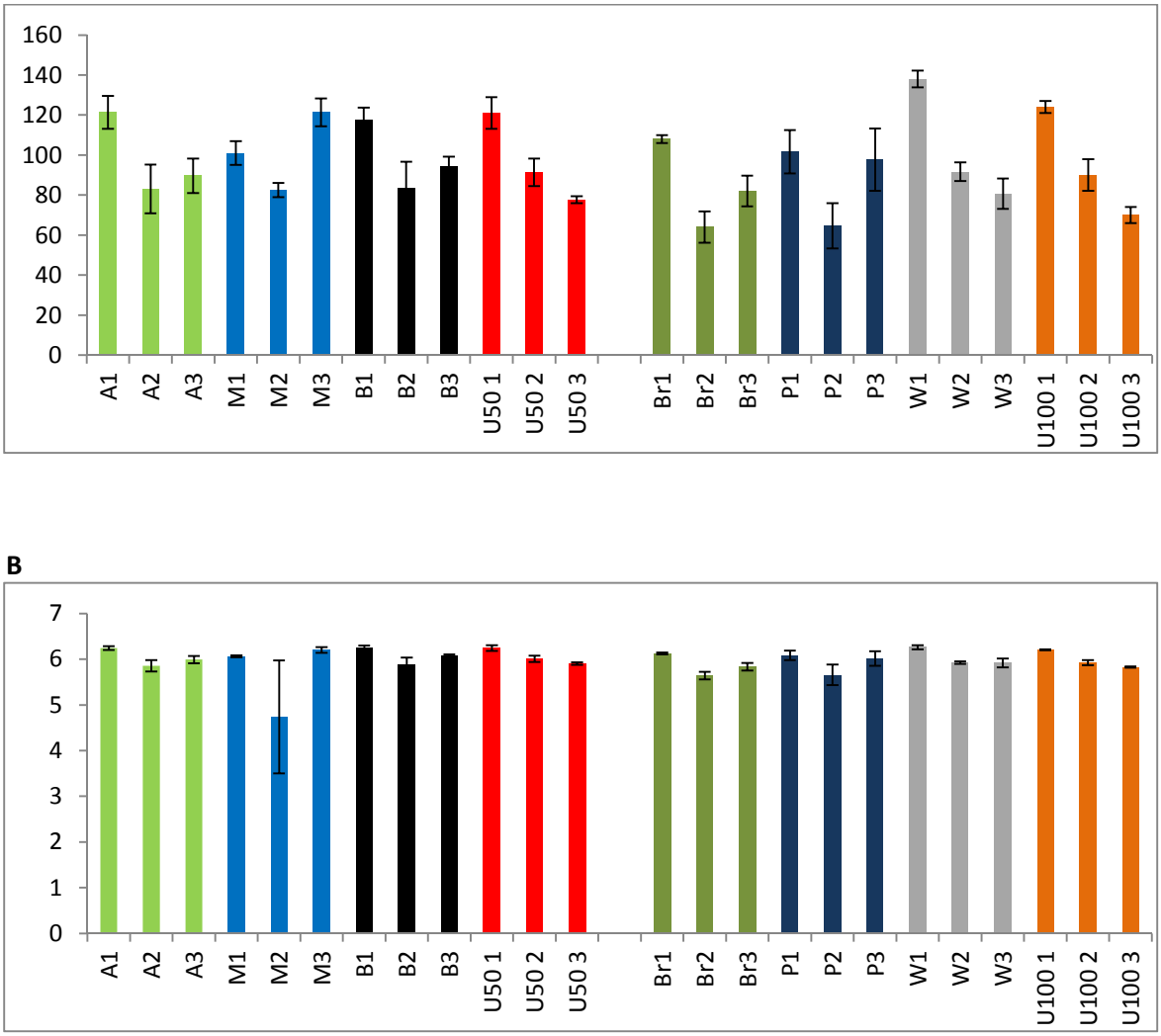


Figure 4.2.9 Richness (A) and Shannon diversity index (B) for the compost experiment based on 454 pyrosequencing data. Richness is presented as the number of different OTUs needed to reach 50% of the total abundance.

Paired test ($p<0.05$) results (X - X not significant, X -*- X significant), for 454 data n=3 each plant/unplanted in each generation.

A) A1 - A2 - A3, M1 - M2 -*- M3, B1 - B2 - B3, U1 -*- U2 - U3

A1 - U1	A2 - U2	A3 - U3
M1 - U1	M2 - U2	M3 -*-U3
B1 - U1	B2 - U2	B3 -*- U3

Br1 -*- Br2 - Br3, P1 - P2 - P3, W1 -*- W2 - W3, U1 -*- U2 - U3

Br1 -*- U1	Br2 - U2	Br3 - U3
P1 - U1	P2 - U2	P3 -U3
W1 - U1	W2 - U2	W3 - U3

B) A1 -*- A2 - A3, M1 - M2 - M3, B1 - B2 - B3, U1 - U2 - U3

A1 - U1	A2 - U2	A3 - U3
M1 - U1	M2 - U2	M3 -U3
B1 - U1	B2 - U2	B3 - U3

Br1 -*- Br2 - Br3, P1 - P2 - P3, W1 -*- W2 - W3, U1 -*- U2 - U3

Br1 - U1	Br2 - U2	Br3 - U3
P1 - U1	P2 - U2	P3 -U3
W1 - U1	W2 - U2	W3 - U3

Data on the abundance of individual ITRs also indicates that model plants did not influence the rhizosphere community as much as crop plants did (Figure 4.2.10). Bar graphs prepared from crop plants data are characterized by a greater number of statistically upregulated ITR sizes, many of which are shared between different plant species. Many ITR sizes are carried over through out the experiment including, 372.05, 513.71, 573.09, 514.08.

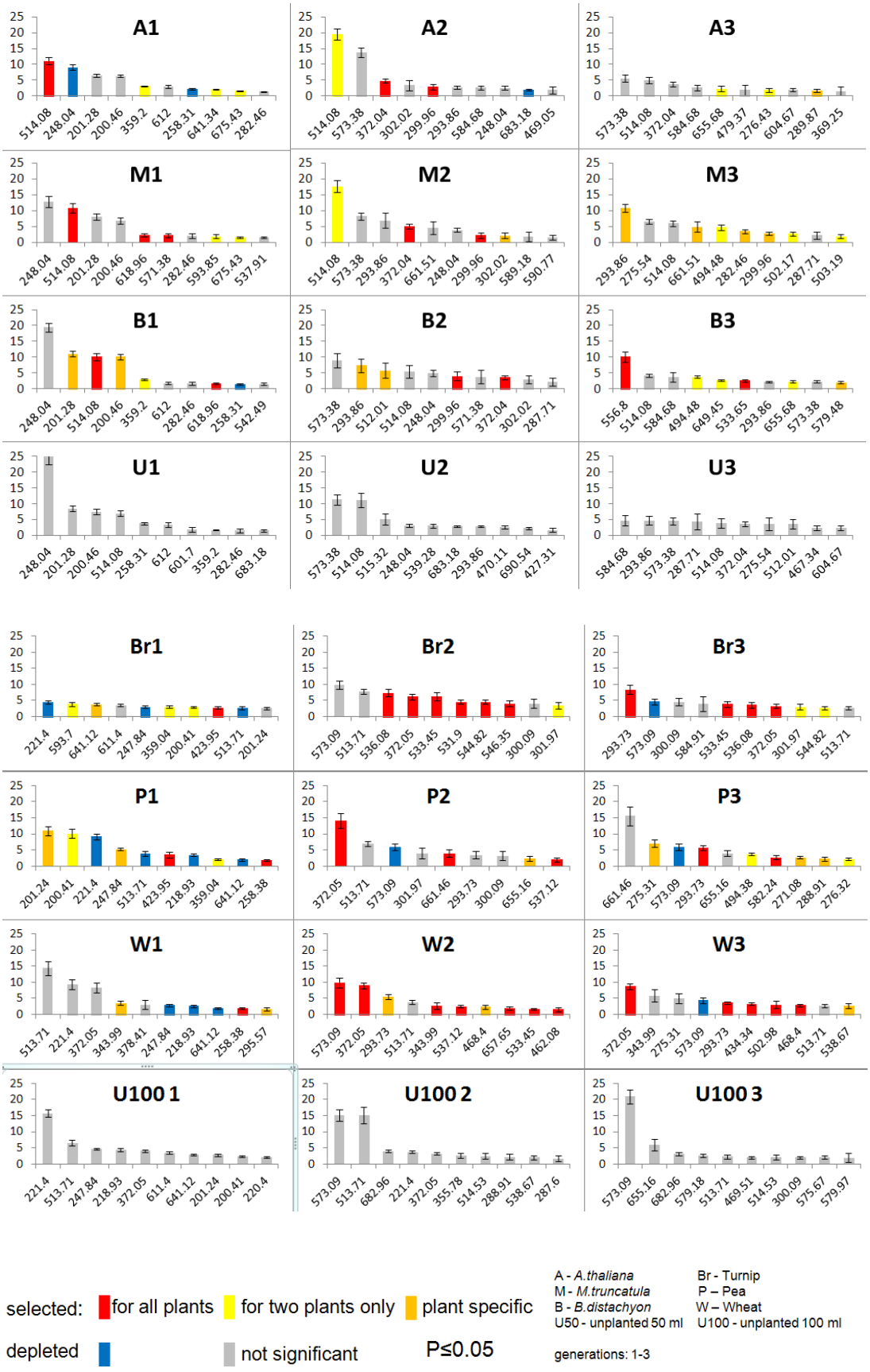
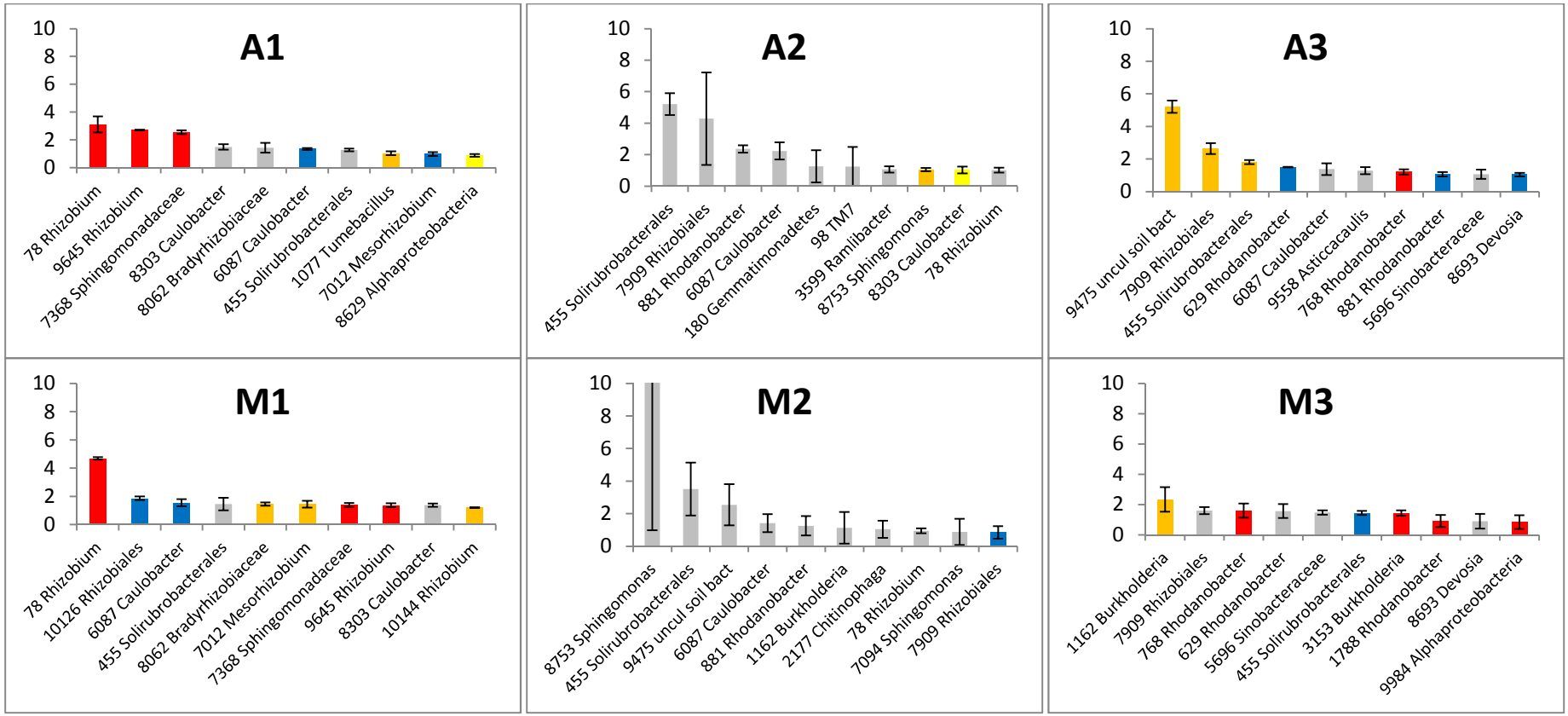
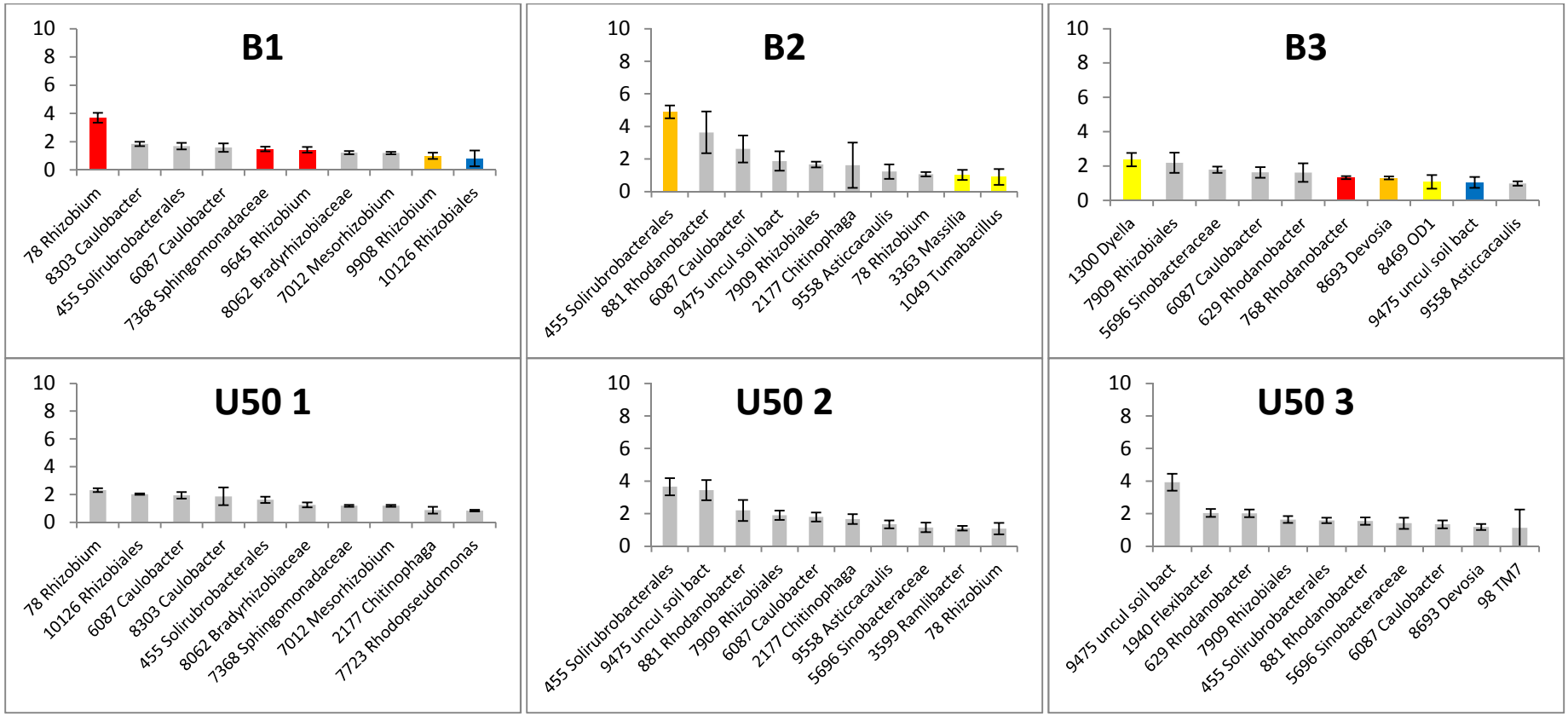
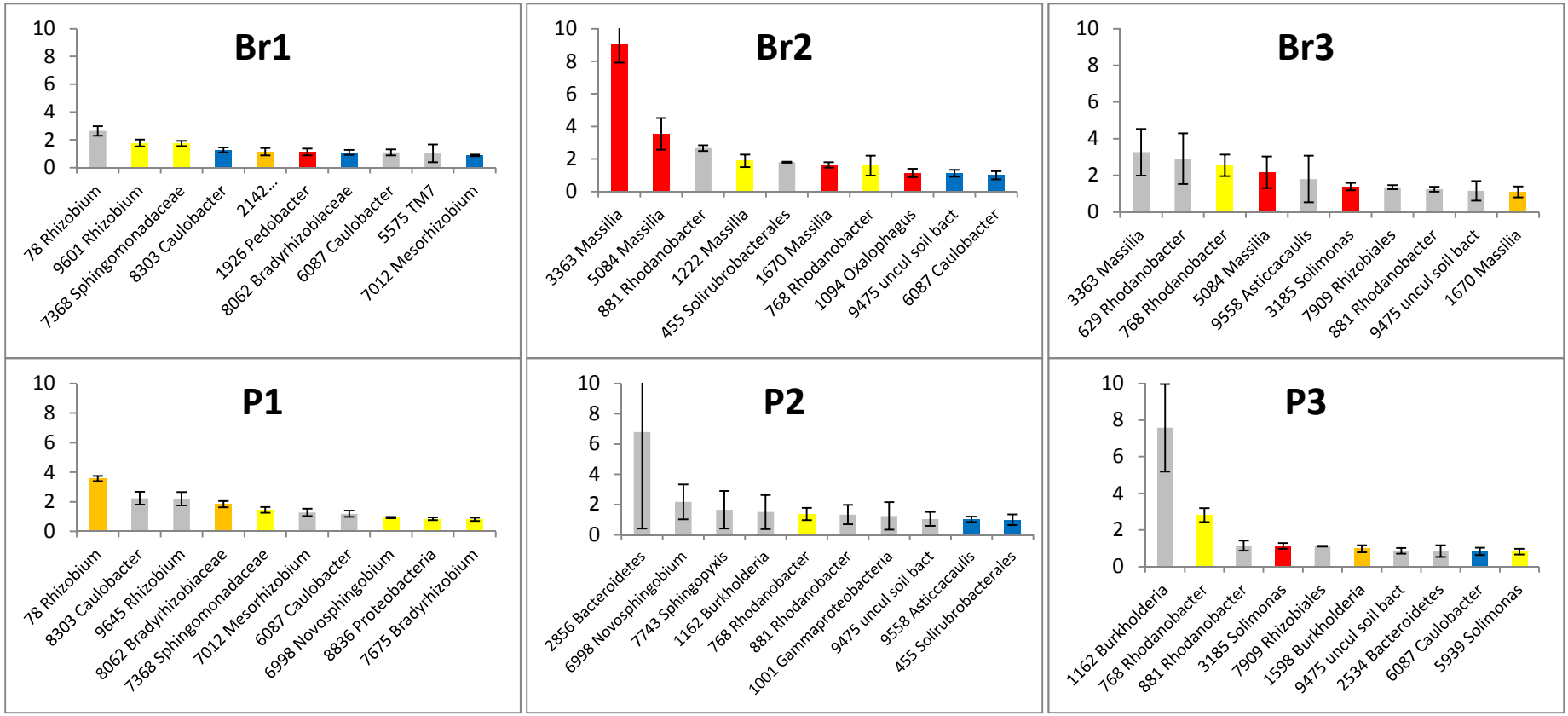


Figure 4.2.10 Bar graphs showing the 10 most abundant bacterial ITR sizes found in the rhizosphere of model and crop plants using ARISA.

Detailed analysis of individual OTUs (Figure 4.2.11) shows that the dominant OTU in all samples in the 1st generation was OTU 78 *Rhizobium*. This species must be very good in early colonization of soil as it became dominant with and without plant influence or it was very abundant to start with. In the 2nd generation OTU 78 can also be found but it lost its dominant position. It suggests that it can not withstand the competition from other organisms. However other *Rhizobium* OTUs are among the dominant species throughout the compost experiment. Model plants rhizospheres were very similar to unplanted controls as they share 5-6 out of 10 dominant OTUs. This confirms again that model plants have a smaller influence on the rhizosphere than crop plants. Crop plants select for specific OTUs that are rare in the unplanted control. In the turnip rhizosphere of the 2nd and the 3rd generation, OTUs belonging to *Massilia* became common (4 and 3 different OTUs in the 2nd and 3rd generation, respectively). Only a single *Massilia* OTU can be found in samples other than the turnip rhizosphere. A strong selection of *Massilia* by turnip may be caused by a plant influence, but may also be a response to a changing fungal community in the rhizosphere (chapter 4.5). In the 3rd generation of the pea rhizosphere OTUs belonging to *Burkholderia* dominated.


A1
10
8
6
4
2
0
78 Rhizobium
9645 Rhizobium
7368 Sphingomonadaceae
8303 Caulobacter
8062 Bradyrhizobiaceae
6087 Caulobacter
455 Solirubrobacterales
1077 Tumebacillus
7012 Mesorhizobium
8629 Alphaproteobacteria
A2
10
8
6
4
2
0
455 Solirubrobacterales
7909 Rhizobiales
881 Rhodanobacter
6087 Caulobacter
180 Gemmatimonadetes
98 TM7
3599 Ramlibacter
8753 Sphingomonas
8303 Caulobacter
78 Rhizobium
A3
10
8
6
4
2
0
9475 uncul soil bact
7909 Rhizobiales
455 Solirubrobacterales
629 Rhodanobacter
6087 Caulobacter
9558 Asticcacaulis
768 Rhodanobacter
881 Rhodanobacter
5696 Sinobacteraceae
8693 Devosia
M1
10
8
6
4
2
0
78 Rhizobium
10126 Rhizobiales
6087 Caulobacter
455 Solirubrobacterales
8062 Bradyrhizobiaceae
7012 Mesorhizobium
7368 Sphingomonadaceae
9645 Rhizobium
8303 Caulobacter
10144 Rhizobium
M2
10
8
6
4
2
0
8753 Sphingomonas
455 Solirubrobacterales
9475 uncul soil bact
6087 Caulobacter
881 Rhodanobacter
1162 Burkholderia
2177 Chitinophaga
78 Rhizobium
7094 Sphingomonas
7909 Rhizobiales
M3
10
8
6
4
2
0
1162 Burkholderia
7909 Rhizobiales
768 Rhodanobacter
629 Rhodanobacter
5696 Sinobacteraceae
455 Solirubrobacterales
3153 Burkholderia
1788 Rhodanobacter
8693 Devosia
9984 Alphaproteobacteria


B1
78 Rhizobium
8303 Caulobacter
455 Solirubrobacterales
6087 Caulobacter
7368 Sphingomonadaceae
9645 Rhizobium
8062 Bradyrhizobiaceae
7012 Mesorhizobium
9908 Rhizobium
10126 Rhizobiales
B2
455 Solirubrobacterales
881 Rhodanobacter
6087 Caulobacter
9475 uncul soil bact
7909 Rhizobiales
2177 Chitinophaga
9558 Asticcacaulis
78 Rhizobium
3363 Massilia
1049 Tumabacillus
B3
1300 Dyella
7909 Rhizobiales
5696 Sinobacteraceae
6087 Caulobacter
629 Rhodanobacter
768 Rhodanobacter
8693 Devosia
8469 OD1
9475 uncul soil bact
9558 Asticcacaulis
U50 1
78 Rhizobium
10126 Rhizobiales
6087 Caulobacter
8303 Caulobacter
455 Solirubrobacterales
8062 Bradyrhizobiaceae
7368 Sphingomonadaceae
7012 Mesorhizobium
2177 Chitinophaga
7723 Rhodopseudomonas
U50 2
455 Solirubrobacterales
9475 uncul soil bact
881 Rhodanobacter
7909 Rhizobiales
6087 Caulobacter
2177 Chitinophaga
9558 Asticcacaulis
5696 Sinobacteraceae
3599 Ramlibacter
78 Rhizobium
U50 3
9475 uncul soil bact
1940 Flexibacter
629 Rhodanobacter
7909 Rhizobiales
455 Solirubrobacterales
881 Rhodanobacter
5696 Sinobacteraceae
6087 Caulobacter
8693 Devosia
98 TM7


Br1
78 Rhizobium
9601 Rhizobium
7368 Sphingomonadaceae
8303 Caulobacter
2142...
1926 Pedobacter
8062 Bradyrhizobiaceae
6087 Caulobacter
5575 TM7
7012 Mesorhizobium
Br2
3363 Massilia
5084 Massilia
881 Rhodanobacter
1222 Massilia
455 Solirubrobacterales
1670 Massilia
768 Rhodanobacter
1094 Oxalophagus
9475 uncul soil bact
6087 Caulobacter
Br3
3363 Massilia
629 Rhodanobacter
768 Rhodanobacter
5084 Massilia
9558 Asticcacaulis
3185 Solimonas
7909 Rhizobiales
881 Rhodanobacter
9475 uncul soil bact
1670 Massilia
P1
78 Rhizobium
8303 Caulobacter
9645 Rhizobium
8062 Bradyrhizobiaceae
7368 Sphingomonadaceae
7012 Mesorhizobium
6087 Caulobacter
6998 Novosphingobium
8836 Proteobacteria
7675 Bradyrhizobium
P2
2856 Bacteroidetes
6998 Novosphingobium
7743 Sphingopyxis
1162 Burkholderia
768 Rhodanobacter
881 Rhodanobacter
1001 Gammaproteobacteria
9475 uncul soil bact
9558 Asticcacaulis
455 Solirubrobacterales
P3
1162 Burkholderia
768 Rhodanobacter
881 Rhodanobacter
3185 Solimonas
7909 Rhizobiales
1598 Burkholderia
9475 uncul soil bact
2534 Bacteroidetes
6087 Caulobacter
5939 Solimonas

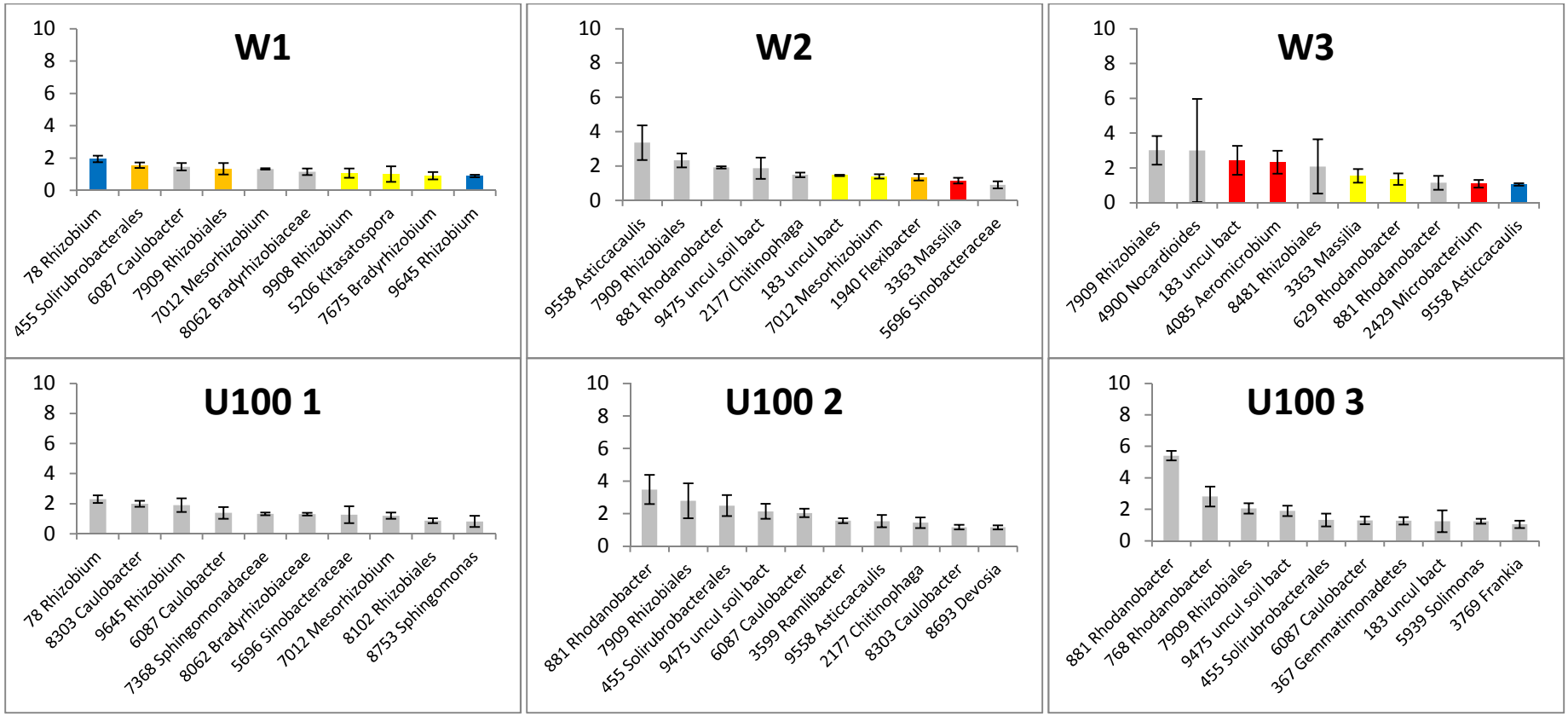
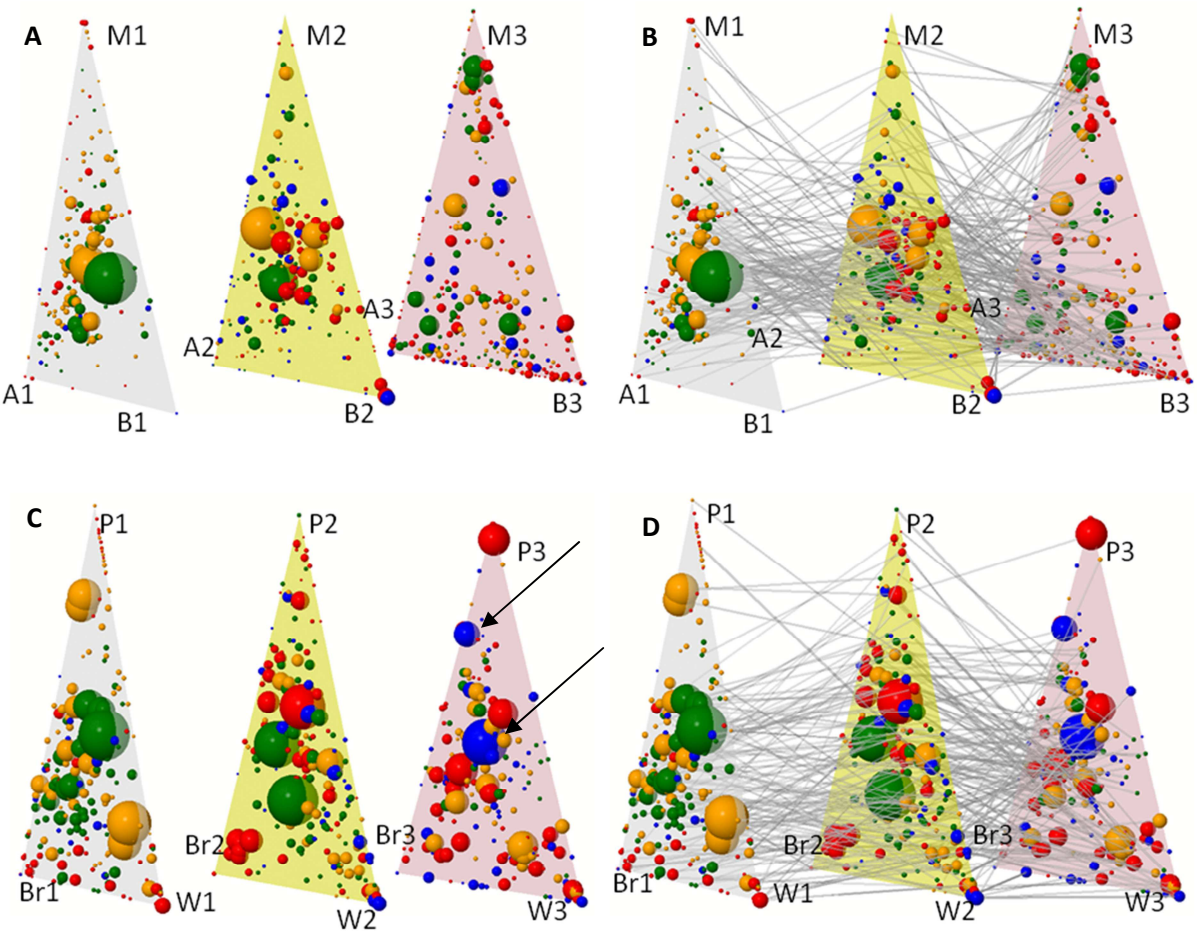


Figure 4.2.11 Ten dominant bacterial OTUs (number and genus annotation) for each plant/unplanted in each generation for model and crop plants grown in the compost experiment.

3D ternary plots confirm that there were a few dominant ITR sizes throughout all 3 generations that are shared between different plant species and unplanted control (Figure 4.2.12 A, B, C, D). They may represent soil organisms recalcitrant to plant influence that remain abundant because of the high organic matter content of compost. Crop plants in their third generation were able to suppress two ITRs represented as big blue balls – one is located in the centre of the triangle and the other one is located in pea corner (see arrows in Figure 4.2.12 C). The crop bacterial communities were dominated by rhizosphere specific bacteria, with ITR sizes annotated as orange and red balls. Most of the ITRs are carried over through successive generations (Figure 4.2.12 B and D).



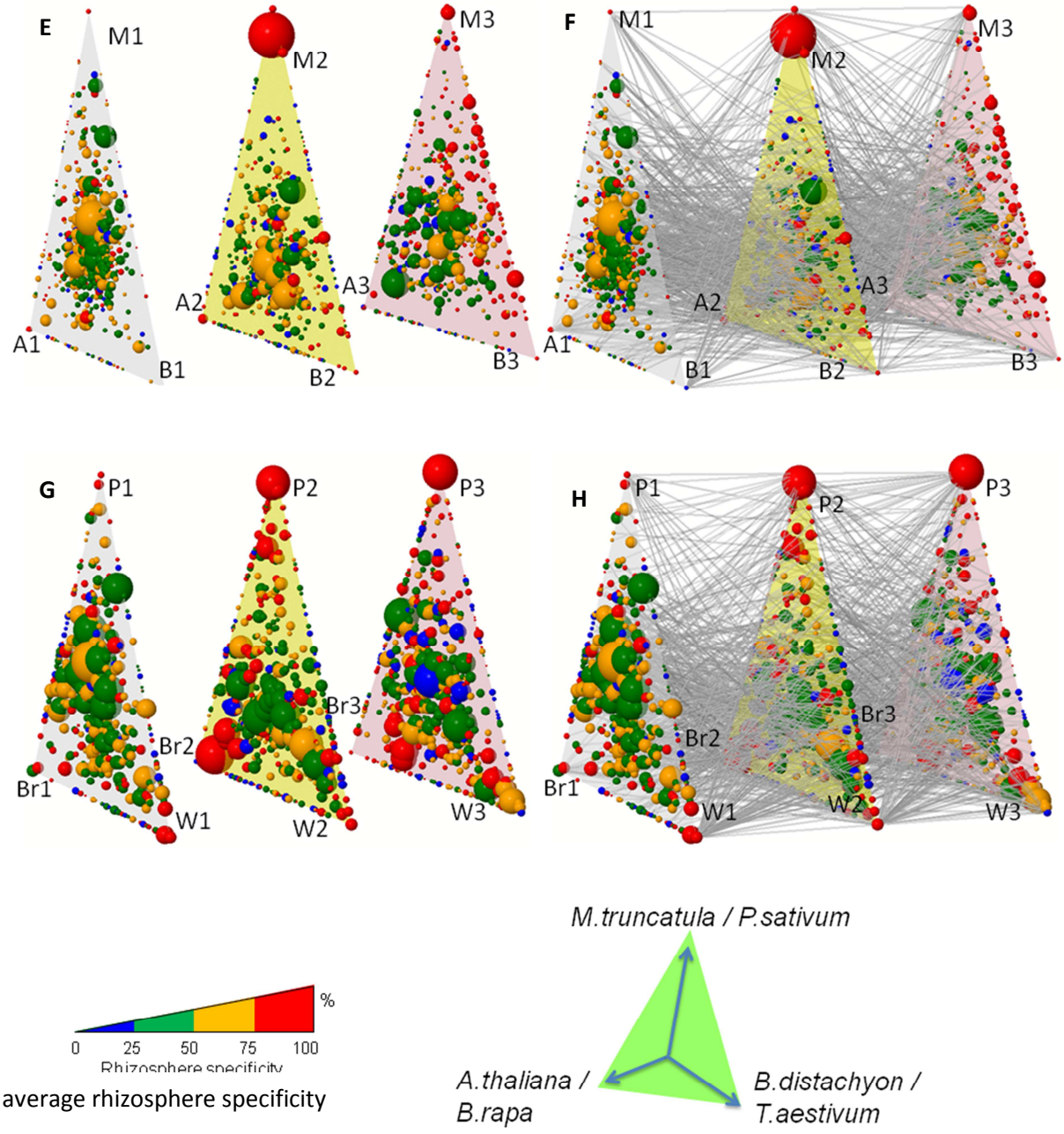


Figure 4.2.12 Two dimensional ternary plots for the compost experiment using ARISA (A,B,C,D) and 454 sequencing (E,F,G,H). Figures A, B, E, F show model plants community and C, D, G, H show crop plants community.

The bacterial community structure in compost, examined using 454 sequencing, was relatively stable over generations as shown on Figure 4.2.12 E, F, G, H. The pea rhizosphere was dominated by *Burkholderia* OTU 1162 (annotated as red ball in the corner) in the 2nd and 3rd generations (Figure 4.2.12 G). *Massilia* OTU 3363 is located in the turnip corner in the 2nd and 3rd generation. The *Medicago* rhizosphere in the 2nd generation was dominated by OTU 8753 *Sphingomonas*. This genus may be invasive, as it cannot be found in the 1st or 3rd generations. The community in the 3rd generation of model plants was clearly divided into highly *Medicago* and *Brachypodium* rhizosphere specific OTUs and soil opportunists (green and orange balls). Even though the selected OTUs were not abundant (balls representing them have a relatively small volume) this suggests that *Medicago* and *Brachypodium* can actively shape the rhizosphere community. This also suggests that the *Arabidopsis* rhizosphere is similar to the unplanted control and this model plant only weakly modifies the soil microbiome. Grey lines connect OTUs that can be found in successive generations (Figure 4.2.12 F and H).

Crop plants selected greater numbers of OTUs in their rhizosphere. This effect may be correlated with greater plant size and therefore larger amount of root exudates influencing the rhizosphere community. Some bacterial taxa are strongly selected: Solirubrobacteraceae, Actinomycetales and Bacteroidetes in all three crop plants (Figure 4.2.13 A). Some taxa are more plant species specific: Caulobacterales in the wheat rhizosphere, *Pseudomonas* and *Burkholderia* in pea rhizosphere. The genus *Massilia* is selected by all crop plants; however it was most abundant in the turnip rhizosphere. Model plants had a weaker effect on the community as not many OTUs were strongly upregulated. Heat maps confirm the findings observed using MDS plots where model plant rhizospheres in compost were located close to unplanted control. A smaller number of depleted OTUs in the model and crop plant rhizospheres in the rhizosphere may indicate that most of the bacteria do not rely on plant exudates and are thriving on soil using nutrients in compost as an energy and nutrient source. Interestingly, six OTUs belonging to Gemmatimonadetes can only be found among the depleted community (Figure 4.2.13 B).

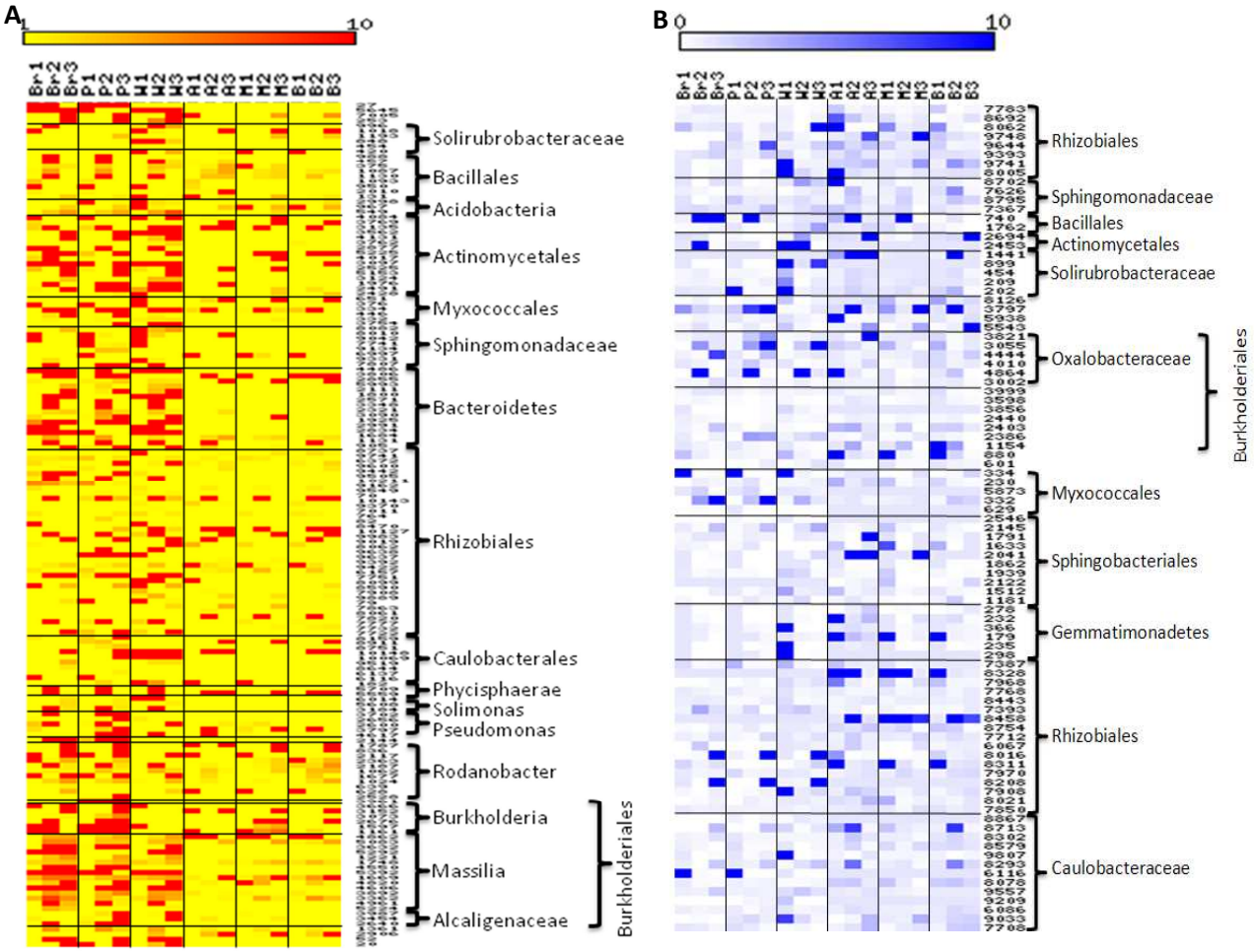


Figure 4.2.13 Heat maps showing A) selected and B) depleted bacterial OTUs in the rhizosphere compared to unplanted control. OTU abundance was compared using t-test against unplanted control. Shown OTUs are selected or depleted in at least one rhizosphere in at least one generation. Colour scale represents fold difference against the unplanted control.

4.2.2 Summary and discussion of the results for ARISA and 454 pyrosequencing of bacterial community of model and crop plants grown in compost

The bacterial community was relatively stable in the compost experiment. However, community drift over generations was still the dominant effect for all samples. There was no major diversity loss or opportunist invasions over generations. Proteobacteria were a dominant phylum, however its abundance did not increase with successive generations (as

happened in the sand experiment). Many bacterial OTUs were not influenced by plants, even though the plant species effect on the rhizosphere bacterial community was significant according to MANOVA analysis of ARISA data (strain/species level). However, plants selected a whole range of different bacterial OTUs. Many of the selected OTUs are annotated as *Massilia*, Burkholderiales and Alphaproteobacteria. Massilia in particular was common in *Brassica* rhizosphere. During the compost experiment crop and model plants were examined. One of the aims of the compost experiment was to test if plants belonging to the same families have a similar effect on the community structure. No such effect could be observed in the analyzed data However, crop plants exerted a stronger bacterial selection than model plants.

4.2.3 Results and discussion of pyrosequencing of fungal succession over 3 generations of plants grown in compost

The fungal community diverged over generations (Figure 4.2.14), with a clear generation shift of the community. The turnip rhizosphere communities in the 2nd and 3rd generation were very different from all other samples, causing them to cluster close to each other, but relatively away from other rhizospheres. The pea rhizosphere also seems to be different, while the wheat community did not change much over generations.

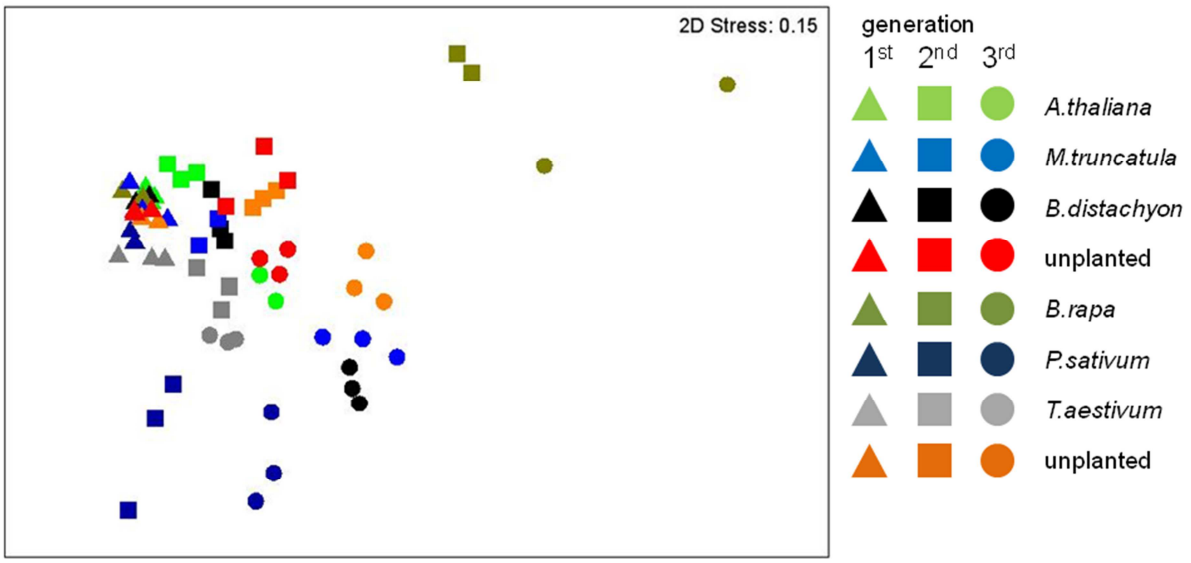


Figure 4.2.14 MDS plot of the model and crop plants grown in compost. Each data point represents fungal rhizosphere community structure based on the 454 pyrosequencing

The fungal community was dominated by Pezizomycotina and Agaricomycotina (Figure 4.2.15). There are some differences between different plant species and generations: pea and *Brachypodium* rhizospheres became dominated by Pezizomycotina in successive generations. Overall, the fungal community in the compost experiment was relatively similar to the community sampled in plants grown on sand.

The turnip rhizosphere was dominated by a single species: *Olpidium brassicae* belonging to either incertae sedis of fungi (UNIPROT taxonomy) or Chytriodiomycotina (Hartwright *et al.*, 2010). More details about the turnip fungal community will be provided later in this chapter.

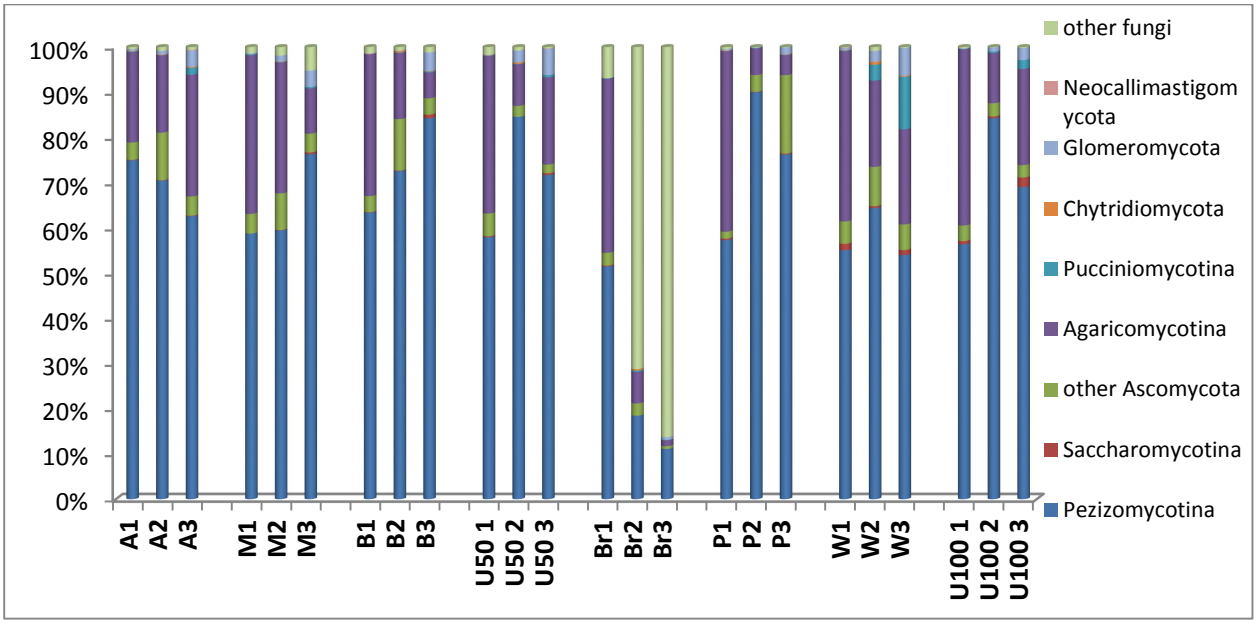


Figure 4.2.15 Fungal community structure in the 3 generations of plants grown in compost. Graph based on GenBank BLAST report uploaded into MEGAN (1% top hit)

Statistics based on ANOVA with Tukey test (significance 0.05)

taxa	Br1	P1	W1	U1	Br2	P2	W2	U2	Br3	P3	W3	U3
Chtridiomycota	a	a	a	a	a	a	a	a	a	a	a	a
Agaricomycotina	a	a	a	a	a	a	a	a	a	a	a	a
other fungi	a	a	a	a	ab	a	a	a	b	a	a	a
Glomeromycota	a	a	a	a	a	a	a	a	a	a	b	ab
Neocallimastigomycota	a	a	a	a	a	a	a	a	a	a	a	a
Pezizomycotina	abc	abc	ab	ab	a	c	abc	abc	a	bc	bc	bc
Pucciniomycotina	a	a	a	a	a	a	a	a	a	a	b	a
Saccharomycotina	ab	ab	abc	ab	a	ab	ab	ab	a	ab	ab	c

taxa	A1	M1	B1	U1	A2	M2	B2	U2	A3	M3	B3	U3
Chtridiomycota	a	a	a	a	a	a	a	a	a	a	a	a
Agaricomycotina	ab	ab	ab	b	ab	ab	a	a	ab	a	a	ab
other fungi	a	a	a	ab	ab	a	a	a	a	b	ab	a
Glomeromycota	a	a	a	a	a	a	a	a	a	a	a	a
Neocallimastigomycota	a	a	a	a	a	a	a	a	a	a	a	a
Pezizomycotina	ab	ab	ab	ab	ab	a	ab	ab	ab	ab	b	ab
Pucciniomycotina	a	a	a	a	a	a	a	a	a	a	a	a
Saccharomycotina	a	a	a	ab	a	a	a	a	a	ab	b	ab

The fungal community was far less diverse than the bacterial community. The same observation was made during the sand experiment. However, the fungal diversity index was at its highest in the rhizosphere of the second generation (even, though this observation is not statistically significant the same trend was observed for all plants except pea and turnip), which is the opposite to that seen for bacteria (Figure 4.2.16). Only the turnip rhizosphere became dominated by a single OTU in the 3rd generation. Unplanted control has a reversed diversity changes comparing to rhizosphere samples, however only statistical differences are observed in the second generation.

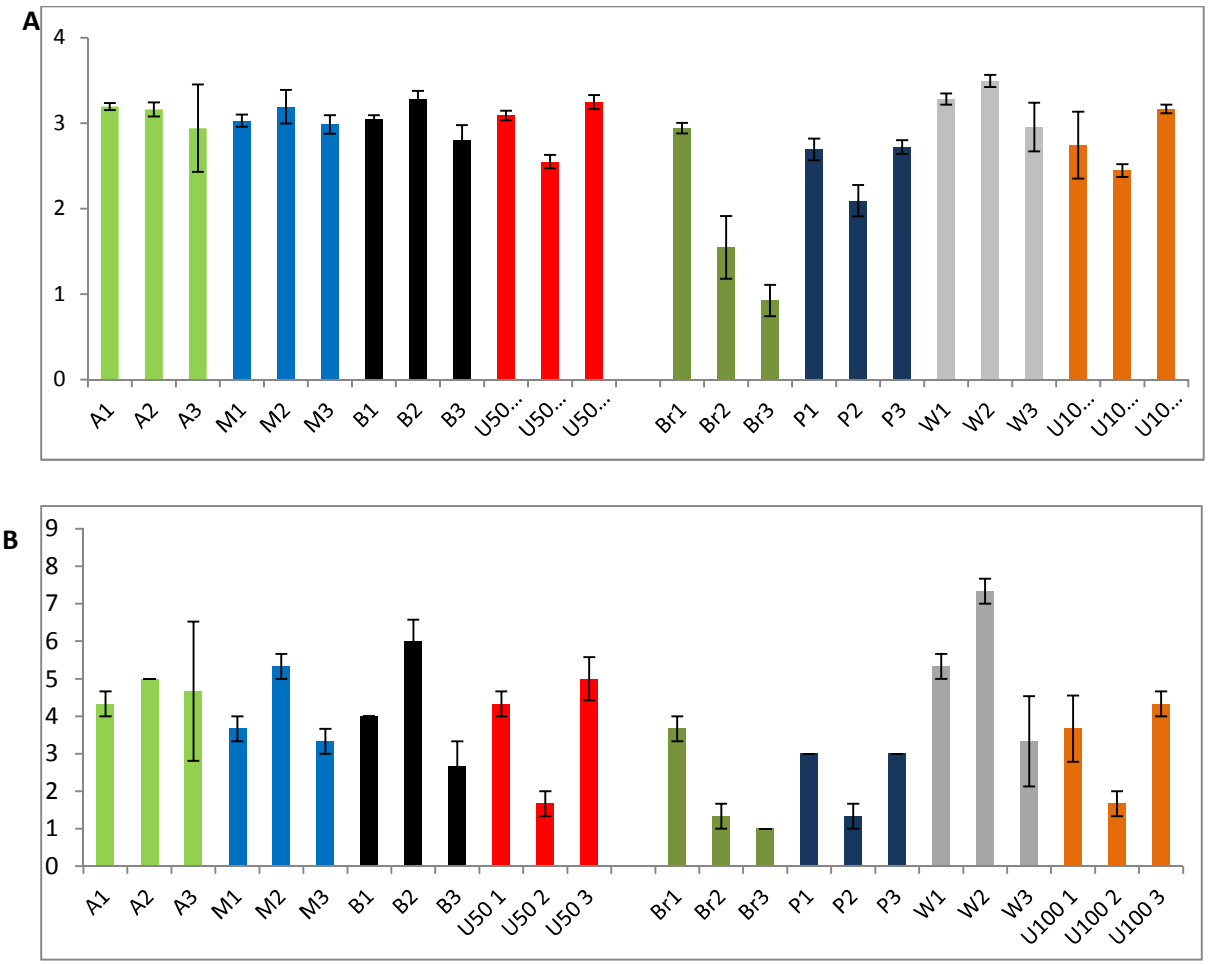


Figure 4.2.16 Shannon diversity index (A) and richness (B) for fungal 454 pyrosequencing data. Richness is presented as the number of different OTUs needed to reach 50% of the total abundance.

Paired test ($p<0.05$) results (X - X not significant, X -*- X significant), for 454 data n=3 each plant/unplanted in each generation.

A) A1 - A2 - A3, M1 - M2 - M3, B1 - B2 - B3, U1 -*- U2 -*- U3

A1 - U1	A2 -*- U2	A3 - U3
M1 - U1	M2 -*- U2	M3 -U3
B1 - U1	B2 -*- U2	B3 - U3

Br1 -*- Br2 - Br3, P1 - P2 -*- P3, W1 - W2 - W3, U1 - U2 -*- U3

Br1 - U1	Br2 - U2	Br3 -*- U3
P1 - U1	P2 - U2	P3 -U3
W1 - U1	W2 - U2	W3 -*- U3

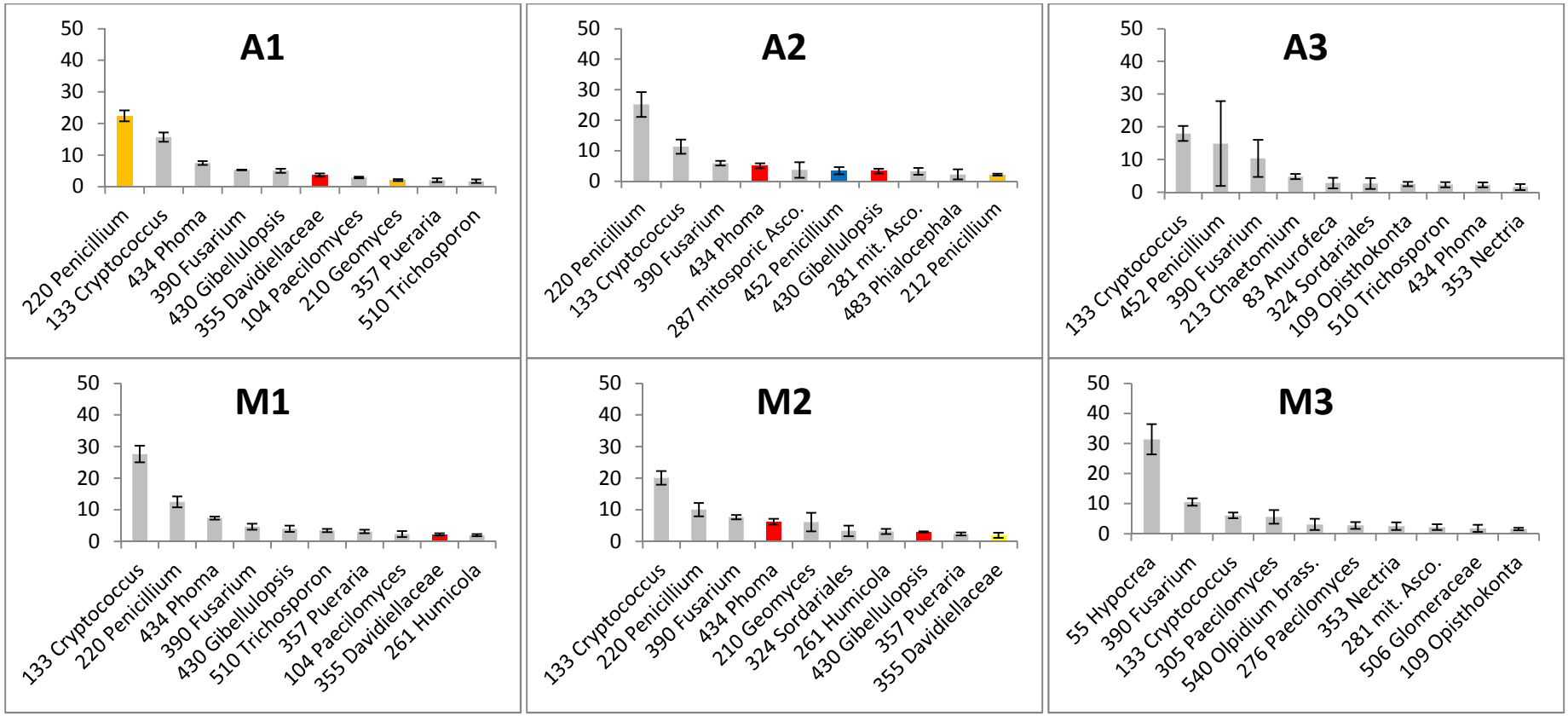
B) A1 - A2 - A3, M1 -*- M2 -*- M3, B1 -*- B2 -*- B3, U1 -*- U2 -*- U3

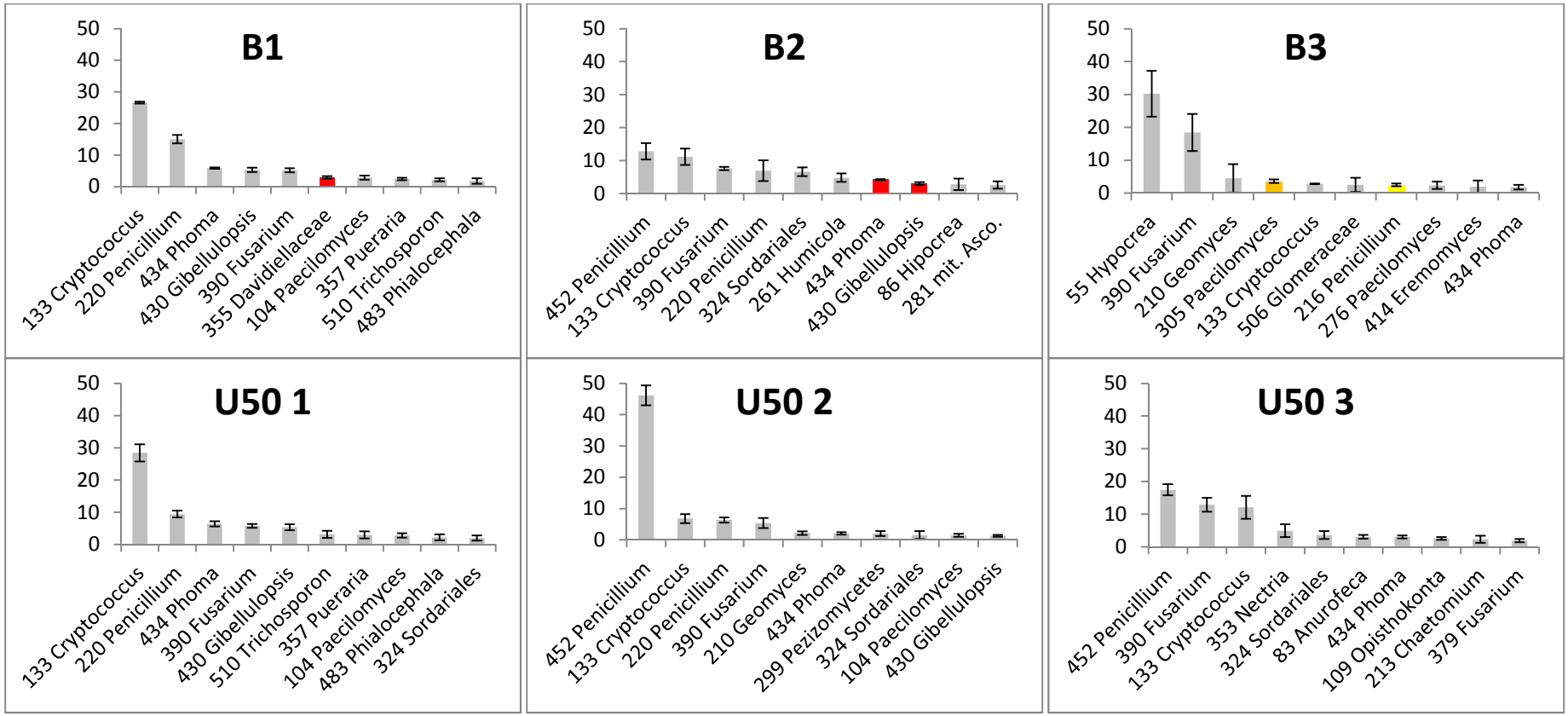
A1 - U1	A2 -*- U2	A3 - U3
M1 - U1	M2 -*- U2	M3 -U3
B1 - U1	B2 -*- U2	B3 - U3

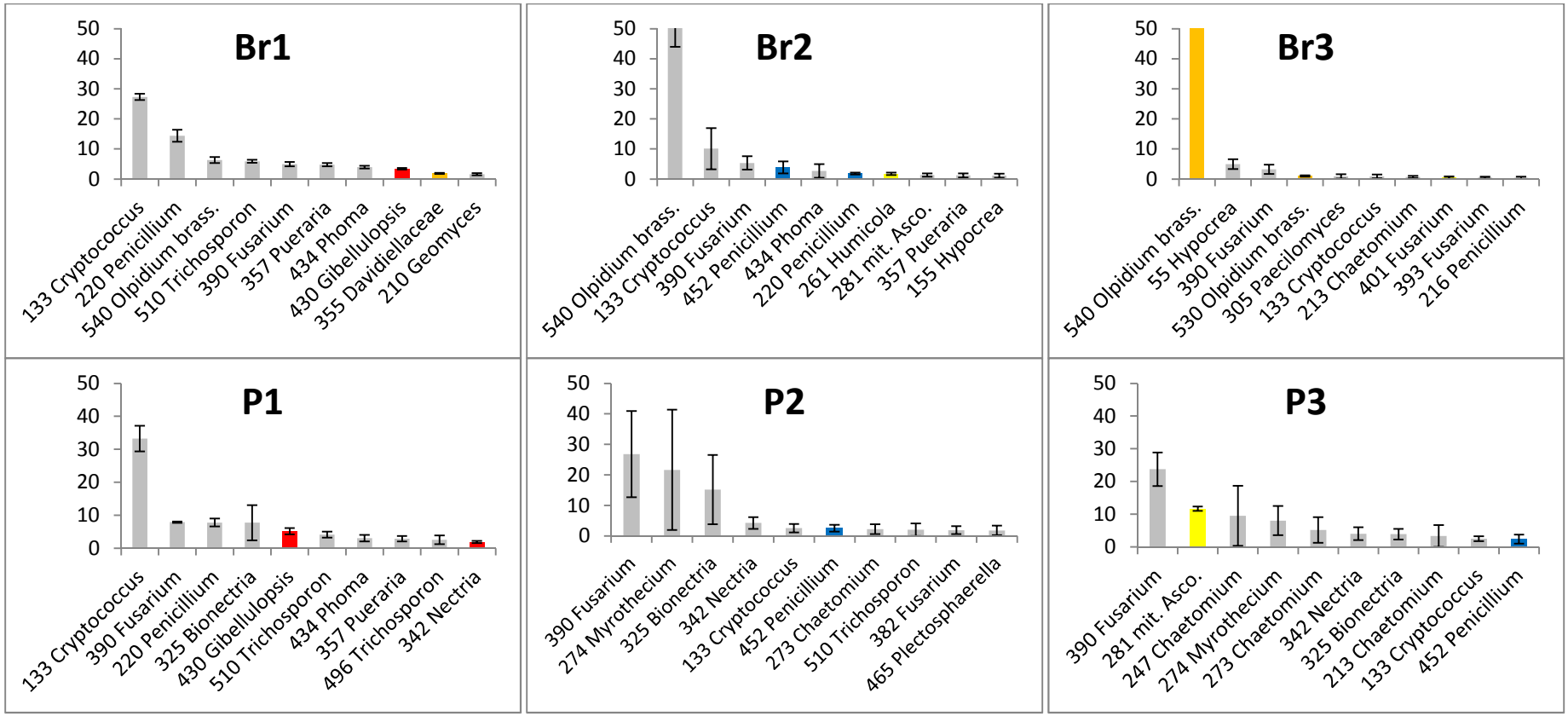
Br1 -*- Br2 - Br3, P1 -*- P2 -*- P3, W1 -*- W2 -*- W3, U1 - U2 -*- U3

Br1 - U1	Br2 - U2	Br3 - U3
P1 - U1	P2 - U2	P3 -U3
W1 - U1	W2 - U2	W3 - U3

The fungal community was dominated by a few OTUs belonging to *Penicillium*, *Cryptococcus*, *Phoma*, *Fusarium* and *Gibellulopsis* genera (Figure 4.2.17). *Olpidium brassicae* became dominant in the turnip rhizosphere. Most of the dominant OTUs can be found in more than one generation. Only the 3rd generation seems to be slightly different as *Medicago* and *Brachypodium* were characterized by higher abundance of *Hypocrea* and pea and wheat by mitosporic Ascomycota OTUs. Due to relatively large differences between replicates only a few dominant OTUs are significantly upregulated or downregulated in the rhizospheres.


A1
50
40
30
20
10
0
220 Penicillium
133 Cryptococcus
434 Phoma
390 Fusarium
430 Gibellulopsis
355 Davidiellaceae
104 Paecilomyces
210 Geomyces
357 Pueraria
510 Trichosporon
A2
220 Penicillium
133 Cryptococcus
390 Fusarium
434 Phoma
287 mitosporic Asco.
452 Penicillium
430 Gibellulopsis
281 mit. Asco.
483 Phialocephala
212 Penicillium
A3
133 Cryptococcus
452 Penicillium
390 Fusarium
213 Chaetomium
83 Anurofeca
324 Sordariales
109 Opisthokonta
510 Trichosporon
434 Phoma
353 Nectria
M1
133 Cryptococcus
220 Penicillium
434 Phoma
390 Fusarium
430 Gibellulopsis
510 Trichosporon
357 Pueraria
104 Paecilomyces
355 Davidiellaceae
261 Humicola
M2
133 Cryptococcus
220 Penicillium
390 Fusarium
434 Phoma
210 Geomyces
324 Sordariales
261 Humicola
430 Gibellulopsis
357 Pueraria
355 Davidiellaceae
M3
55 Hypocrea
390 Fusarium
133 Cryptococcus
305 Paecilomyces
540 Olpidium brass.
276 Paecilomyces
353 Nectria
281 mit. Asco.
506 Glomeraceae
109 Opisthokonta


B1
50
40
30
20
10
0
133 Cryptococcus
220 Penicillium
434 Phoma
430 Gibellulopsis
390 Fusarium
355 Davidiellaceae
104 Paecilomyces
357 Pueraria
510 Trichosporon
483 Phialocephala
B2
452 Penicillium
133 Cryptococcus
390 Fusarium
220 Penicillium
324 Sordariales
261 Humicola
434 Phoma
430 Gibellulopsis
86 Hipocrea
281 mit. Asco.
B3
55 Hypocrea
390 Fusarium
210 Geomyces
305 Paecilomyces
133 Cryptococcus
506 Glomeraceae
216 Penicillium
276 Paecilomyces
414 Eremomyces
434 Phoma
U50 1
133 Cryptococcus
220 Penicillium
434 Phoma
390 Fusarium
430 Gibellulopsis
510 Trichosporon
357 Pueraria
104 Paecilomyces
483 Phialocephala
324 Sordariales
U50 2
452 Penicillium
133 Cryptococcus
220 Penicillium
390 Fusarium
210 Geomyces
434 Phoma
299 Pezizomycetes
324 Sordariales
104 Paecilomyces
430 Gibellulopsis
U50 3
452 Penicillium
390 Fusarium
133 Cryptococcus
353 Nectria
324 Sordariales
83 Anurofeca
434 Phoma
109 Opisthokonta
213 Chaetomium
379 Fusarium


Br1
50
40
30
20
10
0
133 Cryptococcus
220 Penicillium
540 Olpidium brass.
510 Trichosporon
390 Fusarium
357 Pueraria
434 Phoma
430 Gibellulopsis
355 Davidiellaceae
210 Geomyces
Br2
540 Olpidium brass.
133 Cryptococcus
390 Fusarium
452 Penicillium
434 Phoma
220 Penicillium
261 Humicola
281 mit. Asco.
357 Pueraria
155 Hypocrea
Br3
540 Olpidium brass.
55 Hypocrea
390 Fusarium
530 Olpidium brass.
305 Paecilomyces
133 Cryptococcus
213 Chaetomium
401 Fusarium
393 Fusarium
216 Penicillium
P1
133 Cryptococcus
390 Fusarium
220 Penicillium
325 Bionectria
430 Gibellulopsis
510 Trichosporon
434 Phoma
357 Pueraria
496 Trichosporon
342 Nectria
P2
390 Fusarium
274 Myrothecium
325 Bionectria
342 Nectria
133 Cryptococcus
452 Penicillium
273 Chaetomium
510 Trichosporon
382 Fusarium
465 Plectosphaerella
P3
390 Fusarium
281 mit. Asco.
247 Chaetomium
274 Myrothecium
273 Chaetomium
342 Nectria
325 Bionectria
213 Chaetomium
133 Cryptococcus
452 Penicillium

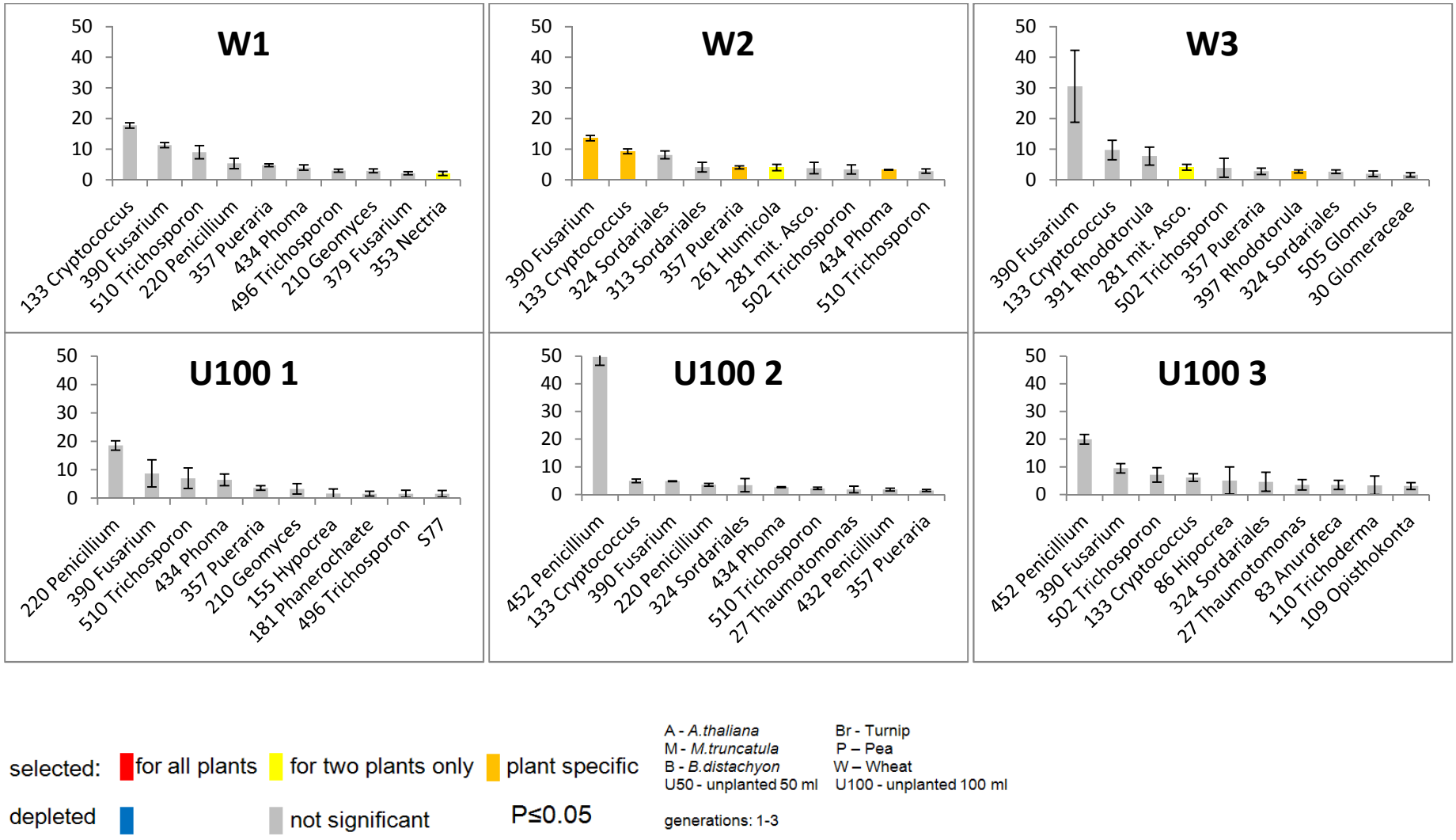


Figure 4.2.17 Ten most common fungal OTUs (number and genus annotation) for each plant/unplanted in each generation for model and crop plants in the compost experiment.

The fungal community was analyzed using lower sequencing depth than the bacterial one. The fungal community was also less diverse producing lower number of OTUs (even for the same number of 454 reads). Three dimensional ternary plots reflect these observations (Figure 4.2.18). There are not many OTUs presented on the graphs. In model and crop plant rhizospheres of the first generation most of the OTUs are located in the central area of the graph and are labelled green or orange. This indicates a very uniform fungal structure among different samples. There were some changes in the 2nd generation. Model plants selected many of the OTUs; however the selection was not strongly plant species specific (orange balls in the central area). Crop plants had a much stronger influence, as most of the community is placed either on the side of the graph or in the corners. Models and crops strongly suppressed OTU 452 (big blue ball on both graphs in the 2nd generation) annotated as *Penicillium citrinum*. This species is known to produce cellulases, xylolases, mycotoxin citrinin and plant hormones like gibberellins (Khan *et al.*, 2008). For unknown reasons this *Penicillium* species can thrive in the unplanted control but can not withstand the plant root influence. The 2nd generation was also characterized by a diversity collapse in turnip as only one OTU became dominant – *Olpidium brassicae*. The pea rhizosphere was very selective as many OTUs can be found in the pea corner only. The third generation of model plants was dominated by a single *Medicago* and *Brachypodium* specific OTU 55 – *Hypocrea* (also found to be very turnip specific). An annotated *Hypocrea* species was previously found in a study focusing on isolation of *Miscanthus* cell wall degrading fungal species (Shrestha *et al.*, 2011). *Arabidopsis* did not exert much influence on the community as most of the OTUs in *Arabidopsis* corner are either green or blue. Almost all other OTUs presented on the crop plants ternary plot were shared between pea and wheat rhizospheres. The most abundant one - *Fusarium* OTU 390 became dominant and it is represented as a large orange ball on the graph (see arrow on 4.2.18).

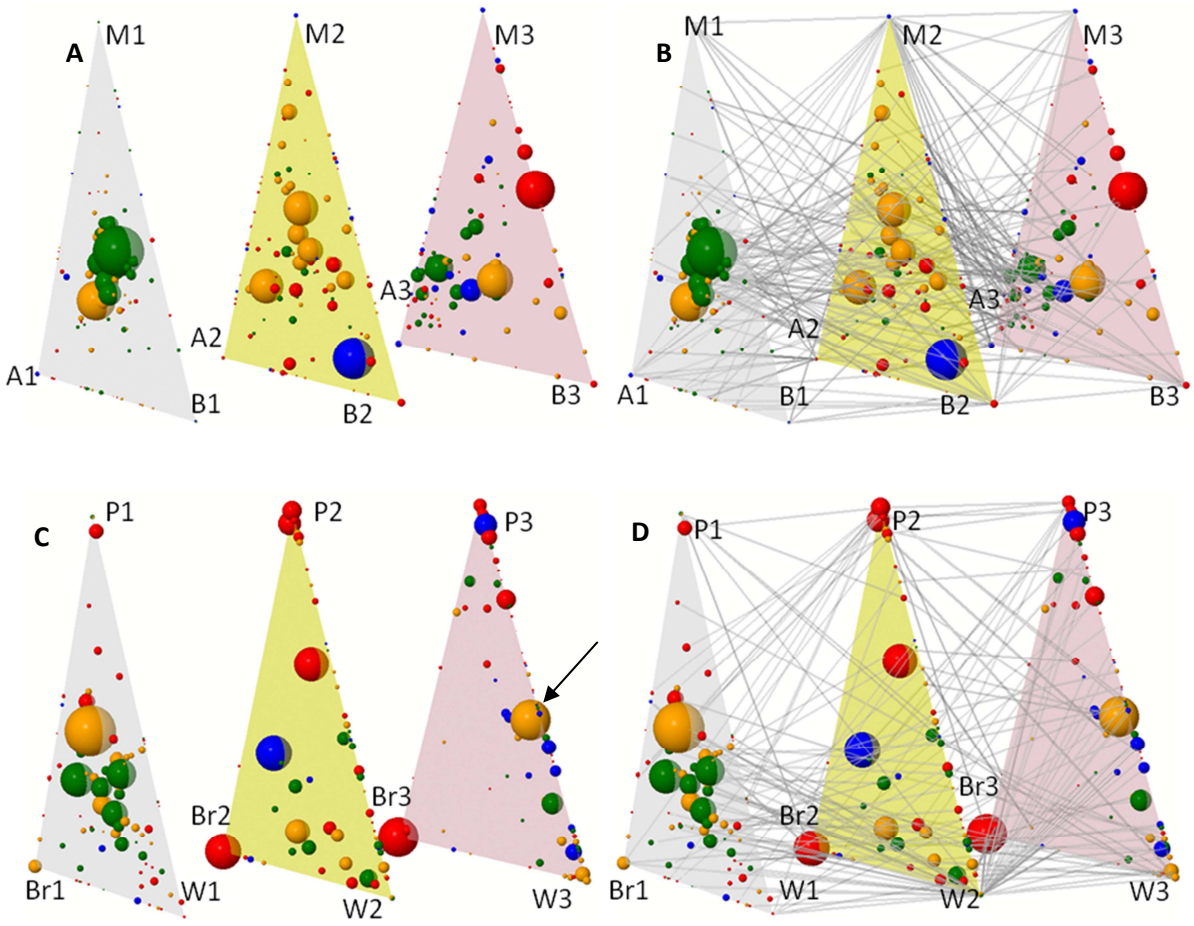


Figure 4.2.18 Three-dimensional ternary plots for the fungal community structure in plants grown in compost using 454 pyrosequencing. A and B – crop plants, C and D – model plants.

Many of the OTUs were either selected or depleted in the rhizosphere (Figure 4.2.19). This observation may be partially caused by low numbers of 454 reads obtained for the fungal community. Low number of reads may produce false positive results, as some species could be present in all samples but were detected only in a few. The turnip rhizosphere, as mentioned above, was dominated by two OTUs belonging to *Olpidium brassicae* in the 2nd and 3rd generation. In the 1st generation this plant selected and suppressed different *Penicillium* OTUs. The pea rhizosphere strongly selected for *Myrothecium* and *Nectria* genera. The wheat rhizosphere selected many different OTUs and strongly suppressed an OTU belonging to the *Nectriaceae* family. *Arabidopsis* in the 1st and 2nd generation was dominated by *Penicillium* OTUs. Interestingly, turnip and *Arabidopsis*, which both belong to Brassicaceae family, selected for the same *Penicillium* OTUs. The *Medicago* and

Brachypodium rhizosphere are relative similar to each other as they both select for *Olpidium* and unclassified *Ascomycota*.

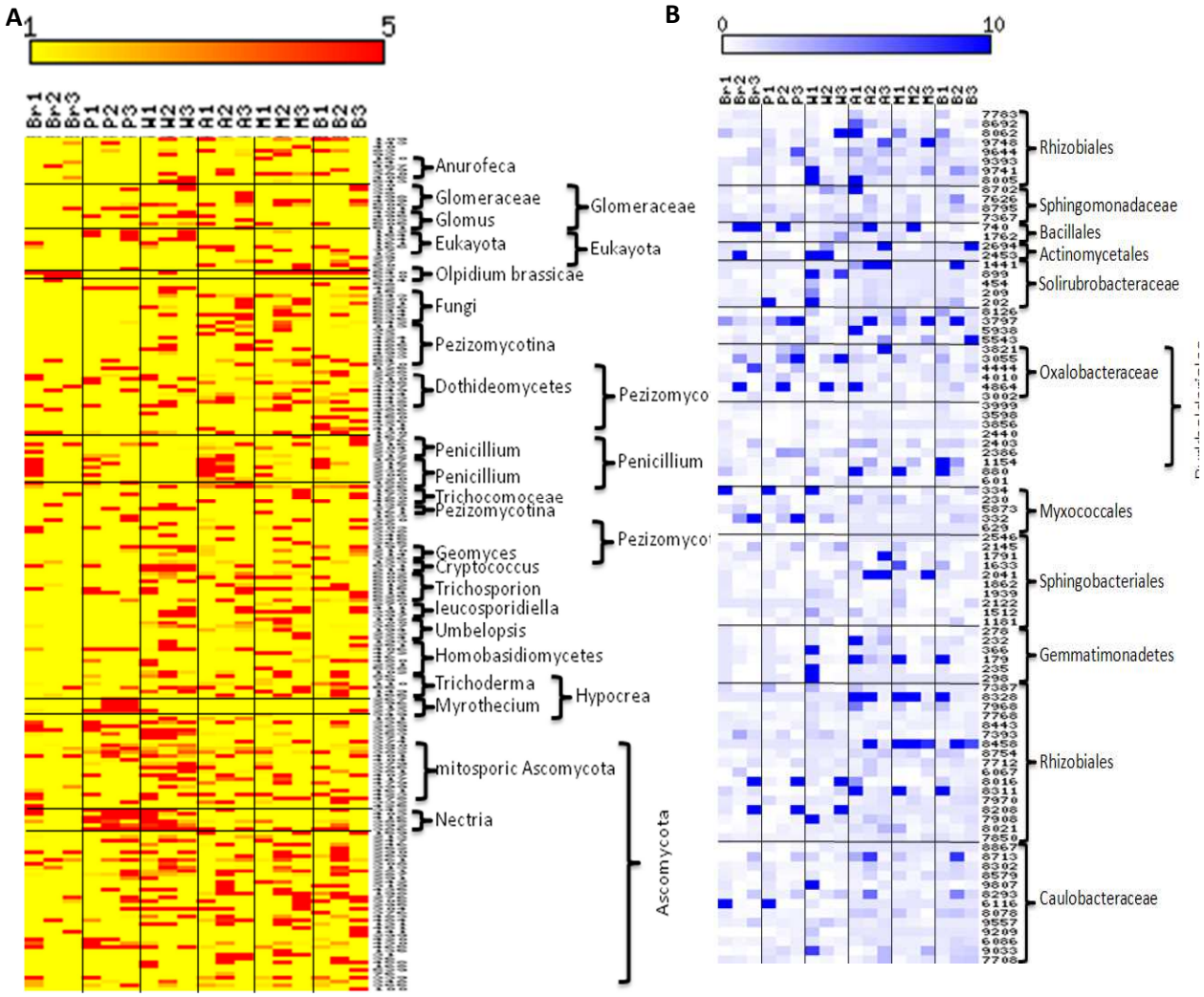


Figure 4.2.19 Heat maps showing A) selected and B) depleted bacterial OTUs in the rhizosphere compared to unplanted control. OTUs abundance was compared using t-tests against unplanted controls. Shown OTUs were selected or depleted in at least one rhizosphere in at least one generation. The colour scale represents the fold difference compared to the unplanted control.

Two OTUs were annotated as *Olpidium brassicae* (OTU 530 and 540). The closest hit in the Genbank database was *Olpidium brassicae* strain GBR7 isolated from *Brassica oleraea* (cauliflower) (Hartwright *et al.*, 2010). *Olpidium brassicae* is a well known obligate root-infecting plant pathogen. It is a vector spreading plant viruses (e.g., *turnip crinkle* virus) in

different hosts including cucumber, lettuce, carrot and the Brassicaceae family (Campbell, 1996; Rochon, 2009). 454 pyrosequencing of the model and crop plant rhizospheres showed that *Brassica rapa* (turnip) is very vulnerable to infection (Figure 4.2.20). The pathogen was also found in the 3rd generation of *Arabidopsis* and *Medicago* and in all other plants, except pea, in at least one generation. However *Olpidium* abundance in the turnip rhizosphere was much higher than in other plant species and it increased over generations. Pathogenic/opportunistic invasions are possible in a rich compost/soil conditions and they are strongly related to a plant influence. The pathogen invasion was caused by a monoculture of turnip plants in the same soil for 3 successive generations. It can be assumed that if a crop rotation was introduced during the experiment the plant species specific pathogens would not build up. It is worth noting that *Olpidium* presence in the rhizosphere did not visually influenced plant growth. The one possibility of this fact is that plants were grown for only 4 weeks (and put into the soil as developed seedlings). Maybe if the plants were kept for longer some disease syndromes would develop. Comparison between bacterial and fungal community structure for *Brassica* showed that *Massilia* is strongly enriched in the rhizosphere and its abundance follows that of *Olpidium*.

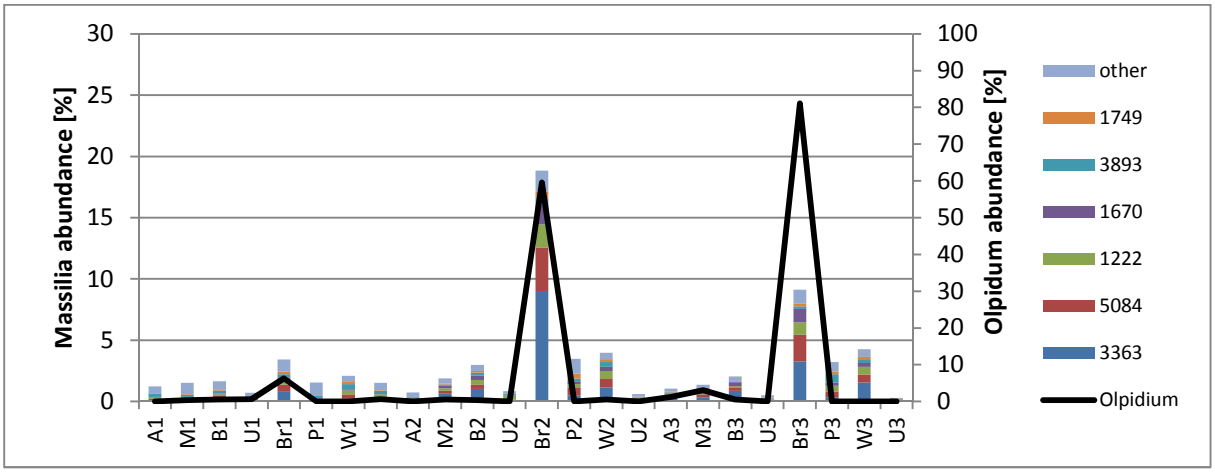


Figure 4.2.20 Abundance of Olpidium brassicae – black line (OTU 530 and 540) and *Massilia* – bar graph (OTU 3363, 5084, 1222, 1670, 3893, 1749 and others) in the rhizosphere of model and crop plants. Abbreviations as on the Table 4.2.1

4.2.4 Summary and discussion of the results for 454 pyrosequencing of fungal community of model and crop plants

The overall picture of the fungal community structure in compost is relatively similar to the bacterial one. There was a clear spread of the community over generations. The community starts very homogenous, with few differences between different plant species and unplanted control. A very interesting phenonenon was the pathogenic invasion of *Olpidium brassicae* in the turnip rhizosphere over generations. This finding underlies the need for crop rotation in real field conditions. *Massilia* abundance increased in the fungal infected rhizosphere. *Massilia timonae* was characterized as able to utilize chitin as the only carbon and nitrogen source *in vitro* (main component of the fungal cell walls) (Adrangi *et al.*, 2010; Faramarzi M.A., 2009). However, *Massilia* is unable to stop the invasion of *Olpidium* in the rhizosphere.

As in the case of the bacterial community *Arabidopsis* was unable to sustain the rich and diverse community in its rhizosphere, especially in the final generation.

4.2.5 Comparison of sand and compost experiment

Three generation experiments were conducted in two different growth media and each experiment was analyzed using two different metagenomic methods (ARISA and 454 sequencing). Plants were grown either in 10 % soil mixed with autoclaved sand or in 10 % soil mixed with autoclaved commercial compost. This chapter focused on comparison of data obtained from succession experiments using these two conditions.

ARISA and 454 pyrosequencing show a similar pattern with large changes from one generation to the next, but within each generation there was considerable similarity between replicate samples from the same community. It indicates that in both cases the core soil community adapted to a new environment after the disruption caused by diluting the soil in sand or compost. There was a bigger difference between the 1st and the 2nd generation than between the 2nd and the 3rd one, suggesting that the community may be approaching equilibrium towards the end of the experiment.

The major difference between these growth conditions is the occurrence of opportunist invasions in the sand experiments. Poor growth conditions made it possible for oligotrophs to gain an advantage over plant dependent species. The microbial community in the sand experiment lost most of its diversity and richness because of the successive reduction in carbon supply (Figure 4.2.21).

4.2.6 Discussion

Compost experiment showed again that the bacterial and fungal community is plant dependent. More focus was laid on bacterial community as it reacts faster to changes. MDS plots and MANOVA statistics confirmed that rhizosphere soil is different from bulk and that every plant species (models and crops) have a distinct microbial community structure at the strain/species level (ARISA), species/genus level (454 sequencing OTU data). There are only a few statistically significant differences at the phylum level between different treatments.

One of the most interesting finding was *Arabidopsis* community structure diverging into three separate consortia. The *A. thaliana* microbiome in sand collapsed in diversity in the final generation and although remaining highly diverse in compost, it divided into one major and two outcast groups with different microbiomes, suggesting stochastic variation. Interestingly, it has already been shown that repeated replanting of *A. thaliana* Landsberg *erecta* in compost for 16 generations lead to significant divergence of plant growth (Swenson *et al.*, 2000). In Swenson' experiment plants were weighed at each generation and soil from the top 10% and bottom 10% of weights was used as a microbiome inoculum for each successive generation. Soil from the heaviest plants lead to enhanced growth in further generations relative to the soil from the lightest plants. This suggests that stochastic variation in the microbiome of plants in successive generations altered growth. The variation we observed in three generations may be an example of the underlying microbial selection process observed by Swenson et al. (Swenson *et al.*, 2000), implying there may be more than one stable population structure for the rhizosphere microbiome. Due to time and budget constrains I could not continue ARISA and 454 sequencing analysis for 16

generations, but it suggests that repeated sub-culturing may lead to very different microbiomes becoming fixed.

Comparing to sand conditions, compost helped maintain a stable diverse community there was invasion over plant generations of the specific fungal pathogen *O. brassicae* (Hartwright *et al.*, 2010) that occurred on its compatible host turnip (*B. rapa)*). Interestingly, there was a corresponding peak in abundance of *Massilia* OTUs suggesting co-selection. This may be due to the ability of *Massilia* to degrade fungal chitin (Adrangi *et al.*, 2010; Faramarzi M.A., 2009), which is normally present in *O. brassicae* (and other fungi) cell wall (James *et al.*, 2006b). It may also at least partially explain the repeated observation that *Massilia* is abundant in the rhizosphere (Bodenhausen *et al.*, 2013).

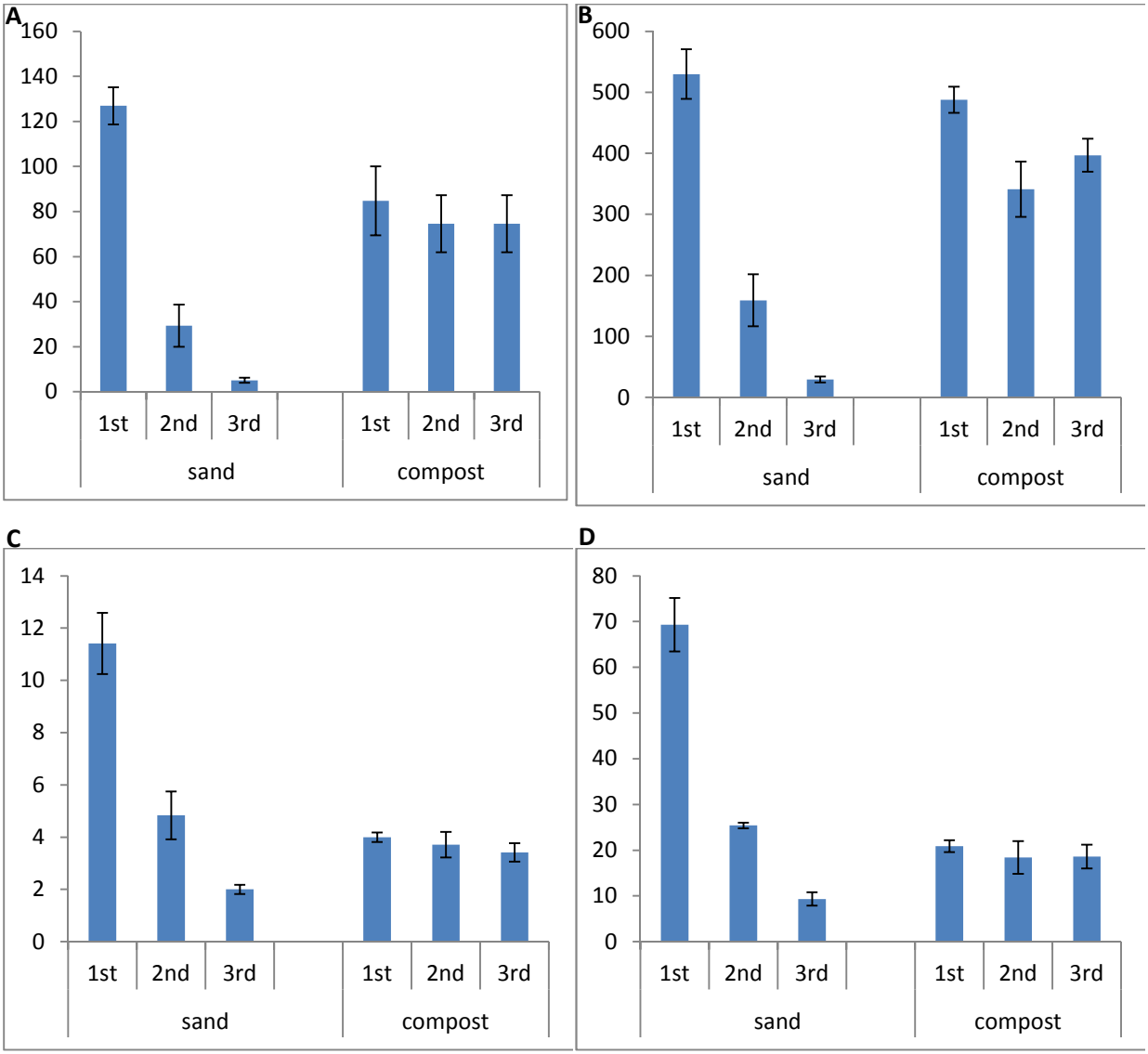


Figure 4.2.21 Richness (A) and Shannon diversity index (B) for the bacterial community and richness (C) and Shannon diversity index (D) for the fungal community over generations in the 3 generation experiments based on the 454 pyrosequencing OTU data.

Paired test ($p<0.05$) results (X - X not significant, X -*- X significant), for 454 data n=3 each plant/unplanted in each generation.

A) sand 1st -*- sand 2nd -*- sand 3rd, compost 1st - compost 2nd - compost 3rd

B) sand 1st -*- sand 2nd -*- sand 3rd, compost 1st - compost 2nd - compost 3rd

C) sand 1st -*- sand 2nd - sand 3rd, compost 1st - compost 2nd - compost 3rd

D) sand 1st -*- sand 2nd -*- sand 3rd, compost 1st - compost 2nd - compost 3rd

Due to the diversity loss and opportunist invasions there are major differences in the community structure between generations in the sand experiment. There are no core bacterial species that thrive in the soil through the whole experiment (Figure 4.2.22). Most of the OTUs are either generation specific or are shared between two of them (but never between 1st and 3rd generation). The compost experiment, on the other hand, allowed most bacterial species to survive through all three generations. This condition is less selective for fast growing, but depending on root exudates or oligotrophic organisms as the community is more diverse boosting competition. However, due to a large proportion of inert community members (e.g. soil opportunists) it is more difficult to focus on the plant root influence as most of the bacteria just thrive in the soil with or without the plants.

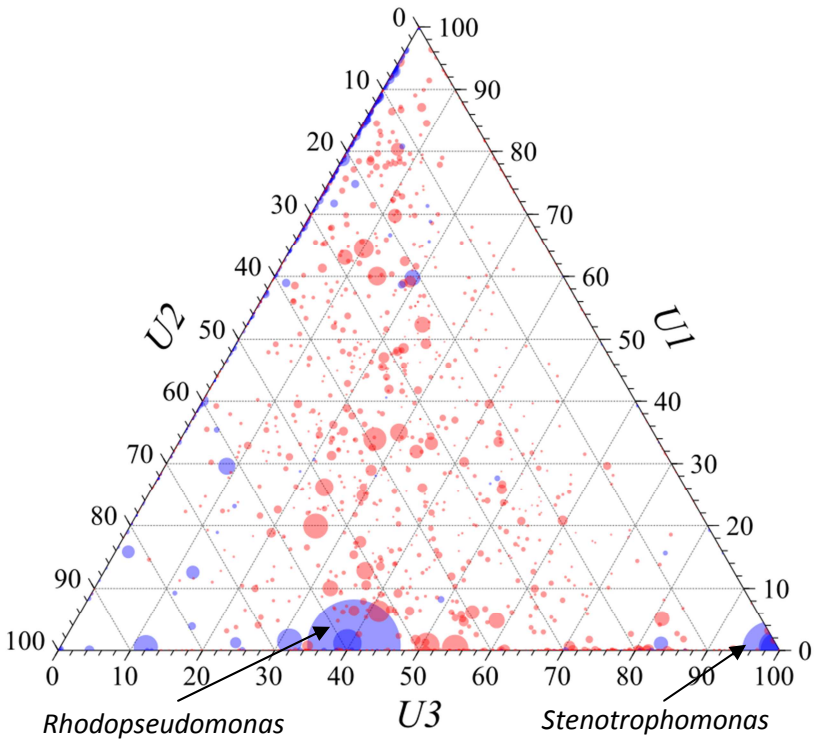


Figure 4.2.22 Ternary plot illustrating the similarity of the unplanted control community over generations in sand (blue dots) and compost (red dots) experiment. U 1,2,3 – unplanted control in the 1st, 2nd and 3rd generation

Each of the growth condition selects for a distinct rhizosphere microbiome with only a small proportion of bacteria that are shared between these two conditions (around 5 % of the total community is equally selected in both conditions) (Figure 4.2.23). Two OTUs belonging

to Rhizobiales and one belonging to Burkholderiales were found to be ubiquitous among different plant species rhizospheres in both sand and compost conditions.

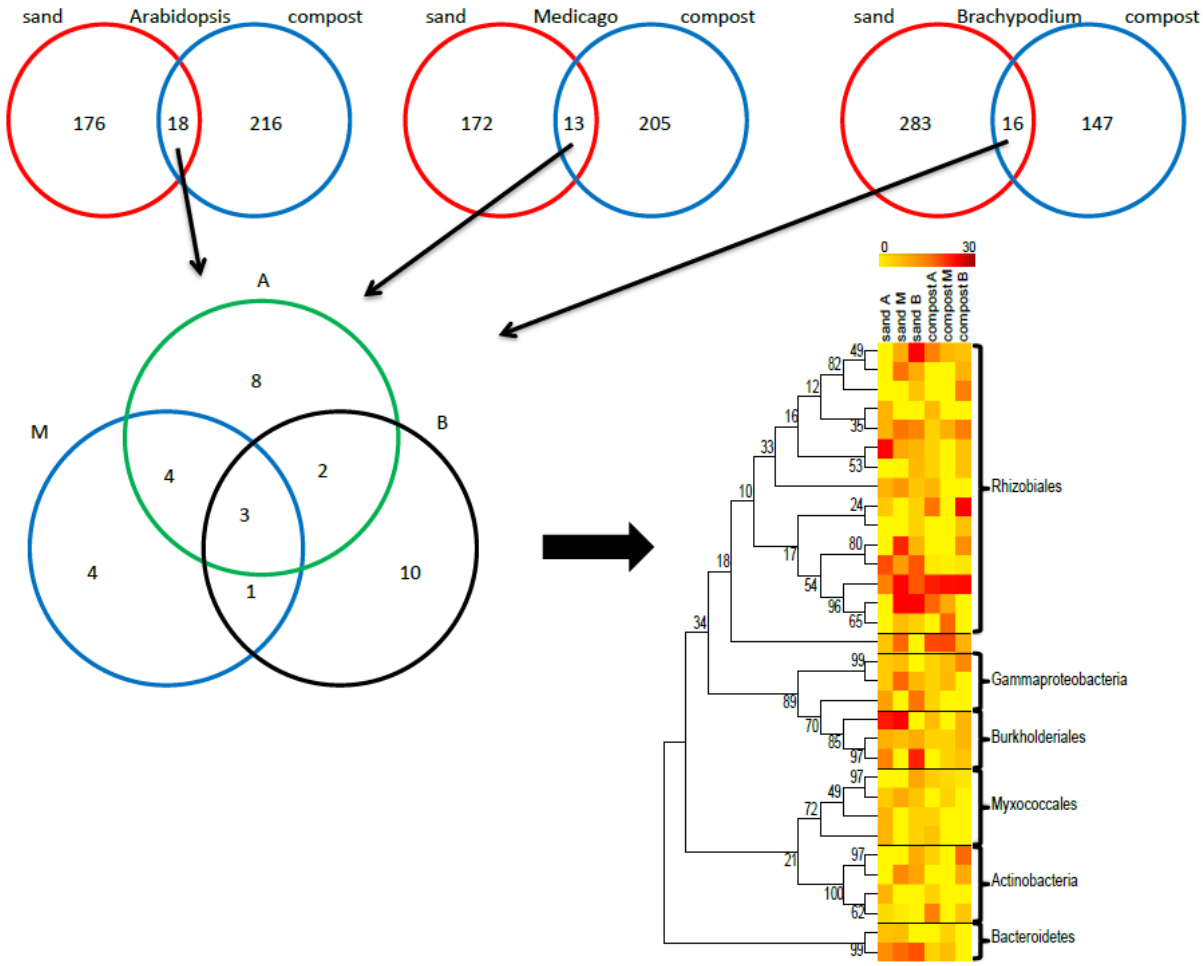


Figure 4.2.23 Rhizosphere selected OTUs. Only OTUs that are found in all 3 sub-replicates and are statistically significant more abundant in both soils in at least one plant species rhizosphere are shown ($p<0.05$ according to t-test). Number of the OTUs selected and their abundance in the total community is shown on the left side of the figure, while the right side shown the OTUs that were selected for both conditions.

Chapter 5: Influence of different plant mutants on the microbial community in the rhizosphere

5.1 Introduction

In the previous chapters it was demonstrated that different plant species are able to modify the bacterial and fungal community in the rhizosphere. To what extent can genetic differences between plants of the same species generate differences of rhizosphere communities? In particular, can single mutation in the plant host genome be responsible for substantial shifts in the rhizosphere community? Previous research has shown that Myc^- and Nod^- mutants of Medicago (Offre *et al.*, 2007) and different genotypes of the same plant species (Bulgarelli *et al.*, 2012; Micallef *et al.*, 2009; Zancarini *et al.*, 2012) have a different bacterial community in the rhizosphere. Even though we know more and more about the soil metagenome structure and function (Delmont *et al.*, 2012) the exact influence of plant metabolites released into the environment is unknown. We hypothesize that plants control the soil microbiota using signalling and antimicrobial compounds secreted along with sugars, amino and organic acids, etc. into the rhizosphere. We aim to understand the influence of selected plant metabolites compounds on the rhizosphere microbiome, where organic acids like quinic acid, lactic acid and maleic acid but not sugars (glucose, sucrose, fructose) are mostly responsible for the observed changes (Shi *et al.*, 2011). However, the influence of signalling and antimicrobial compounds is yet unknown.

In the following experiments the influence of single plant mutations (or multiple mutations in the same operon) on rhizosphere micro-organisms were assessed. We used ARISA to permit a detailed analysis of bacterial community structure differences with large numbers of replicates. 454 sequencing was performed for most of the experiments to identify the bacterial and fungal taxa responsible for the observed changes.

Plant mutants' influence on the rhizosphere was tested using the model plants *Medicago* and *Arabidopsis,* as a number of well characterised mutants were available for these species

and data could be compared with that from the 3 generation experiment using model plants (chapter 3 and 4). *Brachypodium* was not included, because of time constrains. In order to maximize the chance of detecting changes in the rhizosphere community, we chose to examine plants with mutations in the key plant – microbe interaction pathways (in effect, adopting a candidate gene approach). In *Arabidopsis* we focussed on the influence of aliphatic glucosinolates (MYB experiment), PAMP receptors (PAMP experiment) and methyl halides production (HOL experiment) and for *Medicago* the influence of mycorrhization was tested (RAM experiment). All the mutant lines were obtained from collaborators across the John Innes Centre. Detailed background of the plant genotypes is provided in the chapter 1.6.2 and 1.6.3.

5.2 Results and discussion

5.2.1 ARISA and pyrosequencing analysis of bacterial community for the PAMP experiment

The influence of three different *Arabidopsis* mutants on the rhizosphere community was compared (Table 5.2.1). Seeds were obtained from Cyril Zipfel (JIC, Norwich). In order to simplify the system 10 % Bawburgh soil was mixed with autoclaved sand. Such an approach in the growth conditions should make rhizosphere microbiota to be more dependent on the plant root exudates. In this system plants are the major source of carbon and nitrogen compounds. Microorganisms that utilize plant derived compounds are more likely to respond to pathogen associated molecular patterns (PAMP-) triggered immunity response (Table 2.5.1). It is worth noting that this experiment was not designed to mimic natural field soil conditions, but rather to focus on the microbial response to plant mutants influence. Plants were germinated and grown according to protocols used previously in the sand experiment (chapter 3.1). The PAMP experiment was stopped after a single generation of plants and DNA was isolated from the rhizosphere microorganisms. Bacterial ARISA as well as bacterial and fungal 454 pyrosequencing were performed.

mutated gene(s)	corresponding SERK	abbreviation used for the tables and figures
cerk-1	SERK1	single (S)
bak1-5 bkk1-1	SERK3-SERK4	double (D)
bak1-5 bkk1-1 cerk-1	SERK3-SERK4-SERK1	triple (T)
Wild type	N/A	wild type (WT)
Unplanted control	N/A	unplanted (U)

Table 5.2.1 *Arabidopsis* mutants used in the PAMP experiment. Explanation of mutations and PAMP pathway is provided in chapter 1.6.3

An MDS plot based on ARISA fingerprints of the bacterial community shows that the unplanted control was clearly separated from the other data points (Figure 5.2.1 and 5.2.2). The wild type and single mutant rhizospheres clustered closer to the unplanted control than the double and triple mutants. Not all the samples groups are statistically different from each other based on MANOVA (Table 5.2.2). The structure of wild type rhizosphere community was identical to the single mutant rhizosphere (for simplification: WT=S). Rhizospheres of double and triple mutants are also similar to each other (for simplification: D=T). All the rhizospheres are significantly different from the unplanted control (for simplification: WT ≠ U, S ≠ U, D ≠ U, T ≠ U). The differences between wild type-single mutant and double-triple mutants are statistically significant (for simplification: WT = S and D = T, but WT ≠ D, WT ≠ T and S ≠ D, S ≠ T). These samples could be separated from each other using 18-21 biological replicates. The high numbers of samples, needed for MANOVA statistics to show significance, indicate a relatively high similarity between these groups, although they are statistically different. The sand experiment showed that the rhizosphere bacterial communities of different plant species in the same soil conditions (10 % soil / 90 % sand) require 6-9 biological replicates to be separated (Table 3.2.1). So plant species have greater differences in their rhizosphere communities than do mutants of the same species. However in both cases (PAMP and sand experiment) only 6 replicates are needed to separate the plant influence from that of the unplanted control.

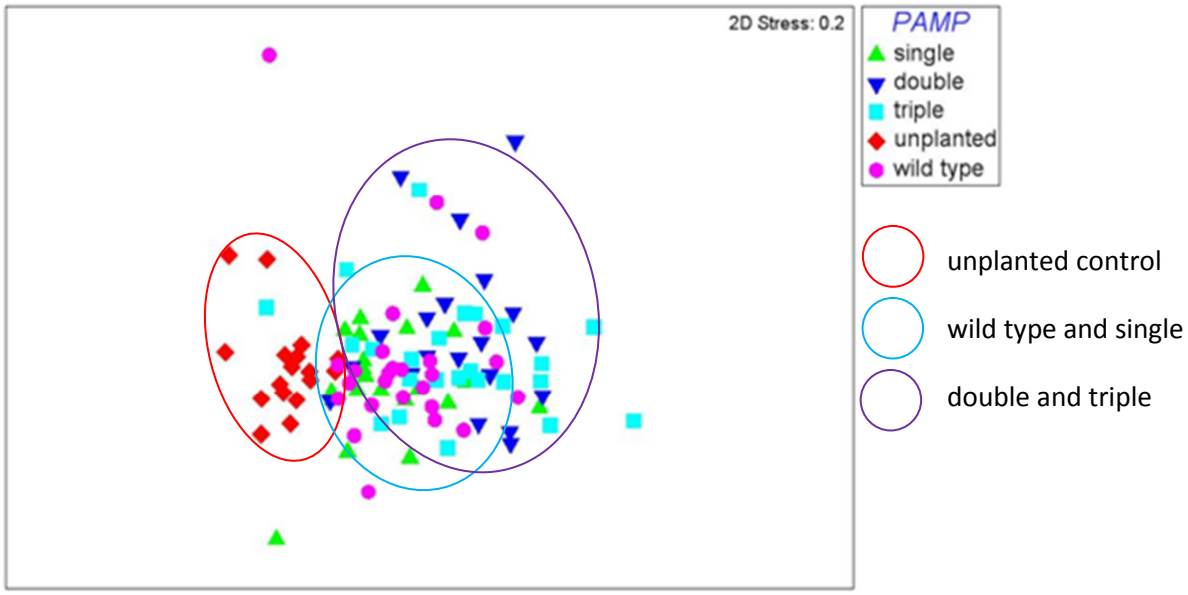


Figure 5.2.1 MDS plot of the influence of PAMP mutants on the rhizosphere community based on ARISA data. The semi-transparent ovals on the graph are aimed to point out the main differences, but are not based on any statistical analysis. *cerk-1* – single, *bak1-5 bkk1-1* – double, *bak1-5 bkk1-1 cerk-1* – triple *Arabidopsis* mutant (each figure represents one bacterial community sampled from an individual plant rhizosphere / unplanted control, n=21 for each plant genotype/unplanted).

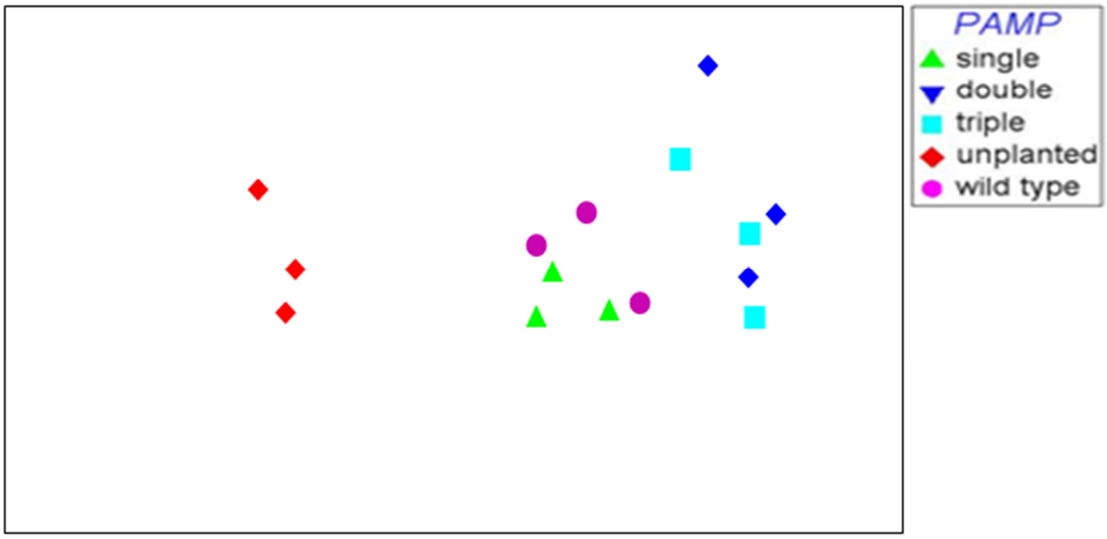


Figure 5.2.2 MDS plot of the influence of PAMP mutants on the rhizosphere community based on ARISA data. For simplicity data points were pooled into pseudo-replicates.

	WT	single	double	triple	unplanted
WT		ns	17	21	7
single	ns		17	20	7
double	17	17		ns	5
triple	21	20	ns		6
unplanted	7	7	5	6	

Table 5.2.2 MANOVA analyses of the PAMP experiment (t-test using p≤0.05, n=21 for each plant genotype/unplanted). The groups that could not be statistically separated are shown in red. *cerk-1* – single, *bak1-5 bkk1-1* – double, *bak1-5 bkk1-1 cerk-1* – triple (p≤0.05, n=21 for each plant genotype/unplanted). ns – not statistically significant

In order to annotate the bacterial and fungal taxa that were influenced by the lack of a PAMP response in the host plants, 454 sequencing was performed (Table S4). Reads were binned into OTUs using 95 % similarity, producing 4078 OTUs for bacteria and 627 OTUs for fungi. These numbers are comparable to the ones obtained in the sand experiment.

Data obtained after the 454 sequencing confirms the overall community differences between different genotypes of host plants. Wild type and single mutants are clustered closer to the unplanted control than the double and triple mutants (Figure 5.2.3).

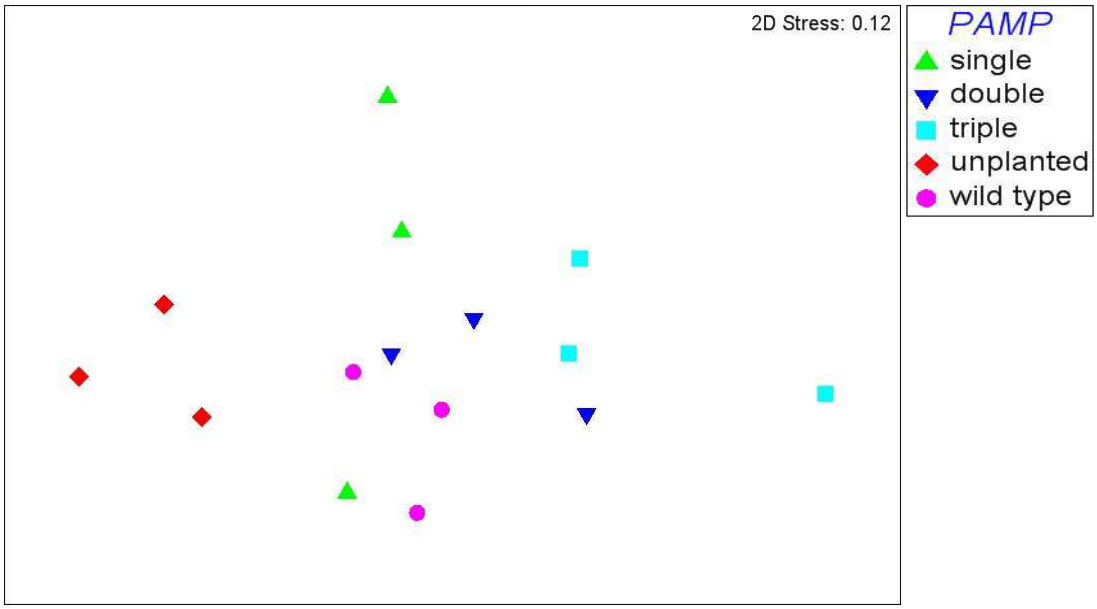


Figure 5.2.3 MDS plot presenting bacterial rhizosphere community of Arabidopsis PAMP experiment analysed using OTU data (based on 454 sequencing). *cerk-1* – single, *bak1-5 bkk1-1* – double, *bak1-5 bkk1-1 cerk-1* – triple (n=3 for each plant genotype/unplanted)

The bacterial community analysis at the phylum level showed that the abundance of Proteobacteria was significantly higher in the double and triple mutant rhizospheres than in the wild type (Figure 5.2.4 A). All mutant rhizospheres were significantly depleted of Actinobacteria, indicating a strong relation between this phylum and plant immunity. Bacteroidetes were significantly enriched in the rhizosphere of the triple mutant.

The rhizosphere of the triple mutant was very different from all other rhizospheres at the subphylum level, with Alphaproteobacteria strongly enriched (statistically significant against

wild type), with a relatively decreasing abundance of the remaining subphyla, especially Betaproteobacteria (Figure 5.2.4 B). It is worth noting that at the subphylum level the triple mutant was relatively similar to the unplanted control.

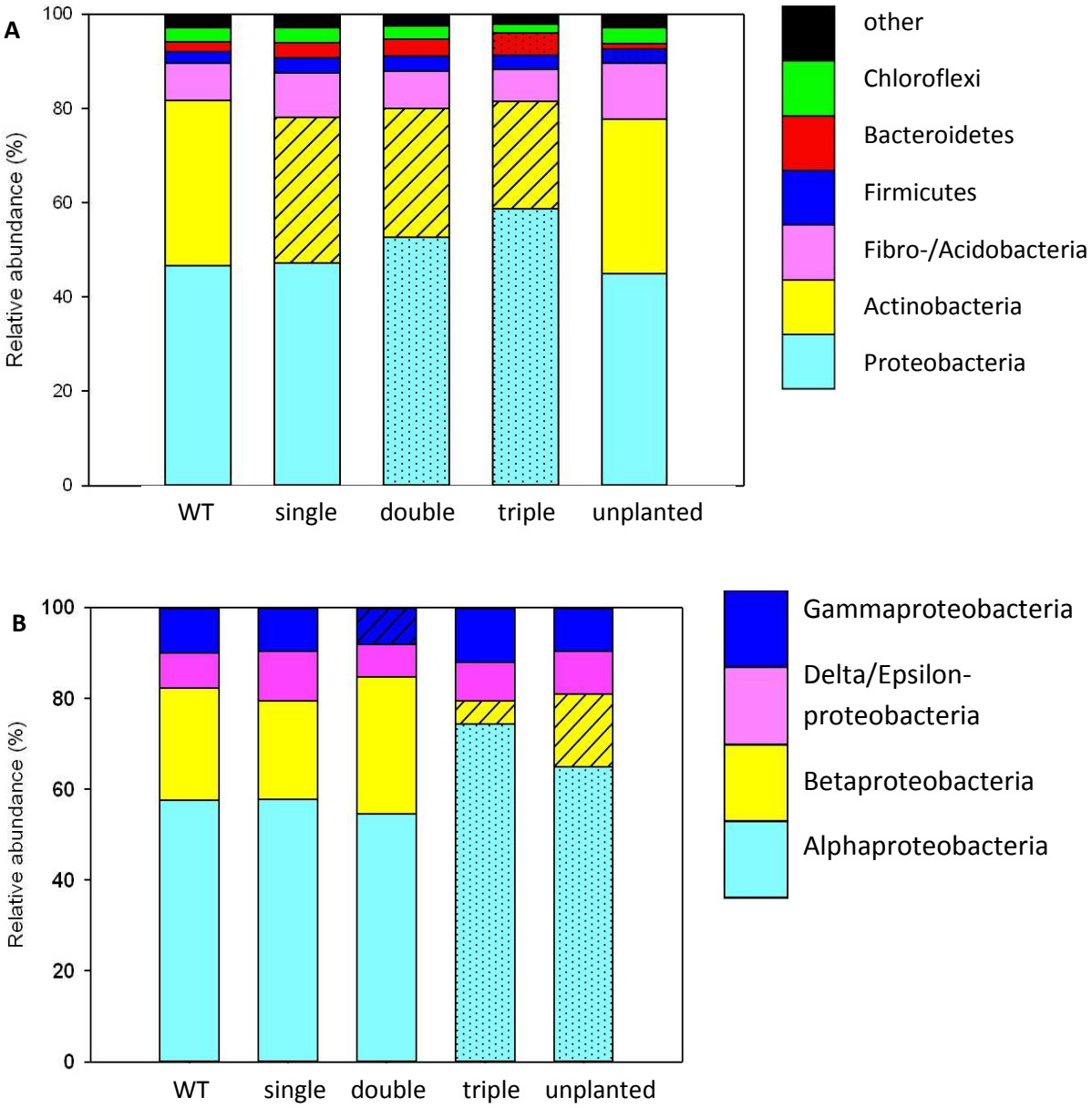


Figure 5.2.4 Bacterial community structure of *Arabidopsis* genotypes tested in the PAMP experiment A) at the phylum level B) subphyla of Proteobacteria – dots and lines indicate phyla and subphyla statistically enriched or depleted against wild type, respectively. *cerk-1* – single, *bak1-5 bkk1-1* – double, *bak1-5 bkk1-1 cerk-1* – triple (n=3 for each plant genotype/unplanted). ANOVA with Tukey test (significance 0.05), dashed lines indicate phyla/subphyla with significant difference (reduced abundance) against wild type and dots indicate phyla/subphyla with significant (more abundant) against wild type.

A phylogram constructed using MEGAN software (Figure 5.2.5) shows that the wild type and single mutant had more Actinobacteria in the rhizosphere. These two genotypes also had an increased abundance of Chloroflexi and Planctomycetes. The double and triple mutant rhizospheres were enriched with Bacteroidetes and Betaproteobacteria.

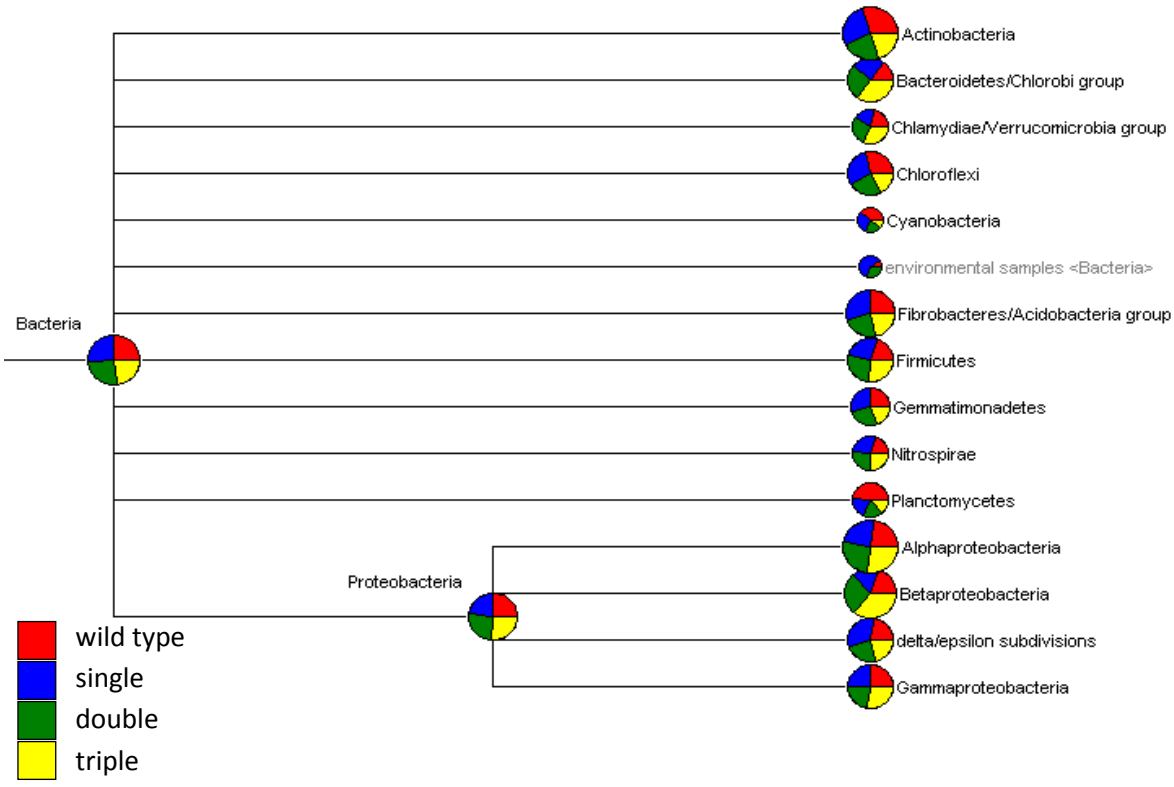


Figure 5.2.5 Phylogram showing phyla and Proteobacterial subphyla for bacterial communities sampled in the PAMP experiment. *cerk-1* – single, *bak1-5 bkk1-1* – double, *bak1-5 bkk1-1 cerk-1* – triple (n=3 for each plant genotype/unplanted)

Double and triple mutant plants reduce the bacterial diversity comparing to wild (Figure 5.2.6; $p \leq 0.05$) however, in overall, values are similar to those observed in the 1st generation of the sand experiment.

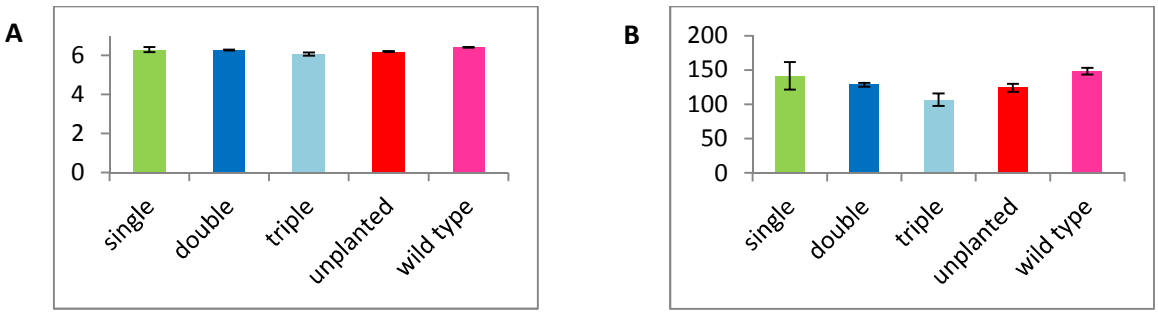


Figure 5.2.6 Analysis of the Shannon diversity index (A) and richness (B) of the bacterial rhizosphere community of Arabidopsis genotypes from the PAMP experiment using 454 sequencing data, *cerk-1* – single, *bak1-5 bkk1-1* – double, *bak1-5 bkk1-1 cerk-1* – triple. (n=3 for each plant genotype/unplanted).

Paired test ($p<0.05$) results (X - X not significant, X -*- X significant), for 454 data n=3 each plant/unplanted in each generation.

A) single – wild type, double -*- wild type, triple -*- wild type, unplanted -*- wild type

B) single – wild type, double -*- wild type, triple -*- wild type, unplanted -*- wild type

Community analysis of the dominant bacterial OTUs showed that *Arabidopsis* wild type and *cerk-1* mutant are very similar to each other in their rhizosphere communities (Figure 5.2.7). Both genotypes are able to suppress OTUs belonging to Rhizobiaceae (compared to unplanted control). In the double and triple mutants, Oxalobacteraceae i.e. *Massilia* and *Duganella* were the most dominant OTUs. They were significantly more abundant than in the unplanted control.

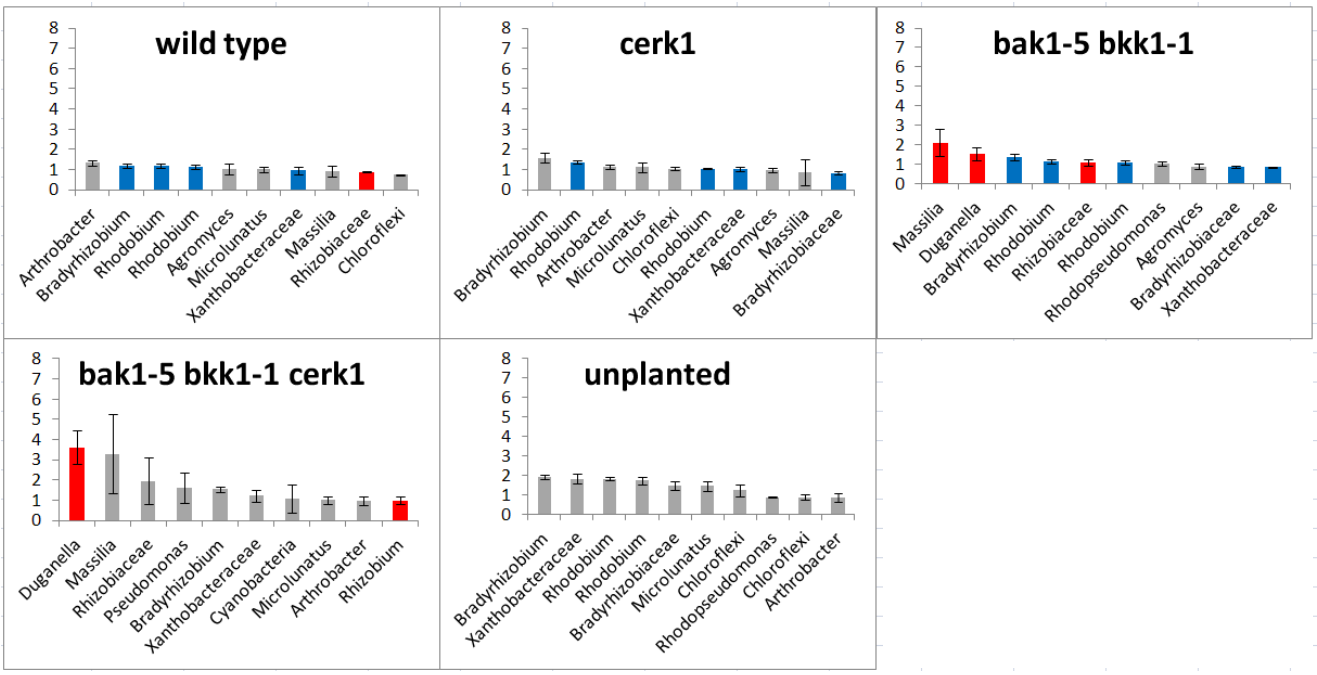
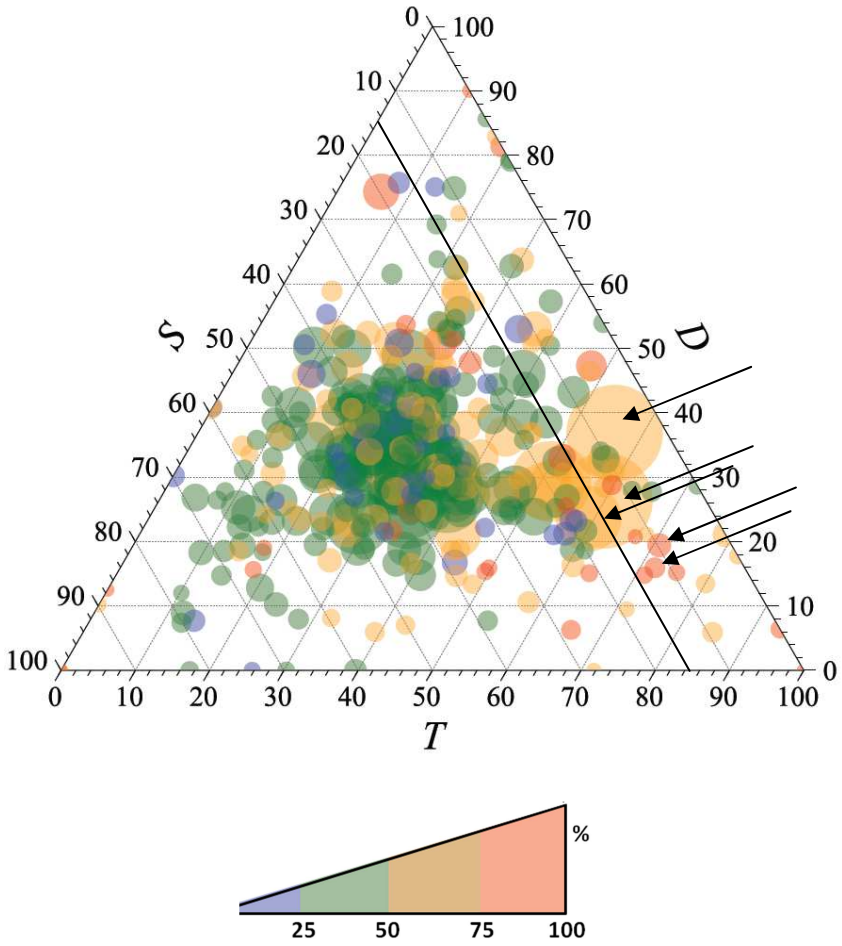


Figure 5.2.7 Bar graphs presenting 10 commonest OTUs of the bacterial rhizosphere community of Arabidopsis PAMP mutants based on 454 sequencing data (red bars show rhizosphere OTUs selected against unplanted control, blue bars show rhizosphere OTUs depleted against unplanted control, grey bars – not statistically significant $p<0.05$) (n=3 for each plant genotype/unplanted).

Most of the bacterial community was shared between different genotypes (Figure 5.2.8). However, there are some OTUs highly selected especially in the double and triple mutant rhizospheres (red and orange spheres in the right corner – see black arrows). The large orange dots belong to Oxalobacteraceae, while the smaller red dots mostly belong to the Bacteroidetes/Chlorobi group. These bacterial species had a clear advantage in the rhizosphere of immunity impaired *Arabidopsis*.



average plant mutant rhizosphere specificity against wild type

Figure 5.2.8 Ternary plot representing the influence of *Arabidopsis* genotypes on the bacterial rhizosphere community. Data based on 454 sequencing reads binned into OTUs. *cerk-1* – S, *bak1-5 bkk1-1* – D, *bak1-5 bkk1-1 cerk-1* – T. The colour of the spheres is calculated on the basis of comparison with the wild type rather than the unplanted control (n=3 for each plant genotype/unplanted). For clarity dots are filled with semi-transparent colours

5.2.2 Pyrosequencing analysis of the fungal community in PAMP mutants

The fungal communities were examined using 454 sequencing. Samples were processed as previously (chapter 2.8)

There are rather small differences in the fungal community between replicates for the wild type, triple mutant and unplanted control, while the rhizospheres of the single and double mutant plants were more heterogeneous (Figure 5.2.9). The data point, located further away from the other points, represents the rhizosphere of the single mutant colonized by *Olpidium* (explained later in the text). Overall, the rhizosphere samples grouped together, away from the unplanted control data points.

Figure 5.2.9 MDS plot presenting bacterial rhizosphere community of Arabidopsis PAMP experiment analysed using OTU data (based on 454 sequencing). *cerk-1* – single, *bak1-5 bkk1-1* – double, *bak1-5 bkk1-1 cerk-1* – triple (n=3 for each plant genotype/unplanted).

The fungal community of the unplanted control and triple mutants were similar to each other at the division/subdivision taxonomic level. The community of wild type rhizospheres was enriched in Chytridiomycota and the community of the double mutant was intermediate between these two groups. The single mutant rhizosphere was enriched with Pezizomycotina, and relatively depleted of all other taxonomic groups (Figure 5.2.10). However, due to the relatively large difference between biological replicates none of these observations are statistically significant.

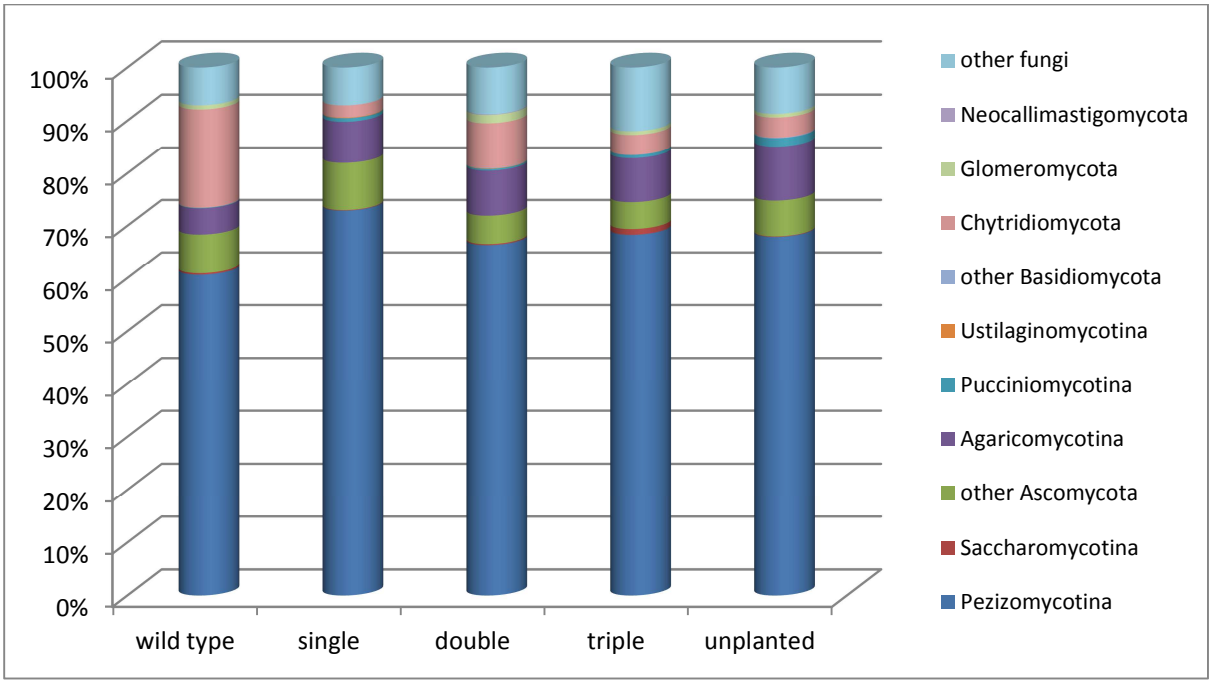


Figure 5.2.10 The fungal community structure in the PAMP experiment. Graph based on GenBank BLAST report uploaded into MEGAN (1 % top hit) *cerk-1* – single, *bak1-5 bkk1-1* – double, *bak1-5 bkk1-1 cerk-1* – triple. (n=3 for each plant genotype/unplanted).

The rhizosphere fungal community was relatively diverse between different genotypes of *Arabidopsis* (Figure 5.2.11). The wild type rhizosphere was enriched with Chytridiomycota and unclassified fungi (MEGAN was able to characterize the reads only to the kingdom level because of a low match to known sequences). Most of the Pucciniomycotina were found in the rhizosphere of the single and triple mutants.

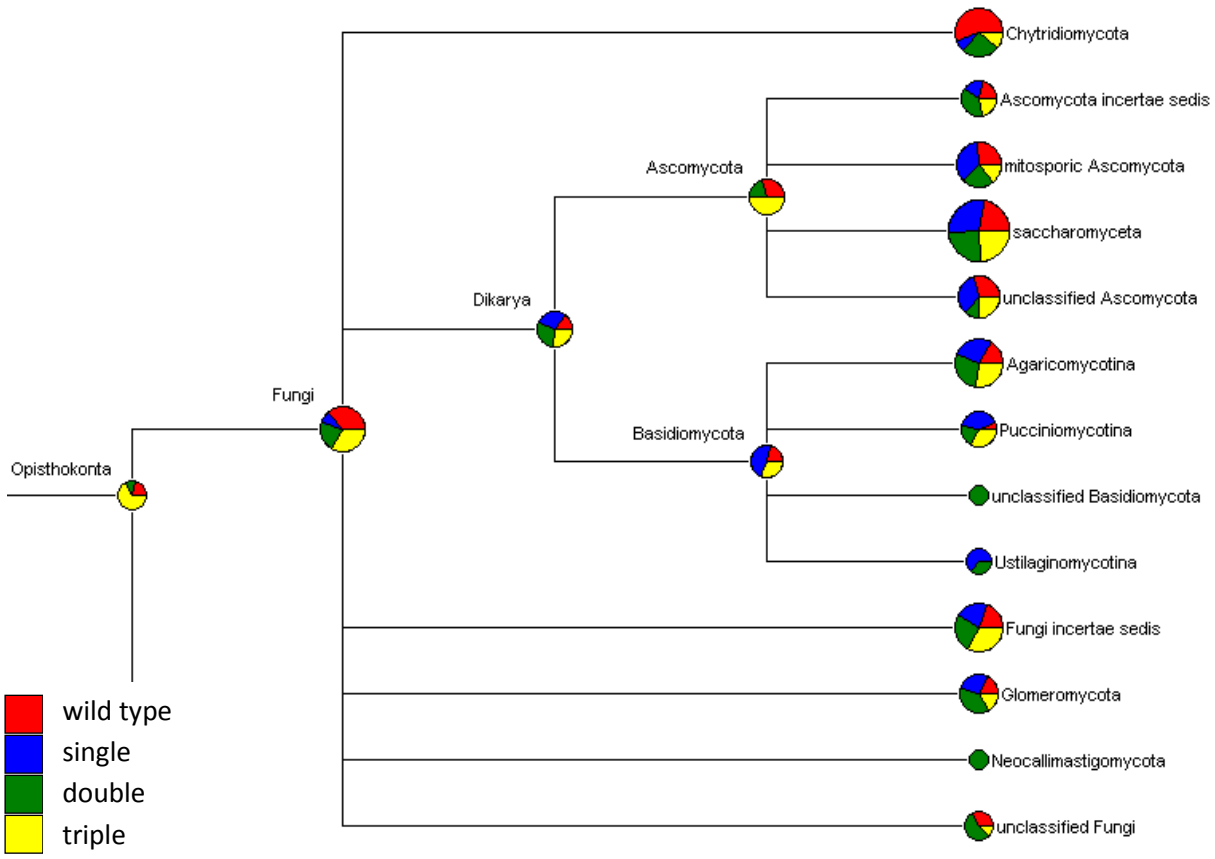


Figure 5.2.11 Phylogram showing phyla, divisions and subdivisions of fungal communities sampled in the PAMP experiment. *cerk-1* – single, *bak1-5 bkk1-1* – double, *bak1-5 bkk1-1 cerk-1* – triple. (n=3 for each plant genotype/unplanted).

The richness and diversity of the fungal communities were much lower than observed for the bacterial communities. The fungal community was less diverse within samples (alpha diversity). The very same observation was already made in case of 3 generation experiments. There are no statistically significant differences of diversity or richness between different plant mutants ($p \leq 0.05$). However, there are differences in the diversity and richness in case of the single mutant rhizosphere, because one of the replicates has reduced these indexes, producing larger standard error (the same sample that clusters away from other points on the MDS plot) (Figure 5.2.12).

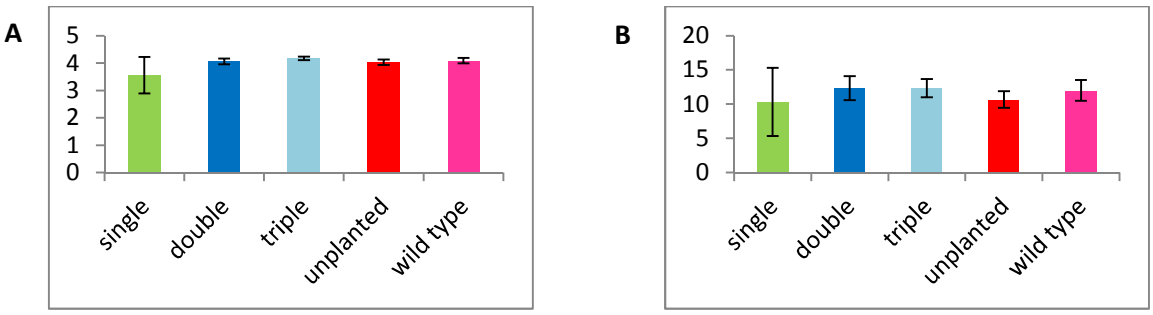


Figure 5.2.12 Shannon index (A) and richness (B) for the fungal community of the PAMP experiment analysed based on 454 sequencing data binned into OTUs. *cerk-1* – single, *bak1-5 bkk1-1* – double, *bak1-5 bkk1-1 cerk-1* – triple. (n=3 for each plant genotype/unplanted).

The common OTUs of the fungal community in the rhizosphere of *Arabidopsis* genotypes were relatively similar to each other. The only statistically valid difference is the enriched abundance of Rhizaria in the rhizosphere of the double and triple mutant (and also in the wild type) (Figure 5.2.13). Rhizaria is an extensive (and phylogenetically uncertain) group of unicellular eukaryotes. They do not belong to the fungal kingdom. However, at least some parts of their rRNA genes operon must be similar to the fungal sequence, as Rhizaria DNA was amplified using fungi specific primers. The single mutant rhizosphere was predominantly occupied by *Olpidium* – a well known fungal pathogen (see chapter 4.2.3).

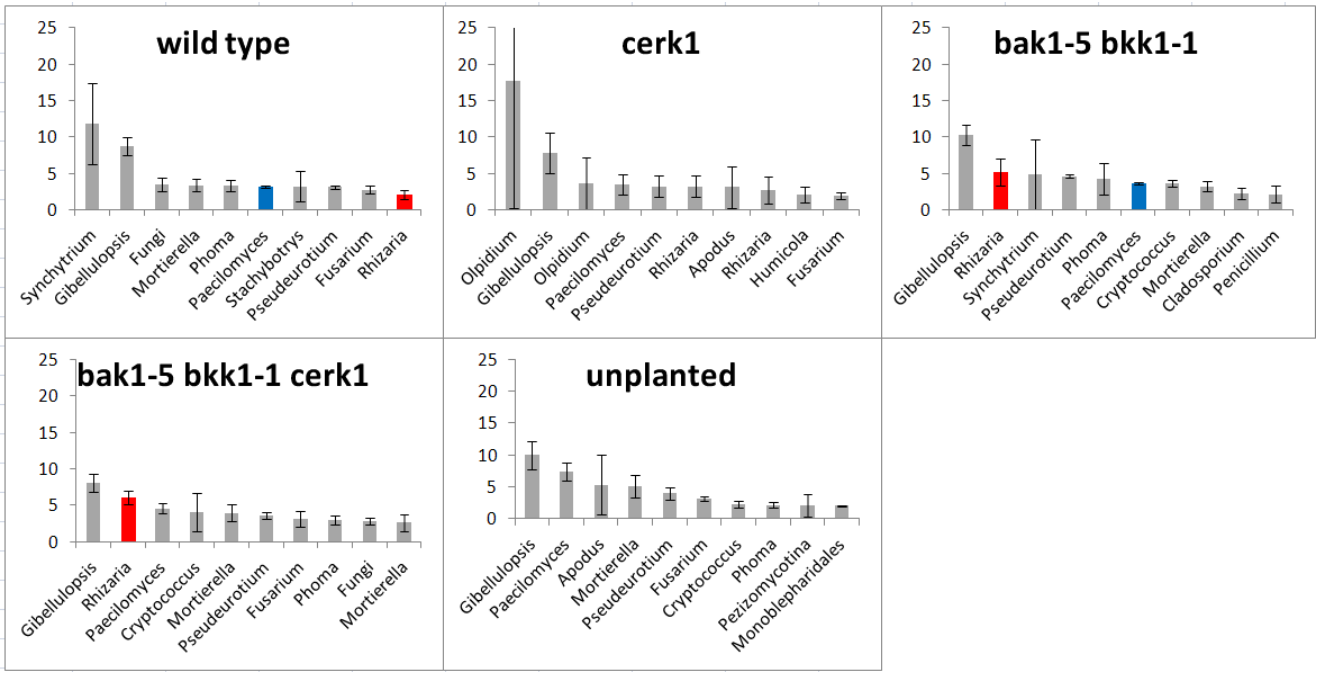
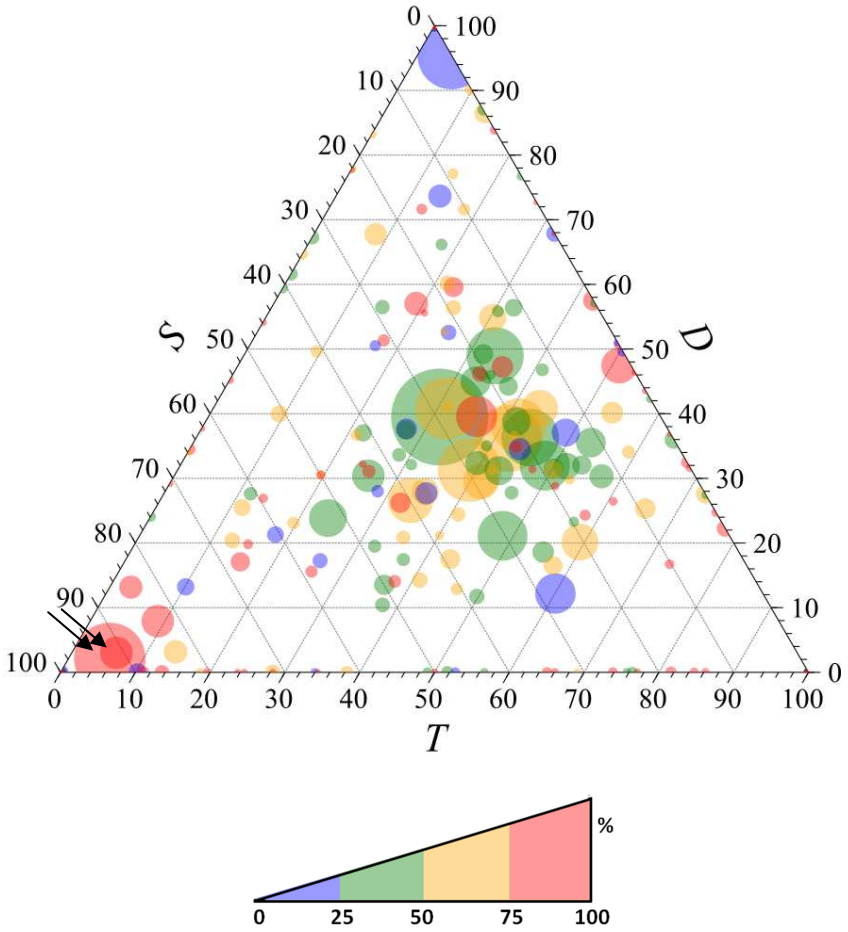


Figure 5.2.13 Bar graphs showing 10 dominant OTUs of the fungal rhizosphere community of Arabidopsis PAMP mutants (red bars present rhizosphere OTUs selected against unplanted controls, blue bars rhizosphere OTUs depleted against unplanted controls, grey bars – not statistically significant $p<0.05$) (n=3 for each plant genotype/unplanted).

The fungal community was similar between different *Arabidopsis* genotypes (Figure 5.3.6). The rhizosphere of the single mutant was enriched in *Olpidium brassicae* (two black arrows in the corner), *Apodus* and *Penicillium* (annotated as red spheres in the S corner). This enrichment caused the remaining OTUs to be located closer to double-triple mutant axis. The large blue sphere in the double mutant corner was annotated as *Synchytrium*. However, the distribution of the fungal community must be treated cautiously because the diversity between replicates of the same plant genotype is much greater than for bacteria. *Olpidium* was only observed in a single *cerk-1* sample, indicating that *Arabidopsis* is susceptible to attack by this pathogen (*Arabidopsis* belongs to the same family as turnip – see chapter 4.2.3).



average plant mutant rhizosphere specificity against wild type

Figure 5.2.14 Ternary plot representing the influence of the Arabidopsis genotype on fungal OTUs. Colour of the spheres is calculated against wild type instead of unplanted control. *cerk-1* – S, *bak1-5 bkk1-1* – D, *bak1-5 bkk1-1 cerk-1* – T. (n=3 for each plant genotype/unplanted).

5.2.3 Summary and discussion of the PAMP experiment

Changes to the PAMP-triggered immune response influenced the rhizosphere microbiome. Mutation in *bak 1-5* and *bkk 1-1* genes reduced the plant immune response against bacteria, while mutations in *cerk1* had a smaller effect (Roux *et al.*, 2011; Zipfel *et al.*, 2006). Even though *Arabidopsis* has a less marked effect on the rhizosphere community than other plant species (see summary of chapter 3 and 4), there are statistical differences between plant rhizospheres and unplanted control. Importantly, most of the mutant rhizosphere bacterial

communities were also different from each other based on ARISA data and MANOVA tests. However, the wild type rhizosphere is indistinguishable from the *cerk1* mutant. Moreover, double and triple mutants have the same effect on the community.

Detailed analysis of the differences between *Arabidopsis* endophytic, rhizosphere and bulk soil bacterial community showed that there are major differences between endophyte and rhizosphere – bulk soil communities. It was suggested that PAMP-triggered immunity is mostly responsible for the observed changes as bacteria that colonize roots have to overcome the plant response to infection (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012). In this PhD project the endophytic community was not examined. However, it is clear that immunity plays a major role in shaping the rhizosphere microbiome, so it can be assumed that endophyte structure is also affected, as it is recruited from the soil surrounding roots.

The rhizosphere of double and triple mutants was enriched with Proteobacteria (especially Alphaproteobacteria in the case of the triple mutant). Actinobacteria were depleted in the mutant rhizospheres. Indirectly, this confirms that the plant immune system selects for Actinobacteria (and/or suppress all other bacteria), as suggested in previous studies (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012). Plants with an impaired immunity response did not select Actinobacteria and the rhizosphere could be occupied by different phyla.

Two Oxalobacteraceae species were highly selected in the double and triple mutant rhizospheres. These OTUs may represent species that utilize plant derived compounds, but can not evade the plant immune system. Interestingly, these two genera have already been commonly found in the *Arabidopsis* rhizosphere, endosphere and phylosphere (Bodenhausen *et al.*, 2013). These genera were also highly abundant in the *Medicago* and *Brachypodium* rhizospheres of the sand and compost experiments. Wild type, single mutant and unplanted controls were colonized by OTU belonging to *Bradyrhizobium.* These bacteria are common in soil (Hirsch & Mauchline, 2012). Overall, the results suggest that *Arabidopsis* immunity suppresses some plant dependent bacteria whether beneficial or harmful.

There were no major differences in the fungal rhizosphere communities between different genotypes of *Arabidopsis*. Interestingly, the pathogen *Olpidium* colonized the rhizosphere of the *cerk-1* mutant, but it was absent from all other rhizospheres. The *cerk-1* gene in rice was shown to be responsible for the defence response against rice blast fungus (Hu *et al.*, 2005).

However, some caution should be taken, as only a single (out of three) *Arabidopsis serk1* samples were infected with *Olpidium*. This observation may be caused by difference in the farm soil inoculum structure (very low abundance of *Olpidium* spores) or may be a result of rhizosphere competition in colonizing plant roots.

The PAMP experiment was conducted in the same way as the sand experiment. Even though the soil used as an inoculum was sampled at a different time and slightly different location (sampling was done at the same farm plot, a few meters apart) the soil communities in the two experiments were very similar to each other. The diversity and dominant bacterial and fungal OTUs are almost identical in both experiments. These findings show that the sand experiment is repeatable and experiment preparation, DNA isolation, PCR and pyro-sequencing method are highly reliable. It is worth noting that all the protocols of plant growth, DNA isolation and PCR were the same, reducing the possibilities of bias towards detection of particular groups of microorganisms (bacteria with a thinner cell wall, stronger attached to soil particles, etc.) (Delmont *et al.*, 2011; Delmont *et al.*, 2012).

5.2.4 ARISA and pyrosequencing analysis of bacterial community for the MYB experiment

Glucosinolate compounds released in the rhizosphere of *Arabidopsis* have an effect on the bacterial community structure. Such an effect was not observed for the fungal community.

This experiment was designed to test the influence of aliphatic glucosinolate production by *Arabidopsis* on rhizosphere bacterial and fungal communities. *Arabidopsis* wild type, *myb28/29* mutant and unplanted control soil was analyzed using bacterial ARISA and bacterial and fungal 454 sequencing. The experiment was run at the same time and using the same growth conditions as the compost experiment. Results for *Arabidopsis* WT and unplanted control are shared between these two experiments. Detailed explanation of glucosinolate role in plant – microbe interactions and Myb transcription factor mutation is provided in chapter 1.6.3.

The ARISA method allowed for a detailed analysis of bacterial community. There are differences between the rhizosphere community of wild type plant and that of the *myb28/29* mutant. Rhizosphere samples were more similar to each other than to unplanted controls, as indicated on the MDS plot (Figure 5.2.15). MANOVA separates the wild type from the *myb28/29* mutant rhizospheres using 17 replicates (Table 5.2.3). The *myb28/29* mutant was more similar to the unplanted control (10 replicates needed for the MANOVA test) than was the wild type (only 6 replicates), indicating that glucosinolates do have an effect on rhizosphere bacteria.

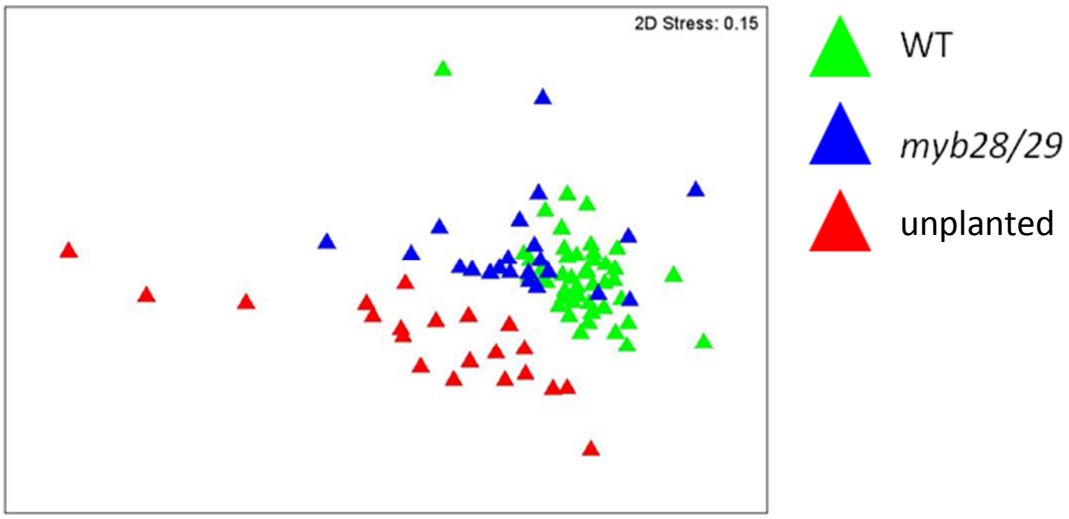


Figure 5.2.15 MDS plot of glucosinolate deficient myb28/29 mutant influence on the bacterial rhizosphere community in the MYB experiment. Graph based on ARISA data (n=21 for each plant genotype/unplanted).

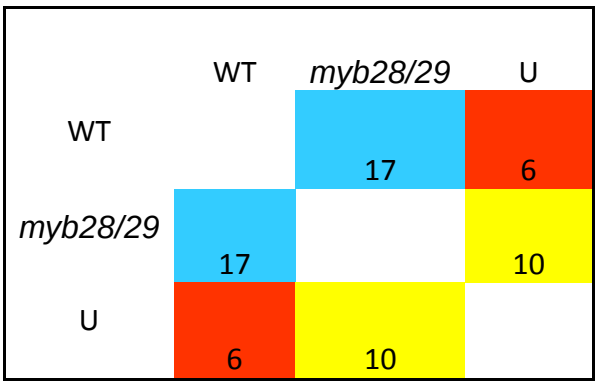


Table 5.2.3 MANOVA analyses run on the Myb experiment based on ARISA data ($p \leq 0.05$, n=21 for each plant genotype/unplanted).

454 pyrosequencing data show that the changes in bacterial community structure were similar to those obtained using ARISA. Again WT and *myb28/29* were next to each other, while control samples were further away (Figure 5.2.16).

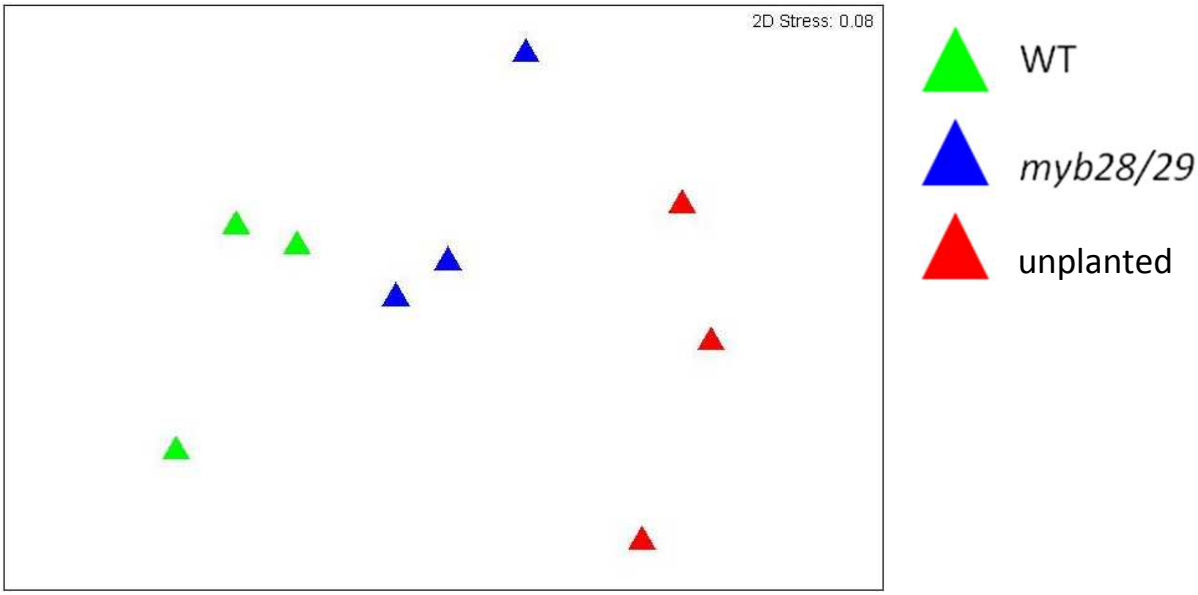


Figure 5.2.16 MDS plot presenting bacterial the rhizosphere community of Arabidopsis Myb mutants analysed using 454 pyrosequencing (n=3 for each plant genotype/unplanted).

There were no significant differences in richness or diversity of the bacterial rhizosphere between the *myb28/29* mutant, the wild type and the unplanted control (Figure 5.2.17).

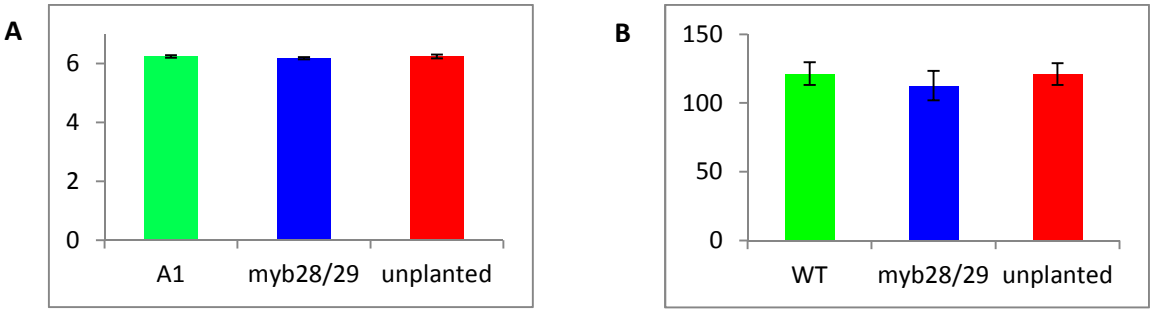


Figure 5.2.17 Analysis of bacterial Shannon index diversity (A) and richness (B) for the bacterial community analysed using 454 sequencing in the MYB experiment (n=3 for each plant genotype/unplanted)

Analysis of bacterial phylum and Proteobacterial subphylum abundances show that the rhizosphere of *myb28/29* plant was slightly enriched with Actinobacteria, although this is not statistically significant, and depleted of Firmicutes (Figure 5.2.18 A and 5.2.19). Differences at the Proteobacteria subphylum level were minimal (Figure 5.2.18 B).

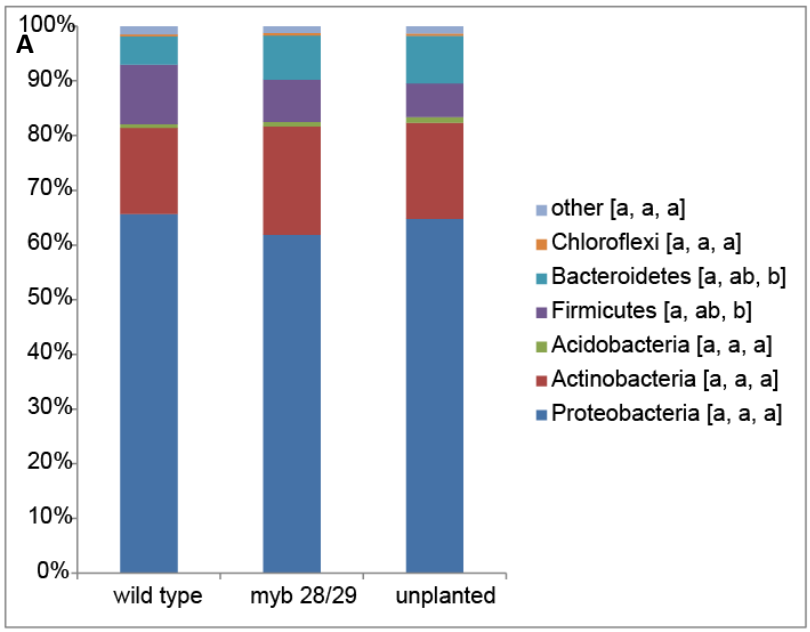


Figure 5.2.18 Bacterial community composition, based on 454 sequencing results, in the Myb experiment A) at the phylum level, (n=3 for each plant genotype/unplanted).t-test ($p < 0.05$) results [wt, *myb28/29*, unplanted].

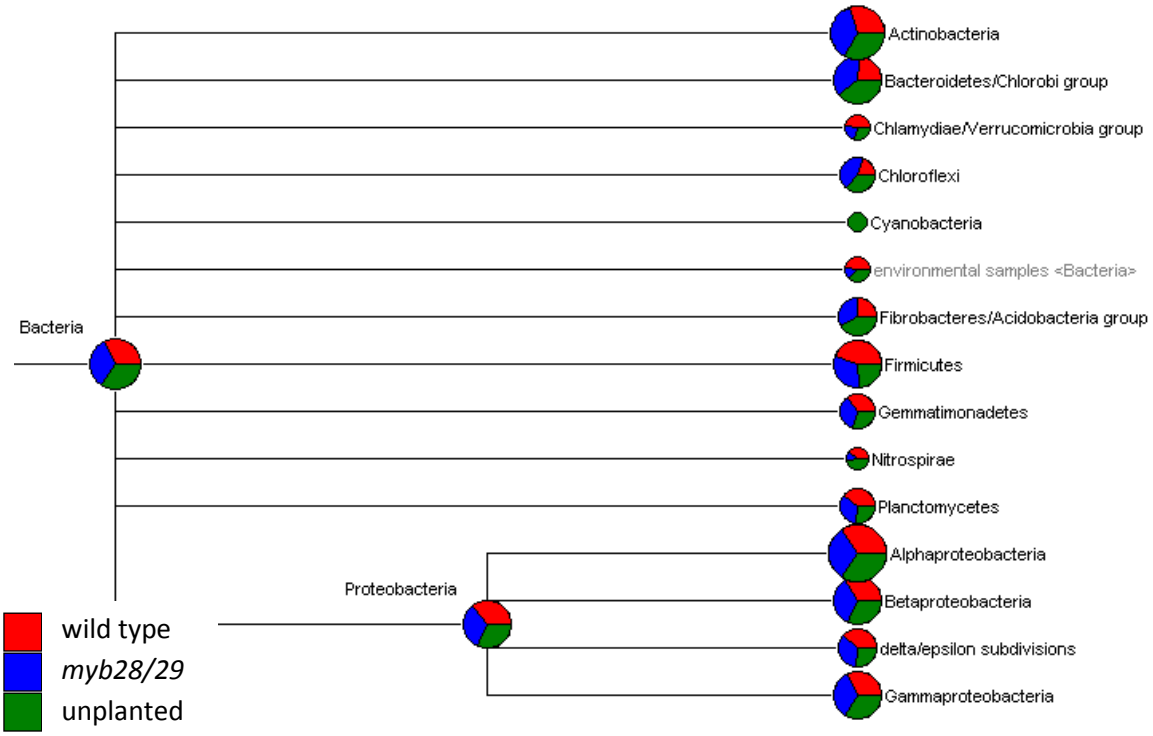


Figure 5.2.19 Phylogram showing phyla and Proteobacteria subphyla for bacterial community sampled in the MYB experiment. (n=3 for each plant genotype/unplanted).

Arabidopsis WT and *myb28/29* rhizosphere shared most of the dominant bacterial OTUs (Figure 5.2.20). *Rhizobium* and Sphingomonadaceae were actively selected in the rhizosphere. However, there were no major differences between these two genotypes at the genus/species level of ten dominant OTUs.

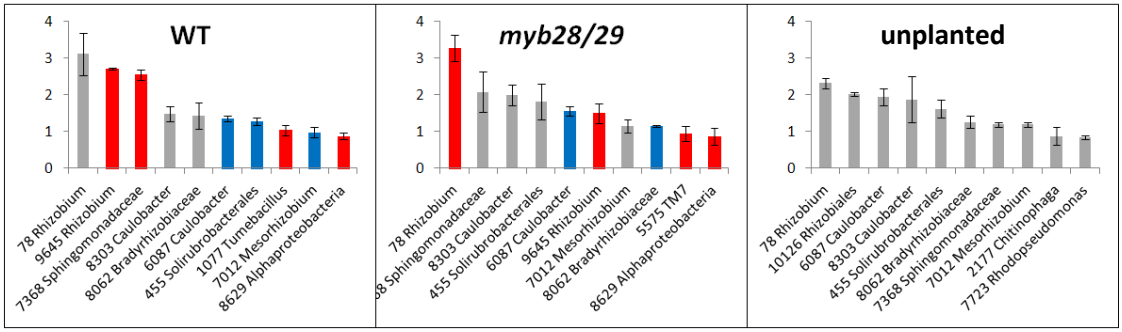


Figure 5.2.20 Bar graphs presenting 10 dominant OTUs in the bacterial community of MYB experiment (Y axis - % of relative abundance). Red bars present rhizosphere OTUs selected against unplanted control, blue bars rhizosphere OTUs depleted against unplanted control ($p<0.05$ for both categories), grey bars – no significant difference from unplanted control.

The ternary plot indicates that most of the OTUs were shared between the rhizosphere and control samples (Figure 5.2.21). However, there is a “tail” towards the unplanted corner, which represents bacterial OTUs that were suppressed in the rhizosphere. *Arabidopsis* genotype specific OTUs were annotated (located in the corners). There are 11 wild type specific OTUs, 5 of which belong to Firmicutes (*Tumebacillus* and *Paenobacillus*), 5 to Alphaproteobacteria and 1 to Actinobacteria. *Myb* plants selected for 6 OTUs, of which 3 belong to Actinobacteria. Firmicutes, Alpha and Beta- Proteobacteria were represented by a single OTU. This indicates a relatively weak association of Firmicutes and Alphaproteobacteria with wild type plants and Actinobacteria with mutant plants.

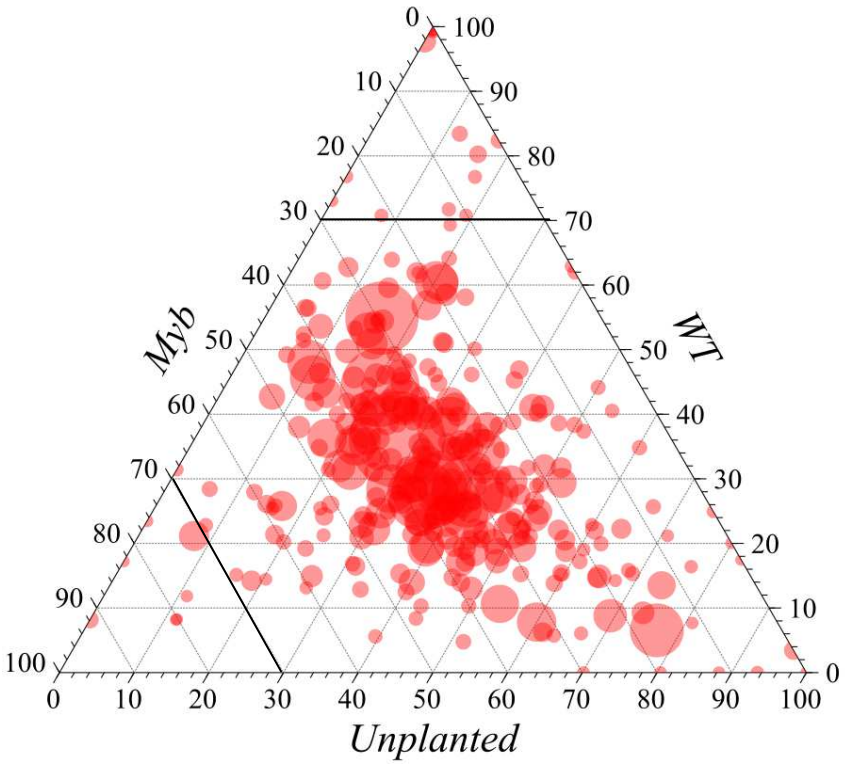


Figure 5.2.21 Ternary plot representing influence of *Arabidopsis* genotypes on bacterial OTUs, based on 454 sequencing. The unplanted control is one axis of the graph, so there is no colour code. The plant genotype specific regions (>70 % of the plant genotype influence) of the graph are separated.

5.2.5 Pyrosequencing analysis of the fungal community for the MYB experiment

The overall fungal community structure was relatively similar between the rhizospheres of wild type plants and the unplanted controls (only two samples for unplanted control were successfully sequenced). The rhizosphere community of *myb28/29* plants seems to be relatively different from the other samples (Figure 5.2.22).

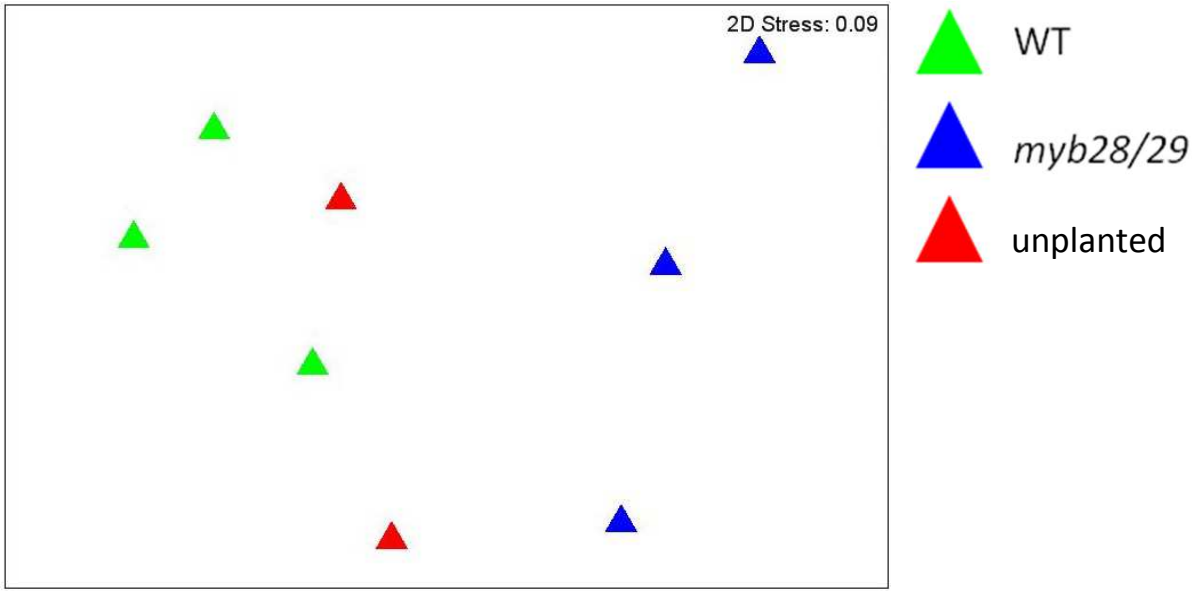


Figure 5.2.22 MDS plot presenting fungal rhizosphere community of *Arabidopsis* Myb experiment analysed using 454 pyrosequencing (n=3 for each plant genotype and n=2 for unplanted).

The richness and diversity of the fungal rhizosphere of *myb28/29* mutant was slightly lower than wild type and unplanted control, however the differences are not statistically significant (Figure 5.2.23). This same pattern was observed for the bacterial community (Figure 5.2.17)

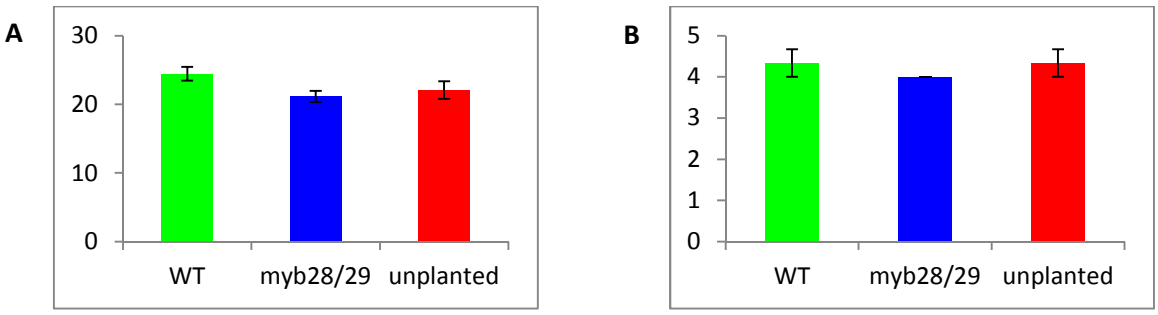


Figure 5.2.23 Analysis of fungal richness (A) and Shannon index diversity (B) for the MYB experiment based on the 454 sequencing data (n=3 for each plant genotype/unplanted).

The fungal community at the broad taxonomic level shows that wild type rhizosphere samples were enriched with Pezizomycotina and Glomeromycota species relative to *myb28/29* plants. Both rhizospheres were depleted of Agaricomycotina compared to the unplanted control (Figure 5.2.24 and 5.2.25).

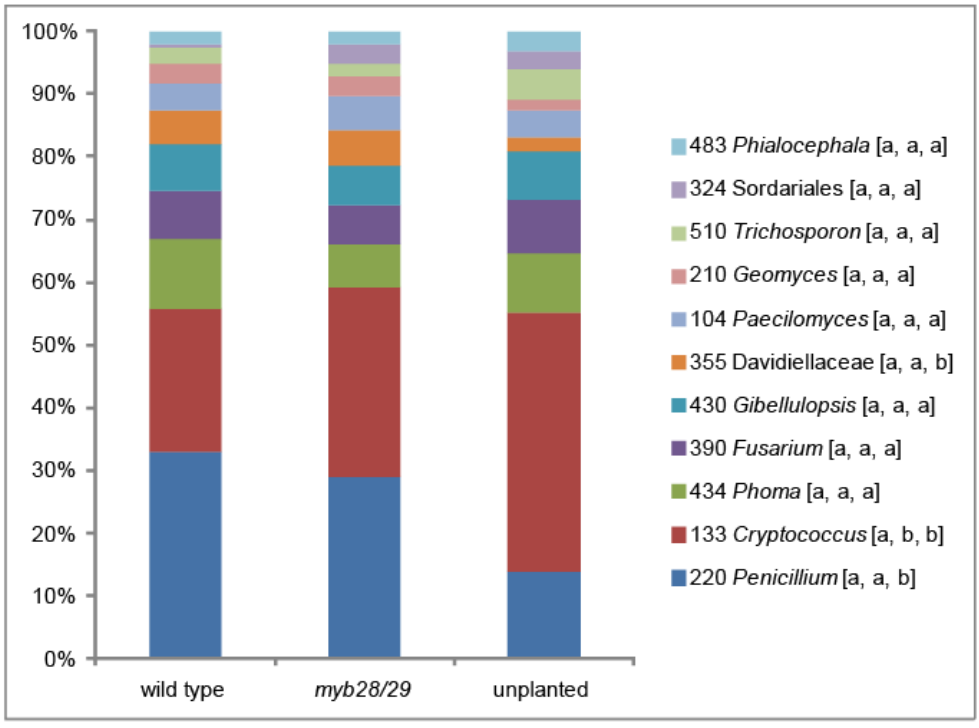


Figure 5.2.24 Fungal community structure in the Myb experiment. The abundance of Pucciniomycotina, Chytiodiomycota and Glomeromyceta was very low and are represented on the bars between Agaricomycotina and other fungi (Glomeromyceta hits are probably caused by some miss-annotation) ($p < 0.05$) results [wt, *myb28/29*, unplanted].

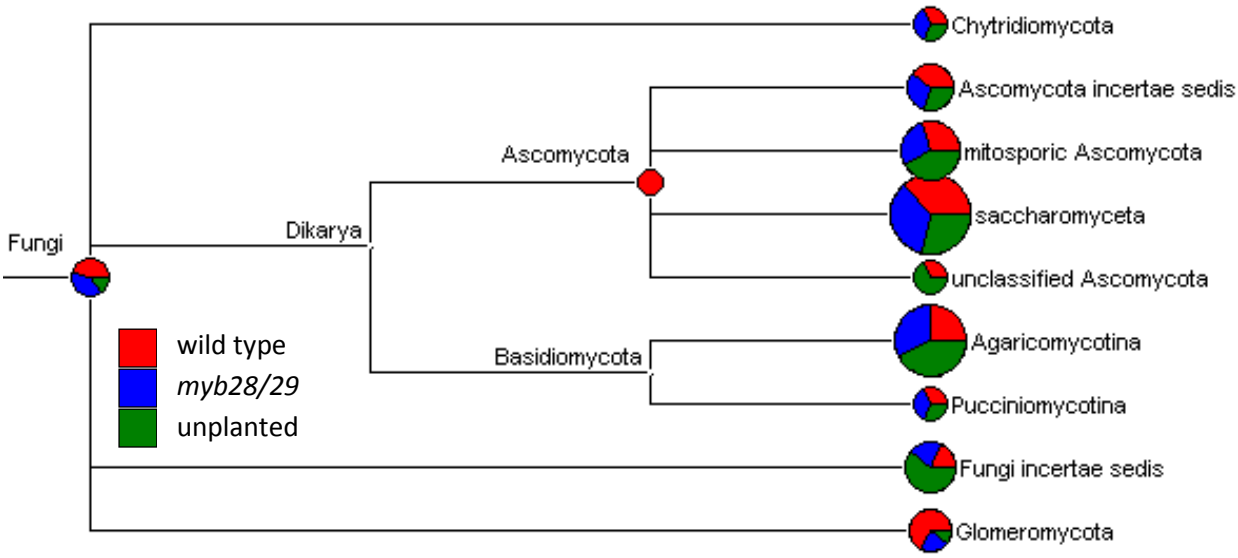


Figure 5.2.25 Phylogram showing phyla, divisions and subdivisions of fungal community sampled in the Myb experiment. The graph is based on 454 sequencing data (n=3 for each plant genotype/unplanted).

The plant rhizosphere environment selected for *Penicillium* and Davidiellaceae species. There were no major differences between plant genotypes in their fungal community structure for the most common OTUs (Figure 5.2.26). All the 10 common fungal OTUs could be annotated to at least the level of order.

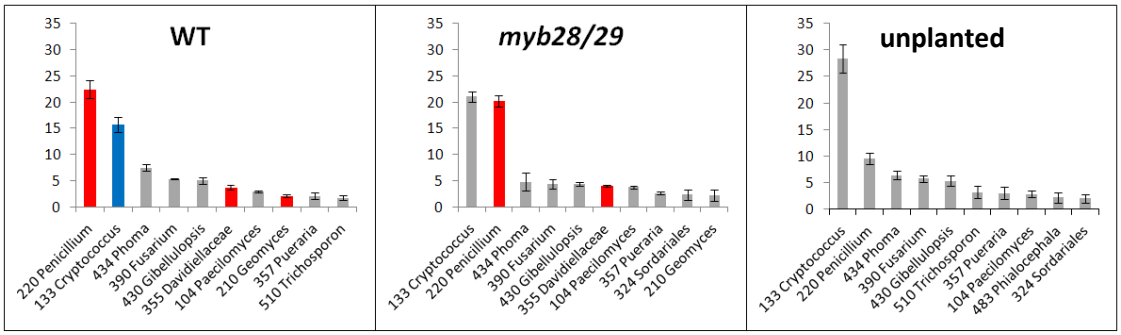


Figure 5.2.26 Bar graphs presenting 10 dominant OTUs in the fungal rhizosphere community of MYB mutants (Y axis - % of relative abundance). Red bars show rhizosphere OTUs selected against unplanted control, blue bars rhizosphere OTUs depleted against unplanted control, $p<0.05$, grey bars – not statistically significant. (n=3 for each plant genotype/unplanted).

Fungal OTUs, whose abundance were most strongly affected by the plant genotype are listed in the Table 5.2.4. Sordariales is a common soil saprophytic group (Kendrick, 2000). *Cryptococcus* genus is also common in soils and it was shown that some members of this genus are able to degrade raw starch (Fonseca *et al.*, 2000). *Penicillium* fungi are able to compete with soil microbiota species producing an array of different antibiotics.

OTU number and annotation	Average abundance in WT (%)	Average abundance in *myb28/29* (%)	Average dissimilarity (%)	Contribution to differences in %
324 *Sordariales*	0.66	1.58	0.69	2.34
188 *Penicillium*	0.78	0	0.58	1.97
133 *Cryptococcus*	3.95	4.66	0.53	1.79
452 *Penicillium*	0.63	0	0.47	1.61
217 *Penicillium*	0.75	0.18	0.43	1.46

Table 5.2.4 Top five fungal OTUs responsible for the community differences between Arabidopsis WT and myb28/29 mutant. Table is based on SIMPER output of the PRIMER 6.0 software.

The fungal communtity was very uniform between samples in the MYB experiment (Figure 5.2.27). Only OTUs of low abundance seem to be more plant genotype specific and this may be the result of insufficient sampling depth.

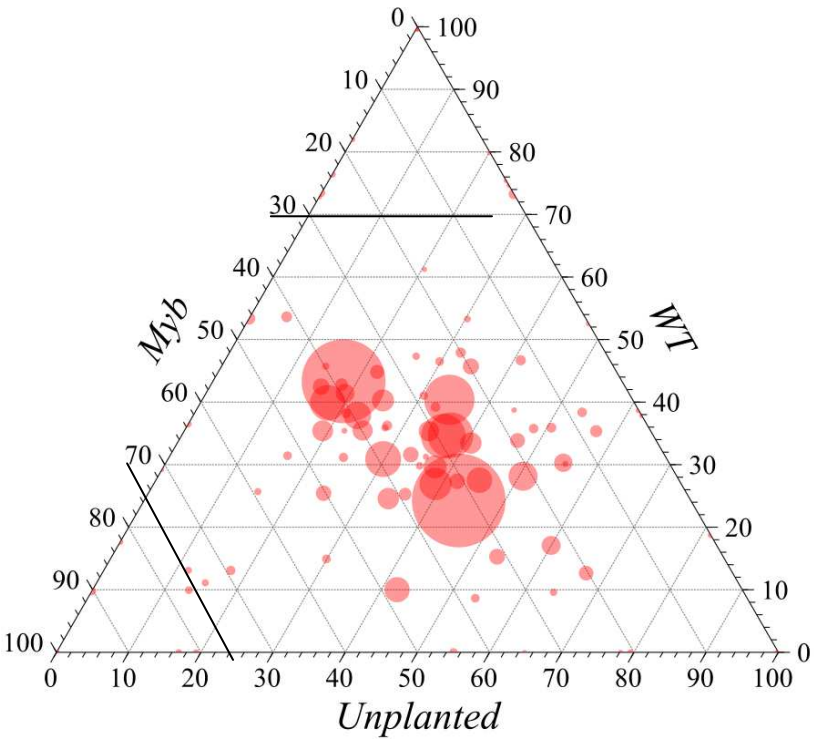


Figure 5.2.27 Ternary plot representing the influence of Arabidopsis genotypes on fungal OTUs. The unplanted control is one axis of the graph, so there is no colour code. The plant genotype specific regions (>70 % of the plant genotype influence) of the graph are separated. (n=3 for each plant genotype/unplanted).

5.2.6 Summary and discussion of the MYB experiment

The MYB experiment was designed to test the influence of aliphatic glucosinolates on the bacterial and fungal community in the rhizosphere of *Arabidopsis*. Plants which do not produce aliphatic glucosinolates are less able to defend themselves against bacterial pathogens than WT *Arabidopsis* (Fan *et al.*, 2011). The experiment showed that the effect of glucosinolates on the rhizosphere is weak but statistically significant. ARISA analysis showed that the bacterial community at the species/strain level was different between wild type

and mutant plants. It was already shown that changes in the glucosinolate secretion profile of *Arabidopsis* by insertion of the sorghum gene CYP79A1 changes the bacterial community in the rhizosphere (Bressan *et al.*, 2009). Our results show that *Arabidopsis* endogenous glucosinolates also have an effect on the rhizosphere, which is the focus of a recent review (Bulgarelli *et al.*, 2013). In ours results the rhizosphere of wild type plants was more different from the unplanted control than *myb28/29* was. Due to the small number of replicates pyrosequencing cannot confirm the findings statistically. However, there are some indications that the glucosinolates have an effect on species belonging to Firmicutes, Actinobacteria and Alphaproteobacteria. Based on 454 pyrosequencing data, glucosinolates have no major effect on the common fungal OTUs. However, there is an influence of the glucosinolate on the less abundant taxa, as concluded from the MDS plot (Figure 5.2.22).

5.2.7 ARISA and pyrosequencing analysis of bacterial community for the RAM experiment

The influence of *ram* mutants on the rhizosphere microbial community was examined using bacterial and eukaryotic ARISA. Bacterial ARISA was described earlier in the material and method chapter. Eukaryotic ARISA was also used for this experiment, as mycorrhization ability of *Medicago* was of special interests to us. In other experiments eukaryotic ARISA was not performed due to time constraints.

A set of primers was used for eukaryotic ARISA: 2243C (5'-GTTTCCGTAGGTGAACCTGC-3') and 3126T (5'-ATATGCTTAAGTTCAGCGGGT-3'). These primers amplify the ITS region between 18S and 28S rRNA genes, including 5.8S rRNA gene (Ranjard *et al.*, 2001; Sequerra, 1997). Primers successfully amplified yeast (*S. cerevisae* NCYC 1026), oomycetes (*Albugo candida)* and plant (*A. thaliana* and *M. truncatula)* ribosomal DNA during ARISA tests. Eukaryotic ARISA produces longer fragments of amplified DNA than bacterial ARISA. The fluorescence noise for the fluorescent dye used here (NED) is greater than in case of the 6FAM dye. The 50 ARISA fragments with the greatest fluorescence intensity were taken into analysis. A fragment of 692 bp was excluded from further analysis as it is the same size as that coming from *Medicago* genomic DNA (Figure 5.2.28). In case of most samples analyzed 692 bp was the dominant fragment.

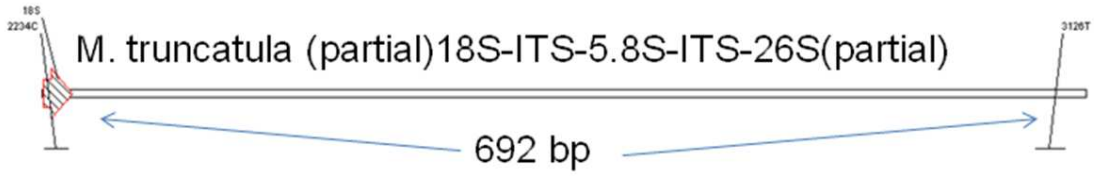


Figure 5.2.28 The ITR size of *Medicago truncatula* of rRNA operon.

Bacterial ARISA shows that the rhizosphere bacterial community of *ram1* and *ram2* plants was different from that of the wild type and was also different from the unplanted control (Figure 5.2.29, Table 5.2.5).

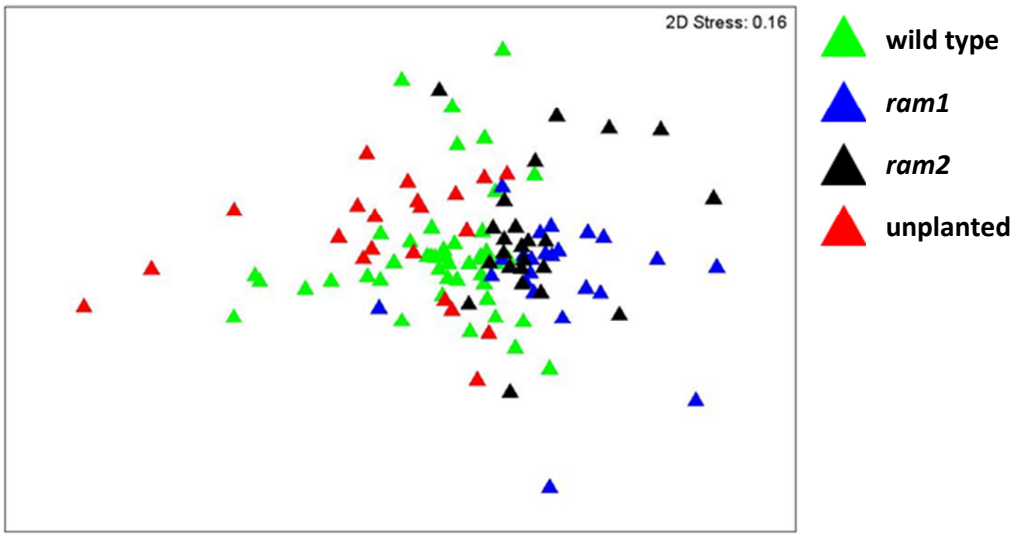


Figure 5.2.29 MDS plot of ram1 and ram2 mutant influence on the rhizosphere bacterial community. Graph based on ARISA data (n=21 for each plant genotype/unplanted).

	WT	*ram1*	*ram2*	U
WT		12	13	10
ram1	12		18	6
ram2	13	18		7
U	10	6	7	

Table 5.2.5 MANOVA analyses for bacterial community in the RAM experiment based on ARISA data (t-test $p \leq 0.05$, n=21 for each plant genotype/unplanted).

In order to annotate the bacterial taxa that were influenced by the lack of arbuscular fungi associations with plants, 454 pyrosequencing of the bacterial 16S rRNA gene fragment (V1-V2 region) was performed (Table S4). Reads were binned into OTUs using 95 % similarity

Bacterial community structure analysed using 454 pyrosequencing gives a similar picture to the analysis of ARISA fingerprints: *ram1* and *ram2* are clustered close to each other, while wild type and unplanted control form separate clusters (Figure 5.2.30).

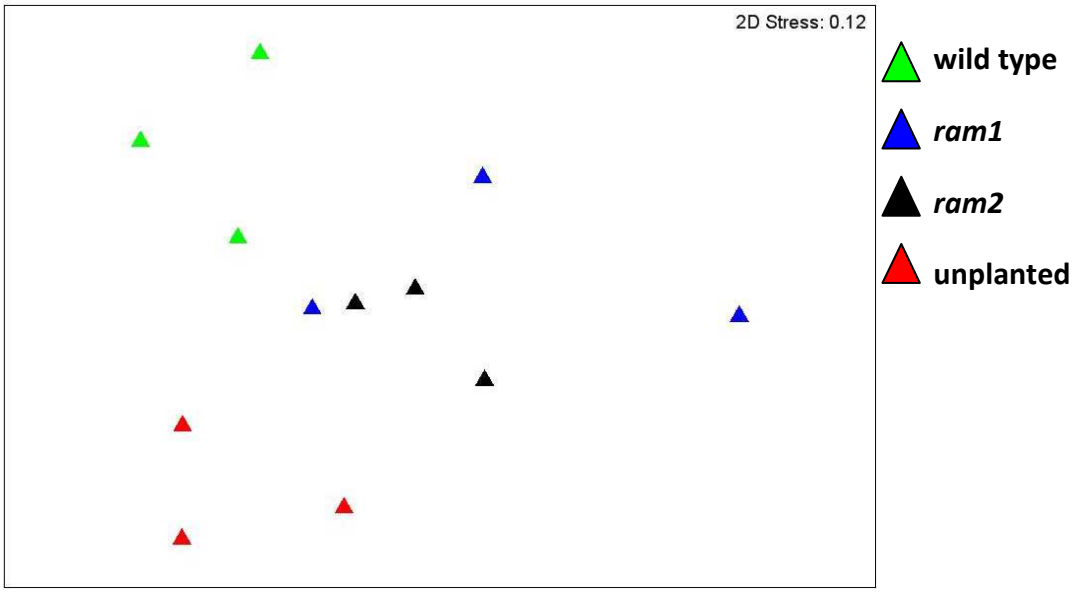
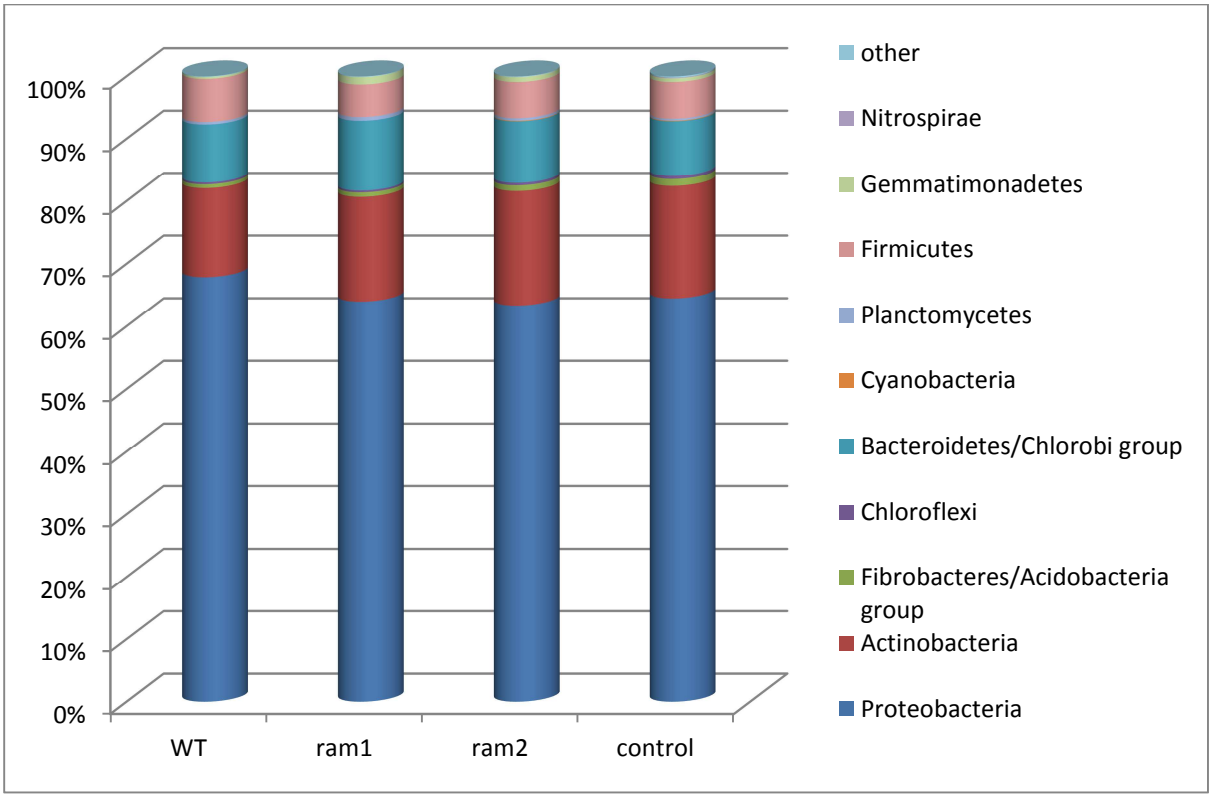


Figure 5.2.30 MDS plot presenting rhizosphere community of *Medicago* RAM experiment analysed using 454 pyrosequencing of bacterial community (n=3 for each plant genotype/unplanted).

There were no statistical differences (t-test p <0.05) in the bacterial community at the phylum and subphylum level between the genotypes of *Medicago* (Figure 5.2.31). Only Proteobacteria, which was the most common phylum in all samples, had increased abundance in the wild type rhizosphere.



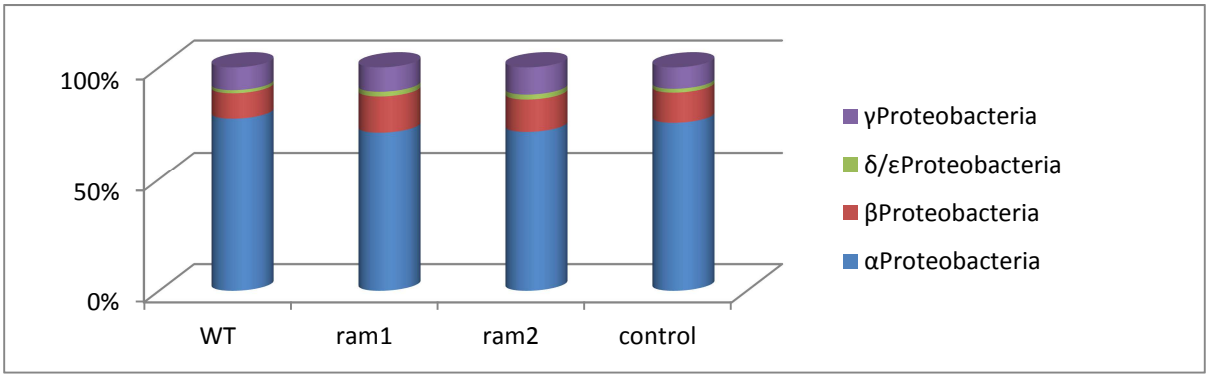


Figure 5.2.31 Bacterial community structure of RAM mutants A) at the phylum level, B) at the subphylum level within Proteobacteria (n=3 for each plant genotype/unplanted).

A more detailed analysis of bacterial structure at the phylum and subphylum level showed that there were no major differences between different samples (Figure 5.2.32). Phyla that were more abundant in the mutant rhizosphere are Gemmatimonadetes and Planctomycetes.

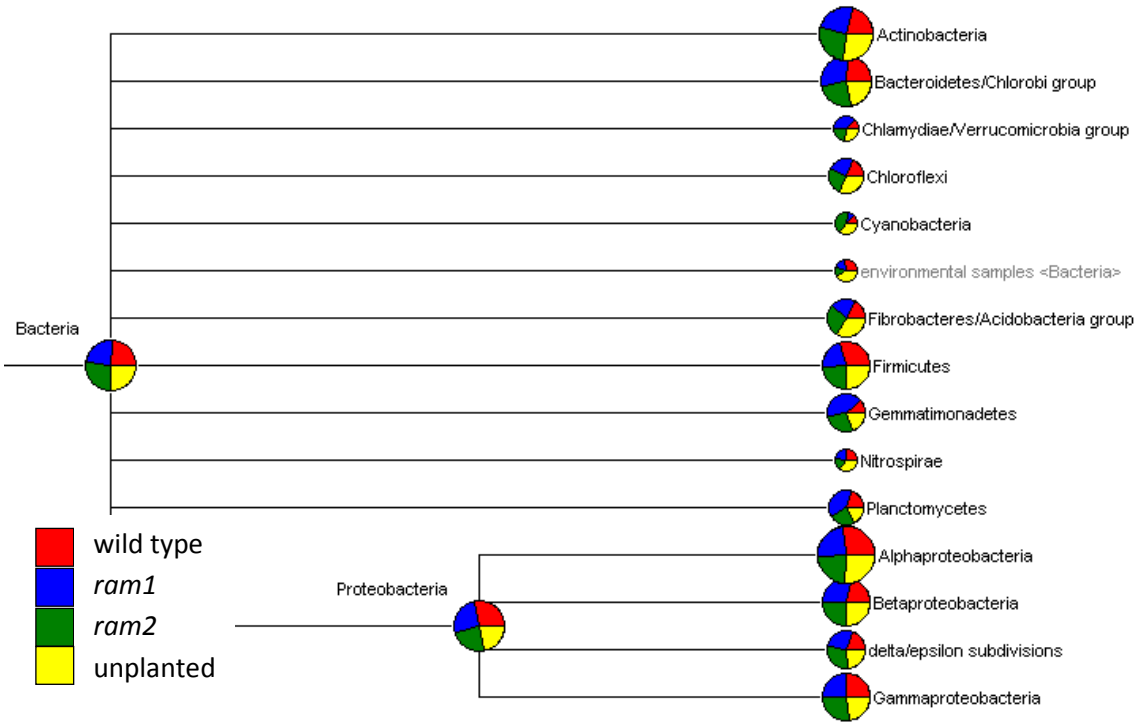


Figure 5.2.32 Phylogram showing phyla and Proteobacterial subphyla for bacterial community sampled in the RAM experiment (n=3 for each plant genotype/unplanted). Figure based on the analysis of the total community (not binned into OTUs) in MEGAN.

The differences in the Shannon index and richness are not statistically significant between any of the analysed *Medicago* mutants (Figure 5.2.33). However these indexes are relatively lower for the wild type plants than for the mutant plants, which may indicate that the formation of a mycorrhizal association reduces bacterial diversity.

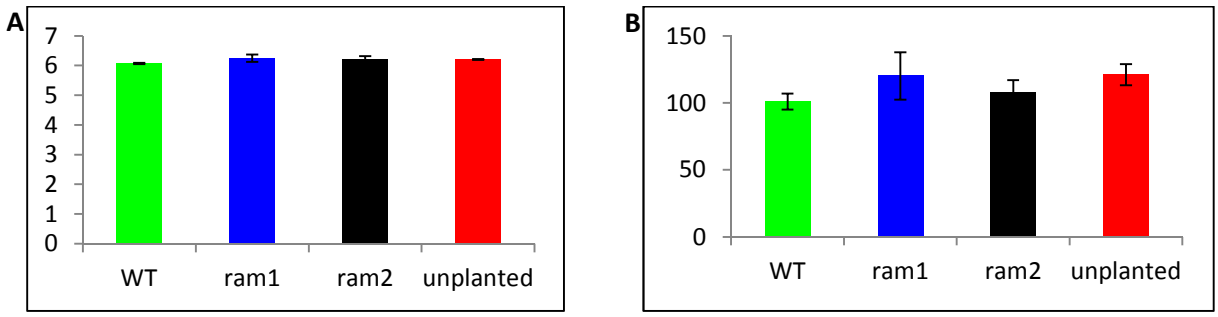


Figure 5.2.33 Analysis of bacterial diversity in the RAM experiment. A) Shannon index B) richness (n=3 for each plant genotype/unplanted). Analysis based on 454 sequencing data. No differences are statistically significant, apart from WT – unplanted pair for the Shannon index (t-test, $p<0.05$).

Most of the abundant bacteria taxa are shared between different genotypes of *Medicago* (Figure 5.2.34). The rhizosphere of wild type strongly selects for the dominant bacterial genera (especially *Rhizobium*), while the mutant rhizosphere was not as selective. Most selected *Rhizobium* OTUs have their closest match with *Rhizobium etli* CIAT 652. *Rhizobium etli* CIAT 652 was sequenced by a group from Mexico (Genome diversity and DNA divergence of Rhizobium etli – accession CP001074), however no further data has been published.

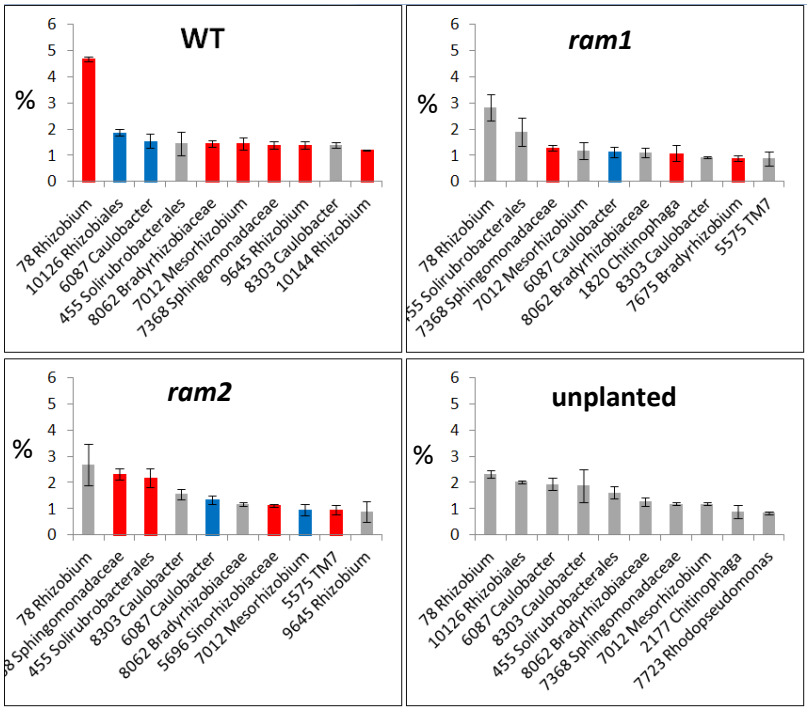


Figure 5.2.34 Bar graphs showing abundance of 10 commonest OTUs in the bacterial rhizosphere community of RAM mutants (Y axis - % of relative abundance). (red bars indicate OTUs selected in the rhizosphere and blue bars OTUs depleted in the rhizosphere both relative to the unplanted control ($p \leq 0.05$). Grey bars represent OTUs not significantly different from the control. n=3 for each plant genotype and control.

Most of the community was shared between the different genotypes of *Medicago*. There were, however a few highly selected bacterial OTUs in the corners of the graph (annotated as red dots) (Figure 5.2.35). *ram1* specific OTUs include Bacteroidetes and *Rhodanobacter* and wild type specific OTUs belong to Firmicutes and Alphaproteobacteria. The two OTUs presented as large green dots in the wild type corner were annotated as Rhizobiales and *Rhizobium*. These OTUs were suppressed in the mutants' rhizosphere.

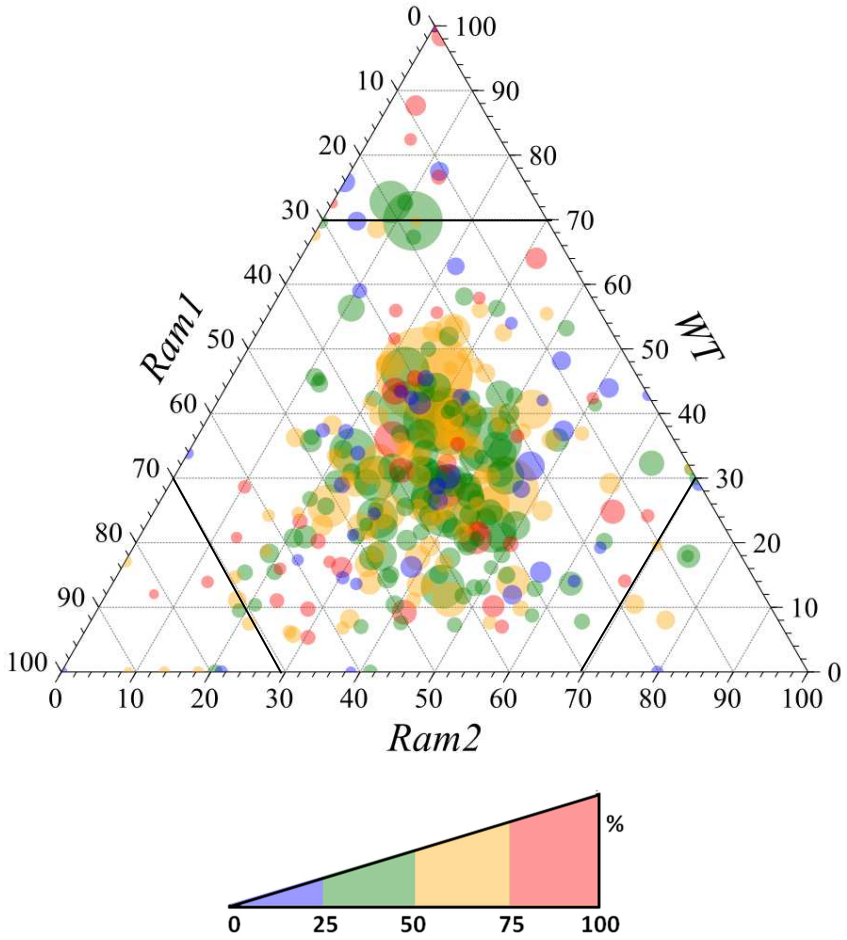


Figure 5.2.35 Ternary plot representing influence of *Medicago* genotypes on bacterial OTUs. The plant genotype specific regions of the graph are separated.

5.2.8 ARISA and pyrosequencing analysis of fungal community for RAM mutants - results and discussion

The fungal rhizosphere community presents a similar overall picture to the bacterial one. The MDS plot based on eukaryotic ARISA data indicates that the fungal rhizosphere community of wild type and unplanted control samples were relatively similar to each other (Figure 5.2.36). The community of both mutant plants are different from those of the wild type and very similar to each other.

MANOVA analysis separated groups from each other using a low number of samples (Table 5.2.6). MANOVA test shows that *ram* mutants were more similar to each other than they are to the wild type or unplanted control. This indicates that lack of a mycorrhizal association has a greater impact on the community than any other differences in the phenotypes of these two mutants. As in case of all previous experiments, the plant rhizosphere is very different from the bulk soil.

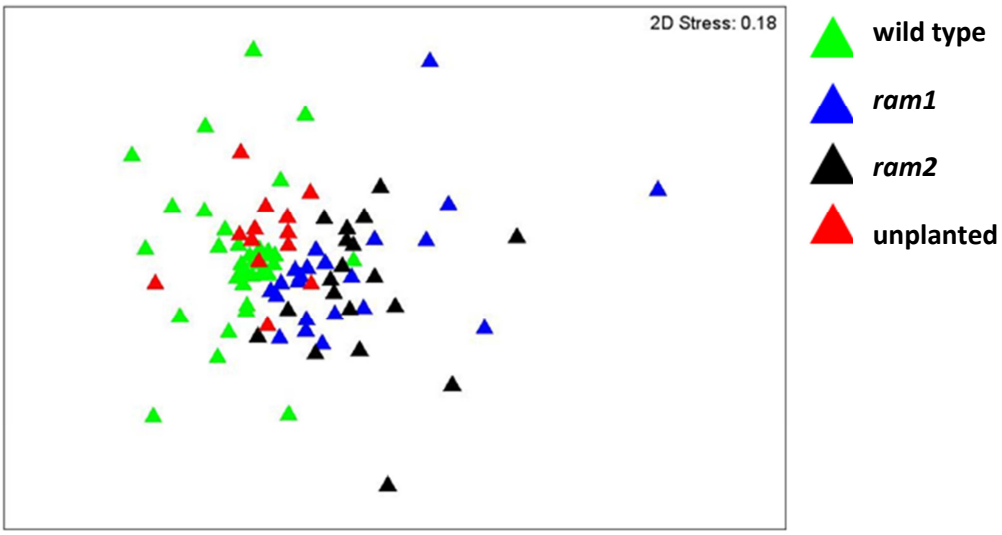


Figure 5.2.36 MDS plot showing the influence of *Medicago* RAM genotypes on the rhizosphere fungal community. Data based on eukaryotic ARISA fingerprinting.

	WT	*ram1*	*ram2*	U
WT		10	8	10
ram1	10		19	8
ram2	8	19		5
U	10	8	5	

Table 5.2.6 MANOVA analyses for fungal community in the RAM experiment based on eukaryotic ARISA data (t-test p≤0.05, n=21 for each plant genotype/unplanted)

There is no clear pattern of fungal community structure based on 454 sequencing data and visualized using MDS plot (Figure 5.2.37). Unplanted control and *ram2* samples have low heterogeneity between replicates and are located relatively close to each other. Wild type samples and *ram1* samples have much greater heterogeneity. In order to better interpret the fungal community a higher number of replicates would need to be sequenced.

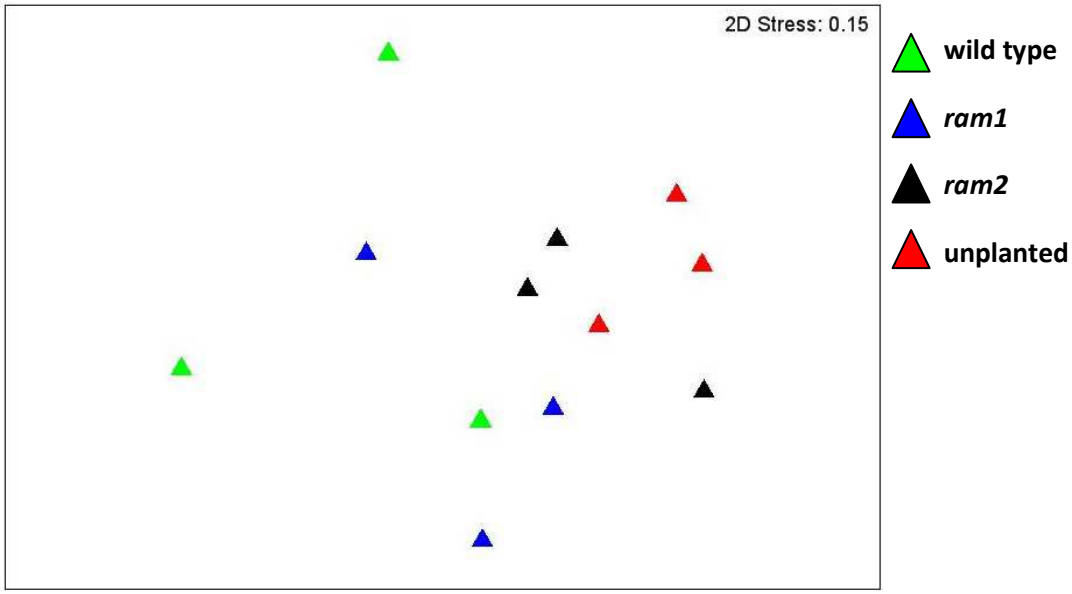


Figure 5.2.37 MDS plot showing *Medicago* genotypes influence on the rhizosphere fungal community. Data based on fungal 454 sequencing (n=3 for each plant genotype/unplanted)

The rhizosphere of two *ram* mutants is slightly enriched with Pezizomycotina compared to the wild type and unplanted control. The higher abundance of Pezizomycotina decreased the relative abundance of Agaricomycotina for the mutant plants samples (Figure 5.2.38). These observations are not statistically significant (t-test $p < 0.05$) and are mentioned here to highlight the trend only.

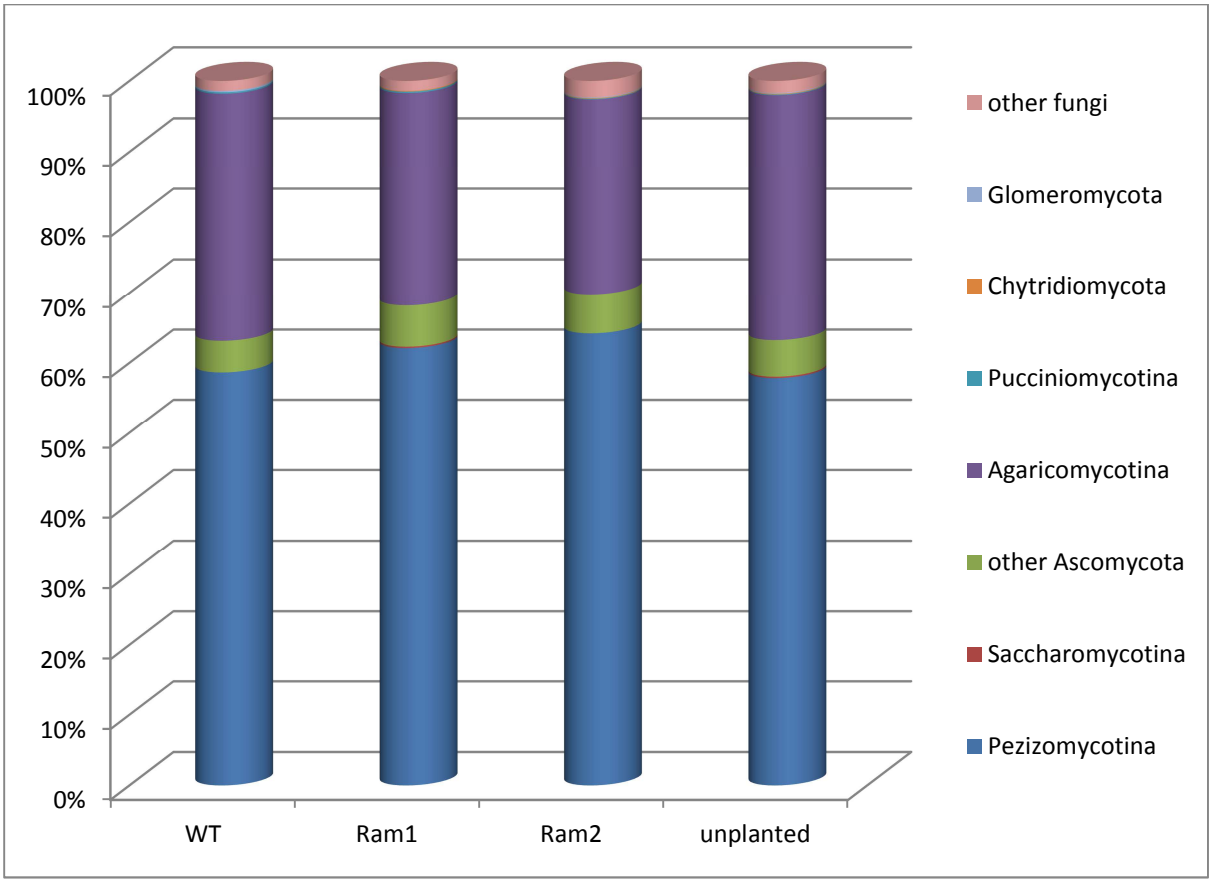
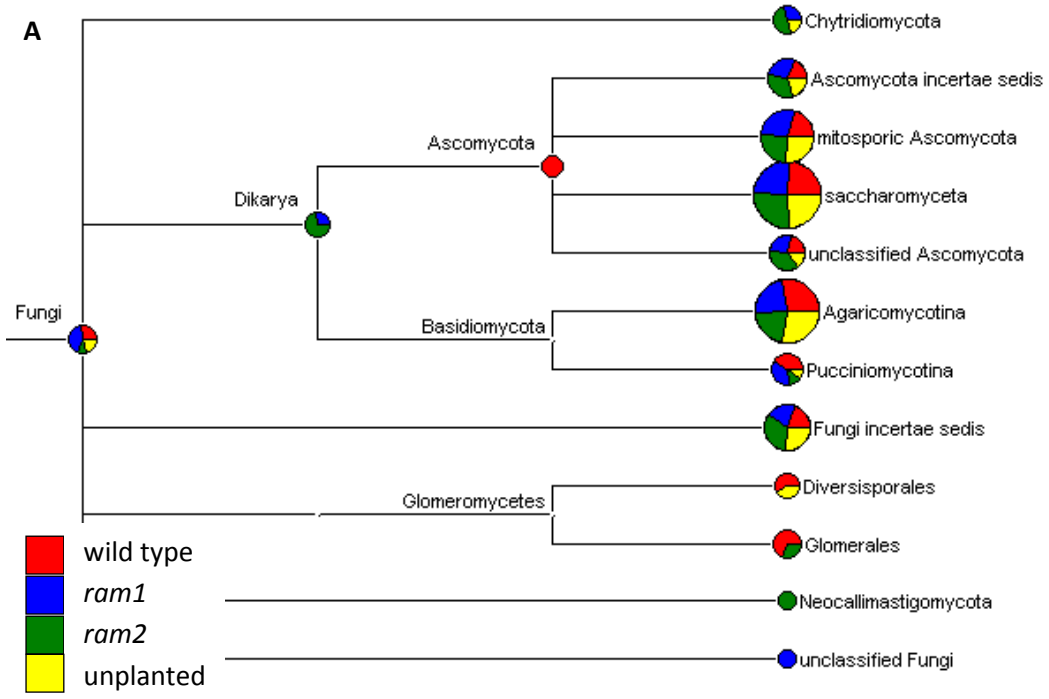


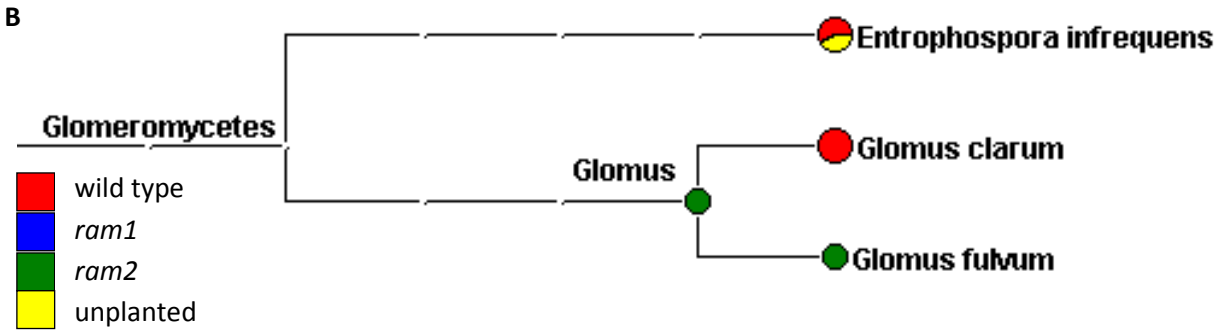
Figure 5.2.38 Fungal community structure in the RAM experiment (n=3 for each plant genotype/unplanted).

The fungal community taxonomic composition was quite similar for all the samples (Figure 5.2.39 A). The phylum Puccinomycotina was more common in the wild type and *ram1* samples. Reads belonging to Glomeromycetes class, which groups all the mycorrhiza fungi, were found only for wild type, *ram2* and unplanted control. This finding is a great confirmation of the mycorrhizal impairment of *ram1* and partially by *ram2* mutant. However the results shown on Figure 5.2.39 B may be misleading as the 454 reads counts were normalized to 10000 in order to level the difference between sequencing depth of different samples. Analysis on absolute number of reads (Figure 5.2.39 C) is less convincing, as there are actually only a few reads belonging to Glomeromycetes. Previous studies found AM fungi to be much more dominant in soils (Davidson *et al.*, 2009; Lovelock *et al.*, 2003; Nuccio *et al.*, 2013). The most probably explanation of this difference is the narrow specificity of the primers used in this study (Buee *et al.*, 2009). Primers were primarily designed for

Basidiomycota and Ascomycota however should also target other fungi. The other explanation of the low Glomeromycetes abundance is the type of sampling protocol used in this study. Samples for DNA isolation were taken from the soil adhering to the roots, but the bacterial and fungal communities thriving inside the root (or being at least firmly anchored) were not analysed.

Of course, it is also possible that Glomeromycetes were very rare in the rhizosphere soil, used as an inoculum, and were not selected by any of the plant genotypes and all observed differences are just stochastic.





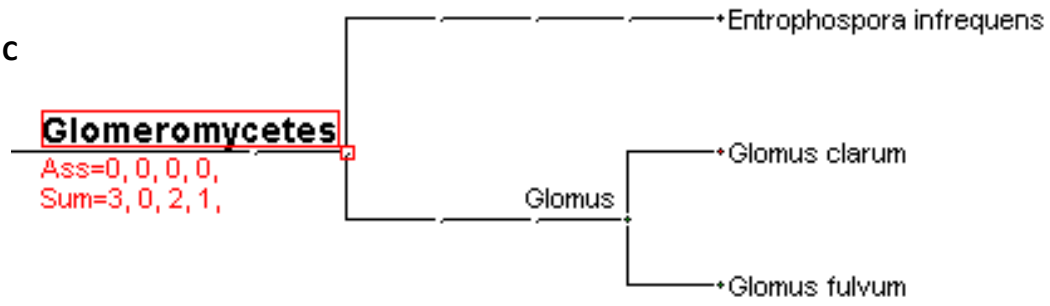


Figure 5.2.39 Phylogram showing phyla, divisions and subdivisions of fungal community sampled in the Ram experiment A) all analyzed phyla, B) Glomeromycetes class, C) Glomeromycetes class – read counts not normalized – the numbers in red indicate how many reads were assigned to Glomeromycetes for WT, *ram1*, *ram2* and unplanted, respectively (n=3 for each plant genotype/unplanted).

The richness and diversity of the fungal community are much lower than for the bacterial communities (Figure 5.2.40). Plant rhizosphere fungal communities have higher diversity than the unplanted control (Shannon and richness), but the wild type plant rhizosphere has the lowest richness (but not Shannon index). The richness index used in this study focuses on the common taxa (first 50 %), while Shannon index takes into an account all the species. Of course, the common community in the richness index is calculated in the relative percentage, so indirectly this index is also influenced by the less common species. The difference between richness and Shannon index values for the wild type plants relatively to the other *Medicago* genotypes is caused by increased relative abundance of the most common taxa in the rhizosphere of wild type. However, the less common taxa have the same abundance in this rhizosphere as in others.

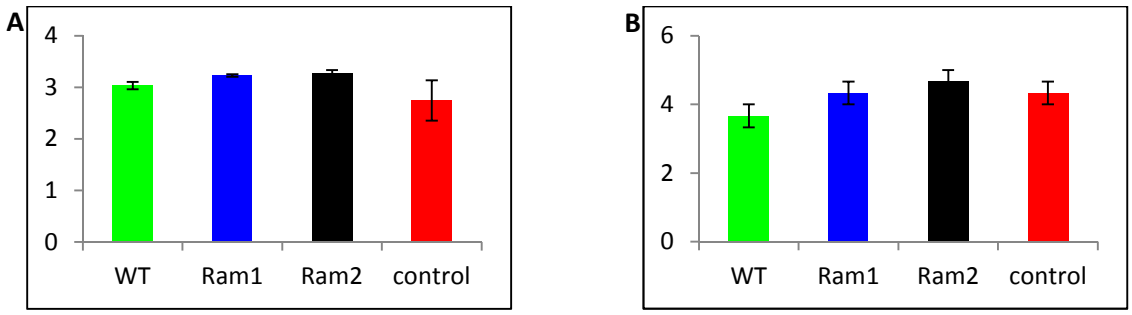


Figure 5.2.40 Analysis of fungal diversity in the RAM mutants. A) Shannon index and B) richness based on 454 sequencing data (n=3 for each plant genotype/unplanted). No differences are statistically significant (t-test, $p<0.05$).

The rhizosphere fungal communities were very similar to each other for the dominant fungal species. *Cryptococcus* was the most dominant genus with the abundance of around 25 – 30 % of the total community. There were no major differences between the rhizosphere samples and the unplanted control (Figure 5.2.41).

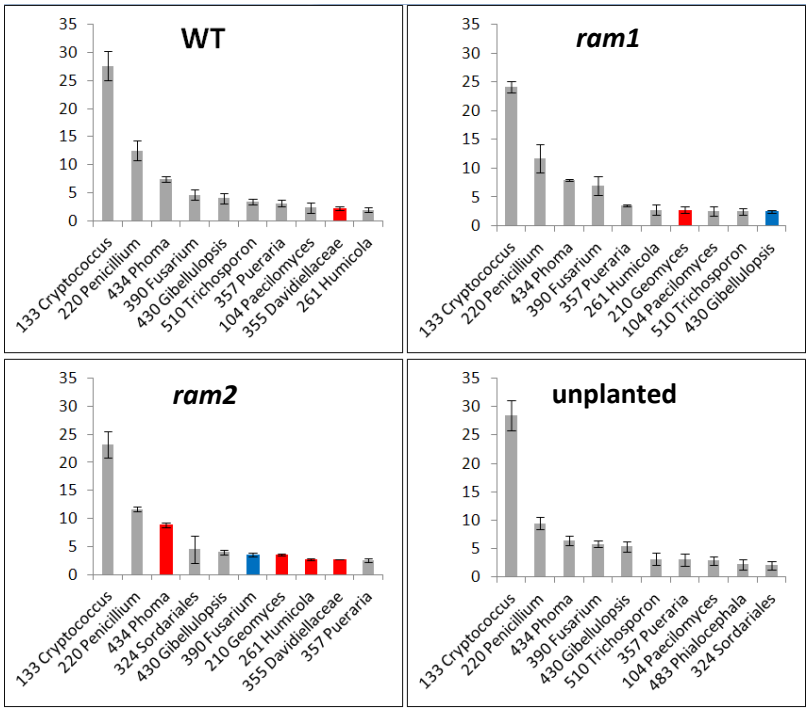


Figure 5.2.41 Bar graphs presenting 10 dominant OTUs in the fungal rhizosphere community of ram mutants (red bars present rhizosphere OTUs selected against unplanted control, blue bars rhizosphere OTUs depleted against unplanted control and grey bars represent OTUs without statistically significant difference, $p\leq0.05$).

Phoma is a common endophyte species, sometimes with plant pathogenic properties and was found to be the dominant species inhibiting needles of Spruce trees based on the culture-independent metagenomic approach (Rajala *et al.*, 2013). *Geomyces* causes a disease called bat white-nose syndrome and was found as one of the dominant fungal species in soils of bat hibernacula based on the culture-dependent methods (Lorch *et al.*, 2013). The relation of this genus with plants is not clear. *Humicola* is able to produce xylanase and cellulase (Boonlue *et al.*, 2003; Masui *et al.*, 2012). This genus probably either metabolizes dead plant wall cells or is a plant pathogen. Members of the Davidiellaceae family have been found to live inside plant leaves and help to degrade leaf litter. Members of this groups are also a common soil species (Zalar *et al.*, 2007).

There were few differences in the fungal community between different genotypes of *Medicago* with most OTUs placed in the middle of the ternary plot (Figure 5.2.42).

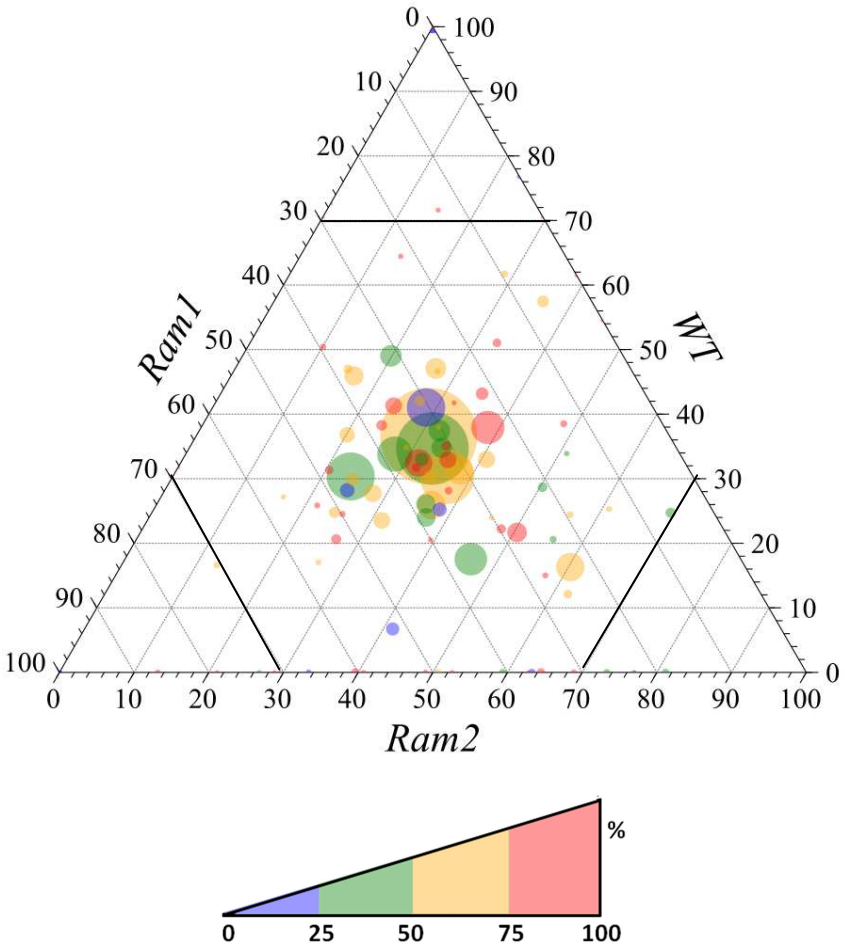


Figure 5.2.42 Ternary plot representing influence of Medicago genotypes on fungal OTUs. The plant genotype specific regions of the graph are separated (n=3 for each plant genotype/unplanted).

5.2.9 Summary and discussion of the RAM mutants

The Ram experiment was designed to test the influence of mycorrhiza on the bacterial and fungal community structure in the rhizosphere of *Medicago*. The experiment showed that inability of the plants to form mycorrhizal interactions has an effect on the rhizosphere. However, we don't know if the effect is caused by the difference in the mycorrhizal fungal abundance, as the primers used in this study proved to be more specific towards

Ascomycota and Basidiomycota. *ram* mutants (*ram1* regulates expression of ram2) do not produce a cutin layer on the root surface. This prevents mycorhizal fungi, but also oomyctes from attaching (Wang *et al.*, 2012). It is very probable that other fungi and bacteria are using this layer for attachment and so their abundance is changed by the lack of it in case of the *ram* mutants (Bolwerk *et al.*, 2005).

ARISA analysis showed that bacteria and especially the fungal community at the species/strain level (ARISA data) were more similar between the two mycorrhizal mutants than between any of these mutants and wild type or unplanted control. The rich compost conditions used in this experiment may not have allowed a strong selection of the bacterial and fungal species. 454 pyrosequencing did not confirm that the rhizosphere community structure was strongly altered by different plant genotypes. Only a few bacterial as well as fungal taxa were affected by the plant genotype. Probably, if the sequencing was performed with a greater depth some changes in less abundant bacterial and fungal species would be observed. Equally important more replication would need to be used.

Unfortunately, plants were not stained for arbuscular mycorrhizal fungi. They were not nodulated, as there was too much of available nitrogen present in the compost growth medium.

The RAM experiment presented in this study is a valuable introduction to further work. At the time of writing this PhD thesis a group of nodulation and mycorrhization mutants are being analysed using ARISA and the detailed annotation of the common taxa are studied using MiSeq sequencing.

5.2.10 ARISA analysis of bacterial community for the HOL experiment - results and discussion

To test whether the production of methyl halides could affect bacterial composition of the microbial community in the rhizosphere of *A. thaliana*, WT, *hol* mutant and *35S::HOL* lines were grown in sterilized compost mixed with soil collected from Bawburgh farm. The *hol*

(*HARMLESS TO OZONE LAYER)* mutant does not produce any methyl halides, while the 35S::*HOL* line overexpressed methyl halides.

To determine how the community changes over time, the rhizosphere was sampled 1, 2 and 4 weeks after planting. Due to the increased number of time points the number of biological replicates was reduced to 7 for the plants and 3 for the unplanted control. At the time of this experiment the normal number of samples needed for statistical comparisons was not known.

Two sets of experiments were started. Initially, the numbers of bacteria (cfu) were compared in the rhizosphere and then the community profiles were obtained using bacterial ARISA.

The first experiment was designed to test the influence of methyl halides on bacterial numbers in the rhizosphere. One gram of rhizosphere soil and soil from the control treatment was suspended in water, vortexed and plated out on a rich medium in order to count the colonies. There were no statistical significant differences between cell counts from the wild type and mutant rhizosphere. It was expected that the *hol* rhizosphere would be enriched and *35S::HOL* would be depleted of bacteria compared to wild type. One of the hypotheses is that methyl halides act as an antimicrobial agent in the rhizosphere, so the difference in the amounts of this compounds present in soil would affect bacterial number there. This assumption was not confirmed in this experiment, as none of the differences in the bacterial (cfu) numbers are statistically significant in the rhizospheres of *Arabidopsis* genotypes (Figure 5.2.43).

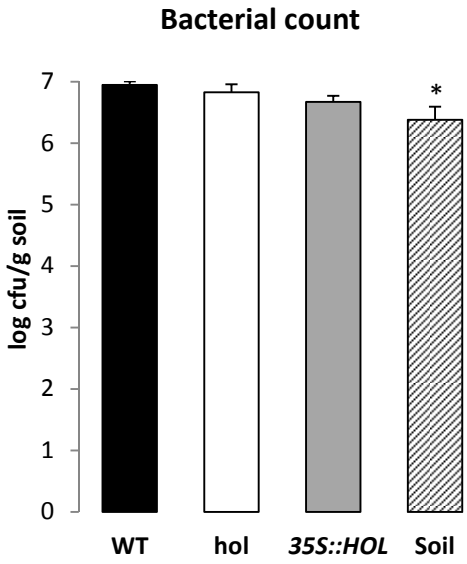


Figure 5.2.43 Bacterial population count in the rhizosphere of WT, hol and 35S::HOL (*35S::HOL*). Soil samples were collected from the rhizosphere or from the unplanted soil control 4 weeks after the start of the experiment. Soil solution was plated on TY agar plates and colony-forming units (cfu) were counted 1 day after inoculation. Asterisks represent significant differences from WT (ANOVA with Fisher's LSD post-hoc test, $p < 0.05$). (n=7 for WT, *hol* and *35S::HOL*, n=3 for soil)

The second experiment focused on comparing bacterial rhizosphere structure in the rhizosphere of *Arabidopsis* genotypes. Bacterial ARISA profiles indicate that there was not a great difference between *Arabidopsis* genotype rhizosphere samples at any time points (5.2.44 A,B,C). However, there was a clear difference in overall community over time (5.2.34 D). This is similar to the effect observed in the 3 generation experiments. The community changed over time as a result of the changing soil conditions. Microbiome was sampled from the field and was probably well adjusted to the condition over there (temperature, soil pH, vegetation cover, etc.). After mixing the soil with rich compost and keeping the soil moist under constant temperature in the growth room the community started to change. We assume that the microbiome is always changing in the real soil conditions as a result of changing of biotic and abiotic conditions. However, addition of rich nutrient source could highly select for fast growing organisms (r-strategists) (MacArthur, 2001)

MANOVA showed no significant difference in the ARISA profiles between genotypes (Table 5.2.7). In the other experiments (sand experiment – chapter 3, compost experiment -

chapter 4) 4-5 biological replicates were enough to successfully separate the microbial community using MANOVA test, so the use of only 3 replicates for the bulk soil greatly reduces the power of the test to detect differences between the rhizosphere and bulk soil. However, the differences were found in the 1st week between rhizospheres and bulk soil. MANOVA test shows that the changes in the community over time are significant for the rhizosphere samples (bulk soil was not separated due to low number of replicates).

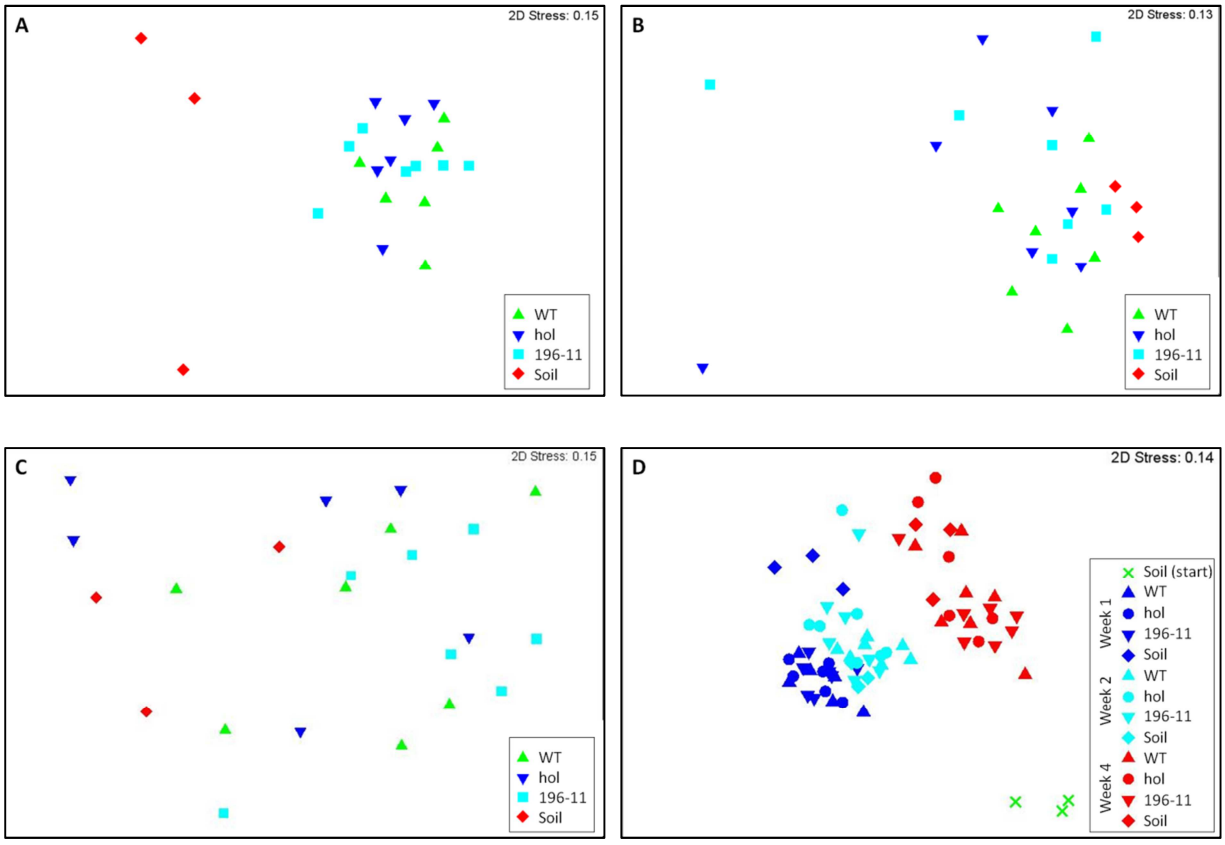


Figure 5.2.44 MDS plots showing rhizosphere community of WT, hol and 35S::HOL plants. A) week 1, B) week 2, C) week 4, D) time comparison (n=7 for WT, *hol* and 196-11, n=3 for unplanted soil). MDS plots based on bacterial ARISA data.

Pairwise comparison	Proportion of significant differences (%)	Pairwise comparison	Proportion of significant differences (%)
Genotype/treatment		**Time point**	
Week 1		**Control**	
Control vs. WT	1	Week 1 vs. Week 2	0
Control vs. *hol*	1	Week 1 vs. Week 4	0
Control vs. *35S::HOL*	1	Week 2 vs. Week 4	0
WT vs. *hol*	0	**WT**	
WT vs. *35S::HOL*	0	Week 1 vs. Week 2	1
hol vs. *35S::HOL*	0	Week 1 vs. Week 4	1
Week 2		Week 2 vs. Week 4	1
Control vs. WT	1	***hol***	
Control vs. *hol*	0	Week 1 vs. Week 2	1
Control vs. *35S::HOL*	0	Week 1 vs. Week 4	1
WT vs. *hol*	0	Week 2 vs. Week 4	1
WT vs. *35S::HOL*	0	**196-11**	
hol vs. *35S::HOL*	0	Week 1 vs. Week 2	1
Week 4		Week 1 vs. Week 4	1
Control vs. WT	0.12	Week 2 vs. Week 4	1
Control vs. *hol*	0		
Control vs. *35S::HOL*	1		
WT vs. *hol*	0		
WT vs. *35S::HOL*	0		
hol vs. *35S::HOL*	0		

Table 5.2.7 MANOVA test run on HOL experiment. Each genotype was analyzed using 7 replicates. Control was analyzed using 3 replicates only. 0 means no significant differences, 1 means all the F-test have a significance value of $p \leq 0.05$

5.2.11 Summary and discussion of the HOL experiment

The HOL experiment showed that methyl halides do not alter the bacterial community. The toxicity of methyl halides is well studied and have an application use in agriculture (soil fumigation using methyl bromide – now banned, and methyl chloride as a herbicide). It was recently showed that high methyl bromide added in high concentration to the soil (1 kg * m^{-3}) suppressed the growth of mycorrhizal fungi (Janos *et al.*, 2013). However, as *Arabidopsis* does not form any mycorrhizal association, it is unlikely that HOL experiment would show any significant differences in the bacterial community structure (manipulated by the abundance of mycorrhiza fungi). There is also some evidence that addition of methyl bromide and methyl iodine changes the bacterial community in the soil. However, normally, the soil would be fumigated with 48 kg ha^{-1} for methyl bromide and 40 kg h^{-1} for methyl iodine (Ibekwe & Ma, 2011). The amount of methyl halides compounds produced by plant is probably not sufficient to alter the bacterial community directly. Probably bacteria able to metabolize methyl halides were affected in the HOL experiment. However, studies focusing on this group of bacteria are using *cmu*A gene sequence (methyltransferase/corrinoid-binding protein CmuA, which carries out the first step in the methyl halide degradation pathway of methylotrophic bacteria) (Cox *et al.*, 2012), rather than ISR fragment for the taxa discrimination. Moreover the analyses presented in this chapter (low resolution ARISA) are not as discriminative as in the case of PAMP, MYB or RAM experiment (high replicated ARISA and 454 sequencing).

Chapter 6: Plant growth promotion properties of the isolated bacterial strains

6.1 Introduction to plant growth promotion by rhizobacteria

Plant-growth promoting rhizobacteria (PGPR) may act directly or indirectly (reviewed in (Lugtenberg & Kamilova, 2009). Direct influence can be divided into biofertilization, rhizoremediation, phytostimulation and stress-control. The most studied biofertilization model includes legume plants and *Rhizobium* and *Bradyrhizobium* species. Bacteria form nodules on plant roots, inside which they convert atmospheric N_2 into plant-available NH_3 and in return feed on carbon compounds released by a plant (Oldroyd *et al.*, 2011; Terpolilli *et al.*, 2012). Other beneficial bacterial rhizosphere processes include solubilization of organic and inorganic bound phosphate (Vassilev *et al.*, 2006), degradation of soil pollutants (Kuiper *et al.*, 2004), phytostimulation by production of auxins and other plant hormones like pyrrolquinoline quinone, gibberellins and cytokinins (Lugtenberg & Kamilova, 2009) and biocontrol. Biocontrol PGPR feed on root exudates and reduce growth of pathogenic micro-organisms by a number of mechanisms including antibiotic production, chelation of Fe^{3+} and competition for nutrients (Shippers *et al.*, 1987). Some PGPR elicit induced systemic resistance (ISR) in host plants which then gain resistance against particular pathogens by producing jasmonic acid and ethylene. Increasing beneficial interactions and suppressing deleterious micro-organisms could have substantial effects on yields. Altering nutrient cycling and uptake could substantially improve the efficiency of fertiliser use or reduce greenhouse gas emissions. A more comprehensive understanding of the nature of plant-microbe interactions is a crucial first step towards achieving this.

Apart from rhizobia, probably the best known PGPR is *Pseudomonas fluorescens*, which may potentially control outbreaks of *Gaeumannomyces graminis* var. *tritici*, a fungus that causes the take-all disease of cereals (Sanguin *et al.*, 2009). This fungus was the cause of the great losses in crop yields in North America and Australia in the early 20th century. It was observed that in cereal monoculture the yield drops dramatically in the second year but is restored

almost to the initial level after a few extra years. In that time the population of the fungus rises, especially in the second year and drops afterwards. Further research suggested that the fungus is being suppressed by 2,4 diacetylphloroglucinol produced by *Pseudomonas fluorescencens* (Keel, 1992; Weller *et al.*, 2007).

6.1.1 Main goals of the PGPR project

The artificial selection experiment conducted using three model plants grown for three generations in sand allowed for strong enrichment of plant dependent bacteria in the rhizosphere. Enrichment was confirmed using 454 sequencing, where three different OTUs (*Massilia, Achromobacter* and *Arthrobacter*) were strongly selected in the rhizosphere of all plants throughout the experiment, but especially in *Brachypodium* and *Medicago* (see chapter 3.2.1). Soil isolation experiments were conducted in order to grow representative strains from the mentioned OTUs of interest. These bacteria managed to colonize the rhizosphere and are actively selected by plants. Details of this relation are of a great interest as PGPR bacteria may interact with the plant using different mechanisms. Phylogenetic relationships were established for the selected isolated strains.

In the later stage of this project it was established that two of the isolated strains are indeed PGPR as they promote growth of plants. However, in order to study this relationship in more details it was necessary to gain insight into their growth requirements and antibiotic resistance. The ultimate test of their PGPR properties is to compare transcriptome difference between rhizosphere and free living (laboratory culture) condition. This approach involves DNA and RNA isolation and mRNA enrichment. Due to the time constraints, the transcriptomic part of this project is not included in this PhD thesis.

6.2 Discussion of the results

6.2.1 Isolation of bacterial strains from the rhizosphere

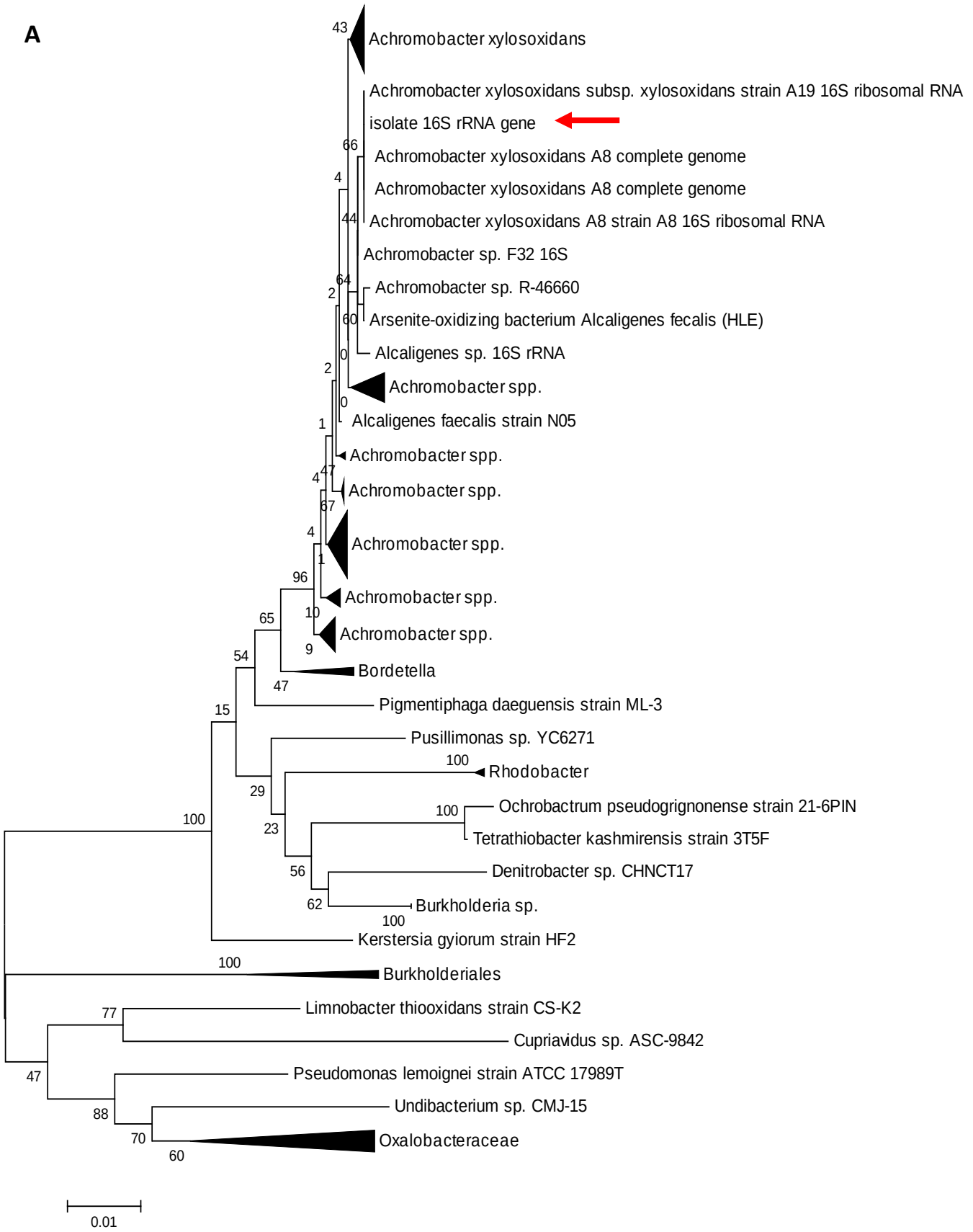
About 50 bacterial colonies were isolated from the rhizosphere of each plant species (*A.thaliana*, *M.truncatula*, *B.distachyon*) after the 3rd generation of growth in sand (Chapter 3.2.1). Colonies were subcultured and ARISA performed on DNA isolated from each. Bacteria with ISR sizes that matched the dominant fragments in the ARISA data obtained from the rhizosphere soil samples were identified (see Chapter 3.1.1) and only one representative of isolates with the same fragment size was identified using 16S rRNA gene sequencing. Amplified 16S rRNA gene was sent for sequencing to the Genome Analysis Centre, Norwich. Primer pair 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT -3') was used. The sequence of the forward primer 27F is the same as used for 454 sequencing (apart from the fact that 454 primer also has a non-target sequence). The reverse primer binds to the end of the 16S rRNA gene (Turner *et al.*, 1999). Amplification of the whole 16S rRNA gene (and sequencing of around 700 – 800 bp using Sanger's method) allowed for a detailed comparison of the obtained sequences against the NCBI GenBank database (Table 6.2.1). Many isolates were identified as *Arthrobacter spp.*, some as *Pseudomonas, Serratia* and others. None of the opportunists from the 3rd generation was isolated. We were especially interested in obtaining bacterial colonies on plates that are also abundant in the rhizosphere (basing on the 454 sequencing data). In order to compare the isolated bacteria to the 454 sequencing data, a representative sequence from each OTU of interest was compared against fragment of 16S rRNA gene sequence of the isolated strains. OTU representative sequence is the first sequence (seed) that was used in the OTU construction. All the remaining sequences in the same OTU are similar to the first one in at least 95 %.

Brachypodium **rhizosphere of 3rd generation**	***Arabidopsis*** **rhizosphere of 3rd generation**	***Medicago*** **rhizosphere of 3rd generation**
Arthrobacter sp. IMMIB L-1606 (2)	*Rhizobium sp*. PSB12 (1)	*Arthrobacter sp*. d9 (1)
Arthrobacter sp. defluvi (1)	*Nocadioides sp*. SCO-A08 (6)	*Arthrobacter sp*. IMMIB L-1606 (4)
Arthrobacter sp. d9 (2)	*Nostoc punctiforme* (2)	*Arthrobacter sp*. WS08(3)
Arthrobacter sp. WPCB190		*Arthrobacter sp*. KA4-2 (2)
Pseudomonas sp. IK-S1 (1)		*Achromobacter xylosoxidans* (1)
Arthrobacter sp. ADG-1 (1)		*Arthrobacter sp.* OTS2-M-217 (2)
Arthrobacter sp. WS08 (1)		*Microbacterium esteromaticum* (2)
Arthrobacter sp. HR110 (1)		*Nocadioides sp*. SCO-A08 (2)
Pseudomonas fluorescence HDY-9 (1)		*Nostoc punctiforme*
Serratia sp. AS12 (2)		
Pseudomonas sp. AV2A (1)		
Pseudomonas fluorescence HDY-8 (1)		
Arthrobacter sp. HZ3 (1)		

Table 6.2.1 Identification of the bacterial colonies isolated from model plant species rhizospheres. Only top hit from the GenBank NCBI database is presented in the table. Number in brackets represents how many strains of the same ARISA profile/16S rRNA sequence were found.

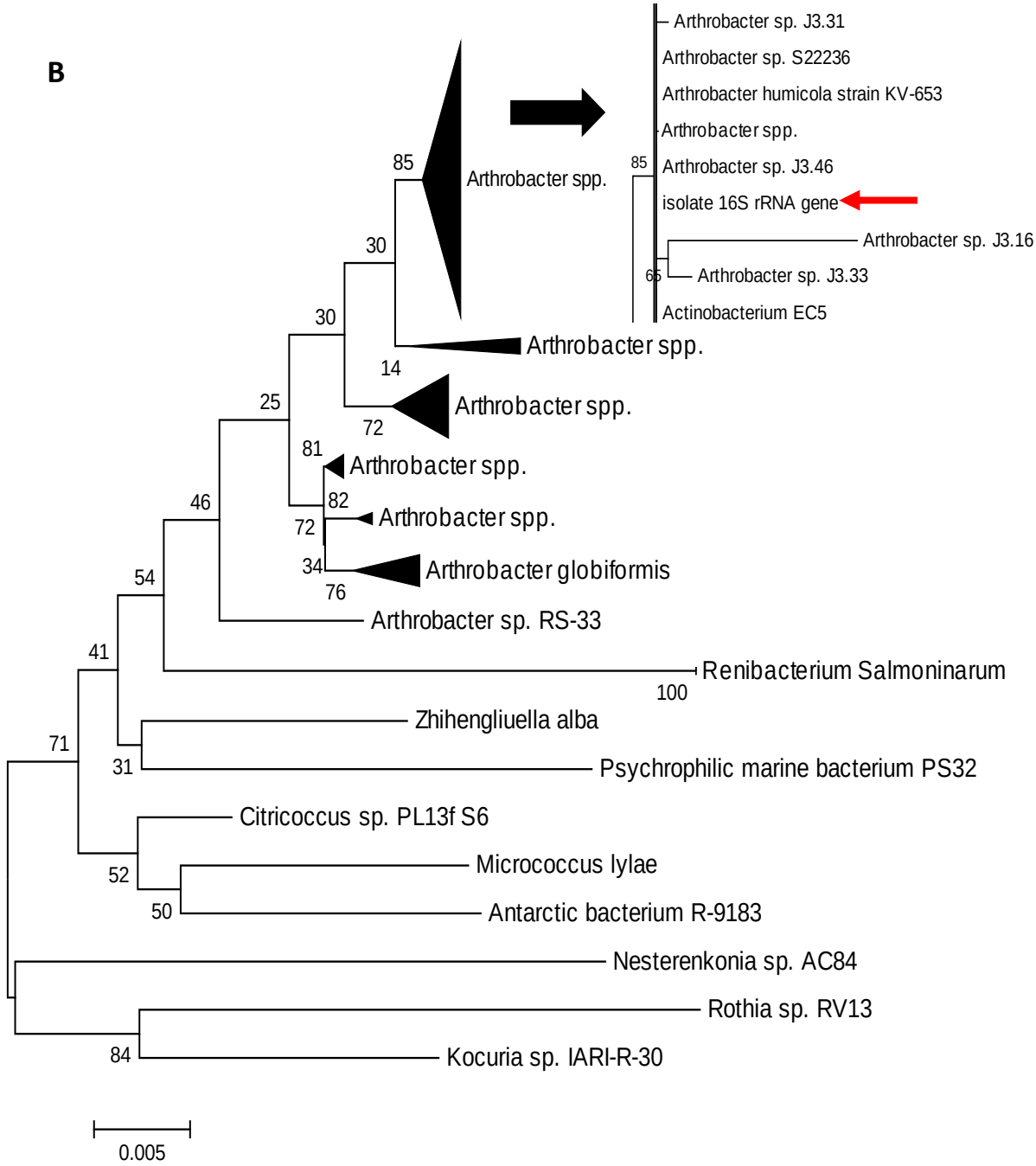
Two of the ribosomal sequence from the bacterial isolates matched to representative sequence of 2 of 3 dominant OTUs assembled from the 454 pyrosequencing data. *Achromobacter xylosoxidans* isolate, when compared using BLAST tool, showed 99 % similarity against a representative sequence from the OTU 19 (score of 544, e value = 1e-159). *Arthrobacter sp.* d9 isolate, when compared using BLAST tool, showed 100 % similarity against a representative sequence from the OTU 39 (score of 556, e value = 7e-163) (Table 6.2.1). Genomic DNA was then isolated from both strains and they were sequenced by TGAC using half a plate of 454Flx each. *Achromobacter* gDNA sequencing resulted with 50 contigs (of which 14 were smaller than 100 bp in length). *Arthrobacter* gDNA sequencing resulted with 48 contigs (of which 17 were smaller than 100 bp in length).

The bacterium isolated from the rhizosphere of *Medicago* was identified as *Achromobacter xylosoxidans* (top hit in the NCBI database – accession number: NR_074754.1, based on the full 16S rRNA sequence – gDNA sequencing – chapter 6.2.6) and the colony isolated from the *Brachypodium* rhizosphere was identified as *Arthrobacter sp.* (top hit in the NCBI database – accession number EF110914.1, based on the full 16S rRNA sequence – gDNA sequencing – chapter 6.2.6) (Figure 6.2.1). Full 16S rRNA gene sequences were obtained from the genomic DNA FASTA file using online RNAmmer 1.2 server (a part of CBS prediction server) (Lagesen *et al.*, 2007). However, using full 16S rRNA gene sequences would skew the results towards fully sequenced strains of these species (see chapter 6.2.6). In order to fully understand phylogenetic place of these strains a partial 16S rRNA gene (1460 and 1486 bp of sequence starting at 27 bp from the start of the gene for *Achromobacter* and *Arthrobacter*, respectively) was compared (BLAST) against GenBank database.


A
Achromobacter xylosoxidans
Achromobacter xylosoxidans subsp. xylosoxidans strain A19 16S ribosomal RNA
isolate 16S rRNA gene
Achromobacter xylosoxidans A8 complete genome
Achromobacter xylosoxidans A8 complete genome
Achromobacter xylosoxidans A8 strain A8 16S ribosomal RNA
Achromobacter sp. F32 16S
Achromobacter sp. R-46660
Arsenite-oxidizing bacterium Alcaligenes fecalis (HLE)
Alcaligenes sp. 16S rRNA
Achromobacter spp.
Alcaligenes faecalis strain N05
Achromobacter spp.
Achromobacter spp.
Achromobacter spp.
Achromobacter spp.
Achromobacter spp.
Bordetella
Pigmentiphaga daeguensis strain ML-3
Pusillimonas sp. YC6271
Rhodobacter
Ochrobactrum pseudogrignonense strain 21-6PIN
Tetrathiobacter kashmirensis strain 3T5F
Denitrobacter sp. CHNCT17
Burkholderia sp.
Kerstersia gyiorum strain HF2
Burkholderiales
Limnobacter thiooxidans strain CS-K2
Cupriavidus sp. ASC-9842
Pseudomonas lemoignei strain ATCC 17989T
Undibacterium sp. CMJ-15
Oxalobacteraceae
0.01

Achromobacter isolate 16S rRNA sequence used for comparison:

ACGCTAGCGGGATGCCTTACACATGCAAGTCGAACGGCAGCACGGACTTCGGTCTGGTGGCGAGTGGCGAACGGGTGAGTAATGTATC
GGAACGTGCCTAGTAGCGGGGGATAACTACGCGAAAGCGTAGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCGCAAGA
CCTTGCACTATTAGAGCGGCCGATATCGGATTAGCTAGTTGGTGGGGTAACGGCTCACCAAGGCGACGATCCGTAGCTGGTTTGAGAG
GACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTG
ATCCAGCCATCCCGCGTGTGCGATGAAGGCCTTCGGGTTGTAAAGCACTTTTGGCAGGAAAGAAACGTCATGGGCTAATACCCCGTGAA
ACTGACGGTACCTGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACT
GGGCGTAAAGCGTGCGCAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGAGCTCAACTTTGGAACTGCATTTTTAACTACCGGGCTA
GAGTGTGTCAGAGGGAGGTGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAAGGCAGCCTCCT
GGGATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTA
GCTGTTGGGGCCTTCGGGCCTTAGTAGCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGG
AATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGTCTGGAAT
TCCGAAGAGATTTGGAAGTGCTCGCAAGAGAACCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCTACGAAAGGGCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGG
GATGACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTCGGGACAGAGGGTCGCCAACCCGCGAGGGGG
AGCCAATCCCAGAAACCCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGCA
TGTCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTTTACCAGAAGTAGTTAGCCTAACCGTA
AGGGGGGCGATTACCACGGTAGGATTCATGACTGGGGTGAAGTCGTAACAA


B
Arthrobacter sp. J3.31
Arthrobacter sp. S22236
Arthrobacter humicola strain KV-653
Arthrobacter spp.
Arthrobacter sp. J3.46
isolate 16S rRNA gene
Arthrobacter sp. J3.16
Arthrobacter sp. J3.33
Actinobacterium EC5
85
85
30
65
30
Arthrobacter spp.
14
Arthrobacter spp.
25
72
81
Arthrobacter spp.
46
82
Arthrobacter spp.
72
34
Arthrobacter globiformis
54
76
Arthrobacter sp. RS-33
41
Renibacterium Salmoninarum
100
Zhihengliuella alba
71
31
Psychrophilic marine bacterium PS32
Citricoccus sp. PL13f S6
52
Micrococcus lylae
50
Antarctic bacterium R-9183
Nesterenkonia sp. AC84
Rothia sp. RV13
84
Kocuria sp. IARI-R-30
0.005

Arthrobacter isolate 16S rRNA sequence used for comparison:

AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGATCCGGTGCTTGCACCGGGGATTA
GTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATATGACTCCT
CATCGCATGGTGGGGGGTGGAAAGCTTTATTGTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAG
GCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAA
GAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCC
GGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACTCCGGATCTGCGGTGGGTACG
GGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAG
GCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACG
TTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGC
TAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTT
GACATGGACCGGACCGGGCTGGAAACAGTCCTTCCCCTTTGGGGCCGGTTCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGA
GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCGCGTAATGGCGGGGACTCATGGGAGACTGCCGGGGT
CAACTCGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGT
TGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCT
AGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTTGGTAACACCCGA
AGCCGGTGGCCTAACCCCTTGTGGGAGGGAGCTGTCGAAGGTGGGACTGGCGATTGGG-ACTAAGTCGTAACAAGGTA

Figure 6.2.1 Tree alignment of fragments of 16S rRNA genes A) *Achromobacter* isolate 16S rRNA gene trimmed to 1460 bp (16S rRNA gene sequence based on gDNA sequencing) alignment against top 100 hits in the GenBank NCBI database and other Burkholderiales species (in order to root the tree). B) *Arthrobacter* isolate 16S rRNA gene trimmed to 1486 bp (16S rRNA gene sequence based on gDNA sequencing) alignment against top 100 hits in the GenBank NCBI database and other Actinomycetales species (in order to root the tree). Sequences were aligned using ClustalW and trees were constructed using MEGA5 software using Neighbor-Joining with Bootstrap of 1000 replicates (bootstrap values are shown on next to the tree nodes) (Felsenstein, 1985; Saitou & Nei, 1987; Tamura *et al.*, 2004; Tamura *et al.*, 2011). Neighbor-Joining tree is commonly used for this kind of analysis (Micallef *et al.*, 2009). For the *Arthrobacter* alignment the node containing isolate 16S rRNA gene sequence is resolved next to the main tree (black arrow).

The genome of *Achromobacter xylosoxidans* is 7.01 Mbp and the one of *Arthrobacter sp.* is 3.815 Mbp. These are minimum sizes because all the contigs have not been closed and there may be some missing DNA.

The genome size for *Achromobacter* is similar to the one obtained for a different *Achromobacter* strain - *A. xylosoxidans* A8, which has a chromosome of 7.36 Mbp and two circular plasmids 98 kbp and 248 kbp (Strnad *et al.*, 2011). However, the *Arthrobacter* genome is around 1 Mbp smaller than most of the others *Arthrobacter* strains – *Arthrobacter sp.* FB24 is 5.07 Mbp (including three plasmids) (Copeland, A., et.al., upublished, NC_008541), *Arthrobacter sp.* Reu61a is 5.08 Mbp (including two plasmids) (Schuldes, J., et.al., unpublished, NC_018531.1), *Arthrobacter phenanthrenivorans* Sphe3 is 4.54 Mbp (including two plasmids ((Kallimanis *et al.*, 2011) NC_015145.1), *Arthrobacter chlorophenolicus* A6 is 4.98 Mbp (including two plasmids (Lucas, S., et.al., unpublished, NC_011886.1), *Arthrobacter aurescens* TC1 5.23 Mbp (including two plasmids) ((Mongodin *et al.*, 2006), NC_008711.1). Only *Arthrobacter arilaitensis* Re117 has a genome size (3.92 Mbp, including two plasmids) comparable to the our strain ((Monnet *et al.*, 2010) NC_014550.1). *A. arilaitensis* has been found to inhabit surface of a cheese, so as it is a specialized bacteria (with a specialized metabolism) living in the relatively simple environment (at least comparing to the soil) it is likely to loose genes that are essential to survive in more harsh environments (e.g. soil). That is why I think that my *Arthrobacter* strain may not be fully sequenced and approximately 1 Mb is missing.

The 16S rRNA gene of the *Achromobacter* isolate was aligned against the genome of *Achromobacter xylosoxidans* A8 (Figure 6.2.1 A). This strain was isolated from soil contaminated with polychlorinated biphenyls (Strnad *et al.*, 2011). 16S rRNA gene sequence is identical between our isolate and A8 strain (100 % coverage and 100 % identity using BLAST).

The influence of the *Achromobacter xylosoxidans* A8 strains on the plants in unknown, however the genus *Achromobacter* is considered to be PGPR, found in the rhizosphere and root endosphere of many plants (Forchetti *et al.*, 2010; Jha & Kumar, 2009). *Achromobacter xylosoxidans* belongs to the family Alcaligenaceae in the order Burkholderiales. All Proteobacteria are Gram-negative, and the *Achromobacter* isolate was confirmed to be Gram-negative (Figure 6.2.3 A).

Phylogeny of the *Arthrobacter* species is not well characterized, as most of the GenBank uploaded sequences are annotated only as *Arthrobacter sp.* without a further assignment into known species level (Figure 6.2.1 B). The species that is most similar to the isolated strain is *Arthrobacter humicola* (Kageyama *et al.*, 2008). This strain was isolated from a paddy soil in Japan.

Some *Arthrobacter* species are known PGPR species (Barriuso *et al.*, 2008). However, other species are soil opportunists that have been shown to survive long periods in cold and nutrient poor conditions (Mongodin *et al.*, 2006). *Arthrobacter* belongs to the family Micrococcaceae in the order Actinomycetales and as like all Actinobacteria, it is Gram-positive (Figure 6.2.3 B).

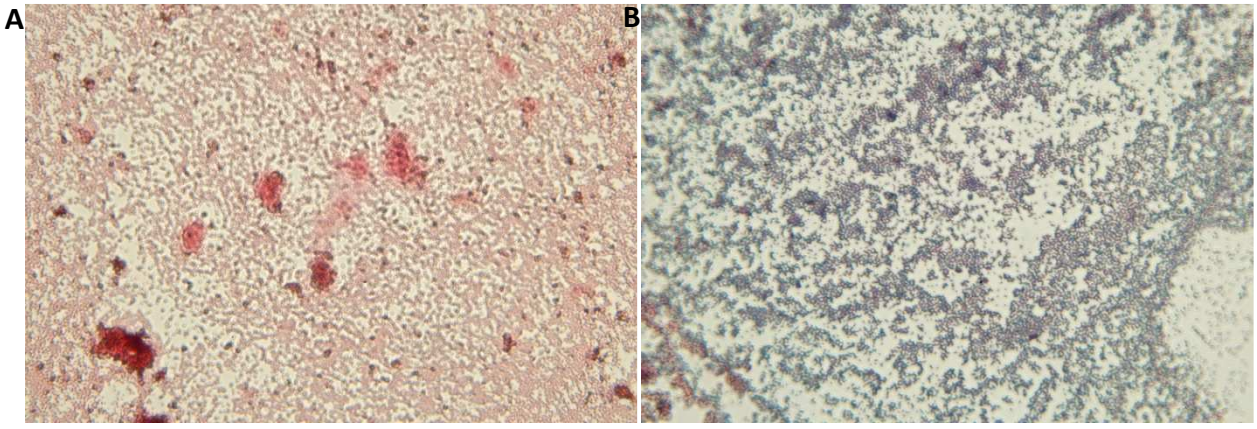


Figure 6.2.3 Gram staining pictures of A) *Achromobacter* isolate and B) *Arthrobacter* isolate. Pictures were taken using bright field microscope – Meiji Techno MT4310H at 100X magnification.

6.2.2 Abundance of OTU 19 *Achromobacter* and OTU 39 *Arthrobacter* in the rhizosphere soil of model plants

Achromobacter and *Arthrobacter* are two (out of three) of the most abundant rhizosphere bacteria found in the sand experiment based on the 454 sequencing data (Figure 6.2.4). Their abundance decreased in the unplanted control over generations, however it stayed very high for all the rhizosphere samples (apart from generation 3 of *Arabidopsis*). This indicates that these bacteria are recruited by the plant from the soil inoculum and their abundance is regulated by the plant.

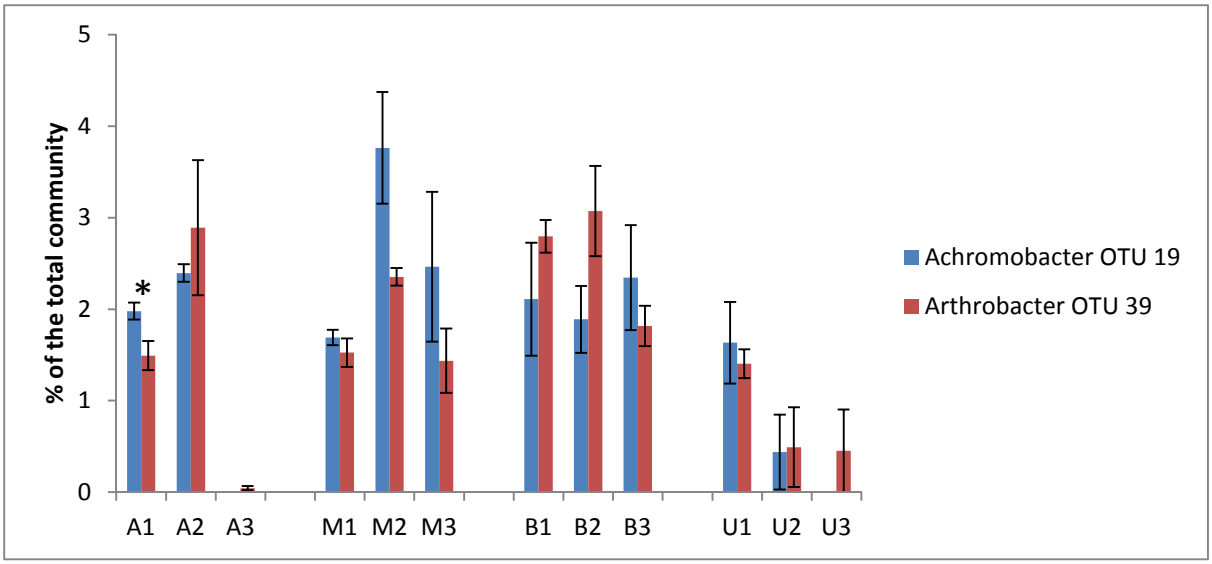


Figure 6.2.4 Abundance of the OTUs in the sand experiment with successfully isolated representative. A – rhizosphere of *Arabidopsis*, M – rhizosphere of *Medicago*, B – rhizosphere of *Brachypodium*, U – unplanted control, 1, 2, 3 – successive generations. Statistically significant differences in abundance between *Achromobacter* and *Arthrobacter* are marked with an asterisk (t-test, $p<0.05$).Analysis based on the 454 sequencing data.

Interestingly, the *Medicago* rhizosphere was enriched with *Achromobacter* (not statistically) and this strain was isolated from the soil of the 3rd generation of *Medicago*. While the *Brachypodium* rhizosphere was enriched with *Arthrobacter* and this strains was isolated from the soil of the 3rd generation of *Brachypodium.*

6.2.3 Effect of isolates on growth of *Arabidopsis*, *Medicago*, *Brachypodium* and *Triticum*

Four different plant species were grown in sterilized Silver sand for 4 weeks. Seeds were surface sterilized at the beginning of the experiment. Plants were watered using sterile, deionised water and once a week 5 ml of standard rooting solution was added. After the first week 10 mg of KNO_3 was added as well (Table 2.4.1). After the first week seedlings were inoculated either with 1.9 x 10^8 cells of *Achromobacter* or 5.3 x 10^7 cells of *Arthrobacter* (numbers based on Miles and Misra plating) or left uninoculated. After three weeks of plant growth, the shoot was cut off, dried and weighed.

Achromobacter increased yield of all plants although this was not statistically significant in the case of *Medicago* and wheat) (Figure 6.2.5). *Arthrobacter* is more plant species specific as it had a positive effect on both grass species (again, not statistically significant in the case of wheat), but did not change the yield of the other plants.

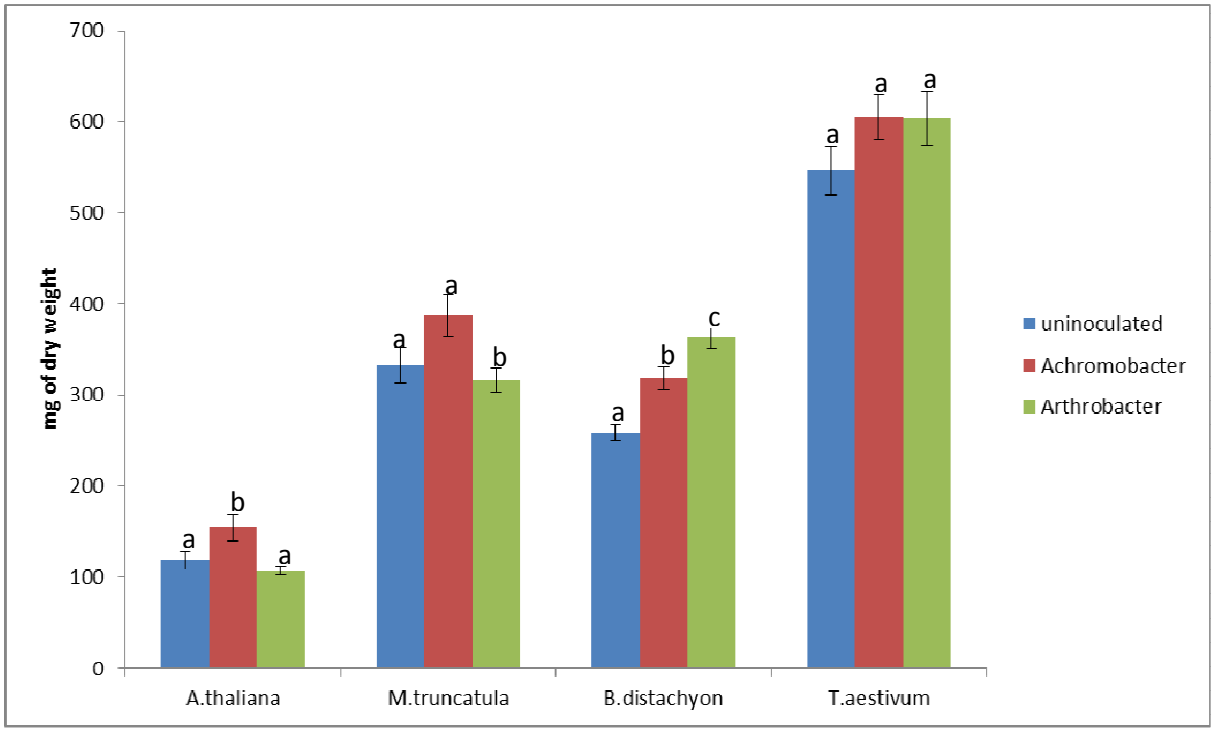


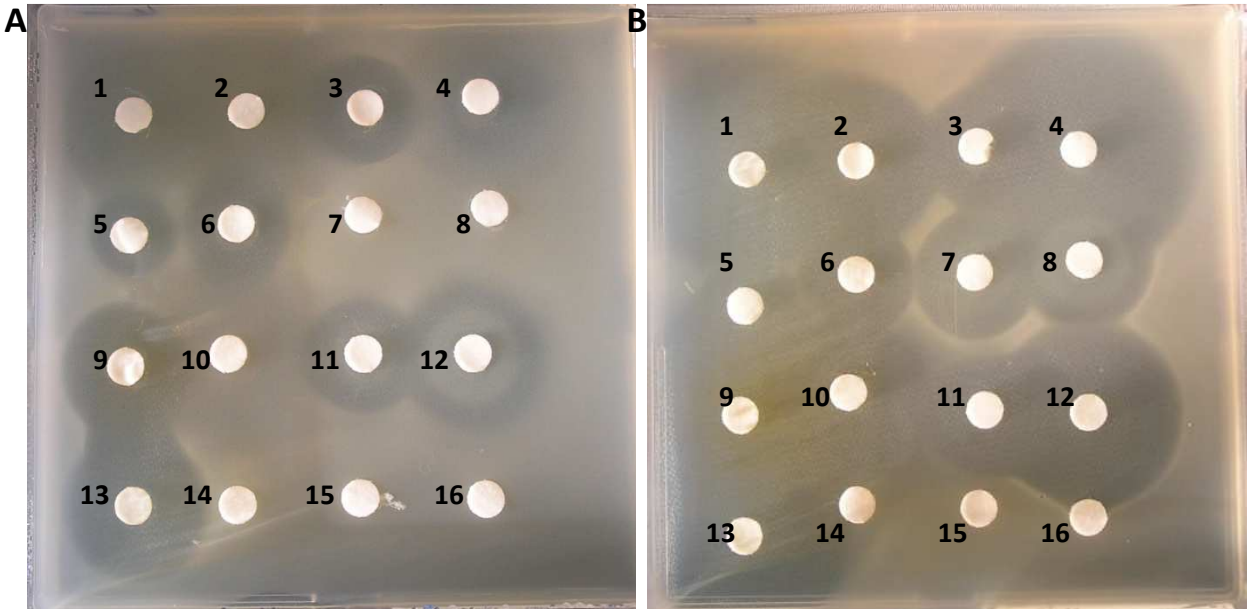
Figure 6.2.5 Plant growth assay using *Achromobacter* and *Arthrobacter* as bacterial inoculum. Bars represent standard error and letters represent the statistical significance using t-test ($p<0.05$). In total 288 plants were harvested (24 plants for each condition).

6.2.4 Growth requirements of *Achromobacter* and *Arthrobacter* isolates

In order to study the bacteria isolated from the rhizosphere soil it was necessary to understand their growth requirements. Both *Achromobacter* and *Arthrobacter* were isolated on TY medium (chapter 2.12). For the CCC project (gDNA sequencing and subsequent mRNA sequencing) it was crucial to grow bacteria on minimal media (in order to correctly interpret transcriptomics data). *Arthrobacter* is able to grow on any minimal media (M9, AMA) supplemented with sugar or organic acid (glucose – 10 mM, glycerol – 30 mM or succinate – 20 mM) and nitrogen source – ammonia 10 mM. *Achromobacter* requires minimal media supplemented with organic acid rather than sugar (here – succinate 20 mM), nitrogen source – ammonia 10 mM and BME (Basal Medium Eagle) vitamin solution. All the bacterial growth tests are preliminary results only and will not be presented in this PhD thesis.

6.2.5 Antibiotic resistance of *Achromobacter* and *Arthrobacter* isolates

For the future experiments using isolated *Achromobacter* and *Arthrobacter* (e.g. mutations) it is essential to know the natural antibiotic resistance of these strains. A. *xylosoxidans* is resistant to neomycin in 80 µg/ml and 200 µg/ml concentrations. This strain is also resistant, but with much slower growth rate to ampicillin, carbenicillin, chloramphenicol and pipercillin. Growth of *Arthrobacter* was inhibited by all tested antibiotics (Figure 6.2.13).



1. amplicillin 2. carbenicillin 3. apramycin 4. chloramphenicol (100, 50, 50, 20 µg/ml)

5. gentamycin 6. kanamycin 7. neomycin 8. neomycin (10, 40, 80, 250 µg/ml)

9. rifamplicilin 10. pipercillin 11. spectomycin 12. streptomycin (10, 20, 50, 250 µg/ml)

13. tetracyclin 14. water 15. water 16. water (5, N/A, N/A, N/A µg/ml)

Figure 6.2.13 Antibiotic resistance assay for the two isolated strains: *Achromobacter* (A) and *Arthrobacter* (B). Bacteria were grown in 0.7 % TY agar poured over 1.5 % TY agar in a square Petri dish. On the top of soft agar autoclaved filter papers were placed and 5 µl of selected antibiotic was added. Plates were incubated for 2 days at 27 °C.

6.2.6 Ongoing and future research involving isolated bacteria

Genomic DNA obtained from the bacteria was submitted for 454 sequencing. Ilumina (HiSeq 2000) sequencing of the cDNA (converted from mRNA) of these two isolates will be performed. Project will focus on comparison of bacterial gene expression in the rhizosphere of *Brachypodium* and wheat relatively to the gene expression observed in the laboratory cultures. The genome sequence will be used as a scaffold for the mRNA reads. Up to the end of this PhD project the genomic DNA data have been retrieved and only partially analysed. Only 16S rRNA gene sequence and the total genome size are taken into consideration in this PhD thesis.

Chapter 7: General discussion

7.1 General discussion of methodology

During this PhD studies many methods have been adopted in dissecting plant microbe interactions in the rhizosphere. At the beginning ARISA was chosen as it is relatively easy, not time-consuming and first of all – cheap. ARISA proved to be a great choice. I have managed to optimize the method and make it a reliable and repeatable method in fingerprinting the microbial community. Thousands of samples were analysed using this method and most of the results were processed and analysed in the later stages. DNA isolation method adapter from (Griffiths *et al.*, 2000) proved to be easy, repeatable and time-efficient. A choice of using 1 gram of soil for DNA sampling also proved to be right (Ranjard *et al.*, 2003) as the results were reproducible. Ranjard showed that using smaller quantities of soil for DNA isolation purposes (and subsequent microbial fingerprinting) leads to much greater variability between samples and non-reproducible results. Soil as an extremely rich and diverse environment needs to be sampled in higher quantity in order to overcome the very local differences in microbial community structure (especially as community structure is also highly soil mineral dependent (Carson *et al.*, 2009)).

During optimization of ARISA (and pre-sequent molecular steps – DNA isolation, PCR) I have compared bacterial fingerprints coming from the same soil sample and only when I was absolutely confident in the method I have started to analyse the samples coming from the rhizosphere experiments (sand and compost experiment and later plant mutant experiments). Moreover, during every experiment I was running the same sample multiple times (for example if I had sampled 5 grams of soil from one plant rhizosphere) and results were compared.

The major problem with ARISA (at least at the beginning) was the amount of data it produces. In order to present the relations between different samples MDS plot were constructed. However, they do not present any statistical value. That is why MANOVA method was adopted. MANOVA was run using R, which was a novel and exciting new language for me to adopt. Luckily, in a short time I was able to generate MANOVA tables

with p values. In order to track the most abundant ITRs in the rhizosphere bar graphs were produced. However, even when using t-test in order to compare their abundance in rhizosphere against unplanted control the results are hard to understand and any significant conclusions are impossible. Ternary plots were a breakthrough in analysing the overall community, still being able to track the abundant ITRs. Ternary plots were a state of art design using Python, Veusz, Jmol and Java. Nowadays, I have developed a pipeline, which is able to update the raw fluorescence data from ARISA (or OTU abundance from 454 sequencing) and produce ternary plots (2D as well as 3D) using a simple command in Python, and the whole process takes only around 1 minute.

Statistical methods implemented during this PhD are standard methods used in science and especially in ecology: t-test, ANOVA, richness, Shannon diversity index. It was possible to use either Microsoft Excel or Genstat 16th edition to calculate all these indices and tests.

The novelty of this PhD project was using high throughout-put sequencing. Our choice was 454Flx sequencing as it was accessible locally with a great help from TGAC. It was essential (due to budgetary reasons) to barcode the samples and pool PCR products in equal-molar quantities. Initially, each PCR product was cleaned and quantified on Nanodrop. Later, I started to use high throughput methods as Qiaexcel and Qubit (data from these two is not presented in this thesis). However, without experience with Nanodrop and the other older methods I would not be able to implement these more advanced methodologies in my research.

454 data was analysed using MEGAN, which reads Blast report files and produce phylogenetic data. Even though this is not perfect software, but for my research was adequate. It was relatively fast (454 data sets may be large) and gives user control of the analysis. Moreover it allows investigating each read and easily spotting any problems.

I have spend much more time in analysing the data than isolating DNA, growing plants, running PCRs and gels. Nowadays, with advanced pipelines in analysing the ARISA and 454 data it would be possible to focus on bigger and more time and effort consuming experiments. The data analysis would take only a part of the project and would focus mostly on troubleshooting rather than on developing new methods of presenting data.

7.2 General discussion of plant – microbe interaction in the rhizosphere

Experiments conducted during this PhD project showed that bacterial and fungal communities of plant rhizospheres are different from the ones found in bulk soil. There are also differences in the microbial community between different plant species. Six different plant species belonging to three different families: Brassicaceae (*Arabidopsis thaliana* and *Brassica rapa*), Fabaceae (*Medicago truncatula* and *Pisum sativum*) and Poaceae (*Brachypodium distachyon* and *Triticum aestivum*) were tested for their influence on the rhizosphere microbiota. Each of these plant species had an influence on bacteria and fungi community, however no plants in the same family were no more similar to each other than other pairs of species.

The main focus of this PhD project was on plant multigeneration experiments. It has been shown that growing the same plant species in the same soil for multiple generation alters the microbial community and leads to changes in plant growth (Swenson *et al.*, 2000). Indeed in the results presented in the previous chapters there are major differences in the rhizosphere microbial community between plant generations. Although there is a drift in community composition over generation, the differences between different plant species remain clear. This indicates that plants are able to modify the microbial community despite processes that underlie the changes over time. In future work, prolonging the experiment for a few additional generations, until the communities stop changing would be of great benefit. This drift was

A more detailed focus on the consequences of changes in the microbial community for plant physiological traits such as biomass, chlorophyll content, leaf area, etc. would also be beneficial. Such an approach combining plant physiological features with the rhizosphere microbiome structure was already presented by Zancarini, where *Medicago* plants were examined for their role in structuring rhizobiome under different soil nitrogen levels (Zancarini *et al.*, 2012). Combining plant physiology (at least plant dry weight) with the knowledge about the rhizosphere (and possibly endosphere) microbiome structure would lead to even better understanding of microbial influence on the plant growth. An emerging

new trend in soil ecology is the analysis of the root endophytic community by culture-independent methods (Bulgarelli *et al.*, 2012; Gottel *et al.*, 2011; Lundberg *et al.*, 2012). Combining endophytic community research with the rhizosphere community would explain the recruitment of the root community from the surrounding soil and indicate possible mechanisms of this process. The plant immunity system response may play a crucial role here, especially since it is already shown in other higher organisms: animals are using PAMP (or rather MAMP) signals to enhance immunity system and recognize beneficial bacterial colonisations (as nicely shown in case of the squid-*Vibrio* symbiosis (Koropatnick *et al.*, 2004)). Other research showed that overexpression of the periculin- 1a (antimicrobial peptide, which production is triggered by the MAMP) in case of the *Hydra spp.* dramatically changed the bacterial community inhabiting polyps, so the immune system is indeed responsible for the selection of the microbiota (Chu & Mazmanian, 2013).

Plants modify the microbial community by selecting and suppressing specific bacteria and fungi via composition of their root exudates (Badri *et al.*, 2009; Dennis *et al.*, 2010). In the sand experiments Proteobacteria became more common in the rhizosphere and bulk soil, probably reflecting their opportunistic life history, plants were able to select a whole array of different species. Two out of three most abundant bacteria selected in the rhizosphere were isolated. These isolates proved to possess plant growth promotion properties. This experiment indicated that plants are able to “farm” the soil in order to enrich it with PGPR species. Berg et.al. has already shown that *Verticillium* (fungal pathogen) can be suppressed by rhizosphere bacteria, where *Pseudomonas* and *Serratia* were the dominant species based on a culture-dependent study (Berg *et al.*, 2006) These isolates are a great example of bacteria that are PGPR and are also very plant species dependent and are abundant in the rhizosphere. More research is needed in order to understand the interactions of these bacteria with the host plants. It is important to understand how their gene expression changes in the rhizosphere compared to the laboratory cultures. Of course different plant species rhizospheres need to be compared in order to fully understand the molecular genetic relationship between these strains and host plants.

Experiments conducted in sand showed that the more nutrient starved the soil becomes the more likely it is that the soil community becomes dominated by opportunists. The third generation rhizosphere community of model plants in the sand experiment was dominated by two groups of soil opportunists: *Stenotrophomonas*, *Variovorax* and *Pseudomonas* (*Medicago*, *Brachypodium*) and *Rhodopseudomonas* (*Arabidopsis*). All these bacterial species were present in the unplanted control, however they were not found together in plant rhizospheres. It indicates that *Arabidopsis* suppresses *Stenotrophomonas*, *Variovorax* and *Pseudomonas* species, while *Medicago* and *Brachypodium* do not allow *Rhodopseudomonas* to become abundant. However, it can not be completely excluded that *Arabidopsis* exudates promote *Rhodopseudomonas* growth (which is thriving in the unplanted control as well) allowing it to outcompete the other bacteria in the soil, and the same is true for *Stenotrophomonas, Variovorax* and *Pseudomonas* in the case of *Medicago* and *Brachypodium*. This observation indicates that even with invasions there is a huge plant influence. Interestingly, in the nutrient rich conditions (compost experiment), the invasions were not observed. Reasons for microbial invasions and their influence on higher organisms and the environment has became a considerable scientific focus in recent years (Badri & Vivanco, 2009; van der Putten *et al.*, 2007).

However, in the compost experiment a fungal pathogen invasion did occur in the rhizosphere of *Brassica rapa* (Hartwright et al., 2010). The pathogen was present in the soil used to start the experiment as it was also found in other rhizospheres and bulk soil in relatively low abundance. However, it was only the *Brassica* rhizosphere that was overtaken by *Olpidium brassicae* and the relative abundance of the pathogen increased over plant generations, reaching more than 80 % of the fungal community. Discovery of the pathogen invasion was only possible thanks to the multigeneration system approach implemented during this PhD project. Increasing abundance of pathogens were observed before in the real soil conditions (Moritz & Odion, 2005), however the farm soil being a very complicated environment plus the influence of the climate, soil animals, pesticides etc. normally found on the farm makes it difficult to elucidate the plant – pathogen interactions. On the other hand inoculation of pathogen spores into the rhizosphere of gnobiotic plants makes the study system very artificial and does not represent the real plant – soil microbiome – plant pathogens interactions (Jupe *et al.*, 2013). The multigeneration approach using controlled

and simplified growth conditions (10 % farm soil mixed with compost) combined with pyrosequencing of the rhizosphere is the best approach in order to understand the complicated plant – soil microorganisms interactions.

We have also found an increase in the abundance of *Massilia* species in the *Brassica* rhizosphere, which was overtaken by *Olpidium* pathogen. At present we cannot definitely say that *Massilia* is in any way suppressive of the pathogen or if it is in any other way helping the plant as the necessary experiments have not been conducted yet. What is already known though, is that *Massilia* is able to grow using chitin (polymer – compound of fungal cell wall) as a carbon and energy source (Adrangi *et al.*, 2010; Faramarzi M.A., 2009). Moreover *Massilia* was found recently as being ubiquitous coloniser of the *Arabidopsis* rhizosphere, root endosphere and phyllosphere (both endo- and epiphytic) (Bodenhausen *et al.*, 2013). *Massilia* was the only bacterial genus found to be very significantly upregulated in its abundance in the *Brassica* rhizosphere over generations. There was a clear correlation between *Olpidium* and *Massilia* abundance in the rhizosphere (*Massilia* was found in the rhizosphere of other plants, but was less common than in the *Brassica*). That is why we believe that *Massilia* increase in the abundance is the first step in transition towards suppressive soil as already seen in case of the pathogen causing "damping off" desease - *Rhizoctonia solani* resistant soils (Mendes *et al.*, 2011). *R.solani* is able to restrict germination or harm seedlings of bean, rice, cabbage and other crops (Wibberg *et al.*, 2012). However, in the Mendes *et al.* study, the bacterial genus/species responsible for the soil suppression was not found. It would be interesting to look into the data obtained by Mendes for the *Massilia* abundance across different soil types (suppressive, conductive, autoclaved, conductive mixed with suppressive). Moreover, it would be necessary to repeat Mendes experiment using multigeneration system in order to confirm that conductive soils are being swept by the pathogen (and use other soils as controls). In such conditions focusing on the bacterial microbiome would allow for identification of the bacterial genera positively correlated in the abundance with the *Rhizoctonia* abundance (assuming their abundance does not increase in the control soils).

Pathogen invasions are seen in agricultural systems and in real soil conditions particularly during monoculture (Schreiner *et al.*, 2010; Sommerhalder *et al.*, 2011). However, I believe this is the first example of such an observation made using total community analysis in an

experimental system. It shows the power of this experimental approach and indicates that multiple environmental parameters could be investigated this way. This would include changing nutrient conditions (e.g. pH, P, N) or biotic conditions (e.g. plant cultivar, addition of PGPR) and assessing how this altered a pathogen invasion.

A comparison of the rhizosphere community between the sand and compost experiments showed that in poor sand conditions the community is more susceptible to opportunist invasions, probably by oligotrophic organisms. *Pseudomonas, Rhodopseudomonas* and *Stenotrophomonas* were among the most common soil opportunists overtaking community in the unplanted control and *Arabidopsis* rhizosphere.

The microbial community in the compost experiment on the others hand was not disturbed by soil opportunists (apart from the fungal pathogen). For the compost experiment, it can be assumed that all observed changes in the microbial community between generations are plant specific. However, the plant selection on the community was weaker, as most of the bacterial and fungal species were as common in the rhizosphere as in the bulk soil. In both growth conditions one bacterial genus was always favoured in the plant rhizospheres: *Massilia*. Unfortunately, despite many attempts no bacteria belonging to the genus of *Massilia* were isolated.

Both growth conditions selected a subset of the bacterial community. Among the most ubiquitous OTUs Rhizobiales and Burkholderiales were found in all plant rhizospheres in the sand and in the compost conditions as well. These OTUs represent highly rhizosphere specific bacteria that can colonize plant roots in any conditions. Rhizobium is actually a model species in studying bacteria root colonisation and attachment (Downie, 2010; Williams *et al.*, 2008). Members of Burkholderiales, like *Massilia* were also found to be very common in the rhizosphere (Bodenhausen *et al.*, 2013).

In both growth conditions one plant species was always different from all other. *Arabidopsis* only weakly influenced the bacterial and fungal rhizosphere community. The explanation of this observation may lie in *Arabidopsis* growth and reproduction strategy. It is a small, annual plant that can grow in a variety of soil conditions and environments. It grows fast and reproduces within 6 weeks (Meinke *et al.*, 1998). This plant is unable to form mycorrhizal associations with fungi (Felten *et al.*, 2010; Reboutier *et al.*, 2002). We

hypothesise that *Arabidopsis* is unable to interact with bacteria at the same level as other plants do (e.g. *Medicago* or *Brachypodium*). The other possibility is the lack of mycorrhiza association is the reason for weaker plant – bacteria interactions as was already shown in case of the RAM experiment (chapter 5.2.8 – 5.2.9). Mycorrhiza influence on the bacterial community has been shown to be one of the dominant factors in shaping the bacterial community structure (Offre *et al.*, 2007). Although recent rhizobiome studies (Bodenhausen *et al.*, 2013; Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2012) used *Arabidopsis* as a host plant, we suggest that this plant should not be considered as a model plant in the plant-soil microbiome research.

In most cases plants were able to shape their rhizosphere community and the differences between biological replicates of the same plant species did not differ greatly in their microbiome, despite the replicates being maintained separately for three generations. However, in the rhizosphere of *Arabidopsis* in the 3rd generation of the compost experiment three different bacterial groups appear as three groups of plants were dominated by different bacterial species. These splinter groups did not influence the plant growth significantly, however such differentiation could continue in the successive generations (this experiment was not continued further). This implies as well that splinters may form stable consortia in which the key roles may be played by different OTUs. Slight differences in the bacterial microbiome could lead into a significant differences over plant generations and have an impact on the plant growth and health (Swenson *et al.*, 2000).

Use of our multi-replicate system allowed for a quick and cost-effective screen of the influence of *Arabidopsis* and *Medicago* mutants. Three groups of *Arabidopsis* mutants were tested: plants unable to produce aliphatic glucosinolates, plants impaired in the PAMP-triggered immune response (using three different mutants with a different degree of impairment) and plants that lacked or over-expressed methyl halides production. It was shown that aliphatic glucosinolates and severe mutations in the PAMP-immune response alter the bacterial community. In the future this research could be continued, testing if addition of plant pathogens and/or PGPR significantly altered the community of the wild type and immune response impaired plants.

Despite known toxicity to bacteria, nematodes and fungi resulting in their commercial application as fumigants, methyl halides did not significantly change the rhizosphere community. If we assume that the methyl halides influence is relatively weak or very localized (only in the very close proximity of the roots), it would be interesting to see if addition of these compounds, being synthesised and in high dose, changes the rhizosphere microbiome. Addition of the methyl halides in higher doses to the rhizosphere and screening for changes in the microbiome structure (for example using ARISA) would be definite test whether these compounds play any role in the plant – microbe interactions. The other possibility would be to focus on abundance of bacterial groups of interest, here able to metabolize methyl halides. Their abundance could be tested using qPCR and focusing on the genes involved in methyl halide metabolism - cmuA (Woodall *et al.*, 2001).

Two mutants of *Medicago* were tested for the impact of mycorrhization ability of plants on the rhizosphere microbiome. Both plants were unable to interact with mycorrhizal fungi, however disruption of this interaction was caused at different levels of mycorrhizal signalling (Gobbato *et al.*, 2012; Wang *et al.*, 2012). The *ram*1 gene is necessary for the plant to form hyphopodia on the surface of the roots and it is required in the Myc factor signalling. Moreover it influences the expression of the *ram*2 gene, which in turn is responsible for cutin biosynthesis on the root surface.

Experiments performed using plant mutants allowed for a better understanding of the effects of specific plant metabolites on the microbial rhizosphere community. These experiments were designed to show if mutation in a particular gene or group of genes of a host plant significantly alters its rhizosphere community. Most of the work was focused on bacterial communities as these organisms quickly react and adapt to different environmental conditions. Further research is needed in order to fully understand the mechanisms behind the community shifts caused by changed plant root exudates composition. Metatranscriptomics of the microbiome would be the best method to study gene expression changes due to the absence of a particular root secretion compound. For example sax genes, which are responsible for utilization of glucosinolates (Fan *et al.*, 2011), would be expected to be less expressed in the *Arabidopsis myb28/29* rhizosphere. The other example is the expression of the fungal genes responsible for mycorrhiza fungi – plant associations. Recent research showed that the “Myc” factors, released by fungi in the

rhizosphere are lipo-chito-oligosaccharides, which stimulates colonisation of roots by arbuscular mycorrhiza in plants (Maillet *et al.*, 2011). However, which fungal genes are responsible for the production of these compounds is not known. Focusing on the nodulation mutants of this plant species on the rhizosphere microbiome would also increase our understanding of the microbial processes taking place in the soil.

This PhD project gave the possibility to study rhizosphere microbiome and its influence on the plants using novel "omics" methods (ARISA, 454 sequencing and as a continuation in the future transcriptomics using Illumina HiSeq). I have discovered opportunists and pathogen invasions in the community over plant generations and soil community changes over time. Moreover this project made it possible for the isolation of PGPR species under high plant selection environment. The project leading for better understanding of the role of these PGPR species in plant growth is already being conducted. Thanks to the collaboration across the John Innes Centre it was possible to study influence of the plant mutants on the rhizosphere microbiome. Some of the plant mutants were only available here (as they were not published at the time of my studies). This gave me a unique chance to understand the role of glucosinolates, mycorrhization, plant immunity and methyl halides on the rhizosphere microbiome.

If this project would be continued by another researcher, I would suggest to further focus on the pathogen invasions during multigeneration experiments. However, this time the main focus should be laid not only on the detailed analysis of the rhizosphere microbiome (probably using Illumina MiSeq as it gives far more reads at a comparable price) but also study endophytic community and plant physiology (dry mass, indication of the diseases). Moreover, it would be interesting to introduce a known plant pathogen in the rhizosphere and screen for the bacterial species that are getting more abundant over infected plant generations. More focus should be laid on the soil bacteria isolation as bacteria selected over plant generations under pathogen presence could possibly have plant growth promotion properties. The other possibility to continue the project would be to the shotgun sequencing on the already isolated DNA and look for the common microbial genes in the

rhizosphere. It would be interesting to know if *Arabidopsis* rhizobiomes have a different gene pool compared to *Medicago* and *Brachypodium* as it was already shown that these plants have different microbial structure. The power of such a study would be to connect the microbial structure with its function. Of course, combining microbial structure with the metatranscriptomic data obtained on the RNA would be even more informative (the method would dependent on the budgetary opportunities). Yet, another option to continue this project would be to focus on metatranscriptomics of the rhizosphere and endophytic community of different plant species.

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Bacterial 16S mid coded 454Flx Titanium primers

338R CATGCTGCCTCCCGTAGGAGT

27F TCAGAGTTTGATCCTGGCTCAG

blue = primer A, green = primer B, red = mid, black = target

Primer name	Sequence
338RM1	CGTATCGCCTCCCTCGCGCCATCAGACACGACGACTCATGCTGCCTCCCGTAGGAGT
338RM2	CGTATCGCCTCCCTCGCGCCATCAGACACGTAGTATCATGCTGCCTCCCGTAGGAGT
338RM3	CGTATCGCCTCCCTCGCGCCATCAGACACTACTCGTCATGCTGCCTCCCGTAGGAGT
338RM4	CGTATCGCCTCCCTCGCGCCATCAGACGACACGTATCATGCTGCCTCCCGTAGGAGT
338RM5	CGTATCGCCTCCCTCGCGCCATCAGACGAGTAGACTCATGCTGCCTCCCGTAGGAGT
338RM6	CGTATCGCCTCCCTCGCGCCATCAGACGCGTCTAGTCATGCTGCCTCCCGTAGGAGT
338RM7	CGTATCGCCTCCCTCGCGCCATCAGACGTACACACTCATGCTGCCTCCCGTAGGAGT
338RM8	CGTATCGCCTCCCTCGCGCCATCAGACGTACTGTGTCATGCTGCCTCCCGTAGGAGT
338RM9	CGTATCGCCTCCCTCGCGCCATCAGACGTAGATCGTCATGCTGCCTCCCGTAGGAGT
338RM10	CGTATCGCCTCCCTCGCGCCATCAGACTACGTCTCTCATGCTGCCTCCCGTAGGAGT
338RM11	CGTATCGCCTCCCTCGCGCCATCAGACTATACGAGTCATGCTGCCTCCCGTAGGAGT
338RM12	CGTATCGCCTCCCTCGCGCCATCAGACTCGCGTCGTCATGCTGCCTCCCGTAGGAGT
338RM13	CGTATCGCCTCCCTCGCGCCATCAGAGACTCGACGTCATGCTGCCTCCCGTAGGAGT
338RM14	CGTATCGCCTCCCTCGCGCCATCAGAGTACGAGAGTCATGCTGCCTCCCGTAGGAGT
338RM15	CGTATCGCCTCCCTCGCGCCATCAGAGTACTACTATCATGCTGCCTCCCGTAGGAGT
338RM16	CGTATCGCCTCCCTCGCGCCATCAGAGTAGACGTCTCATGCTGCCTCCCGTAGGAGT
338RM17	CGTATCGCCTCCCTCGCGCCATCAGAGTCGTACACTCATGCTGCCTCCCGTAGGAGT
338RM18	CGTATCGCCTCCCTCGCGCCATCAGAGTGTAGTAGTCATGCTGCCTCCCGTAGGAGT
338RM19	CGTATCGCCTCCCTCGCGCCATCAGATAGTATACGTCATGCTGCCTCCCGTAGGAGT
338RM20	CGTATCGCCTCCCTCGCGCCATCAGCAGTACGTACTCATGCTGCCTCCCGTAGGAGT
338RM21	CGTATCGCCTCCCTCGCGCCATCAGCGACGACGCGTCATGCTGCCTCCCGTAGGAGT

Table S1 Table of 454 pyrosequencing primers

Primer name	Sequence
338RM22	CGTATCGCCTCCCTCGCGCCATCAGCGACGAGTACTCATGCTGCCTCCCGTAGGAGT
338RM23	CGTATCGCCTCCCTCGCGCCATCAGCGATACTACGTCATGCTGCCTCCCGTAGGAGT
338RM23	CGTATCGCCTCCCTCGCGCCATCAGCGATACTACGTCATGCTGCCTCCCGTAGGAGT
338RM24	CGTATCGCCTCCCTCGCGCCATCAGCGTACGTCGATCATGCTGCCTCCCGTAGGAGT
338RM25	CGTATCGCCTCCCTCGCGCCATCAGCTACTCGTAGTCATGCTGCCTCCCGTAGGAGT
338RM26	CGTATCGCCTCCCTCGCGCCATCAGGTACAGTACGTCATGCTGCCTCCCGTAGGAGT
338RM27	CGTATCGCCTCCCTCGCGCCATCAGGTCGTACGTATCATGCTGCCTCCCGTAGGAGT
338RM28	CGTATCGCCTCCCTCGCGCCATCAGGTGTACGACGTCATGCTGCCTCCCGTAGGAGT
338RM29	CGTATCGCCTCCCTCGCGCCATCAGACACAGTGAGTCATGCTGCCTCCCGTAGGAGT
338RM30	CGTATCGCCTCCCTCGCGCCATCAGACACTCATACTCATGCTGCCTCCCGTAGGAGT
338RM31	CGTATCGCCTCCCTCGCGCCATCAGACAGACAGCGTCATGCTGCCTCCCGTAGGAGT
338RM32	CGTATCGCCTCCCTCGCGCCATCAGACAGACTATATCATGCTGCCTCCCGTAGGAGT
338RM33	CGTATCGCCTCCCTCGCGCCATCAGACAGAGACTCTCATGCTGCCTCCCGTAGGAGT
338RM34	CGTATCGCCTCCCTCGCGCCATCAGACAGCTCGTGTCATGCTGCCTCCCGTAGGAGT
338RM35	CGTATCGCCTCCCTCGCGCCATCAGACAGTGTCGATCATGCTGCCTCCCGTAGGAGT
338RM36	CGTATCGCCTCCCTCGCGCCATCAGACGAGCGCGCTCATGCTGCCTCCCGTAGGAGT
27FPB	CTATGCGCCTTGCCAGCCCGCTCAGTCAGAGTTTGATCCTGGCTCAG

Table S1 Table of 454 pyrosequencing primers

Eukaryotic 18S (ITS) mid coded 454Flx Titanium primers

ITS1 5’-TCCGTAGGTGAACCTGCGG-3’

ITS2 5’-GCTGCGTTCTTCATCGATGC-3’

blue = primer A, green = primer B, red = mid, black = target

Primer name	Sequence
ITS2M1	CGTATCGCCTCCCTCGCGCCATCAGACACGACGACTGCTGCGTTCTTCATCGATGC
ITS2M2	CGTATCGCCTCCCTCGCGCCATCAGACACGTAGTATGCTGCGTTCTTCATCGATGC
ITS2M3	CGTATCGCCTCCCTCGCGCCATCAGACACTACTCGTGCTGCGTTCTTCATCGATGC
ITS2M4	CGTATCGCCTCCCTCGCGCCATCAGACGACACGTATGCTGCGTTCTTCATCGATGC
ITS2M5	CGTATCGCCTCCCTCGCGCCATCAGACGAGTAGACTGCTGCGTTCTTCATCGATGC
ITS2M6	CGTATCGCCTCCCTCGCGCCATCAGACGCGTCTAGTGCTGCGTTCTTCATCGATGC
ITS2M7	CGTATCGCCTCCCTCGCGCCATCAGACGTACACACTGCTGCGTTCTTCATCGATGC
ITS2M8	CGTATCGCCTCCCTCGCGCCATCAGACGTACTGTGTGCTGCGTTCTTCATCGATGC
ITS2M9	CGTATCGCCTCCCTCGCGCCATCAGACGTAGATCGTGCTGCGTTCTTCATCGATGC
ITS2M10	CGTATCGCCTCCCTCGCGCCATCAGACTACGTCTCTGCTGCGTTCTTCATCGATGC
ITS2M11	CGTATCGCCTCCCTCGCGCCATCAGACTATACGAGTGCTGCGTTCTTCATCGATGC
ITS2M12	CGTATCGCCTCCCTCGCGCCATCAGACTCGCGTCGTGCTGCGTTCTTCATCGATGC
ITS2M13	CGTATCGCCTCCCTCGCGCCATCAGAGACTCGACGTGCTGCGTTCTTCATCGATGC
ITS2M14	CGTATCGCCTCCCTCGCGCCATCAGAGTACGAGAGTGCTGCGTTCTTCATCGATGC
ITS2M15	CGTATCGCCTCCCTCGCGCCATCAGAGTACTACTATGCTGCGTTCTTCATCGATGC
ITS2M16	CGTATCGCCTCCCTCGCGCCATCAGAGTAGACGTCTGCTGCGTTCTTCATCGATGC
ITS2M17	CGTATCGCCTCCCTCGCGCCATCAGAGTCGTACACTGCTGCGTTCTTCATCGATGC
ITS2M18	CGTATCGCCTCCCTCGCGCCATCAGAGTGTAGTAGTGCTGCGTTCTTCATCGATGC
ITS2M19	CGTATCGCCTCCCTCGCGCCATCAGATAGTATACGTGCTGCGTTCTTCATCGATGC
ITS2M20	CGTATCGCCTCCCTCGCGCCATCAGCAGTACGTACTGCTGCGTTCTTCATCGATGC
ITS2M21	CGTATCGCCTCCCTCGCGCCATCAGCGACGACGCGTGCTGCGTTCTTCATCGATGC

Table S1 Table of 454 pyrosequencing primers

Primer name	Sequence
ITS2M22	CGTATCGCCTCCCTCGCGCCATCAGCGACGAGTACTGCTGCGTTCTTCATCGATGC
ITS2M23	CGTATCGCCTCCCTCGCGCCATCAGCGATACTACGTGCTGCGTTCTTCATCGATGC
ITS2M24	CGTATCGCCTCCCTCGCGCCATCAGCGTACGTCGATGCTGCGTTCTTCATCGATGC
ITS2M25	CGTATCGCCTCCCTCGCGCCATCAGCTACTCGTAGTGCTGCGTTCTTCATCGATGC
ITS2M26	CGTATCGCCTCCCTCGCGCCATCAGGTACAGTACGTGCTGCGTTCTTCATCGATGC
ITS2M27	CGTATCGCCTCCCTCGCGCCATCAGGTCGTACGTATGCTGCGTTCTTCATCGATGC
ITS2M28	CGTATCGCCTCCCTCGCGCCATCAGGTGTACGACGTGCTGCGTTCTTCATCGATGC
ITS2M29	CGTATCGCCTCCCTCGCGCCATCAGACACAGTGAGTGCTGCGTTCTTCATCGATGC
ITS2M30	CGTATCGCCTCCCTCGCGCCATCAGACACTCATACTGCTGCGTTCTTCATCGATGC
ITS2M31	CGTATCGCCTCCCTCGCGCCATCAGACAGACAGCGTGCTGCGTTCTTCATCGATGC
ITS2M32	CGTATCGCCTCCCTCGCGCCATCAGACAGACTATATGCTGCGTTCTTCATCGATGC
ITS2M33	CGTATCGCCTCCCTCGCGCCATCAGACAGAGACTCTGCTGCGTTCTTCATCGATGC
ITS2M34	CGTATCGCCTCCCTCGCGCCATCAGACAGCTCGTGTGCTGCGTTCTTCATCGATGC
ITS2M35	CGTATCGCCTCCCTCGCGCCATCAGACAGTGTCGATGCTGCGTTCTTCATCGATGC
ITS2M36	CGTATCGCCTCCCTCGCGCCATCAGACGAGCGCGCTGCTGCGTTCTTCATCGATGC
ITS1PB	CTATGCGCCTTGCCAGCCCGCTCAGTCCGTAGGTGAACCTGCGG

Table S1 Table of 454 pyrosequencing primers

no	domain	MID barcode	plant species	generation	rep.	DNA source	reads before filtering	reads after filtering	Fraction kept
1	fungi	ACACGACGACT	*A.thaliana*	1st	A	1-8	2143	1857	0.866
2	fungi	ACACGTAGTAT	*A.thaliana*	1st	B	9-16	3876	3471	0.895
3	fungi	ACACTACTCGT	*A.thaliana*	1st	C	17-24	2898	2349	0.81
4	fungi	ACGACACGTAT	*M.truncatula*	1st	A	1-8	3119	2663	0.853
5	fungi	ACGAGTAGACT	*M.truncatula*	1st	B	9-16	2455	2049	0.834
6	fungi	ACGCGTCTAGT	*M.truncatula*	1st	C	17-24	1820	1566	0.86
7	fungi	ACGTACACACT	*B.distachyon*	1st	A	1-8	2266	1906	0.841
8	fungi	ACGTACTGTGT	*B.distachyon*	1st	B	9-16	1675	1505	0.898
9	fungi	ACGTAGATCGT	*B.distachyon*	1st	C	17-24	1883	1687	0.895
10	fungi	ACTACGTCTCT	unplanted	1st	A	1-8	2102	1911	0.909
11	fungi	ACTATACGAGT	unplanted	1st	B	9-16	5826	5078	0.871
12	fungi	ACTCGCGTCGT	unplanted	1st	C	17-24	3535	3147	0.89
13	fungi	AGACTCGACGT	*A.thaliana*	2nd	A	1-8	3118	2786	0.893
14	fungi	AGTACGAGAGT	*A.thaliana*	2nd	B	9-16	4079	2240	0.549
15	fungi	AGTACTACTAT	*A.thaliana*	2nd	C	17-24	1763	1275	0.723
16	fungi	AGTAGACGTCT	*M.truncatula*	2nd	A	1-8	2022	1629	0.805
17	fungi	AGTCGTACACT	*M.truncatula*	2nd	B	9-16	4921	3595	0.73
18	fungi	AGTGTAGTAGT	*M.truncatula*	2nd	C	17-24	3532	3134	0.887
19	fungi	ATAGTATACGT	*B.distachyon*	2nd	A	1-8	3870	3453	0.892
20	fungi	CAGTACGTACT	*B.distachyon*	2nd	B	9-16	4835	2412	0.498
21	fungi	CGACGACGCGT	*B.distachyon*	2nd	C	17-24	1994	1645	0.824
22	fungi	CGACGAGTACT	unplanted	2nd	A	1-8	2223	1294	0.582
23	fungi	CGATACTACGT	unplanted	2nd	B	9-16	730	604	0.827
24	fungi	CGTACGTCGAT	unplanted	2nd	C	17-24	5642	4267	0.756
25	fungi	CTACTCGTAGT	*A.thaliana*	3rd	A	1-8	9242	7563	0.818
26	fungi	GTACAGTACGT	*A.thaliana*	3rd	B	9-16	1520	626	0.411
27	fungi	GTCGTACGTAT	*A.thaliana*	3rd	C	17-24	2478	707	0.285
28	fungi	GTGTACGACGT	*M.truncatula*	3rd	A	1-8	4295	3314	0.771
29	fungi	ACACAGTGAGT	*M.truncatula*	3rd	B	9-16	6623	3595	0.542
30	fungi	ACACTCATACT	*M.truncatula*	3rd	C	17-24	7536	6820	0.904
31	fungi	ACAGACAGCGT	*B.distachyon*	3rd	A	1-8	5519	4451	0.806
32	fungi	ACAGACTATAT	*B.distachyon*	3rd	B	9-16	5174	4322	0.835
33	fungi	ACAGAGACTCT	*B.distachyon*	3rd	C	17-24	7460	6445	0.863
34	fungi	ACAGCTCGTGT	unplanted	3rd	A	1-8	1882	1299	0.69
35	fungi	ACAGTGTCGAT	unplanted	3rd	B	9-16	2324	562	0.241
36	fungi	ACGAGCGCGCT	unplanted	3rd	C	17-24	2558	1386	0.541

Table S2 454 pyrosequencing primer used in the sand experiment and sequencing efficiency results

1	bact	ACACGACGACT	*A.thaliana*	1st	A	1-8	2957	2721	0.92
2	bact	ACACGTAGTAT	*A.thaliana*	1st	B	9-16	3943	3571	0.905
3	bact	ACACTACTCGT	*A.thaliana*	1st	C	17-24	2764	2543	0.92
4	bact	ACGACACGTAT	*M.truncatula*	1st	A	1-8	2487	2261	0.909
5	bact	ACGAGTAGACT	*M.truncatula*	1st	B	9-16	3506	3248	0.926
6	bact	ACGCGTCTAGT	*M.truncatula*	1st	C	17-24	2634	2417	0.917
7	bact	ACGTACACACT	*B.distachyon*	1st	A	1-8	3749	3462	0.923
8	bact	ACGTACTGTGT	*B.distachyon*	1st	B	9-16	1961	1795	0.915
9	bact	ACGTAGATCGT	*B.distachyon*	1st	C	17-24	1660	1511	0.91
10	bact	ACTACGTCTCT	unplanted	1st	A	1-8	1816	1657	0.912
11	bact	ACTATACGAGT	unplanted	1st	B	9-16	1699	1544	0.908
12	bact	ACTCGCGTCGT	unplanted	1st	C	17-24	2664	2392	0.897
13	bact	AGACTCGACGT	*A.thaliana*	2nd	A	1-8	3518	3319	0.943
14	bact	AGTACGAGAGT	*A.thaliana*	2nd	B	9-16	2576	2414	0.937
15	bact	AGTACTACTAT	*A.thaliana*	2nd	C	17-24	3989	3789	0.949
16	bact	AGTAGACGTCT	*M.truncatula*	2nd	A	1-8	2647	2498	0.943
17	bact	AGTCGTACACT	*M.truncatula*	2nd	B	9-16	3343	3199	0.956
18	bact	AGTGTAGTAGT	*M.truncatula*	2nd	C	17-24	1166	1098	0.941
19	bact	ATAGTATACGT	*B.distachyon*	2nd	A	1-8	3358	3189	0.949
20	bact	CAGTACGTACT	*B.distachyon*	2nd	B	9-16	4151	3956	0.953
21	bact	CGACGACGCGT	*B.distachyon*	2nd	C	17-24	3109	2925	0.94
22	bact	CGACGAGTACT	unplanted	2nd	A	1-8	2002	1830	0.914
23	bact	CGATACTACGT	unplanted	2nd	B	9-16	2594	2423	0.934
24	bact	CGTACGTCGAT	unplanted	2nd	C	17-24	3516	3325	0.945
25	bact	CTACTCGTAGT	*A.thaliana*	3rd	A	1-8	1395	1341	0.961
26	bact	GTACAGTACGT	*A.thaliana*	3rd	B	9-16	1823	1724	0.945
27	bact	GTCGTACGTAT	*A.thaliana*	3rd	C	17-24	1774	1699	0.957
28	bact	GTGTACGACGT	*M.truncatula*	3rd	A	1-8	1746	1679	0.961
29	bact	ACACAGTGAGT	*M.truncatula*	3rd	B	9-16	2272	2165	0.952
30	bact	ACACTCATACT	*M.truncatula*	3rd	C	17-24	1918	1838	0.958
31	bact	ACAGACAGCGT	*B.distachyon*	3rd	A	1-8	2319	2221	0.957
32	bact	ACAGACTATAT	*B.distachyon*	3rd	B	9-16	2367	2275	0.961
33	bact	ACAGAGACTCT	*B.distachyon*	3rd	C	17-24	1530	1453	0.949
34	bact	ACAGCTCGTGT	unplanted	3rd	A	1-8	2586	2488	0.962
35	bact	ACAGTGTCGAT	unplanted	3rd	B	9-16	1567	1490	0.95
36	bact	ACGAGCGCGCT	unplanted	3rd	C	17-24	1608	1550	0.963

Table S2 454 pyrosequencing primer used in the sand experiment and sequencing efficiency results

no	do-main	MID barcode	plant species	gener-ation	rep.	DNA source	Reads before filtering	Reads after filtering	Fraction kept
1	bact	ACACGACGACT	*A.thaliana*	1st	A	1-8	2368	1968	0.83
2	bact	ACACGTAGTAT	*A.thaliana*	1st	B	9-16	3565	3027	0.85
3	bact	ACACTACTCGT	*A.thaliana*	1st	C	17-24	3420	2940	0.86
4	bact	AGACTCGACGT	*A.thaliana*	2nd	A	1-8	2558	2145	0.84
5	bact	AGTACGAGAGT	*A.thaliana*	2nd	B	9-16	2337	1899	0.81
6	bact	AGTACTACTAT	*A.thaliana*	2nd	C	17-24	2240	1868	0.83
7	bact	CTACTCGTAGT	*A.thaliana*	3rd	A	1-8	2621	2242	0.86
8	bact	GTACAGTACGT	*A.thaliana*	3rd	B	9-16	3085	2678	0.87
9	bact	GTCGTACGTAT	*A.thaliana*	3rd	C	17-24	2126	1839	0.87
10	bact	ACGTACACACT	*B.distachyon*	1st	A	1-8	2728	2336	0.86
11	bact	ACGTACTGTGT	*B.distachyon*	1st	B	9-16	2775	2379	0.86
12	bact	ACGTAGATCGT	*B.distachyon*	1st	C	17-24	3771	3310	0.88
13	bact	ATAGTATACGT	*B.distachyon*	2nd	A	1-8	2463	2120	0.86
14	bact	CAGTACGTACT	*B.distachyon*	2nd	B	9-16	2610	2252	0.86
15	bact	CGACGACGCGT	*B.distachyon*	2nd	C	17-24	2157	1819	0.84
16	bact	ACAGACAGCGT	*B.distachyon*	3rd	A	1-8	2596	2264	0.87
17	bact	ACAGACTATAT	*B.distachyon*	3rd	B	9-16	2589	2319	0.90
18	bact	ACACGACGACT	*B.distachyon*	3rd	C	17-24	2625	2149	0.82
19	bact	ACGAGTAGACT	*B.rapa*	1st	A	1-8	2708	2081	0.77
20	bact	ACGCGTCTAGT	*B.rapa*	1st	B	9-16	2057	1606	0.78
21	bact	ACGTACACACT	*B.rapa*	1st	C	17-24	2373	1803	0.76
22	bact	AGTCGTACACT	*B.rapa*	2nd	A	1-8	2366	1843	0.78
23	bact	AGTGTAGTAGT	*B.rapa*	2nd	B	9-16	2173	1664	0.77
24	bact	ATAGTATACGT	*B.rapa*	2nd	C	17-24	2170	1732	0.80
25	bact	ACACAGTGAGT	*B.rapa*	3rd	A	1-8	2049	1623	0.79
26	bact	ACACTCATACT	*B.rapa*	3rd	B	9-16	2132	1725	0.81
27	bact	ACAGACAGCGT	*B.rapa*	3rd	C	17-24	1736	1395	0.80
28	bact	ACGACACGTAT	*M.truncatula*	1st	A	1-8	2325	2037	0.88
29	bact	ACGAGTAGACT	*M.truncatula*	1st	B	9-16	2624	2268	0.86
30	bact	ACGCGTCTAGT	*M.truncatula*	1st	C	17-24	2188	1906	0.87
31	bact	AGTAGACGTCT	*M.truncatula*	2nd	A	1-8	1273	1169	0.92
32	bact	AGTCGTACACT	*M.truncatula*	2nd	B	9-16	3440	2890	0.84
33	bact	AGTGTAGTAGT	*M.truncatula*	2nd	C	17-24	2751	2370	0.86
34	bact	GTGTACGACGT	*M.truncatula*	3rd	A	1-8	2066	1772	0.86
35	bact	ACACAGTGAGT	*M.truncatula*	3rd	B	9-16	2629	2291	0.87
36	bact	ACACTCATACT	*M.truncatula*	3rd	C	17-24	2393	2106	0.88
37	bact	ACGTAGATCGT	*A.thaliana*		A	1-8	3362	2920	0.87
38	bact	ACTACGTCTCT	*A.thaliana*		B	9-16	2990	2592	0.87
39	bact	ACTATACGAGT	*A.thaliana*		C	17-24	2216	1911	0.86
40	bact	ACGTACTGTGT	*P.sativum*	1st	A	1-8	2662	2141	0.80

41	bact	ACGTAGATCGT	*P.sativum*	1st	B	9-16	2600	2057	0.79
42	bact	ACTACGTCTCT	*P.sativum*	1st	C	17-24	2064	1645	0.80
43	bact	CAGTACGTACT	*P.sativum*	2nd	A	1-8	2185	1817	0.83
44	bact	CGACGACGCGT	*P.sativum*	2nd	B	9-16	2452	1877	0.77
45	bact	CGACGAGTACT	*P.sativum*	2nd	C	17-24	2208	1814	0.82
46	bact	ACAGACTATAT	*P.sativum*	3rd	A	1-8	2640	2132	0.81
47	bact	ACACGACGACT	*P.sativum*	3rd	B	9-16	3279	2864	0.87
48	bact	ACACGTAGTAT	*P.sativum*	3rd	C	17-24	2207	1943	0.88
49	bact	ACTCGCGTCGT	*M.truncatula*		A	1-8	2216	1930	0.87
50	bact	AGACTCGACGT	*M.truncatula*		B	9-16	2069	1800	0.87
51	bact	AGTACGAGAGT	*M.truncatula*		C	17-24	1916	1681	0.88
52	bact	AGTACTACTAT	*M.truncatula*		A	1-8	2274	2003	0.88
53	bact	AGTAGACGTCT	*M.truncatula*		B	9-16	2396	2117	0.88
54	bact	AGTCGTACACT	*M.truncatula*		C	17-24	2497	2179	0.87
55	bact	AGTACGAGAGT	unplanted 100ml	1st	A	1-8	1662	1433	0.86
56	bact	AGTACTACTAT	unplanted 100ml	1st	B	9-16	2618	2253	0.86
57	bact	AGTAGACGTCT	unplanted 100ml	1st	C	17-24	2000	1756	0.88
58	bact	GTACAGTACGT	unplanted 100ml	2nd	A	1-8	1916	1694	0.88
59	bact	GTCGTACGTAT	unplanted 100ml	2nd	B	9-16	2222	1981	0.89
60	bact	GTGTACGACGT	unplanted 100ml	2nd	C	17-24	2599	2289	0.88
61	bact	ACGCGTCTAGT	unplanted 100ml	3rd	A	1-8	2478	2169	0.88
62	bact	ACGTACACACT	unplanted 100ml	3rd	B	9-16	2621	2289	0.87
63	bact	ACGTACTGTGT	unplanted 100ml	3rd	C	17-24	2587	2208	0.85
64	bact	ACTACGTCTCT	unplanted 50ml	1st	A	1-8	2536	2215	0.87
65	bact	ACTATACGAGT	unplanted 50ml	1st	B	9-16	3275	2906	0.89
66	bact	ACTCGCGTCGT	unplanted 50ml	1st	C	17-24	2407	2057	0.85
67	bact	CGACGAGTACT	unplanted 50ml	2nd	A	1-8	2782	2433	0.87
68	bact	CGATACTACGT	unplanted 50ml	2nd	B	9-16	4278	3737	0.87
69	bact	CGTACGTCGAT	unplanted 50ml	2nd	C	17-24	3383	2987	0.88
70	bact	ACACGTAGTAT	unplanted 50ml	3rd	A	1-8	2124	1573	0.74
71	bact	ACACTACTCGT	unplanted	3rd	B	9-16	2388	1822	0.76

			50ml						
72	bact	ACGACACGTAT	unplanted 50ml	3rd	C	17-24	2832	2172	0.77
73	bact	ACTATACGAGT	*T.aestivum*	1st	A	1-8	2409	1870	0.78
74	bact	ACTCGCGTCGT	*T.aestivum*	1st	B	9-16	1902	1462	0.77
75	bact	AGACTCGACGT	*T.aestivum*	1st	C	17-24	1984	1571	0.79
76	bact	CGATACTACGT	*T.aestivum*	2nd	A	1-8	1937	1724	0.89
77	bact	CGTACGTCGAT	*T.aestivum*	2nd	B	9-16	2182	1892	0.87
78	bact	CTACTCGTAGT	*T.aestivum*	2nd	C	17-24	2469	2179	0.88
79	bact	ACACTACTCGT	*T.aestivum*	3rd	A	1-8	2489	2110	0.85
80	bact	ACGACACGTAT	*T.aestivum*	3rd	B	9-16	2776	2400	0.86
81	bact	ACGAGTAGACT	*T.aestivum*	3rd	C	17-24	2473	2131	0.86
82	bact	ACACTCATACT	*A.thaliana*		A	1-8	2816	2403	0.85
83	bact	ACAGACAGCGT	*A.thaliana*		B	9-16	2298	1950	0.85
84	bact	ACAGACTATAT	*A.thaliana*		C	17-24	2823	2453	0.87
85	bact	CGTACGTCGAT	*A.thaliana*		A	1-8	2464	1956	0.79
86	bact	CTACTCGTAGT	*A.thaliana*		B	9-16	2342	1848	0.79
87	bact	GTACAGTACGT	*A.thaliana*		C	17-24	2745	2189	0.80
88	bact	CGACGACGCGT	*A.thaliana*		A	1-8	2049	1556	0.76
89	bact	CGACGAGTACT	*A.thaliana*		B	9-16	2043	1482	0.73
90	bact	CGATACTACGT	*A.thaliana*		C	17-24	2497	1879	0.75
91	bact	GTCGTACGTAT	*A.thaliana*		A	1-8	1763	1361	0.77
92	bact	GTGTACGACGT	*A.thaliana*		B	9-16	1862	1455	0.78
93	bact	ACACAGTGAGT	*A.thaliana*		C	17-24	2130	1706	0.80
94	bact	AGTGTAGTAGT	*A.thaliana*		A	1-8	2671	2338	0.88
95	bact	ATAGTATACGT	*A.thaliana*		B	9-16	2396	2081	0.87
96	bact	CAGTACGTACT	*A.thaliana*		C	17-24	3198	2748	0.86
1	Fungi	ACACGACGACT	*A.thaliana*	1st	A	1-8	944	819	0.87
2	Fungi	ACACGTAGTAT	*A.thaliana*	1st	B	9-16	872	767	0.88
3	Fungi	ACACTACTCGT	*A.thaliana*	1st	C	17-24	743	668	0.90
4	Fungi	AGACTCGACGT	*A.thaliana*	2nd	A	1-8	425	383	0.90
5	Fungi	AGTACGAGAGT	*A.thaliana*	2nd	B	9-16	428	371	0.87
6	Fungi	AGTACTACTAT	*A.thaliana*	2nd	C	17-24	839	753	0.90
7	Fungi	CTACTCGTAGT	*A.thaliana*	3rd	A	1-8	192	174	0.91
8	Fungi	GTACAGTACGT	*A.thaliana*	3rd	B	9-16	973	853	0.88
9	Fungi	GTCGTACGTAT	*A.thaliana*	3rd	C	17-24	30	27	0.90
10	Fungi	ACGTACACACT	*B.distachyon*	1st	A	1-8	351	317	0.90
11	Fungi	ACGTACTGTGT	*B.distachyon*	1st	B	9-16	810	735	0.91
12	Fungi	ACGTAGATCGT	*B.distachyon*	1st	C	17-24	1055	936	0.89
13	Fungi	ATAGTATACGT	*B.distachyon*	2nd	A	1-8	512	462	0.90
14	Fungi	CAGTACGTACT	*B.distachyon*	2nd	B	9-16	544	476	0.88
15	Fungi	CGACGACGCGT	*B.distachyon*	2nd	C	17-24	580	527	0.91
16	Fungi	ACAGACAGCGT	*B.distachyon*	3rd	A	1-8	737	677	0.92

17	Fungi	ACAGACTATAT	*B.distachyon*	3rd	B	9-16	734	658	0.90
18	Fungi	ACACGACGACT	*B.distachyon*	3rd	C	17-24	661	581	0.88
19	Fungi	ACGAGTAGACT	*B.rapa*	1st	A	1-8	615	554	0.90
20	Fungi	ACGCGTCTAGT	*B.rapa*	1st	B	9-16	535	481	0.90
21	Fungi	ACGTACACACT	*B.rapa*	1st	C	17-24	661	594	0.90
22	Fungi	AGTCGTACACT	*B.rapa*	2nd	A	1-8	45	42	0.93
23	Fungi	AGTGTAGTAGT	*B.rapa*	2nd	B	9-16	124	106	0.85
24	Fungi	ATAGTATACGT	*B.rapa*	2nd	C	17-24	173	162	0.94
25	Fungi	ACACAGTGAGT	*B.rapa*	3rd	A	1-8	717	649	0.91
26	Fungi	ACACTCATACT	*B.rapa*	3rd	B	9-16	94	87	0.93
27	Fungi	ACAGACAGCGT	*B.rapa*	3rd	C	17-24	310	288	0.93
28	Fungi	ACGACACGTAT	*M.truncatula*	1st	A	1-8	517	463	0.90
29	Fungi	ACGAGTAGACT	*M.truncatula*	1st	B	9-16	360	321	0.89
30	Fungi	ACGCGTCTAGT	*M.truncatula*	1st	C	17-24	652	589	0.90
31	Fungi	AGTAGACGTCT	*M.truncatula*	2nd	A	1-8	68	63	0.93
32	Fungi	AGTCGTACACT	*M.truncatula*	2nd	B	9-16	815	727	0.89
33	Fungi	AGTGTAGTAGT	*M.truncatula*	2nd	C	17-24	608	553	0.91
34	Fungi	GTGTACGACGT	*M.truncatula*	3rd	A	1-8	603	538	0.89
35	Fungi	ACACAGTGAGT	*M.truncatula*	3rd	B	9-16	691	623	0.90
36	Fungi	ACACTCATACT	*M.truncatula*	3rd	C	17-24	445	397	0.89
37	Fungi	ACGTAGATCGT	*A.thaliana*		A	1-8	163	143	0.88
38	Fungi	ACTACGTCTCT	*A.thaliana*		B	9-16	637	589	0.92
39	Fungi	ACTATACGAGT	*A.thaliana*		C	17-24	898	816	0.91
40	Fungi	ACGTACTGTGT	*P.sativum*	1st	A	1-8	813	747	0.92
41	Fungi	ACGTAGATCGT	*P.sativum*	1st	B	9-16	856	788	0.92
42	Fungi	ACTACGTCTCT	*P.sativum*	1st	C	17-24	92	78	0.85
43	Fungi	CAGTACGTACT	*P.sativum*	2nd	A	1-8	821	739	0.90
44	Fungi	CGACGACGCGT	*P.sativum*	2nd	B	9-16	528	493	0.93
45	Fungi	CGACGAGTACT	*P.sativum*	2nd	C	17-24	754	691	0.92
46	Fungi	ACAGACTATAT	*P.sativum*	3rd	A	1-8	598	548	0.92
47	Fungi	ACACGACGACT	*P.sativum*	3rd	B	9-16	1037	943	0.91
48	Fungi	ACACGTAGTAT	*P.sativum*	3rd	C	17-24	1035	942	0.91
49	Fungi	ACTCGCGTCGT	*M.truncatula*		A	1-8	826	714	0.86
50	Fungi	AGACTCGACGT	*M.truncatula*		B	9-16	971	846	0.87
51	Fungi	AGTACGAGAGT	*M.truncatula*		C	17-24	916	781	0.85
52	Fungi	AGTACTACTAT	*M.truncatula*		A	1-8	553	457	0.83
53	Fungi	AGTAGACGTCT	*M.truncatula*		B	9-16	493	387	0.78
54	Fungi	AGTCGTACACT	*M.truncatula*		C	17-24	695	573	0.82
55	Fungi	AGTACGAGAGT	unplanted 100ml	1st	A	1-8	296	232	0.78
56	Fungi	AGTACTACTAT	unplanted 100ml	1st	B	9-16	1040	920	0.88
57	Fungi	AGTAGACGTCT	unplanted	1st	C	17-24	832	663	0.80

			100ml						
58	Fungi	GTACAGTACGT	unplanted 100ml	2nd	A	1-8	741	601	0.81
59	Fungi	GTCGTACGTAT	unplanted 100ml	2nd	B	9-16	960	758	0.79
60	Fungi	GTGTACGACGT	unplanted 100ml	2nd	C	17-24	762	593	0.78
61	Fungi	ACGCGTCTAGT	unplanted 100ml	3rd	A	1-8	955	769	0.81
62	Fungi	ACGTACACACT	unplanted 100ml	3rd	B	9-16	965	771	0.80
63	Fungi	ACGTACTGTGT	unplanted 100ml	3rd	C	17-24	923	759	0.82
64	Fungi	ACTACGTCTCT	unplanted 50ml	1st	A	1-8	1162	1049	0.90
65	Fungi	ACTATACGAGT	unplanted 50ml	1st	B	9-16	859	777	0.90
66	Fungi	ACTCGCGTCGT	unplanted 50ml	1st	C	17-24	959	862	0.90
67	Fungi	CGACGAGTACT	unplanted 50ml	2nd	A	1-8	869	780	0.90
68	Fungi	CGATACTACGT	unplanted 50ml	2nd	B	9-16	886	782	0.88
69	Fungi	CGTACGTCGAT	unplanted 50ml	2nd	C	17-24	1749	1558	0.89
70	Fungi	ACACGTAGTAT	unplanted 50ml	3rd	A	1-8	29	25	0.86
71	Fungi	ACACTACTCGT	unplanted 50ml	3rd	B	9-16	421	389	0.92
72	Fungi	ACGACACGTAT	unplanted 50ml	3rd	C	17-24	702	625	0.89
73	Fungi	ACTATACGAGT	*T.aestivum*	1st	A	1-8	556	509	0.92
74	Fungi	ACTCGCGTCGT	*T.aestivum*	1st	B	9-16	343	315	0.92
75	Fungi	AGACTCGACGT	*T.aestivum*	1st	C	17-24	477	438	0.92
76	Fungi	CGATACTACGT	*T.aestivum*	2nd	A	1-8	799	722	0.90
77	Fungi	CGTACGTCGAT	*T.aestivum*	2nd	B	9-16	857	781	0.91
78	Fungi	CTACTCGTAGT	*T.aestivum*	2nd	C	17-24	877	787	0.90
79	Fungi	ACACTACTCGT	*T.aestivum*	3rd	A	1-8	663	599	0.90
80	Fungi	ACGACACGTAT	*T.aestivum*	3rd	B	9-16	627	565	0.90
81	Fungi	ACGAGTAGACT	*T.aestivum*	3rd	C	17-24	830	741	0.89
82	Fungi	ACACTCATACT	*A.thaliana*		A	1-8	605	543	0.90
83	Fungi	ACAGACAGCGT	*A.thaliana*		B	9-16	271	245	0.90
84	Fungi	ACAGACTATAT	*A.thaliana*		C	17-24	693	642	0.93

85	Fungi	CGTACGTCGAT	*A.thaliana*		A	1-8	511	453	0.89
86	Fungi	CTACTCGTAGT	*A.thaliana*		B	9-16	468	419	0.90
87	Fungi	GTACAGTACGT	*A.thaliana*		C	17-24	391	349	0.89
88	Fungi	CGACGACGCGT	*A.thaliana*		A	1-8	138	129	0.93
89	Fungi	CGACGAGTACT	*A.thaliana*		B	9-16	643	571	0.89
90	Fungi	CGATACTACGT	*A.thaliana*		C	17-24	641	575	0.90
91	Fungi	GTCGTACGTAT	*A.thaliana*		A	1-8	661	590	0.89
92	Fungi	GTGTACGACGT	*A.thaliana*		B	9-16	317	284	0.90
93	Fungi	ACACAGTGAGT	*A.thaliana*		C	17-24	510	451	0.88
94	Fungi	AGTGTAGTAGT	*A.thaliana*		A	1-8	762	697	0.91
95	Fungi	ATAGTATACGT	*A.thaliana*		B	9-16	886	789	0.89
96	Fungi	CAGTACGTACT	*A.thaliana*		C	17-24	1005	898	0.89

Table S3 454 pyrosequencing primer used in the compost experiment and sequencing efficiency results